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Implications of cytoskeletal interactions for cellular architecture and behaviour

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[Plates 1–7]

The three major filamentous components of the cytoskeleton (microfilaments, microtubules and intermediate filaments) do not just coexist in the cell, but interact with each other in various ways. This paper discusses some examples of structural interactions visualized in critical-point-dried cells by stereoscopic high-voltage electron microscopy. The relative contribution of two classes of interactions to the consolidation of different cytoskeletal domains is considered. One class is represented by T-junctions (end-to-side contacts) of actin filaments with other filaments, and the other by 3 nm links. Attention is then turned to what may be called the behavioural consequences of cytoskeletal interactions. As an example of a coordinated interplay between events at the cell membrane and the cytoskeleton, we discuss changes in cytoskeletal organization of polymorphonuclear leucocytes upon stimulation with a chemotactic factor. These changes culminate in some cells in centriole separation and the establishment of two microtubule asters, each centred around a single centriole.

Introduction

In recent years cell biologists have become more and more aware of the possibility that the major cytoskeletal components (microfilaments, microtubules and intermediate filaments) do not just coexist in the cell, but also are linked to one another, thus influencing each other's location, organization and activity. They may also cooperate in a number of cellular activities, notably those related to motility. The interaction of subcellular components is evident not only in highly specialized cell structures such as heliozoan axopodia, where microtubules are linked into a highly ordered axonemal bundle (Tilney & Porter 1965), intestinal microvilli containing parallel arrays of actin filaments (Mooseker & Tilney 1975), or neuronal processes with their microtubule–neurofilament complexes (Wuerker & Kirkpatrick 1972); 'cross-talking' of cytoskeletal filaments is also expressed in less well defined cell structures such as the three-dimensional cytoskeletal networks of cultured cells. Many questions about the exact nature of the interactions and their regulation remain to be answered; even so, considerable advances in the identification and characterization of proteins that interact with the major cytoskeletal filaments have been made in the past few years. These advances are probably best illustrated by the large number of proteins recently identified and characterized that have been shown to interact with actin (Schliwa 1981). Such actin-associated proteins include not only factors that

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one would intuitively classify as 'cytoskeletal' since they can directly modify the state of actin, but also seemingly 'non-cytoskeletal' proteins of the cytoplasmic matrix such as enzymes of intermediary metabolism (Clarke & Masters 1976; Brady & Lasek 1981), large complexes such as ribosomes (Lenk *et al.* 1977), or proteins that are associated with the cell membrane (Geiger 1979; Burridge & Feramisco 1980). The possibility of extensive interactions between cytoplasmic constituents suggests that the cytoskeleton is in essence a structural continuum. This view has found its most consequent expression in the microtrabecular network hypothesis formulated by Porter and his colleagues (Buckley & Porter 1975; Wolosewick & Porter 1976, 1979; Byers & Porter 1977; Schliwa *et al.* 1981*a*; Porter & Tucker 1981) and has led to the proposal that the cytoplasmic matrix, of which the cytoskeletal network is an important part, is a giant, self-contained and self-regulated structural and functional unit, the cytoplast (Porter & Tucker 1981).

In this paper we present evidence for the existence of different types of structural interaction between filaments of the cytoskeleton and discuss their relative contribution to the formation and consolidation of different cellular domains within the cytoskeletal network. In the second part of this paper we discuss a unique example of a coordinated interaction that links cell membrane events, the contractile machinery and the microtubule system.

Supramolecular organization of cytoskeletal associations

When cultured epithelial cells (African green monkey kidney, strain BSC-1) are extracted with Triton X-100 (10–20 g l^{-1}) in a stabilization buffer (Schliwa & van Blerkom 1981), the cell residue left behind on the substratum (the cytoskeleton) consists of a network of the three major filament types, the nuclear remnant, ribosomes and other unextractable material, part of which presumably represents residues of secondary lysosomes. When these cytoskeletal preparations are examined by two-dimensional gel electrophoresis, more than 100 polypeptides are resolved, the most prominent being β and γ actin, α and β tubulin, and the intermediate-filament protein, vimentin (Schliwa & van Blerkom 1981). Also present are small quantities of α-actinin, tropomyosin and myosin. Most of the other polypeptides have not yet been identified. Careful comparison of extracted and unextracted cells by immunofluorescence microscopy using antibodies against tubulin demonstrates that the microtubule system, the most labile of the cytoskeletal components, is well preserved after extraction (Schliwa *et al.* 1981). This has led us to believe that the buffer used for extraction results in adequate preservation of the less labile components as well.

Figure 1, plate 1, shows a low-magnification overview of part of a BSC-1 cell to demonstrate the overall organization of the cytoskeleton as revealed by a combination of detergent extraction, critical-point drying, and stereoscopic high-voltage electron microscopy. Although the network character of the filament system can already be appreciated at this relatively low magnification, the nature of the interaction between the components of the network is faithfully depicted only at higher magnification. For this analysis, we photographed stereo pairs in a cell region about halfway between the nucleus and the cell periphery, where all three major cytoskeletal fibres are abundant. As a rule, three categories of associations can be distinguished (figure 2, plate 2): (i) lateral contacts between filaments (crossover pattern); (ii) end-to-side associations (T- or Y-junctions); and (iii) linkages by small (3 nm) filaments. Of these, the first will not be considered here because it is impossible to distinguish by morphological criteria

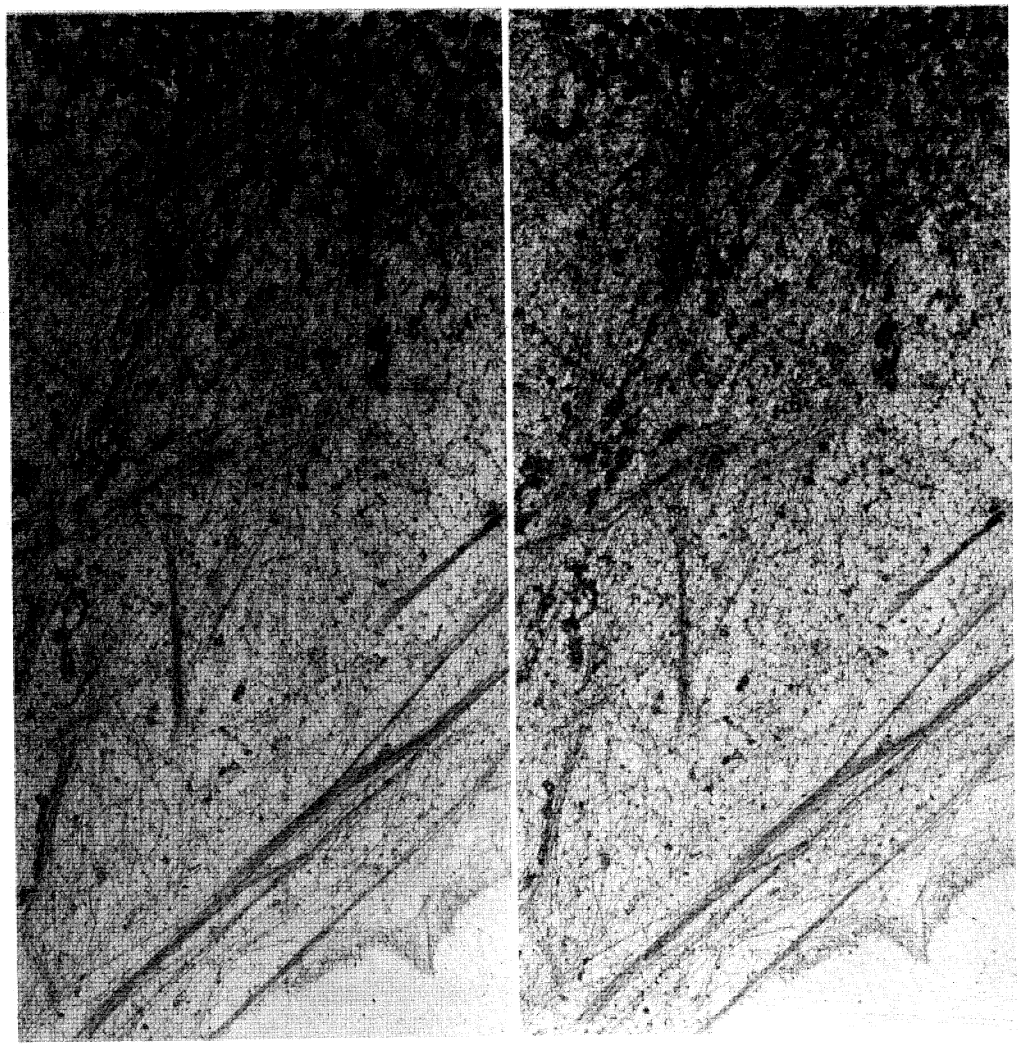


FIGURE 1. Overview of the cytoskeleton of a BSC-1 cell. The perinuclear area is at the top of the micrograph. Note substrate-associated filament bundles and the three-dimensional network extending throughout the cell body. (Magn. × 5000.)

(*Facing p.* 200)

