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Filament arrangements in negatively stained cultured cells: the organization of actin

Filamentanordnungen in Gewebekulturzellen nach Anwendung der Negativ-staining-Technik: Die Verteilung des Actins

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Abstract

Actin – microtubules – cell motility – cytoskeleton

Treatment of spread, cultured cells with Triton X-100 followed by negative staining reveals the organization of the unextracted intracellular filamentous elements: actin, microtubules and the 100 Å filaments. The present report describes the organization of the actin-like filaments in human skin fibroblasts and mouse 3 T 3 cells.

As shown in earlier studies, the cytoplasmic stress fibres were seen to be composed of bundles of colinear actin-like filaments. In addition to these large stress fibres much smaller bundles of thin filaments as well as randomly oriented thin filaments were also observed. A thick bundle of thin filaments, 0.2 μm to 0.5 μm in diameter, was found to delimit the concave cell edges most prominent in well-spread stationary cells.

The leading edge and ruffled border of human skin fibroblasts appeared as a broad web, or meshwork of diagonally oriented thin filaments interconnecting radiating, linear bundles of thin filaments about 0.1 μm in diameter. These bundles corresponding to the microspikes described earlier ranged from about 1.5 μm to 6.0 μm in length and were separated by 1 μm

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Fig. 1. Survey micrograph of a well spread 3 T 3 cell showing stress fibres and concave cell edge delimited by a thick bundle of actin filaments (see Fig. 5, inset). Densely staining area around cell nucleus corresponds to region occupied by the cytoplasmic 100 Å filaments. – Bar 10 μm.


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to 3 μm laterally. The leading edge of 3 T 3 cells showed a similar organization but with fewer radiating thin filament bundles. Both the filaments in the bundles and in the meshwork formed arrowhead complexes with smooth muscle myosin subfragment – 1 which were unipolar and directed towards the main body of the cell. The findings are discussed in relation to the mechanism of non-muscle cell motility.

Introduction

The demonstration of the presence of actin and myosin in numerous non-muscle cell types has led to various speculations about the involvement of these proteins in both cellular and subcellular motility (see reviews [18, 29]). In cultured cells actin commonly forms prominent fibrils or "stress fibres" [11, 24] which are resolvable in the light microscope and which have been demonstrated rather dramatically by the use of the indirect immunofluorescent technique [14, 23]. Related studies suggest that myosin is also present within these stress fibres [13, 20, 34] although not in a filamentous form. In addition to actin, two other filament types may be distinguished in cells in the electron microscope and, like actin, occur in varying amounts according to the cell type; these are the microtubules and the 100 Å filaments. The different roles that these three types of filament play in the maintenance of cell shape, in cell locomotion and in membrane fluidity [26] are at present the subject of intense investigation.

While the immunofluorescent method has proved invaluable for defining the general arrangement of the filamentous components of cultured cells, studies by this technique are necessarily limited by the resolution of the light microscope. The advantage of the increased resolution of the electron microscope is, on the other hand, to a large extent outweighed if investigations are restricted to ultrathin sections. With this limitation in mind Buckley and Porter [10, 11] grew cultured cells on electron microscope support films and showed that, after fixation and critical point drying, the cell organelles and filament matrix could be resolved in the thinner regions of the cytoplasm. Subsequently, similar preparations were observed in a high voltage electron microscope which allowed visualization of the entire cytoplasmic components at a resolution comparable to that attained in thin section material [12, 37].

In our own investigations we were specifically interested in observing the filamentous components of cultured cells and for this reason we considered an alternative method based on that successfully applied to the observation of filaments in isolated smooth-muscle cells [32]. The procedure consists in dissolving the cell membranes with Triton X-100 under conditions known to preserve the contractile apparatus of the smooth muscle cell [31] followed by glutaraldehyde fixation and negative staining. Under these conditions all three filamentous elements, actin, microtubules and the 100 Å filaments could be readily distinguished and their distribution and interrelationships investigated. In this report we describe the details of the technique and present results concerning the organization of the actin-like filaments in the stress fibres and in the leading edge [1] of human skin fibroblasts and mouse 3 T 3 cells. Elsewhere we report observations on the organization of the 100 Å filaments (Small and Celis, in preparation) as well as on the filament organization in certain malignant cell types (Celis et al., in preparation).

During preparation of this manuscript a report appeared by Brown, Levinson and Spudich [8] describing the use of a similar technique for the observation of cultured cells. The method described by these authors, however, failed to preserve both the microtubular elements and the delicate network of filaments in the leading edge.

Fig. 2. Enlargement of part of cell shown in Figure 1. – Bar 2 μm.


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Materials and methods

Cell cultures

Human skin fibroblasts and mouse 3 T 3 cells were grown as monolayer cultures in Eagle's modified minimal medium containing 10 % fetal calf serum and antibiotics (penicillin 100 UI/ml; streptomycin 50 µg/ml).

Electron microscopy

Silver electron microscope grids (167/192; Maser & Morton, London) were placed, in rows of three to six, on sections of carbon-coated parlodion film floated onto a water surface and were picked up on glass coverslips (24 mm × 8 mm). To ensure firm binding of the film, the coverslips were coated on the edges with a solution of 1 % polybutene in toulene and allowed to dry before use. Excess water was drained from the film with filter paper and the grids stored after drying, at 4° C. Prior to transfer to the culture dishes the grids with coverslips were sterilized with ultraviolet radiation.

