

Stable Polymers of the Axonal Cytoskeleton: The Axoplasmic Ghost

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ABSTRACT We have examined the monomer-polymer equilibria which form the cytoskeletal polymers in squid axoplasm by extracting protein at low concentrations of monomer. The solution conditions inside the axon were matched as closely as possible by the extraction buffer (buffer P) to preserve the types of protein associations that occur in axoplasm. Upon extraction in buffer P, all of the neurofilament proteins in axoplasm remain polymerized as part of the stable neurofilament network. In contrast, most of the polymerized tubulin and actin in axoplasm is soluble although a fraction of these proteins also exists as a stable polymer. Thus, the axoplasmic cytoskeleton contains both stable polymers and soluble polymers. We propose that stable polymers, such as neurofilaments, conserve cytoskeletal organization because they tend to remain polymerized, whereas soluble polymers increase the plasticity of the cytoskeleton because they permit rapid and reversible changes in cytoskeletal organization.

Cytoskeletal networks are predominantly constructed from three types of protein polymers: microtubules (MT), microfilaments (MF), and intermediate filaments (IF). It is commonly assumed that MT and MF are in a steady state equilibrium with their unpolymerized subunits in the cell (4, 20, 33, 50). Similar data do not exist for IF primarily because IF are insoluble in physiologic buffers (16, 42, 44). However, IF do depolymerize in unphysiologic solvents and their subunits polymerize following kinetics which are similar to those of other cytoskeletal polymers (46, 54, 55). The relative stability of cytoskeletal polymers *in vitro* is defined by the kinetics of monomer-polymer exchange, so polymers with slower exchange rates are said to be more stable than polymers which undergo rapid monomer-polymer exchange (28). *In vivo*, the relative stability of cytoskeletal polymers is assayed indirectly by measuring the amount of polymer after changes in temperature, cellular metabolism, or during drug exposure (6, 36, 51). For example, mitotic spindles contain both labile and stable MT when the cell is exposed to low temperature (6). MF and IF can also vary in their relative stability (16, 52). Cytoskeletal networks which are constructed from stable polymers and those constructed from polymers of lesser stability should confer different properties upon the cell. Differences in cytoskeletal stability could regulate the plasticity of cellular morphology during ontogeny.

The relationships between the detailed properties of cyto-

skeletal polymers and cellular morphology have been analyzed in specialized regions of cells, notably, intestinal cell microvilli, the sperm acrosome, and cilia (13, 30, 48). This report characterizes the stability of cytoskeletal polymers in another specialized region of the cell, the nerve cell axon, which offers the particular advantage of its length. In addition, the axon contains a relatively simple fraction of nerve cell cytoplasm which includes parallel arrays of MT and IF (in nerve cells called neurofilaments, NF), a meshwork of MF, but is devoid of ribosomes. During growth and maturation, axons exhibit changes in their cytoskeletal composition and diameter (12, 35). Understanding the stabilities of the cytoskeletal polymers in axoplasm could provide insight into the roles that different polymers have during this differentiation of neuronal morphology.

Axoplasm from the squid giant axon is particularly well suited for an analysis of cytoskeletal organization. It contains a typical complement of MT, NF, and MF. More importantly, large quantities of pure axoplasm (4–8 μ l) can be rapidly obtained from a single axon by extrusion (Fig. 1) without the use of detergents to solubilize membranes (25). Furthermore, the data on the low molecular weight components of squid axoplasm is rather complete so that an axoplasmic buffer (buffer P) can be constructed which simulates the solution conditions in the axon (10, 11). Using buffer P, it is possible to extract the soluble proteins from axoplasm and reveal the more

stably polymerized elements of the cytoskeleton in the axon. Some of this work has been previously reported in the form of an abstract (31).

MATERIALS AND METHODS

Isolation of Axoplasm

Squid (*Loligo pealei*) were supplied by the Marine Biological Laboratory at Woods Hole, Mass., and were used in these experiments <48 h after their capture at sea. Axoplasm was obtained by extrusion from the medial pair of giant axons (Fig. 1) using a modification of the procedure described by Lasek (25). The axon was tied off at proximal and distal points with thread before being dissected from the squid mantle. From this step on, all manipulations were performed with the aid of a dissecting microscope to permit a more careful dissection of the axon. Small axons and connective tissue were cleaned from the proximal 2 cm of the giant axon. Next, the axon was briefly rinsed in Ca⁺⁺-free artificial sea water (425 mM NaCl, 9 mM KCl, 60 mM MgCl₂, 10 mM Tris HCl, pH 7.6) and carefully blotted on paper to avoid contaminating the axoplasm with Ca⁺⁺. The proximal end was severed with fine dissecting scissors, and the axon was placed on a cleaned glass slide with its cut end hanging over the edge. Axoplasm was then extruded directly into 1 ml of buffer P using a short piece of polyethylene tubing (Intramedic, PE 200, Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.) as a moving press. Extrusion was stopped just short of the axon's cut end to avoid contamination from the sheath. Finally, the partially extruded axoplasm was cut with scissors just above the point where it entered the buffer. The cleaning of the axon and extrusion altogether takes ~15 min per axon after it is removed from the squid.

