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Polymorphism of Brain Tubulin†

Henry J. George, Lily Misra, Deborah J. Field, and James C. Lee*

ABSTRACT: Calf brain tubulin was subjected to isoelectric focusing and tryptic peptide map analysis. Results from isoelectric focusing experiments showed a total number of 17 well-resolved protein peaks. The number of peaks and the mass distribution under each peak remained the same when the concentration of protein or ampholyte was altered. When the protein was subjected to two-dimensional isoelectric focusing, a diagonal pattern was observed, indicating that the multiple peaks observed are not a manifestation of tubulin–ampholyte interaction. Further investigation by isolating these individual subspecies and subjecting them to isoelectric focusing yielded single peaks corresponding to the original ones without generating the initial pattern of multiple peaks. Tryptic peptide maps showed that among the subspecies of the α subunit there are 26 spots that are common among them. There are, however, 7 ± 1 spots that are unique in each subspecies. Similar observations were obtained for the subspecies of the β subunit although there are only 2 ± 1 unique spots in each subspecies. These results suggest that tubulin subunits probably consist of polypeptides with both constant and variable regions in their sequences. Identical results were obtained for canine and rabbit brain tubulin, indicating that tubulin polymorphism is common among brain tissues. Tubulin isolated by either the polymerization–depolymerization or the modified Weisenberg procedures yielded identical results. These results show that the same subspecies of tubulin are extracted by both isolation procedures.

Microtubules constitute a major portion of cytoplasmic proteins in nerve cells (Hiller & Weber, 1978). They have been implicated to play a central role in axonal transport, neurotransmitter release, neurite outgrowth, and synaptogenesis (Shelanski & Feit, 1972). A cell is able to control the polymerization and organization of microtubules, thus influencing the cellular processes with which microtubules seem to be associated. The mechanism of control is believed to be through an influence on the dynamic equilibrium of microtubules with its subunit protein, tubulin. For many years, tubulin isolated from various mammalian brain tissues has been shown to exist as a complex of two nonidentical polypeptide chains, α and β. These polypeptide chains have a molecular weight of about 54 000 (Lee et al., 1973) and have been shown to possess different amino acid sequences (Luduena & Woodward, 1973). Recently, however, there has been an increasing number of reports implying that brain tubulin exhibits extensive heterogeneity when subjected to isoelectric focusing (Gozes & Littauer, 1978; Gozes et al., 1979; Marotta et al., 1978; Feit et al., 1977; Forgue & Dahl, 1979; Dahl & Weibel, 1979; Nelles & Bamburg, 1979). The number of subspecies reported varies from three to nine. Although the cause for the reported heterogeneity remains unknown, it has been proposed that the number of subspecies

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are determined by development (Gozes & Littauer, 1978; Dahl & Weibel, 1979) and are in part regulated at the messenger ribonucleic acid (mRNA) level (Gozes et al., 1980). It has also been suggested that posttranslational modifications such as phosphorylation, glycosylation, deamidation, and tyrosylation may give rise to multiple subspecies (Dahl & Weibel, 1979; Nelles & Bamburg, 1979). Rigorous proof for any such modifications as the cause of multiple subspecies has yet to be provided, and conflicting reports on the number of observable subspecies have rendered the matter of heterogeneity questionable at present.

The presence of multiple peaks during isoelectric focusing does not necessarily indicate heterogeneity of the protein sample. Cann and co-workers (Hare et al., 1978; Cann & Stimpson, 1977; Stimpson & Cann, 1977; Cann et al., 1978; Cann & Gardiner, 1979) have elegantly demonstrated by computer stimulation that even for a homogeneous sample of macromolecule a pattern showing multiple well-resolved peaks can be observed if the system undergoes a reversible carrier ampholyte–macromolecule interaction, e.g., $P + A \rightleftharpoons PA$. Such an interaction may include ligand-induced isomerization, association–dissociation of the macromolecule, or pH-dependent conformational transitions. The number of peaks then reflects the presence of various ampholyte–macromolecule complexes but not necessarily the intrinsic heterogeneity of the macromolecule. Several examples are found in the literature, and these include an acidic protein from wool (Frater, 1970), bovine serum albumin (Kaplan & Foster, 1971; Wallevik, 1973), myoglobin (Felgenhauer et al., 1971), and transfer RNA (tRNA) (Drysdale & Righetti, 1972; Galante et al., 1976).

In the absence of evidence provided by rigorous tests, it may be premature to conclude that the multiple components observed for tubulin represent intrinsic heterogeneity. In an effort to resolve this ambiguity and in light of the potential physiological significance of heterogeneity in tubulin, an investigation was initiated. This paper presents the results of such a study.

Materials and Methods

Iodoacetamide, β-lactoglobulin (lot 106C-8072), and bovine pancreas trypsin (type XI, treated with diphenylcarbamoyl chloride; lot 106C-81101) were purchased from Sigma. Ultrapure-grade urea (lot 1755) and acrylamide (lot 1748) were supplied by Bethesda Research Laboratories, Inc. Sodium dodecyl sulfate ($NaDodSO_4$)[1] (lot 20177) and ultrapure-grade urea (lot EZ-3428) were obtained from Bio-Rad and Schwarz/Mann, respectively. Fluorescamine was purchased from Roche whereas monobromobimane (Thiolyte MB lot 940096) was from Calbiochem. [^{14}C]Iodoacetamide, ampholine, and silica gel (60) plates without fluorescent indicator were obtained from Amersham, LKB, and Brinkmann, respectively. Phosphocellulose P11 was supplied by Whatman.

