Improved Preservation and Staining of HeLa Cell Actin Filaments, Clathrin-coated Membranes, and Other Cytoplasmic Structures by Tannic Acid-Gluteraldehyde-Saponin Fixation

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ABSTRACT Fixation of HeLa cells with a mixture of 100 mM glutaraldehyde, 2 mg/ml tannic acid and 0.5 mg/ml saponin allows the tannic acid to penetrate intact cells without disruption of membranes or extraction of the cytoplasmic matrix. After subsequent treatment with OsO₄, cytoplasmic structures are stained so densely that fine details are visible even in very thin (dark gray) sections. Actin filaments are protected from disruption by OsO₄ so that straight, densely stained filaments are seen in the cell cortex, filopodia, ruffling membranes, and stress fibers. Stress fibers also have 15-18-nm densities similar in appearance to myosin filaments. Tannic acid staining reveals that the coats of coated vesicles, pits, and plaques have a 12-nm layer of amorphous material between the membrane and the clathrin basketwork. HeLa cells have very large clathrin-coated membrane plaques on the basal surface. These coated membrane plaques appear to be a previously unrecognized site of cell-substrate adhesion.

Work on the ultrastructure of the cytoplasmic matrix has been hampered by the continuing problem that methods of specimen preparation that are acceptable for organelles can destroy or alter major cytoplasmic fibers, especially actin filaments and microtubules. In early work both microtubules and actin filaments were destroyed during fixation with OsO₄ or KMnO₄. Fixation with glutaraldehyde followed by OsO₄ (42) solved the problem with microtubule preservation, but glutaraldehyde can damage actin filaments (29) and OsO₄ can severely disrupt actin filaments even after glutaraldehyde treatment (32). Additional problems with traditional chemical fixation and embedding techniques are that fibrous elements may have poor contrast compared with the embedding medium (54), and that they may be obscured by fixed soluble constituents of the matrix.

These deficiencies of conventional chemical fixation and embedding stimulated the development of four new methods to prepare cells for electron microscopy of the cytoplasmic matrix: (a) preparation of whole amounts of intact cells by chemical fixation, dehydration, and critical point drying (14, 54); (b) physical or chemical lysis of cultured cells followed by negative staining (12, 16, 30, 47); (c) physical lysis, freezedrying, and rotary shadowing (9); and (d) rapid freezing followed by freeze-substitution or by freeze-fracturing, deepetching, and rotary shadowing (21, 22, 24). These new methods have contributed most of the recent information about the organization of the cytoplasmic matrix. However, each of these methods has limitations, which we consider in the Discussion.

We felt that thin sections offer enough advantages over the various whole mount procedures to justify a new effort to overcome the difficulties with chemical fixation and poor specimen contrast. We focused our efforts on methods to introduce tannic acid into the cell during fixation, for two reasons. First, it is well established that tannic acid is an excellent electron-dense stain for both membranes (45) and cytoplasmic fibers (10, 19, 27, 49). Second, we found that tannic acid also protects purified actin filaments from fragmentation by OsO₄ (38). Unfortunately, intact cells are impermeable to tannic acids (35). We report here that fixation of HeLa cells with a mixture of glutaraldehyde, tannic acid, and saponin allows the penetration of tannic acid without disrupting membranes. The actin filaments are well preserved, and most cytoplasmic structures, including large sheets of clathrin coating the basal plasma membrane, are densely stained, allowing one to study very thin sections. These advantages of the fixation...
method have allowed us to make a number of new or improved observations on HeLa cell fine structure.

MATERIALS AND METHODS

HeLa Cell Culture

HeLa cells were grown on 35 x 10 mm plastic petri dishes (Falcon #3001, Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, CA) in DME with 10% fetal calf serum.

