

# Axonal Tubulin and Axonal Microtubules: Biochemical Evidence for Cold Stability

SCOTT T. BRADY, MICHAEL TYTELL, and RAYMOND J. LASEK

*Department of Developmental Genetics and Anatomy, Case Western Reserve University, Cleveland, Ohio 44106. Dr. Tytell's present address is Department of Anatomy, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27109.*

**ABSTRACT** Nerve extracts containing tubulin labeled by axonal transport were analyzed by electrophoresis and differential extraction. We found that a substantial fraction of the tubulin in the axons of the retinal ganglion cell of guinea pigs is not solubilized by conventional methods for preparation of microtubules from whole brain. In two-dimensional polyacrylamide gel electrophoresis this cold-insoluble tubulin was biochemically distinct from tubulin obtained from whole brain microtubules prepared by cold cycling. Cleveland peptide maps also indicated some differences between the cold-extractable and cold-insoluble tubulins. The demonstration of cold-insoluble tubulin that is specifically axonal in origin permits consideration of the physiological role of cold-insoluble tubulin in a specific cellular structure. It appears likely that much of this material is in the form of cold-stable microtubules. We propose that the physiological role of cold-insoluble tubulin in the axon may be associated with the regulation of the axonal microtubule complexes in neurons.

Much of our knowledge about the properties of tubulin and microtubules is based on studies of that fraction of microtubule protein solubilized in low temperature homogenates of whole brain. Such studies treat the brain as a homogeneous tissue even though the central nervous system is cytologically complex. Not only is the brain composed of many different cell types containing microtubules, but ultrastructural studies indicate that different regions within the same cell may have differently organized cytoskeletal elements (39). Standard procedures for preparation of tubulin do not solubilize a substantial fraction of the tubulin in whole brain (48), but little has been known about the function or cellular source of either cold-extractable or cold-insoluble tubulin in vertebrate brain. It is clear, however, that microtubules are not all equivalent. They may differ with respect to ultrastructural details, resistance to destabilizing conditions, cellular distribution, and associated proteins (for reviews see references 16 and 20). Tubulins are a multigene family of closely related polypeptides in which the expression of a specific gene is carefully regulated according to cell type and stage of development (20, 27, 32). Tubulin is also subject to several types of posttranslational modification (for examples see references 16, 20, 27, 32, 41). The range and significance of this heterogeneity has only begun to be investigated in detail. A better understanding of tubulin heterogeneity is important for understanding the

physiological roles microtubules play in cells of the nervous system.

The ability to selectively label axonal proteins by axonal transport is the basis of a powerful method for the study of cytoskeletal elements in a defined subcellular region, the axon, of a defined cell type, the neuron (9). Use of this paradigm has already provided the initial identification of the subunit proteins of neurofilaments (23) as well as insights into the organization and associations of several other neuronal proteins (4, 8, 10). Analyses of the axonal transport of microtubule-associated proteins (MAPs)<sup>1</sup> showed that axonal microtubules of the retinal ganglion cells are a distinct population of brain microtubules which contain tau proteins, but not high molecular weight MAPs (47). In the course of these studies, we noted that a substantial fraction of the axonal tubulin is not solubilized by the conventional methods for preparing tubulin from whole brain. The bulk of the axonal tubulin continues to pellet during high speed centrifugation

<sup>1</sup> *Abbreviations used in this paper:* IEF, isoelectric focusing; MAP, microtubule-associated protein; MTOC, microtubule-organizing center; MTG buffer, 100 mM 2-(*N*-morpholino)ethane sulfonic acid, pH 6.4, 1 mM EGTA, 1 mM GTP, and 0.5 mM MgCl<sub>2</sub>; S<sub>Ca</sub>, slow component a; 2D PAGE, two-dimensional polyacrylamide gel electrophoresis.

after homogenization and incubation in the cold. Initial characterization of the cold-insoluble fraction of axonal tubulin has begun to reveal distinctive properties of this fraction. Differential extraction in the cold and with  $\text{Ca}^{2+}$ -containing buffers suggests that axons contain at least two biochemically distinct pools of tubulin, which differ in both their solubility and subunit composition.

## MATERIALS AND METHODS

Axonal tubulin and associated proteins were labeled in the retinal ganglion cell axons of adult guinea pigs (Hartley strain, 200–300 g) (for a few experiments adult male Sprague-Dawley rats [Zivic-Miller, 150–250 g] were employed for comparison with the guinea pigs) by injection of 500  $\mu\text{Ci}$  of [ $^{35}\text{S}$ ]methionine (New England Nuclear, Boston, MA) into the vitreous of the eye. Before injection, the labeled methionine had been concentrated down by lyophilization in a vacuum centrifuge and resuspension in 10  $\mu\text{l}$  of glass-distilled water. 40–60 d after injection, the animal was killed and the optic nerves and tracts of injected eyes were dissected. At this injection/sacrifice interval the only axonal proteins labeled are those moving as part of the Slow Component a (SCa) complex of proteins. In the optic system, this includes essentially all of the labeled tubulin and neurofilament proteins that enter the axon. The nerve and tract were separated and each was homogenized in 500  $\mu\text{l}$  of MTG buffer (100 mM 2-(*N*-morpholino)ethane sulfonic acid, pH 6.4, 1 mM EGTA, 1 mM GTP, and 0.5 mM  $\text{MgCl}_2$ ) in glass on glass microhomogenizers at 0–4°C. The homogenate was incubated 30 min on ice and then centrifuged for 30 min at 130,000 *g* at 2°C (45,000 rpm in a Beckman Ty 65 rotor using a Beckman L2-65B ultracentrifuge; Beckman Instruments, Inc., Palo Alto, CA). The supernatant (S1), containing the cold-soluble or -extractable polypeptides of the nerve, was retained and precipitated by being brought to 10% trichloroacetic acid, incubated at 4°C for 1–2 h, and centrifuged at 15,000 rpm in a Sorvall RC2B SS-34 rotor (4°C E. I. DuPont de Nemours & Co., Newtown, CT). The trichloroacetic acid pellet was washed in 100% ethanol and resuspended in BUST (2% 2-mercaptoethanol, 8 M urea, 1% SDS, 0.2 M Tris, pH 6.8, and 0.02 mg/ml phenol red) and incubated overnight at room temperature to complete solubilization.