Preparation of Tubulin. Calf brain tubulin was prepared by two methods, namely, the modified Weisenberg procedure (Weisenberg et al., 1968; Weisenberg & Timasheff, 1970; Lee, 1980) and the polymerization–depolymerization method (Shelanski et al., 1973) as modified by Weingarten et al. (1974) with 90-min periods of centrifugation at 106000*g* and 4 °C (Runge et al., 1979). Rabbit and canine brain tubulin was also purified by the polymerization–depolymerization method. Tubulin prepared by the latter method was further purified by phosphocellulose chromatography according to the procedure of Weingarten et al. (1975). Tubulin samples obtained by these three methods will be referred to as W-, c-, and PC-tubulin, respectively. Tubulin concentrations were determined spectrophotometrically in 6 M guanidine hydrochloride by using an absorptivity of 1.03 L/(g·cm) at 275 nm.[2]

The concentrations of tubulin labeled with mBBr were determined by the method of Bradford (1976) with bovine serum albumin as standard.

Chemical Modifications of Tubulin. Carboxyamidomethylated derivatives of tubulin were prepared by dissolving W- or PC-tubulin to a concentration of 5 mg/mL in 6 M Gdn-HCl, 0.2 M Tris-HCl, and 0.1 mM EDTA at pH 8.4. The protein was reduced with DTT at a 4-fold molar excess with respect to the concentration of cysteine residues. The solution was degassed with N_2, placed in a 100 °C water bath for 5 min, and then allowed to cool to room temperature. The protein solution was alkylated at pH 8.4 by adding iodoacetamide solution (100 mg/mL) in 2.0 M Tris-HCl to a final concentration equivalent to a 2-fold excess over that of DDT. The reaction was carried out for 30 min in the dark at room temperature and stopped with an excess of β-mercaptoethanol. The excess reagents and reaction byproducts were removed by extensive dialysis against deionized water at 4 °C in the dark. The protein solution was lyophilized and stored desiccated at −20 °C. For radioactive labeling, [^{14}C]iodoacetamide (sp act. 40 μCi/mmol), dissolved in 200 μL of 2.0 M Tris-HCl, pH 8.4, was first added to the protein solution. After a 15-min incubation, cold iodoacetamide in excess with respect to the concentration of cysteine residues was added, and the mixture was incubated for an additional 15 min.

W-Tubulin was labeled with the fluorescent compound mBBr by a modification of the procedure described by Kosower et al. (1979). A 10-mg sample of W-tubulin was dissolved in 2 mL of 6 M Gdn-HCl and 0.04 M sodium phosphate buffer, pH 7.4, and sufficient DTT was added to give a 2-fold molar excess with respect to the concentration of cysteine residues. The protein solution was heated for 5 min in a 100 °C water bath and allowed to cool to room temperature. A 370-μL aliquot of 10 mM mBBr in 10 mM sodium phosphate at pH 7.4 made fresh from a 5× stock of mBBr in acetonitrile was then added and mixed with the protein solution. The reaction was carried out at room temperature for 30 min in the dark. Excess reagents and reaction byproducts were removed by exhaustive dialysis against deionized water in the dark at 4 °C. The protein was lyophilized, stored in the dark, and desiccated at −20 °C.

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–urea polyacrylamide slab gel electrophoresis was carried out according to a modified method of Laemmli (1970). Slab gels (20 × 14 × 0.1 cm) were cast by using a 4–12% (w/v) acrylamide gradient with a constant ratio for acrylamide/bis(acrylamide) of 30:0.8 in 6 M urea, 0.1% (w/v) $NaDodSO_4$, and 0.375 M Tris-HCl at pH 8.8 with a 0–10% (w/v) sucrose gradient. A 2-cm stacking gel containing 3% (w/v) acrylamide in 0.1% (w/v) $NaDodSO_4$ and 0.0675 M Tris-HCl at pH 6.8 was added onto the running gel. Tubulin samples were solubilized in a sample buffer containing 6 M urea, 0.02 M Tris-HCl, 0.1 mM β-mercaptoethanol, and 1% (w/v) $NaDodSO_4$ at pH 6.8. The samples were boiled for 5 min and layered onto the gels. Upper and lower chamber buffers contained 0.025 M Tris–glycine and 0.1% (w/v) $NaDodSO_4$ at pH 8.8. Electrophoretic experiments were conducted for

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; Gdn-HCl, guanidine hydrochloride; mBBr, monobromobimane; cam-tubulin, carboxyamidomethylated tubulin.

[2] G. C. Na and S. N. Timasheff, unpublished experiments.

20 h at 150 V with an initial amperage of 15 mA. At the completion of the experiment, gels were stained in 0.1% (w/v) Coomassie Blue G-250 in a solvent system of methanol/H_2O/acetic acid (5:5:1 v/v/v), destained in a solvent system of methanol/H_2O/acetic acid (3:7:1 v/v/v), and dried under vacuum on a sheet of 3 MM chromatography paper.

Isoelectric Focusing. Isoelectric focusing of tubulin under denaturing conditions were performed by the method described by O'Farrell (1975) in tube gels containing 4% (w/v) acrylamide [at a ratio for acrylamide to bis(acrylamide) of 30:08], 2% (v/v) ampholine (pH range 4–6), and 9.16 M urea. Isoelectric focusing sample buffer (50 μL) containing 9.16 M urea, 2% ampholine (pH 4–6), and 5% β-mercaptoethanol was layered onto the gel surface, and the gels were prefocused at 200 V for 15 min, 300 V for 30 min, and 400 V for 1 h, or until the amperage dropped below 1 mA. Upon completion of prefocusing, the overlay was removed, and protein samples, solubilized at room temperature in the sample buffer, were layered onto the gels.