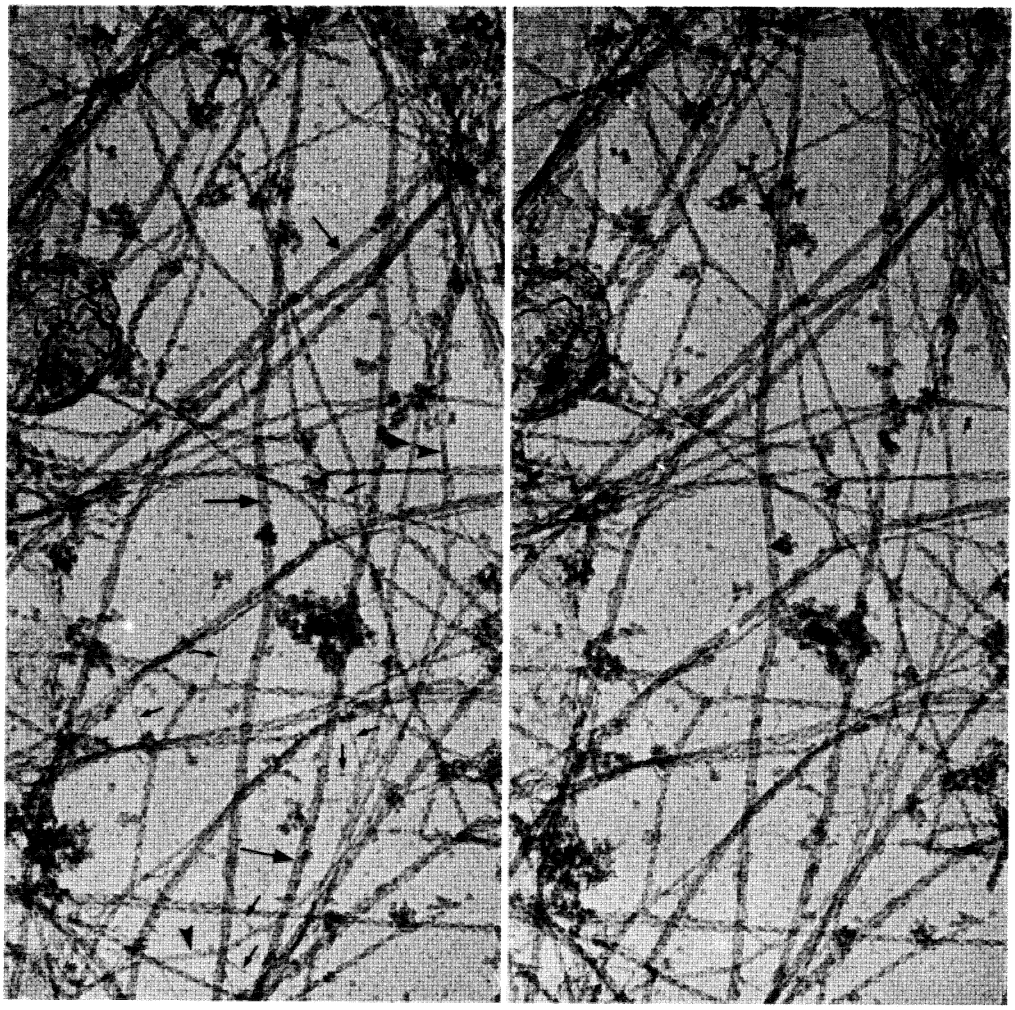


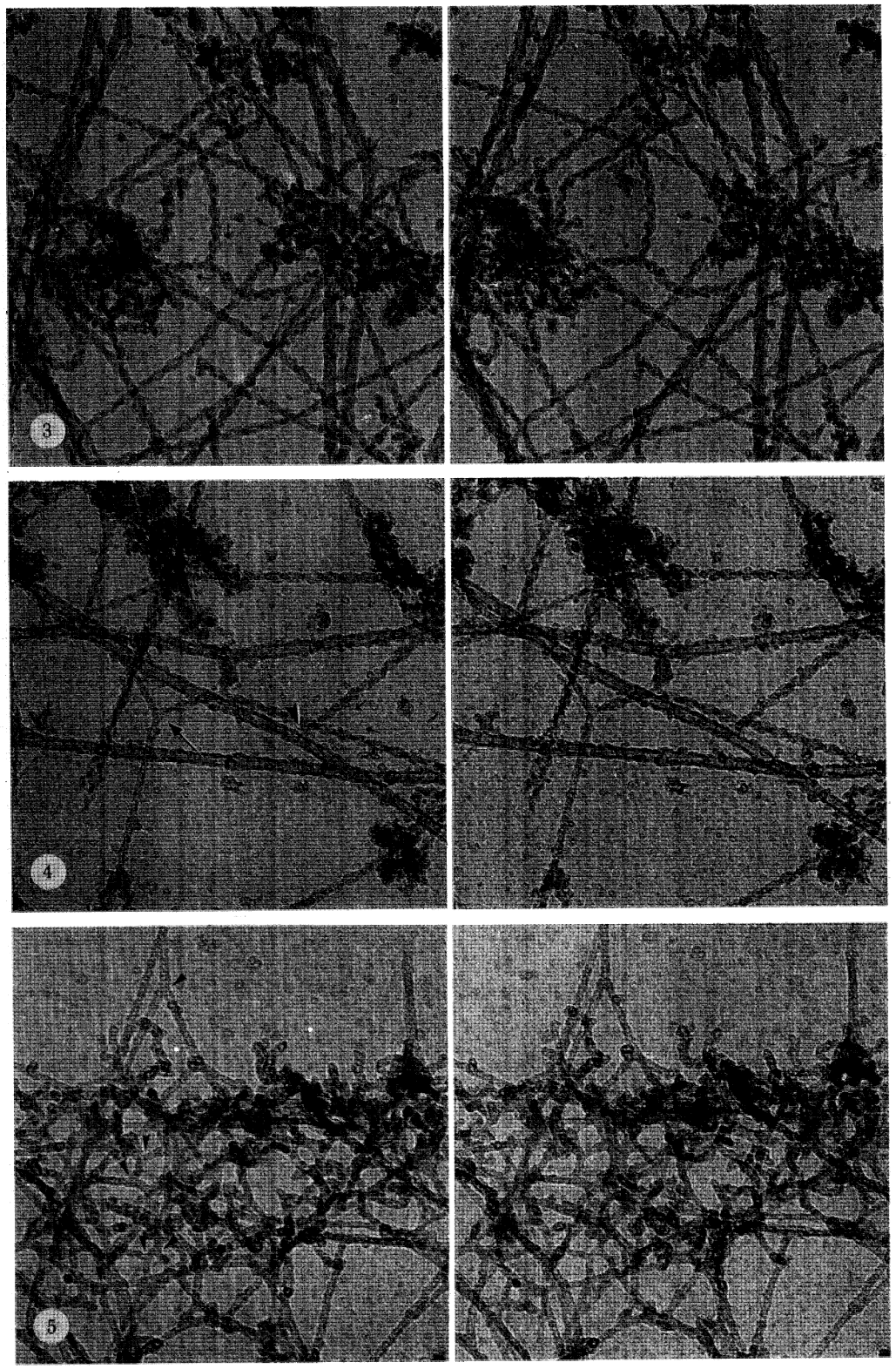
FIGURE 2. Cytoskeletal network in a cell region half-way between the nucleus and the pheriphery. Actin filaments are decorated with heavy meromyosin – subfragment 1 to distinguish them from unlabelled intermediate filaments (arrowheads), and to visualize their polarity. Note the presence of numerous 3 nm filaments (small arrows). Also shown are microtubules (large arrows). (Magn. × 90000.)

DESCRIPTION OF PLATE 3

FIGURE 3. T-junction of two actin filaments. Note that contact is made through the 'pointed' filament end (small arrows). In this and subsequent figures, small arrows indicate the polarity of decorated actin filaments. (Magn. × 120000.)

FIGURE 4. T-junction of an actin filament with an intermediate filament (arrow) and of an actin filament with a microtubule (arrowhead). (Magn. × 120000.)

FIGURE 5. Filament network in the periphery of a BSC-1 cell (= small ruffle). This preparation has not been labelled with heavy meromyosin to visualize the extensive interconnection of actin filaments via T-junctions in this cell region. In heavy meromyosin-decorated preparations, the filaments in this cell region are identified as actin. (Magn. × 120000.)



FIGURES 3–5. For description see opposite.

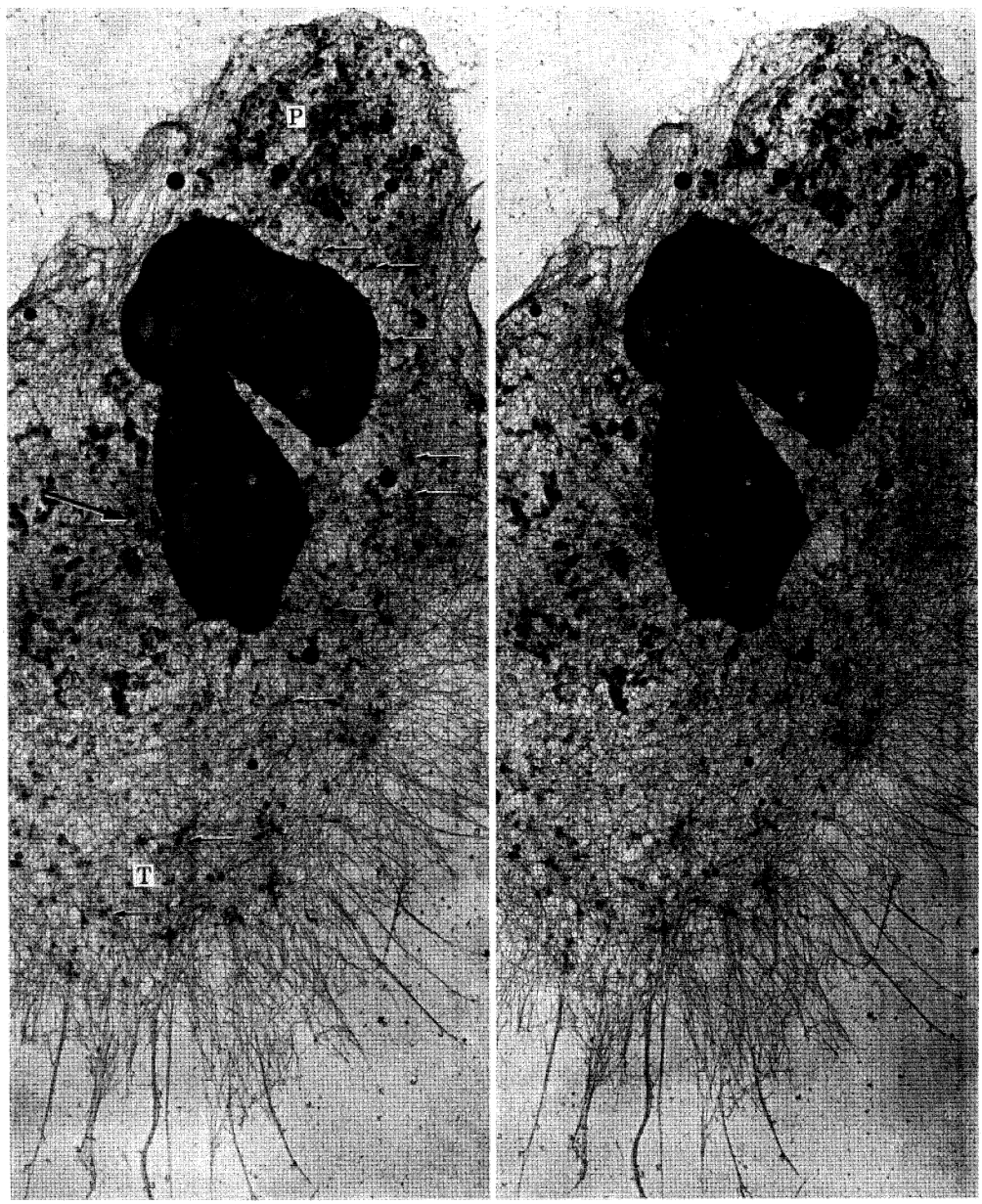


FIGURE 6. Overview of an extracted neutrophil showing the major cytoskeletal domains: advancing pseudopod with ruffles extending from it (P); cell posterior with tail fibres (T); and substrate-associated dense foci (arrows). Many granules, which probably correspond to the azurophils, are not completely extracted and remain suspended between the upper and lower cell cortex. The centrosome with microtubules extending from it is, in this cell, located in a lateral nuclear pocket (long arrow). (Magn. × 6000.)

