

CHANGING PATTERNS OF PLASMA MEMBRANE-ASSOCIATED FILAMENTS DURING THE INITIAL PHASES OF POLYMORPHONUCLEAR LEUKOCYTE ADHERENCE

JANET BOYLES and DOROTHY F. BAINTON

From the Department of Pathology, University of California, San Francisco, California 94143

ABSTRACT

By utilizing a combination of several ultrastructural techniques, we have been able to demonstrate differences in filament organization on the adherent plasma membranes of spreading and mobile PMN as well as within the extending lamellipodia. To follow the subplasmalemmal filaments of this small amoeboid cell during these kinetic events, we sheared off the upper portions of cells onto glass and carbon surfaces for 30 s–5 min. The exposed adherent membranes were immediately fixed and processed for high-resolution SEM or TEM. Whole cells were also examined by phase contrast microscopy, SEM, and oriented thin sections. Observed by SEM, the inner surface of nonadherent PMN membranes is free of filaments, but within 30 s of attachment to the substrate a three-dimensional, interlocking network of globular projections and radiating microfilaments—i.e., a subplasmalemmal filament complex—is consistently demonstrable (with or without postfixation in OsO₄). Seen by TEM, extending lamellipodia contain a felt of filamentous and finely granular material, distinct from the globule/filament complex of the adjacent adherent membrane. In the spread cell, this globule-filament complex covers the entire lower membrane and increases in filament-density over the next 2–3 min. By 3–5 min after plating, as the PMN rounds up before the initiation of amoeboid movements, another pattern emerges—circumferential bands of anastomosing filament bundles in which thick, short filaments resembling myosin are found. This work provides structural evidence on the organization of polymerized contractile elements associated with the plasma membrane during cellular adherence.

KEY WORDS PMN · microfilaments · adherence · SEM · plasma membrane

Granulocyte adherence is an important step in the development of the acute inflammatory reaction in host defense. Circulating heterophilic polymorphonuclear leukocytes (PMN) must adhere to ves-

sel walls before they can emigrate into tissues where they function as phagocytes. When collected in suspension from peritoneal exudates, the cells rapidly and tenaciously adhere in vitro to many artificial surfaces, in a manner similar to their behavior in vivo. Because of such properties, they are an ideal small amoeboid cell for exam-

ining the early events of adherence, and specifically the interaction between contractile filaments and the plasma membrane.

In PMN, localized polymerization of actin has been proposed to occur on the plasma membrane in response to adherence (2), possibly in conjunction with actin-binding protein (4). The assembly of myosin filaments and their interaction with polymerized actin would then supply the contractile force for motility (39). Biochemical evidence of the participation of contractile proteins in adherence and locomotion has been provided by isolating these proteins from PMN (2, 4, 39). In addition, by the use of heavy meromyosin and antibodies, respectively, both actin filaments and myosin have been observed to be associated with the plasma membrane (2, 31, 35) as well as with the cytoplasm (2, 4, 36). Actin-binding protein has been localized on the plasma membrane by antibody techniques (3). Yet the organization of these contractile elements and any alterations of organization with changing cellular activities remain unknown.

In the present study of PMN adherence, a novel

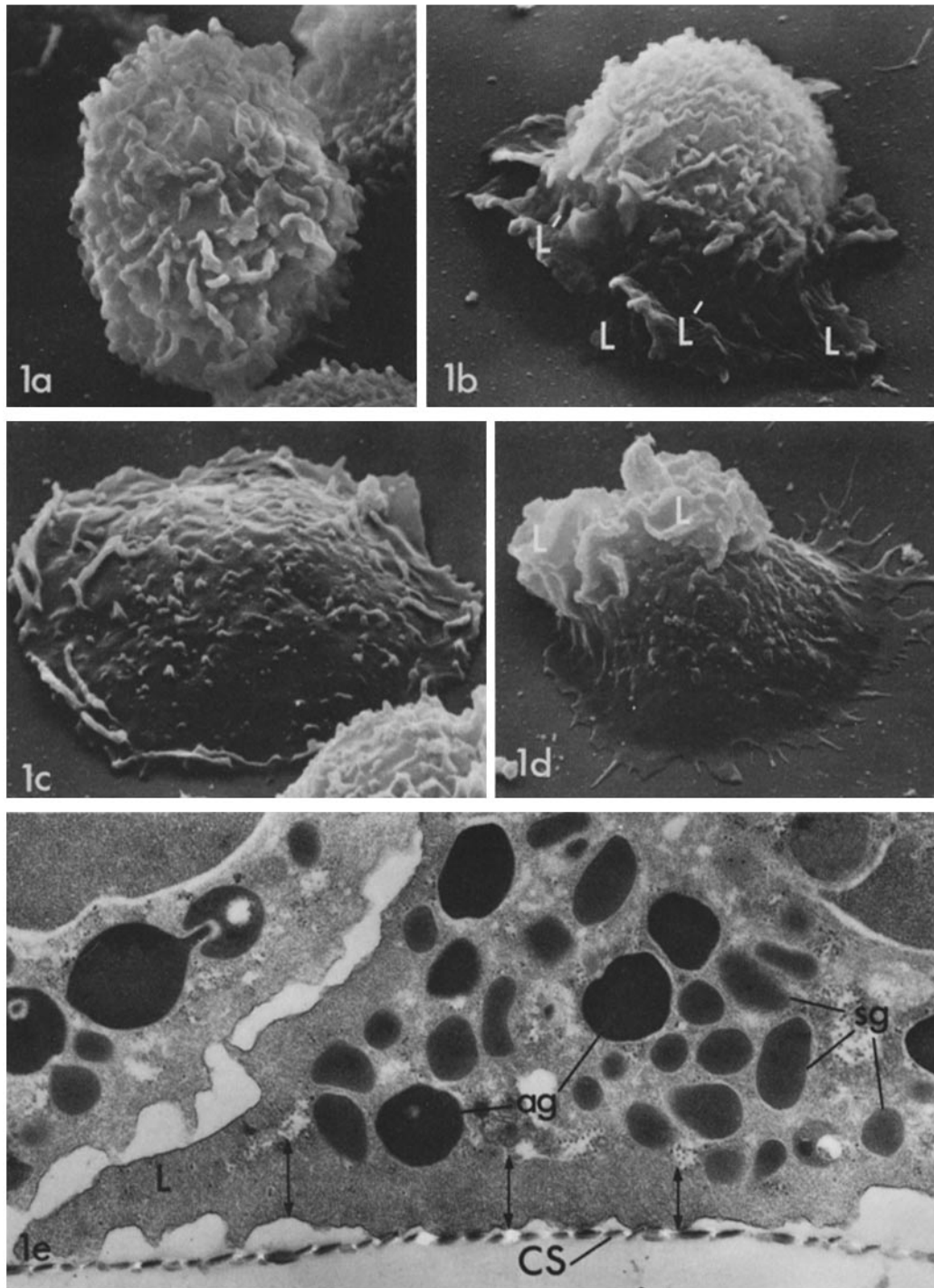
technique was utilized and adapted to this cell type: a procedure used by Clarke et al. (8) with *Dictyostelium discoideum* amoebae. After rupturing the amoebae with a shearing jet of buffered salt solution, these investigators were able to demonstrate actin-containing filaments on the exposed cytoplasmic surfaces of adherent membranes. Similarly, we have followed the cytoplasmic filaments which associate with the plasma membranes of PMNs using the three dimensionality of high-resolution scanning electron microscopy (SEM) and transparency of critical-point-dried material in transmission electron microscopy (TEM). Parallel samples of adhering PMN were also scrutinized by phase-contrast microscopy and fixed for conventional thin-sectional TEM and whole-cell SEM, so that the structures observed on the exposed plasma membranes could be correlated with the more common aspects of cellular ultrastructure and specific cellular activities.

Using these multiple techniques with PMN, we were able to view the changing patterns of filamentous structures which are associated with the plasma membrane during lamellipodial extension

Key to Symbols

<i>ag</i>	azurophil granule	<i>g</i>	granules
<i>b</i>	band of filaments	<i>L</i>	lamellipodium
<i>CMS</i>	cytoplasmic membrane surface	<i>pm</i>	plasma membrane
<i>CS</i>	carbon surface	<i>r</i>	ribosomes
<i>EMS</i>	external membrane surface	<i>S</i>	empty sac of membrane
<i>f</i>	filaments	<i>sg</i>	specific granule

FIGURE 1 (a-d) Scanning electron micrographs of rabbit exudate PMN. (a) A cell fixed in suspension is round and studded with short, irregular projections and membrane folds. $\times 8,000$. (b) A PMN allowed to adhere for 1-2 min to a carbon-coated coverslip. Waves of lamellipodia or "ruffles" radiate from the spreading cell, both at the point of contact (*L*) and above (*L'*), as the cell flattens itself against the carbon surface. $\times 7,000$. (c) A cell after 2-3 min contact with a glass coverslip. This PMN has fully spread to a flattened hemisphere. (Not all cells spread to such an extreme but all at least become hemispheres.) Ruffles no longer rim the cellular border; only ridges of membrane are found there and the remainder of the surface is smoother than in cells from earlier intervals. $\times 5,000$. (d) A PMN plated onto a glass coverslip for 3 min. In this cell the cytoplasm has withdrawn from the periphery, leaving only a thin ragged border of adherent membrane to mark the extent of previous spreading. Concurrent with this rounding up, the cell has also begun ruffling—projecting lamellipodia (*L*) from its upper surface. Specimens fixed in 2% glutaraldehyde at 37°C in SBS (stored overnight in the same fixative at 22°C), post-osmicated, treated with uranyl acetate, dehydrated, dried with CO₂, and coated with platinum-carbon. $\times 5,500$. (e) Conventional thin section of a PMN allowed to settle on a carbon-coated surface (*CS*) for 2 min before fixation. The cell's two granule-types—specifics (*sg*) and azurophils (*ag*)—are suspended in the dense cytoplasmic matrix. In the subplasmalemmal region above the area of cell/carbon contact, a wide zone of homogeneous cytoplasm (double-headed arrows) prevents these granules from closely approaching the membrane and continues into the lamellipodium (*L*). A thinner zone also separates the cell's granules from the non-adherent membrane above the lamellipodium. Specimen fixed for 30 min at 37°C in 1.5% glutaraldehyde in 0.1 M sodium cacodylate plus 1% sucrose, held overnight at 4°C, post-osmicated in 1% OsO₄ in 0.1 M Na₂PO₄-NaHPO₄ buffer plus 4% sucrose for 1 h, en bloc stained with uranyl acetate, dehydrated, and embedded in Spurr's resin. $\times 19,000$.



and flattening of the cell body with membrane adherence to a substrate, together with the rounding up of the cell before the initiation of locomotion. Our work provides ultrastructural evidence that cellular adherence and resultant locomotion are dependent upon polymerized contractile elements. Additionally, the fluctuating patterns and organization of these elements, combined with earlier biochemical work, afford some clues which may explain how the polymerization and interaction of contractile and accessory proteins are orchestrated in this small amoeboid cell so that movement can be generated.