Electrofocusing of the samples was then performed at 300 V for 18 or 72 h, as specified, at 8 °C. Upon completion of the experiment, gels that were not used for isolation of subspecies were fixed in 5% (w/v) Cl_3CCOOH for 1 h, washed in 5% (w/v) acetic acid for 10–16 h, and stained as described under Polyacrylamide Gel Electrophoresis. After being stained, gels were scanned at 580 nm on a Gilford Model 250 spectrophotometer with a Gilford Model 2520 20-cm gel scanner. For determination of the pH gradients, duplicate gels were sliced into 2-mm slices immediately after completion of focusing and incubated in 1 mL of deionized, distilled water overnight, and the pH of the solution was measured with a Radiometer PHM 64 research pH meter.

Two-dimensional isoelectric focusing was performed by first focusing 100 μg of cam-W-tubulin on tube gels as described. The focused tube gel was then placed on top of a slab gel containing 4% acrylamide, 8 M urea, and 2% ampholine, pH 4–6. Enough gel solution was added to seal the tube gel to the slab gel. After polymerization of the slab gel, isoelectric focusing was performed under similar conditions as described for tube gels.

Isolation of Tubulin Subspecies. Cam-W-tubulin, mixed with 10% (w/w) tubulin labeled with mBBr serving as tracer, was subjected to isoelectric focusing for 72 h by using the procedure described. Separation of bands could be monitored by visual inspection with a UV lamp. After completion of a run, gels were carefully removed from the tubes and placed on a glass plate. Individual bands visualized under a UV lamp were sliced from the gels. These subspecies were then electrophoretically eluted from the gel slices by the method of Stephens (1975) and dialyzed against deionized, distilled water to remove $NaDodSO_4$. They were then lyophilized and stored desicated in the dark at −80 °C.

Tryptic Peptide Mapping. A protein solution at 1.1 mg/mL in 0.1 M NH_4HCO_3 was digested with trypsin at an enzyme/protein ratio of 1:100 at room temperature for 25 h with continuous stirring. The sample was evaporated to dryness with purified dry air. The dried sample was resuspended in 30 μL of 0.1 M NH_4HCO_3 to yield a final concentration of 0.33 nmol/μL.

In order to assure a clear background, the silica gel plate (20 cm × 20 cm × 0.25 mm) was prechromatographed for 16–24 h in a solvent system of 1-butanol/pyridine/acetic acid/water (50:33:1:40 v/v/v/v) at pH 5.9. The plate was then dried at 100 °C for 30 min before 1–3 μL of the digested protein solution was spotted at the corner of the plate with a blunt-tip Hamilton syringe so that the spot size was no more than 1 mm in diameter.

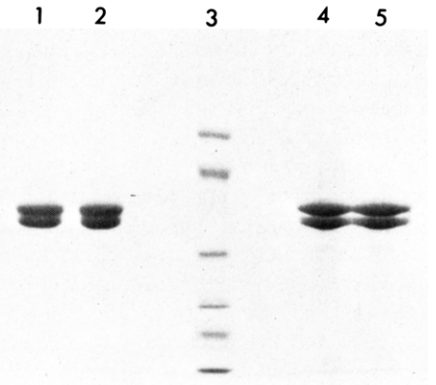

FIGURE 1: $NaDodSO_4$–urea polyacrylamide gel electrophoresis of purified tubulin. Identity and the amount of protein are as follows: (1) PC-tubulin, 10 μg; (2) W-tubulin, 10 μg; (3) standard proteins—phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme, 40 μg; (4) cam-PC-tubulin, 10 μg; (5) cam-W-tubulin, 10 μg.

Electrophoresis was performed at pH 3.5 with a solvent system of pyridine/acetic acid/water (2:20:978 v/v/v) on a Desaga TLE double-chamber apparatus. The plate was wetted with buffer and kept at 4 °C with a Neslab RTE-8 circulating bath. Two strips of Whatman 3 MM paper (6 × 20 cm) dampened with solvent were employed to connect the plates to electrode troughs. Electrophoresis was carried out at 1000 V for 1.5 h. The plate was removed, dried at 100 °C for 30 min, and then subjected to chromatography.

The chromatographic solvent system employed was 1-butanol/pyridine/acetic acid/water (50:33:1:40 v/v/v/v) at pH 5.9. Chromatography was stopped when the solvent front was 1 cm below the top of the plate, which was then dried at 100 °C for 30 min and cooled to room temperature. Before being sprayed with fluorescamine, fluorescent spots viewed under UV illumination were also marked. These represent the peptides containing bimane. The plate was then sprayed sequentially with solutions of acetone containing 0.01% (w/v) fluorescamine and 5% (v/v) triethylamine. Peptide spots were observed and marked under UV illumination.

Results

The homogeneity of calf brain tubulin prepared by both the modified Weisenberg and the polymerization–depolymerization procedures was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. Figure 1 shows that no proteins other than tubulin are present in these preparations. Furthermore, carboxyamidomethylation does not alter the migration nor the resolution of tubulin into its α and β subunits.

