Characterization of Tubulin Modified with the Sulfhydryl-Reactive Fluorochrome Monobromo(trimethylammonio)bimane†

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ABSTRACT: Microtubules assembled in vitro have been fluorescently labeled with the highly fluorescent probe monobromo(trimethylammonio)bimane (bimane), which binds covalently to accessible sulfhydryl groups. The labeling procedure is carried out while the microtubules are in the assembled state so as to protect from reaction with the bimane those sulfhydryl groups critical for formation of a microtubule. The resulting fluorescent microtubules are cold labile and remain competent to participate in further rounds of the temperature-dependent assembly–disassembly reaction in vitro and display an excitation maximum at 385 nm and an emission peak at 460 nm. Negative stain electron microscopy confirms that morphologically normal microtubules are formed from the fluorescent subunits, and the kinetics of their assembly are identical with those of a control, unlabeled preparation when both are compared at the same protein concentration. In addition, and perhaps more importantly, the fluorescent microtubule proteins can compete quantitatively with a large excess of unlabeled microtubule proteins, indicating that the derivatized proteins assemble with the same efficiency as their unlabeled counterparts. Examination of unstained sodium dodecyl sulfate–polyacrylamide gels illuminated with long-wavelength ultraviolet light demonstrates that both the α and β subunits of the tubulin dimer are labeled as are the high molecular weight microtubule-associated proteins MAP 1 and MAP 2. Since the bimane has been demonstrated previously to be specific for reactive sulfhydryl groups, the extent of labeling of phosphocellulose-purified tubulin was determined by titration of sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid) under denaturing conditions. Comparison of the total sulfhydryl titer of control and fluorescent tubulin dimers indicates that an average of 1 mol of fluorochrome is bound per mol of tubulin dimer. The results are discussed in light of the use of these fluorescent derivatives as easily detectable molecular cytochemical probes for studying the dynamics of microtubule assembly and disassembly within living cells.

The spatial and temporal distribution of microtubules within cells has been correlated with a variety of cell motility events such as mitosis, axonal transport, and the development and maintenance of cell form [see Snyder & McIntosh (1976) and Stephens & Edds (1976) for a review]. A basic understanding of the role of microtubules in such varied phenomena has been gained by several morphological techniques. Both electron microscopy (Porter, 1966; Tilney, 1971) and immunofluorescence microscopy (Weber et al., 1975; Sato et al., 1976; Connolly et al., 1978) have been used to study the distribution of microtubules in a variety of cell types; however, since these techniques require fixed and stained preparations, the dynamic nature of the motile event is lost. Alternatively, the light microscope has been used to observe motility in living cells by using such sensitive optical techniques as differential interference contrast and polarization microscopy. Indeed, the latter has been used most elegantly by Inoué & Sato (1967) in studies on the assembly and function of the mitotic apparatus. Yet these light microscopic techniques are limited by the resolution limits of the light microscope and also, in the case of polarization optics, by the weak birefringence of most biopolymers.

An alternative approach has thus been to use microtubules purified in vitro to investigate the control of microtubule assembly. From such studies, the ionic and nucleotide requirements have been determined (Olmsted & Borisy, 1975), as well as the role of such varied parameters as sulfhydryl groups, microtubule organizing centers [MTOCs, see Pickett-Heaps (1969)], and various nontubulin proteins termed collectively microtubule-associated proteins (MAPs)[1] (Sloboda et al., 1975, 1976; Weingarten et al., 1975). However, despite these detailed morphological and biochemical investigations, the manner in which the cytoskeleton is assembled and functions within cells remains elusive.

In order for this latter point to be addressed more directly, several laboratories have begun synthesizing fluorescent derivatives of purified native cellular proteins to be introduced into suitable cells or lysed cell models. This technique, molecular cytochemistry, was first described by Taylor & Wang (1978) and is a powerful new approach to the study of the cytoskeleton. Fluorescent derivatives of actin (Taylor & Wang, 1978; Kreis et al., 1979; Gawlitta et al., 1980), α-actinin (Feramisco, 1979), 130K protein (Burridge & Feramisco, 1980), and tubulin (Travis et al., 1980; Keith et al., 1981) have been prepared and injected into various cell types. The distribution of these molecules is then followed by using the fluorescence microscope coupled to an image intensification video camera (Willingham & Pastan, 1978; Reynolds & Taylor, 1980). With this approach in mind, a derivative of tubulin has been prepared which is stoichiometrically labeled with the sulfhydryl-reactive fluorochrome monobromo(trimethylammonio)bimane [bimane; see Kosower et al. (1979, 1980)]. This native, highly fluorescent probe is suitable for the technique of molecular cytochemistry and therefore should be relevant for use in experiments designed to follow the spatial

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[1] Abbreviations: microtubule proteins, a term used in this paper to signify that microtubules assembled in vitro from brain homogenates contain a number of associated proteins in addition to the major component, tubulin; MAP, microtubule-associated protein; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GTP, guanosine 5′-triphosphate; Pipes, piperazine-*N,N′*-bis(2-ethanesulfonic acid); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; PCMPS, *p*-(chloromercuri)benzenesulfonate; DTT, dithiothreitol.

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and temporal distribution of microtubules in living cells and lysed cell models. In this paper, the preparation and characterization of the labeled tubulin are described. Portions of this work have appeared in abstract form (Wadsworth & Sloboda, 1980).