Buffer P

Buffer P was designed from data in the literature (10, 11) as a simulation of the solution conditions in the squid giant axon. Initially, buffer P contained 16 amino acids (ala, ile, leu, met, phe, pro, val, gly, ser, thr, tyr, asp, glu, arg, lys, and taurine), six organic metabolites (isethionic acid, glycerol, citrulline, hypoxanthine, myo-inositol, and ornithine), two carbohydrates (glucose and fructose), and five inorganic ions (K⁺, Na⁺, Mg⁺⁺, Cl⁻, and P_i), all at their reported physiologic concentrations. Subsequently, in a simplification of this buffer, alanine, glycine, aspartic acid, and arginine were used in the appropriate amounts to substitute for their nonpolar, polar, acidic, or basic counterparts. Excess taurine was added to substitute for homarine and cysteic acid. Also, citrulline, hypoxanthine, myo-inositol, and ornithine were eliminated from the buffer. No differences in the results were detected after these changes were made. ATP and GTP are included in buffer P at their approximate physiologic levels to fulfill the energy requirements of axoplasm. EGTA and PMSF were added to chelate Ca⁺⁺ and inhibit proteolysis, respectively. Table I summarizes the composition of axoplasm and compares it to buffer P. Note that axoplasm contains >0.3 M amino acids and, like buffer P, is at pH 7.0. It is also of interest to note that axoplasm contains ~2% protein by weight (19), an ingredient which is missing from buffer P. Buffer P was made fresh from refrigerated or frozen stock solutions, brought to room temperature, and millipore-filtered immediately before use.

Electron Microscopy

Samples of axoplasm were prepared for electron microscopy using 2.5% glutaraldehyde in 20 mM phosphate buffer with 1 M glucose (pH 7.0) as a fixative. Axoplasm was either extruded directly into fixative or transferred into fixative after the soluble proteins had been extracted in buffer P. Fixed samples were treated with 2% osmium tetroxide and 1.5% potassium ferrocyanide before dehydration and embedding in 100% Araldite. Light gold-to-silver sections were cut with a diamond knife and stained with uranyl acetate-lead citrate stain before examination in the electron microscope.

SDS PAGE

All samples for electrophoresis were solubilized in BUST (1% SDS, 5% beta-Mercaptoethanol, 10 mM Tris, 8 M Urea, at pH 6.8). Samples of extracted axoplasm were homogenized directly in BUST while aqueous samples of protein were first precipitated in 10% trichloroacetic acid and washed with ether: ethanol (1:1). Electrophoresis was performed in slab gradient (5–16%) polyacrylamide gels using the buffer system of Laemmli (22). Gels were run at constant current (50 mA) until the bromophenol blue tracking dye just reached the bottom of the gel. Molecular weights were determined by running protein standards (myosin, 200,000; phosphorylase a, 93,000; bovine serum albumin, 68,000; pyruvate kinase, 57k; ovalbumin, 43,000; and lysozyme, 14,000) on an adjacent column of the

TABLE I
Comparison of Axoplasm and Buffer P

	Squid Axoplasm mM	Buffer P mM
Amino acids		
Non Polar amino acids (Alanine)	16.14	16.14
Polar amino acids (Glycine)	18.41	18.41
Acidic amino acids (Aspartic acid)	100.29	100.29
Basic amino acids (Arginine)	6.06	6.06
Betaine	73.7	73.6
Taurine	106.7	132.0
Homarine	20.4	*
Cysteic acid	4.9	*
Organic metabolites		
Isethionic acid	164.6	164.6
Glycerol	4.35	4.35
Others	11.02	‡
Carbohydrates		
Glucose	0.24	1.16
Mannose	0.92	*
Fructose	0.24	0.48
Sucrose	0.24	*
Inorganic ions		
Potassium	344.0	344.0
Chloride	151.2	147.2
Sodium	65.0	65.0
Phosphate	17.8	17.8
Magnesium	10.0	10.0
Sulfate	7.5	§
Calcium	3.5	§
Nucleoside triphosphates		
ATP	1.0	1.0
GTP	NA	0.5
Others		
EGTA	§	1.0
PMSF	§	0.1
Protein	2% (By weight)	§

A comparison of axoplasm and buffer P. Note that buffer P simulates the solution conditions in axoplasm carefully. However, axoplasm contains 2% protein and buffer P has none. NA, data not available.