The purified tubulin samples were subjected to isoelectric focusing. Under conditions similar to those of Gozes & Littauer (1978), about nine well-resolved peaks were observed, as shown in lane 1 of Figure 2A. Upon closer observation, the presence of additional peaks is evident though they are not well resolved. Such an observation is very similar to that reported by Gozes & Littauer (1978). An effort was made to improve on the resolution of these peaks. The resolution was improved significantly by using a shallower pH gradient and also by increasing the gel length and the duration of focusing to 16.5 cm and 72 h, respectively, as shown in Figure 2A. A total of 17 well-resolved peaks were observed. The separation of peaks is more easily detected by scanning the gels as shown in Figure 2B. It is evident that the pH gradient was not linear throughout the gel after 72 h of focusing, al-

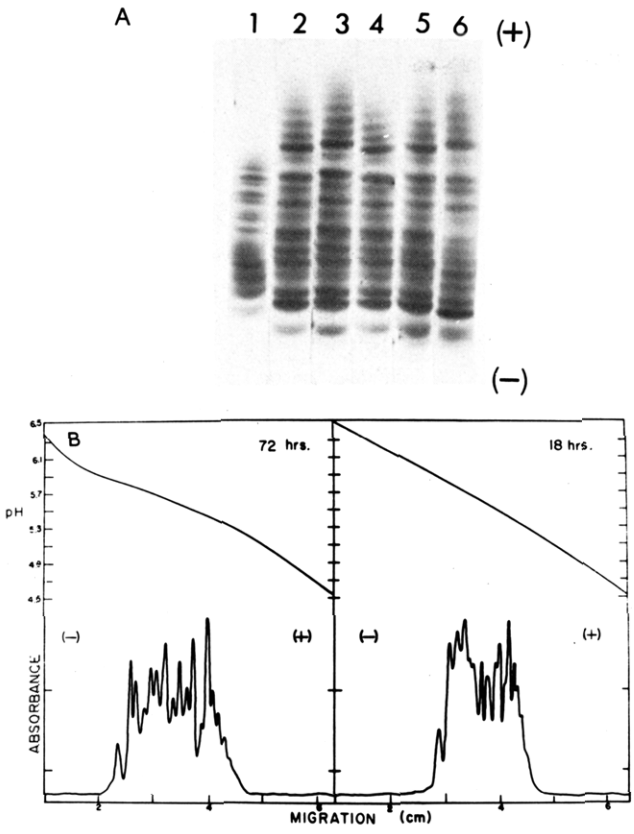

FIGURE 2: (A) Isoelectric focusing patterns of tubulin. Experimental conditions were the following: (1) total duration of experiment was 18 h with an ampholine mix of 1.2% (v/v), pH range 4–6, and 0.8% (v/v), pH range 5–7; (2–6) total duration of experiments was 72 h with a 2% (v/v) ampholine mix of pH range 4–6 at 8 °C. A 100-μg sample of protein at 3.0 mg/mL was applied to each gel. The protein samples are as follows: (1 and 2) cam-W-tubulin; (3) W-tubulin; (4) cam-PC-tubulin; (5) PC-tubulin; (6) bimane-labeled tubulin. (B) Densitometric tracings of isoelectric focusing gels 1 and 2.

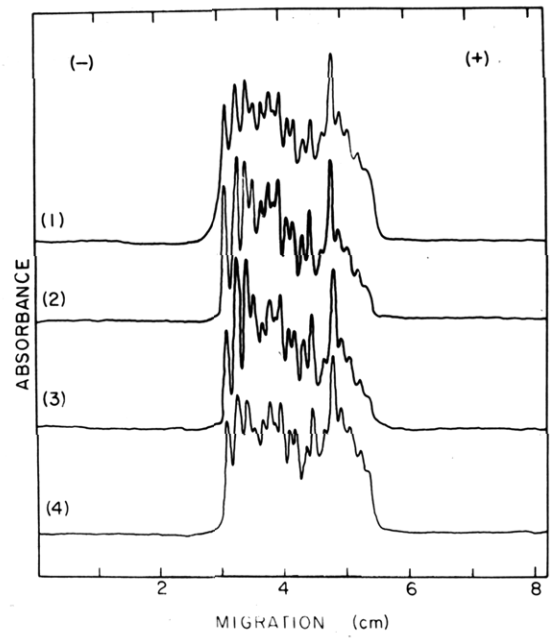

FIGURE 3: Densitometric tracings of isoelectric focusing gels as a function of tubulin concentration. Experimental conditions were the following: 2% (v/v) ampholine of pH range 4–6 at 8 °C for 72 h. A 45-μg sample of W-tubulin was applied to each gel, and their concentrations in mg/mL were the following: (1) 3.0; (2) 2.25; (3) 1.5; (4) 0.75.

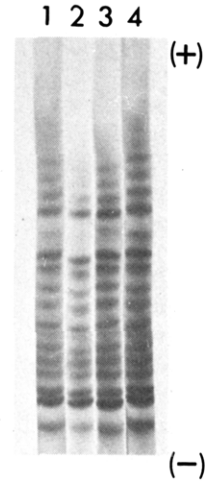

FIGURE 4: Isoelectric focusing patterns of tubulin as a function of total protein. The experimental conditions were the same as in Figure 3. Cam-W-tubulin at 3.0 mg/mL was applied to the gels. The total amounts in μg applied are as follows: (1) 30; (2) 45; (3) 75; (4) 105.

though it remained linear within the range where tubulin is distributed. Similar isoelectric focusing patterns were observed for tubulin purified by both procedures. Furthermore, blocking of the sulfhydryl residues did not alter the pattern as shown in Figure 2A. Under either condition, the same number of peaks was resolved, and the relative area under each peak was unchanged. The presence of these peaks leads to the following question: Do these peaks represent heterogeneous species of tubulin or are they consequences of ampholyte–protein interaction?