Materials and Methods

Protein Purification. Microtubule proteins were purified from 2-day-old chick brains according to Sloboda & Rosenbaum (1979). Briefly, brains were homogenized in polymerization buffer (PM) consisting of 100 mM Pipes, pH 6.9, 1 mM $MgSO_4$, 2 mM EGTA, and 1 mM GTP, at a ratio of 0.8 mL of buffer per g of brain. The supernatant obtained after centrifugation at 35000*g* for 80 min was warmed at 37 °C for 30 min. The microtubules which formed were collected by centrifugation at 10000*g* for 30 min. Sedimented microtubules were resuspended in PM, depolymerized at 0 °C for 30 min, and centrifuged at 10000*g* for 15 min to remove any cold-stable aggregates. The resulting supernatant was again warmed to 37 °C, and the microtubules were sedimented, overlaid with fresh PM, and stored at −70 °C. Stored microtubule proteins were routinely subjected to one complete assembly–disassembly cycle before use in an experiment to ensure that only assembly-competent proteins were present. Tubulin was prepared free of microtubule-associated proteins by phosphocellulose chromatography (Weingarten et al., 1975; Sloboda & Rosenbaum, 1979). Columns (1.5 × 10 cm) were equilibrated and developed in column buffer (CB) containing 50 mM Pipes, pH 6.9, 0.5 mM $MgSO_4$, 1 mM EGTA, and 0.1 mM GTP. Tubulin-containing fractions were adjusted to 1 mM GTP and 1 mM $MgSO_4$ immediately after elution from the column (Sloboda & Rosenbaum, 1979). The MAP fraction was eluted in a single-step wash of 0.8 M KCl in CB and desalted into CB by Sephadex G-25 chromatography. Proteins were concentrated with an Amicon ultrafiltration device using YM-30 filters, frozen in liquid nitrogen, and stored at −70 °C.

Labeling. The fluorescent labeling reagent monobromo(trimethylammonio)bimane (bimane) was used to label microtubules. Bimane (sold by Calbiochem under the trade name Thiolyte MQ) from a freshly prepared 1–5 mM stock solution in PM was added to a solution of polymerized microtubules at 37 °C with rapid pipetting to ensure mixing. The fluorochrome was added at a 4:1 molar excess to the protein present in the sample, assuming an average molecular weight of 110000. Since not only MAPs but also numerous other protein species are present in minor amounts in a solution of in vitro assembled brain microtubules, this average calculation ensures that bimane is present in slight molar excess to the protein. The duration of the labeling reaction varied with the experiment (see Results). Labeled microtubules were then collected by centrifugation at 10000*g* for 30 min at 25 °C and were resuspended in fresh PM. This step removed the bulk of the unreacted fluorochrome. After disassembly at 0 °C for 30 min, the labeled protein was centrifuged at 10000*g* for 15 min to remove any cold-stable aggregates. The fluorescently modified proteins were cycled again before use in the experiments reported here, either immediately or after storage at −70 °C. For the preparation of fluorescently labeled, purified tubulin dimers, the microtubule proteins were applied to a phosphocellulose column without a further cycle of temperature-dependent assembly and disassembly; any free bimane present is retained by the column, and the labeled proteins were collected, concentrated, and stored as described above.

Sulfhydryl Determinations. Sulfhydryl content was determined by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as originally described by Ellman (1959). Prior to titration, the protein samples were adjusted to 50 mM DTT and allowed to incubate at 4 °C for 30 min. This step was performed to ensure that all half-cystines on the tubulin would be present in the reduced form. The samples were then desalted on columns of Sephadex G-25 and immediately titrated with DTNB. Reaction mixtures contained 1.25×10^{-3} M EDTA, pH 8.0, 4 M urea in 0.1 M sodium phosphate, pH 8.0, 5–10 μM protein in PM, and 2×10^{-3} M DTNB, in a final volume of 0.25 mL. Three times recrystallized DTNB (a gift of Dr. Lionel I. Rebhun), from a freshly prepared stock solution of 20 mM DTNB in 0.1 M phosphate, pH 8.0, was added last, and the samples were vortexed vigorously. The absorbance was determined at 412 nm vs. a protein blank to give the apparent absorbance. The absorbance of a reagent blank was subtracted from this value to obtain the net absorbance (Habeeb, 1972). The change in absorbance vs. time was monitored in a Gilford Model 250 recording spectrophotometer; color development was complete within 1 min and stable for at least 1 h. Samples were therefore routinely read 10 min after the addition of DTNB. Sulfhydryl content was determined by using a molar extinction coefficient of 13600 M^{-1} cm^{-1} (Ellman, 1959). Protein concentration was determined by the method of Lowry et al. (1951) as modified by Schacterle & Pollack (1973).

Electron Microscopy. Diluted solutions of microtubules were applied to carbon over formvar-coated copper grids and allowed to adsorb for 30 s. The grids were then rinsed with several drops of cytochrome *c* in 1% *n*-amyl alcohol, stained with 1% aqueous uranyl acetate, and air-dried (Dentler et al., 1975). The grids were examined in a JEOL 100-CX electron microscope operated at 60 kV.

Other Methods. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Proteins were stained with Coomassie Brilliant Blue R according to Fairbanks et al. (1971). Unstained gels were fixed in 10% acetic acid, washed in 50 mM Tris, 150 mM NaCl, and 0.02% NaN_3, pH 7.5 (Burridge, 1976), and photographed under long-wavelength ultraviolet illumination with a no. 15 orange filter. Turbidity measurements (Gaskin et al., 1974) were performed with a Gilford Model 250 recording spectrophotometer fitted with a thermal cuvette holder. Fluorescence measurements were made with a Turner Model 430 spectrofluorometer equipped with an Aminco photon counter and a 150-W xenon lamp. Viscosity was measured in Ostwald high-shear viscometers in a water bath at 37 °C.

