

# Stable Complexes of Axoplasmic Vesicles and Microtubules: Protein Composition and ATPase Activity

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**Abstract.** Fast transport of axonal vesicles and organelles is a microtubule-associated movement (Griffin, J. W., K. E. Fahnestock, L. Price, and P. N. Hoffman, 1983, *J. Neuroscience*, 3:557-566; Schnapp, B. J., R. D. Vale, M. P. Sheetz, and T. S. Reese, 1984, *Cell*, 40:455-462; Allen, R. D., D. G. Weiss, J. H. Hayden, D. T. Brown, H. Fujiwake, and M. Simpson, 1985, *J. Cell Biol.*, 100:1736-1752). Proteins that mediate the interactions of axoplasmic vesicles and microtubules were studied using stable complexes of microtubules and vesicles (MtVC).<sup>1</sup> These complexes formed spontaneously in vitro when taxol-stabilized microtubules were mixed with sonically disrupted axoplasm from the giant axon of the squid

*Loligo pealei*. The isolated MtVCs contain a distinct subset of axoplasmic proteins, and are composed primarily of microtubules and attached membranous vesicles. The MtVC also contains nonmitochondrial ATPase activity. The binding of one high molecular mass polypeptide to the complex is significantly enhanced by ATP or adenylyl imidodiphosphate. All of the axoplasmic proteins and ATPase activity that bind to microtubules are found in macromolecular complexes and appear to be vesicle-associated. These data allow the identification of several vesicle-associated proteins of the squid giant axon and suggest that one or more of these polypeptides mediates vesicle binding to microtubules.

**S**EVERAL forms of intracellular organelle and vesicle translocations are dependent on microtubules (Murphy and Tilney, 1974; Lasek and Hoffman, 1976). In most of these studies, microtubule involvement was inferred from ultrastructural observations or from the fact that drug depolymerization of tubules is accompanied by a cessation of organelle movement (for review see Grafstein and Forman, 1980; Schliwa, 1984). Recently, more direct evidence has appeared with regard to fast transport of vesicles and organelles in nerve axoplasm. Several investigators have observed bidirectional organelle movement along microtubules in disrupted axoplasm from the giant axon of the squid *Loligo pealei* (Schnapp et al., 1984; Allen et al., 1985; Brady et al., 1985). In addition, Griffin and co-workers (1983) have shown that after  $\beta, \beta'$ -iminodipropionitrile-induced rearrangement of peripheral nerve axon cytoskeletal structures in vivo, transported organelles segregate almost quantitatively with microtubules. Furthermore, Schnapp et al. (1984), through the ingenious use of correlative light and electron microscopy, have unequivocally demonstrated the direct interaction of moving vesicles with single microtubules in the squid giant axon.

Vesicle transport in nerve axons requires ATP (Adams, 1982; Forman et al., 1983) and it is well accepted that or-

ganelle movement in general is energy dependent. Two basic requirements of microtubule-vesicle transport are, therefore, (a) a specific interaction between microtubules and organelles, and (b) a mechanochemical transducer or "molecular motor." Several research groups have recently identified a new class of proteins that have the characteristics of vesicle transport motors. These proteins have been found in soluble fractions (100,000 g supernatant) of squid giant axon (Vale et al., 1985), squid, bovine, and chick brain (Vale et al., 1985; Brady, 1985), and sea urchin eggs (Scholey et al., 1985). All of these proteins have been purified on the basis of nucleotide-dependent binding to microtubules and all are between 110,000 and 140,000 *M<sub>r</sub>*. In one case, the 130,000 protein isolated from bovine brain appears to have substantial ATPase activity (Brady, 1985). Vale and co-workers (1985) have applied the name kinesin to this new class of microtubule translocators. Purified brain and sea urchin egg kinesin generate ATP-dependent movement of microtubules (prepared free of microtubule-associated proteins [MAPs]) across a glass surface, as well as the movement of polystyrene beads along microtubules (Vale et al., 1985; Scholey et al., 1985). The kinesin family of proteins are the best candidates at present for the role as a motor for orthograde vesicle transport.

However, kinesin-associated movement is unidirectional (Vale et al., 1985), while axonal transport is bidirectional (Forman et al., 1983; Schnapp et al., 1984; Allen et al., 1985; Gilbert and Sloboda, 1984). This suggests that other

1. *Abbreviations used in this paper:* AMP-PNP, adenylyl imidodiphosphate; ImB, Imidazole buffer; MAPs, microtubule-associated proteins; MtVC, microtubule-vesicle complexes.

molecules can also mediate vesicle motility. In addition, Gilbert et al. (1985) have shown that purified squid vesicles move at *in vivo* rates along flagellar outer doublet microtubules in the absence of added kinesin. If these isolated vesicles are first treated with trypsin to remove associated proteins, they are no longer transported either along flagellar microtubules, or in axoplasm where there should be ample kinesin (Gilbert and Sloboda, 1984; Gilbert et al., 1985). Vale and co-workers (1985) also report that kinesin is much less effective at eliciting or promoting vesicle movement (vs. bead movement) in an *in vitro* system composed of squid axoplasmic organelles and MAP-free brain tubules. Moreover, neither purified kinesin nor the high speed brain supernatant can induce microtubule-associated movement of trypsinized organelles or pure lipid vesicles in the *in vitro* assay (Vale et al., 1985). These data suggest that other proteins, most likely vesicle-associated, may mediate the interaction of vesicles with microtubules and/or of kinesin with vesicles. MAPs have been implicated in the attachment of secretory granules (Sherline et al., 1977; Suprenant and Dentler, 1982) to brain microtubules *in vitro*. The functional specificity of this interaction is not clear however, since the results discussed above (Gilbert et al., 1985; Vale et al., 1985) demonstrate that axoplasmic vesicles and kinesin-coated beads will move on MAP-free microtubules.

The study reported here was designed to identify proteins in the squid giant axon that could link vesicles and microtubules. After incubation of squid brain microtubules and sonically disrupted axoplasm, stable associations are formed between the tubules and axonal vesicles. The resulting microtubule-vesicle complexes (MtVC) have been isolated and found to contain a consistent subset of axoplasmic proteins and nonmitochondrial ATPase activity. The preparation of the MtVC allows the identification of several squid axoplasmic vesicle proteins, one or more of which may serve as a microtubule-vesicle bridge, and provides a molecularly defined model system containing native organelles in which to investigate the parameters of vesicle transport.

## Materials and Methods

### Experimental Animals

Squid (*Loligo pealei*) were collected daily by the Marine Resources Center at the Marine Biological Laboratory (Woods Hole, MA). The animals were sacrificed within 36 h of capture and axoplasm or brain proteins were prepared as described from fresh tissue.

### Solutions

PEM consisted of 0.1 M Pipes (pH 6.8), 5 mM EGTA, 1 mM MgCl<sub>2</sub>, 0.1 mM dithiothreitol, 0.1 mM GTP, 1 µg/ml leupeptin, and 1 µg/ml soybean trypsin inhibitor. Imidazole buffer (ImB) consisted of 10 mM Imidazole, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 250 mM sucrose, 0.1 mM dithiothreitol, 1 µg/ml leupeptin, and 1 µg/ml soybean trypsin inhibitor. Buffer X (Morris and Lasek, 1982) consisted of 350 mM potassium aspartate, 130 mM taurine, 70 mM betaine, 50 mM glycine, 12.5 mM MgCl<sub>2</sub>, 10 mM EGTA, 3 mM CaCl<sub>2</sub>, 1 mM glucose, 1 mM ATP, and 20 mM Hepes (pH 7.2).

### Preparation of Squid Brain and Axoplasmic Microtubules

MAP-free squid brain microtubules were prepared essentially by the technique designed by Vallee (1982) using taxol. The final pellet of MAP-free microtubules was resuspended in PEM containing 20 µM taxol and after estimating the protein concentration (see below) the microtubules were split into 1.5 ml Eppendorf tubes such that each tube contained 100–200 µg of tubulin. The microtubules were pelleted and stored "dry" at –80°C.

Microtubules and MAPs were prepared from squid giant axon by the same procedure (Vallee, 1982) with minor modifications. An axoplasmic homogenate was centrifuged at 100,000 g for 30 min in a rotor (model Ti50; Beckman Instruments, Inc., Fullerton, CA) and the supernatant was brought to 1 mM GTP and 20 µM taxol. After a 20-min incubation at room temperature, the microtubule-MAP pellet was collected by centrifugation. The pellet was washed once with PEM containing 0.35 M NaCl and 20 µM taxol and the final pellet of microtubules without MAPs was resuspended in PEM without taxol.