In order to provide answers to the question, more isoelectric focusing experiments were conducted under a variety of conditions. If the multiple number of peaks observed was a true reflection on the number of heterogeneous subspecies, then the total number of peaks and the relative area under each peak should remain unchanged upon perturbation of either protein concentration, total protein amount, or ampholyte concentration. However, if there were indeed ligand-induced changes in the macromolecule, then on the basis of the LaChatelier's principle the number of peaks resolvable by isoelectric focusing and the relative area under each peak should vary as a function of protein or ampholyte concentration. Isoelectric focusing experiments were, therefore, carried out as a function of initial protein concentration. The amount of protein in each sample layered on the gel was kept constant so as to eliminate potential differences in the buffering effect due to the presence of varying amounts of protein. Typical results of these experiments are shown in Figure 3. Within the protein concentration range of 0.75–3.0 mg/mL, the number of resolvable peaks remains the same. The relative area encompassed by each peak remains approximately the same throughout the range of protein concentrations tested. Thus, the results seem to indicate that the presence of these peaks is a true reflection on the number of heterogeneous species in tubulin. The effect of protein amount on the isoelectric focusing pattern was also investigated. The result of such a study is shown in Figure 4. It is evident that within the range of 30–105 μg of protein employed there is no detectable difference in the number of peaks nor the relative mass distribution of these components. The relative area encompassed by each peak is proportional to the total amount of protein employed in each experiment. These results imply that there is no detectable interaction among the species resolved on isoelectric focusing.

The effect of ampholyte concentration was then tested. At ampholyte concentrations of less than 1% (v/v), the patterns

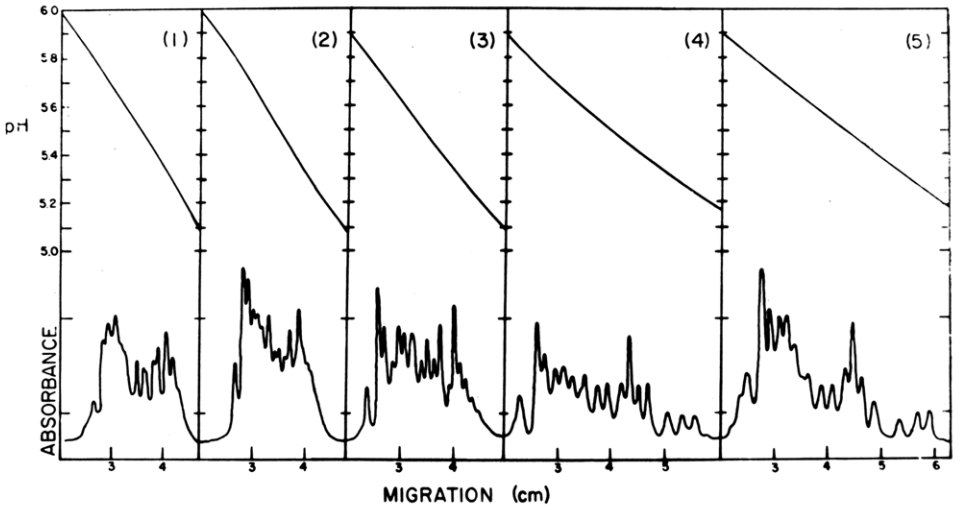

FIGURE 5: Densitometric tracings of isoelectric focusing gels of tubulin as a function of ampholyte concentration. The experimental conditions were the same as those in Figure 3. A 45-μg sample of cam-W-tubulin at 3.0 mg/mL was applied to each gel. The ampholyte concentrations in percent (v/v) are the following: (1) 1.0; (2) 1.5; (3) 2.0; (4) 2.5; (5) 3.0.

showed no resolvable peaks, while at a concentration greater than 3.5% (v/v), the protein would precipitate in the sample buffer. Meaningful results could only be obtained within the range of 1–3% ampholyte concentration. Figure 5 shows the densitometric scans of isoelectric focusing gels obtained from experiments carried out as a function of ampholyte concentration. The resolution increased with increasing ampholyte concentration. It is evident that the pH gradient becomes shallower with increasing ampholyte concentration and thus allows a better resolution of these protein components. The greater number of identifiable peaks, therefore, is most likely not due to a ligand-induced isomerization of association–dissociation of tubulin. The measured p*I* of the identifiable peaks remained the same.

Although all of the tests completed so far yield results indicating the presence of multiple noninteracting species in brain tubulin, they may all suffer from a common assumption. It is assumed that all the protein that was layered onto the gel migrated into the gel matrix. Since at ampholyte concentration of 3.5% (v/v) tubulin was found to be precipitated out of solution, tests were therefore conducted to investigate the amounts of protein that migrate into the gel matrix as a function of protein concentration. In order to increase the sensitivity and facilitate the monitoring of protein content, tubulin was labeled with [^{14}C]iodoacetamide. The labeled samples of tubulin were subjected to isoelectric focusing at 2.0% (v/v) ampholyte concentration. Upon completion of the experiment, the gels were sliced into fragments and assayed for radioactivity. After correcting for quenching due to the presence of gel and ampholytes, it was found that between 12 ± 5% to 60 ± 5% of the labeled tubulin did not enter the gel and was actually retained at the top of the gel. The amount retained is a function of protein concentration, with a greater amount not entering the gels at higher protein concentrations, although the focusing patterns remained unchanged. A test with five other proteins, including β-lactoglobulin, bovine serum albumin, jack bean urease, γ-globulin, and silkmoth chorion proteins, showed that a high percentage of these proteins also does not enter the gel under the present experimental conditions. The exact protein amount that migrated into the gel was certainly not equivalent to that layered onto the gel, but is apparently dependent on the nature of the protein. It is apparent, therefore, that the results of the tests by varying protein and ampholyte concentrations, while a necessary condition for polymorphism, are not sufficient. Thus,