MATERIALS AND METHODS

Collection of Cells

Exudates of 95% neutrophils were produced and harvested according to the method of Hirsch (15). For use in experiments, a portion of the exudate was centrifuged at low speed so that the cells formed a loose pellet, which was resuspended and then washed twice in Hanks' balanced salt solution (HBSS), gassed with 95% air-5% CO₂, and used immediately. The cells were held at 37° or 22°C, including the period of initial fixation.

Adherent Membranes and Whole Cells

Cells were allowed to settle on acid-cleaned coverslips at 22° and 37°C. Some coverslips were coated with evaporated carbon and ionization-cleaned before use. After 1 min, the coverslips were rinsed of nonadherent cells. At intervals of 30 s–15 min, samples were fixed for whole-cell morphology or the cells were ruptured with a jet of standard breaking solution (SBS) (150 mM KCl, 2 mM MgCl₂, 20 mM Na₂PO₄-NaHPO₄) delivered from a syringe with a 25-gauge needle and the coverslips immediately immersed in fixative.

Membranes from Cells Disrupted in Suspension

To rupture the cells, a small drop of very loose cell pellet was placed on the edge of a coverslip; then a

second coverslip was dragged across the first, shearing cells between the two surfaces. Both coverslips were dipped in SBS to remove excess cells and subsequently immersed in glutaraldehyde fixative.

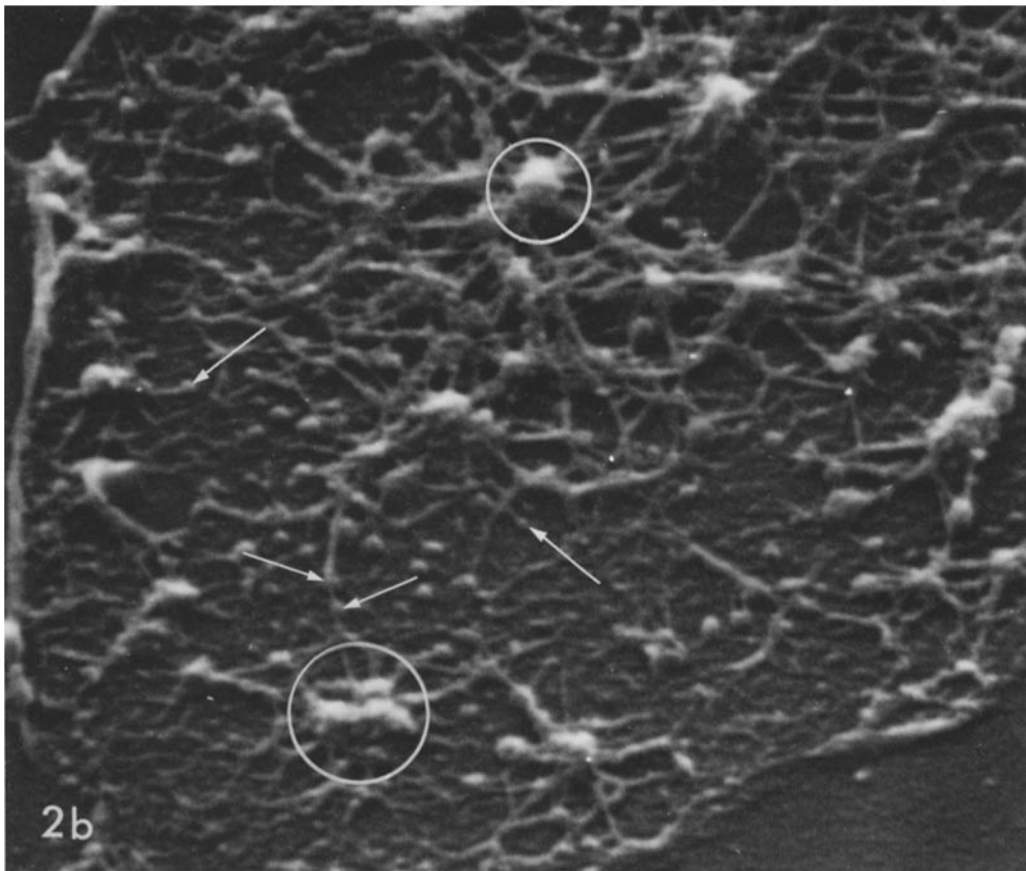
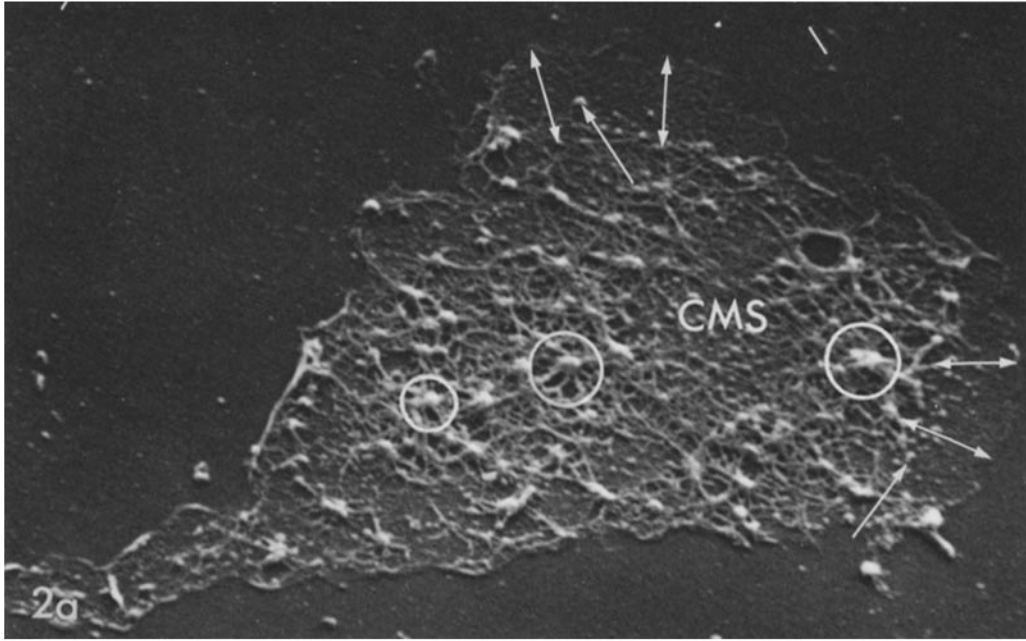
Fixation

Samples initially placed in glutaraldehyde solution (1:1 breaking solution and 4% glutaraldehyde [Electron Microscope Sciences, Fort Washington, Pa.] in 20 mM Na₂PO₄-NaHPO₄, pH 7.4, or in 1.5% glutaraldehyde plus 1% sucrose in 0.1 M sodium cacodylate buffer, pH 7.4, at 37°C) were allowed to cool to room temperature and held overnight or up to 3 d. Samples for thin sectioning were held at 4°C overnight. Then, without a buffer rinse, they were post-osmicated for 1 h in 1% OsO₄ in 100 mM Na₂PO₄-NaHPO₄, pH 7.4, plus 4% sucrose; rinsed in Michaelis buffer, pH 6.0, plus 7% sucrose; and submerged for 1 h in 0.5% uranyl acetate in the same buffer plus 3% sucrose. Uranyl acetate was used not only to enhance the membranes in thin sections but also to remove the glycogen, which otherwise in SEM and TEM samples coats the exposed internal surfaces of PMN and the glass or film supports they rest upon. Osmication was omitted in some samples to test its effects. Ethanol dehydration preceded embedding in either Araldite or Spurr's resins, or critical-point drying in either CO₂ or Freon at 40°C. Certain samples were freeze dried from chloroform-saturated distilled water.

SEM of Critical-Point-Dried Cells

After drying, the coverslips were mounted on aluminum stubs with conductive paint and coated that same day. (Samples must be coated on this day or the exposed filaments tend to be indistinct as if partially dissolved or covered by a thin layer of substance. After they have been coated, however, specimens remain stable for months.) Platinum-carbon (5–7 nm) was evaporated onto the samples in a tilting Omnirotor (Denton Vacuum Inc., Cherry Hill, N. J.). The source of evaporation was platinum wire wound between two sharpened carbon rods and melted to form a droplet. If necessary, the rods were turned so that the platinum droplet always faced the

FIGURE 2 Scanning electron micrographs of the adherent portion of a plasma membrane from a PMN allowed to settle on glass for 30 s–1 min, then opened with a shearing stream. (a) The external membrane-surface is not seen here—only the adherent cytoplasmic membrane-surface (CMS). Freely branching filaments radiating from irregular projections (circles) form an interlocking network which dominates the exposed internal membrane surface. At the borders of the membrane, where lamellipodia are expected, on the right side and upper edge (double-headed arrows), this subplasmalemmal filament complex does not reach as far as the rim. Here a few spheres (arrows) and a barren region of membrane can be discerned. $\times 12,000$. (b) Enlargement of an area from the figure above. Variable amounts of branched filaments radiate from the large, irregular globules (circles), which appear to be composed of multiple units at this magnification. Filaments also run atop and between smaller spheres (arrows) on the membrane. $\times 50,000$. Specimen fixed at 37°C in 2% glutaraldehyde in SBS, stored for 3 d at 22°C, postfixed with uranyl acetate before dehydration, dried, and thinly coated with 5–7 nm of platinum-carbon.



samples during the evaporation process. White cards were used to monitor the thickness of the coat.

The coating used was equivalent to the shadowing procedure normally used for freeze-fracture replicas. All samples were viewed on a Coates and Welter Quik Scan (Model 50 Special; Coates & Welter Instrument Corp., Sunnyvale, Calif.) with a proven resolution of 6 nm. Coatings thinner than 5 nm were not useful because of poor secondary signal and intermittent charging. For photographing whole cells, as opposed to flat membranes, additional coating was required (10–15 nm).

TEM of Critical-Point-Dried Cells

Nickel grids were placed on 0.2% Formvar films and picked up on coverslips. These grids and film-coated coverslips were carbon-coated and ionization-cleaned and then used in experiments. (Most grids will remain adherent to the coverslips through processing if handled gently.) After critical-point drying, single grids were freed from their coverslip supports and viewed at 40 or 80 kV.

Oriented Thin Sections

Cells were plated onto carbon-coated and ionization-cleaned Thermanox coverslips (No. 1-1/2, Lux Scientific, Newbury Park, Calif.) at 22° and 37°C and fixed at various time intervals.

To produce sections cut parallel to the surface, pieces of Thermanox coverslips were put on top of filled Beam capsules and then polymerized. Immediately after cooling, the coverslips were snapped off and block "faces" were cut from the desired areas. For sectioning, blocks were mounted at an angle so that each section contained a continuum of cells cut at successive distances above the surface from one edge to the opposite one.

For sections cut perpendicular to the surface, the specimens were polymerized in a thin layer of resin. After cooling, coverslips and cell layers were separated. Two pieces of the free cell layers were re-embedded *vis-a-vis* in a groove cut in the top of a blank block and

re-polymerized. Each of these sections contained two layers of cells.