* Excluded from simplified buffer P.

‡ Replaced by the component above.

§ Not included in buffer P.

same gel. The gels were then stained with Coomassie Blue. Densitometric analysis of stained protein was performed with a Zeiss scanning densitometer and peak areas representing polypeptides of interest were measured using gravimetric methods or digitization planimetry.

RESULTS

Axoplasm was examined before and after extrusion into buffer P to determine the effects of removing axoplasm from its cell membrane and collagenous glial cell sheath (Fig. 1). Axoplasm is transparent both before and after extrusion when viewed with transmitted light, but it is easily visualized with dark-field optics because of its light scattering properties. As shown in Fig. 2, axoplasm retains its original cylindrical shape after extrusion into buffer P. Mitochondria in the extruded axoplasm are spherical (their normal shape) and do not undergo brownian movement. In some cases, helical bundles of fibrils may be seen coursing through the extruded axoplasm. These correspond to bundles of axonal NF (15). Axoplasm extruded directly into fixative is ultrastructurally identical to axoplasm fixed in the axon, indicating that extrusion itself does not significantly disturb the organization of the axonal cytoskeleton

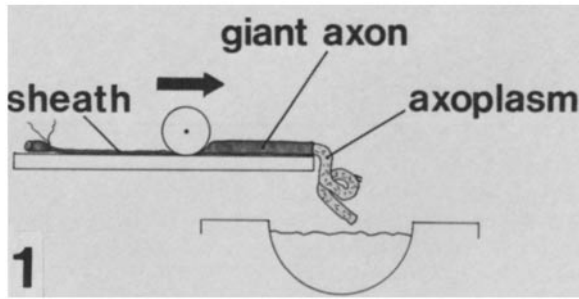


FIGURE 1 Axoplasm is extruded from the giant axon directly into a buffer P bath. This separates pure axoplasm from its limiting axolemma and glial cell sheath without the use of detergents to solubilize lipids.

(J. Metzuzals, A. J. Hodge, and R. J. Lasek, manuscript in preparation).

The border between buffer P and extruded axoplasm is sharp in the light microscope (Fig. 2). This observation suggests that the process of extrusion removes axoplasm along a natural cleavage plane which is an annular ring in the cortex of the axon. Ultrastructural studies by others have shown that extrusion removes >95% of the axoplasm and leaves behind a 10- μ m thick layer of cortical axoplasm (1, 29). This cortical layer is similar in composition to the extruded axoplasm, especially with regard to the cytoskeletal proteins (2). The integrity of the cortical layer is important in the maintenance of axonal excitability (29). After the extrusion of axoplasm, the sheath containing this cortical layer may be "reinflated" with buffers to study the normal electrophysiologic properties of the axon (1, 47). All the above results indicate that extrusion itself does not significantly alter the properties of either axoplasm or the sheath.

The chief difference between axoplasm in the axon and extruded axoplasm is that the extruded axoplasm is slightly wider and shorter than the dimensions of the giant axon from which it came. This difference was examined in greater detail. We found that axoplasm undergoes two types of shape change after extrusion. The first is rapid and occurs as the axoplasm is extruded into buffer P or onto a piece of dry parafilm. The diameter of the extruded axoplasm increases with a simultaneous and proportionate decrease in length so that the volume of the axoplasm remains constant. The diameter increases by a factor of 1.6 ± 0.1 over the diameter of the intact axon. This value was determined by direct measurement of five axons using a calibrated reticle in the microscope eyepiece. We interpret this change as an elastic recoil of axoplasm that occurs as it is extruded. It suggests that axoplasm is normally under compression in the axon. The second type of shape change is a much subtler slow swelling. This increases the diameter of the axoplasm by a further 15–20% during the first 2 h in buffer P.

Other than showing the slow swelling, the extruded axoplasmic cylinder remains apparently unchanged for >24 h at 20°C in buffer P. Axoplasm extruded into buffer P may be