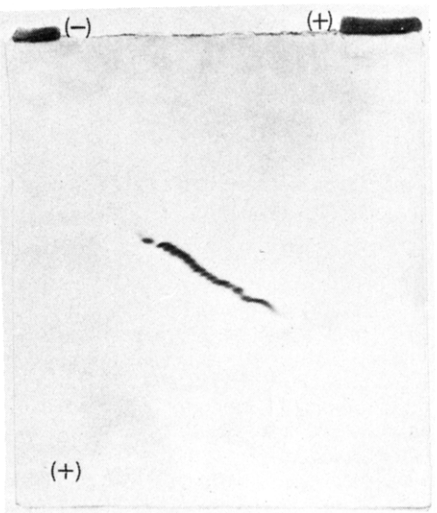

FIGURE 6: Two-dimensional isoelectric focusing of tubulin. 100 μg of cam-W-tubulin was subjected to isoelectric focusing under the same experimental conditions as those in Figure 3. Isoelectric focusing in the second dimension was conducted under the same conditions on a slab gel (15.5 × 14.5 × 0.1 cm) for 24 h.

when taken alone, they do not allow one to conclude with certainty that the identity of the isoelectric focusing patterns indicates the presence of polymorphism in brain tubulin.

Due to the failure to obtain unequivocal results by using the experimental tests described, two-dimensional isoelectric focusing experiments were conducted. Briefly, isoelectric focusing in tube gels constitutes the first dimension, and it is followed by focusing in slab gels under identical conditions for the second dimension. If ampholyte-induced changes in the macromolecules are the cause of the presence of multiple bands in the first dimension, then it is expected that each band would further resolve into multicomponents in the second dimension, leading to a pattern of multiple bands on either side of the diagonal on the slab gel. Conversely, if the multiple peaks do indeed represent genuine polymorphic components of brain tubulin, then the pattern should show the bands distributing along the diagonal on the slab gel. When cam-tubulin was subjected to two-dimensional isoelectric focusing, the results are shown in Figure 6. It is evident that no additional bands were generated in the second dimension, and the protein bands are distributed along the diagonal on the slab gel. The pH gradient generated on a slab gel was usually

Table I: Apparent Isoelectric Points of Tubulin Subspecies

band[a]	p*I*	band[a]	p*I*
1	5.83 ± 0.03	10	5.56
2	5.77	11	5.52
3	5.74	12	5.49
4	5.72	13	5.47
5	5.69	14	5.44
6	5.65	15	5.41
7	5.64	16	5.37
8	5.61	17	5.34
9	5.58		

[a] The tubulin subspecies are given the arbitrary designations of bands 1 through 17, with band 1 being the most basic species.

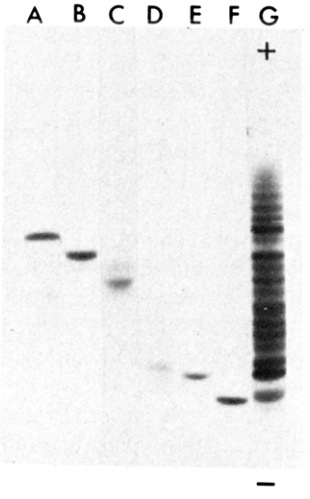

FIGURE 7: Isoelectric focusing of isolated subspecies of tubulin. The experimental conditions were the same as those in Figure 3. (A–F) Isolated subspecies 14, 12, 10, 3, 2, and 1, respectively. A 10-μg sample of each subspecies at 3.0 mg/mL was applied. 45 μg of cam-W-tubulin at 3.0 mg/mL was applied to gel G.

not as linear as in a tube gel, thus leading to the slight curvatures in the gel pattern. When the experiment was conducted with a steeper pH gradient or for a shorter duration, the pattern became straighter with less resolution between protein bands.

A conclusive demonstration of heterogeneity is to isolate individual bands, concentrate to initial protein concentrations, and repeat the isoelectric focusing experiments for each isolated band under identical experimental conditions. For facilitation of visualization and isolation of these bands, brain tubulin was labeled with the sulfhydryl-specific fluorescent probe mBBr. Brain tubulin labeled with mBBr was subjected to isoelectric focusing, and the resultant gel was scanned. The results are shown in Figure 2A,B. It is evident that the introduction of the fluorescent probe does not alter the resolution of brain tubulin into multiple bands. The isoelectric points and relative mass distribution of these species remain the same, as shown in Table I and Figure 2A, respectively. With the establishment that the covalent linkage of the fluorescent probe does not apparently modify the behavior of tubulin, the bimane-labled protein was employed as a tracer. Six representative protein bands were sliced from eight identical gels. These were electrophoretically eluted, exhaustively dialyzed against deionized water, and lyophilized. These isolated protein fractions were resuspended in sample buffer and adjusted to a concentration equal to the initial unfractionated protein sample. Subsequently, they were subjected to isoelectric focusing, and the results are shown in Figure 7. Each of these six tubulin fractions shows no indication of heterogeneity. The isoelectric points of the isolated protein bands are the same

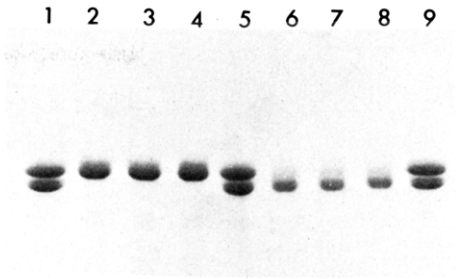

FIGURE 8: $NaDodSO_4$–urea polyacrylamide gel electrophoresis of isolated subspecies of tubulin. Lanes 1, 5, and 9 each contained 10 μg of bimane-labled W-tubulin. Lanes 2, 3, 4, 6, 7, and 8 contained 5 μg of subspecies 1, 2, 3, 10, 12, and 14, respectively.

as the corresponding components in the unfractionated sample. If the protein bands were labeled from 1 to 17, starting from the basic region, the isolated fractions correspond to 1, 2, 3, 10, 12, and 14. The presence of an additional band in lane C of Figure 7 is most likely due to contamination in the initial isolation procedure of the component. It may, therefore, be concluded that at least for the six protein fractions tested these components represent genuine polymorphic species of brain tubulin. These components were further subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis as shown in Figure 8. The three most basic bands correspond to α-tubulin whereas the three most acidic bands to β-tubulin.

