Reactivation of Organelle Movements along the Cytoskeletal Framework of a Giant Freshwater Ameba

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Abstract. The peripheral feeding network of the giant freshwater ameba *Reticulomyxa* can be easily and rapidly lysed to produce an extensive, stable, and completely exposed cytoskeletal framework of colinear microtubules and microfilaments. Most of the organelles that remain attached to this framework resume rapid saltatory movements at rates of up to 20 μm/s if ATP is added. This lysed model system is also capable of other forms of motility, namely an active splaying of microtubule bundles and bulk streaming. Reactivation does not occur with other nucleoside triphosphates, requires Mg ions, is insensitive to even high concentrations of erythro-9-(3-[2-hydroxynonyl]) adenine, is sensitive to vanadate only at concentrations of ~100 μM, and is inhibited by *N*-ethylmaleimide at concentrations >100 μM. The physiology of this reactivation suggests an organelle transport motor distinct from cytoplasmic dynein and possibly the recently described kinesin. This system can serve as a model for elucidating the mechanisms of intracellular transport and, in addition, provides a unique opportunity to examine associations between microtubules and microfilaments.

RECENT advances in computer-processed light microscopy (1, 2, 14, 38) and the development of in vitro models of intracellular transport (3, 13, 22, 28, 29, 31, 32, 34–36) have been invaluable toward understanding, on a molecular level, the relationships between moving organelles and the cytoskeleton (reviewed in reference 25). There now exist well characterized model systems for both microtubule-based and microfilament-based organelle transport. The squid giant axon is a good example of microtubule-based motility because it provides both an in vitro model, the extruded axoplasm (3, 34), as well as sufficient starting material for the biochemical dissection of the transport machinery. Its analysis has led to the identification of at least one novel translocator, kinesin, and the development of a reconstituted in vitro motility system of latex beads, kinesin, and purified microtubules (36). Model systems for microfilament-dependent movements have involved myosin-coated beads and ordered native or reassembled arrays of actin filaments (29, 31, 32). Permeabilized cell models have also been valuable in examining the physiological parameters of organelle movements (7, 9, 23, 33). Despite these advances, information on the molecular nature and universality of organelle–cytoskeletal interactions or their regulation in vivo is still scarce. Even less is known of the possible coexistence and function of different transport systems in the same cell (see however, references 7, 19 and 20). Whereas most in vitro models are well defined, they are at present limited to either the microtubule-based or the actin-based transport system and do not allow examination of interactions between them.

This paper presents a model system of intracellular transport consisting of endogenous organelles and a relatively simple, native cytoskeletal framework of both microtubules and microfilaments. This model appears to retain some of the native structural complexities that are lacking in present reconstituted in vitro systems while, at the same time, it is less complicated and more accessible experimentally than previous cell models. In addition, both microtubule-based and perhaps microfilament-based forms of organelle transport are expressed.

Reticulomyxa filosa is a giant freshwater ameba (class Rhizopoda, order Granuloreticulosea) that exhibits rather striking intracellular transport (15, 21, and Koonce, M., U. Euteneuer, K. McDonald, D. Menzel, and M. Schliwa, manuscript in preparation). Extraction of the peripheral reticulopodial network with a buffer containing a nonionic detergent and other stabilizing agents yields a highly ordered, stable cytoskeletal framework that is completely open to the environment. Many organelles remain attached to this framework and upon the addition of ATP, resume rapid (up to 20 μm/s) movements that very closely resemble in vivo motility. Two other motile phenomena are also reactivated with ATP. First, microtubule bundles bend, undulate, and splay apart, often resulting in a dense meshwork of what appear to be single microtubules. Second, slow bulk movements of strands and particle clusters occur, a phenomenon visibly distinct from the rapid movements of individual organelles. This paper emphasizes the physiology of reactivated individual organelle movements.

Materials and Methods

Light Microscopy

Stock cultures of *Reticulomyxa* were maintained in petri dishes containing tap water and were fed wheat germ. For light microscopy experiments, small pieces of cell bodies were placed onto polylysine-coated glass cover-

slips in dishes containing 10 mM Hepes and 2 mM $MgCl_2$ (pH 7.0), and allowed to spread, forming a large network within 1 h. The cell body was removed with a pipette, leaving just the network attached to the coverslip which was then inverted over a slide with coverslip pieces as spacers. The longitudinal sides of this sandwich were sealed with valap (1:1:1 mixture of Vaseline, lanoline, and paraffin), leaving the ends open for perfusion of solutions. All manipulations were performed at room temperature.

The preparations were viewed with a Zeiss Photomicroscope III equipped with phase-contrast, differential interference contrast (DIC),[1] or fluorescence optics. DIC illumination was provided by a 100 W heat filtered mercury arc made monochromatic with a 550-nm interference filter. A 100X Zeiss plan objective projected the image into a series 67 video camera (Dage-MTI Inc., Wabash, MI) whose signal was fed into an Interactive Video Systems Image I processor (Interactive Video Systems Inc., Concord, MA). Using the image processor, the analog gain and black levels of the image were optimized, and a defocused background image was subtracted from the live primary image. The contrast of the subtracted live image was digitally expanded to produce the final image (2, 14). Images were displayed on a video monitor with a final magnification of 5000 and recorded in real time with a ½-inch Panasonic video recorder (model nv-8050). Photographs were taken directly from the video monitor with a 35-mm camera and Kodak Plus-X film.

