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β_2-Tubulin, a Form of Chordate Brain Tubulin with Lesser Reactivity toward an Assembly-Inhibiting Sulfhydryl-Directed Cross-Linking Reagent†

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ABSTRACT: β_1 and β_2 are the designations given to two forms of β-tubulin that have different electrophoretic mobilities on discontinuous polyacrylamide gels in the presence of sodium dodecyl sulfate [Little, M. (1979) *FEBS Lett. 108*, 283–286]. β_1 and β_2 constitute respectively 75% and 25% of the total β-tubulin in bovine brain. Although β_1 appears to be ubiquitous in animals, β_2 has so far only been found in the brains of cows, pigs, deer, rats, chicks, and dogfish but not in squid brain. β_2 is not found in bovine kidneys, in porcine lungs, or in any nonchordate tubulin that has been examined. When tubulin is reacted with the sulfhydryl-directed reagent *N*,*N*′-ethylenebis(iodoacetamide) (EBI), β_1, but not β_2, is converted to a faster moving form, β^*. The yield of β_2 in this reaction is not altered by the presence of drugs. When [^{14}C]EBI is used as a probe, most of the label is incorporated into β_1 rather than β_2. Tubulin molecules that have reacted with EBI to form β^* are much less likely to polymerize into microtubules than are molecules that have not formed β^*. In view of the observation that only β_1, and not β_2, can form β^*, it is possible that β_1 represents a form of tubulin whose assembly may be regulated by a mechanism involving sulfhydryls. In contrast, β_2 may represent a form of tubulin whose assembly is regulated by some other mechanism.

Microtubules are long cylindrical organelles that play critical roles in a variety of cellular processes, including mitosis, secretion, axonal transport, and ciliary and flagellar motion (Roberts & Hyams, 1979). They are composed of a structural protein, tubulin (M_r 100 000), a heterodimer of two M_r 50 000 polypeptide chains (Ludueña et al., 1977), designated α and β. The hypothesis that there may be multiple forms of α- and β-tubulins, with different amino acid sequences, has long been an attractive way to account for the different functional roles of microtubules and for the probable complexity of regulating their assembly in vivo (Fulton & Simpson, 1979). Evidence has been presented, on the basis of peptide mapping, that sea urchin eggs, embryos, and sperm contain a total of six forms of α and six forms of β (Stephens, 1978); similarly, the existence of at least three forms of α and two of β, each with unique electrophoretic mobilities and isoelectric points, has been postulated in mammalian brains (Marotta et al., 1978, 1979a,b). In some of these cases, however, incomplete post-translational modifications or preparative artifacts cannot be rigorously excluded as possible explanations for the apparent heterogeneity. Recently, George et al. (1981), using calf brain tubulin prepared in a variety of ways, have found 17 isoelectric variants of tubulin. They suggest that some of the heterogeneity could arise from partial deamidation of glutamine and asparagine residues, a relatively common phenomenon in proteins (Robinson & Rudd, 1974). The clearest, most direct evidence for heterogeneity comes from the work of Ponstingl and his colleagues (Ponstingl et al., 1981; Krauhs et al., 1981; Little et al., 1981), who showed, by amino acid sequencing of the complete α and β chains, that there exist at least four forms of α and two of β in porcine brain tubulin. In none of the cases of tubulin heterogeneity that have been observed, however, have any of the structural differences between the different isotubulins been able to account for any kind of

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functional difference. Nevertheless, the fact that tubulin is highly conserved in evolution (Ludueña & Woodward, 1973) suggests that any sequence difference between two forms of tubulin should have a functional correlation; this is particularly true for the β chain, which appears to be conserved significantly more than the α chain (Little et al., 1981).

Little (1979), in his preparations of porcine brain tubulin, observed a protein migrating slightly slower than β-tubulin in discontinuous sodium dodecyl sulfate gels (Laemmli, 1970) and eluting after β-tubulin on hydroxylapatite chromatography. By peptide mapping, he showed that this band was a form of β and he designated it β_2, in contrast to the majority species, which he designated β_1. He reported that β_1 and β_2 constitute respectively about 72% and 28% of the total β-tubulin population. In the work reported here, we have examined the tissue and phyletic distributions of β_1 and β_2 and have compared their abilities to react with a sulfhydryl alkylating agent, *N,N'*-ethylenebis(iodoacetamide) (EBI).[1] We have found that, whereas β_1 appears to be ubiquitious among the animals, β_2 may be restricted to chordate brains. We have also found that EBI converts β_1, but not β_2, into a form, designated β^*, that migrates faster than β_1 on discontinuous $NaDodSO_4$ gels. Using [^{14}C]EBI, we have shown that β_2 reacts much less well with EBI than does β_1. Since EBI inhibits microtubule assembly in vitro (Palanivelu et al., 1981; Palanivelu & Ludueña, 1982), it is possible that β_1 represents a type of tubulin dimer, $\alpha\beta_1$, whose assembly may be regulated by a mechanism involving sulfhydryl groups. In contrast, β_2 may represent a form of tubulin, $\alpha\beta_2$, whose assembly may be regulated by some other mechanism.

Experimental Procedures

Materials. Sodium iodoacetate, iodoacetamide, fast green FCF, colchicine, reduced glutathione, and GTP were from Sigma Chemical Co., St. Louis, MO; Coomassie brilliant blue G250 was from Ames Co., Elkhart, IN; acrylamide and *N,N'*-methylenebis(acrylamide) were from Eastman Chemical Co., Rochester, NY; sodium dodecyl sulfate was from Accurate Chemical and Scientific Corp., Hicksville, NY; ethylenediamine, 1,2-dichloroethane, podophyllotoxin, and nocodazole were from Aldrich Chemical Co. Inc., Milwaukee, WI; iodoacetyl chloride was from ICN Pharmaceuticals, Plainview, NY; iodoacetic anhydride was from Alfa Products, Danvers, MA. Vinblastine sulfate and vincristine sulfate were kind gifts of the Eli Lilly Co., Indianapolis, IN; maytansine (NSC 153858) was the kind gift of Dr. John Douros of the Developmental Therapeutics Program, Chemotherapy, The National Cancer Institute. [U-^{14}C]Ethylenediamine hydrochloride and NCS tissue solubilizer were from Amersham Corp., Arlington Heights, IL; Omnifluor and L-[*glycine*-2-^{3}H]glutathione were from New England Nuclear, Boston, MA. The [^{3}H]glutathione was diluted with unlabeled GSH to a final specific activity of 1.38 Ci/mol.