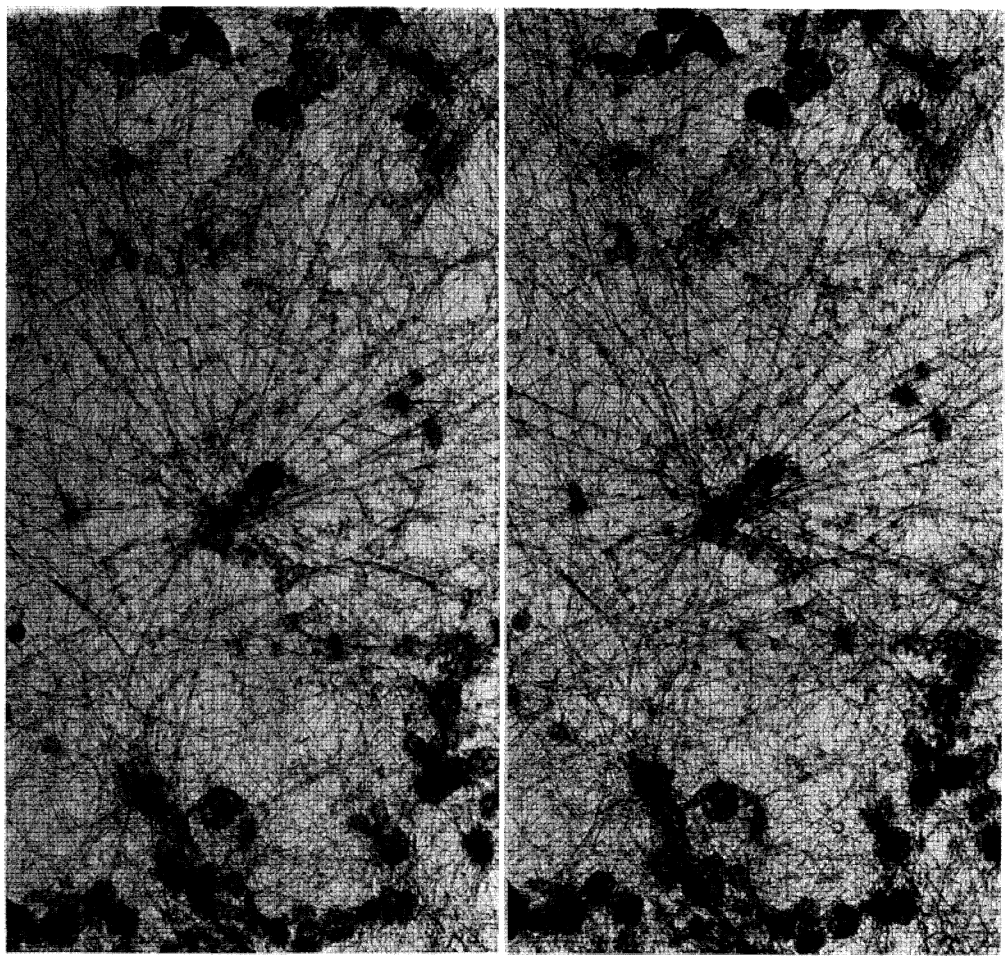


FIGURE 7. Higher magnification of a neutrophil centrosome with aster located approximately 2 μm from the nucleus. The microtubule system of this randomly migrating cell counts 37 microtubules. Also visible are several substrate-associated filament foci (arrows). (Magn. × 18000.)

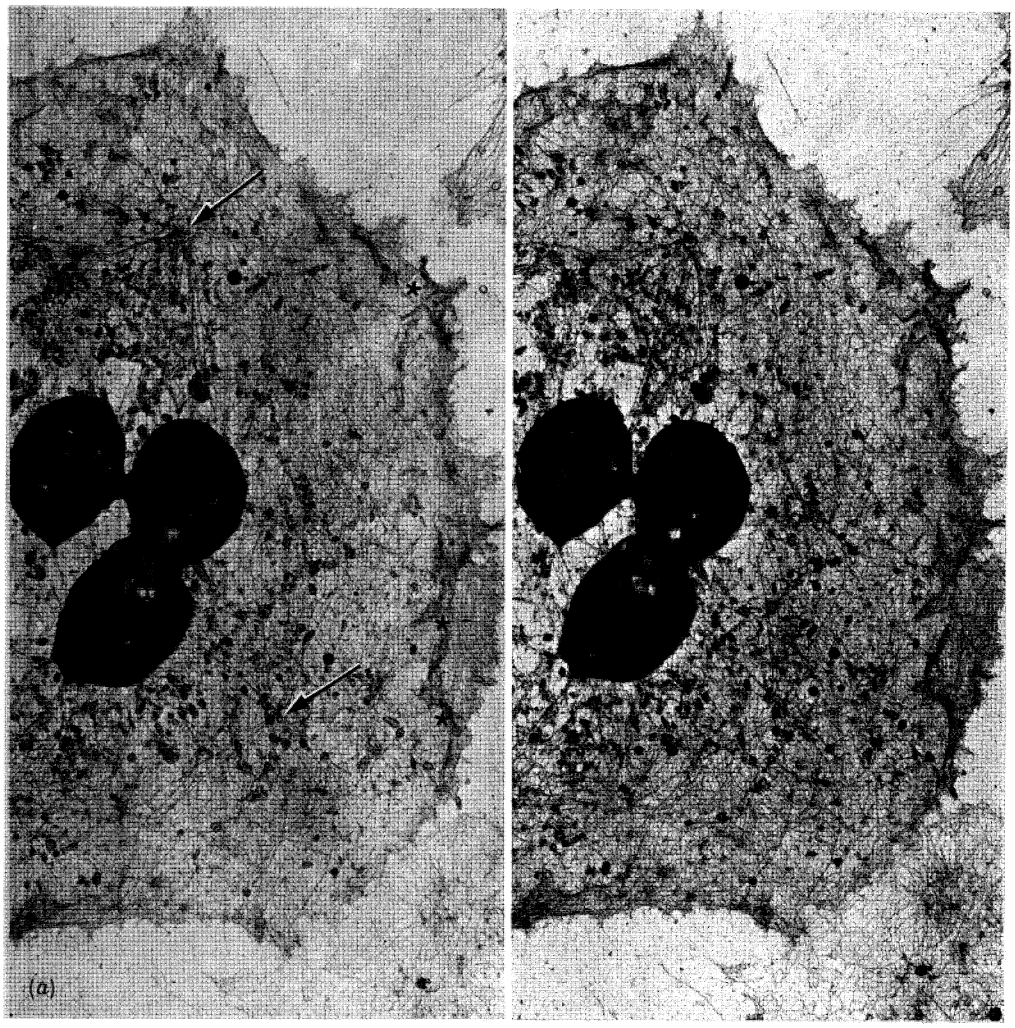


FIGURE 8. (*a*) Neutrophil after stimulation with zymosan-activated serum. The cell has substantially spread on the substratum and numerous ruffles have developed in the cell periphery (asterisks). The two centrioles with associated microtubules (arrows) are, in this cell, separated by a distance of approximately 10 μm. The two asters are shown at higher magnification in (*b*) and (*c*). (Magn. × 6500.)

(*b*, *c*) The two separated centrioles of the cell shown in (*a*) at higher magnification. (*b*) The top centriole has 25 microtubules associated with it. (*c*) The bottom centriole is surrounded by 24 microtubules. Note that essentially all microtubules associated with this centriole focus on a point at the lower end of the centriole facing the substrate. The series of small arrows denote a microtubule bent very sharply, thereby changing its course by 180°. (Magn. × 18000.)

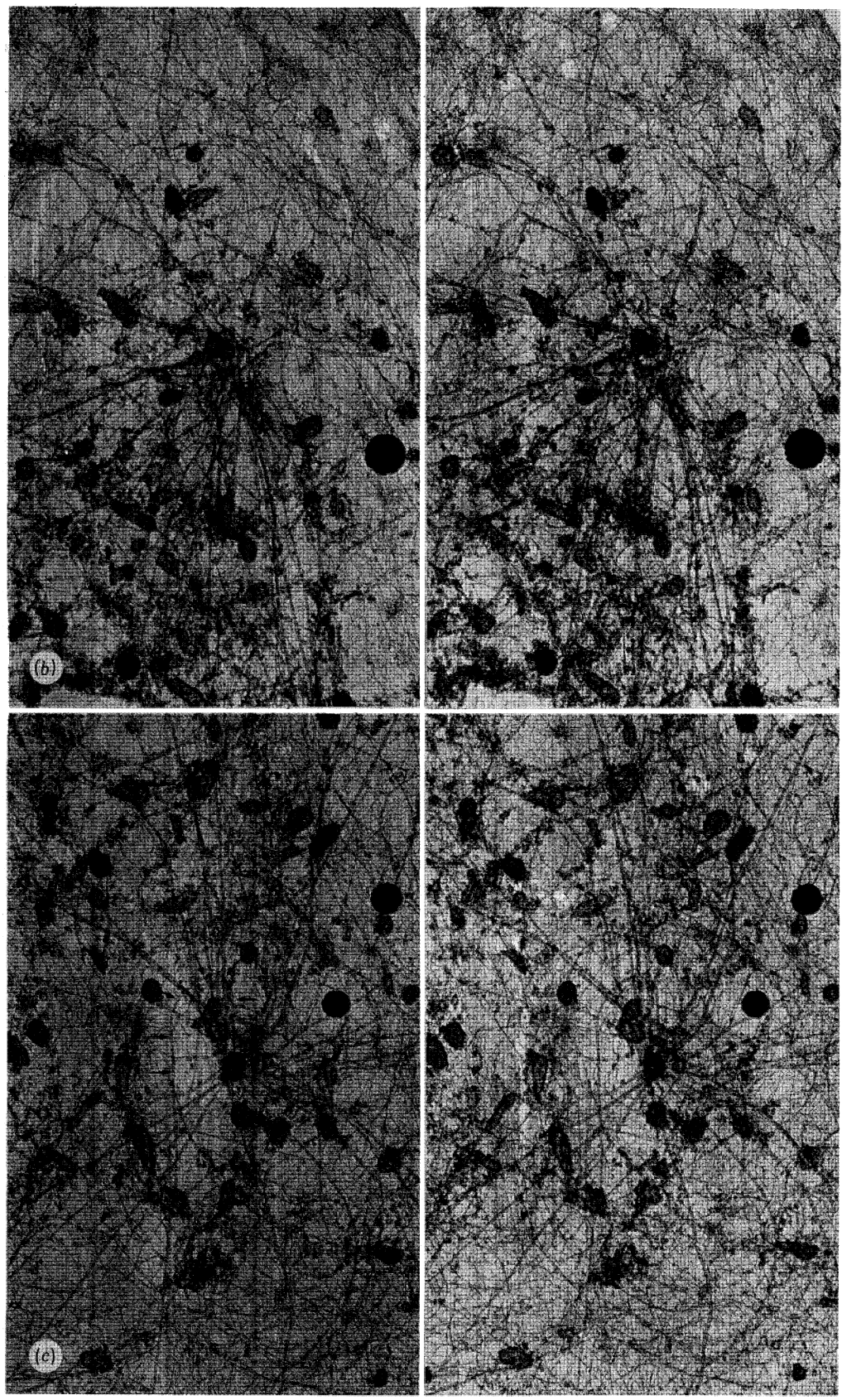


FIGURE 8 (*b*, *c*). For description see opposite.

between specific interaction mediated by a cross-linking molecule and mere apposition without interaction. We shall therefore concentrate on the latter two types of interactions.

As discussed earlier (Schliwa & van Blerkom 1981), actin filaments will associate with other actin filaments in an end-to-side fashion which in electron micrographs appear as Y- or T-shaped branching points (figure 3, plate 3). There are reasons to believe that these junctions represent a specific association between two actin filaments and not just accidental 'touching', mainly on two grounds: (i) essentially all contacts are made through the pointed filament end, as demonstrated by decoration with heavy meromyosin-subfragment 1; (ii) T-junctions have recently been demonstrated *in vitro* in mixtures of purified gelation factors and actin filaments (Hartwig *et al.* 1980; Condeelis 1981). *In vitro*, as in the cytoskeleton, contact is made through the pointed filament end. Thus there seems to be excellent agreement between studies *in vitro* with purified components and electron microscopic observations of extracted cells. It remains to be demonstrated, however, whether the junctions in cell models are likewise mediated by an *F*-actin cross-linking factor. Small filaments that could represent actin binding protein molecules linking the two actin filaments at a point of contact, as demonstrated in the rotary-shadowed *in vitro* preparations of Hartwig *et al.* (1980), have not been observed in extracted cells with the technique used here.

Unexpectedly, actin filaments also form T-junctions with microtubules and intermediate filaments. As in the case of actin–actin associations, contact with the surface of microtubules or intermediate filaments is made through the pointed filament end (figure 4, plate 3). It is worth noting that the frequency of T-junctions with microtubules and intermediate filaments is substantially lower than that with actin filaments; for every actin–intermediate filament contact there are approximately 15 actin–microtubule and 200 actin–actin junctions. It should also be mentioned that actin–actin junctions are far more abundant in the cell periphery, notably in ruffles (figure 5, plate 3). Here the actin mesh is sometimes so tightly 'woven' that the mean free path of an actin filament, i.e. the length it runs without forming a T-junction with another actin filament, is only 0.1–0.2 μm!