After sufficient overgrowth of the cells onto the grids had taken place each coverslip with attached grids was removed from the culture dish, rinsed in phosphate buffered saline (PBS) and transferred to 0.1 % Triton X-100 in a solution of the following composition in mM: NaCl, 137; KCl, 5; Na_2HPO_4, 1.1; KH_2PO_4, 0.4; $NaHCO_3$, 4; glucose, 5.5; $MgCl_2$, 2; EGTA, 2; PIPES, 5 mM *p*H 6.0. After 45 to 60 seconds the coverslip was rinsed in the same buffer without Triton and transferred to 2.5 % glutaraldehyde, again in the same buffer. The preceding processing steps were carried out at room temperature. The period of fixation was not critical and varied from about 5 to 30 minutes, after which time the grids were separated from the coverslip and processed for electron microscopy as follows. Each grid was rinsed in two changes of distilled water, dried briefly on filter paper on the cell-free side and inverted on a drop of buffer to maintain the cells in a hydrated state. The drop with grid was then transferred to a cold room (4°) and the grid negatively stained with 1 % uranyl acetate following a rinse with several drops of 0.2 mg/ml cytochrome C in 0.1 % amyl alcohol [28].

The preparations were examined and photographed in a Siemens Elmiskop 101 operating at 80 kV.

Treatment with myosin subfragment-1

Myosin subfragment-1 (S-1) was prepared from purified smooth muscle myosin [33] according to the method of Margossian and Lowey [27].

Following the washing step to remove Triton, coverslips with attached grids were transferred to a solution containing 1 mg/ml S-1 (in: 60 mM KCl, 1 mM $MgCl_2$, 1 mM cysteine, 20 mM imidazole, *p*H 6.8) for 30 seconds to 1 minute at room temperature. The coverslips were then rinsed in the same buffer without S-1 and transferred to fixative, as before, prior to negative staining.

Indirect immunofluorescence

Cells grown on glass coverslips were washed twice with Dulbecco's phosphate buffered saline (PBS) and treated for 45 to 60 seconds in a 0.1 % Triton X-100 solution in Pipes buffer (as above). After washing twice in Triton-free buffer the cells were fixed for 20 minutes in 3.5 % formaldehyde in the same buffer. After washing extensively the coverslips were covered with 10 µl of anti-actin antibody (human smooth-muscle antibody; Anderson, Small, and Sobieszek [6]) and incubated for 45 minutes at 37° C in a humid environment. The coverslips

Fig. 3. Selected area of cell from Figure 1 showing distribution of filaments and microtubules in an intermediate area of the cytoplasm. – Bar 2 µm.


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were then washed several times with PBS and covered with 100 µl of fluoresceine isothiocyanate conjugated rabbit anti-human IgG (DAKO, diluted 1/20). After 45 minutes incubation at 37° C in a humid environment the coverslips were washed thoroughly with PBS. Observations were made on a Zeiss photomicroscope equipped with fluorescence and phase contrast optics.

Results

For 3 T 3 cells well spread on the substrate the negative staining method revealed the general arrangement of filamentous elements over much of the cell area (Figs. 1, 2). However, from the nature of the method the detailed features of the filaments were most readily visualized only in the thinner areas of the cell, towards the cell edges. In the thicker, perinuclear region filaments in the upper part of the cell could be observed, but even under the most favourable conditions they were rather poorly contrasted. Figures 3 and 4 show in more detail intermediate and peripheral regions in the cytoplasm of the 3 T 3 cell shown in Figure 1. Microtubules, stress fibres containing actin filaments and free actin-like filaments are readily visualized. The concave edges of spread cells, commonly found in preparations of 3 T 3 cells were seen to be composed of a thick bundle, about 0.2 µm to 0.3 µm in diameter, of colinear actin-like filaments (Fig. 5). Such bundles stained strongly after application of the immunofluorescent technique with actin-specific antibody (Fig. 5, inset). The same bundles were also observed earlier in studies on thin section material [9, 15] and seem to occur at regions of the cell periphery not bound strongly to the underlying substrate [16]. In addition to being present in the stress fibres actin-like filaments were present in much smaller bundles (see Fig. 6), not resolvable in the light microscope, and also occurred singly and randomly arranged.

In the perinuclear area a densely stained, often punctuated region was always found and was particularly prominent in skin fibroblasts (Fig. 9). As shown elsewhere (Small and Celis, in preparation) this region corresponded to that occupied by the cytoplasmic 100 Å filaments.

The leading edge

Further, specialized arrangements of actin-like filaments were observed at the peripheral regions of the cells corresponding to the "leading edge" described in the light microscope [1]. In these regions a broad band containing actin-like thin filaments was observed (Figs. 6, 7, 8, 10) which was clearly delineated from the abutting cytoplasm and from which microtubules and 100 Å filaments were excluded. In skin fibroblasts this band, which varied from 2 µm to 5 µm in width, was composed of a web or meshwork of diagonally oriented thin filaments interconnecting radiating linear bundles of thin filaments about 0.1 µ in diameter (Figs. 6, 8). The bundles ranged from about 1.5 µm to 6.0 µm in length and were separated by 1 µm to 3 µm laterally. As shown in Figure 7 the filaments within the bundles exhibited the same characteristic substructure

