Organization of Actin in the Leading Edge of Cultured Cells: Influence of Osmium Tetroxide and Dehydration on the Ultrastructure of Actin Meshworks

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ABSTRACT The ordered structure of the leading edge (lamellipodium) of cultured fibroblasts is readily revealed in cells extracted briefly in Triton X-100-glutaraldehyde mixtures, fixed further in glutaraldehyde, and then negatively stained for electron microscopy. By this procedure, the leading edge regions show a highly organised, three-dimensional network of actin filaments together with variable numbers of radiating actin filament bundles or microspikes. The use of Phalloidin after glutaraldehyde fixation resulted in a marginal improvement in filament order.

Processing of the cytoskeletons through the additional steps generally employed for conventional electron microscopy resulted in a marked deterioration or complete disruption of the order of the actin filament networks. In contrast, the actin filaments of the stress fiber bundles were essentially unaffected. Thus, postfixation in osmium tetroxide (1% for 7 min at room temperature) transformed the networks to a reticulum of kinked fibers, resembling those produced by the exposure of muscle F-actin to OsO_4 in vitro (P. Maupin-Szamier and T. D. Pollard. 1978. *J. Cell Biol.* 77:837–852). While limited exposure to OsO_4 (0.2% for 20 min at 0°C) obviated this destruction, dehydration in acetone or ethanol, with or without post-osmication, caused a further and unavoidable disordering and aggregation of the meshwork filaments. The meshwork regions of the leading edge then showed a striking resemblance to the networks hitherto described in critical point-dried preparations of cultured cells. I conclude that much of the "microtrabecular lattice" described by Wolosewick and Porter (1979. *J. Cell Biol.* 82:114–139) in the latter preparations constitutes actin meshworks and actin filament arrays, with their associated components, that have been distorted and aggregated by the preparative procedures employed.

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While the presence of actin in the ordered bundles, or "stress fibers," of cultured cells has been exhaustively documented (8, 12, 22) the other organizational states of this major cellular protein have been less well defined. This has been largely due to the lack of adequate procedures to preserve actin filaments in their more transitory, less stabilized assemblies within the cell. For substrate-attached cultured cells, an example of such an assemblage of actin can be found in the motile and normally convex-shaped regions of the cell periphery generally referred to as the "leading edge" or lamellipodium (1, 21). Earlier electron microscopy studies of embedded material had indicated the presence of a net of "microfilaments" in such regions (8, 35) but, because of the lack of structural order, little insight could be gained into the role played by the filaments in cell motility.

In the course of investigations of the organization of cytoplasmic filaments within cultured cells, I and my colleagues (32, 33, 34), as well as others (7, 18, 27), have developed techniques using detergent extraction to visualize the intracellular filament systems directly in the electron microscope. We were able to demonstrate that under appropriate conditions, namely using a balanced salt solution buffered at low pH (close to 6.0) and with added EGTA and Mg^{2+}, a well-defined network of actin filaments is preserved within the region corresponding to the leading edge. The breadth and contours of the meshwork correlated with images obtained in the fluorescence microscope of a band of material at the leading edge reacting with antibodies to actin (see e.g., reference 23). In

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further studies aimed at elucidating the mechanism of locomotion of vertebrate cells, we have been analysing the structure and composition of the leading edge region in more detail. At the same time, it has become apparent that the adequate preservation of the complex assemblies of actin in this region are very susceptible to the preparative procedures employed. In particular, we were concerned that in cells prepared by the critical point–drying procedure little trace remained of the highly ordered networks seen after detergent extraction, glutaraldehyde fixation, and negative staining.

By analysing the structure of the leading edge after each of the steps normally used before critical point–drying, or indeed before conventional plastic embedding, we have been able to show where the distortions arise. The data, which confirm and extend the findings of Maupin-Szamier and Pollard (25) on purified muscle F-actin, show that osmium tetroxide as a postfixative has a primary destructive effect on actin within the leading edge, even in the presence of actin accessory proteins such as filamin and α-actinin. The mushroom toxin Phalloidin (39), used after glutaraldehyde fixation, produced a marginal improvement in the order of the negatively stained actin meshworks and also provided partial stabilisation against osmium tetroxide. However, further and unavoidable aggregation and distortion resulted from dehydration in organic solvents and was thus always reflected in the critical point–dried preparations. In short, ordered actin networks as seen after glutaraldehyde fixation were converted, after dehydration and critical point–drying, into irregular fibrous lattices reminiscent of the "microtrabecular lattice" described by Wolosewick and Porter (41).

MATERIALS AND METHODS

Cells

Embryonic chick fibroblasts were obtained by trypsinisation of hearts from 14- to 16-d embryos and grown at 37°C in Earle's minimal essential medium containing 10% fetal calf serum and in the presence of 5% CO_2. For electron microscopy, the cells, taken after the second to fourth plating, were grown on Parlodion-carbon or Formvar-coated silver or gold hexagonal support grids (150-mesh; Teepe Brandsma, Holland) and used after 12–48 h.

Preparation of Triton X-100-Glutaraldehyde Cytoskeletons

Cytoskeletons were prepared using mixtures of Triton X-100 and glutaraldehyde (18) in a buffer system described previously (32) and to which we shall refer as "cytoskeleton buffer." This buffer corresponds to a Hanks balanced salt solution lacking Ca^{2+}, with added Mg and EGTA and with the pH reduced to ~6.1. The components were: NaCl, 137 mM; KCl, 5 mM; Na_2HPO_4, 1.1 mM; KH_2PO_4, 0.4 mM; $NaHCO_3$, 4 mM; glucose, 5.5 mM; $MgCl_2$, 2 mM; EGTA, 2 mM; PIPES, 5 mM; pH 6.0–6.1.

For this study, the Triton X-100-glutaraldehyde mixture consisted of 0.5% Triton X-100 and 0.25% glutaraldehyde. Extraction-fixation was carried out with room temperature solutions in the following way: The grids carrying the cells were removed from the culture tube, washed briefly in Tris-buffered saline (TBS), followed by cytoskeleton buffer, and then transferred to the Triton X-100-glutaraldehyde mixture for 1 min. After a brief wash in cytoskeleton buffer the grids were stored on the cover slips in the same buffer containing 2.5% glutaraldehyde, before negative staining or further processing for electron microscopy. In some cases, a passage through 2×10^{-5} M Phalloidin (a gift from Th. Wieland, Max Planck Institute) in the same buffer (2 min) preceded the second fixation in 2.5% glutaraldehyde.

