

Bidirectional Transport of Fluorescently Labeled Vesicles Introduced into Extruded Axoplasm of Squid *Loligo pealei*

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ABSTRACT A reconstituted model was devised to study the mechanisms of fast axonal transport in the squid *Loligo pealei*. Axonal vesicles were isolated from axoplasm of the giant axon and labeled with rhodamine-conjugated octadecanol, a membrane-specific fluorescent probe. The labeled vesicles were then injected into a fresh preparation of extruded axoplasm in which endogenous vesicle transport was occurring normally. The movement of the fluorescent, exogenous vesicles was observed by epifluorescence microscopy for as long as 5 min without significant photobleaching, and the transport of endogenous, nonfluorescent vesicles was monitored by video-enhanced differential interference-contrast microscopy. The transport of fluorescent, exogenous vesicles was shown to be bidirectional and ATP-dependent and occurred at a mean rate of $6.98 \pm 4.11 \mu\text{m/s}$ (mean \pm standard deviation, $n = 41$). In comparison, the mean rate of transport of nonfluorescent, endogenous vesicles in control axoplasm treated with vesicle buffer alone was $4.76 \pm 1.60 \mu\text{m/s}$ ($n = 64$). These rates are slightly higher than the mean rate of endogenous vesicle movement in extruded axoplasm ($3.56 \pm 1.05 \mu\text{m/s}$, $n = 40$) not subject to vesicles or vesicle buffer. Not all vesicles and organelles, exogenous or endogenous, were observed to move. In experiments in which proteins of the surface of the fluorescent vesicles were digested with trypsin before injection, no movement of the fluorescent vesicles was observed, although the transport of endogenous vesicles and organelles appeared to proceed normally. The results summarized above indicate that isolated vesicles, incorporated into axoplasm, move with the characteristics of fast axonal transport. Because the vesicles are fluorescent, they can be readily distinguished from nonfluorescent, endogenous vesicles. Moreover, this system permits vesicle characteristics to be experimentally manipulated, and therefore may prove valuable for the elucidation of the mechanisms of fast axonal transport.

The neuron, because of its elongate form, has proven to be a popular system for the study of movement of intracellular membranous organelles. This rapid movement, called fast axonal transport, occurs at rates from 1 to $5 \mu\text{m/s}$ in either the orthograde direction (from the cell body to the axon terminal) or in the retrograde direction (from axonal extremities to the cell body; reviewed in references 10, 15, 34). Axoplasmic transport can be blocked, for example, by constriction (27) or by locally applied cold temperatures (12, 30). Electron microscopical studies (27, 30) have shown that the membranous organelles that accumulate in the vicinity of such a block include, in the case of orthograde movement, dense core granules, small tubules, and large numbers of vesicles, collectively called tubulovesicular membranous or-

ganelles. The organelles that are moving in the retrograde direction are different in nature, and include multivesicular and lamellar bodies (30).

Models have been developed in an attempt to explain this extreme example of intracellular particle movement. Because organelle movements are not dependent on action potentials or on ion flux across the membrane (11, 21), it is possible to separate by mechanical extrusion the axoplasm from the surrounding connective tissue (6, 11, 20) or to permeabilize the plasma membrane with detergents (14) or electrical discharge (1, 2). With the normal permeability barrier of the axon removed or destroyed, and with the use of appropriate buffers, light microscopy was used to observe the movement of various organelles; furthermore, these movements were

shown to occur at normal rates and to require ATP hydrolysis (1, 11, 14).

Another approach to study the mechanisms of intracellular organelle movement is microinjection of synthetic particles into cells and characterization of the exogenous particle movement in comparison with endogenous organelle transport. Adams and Bray (3) have shown that synthetic particles with sizes up to 0.5 μm in diameter microinjected into intact crab axons were transported with movements indistinguishable from endogenous organelles moving in the orthograde direction. They concluded that a transport system exists in axons that has the capability of carrying any particle of suitable physical properties in the orthograde direction. Similarly, Beckerle and Porter (7) have demonstrated that the transport of microinjected fluorescent polystyrene beads in tissue culture cells does not require membrane-encoded information. They found that the movement of the beads could be reversibly inhibited by the microtubule inhibitor nocodazole, while at the same time the F-actin that stained with nitrobenzoxadiazol-phalloidin remained intact. In interpreting these studies, the investigators stated that they did not exclude the possibility that the exogenous particles became coated with an endogenous force-generating protein such as myosin or dynein.

In studies designed to correlate intracellular membranous organelle movement with cytoskeletal structures, Travis and Allen (29) and Hayden et al. (16) demonstrated that these organelles move in close association with bundles of microtubules. By combining video-enhanced differential interference contrast (DIC)¹ and immunofluorescence light microscopy with electron microscopy, it has very recently been shown that organelle movement can take place along a single microtubule. (John H. Hayden, personal communication).

Using another approach, Sheetz and Spudich (26) have developed an in vitro model that employs myosin-coated synthetic beads that have the ability to move on subcortical actin bundles of the alga *Nitella*. The F-actin filaments within the bundles are aligned with the same polarity, and the myosin-coated spheres attached and moved unidirectionally along the actin cables. This assay permits the study of the movement of synthetic particles or cellular organelles in an isolated actomyosin-dependent system.

In the experiments described here, axonal vesicles were isolated from the axoplasm of the squid *Loligo pealei* and labeled with rhodamine-conjugated octadecanol (R-C₁₈), a membrane specific fluorescent probe. These labeled vesicles were then injected into another preparation of extruded axoplasm in which endogenous particle movement was proceeding normally. The results demonstrate that the labeled, exogenous vesicles move with the characteristics of fast axonal transport. Because the vesicles are fluorescent, they can be distinguished from endogenous membranous organelles. The vesicle characteristics can thus be manipulated in experiments designed to elucidate the mechanisms of fast axonal transport.