### Preparation of the Microtubule-Vesicle Complex (MtVC)

The giant axons from 3–5 squid were removed and kept in chilled calcium-free sea water. The axons were dissected free of adhering connective tissue in a calcium-free sea water bath under a dissecting microscope, rinsed in half strength ImB, and blotted on filter paper. The axoplasm was extruded onto Parafilm as described (Brady et al., 1985) and transferred in the tip of a Pasteur pipet to a 1.5 ml Eppendorf tube containing 0.5 ml of ImB at 4°C. The axoplasm from 8–10 axons was suspended in ImB at a ratio of 100 µl ImB/axoplasm, and was disrupted by sonication using a microprobe tip and a sonicator (model W10; Heat Systems-Ultrasonics, Inc., Farmingdale, NY) set at full power, for 10 1–2-s intervals and two 5-s intervals. Undisrupted axoplasm was pelleted by centrifugation at 10,000 rpm (SS-34 rotor; E.I. DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newton, CT) for 5 min. The supernatant from this spin was adjusted to a protein concentration of 0.5–1.0 mg/ml with ImB and the final solution is referred to throughout this report as axoplasm or whole axoplasm.

For preparation of MtVCs, 100–200 µg of pelleted microtubules were resuspended in 0.3–0.4 ml of whole axoplasm to obtain a protein weight ratio of ~5:1, axoplasm/microtubules. In most cases the axoplasm contained 20 µM taxol. This axoplasm-microtubule mixture was incubated at 21–23°C (room temperature) for 30 min and then 0.25–0.35 ml was layered over 1 ml of a solution of ImB containing 1.0 M sucrose and 20 µM taxol in a 4.4-ml nitrocellulose tube and spun at 20,000 rpm for 30 min in a (model SW60; Beckman Instruments, Inc.) rotor. An equal volume to that loaded was removed from the solution above the sucrose and the interface and this fraction is referred to as the supernatant. The sucrose layer was removed and discarded. The pellet was resuspended in one-half the loaded volume of ImB containing 20 µM taxol and is referred to as the pellet or MtVC.

### Gel Filtration Chromatography

Gel filtration was carried out at 4°C using either Ultrogel 22 (LKB Instruments Inc., Bromma, Sweden), which has an exclusion limit of 3,000,000 D and a fractionation range of 100,000–1,200,000 D, or Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ), which has a fractionation range of 10,000–4,000,000 D. The void volume of the column was determined by the elution position of Blue Dextran 2000 and the total volume was marked by the elution volume of ATP.

### Sucrose Density Gradient Centrifugation

The MtVC sample was suspended in 50% sucrose, 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.1% β-mercaptoethanol, 0.1 µg/ml leupeptin, 0.1 µg/ml soybean trypsin inhibitor, and dialyzed against the same buffer overnight. The sample (0.2 ml) was loaded into an 1.3 cm × 5 cm nitrocellulose tube and overlaid with a gradient of 15–40% sucrose in the same buffer. The tubes were filled by adding 0.2 ml of 5% sucrose in the same buffer. The gradients were spun at 32.5K g for 8 h in a Beckman SW50.1 rotor at 4°C. 11 fractions (0.5 ml each) were collected by pumping out the gradient from the bottom.

### Electron Microscopy

For negative staining, 300-mesh copper grids were coated with 0.25% formvar or 3% parlodian and overlaid with evaporated carbon. Protein samples were diluted if necessary to <0.2 mg/ml in ImB, placed on grids, washed gently with diluted buffer and distilled water, and stained with 1–2% uranyl acetate. The grids were air-dried and viewed and photographed within 24 h.

### SDS PAGE

SDS PAGE was carried out using 0.75-mm slab gels in an electrophoresis apparatus (Protean 16; Bio-Rad Laboratories, Richmond, CA). The gels

contained a 30:0.8 ratio of acrylamide/bis-acrylamide in a final concentration gradient of 5–15% or 6–12% polyacrylamide. The discontinuous system described by Laemmli (1970) was used and in some cases was modified so that the separating gel contained a gradient of 2–8 M urea. The gels were typically run at 15 mA (constant current) for 3–3.5 h. Proteins were visualized either by silver staining as previously described (Pratt et al., 1984), or by staining in 0.1% Coomassie Brilliant Blue, 40% methanol, and 10% acetic acid for 6–12 h, followed by destaining in 10% acetic acid. Glycoproteins were visualized by staining with dansyl-hydrazine according to the method of Eckhardt et al., 1976.

For determining protein content of individual bands, densitometric tracings of the Coomassie Blue-stained gels were prepared in an Ortec model 4310 scanning densitometer. Peaks of interest were excised and weighed and the ratios of any two peaks constituted an estimated protein weight ratio of the two bands.

### Protein and Enzyme Assays

Protein concentrations were estimated by the method of Lowry et al. (1951) using BSA as a standard.

ATPase activity was measured as previously described by colorimetric determination of inorganic phosphate released over time (Pratt et al., 1984). Unless otherwise noted, the buffer conditions were 30 mM Tris-HCl (pH 8.0), 2 mM MgCl<sub>2</sub>, 0.2 mM EGTA, 0.3 M KCl, and 0.5 mM ATP that was added to start the reaction. One unit of ATPase activity will generate 1.0 μmol inorganic phosphate/min.

Cytochrome oxidase activity was measured by a modification of the method described by Silver et al. (1983). An aliquot of the protein sample to be tested was added to a solution of reduced cytochrome C in ImB so that the final volume was 1 ml and the concentration of cytochrome C was 0.1 mg/ml. A blank tube was prepared with reduced cytochrome C alone. The OD<sub>550</sub> of the blank tube and of the reaction tubes was determined just after sample addition and again at 10- and 20-min intervals. The change in OD<sub>550</sub>/min less any change in the blank was calculated and compared with a standard curve prepared using purified cytochrome oxidase. One unit of cytochrome oxidase will oxidize 1.0 μmol reduced cytochrome C/min at pH 7.0. Specific activity is defined as units per milligram protein. A preparation enriched in mouse liver mitochondria, generously supplied by R. Sloboda and S. Gilbert (Dartmouth College, Hanover, NH) was assayed for comparison with axoplasmic samples.

## Results

### Protein Composition of the Microtubule-Vesicle Complex

As a first approach to the identification of proteins in the squid giant axon that might play a role in microtubule-based vesicle transport, experiments were designed to catalogue axoplasmic components that would bind specifically and stably to microtubules. Extruded axoplasm was disrupted by sonication in a modification of ImB designed by Gilbert and Sloboda (1984) that is known to support vesicle movement. Negative stain electron microscopy revealed that the clarified axoplasmic homogenate contained globular masses of various sizes, intermediate filaments (neurofilaments), and a heterogeneous population of membrane vesicles, but microtubules were extremely scarce. None of the axoplasmic components would pellet through 50% sucrose in ImB when spun at 20,000 rpm (42,000 g) for 30 min in a Beckman SW60 rotor (Fig. 1). Taxol-stabilized squid brain microtubules containing no MAPs pelleted quantitatively under identical conditions.

When MAP-free squid brain microtubules are incubated with sonically disrupted squid axoplasm and then recovered by centrifugation, a defined subset of axoplasmic polypeptides co-pellet with the tubules (Fig. 1). Comparison of data from seven experiments allows the identification of eight nontubulin polypeptides that are consistently found in the

pellet. These axoplasmic proteins, designated by number in Fig. 1 A, have the following *M<sub>r</sub>* as estimated by SDS PAGE: protein No. 3, 250,000; No. 6, 150,000; No. 7, 120–130,000; No. 8, 100,000; No. 9, 80,000; No. 10, 43,000; No. 11, 37,000; and No. 12, 33,000. Polypeptide No. 7 is identified with a molecular mass range because it appears as a broad and diffuse band. Other proteins that are found reproducibly but in variable amounts in different preparations are Nos. 1 and 5, which comigrate with the neurofilament proteins 1 and 2 (NF1 and NF2), respectively. No. 4, a doublet designated A and B (Fig. 1 A), which co-migrates with fodrin (Levine and Willard, 1981), and an additional high molecular mass protein of 250,000 (No. 2) (Fig. 1 A). Occasionally, other polypeptides may be found in a single preparation (Compare lanes Ax + Mt - P and MtVC in Fig. 1 A). If taxol alone (in the absence of brain microtubules) is added to axoplasm before centrifugation through sucrose, smaller amounts of the specific MtVC proteins can appear in the pellet, but only if tubulin is found there also.