The high speed pellet (P1) was resuspended in 500  $\mu\text{l}$  CMTG buffer (MTG buffer in which the 1 mM EGTA was replaced with 5 mM  $\text{CaCl}_2$ ) and extracted for 30 min at room temperature. The calcium-extracted material was again centrifuged at 130,000 *g* as above, but at 25°C. The supernatant (S2) contained the cold-insoluble and  $\text{Ca}^{2+}$ -extractable material and was processed as described above for S1. The second high speed pellet (P2), which represents the cold- and  $\text{Ca}^{2+}$ -insoluble fraction of the nerve, was resuspended in SUB (0.5% SDS, 8 M urea, and 2% 2-mercaptoethanol) and solubilized by overnight incubation at room temperature. Three fractions were generated: the first supernatant, S1, which contains the cold-extractable material of the nerve; the second supernatant, S2, which is the cold-insoluble,  $\text{Ca}^{2+}$ -extractable material, and the pellet, P2, which corresponds to the cold- and  $\text{Ca}^{2+}$ -insoluble material.

All fractions were analyzed by one-dimensional SDS polyacrylamide gel electrophoresis (SDS PAGE) modified slightly from the method of Laemmli (29). Most were also examined by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) using a procedure modified slightly from O'Farrell (37). For 2D PAGE analysis, the SDS-containing samples were diluted in 1–2 vol of lysis buffer (8% Triton X-100, 1.6% pH 5–7 Pharmalytes, 0.4% pH 3–10 Pharmalytes, 5% 2-mercaptoethanol, and 9 M urea) and focused for 6,000–6,500 V-h. Both SDS PAGE and 2D PAGE second-dimension gels were 6–20% acrylamide slab gels with 4% acrylamide stacking gels. Proteins were visualized by staining with 0.7 g/liter Serva Blue R in 35% methanol and 7% acetic acid followed by destaining in 35% methanol and 7% acetic acid. Radioactive polypeptides were localized in gels by fluorography (6). Radioactivity associated with a band in SDS PAGE gels was quantitated by cutting out the appropriate region of the gel, solubilizing the gel with 0.75 ml 30% hydrogen peroxide, and counting in Beckman LS 335 scintillation counter using Formula 963 scintillant (New England Nuclear). Peptide maps were made by the method of Cleveland et al. (14) using the V8 protease from *Staphylococcus aureus* (Boehringer Mannheim Biochemicals, Indianapolis, IN). Samples were run on SDS PAGE as described above and the region of the gel containing the appropriate molecular weight polypeptides was excised. Polypeptides were digested and analyzed on 10–20% acrylamide slab gels with 4% acrylamide stacking gels and peptide fragments visualized by fluorography.

## RESULTS

Injection of [ $^{35}\text{S}$ ]methionine into the vitreous of the guinea pig eye 40–60 d before dissection of the optic nerve specifically labels a small subset of the total complement of protein in

the axons of the retinal ganglion cells (Fig. 1). The labeled proteins moving through the optic nerve and tract at this injection/sacrifice interval have been termed Slow Component a (SCa) (31). In the optic nerve, 75–80% of the radioactivity associated with SCa is distributed among five major cytoskeletal proteins: the three neurofilament subunit proteins and the two tubulins (5, 31). Thus, axonal transport may be used to specifically label the cytoskeletal elements of the axon (9, 10). This permits biochemical characterization of the labeled cytoskeleton and associated proteins which are restricted to the axon without extensive purification procedures (4, 8–10, 47).

During studies on the MAPs of retinal ganglion cell axons (47), guinea pig optic nerve and tract containing labeled SCa proteins were extracted at 0–4°C according to a standard procedure for preparation of microtubules from whole brain (43). Cold extraction under these conditions did not solubilize the bulk of the radioactivity in the nerve. Most of the radioactivity in this cold-insoluble fraction of SCa-labeled optic nerve co-migrated with tubulin in one-dimensional SDS PAGE as seen in Fig. 2. This raised the possibility that axonal tubulin was a major source of cold-insoluble tubulin in vertebrate brain. A series of experiments were designed to begin characterization of the cold-insoluble fraction of axonal tubulin.

A differential extraction procedure was devised based on standard methods for preparation of microtubules from whole brain. Three fractions are generated: the first supernatant, S1, which contains the cold-extractable material of the nerve; the second supernatant, S2, which is the cold-insoluble,  $\text{Ca}^{2+}$ -extractable material, and the pellet, P2, which corresponds to the cold- and  $\text{Ca}^{2+}$ -insoluble material. Fig. 2 shows the labeled polypeptide composition for each of these fractions as resolved in SDS PAGE. Polypeptides of molecular weight 57,000 and 53,000 are the major labeled species in all three fractions.

Peptide maps of the 57,000- and 53,000-mol-wt proteins were made using the method of Cleveland et al. (14). For peptides from guinea pig SCa-labeled optic nerve, the major polypeptide at these molecular weights for all three fractions was homologous to alpha-tubulin and beta-tubulin, respectively. No significant differences were detected between the staining pattern obtained by digestion of the appropriate subunit of tubulin from cold-cycled microtubules and the labeled pattern for any of the three fractions from guinea pig optic nerve (7). However, when SCa-labeled tubulin from rat optic system was used as the source material, small differences could be detected between peptide maps of alpha-tubulin from S1 and P2 (Fig. 3). The fraction of tubulin in the S1, S2, and P2 fractions is similar for rat and guinea pig. Peptide maps for alpha- and beta-tubulin for all three fractions as well as actin from the S1 and P2 fractions were compared. In rat and guinea pig, for both alpha- and beta-tubulin, the overall pattern of [ $^{35}\text{S}$ ]methionine-containing peptide fragments is similar for S1, S2, and P2 polypeptides. Filled stars indicate major bands or groups of bands that can be detected in all three patterns for a given molecular weight protein. Open stars indicate a band in the alpha pattern that may be reduced in intensity in the P2 fraction and the circle with inset star indicate bands that are new or increased in intensity in the P2 fraction. No significant differences were found between the peptide maps for actin from S1 and P2 indicating that the differences are not due to processing. Differences were reproducible in three different digestions, suggesting a small but