MATERIALS AND METHODS

Membrane Probe: The R-C₁₈ was prepared by Dr. Wendy F. Boss (North Carolina State University), using a modification of the procedure of Boss et al. (9) and characterized further as described below. R-C₁₈ is a nonspecific membrane probe with a hydrocarbon chain that allows it to partition into membranes. The reaction mixture was extracted with chloroform, and the final

¹ *Abbreviations used in this paper:* DIC, Differential interference contrast; DNP, 2,6-dinitrophenol; R-C₁₈, rhodamine-conjugated octadecanol.

product was purified by thin layer chromatography (18). The R-C₁₈ product was scraped from the plate, dissolved in ethanol, and the silica gel removed by centrifugation. An aliquot of R-C₁₈ was dried under N₂, and the resulting oil was analyzed by infrared spectroscopy (Perkin-Elmer 599, Perkin-Elmer Corp., Ridgefield, CT), mass spectroscopy (Finnigan 4000, Finnigan Corp., San Jose, CA), and chemical analysis (Atlantic Microlabs, Atlanta, GA). The infrared spectrum of the R-C₁₈ showed a carbonyl ester stretching frequency of 1,715 cm⁻¹ as compared to 1,685 cm⁻¹ for the free acid starting material, rhodamine B. The higher stretching frequency of the carbonyl ester was as expected, and no free acid was present in the R-C₁₈ sample. The mass spectrum and chemical analysis indicated contamination by imidazole, a by-product of the synthesis reaction; this was thought not to be a problem since the probe was used in an imidazole buffer. For use, the R-C₁₈ was diluted 1:500,000 in 95% ethanol, and free molecules of probe were separated from labeled membranes by sucrose density gradient centrifugation (see below).

Purification and Labeling of Vesicles: Squid (*L. pealei*) obtained from the Department of Marine Resources, Marine Biological Laboratory, Woods Hole, MA, were used for experiments <48 h after their capture at sea. The dorsal giant nerve fibers were removed, and axoplasm from 10 axons was collected by mechanical extrusion (20). The extruded axoplasm was diluted in 100–200 μl of buffering medium (250 mM sucrose, 250 mM KCl, 10 mM MgCl₂, 1 mM EGTA, 1 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 10 mM imidazole, pH 7.4) at 4°C and homogenized gently in a glass-Teflon homogenizer (Wheaton Scientific, Millville, NJ). The homogenate was incubated with 5 μl R-C₁₈ for 10 min on ice, and layered on top of a three-step sucrose gradient of 150 μl of 0.3 M sucrose, 150 μl of 0.6 M sucrose, 100 μl of 1.5 M sucrose. The sample was overlaid with 50 mM sucrose to fill the 0.8 ml Ultraclear centrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA). The sucrose solutions were prepared in a buffer containing 100 mM KCl, 10 mM MgCl₂, 1 mM EGTA, 1 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 10 mM imidazole, pH 7.4. The samples were centrifuged at 22,000 rpm for 5 h at 4°C in a SW50.1 rotor (Beckman Instruments, Inc.). The gradients were then fractionated into ~200- μl fractions. Each fraction was monitored by epifluorescence microscopy and by negative-stain electron microscopy using 1% uranyl acetate. The first 250–300 μl from the top of the tube contained R-C₁₈ that was not incorporated into membrane; therefore these fractions were discarded. The next two fractions contained large numbers of fluorescently labeled vesicles, and these fractions were combined for microscopy and biochemical studies. The vesicle preparation was stored at 4°C until use.

Light Microscopy: Axoplasm from the giant axon was extruded mechanically onto a cover glass (11). Labeled, exogenous vesicles were introduced into the midregion of the cylinder of axoplasm using a pulled, glass pasteur pipette. The long axis of the pipette was held perpendicular to the cylinder of axoplasm and parallel to the surface of the slide during the injection. The preparation was incubated for 10 min at 10–16°C to give the exogenous vesicles time to associate with the transport apparatus. Subsequently, the axoplasm was washed with buffer X (350 mM aspartate, 130 mM taurine, 70 mM betaine, 50 mM glycine, 12.9 mM MgCl₂, 10 mM EGTA, 3 mM CaCl₂, 1 mM ATP, 1 mM glucose, 20 mM HEPES, pH 7.2) (a modification of buffer P, see reference 23) to remove excess, unassociated vesicles. The preparation was transferred to an inverted Zeiss Axiomat equipped with 50 \times /1.0 N.A. and 100 \times /1.3 N.A. planapochromatic objectives. The exogenous vesicles were examined by epifluorescence microscopy with a 100 W Hg light source, 515–560 nm excitation filter, 580 nm dichroic reflector, and 590 nm barrier filter (Carl Zeiss, Inc., Thornwood, NY). The fluorescent images were recorded with a Hamamatsu C-1000-12 silicon-intensified target camera coupled to a SONY VO-5800 3/4 inch videocassette recorder. Endogenous vesicle transport was monitored by video-enhanced contrast, DIC microscopy (4). For reproduction of images from the videotape, micrographs were photographed directly from the video monitor onto 35-mm Kodak Plus-X film which was developed in Microdol-X.

The motility of the labeled, exogenous particles was analyzed as described previously (5, 16), using a Graf/Pen digitizer (Science Accessories Corp., Southport, CT) connected to a NEDCO Able 60 computer (New England Digital Corp., White River Junction, VT). The measurements were made on vesicle images that were stored on videotape and played at normal speed (60 fields/s; 30 frames/s after 2-field interlace). For measurements of movement of endogenous, nonfluorescent vesicles, only those vesicles that were spherical and moving were measured. This helped to ensure that movements of mitochondria (which were generally oblong by rhodamine 123 staining, data not shown) were not counted in the measurements. In the fluorescence observations, only spherical, moving particles were included in the experimental sample. Thus, both endogenous and fluorescent vesicles were chosen using the same criteria. The rates reported are of individual vesicles that traveled continuously a distance (micrometers) per unit time (seconds), and rates of translocations include in the measurement no pause times (if any) of the particles. Each of the three experimental samples (see Fig. 3) was composed of data from vesicles moving in the orthograde direction and from vesicles moving in the retrograde direction.