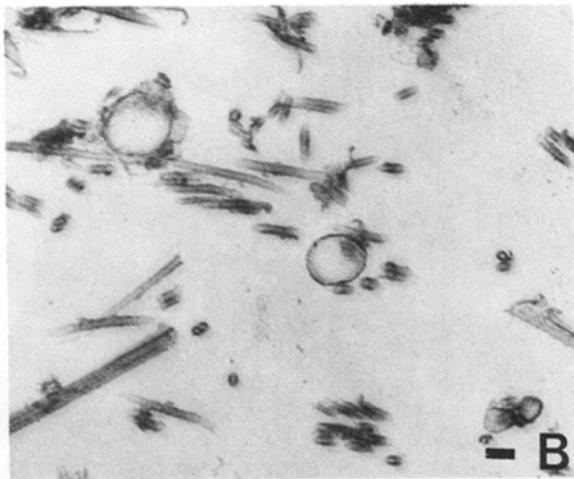
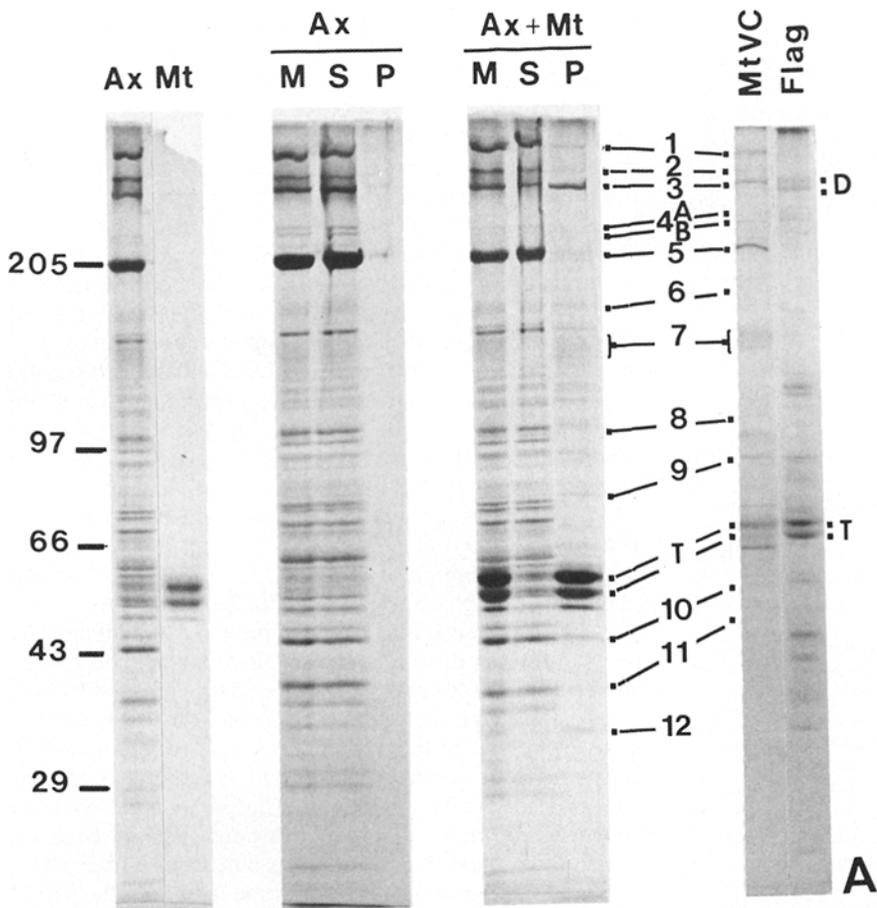
The most prominent polypeptide co-pelleting with the microtubules is No. 3 (Fig. 1 A). This band comigrates with the larger of the two major dynein heavy chains (dynein A) (Fig. 1 A). It is distinctly smaller than polypeptide No. 2, which is a variable component of the microtubule pellet (see below). The high molecular mass protein No. 3 is present in a weight ratio to tubulin dimer of 1:17, as determined by densitometry of Coomassie Blue-stained gels for seven separate preparations of complexes. The polypeptide contains bound carbohydrate as judged by staining of the band on SDS-polyacrylamide gels with dansyl hydrazine after periodate treatment (data not shown). Some tubulin in the MtVC is also stained by this reagent and may be a glycosylated vesicle-associated form of the protein.

### Morphology of the Microtubule-Vesicle Complex

Thin section and negative stain electron microscopy reveal that the pellet collected after incubation of tubules and axoplasm contains variable sized aggregates of microtubules and membrane-bound vesicles (Figs. 1 B and 2). This morphology gave rise to the appellation "microtubule-vesicle complex" or "MtVC." Some amorphous globular material and some filaments are also present (Fig. 2). As judged by negative stain electron microscopy, the vesicles are closely juxtaposed to the microtubule surface even in the axoplasm-microtubule mixture. It is unlikely that this is due to superimposition since examination of the MtVC using video-enhanced differential interference contrast microscopy reveals filamentous elements (microtubules) associated with globular structures the size of axoplasmic vesicles (Brady et al., 1985; Gilbert and Sloboda, 1984) (data not shown). These structures, resembling beads on a string, are stable to flow. Ultrastructurally, the microtubules recovered from axoplasm exhibit small globular or filamentous projections but these are of variable size and spacing. The MAP-free squid brain microtubule preparations before and after centrifugation through sucrose in the absence of axoplasm contain only smooth-walled microtubules and no vesicles by negative stain electron microscopy (Fig. 2 A).

### Factors Affecting Formation of the MtVC

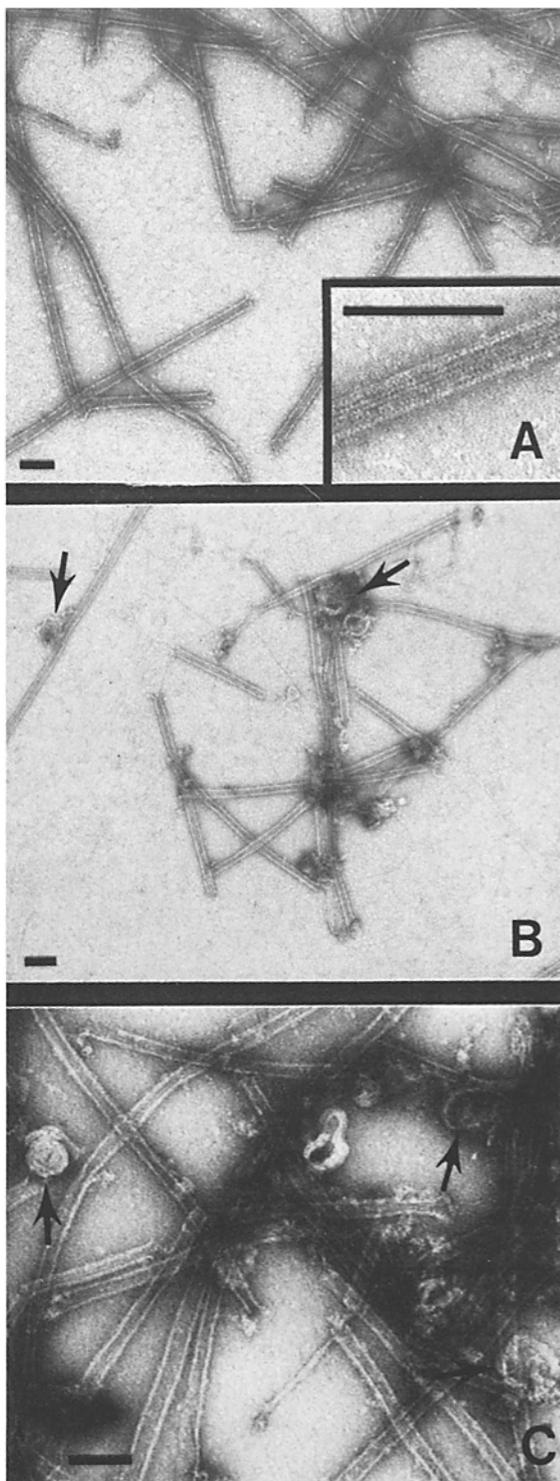
Standard conditions for the preparation of the MtVC include ImB at pH 7.2–7.4, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 20 μM