Lysis

The network strands were lysed by perfusion of 5% hexylene glycol, 1 mM sodium orthovanadate, and 0.15% Brij 58 in a buffer (50% PHEM [28]) consisting of 30 mM Pipes, 12.5 mM Hepes, 4 mM EGTA, and 1 mM $MgCl_2$. A pH of 7.0 was used in most cases (see Results). Though not absolutely necessary, a protease inhibitor cocktail of 10 μg/ml soybean trypsin inhibitor, 10 μg/ml *N*α-*p*-tosyl-L-arginine methyl ester, 10 μg/ml benzyl arginylmethyl ester, 1 μg/ml leupeptin, and 1 μg/ml pepstatin (adapted from reference 8) was included in the lysis mixture. After lysis (usually <1 min), the preparations were rinsed with 5 vol of buffer alone and then with fixative or with the experimental solutions described in Results. For actin decoration, the S1 portion of rabbit skeletal muscle myosin (provided by Dr. W. Z. Cande, University of California, Berkeley, CA) was incubated with the lysed preparations for 15 min, then fixed and processed for electron microscopy.

Fluorescence Microscopy

Lysed strands were fixed with 1% glutaraldehyde in 50% PHEM and processed as described in (26). The microtubules were reacted with a monoclonal antibody to *Drosophila* alpha tubulin (provided by Dr. Margaret Fuller, University of Colorado, Boulder, Colorado), and a fluoresceinated secondary antibody (Cappel Laboratories, West Chester, PA). Microfilaments were visualized with rhodamine-phalloidin (provided by Dr. T. Wieland, Max Plank Institut für Medizinische Forschung, Heidelberg, Germany).

Electron Microscopy

For whole-mount lysed preparations, small pieces of ameba were spread on formvar-covered gold finder grids attached to coverslips. All lysis and fixation manipulations were monitored in the light microscope. Lysed strands were fixed with 1% glutaraldehyde in 50% PHEM for at least 15 min, postfixed with 0.4% OsO_4 containing 0.8% KFeCN (18) (4 min), treated with 0.2% tannic acid (1 min), dehydrated with ethanol, critical point dried, and carbon coated. The preparations were observed in a Kratos high voltage electron microscope (HVEM) (The National Center for Electron Microscopy, Lawrence Berkeley Laboratories, Berkeley, CA) operated at 1,500 kV. Stereo pairs were made of all micrographs with tilt angles of 12°.

Results

Network Lysis

Perfusion of the peripheral network of *Reticulomyxa* with buffer containing Brij 58, hexylene glycol, and vanadate results in the immediate arrest of all motility and a rapid

1. *Abbreviations used in this paper*: AMPPNP, 5′ adenylyl-imidodiphosphate; DIC, differential interference contrast; DTT, dithiothreitol; HVEM, high voltage electron microscopy.