Evidence for heterogeneity was further acquired by peptide mapping. On the basis of the lysine and arginine content of calf brain tubulin (Lee et al., 1973), the expected number of spots of a tryptic digest would be 43 if tubulin consisted of identical subunits. A total of 86 spots could be anticipated if tubulin consisted of two subunits with totally nonidentical sequences. A total of 90 ± 5 spots can be observed, indicating the presence of heterogeneity in brain tubulin. The presence of 10% (w/w) bimane-labeled tubulin did not generate any additional spots. Control experiments showed that no distinct spots can be detected at that concentration. The isolated subspecies of tubulin were also subjected to the tryptic digest, and the composite tracings of multiple experiments are shown in Figure 9. Panels a, b, and c correspond to the tryptic peptide maps of bands 1, 2, and 3, respectively, of the α subunit, whereas panels d, e, and f correspond to those of bands 10, 12, and 14, respectively, of the β subunit. It is evident that there are significant differences among subspecies of the same tubulin subunit although there are many peptides that are common among them. Among the subspecies for the α subunit, there are 26 spots that are common among them. There are a total of 46, 46, and 42 spots for bands 1, 2, and 3, respectively. There are, however, 7 ± 1 spots that are unique in each subspecies. Similarly, among the subspecies of the β subunit, there are 28 spots that are common among the three subspecies tested. There are a total of 45, 46, and 42 spots for bands 10, 12, and 14, respectively. In contrast to those for the α subunit, there are only 2 ± 1 spots that are unique in each subspecies. The presence of many apparently unique peptides instead of one for each subspecies implies that the differences among subspecies of the same subunit are caused by major changes in the primary sequences rather than single amino acid substitutions. The total number of spots varies from 42 to 46, and it is in good agreement with the expected values based on the averaged amino acid composition. In order to test for the sensitivity of our experimental procedure for peptide mapping, individual species of bovine β-lactoglobulin A and B fractionated by DEAE-cellulose (Piez et al., 1961) were subjected to analysis by the procedure. It was found that it is possible to detect the difference between these two genetic

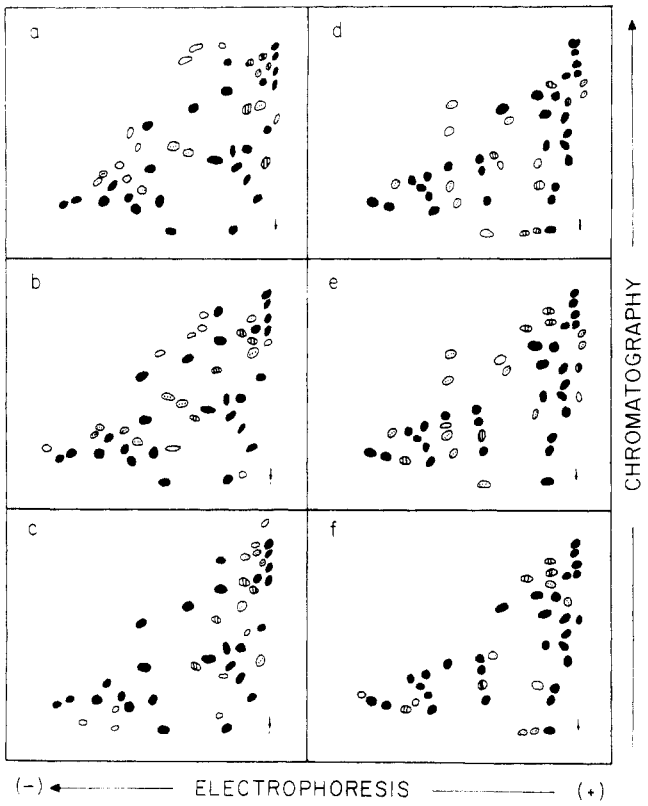

FIGURE 9: Tryptic peptide maps of isolated subspecies of tubulin. Panels a–c represent the composite maps of α-tubulin subspecies 1–3, respectively. Panels d–f represent those of β-tubulin subspecies 10, 12, and 14, respectively. Symbols of the spots are as follows: (●) common spots among all three subspecies; (○) unique spots for the subspecies; (⊙ and ⊕) common spots between two of the three subspecies.

variants of β-lactoglobulin. Hence, the procedure employed by this investigation is sensitive enough to detect changes represented by single amino acid substitutions.

Brain tubulin from other mammalian species was also subjected to isoelectric focusing. It was found that brain tubulin from canine and rabbit exhibits the same pattern with an identical number of resolvable components as that of calf brain protein. It may be concluded that at least with three mammalian species tested these brain tissues contain the same polymorphic forms of tubulin.

Discussion

Since the introduction of isoelectric focusing as a technique to separate protein molecules, it has become routine to employ this method to characterize isolated and purified protein samples. Cann and co-workers have clearly pointed out the potential pitfalls of the technique in characterizing and identifying the apparent heterogeneity of the protein sample. Most of the examples cited (Hare et al., 1978) are experiments conducted in native conditions. Although the initial reports of heterogeneity in brain tubulin were observations under denaturing conditions, one cannot rule out the possibility of ampholyte-induced changes in the macromolecule as a source of the multiple peaks observed, especially after an earlier observation in our laboratory that tubulin in 9 M urea can be precipitated out of solution at higher ampholyte concentrations. The results of this investigation demonstrate that brain tubulin does exist as a mixture of isoelectrically heterogeneous species. Let us examine the results which lead to the conclusion.