Syntheses of EBI and [^{14}C]*EBI.* EBI was synthesized from iodoacetyl chloride and ethylenediamine by the method of Ozawa (1967) as previously described (Ludueña & Roach, 1981a). ^{14}C-Labeled EBI was synthesized as follows: 250 μCi of [U-^{14}C]ethylenediamine hydrochloride (25 Ci/mol) was diluted 1:10 with cold ethylenediamine and the pH adjusted to 7.0 with 1 N NaOH. The solution was mixed with 0.22 M iodoacetic acid anhydride in 1,2-dichloroethane. The solution was vortexed for 5 min and the resulting precipitate collected by centrifugation at 20000*g* for 30 min at 1 °C. The precipitate, which was [^{14}C]EBI, was dried by lyophilization. The structure of [^{14}C]EBI was not verified directly. However, unlabeled EBI was synthesized by the same procedure and its structure verified by mass spectrometry. The specific activity of [^{14}C]EBI was determined by reacting it with [^{3}H]GSH by using a modification of the procedure used by Ludueña & Roach (1981a) to calibrate the specific activity of [^{14}C]-iodoacetamide. The method was as follows: 2.5 mM [^{3}H]-GSH was reacted for 2 h at 25 °C in the dark with an 11.4-fold molar excess of [^{14}C]EBI in a solution of 0.04 M ammonium acetate, pH 7.0, in 58% dimethyl sulfoxide. Aliquots of the reaction mixture were applied to 20 cm × 20 cm Anasil microcrystalline cellulose 250-μm thin-layer plates (Analabs, North Haven, CT) and subjected to electrophoresis at 650 V in a 900:100:4 water:pyridine:glacial acetic acid solvent in a Brinkmann double-chamber thin-layer electrophoresis apparatus. Unlabeled GSH and GSSG were used as standards. The electrophoresis was continued until the picric acid marker had migrated 10 cm. The plates were then dried under a stream of hot air and sprayed with a solution of 0.5% ninhydrin in 95% acetone. When the spots had appeared, the plates were scored and the areas containing spots scraped into scintillation vials. The vials were then filled with toluene–Omnifluor scintillation fluid and counted in a Beckman LS 7000 liquid scintillation counter. When the results were obtained, it was found that about 55% of the ^{3}H counts in the [^{14}C]EBI/[^{3}H]GSH reaction mixture migrated with the mobility of GSH but did not stain with ninhydrin. Since this area did not contain ^{14}C counts, it was assumed to represent an unreactive contaminant in the [^{3}H]GSH, and the reacted glutathione was assumed to be distributed among the ninhydrin-positive spots proportionally according to their ^{3}H counts. A total of 77–81% of the glutathione was in a heavily stained spot with a mobility equal to 65% of that of unreacted GSH. Since no spot was observed in the region of GSH and because of the large excess of EBI over GSH in the reaction mixture, this spot was assumed to represent a monomeric EBI–glutathione product. On the basis of these considerations, the specific activities of the different batches of [^{14}C]EBI were estimated, ranging from 1.8 to 2.0 Ci/mol. These values were equal to 72–81% of the expected specific activity of 2.5 Ci/mol based on the 1:10 dilution of the original [^{14}C]ethylenediamine.

Tubulin Preparations. Cow brains were obtained from Roegelein Co., San Antonio, TX. Adult male Sprague-Dawley rats were decapitated and the brains removed immediately. A doe of the species *Odocoileus virginianus* (white-tailed deer) was shot on December 1, 1981, at the Walters Ranch, Kerr County, TX; the doe's brain was removed 3 h after her death. Microtubule protein was prepared from the cerebra of cows, rats, and deer by a cycle of assembly in the presence of glycerol according to Fellous et al. (1977). The same method was also used to prepare microtuble protein from bovine cerebellum and from preparations of gray matter and white matter from bovine cerebra. Tubulin was purified from microtubule protein by chromatography on phosphocellulose (Fellous et al., 1977). Unless otherwise indicated, all the tubulin used in experiments described in this paper was prepared from bovine cerebra by this procedure. Microtubule protein was also prepared from cow brains by three cycles of assembly and disassembly in the absence of glycerol, according to Asnes & Wilson (1979).

[1] Abbreviations: MAPs, microtubule-associated proteins; MAP 2, the high molecular weight MAP; buffer A, 100 mM 2-(*N*-morpholino)-ethanesulfonic acid, pH 6.4, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM $MgCl_2$, and 1 mM guanosine 5'-triphosphate (GTP); EBI, *N,N'*-ethylenebis(iodoacetamide); $NaDodSO_4$, sodium dodecyl sulfate; GSH, reduced glutathione.

Tubulin was purified from embryonic chick brains and from pig brains by the procedure of Eipper (1972). Tubulins purified by two cycles of assembly and disassembly (Langford, 1978) from the brain of the smooth dogfish shark *Mustelus canis* and from the brain of the squid *Loligo pealei* were the kind gifts of Dr. George Langford, the University of North Carolina, Chapel Hill, NC. Tubulin from bovine kidneys, prepared by recycling in the presence of glycerol (Barnes & Roberson, 1979), was the kind gift of Dr. Larry Barnes, the University of Texas Health Science Center, San Antonio, TX. Tubulin from porcine lungs, prepared by recycling, was the kind gift of Dr. Jesûs Avila, Universidad Autônoma de Madrid, Madrid, Spain. Tubulin prepared by vinblastine precipitation from extracts of eggs of the sea urchin *Strongylocentrotus purpuratus* was the kind gift of Dr. Clara Asnes, Friday Harbor Laboratories, Friday Harbor, WA, as was outer doublet tubulin from the sperm flagella of the sea anemone *Tealia crassicornis* and the polychaete worm *Schizobranchia insignis*. Tubulin prepared by recycling (Farrell & Wilson, 1978) from the outer doublets of the sperm flagella of *S. purpuratus* was the kind gift of Dr. Kevin Farrell, the University of California, Santa Barbara, CA. Outer doublet tubulins were isolated from sperm flagella of the echiuroid *Urechis caupo*, the tunicate *Ciona intestinalis*, and the abalone *Haliotis rufescens* and from gill cilia of the clam *Mercenaria mercenaria* and the brachiopod *Laqueus californicus* as previously described (Little et al., 1981; Ludueña & Little, 1981). Flagellar central pair microtubule tubulins were prepared by the method of Stephens (1970) from the sea urchin *Lytechinus pictus* and from *Urechis*, *Haliotis*, and *Ciona*. Ciliary central pair microtubule tubulins from *Mercenaria* were also prepared by this method. All tubulins used for determination of the presence of β_2 were reduced and carboxymethylated with sodium iodoacetate (Crestfield et al., 1963). Heat-stable bovine brain MAPs as well as bovine τ and MAP 2 were prepared as previously described (Fellous et al., 1977).