Electron Microscopy

The standard fixation method was to replace the culture medium with a 37°C solution of 100 mM (1%) glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA), 0.5 mg/ml saponin (Merck and Co., Inc., Rahway, NJ), and 2 mg/ml tannic acid (Malinckrodt #1764, Malinckrodt Inc., St. Louis, MO) in 100 mM sodium phosphate, 50 mM KCl, 5 mM MgCl₂, pH 7.0 (Buffer A). After 30 min at room temperature the dish was rinsed briefly with Buffer A at pH 6.0 and treated with 40 mM (1%) OsO₄ in Buffer A, pH 6.0, for 20 min at room temperature. The cells were dehydrated over 45 min at room temperature with ethanol (50%, 70%, 95%, three changes of absolute) and then infiltrated with a 1:1 mixture of ethanol and epoxy resin (Luft mixture with Epon 812) for 1 h followed by 100% epoxy resin for 1 h. Fresh epoxy was poured into the dishes to a height of 2-3 mm and cured at 60°C for 2 d. For comparison, the primary fixation was varied as follows: (a) as described above without saponin; (b) in 400 mM glutaraldehyde, 80 mg/ml tannic acid in Buffer A; and (c) in 400 mM glutaraldehyde, 80 mg/ml tannic acid in 100 mM cacodylate buffer, pH 7.2, for 2 h at room temperature.

We separated the epoxy disks from the plastic dish by hammering away the sides of the plastic dish, then holding the resultant disk perpendicular to a hard surface and striking with a hammer the edge of the disk vertically tangential to the epoxy-dish interface. This leaves a smooth surface (no stress marks) so that single cells may be evaluated, marked, and photographed in a phase-contrast microscope. Identified cells were cut out of the epoxy disk and mounted on epoxy plugs in the desired orientation. For sectioning perpendicular to the growth surface, the specimen was reembedded.

Silver-gray (75 nm) to dark gray (35 nm) sections were cut, collected on nitrocelulose and carbon-coated single hole grids, and stained with lead citrate (52). Micrographs were taken with a JEOL 100 CX or Zeiss EM 10A electron microscope. Paracrystals of skeletal muscle tropomyosin (39.5-nm periodicity) were used as magnification standards.

RESULTS

Effects of Tannic Acid on Actin Filaments

Fixation of actin filaments with tannic acid and glutaraldehyde strongly inhibits their disruption during subsequent treatment with OsO₄. Actin filament pellets prepared as in reference 32 can survive mild OsO₄ treatment (32; Fig. 1a), but even after glutaraldehyde fixation actin filaments are disrupted by exposure to OsO₄ under conditions traditionally used for fixation (Fig. 1b), unless tannic acid is present during primary fixation with glutaraldehyde (Fig. 1c). Unfortunately, it is not possible to quantitate this effect of tannic acid by viscometry because, even after the samples are dialyzed to remove free glutaraldehyde and tannic acid, a dense black precipitate forms upon the addition of OsO₄.

Tannic acid treatment also increases the electron density of actin filaments (Fig. 1c and d) as originally emphasized by LaFountain et al. (27). The extra density is due, in part, to the fact that glutaraldehyde-tannic acid-fixed filaments reduce four to five times more OsO₄ than native or glutaraldehyde-fixed actin filaments (data not shown). In addition, tannic acid itself must contribute part of the density, because it increases density even when applied after OsO₄ treatment (Fig. 1d). After exposure to tannic acid, the diameter of embedded actin filaments is 7-10 nm depending on the concentration of tannic acid. This is larger than without tannic acid.

Introduction of Tannic Acid into Cells During Fixation

Using HeLa cells we confirmed the observation of LaFountain et al. (27) that many cells are penetrated by the tannic acid and have densely stained cytoplasmic fibers and membranes after fixation with a high concentration (80 mg/ml) of tannic acid and 400 mM glutaraldehyde in 100 mM cacodylate buffer. However, there is always some extraction of the cytoplasmic matrix in the penetrated cells. This might be considered an advantage for visualizing individual filaments, but cellular structure is altered by extraction. Very few cells are penetrated by tannic acid when 100 mM phosphate buffer, pH 7, is substituted for cacodylate buffer, so we suspect that penetration by tannic acid in cacodylate is attributable, at least in part, to damage to the plasma membrane in the presence of cacodylate.