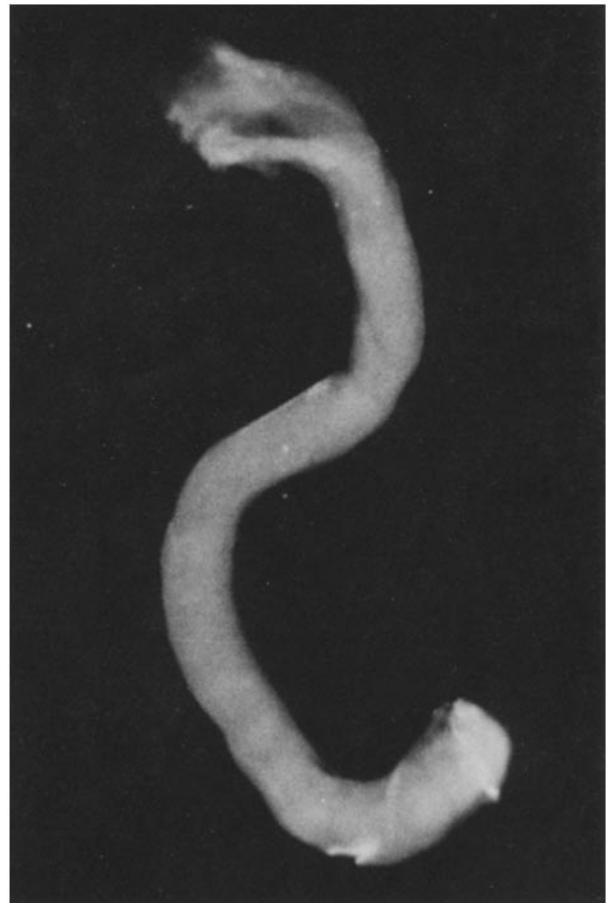
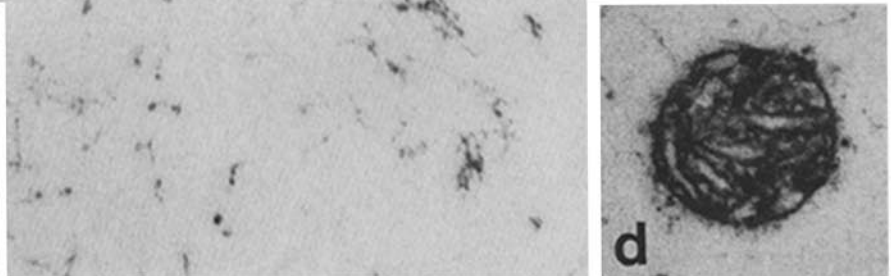
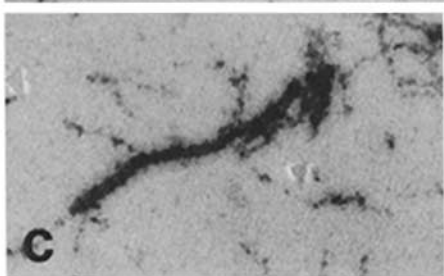