Results

When monobromo(trimethylammonio)bimane (bimane) is added to a solution of assembled microtubules, the reaction between protein sulfhydryl groups and the fluorochrome is rapid, as shown by the time course of the labeling reaction. As seen in Figure 1A, a rapid rise in fluorescence specifically associated with polymerized protein occurs within the first 30 s of the reaction. The increase in fluorescence obtained during the following 20 min is linear; however, the slope of this portion of the curve is significantly lower. This slow, steady increase in fluorescence is probably due to modification of protein SH groups that react with the bimane with slower kinetics than those that react initially. Furthermore, since only sedimentable microtubules are being assayed for fluorescence, any modification of free dimers that occurs is not contributing to the fluorescence measured in Figure 1. The increase in fluorescence observed with the spectrofluorometer (Figure 1A) was quantitated further by calculating the specific fluorescence (moles of fluorochrome per mole of protein) at various times

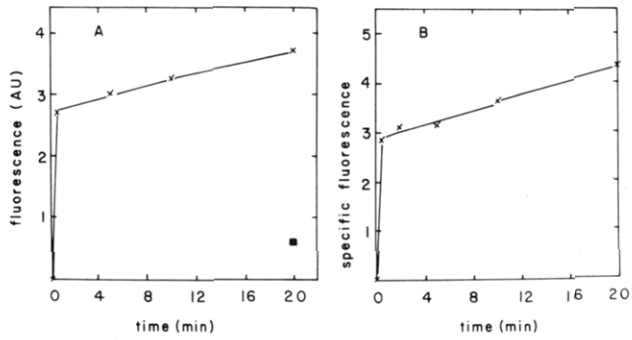

FIGURE 1: Time course of the fluorescent labeling reaction. (A) Polymerized microtubules, incubated with bimane for the times indicated, were collected by centrifugation, resuspended in PM, cold depolymerized, and clarified by centrifugation. The samples were then adjusted to identical protein concentration and assayed in the spectrofluorometer. The filled square represents the amount of fluorescence obtained when the proteins are first treated with 1 mM NEM (see Results) to block available SH groups. (B) Specific fluorescence (moles of bimane per mole of protein) of microtubule proteins labeled as in (A). The amount of bimane was calculated at an absorbance of 385 nm and an extinction coefficient of 4000 M^{-1} cm^{-1}, against a protein blank; protein concentration was determined as described under Materials and Methods, and the number of moles was estimated as described under Results.

during the labeling reaction by using the molar extinction coefficient for bimane of 4000 M^{-1} cm^{-1} (Kosower et al., 1979). Such an analysis shows that the kinetics observed with the spectrofluorometer are identical with those obtained when the bimane to protein ratio is determined at specific time points during the labeling reaction (compare panels A and B of Figures 1). If one takes as a reasonable approximation a MAP content of 20% with an average molecular weight of 300 000 (Sloboda & Rosenbaum, 1979) with the remainder of the protein tubulin dimers having a molecular weight of 110 000, then the microtubules become labeled with 2.8 mol of bimane per mol of protein within 30 s after addition of the fluorochrome; at 20 min, this ratio has increased to approximately 4.3 mol of bimane per mol of protein. However, it should be noted that these are estimates, and thus methods are reported in the following section that quantitate more precisely the exact stoichiometry of the labeling reaction.

The specificity of the bimane for sulfhydryl (SH) groups was tested by blocking the available SH groups with the SH-specific reagent *N*-ethylmaleimide (NEM) (Benesch & Benesch, 1962) prior to labeling. Pretreatment of the microtubules with 1 mM NEM for 15 min before labeling with bimane reduced the amount of label incorporation by 84%, as compared to a control sample labeled in the usual manner (Figure 1). When equivalent amounts of protein were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, labeled proteins were fluorescent (see Figure 5) while NEM-treated proteins were not fluorescent (data not shown).

For further characterization of the spectral characteristics of these fluorescent microtubule proteins, the excitation–emission spectrum of a labeled sample was determined, and the data are presented in Figure 2. Here, the excitation spectrum of the fluorescent protein is plotted along with the corresponding wavelength at which the fluorescent emission was maximum. Thus, the excitation and emission maxima of bimane-labeled microtubule proteins are 385 and 460 nm, respectively.

Assembly Characteristics of the Fluorescent Proteins. Several additional experiments were performed to demonstrate that the labeled proteins retain their native properties. First, the assembly kinetics of the labeled proteins were compared with control, unlabeled samples. As shown in Figure 3, samples labeled with bimane for either 10 or 20 min assembled with both a rate and final extent of assembly identical with those of the control samples when each was compared at equivalent protein concentrations. Negative stain electron microscopy of the assembled fluorescent proteins revealed

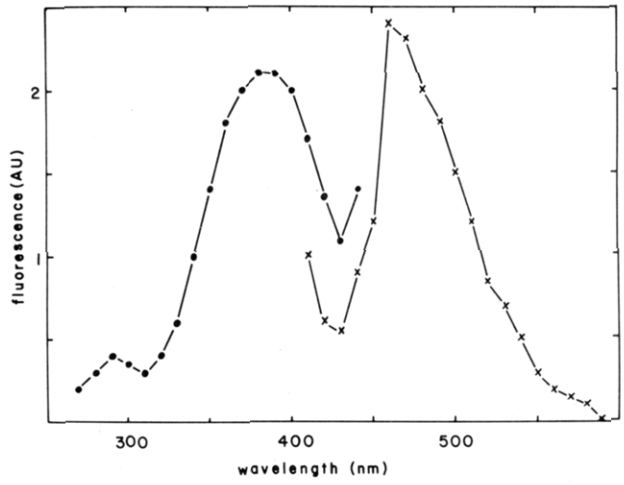

FIGURE 2: Excitation/emission spectrum of bimane-labeled microtubule proteins. Excitation (●) is plotted along with the corresponding fluorescence emission (×), yielding excitation and emission maxima of 385 and 460 nm, respectively. AU = arbitrary units.