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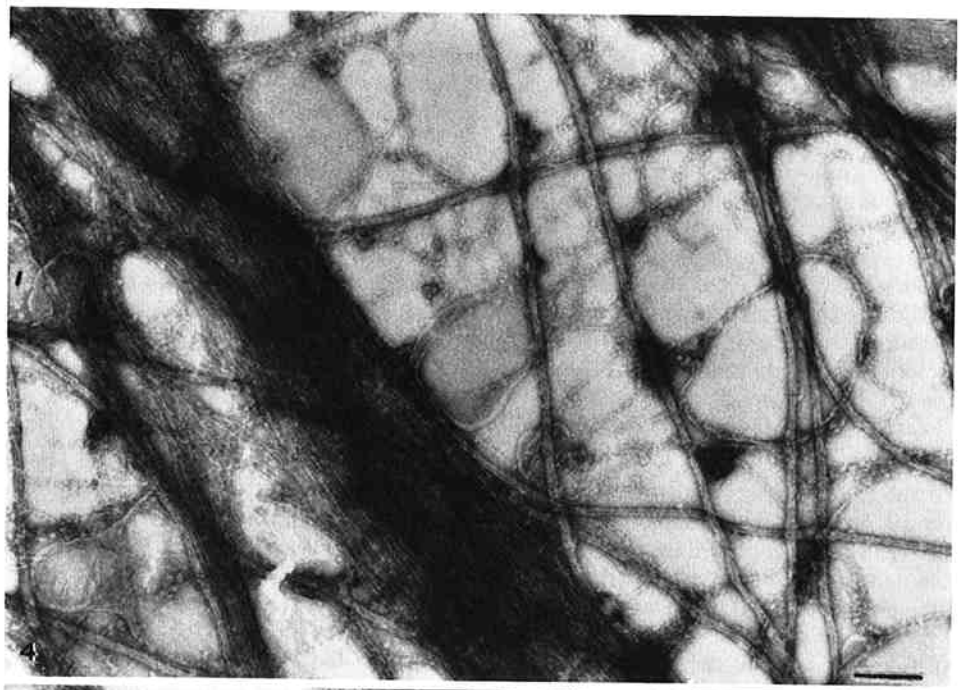
Figs. 4 and 5. Regions towards and at the cell edge of the 3 T 3 cell in Figure 1 showing microtubules, actin filament bundles and single actin filaments. Figure 5 shows the thick bundle of actin filaments found at the cell periphery of stationary cells and which stains strongly with actin-specific antibody *(inset)*. – Bars 0.2 µm. – Inset bar 10 µm.



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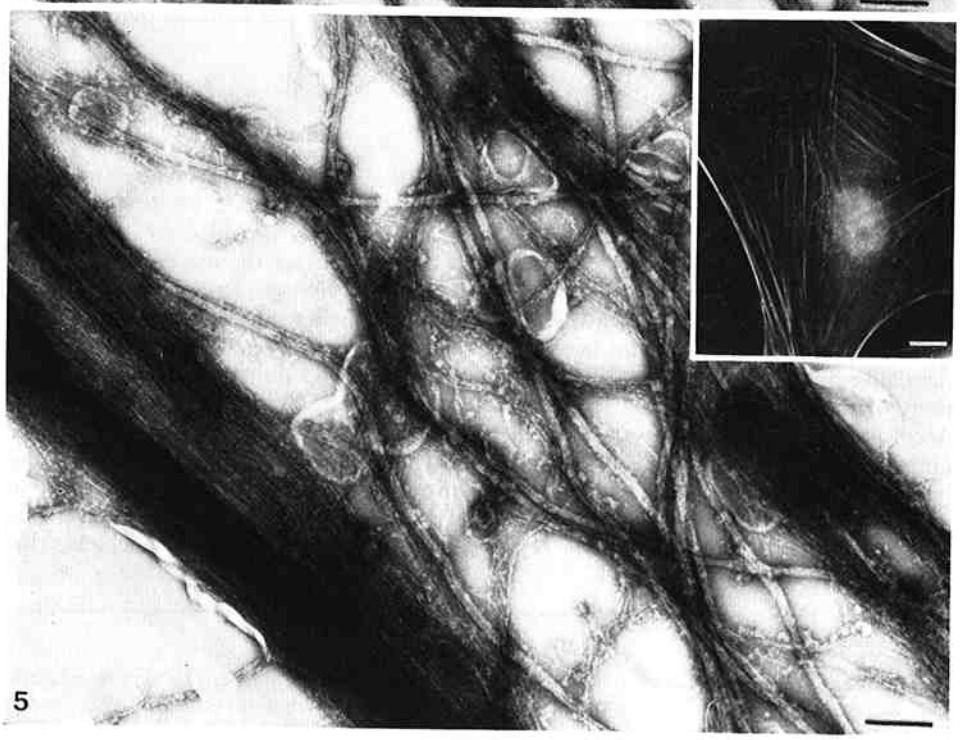

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as muscle actin filaments. In favourable instances the same globular substructure could also be recognized in the filaments within the adjacent meshwork, although here it was less apparent. In 3 T 3 cells the leading edge showed the same meshwork as in the skin fibroblasts but differed in exhibiting fewer thin filament bundles (Fig. 10). After application of the immunofluorescent method with antiactin antibody the leading edge appeared as a brightly fluorescent band containing radiating linear "spikes", the latter evidently corresponding to the linear bundles of thin filaments and to the microspikes described by others (for references see e. g. [4]).