OsO_4 Postfixation and Dehydration

To test the effect of OsO_4, glutaraldehyde-fixed cytoskeletons were prepared as described above and then treated with 0.5% Triton X-100 in cytoskeleton buffer for 15 min at room temperature. This additional Triton X-100 treatment was found necessary to allow final penetration of the negative stain after OsO_4; it had no visible effect on the order of filaments seen after glutaraldehyde fixation. Postfixation was then performed in 1% OsO_4 in 0.1 M sodium cacodylate buffer for 5–15 min at room temperature or in 0.2–1% OsO_4 in 0.1 M sodium phosphate-buffer, pH 6.3 (2, 25, 36), for 20 min at 0°C. After washing in cytoskeleton buffer, negative staining was performed in 1% aqueous uranyl acetate, as described below.

The dehydration of cytoskeletons fixed as described above was performed with Formvar-coated grids using a graded series, 15–100%, of alcohol or acetone and a total passage time of 10–20 min at room temperature. The grids were then negatively stained in 1% uranyl acetate in 100% methanol or, alternatively, rehydrated through the same series and stained in aqueous uranyl acetate.

Negative Staining and Critical Point–drying

Negative staining with uranyl acetate was carried out either in the cold using a 1% aqueous solution, as described previously (32), or at room temperature, using a solution of 1.0% uranyl acetate in 100% methanol. For the latter staining, grids were immersed in the stain solution for 30 s, drained of excess stain with filter paper, and air-dried.

Staining in sodium silicotungstate (18, 38) was carried out at room temperature as follows: Grids were rinsed two times in water and transferred sequentially through 4 drops of bacitracin (40 μg/ml in water; Sigma Chemical Co., St. Louis, Mo.; 13) in a plastic petri dish and drained briefly on the edge with filter paper; they were then passed through 4 drops of 3% sodium silicotungstate and finally drained of excess stain and allowed to dry. A stain combination was also employed which involved positive staining (immersion) in 1% aqueous uranyl acetate followed by rinsing in water and negative staining in sodium silicotungstate.

Critical point–drying in CO_2 was carried out after rapid dehydration in acetone in a Balzers critical point-drying apparatus (Balzers, Liechtenstein).

RESULTS

Organization of the Leading Edge after Glutaraldehyde Fixation

The general appearance of a heart fibroblast extracted in a mixture of 0.05% Triton X-100 and 0.25% glutaraldehyde and negatively stained in aqueous uranyl acetate is shown in Fig. 1 *a*. Fig. 1 *b* shows a cell extracted in the same way but processed through acetone and critical point–dried. The broad band occurring at the convex region of the cells and corresponding to the leading edge is clearly defined and is shown at higher magnification in Fig. 1 *c*. As shown previously (32, 33) this band characteristically exhibits a criss-cross network of actin filaments together with variable numbers of radiating filament bundles, or microspikes.

The organization of the filaments as seen with two different staining procedures is shown in Figs. 2 *a* and *b*. The micrographs have been chosen to show the dense packing of actin filaments within the filament meshworks as well as the nature of the microspike bundles. Filaments within the meshwork commonly converge in groups onto sites containing extra material at the cell periphery (see also references 18, 33, 34) and are, in other situations, seen to merge into the microspike bundles of actin filaments (Figs. 2 *a* and 3 *a* and *b*). This arrangement of filaments is seen not only in the leading edge of heart fibroblasts but is characteristic for the corresponding regions in various other cultured cell types so far investigated (18, 31–33). The images suggest that the microspikes form via a coalescence of the meshwork filaments and that the reverse process, the divergence of bundles into meshworks, may also occur. The diameter of the filament bundles ranges from ~0.1 μm to 0.24 μm and the number of visualized actin filaments within them from ~13 to 30. A better idea of the total number of filaments in the bundles may be obtained in situations where the bundles splay apart at their base, towards the rear of the leading edge; in these regions, up to as many as 40 filaments may be counted.

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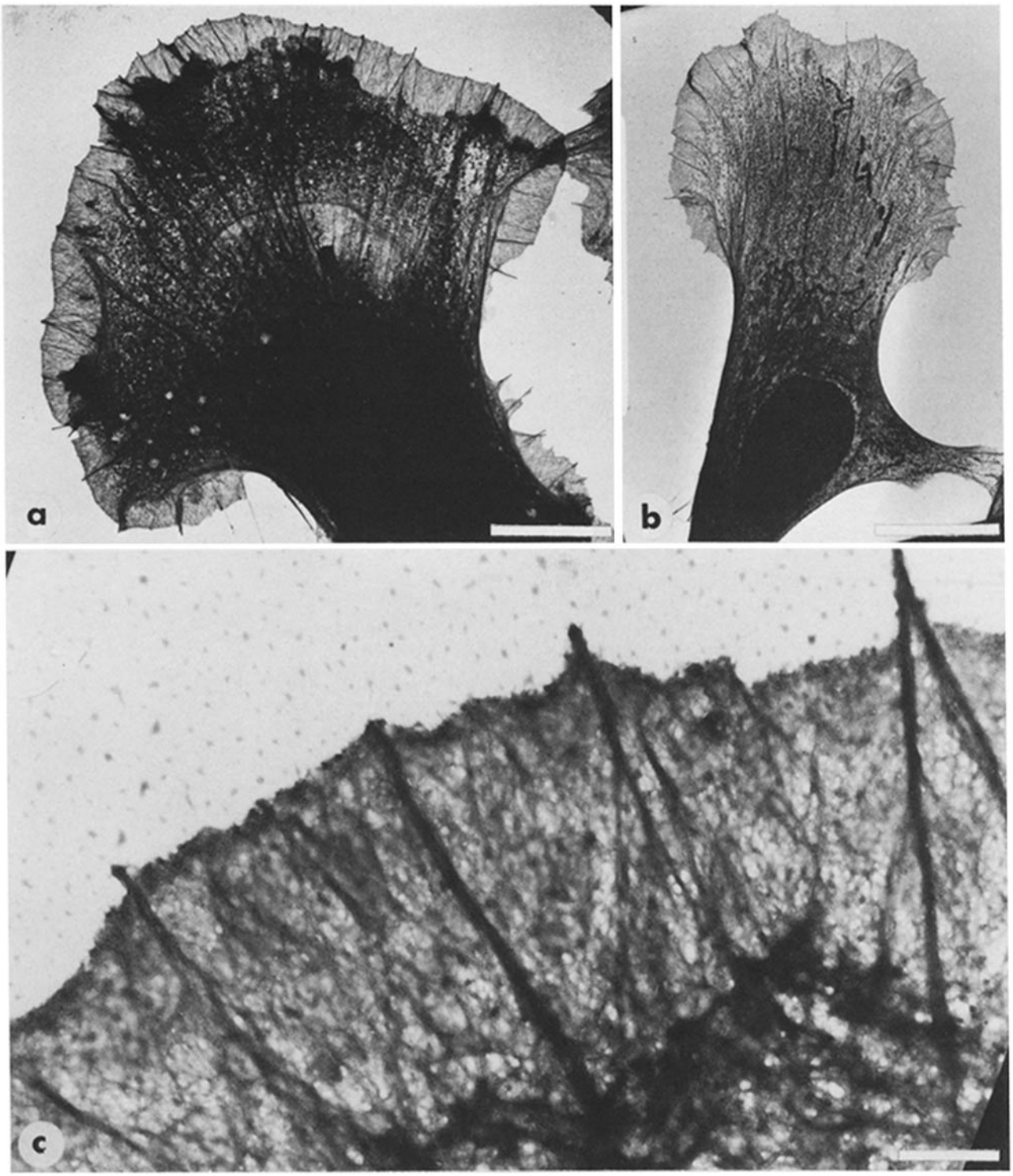