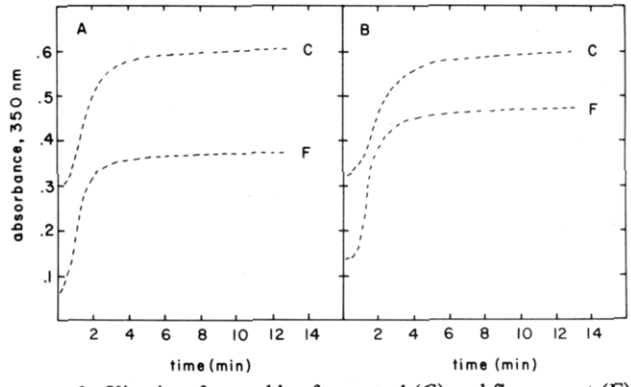

FIGURE 3: Kinetics of assembly of a control (C) and fluorescent (F) sample of microtubule proteins, each at 2.3 mg/mL. In graph A, the labeling reaction had been carried out for 10 min and in graph B for 20 min (see Figure 1) prior to testing the ability of the sample to assemble at 37 °C. In both, the initial A_{350} values have been offset along the ordinate by using the controls on the spectrophotometer.

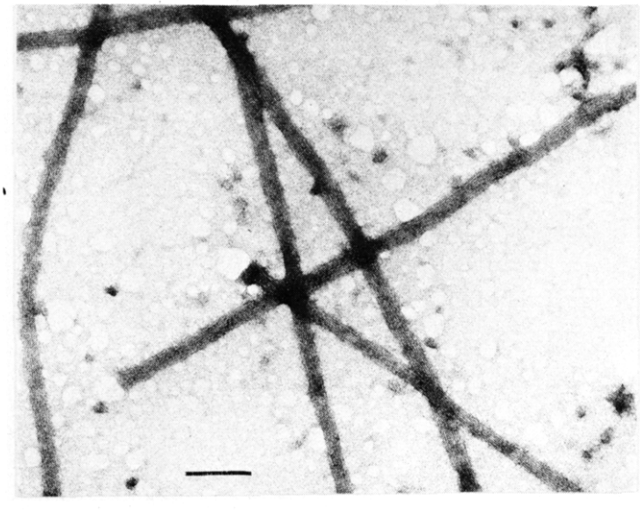

FIGURE 4: Negative stain electron micrograph of assembled, fluorescent microtubules showing the normal morphology of the polymer is negative stain. Bar = 100 nm.

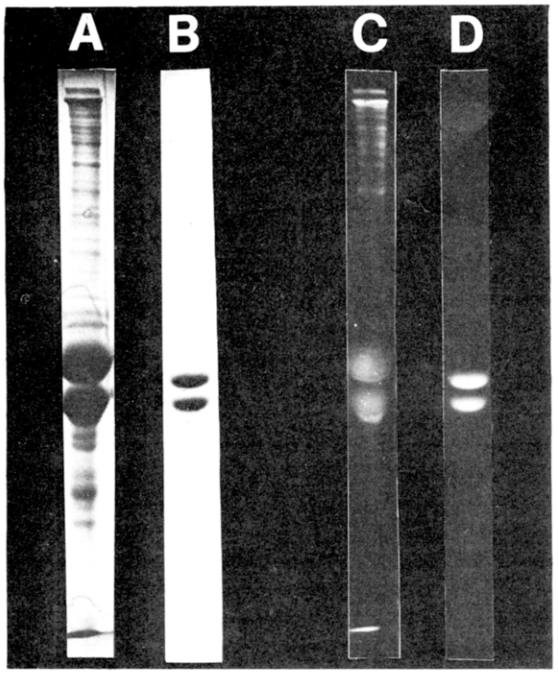

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of fluorescent microtubule proteins and purified tubulin dimers. Corresponding Coomassie Blue staining pattern (A, B) and fluorescent image (C, D) of the same gels photographed prior to staining. Samples A and C are microtubule proteins, and samples B and D are phosphocellulose-purified tubulin dimers. The majority of the labeling occurs coincident with the α and β subunits of tubulin and the high molecular weight MAPs.

normal microtubules that were indistinguishable from controls (Figure 4). Thus, the labeled proteins retain the ability to form morphologically normal microtubules with the same assembly kinetics as unlabeled preparations.

Unstained polyacrylamide gels of the labeled proteins revealed that both the α and the β subunits of tubulin were fluorescent as well as the high molecular weight MAPs. The same gels were then stained for protein to confirm that the tubulin subunits and MAPs were the major components of the preparation and that the fluorescence comigrated with the protein (Figure 5). Since these are $NaDodSO_4$ gels run under denaturing and reducing conditions, these results also indicate that the fluorochrome is bound covalently to the proteins.