When saponin is included in the tannic acid-glutaraldehyde fixative, the fraction of HeLa cells penetrated by tannic acid depends on the concentration of saponin, the duration of fixation, and the stage of the cell cycle. Cells penetrated by tannic acid are readily identified by light microscopy (Fig. 2a)

1 Although we used 40 mM OsO₄ in this study, we have subsequently determined that 4 mM OsO₄ is adequate. This lower OsO₄ concentration has the theoretical advantage that it is less likely to alter cellular structure by oxidizing proteins.

![Figure 1](https://example.com/image1.jpg)

**Figure 1** Thin sections of actin filament pellets fixed by different methods to illustrate effects of OsO₄ and of tannic acid. Small pellets of actin filaments were fixed as follows: (a) 100 mM glutaraldehyde, pH 7.0, for 30 min at room temperature followed by 4 mM OsO₄, pH 6.0, for 10 min at 2°C; (b) same glutaraldehyde treatment as (a) followed by 40 mM OsO₄, pH 7.0, for 1 h at room temperature; (c) 100 mM glutaraldehyde with 2 mg/ml tannic acid, pH 7.0, for 45 min at room temperature followed by 40 mM OsO₄, pH 6.0 for 20 min at room temperature; (d) 100 mM glutaraldehyde, pH 7.0, for 45 min at room temperature followed by the same OsO₄ treatment as (c) then by 2 mg/ml tannic acid, pH 6.0, for 10 min at room temperature. Buffer: (a and b) 50 mM or (c and d) 100 mM sodium phosphate, 50 mM KCl, 5 mM MgCl₂. All samples were dehydrated with ethanol and propylene oxide. The filament diameters were (SD <1, n 20 in each) a, 7 nm; b, 7 nm; c, 9 nm; d, 10 nm. × 90,000.
even after embedding, because they stain a deep brown. Likewise, penetrated cells have a much higher electron density than unpenetrated neighbors (Fig. 2b). For most of our work we used 100 mM glutaraldehyde, 0.5 mg/ml saponin, and 2 mg/ml tannic acid for 30 min, because this treatment provides a reasonable number of unpenetrated cells for comparison. When the saponin concentration is 0.1 mg/ml or less, very few cells are penetrated by tannic acid even after 30 min. At saponin concentrations ≥0.3 mg/ml, >90% of the cells are penetrated by tannic acid within 10 min. All cells are penetrated after treatment with 0.7 mg/ml saponin for 10 min. Two Cells in mitosis are penetrated at lower saponin concentrations than cells in interphase.

Cortical Actin Filaments

The actin filaments of HeLa cells are well preserved and densely stained after tannic acid-glutaraldehyde-saponin fixation. For example, the filopodia (Fig. 3a) of cells penetrated by tannic acid contain a regular bundle of ~19 (SD 2, n 30) straight actin filaments with diameters of 10 nm (SD < 1 nm, n 20) with occasional densities between the filaments suggesting cross-links. There is some ill-defined granular material between the filament bundle and the plasma membrane. The surrounding membrane is sometimes swollen away from the bundle of filaments, a distortion that probably occurs during fixation. Adjacent cells not penetrated by tannic acid have a lightly stained, irregular network of fine, branching 6-nm (SD 1 nm, n 15) filaments in their filopodia (Fig. 3e) that are similar to pure actin filaments exposed to OsO4 without prior treatment with tannic acid (Fig. 1b).

The cortex of HeLa cells contains largely random networks of straight, densely stained actin filaments (Fig. 3c). Many, perhaps most, of these cortical actin filaments are obscured by the surrounding amorphous components of the cytoplasmic matrix, but many can be identified, particularly if their paths are traced from near the plasma membrane or from bundles of actin filaments. Occasionally, small bundles of cortical actin filaments appear to attach end-on to the plasma membrane (Fig. 3c). In some cases, a small amount of dense amorphous material is associated with the attachment site. Morphologically similar attachment site specializations are seen in negatively stained cells (30, 47). In cells not penetrated by tannic acid, the stress fiber actin filaments are relatively intact (Fig. 3a), but cortical actin filaments are difficult to identify.