Alkylation Reactions. Bovine brain tubulin was used for alkylation experiments immediately after purification on phosphocellulose. The tubulin samples were incubated with EBI or [^{14}C]EBI, as indicated, dialyzed against buffer A containing 1 mM β-mercaptoethanol, and then reduced and carboxymethylated and run on discontinuous 6% polyacrylamide gels containing $NaDodSO_4$ (Laemmli, 1970). Unless otherwise indicated, all gel electrophoreses were done in this system. For some experiments, 6% polyacrylamide gels in the system of Yang & Criddle (1970) were used. Tube gels were stained in fast green and scanned at 640 nm in a Gilford 250 spectrophotometer equipped with linear transport. Slab gels were stained with Coomassie brilliant blue G250 and scanned on a Helena quick-scan densitometer. The areas of the peaks on the gel scans were determined by planimetry. When it was desired to know the amount of aggregated cross-linked β-tubulin generated by EBI, the alkylation reaction was done in the presence of reduced and carboxymethylated conalbumin (Ludueña & Roach, 1981a). The amount of aggregated β-tubulin (β_A) was determined by the following formula: $\beta_A = R_\beta C - \beta_1 - \beta_2 - \beta^*$, where C, β_1, β_2, and β^* represent respectively the areas of conalbumin, β_1, β_2, and β^* peaks in the sample and R_β is the ratio of the sum of the areas of the β_1 and β_2 peaks to that of the conalbumin peak in a control sample not treated with EBI. Radioactive gels were sliced, and the radioactivity of the slices was determined as previously described (Ludueña & Roach, 1981a). Since small amounts of cross-linked aggregate could be formed by [^{14}C]EBI, a sample of reduced and carboxymethylated tubulin, of known protein concentration, that had not been treated with EBI was always run in the same set of gels as the EBI-treated samples in order to determine the ratio between peak size on the gel and the amount of protein in the peak.

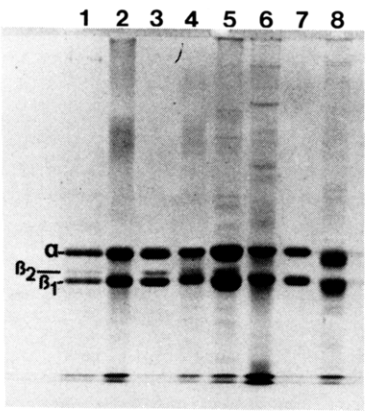

FIGURE 1: Electrophoresis of various tubulins. A set of reduced and carboxymethylated tubulins from various sources were subjected to electrophoresis on a 6% slab gel with the system of Laemmli (1970). The gel was stained with Coomassie brilliant blue G-250. Tubulin samples are as follows: (1) bovine cerebra, (2) bovine kidney, (3) rat brain, (4) chick brain, (5) dogfish brain, (6) sea urchin (*S. purpuratus*) egg, (7) *S. purpuratus* sperm outer doublets, and (8) squid brain. The α, β_1, and β_2 bands are indicated.

Other Methods. Microtubule assembly at 37 °C was monitored by turbidimetry at 350 nm in a Gilford 250 spectrophotometer equipped with an automatic cuvette programmer. In some experiments, the extent of microtubule assembly was estimated by centrifuging the microtubule suspension at 39000*g* for 40 min at 30 °C and then resuspending the pellet in water and measuring the protein concentrations. All protein determinations were done by the method of Lowry et al. (1951).

Results

Distribution of β_1- and β_2-Tubulins. Analysis of the various tubulins showed that the β_1 band was present in brain tubulin from rats, pigs, cows, deer, chicks, dogfish, and squid, as well as in bovine kidney and porcine lung tubulins. It was also present in outer doublet tubulins from *Strongylocentrotus*, *Ciona*, *Urechis*, *Mercenaria*, *Haliotis*, *Laqueus*, *Schizobranchia*, and *Tealia* and in central pair tubulins from *Lytechinus*, *Ciona*, *Urechis*, *Mercenaria*, and *Haliotis*. The β_2 band, however, was not present in any nonchordate examined. In fact, no nonchordate tubulin contained more than one β band. In contrast, β_2 was present in all the chordate brain tubulins examined but not in bovine kidney or porcine lung tubulin (Figure 1). The facile demonstration of the presence of β_2 required that the $NaDodSO_4$ gel system of Laemmli (1970) be used. When the closely related system of Yang & Criddle (1970) was used, β_1 and β_2 migrated so closely together that they could only be resolved when very small amounts of tubulin were run on the gels. Similarly, when tubulin was alkylated with iodoacetamide instead of iodoacetate, β_1 and β_2 comigrated. The yield of β_2 expressed as a percentage of the total of the β_1 and β_2 bands and as measured in Coomassie blue stained gels varied with the source organism as follows: cow brain (15%), rat brain (19%), chick brain (14%), and dogfish brain (8%). When the more quantitative stain fast green was used, the yield of β_2 in bovine cerebral tubulin prepared by the method of Fellous et al. (1977) was 25.8 ± 2.6%. Similarly, the yield of β_2 in bovine cerebral tubulin prepared by the method of Asnes & Wilson (1979) was 24.8 ± 0.7%. The yield of β_2 in deer brain tubulin was 20.2 ± 1.6%.