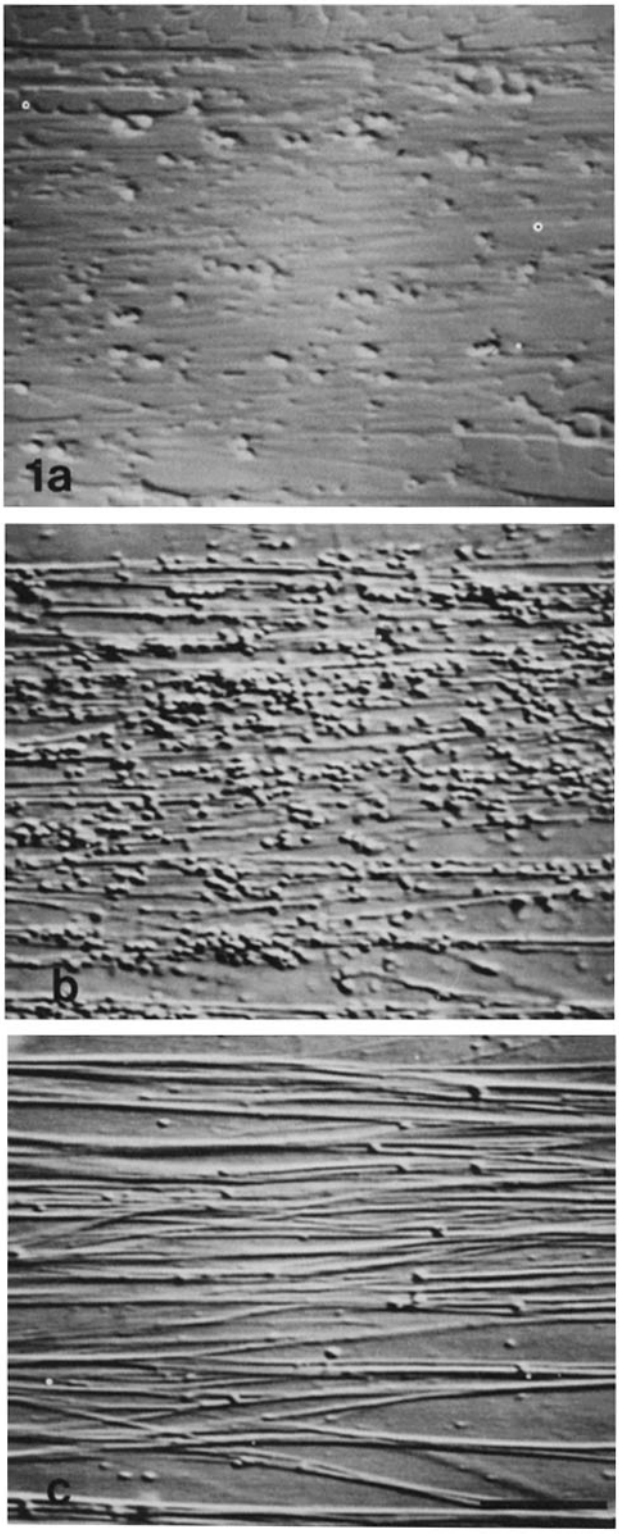


Figure 1. DIC videomicrograph sequence demonstrating the lysed network of *Reticulomyxa*. (*a*) A flattened region of network, live, before lysis. Organelles were moving horizontally in both directions. (*b*) Same region as in *a* after lysis and video enhancement. The structural elements are more visible and, due to their lack of movement, more organelles appear present. Typically, ATP is added at this point. (*c*) Same region after treatment with 1% Triton X-100. Most remaining organelles have been solubilized, yielding a relatively clean cytoskeletal framework. Stripped networks such as these can extend for several millimeters across a coverslip. Bar, 10 μm.

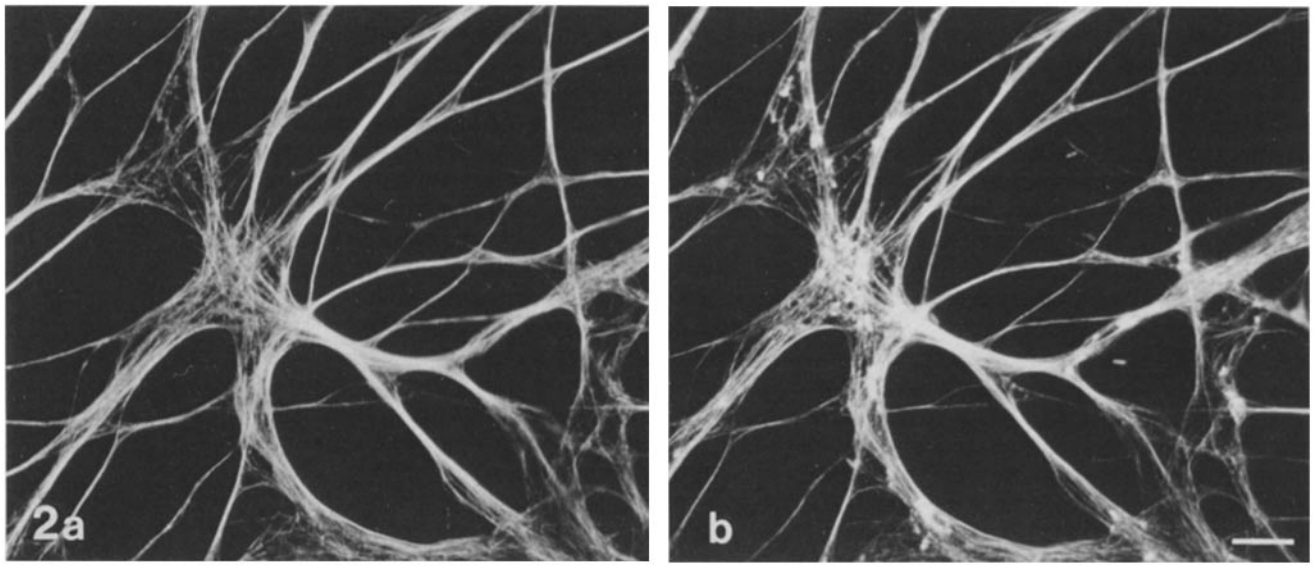


Figure 2. Microtubule (*a*) and microfilament (*b*) patterns after lysis. Strands were lysed, rinsed with buffer for 5 min, then fixed and labeled with a tubulin antibody and rhodamine-phalloidin. Note the close correspondence in staining patterns between the two cytoskeletal elements. Bar, 10 μm.

solubilization of the plasma membrane. The lysis is complete within 1 min and yields an extensive, entirely exposed cytoskeletal framework with many attached organelles (Fig. 1). This preparation is stable in buffer at room temperature and remains fully reactivatable for at least 1 h after lysis (longest time tested). Virtually all of the initial network, which can easily extend over an entire 22-mm square coverslip, is retained after lysis. Both microtubules and microfilaments remain intact, retaining their colinear organization (Fig. 2).

Vanadate and hexylene glycol are essential components of the lysis buffer. In their absence, the network disintegrates. 1 M hexylene glycol provides a faster and, in the thicker strands, a more complete lysis, but motility is not reactivatable. Vanadate causes the immediate cessation of in vivo movement and promotes the attachment of many organelles to the cytoskeleton. Substitution of vanadate with 5 mM 5′ adenylyl-imidodiphosphate (AMPPNP) (17) results in substantial network preservation but many strands are broken and appear frayed. Furthermore, AMPPNP-lysed networks disintegrate upon the addition of ATP.

Whole-mount electron microscopy of lysed preparations shows that all of the plasma membrane in the fine strands and flattened areas have been removed (Fig. 3). Surprisingly, however, the organelle membranes appear to remain intact. Most of the organelles retained along the network (both mitochondria and small clear vesicles) appear attached to microtubules, often at multiple points (Fig. 4). Although large numbers of actin filaments remain after lysis (as shown by rhodamine-phalloidin staining, see Fig. 2), many filaments appear to be lost during the fixation and subsequent processing for electron microscopy. However, decoration with myosin-S1 seems to stabilize actin filaments during processing for electron microscopy as a significant number of actin filaments can be found in these preparations (Fig. 5).