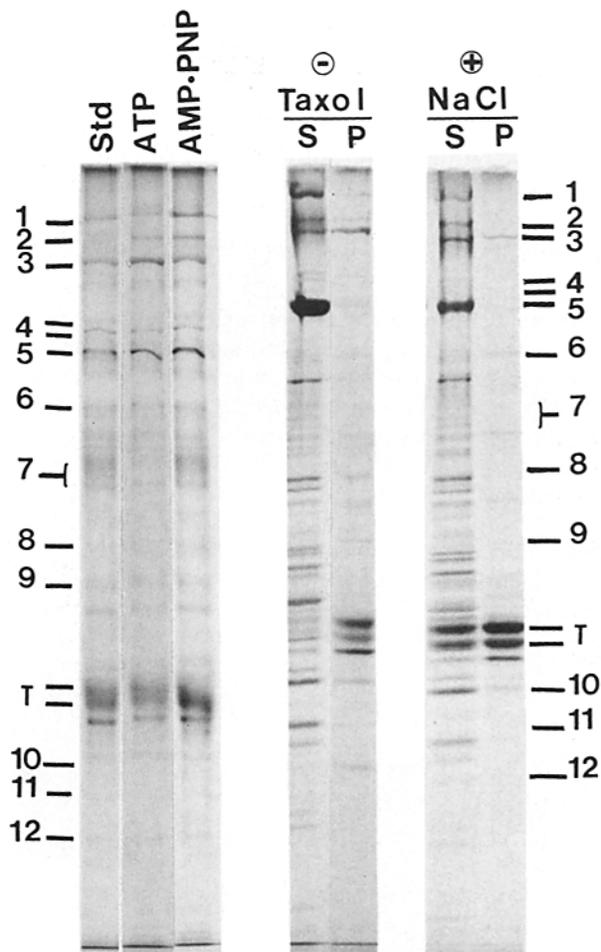
**Figure 1.** Protein composition and morphology of the MtVC. (A) Polypeptide composition as revealed by SDS PAGE of whole squid axoplasm (Ax) and taxol-stabilized squid brain microtubules (Mt). Either the axoplasm alone (Ax) or a mixture of axoplasm and microtubules (Ax + Mt) were layered over a sucrose cushion and centrifuged. Polypeptide compositions of the mix (M), supernatant (S), and pellet (P) from each experiment are shown. Supernatants and pellets were loaded stoichiometrically (equal load volumes from samples of the same size). Bands that are consistent components of the MtVC are labeled by numbers on the right, which designate corresponding polypeptides in two separate MtVC preparations (Ax + Mt-P, and MtVC) and tubulin are indicated (T). A lane of flagellar axoneme protein (Flag) is included for comparison. The positions of the dynein (D) and tubulin (T) doublets are indicated. The positions of molecular mass markers are denoted in kilodaltons at the left. All gels are stained with silver nitrate. (B) A thin section electron micrograph of an MtVC pellet showing both microtubules and vesicles. Bar, 100 nm.

taxol, and 0.25 M sucrose to make the solution isosmotic with axoplasm. The solution also contains a ~1:20 dilution (10  $\mu$ l axoplasm in 200  $\mu$ l buffer) of axoplasmic proteins, salts, and nucleotides to give an ATP concentration of ~50  $\mu$ M. Taxol can be omitted from the axoplasm-microtubule mixture without significantly affecting protein composition of the MtVC (Fig. 3). The formed MtVC is stable to washing by resuspension and recentrifugation in ImB plus taxol. If the

pellet is washed in ImB-taxol containing 0.35 M NaCl, up to 50% of the bound axoplasmic proteins and 20% of the tubulin are extracted. Negative stain electron microscopic examination of the salt-extracted pellet reveals some dispersion of the MtVC aggregates that are generally smaller in size but the remaining microtubules retain a few associated vesicles. If, instead, 250 mM NaCl is added to the standard buffer or if ImB is replaced with Buffer X during MtVC for-



**Figure 2.** Morphology of brain microtubules and the MtVC as revealed by negative stain electron microscopy. (A) Taxol-stabilized squid brain microtubules before mixing with axoplasm. Note the smooth walls and absence of vesicles. Inset shows microtubules at higher magnification. (B) Low magnification view of the MtVC pellet. Vesicles are denoted by arrows. (C) High magnification view of the MtVC pellet. Arrows designate several vesicles. Bars, 100 nm.



**Figure 3.** Influence of buffer conditions on protein composition of the MtVC. Proteins consistently found in the MtVC are numbered as in Fig. 1. The first three lanes show MtVCs formed under standard conditions (Std) or in the presence of ATP (ATP) or AMP-PNP (AMP-PNP). The last four lanes show supernatants (S) and pellets (P) from MtVC preparations in which taxol was omitted ( $\ominus$  Taxol) or 0.35 M NaCl was added ( $\oplus$  NaCl) during incubation of the microtubules with axoplasm. Supernatants and pellets are loaded stoichiometrically (equal load volumes from samples of the same size). All gels were stained with silver nitrate.

mation, the amount of protein in the pellet including tubulin is substantially reduced (Fig. 3). Ultrastructurally, these pellets contain mostly single microtubules (not bundled) with many fewer vesicles attached than in the standard preparation.

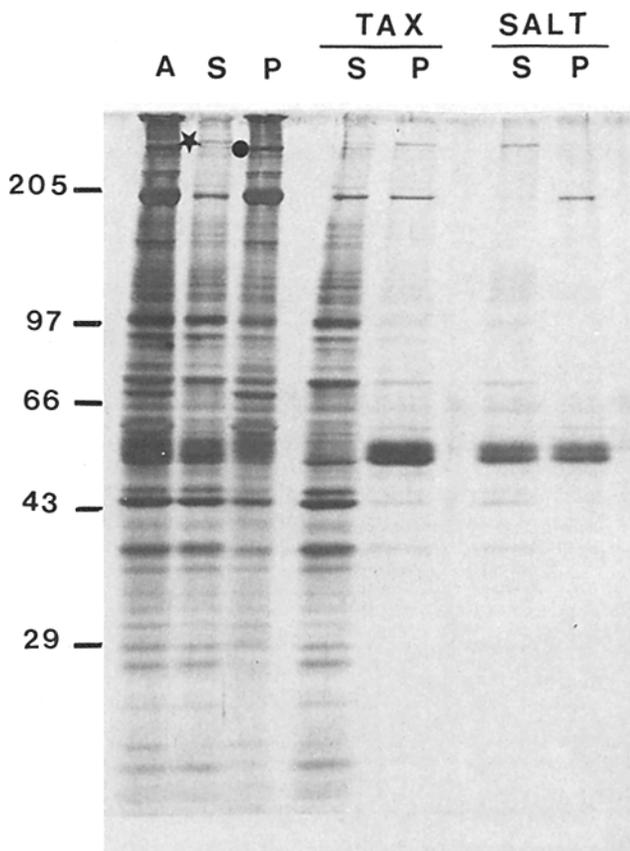
The association of two polypeptides, Nos. 7 and 2, with the MtVC shows nucleotide dependence. Adding 10 mM ATP during MtVC formation leads to a diminution in the amount of the No. 7 group of polypeptides found in the pellet (Fig. 3). However, reduction of the nucleotide concentration in axoplasm by dialysis before MtVC formation does not significantly change the protein composition of the pellet. If AMP-PNP is present during MtVC formation, the amount of the group No. 7 polypeptides in the pellet is the same as under standard conditions (Fig. 3). With regard to polypeptide No. 2, the presence of either 10 mM ATP or 10 mM adenylyl imidodiphosphate (AMP-PNP) during MtVC formation causes a dramatic increase in the quantity found in the pellet.

In the presence of either of these nucleotides, protein No. 2, typically present in low amount or absent, is recovered almost quantitatively in the MtVC as judged by its absence from the supernatant fraction (Fig. 3). Negative stain and thin section electron microscopy reveal that these pellets contain more globular material and vesicles than the standard MtVC.

#### Axoplasmic Microtubule-associated Proteins (MAPs)

To determine whether any of the MtVC proteins associates with microtubules in the absence of vesicles, we polymerized microtubules from a high speed supernatant (soluble fraction) of axoplasm using taxol, a microtubule stabilizing drug. MAPs were defined as polypeptides that pelleted with tubulin after taxol polymerization and could be extracted with 0.35 M NaCl in the presence of taxol (Vallee, 1982).