Actin filaments predominate in membrane ruffles (lamellipodia) at the margins of the cell (Fig. 3b). As in other parts of the cortex, these actin filaments are arranged in crisscrossed networks rather than in the regular bundles found in filopodia. In earlier electron micrographs of the lamellipodia (2, 11), the actin filaments were so poorly preserved after fixation and embedding that the matrix was described as "granular... with vague and irregular filaments" (2).

Stress Fibers

Stress fibers of HeLa cells fixed with tannic acid are composed of straight, roughly parallel thin filaments with diameters of 10 nm (Fig. 4). In cross sections (Fig. 5), it is clear that the thin filaments are not organized in a regular array but instead have variable center-to-center spacing (range 10–21 nm; mean ~19 nm, SD 3 nm, n 35). As a consequence of this tight packing, most of what appear to be individual actin filaments in routine longitudinal thin sections (60–75 nm thick; Fig. 4a–b) are actually two or more superimposed filaments as illustrated by the inset in Fig. 4b. Consequently, very thin sections such as Fig. 4c are necessary to visualize individual actin filaments in longitudinal sections. In such thin sections, it is seen that the individual filaments are generally aligned with the long axis of the fiber but may be skewed up to 35°.

Stress fibers stained with tannic acid have two different types of densities. One type consists of amorphous material between the actin filaments and has a rounded shape (Fig. 4). They are probably comparable to the stress fiber densities observed by Goldman et al. (19) in BHK-21 cells after fixation with tannic acid and glutaraldehyde. The other dense structure is a filament ~19 nm (SD 3 nm, n 15) wide and up to 250 nm long, which is best seen in very thin sections (Fig. 4c). In cross sections, it is difficult to identify with certainty but there are a number of filament 17 nm (SD 1 nm, n 15) in diameter among the 10-nm thin filaments (Fig. 5a and c). These thicker filaments are the same size as synthetic platelet myosin filaments (34).

Most of the stress fibers in HeLa cells are located near the basal plasma membrane oriented parallel to the substrate. A few stress fibers are located near the free surface (Fig. 5c) or course diagonally through the cytoplasm. For the most part, the stress fibers located near the plasma membrane do not have any structural specializations linking them to the adjacent membrane, and the membrane is not closely associated with the underlying substrate (Fig. 5).

Substrate Adhesion Sites

We find that HeLa cells have at least two morphologically distinct substrate adhesion sites: the classical adhesion plaques, called focal contacts, at the ends of stress fibers (1, 25), and adjacent plaques where the plasma membrane is coated with material which is morphologically identical to clathrin of coated pits and vesicles (Fig. 6). focal contacts: Stress fibers terminate on the plasma membrane at specialized adhesion plaques where the membrane is separated from the substrate by a uniform gap of 10 nm (SD 2 nm, n 25) (Fig. 6). The gap is bridged by fine strands ~6 nm wide (SD < 1 nm, n 15), spaced at 14-nm (SD 4 nm, n 15) intervals, with tannic acid (Fig. 1b).

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FIGURE 4  Electron micrographs of longitudinal sections of stress fibers. All are the first or second section parallel to the substrate. Note the stress fibers (SF) with dense areas (some circled), intermediate filament bundles (IFB), coated pits (CP), nuclear envelope (N), and filopodia. Individual thin filaments (width 10 nm) and thicker filaments (arrowheads, diameter 17 nm) can be seen in b and c. Insets in b and c are tracings of stress fiber cross sections showing the packing density of the actin filaments and the approximate thickness of these two sections to illustrate the extent of actin filament superimposition expected in these longitudinal sections. Section thickness: (b) silver gray, ~75 nm; (c) light gray, ~45 nm. a, × 5,200; b and c, × 56,000.