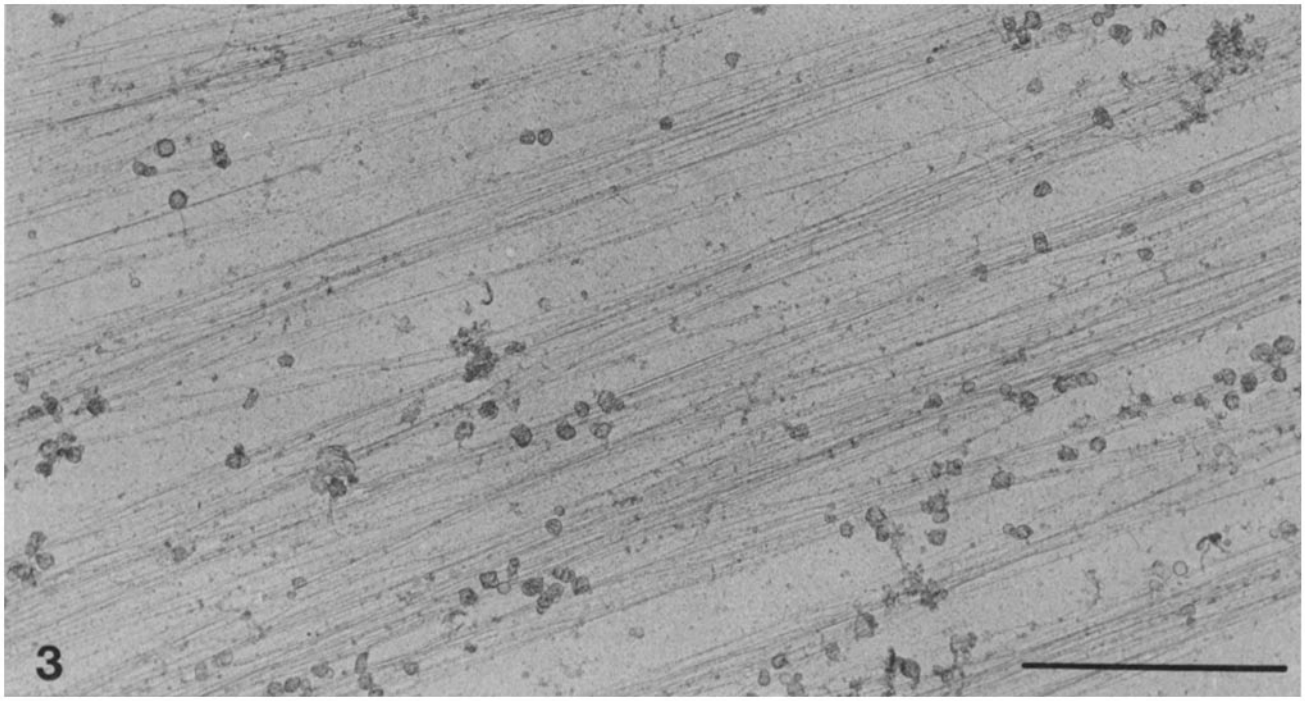
Network Reactivation

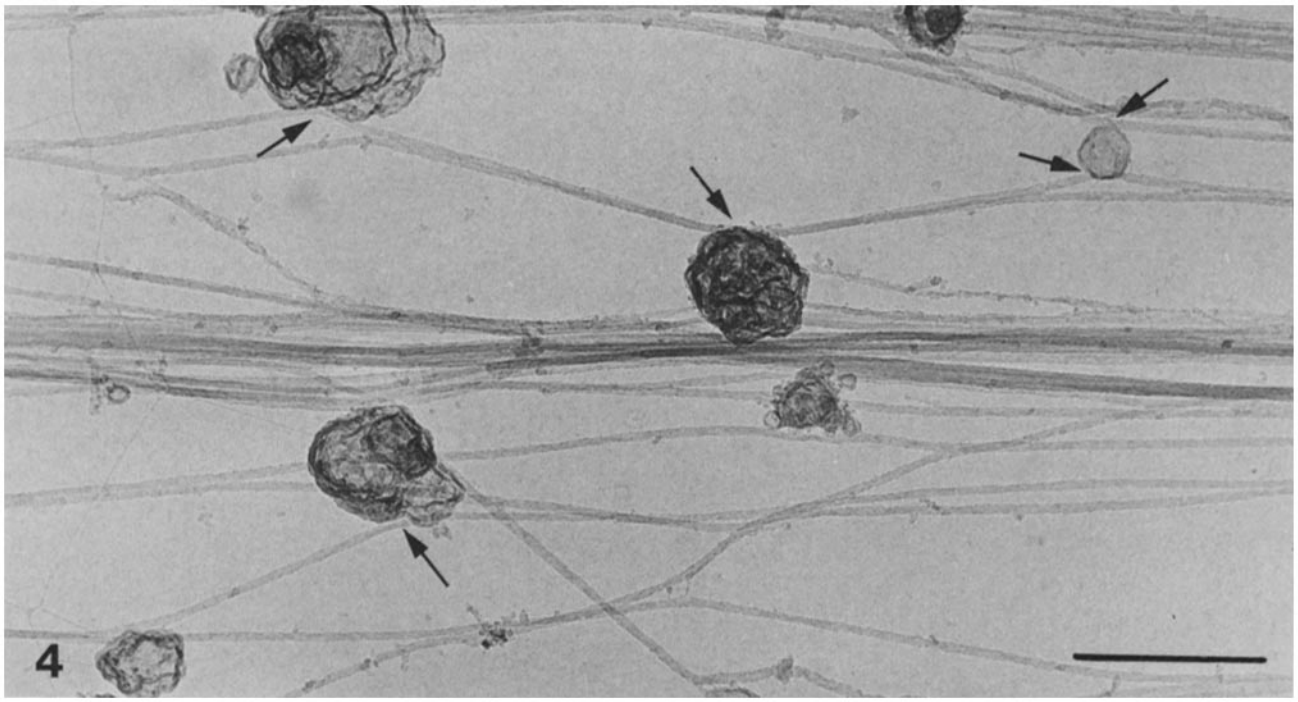
Upon the addition of ATP, three distinct forms of motility are observed in the lysed strands of *Reticulomyxa*: organelle movements along linear tracks, splaying and bending of microtubule bundles, and bulk streaming of organelle clusters.