Stoichiometry of Labeling. Since the reaction of the bimane with protein is specific for available SH groups [Figure 1 and Kosower et al. (1979)], the stoichiometry of labeling of tubulin was determined by titration of SH groups with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) both before and after labeling. Since the bimane covalently modifies available SH groups, the fluorescently modified SH groups are not available for disulfide exchange reactions with the DTNB. Thus, the extent of labeling can be quantitated by measuring the difference in the SH titer between control and experimental samples. Tubulin was prepared free of MAPs by phosphocellulose chromatography, and the SH content was subsequently measured under denaturing conditions. Unlabeled dimers contained 17.16 ± 0.40 (N = 6) mol of SH per mol of dimer, based on a molecular weight of 110 000. Labeling with the bimane reduced the titer of available SH groups; after 5 min of labeling, the number of SH groups that reacted with DTNB was 16.14 ± 0.43 (N = 6) per tubulin dimer. Thus, labeling blocked an average of 1.02 mol of SH per mol of dimer.

The following experiment was performed to demonstrate that assembly-competent tubulin, not a subset of denatured tubulin, was modified by the bimane. Labeled microtubule proteins were subjected to an additional round of assembly and disassembly prior to purification of the tubulin by phosphocellulose chromatography. This step ensured that only assembly-competent proteins would be analyzed. Tubulin purified in this manner is also fluorescent, thus demonstrating that native, assembly-competent tubulin, not a subset of denatured dimers, is the major labeled species present.

Table I: Ratios of Bimane to Protein in Various Preparations, Assayed Using the Reported Extinction Coefficient of Bimane[a]

prepn	labeling time (min)	bimane/protein molar ratio
microtubule proteins	5	3.16
	10	3.60
high-speed supernatant[b]	5	0.83
	10	1.30
phosphocellulose-purified MAP fraction	5	4.10

[a] 4000 M^{-1} cm^{-1} (Kosower et al., 1979). [b] Obtained by centrifuging a solution of microtubule proteins at 102000g for 60 min at 4 °C.

Table II: Effect of Bimane on the Relative Concentration of Protein in Monomer and Polymer Fractions[a]

expt	sample	concn (mg/mL) control	concn (mg/mL) bimane
1	supernatant	0.77	0.74
	pellet	1.22	1.08
2	supernatant	0.77	0.78
	pellet	1.17	1.05

[a] Concentrations of monomer and polymer were determined by the sedimentation assay of Johnson & Borisy (1975) and are expressed as mg of protein per mL of solution.

The stoichiometry of labeling of tubulin and the MAPs was also estimated by using the extinction coefficient of the bimane as reported by Kosower et al. (1979). As shown in Table I (see also Figure 1B), greater than 3 mol of fluorochrome are bound per mol of microtubule protein after 5 or 10 min of labeling. The amount of fluorochrome bound per mole of tubulin dimer was estimated by using a high-speed supernatant of microtubule protein which was depleted in MAP content. By this somewhat less quantitative analysis, on the order of 1 mol of bimane is bound per mol of tubulin dimer, a value that agrees quite well with the value obtained by titration of purified labeled dimers with DTNB. Similarly, the MAP fraction obtained by phosphocellulose chromatography of labeled microtubule proteins contained approximately 4.1 mol of bimane bound per mol of MAPs [assuming an average molecular weight of 300 000; see Sloboda et al. (1975)].

Effect of Bimane on the Tubulin Monomer–Polymer Distribution. Previous investigations on the role of SH groups in microtubule polymerization (Mellon & Rebhun, 1976; Kuriyama & Sakai, 1974) indicated that the addition of mercaptide-forming reagents to microtubules both inhibited polymerization and caused formed microtubules to depolymerize as measured by viscosity. Moreover, microtubule disassembly was accompanied by a reduction in the SH content of the protein (see Discussion). Thus, the effect of bimane labeling on the assembly state of the microtubules was determined by using two techniques. First, a sedimentation assay was used to investigate the effect of labeling on the relative monomer–polymer distribution at apparent equilibrium. Formed microtubules were sedimented, and the relative con-

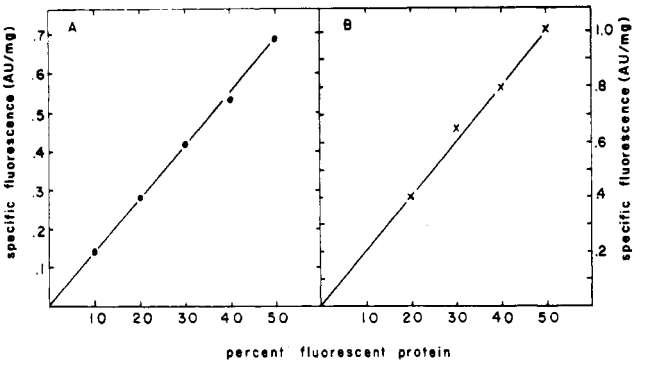

FIGURE 6: Specific fluorescence of assembled microtubules as a function of the percentage of fluorescent microtubule proteins (A) or purified tubulin (B) in the starting mixture. See text for details. Specific fluorescence is here defined as the fluorescence, in arbitrary units (AU), divided by milligrams of protein.

centration of protein remaining in the supernatant (monomer) and pellet (polymer) fractions was determined (Johnson & Borisy, 1975). In two separate experiments (Table II), the relative concentrations of monomer and polymer were not altered by bimane labeling.