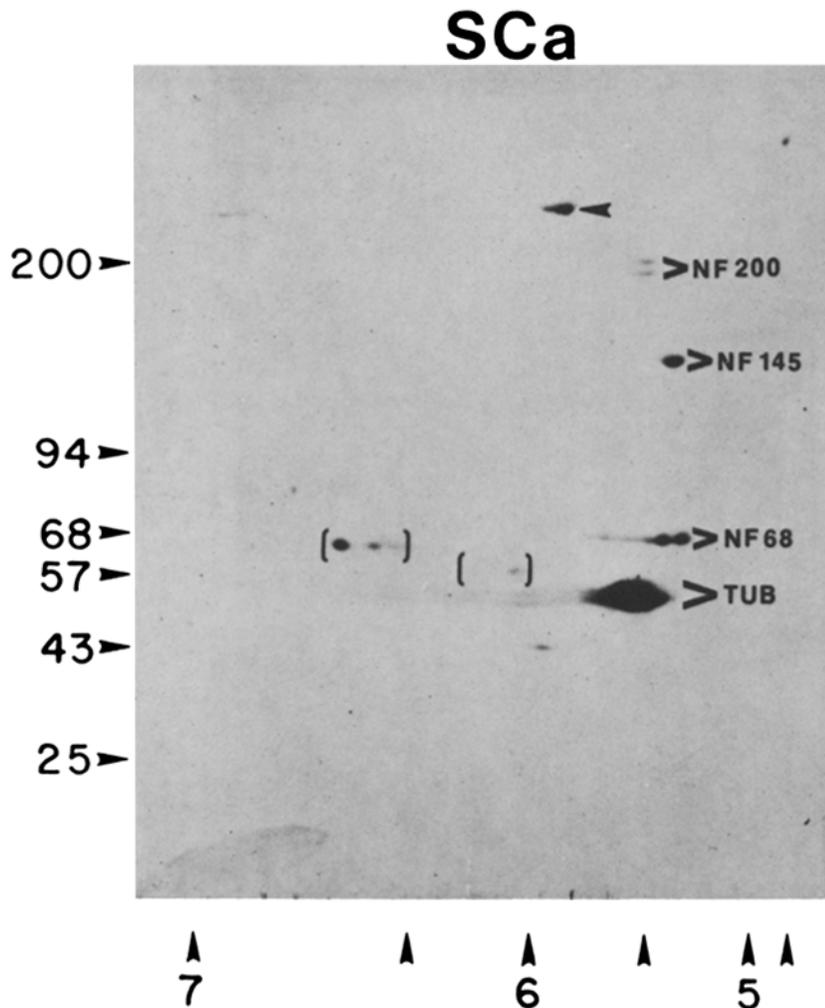


FIGURE 1 Protein composition of SCa. A fluorograph of labeled proteins moving as part of SCa in the guinea pig optic nerve analyzed by two-dimensional gel electrophoresis. The proteins were labeled by injection of [ $^{35}$ S]methionine into the vitreous of the eye and killing the animal 40–60 d after injection. At this injection/sacrifice interval only SCa proteins are labeled in the optic nerve. 75–80% of the total radioactivity is associated with tubulin (*TUB*) or the neurofilament proteins (*NF68*, *NF145*, and *NF200*). Minor constituents of SCa include brain spectrin (arrowhead) and the putative tau proteins (enclosed in brackets).

real difference between tubulins associated with the cold-insoluble and cold-extractable fractions in the rat. The lack of detectable differences in guinea pig tubulin may reflect limitations in the resolution of Cleveland peptide mapping methods. Further information about these differences will require two-dimensional peptide mapping analysis. Homologies between alpha-tubulin from cold-extractable and cold-insoluble fractions and between beta-tubulin from cold-extractable and cold-insoluble fractions do indicate that the major labeled 57,000- and 53,000-mol-wt proteins in each fraction correspond to alpha- and beta-tubulin.

The appropriate regions of the gel were cut out after fluorography and the amount of radioactivity associated with the major polypeptides in each fraction was quantitated in a liquid scintillation counter. Table I shows the percent of recovered counts associated with the tubulin regions of gels for the cold-extractable (S1), cold-insoluble and  $\text{Ca}^{2+}$ -extractable (S2), and cold and  $\text{Ca}^{2+}$ -insoluble (P2) fractions. For both alpha- and beta-tubulin almost 60% of the labeled protein is cold-insoluble and nearly 50% continues to pellet after a further extraction with 5 mM  $\text{Ca}^{2+}$  buffers. Either of these treatments would be sufficient to completely solubilize tubulin in the form of microtubules prepared by cycling from whole brain homogenates (16, 28). Under these conditions, >90% of the radioactivity associated with neurofilament proteins is present in the P2 fraction.

2D PAGE was used to analyze these fractions in greater

detail. Fig. 4 shows the fluorographs obtained from 2D PAGE analysis of the cold-extractable (S1) and cold- and  $\text{Ca}^{2+}$ -insoluble (P2) fractions of SCa-labeled nerve. These are 2D PAGE gels of the same samples analyzed by SDS PAGE in Fig. 2. Several differences in the composition of the S1 and P2 fractions of SCa can be seen. As expected, effectively all of the radioactivity associated with neurofilament triplet proteins is found in P2 (arrows on gel P2 of Fig. 4), because neurofilaments are known to be insoluble in physiological buffers (36, 49). Other proteins partition differently. One of the tau proteins that has recently been identified as a major MAP of the retinal ganglion cell axon (47) preferentially associates with the cold-extractable (S1) fraction of SCa, while the other tau appears to be present in all three fractions to some extent (Figs. 2 and 4).