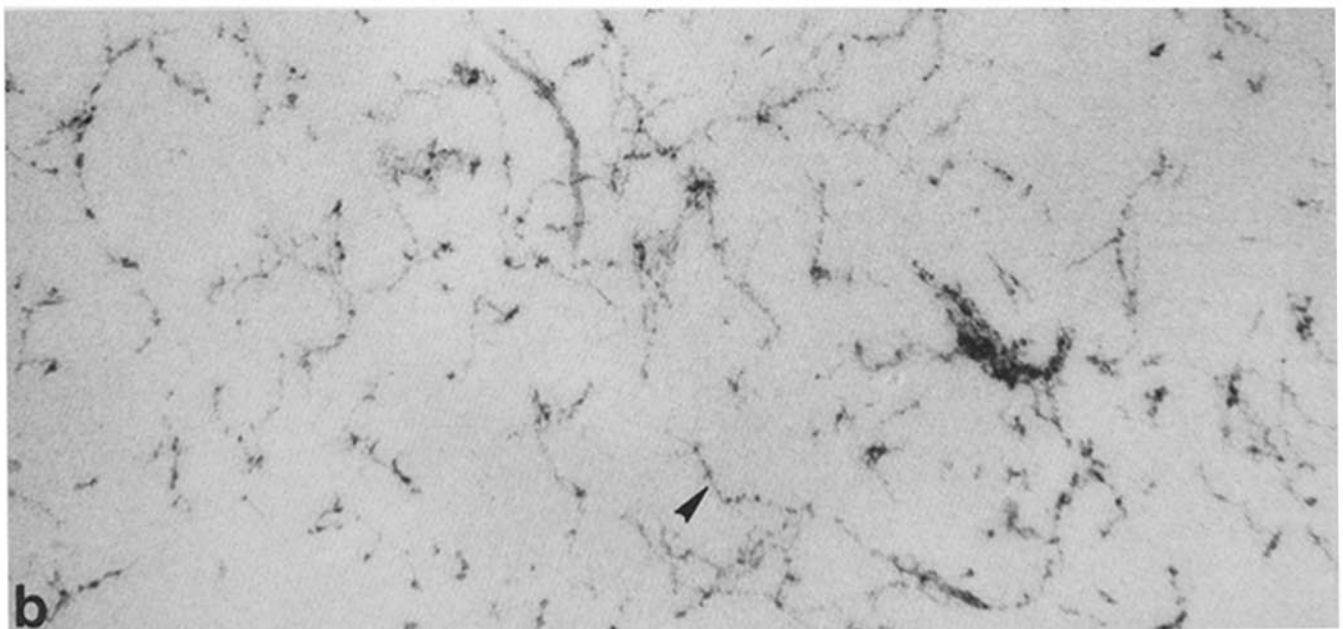
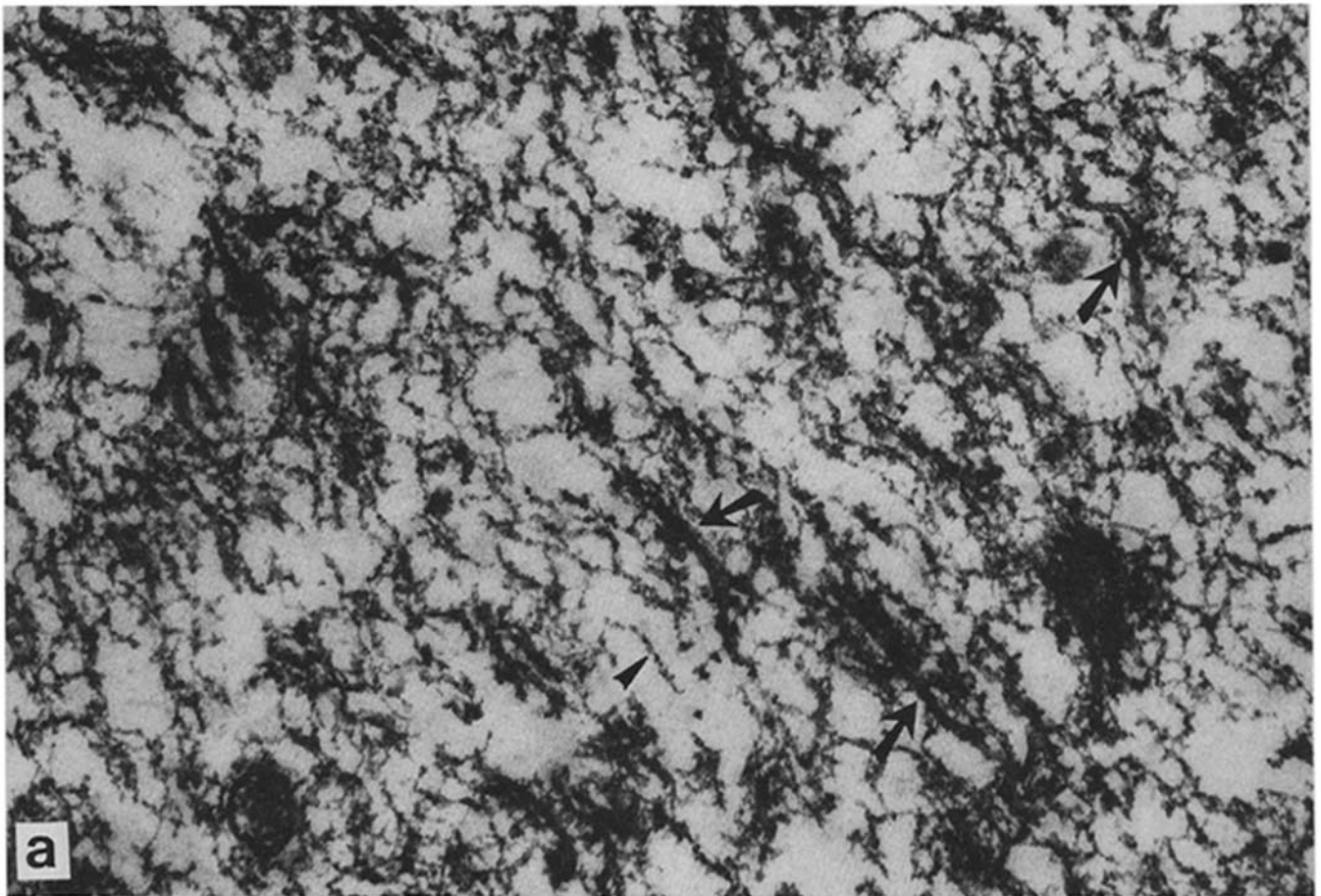