SEM of Actin Filaments

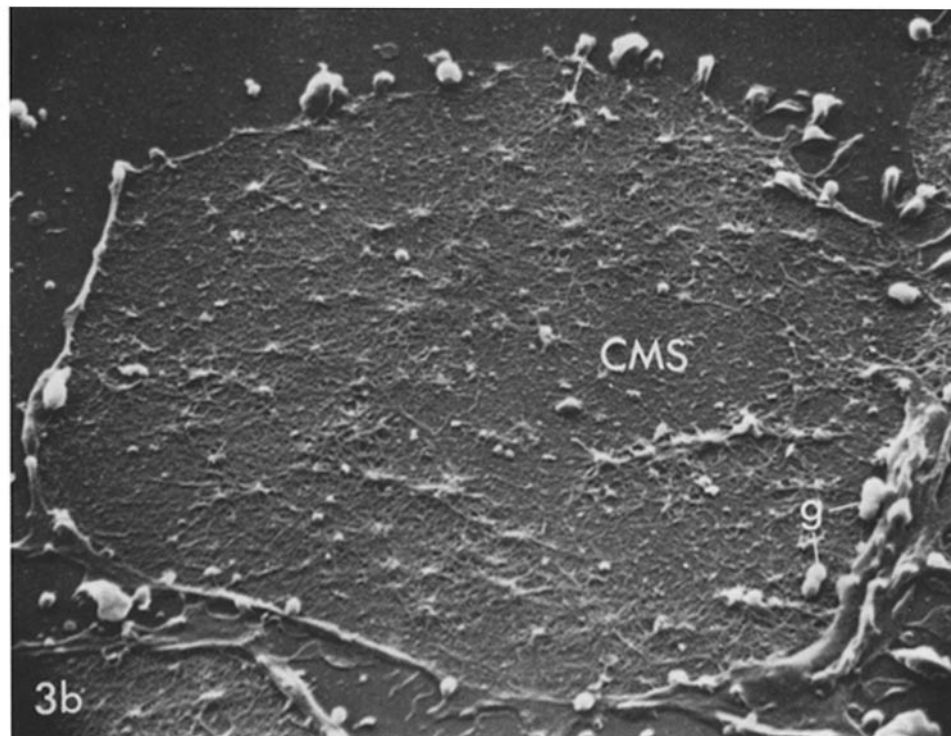
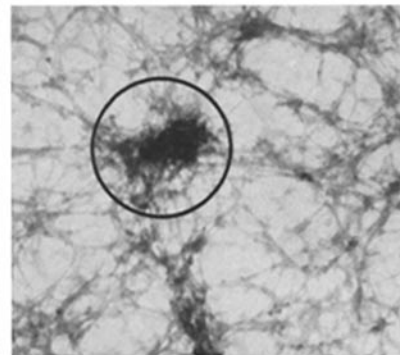
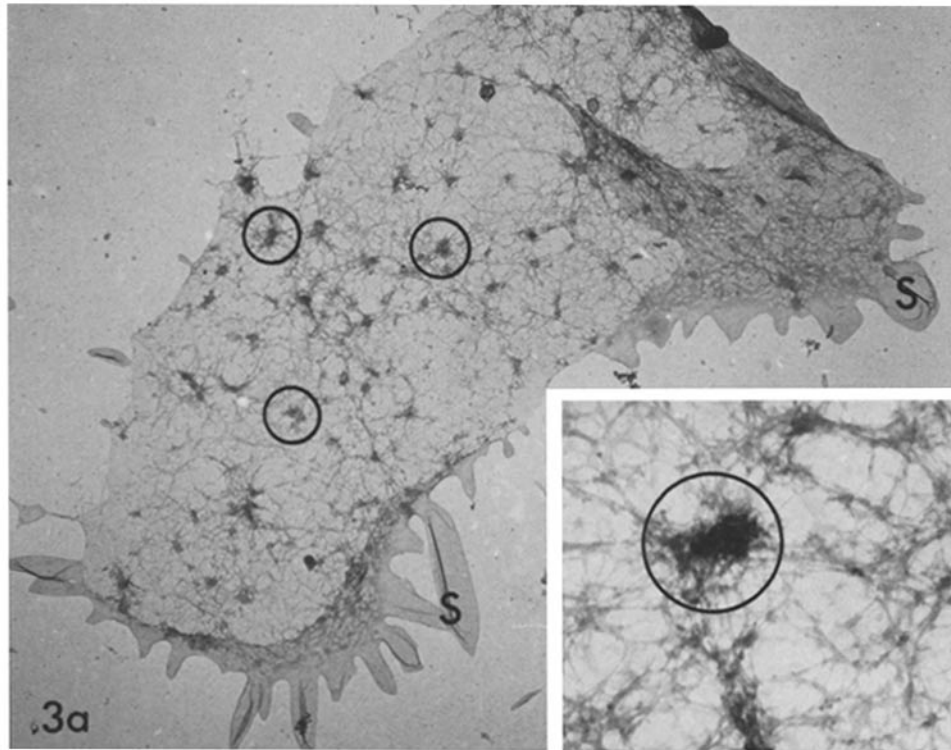
Purified actin from rabbit muscle was provided by Ron Meeusen of the Botany Department, University of California, Berkeley. Actin filaments at a protein concentration of 0.5 mg/ml (in 100 mM KCl, 2 mM MgCl₂, and 8 mM [4-(2-hydroxyethyl)-piperazinepropane sulfonic acid] [EPPS], pH 8.6) were allowed to adhere to coverslips for 1 min, then washed with SBS and fixed at room temperature. The remainder of the processing was exactly as in the case of all other samples for SEM.

RESULTS

Subplasmalemmal Filament Complex

TIME-COURSE AND DESCRIPTION OF PMN ADHERENCE: The quick response of rabbit PMN to surfaces is demonstrated by SEM in Fig. 1 *a-d*. In suspension, the cells are round and evenly covered with stubby projections and membrane folds (Fig. 1 *a*). When they have been plated on glass coverslips (with or without carbon as a coating) for only 1 min, the already adhering cells exhibit layers of lamellipodia or "ruffles," reaching out from above earlier points of contact (Fig. 1 *b*), producing new cell/surface contacts. This stage, observed by phase-contrast microscopy, shows a cell actively tossing out a circumferential veil of hyaline cytoplasm free of organelles. The granules flow into this veil, filling and obliterating it as the cell body flattens against the surface. The results of such flattening is a spread cell like that shown by SEM in Fig. 1 *c*. When spreading has reached its limits (~3 min after plating), the cell retracts from the spread state and, simultaneously, may begin a second wave of localized "ruffling" anywhere on its surface. That is, the granules which

FIGURE 3 (a) Transmission electron micrograph of a critical-point-dried adherent membrane from a PMN settled on a carbon-Formvar film for 1–2 min before disruption. At the cellular periphery, large areas of the upper cell-surface membrane persist, increasing the electron density over filamentous regions and forming filament-free membrane sacs (*S*). The electron-dense centers (circles) visible in this type of preparation are assumed to be the multistructured globules observed by SEM (Fig. 2). Specimen prepared as in Fig. 1 except for the omission of post-osmication and the conductive coating required for SEM. *Inset*: Another membrane preparation, in which post-osmication was included to increase the contrast. Only scant details of the filament/center association (circle), however, can be detected. $\times 14,000$; *inset* $\times 50,000$. (b) Scanning electron micrograph of the adherent membrane of a PMN plated onto glass for 3 min before disruption. The subplasmalemmal filament complex forms a thick mat which covers the entire cytoplasmic membrane surface (*CMS*) except for a thin region in the center of the cell. The globular centers so prominent at earlier times are now essentially obliterated by the thickened mat of interconnected and branched filaments. Specimen prepared as in Fig. 1 except for the omission of osmication and the use of a thin coating as in Fig. 2. $\times 11,000$.



streamed into the peripheral areas become fixed and are usually withdrawn from the entire border leaving a ragged edge of membrane at the periphery. The cell in Fig. 1*d* is an example of the morphology of PMN fixed at this time. If these second lamellipodia result in attachments, the cell's granules then pour into these new areas of hyaline cytoplasm as the cell begins locomotion, but this does not occur in all cells. Rather, most cease ruffling and/or granule withdrawal only to repeat the same processes without any translocational progress across the glass surface. By 5 min after plating only ~20% of these cells show the elongate shape of locomotion.

Thin sections of early adherent PMN cut perpendicular to the surface expose a thick zone of homogeneous cytoplasm adjacent to the point of cell/surface contact, continuing onward into the lamellipodia (Fig. 1*e*). All organelles, including microtubules and intermediate filaments are excluded from this zone. But rarely are the microfilaments expected in this region of the cytoplasm visible; they can be unmasked, however, by disrupting the cell and using other techniques to view this area.

PMN DISRUPTED 30S-3 MIN AFTER PLATING: Few adherent membranes can be seen on coverslips when the cells have been allowed to settle for only 30 s-1 min before rupture, and those detected are generally small patches of membrane. Both the diminutive membranous fragments and the seldom-encountered large expanses are dominated by a complex pattern of branching filaments and globular projections. These randomly spaced, irregular projections or globules constitute the centers for branching filaments that vary in length and may or may not mingle with filaments from other centers to form a continuous network—a subplasmalemmal filament complex. At the peripheries of only a few membranes from adherent cells and at the centers of others, there may be areas devoid of filaments. Usually, such areas are not smooth but are strewn with spheres of assorted sizes (Fig. 2*a*).

When examined in greater detail at higher magnification, the large irregular globules (150-200 nm) appear to be aggregates of some smaller subunits. In addition to the large globules with their radiating networks of small filaments, smaller spheres are present on the membrane, with single filaments running between some of them (Fig. 2*b*).¹

¹ The inaccuracy and imprecision of the magnifications

TEM of critical-point-dried samples was used to complement and verify the results of SEM of disrupted cells. At 1-2 min, heavily stained centers with radiating filaments of 4- to 7-nm diameter are very distinct in this type of preparation and, because of their distribution, size, morphology, and multiplicity, are assumed to be the large, many-componented globules observed by SEM. However, superimposition of the images of surrounding filaments and possibly beam damage cloud the finer details of these structures (Fig. 3*a*). Additionally, the smaller membrane/filament associations seen by SEM as spheres are not readily identifiable.

By 2-3 min, greater areas of adherent membrane remain on the glass after the cell has been broken. The number of cells rupturing and leaving their membranes behind on the glass increases to a maximum also. Although the topography of the filament complex has undergone no changes, a notable enhancement in the amount of filaments is evident. After 2 min, a net of interlocking filaments covers the entire adherent-membrane surface, usually being most fragile at the center of the cell. By 3 min, the globular projections, predominant features of the 1- to 2-min-adherent PMN, are now often concealed by the progressively dense mat of filaments (Fig. 3*b*).

PMN DISRUPTED 3-5 MIN AFTER PLATING: Beginning with 3-min samples, in some portions of the mat, filaments aggregate into bands. Circumferential groups of anastomosing bands of long filaments can be found in virtually every cell by 5 min (Fig. 4*a*), but actually the formation of such bands, or bundles, occurs throughout the filament complex. Occasionally, in these samples, we again encounter areas of membrane free of filaments and either smooth or sprinkled with spheres (as was sometimes noted in 30-s to 1-min specimens) at one border of the cell.