The evidences provided by this study are the following: (1) invariant isoelectric focusing patterns were observed with varying protein or ampholyte concentrations, (2) a diagonal pattern was observed when brain tubulin was subjected to two-dimensional isoelectric focusing, and (3) the isolated individual species did not resolve into multiple components. These results indicate that the multiple components resolved under the present experimental conditions are not a consequence of a rapid reversible interaction of brain tubulin with components employed in the isoelectric focusing experiment. The presence of multiple components may, therefore, represent the consequence of a strong irreversible interaction of brain tubulin with some ligands, or the purified tubulin sample is a mixture of polypeptides with distinctive differences in their primary structures.

It is conceivable that some contaminants in the chemicals employed in our isoelectric focusing system may interact with the protein irreversibly to induce the formation of multiple separable components. These ligands cannot be present in a random fashion in urea, acrylamide, or ampholyte employed. Experiments were carried out in the presence of different lots of these chemicals, and identical results were obtained. Recrystallization of urea and acrylamide did not lead to any observable difference in the number of resolvable peaks nor the mass distribution of them. Any charged ligands that are free to migrate would be removed by the prefocusing procedure routinely employed in this study. It is unlikely that formation of carbamoyl groups is the cause of heterogeneity. The protein samples employed for isoelectric focusing experiments were not exposed to urea until just before layering onto the gel after being dissolved in the sample buffer at room temperature. It may, therefore, be concluded that the multiple components observed cannot be due to an artifact generated by irreversible modification of tubulin by contaminants which are present in spite of the efforts of recrystallization and prefocusing. It is unlikely that contaminants in the ampholyte can be the cause since the same mass distribution of subspecies was observed as a function of ampholyte concentration.

Another potential cause of polymorphism could be originated from the procedure of purification. Tubulin was isolated by two procedures which differ significantly from each other. The polymerization–depolymerization method conceivably could selectively isolate the pool of tubulin that is competent in forming the microtubule, and since glycerol was included in the buffer system it is possible that contaminants in glycerol may chemically modify tubulin (Detrich et al., 1976; Bello & Bello, 1976), thus leading to the presence of multiple resolvable components. On the other hand, the Weisenberg procedure utilizes conventional protein fractionation principles and is most likely to isolate proteins with similar physicochemical properties. It is evident that tubulin samples isolated by either procedure yield the same pattern (Figure 2A), indicating that the same population of tubulin was isolated and the protein is expected to exhibit identical behavior. This conclusion is in good agreement with an earlier report by Lee et al. (1978). Furthermore, it is interesting to note that brain tubulin from dog and rabbit yields a pattern identical with that of calf. It seems, therefore, that the procedures of isolation and the source of the brain are unlikely the cause of polymorphism in brain tubulin. If there were any chemical modification on tubulin, it was not detected by isoelectric focusing or $NaDodSO_4$–polyacrylamide gel electrophoresis.

With the establishment that at the least 88% of brain tubulin does exist in polymorphic forms, the effort of investigation is then focused on the nature of such heterogeneity. The results obtained from comparing the tryptic peptide maps of isolated subspecies of both α- and β-tubulin subunits indicate that substantial homology exists in the amino acid sequences be-

tween the subspecies of each subunit. However, between each pair of subspecies, there are differences of up to 10% of the total number of spots. It indicates that there must be regions in the sequences that are quite different. Since the average number of unique spots for the subspecies in α-tubulin is seven as compared to two in β-tubulin, it seems that α-tubulin is composed of polypeptide chains of greater differences in their amino acid sequences. Ponstingl et al. (1979) reported the amino acid sequence of 78 residues from the C-terminal region in α-tubulin and concluded that a minor difference in the sequence is detected in this region. It is conceivable that greater differences would be encountered when the sequencing effort is extended further into the polypeptide chain. The conclusion that tubulin exists in polymorphic forms based on biochemical studies as presented in this report is consistent with genetic evidences (Cleveland et al., 1980; Gozes et al., 1980). It is not known, however, at present the source of the polymorphic forms of tubulin. These forms may be a reflection on the vast number of cell types present in the brain tissues, they might be related to the development and maturity of these cells, or they might be the manifestations of a nonsynchronized cell population. Much effort is yet required to elucidate the biological significance of these polymorphic forms of tubulin.

The isolation of individual subspecies is facilitated by the chemical modification of sulfhydryl residues with mBBr. The protein has to be excised from the polyacrylamide gel without undergoing any further chemical alterations. The usual routine procedure of staining with Coomassie Blue or precipitation with Cl_3CCOOH would most likely generate undesirable changes on the protein. However, the presence of bimane-labeled tubulin can be detected by UV illumination. The advantages of mBBr over other fluorescent probes are the following: (1) the net charge of the protein molecule has not been altered; (2) the chemical modification is specific for sulfhydryl residues which have to be chemically blocked anyway; (3) the extreme photostability enables one to conduct manipulations which are time consuming without causing photobleaching; (4) the high quantum yield of the protein–probe complex increases the sensitivity of detection.

In conclusion, brain tubulin has been demonstrated by the technique of isoelectric focusing and tryptic peptide mapping to be polymorphic. There are constant regions among the amino acid sequences of the subspecies; however, there are apparently also variable regions among them. The source and significance of such polymorphism are presently under investigation.

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