FIGURE 1 Survey views of Triton X-100-glutaraldehyde cytoskeletons of chick heart fibroblasts: *a* and *c*, negatively stained with aqueous uranyl acetate; *b*, dried by the critical point procedure after dehydration in acetone. The higher magnification of the leading edge region in *c* shows the general appearance of the radiating actin filament bundles (microspikes) and the intervening actin meshwork. Bars, *a* and *b*, 10 μm; *c*, 1 μm. *a* and *b*, × 2,100; *c*, × 17,000.

Cytoskeletons treated after the initial Triton X-100-glutaraldehyde mixture with phalloidin (in cytoskeleton buffer) consistently exhibited particularly well-ordered filament networks in the leading edge (Figs. 2*a* and 3*a* and *b*), suggesting that this toxin may partly stabilize the actin filaments against any disordering that occurs during drying of the stain. That phalloidin binds to the glutaraldehyde-fixed cytoskeletons was shown by separate experiments (34) using fluorescently-labeled phalloidin (43).

The observation of stereo pairs demonstrated the existence of a considerable degree of three-dimensional order in the negatively stained preparations (Fig. 3*a* and *b*). Within the meshwork regions the filaments could be seen to be extensively interconnected and extra globular material was present on the

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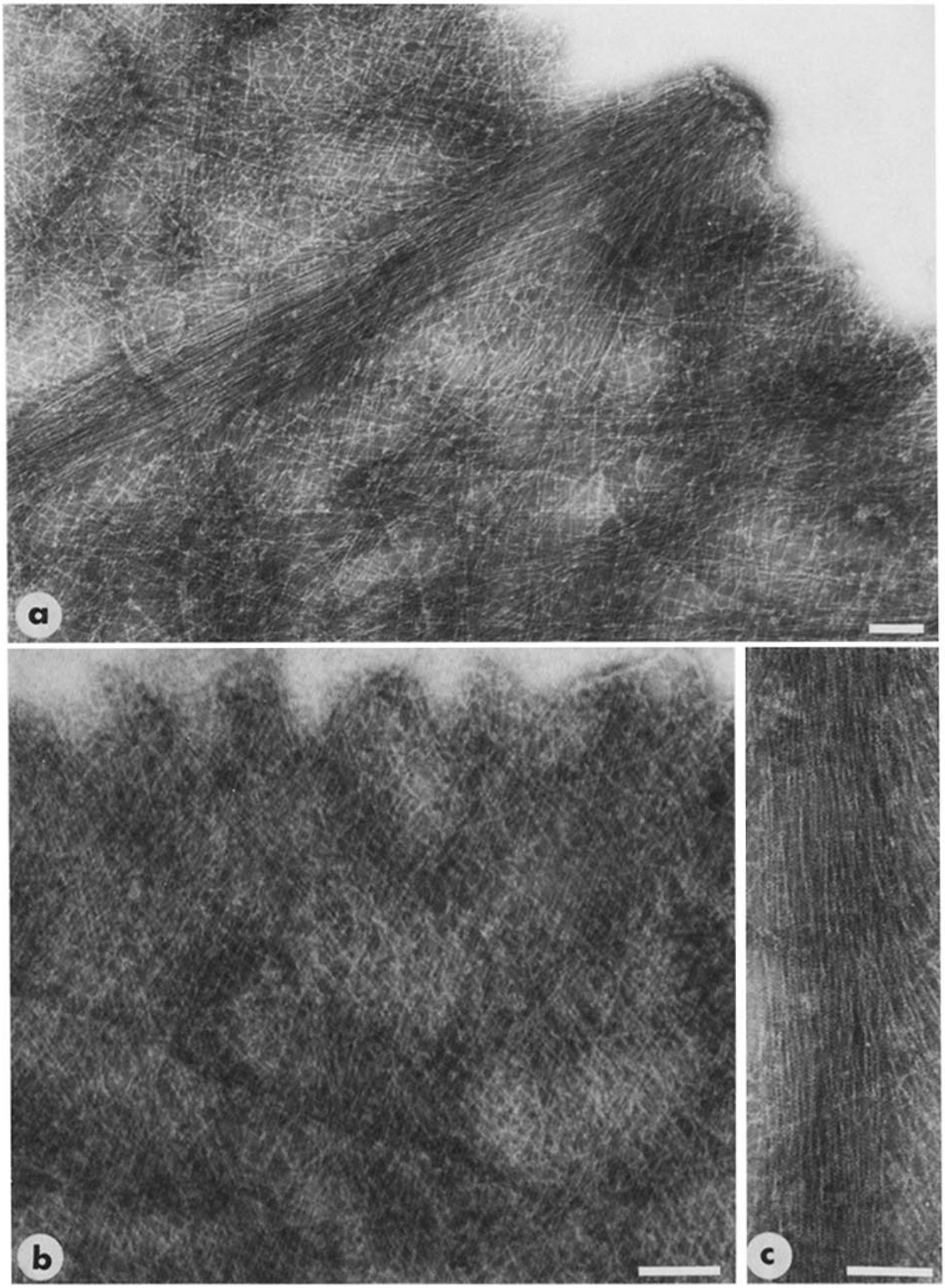

FIGURE 2 Details of the leading edge region of negatively stained Triton X-100-glutaraldehyde cytoskeletons: *a*, stained with sodium silicotungstate (cytoskeleton post-treated with phalloidin—see Materials and Methods); *b*, positively stained with aqueous uranyl acetate and then negatively stained with sodium solicotungstate; *c*, enlargement of microspike in cell prepared as for *a* showing characteristic substructure of F-actin. Note abundance of filaments within the meshwork and the microspike bundles and, in *b*, the convergence of the meshwork filaments at foci situated at the cell front. Bars, all 0.1 μm. *a*, × 96,000; *b*, × 142,000; *c*, × 150,000.