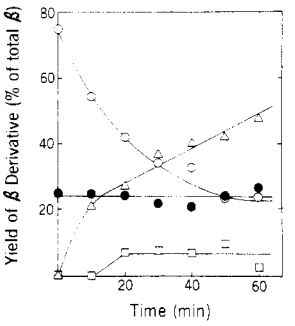

FIGURE 2: Effect of EBI incubation on yields of β_1, β_2, β^*, and aggregated β. 255-μL aliquots of phosphocellulose-purified tubulin (0.76 mg/mL) containing reduced and carboxymethylated conalbumin (0.2 mg/mL) were incubated at 30 °C with 0.67 mM EBI. At the indicated times, the samples were processed and analyzed as described under Experimental Procedures. The relative amounts of β_1 (○), β_2 (●), β^* (△), and aggregated β (□) were determined. The results are expressed as a percentage of the total β content. Each time point represents a separate incubation.

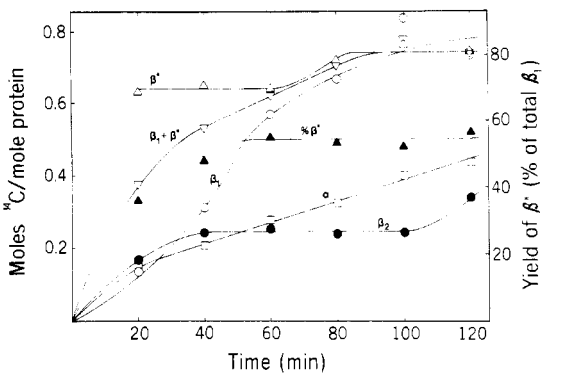

FIGURE 3: Reaction of α-, β_1-, and β_2-tubulins with [^{14}C]EBI. 270-μL aliquots of phosphocellulose-purified tubulin (0.69 mg/mL) containing reduced and carboxymethylated conalbumin (0.19 mg/mL) were incubated with 0.63 mM [^{14}C]EBI (1.8 Ci/mol) at 30 °C. At the indicated times samples were processed and analyzed as described under Experimental Procedures. The incorporation of label into each band was determined as described. Each time point represents a separate incubation. The graph shows the incorporation of label into the α (□), residual β_1 (○), β_2 (●), and β^* (△) bands as well as the total β_1 (▽), consisting of β^* and residual β_1. The graph also shows the yield of the β^* band (▲) as a percentage of the total β_1. The percent $\beta^* = [\beta^*/(RC_n)] \times 100$, where C_n and β^* represent the areas of the conalbumin and β^* bands on the gel and R is equal to the ratio of the areas of β_1 and conalbumin on a control gel of a sample not treated with EBI.

Analysis of subfractions of brain tubulin showed no significant difference between bovine cerebral and cerebellar tubulin, the yield of β_2 in the latter being 24.1 ± 2.2%. However, a small difference in distribution of β_2 between cerebral gray matter and white matter was observed, with gray matter having a β_2 yield of 25.7 ± 0.7% and with white matter having a yield of 20.7 ± 0.5%. The gray matter tubulin recycled with MAP 2, while the white matter tubulin did not. This observation is similar to that of Vallee (1982), who found significantly more MAP 2 in gray matter tubulin than in white matter tubulin.

Reaction of β_1 and β_2 with EBI. When freshly prepared bovine brain tubulin is incubated with EBI, a substantial portion of β-tubulin is converted to a form, designated β^*, that migrates faster than β_1 on NaDodSO$_4$ gels; β^* is apparently an intrachain cross-linked form of β (Ludueña & Roach, 1981a). Close examination of gels of samples generated in a typical experiment with EBI shows that the β^* band comes almost exclusively from the β_1 band; the yield of the β_2 band stays fairly constant during this reaction (Figure 2). Even much of the low yield of aggregated cross-linked β seems to arise from β_1 rather than from β_2. Ludueña & Roach (1981b,c) showed that the production of β^* by EBI is markedly influenced by the presence of drugs; vinblastine and maytansine enhance the yield of β^*, while colchicine and podophyllotoxin prevent β^* production. However, in an experiment where tubulin was incubated with 100 μM concentrations of drugs prior to EBI treatment, the yield of β_2 was not significantly affected by the drugs. The yields of β_2 in the presence of each drug were as follows: colchicine, 20.4 ± 1.1%; podophyllotoxin, 21.3 ± 0.3%; vinblastine, 21.3 ± 0.9%, maytansine, 20.6 ± 0.6%; vinblastine and colchicine together, 23.1 ± 0.4%. In the absence of added drug, the yield was 21.1 ± 0.5%. The only significant effect of the drugs was the slight increase in the yield of β_2 in the presence of colchicine and vinblastine together. This probably reflects the fact that this drug combination is the most potent suppressor of the formation of cross-linked aggregate by EBI (Ludueña & Roach, 1981b).

Reaction of α, β_1, and β_2 with [^{14}C]EBI. The observation that β^* comes from β_1 and not from β_2 does not necessarily imply that EBI reacts better with β_1 than with β_2, only that its presumed reaction product with β_2 does not have an altered electrophoretic mobility. So that the abilities of β_1 and β_2 to react with EBI could be compared, phosphocellulose-purified tubulin was incubated with [^{14}C]EBI, and the extent of incorporation of ^{14}C into each chain was determined with respect to incubation time (Figure 3). After 20 min of reaction, the β_1 chain has incorporated 2.3 times more label than has the β_2 chain and 2.6 times more than has the α chain. After 40 min, incorporation into β_2 stays constant for about 1 h at a value of 0.25 mol of label/mol of tubulin while the incorporation into both residual β_1 and α continues to increase. As the reaction proceeds, part of the β_1 population is converted to β^*. The increase in the yield of β^* is roughly paralleled for the first hour by an increase in the specific radioactivity of the total β_1 (including both β^* and the residual β_1). Although the yield of β^* increases in the first hour, the specific radioactivity of β^* stays constant at about 0.64 mol/mol. Thereafter, the yield of β^* stays constant while its specific radioactivity increases. These results are consistent with the model that EBI converts β_1 to β^* by a reaction with specific sulfhydryl groups (Ludueña & Roach, 1981a) and that further alkylation of other sulfhydryl groups can occur in both β^* and the residual β_1 when the first group of sulfhydryls have reacted with EBI. The results in Figure 3 also indicate that β_1 reacts more quickly with EBI than does β_2.