When the regions corresponding to tubulin from two-dimensional gels of S1 and P2 are compared, a striking and unexpected difference is seen. Labeled polypeptides co-migrating with alpha-tubulin can be seen in the cold-extractable fraction (*a* on gel S1, Fig. 4), but the corresponding region of the gel in the cold-insoluble fraction (*a* on gel P2, Fig. 4) is only minimally labeled. The beta-tubulin regions of the cold-extractable and the cold-insoluble fractions (*b* on both gels) appears similar for both fractions. A major labeled polypeptide with the molecular weight of alpha-tubulin is present in SDS PAGE analysis of the P2 cold-insoluble material (Fig. 2), but it is apparently lost during 2D PAGE (Fig. 5). Analysis

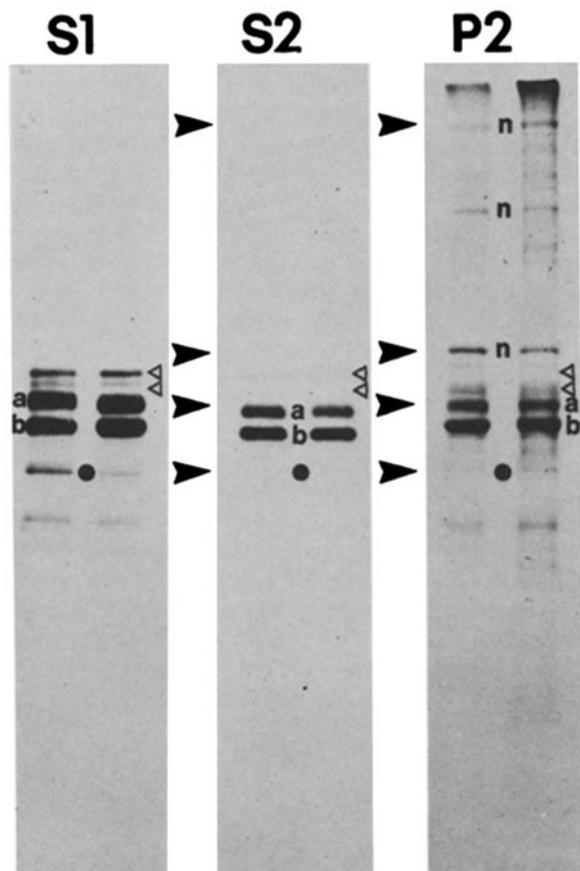


FIGURE 2 Fractionation of SCa proteins. Optic nerve with labeled SCa proteins was homogenized in MTG buffer at 4°C and centrifuged as described in the text to obtain the cold-extractable fraction (S1). The pellet from this step was rehomogenized at room temperature in CMTG buffer with 5 mM CaCl<sub>2</sub> and recentrifuged to obtain the cold-insoluble and Ca<sup>2+</sup>-extractable fraction (S2) and the cold- and Ca<sup>2+</sup>-insoluble fraction (P2). For each fraction, the left-hand column is material from the optic nerve and the right-hand column is material from the optic tract. Note that the major proteins in each fraction are alpha- (a) and beta- (b) tubulin. All detectable neurofilament protein (n) is in the P2 fractions. Other proteins also partition preferentially with one fraction. Actin (filled dot) and the higher molecular set of tau proteins (open triangles) are predominantly associated with the S1 fraction. Molecular weight markers (arrowheads) correspond to 43,000, 57,000, 68,000, and 200,000.

by 2D PAGE of the SCa-labeled S2 fraction indicates that the tubulin in S2 generally behaves like the tubulin of P2 (data not shown). This similarity serves as an internal control for the possibility that changes in the behavior of the alpha-tubulin in 2D PAGE is generated by processing of the samples. The S1 and S2 fractions are handled identically, while P2 is subject to less harsh treatments (no precipitation with trichloroacetic acid and lower SDS concentrations). It is apparent that the tubulin associated with these two cold-insoluble fractions differs from the cold-extractable tubulin not only in solubility properties, but also in behavior during 2D PAGE analysis. The simplest explanation is that the alpha-tubulin of the cold-insoluble P2 fraction of SCa is biochemically distinct from the alpha-tubulin of the cold-extractable S1 fraction of SCa and from the alpha-tubulin of cycled microtubules from whole brain.

The anomalous behavior of the cold-insoluble alpha-tubulin in 2D PAGE raised questions about how this protein