FIGURE 2 This shows a sample of axoplasm (~1 mm in diameter) in buffer P. Axoplasm retains its cylindrical shape for the duration of the experiment.

transferred with forceps to another solution, without incurring obvious changes in its shape. This indicates the strength of the axoplasmic gel in buffer P. The apparent insolubility of axoplasm in buffer P contrasts with the rapid dissolution which occurs in minutes when axoplasm is placed in a solution of high ionic strength or is in the presence of millimolar concentrations of Ca^{++} (3, 14, 17, 34).

Electron microscopy was used to characterize the insoluble cylinder in greater detail and to compare it to unextracted axoplasm (extruded directly into fixative). In the former case, axoplasm was extracted in buffer P for 120 min before being transferred into fixative. This incubation time was selected because SDS PAGE showed that essentially all of the soluble proteins were extracted from axoplasm by 2 h. Axoplasm which has been fixed immediately after extrusion contains many MT, NF, and electron-dense regions (Fig. 3 a). Most of the MT and electron-dense regions are not found in axoplasm after it is extracted in buffer P (Fig. 3 b). On the other hand, NF are a

FIGURE 3 Several electron micrographs of extruded axoplasm. (a) Axoplasm which has been extruded directly into fixative. Arrows point to MT which typically are embedded in an electron-dense matrix. The arrowhead points to a NF with an irregular profile. $\times 45,000$. (b) Axoplasm which has been extracted in buffer P for 2 h before fixation. MT are difficult to find and NF (with smooth and irregular profiles) form an extensive network (arrowhead). $\times 45,000$. (c) A MT found in a sample of extracted axoplasm. $\times 48,000$. (d) An intact mitochondrion in a sample of extracted axoplasm. $\times 46,000$.



prominent feature of axoplasm both before and after the extraction in buffer P (arrowheads, Fig. 3*a* and *b*). Before extraction, the NF have irregular profiles and interconnect to form a three-dimensional network that extends throughout the axoplasm. After extraction, the cross-bridging network is preserved and the NF have smoother profiles in general. The size of the spaces between adjacent NF does not appear to change greatly. This suggests that the density of the NF network is unaltered during the loss of the soluble components of axoplasm. Several other ultrastructural features of axoplasm are relatively unchanged after extraction in buffer P. Mitochondria and smooth endoplasmic reticulum are retained in the insoluble cytoskeleton (Fig. 3*d*). Also, a small number of MT can be found in axoplasm which has been extracted in buffer P (Fig. 3*c*). Thus, electron microscopy demonstrates that profound changes occur in the ultrastructure of axoplasm during extraction in buffer P although some features also seem to resist change under these conditions.

SDS PAGE was used to characterize the solubilities of axoplasmic proteins in buffer P. At least 2 h after extrusion, the insoluble cylinder was removed from the buffer P bath using forceps. The insoluble cylinder and an aliquot of the buffer P bath were prepared for electrophoresis. After the electrophoretic gels were stained, axoplasmic tubulin and actin were identified on the basis of their similar behavior with either cycled squid brain tubulin or purified chicken gizzard actin on one- and two-dimensional gels (data not shown). The NF proteins were identified on the basis of their copurification with squid NF as reported by others who used ionic strength cycling, column chromatography, centrifugation, and electrophoresis on gel systems similar to the one used here (24, 54). Fig. 4 shows two columns in one gel which contains samples of the insoluble cytoskeleton and the buffer P bath. It is apparent that axoplasmic proteins are differentially soluble in buffer P; some are insoluble, some are highly soluble, and others have both soluble and insoluble forms. The most conspicuous members of the set of insoluble proteins are the subunit proteins of squid NF. These are only detected in the column labeled GHOST and have molecular weights of 60,000, 200,000, and >500,000 (the latter is labeled NF1 in Fig. 4). Most of the other axoplasmic proteins are essentially completely soluble and are only detected in the buffer P bath. Some conspicuous members of this set are found at 14,000, 35,000, and 90,000 daltons. Finally, there are several proteins which have both soluble and insoluble forms in axoplasm. Tubulin and actin are the most prominent members of this set. They are detected in significant amounts both in the insoluble cylinder and in the buffer P bath.

The proteins which make a significant contribution to the insoluble cytoskeleton may be considered as a separate set. This set consists of the NF proteins, alpha and beta tubulin, and actin. These polypeptides were found in similar amounts each time axoplasm was extracted using buffer P. The cylindrical structure they comprise contains the stable polymers of the axonal cytoskeleton. We subsequently refer to this extracted preparation as the "axoplasmic ghost."