The nature of the end-to-side interactions of actin filaments with microtubules and intermediate filaments remains to be determined. *In vitro*, microtubules will interact with actin filaments to form a gel if microtubule-associated proteins are present (Griffith & Pollard 1978), but the morphology of this interaction is only poorly defined. As with actin–actin junctions, immunoelectron microscopy should help answer the question whether this intriguing example of association involves specific molecules.

Cytoskeletal components may also interact via a different class of structures represented by short, thin filaments of about 3 nm diameter and up to 300 nm length. They seem to act exclusively as linkers of other filaments; rarely are they linked to one another in a Y pattern. If they are, it is in such a way that two of the branches make contact with the surface of one filament (e.g. an intermediate filament) while the third is connected to another (e.g. a microtubule). They are only defined as a structural entity, and the possibility that they are not a homogeneous class of filaments by biochemical criteria can not be excluded. Attempts to extract these filaments selectively from cytoskeletons and to determine their composition have failed so far.

The 3 nm filaments have been found to link the major cytoskeletal fibres in every conceivable combination. They are most frequently associated with intermediate filaments and less so with actin (table 1). Further analysis of the 'pairing frequencies' shows that they prefer certain

combinations (table 2): linkages of intermediate filaments with microtubules and other intermediate filaments are by far the most frequent, whereas actin–microtubule connections are the rarest, representing only 5 % of all links observed.

Our ignorance of the biochemical composition of 3 nm filaments makes a consideration of the nature of these linkages very difficult. Theoretically, 3 nm filaments could represent molecules of actin-binding protein (filamin) or myosin; the former has been shown to be a highly

TABLE 1. ASSOCIATION OF 3 nm FILAMENTS WITH ACTIN FILAMENTS, MICROTUBULES AND INTERMEDIATE FILAMENTS

(The number of associations of 3 nm filaments with the major cytoskeletal filaments was determined in cell regions of BSC-1 cells at least 5 μm peripheral to the nucleus and about 5 μm inside the cell perimeter.)

actin filaments	81 (18 %)
microtubules	147 (32 %)
intermediate filaments	228 (50 %)

TABLE 2. FREQUENCIES OF FILAMENT COMBINATIONS LINKED BY 3 nm FILAMENTS

(Counts were made in the same cell region as specified in table 1.)

actin–actin	41 (9 %)
actin–intermediate filament	55 (12 %)
actin–microtubule	24 (5 %)
microtubule–intermediate filament	170 (37 %)
microtubule–microtubule	50 (11 %)
intermediate filament–intermediate filament	116 (25 %)

flexible, asymmetric molecule of up to 160 nm length (Hartwig & Stossel 1981). However, the apparent sparsity of 3 nm filaments in association with *F*-actin makes this difficult to believe, particularly since they are rarely found in ruffles, cell regions with the most highly interconnected actin filament complex. Another possibility is that there are different classes of 3 nm filaments forming different types of linkage, and that they differ biochemically despite similar morphology. Whatever their biochemical nature, the apparent preference for the microtubule–intermediate filament complex is remarkable. On the basis of this preference, it is tempting to speculate that they are responsible for the apparent association of these two major cytoskeletal components noted in previous studies (e.g. Wang & Goldman 1978; Geiger & Singer 1981). The abundance of 3 nm crosslinks between microtubules and intermediate filaments may explain why the organization of the latter is profoundly affected when microtubules are depolymerized: the vimentin filament network collapses into a perinuclear aggregate or cap (see, for example, Gordon *et al.* 1978; Franke *et al.* 1979). It will be of interest to analyse the time course of this process with the methods used here, and to determine why only the vimentin and not the pre-keratin network is affected so profoundly by the removal of microtubules (Osborn *et al.* 1980).

The precise role(s) of the various types of associations between cytoskeletal filaments remain a matter of speculation. To start with, they can be expected to consolidate the cytoskeletal network and to increase cytoplasmic rigidity. However, it would be dangerous to conclude that the cytoskeleton is a rigid framework. Often static electron micrographs can make one forget the dynamics of many cellular events in which cytoskeletal complexes are involved (e.g. cell spreading, locomotion, intracellular transport, ruffling). Consider, for instance, that the struc-

ture that appears most highly interconnected (through actin–actin T-junctions), the ruffle, is also one of the most dynamic and flexible cytoskeletal assemblies! Secondly, structural interaction may also be considered as a means of transmitting information or signals from one cell region to another. 'Cross-talking' is the term we have used previously in an attempt to outline such a role (Schliwa & van Blerkom 1981). Thus this highly interconnected continuum may serve as a vehicle for the transmission of stimuli from the cell periphery to the cell centre, including the nucleus, and may perform morphological changes in response to these stimuli. If this view were correct, then the cytoplasmic network would function as a receptor and transducer of stimuli as well as an effector of cell morphological and contractile responses.

Stimulus–motility coupling: description of an unusual phenomenon in polymorphonuclear leucocytes

As an example of a concerted interaction between the cell surface and the cytoskeleton that could serve to illustrate the presumptive role of interactions just mentioned, we shall discuss briefly a phenomenon related to the activity of human polymorphonuclear leucocytes (neutrophils). Neutrophils are highly motile, amoeboid cells that function in host defence against bacterial and fungal infections. Because of the importance of the cytoskeleton in a variety of neutrophil activities, the motility of these cells has been the subject of numerous studies (summaries are given by Zigmond (1977) and Gallin (1980)). Neutrophils migrating on artificial substrates assume a definite orientation. The leading edge is characterized by a lamellipodium with numerous ruffles; the nucleus is located in the cell centre or in the posterior cell portion, while the bulk of the secretory granule-containing cytoplasm resides between the nucleus and the leading edge. Tail or retraction fibres extend from the rear (uropod) of the cell. As a rule, three different conditions or states of activation of neutrophils migrating *in vitro* are distinguished: (i) random migration in media without additions, (ii) activated random migration or chemokinesis in media containing a uniform concentration of chemoattractant, and (iii) chemotaxis along a gradient of chemoattractant. Here we are concerned with the transition from random migration to chemokinesis (i → ii).

Figure 6, plate 4, is an overview of a neutrophil kept under conditions of random migration before extraction and fixation. A more complete account of the cytoskeletal organization of these cells is beyond the scope of this paper and will be given elsewhere (Pryzwansky *et al.* 1982). Suffice it to say for the purpose of this paper that the clarity with which the microtubule system is displayed in these preparations attracted our attention. For the first time it seemed possible to obtain an overview of the centrosome and essentially all microtubules of a single cell at the electron-microscopic level, with all the detailed information provided by this method (figure 7, plate 5). It is the organization of the centrosome that is dramatically altered when randomly migrating cells are activated by the addition of a chemoattractant, for instance a synthetic chemotactic peptide or zymosan-activated serum. In addition to substantial flattening and spreading of the cells on the substrate (Zigmond & Sullivan 1979), a variable number of cells exhibit separation of the centrosome into two solitary centrioles, each associated with an aster of microtubules (figure 8, plates 6 and 7). Centrioles may separate for a few micrometres only, but quite frequently the two asters may be found at almost opposite ends of the cell, as shown in figure 8. Centrosome splitting is accompanied by increased ruffling activity that seems to be even more accentuated along that portion of the cell margin that is next to asters. This and

other morphological criteria to be outlined in more detail elsewhere (Schliwa *et al.* 1982) have led us to believe that the cell appears to attempt to migrate in two directions at the same time. This attempt seems to be coupled with a profound reorganization of the internal cell architecture, including the cytoplasmic microtubule system, a reorganization that culminates in some cells in the establishment of two seemingly independent asters each centred around a single centriole. Thus an interrelation may exist between the direction of cell migration and the orientation of the microtubule system. In fact, it is likely that in cells migrating in a chemotactic gradient, the centrosome attains a position between the nucleus and the leading front (Bessis & Breton-Gorius 1967; Malech *et al.* 1977). The observation that centrioles and associated microtubule asters may separate in cells exposed to a sudden increase in the concentration of a chemoattractant acting from all sides on the cell may suggest that the cell is temporarily confused and cannot decide which way to move.

It is not entirely clear from our studies whether the profound reorganization of the microtubule system is a consequence of cell movement or whether it determines the direction of movement and thus actually precedes locomotion. Two observations suggest, however, that the latter may be correct. Firstly, a rapid increase in the concentration of a chemoattractant stops cell locomotion while at the same time inducing flattening and spreading (Zigmond & Sullivan (1979), and our own observations). Thus, at the time when centrosome separation occurs the cells are not translocating over the substrate. Secondly, a reversal of the direction of a chemotactic gradient induces a dramatic reorganization of internal morphology, including a shift in the position of the centrosome, even under conditions where the cells do not locomote (Malech *et al.* 1977). Thus relocation of the centrosome can occur in the absence of significant cell translocation.

Although the precise sequence of events leading to this unique phenomenon remains to be determined, it represents in our opinion an example of a coordinated interaction between cell membrane receptors, the contractile system and the microtubule complex. The molecular details of the linkage of these three systems are not known, and more experiments are needed. Our studies call attention to the centrosome and microtubules as important factors in the regulation of cell orientation and locomotion.

Finally, we wish to stress that the ability of centrioles of a single pair to separate by several micrometres is very interesting in its own right. Centrioles of interphase cells are commonly envisaged as being closely associated with the nucleus; this association is so tight that the two can be isolated together as a centrosome–nucleus complex (see, for example, Bornens 1977; Nadezhdina *et al.* 1978). More importantly, they always come as a tightly linked unit, the diplosome. Bessis and coworkers (Bessis & Locquin 1950; Policard & Bessis 1953; Bessis & Breton-Gorius 1967; Breton-Gorius 1968) have called attention to the observation that the centrosome of neutrophils is a highly active, movable organelle that can be located several micrometres away from the nucleus. They have also described centriole separation in 'resting' cells. Unfortunately, their initial observations were not followed up. It is hoped that future studies with the technique applied here will help understand cause, mechanism and function of centriole separation.