Taxol-induced microtubule polymer consists of tubulin and a small subset of axoplasmic proteins (Fig. 4). Most of the nontubulin proteins in the microtubule fraction, with the exception of a 200,000  $M_r$  and a 58,000  $M_r$  polypeptide, were extracted by 0.35 M NaCl, indicating that they are



**Figure 4.** Axoplasmic MAPs. Axoplasmic microtubules were polymerized using taxol and extracted with high salt as described in Materials and Methods. The figure shows the protein composition as revealed by SDS PAGE of whole axoplasm (A), the first cold supernatant (S), the discarded pellet (P), the supernatant and pellet after taxol addition (TAX S and TAX P), and the supernatant and pellet after salt extraction of the taxol pellet (SALT S and SALT P). The two large polypeptides found in the MtVC, Nos. 2 (★) and 3 (●) are marked. The positions of several molecular mass markers are noted in kilodaltons at the left margin. The gels were stained with silver nitrate.

**Table I.** ATPase Activity in Axoplasmic Fractions and MtVC Pellet

Sample	ATPase		Total Units <sup>‡</sup>
	<i>u</i>	<i>sp act</i> <sup>*</sup>	
Axoplasm/ microtubule mix	0.0045 + 0.0022 <sup>§</sup>	0.0067 + 0.0037	
Supernatant	0.0013 + 0.0011	0.0052 + 0.0022	28.8
Pellet	0.0033 + 0.0025	0.0115 + 0.0025	73.3

\* U/mg-min

<sup>‡</sup> This value is calculated using total unit values in column 1.

<sup>§</sup> All numbers represent the mean + SD for five different MtVC preparations.

MAPs. This MAP fraction contains six of the 12 MtVC proteins, Nos. 2, 7, 8, 9, 10, and 11 (Fig. 4). It is important to note, however, that none of the MtVC proteins were quantitatively solubilized in the axoplasmic homogenate. In fact all of them appeared in the discarded high speed pellet, including a significant amount of polypeptide No. 3 (Fig. 4). Polypeptide No. 2, a variable component of the MtVC was the most prominent of the axoplasmic MAPs (Fig. 4). One protein of 62,000  $M_r$  was recovered in the MAP fraction (Fig. 4), but never appeared in the MtVC.

Morphological examination of the salt-washed axoplasmic microtubules by negative stain electron microscopy revealed smooth-walled microtubules and some sheets of protofilaments but no vesicles.

#### ATPase Activity of Axoplasm and MtVC

If axoplasm is disrupted either by Dounce homogenization or sonication in various buffers of different ionic strength, and pH, >50% of the total  $Mg^{++}$ ATPase units in the homogenate are pelleted at 100,000 g for 30 min (data not shown). This activity appears, therefore, to be associated with axoplasmic particulates. The pelleted MtVC contains >50% of the  $Mg^{++}$ ATPase activity present in the parent axoplasm-microtubule mix (Table I). When axoplasm with-

**Table II.** Characteristics of MtVC ATPase Activity

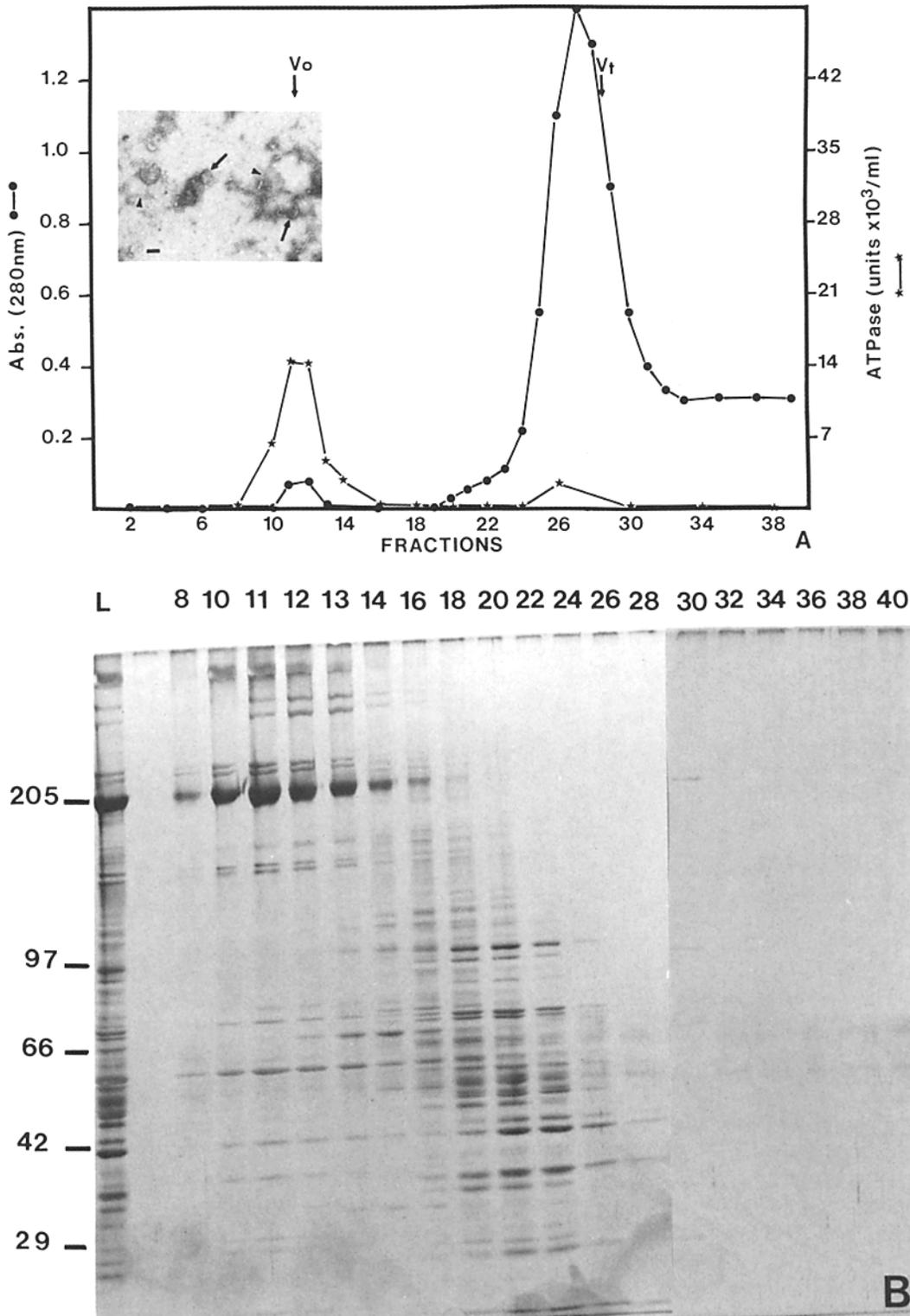
Assay conditions	MtVC	MtVC Void pool <sup>‡</sup>
<i>Buffer 1</i> <sup>*</sup>	% of $Mg^{++}$ ATPase	
$Mg^{++}$ (EGTA)	100	100
$Ca^{++}$	100	90
$K^+$ -EDTA	6	0
$Mg^{++}$ plus		
100 $\mu$ M Vanadate	62	60
25 $\mu$ g/ml Oligomycin	136	85
100 $\mu$ M Oubain	163	80
20 mM NaF	64	ND <sup>§</sup>
1 mM Azide	102	98
<i>Buffer 2</i> <sup>  </sup>		
- Actin	100	ND
+ 0.2 mg/ml F-Actin	111	ND

\* 30 mM Tris-HCl (pH 8.0), and 2 mM divalent cation or EDTA, 0.3 M KCl, and 0.5 mM ATP.

<sup>‡</sup> Pool of fractions from the first peak as in Fig. 6, containing axoplasmic proteins of the MtVC.

<sup>§</sup> Not determined.

<sup>||</sup> 20 mM Imidazole (pH 7.0), 5 mM  $MgCl_2$ , 1 mM EGTA, 50 mM KCl, 1 mM ATP, and 0.16 mg/ml MtVC.