Effect of EBI on Microtubule Assembly in Vitro. Palanivelu et al. (1981) and Palanivelu & Ludueña (1982) have previously shown that the reaction of EBI with the β chain to generate β^* will inhibit microtubule assembly, and they have suggested that the assembly-critical sulfhydryls are the ones involved in β^* formation. In the previous study, no attempt was made to discriminate between the two β chains; thus, even though the results presented above show that β_1 reacts faster with EBI at 30 °C than does β_2, it is necessary to determine if the difference in reaction rates is also present under conditions where assembly inhibition may be measured. Due to the lability of pure tubulin, it is necessary to do the reaction with EBI at 0 °C and then reconstitute it with MAPs and measure its ability to assembly at 37 °C. Such an experiment is shown in Figure 4. Of the three tubulin chains, β_1 is the most reactive with EBI; it reacts about 1.6 times and 2.3 times as rapidly as do β_2 and α, respectively. Figure 4 shows that assembly inhibition, the yield of β^*, and the incorporation of ^{14}C into the α, β_2, and β_1 chains all increase with time. A

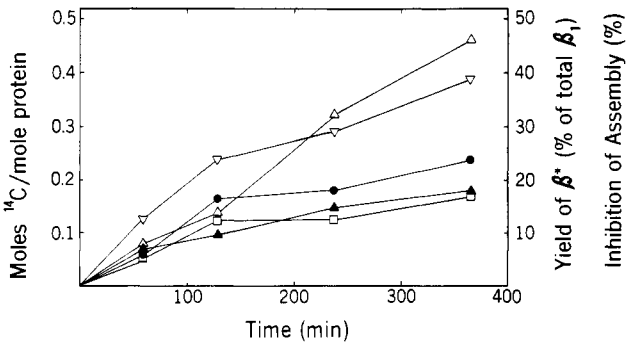

FIGURE 4: Effects of preincubation with [^{14}C]EBI on microtubule assembly and alkylation of different tubulin chains. Two samples of phosphocellulose-purified tubulin (1.09 mg/mL) were incubated at 0 °C in the presence or absence of 0.3 mM [^{14}C]EBI (1.7 Ci/mol). Both samples were 2% in dimethyl sulfoxide. At the indicated times aliquots were removed from each sample and diluted with GSH and heat-stable MAPs to give the following final concentrations: tubulin, 1.0 mg/mL; GSH, 2 mM; MAPs, 0.14 mg/mL. A 200-μL portion of the EBI-treated aliquot was immediately dialyzed at 1 °C against buffer A containing 1 mM β-mercaptoethanol. The remainders of the aliquots were incubated at 37 °C in a Gilford 250 spectrophotometer. Microtubule assembly was monitored by turbidimetry at 350 nm. The change in absorbance of each sample after 20 min of incubation was determined for both aliquots. The percent inhibition of assembly at each time point due to EBI pretreatment is shown in the figure (△). The dialyzed aliquots were reduced and carboxymethylated and analyzed on gels. The gels were stained, scanned, and sliced as described under Experimental Procedures. The figure shows the yield of β^* (▲) as a percentage of the total β_1 and the specific activities of the α (□), β_2 (●), and β_1 chains (▽). The specific activity of β_1 includes the contribution from both β^* and residual β_1.

simple extrapolation of the data in Figure 4 suggests that 50% inhibition of assembly would be attained at about 400 min of incubation with EBI, at a time when the yield of β^* would be 19% and the specific radioactivities of α, β_2, and β_1 would be 0.18, 0.26, and 0.42 mol of ^{14}C/mol of tubulin, respectively. In order to determine which of these reactions with EBI is the most critical in determining whether or not a tubulin molecule can assemble, it is necessary to collect the microtubules in a sample whose assembly has been partially inhibited by EBI and compare the extents to which the different polypeptide chains have reacted with EBI. The microtubules can best be collected by centrifugation at 30 °C. In one such experiment, where phosphocellulose-purified tubulin was preincubated with 0.89 mM [^{14}C]EBI for 15 min at 0 °C and then reconstituted with τ to give final concentrations of tubulin and τ of 1.0 and 0.1 mg/mL, respectively, assembly was found to be inhibited by 38%. The specific radioactivities of the α, β_2, and β_1 chains in the supernatant were respectively 0.06, 0.03, and 0.17 mol of ^{14}C/mol of tubulin and in the pellet were respectively 0.05, 0.01, and 0.10 mol of ^{14}C/mol of tubulin. The differences in specific activities between the supernatant and the pellet for α, β_2, and β_1 were respectively 0.01, 0.02, and 0.07 mol of ^{14}C/mol of tubulin. In a similar experiment where tubulin was preincubated with 0.89 mM [^{14}C]EBI for 15 min at 0 °C and then reconstituted with MAP 2 to give final concentrations for tubulin and MAP 2 of 0.94 and 0.19 mg/mL, respectively, assembly was found to be inhibited by 50%. The specific activities of the α, β_2, and β_1 chains were respectively 0.06, 0.16, and 0.19 mol of ^{14}C/mol of tubulin in the supernatant and 0.05, 0.16, and 0.07 mol of ^{14}C/mol of tubulin in the pellet. The differences in specific activities between supernatant and pellet for α, β_2, and β_1 were respectively 0.01, 0, and 0.12 mol of ^{14}C/mol of tubulin. In each of these two cases, the chain showing the largest difference between the supernatant and the pellet was β_1. The most striking difference between the supernatant and pellet in this type of experiment, however,