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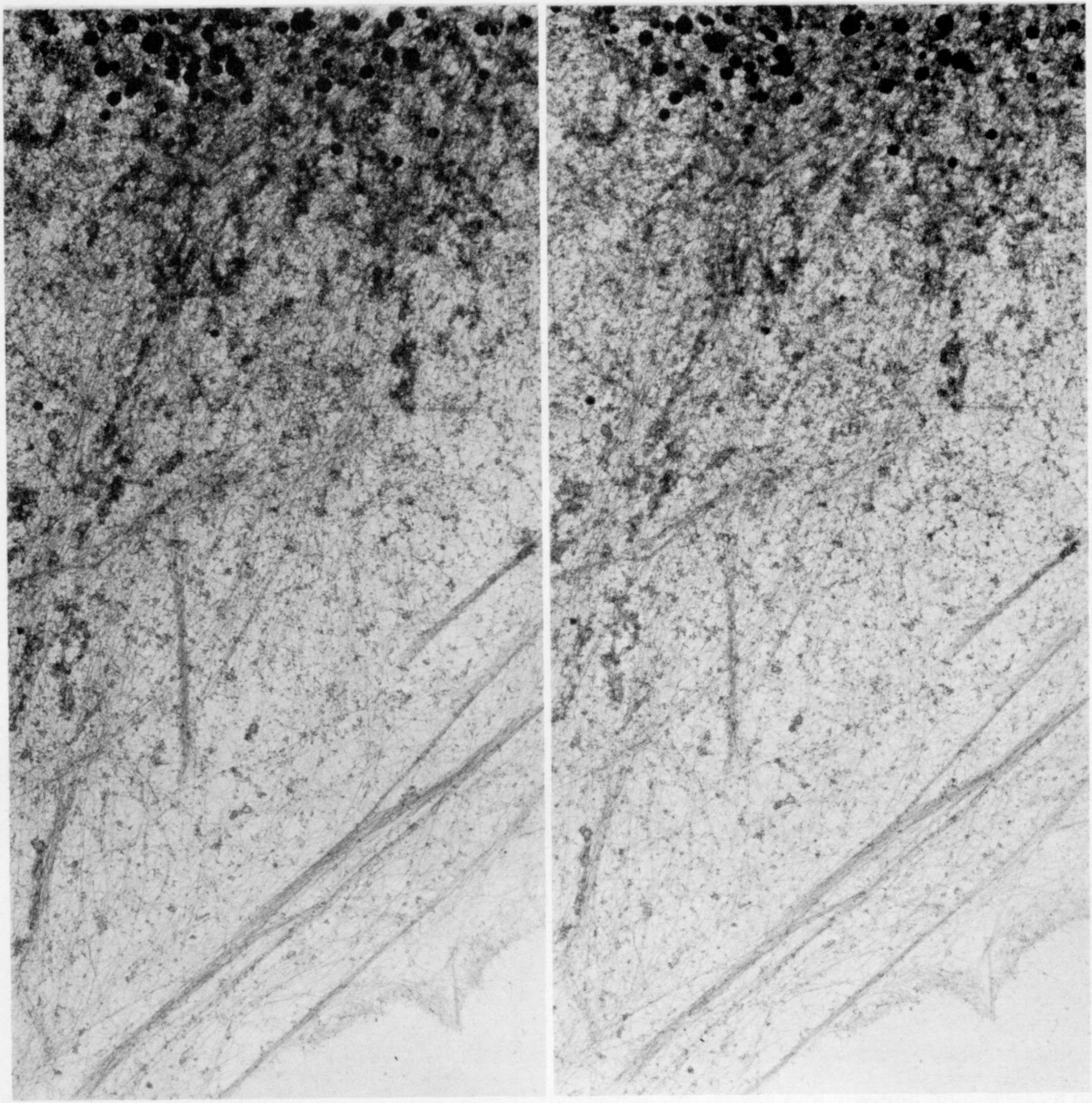