Organelle Movements along Linear Tracks. When lysed networks are rinsed with buffer containing 1 mM ATP (but no hexylene glycol, vanadate, or Brij), ~50–70% of the remaining organelles resume movement that very closely resembles motility in vivo (Fig. 6). Depending on the conditions, reactivation results in average organelle rates of up to 15 μm/s (Fig. 7). No difference in rate or behavior can be seen between the mitochondria and smaller vesicles. In general, most reactivated models display equal frequencies and rates of motility in the anterograde (away from the cell body) and retrograde directions (see legend to Fig. 7).

Organelle movement occurs only in association with the visible linear elements; unattached organelles undergo Brownian motion. Individual organelles can be followed for >200 μm, although most tend to travel ~50 μm before detaching from the framework. Single particles or small aggregates of organelles move predominantly in one direction along any given linear element, but instances of two organelles moving in opposite directions or one reversing direction along the same pathway are not uncommon. Organelle movements are most prominent within the first 5 min of reactivation, but moving organelles can still be found at least 30–40 min later.

Splaying and Bending of Microtubule Bundles. In addition to organelle movements, ATP causes many of the parallel microtubule bundles to detach from the coverslip and undergo active splaying and bending motions. This results in a random and often dense microtubule meshwork (Fig. 8) that continues to support organelle movements. If a lysed network is rinsed with buffer containing 1% Triton X-100, most remaining organelles are solubilized (see Fig. 1 *c*), and, upon ATP addition, the splaying and bending of these bundles is greatly reduced. However, active splaying is still prominent in the few areas where organelles remain and continue to move.





Figures 3 and 4. (*Fig. 3*) HVEM overview of a lysed strand demonstrating the lack of any visible plasma membrane and long linear cytoskeletal bundles with associated organelles. Bar, 5 μm. (*Fig. 4.*) High magnification HVEM micrograph of a lysed strand showing microtubule–organelle associations (*arrows*). This preparation was fixed in the presence of vanadate. Note how the microtubules bend to contact the organelle surface, suggesting firm associations. Bar, 0.5 μm.

Bulk Streaming of Organelle Clusters. A third motility component of reactivated *Reticulomyxa* strands is a steady streaming motion of entire strands, parts of strands, and aggregates of organelles at rates of ~1–4 μm/s (Fig. 9). This movement is usually quite smooth and is visually distinct from the movement of individual organelles or bundle splaying. After several minutes, many organelles and particles have condensed into discrete clusters or foci which continue slow but steady movement.

Physiology of Reactivation

Reactivated motility requires ATP and is not induced by the nonhydrolyzable analogues ATPγS or AMPPNP (1 mM). Similarly, neither movement nor organelle release is pro-

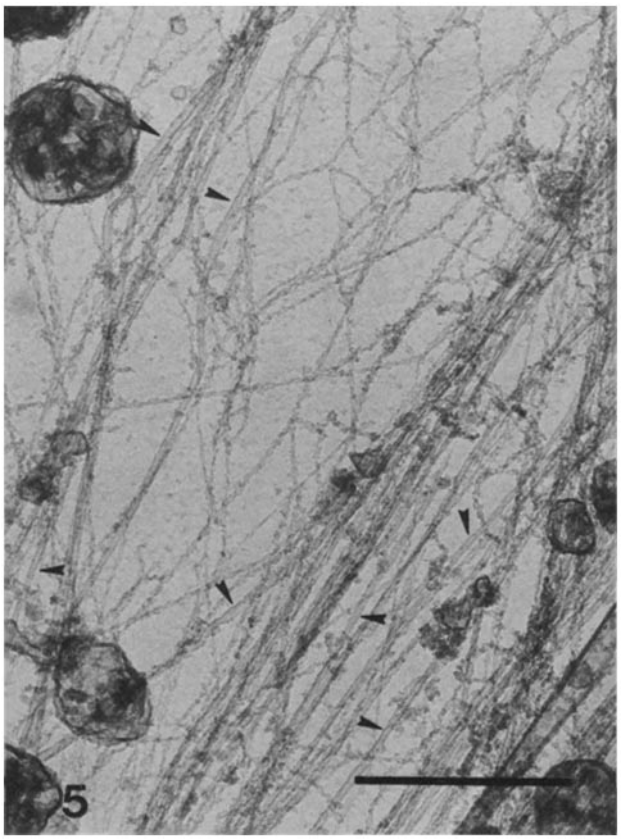


Figure 5. HVEM micrograph of a lysed preparation followed by S1-myosin decoration. Decorated filaments appear much more stable during electron microscopic processing and are more abundant. Several microtubules are also visible (*arrowheads*). Bar, 0.5 μm.

moted by the nucleotides GTP, CTP, UTP, ITP, or ADP (1 mM). ATP concentrations as low as 10 μM produce organelle movements but they are slower and increasingly saltatory. Equimolar concentrations (1 mM each) of ATP and AMPPNP (or ATPγS) produce a slow and very saltatory motility, suggesting competition for an ATP binding site.