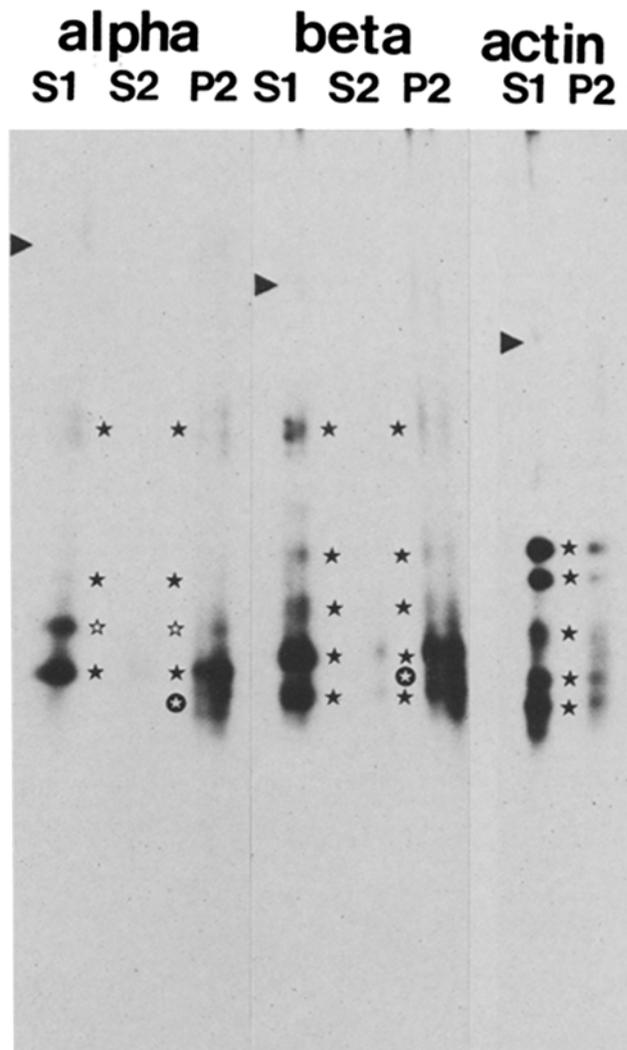


FIGURE 3 Peptide maps of alpha- and beta-tubulin and of actin. SCa proteins were labeled in rat visual system with [<sup>35</sup>S]methionine as a precursor and fractionated as for Fig. 2. Polypeptides with molecular weights corresponding to alpha- (57,000) and beta- (53,000) tubulin from the S1, S2, and P2 fractions and to actin (43,000) from the S1 and P2 fractions were excised from SDS PAGE slab gels and subjected to limited proteolysis with *S. aureus* V8 protease according to the Cleveland method. Comparison of the major peptide fragments indicate that alpha- and beta-tubulin are different from each other and different from actin. Comparison of patterns obtained from the same molecular weight polypeptide in different fractions, however, indicate that significant homology exists between proteins with the same molecular weight in different fractions. Bands common to all three fractions (S1, S2, and P2) are indicated by a filled star. The presence of multiple common bands between the fractions is evidence of homology for the major polypeptides of that molecular weight for all three fractions. Open stars indicate positions of a band in the alpha pattern that is present at reduced levels in the P2 fraction and circles with an inset star indicate bands in the P2 alpha or beta patterns that are not present in corresponding S1 pattern. When guinea pig SCa tubulin was analyzed in the same manner, no differences could be detected between the S1 and P2 patterns for either alpha- or beta-tubulin (data not shown). The minor differences in the rat patterns raise the possibility that different gene products are enriched in the cold-insoluble tubulin fraction, but are consistent with the identification of the major 57,000- and 53,000-mol-wt polypeptides in SCa as alpha- and beta-tubulin, respectively. The triangles indicate the position at which undigested proteins would be expected to be found.

differs from the more conventional cold-extractable alpha-tubulin. Because the P2 alpha-tubulin has a molecular weight in SDS PAGE similar to that of the S1 alpha-tubulin (Fig. 2) and was homologous in the peptide mapping studies, it seemed likely that the change was due to differences in the native charge of the molecule. To test this hypothesis, we

TABLE I  
Percent of Axonal Tubulin Extracted by Cold and  $Ca^{2+}$

	S1 (cold ex- tracted)	S2 ( $Ca^{2+}$ ex- tracted)	P2 (cold and $Ca^{2+}$ insoluble)
57,000	41.88 ± 4.45	9.83 ± 1.17	48.29 ± 4.83
53,000	41.32 ± 4.45	9.40 ± 1.143	49.27 ± 3.94

Tubulin of the optic nerve and tract of guinea pigs was labeled with [ $^{35}S$ ]-methionine by axonal transport as described in Materials and Methods. At 40–60-d injection/sacrifice intervals, the nerves were dissected out and processed as described in the text. Fractions were analyzed in SDS PAGE and the location of radioactively labeled polypeptides was determined by fluorography. The regions of the gels corresponding to proteins of 57,000 mol wt (includes alpha-tubulin) and 53,000 mol wt (includes beta-tubulin) were excised from the gels and solubilized with 30% hydrogen peroxide. 2D PAGE and peptide maps following limited proteolysis indicate that only tubulin was labeled in these molecular weight ranges at these times. The number of counts at these molecular weights were determined for all three fractions in each nerve and the total was termed the recovered radioactivity associated with that molecular weight for that nerve. The amount of material present in each fraction was then expressed as the percent of recovered counts at that molecular weight. S1 represents the fraction of alpha- or beta-tubulin that was extracted by incubation in cold MTG buffer, S2 represents the amount of material that was extracted by resuspension of the cold-insoluble fraction in 5 mM  $Ca^{2+}$ -containing buffer (CMTG), and P2 is the fraction of material that was both cold and  $Ca^{2+}$  insoluble. All values are expressed as percent recovered counts ± SEM,  $n = 10$ .

evaluated the behavior of Sca proteins during the course of the isoelectric focusing (IEF) step of 2D PAGE. A series of tube gels for IEF was set up according to the procedures normally used in the first dimension of 2D PAGE (pH 5–7, 4% acrylamide) and equal aliquots of 58-d [ $^{35}S$ ]-methionine-labeled Sca were loaded onto each gel. After 2, 4, 8, and 12 h of focusing at 500 V (constant voltage), gels were removed and run in the second dimension of 2D PAGE conventionally. Fig. 5 shows the series of fluorographs that were produced by these gels. At 2 h (1,000 V-h), all of the labeled proteins have entered the IEF gel, but are still near the top of the gel. Bands corresponding in molecular weight to both alpha- and beta-tubulin can be seen at comparable positions in the IEF gel step. By 4 h (2,000 V-h), the beta-tubulin and many of the other labeled proteins have migrated more than half the length of the IEF gel, well on the way to positions at which they normally focus in these gels. However, the bulk of the labeled material with the molecular weight of alpha-tubulin remains near the top of the gel which represents the more basic region of the pH gradient. At 8 and 12 h (4,000 and 6,000 V-h), the beta-tubulin and other proteins of Sca continue to focus and form the patterns seen in 2D PAGE analyses of Sca, but the alpha-tubulin has apparently been lost from the IEF gel, presumably by migration into the upper reservoir buffer. This could be due to the tendency of the pH gradient on the basic end of the IEF gel to be unstable, resulting in pH at the top of the gel lower than expected if the gradient remained linear (13). It is important to note that both alpha- and beta-tubulin enter the IEF gel during the initial phase of IEF and it is only