The relative amounts of the insoluble proteins in the axoplasmic ghost and the soluble proteins extracted in buffer P were measured by scanning stained gels with a densitometer. Representative gels from five axons similar to the one in Fig. 4 were used in this phase of analysis. The standard error for these samples was <10% of the values obtained. Altogether, 80% of the axoplasmic protein is soluble in buffer P, leaving 20% insoluble as the axoplasmic ghost. Data which further

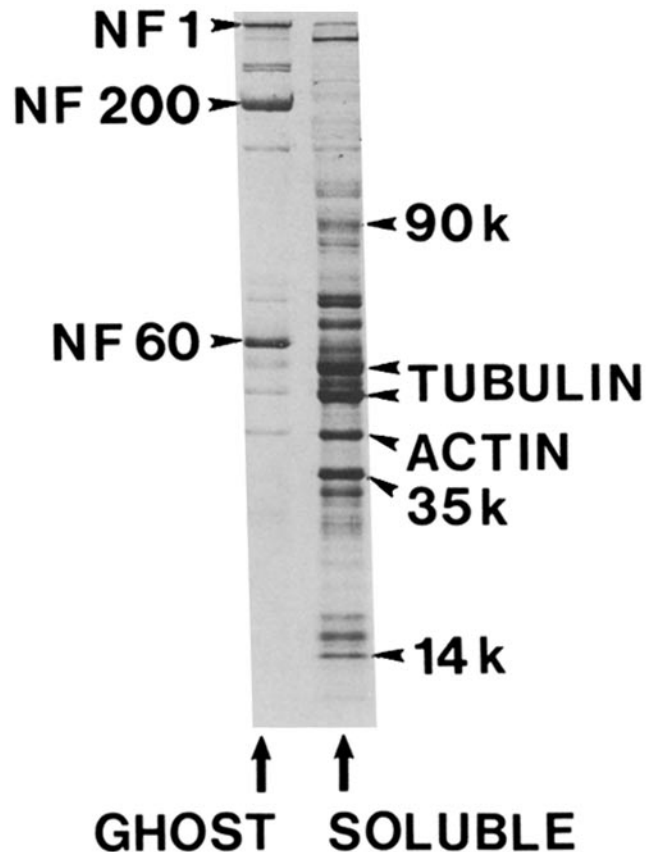


FIGURE 4 An SDS PAGE comparing the soluble proteins of axoplasm with those in the axoplasmic ghost. Note that the NF proteins are insoluble while tubulin and actin have both soluble and insoluble forms.

define the composition of the axoplasmic ghost are presented in Table II. As expected, essentially all of the NF protein (>95%) remains associated with the stable axonal cytoskeleton. Most of the actin and tubulin is soluble in buffer P. Only 27% of the actin and 15% of the tubulin remain as part of the axoplasmic ghost. NF proteins constitute 54% of this insoluble structure while the NF proteins, tubulin, and actin comprise >70% of the protein in the axoplasmic ghost.

DISCUSSION

We have studied the solubilities of axonal cytoskeletal proteins under solution conditions which approximate those inside the axon. Careful attention to solution conditions is important in studies like this one because protein-solvent interactions can significantly alter the conformations of polypeptides and change their solubilities (53).

Buffer P represents our attempt to simulate in detail the natural solvent conditions inside the squid giant axon. We presume that buffer P maintains the native conformations of axoplasmic proteins and supports the types of protein-protein associations that normally occur in axoplasm. Nonetheless, buffer P does not replicate axoplasm in at least one important respect: axoplasm contains ~2% protein while buffer P does not contain any protein (Table I). When axoplasm is extruded into buffer P, the concentration of axoplasmic proteins is rapidly lowered by several orders of magnitude because the volume of buffer P (1 ml) is large in comparison to the volume of the axoplasmic cylinder (~5 μ l). Any polymers which undergo an active exchange with their subunits will rapidly

TABLE II
Protein Composition of the Axoplasmic Ghost

Protein (mol wt)	Protein remain-	Ghost protein
	ing in ghost	
	%	%
Neurofilament proteins (60,000; 200,000; 500,000)	>95	54
Tubulin dimer (53,000; 57,000)	15	12
Actin (43,000)	27	5

Essentially none of the NF proteins are extracted in buffer P while most of the tubulin and actin is extracted. The NF proteins, tubulin, and actin comprise 70% of the protein in the axoplasmic ghost.

depolymerize, while those which have a less active exchange should remain polymerized for longer times, perhaps remaining insoluble.

Here we define "stable polymers" as those polymers which remain polymerized at low subunit concentrations. Those polymers which disassemble under the same conditions are called "soluble polymers." Morphologically, the stable polymers correspond to the insoluble structures of extracted axoplasm (Fig. 3*b*) while the soluble polymers are lost from axoplasm during extraction (Figs. 3*a* and *b*).