The reactivated movements occur within a pH range of 6.0–8.0 (Fig. 7). Buffers adjusted to pH 6.0 provide good preservation during lysis but yield very slow reactivated movement. Lysis at pH 8.0 takes longer (>1 min), does not completely remove the plasma membrane of many fine strands, and results in the release of most organelles. Lysis at pH 7.0 followed by reactivation at pH 8.0 results in most organelles immediately detaching. The few organelles that remain show rapid movement before falling off. Lysis at pH 7.0 with reactivation at pH 7.5 appears optimal for retaining organelles and a maximum rate of motility. Interestingly, if the lysed networks are first rinsed with buffer at pH 8.5, (without ATP, organelles do not detach), then reactivated at pH 7.5, the average rate increases by 33% (10–15 μm/s) with a notable reduction in the saltatory nature of movement.

Reactivated motility is insensitive to 5 mM erythro-9-(3-[2-hydroxynonyl])adenine and sensitive only to relatively high concentrations (100 μM) of vanadate (Table I). Substitution of EDTA for EGTA in the reactivation buffer completely inhibits motility. This inhibition can be overcome by adding magnesium (4 mM) to the ATP-EDTA mixture or by subsequently rinsing the network with the standard Mg-ATP buffer, thus indicating a magnesium requirement. Calcium is not necessary for reactivation and free calcium >10 μM significantly inhibits motility and decreases network stability in the presence of ATP. Concentrations of *N*-ethylmalemide >100 μM noticeably inhibit movement. Incubation of the lysed network with 2.5 mM N-ethylmalemide (5 min), even if followed by a 5-min period of inactivation with 5 mM dithiothreitol (DTT) completely prevents reactivation. 1 mM DTT alone did not enhance or otherwise affect reactivated motility. None of these treatments differentially affect anterograde or retrograde movements. In addition, taxol (20 μM), phalloidin (10 μg/ml), nocodozole (10 μg/ml), or cytochalasin D (10 μg/ml) had no visible effect on either the cytoskeletal structure or reactivated movements in these lysed preparations.

Discussion

Lysis of the peripheral network of *Reticulomyxa* yields an extensive cytoskeletal framework that retains its ability to sup-

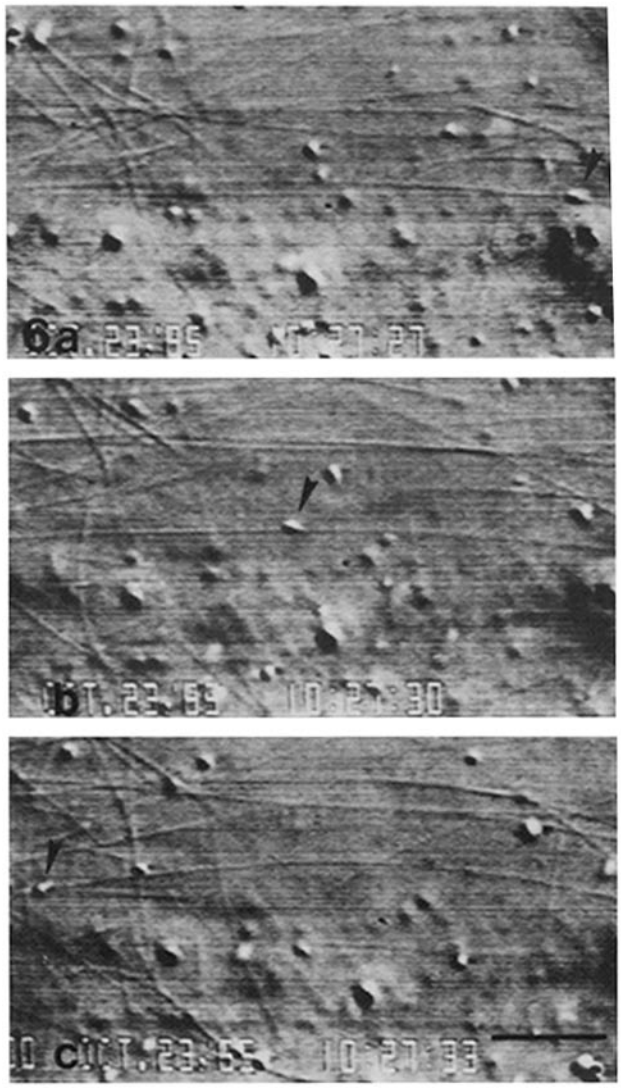


Figure 6. DIC videomicrograph sequence of reactivated organelle movement. A mitochondrionlike particle (*arrowhead*) is moving along a small bundle of microtubules at ~8 μm/s. Time is displayed in seconds. Bar, 10 μm.