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filaments and often located at apparent junction sites. In favorable situations the helical substructure of actin was also evident, being most clear in the microspike bundles (Fig. 2 *c*).

Fig. 3 *c* has been included to illustrate the appearance of the other filaments, the microtubules, and 10-nm filaments that occur with actin in regions behind the leading edge and gen-

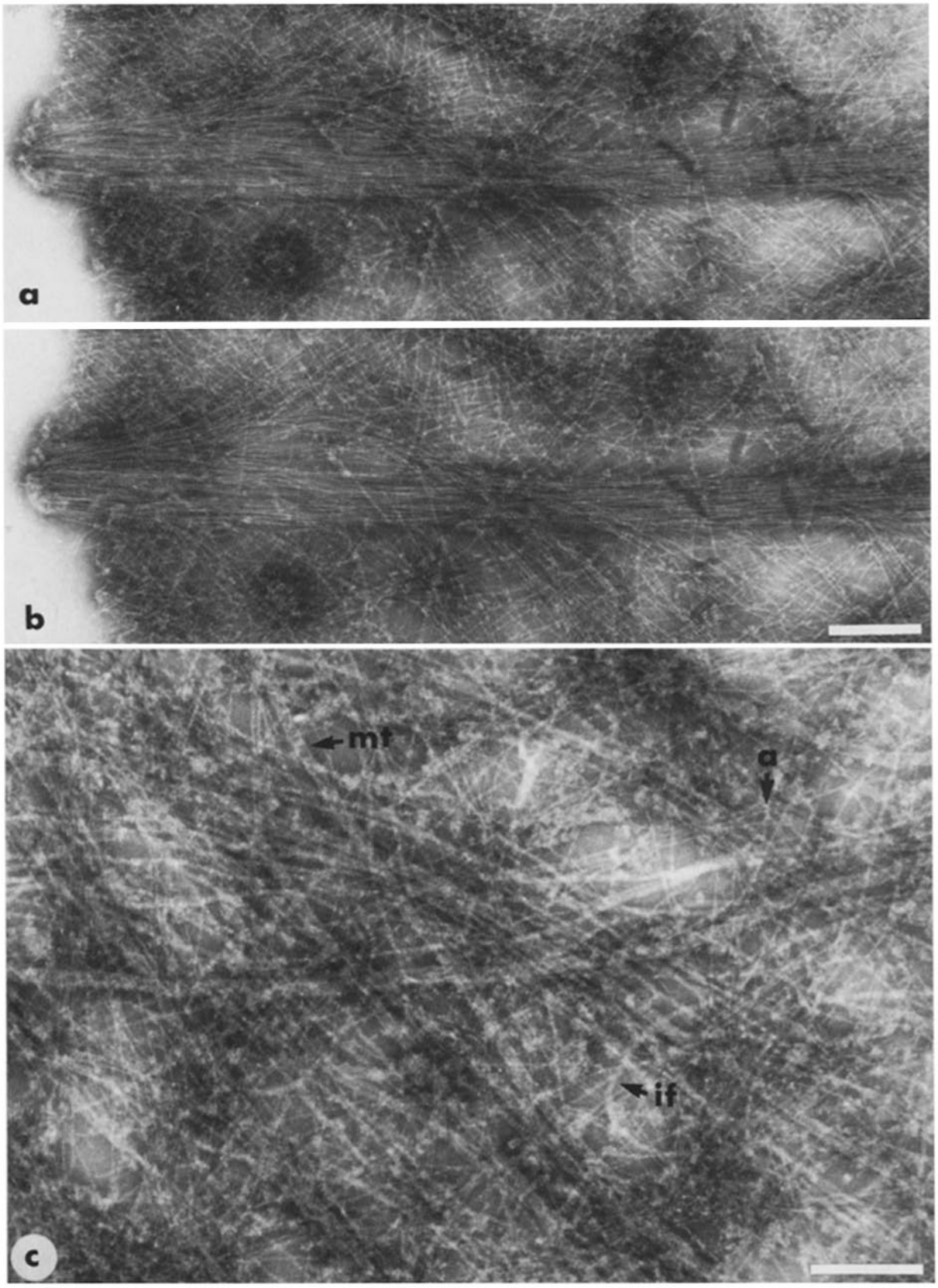

FIGURE 3 *a* and *b*, stereo pair of leading edge region (prepared as Fig. 2 *a*) illustrating the three-dimensional nature of the actin meshworks and microspikes. Bar: 0, 2 μm. × 80,000. *c*, inner region of cell prepared as Fig. 2 *a* showing the occurrence and general arrangement of actin filaments (*a*), intermediate filaments (*if*), and microtubules (*mt*). Bar, 0.2 μm. × 95,000.

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erally throughout the remaining cytoplasm. The penetration of some microtubules into the leading edge and occasionally along the filament bundles was also observed (data not shown).

Effect of OsO_4 Postfixation

After postfixation of glutaraldehyde-fixed cytoskeletons in 1% OsO_4 for 7 min at room temperature and negative staining in aqueous uranyl acetate, little trace of the regular organization of filaments within the leading edge remained (Fig. 4*a*). Rather than appearing as a criss-crossing net of linear filaments, the leading edge region presented itself more as a reticulum of short irregular fibers. This appearance of the net could be attributed mainly to a kinking of the filaments, recognised most easily in the filament bundles (microspikes) within the meshwork. As might have been expected from earlier electron microscope studies of section material (8, 35), such a kinking effect was not evident in the filaments of the stress fibers (Fig. 4*c*).