**Figure 5.** Fractionation of whole axoplasm by chromatography over Ultrogel 22. (A) Whole axoplasm (seven axons in 0.4 ml buffer) was loaded on a 9.5-ml column in a 10-ml pipette. The homogenization, equilibration, and elution buffer was ImB containing 1 mM ATP and 250 mM KCl. The flow rate was 8–10 ml/h, and the void ( $V_o$ ) and total ( $V_t$ ) volumes are indicated. The figure shows protein and ATPase distribution across the column. The inset is a negative stain electron micrograph showing the constituents of fraction II; vesicles (*arrows*) and filaments (*arrowheads*) are noted. Bar, 200 nm. (B) SDS PAGE showing protein composition of the whole axoplasm in the column load (L) and certain fractions (as numbered) from the column. The positions of several molecular mass markers are noted in kilodaltons at the left margin. Note that there is little detectable protein in the second large peak. The gels were stained with silver nitrate.

out added microtubules is centrifuged as in an MtVC preparation, the pelleting of ATPase activity is dependent on the presence of taxol to polymerize "native" microtubules. In the absence of microtubules, either added or native, no ATPase activity appears in the pellet.

The MtVC pelleted activity was examined for similarity to known ATPases, including dynein, myosin, Na<sup>+</sup>K<sup>+</sup>ATPase, mitochondrial F<sub>1</sub> ATPase, and general phosphatases. Table II delineates some of the characteristics of the enzymatic activity. Inorganic phosphate release is equally stimulated by Ca<sup>++</sup> or Mg<sup>++</sup>, though it does not require calcium ions. Some divalent cation is required because the activity disappears in the presence of potassium and EDTA. Enzymatic activity is inhibited moderately by 100 μM sodium vanadate, which has been shown to halt vesicle transport in lobster axons (Forman et al., 1983). Vanadate is also known as an inhibitor of dynein ATPase, but the MtVC ATPase is less sensitive than either the flagellar or cytoplasmic forms of dynein (Gibbons et al., 1978; Cande and Wolniak, 1978; Pratt et al., 1984). Inorganic phosphate release by the MtVC is also inhibited by NaF, an inhibitor of phosphatases. The activity is unaffected by the presence of F-actin in buffers where myosin ATPase would be stimulated up to 10-fold (Table II). Neither the detergent Triton X-100, a general hydrophobic perturbant and stimulator of several enzyme activities, nor 6 μM calmodulin in the presence or absence of millimolar calcium affected ATPase activity (data not shown). The enzyme is not affected by ouabain, an inhibitor of Na<sup>+</sup>K<sup>+</sup>-ATPase, or by either of the mitochondrial ATPase inhibitors (Table II).

To further determine whether this ATPase could be mitochondrial in origin, cytochrome oxidase activity (a marker for mitochondria) in the MtVC was measured and compared with values obtained for an enriched fraction of mouse liver mitochondria. The specific cytochrome oxidase activity of the MtVC is 16 times lower than that of the mitochondrial fraction (Table III). More importantly, ~75% of the mitochondrial enzyme activity found in the axoplasm-mitochondrial mixture is recovered in the supernatant after pelleting of the MtVC (Table III) and both fractions contain six to nine times as much cytochrome oxidase activity as the MtVC.

### Fractionation of Whole Axoplasm

To determine the origin of the proteins pelleting with microtubules, and in particular to determine which of them might be vesicle-associated, whole axoplasm was fractionated by gel filtration and the fractions were analyzed by SDS PAGE for the presence of MtVC proteins. Fig. 5 shows a typical elution pattern when the column is packed with Ultrogel 22. One small peak of A<sub>280</sub> absorbing material appears at the column void volume, followed by a second large peak. Spot-

ting of fractions on filter paper followed by Coomassie Blue staining, measurement of A<sub>260</sub>, and SDS PAGE reveal that the second peak contains two smaller overlapping peaks, one of protein and one of nucleotides. When Sepharose CL-6B is used as the gel filtration matrix, the second protein peak appears as a leading shoulder on the much larger peak of A<sub>260</sub> absorbing material.

Nearly all of the axoplasmic Mg<sup>++</sup>ATPase activity also appears in the first small protein peak (Fig. 5 A). This activity includes the ATPase of mitochondrial origin since the peak also exhibits cytochrome oxidase activity of the level found in whole axoplasm (Table III).

Most of the MtVC proteins appear in the small voided peak (Fig. 5 B), which suggests that they are associated with macromolecular structures. This axoplasmic fraction also contains large amounts of neurofilament proteins in addition to several polypeptides that are not found in the MtVC. The major morphological constituents of this fraction, as determined by negative stain electron microscopy, are neurofilaments and vesicles of the type found in the MtVC (Fig. 5 A, inset).

### Fractionation of the MtVC by Gel Chromatography

Because of the contamination of the voided peak of axoplasmic protein with non-MtVC proteins, the MtVC itself was subjected to gel filtration to better examine the origin and native molecular weight of the proteins in the complex. The MtVC aggregates were dispersed by depolymerizing the microtubules by dialyzing the resuspended pellet against 20 mM Tris (pH 8.0) 0.1 mM EDTA, 0.1% β-mercaptoethanol, 1 M sucrose, and proteolysis inhibitors but without taxol (Tris/sucrose), for 12 h at 4°C. It was predicted that this treatment would also release nonvesicle proteins into a "soluble" fraction that would enter the included volume of the column. The dialysate was clarified by centrifuging at 30,000 g in a Sorvall SS-34 rotor for 20 min and then fractionated on a column of Sepharose CL-6B.

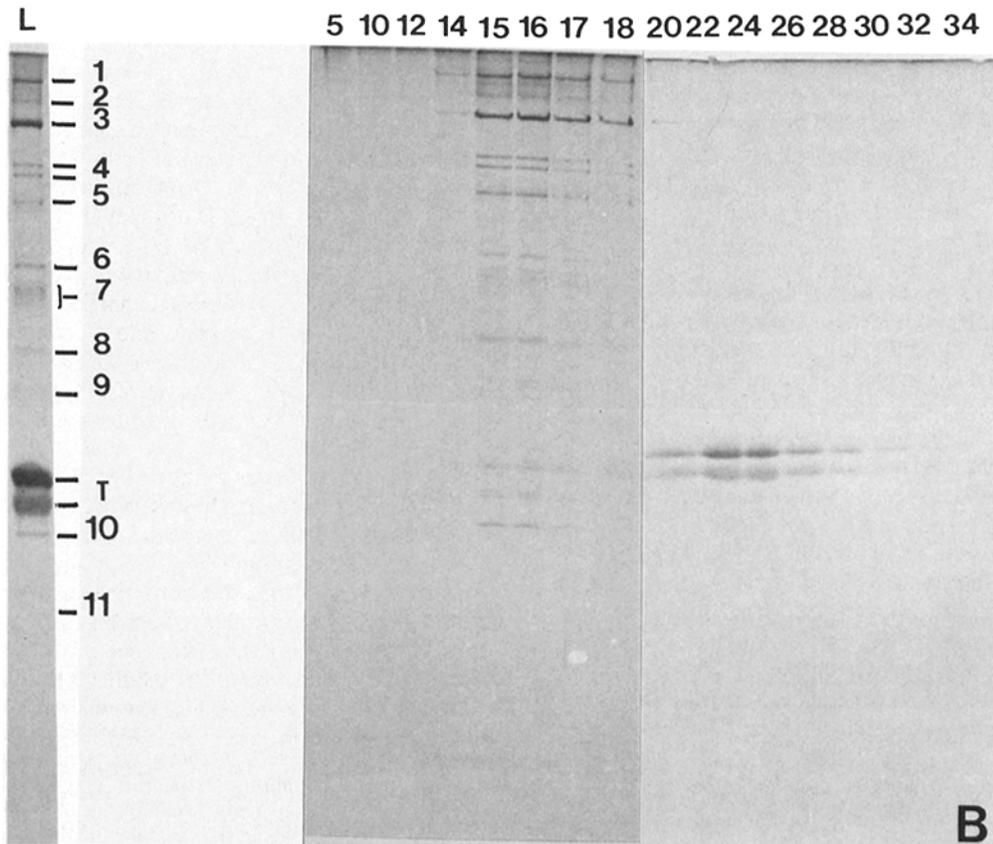
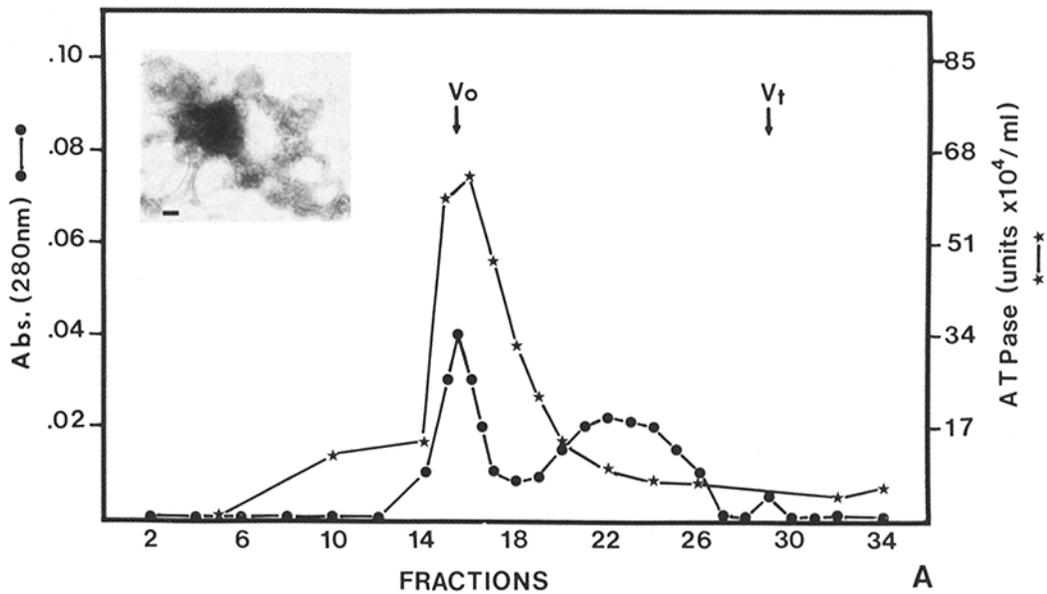
Fig. 6 shows a typical fractionation pattern. Two peaks of material absorbing at 280 nm are clearly separated, one appearing at the column void volume and one included that elutes before the total column volume (Fig. 6 A). Analysis of the fractions by SDS PAGE shows that both peaks contain protein in amounts corresponding to their relative A<sub>280</sub> (Fig. 6 B). The first peak of voided material contains all of the MtVC proteins including a small amount of tubulin. Most of the tubulin is found in the second peak. Negative stain electron microscopy confirms that the first peak contains membrane-delimited vesicles as the major morphological entity (Fig. 6 A, inset). All of the MtVC ATPase activity also appears in the peak voided from the gel filtration column.