The transparency of the filament complexes viewed by TEM is useful for demonstrating that the dense centers (the globules discerned by SEM) have not disappeared in these specimens; rather, they are hidden by overlying filaments, except at the cell's border where they are not found in the fingers of membrane left when the cell rounds up. When bands have formed, they exist independent of the large, irregular centers (Fig. 4*b*). Although

recorded on our SEM instrument, as well as variations in the thickness of the conductive coating, do not allow exact comparison of minute features in one micrograph with those of another.

the minutiae of filament-band structure cannot be detected by this technique, many filaments appear thicker than in earlier samples or aggregated (*inset*, Fig. 4*b*). By SEM, the peripheral bands are obviously composed of multiple filaments, many of which are thicker² than the branched filaments seen in earlier samples and irregularly beaded (Fig. 5).

NONDISRUPTED PMN: After noting the planar arrangements of filaments in the subplasmalemmal complex by SEM, we returned to thin sections of undisrupted cells. By cutting the sections just within and parallel to the adherent membrane or perpendicular to the surface, we hoped to verify the patterns we have already observed in sheared cells in these undisrupted PMN.

In samples adherent for 3 min or less, our findings were disappointing: we saw only homogeneous cytoplasm adjacent to the adherent membrane, not the anticipated complex of filaments. Only in parallel sections from later samples adherent for 5 min or longer at 37°C, when the circumferential filament bands were present in these cells, could we identify filaments (Fig. 6*a* and *b*) along with occasional densities (Fig. 6*b*), possibly analogous to the multistructured globules evident by SEM and the dense, filamentous centers seen by TEM. The filaments observed at this later time were thick, measuring 10- to 12-nm wide and 200- to 300-nm long. The general density of the cytoplasmic matrix, however, made it difficult to detect even these larger and aligned filaments.

Parallel thin sectioning of disrupted cells was also performed, in the hope of using the attenuation and higher resolution of this technique to better define the filament/membrane associations once the obscuring fog of dense cytoplasm had been washed away. Fig. 6*c* illustrates such a membrane preparation. Here we found a stellate complex of transparent groundwork with fine, radiating filaments in sections containing the membrane. This structure probably corresponds to the irregular globules demonstrable by SEM and the dense centers seen by TEM. However, we were unable to detect the spheres which linked single filaments to the membrane in SEM samples. In these specimens, it was again apparent that at late intervals many of the filaments found are thick, 10- to 12-

nm filaments while others were thin and wispy, 4- to 6-nm branched filaments.

Evolution of the Subplasmalemmal Filament Complex

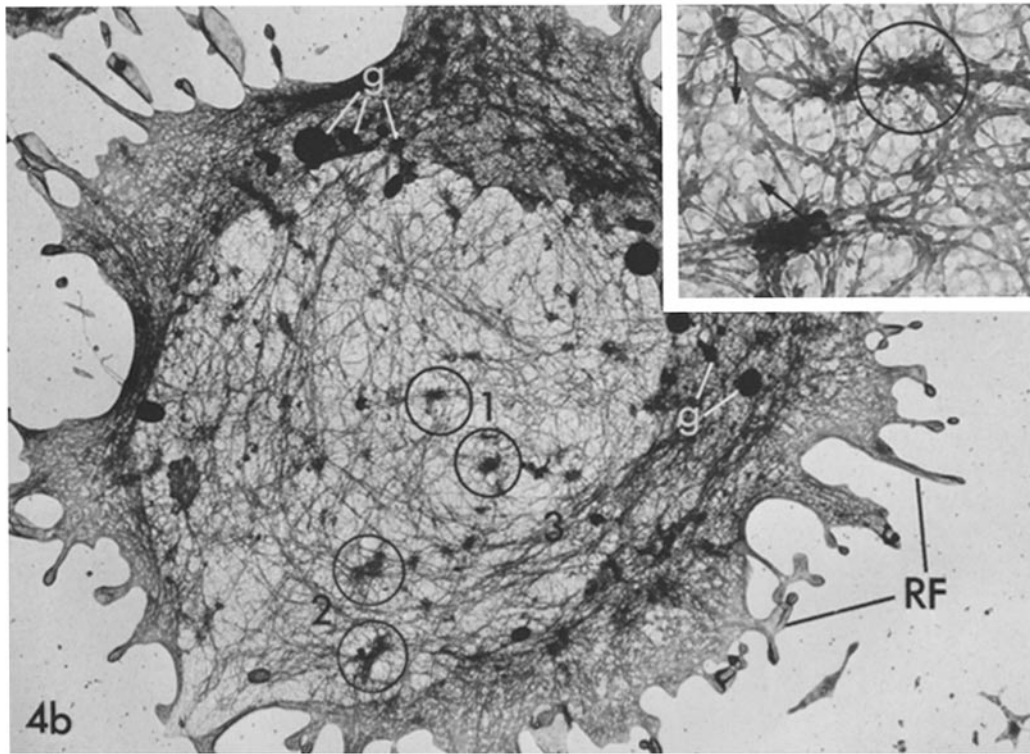
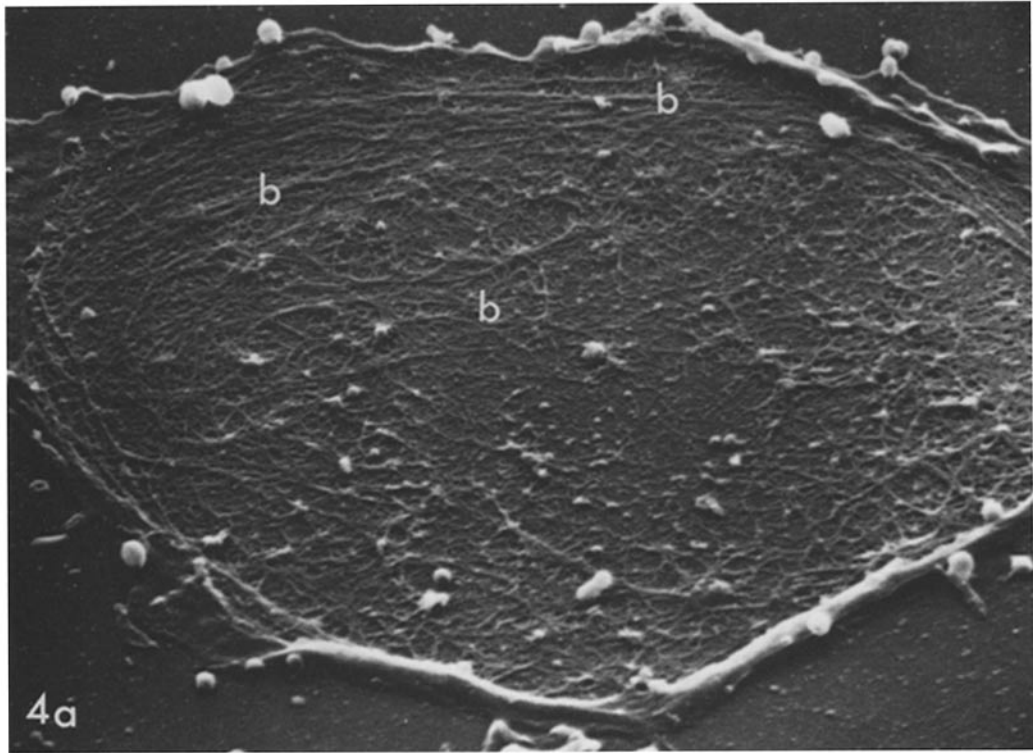
PMN DISRUPTED IN SUSPENSION: To determine whether filaments are associated with the membranes of round cells in suspension or are a consequence of membrane adherence, we investigated the membranes of cells like that depicted in Fig. 1*a*. These cells were opened by rolling between two glass coverslips or carbon-coated glass coverslips, rather than by a jet of buffer as is possible with adherent PMN. The coverslips with the absorbed plasma membranes were then immediately fixed and processed for SEM. (While the membranes from cells in suspension tightly stick to both glass and carbon and therefore cannot be considered truly nonadherent membranes, neither are they adherent membranes in the sense that they are not from actively spreading adherent cells as have been the membranes described in previous sections.)

The cytoplasmic surfaces of these membranes are studded with a great diversity of projections, including regular spheres and knobs of all sizes (~15–200 nm) (Fig. 7*a* and *b*). Not infrequently, the membranes disclose border regions which are free of projections and either smooth (depending on the fixation protocol) or finely pebbled with small particles. From sample to sample, a variable but small percentage of plasma membranes manifest some slender filaments. Commonly, these are few and associated with spheres (*inset*, Fig. 7*a*), as occasionally observed on the peripheries and within the interiors of some 30-s to 1-min adherent cells. In rare instances, a small mat or tangled skein of filaments is found in association with granules; the irregular multistructured globule is almost universally absent, however.

ADHERENCE AT A LOWER TEMPERATURE: We allowed some cells to cool to room temperature (22°C) in the belief that their slower responses would enable us to trace the developments which link the usually filament-bare but sphere-strewn surfaces of cells in suspension (Fig. 7) with the central-globule/radiating-filament complex seen in the earliest-spreading cells (Fig. 2).

At this nonphysiologic temperature, the body of the spreading cell as noted by SEM is not surrounded by a series of thin ruffles but by a single lamellipodial "skirt" (*inset*, Fig. 8*a*). The cell spreads by extension of this lone lamellipodium

² The relatively poor resolution of this technique (6 nm or less) makes it impossible to differentiate between a single large fiber and two or more intimately entwined thinner filaments.



and simultaneous flattening of the cell body to fill the projection. The cytoplasmic surfaces of these membranes displayed an admixture of all the distinctive features of nonadherent membranes and adherent membranes at 37°C. Often, the open membranes from these cells, plated 30 s–1 min earlier, were completely or partially bordered by closed regions of the peripheral lamellipodial skirt.

By 2–3 min, cells at both temperatures (22° and 37°C) are comparable—that is, the lamellipodia have disappeared from the cell margins as the cell body has completed its spreading and the filament complex covers the entire membrane. The filament complexes of both cell groups are also identical, although bands of anastomosing filament bundles fail to evolve in the cells maintained at room temperature until they have contacted the surface for 10–12 min, just as they require 10–12 min to retract and round up, slowly beginning movements, as observed by phase microscopy.

MORPHOLOGY OF LAMELLIPODIA: Because large skirts of lamellipodia frequently remained intact after cell rupture, we seized this opportunity to examine the structure of the lamellipodia of similar critical-point-dried specimens by TEM.

Within the lamellipodia, filaments do not fashion an orderly, distinct complex as they do on the adherent membrane surface beneath the body of the cell. Here is found a tangled felt of filamentous and granular material (Fig. 8). The visibility of this felt is quite variable, evidently depending on the extent of soluble cytoplasm leaching before fixation in glutaraldehyde. Occasionally the lamellipodia of opened cells are as uniformly dense as those from nearby unopened cells.