After the earlier reports which indicated that brief OsO_4 treatment in the cold is less damaging to F-actin (2, 25, 36), we determined the limiting concentration of OsO_4, used for 20 min at 0°C, that leads to visible morphological changes in actin at the leading edge. With postfixation in 0.2% OsO_4, actin within the meshwork as well as in the microspikes appeared essentially as in the glutaraldehyde-fixed controls except for an apparent change in contrast (Fig. 4*b*). Increasing the OsO_4 concentration above ~0.4% produced a noticeable distortion of actin and with 0.8% OsO_4 this distortion was nearly as extensive as with 1% OsO_4 used at room temperature.

Since previous studies have indicated the absence of tropomyosin in the leading edge (23) as well as the stabilizing effect, under certain conditions, of this protein against the destruction of muscle actin by OsO_4 (25), it was of interest to test to what extent tropomyosin may stabilize the leading edge filaments against OsO_4 postfixation. For these experiments muscle tropomyosin was added to unfixed cytoskeletons, suitably produced by extraction of the living cells in 0.05% Triton X-100 in cytoskeleton buffer for 30 s. The incubation of these cytoskeletons in 1 mg/ml skeletal muscle tropomyosin for 2 min (room temperature, cytoskeleton buffer), followed by washing in buffer and processing in the Triton X-100-glutaraldehyde mixture for 40 s, gave leading-edge regions in which the actin filaments of the meshwork were well preserved and defined with particular clarity. However, in preparations postfixed with OsO_4 (1% for 10 min at room temperature), the network was disrupted and resembled that seen with the preparations not treated with tropomyosin.

Effects of Dehydration and Critical Point–drying

To trace the changes that occurred during the dehydration step, negative staining following acetone or alcohol dehydration was performed using a dehydrating agent as solvent for the stain. Tests showed methanol to be rather suitable in this respect; uranyl acetate is readily solubilised in this solvent and the resulting staining was adequate and reproducible.

Fig. 5*a* shows the result obtained with cells extracted with the Triton X-100-glutaraldehyde mixture, fixed in 2.5% glutaraldehyde for 15 min, dehydrated in acetone, and stained in methanol–uranyl acetate. A noticeable distortion in the filament net has occurred; the filaments are more variable in thickness (measured range of diameters 6.8–17.0 nm) and relatively few filaments may be counted in the microspike bundles. The circular inset in this figure shows, for comparison, the dimensions of the "intact" network, as seen in the cytoskeletons, after glutaraldehyde fixation. In an alternative approach, dehydrated specimens were rehydrated and stained in aqueous uranyl acetate (Fig. 5*b*). Again, a considerable rearrangement and aggregation of the actin filament net was evident. In this example (Fig. 5*b*) the cytoskeleton was fixed in glutaraldehyde followed by OsO_4 (1%, 7 min, room temperature) before dehydration. For cytoskeletons fixed in glutaraldehyde alone, the effect was similar but with the result that the fibers were somewhat more regular in diameter with fewer constrictions. Phalloidin, used after Triton X-100-glutaraldehyde treatment, did not protect the actin meshworks against these distortions arising from dehydration, although a somewhat lesser aggregation of the meshwork filaments was apparent (Fig. 5*c*).

Leading edge regions of Triton X-100-glutaraldehyde cytoskeletons that were critical point–dried after dehydration in acetone are shown in Fig. 6. The lattice observed and typical of that described by Wolosewick and Porter (41) resembles in its general morphology the image seen after dehydration. Again, the inset shows the level of detail seen in cytoskeletons prepared as for Fig. 2*a* to illustrate the loss of structural order that has occurred. In the critical point–dried preparations the difference between cells treated for different times in OsO_4 was not significant, presumably due to the overriding changes that took place during the dehydration step.

DISCUSSION

Cytoskeletal Preparations

It is appropriate to comment first on the relation of the filament organization of the cytoskeletons with that of the "intact" cell. In earlier studies we extracted cells briefly in Triton X-100 alone, before glutaraldehyde fixation (32, 33). Under these conditions it could be established, for HeLa cells, that ~50% of the total actin was lost in the supernate, accompanied by ~70% of the tubulin (α and β) and 60% of the α-actinin (6). According to other studies (4, 5, 17; see also review article 24, 37), a high proportion of the released actin is likely to be in an unpolymerised form, although the ratios between monomeric and polymeric actin must clearly depend on the conditions employed (on the presence or absence of calcium, on actin-associated proteins, etc.). The addition of glutaraldehyde to our extraction buffer, a modification introduced by Höglund et al. (18), resulted, with heart fibroblasts, in a denser packing of filaments in the leading edge, suggesting that the loss of pre-existing F-actin had been mainly prevented. In a parallel study, it has been demonstrated further that such Triton X-100-glutaraldehyde mixtures produce an immediate cessation of motile activity of the leading edge of chick heart fibroblasts with no detectable retraction, at least at the limit of resolution of the light microscope (34). The structural arrangements observed thus bear a real relation to the phases of movement of the leading lamella.

We cannot now rule out the possible release of some filaments from the dorsal surface of the leading edge during "fixation-extraction", but consider that this may be minimal. Thus, with the use of lower Triton X-100 concentrations for which the contours of the membrane delimiting the edge of the cell could still be observed, the density of filament packing was not noticeably greater. Nevertheless, the removal of at least a submembranous layer could be inferred from the loss of the microvilli that project upwards from the dorsal cell surface. Some actin binding proteins must also inevitably be lost, but