Stable Cytoskeleton of the Axon

Axoplasm which has been extruded into buffer P remains cylindrical for long periods of time (Fig. 2). Electron microscopy demonstrates that the major ultrastructural component of the stable cylinder is an extensive NF network (Fig. 3*b*). Complementing the morphological evidence, our biochemical data indicate that only a few percent of the NF protein in the axon is soluble in buffer P (Fig. 4 and Table II). Although it remains possible to argue that the NF are stable in buffer P but are not similarly stable in the axon, this is unlikely given the low solubility of NF and other IF in physiologic buffers (7, 16, 42, 44). Also, the long incubation time (24 h in buffer P) is sufficient for NF protofilaments to diffuse out of a cylinder with the size and porosity of extruded axoplasm (54; Fig. 3; and unpublished calculations). Instead, we conclude that little NF protein is normally dissociated from the NF network inside the axon. Thus, NF can be considered members of the set of naturally stable polymers.

NF Are Naturally Stable Polymers in the Axon

The idea that NF form naturally stable polymers is consistent with the structure and the properties of the NF protein subunits. NF are members of the class of stable fibrillar polymers that also includes tonofilaments and keratin (21). As a rule, such polymers have subunits with a high degree of alpha-helical secondary structure, and they tend to be rod shaped (16, 43, 45). The NF subunits associate via hydrogen bonds and hydrophobic interactions (54). During NF polymerization in vitro, the rod-shaped NF subunits associate laterally to form fibrous protofilaments which then wrap around each other to form the NF (21, 54, 55). It is likely that the many interactions between the NF subunits in this type of polymer contribute substantially to the natural stability of the NF inside nerve cells. Furthermore, axonal transport experiments in vertebrates show that the NF proteins move together, suggesting that these proteins remain polymerized during the transport process (18). Thus, both in vivo and in vitro analyses of axonal NF organi-

zation support the idea that NF are naturally stable polymers in axoplasm. We hypothesize that this natural stability is a general property of axonal NF networks because the stability of NF has been demonstrated in the axons of molluscs and vertebrates.

A Fraction of the Tubulin and Actin Is Stably Polymerized in Axoplasm

SDS PAGE reveals that 15% of the tubulin and 27% of the actin are not extracted from axoplasm by buffer P (Table II). The insoluble tubulin can be accounted for by a set of stable MT which we have discovered in electron microscope analyses of the axoplasmic ghost (Fig. 3*c*). The stable MT which we find in the axoplasmic ghost differ from most MT examined in vitro because the stable MT do not depolymerize at low subunit concentrations (32). MT which are stable at low subunit concentrations have also been observed near the nucleus in detergent-extracted 3T3 cells and in demembrated cilia (13, 37, 49).

On the other hand, electron microscope analysis has not yet demonstrated a clear ultrastructural correlate for the actin that is part of the axoplasmic ghost. This is not surprising because there is only a small amount of stable actin (5% of the ghost protein) and because actin as MF consists of typically short, thin polymers which are difficult to identify without a specific stain (9, 26, 29). However, insoluble actin MF have been identified in the subaxolemmal network of the squid giant axon and the stable actin in the axoplasmic ghost may be in a similar set of stable MF (29).

The insoluble tubulin and actin are candidates for sites at which the soluble forms of these proteins could polymerize in vivo. In this capacity, the insoluble tubulin and actin could function as stable nucleation sites for MT or MF formation inside the axon. Previously, all cytoskeletal nucleation sites have been found in the cell body, near the nucleus (40, 41). Nucleation sites in the cell body, being far from most of the axon, probably have only indirect effects on the organization of the axonal cytoskeleton. It is possible that cytoskeletal organization is locally controlled from nucleation sites which are woven into the axonal cytoskeleton. This idea is consistent with recent studies showing that, although axonal MT can be macroscopic in length, they are discontinuous (5, 8). Cytoskeletal organizing sites which are distributed through the axon could locally maintain the morphology of the cytoskeleton (23).

Stable Polymers Are Involved in the Stabilization of Neuronal Circuitry

Stable polymers, like the NF and the stable MT, could act to conserve cytoskeletal organization because they tend to remain polymerized. Thus, stable polymers may be important intracellular factors that constrain the capacity of the neuron to change morphology. This capacity is often referred to as neuronal plasticity. Soluble polymers, like the soluble MT, may increase neuronal plasticity because their subunit equilibria permit rapid and reversible changes in cytoskeletal organization. The idea that the stability of the cytoskeleton regulates neuronal plasticity is compatible with developmental studies of mammalian neurons. During development, neurons exhibit significant plasticity until they assume their final morphologies which are relatively constant for the adult life of the organism. Studies have shown that the NF:MT ratio increases in axons

and dendrites as neurons assume their mature shapes (27, 35, 38, 39). Smith has proposed that the continued addition of NF to the neuronal cytoskeleton contributes to the natural loss of neuronal plasticity which occurs during development (39). Our study supports this hypothesis by providing evidence that NF are stable polymers under physiologic conditions.

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