In these TEM samples, the subplasmalemmal filament complex often blends with the fabric of the lamellipodia. Rarely, the upper surface from a portion of the periphery is lost and a tangle of filaments exposed. More commonly, empty membrane sacs as well as cell borders with barren lower membranes or with only single isolated filaments are found. Because of their location, all these regions appear to be the remnants of lamellipodia disrupted by the shearing jet.

After easily identifying lamellipodia in these cooled cells, we re-examined cells at 37°C by TEM to ensure that their smaller and more plentiful ruffles were structurally similar to the large skirt of lamellipodia. Although only small areas of ruffling are retained in rare disrupted cells, they do exist and they contain the same weaving of filaments and granular material. Similarly, the smooth areas of membrane at the borders of occasional cells are probably remnants of lamellipodia (Fig. 2).

Effects of Post-Osmication

Because adherent membrane specimens can be prepared either with or without osmium for SEM and TEM of critical-point-dried material, we decided to assess the effects of osmication on the PMN subplasmalemmal filament complex and similarly processed, purified rabbit-muscle actin. The use of SEM and TEM also circumvents any potential changes during resin infiltration and polymerization as well as in the sectioning and post-staining of samples prepared for traditional thin-section microscopy.

FIGURE 4 (a) Scanning electron micrograph of an adherent membrane from a PMN allowed to settle on glass for 4 min and then sheared open. By this time, the dense filament mat of the cell exhibits peripheral (and to a lesser extent, interior) bands of long anastomosing filaments (b). Specimen preparation as in Fig. 1 except for Freon drying and a thin coating as in Fig. 2. $\times 15,000$. (b) Transmission electron micrograph of a critical-point-dried membrane from a PMN plated for 5 min on a carbon-Formvar film. At the cellular borders, sizable rims of upper-surface membrane remain, trapping granules (g) but not forming the filament-free sacs observed in earlier-interval preparations. Specimens such as this afford the best view of microstructures and their inter-relationships within the dense meshes and bands of filaments appearing at later intervals. The electron-dense centers (circles) are situated in the central part of the membrane lacking filament bundles (1) and within these bundles (2). Filament bundles may also exist without these densities (3) but the densities are missing from the borders of membrane beyond the ring of filament bands. This sample and that in the inset were prepared as in Fig. 1 except for omission of the conductive coating. *Inset:* Thicker filaments as well as thin ones are found at this time in conjunction with the dense centers (circle). The membrane beneath (arrow) is visible in this preparation, for which we used 40 kV accelerating voltage rather than the more common 80 kV used in the inset of Fig. 3a. $\times 12,000$; *inset* $\times 50,000$.

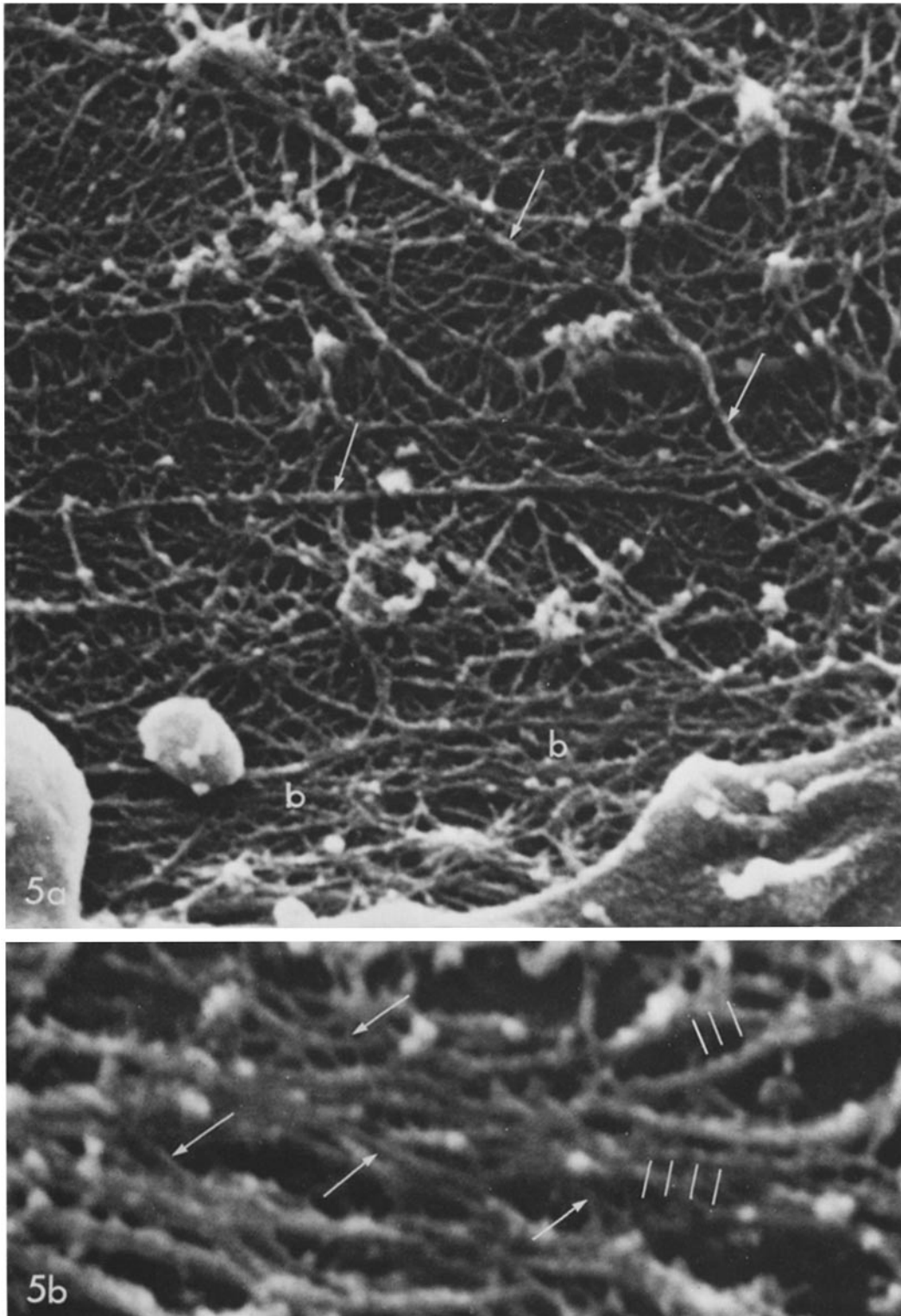


FIGURE 5 High magnification scanning electron micrographs of a portion of the adherent membrane from a PMN in contact with the glass coverslip for 4-5 min. (a) Anastomosing bands of filaments (b) are evident near the edge of the cell at the bottom of the micrograph, and additionally criss-crossing the interior surface of the filament complex (arrows). $\times 50,000$. (b) Enlargement of a peripheral band of filaments from the same sample as above. The beaded nature of the larger filaments (slanted lines) found in these bands is visible. Compare them with the finer filaments (arrows) also present. $\times 75,000$. Specimen preparation as in Fig. 3 b.

SUBPLASMALEMMA FILAMENT COMPLEX: Osmium provoked only negligible damaging of the filament complex as observed by SEM and TEM (compare the specimens pictured in Figs. 2, 3, and 5 prepared without the osmium, and those in Figs. 3 *a* (inset), 4, 7 *a* (inset), and 8 exposed to osmium). Even dangling individual filaments, only cursorily supported by membrane-bound spheres at a few points, appeared to be unbroken and unbent (inset, Fig. 7 *a*). Also, as illustrated in Fig. 2, in which osmication of the specimen was omitted, these filaments form a naturally branching network. The most significant effects of osmium were to increase the signal of thinly coated samples (thus sharpening the images) and to protect whole cells from beam damage. Moreover, isolated membranes were better preserved, with enhanced rigidity, smoothness, and a notable reduction in the number of small particles. In TEM samples, osmication increased the contrast, facilitating distinction of the filaments against the background membrane. In fact, osmium was essential for defining filaments when the upper part of the membrane persisted. The minimal breakage associated with this type of preparation usually occurs during initial viewing, before micrographs can be obtained.

PURIFIED RABBIT-MUSCLE ACTIN FILAMENTS: Both to be sure that our SEM techniques could resolve single actin filaments and to test the effects of osmication, we put pure actin filaments on coverslips and processed them exactly as we had prepared the membrane samples. The fixation protocol included either the use or omission of post-osmication in varying preparations. While we had observed no destructive effects of post-osmication on the filament complex, our experience with purified muscle-actin filaments resulted in entirely different pictures, depending on the manner of fixation. Actin filaments are easily identified against the background as long and straight or gently curved if osmium is omitted from the fixation process (Fig. 9 *a*). However, if OsO_4 is added as a postfixative for 1 h, extensive havoc is wrought—i.e., strings of remnants form “dots and dashes” caricature of filaments. Such remains of filaments show no evidence of branching, network formation, or other rearrangements, but only disintegration (Fig. 9 *b*).