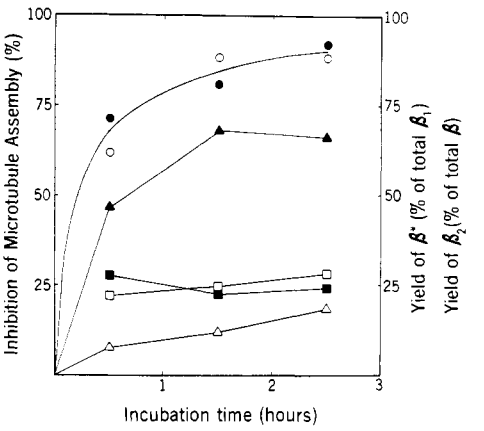

FIGURE 5: Distribution of β^*- and β_2-tubulins in supernatants and pellets of samples of microtubule protein treated with EBI prior to microtubule assembly. Two 2.04-mL samples of microtubule protein (0.98 mg/mL) were incubated at 0 °C in the presence or absence of 0.59 mM EBI. Both samples were 2% in dimethyl sulfoxide. At the indicated times, 0.5-mL aliquots were withdrawn from each sample, made 2 mM in dithiothreitol, and incubated for 30 min at 37 °C to permit microtubule assembly. At the end of 30 min each aliquot was centrifuged at 39000g for 40 min at 30 °C. Each pellet was rinsed in warm buffer A and resuspended in 5.2 mM dithiothreitol. An aliquot was removed from each pellet and supernatant for protein determination. The pellets and supernatants were dialyzed against buffer A, reduced and carboxymethylated, and run on 6% gels (Laemmli, 1970). The gels were stained and scanned and the areas of the α, β_2, β_1, and β^* peaks determined by planimetry. The percent β^* on each gel was calculated from the formula percent $\beta^* = [\beta^*/(\beta_1 + \beta^*)] \times 100$. The percent β_2 on each gel was calculated from the formula percent $\beta_2 = [\beta_2/(\beta_1 + \beta_2 + \beta^*)] \times 100$. The figure shows the percent β^* in each supernatant (▲) and pellet (△) and the percent β_2 in each supernatant (■) and pellet (□). The ratio of protein content to peak area on the gel scan was calculated by using a sample of tubulin that had not been treated with EBI. This value was used to estimate the fraction of tubulin in the EBI-treated samples that was in the form of a cross-linked aggregate that did not enter the gels. Most of the cross-linked aggregate pelleted with the microtubules. The percentage of tubulin that was in the form of cross-linked aggregate increased to 76% in the pellet and to 16% in the supernatant in the course of the incubations. The extent of tubulin polymerization was measured directly from the protein concentration in the resuspended pellets and indirectly from the protein concentration in the supernatants and was corrected for the fraction of pellet that consisted of cross-linked aggregate. The inhibition of assembly due to reaction with EBI was determined at each time point by comparison of the extent of microtubule assembly in the EBI-treated sample and the control sample. The figure shows the percent inhibition as derived starting both with the supernatants (●) and the pellets (○).

is the high yield of β^* in the supernatant and its low yield in the pellet. As can be seen from Figure 5, the percent β^* in the supernatants increased from 47% to 79% in a period of 3.5 h, while the percent β^* in the pellets increased from 8% to 18% in that same period of time. Electrophoretic analysis of the pellets showed that the percentage of high molecular weight cross-linked aggregate increased from 47% to 76% in this time. The presence of large amounts of cross-linked aggregate in the pellets suggests that perhaps the small amount of β^* in the pellets could represent trapped supernatant. Figure 5 also shows that incubation with EBI had little or no effect on the distribution of β_2 between supernatant and pellet, suggesting that the higher reactivity of β_1 with EBI did not lead to an enrichment for β_2 in the microtubules.

Interactions of Putative $\alpha\beta_2$ Dimer with Antitubulin Drugs. The fact that the total of the β_2 and β_1 contents in a tubulin sample is equal to the content of α (Little, 1979) suggests that the tubulin population must consist of at least two types of tubulin dimer, here designated $\alpha\beta_1$ and $\alpha\beta_2$. Since β_2 could not be separated from β_1 without undergoing reduction and

Table I: Effect of Drugs on Reaction of α, β_1, and β_2 Chains with [^{14}C]EBI[a]

drug added	incorporation of ^{14}C (% of control)		
	α	β_1	β_2
none	100 ± 2[b]	100 ± 4[c]	100 ± 1[d]
colchicine	88 ± 5	20 ± 4	51 ± 14
podophyllotoxin	89 ± 9	29 ± 5	96 ± 21
nocodazole	83 ± 6	15 ± 1	41 ± 10
vinblastine	44 ± 5	85 ± 7	65 ± 17
vincristine	31 ± 4	81 ± 9	38 ± 14
maytansine	89 ± 4	87 ± 8	84 ± 10
colchicine and vinblastine	26 ± 3	16 ± 5	32 ± 6

[a] 265-μL aliquots of tubulin (0.71 mg/mL) were incubated at 37 °C for 20 min in the presence of 100 μM concentrations of the indicated drug. The samples were then made 0.63 mM in [^{14}C]EBI (1.8 Ci/mol) and incubated at 30 ° for 60 min. Samples were then processed and analyzed as described under Experimental Procedures, and the incorporation of label into the α, β_1, and β_2 chains was determined. Incorporation into β_1 reflects contributions from both β* and residual β_1. All incubations were done in triplicate; standard deviations are given. [b] Control value = 0.29 mol of ^{14}C/mol of α. [c] Control value = 0.72 mol of ^{14}C/mol of β_1. [d] Control value = 0.23 mol of ^{14}C/mol of β_2.

Table II: Effect of Maytansine on Alkylation of α, β_1, and β_2 Chains by [^{14}C]EBI[a]

drug added	incorporation of ^{14}C (% of control)		
	α	β_1	β_2
none	100 ± 3[b]	100 ± 3[c]	100 ± 9[d]
10 μM vincristine	44 ± 3	89 ± 7	62 ± 10
10 μM maytansine	92 ± 8	99 ± 7	84 ± 17
10 μM vincristine and 10 μM maytansine	106 ± 7	104 ± 9	84 ± 9
100 μM vincristine	34 ± 3	85 ± 7	46 ± 9
100 μM vincristine and 100 μM maytansine	95 ± 4	115 ± 9	98 ± 21

[a] 260-μL aliquots of phosphocellulose-purified tubulin (0.72 mg/mL) were reacted for 15 min at 37 °C with the indicated drug and then for 60 min at 30 °C with 0.64 mM [^{14}C]EBI (1.8 Ci/mol). The samples were then processed as described under Experimental Procedures, and the incorporation of label into the α, β_1, and β_2 chains was determined. All incubations were done in triplicate. Standard deviations are indicated. [b] Control value = 0.62 mol of ^{14}C/mol of α. [c] Control value = 1.15 mol of ^{14}C/mol of β_1. [d] Control value = 0.56 mol of ^{14}C/mol of β_2.