Vesicle Treatments: For some experiments the vesicle preparations were pretreated with trypsin (1 $\mu\text{g}/\text{ml}$, Type V-S, Sigma Chemical Co., St. Louis, MO) for 10 min followed by treatment with soybean trypsin inhibitor (5–10 $\mu\text{g}/\text{ml}$, Type II-S, Sigma Chemical Co.). The proteins of the vesicles were analyzed by SDS PAGE.

For another series of experiments, the axoplasm, containing labeled, exogenous vesicles, was treated with 2,6-dinitrophenol (DNP, 200 μM), an uncoupler of oxidative phosphorylation, in the presence and absence of added ATP (1 mM).

Electron Microscopy: Isolated, labeled vesicles were collected by centrifugation for 2 h at 45,000 rpm in a Beckman SW50.1 rotor at 4°C. The supernatant was removed, and the pellet containing vesicles was fixed for 3 h at 4°C in 3% glutaraldehyde, 1 M sucrose, 0.1 M cacodylate buffer, pH 7.4. The pellet was washed in 1 M sucrose, 0.1 M cacodylate buffer, pH 7.4, and postfixed for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, at 4°C. The sample was then dehydrated in ethanol, transferred through three changes of propylene oxide, and embedded in LX-112 (Ladd Research Industries, Burlington, VT).

For transmission electron microscopy, thin sections (60–70 nm) were cut with a glass knife and stained with saturated aqueous uranyl acetate followed by lead citrate (24). The sections were observed with a JEOL-100CX electron microscope at an accelerating voltage of 60 kV.

SDS PAGE: Vesicles were collected by centrifugation for 2 h at 45,000 rpm in a Beckman SW50.1 rotor at 4°C. The proteins of the pellet were resolved in 6–10% acrylamide and 12–48% urea linear gradient slab gels according to the method of Laemmli (19). Gels were stained for protein either with Coomassie Blue (13) or with Kodavue (Eastman Kodak Co., Rochester, NY).

RESULTS

The purification procedure described in Materials and Methods yields highly fluorescent vesicles that can be continuously observed by epifluorescence microscopy for up to 5 min without significant bleaching. Because the R-C₁₈ partitions into membranes nonspecifically (18), the labeled vesicles presumably represent the general population of membranous organelles found in axoplasm. Fig. 1 illustrates that the population of fluorescent vesicles was heterogeneous, having a range of diameters of 38–475 nm. In addition to vesicles, the fraction contained some mitochondria, as well as filamentous aggregates which were probably of cytoskeletal origin.

The R-C₁₈ vesicles were observed to move in both the orthograde and retrograde directions after injection into extruded axoplasm. In Fig. 2, the vesicle denoted as Y moved

in the retrograde direction at a rate of 8.2 $\mu\text{m}/\text{s}$, while vesicle X moved in the orthograde direction at 8.8 $\mu\text{m}/\text{s}$. Their paths of movement were within 14 μm of each other. The vesicle denoted as Z moved in the retrograde direction at a slower rate (2.1 $\mu\text{m}/\text{s}$) and was observed to pause or hesitate three times during the period of observation. In Fig. 2e, the photograph was produced by a continuous 10-s exposure of the videomonitor while the image was displayed at normal time. The streaks illustrating vesicle movement show sequential areas of bright, punctate fluorescence alternating with less bright areas. One has the impression from such images that the vesicle movement occurs in a discontinuous, steplike fashion, although when one views the videotape, the movement appears continuous.

Most of the observations made on the video monitor were of a single, fluorescent R-C₁₈ vesicle moving across a dark screen rather than of many fluorescent vesicles moving in the orthograde and retrograde directions at the same time. This occurred because there were few fluorescent vesicles in the axoplasmic preparation in comparison to the possible number of fields of focus available for examination. That is, few if any fluorescent vesicles occupied a given field and plane of focus at any one time of observation. For example, because the cylinder of axoplasm was 1.5 cm long, and the microscope field width was 185 μm , 80 individual areas could potentially be examined. In addition, each of the eighty fields had numerous planes of focus (depth of focus, ~ 410 nm) due to the thickness of the preparation of extruded axoplasm (~ 1 mm).

The rate of R-C₁₈-vesicle movement was 6.98 ± 4.11 $\mu\text{m}/\text{s}$ (mean \pm standard deviation, $n = 41$), whereas endogenous vesicles from control extruded axoplasm treated with R-C₁₈-vesicle buffer alone demonstrated a mean rate of 4.76 ± 1.60 $\mu\text{m}/\text{s}$, $n = 64$. By contrast the mean rate of unlabeled, endogenous vesicles from untreated extruded axoplasm was 3.56 ± 1.05 $\mu\text{m}/\text{s}$, $n = 41$ (Fig. 3). The means were significantly different from one another ($P < 0.01$) as determined by the Student's *t* test. The data suggest that the vesicle buffer affected the axoplasm to allow faster rates of movement (see Discussion).