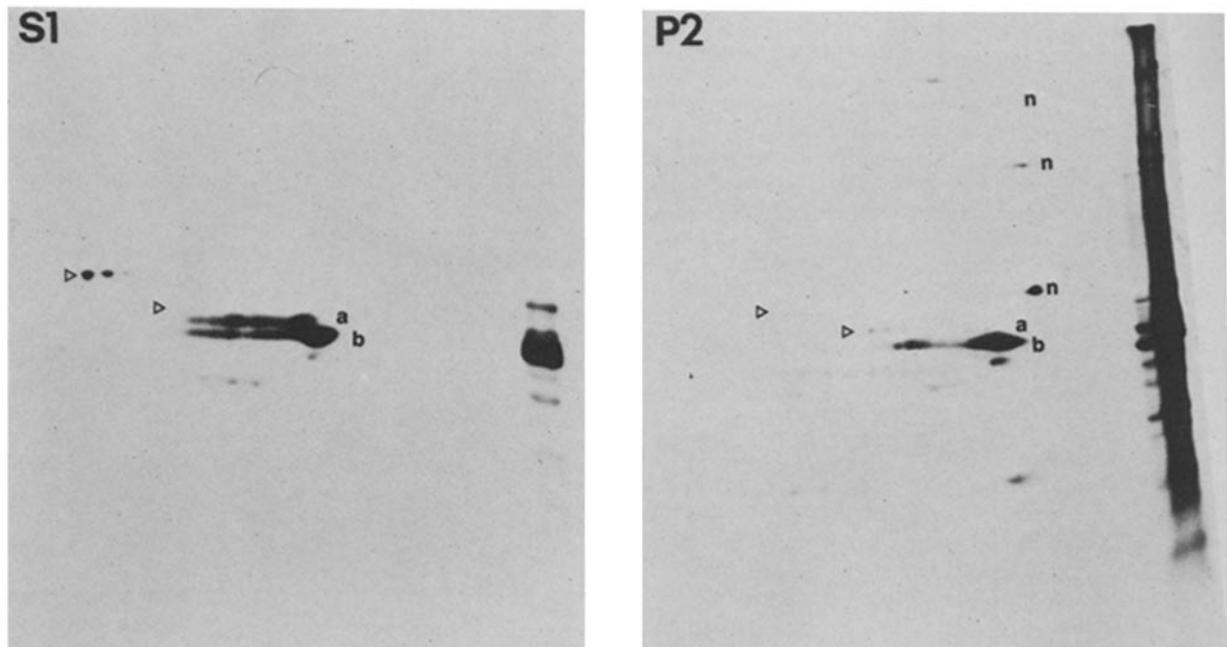


FIGURE 4 Two-dimensional gel analysis of cold-extractable and cold- and  $Ca^{2+}$ -insoluble fractions of Sca-labeled optic nerve. The S1 and P2 fractions from above were analyzed by two-dimensional gel electrophoresis. Note again that the higher molecular weight set of tau MAPs (upper arrowhead on both fluorographs) is almost entirely in the S1 fraction, while the other is predominantly in the P2 fraction. The neurofilament proteins ( $n$ ) are found only in P2. The region of the gel associated with tubulin exhibits the most striking difference between the S1 and P2 fractions. The S1 tubulin pattern is very similar to the Coomassie Brilliant Blue-stained pattern seen in the same gel with carrier cold-cycled tubulin prepared from whole brain. Both alpha- and beta-tubulin spots are prominent. However, in the P2 fraction, little labeling is found in the region of the gel that normally corresponds to beta-tubulin (a) while the beta-tubulin spot (b) is comparable to that seen in S1. The stained pattern produced by carrier tubulin in the same gels are indistinguishable from the S1 pattern and labeled alpha-tubulin is clearly present in the one-dimensional SDS PAGE analysis (see track at right of P2 two-dimensional gel and Fig. 2). Presumably the radioactivity associated with alpha-tubulin either fails to focus or is lost from the gel.

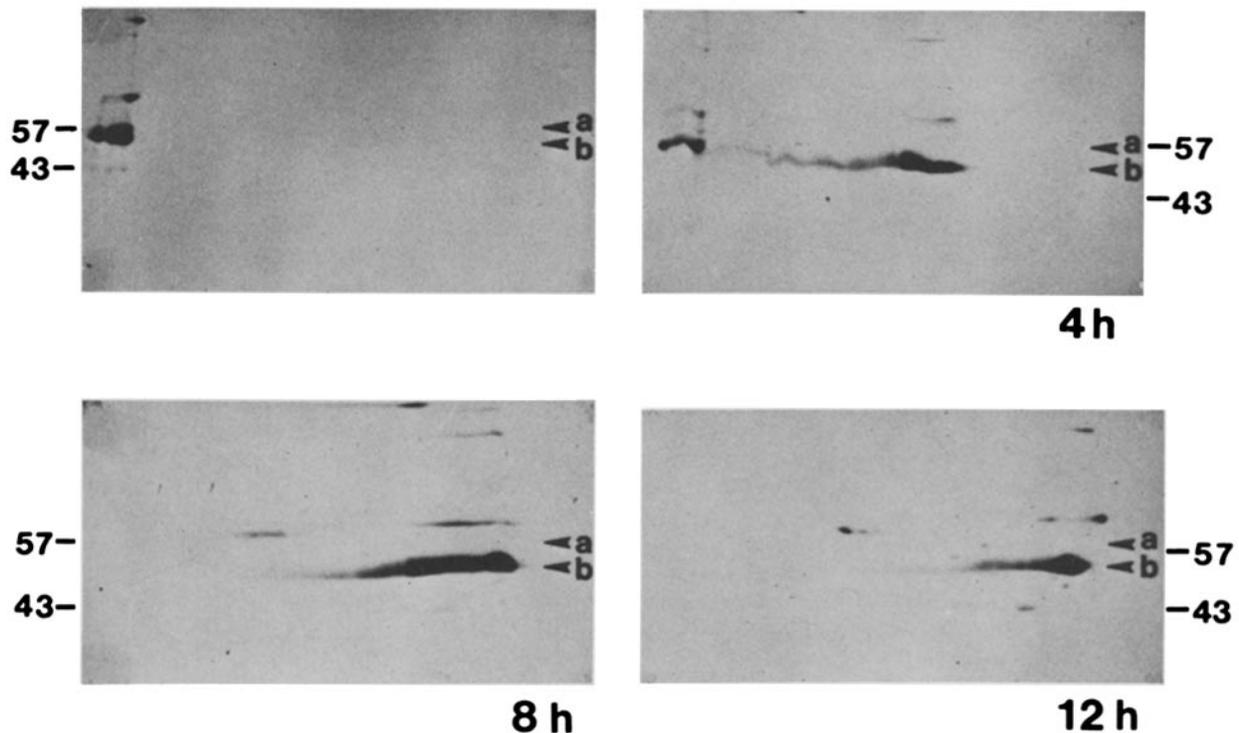


FIGURE 5 Time course IEF in two-dimensional gel electrophoresis. In this experiment, whole nerve was homogenized directly into SUB (0.5% SDS, 8 M urea, and 2% 2-mercaptoethanol) and then prepared for 2D PAGE. Identical samples were loaded into eight identical tubes and run at 500 V constant voltage. At the stated intervals two tubes were removed and run in the second dimension by the standard procedures. These fluorographs illustrate the behavior of the labeled proteins during the course of IEF. At 2 h, there are two bands (a and b) in the tubulin molecular weight range that have just entered the IEF gel. At 4 h, the lower of these two bands, corresponding to beta-tubulin, has migrated further into the IEF gel and is approaching the appropriate isoelectric point as are the neurofilament proteins. The band with the molecular weight corresponding to alpha-tubulin has not moved from the position it occupied at 2 h. At both 2 and 4 h of focusing, the alpha-tubulin has entered the IEF gel and migrates conventionally in SDS direction on these 2D PAGE gels. At 8 and 12 h, the beta-tubulin and other Sca proteins proceed to their isoelectric points and focus normally, but the band corresponding to alpha-tubulin appears to be lost from the gel. The final pattern after 6,000 V-h (12 h) is comparable to that seen in Fig. 1 and in Fig. 3 P2. A longer exposure for fluorography indicates that some labeled material does focus at the position of alpha-tubulin, but it represents only a fraction of the amount expected from one-dimensional PAGE analysis. For further discussion, see text.