FIGURE 3  Electron micrographs of cortical actin filaments. (a) Longitudinal sections of filopodia sections showing the difference in preservation and staining of actin filaments in a cell that was penetrated by tannic acid and another that was not penetrated. Inset: a cross section of a filopodium stained with tannic acid showing the actin filament core with several fine fibers between it and the membrane. (b) Three ruffling membranes (lamellipodia) at the leading edge of a HeLa cell. The section is perpendicular to the growth substrate (thin line) and parallel to the long axis of the cell. All three membrane ruffles contain actin filament networks and are attached to the leading lamella in other planes of section. Insets: sequential sections illustrating longitudinal and cross sections of actin filaments and their relationship to the membrane. (c) A cell penetrated by tannic acid with many densely stained actin filaments in the cortex. Several filaments insert into the plasma membrane at two discrete foci (arrows). The majority of the cortical actin filaments are randomly arranged so that only short pieces or cross sections (arrowheads) are seen in a thin section. ▲ marks the bifurcation of a small actin filament bundle. (d) A cell that was not penetrated by tannic acid from the same dish as c. Actin filaments are distinguishable in the stress fiber (SF), but only indistinct microfilament networks are present elsewhere in the cortex. g, glycogen; r, ribosome. a, × 73,000; b, × 26,000; insets, × 62,000; c and d, 67,000.
15) intervals. In conventional thin sections (Fig. 6d–g), the actin filaments on the cytoplasmic surface of the plaque membrane appear to be embedded in amorphous dense material which is attributable, at least in part, to the high concentration of the actin filaments themselves, because in thinner sections (Fig. 6 h and i) only actin filaments or other fibers of similar size and staining properties are visible.

**Coated Membrane Plaques:** The second type of close association of the plasma membrane with the underlying substrate occurs in regions where the cytoplasmic surface of the membrane is coated exactly like coated pits and vesicles (Fig. 6). Tannic acid increases the electron density of these coats so that these membranes are readily distinguished from uncoated membranes. The extracellular surface of the coated membrane plaques is closely apposed to the substrate. The 12-nm (SD 2 nm, n 25) gap between the membrane surface and the substrate is usually spanned by projections about 8 nm (SD <1 nm, n 10) wide (Fig. 6a–c and g–i). The cytoplasmic surface of the coated membrane has two layers. Directly associated with the membrane is a very dense amorphous layer 12 nm (SD <1 nm, n 10) thick. Extending into the cytoplasm from the dense layer at regular intervals of 28 nm (SD 1 nm, n 20) are projections 9 nm (SD <1 nm, n 25) long and 16 nm (SD 3 nm, n 15) wide. Sections parallel to the substrate show that these projections are elements of extended arrays of polygons with center-to-center spacing of 28 nm (SD <1 nm, n 25). Most of the polygons are hexagons, but a few of them are pentagons. Frequently, there is an electron-dense spot in the center of the polygon. These coated membrane plaques are most frequently located adjacent to stress fibers (Fig. 7a and b), especially near the focal contacts. The largest plaques consist of >1,000 polygons and altogether they cover substantial areas of the basal plasma membrane.

**Other Coated Membranes**

The flat membrane plaques are continuous with coated pits with a mean radius of curvature of 54 nm (SD 18 nm, n 10), an amorphous dense layer, and projections spaced somewhat closer together (23 nm, SD 3 nm, n 15) than in the flat areas. One section parallel with the substrate can provide views of both the amorphous and polygonal layers of the coat when it grases the top of one of the pits (Fig. 7d).

Coated vesicles with the same amorphous layer and surface projections are found throughout the cytoplasm of HeLa cells. Those >1 μm from the plasma membrane are seen in serial sections to be separated from the plasma membrane.

**Intermediate Filaments**

The tannic acid procedure stains intermediate filaments very densely and, as a consequence, small bundles of 5–40 filaments stand out clearly in both longitudinal (Fig. 4a) and cross sections (Fig. 5a). After tannic acid staining, the intermediate filaments are 16 nm (SD 1 nm, n 25) in diameter and their average center-to-center spacing in small bundles is 21 nm (SD 3 nm, n 65). The intermediate filament bundles are usually located slightly deeper in the cytoplasm than the majority of the actin filaments and are concentrated around the nucleus (Fig. 5a).