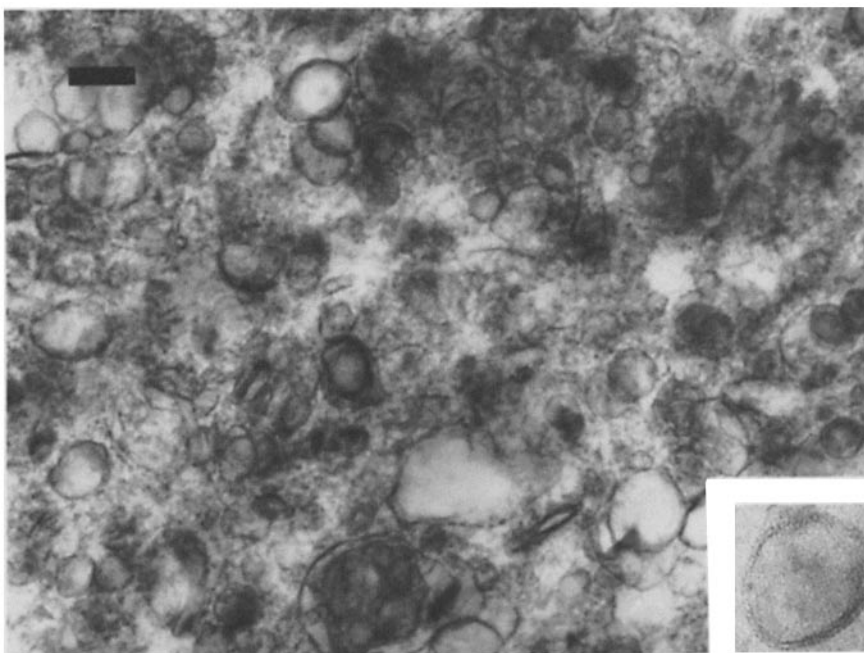


FIGURE 1 Transmission electron micrograph illustrating the heterogeneity of the vesicle fraction. In addition to vesicles of different sizes (38–475 nm), there are filamentous aggregates probably of cytoskeletal origin. The vesicle in the inset (60 nm in diameter) demonstrates the characteristic trilaminar appearance of unit membranes. Bar, 105 nm. $\times 53,000$. (Inset) $\times 225,000$.

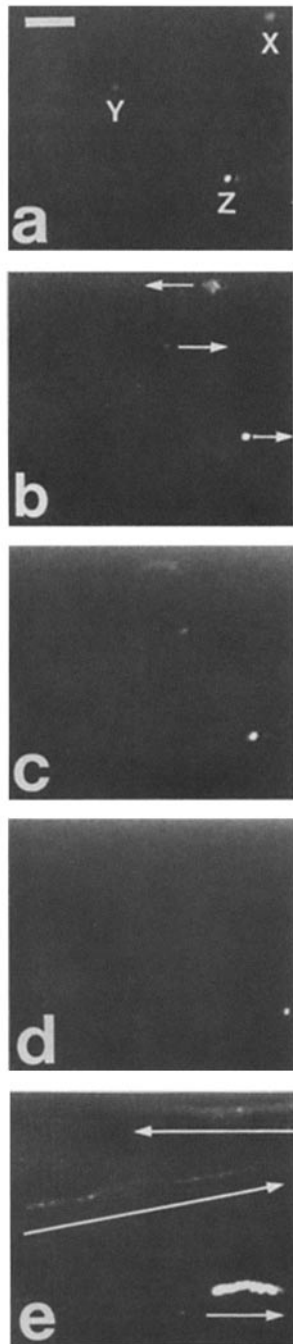


FIGURE 2 Epifluorescence micrographs of R-C₁₈-vesicle movement. (a-d) Demonstration of bidirectional movement during a 14-s time interval; each micrograph was obtained with a ¼-s exposure. Vesicle Y moved in the retrograde direction at a rate of 8.2 μm/s, while vesicle X moved in the orthograde direction at 8.8 μm/s. Vesicle Z moved in the retrograde direction at 2.1 μm/s. Vesicles X and Y had moved out of the plane of focus by the time frame *d* was obtained. In *e*, the photograph was produced by a continuous 10-s exposure of the video monitor while the videorecorder played at a rate representing normal time. Arrows indicate the direction of vesicle movement. Bar, 10 μm. × 600.

Some R-C₁₈ vesicles moved continuously while others were observed to pause in transit; still others were not observed to move. Also, the paths of movement were linear, although some fluorescent vesicles were observed to deviate from one linear trajectory to another as has been observed for endoge-

nous membranous organelles in intact giant axons (5). In addition to these similarities, there were differences. Neither the endogenous axonal transport nor the R-C₁₈-vesicle movements could be observed after ~30 min. The preparations of axoplasm that received R-C₁₈ vesicles or controls that received vesicle buffer alone were not viable for as long as extruded axoplasm with no added buffer or with added buffer X (see Methods and also reference 23).

To show that the movement of the exogenous, fluorescent vesicles was an energy dependent process, the following experiment was performed. R-C₁₈ vesicles were injected into extruded axoplasm. After 10 min, a time that would assure that the fluorescent vesicles were moving, the axoplasm was washed with buffer X containing 200 μM DNP in the presence or absence of added 1 mM ATP. In axoplasm treated with DNP in the absence of added ATP, the R-C₁₈ vesicles stopped moving within 15 min. They were stationary and did not show Brownian movement, suggesting that they remained attached to the transport apparatus. In addition, the transport of the endogenous, nonfluorescent vesicles and organelles also ceased, and the mitochondria appeared swollen. However, in the presence of exogenously supplied ATP, the movement of the R-C₁₈ vesicles was comparable to that observed in the absence of DNP, and the transport of endogenous vesicles appeared to proceed normally. The mitochondria, though, remained swollen and stationary. Such results suggest that the movement of injected, exogenous, fluorescent vesicles occurs via an ATP dependent transport process.

To determine if membrane-associated proteins were of functional significance in R-C₁₈-vesicle movement, we pre-treated the fluorescent vesicles with trypsin as described in Materials and Methods, followed by soybean trypsin inhibitor. The fluorescent vesicles that had been exposed to trypsin were incorporated into axoplasm and allowed to incubate 10 min before washing with buffer X. The exogenous, fluorescent vesicles remained stationary and did not undergo Brownian motion. However, endogenous axoplasmic transport was observed to occur in the same regions of axoplasm in which the stationary fluorescent vesicles were seen.

Fig. 4 illustrates the protein composition of axoplasm and of isolated vesicles, as determined by SDS PAGE, followed by Kodavue stain; the lanes were from a single slab gel. Lanes *a*, *b*, and *c* in Fig. 4 contain various standards. Lane *d* demonstrates the polypeptide composition of the vesicles isolated by centrifugation of the vesicle fraction initially obtained by sucrose gradient centrifugation. In Fig. 4, lane *e*, the polypeptide composition of the sedimented vesicles after trypsin digestion is shown for comparison. The trypsin treatment resulted in a loss of approximately six minor bands and four major bands, the latter indicated with arrows in Fig. 4, lane *d*. One of these co-migrated with the brain microtubule-associated protein MAP 2 (compare lanes *c* and *d*). The arrowhead in lane *e* indicates the band of one prominent fragment that was generated by the tryptic digestion (*M_r* 256,000).