Second, the effect of the bimane on the relative viscosity of polymerized microtubules was measured. Microtubules were polymerized in Ostwald viscometers at 37 °C, and after an apparent equilibrium had been reached, buffer (control) or bimane was added, the samples were mixed, and the viscosity was further monitored for an additional 15 min (data not shown). During this time, the labeling reaction goes essentially to completion (Figure 1). Since after 15 min the viscosity of both control and experimental samples remained constant and identical, the results show that labeling with bimane does not alter the relative viscosity of microtubules at 37 °C. Thus, both the sedimentation and viscosity experiments suggest that the labeling reaction does not drastically alter the monomer–polymer distribution for the length of time and concentration of bimane examined here.

Suitability of Fluorescent Tubulin as an Intracellular Probe. In order for a fluorescently labeled protein to serve as a useful cytochemical probe, several criteria must be met. First, the molecule should retain its native conformation as judged by the ability to function in vitro in a manner identical with that of the unlabeled molecule. As described above, the bimane-labeled tubulin behaves in a manner identical with unlabeled protein with respect to its cold lability, molecular weight, kinetics of polymerization, and morphology. In addition, and perhaps more importantly, the labeled molecule must be able to compete quantitatively with an excess of unlabeled tubulin in the assembly reaction. Such a situation would be encountered, for example, in an experiment in which labeled tubulin was microinjected into a cell containing an endogenous pool of tubulin. Thus, the following experiments were performed to determine the efficiency with which labeled tubulin competes with unlabeled tubulin. Increasing amounts of either fluorescent microtubule proteins or purified fluorescent tubulin dimers were added to decreasing amounts of the respective unlabeled proteins, adjusting the relative concentrations of each sample so that the total protein concentration remained constant. The mixtures were then warmed to 37 °C, and after an apparent equilibrium had been reached, the resulting hybrid microtubules were collected by centrifugation and assayed for fluorescence. As shown in Figure 6, the amount of fluorescence measured increased linearly with respect to the percentage of fluorescent microtubule proteins (Figure 6A) or fluorescent tubulin dimers (Figure 6B) present in the original mixtures. Therefore, these data demonstrate that labeled microtubule proteins, which contain tubulin and MAPs, as well as purified tubulin can compete quantitatively with an excess of the respective unlabeled protein for assembly into the polymer. This fact is demonstrated by the linear slopes of the curves in Figure 6. Such a characteristic is an essential criterion for use of any fluorescent derivative in molecular cytochemistry.

Discussion

This paper describes the preparation of native, fluorescently labeled tubulin and MAPs using the sulfhydryl-reactive fluorochrome monobromo(trimethylammonio)bimane (bimane). The labeling procedure described is rapid, and the resulting fluorescent proteins are stable under ultraviolet illumination. The stoichiometry of labeling is reported using two different methods with consistent results. The duration of the labeling can be varied to modify this stoichiometry (Figure 1). The labeling procedure effectively modifies the tubulin dimers without disrupting the monomer–polymer distribution for the times and concentrations examined (Table II). This is in contrast to other reports demonstrating the depolymerization of microtubules and concomitant reduction in the sulfhydryl (SH) titer by SH-active reagents. The possible reasons for this discrepancy are discussed below.

The reversible inhibition of microtubule assembly by SH-active agents has been observed by several groups of workers. As reported by Kuriyama & Sakai (1974), inhibition of assembly was maximal when 2 mol of titratable SH groups per mol of monomer was modified by DTNB, NEM, or PCMPS before initiation of the assembly reaction. A reversible reduction in viscosity was also observed when formed microtubules were treated with SH reagents. Half-maximal reduction was correlated with the loss of one SH group per monomer. In a similar series of experiments, Mellon & Rebhun (1976) examined the effects of diamide on unfractionated microtubule proteins. Inhibition of assembly by diamide was accompanied by a 50% reduction in the SH content of the protein. However, the concentration of diamide used in experiments to calculate the SH content was greater than the concentration needed to inhibit assembly maximally. Using platelet tubulin, Ikeda & Steiner (1978) found complete inhibition of assembly when 3 mol of SH were modified per monomer and a 25% inhibition with modification of only 1 mol of SH per mol of monomer.

These reports strongly suggest that reaction of tubulin in solution with SH-active reagents inhibits assembly and that excess reducing agent can at least partially reverse the effect. The data indicate that some inhibition occurs when only one SH group per monomer is modified. The effect of SH reagents on polymerized microtubules is less clear. Although the data of Kuriyama & Sakai (1974) suggest high sensitivity of formed microtubules to SH reagents, the comments of Mellon & Rebhun (1976) are noteworthy in light of the observations reported here. These authors tested the sensitivity of the SH groups of polymerized microtubules to diamide at a concentration in excess of that needed to abolish completely the polymerization of microtubules. The sensitivity of the formed microtubules to the diamide was extremely variable such that these authors reported that disassembly in the presence of diamide occurred in times as short as 5 min or as long as 2 h.

In the experiments reported here, preformed microtubules were reacted with bimane; thus, SH groups essential for polymerization presumably were not immediately available for modification. In addition, an average of 1 mol of SH per mol of tubulin dimer was modified. Fluorescent labeling of the

tubulin dimers to this extent did not disturb the assembly state or the ability of the dimers to undergo further rounds of assembly–disassembly in vitro (Figure 3; Table II).