The intermediate filament bundles are associated with the plasma membrane at structures similar to hemidesmosomes, which stain intensely with tannic acid (Fig. 8). The hemidesmosomes are found on all surfaces of the cell. On the cytoplasmic surface of the membrane is a very dense plaque ~24 nm (SD 2 nm, n 10) thick and 130–190 nm wide. Interior to the plaque is a bundle of intermediate filaments ~153 nm (SD 21 nm, n 20) in diameter. The individual intermediate filaments

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**Figure 5** Electron micrographs of cross sections of stress fibers. The cells in a and c were fixed by the standard method. The cell in b was fixed with 400 mM glutaraldehyde, 80 mg/ml tannic acid, 100 mM cacodylate buffer, pH 7.2. Stress fibers are bracketed. Thin filament diameters are 10 nm in a and c. The higher concentration of tannic acid used in b increases the thin filament diameter compared with a and c. Cross sections of some thicker filaments (diameter 17 nm) are indicated with arrowheads. IFB, intermediate filament bundle; CV, coated vesicle; NP, nuclear pore. Magnifications: a and b, × 56,000; c × 122,000.
in these bundles are difficult to resolve, even in very thin sections parallel (Fig. 8 g and h) or perpendicular (Fig. 8 a-f) to the bundle, because the filaments are tightly packed and embedded in electron-dense material. The area of contact between the filament bundle and the plaque is very limited, because only one or two sections in a series show any connection. On the extracellular surface of the plaque membrane are irregularly spaced projections 11 nm (SD < 1 nm, n 10) long, but these hemidesmosomes rarely make contact with the substrate (Fig. 8). Therefore, it is not likely that the intermediate filament system of HeLa cells is involved with anchorage to the substrate.

**FIGURE 6** Electron micrographs of cross sections of two types of adhesion plaques. Cells were fixed by the standard method except for d, which was prepared with a high concentration of tannic acid as in Fig. 5 (b). The thin dense line beneath the cell is the substrate. Some adhesion plaques are associated with stress fibers and have a fairly uniform gap of 10 nm between the plasma membrane and the substrate. These “focal contacts” are indicated with ▲▲. Other plaques have a distinct electron dense coat on the cytoplasmic surface of the plasma membrane and are separated from the substrate by a gap of 12 nm. These coated membrane plaques are indicated with △△. Micrographs (g-i) illustrate both types of plaques in three consecutive serial sections ranging in thickness from (g) ~75 nm (silver-gray) to (i) ~35 nm (very dark gray). Amorphous densities in the stress fiber are noted with arrows. CP, coated pit; U, unilamellar membrane vesicle. a-f, × 78,000; g-i, × 70,000.
Tannic Acid Staining of Other Cellular Structures

The effects of tannic acid staining on the structure and electron density of various cellular components can be appreciated by comparing penetrated with unpenetrated neighboring cells, especially in very thin sections where superimposition is minimal (Fig. 9). Most of the cytoplasmic matrix consists of densely stained fibrogranular material (Figs. 3c and 9d). The concentration of this matrix material varies among cell types. For example, in HeLa cells its concentration is low enough to allow one to identify the matrix filaments, whereas in hepatocytes its concentration is so high that no details of matrix structure can be identified. Free ribosomes and glycogen particles are identifiable on the basis of their size and shape (Fig. 3c and d). Plasma membranes are 12 nm (SD 2 nm, n 30) thick and densely stained on both surfaces, which allows resolution of the three layers. This is difficult or impossible with conventional fixation and staining methods. Tannic acid also brings out the trilaminar appearance of most internal membranes including those of the endoplasmic reticulum, Golgi apparatus, and nuclear envelope (all 7-nm thick, SD <1, n 30). Exceptions are the mitochondrial membranes, in which only the outer leaflet is densely stained, and a considerable number of vesicles 70–100 nm in diameter that are bounded by a "membrane" that appears as a single dense line (5 nm thick, SD <1, n 25).
DISCUSSION

Of the various methods used to study cellular architecture, thin sections of fixed, embedded cells have suffered from poor preservation and low contrast of cytoplasmic matrix elements. The tannic acid-glutaraldehyde-saponin fixation remedies some of these problems. Actin filaments are preserved better than with conventional fixatives, and the dense staining of membranes, membrane coats, filaments, and other elements of the cytoplasmic matrix allows one to visualize fine details even in very thin sections. For introducing tannic acid during fixation of cultured cells, this saponin-glutaraldehyde method is superior to OsO₄ vapor pretreatment (39), which is less consistent and causes some extraction of the cytoplasmic matrix.