Bands presumably representing the axonal cytoskeletal proteins were also present in the vesicle pellet (Fig. 4, lane *d*). These included the neurofilament proteins (*M_r* 200,000 and *M_r* 60,000), the fodrin doublet (*M_r* 230,000), α- and β-tubulins, and actin (*M_r* 43,000). After trypsin treatment bands representing the fodrin doublet, α- and β-tubulins, and actin appeared unchanged. This observation may indicate that at least these proteins were protected from or resistant to the

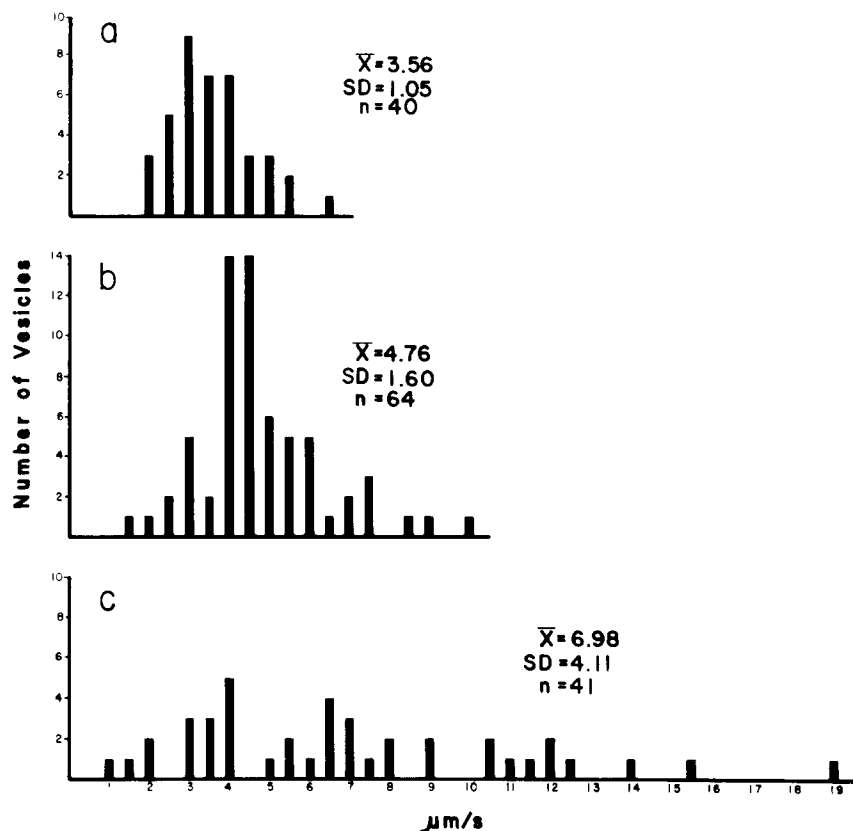


FIGURE 3 Rates of vesicle movement. The distributions of rates of endogenous vesicles from untreated, extruded axoplasm (a), extruded axoplasm treated with R-C₁₈-vesicle buffer (b), and rates of R-C₁₈ vesicles (c) demonstrate significantly different mean rates ($P < 0.01$). The mean (\bar{X}), standard deviation (SD) and sample size (n) are indicated for each histogram.

hydrolytic activity of trypsin. However, this result would also be obtained if these proteins were present both before and after trypsin treatment at a concentration above that in which the extent of staining by Kodavue is linearly related to the amount of protein present. Thus, while the conclusion that fodrin, α - and β -tubulins, and actin are resistant to the activity of trypsin is consistent with the data of Fig. 4 (see also reference 32), there is a possible alternative explanation.

DISCUSSION

The results presented here demonstrate that the R-C₁₈ intensely labeled the membranes of axonal organelles. Furthermore, when injected into extruded axoplasm, these fluorescent vesicles began moving with characteristics comparable to fast axonal transport. These fluorescent, membranous organelles were observed, using epifluorescence microscopy, for as long as 5 min without significant photobleaching. They moved with rates and trajectories similar to endogenous, nonfluorescent membranous organelles. The observed movement of the R-C₁₈ vesicles could be explained by one or more of several hypotheses. First, the exogenous, labeled vesicle movement could occur by the direct interaction of the labeled vesicle with the endogenous transport machinery. Second, the exogenous vesicles, once introduced into the extruded axoplasm, could have bound endogenous proteins and/or vesicles; therefore, fluorescent vesicle movement might have been by an indirect mechanism. Third, the exogenous vesicles might conceivably have been phagocytosed by an axonal endosome, and the movement observed was that of the endogenous endosome. The experimental results presented here do not directly distinguish between these hypotheses. However, the characteristics of movement of the fluorescent vesicles indicate that probe redistribution or passive flow were

unlikely causes of the bidirectional movements observed. These conclusions are supported by the results discussed below.

The infrared spectra indicated that there was no free rhodamine B in the probe sample (Materials and Methods). In addition, the labeling and isolation procedure presented in Materials and Methods was designed to remove any remaining R-C₁₈ molecules that were not incorporated into membrane. In the light microscopy experiments the number of fluorescently labeled, exogenous vesicles present in the axoplasm was small compared to the number of endogenous vesicles and membranous organelles. For endogenous organelles to become fluorescently labeled, it would require large numbers of probe molecules to leave the hydrophobic lipid bilayer of the exogenous membranes, cross through an aqueous, hydrophilic cytoplasm, and incorporate into another membrane. This process is thermodynamically unfavorable. Also, if the probe molecules did leave the exogenous vesicle membranes, there would be such a large dilution factor that it is unlikely a newly labeled endogenous membrane would be detected by epifluorescence microscopy. In addition, if the probe molecules were moving from one membrane to another, one would expect to observe other vesicles, in the region of an exogenous, R-C₁₈ vesicle, become fluorescent. This observation was never made. These data do not support probe redistribution as an explanation for the observed movement of fluorescent vesicles.