carboxymethylation, the drug-binding properties of the putative $\alpha\beta_2$ dimer, in its native configuration, could not be directly determined. They could, however, be examined indirectly by measuring the effects of drugs on the reactions of the α, β_1, and β_2 chains with [^{14}C]EBI. The rationale for these experiments is that a drug that affects the alkylation of β_2 must bind to the putative $\alpha\beta_2$ dimer. As is shown in Table I, colchicine, podophyllotoxin, and nocodazole have little effect on α but strongly inhibit the reaction of β_1 with [^{14}C]EBI. Colchicine and nocodazole also inhibit the reaction of β_2 with [^{14}C]EBI. In contrast, vinblastine and vincristine strongly suppress the reaction of α with [^{14}C]EBI but have a very small effect on β_1. They do, however, markedly inhibit the reaction of β_2 with [^{14}C]EBI. Maytansine is a drug that, when used alone, does not affect the alkylation of tubulin, but when it is used in combination with vinblastine, it abolishes the inhibitory effect of vinblastine (Ludueña & Roach, 1981c). Table II shows that maytansine can abolish the inhibitory effects of vincristine on α, β_1, and β_2. These results suggest that the putative $\alpha\beta_2$ dimer is capable of interacting with colchicine, nocodazole, vinblastine, vincristine, and maytansine but do not tell us whether the drug-binding affinity of $\alpha\beta_2$ differs from that of $\alpha\beta_1$. An experiment designed to test this in the case of vincristine is shown in Figure 6. Here tubulin was alkylated with [^{14}C]EBI in the presence of a series of vincristine concentrations. The results show that increasing vincristine concentrations suppress the alkylation of all three chains. Although the standard deviations are large, the results suggest that half-maximal suppression of alkylation for each chain is obtained at a vincristine concentration around 5 μM. This implies that there is no major difference in the drug-binding affinities for vincristine between $\alpha\beta_1$ and $\alpha\beta_2$.

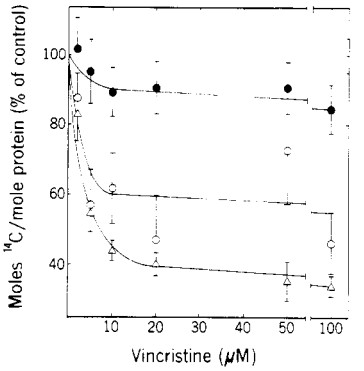

FIGURE 6: Effect of vincristine concentration on alkylation of α, β_1, and β_2 chains by [^{14}C]EBI. 260-μL aliquots of phosphocellulose-purified tubulin (0.72 mg/mL) were incubated for 15 min at 37 °C with the indicated concentrations of vincristine and then for 60 min at 30 °C with 0.64 mM [^{14}C]EBI (1.8 Ci/mol). The samples were then processed and analyzed as described under Experimental Procedures, and the specific activities of the α (△), β_2 (○), and β_1 (●) chains determined. Each incubation was done in triplicate. Standard deviations are shown. The control values for incorporation of ^{14}C into each chain were as follows: α, 0.65 mol of ^{14}C/mol of α; β_1, 1.21 mol of ^{14}C/mol of β_1; β_2, 0.65 mol of ^{14}C/mol of β_2.

Discussion

This paper reports that β_2-tubulin, which constitutes about 25% of the total β-tubulin in bovine cerebra, is restricted in both its tissue and organismal distribution and differs from β_1, the majority species, in its ability to react with EBI, a sulfhydryl-directed bifunctional alkylating agent that is a potent inhibitor of microtubule assembly in vitro. The fact that β_2 was present only in vertebrate brain tubulin, prepared by any of various isolation procedures, and was not seen in tubulins from other sources, even when these latter were prepared by procedures similar to those used for brain tubulin, clearly indicates that β_2 is not an artifact of the isolation procedure. Since the total of the yields of β_1 and β_2 is equal to that of α (Little, 1979), we must postulate the existence, in vertebrate brains, of at least two kinds of tubulin dimer, here designated as $\alpha\beta_1$ and $\alpha\beta_2$.

There have been many reports of heterogeneity in vertebrate brain tubulin in which there appear to be multiple forms of both α and β, differing from each other in their isoelectric points and, to a lesser extent, their electrophoretic mobilities on NaDodSO$_4$ gels (Marotta et al., 1978, 1979a; Gozes & Littauer, 1978; Gozes et al., 1979; Nelles & Bamburg, 1979; George et al., 1981). The heterogeneity has been in some cases correlated with cell type or developmental stage (Dahl & Weibel, 1979; Gozes et al., 1979; Marotta et al., 1979b). It would naturally be of interest to correlate the β_1/β_2 heterogeneity that we observe with these other reported heterogeneities. However, in some of these reports, the tubulin was not alkylated prior to analysis (Marotta et al., 1978; Gozes & Littauer, 1978; Gozes et al., 1979; Dahl & Weibel, 1979). This has the potential disadvantage, already alluded to above, of not reversing partial oxidation of sulfhydryl groups that may have occurred during the isolation procedure or of generating

such groups during the analytical procedure, in either case conceivably producing an artifactually large number of tubulin species. The other studies on tubulin heterogeneity in brain have used tubulin that was alkylated with iodoacetamide rather than iodoacetate, which is used in this paper (Nelles & Bamburg,1979; George et al., 1981). As we have shown, β_1 and β_2 have identical electrophoretic mobilities on $NaDodSO_4$ gels when they have been alkylated with iodoacetamide. Undoubtedly, tubulin molecules that contain 8–11 acidic carboxymethyl groups will have different isoelectric points than tubulin molecules whose sulfhydryls have been substituted with neutral carboxamidomethyl groups. It is thus not yet possible to correlate the β_1/β_2 heterogeneity described in this paper with the heterogeneities reported by others. It is certainly possible, however, that β_1 and β_2 each correspond to a family of isotubulins.

We have previously shown that EBI reacts with native tubulin to generate an intrachain cross-linked form of β, designated β^*, that migrates ahead of β on discontinuous $NaDodSO_4$ gels (Ludueña & Roach, 1981a). Close analysis of the stoichiometry of this reaction indicates that the increase in the yield of β^* is correlated with a decrease in the yield of β_1, whereas the yield of β_2 stays roughly constant (Figure 2). This implies that β^* is generated from β_1 and not from β_2. Therefore, when assessing the extent of reaction of the tubulin chains with [^{14}C]EBI, it seems reasonable to consider β^* and the residual β_1 chain as one unit, as we have done in Figures 3–6 and Tables I and II.