after prolonged focusing that a substantial fraction of the alpha-tubulin appears to be lost from the gel. The alpha-tubulin is not prevented from entering the IEF gel because of some aggregation into a complex too large to pass through the pores of the gel. Fluorographs from longer exposures of these gels shows that a fraction of the alpha-tubulin in this unfractionated Sca does focus in the conventional position of alpha-tubulin, but most is lost from the gels. The biochemical change in the alpha-tubulin that causes this anomalous behavior in 2D PAGE is not yet known.

## DISCUSSION

### *Cold-insoluble Tubulin*

Current understanding of tubulin and microtubules is in large part the result of studies on microtubules prepared from whole brain homogenates by cold extraction and purified by alternating cycles of warm and cold incubations (16). These preparations contain a number of different isoforms of tubulin, but are derived from many different cell types and cell structures. The existence of cold-insoluble tubulin has been recognized (17, 38, 48), but not well characterized. Little information has been available about its biochemistry, cellular localization, or physiological role in vertebrate brain. Margolis

and co-workers (24, 25, 33, 34) have looked at the acquisition of cold stability by the tubulin in extracts and have partially characterized a complex of proteins that may be involved in cold stability. However, their fractions do not include endogenous cold-insoluble tubulin. The use of axonal transport to label specifically axonal tubulin permits us to address for the first time the biological role and properties of cold-insoluble tubulin in the axon.

Most axonal tubulin is insoluble when nerves containing Sca-labeled axonal tubulin are subjected to extractions in the cold (Fig. 2 and Table I). Several lines of evidence suggest that this cold-insoluble tubulin is a discrete physiological pool of axonal tubulin rather than an artifact of handling. Some proteins are found predominantly in the cold-extractable fraction while others partition preferentially with the cold- and  $\text{Ca}^{2+}$ -insoluble fraction. For example, one of the tau proteins is found primarily in S1 (Figs. 2 and 4). Resuspension of the cold-insoluble material and a second cold extraction does not significantly increase the amount of tubulin solubilized (data not shown) unless millimolar levels of  $\text{Ca}^{2+}$  are added to the second extraction buffer (Table I). Addition of 1% Triton X-100 to the homogenization and extraction buffers does not increase the amount of cold-extractable tubulin and may even decrease it (7). It is, therefore, unlikely that the different

fractions of axonal tubulin result from incomplete extraction. Finally, the anomalous behavior of the alpha-tubulin in gel electrophoresis suggests that the basis of cold insolubility in axonal tubulin is related to biochemical properties of the tubulin. These observations demonstrate the existence of a pool of tubulin in axons that has not been included in studies on cold-extractable tubulin from whole brain.

The studies of Black et al. (2) on the effects of various destabilizing treatments on microtubules in neurite-bearing cells further support this conclusion. Using detergent extractions of rat sympathetic neurons in primary culture, they have found comparable fractions of tubulin, although the relative amount of tubulin in each fraction differs. 23–35% of the neuronal tubulin is extractable under stabilizing conditions, 60–70% more is solubilized by destabilizing conditions, and 5–7% of the tubulin was cold- and  $\text{Ca}^{2+}$ -insoluble (analogous to P2). Electron microscopy of the cold and  $\text{Ca}^{2+}$ -insoluble material demonstrated that some microtubules were still present after extraction, but the location in the neuron could not be identified because the plasmalemma and other landmarks were removed by detergent extraction. The fraction of tubulin that is extractable in cultured neurons and SCa-labeled optic axons is remarkably similar given the differences in the two preparations (adult sensory nerve axons versus detergent-treated whole embryonic sympathetic neurons). The difference in amount of tubulin that is both cold and  $\text{Ca}^{2+}$  insoluble may be significant. For example, growing neurons and mature neurons could differ in the relative stability of their cytoskeletal elements.

Axonally transported tubulin in rat optic nerve, ventral motor neurons, and dorsal root ganglion cells and tubulin in squid axoplasm (S. T. Brady, unpublished data) also contain a substantial fraction of cold-insoluble tubulin. Electrophoretic analyses suggest that the behavior of the alpha subunit of axonally transported tubulin in 2D PAGE may be variable in different species or nerves, but is clearly distinct from the behavior of alpha-tubulin from whole brain cold-cycled microtubules. Tashiro and Komiya (45) confirm the existence of an electrophoretic variant for alpha-tubulin specific to axonally transported tubulin in rat vagus nerve, but do not report on the solubility properties. Several conclusions about cold-insoluble tubulin in brain may be reached from these observations. First, cold-insoluble tubulin is a significant fraction of neuronal tubulin, which has not been well characterized in previous studies. Second, axons are a major source of cold-insoluble tubulin in mammalian brain. Finally, much of the tubulin obtained from cold-cycled microtubules from whole brain is derived from nonaxonal regions of the brain.