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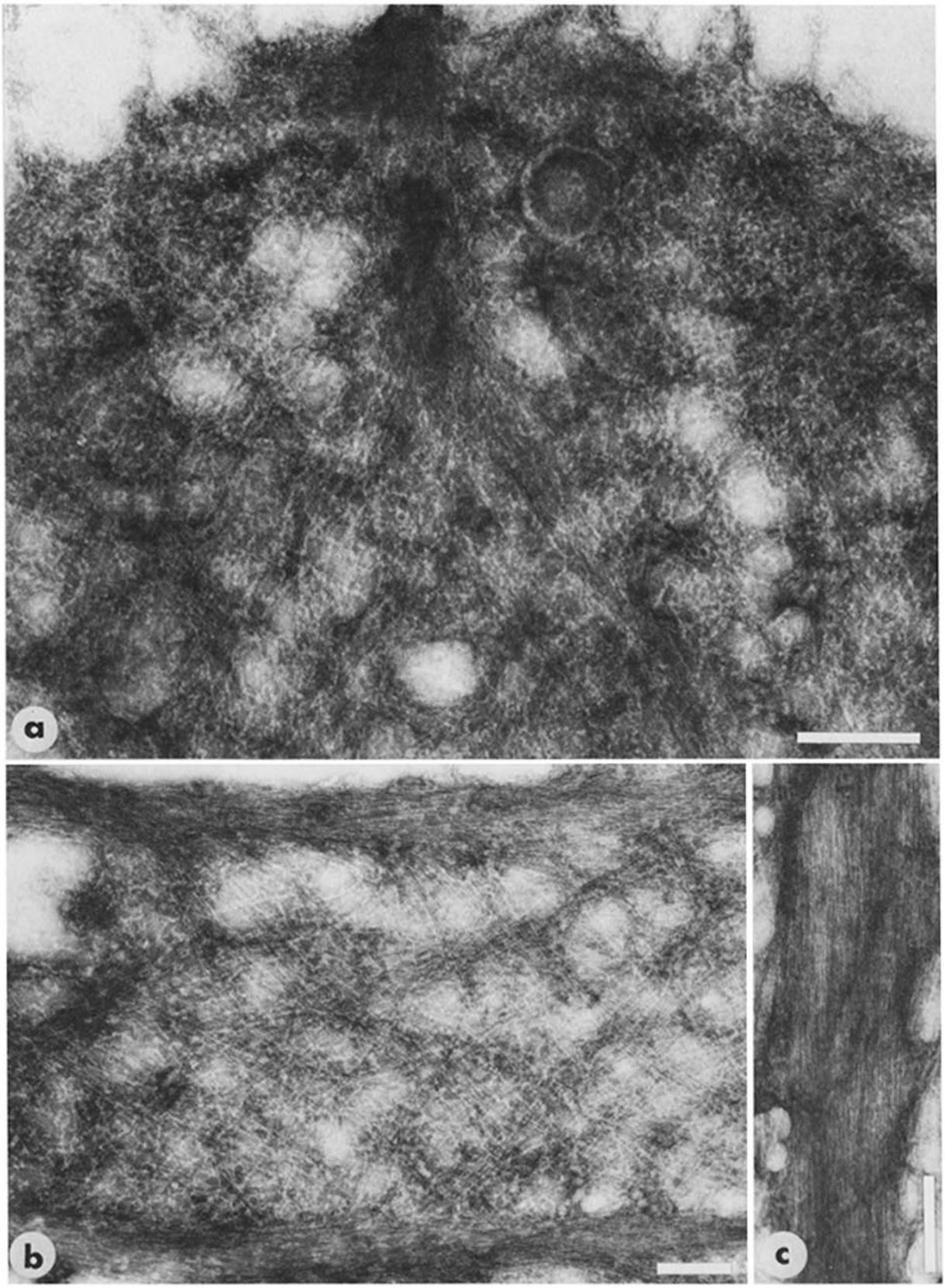

FIGURE 4 Effect of OsO_4 postfixation on actin filaments in the leading edge and the stress fibers of Triton X-100-glutaraldehyde cytoskeletons. *a*, leading edge region exposed to 1% OsO_4 for 7 min at room temperature and stained in aqueous uranyl acetate. *c*, stress fiber in similarly treated cell. Note that actin in the stress fibers resists distortion by OsO_4, whereas the actin filaments in the leading edge are grossly distorted. *b*, leading edge region of cytoskeleton postfixed in 0.2% OsO_4 (phosphate buffer, pH 6.3) for 20 min at 0°C. The actin filaments are still intact after this limited exposure. Bars, 0.2 μm. *a*, × 104,000; *b*, × 63,000; and *c*, × 82,000.

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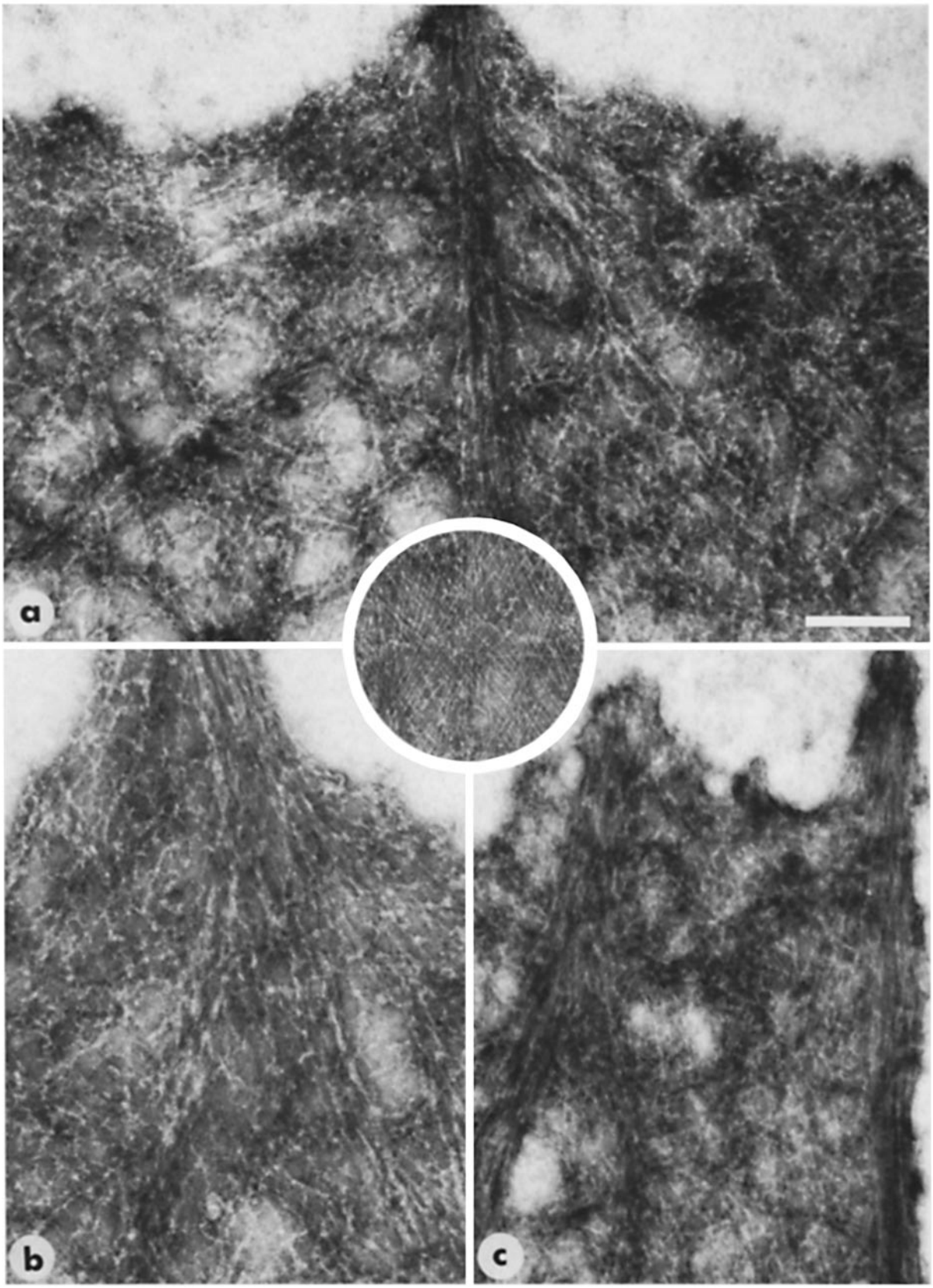