Passive flow—the movement of the exogenous vesicles in a stream of buffer or liquified axoplasm created by the experimental procedure—is also an unlikely explanation for R-C₁₈-vesicle movement for several reasons. First, the method of R-C₁₈-vesicle incorporation that was used helped to avoid biasing the vesicle movement in the orthograde or retrograde direction. It allowed the axoplasm on each side of the incorporation

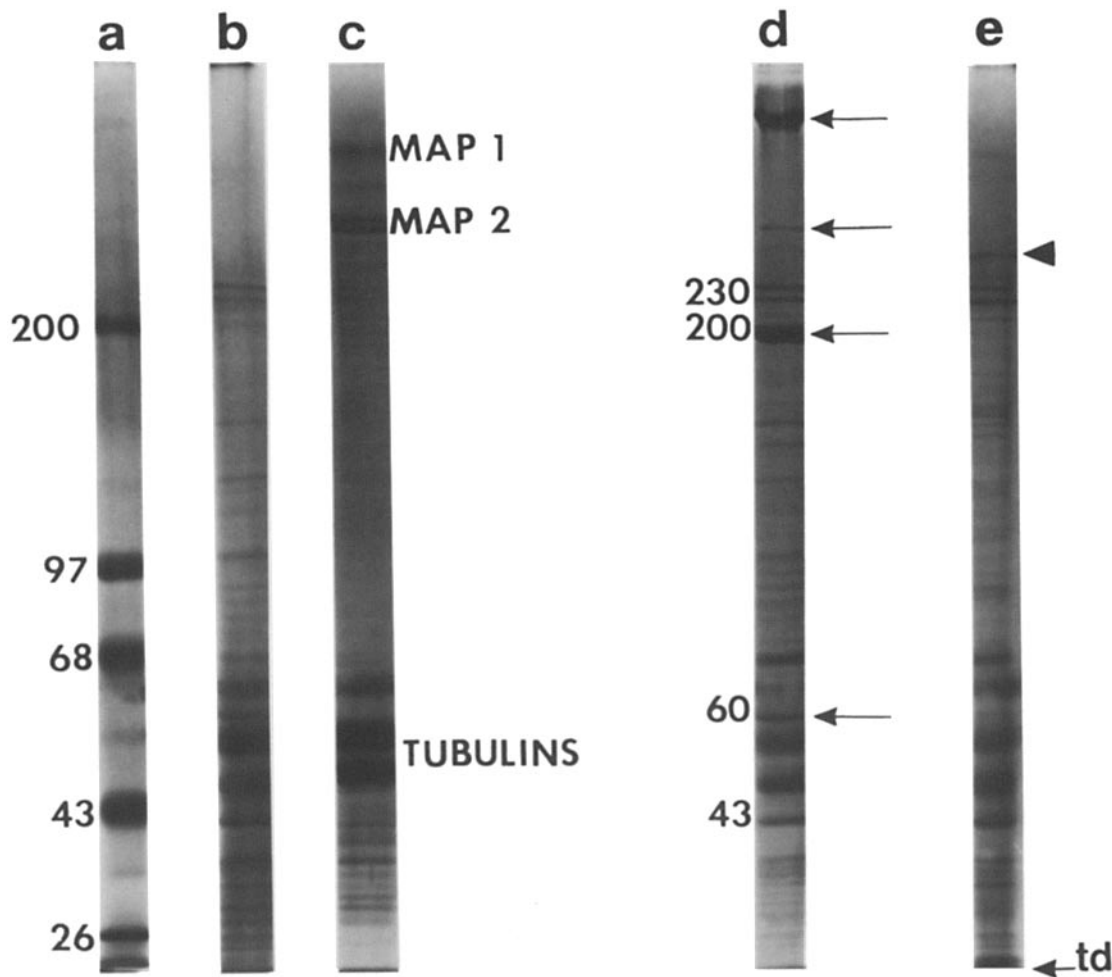


FIGURE 4 SDS PAGE analysis of the protein composition of axoplasm and of isolated vesicles. All lanes are from the same slab gel and are stained with Kodavue. Lanes *a*, *b*, and *c* represent standard lanes: (lane *a*) containing molecular weight markers ($\times 10^{-3}$); (lane *b*) whole axoplasm; and (lane *c*) purified mammalian brain microtubules. Lane *d* demonstrates the polypeptide composition of the vesicle pellet before the trypsin treatment. The arrows in lane *d* indicate the four main bands that were lost after trypsin treatment. The arrowhead (lane *e*) indicates a fragment of M_r 256,000 generated by the tryptic digestion. *td*, tracking dye.

site to remain intact and undisrupted. Furthermore, observations were not made at the site of incorporation, but rather several field widths to both sides of the injection site. Second, two vesicles traveling in opposite directions were observed to pass within $\sim 14 \mu\text{m}$ of each other (Fig. 2). It is difficult to imagine streams of flow from disruption occurring in opposite directions, close together in distance, and at the same time. Also, some R-C₁₈ vesicles remained stationary in regions where other fluorescent vesicles were moving. Similar results were obtained when observing endogenous vesicle movement. Finally, the movement of exogenous, R-C₁₈ vesicles was demonstrated to be an energy-dependent process, using the oxidative phosphorylation uncoupling agent, DNP in the presence and absence of added ATP. Such a result is consistent with that of Brady et al. (11), who treated extruded axoplasm of the squid giant axon with $200 \mu\text{M}$ DNP in the absence of added ATP and observed that transport of all membranous organelles stopped. In addition, the mitochondria appeared swollen. When ATP was added in the presence of DNP, the vesicles moved normally, but the mitochondria remained swollen and did not move. Because the R-C₁₈-vesicle movement occurred bidirectionally in areas in which other fluorescent vesicles did not move, and because the movement was

ATP dependent, the movement of the R-C₁₈ vesicles appears to be effected by the transport mechanism of axoplasm.