Table III. Comparison of ATPase and Cytochrome Oxidase Activities in Mitochondrial and Axoplasmic Fractions

Sample	ATPase (total units)	Specific ATPase activity	Cytochrome oxidase (total units)	Specific cytochrome oxidase activity
Mitochondria	NA*	0.041	NA	10
Axoplasm/microtubule mix	0.0055	0.0033	3.15	1.88
Supernatant	0.0022	0.0019	2.34	2.06
Pellet	0.0071	0.012	0.36	0.63
Column void‡	NA	0.015	NA	4.74

\* Not applicable.

‡ This sample is a pool of the voided peak after column chromatography of whole axoplasm as shown Fig. 5.



**Figure 6.** Fractionation of the MtVC by chromatography over Sepharose CL-6B. (A) MtVC proteins (0.4 mg in 0.25 ml) were loaded onto a 12.5-ml column (0.9 cm × 20 cm) equilibrated and eluted with Tris/sucrose (see text). The flow rate was 3–5 ml/h. The void ( $V_o$ ) and total ( $V_t$ ) volumes are indicated. The figure shows protein and ATPase distribution across the column. The inset is a negative stain electron micrograph showing the vesicles found in fraction 15. Bar, 100 nm. (B) SDS PAGE showing protein composition of the MtVC proteins in the column load (L) and certain fractions (as numbered) from the column. The standard MtVC proteins in the load fraction are labeled by number as in Fig. 1. The gels were stained with silver nitrate.

The protein concentration of this fraction is too low to measure by colorimetric assay but if we assume it is the lowest measurable concentration then the specific ATPase activity of the pool of voided protein is  $>0.15 \mu\text{mol P/mg min}$ . This

ATPase is unlikely to be mitochondrial in origin because, as demonstrated above, the MtVC contains little if any mitochondrial ATPase. The enzymatic activity has the same characteristics as the total MtVC ATPase (Table III).

The MtVC was also subjected to gel filtration in the presence of 0.4 M KCl in ImB. The elution pattern was nearly identical to that shown in Fig. 6 except that the two peaks of protein overlapped slightly, and more tubulin appeared in the voided peak. All of the ATPase activity was recovered in the voided peak, and negative stain electron microscopy confirmed the presence of vesicles in those fractions.

### Fractionation of the MtVC by Equilibrium Gradient Centrifugation

Fractionation of the MtVC by gel filtration suggested that all of the proteins were vesicle-associated. Since the column fractionates by size only, it was possible, however, that some of the voided proteins were found in macromolecular aggregates of  $>200,000 M_r$ , and co-eluted adventitiously with the vesicles. To address this question, the MtVC was fractionated by equilibrium gradient centrifugation (Fig. 7), which separates macromolecular structures on the basis of density. When the complex is loaded at the bottom of a sucrose gradient that is then spun to equilibrium, all of the MtVC proteins float near the top of the gradient, at a sucrose density of 1.05–1.06 (Fig. 7). Negative stain electron microscopy confirms the presence of vesicles in these top fractions (Fig. 7 A, inset). Some of the tubulin disperses into several fractions in the bottom half of the gradient, and some of it floats to the top (Fig. 7 B). The latter result further suggests that a fraction of MtVC tubulin is vesicle-associated. Several proteins in the loaded sample that are not consistent MtVC components are found in the gradient pellet (Fig. 7 B).

### Discussion

In vitro construction of a complex of microtubules and vesicles (the MtVC) has allowed the identification of a group of polypeptides from the squid giant axon that form stable interactions with both microtubules and vesicles, and that can hydrolyze ATP. Although small quantities of the MtVC polypeptides appear in taxol-induced preparations of microtubule protein, these proteins do not have the characteristics of typical MAPs. They are not readily solubilized from axoplasm under conditions that extract tubulin; neither are they released into solution with tubulin upon depolymerization of MtVC microtubules. The axoplasmically derived MtVC proteins have instead, the characteristics of vesicle-associated proteins. Upon either gel filtration chromatography or equilibrium gradient centrifugation, they co-fractionate with vesicle-containing fractions. These data show that the MtVC proteins associate with macromolecular complexes of both the size and density of membrane vesicles. Some tubulin also appears to be vesicle associated because it is present in the low density fractions of the sucrose gradient. All of the MtVC proteins are also found in an axoplasmic vesicle fraction isolated by Gilbert and Sloboda (1986) by a different procedure.

The most prominent axoplasmic polypeptide in the MtVC is the one designated No. 3. This protein associates tightly with the MtVC and some evidence suggests that it may be an integral membrane protein. It is not readily extracted from the MtVC by either low or high ionic strength buffers that would remove peripheral proteins, or by 0.1% Triton X-100 that leaves the vesicles intact. In addition, the protein is glycosylated. Recent ultrastructural studies have revealed projections from axoplasmic vesicles that mediate the inter-