FIGURE 5 Effect of dehydration on actin filaments in the leading edge of Triton X-100-glutaraldehyde cytoskeletons. *a*, cytoskeleton dehydrated in acetone, after final glutaraldehyde fixation, and stained in uranyl acetate in absolute methanol. *b*, cytoskeleton postfixed in OsO_4 (1%, 7 min, room temperature), dehydrated in acetone, then rehydrated and stained in aqueous uranyl acetate. *c*, cytoskeleton post-treated with phalloidin, as in Fig. 2 *a*, dehydrated in ethanol, and negatively stained in methanol-uranyl acetate. In all cases dehydration results in a similar disorganisation and/or aggregation of the actin filaments. Circular inset shows at the same magnification the leading edge meshwork of cytoskeletons prepared as for Fig. 2 *a*, for comparison. Bar, 0.2 μm. × 90,000.

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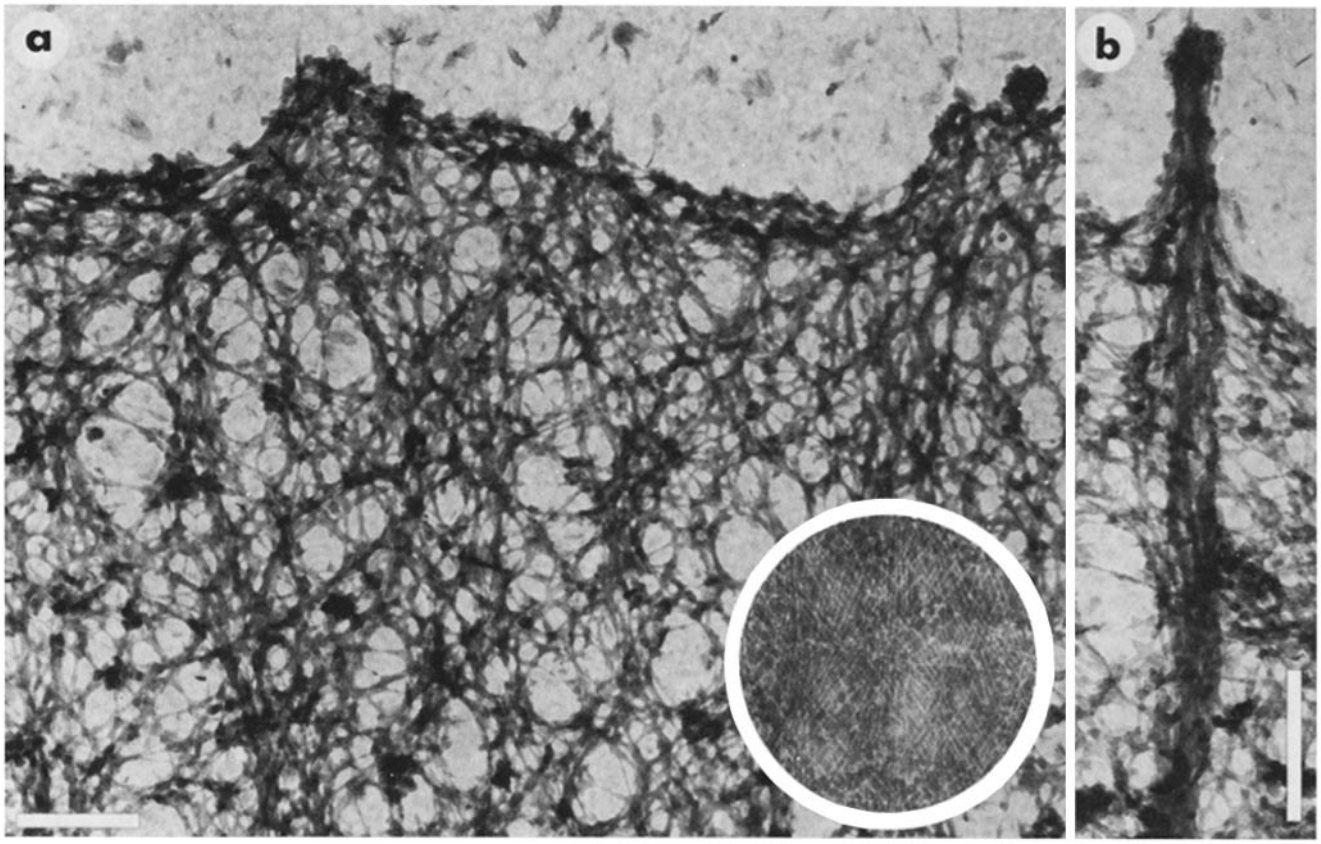

FIGURE 6 Leading edge region of critical point-dried Triton X-100-glutaraldehyde cytoskeletons. *a*, meshwork region postfixed with 0.2% OsO_4 for 20 min at 0°C (see text). *Inset* shows, for comparison, meshwork region in cell prepared as for Fig. 2 *a* printed at the same magnification. *b*, microspike postfixed with 1% OsO_4 at room temperature. Bars, 0.2 μm. *a*, × 80,000; *b*, × 100,000.

antibody staining experiments showed that a significant amount of both α-actinin and filamin remain in the leading edge after the glutaraldehyde-Triton X-100 treatment (42).