However, it should be noted that while the axoplasm was viewed for endogenous vesicle transport prior to epifluorescence observations, the converse experiment was not performed. That is, the same fluorescent vesicle was not viewed first by epifluorescence and then by DIC microscopy to compare its movement with the movements of endogenous vesicles in the immediate surroundings. Such an observation, if made, would have strengthened considerably the conclusion that the fluorescent vesicles move due to axonal transport rather than due to passive flow. Such a dual observation was not made, however, because it required at least 5 min to change the optical equipment from epifluorescence to DIC microscopy. Rather, the extruded axoplasm was observed by DIC microscopy after all fluorescent observations were completed.

The data of Fig. 3 show that the mean rate of R-C₁₈-vesicle movement was significantly faster than the mean rate of movement of unlabeled, endogenous vesicles in extruded axoplasm treated with R-C₁₈-vesicle buffer alone. Moreover, both of these rates were significantly greater than the mean rate of movement of vesicles in extruded axoplasm not treated

with buffer. By comparison, the rate of movement of vesicles in the intact squid giant axon, reported by Allen et al. (5), ranged from 0.5 to 5 $\mu\text{m/s}$. The results presented here, therefore, must be interpreted in light of these varying rates observed under different experimental conditions. The easiest explanation, but not necessarily the correct one, for the faster movements noted in the R-C₁₈-vesicle experiments is that the mean rate is shifted to a higher value due to the inclusion of a small population of vesicles that move at very high rates (see distribution in Fig. 3c). These could conceivably represent vesicles moving due to passive flow rather than due to the endogenous transport machinery. Yet several other factors relative to the manner in which the data summarized above were collected could also explain the variation in mean rates. The fluorescent labeling technique may have allowed detection of a faster moving population of vesicles than has been observed previously using methods based on phase differences of vesicles (phase-contrast and DIC microscopy). Because a fluorescent vesicle appears as a bright spot on a black background, the vesicle is easy to follow for very long distances (at least 185 μm) not only axially along the axon, but also at different focal planes within the axoplasm. Conversely, motion analysis of unlabeled, endogenous vesicles can only be done for a short distance (4–8 μm) before the vesicle moves out of the plane of focus and becomes lost in the surrounding axoplasm that is composed in part of numerous other vesicles that look and move in a similar manner (see references 1, 2). Therefore, the visual characteristics of the vesicles in the fluorescence and DIC experiments are quite different.

The results reported may also reflect the effects of the buffer employed. The buffer that favored the R-C₁₈-vesicle isolation did not stimulate the solution condition of the axoplasm (23) in that it was hypotonic and contained a high concentration of chloride ions. Chloride ion in high concentration has been determined to have a chaotropic effect on intracellular structure and function (17, 28, 33). It is conceivable that the buffer conditions disrupted the axoplasm somewhat and may have allowed faster particle movement to occur. The data of Fig. 3, a and b, support this explanation because the mean rate of movement of endogenous vesicles of axoplasm treated with R-C₁₈-vesicle buffer is significantly higher than the mean rate of movement of vesicles of extruded axoplasm alone. Also, Adams and Bray (3) reported that large particles (0.8 μm in diameter) microinjected into crab axons could be made to move by reducing the osmotic strength of the buffer, which causes the axon to swell. Thus, the higher rates of the R-C₁₈ vesicles reported here may be due not only to the buffer conditions employed, but also to the improved ability to detect vesicles as a result of using the fluorescent membrane probe.

The trypsin experiments indicated that the movement of the R-C₁₈ vesicles was dependent upon membrane-associated proteins. The vesicles that were treated with trypsin followed by soybean trypsin inhibitor did not move when incorporated in axoplasm, yet the transport of endogenous nonfluorescent vesicles proceeded normally. SDS PAGE revealed a loss of four major bands, indicated by arrows in Fig. 4, lane d, as well as approximately six minor bands. Bands having molecular weights similar to the neurofilament proteins were lost while bands presumably representing the fodrin doublet, α - and β -tubulins, and actin were still strongly evident. One of the major bands that was lost with trypsin digestion migrated with a mobility similar to brain MAP 2 (Fig. 4, compare lanes

c and c), while Fig. 4, lane e, demonstrates that a 256,000-mol-wt band was generated by the trypsin digestion (arrowhead, Fig. 3e). Vallee and Borisy (32) reported the removal of the projections from isolated microtubules with trypsin, generating fragments having M_r of 255,000, 148,000, and 116,000. They concluded from their results that the trypsin selectively destroyed high molecular weight proteins associated with microtubules. A MAP 2-like molecule has not been previously reported to be a component of the axonal cytoskeleton (8, 22, 31). However, previous reports on the protein composition of the axon dealt with the entire axoplasm, while this report uses a vesicle preparation that is presumably enriched in vesicle membrane-associated proteins. It must be remembered, though, that the vesicle fraction is not a pure membrane fraction. Fig. 1 demonstrates its heterogeneity, including filamentous aggregates probably representing cytoskeletal proteins.

Finally, the bright, punctate areas in the fluorescence streaks illustrating vesicle movement (Fig. 2e) deserve comment. This observation was completely unexpected since views of moving vesicles give the impression that the movement is continuous with no changes in brightness and no Brownian motion. This punctate pattern could indicate a mechanism in which a vesicle moves in a steplike, thrusting fashion. Schmitt (25) proposed such a model in which vesicles would move along microtubules or neurofilaments in this fashion. The evidence to support such a model will be the subject of another paper (S. P. Gilbert, R. D. Sloboda, and R. D. Allen, manuscript in preparation).

The results presented here do not distinguish or provide evidence in support of a specific molecular mechanism, but rather report a reconstituted model that can be used to study intracellular particle movement and fast axonal transport. It is hoped that this homologous system can be used in experiments designed to perturb the surface of the labeled vesicles in an attempt to localize and characterize the force transducing molecules involved in fast axonal transport.