In some regions of the cell periphery extensive overlap of the peripheral border occurred (Figs. 9, 10); these regions were interpreted as sites of membrane ruffling [19]. Although the high density of the ruffles tended to preclude an assessment of their detailed structure it appeared that the filamentous organization was the same as that described above for the leading dege.

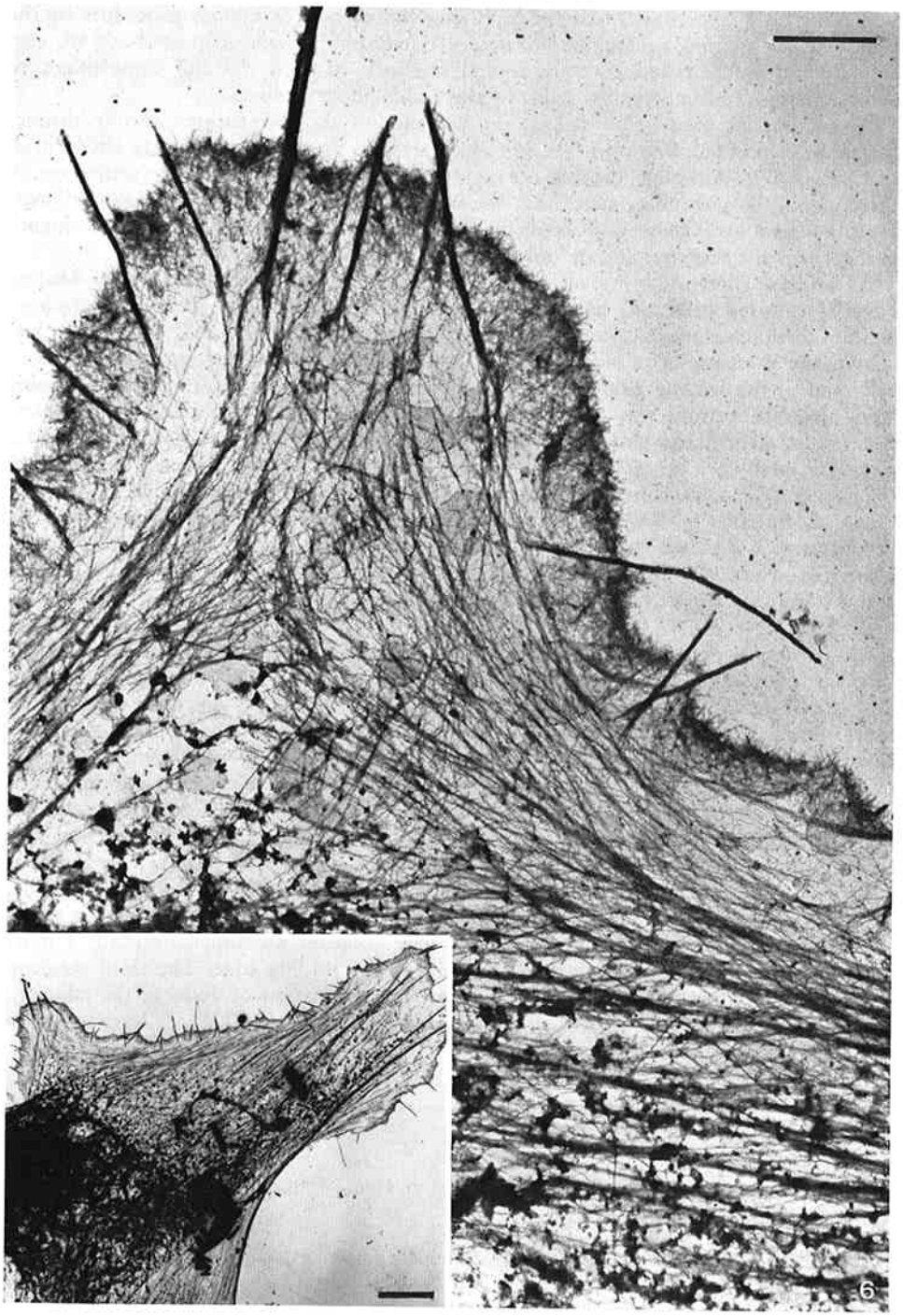
Polarity of actin filaments in the leading edge

Myosin subfragment-1 bound to thin filaments in all the situations described above; to the stress fibres, small filament bundles, randomly arranged thin filaments and to the filaments in the leading edge. We shall consider here only the polarity of filaments in the leading edge. In this region arrowhead complexes were formed both with the filaments in the broad meshwork and in the associated filament bundles (Figs. 12 to 15). While the polarity of the arrowheads was difficult to discern in the regions of high filament density, observation at the tips of the filament bundles (Figs. 13, 14) and on the anterior edge of the meshwork (Fig. 12) showed that the arrowheads were directed towards the cell body. This could also be seen at the ends of the filament bundles where they originated in the cytoplasm (Fig. 15).