Although the order in the current preparations might be expected to be modified by a flattening during drying in the negative stain, we have shown in fact that a good degree of three-dimensional order is retained (see also 34). Presumably, the leading edge networks are cross-linked to such an extent as to resist collapse onto the support film.

Effects of Chemical Fixation on Cytoplasmic Actin

For technical reasons the leading edge of cultured cells and likewise the spreading zones shown by other cells when attached to solid substrates (1, 3, 10, 14, 28) are ideal subjects for the structural investigations of actin meshworks and the factors that affect their preservation. These thin, broad zones of actin filaments, sandwiched only by the plasmalemma, are readily accessible to study *in toto* by diverse electron microscope techniques, using conventional accelerating voltages.

In considering the problems related to the preservation of actin filaments we can turn first to the long experience of the investigators of muscle fine structure. Their x-ray studies have shown that the integrity of the thin filament, of the packing of actin monomers in the double helix, is disturbed neither by detergent extraction nor by chemical fixation in glutaraldehyde (20, 29, 42). Indeed, glutaraldehyde-fixed actin is still capable of activating the ATPase activity of muscle myosin (26). Related studies have also established the negative staining method as the most suitable for revealing the fine structure of actin filaments in the electron microscope (15, 19).

Osmium tetroxide, on the other hand, when used as a postfixative produces a loss of the fine structure of sarcomeric actin (20, 30), although the orientation of the filaments is maintained within the myofibril. The filaments of the stress fibers of nonmuscle cells show a similar resistance to total disruption by OsO_4 by maintaining their linearity. Perhaps for this reason alone many authors have been led to assume that actin elsewhere in the cell would be equally well preserved. But, as we show here, the difference in sensitivity to OsO_4 of the filaments in the stress fibers and those existing in the peripheral lamellae is dramatic. Exposure to 1% OsO_4 after glutaraldehyde fixation for shorter times than generally employed for conventional electron microscopy (5–10 min) results in a more or less total destruction of free actin filaments and actin meshworks. The distortion and kinking of the filaments that is observed corresponds almost exactly to that seen with purified muscle actin treated similarly in vitro (25).

Dehydration, Critical Point-drying, and the Microtrabecular Lattice in the Leading Edge

As we have seen, dehydration in organic solvents has a further deleterious effect on the morphology of the leading edge meshwork, causing additional distortions and a thickening or aggregation of the filaments. It is particularly noteworthy that rather few filaments, up to ~8 or 10, may be counted in the microspikes of dehydrated and critical point-dried cells (9 and this study) compared with a maximum of 30–40 filaments

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as seen after glutaraldehyde fixation and negative staining. From their studies with freeze-dried cytoskeletons, Heuser and Kirschner (16) cite a similar aggregation process occurring as a result of dehydration after glutaraldehyde fixation. These latter authors also used 10% methanol as a routine cryoprotectant, which prompted us to check whether in fact some changes already occur even at this low methanol concentration and which might have been overlooked. In a preliminary test, no gross distortions could be detected in the order of leading edge meshworks when 10% methanol was included in the negative-staining solutions. From the close identity of the negatively stained images obtained at the end of the dehydration series and after critical point–drying we conclude that the critical point–drying step itself introduces no additional distortions of the actin meshwork.

Wolosewick and Porter (41) have recently considered at length the relation that their microtrabecular lattice observed in critical point–dried cells may bear to the organization of the cytoplasm in vivo. The microtrabecular lattice is an irregular, three-dimensional filamentous net, taken to represent the "cytoplasmic ground substance," that embraces and interconnects the cell organelles and membranous components and extends into the peripheral cytoplasmic regions (9, 40, 41). In the latter peripheral lamellae that are engaged in motile activity this lattice and the microspike bundles are the only structural components recognised (9). Since it is now clear that ordered actin meshworks exist in such regions, we cannot escape the conclusion that the microtrabecular lattice, at least in these peripheral zones, is in large part the distorted remains of pre-existing, organised actin filaments. Additional components may exist in the complete lattice that are lost during detergent extraction, but even so, our own data on critical point–dried whole cells and cytoskeletons revealed little difference in appearance between the elements seen after critical point–drying in the two types of preparations. Of the lattice in the remaining part of the cytoplasm we can only infer that much of this may also be a distorted form of filamentous actin, together with the 10-nm filaments and filament-associated proteins (see also 16).

We would thus endorse the conclusion of Wolosewick and Porter (41) that the images hitherto obtained by the critical point method probably correspond closely to those derived from plastic-embedded material. But in contrast to their view we conclude that the actin filament meshworks in both types of preparations are largely destroyed. This destruction results from the postfixation in OsO_4, as could be predicted from earlier work (25), and also from the dehydration in organic solvents. Wolosewick and Porter (41) did note a "decay" in the lattice when OsO_4 was used as a primary fixative, but failed to detect any changes resulting from postfixation in OsO_4 after glutaraldehyde. From these studies I conclude that these changes were masked in part by the additional distortions caused by dehydration.

Future Studies of Actin Meshworks

Since OsO_4 has long been recognized as a useful postfixative for conventional electron microscopy (see reference 41), I attempted, as did Maupin-Szamier and Pollard (25) with isolated F-actin, to establish the concentrations and exposures tolerated by the cytoplasmic actin meshworks. As I indicated, treatment with a 0.2% solution (pH, 6,2 phosphate buffer) at 0°C for 20 min had no visible destructive effect. Thus, for studies where the cell in its entirety must be observed, the desirability of using such limited exposure to OsO_4 should not be overlooked. Needless to say, improvements beyond this will be required for preserving order in embedded or critical point–dried specimens. Scope in this direction may be offered by the use of phalloidin (39) to stabilize actin filaments against OsO_4 (11) and combining this with the glutaraldehyde-tannic acid procedure described by Begg et al. (2). But, as these results show, the most difficult barrier to surmount is clearly the dehydration step. One may only suggest that for studies aimed at resolving this problem, the actin meshworks of substrate-attached cells offer themselves as a useful test system.

For further studies of the thin regions of cultured cell cytoskeletons, the use of glutaraldehyde-detergent mixtures followed by negative staining offers some obvious advantages which have yet to be fully exploited. It is now of particular interest to establish how the structure seen after negative staining compares with that observed in the same cytoskeletons prepared by the freeze-drying-replication method of Heuser and Kirschner (16). The use of either or both of these methods should now open the way for detailed analysis of the structural interrelationships that occur between actin-associated proteins and cytoplasmic actin and which form the basis of actin-mediated phenomena in the cell.

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