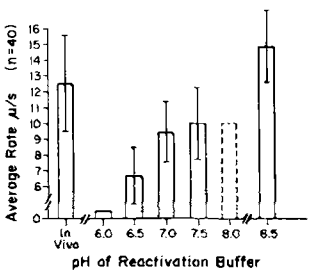


Figure 7. Effect of pH on reactivated motility. In vivo rates average 12.5 µm/s and range up to 25 µm/s. Reactivated rates range up to 20 µm/s. Rates were determined from organelles that moved continuously for at least 5 µm. Reactivation at pH 8.0 results in most organelles detaching, although motility of the few remaining organelles (not measured) appears identical to movement at pH 7.5. The bar above pH 8.5 represents strands prerinsed with buffer (no ATP) at pH 8.5 and reactivated at pH 7.5. Anterograde and retrograde motility was measured at pHs 7.0 and 7.5 and showed no significant difference in rate (pH 7.0, anterograde = 9.2 ± 1.5 µm/s, retrograde = 9.6 ± 1.9 µm/s; pH 7.5, anterograde = 10.3 ± 2.3 µm/s, retrograde = 9.7 ± 1.9 µm/s. Results represent movement of at least three preparations at each pH value.

port endogenous organelle movements. This model system differs from many previously described permeabilized or lysed cell transport systems in several respects. First, based on electron microscopy, it is completely open to the environment, allowing the unhindered access of labeled probes, large molecules, or other organelle complexes, a stringent control of environmental conditions, and the rapid removal of all soluble cytoplasmic components. Second, the lysed networks behave in a consistently reproducible fashion in reactivation experiments and are remarkably stable. Reactivation initiated as long as 1 h after lysis is essentially indistinguishable from that induced immediately after extraction. This is a significant advantage over reactivatable cell models which decay rapidly. Third, by removing the endogenous organelles, this system provides a simple and ordered colinear arrangement of both microtubules and microfilaments that is not complicated by large internal membrane systems, nuclei, or intermediate filaments. This unique arrangement should serve as a convenient motility assay system for organelles isolated from other cell types or coated beads. It should also be a useful system with which to study both structural and functional relationships between microtubules and microfilaments. Finally, the large size of this network (single lysed strands can easily extend continuously for several millimeters) makes this system amenable to biochemical dissection.

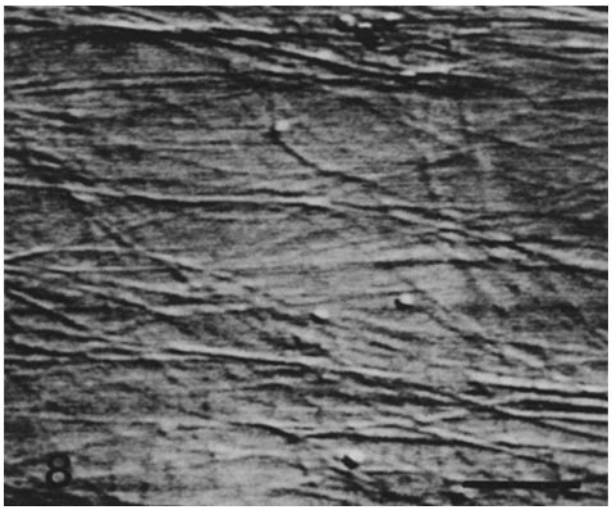


Figure 8. Video-enhanced DIC micrograph of a moderate microtubule meshwork. Compare this image with Fig. 1, *b* or *c*. This micrograph was taken ~5 min after reactivation. Bar, 10 µm.

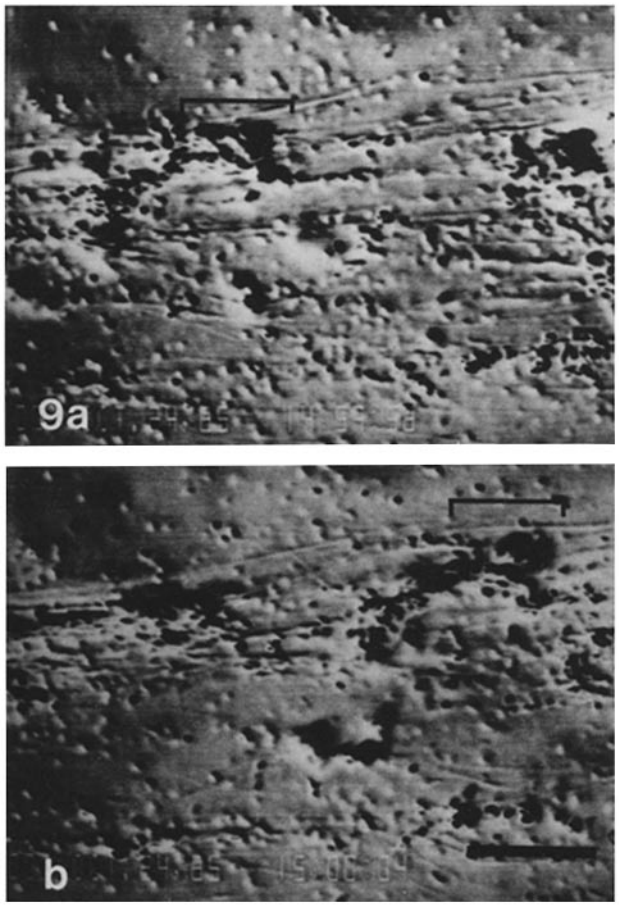


Figure 9. Sequence of videomicrographs taken 6 s apart that demonstrate the reactivated streaming of organelle aggregates. Clumps of organelles (brackets) are moving ~3 µm/s from left to right (toward the cell body). Bar, 10 µm.