Discussion

The present method is clearly most suited to the study of the thinner lamellae of cultured cells and cannot give much more than two dimensional information about filament arrangements. Even so, with the higher resolution provided by the negative staining technique the method offers a distinct advantage over the use of critical-point-dried cells [10, 12, 37]. While the latter studies showed very elegantly the three dimensional interrelationships between the intracellular organelles and filamentous networks the individual filaments in the lamella regions of the cells were less clearly resolved than after Triton extraction and negative staining. As yet we do not know what proportions of the filament proteins are lost as a result of the Triton extraction procedure. According to other studies, cultured cells contain a considerable proportion of loosely bound or monomeric actin [7, 8] which is readily extracted and which must also have been lost from our own preparations. However, in adopting a procedure known to minimise the removal of contractile proteins from smooth muscle cells [31] we have apparently preserved a considerably larger proportion of the filamentous elements than

Fig. 6. The leading lamella of a human skin fibroblast showing the peripheral web of actin-like filaments at the leading edge (see also Fig. 7) and the radiating thin filament bundles. Thin bundles of actin-like filaments occur in the cytoplasm. – Bar 2 μm. – Inset bar 10 μm.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was achieved by previous methods [8]. Using the present extraction procedure as the initial step in staining for immunofluorescence microscopy with actin antibody we also found that, as compared to conventional methods, at least the full complement of actin-containing structures observable by this technique, is retained.

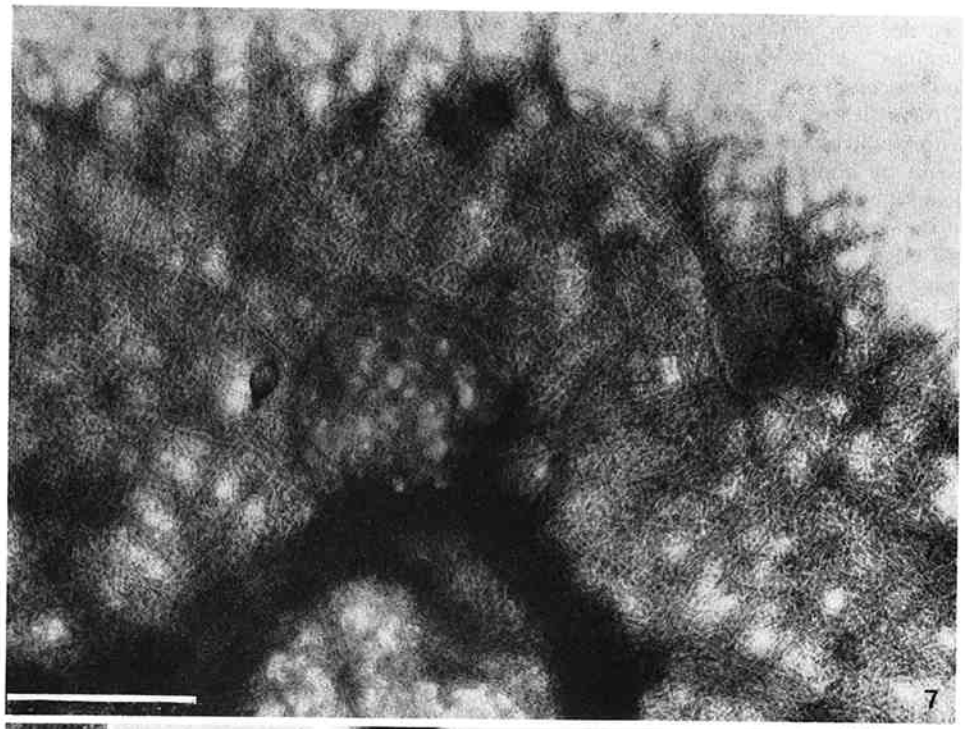
Recent studies have demonstrated the presence of three electrophoretically distinct forms of actin in cultured cells [30, 36] which are also present in fibroblasts after triton-extraction [30]. Although data is not as yet available on the assembly properties of these actins the possibility exists that the noted differences are related to the different morphological arrangements of actin filaments; in the stress fibres, the actin meshwork and perhaps the actin bundles or "microspikes" at the leading edge.

As we have shown, the present method is very well suited to the study of the leading edge of cultured cells and has provided new information about the organization of actin in this important location. Whereas earlier studies (see e. g. [5, 11, 15, 21, 35]) had shown the presence of a meshwork of actin-like filaments at the surface of various cells and at the leading edge of cultured fibroblasts [2, 9] the information obtainable from ultrathin sections was rather limited. From the present observations the organization of the actin filaments in the leading edge is more clear. In addition to showing a diagonal meshwork of actin-like filaments interconnecting the filament bundles, or microspikes, we have been able to show that the filaments in the leading edge possess only a single polarity. With regard to the direction of the formed arrowhead complexes this polarity is directed away from the direction of cell movement. The same, unipolar organization of actin has, more lately, been shown to occur in the outgrowing regions and growth cones of cultured neuroblastoma cells (Isenberg and Small, manuscript in preparation).

While such a polarity of actin filaments is consistent with an advancement of the cytoplasm according to a process of actomyosin shearing as earlier outlined by Huxley [18] certain modifications of this scheme are indicated by the present observations. The first is that at least a large proportion of the thin filaments must remain in close association with the substrate. Indeed it has already been shown by interference reflection microscopy that the sites of closest cell-to-substrate contact correspond in position to the filament bundles or microspikes at the leading edge [3, 25]. The second concerns the form of the myosin component. So far myosin in the form of filaments has not been seen and if myosin filaments are present *in vivo* then they must be either very small or even more labile than the thick filaments of smooth muscle which are visualized under the present conditions [31]. With only a single direction of movement taking place, together with unipolar actin filaments, we may consider monomeric myosin a more attractive candidate for the actomyosin system at the leading edge. The third concerns the possible involvement of the unidirectional polymerization of actin in the advancement of the leading edge. Studies of actin polymerization *in vitro* [17, 38] have indicated

Fig. 7. Part of leading edge of skin fibroblast in Figure 9 showing diagonal meshwork of actin filaments. – Bar 0.5 μm.