Compared with whole cell mounts and replicas of extracted cells, thin sections do not provide easy access to three-dimensional interrelationships in the cytoplasmic matrix, but sections make it possible to visualize details in dense structures such as stress fibers and membrane coats, which are obscured in the other specimens. Furthermore, extraction is not necessary before fixing and embedding. As emphasized by Buckley (13), detergent extraction of soluble constituents results in the loss of some and the disorganization of other cytoplasmic actin filaments. Our fixation procedure appears to preserve and stain most of the components of the cytoplasmic matrix including the ground substance. This material is extracted in most replicating and negative-staining procedures and is mysteriously absent after critical point drying intact cells.

The advantages of tannic acid-stained cells make thin sections a valuable addition to the other methods for preparing cells for electron microscopy. None of the methods is without potential artifacts, so we will compare them in the following sections. Consistent results from several methods provide confidence that an accurate picture of the cell has been obtained.

**Morphology of Actin Filaments in the Intact Cell**

Cytoplasmic actin filaments fixed by our new method are long, straight, and constant in diameter. They are similar to the cytoplasmic actin filaments in lysed cells prepared for electron microscopy by rapid freezing, deep etching and shadowing (22); by freeze-drying and shadowing (9); by chemical fixation and critical point drying (44); and by negative staining (30, 47). The actin filaments in all of these preparations are different from the microtrabeculae of critical-point-dried whole mounts (54), but are similar to filaments in critical-point-dried whole mounts in which extra caution was taken to remove all traces of water before drying (40).

Cortical filaments seem to be the most damaged during conventional fixation and embedding. This gave rise to earlier descriptions of microfilament networks in the cortex (55) and ruffling membranes (2, 11) of cultured cells. Although it is true that the cortex has a network of actin filaments, our work and that of Small (47) show that the network consists of relatively long straight filaments of uniform width, not the branched, irregular fibers that comprise the microfilament networks observed after conventional fixation. We suggest that the tannic acid-glutaraldehyde-saponin fixation is capable of preserving, to a degree not possible with earlier fixation-embedding methods, the natural structure of individual actin filaments in the cytoplasm of HeLa cells.

This new fixation method also provides the best available picture in thin sections of actin filament assemblies such as stress fibers, filopodia, ruffling membranes, and cortical net-
works. In each case the arrangement in our sectioned material is most similar to that seen in replicas of extracted, quick frozen, etched cells (22), but some new features are revealed in sections of intact cells. In stress fibers there are two types of dense material in addition to the actin filaments. One is the stress fiber dense body (19, 43) thought to contain alpha-actinin (28). The other can only be appreciated in very thin sections and seems to be a short filament similar in size to platelet myosin filaments (34). These myosin-like filaments may be responsible for the staining of some stress fiber densities by ferritin-labeled anti-myosin (20).

**Cytoplasmic Ground Substance**

The fibrous elements are not the major component of the cytoplasmic matrix of HeLa cells. Rather, it is a poorly characterized fibrogranular material that is continuous throughout the matrix. A similar cytoplasmic ground substance is preserved by rapid freezing, etching, and shadowing of intact cells (22), but is absent in critical-point-dried whole mounts (54), in which it may become associated with the fibrous elements during fixation, dehydration, or drying. In life this ground substance is probably a solution, because a large fraction of total cellular proteins salts and metabolites are soluble after gentle cell lysis (18) and because the diffusion coefficient of small protein molecules is only slightly less in the cytoplasm than in aqueous solution (53). The gel-like consistency of cytoplasm is attributable to the fibrous elements which form a continuous network in the cytoplasm (48). As illustrated here, the spaces between the filaments and microtubules are filled with amorphous ground substance. These pores are large enough for small molecules to diffuse more or less freely, but small enough to restrict the movement of larger particles such as organelles.