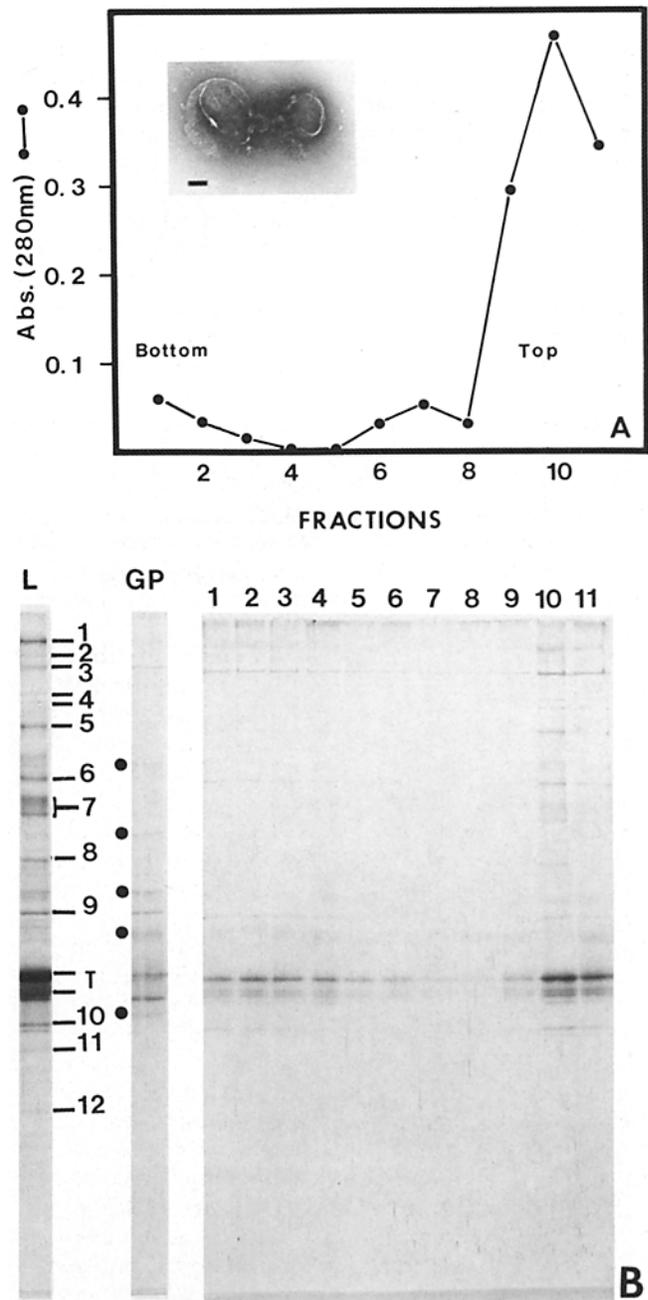


Figure 7. Fractionation of the MtVC by equilibrium gradient centrifugation. (A) MtVC proteins in 50% sucrose were loaded at the bottom of a tube, overlaid with a 15–40% sucrose gradient, and centrifuged to equilibrium. The amount of material absorbing at 280 nm is plotted for each fraction. The negative stain electron micrograph inset shows vesicle components in fraction No. 10 at the top of the gradient. Bar, 100 nm. (B) SDS PAGE showing protein composition of the MtVC loaded fraction (L), the pellet after centrifugation (GP), and fractions from the gradient (as numbered). MtVC components are identified in the load fraction by numbers, as in Fig. 1. Proteins that are not consistent components of the MtVC and are found in the gradient pellet are designated (•). The gels were stained with silver nitrate.

actions with microtubules (Gilbert et al., 1985; Miller and Lasek, 1985). One possibility is that polypeptide No. 2 forms these tubule-vesicle bridges, although we have no direct evidence to support this hypothesis.

The interaction between the entire group of axoplasmic proteins and microtubules appears to be largely ionic since an increase in the ionic strength of the buffer in which axoplasm and microtubules are mixed leads to a reduction in the amount of vesicles and MtVC proteins that pellet. In addition, incubation of the MtVC with 0.35 M NaCl extracts some of each of the MtVC proteins.

Because microtubule-associated vesicle transport requires ATP, it was of interest to examine the role of ATP in MtVC interactions. In general, associations within the MtVC are not nucleotide dependent. This property distinguishes the microtubule binding in the MtVC from the interactions found in preparations of taxol-stabilized brain microtubule protein (Turner and Margolis 1984).

Two MtVC proteins are of particular interest because, in contrast to the others, their associations with the MtVC are affected by the presence of added nucleotide. The first, polypeptide No. 7, has several features in common with kinesin. It associates with the MtVC in the absence of added ATP or in the presence of AMP-PNP, but is released by added ATP, in a manner similar to kinesin-microtubule interaction (Vale et al., 1985; Brady et al., 1985). In addition, the polypeptide group No. 7 falls in a molecular mass range similar to that reported for kinesin, 110–130 kD (Brady et al., 1985; Vale et al., 1985). The second, polypeptide No. 2, has a very different nucleotide dependence, associating with the MtVC only in the presence of ATP or AMP-PNP. This observation is consistent with the observation that the protein (also designated 292-kD protein by Gilbert and Sloboda, 1986) binds ATP (Gilbert and Sloboda, 1986). Polypeptide No. 2 (292 kD) may associate exclusively with either microtubules or vesicles, but there is evidence to suggest that it can bind both structures. First, the protein can be solubilized from axoplasm and will fractionate as a MAP in a typical taxol microtubule preparation. It behaves as a vesicle-associated protein, however, in the isolation procedure of Gilbert and Sloboda (1986). This can be explained by the fact that these investigators always prepare vesicles in the presence of ATP. Hence, the protein appears to have a weak microtubule binding site, in part ionically dependent since it can be removed with high salt, and an ATP-dependent vesicle binding site. Gilbert and Sloboda (1986) have demonstrated that protein No. 2 (292 kD) crossreacts with an antibody to brain MAP2. *In vitro* studies of pancreatic vesicle binding to microtubules suggested a role for MAPs, in particular MAP2, as linkage proteins (Suprenant and Dentler, 1982), and our data would support that interpretation. Although microtubule-vesicle interactions occur in the presence of small quantities of polypeptide No. 2, they are enhanced when larger quantities of the protein appear in the MtVC, in response to the addition of ATP.

The MtVC ATPase is enzymatically very similar to a  $Mg^{++}$  or  $Ca^{++}$ ATPase identified in squid giant axon by Shecket and Lasek (1982). These investigators also reported that the enzymatic activity was enriched in a rapidly sedimenting fraction of axoplasm, and suggested an association with either the cytoskeleton or a membranous vesicular structure. It is very likely that the MtVC ATPase is the same as the one reported by Shecket and Lasek (1982).

Little if any of the ATPase in the MtVC is mitochondrial. When the axoplasm-microtubule mix and mitochondrial samples are standardized for the amount of  $Mg^{++}$ ATPase present, the levels of cytochrome oxidase in the former are

~30% of those in the latter. It follows therefore that some 30% of the ATPase activity in the axoplasm could be mitochondrial in origin. Because 25% of the axoplasmic cytochrome oxidase activity appears in the MtVC, only 7.5% (25% of 30%) of the ATPase activity in the MtVC could be mitochondrial in origin. The actual amount is probably even less than this since the  $Mg^{++}$ ATPase activity of the MtVC is not inhibited by either oligomycin or sodium azide ( $NaN_3$ ). Also, the MtVC ATPase activity does not have the characteristics of the  $F_1$ -like enzyme identified in vesicles which copurify with brain microtubules (Murphy et al., 1983).

The ATP-hydrolyzing activity associated with the MtVC is not enzymatically similar to myosin or  $Na^+K^+$ ATPase. Some properties of the enzyme and the MtVC, however, suggest the presence of dynein-like activity. The ATPase activity is activated almost equally by calcium and magnesium, abolished in  $K^+$ -EDTA, and inhibited by sodium vanadate, all properties of dynein ATPase (Pratt et al., 1984). In addition, though the ATPase activity cannot be assigned to any one protein in the MtVC, the preparation does contain a prominent polypeptide (high molecular mass) that comigrates with flagellar dynein on SDS PAGE. This protein may be analogous to the membrane-associated dynein-like ATPase that appears to be part of a bridge between ciliary outer doublet microtubules and ciliary membranes (Dentler et al., 1980), although this possibility has not been directly investigated.

A major question is whether the ATPase activity of the MtVC is likely to play a role in vesicle movement, particularly as a mechanochemical transducer. We have no direct evidence bearing on this point, but because the kinesin proteins appear to be the motor for orthograde transport (Vale et al., 1985), the most likely possibility is that the MtVC ATPase may mediate retrograde transport. This interpretation is consistent with the report by Vale and co-workers (1986) that the motors for vesicle movement in each of the two directions are distinct, and that the retrograde factor is found associated with insoluble axoplasmic components that fractionate with organelles on sucrose step gradients (Vale et al., 1985, 1986).

The specific ATPase activity of the MtVC is not high and an alternative interpretation is that the low level of inorganic phosphate release measured is due to a coupling of kinase and phosphatase activities. This interpretation is consistent with the inhibition of phosphate release by NaF. If this were true, then the hydrolysis of ATP would not be expected to produce energy for movement. Phosphorylation of motility coupled proteins is regulatory in several actomyosin systems (Adelstein, 1982), however, and phosphorylation of MAPs can mediate their interactions with microtubules (Burns and Islam, 1986). One possibility is that a similar regulatory mechanism may be operating to mediate the interaction of polypeptide No. 2 with the MtVC.

In conclusion, the MtVC is an excellent model system for further studies of vesicle-microtubule interaction and transport. Fractionation of the complex provides a pure population of axoplasmic membrane vesicles uniquely free of neurofilaments. These vesicles are ideal starting material for cataloguing integral and peripheral proteins which may serve as binding sites for kinesin. In addition, the MtVC provides an isolated and defined system of native vesicles and microtubules in which to study conditions required for both orthograde and retrograde transport.

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