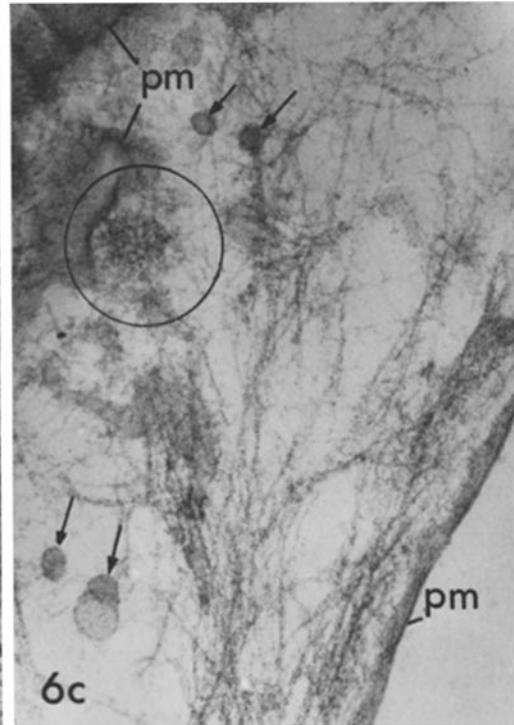
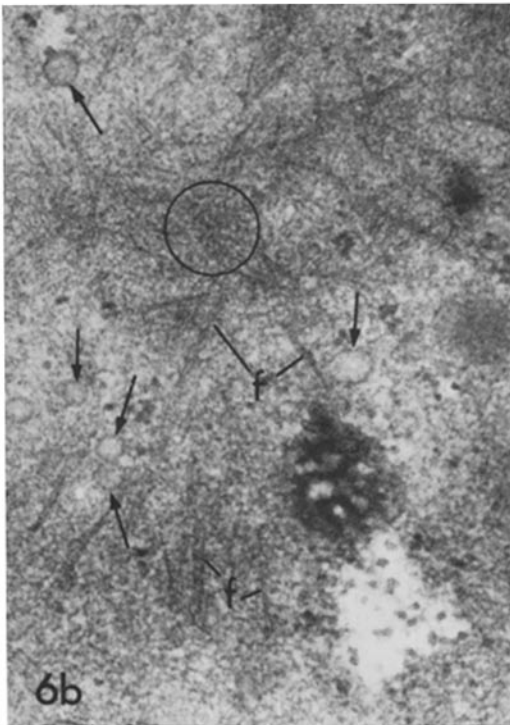
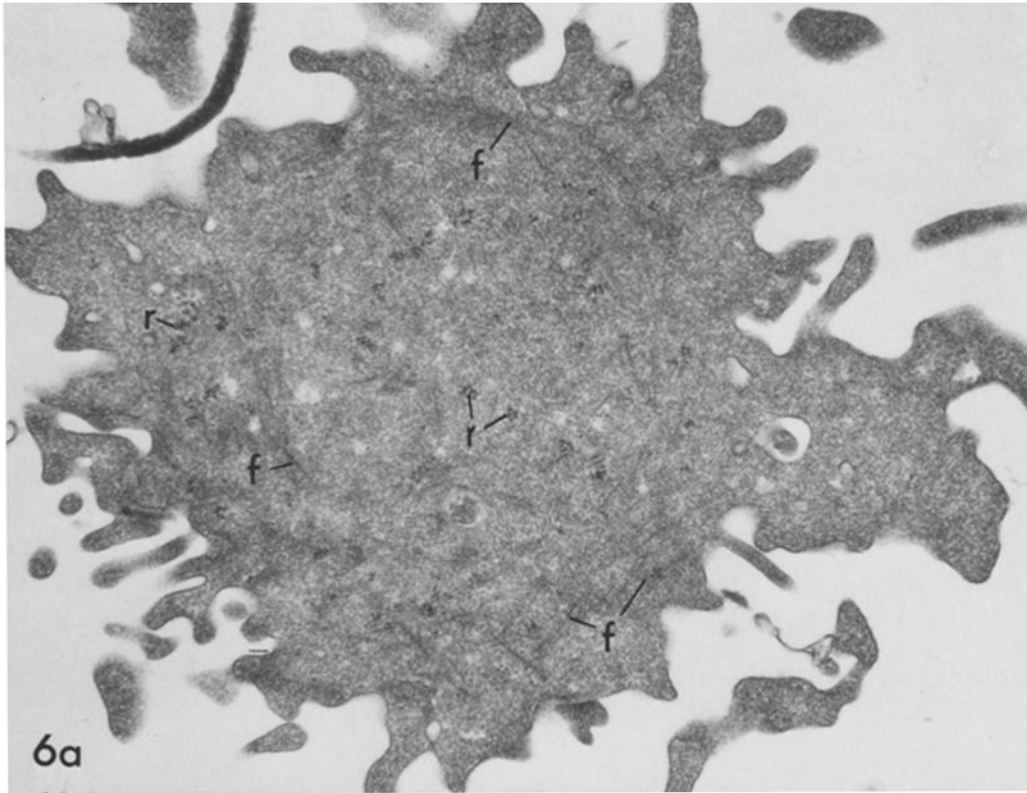
DISCUSSION

General Comments

The intent of this study was to examine the

cytoplasmic surface of the PMN plasma membrane during initial phases of cellular adherence and spreading. Contractile proteins are assumed to be the causative force behind these processes, but their organization (with alterations of cellular activities) has remained obscure. Scanning electron microscopy of disrupted cells, a novel technique introduced by Vacquier (41) and further explored by Clarke et al. (8), enables the viewing of interior plasmalemmal surface topography and portions of the cytoskeleton which are tightly bound to the membrane. Observed by high resolution SEM, whereas the inner surface of the nonadherent PMN membrane is free of filaments, within 30 s of attachment to an artificial surface a three-dimensional, interlocking network of globular projections and radiating, thin, short filaments—i.e., a subplasmalemmal filament complex—is consistently demonstrable. TEM of the same type of preparation permits penetration of the overlying upper cell-surface membrane and exposes the cytoplasmic elements beneath. By this technique, one can see that the lamellipodia of spreading cells contain filamentous and granular material, quite distinct from the globule/filament complex of the adjacent adherent membrane. This complex, once formed on the membrane, is not static but increases in filament density over the next 2–3 min. By 3–5 min, as the cells round up before commencing amoeboid movements, another pattern emerges: circumferential bands of anastomosing long filaments in which another thicker, beaded filament is found. This sequence of events is shown diagrammatically in Fig. 10.

The primary use of ruptured cells in this study was necessary because PMN present some problems which diminish the usefulness of many morphological techniques; i.e., this is a small cell (5–10 μm) with dense cytoplasm which does not extensively flatten upon adherence. The dense cytoplasm limits the information to be gained from thin sections, although thin sectioning has proved of great value in studies of filamentous structures in much larger and more translucent cultured cells (1, 10, 12, 13, 25, 26, 37, 45). Another method of contractile protein localization which has been primarily used in much larger cells is the application of fluorescent antibodies or heavy mero-myosin to whole cells (14, 20–22, 32). While it was not attempted in this study, the small size of the PMN coupled with its tendency not to flatten extensively could be expected to greatly constrict the data to be gained through these techniques. Even without the interference of the PMN's cyto-



plasmic organelles, the limited resolution of fluorescence microscopy would be incapable of demonstrating the fine details of the patterns seen on the adherent membrane by SEM and TEM. The use of specific antibodies, however, affords an unexplored potential for identifying the components of these patterns on the exposed membrane.

Effect of Post-Osmication on the Subplasmalemmal Filament Complex

Actin is associated with the plasma membranes of diverse cell types (2, 7, 8, 10, 12, 35, 43; reviews 16, 19, 30); it is reasonable to assume that the filament networks associated with the plasma membranes of early adherent PMN described in this work are, similarly, at least partially composed of actin. This assumption is consistent with the filament diameter of 4–7 nm seen in thin sections (Fig. 6c) and TEM (Fig. 3a).

That similar microfilament networks which have been described in many other cells (5–7, 25, 37, 44) are a true representation of cellular actin morphology has been recently challenged. Osmium postfixation has been postulated by Maupin-Szamier and Pollard to break and rearrange rectilinear cellular actin filaments unprotected by associated proteins, thereby producing artifactual networks of branched filaments (24, 28, 29). Because they do not require osmium postfixatives, critical-point-dried samples can be used with SEM and TEM to test this hypothesis.

The results of our experiments indicate that the branched networks of filaments observed on the adherent membrane surfaces of PMN are not an artifact of osmication. Rather, without osmication these filaments branch and form a net (Figs. 2 and 3). In thin sections of whole cells, however, this

same region appears as a zone of organelle exclusion, or “fuzz,” lacking any obvious thin filaments. Such an image is probably a consequence of this cell’s dense cytoplasmic matrix obscuring the irregular meshwork.

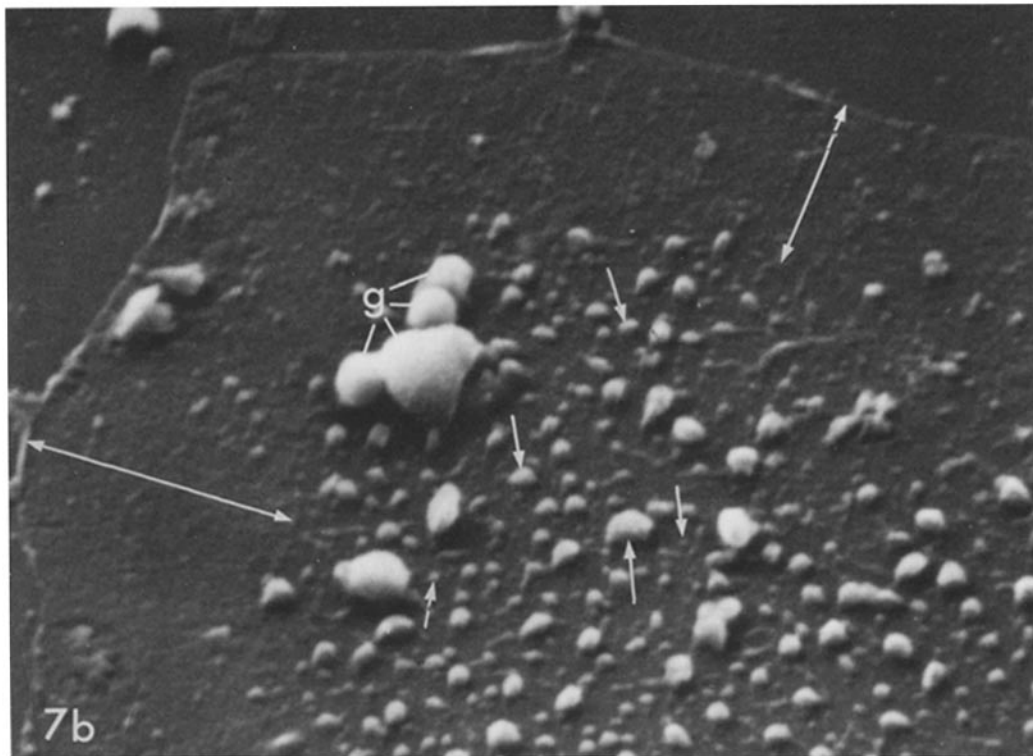
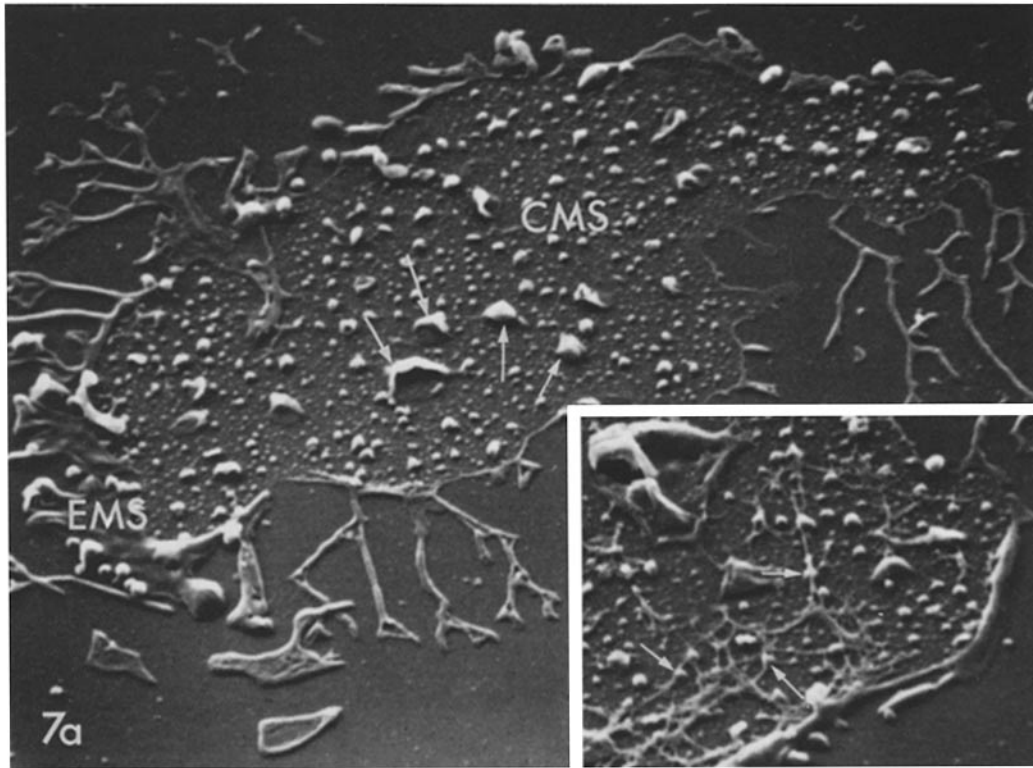
We agree, however, that osmium effectively destroys glutaraldehyde-fixed, purified, skeletal-muscle actin filaments, suggesting that subplasmalemmal filaments are not composed of pure actin but possess other bound proteins and thus are protected from breakage by osmium (24, 28, 29).