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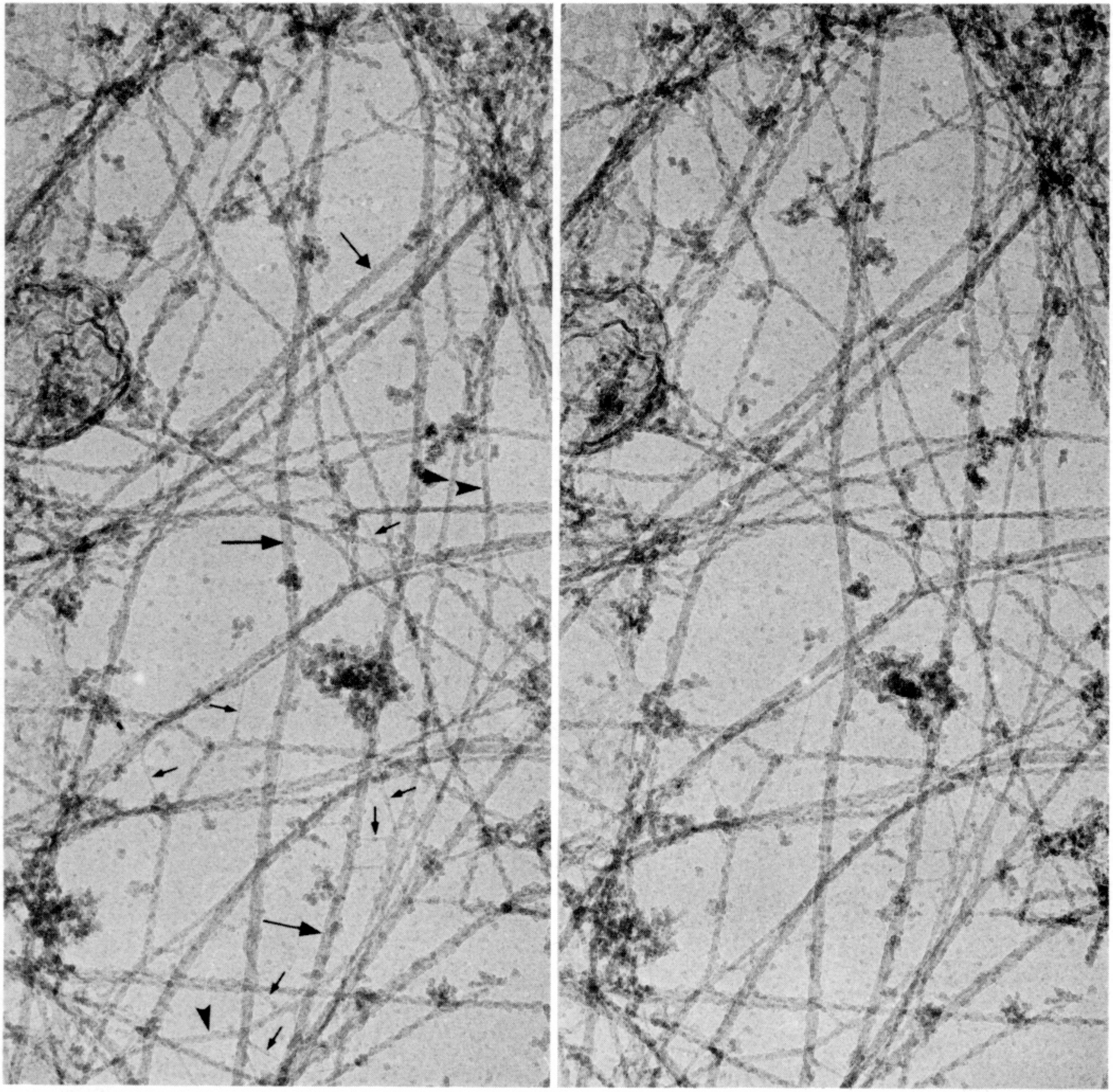
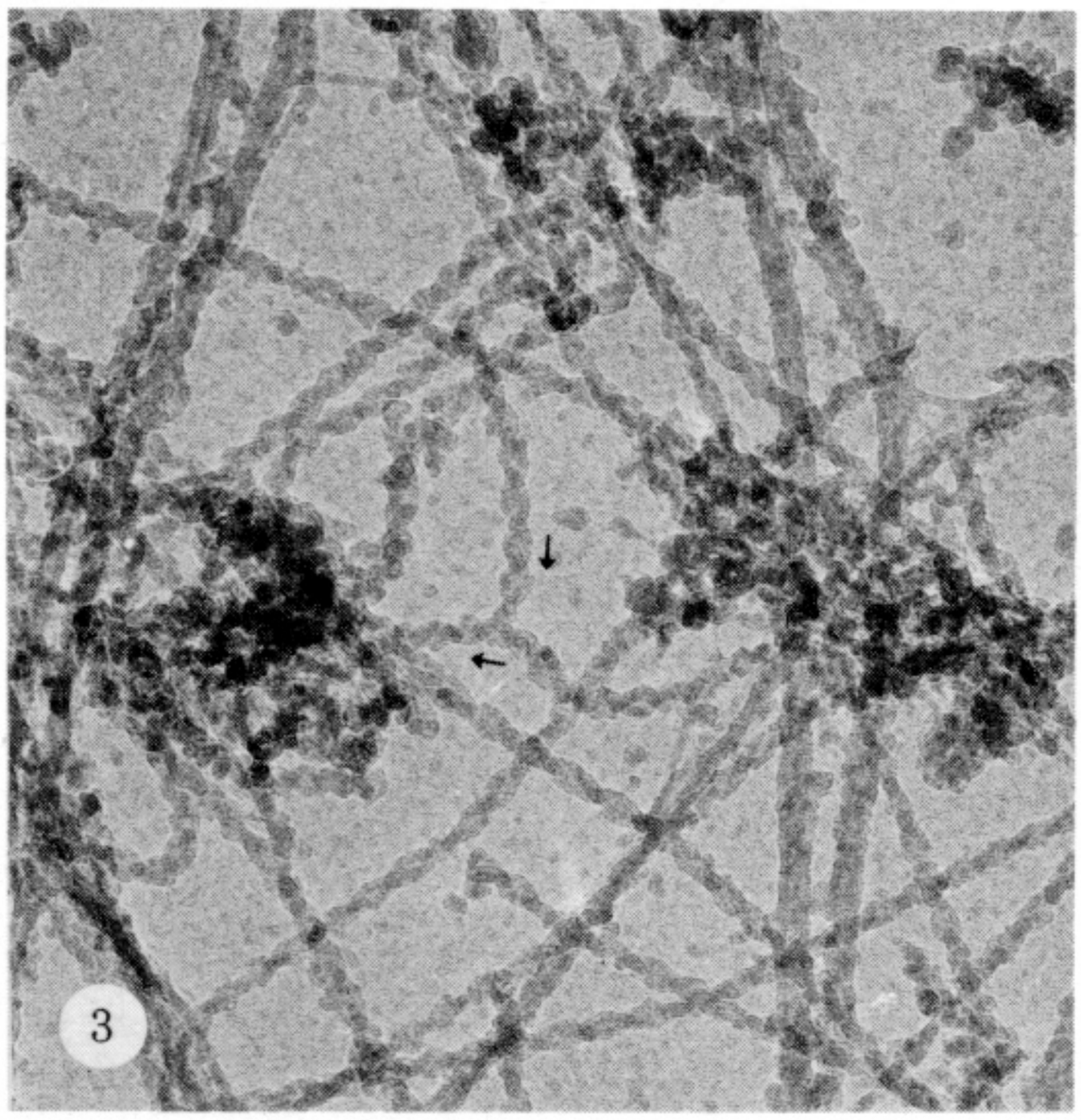
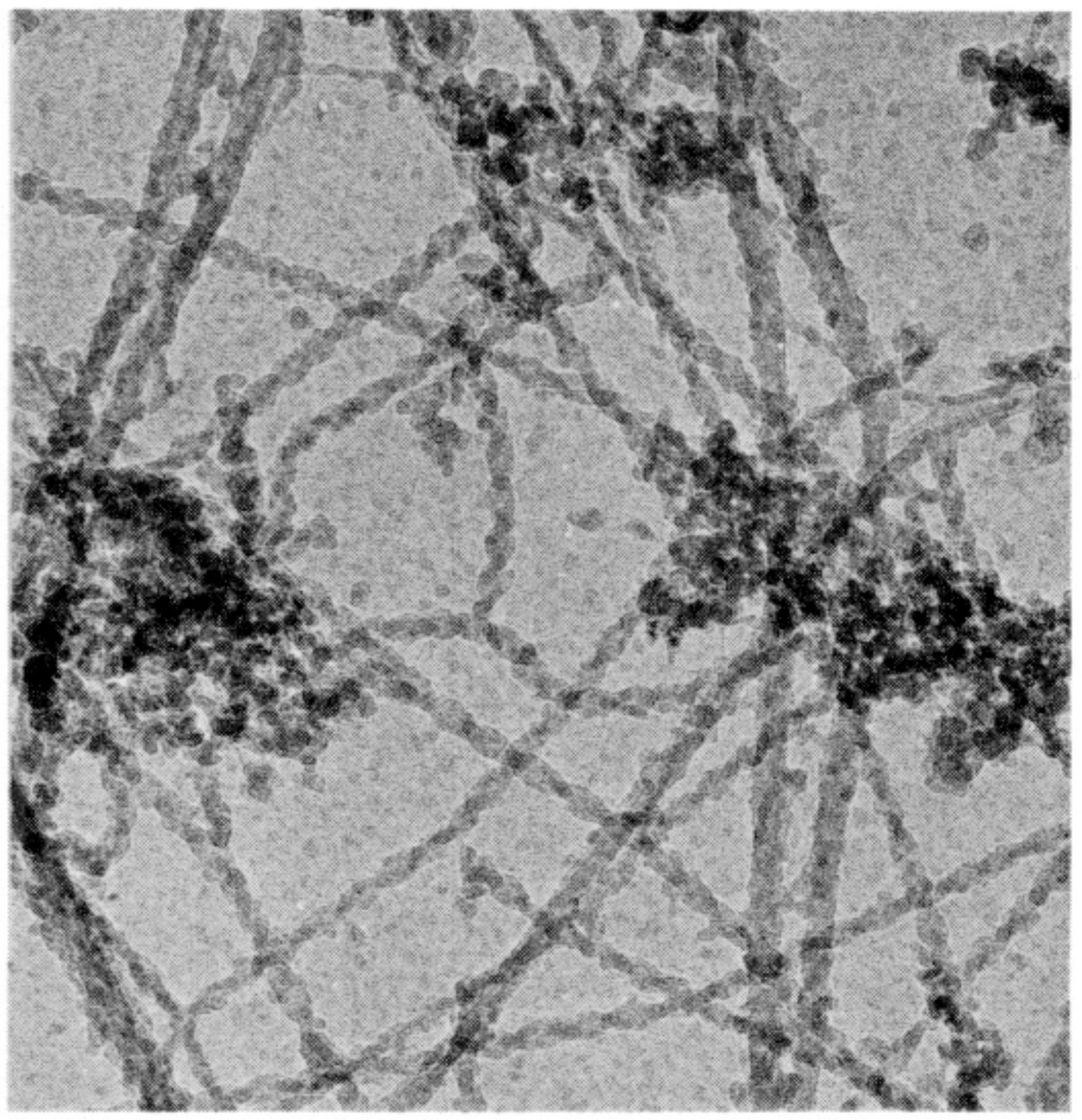
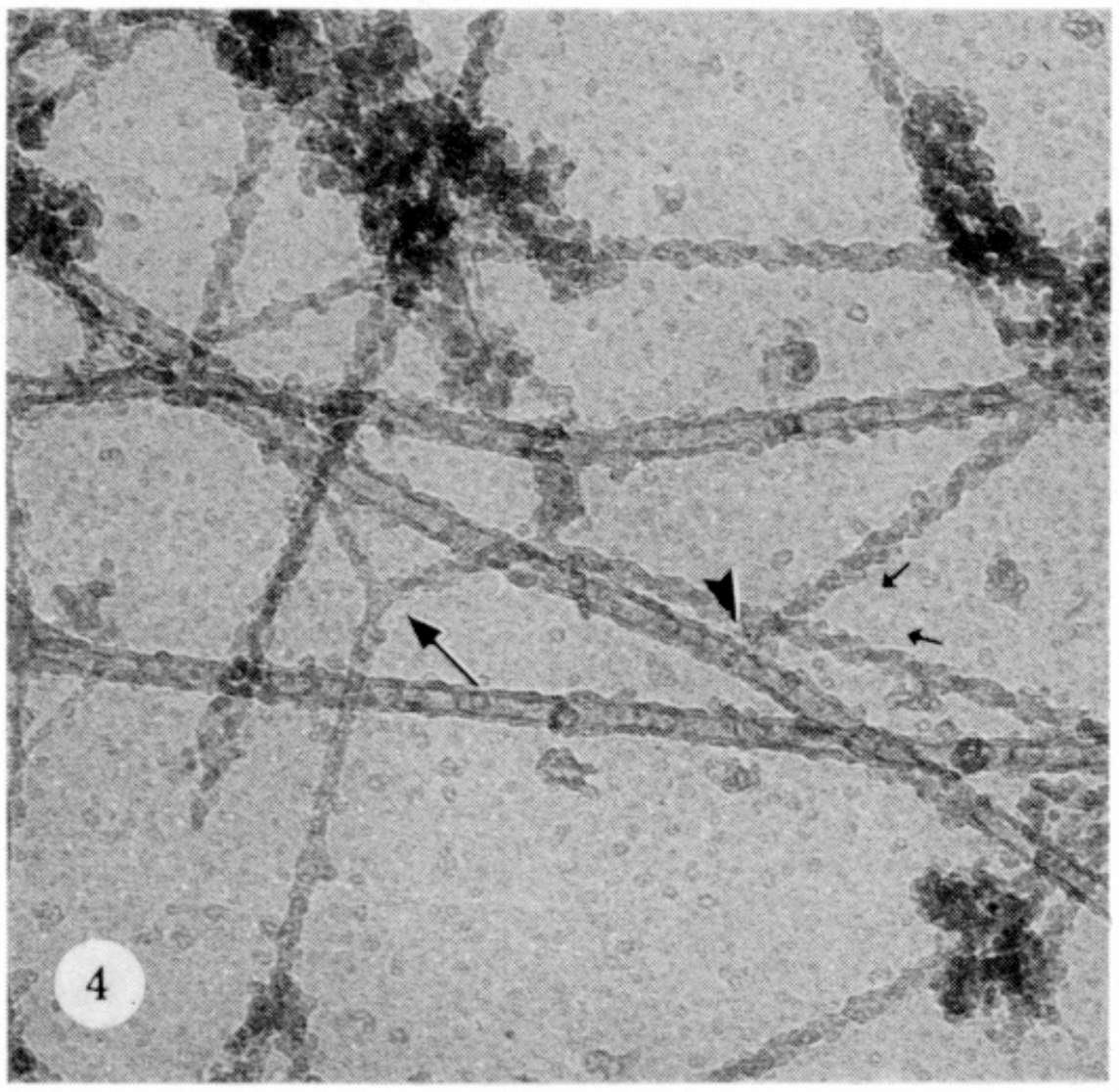
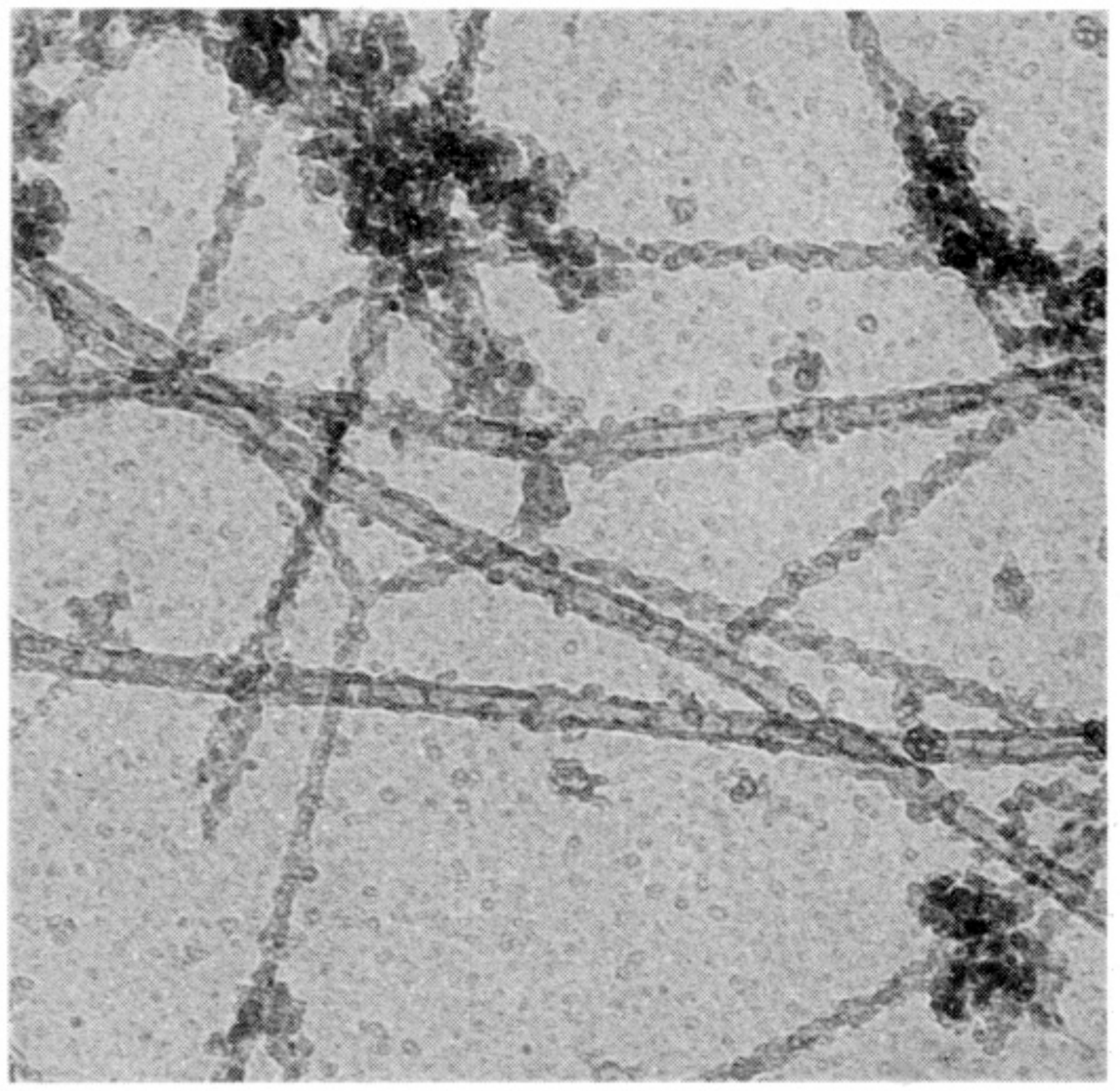