**Stress Fiber Attachment Plaques**

Considering the recent interest in the localization of alpha-actinin, vinculin, and fibronectin on either side of the plasma membrane at the points where stress fibers are anchored to the membrane (7, 15, 46), very little is known about the fine structure of these plaques. In earlier thin section studies the preservation was not adequate to reveal much beyond the presence of actin filaments (2, 8, 37). In critical-point-dried whole mounts no details are visible at these sites and no work has been reported with frozen, etched replicas.

We find that the membrane of HeLa focal contacts is separated from the substrate by a fairly uniform gap of 10 nm that is spanned by small projections that may be the cell-substrate attachment molecules. The membrane itself appears no different from other parts of the plasma membrane. In particular, it does not have a conspicuous electron-dense coat on its cytoplasmic surface comparable to the dense plaque where actin filaments insert at the tips of microvilli (33). The cytoplasmic surface of the membrane is dense, but this is due largely to the high concentration of actin filaments, which may be accompanied by other fibrous material.

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**Figure 9** Electron micrographs comparing an unpenetrated cell (a) with cells penetrated by tannic acid (b, c and d). Sections a, b, and d were bright gray (~60 nm thick). Section c was dark gray (~35 nm thick). CM, coated membrane; ER, endoplasmic reticulum; G, Golgi apparatus; I FB, intermediate filament bundle; L, lipid droplet; M, mitochondria; U, unilamellar membrane vesicle; n, 11-nm nuclear granule; NP, nuclear pore; RNP, ribonucleoprotein particle; \( \), stress fiber; \( \bigtriangleup \), coated membrane; \( \bigtriangledown \), 11-nm chromatin filament; \(< >\), 23 nm chromatin fiber. \( \times 55,000.\)
Coated Membranes

The increased density of the membrane coats after fixation in tannic acid makes it possible to identify these coats with ease and to discern new structural features. The polygonal nature of the coat has always been very clear by negative staining (26), but the appearance of the coat in thin sections varies with the fixation method (5, 23, 41). The major component of the polygonal coat is a protein called clathrin (36) that can self-assemble into triskelions and polygonal baskets (17, 51). Rotary shadowing preserves beautifully the polygonal clathrin structure on coated pits and vesicles (21) and also reveals small flat sheets of clathrin hexagons on the cytoplasmic surface of the plasma membrane of disrupted cells (3, 21). In membrane coats there is a 12-nm thick, electron-dense layer between the membrane and the clathrin basket work, that has not been observed without tannic acid. The composition of this amorphous layer is not known, but it could very well include the 100,000 mol wt coated vesicle polyepitide that is necessary for binding clathrin to the membrane (50). This amorphous layer may be the reason Heuser (21) found no evidence in replicas for a basket work on coated pits viewed from outside Triton-extracted cells. Likewise, the 19-nm thickness of the "membrane" of cross fractured and replicated coated pits (21) is accounted for by the combined thickness of the membrane and the amorphous layer of the coat.

The coated membrane plaques in HeLa cells are considerably larger than the small sheets of clathrin observed in other cells (3, 21). The large sheets might be peculiar to HeLa cells, but they could have been missed in previous studies, particularly those with fluorescent anticalthin (6). The high contrast in fluorescent microscopy can give the false impression of large differences in the fluorescence of intensity where the actual difference is only a factor of four or five. See Herman and Pollard (20) for such an example. In the case of clathrin sheets and coated pits observed normal to the sheets, the local concentration of clathrin is easily five times higher in the pits than in the sheets, so the sheets could be missed unless the film is overexposed with respect to the pits and vesicles. The existence of large sheets with multiple internal pits puts specializations, and cytoplasmic filaments of cultured cells. Z

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