Lamellipodia

Lamellipodia are distinct organelles—thin (0.1–0.2 μm) sheets of hyaline cytoplasm in which actin is concentrated (14, 20, 21, 32)—which extend during adherence and locomotion. The lamellipodia of PMN are short compared to those of some tissue-culture lines, though they are probably analogous organelles. In PMN, the soluble components of the cytoplasm must be dispersed by rupturing the cell, before the morphology of the filaments and granular material which comprise the organelle becomes evident (Fig. 8). The filamentous material rendered visible by this technique resembles that described in the more translucent, cultured nerve cell and fibroblast, prepared as whole cells for 80–100 kV TEM (5, 6, 18). The similarity of the lamellipodia in these different cell types, processed by various procedures, strengthens the argument that these procedures preserve the true lamellipodial structure and that the images seen do not result as an artifact of either cell rupture or of fixation.

Additionally, the structure of this felt resembles that of cytoplasmic gels (4, 9, 27, 40) primarily

FIGURE 6 (a) Conventional thin section, cut parallel to the surface, and just within the adherent membrane of an intact PMN permitted to settle for 5 min on a carbon-coated plastic coverslip. Short filaments (*f*) tend to form a circumferential band similar to that seen in Fig. 4. A few ribosomes (*r*) are also present. Sample fixed as in Fig. 1. $\times 8,000$. (b) Thin section at higher magnification, also cut parallel to the surface, just within the membrane of an intact PMN adherent for 8 min. A density (circle) is discernible inside a band of thick (10–12 nm) filaments (*f*). Note the abundance of small vesicles (arrows). Specimen fixation as above. $\times 50,000$. (c) Thin section, also cut parallel to the surface and taken just within the membrane, of a disrupted PMN allowed 12-min contact with the surface at 22°C. With the cytoplasm washed away, a globular center (circle) stands out in negative relief against the fine filaments radiating from it, clearly defining its subunit morphology. Two types of filaments are displayed in this sample—the delicate, wispy filaments predominate in the upper right-hand corner, while the wider ones are more prominent in the lower left-hand corner. Dense vesicles (arrows) are again seen here—between the plasma membrane (*pm*) in the upper left corner and the overlying filament bands. Specimen fixation as above. $\times 50,000$.



consisting of actin and actin-binding protein. The biochemical (14, 42) and morphological similarities of lamellipodial contents and gels are beginning to strongly support the hypothesis that growth or formation of lamellipodia results from a process of controlled gelation or polymerization of soluble components (2, 14, 27, 38, 40).

The lamellipodial felt of filaments is not so tightly bound to the membrane as the subplasmalemmal filament complex, because the feltwork of filaments within lamellipodia is frequently lost with the cell contents during disruption of the cell. Similarly, because the membranes of cells in suspension (Fig. 7) are identical to those of lamellipodia (Fig. 2*a*), and there are a few filaments occasionally found on these membranes in addition to the presence of a narrow zone of organelle exclusion separating the cell content from the membrane in thin section (46), filaments which are not tightly bound may also be present beneath these membranes.

The similarity of the two membranes, likewise, may reflect the rapidity of the plasma-membrane response to surface contact. Cells in suspension were broken open simultaneously with contact. The smooth borders of their membranes and those of lamellipodial membranes may reflect areas lacking contact with the surface until after fixation, whereas the region strewn with spheres and a few filaments may represent the first response to adherence in both.

Subplasmalemmal Filament Complex

The origin and identification of the irregular, multistructured centers and their associated filaments which form the subplasmalemmal filament complex remains unresolved by this study. The

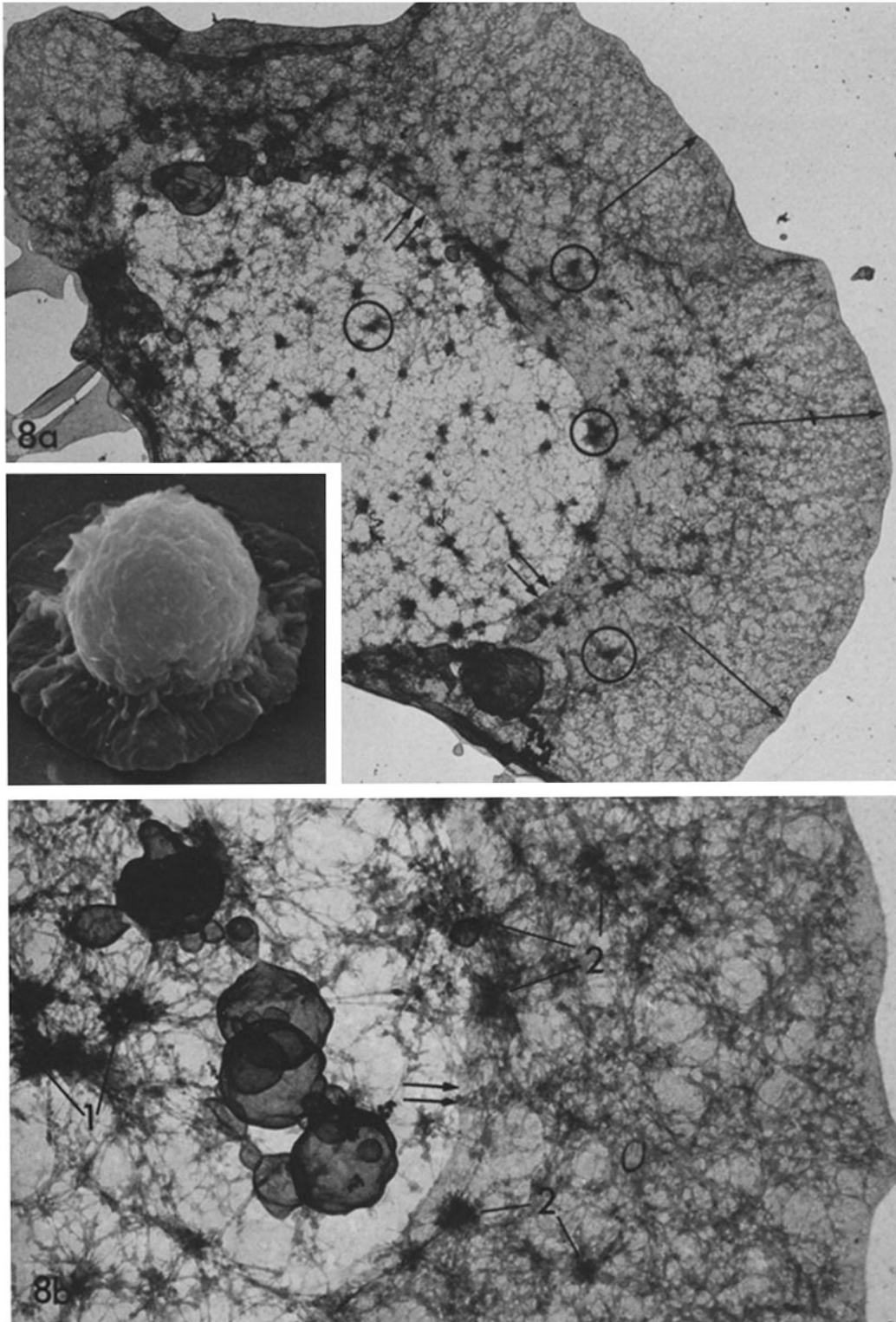
centers may be aggregations of cytoplasmic constituents upon which filaments polymerize, entirely distinct from the feltwork of the lamellipodia. They appear, however, to be an accretion of certain of the multiple spheres and granular material initially present in the lamellipodia; thus the filament complex represents a new organization of the original felt. Whatever the derivation of these centers and their radiating filaments, they clearly assemble on the membrane in response to adherence concurrent with the spreading of the cell's cytoplasm into the lamellipodia.

While the filament complex is tightly bound to the membrane, possibly via these centers, we have no evidence that they are adhesive organelles similar to those reported in large cultured cells (1, 12, 25, 26). In fact, it would appear that membrane regions free of dense centers may be adherent (Figs. 2*a*, 3*a*, 4*b*, and 7). In a recent study of adherent human PMN fixed in a different manner from the one which we used, Malech et al. (23) found dense plaques with associated "fuzz" resembling adhesive plaques. However, using similar methods—i.e., oriented thin sections—we could not unequivocally localize such plaques in perpendicular sections of rabbit PMN, where we found large areas of membrane closely adherent to the substrate. In parallel sections of disrupted cells, we did find a crystalline structure somewhat resembling the crystalline arrays (12) noted in other cell types, which we believe corresponds to the irregular centers seen by TEM and SEM.