Fig. 8. Leading edge of a human skin fibroblast showing diagonal meshwork of actin filaments together with radiating thin filament bundles. Inset shows characteristic structure of actin in the filaments within the bundles. – Bar 0.5 μm. – Inset 0.1 μm.



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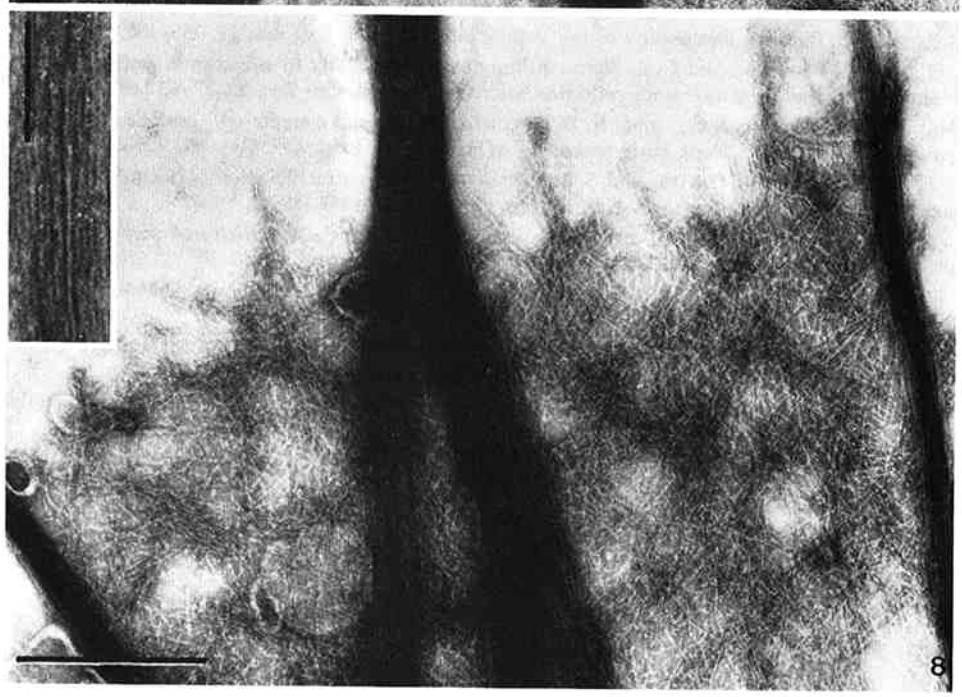

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that this process occurs preferentially in the direction opposite to that of the "arrowhead complex" which would correspond, for cultured cells, to the direction of net forward movement.

As indicated by Abercrombie et al. [1], membrane ruffling is commonly associated with a retraction of the leading edge. In this case the ruffling, presumably brought about by intensive actomyosin shearing [18] may serve to loosen the adhesions of the leading edge to the substrate and enable retraction via internal contractile forces either in the stress fibres [20] or in smaller actomyosin filament bundles. Advancement of the cell edge, on the other hand, could be brought about by the forward transfer of actin rich cytoplasm by actomyosin shearing (in the absence of ruffling) coupled with local polymerization of actin at the anterior edge. The relative contributions of actin polymerization and actomyosin shearing to cell locomotion and the specialized roles of the actin meshwork and the actin bundles in the motile mechanism are clearly important areas for further investigation.

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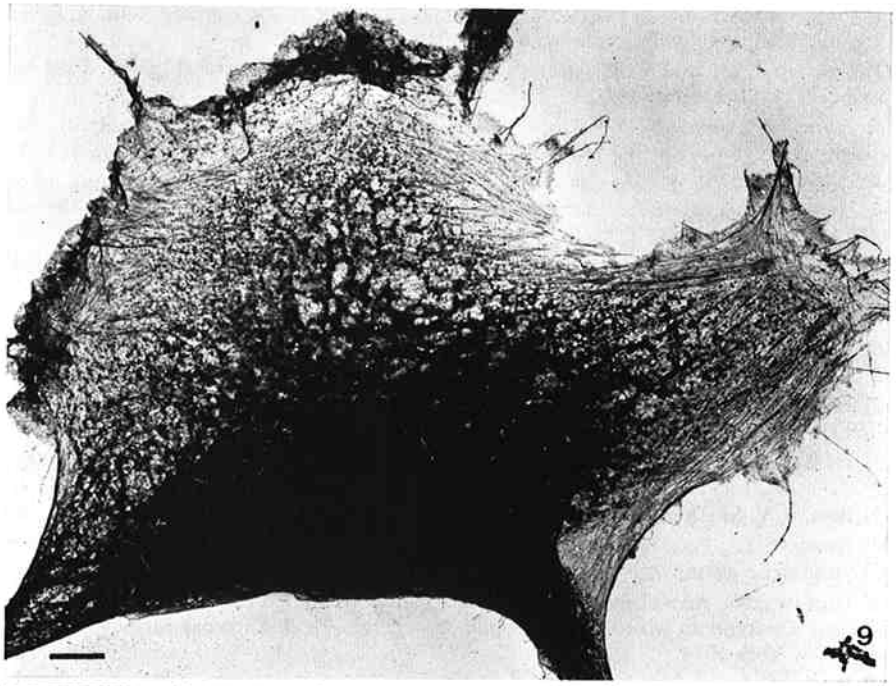
Fig. 9. Human skin fibroblast showing two regions of membrane ruffling. – Bar 5 μm.