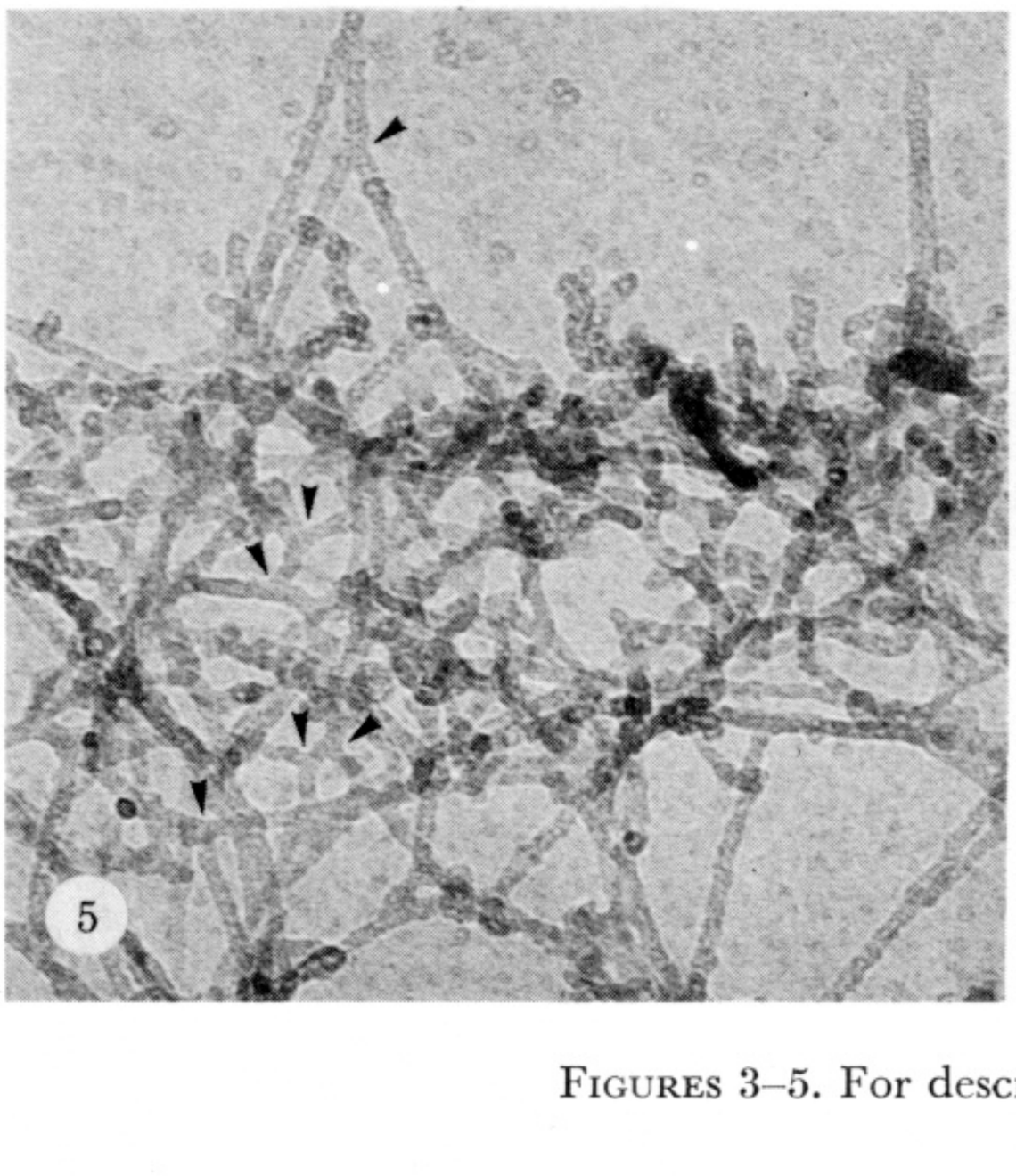
FIGURE 2. Cytoskeletal network in a cell region half-way between the nucleus and the pheriphery. Actin filaments are decorated with heavy meromyosin – subfragment 1 to distinguish them from unlabelled intermediate filaments (arrowheads), and to visualize their polarity. Note the presence of numerous 3 nm filaments (small arrows). Also shown are microtubules (large arrows). (Magn. × 90000.)

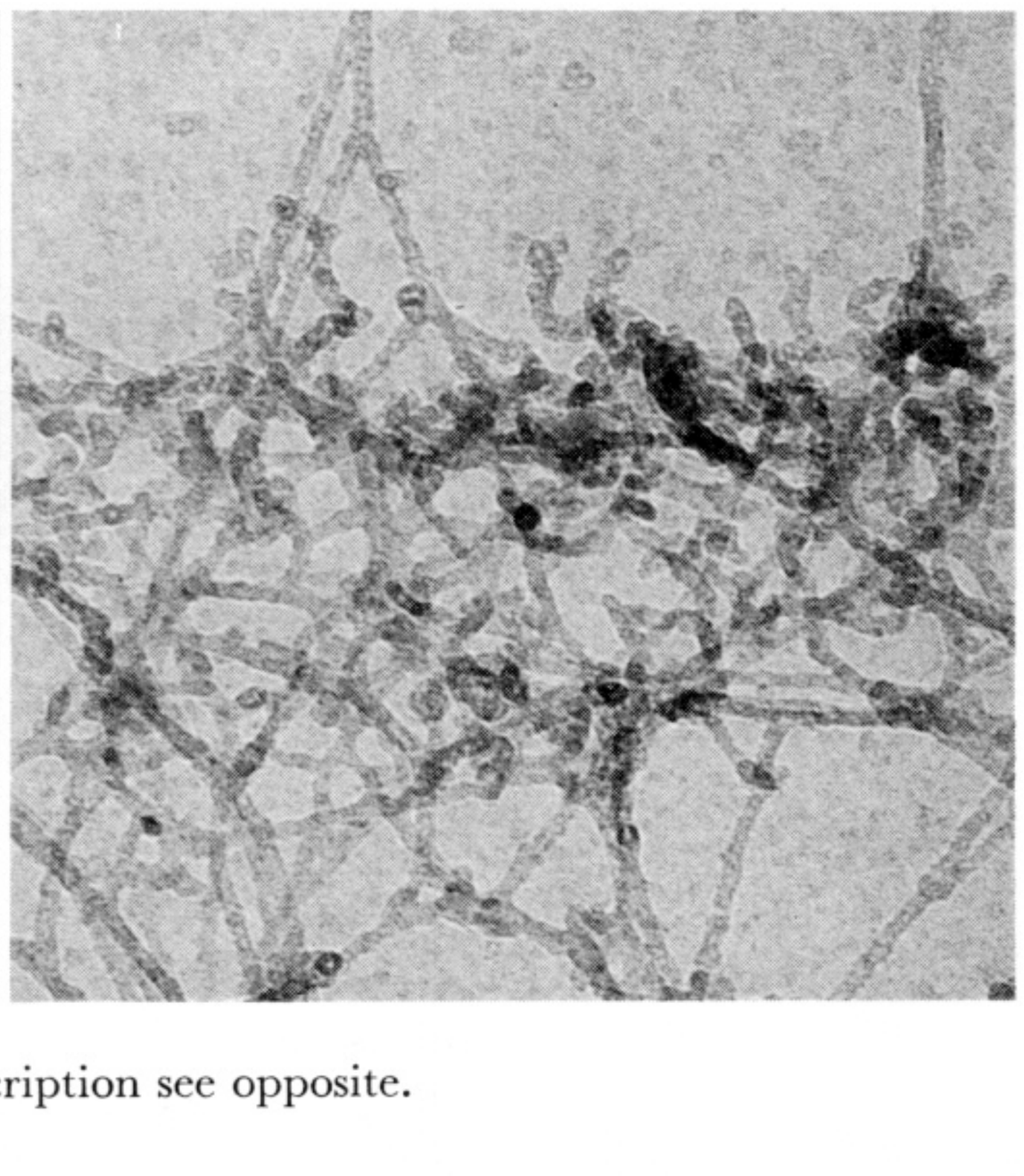












FIGURES 3–5. For description see opposite.

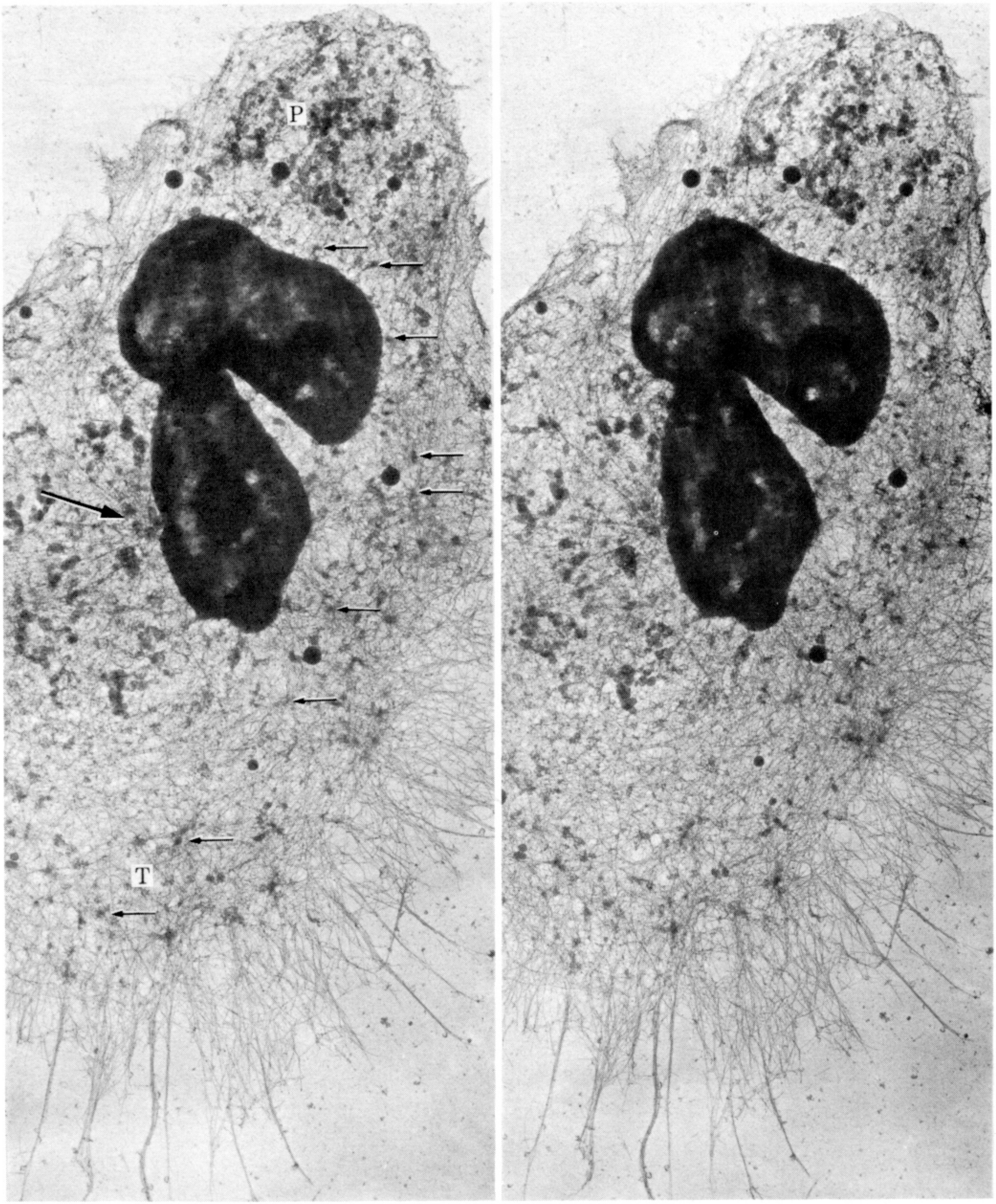


FIGURE 6. Overview of an extracted neutrophil showing the major cytoskeletal domains: advancing pseudopod with ruffles extending from it (P); cell posterior with tail fibres (T); and substrate-associated dense foci (arrows). Many granules, which probably correspond to the azurophils, are not completely extracted and remain suspended between the upper and lower cell cortex. The centrosome with microtubules extending from it is, in this cell, located in a lateral nuclear pocket (long arrow). (Magn. × 6000.)