Filament Bundles

Concomitant with the rounding up of the spread PMN, the subplasmalemmal complexes on the majority of adherent membrane surfaces become

FIGURE 7 Scanning electron micrographs of the membranes from PMN in suspension which were sheared open between glass coverslips. (*a*) The external membrane surface (*EMS*) can only be seen at the periphery of this specimen. We are looking down on the cytoplasmic membrane surface (*CMS*), which is completely covered with spheres of all sizes. Generally, no filaments are found on the membrane. The numerous blebs (arrows) indicate that the cell had not spread on the coverslip before disruption. *Inset*: Part of another membrane from the same preparation. This membrane exhibits a few filaments associated with spheres (arrows) on one area of its surface. They are not lying on the membrane itself, but rather are associated with spheres similar to those visible on the filament-free membrane above. $\times 13,000$; *inset* $\times 15,000$. (*b*) Higher magnification of the cytoplasmic surface of another suspended cell membrane on glass. This specimen exhibits a finely granular border (double-headed arrows). Only the interior of the membrane is strewn with the wide variety of projections or spheres (arrows) that entirely cover the membrane above. Additionally, occasional granules (*g*) released from the disrupted cells litter the membrane and the glass surfaces. In general, a "grainy" membrane rather than a smooth one is found in samples fixed without osmium, or left in the initial fixative for >1 d before osmication as in this sample. $\times 37,000$. Specimen prepared as in Fig. 2 except for the inclusion of post-osmication during fixation.



organized into bands of filament bundles. The additional presence (but not at earlier time intervals) of thick filaments (Fig. 6 *a* and *b*) with dimensions consistent with those of isolated PMN myosin (4, 39) and morphology by SEM similar to that of myosin filaments in muscle (33) leads us to infer that this pattern is a result of the interaction of myosin filaments and the filament complex in

a contractile event. The retraction, or rounding up, of the cell at this stage can be viewed as a contraction on the entire filament complex. While only the biochemical or immunological identification of myosin in the complex at this time (and not in earlier patterns) would allow verification of this theory, our conclusion gains additional support from the fact that myosin is the only protein

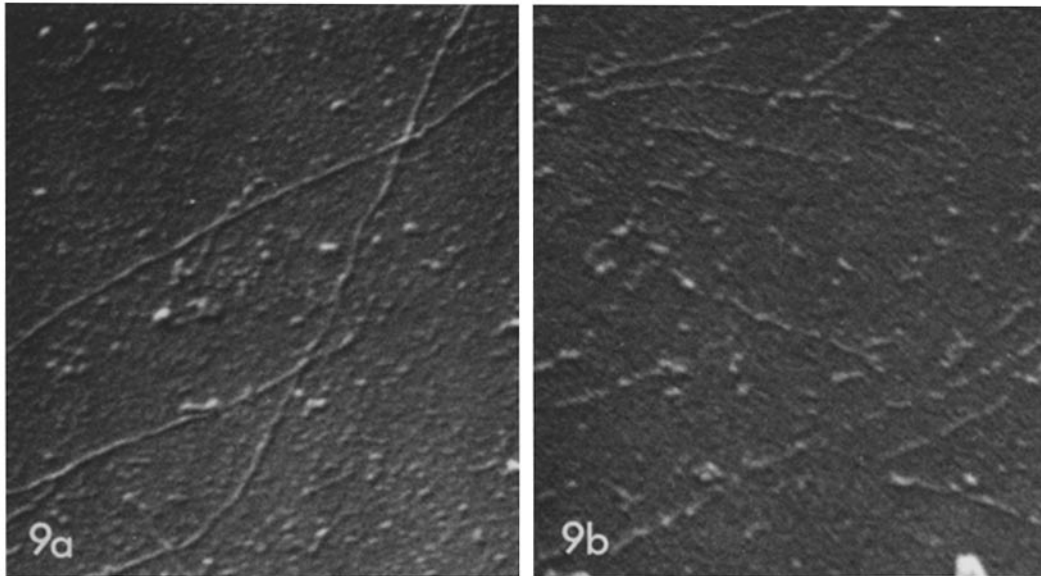


FIGURE 9 Scanning electron micrographs of pure rabbit-muscle actin on glass coverslips. (*a*) This specimen was prepared for SEM in exactly the same manner as were earlier membrane samples processed for SEM without post-osmication. The filaments are long, and there is an occasional hint of the actin helix repeat along the strands. $\times 50,000$. (*b*) A sample of actin fixed in glutaraldehyde and post-osmicated before subsequent processing for SEM. When detectable, the actin filaments are broken and irregular. Large areas of the coverslip often reveal only a scattering of remnants unrecognizable as filaments. $\times 50,000$.

FIGURE 8 Transmission electron micrographs of a critical-point-dried, adherent membrane from a PMN settling on a carbon-coated Formvar film for 1 min at 22°C and then disrupted. (*a*) A large expanse of lamellipodia (long arrows) remains at the perimeter of this cell. The overlying upper surface membrane (edge at short arrows), which causes an increase in electron density, continues beyond the limit of the lamellipodia and blankets a considerable portion of the lower surface membrane once overlain by the cell body but now lost. Note that the dense centers (circles) do not extend into the lamellipodia. *Inset*: Cell plated at 22°C for 1 min on glass. The single circumferential, lamellipodial "skirt" of this cell contrasts with the ruffles of cells settling at 37°C (Fig. 1 *b*). Specimen prepared as in Fig. 1 except for freeze-drying of this sample. Critical-point-dried parallel samples are similar, but their lamellipodia are thinner and flattened against the glass. $\times 12,000$; *inset* $\times 5,000$. (*b*) Enlargement of one area of a PMN membrane from the same sample as above. The enhanced density over the left-hand portion of the membrane results from the upper surface membrane. The edge of this ruptured membrane (arrows) lies approximately at the junction of the electron-dense centers and the lamellipodia proper. The dense centers on the open membrane (1) are surrounded by a distinct filamentous network. (Compare this with the felt of filamentous and granular material within the lamellipodia.) At the interface of these two regions of the membrane, the dense centers may be forming at foci of increased density (2). $\times 40,000$. Samples prepared as in Fig. 1 except for the omission of conductive coating.

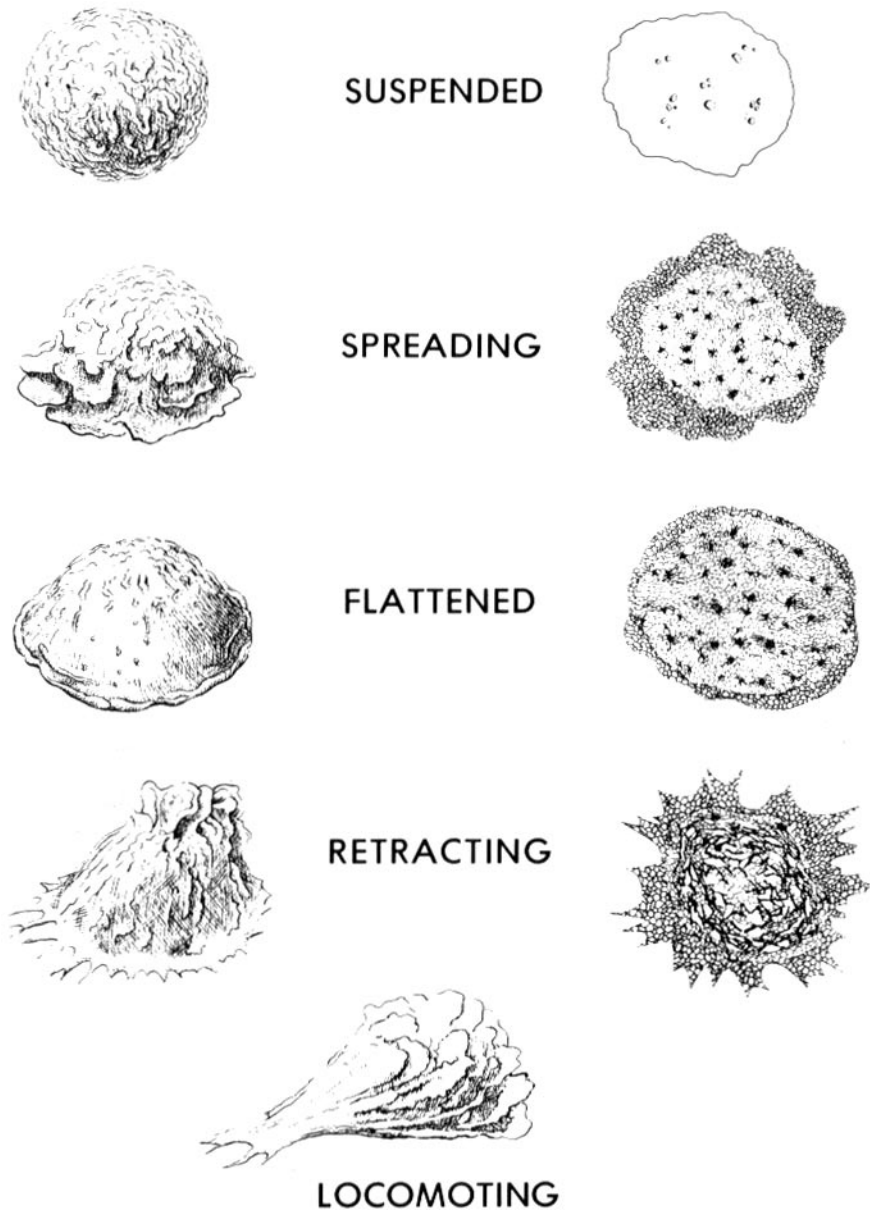


FIGURE 10 A diagrammatic summary of the sequential events which occur at 37°C as a PMN in suspension adheres to an artificial substrate, spreads, and begins random amoeboid movements. On the left, the whole cell is depicted as it appears by SEM during these processes; on the right is the lower part of the plasma membrane with its associated filamentous structures as they appear by TEM after the cell body has been removed. The Suspended PMN is round, with surface ridges and short projections. The plasma membrane is generally free of bound filaments. The Spreading cell (30 s–2 min), in response to contact and adhesion to the substrate, throws out lamellipodia which extend the plasmalemma over the surface, providing additional sites for adherence. A felt of fine filamentous and granular material fills the lamellipodia, while on the substrate-adherent portion of the plasma membrane beneath the cell body a complex of dense centers with radiating networks of thin branched filaments develops. A Flattened form (2–3 min) results from the spreading of the cell body and filling of the outstretched lamellipodia. The complex of dense centers and branched filaments also spread to cover the entire lower part of the membrane surface. The total number of filaments in the complex increases during this period. A Retracting morphology (3–5 min) is observed when the spread PMN draws away from the surface, leaving fingers of membrane at its base. At the same time, the cell usually begins to extend a new wave of lamellipodia elsewhere on its surface. Beneath the cell body, anastomosing bundles of filaments form on the adherent membrane. In addition to elements of the complex, they also contain a new type of filament, short and thick. If the new wave of lamellipodia makes contact with the substrate and new adhesions develop as the original adhesions are lost, the PMN assumes the elongate configuration of a Locomoting cell (no open membrane is shown).

presently known to cause a contraction of an actin net, and the fact that in fibroblasts and lymphocytes, a great deal of evidence has accumulated pointing to the retraction of the posterior of the moving cell as contractile in character (11, 12, 17, 34, 37).

A reorganization appears to be occurring in the filament complex of PMN at this time. Contraction heralds its dissolution. The cell's borders contain few filaments, and have lost the dense centers which can still be found in the interior of the membrane. Additionally, rather than the complex condensing with contraction, which might be expected because its highly branched filaments can present only short regions of F actin or myosin filament interaction, long anastomosing ropes or bundles of filaments are formed. A paper by Condeelis and Taylor (9) proposed recently that in cytoplasmic gels of *D. discoideum* one limit to contraction may be the formation of F actin filaments. Conceivably, long actin filaments are being produced and it is these, cross-linked by the myosin filaments, that form the bands of filament bundles.

CONCLUSION

The series of events described here in adhesive PMN—that is: (a) surface contact, which initiates lamellipodial extension; (b) the resulting surface adherence of the plasma membrane which induces the organization of a filament complex and flattening of the cell body; and (c) the later contraction of the filament complex which is concurrent with its disappearance and the breaking of these adhesions—may comprise one general cellular response to adherence. Somewhat similar events have been described in the chick fibroblast when the leading lamella of one cell adheres to that of another (13). If this is the case, then isolated adherent membranes may be good models for the study of locomotion which can be thought of as a series of sequential adhesions.

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