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REFERENCES

1. Adams, R. J. 1982. Organelle movement in axons depends on ATP. *Nature (Lond.)* 297:327–329.

2. Adams, R. J., P. F. Baker, and D. Bray. 1982. Particle movement in crustacean axons that have been rendered permeable by exposure to brief intense electric fields. *J. Physiol. (Lond.)* 326:7P.
3. Adams, R. J., and D. Bray. 1983. Rapid transport of foreign particles microinjected into crab axons. *Nature (Lond.)* 303:718-720.
4. Allen, R. D., and N. S. Allen. 1983. Video-enhanced microscopy with a computer frame memory. *J. Microsc.* 129:3-17.
5. Allen, R. D., J. Metzuzals, I. Tasaki, S. T. Brady, and S. P. Gilbert. 1982. Fast axonal transport in squid giant axon. *Science (Wash. DC)* 218:1127-1129.
6. Bear, R. S., F. O. Schmitt, and J. Z. Young. 1937. Investigations on the protein constituents of nerve axoplasm. *Proc. R. Soc. Lond. Ser. B. Biol. Sci.* 123:520-529.
7. Beckerle, M. C., and K. R. Porter. 1983. Microinjected fluorescent polystyrene beads exhibit saltatory motion in tissue culture cells. *J. Cell Biol.* 97(2, Pt. 2):193a. (Abstr.)
8. Black, M. M., and J. T. Kurdyla. 1983. Microtubule-associated proteins of neurons. *J. Cell Biol.* 97:1020-1028.
9. Boss, W. F., C. J. Kelley, and F. R. Landsberger. 1975. A novel synthesis of spin label derivatives of phosphatidylcholine. *Anal. Biochem.* 64:289-292.
10. Brady, S. T., and R. J. Lasek. 1982. Axonal transport: a cell-biological method for studying proteins that associate with the cytoskeleton. *Methods Cell Biol.* 25:365-398.
11. Brady, S. T., R. J. Lasek, and R. D. Allen. 1982. Fast axonal transport in extruded axoplasm from squid giant axon. *Science (Wash. DC)* 218:1129-1131.
12. Brimijoin, S. 1975. Stop flow: a new technique for measuring axonal transport, and its application to the transport of Dopamine- β -hydroxylase. *J. Neurobiol.* 6:379-394.
13. Fairbanks, G. T., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606-2617.
14. Forman, D. S., K. J. Brown, and D. R. Livengood. 1983. Fast axonal transport in permeabilized lobster giant axons is inhibited by vanadate. *J. Neurosci.* 3:1279-1288.
15. Grafstein, B., and D. S. Forman. 1980. Intracellular transport in neurons. *Physiol. Rev.* 60:1167-1283.
16. Hayden, J. H., R. D. Allen, and R. D. Goldman. 1983. Cytoplasmic transport in keratocytes: direct visualization of particle translocation along microtubules. *Cell Motility.* 3:1-19.
17. Inoue, I., H. C. Pant, I. Tasaki, and H. Gainer. 1976. Release of proteins from the inner surface of the squid axon membrane labeled with tritiated N-ethylmaleimide. *J. Gen. Physiol.* 68:385-395.
18. Keller, P. M., S. Person, and W. Snipes. 1977. A fluorescence enhancement assay of cell fusion. *J. Cell Sci.* 28:167-177.
19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature (Lond.)* 227:680-685.
20. Lasek, R. J. 1974. Biochemistry of the squid giant axon. In *A Guide to the Laboratory Use of the Squid*. J. M. Arnold, W. C. Summers, and D. L. Gilbert, editors. Marine Biological Laboratory, Woods Hole, MA. 69-74.
21. Lux, H. D., P. Schubert, G. W. Kreutzberg, and A. Globus. 1970. Excitation and axonal flow: autoradiographic study on motor neurons intracellularly injected with a ^3H -amino acid. *Exp. Brain Res.* 10:197-204.
22. Matus, A., R. Bernhardt, and T. Hugh-Jones. 1981. High molecular weight microtubule-associated proteins are preferentially associated with dendritic microtubules in brain. *Proc. Natl. Acad. Sci. USA.* 78:3010-3014.
23. Morris, J. R., and R. J. Lasek. 1982. Stable polymers of the axonal cytoskeleton: the axoplasmic ghost. *J. Cell Biol.* 92:192-198.
24. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208-211.
25. Schmitt, F. O. 1968. Fibrous proteins neuronal organelles. *Proc. Natl. Acad. Sci. USA.* 60:1092-1101.
26. Sheetz, M. P., and J. A. Spudich. 1983. Movement of myosin-coated fluorescent beads on actin cables *in vitro*. *Nature (Lond.)* 303:31-35.
27. Smith, R. S. 1980. The short term accumulation of axonally transported organelles in the region of localized lesions of single myelinated axons. *J. Neurocytol.* 9:39-65.
28. Tasaki, I., I. Singer, and T. Takenaka. 1965. Effects of internal and external ionic environment on excitability of squid giant axon. *J. Gen. Physiol.* 48:1095-1123.
29. Travis, J. L., and R. D. Allen. 1981. Studies on the motility of the foraminifera. I. Ultrastructure of the reticulopodial network of *Allogromia laticollaris* (Arnold). *J. Cell Biol.* 90:211-221.
30. Tsukita, S., and H. Ishikawa. 1980. The movement of membranous organelles in axons. EM identification of anterogradely and retrogradely transported organelles. *J. Cell Biol.* 84:513-530.
31. Vallee, R. B. 1982. A taxol-dependent procedure for the isolation of microtubules and microtubule-associated proteins (MAPs). *J. Cell Biol.* 92:435-442.
32. Vallee, R. B., and G. G. Borisy. 1977. Removal of the projections from cytoplasmic microtubules *in vitro* by digestion with trypsin. *J. Biol. Chem.* 252:377-382.
33. von Hippel, P. H., and T. Schleich. 1969. The effects of neutral salts on the structure and conformational stability of macromolecules in solution. In *Structure and Stability of Biological Macromolecules*. S. N. Timasheff and G. D. Fasman, editors. Marcel Dekker, Inc., New York. 417-574.
34. Weiss, D. G. 1982. Axoplasmic transport. Springer-Verlag, New York. 478 pp.