Fig. 10. Area of membrane ruffling at the leading edge of cell shown in Figure 9. – Bar 2 μm.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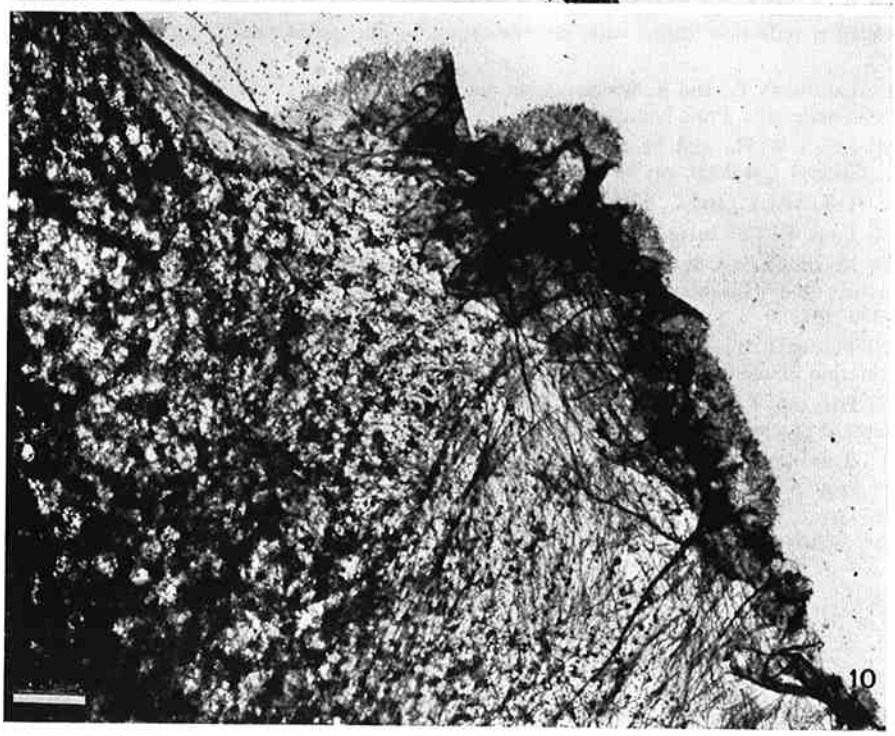
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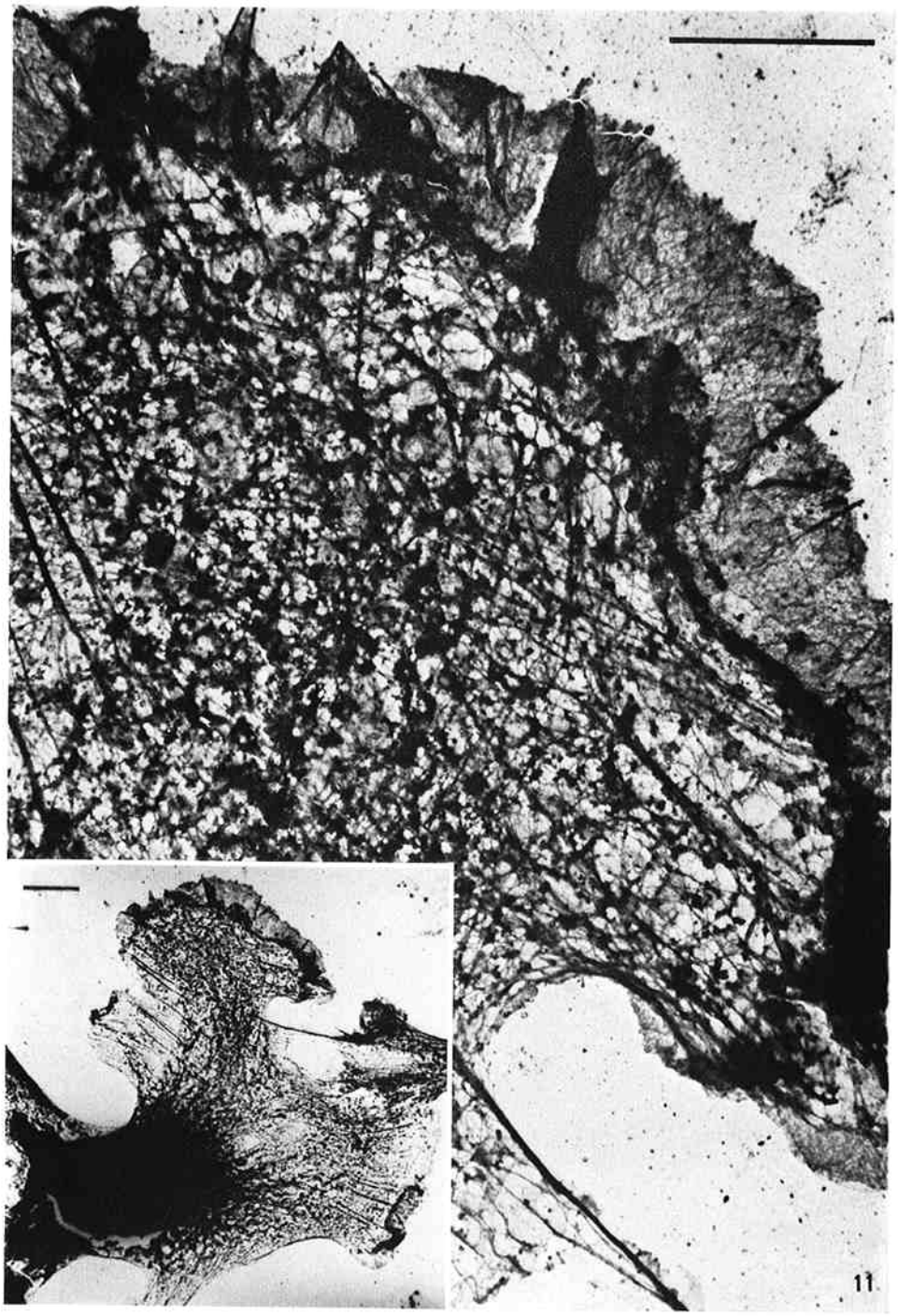
Fig. 11. Leading edge of migrating 3 T 3 cell shown in inset. – Bar 5 μm. – Inset 10 μm.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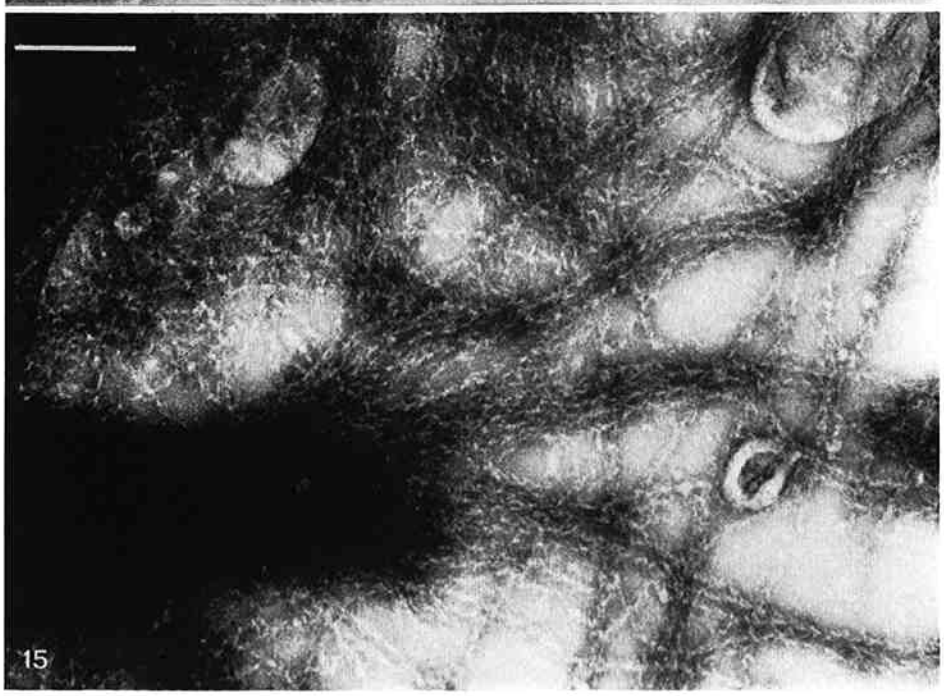
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Figs. 12 to 15. Selected areas of leading edge of skin fibroblasts after labelling with myosin subfragment-1. The S-1-actin arrowhead complexes are directed towards the main body of the cell. Figure 15 shows the origin of a radiating thin filament bundle in the cytoplasm. The connecting thin filaments are again polarized towards the main cell body. – All the same magnification bar 0.2 μm.