The SH content of tubulin has also been examined by several laboratories; the values obtained range from 4 to 12 SH groups per 55 000 daltons (Eipper, 1973; Lee et al., 1973; Mellon & Rebhun, 1976; Kuriyama & Sakai, 1974; Ikeda & Steiner, 1978). Eipper (1973) reported 11 SH groups, all of which could be titrated with 4,4′-dithiopyridine. Lee et al. (1973) found 8 easily titratable SH groups and 11 half-cystines by amino acid analysis, suggesting one disulfide bond per monomer. The data of Mellon & Rebhun (1976) are in agreement with those of Kuriyama & Sakai (1974) with seven titratable SH groups per monomer in brain tubulin. Using platelet tubulin extracted under nitrogen, Ikeda & Steiner (1978) found 12 free SH groups per monomer and confirmed the existence of an intrachain disulfide bond.

The value of 17.16 titratable SH groups per dimer reported here is in the range of values reported for spectrophotometric determinations of SH content. Differences can be accounted for in part by variations in the source of the protein, as most tubulins show similar, yet not identical, amino acid compositions (Stephens, 1971). Also the half-cystine content measured by titration can vary with the type of colorimetric assay employed (Janatova et al., 1968). However, two recently published reports of the amino acid sequence of tubulin suggest that the SH titer reported here for unlabeled tubulin is more nearly correct. Specifically, Ponstingl et al. (1981) have reported 12 half-cystine residues in the α-tubulin from porcine brain, determined by conventional amino acid analysis. By sequencing cloned cDNAs obtained from embryonic chicken mRNAs for α- and β-tubulin, Valenzuela et al. (1981) reported eight half-cystine residues in the β subunit and eight residues in a partial sequence from the α-gene clone. Thus, brain tubulin contains on the order of 20 half-cystine residues per dimer. The value of 17.16 per dimer reported here approaches this value and is slightly higher than most other values reported spectrophotometrically. This may be explained at least in part by the manner in which the titrations reported here were performed. Since the protein was treated with 50 mM DTT and then was subjected to Sephadex G-25 chromatography immediately prior to titration, this would have reduced completely the available cysteines, thus maximizing the amount capable of reaction with DTNB. This step serves to make SH titration from sample to sample and day to day more reproducible, therefore decreasing the variability from one preparation to the next.

The purity of the proteins used will also influence the value obtained. The data presented here demonstrate that the MAPs are labeled by bimane and thus contain free SH groups. Therefore, microtubule protein preparations containing the MAPs will yield different SH titers than purified tubulin dimers. The method of preparation of the protein has also been shown to influence the SH content of tubulin. Microtubule proteins prepared without glycerol contain 4.0 titratable SH groups per monomer; however, when isolated in the presence of glycerol this value is increased to 7.0 (Mellon & Rebhun, 1976). Zeremba & Irwin (1981) further examined this effect by using purified tubulin dimers. Their results show 8.2 titratable SH groups per monomer in the absence of glycerol and 12.06 SH groups per monomer in the presence of glycerol. Their data also demonstrate that storage decreases the number of free SH groups without regard to the method of preparation.

The MAPs which make up the projections from the wall of microtubules assembled in vitro (Dentler et al., 1975; Murphy & Borisy, 1975; Sloboda et al., 1976; Kim et al., 1979) and which are associated with microtubules in situ (Sherline & Schiavone, 1977, 1978; Sheterline, 1978; Sloboda & Dickersin, 1980) are also modified by the bimane. This demonstrates the presence of reactive SH groups on these proteins. Titration of the MAP fraction with DTNB gave inconsistent results, and this can be explained in part by the heterogeneity of the MAP fraction prepared by phosphocellulose chromatography. Thus, current experiments are under way to determine the SH content and extent of labeling of a purified component of the MAP fraction (MAP 2).

The stoichiometry of labeling of the purified dimers, rather than total mitotubule protein (which contains MAPs in addition to tubulin) has been examined in detail for several practical reasons. Tubulin dimers purified from neural tissue copolymerize with microtubules from a variety of sources including flagellar axonemes, isolated mitotic apparatuses, basal bodies, and centrioles and kinetochores from cultured cells (Binder et al., 1975; McGill & Brinkley, 1975; Snell et al., 1974; Telzer et al., 1975; Rebhun et al., 1974; Bergen et al., 1980). Although different tubulins do exist, a conclusion based on slight variations in amino acid sequence data (Luduena & Woodward, 1973; Sanchez et al., 1980), the ability to copolymerize tubulins from different sources has been conserved among the proteins studied to date. Thus, the bimane tubulin described in this paper is a suitable candidate for use as a probe in molecular cytochemistry. However, the presence of MAPs in preparations of microtubules assembled in vitro tends to complicate the matter somewhat. The in vitro data of Griffith & Pollard (1978) and of Runge et al. (1979) indicate that the MAPs may interact with other cytoskeletal elements including microfilaments and intermediate filaments. Components other than, or in addition to, microtubules could then be labeled by fluorescent MAPs in vivo. Moreover, MAPs can also compete with dynein for binding to outer doublet microtubules (Haimo, 1980). This suggests that MAPs could associate both with newly growing microtubules as well as with preexisting microtubules by competition with endogenous MAPs for sites on assembled microtubules. For these reasons, it is essential that purified fluorescent tubulin dimers be used in the technique of molecular cytochemistry to avoid the problems presented by the presence of the MAPs discussed above.