### *Biochemical Basis of Cold Insolubility*

The anomalous behavior of the cold-stable alpha-tubulin in 2D PAGE suggests that the difference in solubility properties for S1 and P2 tubulin results from a biochemical difference between the alpha-tubulins in these fractions. One possible explanation is that alpha-tubulin is known to be subject to posttranslational modifications of various types (16, 20, 32, 41). Alternatively, slight differences in the peptide maps for alpha-tubulin from the different fractions (Fig. 3) could reflect different gene products in different fractions. Other possibilities, such as interactions with other proteins like neurofilaments or MAPs, may also be important for conferring cold insolubility on the axonal tubulin. For example, electron microscopy has shown that neurofilaments

interact with microtubules by cross-bridges (39, 44); neurofilament triplet proteins and tubulin move coordinately during axonal transport (5, 23); and neurofilament protein has been shown to be a minor component of cycled microtubule protein in some preparations (1, 44). The cold-insoluble fraction might therefore represent tubulin that interacts with the neurofilaments of the axon. It is noteworthy that the relative number of neurofilaments in the axon increases during maturation (19), which could be related to the observed differences in the amount of cold- and  $\text{Ca}^{2+}$ -insoluble tubulin in mature nerve fibers and cultured embryonic neurons. The tau fraction from whole brain also affects the stability of microtubules (15). The modulator proteins of Margolis and his associates (24, 33, 34) include two phosphorylatable proteins similar in molecular weight to the two tau proteins of optic nerve. These tau proteins can be phosphorylated under conditions that also favor phosphorylation of the neurofilament triplet (G. Schekett and S. Brady, unpublished observations). Some or all of these factors may contribute to the altered solubility of axonal tubulin.

### *What Is the Morphological Form of Axonal Cold-insoluble Tubulin?*

Several possible morphological forms could be associated with the tubulin in S1, S2, and P2. At least a portion of the axonal tubulin associated with S1 would be expected to exist in the form of unpolymerized dimer. The cold-insoluble tubulin in S2 and P2 presumably exists either as part of a microtubule, in association with membranous structures, or in the form of an oligomer that is not recognizable as a microtubule. A membrane association is unlikely because Triton X-100 treatment does not solubilize cold-insoluble tubulin (7). The large number of microtubules in these axons requires that a significant fraction of the total axonal tubulin be assembled into microtubules. Biochemical studies cannot distinguish between cold-insoluble tubulin in axonal microtubules or in some other oligomeric form, but they do demonstrate that the cold-insoluble tubulin does not exchange freely with cold-extractable tubulin. Several lines of evidence indicate that some or all of this cold-insoluble tubulin is in the form of cold-stable microtubules.

Microtubules or segments of microtubules stable to cold, prolonged extraction, and pharmacological manipulation have been reported in the literature (2, 12, 26, 36). Using a combination of biochemical and morphological approaches, Morris and Lasek (36) find three pools of tubulin in axoplasm from the squid giant axon: tubulin dimers, microtubules in equilibrium with the dimeric tubulin, and microtubules that are not free to exchange with the dimeric tubulin. Black et al. (2) also find a number of cold-stable microtubules and a smaller fraction of microtubules that are both cold and  $\text{Ca}^{2+}$  stable. Homogenizations during preparation of the S1, S2, and P2 fractions of SCa tubulin make these fractions unsuitable for the ultrastructural determination of microtubules in axons of the optic nerve. To address the question of a morphological correlate for cold-insoluble tubulin in optic axons, Sahenk and Brady (42) used a modified procedure that does not include homogenization to show that cold-stable microtubules are present in the mammalian optic axons in substantial numbers. These cold-stable microtubule segments are also resistant to depolymerization by antimetabolic agents. It is likely, therefore, that some and perhaps all of the cold-insoluble

tubulin of the axon is in the form of stable microtubule segments.

### Biological Role of Axonal Cold-insoluble Tubulin

A substantial literature exists on the structure and physiology of the axon that can assist in understanding the role of cold-insoluble tubulin in the axon. Microtubules of the axon all seem to have the same polarity—the “+” end being the most distal (21)—and are organized into bundles (30, 39). They are discontinuous and do not extend the length of the axon (11, 46), but may be extremely long, 100  $\mu\text{m}$  or more (46). The coherent movement of tubulin in axonal transport (5), the co-transport of tubulin and MAPs (47), and the existence of cold-stable microtubules (42) indicate that some or all of the tubulin moving in axonal transport is in the form of microtubules. The existence of discontinuous, moving microtubules in the axon has several important implications for the properties of those microtubules. One of these concerns the regulation of microtubule structures in the axon.

Regulation of microtubule structures in other cells appears to be associated with a cellular structure termed a microtubule-organizing center (MTOC) (40). MTOCs are usually near the nucleus, often associated with centrosomes or basal bodies. MTOCs are important for regulating the size of the microtubules and shape of the cytoplasmic microtubule complex (12, 28, 40). Segments of microtubules in the vicinity of an MTOC tend to be more stable to destabilizing treatments such as cold (12). It is thought that one mode of action is to cap an end of the microtubule and consequently alter the equilibria for assembly and disassembly of the microtubule (28). Such anchoring of the microtubule may be the basis for regulating the length, the stability, and the organization of the cytoplasmic microtubule complex. Continuity between the microtubule and the MTOC is therefore essential for the microtubule to be regulated by the MTOC. This criterion is clearly not met by the discontinuous microtubules of the axon (11, 46). However, when axonal microtubules are depolymerized and allowed to reform, they do so in a proximal direction (18) and the resultant microtubules retain the original polarity (23). Some regulatory process apparently maintains the size, distribution, and polarity of the highly ordered microtubule complex of the axon.

What is the mechanism by which the highly ordered microtubules of the axon are regulated in the absence of interactions with defined MTOCs? One result of the association with a MTOC (28, 40) is stabilization of the microtubule, but the discontinuity between axonal microtubules and the cell body requires that any MTOC-analogue for axonal microtubule be independent of the perikaryal MTOC. Axonal microtubules, or at least regions of them, also appear to be unusually resistant to destabilizing treatments (2, 3, 12, 26, 36, 42). Stable domains within the microtubule, (particularly at one or both ends) could serve a function in the axon analogous to the MTOC in the perikaryon. Whether such domains are generated by modification of the tubulins or by interaction of the microtubule with other proteins or structures, they would have to be moving within the axon, a transportable microtubule organizing complex.

The presence of transportable microtubule-organizing complexes could explain a number of the properties of the axonal cytoskeleton. They would permit precise regulation of the size and stability of axonal microtubules without reference to the cell body by altering the rates of assembly and/or disassembly

for the individual microtubules. This would reduce the requirement for a large pool of tubulin monomer along the axon. Equally important, they could seed new microtubule growth while maintaining appropriate orientation and polarity in cases where the axonal microtubules were interrupted (18, 22) or reorganized. The stability of mature axonal morphologies suggests that axonal cytoskeletons are relatively stable, but the rapid reorganization of the cytoskeletal elements in the axon seen during sprouting and regeneration of axons also indicates the necessity of a potential for change (31, 35).

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