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Filamentous actin, 100 Å filaments and microtubules in neuroblastoma cells. Their distribution in relation to sites of movement and neuronal transport

Aktinfilamente, 100 Å Filamente und Mikrotubuli in Neuroblastomzellen. Ihre Verteilung in bezug auf Motilität und neuronalen Transport

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Abstract

Actin – 100 Å filaments – microtubules – nerve cells – neuronal transport

The distribution of actin filaments, 100 Å filaments and microtubules has been studied in whole neuroblastoma cells after Triton X-100 extraction and negative staining of monolayers grown on electron microscope grids. Observation of the spatial arrangements of actin and 100 Å filaments within the neurite was facilitated by removal of the microtubules with Ca^{++}.

Actin filaments were the only filamentous components in the leading edges of the cells and at the tips of the neurites and showed at least two supramolecular aggregation states: a planar filament meshwork and paracrystalline-like filament bundles, corresponding to the microspikes.

Decoration with myosin subfragment-1 showed the actin in the microspikes to have a single polarity, directed towards the base of the neurite. Microtubules and 100 Å filaments (neurofilaments) were found to occupy the core of mature neurites. Actin filaments were not observed in association with microtubules. They were absent from the neurite core and occurred only in small bundles at the edges of the neurites. The data are discussed in terms of both the motile phenomena associated with neuronal outgrowth and the mechanism of neuronal transport.

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