The fluorescent tubulin described in this paper fills all of the criteria essential for a molecular cytochemical probe to study the assembly and disassembly of microtubules in living cells. The tubulin can be labeled stoichiometrically, and it can be purified free from contaminating MAPs. On the basis of the evidence summarized above, bimane-labeled tubulin should be capable of adding on to preexisting microtubule organizing centers when used in lysed cell models of, for example, the mitotic apparatus. The data reported here show that the bimane-labeled fluorescent tubulin retains all of the characteristics of native tubulin in vitro with respect to its ability to assemble into morphologically normal microtubules. In addition, the bimane tubulin should be able to copolymerize with the endogenouss tubulin pool after microinjection into a living cell, since, as described here, fluorescent tubulin can compete quantitatively with unlabeled tubulin during assembly (Figure 6B). This is an important prerequisite for in vitro labeled fluorescent protein destined for use as a molecular cytochemical probe.

One additional use for the fluorescent tubulin should also be mentioned. Since individual fluorescent microtubules can be viewed with an image intensification video camera coupled

to high-resolution fluorescence optics, it may be possible to study in vitro the phenomenon of treadmilling (Margolis & Wilson, 1978). This event is associated with the normal head to tail polymerization of microtubules in vitro and results in a flow of subunits through the microtubule. One could employ bimane tubulin as a marker to test, for example, the effect of various agents such as ATP (Margolis & Wilson, 1979) on the rate of treadmilling and confirm whether or not the phenomenon occurs in vivo as well as in vitro. Various experiments addressing these possible uses for fluorescent tubulin are currently in progress.

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5-(Dimethylamino)naphthalene-1-sulfonic Acid, a Fluorescent Probe of the Medium Chain Fatty Acid Binding Site of Serum Albumin†

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ABSTRACT: Reversible binding of 5-(dimethylamino)-naphthalene-1-sulfonic acid (DNS) to human and bovine serum albumin has been monitored by changes in fluorescence intensity, wavelength maxima, and polarization. DNS has only one major binding site ($k_a = 5 \times 10^6$) and one minor site ($k_a = 3 \times 10^5$) on these proteins. The probe is competitively displaced from its high-affinity site by medium chain fatty acids and by *N*-acetyl-L-tryptophan. The binding site for DNS is highly hydrophobic and is considerably less polar than the hydrocarbon region of lipid bilayers. Resonance energy transfer indicates that the binding site is located 20.7 ± 2 Å from the single tryptophan residue of human serum albumin.

Serum albumin is the intravascular transport protein for cytotoxic lipolytic products, such as fatty acid, lysolecithin, and monoacylglycerol, as well as for many pharmacological agents and metabolites (Switzer & Eder, 1965; Arvidson & Belfrage, 1969; Sollenne & Means, 1979). The specificity of binding of fatty acids to albumin has been studied extensively (Tanford, 1980). The model that best accounts for experimental binding data has been described by Spector and colleagues (Fletcher et al., 1970; Spector et al., 1971; Ashbrook et al., 1972). According to this model, the binding of any fatty acid species to albumin occurs by a stepwise process at multiple individual binding sites that have progressively lower affinity with increasing molar amounts of fatty acid. Furthermore, the affinity of albumin for fatty acids decreases as the chain length decreases. A concise summary of previous studies has been prepared by Tanford (1980).

The interpretation of fatty acid binding data utilizing this model raises several interesting points. It is known that the decrease in affinity for the binding sites is complex with respect to alkyl chain length. A sigmoidal relationship exists between the logarithms of the first two stepwise affinity constants and chain length; however, similar plots of the stepwise binding constants for the sites of lower affinity do not exhibit this behavior. In addition, the affinity of albumin for fatty acids cannot be fully predicted on the basis of chain length alone, as would be expected from hydrophobic partitioning (Karush, 1954). Competition of octanoate acid with either *cis*-octadec-9-enoate or hexadecanoate to albumin shows apparent nonideal behavior (Ashbrook et al., 1972). Binding of the first mole of octanoate is not significantly affected by the long chain acids when the molar ratio of free fatty acid to albumin is less than 2. However, the binding of octanoate decreases substantially in the presence of high ratios of long chain fatty acid to albumin. Double-reciprocal plots of these data are nonlinear and do not have points of intersection in common for the binding isotherm of octanoate at different concentrations of hexadecanoate and *cis*-octadec-9-enoate (Ashbrook et al., 1972). By contrast, a double-reciprocal plot of the displacement of hexadecanoate by octanoate is linear and passes through zero (Meisner & Neet, 1978). The nonideal behavior in this system has been attributed to differences in configurational adaptability of the albumin molecule to fatty acids with different chain lengths (Spector, 1975) and to possible competitive allosterism (Meisner & Neet, 1978).

An alternate explanation for these observations is that albumin has one or more high-affinity sites with specificity for the medium chain fatty acids. Data that support this hypothesis have appeared (Cunningham et al., 1975; Doody & Smith, 1978; Koh & Means, 1979; Lee & McMenamy, 1980; Santos & Spector, 1974; Soltys & Hsia, 1978a,b). In a report of spin-label displacement studies, Soltys and Hsia identified three low-affinity bilirubin binding sites on human albumin that bind the spin probe and medium chain fatty acids, but not long chain fatty acids. No data on the relative binding affinities of these sites for individual fatty acids were reported. Santos & Spector (1974) have noted qualitative differences in the abilities of fatty acids of different chain length to perturb the fluorescence spectrum of 8-anilino-1-naphthalenesulfonic acid (ANS)[1] bound to multiple sites on human serum albumin. Cunningham et al. (1975) noted an effect of chain length on displacement of tryptophan from its binding site on albumin

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; BSA, bovine serum albumin; HSA, human serum albumin; DNS, 5-(dimethylamino)naphthalene-1-sulfonic acid; Q_0, fluorescence quantum yield; $\bar{r}$, ratio of the molar concentrations of the bound ligand and of albumin.

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