Table I. Effect of Various Inhibitors on Reactivated Motility

Treatment		Motility
	µM	
Vanadate	100	No movement
	50	Very saltatory and slow
	25	Almost normal
	10	Normal
	mM	
EHNA	5	Normal
	1	Normal
N-ethylmaleimide	2.5	No movement
	0.1	Almost normal
DTT	1	Normal

Lysed and rinsed networks were perfused with 50% PHEM containing vanadate or DTT plus 1 mM ATP. erythro-9-(3-[2-hydroxynonyl])adenine experiments received 0.1 mM ATP. Networks were treated with *N*-ethylmaleimide in buffer for 5 min then rinsed with buffer + 1 mM ATP. Motility results were obtained from at least three preparations. Almost normal motility describes the slightest inhibitory effect and probably reflects the threshold concentration of inhibitor.

Hexylene glycol and vanadate are essential stabilizing agents in this lysis procedure. Hexylene glycol, introduced by Kane in 1965 (15), is a poorly understood though often used microtubule stabilizer. Its effects on *Reticulomyxa* cannot be mimicked by glycerol, polyethylene glycol, or taxol. We believe the stabilizing effects of vanadate are twofold. First, by immediately arresting the rapid in vivo movement, the instability created by the movement alone is eliminated. Second, it appears that vanadate "locks" organelles onto the cytoskeleton in a manner analogous to the described action of AMPPNP on fast axonal transport (17). This effect contrasts with the reported inhibitory action of vanadate on sea urchin dynein which acts to "freeze" dynein at a crossbridge step after detachment from the B-subfiber microtubule (24, 30). Since single organelles appear able to bind more than one transport filament, these organelle–cytoskeletal attachments might serve to reversibly cross-link entire strands and thus provide additional stability. It is puzzling that the action of vanadate cannot be mimicked by AMPPNP as in other systems (5, 28, 36) and therefore we cannot rule out additional inhibitory effects.

Reactivation with ATP restores forms of motility that closely resemble the rapid movements of individual organelles in vivo, a splaying of the microtubule bundles, and smooth, slower bulk movements of strands and particle clusters. Based on fine structural evidence that predominantly shows organelle–microtubule rather than organelle–microfilament associations, and preliminary actin fragmentation experiments with gelsolin (6) (not shown), the movement of individual organelles in *Reticulomyxa* appears to be a microtubule-based process. Similar organelle movements in other systems (3, 22, 28, 34) have also been shown to be based solely on microtubules.

The apparent splaying of microtubule bundles might be the result of several overlapping phenomena and must be cautiously interpreted. First, the dynamic network splaying and extension seen in vivo (16, Koonce, M., U. Euteneur, K. McDonald, D. Menzel, and M. Schliwa, manuscript in preparation.) suggests some sort of lateral interactions (sliding or zipping?) exist between microtubules that might be reactivated in the lysed preparations. Second, in the absence of a confining plasma membrane, the organelle movements themselves could indirectly push apart microtubules or, conversely, impeded organelle progress with the motor (either on the organelle surface or microtubule) still running could result in direct physical displacement of the tracks. Finally, a contractile system involving the microfilaments could also play a role in this process. Based on the available evidence, we cannot distinguish between these possibilities or rule out that this phenomenon is a cumulative effect involving all these mechanisms.

The slow, smooth bulk movements of organelle aggregates closely parallels the bulk cytoplasmic streaming seen in vivo (Koonce, M., U. Euteneur, K. McDonald, D. Menzel, and M. Schliwa, manuscript in preparation.). These movements also share a striking similarity to the behavior of reactivated rhodamine–phalloidin-labeled actin filaments in this system as visualized by low light level video fluorescence microscopy (not shown). In these preliminary experiments, actin filaments slowly condense from long linear bundles into discrete foci, an observation consistent with the idea that an actin-based system mediates these smooth bulk movements. These lysed preparations therefore seem to preserve more than one transport system, and current experiments address this possibility.

Our pharmacological data suggest that the organelle transport motor of *Reticulomyxa* differs from other known microtubule-associated ATPases. The insensitivity to low concentrations of vanadate and EHNA (4, 12) is inconsistent with a role for a dynein-like motor in powering transport. There are also differences to squid axon kinesin (36) and other kinesin-like molecules (5, 10, 22, 28). Though both share a similar relative insensitivity to vanadate, the *Reticulomyxa* translocator moves organelles both in vivo and in the reactivated system an order of magnitude faster than kinesin (35, 36). In addition, GTP will not reactivate motility in *Reticulomyxa*, while kinesin is able to use it as an energy source (36). It is plausible that a modified kinesin powers transport in *Reticulomyxa*, however, the presence of a different translocator can not yet be excluded. Experiments are currently under way to identify the *Reticulomyxa* translocator(s) and to compare it in greater detail with kinesin and other components of the motile machinery in the squid giant axon (37). In addition, efforts are under way to selectively remove either the microtubules or the microfilaments to examine the respective contributions of these two cytoskeletal systems to the motility processes in *Reticulomyxa*.

We thank Drs. W. Z. Cande, M. Fuller, and T. Wieland for generously providing antibodies, labeled phalloidin, and nucleotides. We also thank Drs. W. Z. Cande, U. Euteneuer, and B. Burnside for many helpful discussions, suggestions, and critical reading of the manuscript. A special thanks goes to Dr. D. Menzel for his "naturalist's eye" and many helpful suggestions and comments.

This work was supported by National Institute of General Medical Sciences grant 31041.

Received for publication 27 February 1986, and in revised form 22 April 1986.

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