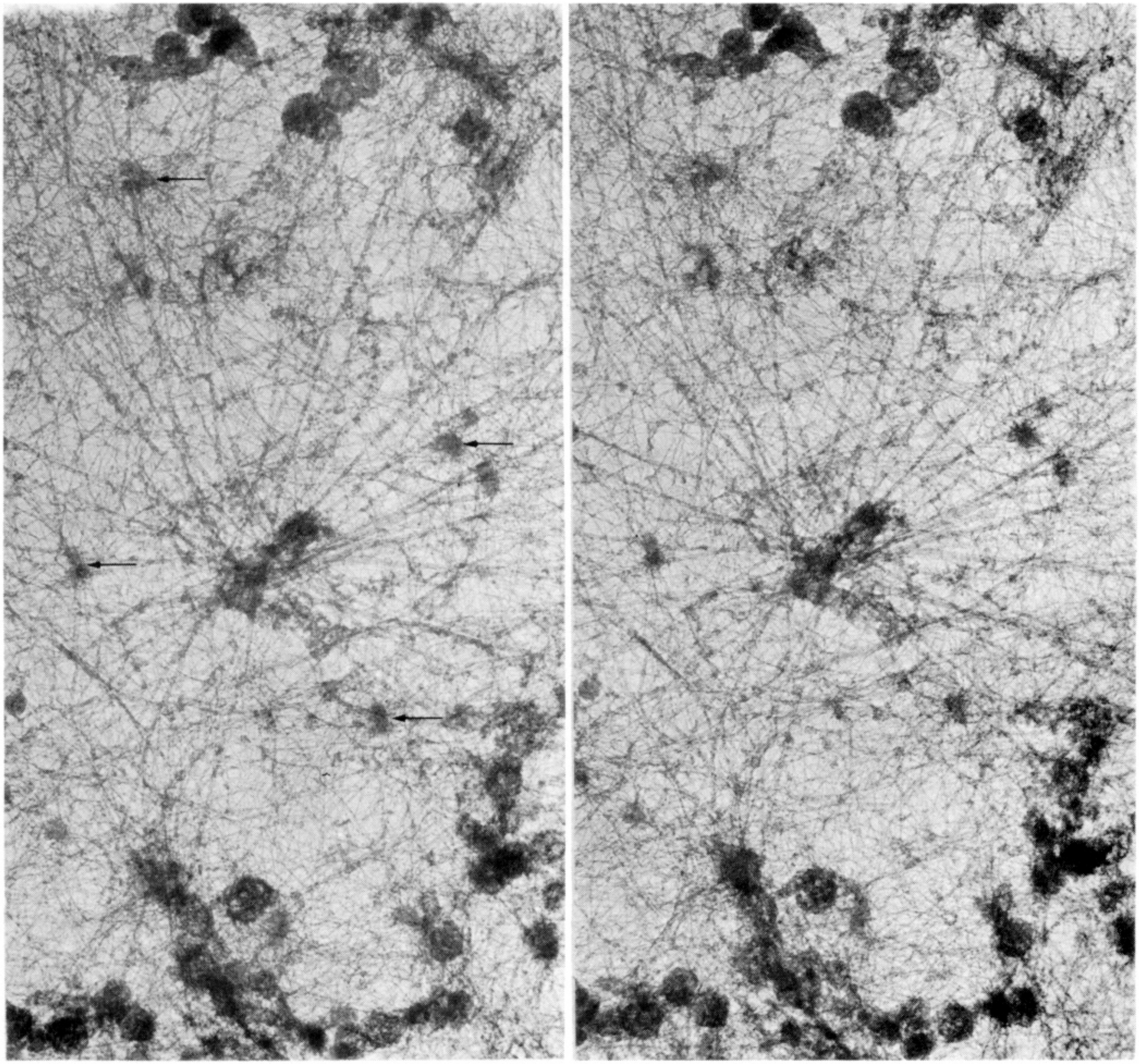


FIGURE 7. Higher magnification of a neutrophil centrosome with aster located approximately 2 μm from the nucleus. The microtubule system of this randomly migrating cell counts 37 microtubules. Also visible are several substrate-associated filament foci (arrows). (Magn. × 18 000.)

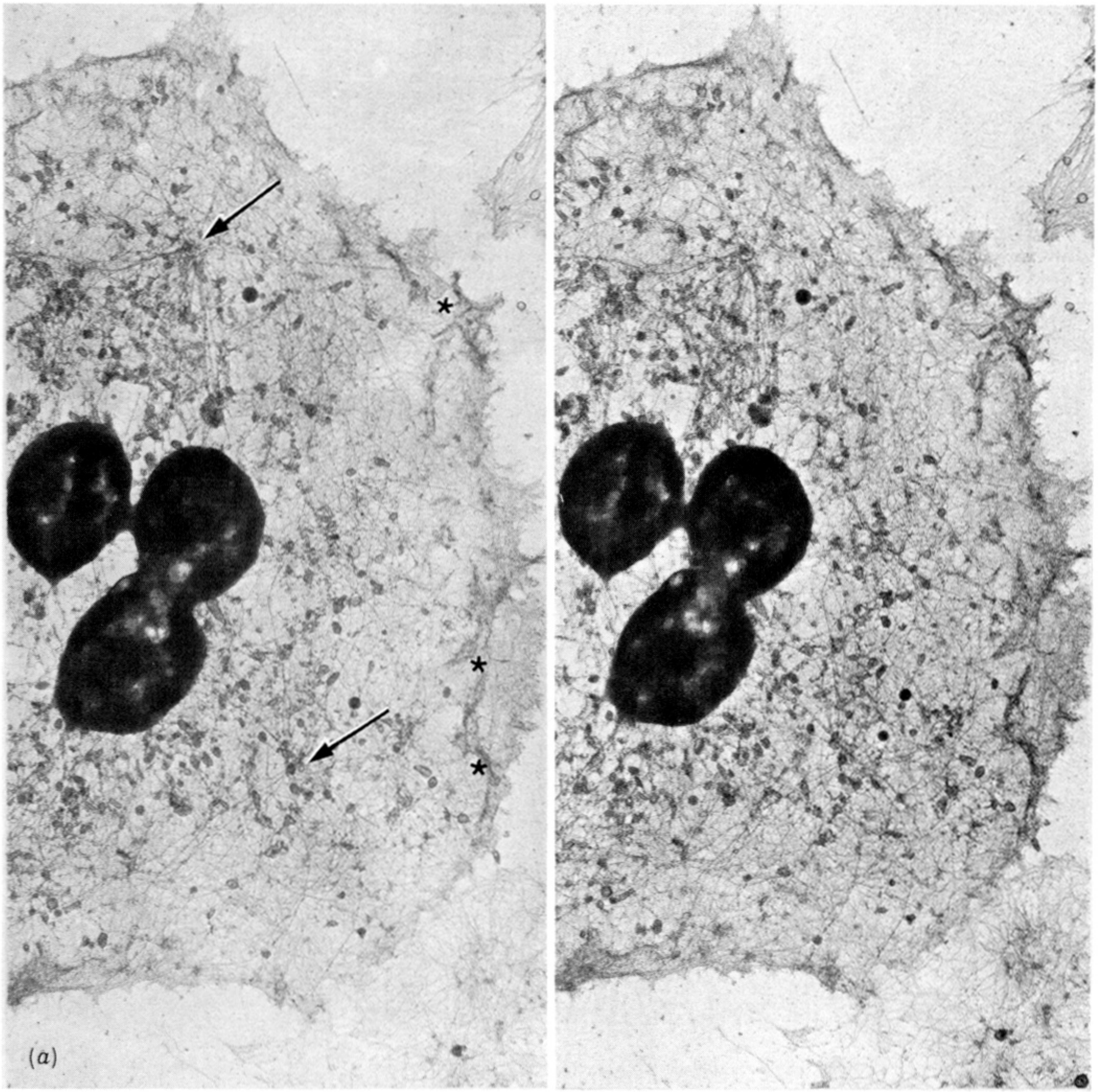


FIGURE 8. (*a*) Neutrophil after stimulation with zymosan-activated serum. The cell has substantially spread on the substratum and numerous ruffles have developed in the cell periphery (asterisks). The two centrioles with associated microtubules (arrows) are, in this cell, separated by a distance of approximately 10 μm. The two asters are shown at higher magnification in (*b*) and (*c*). (Magn. × 6500.)

(*b*, *c*) The two separated centrioles of the cell shown in (*a*) at higher magnification. (*b*) The top centriole has 25 microtubules associated with it. (*c*) The bottom centriole is surrounded by 24 microtubules. Note that essentially all microtubules associated with this centriole focus on a point at the lower end of the centriole facing the substrate. The series of small arrows denote a microtubule bent very sharply, thereby changing its course by 180°. (Magn. × 18000.)

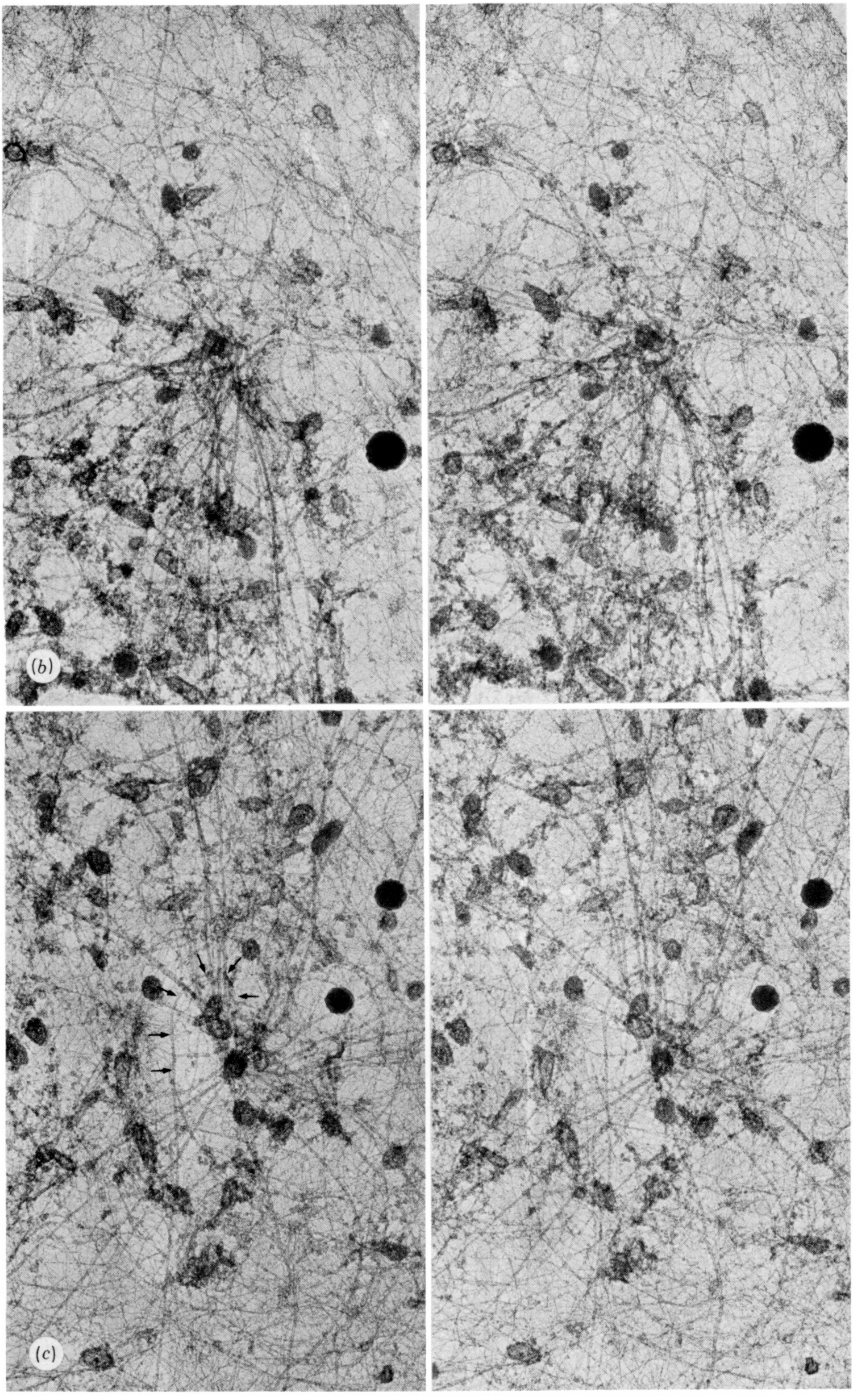


FIGURE 8 (*b*, *c*). For description see opposite.