There are two possible ways to account for this difference between β_1 and β_2. One way is to postulate that the sulfhydryl groups in β_2 are located in different places in the tertiary structure such that EBI cannot form an intrachain cross-link between them, whereas in β_1 they are appropriately located for such a cross-link. A second possibility is that at least one of the sulfhydryls involved in β^* formation is absent or inaccessible in β_2. The observation shown in Figure 3 that [^{14}C]EBI reacts much faster with β_1 than with β_2 supports the second possibility, namely, that one of the sulfhydryls involved in β^* formation is missing or inaccessible in β_2. One could of course argue that the first possibility could be true and that EBI's inability to form a cross-link in β_2 would slow down its reaction with β_2 as compared to its reaction with β_1, thereby accounting for the results in Figure 3. However, the same figure shows that α and β_2 react with [^{14}C]EBI at about the same rate. Ludueña & Roach (1981a) have shown by peptide mapping that EBI generates at least one intrachain cross-link in α, although without altering its electrophoretic mobility. If the ability of EBI to generate an intrachain cross-link were the determining factor in its more rapid rate of reaction with β_1, then it is hard to explain why α does not react fast as well. It appears, therefore, that the different rates of reaction of β_1 and β_2 with [^{14}C]EBI cannot be explained solely by EBI forming a cross-link in β_1 and not in β_2. Perhaps the most telling evidence bearing on this point is that the residual β_1 band has a much higher specific activity, after 40 min of reaction, than does the β_2 band (Figure 3). Since the residual β_1 band presumably represents β chains that have reacted with EBI without forming an intrachain cross-link, the fact that they react faster than does β_2 strongly suggests that β_1 has a reactive sulfhydryl that is absent or inaccessible in β_2.

The published amino acid sequence of tubulin supports the hypothesis that β_1 and β_2 differ in their sulfhydryl contents. There are seven positions at which the sequence of β is heterogeneous; only one of them involves a cysteine—a Cys/Ser heterogeneity at position 201 (Little et al., 1982). Since the β-tubulin used for sequencing consisted of an approximately 1:1 mixture of β_1 and β_2, it is not yet possible to assign with certainty the amino acid residues at the heterogeneous positions to one or the other of the two chains. However, the fact that one of the heterogeneous positions involves a cysteine is certainly consistent with our observation that β_1 reacts more rapidly with a sulfhydryl-directed reagent than does β_2.

Although many cases of tubulin heterogeneity have been reported, little is known about the functional significance of tubulin heterogeneity. However, the β_1/β_2 heterogeneity that we describe here may be an exception. The fact that EBI potently inhibits microtubule assembly in vitro and preferentially reacts with β_1 over β_2 raises the possibility that the $\alpha\beta_1$ and $\alpha\beta_2$ dimers may respond differently to putative sulfhydryl-directed assembly-regulating agents in vivo. It is clear from Figure 4 that inhibition of microtubule assembly by [^{14}C]EBI is correlated with the reaction of EBI with the α, β_2, and β_1 chains, as well as with the production of β^*. It is not possible, from this figure alone, to decide which of the tubulin chains has a sulfhydryl group critical for microtubule assembly. However, the fact that β^* produced in the reaction ends up largely in the supernatant and not in the pellet (Figure 5) indicates that a tubulin molecule that reacts with EBI to form the intrachain cross-linked β^* is unable or less able to assemble into or onto a microtubule. This suggests that one or both of the sulfhydryl groups involved in β^* formation may be critical for assembly. Since β^* derives from β_1, then it is possible that β_1 has an assembly-critical sulfhydryl group that is missing or inaccessible in β_2. It could still be argued, however, that there is an assembly–critical sulfhydryl group common to β_1 and β_2 that is not directly involved in β^* formation in β_1 but whose reaction with EBI somehow facilitates the subsequent formation of β^*. In such a scheme, β^* would end up largely in the supernatant without either of the sulfhydryl groups involved in the intrachain cross-link having to be critical. This argument is significantly weakened, however, by the fact that β^* consistently incorporates at most 1.0 mol of ^{14}C/mol of protein except after prolonged reaction times, whereas the scheme just described would require that it incorporate at least 2 mol.

Until the alkylated tubulin chains of an experiment such as the one in Figure 4 are subjected to proteolytic digestion and the rates of reaction of each individual sulfhydryl group with [^{14}C]EBI are determined, it will be impossible to know the order in which the individual sulfhydryl groups react with [^{14}C]EBI. Presumably those that react fastest would be good candidates for being critical sulfhydryls. Although this analysis has not yet been performed, one can approach this issue by taking advantage of the fact that β^* formation segregates, at least in part, reacted and unreacted molecules of β_1. On the basis of this fact, a careful analysis of the data in Figure 4 shows that the sulfhydryls involved in β^* formation are the most reactive in the tubulin molecule. For example, after 6 h of reaction with [^{14}C]EBI, assembly is inhibited 46% and the yield of β^* is 18%. This means that at this time, in at least 18% of the tubulin molecules, each of the two sulfhydryl groups in β_1 involved in the intrachain cross-link has reacted with EBI. At the same time, the specific radioactivities of α and β_2 are 0.17 and 0.24 mol of ^{14}C/mol of protein. Even with the assumption that all of the radioactivity in α is accounted for by the reaction of a single sulfhydryl group, at most only 17% of the population of that sulfhydryl group could have reacted. In the case of β_2, at most 24% of the population of a single sulfhydryl group could have reacted at this time. If we postulate that β_2 has a sulfhydryl group as reactive as the ones

in β_1 that make β^*, we have to assume that 75% of the reaction of β_2 with EBI is accounted for by that sulfhydryl group that the other sulfhydryl groups can account for at most 0.06 mol of ^{14}C/mol of protein. However, although EBI may be selective, it is unlikely to be that selective; in β_1, for example, reactions other than that involved in β^* formation account for 0.16 mol/mole of the radioactivity in β_1. In short, it appears likely that the two sulfhydryl groups involved in β^* formation are more reactive than any other sulfhydryls in the tubulin molecule and are therefore likely candidates for being critical in regulating microtubule assembly in vitro.

It has been proposed that the sulfhydryl groups in tubulin may play critical roles in regulating microtubule assembly in vitro and in vivo (Kuriyama & Sakai, 1974; Burchill et al., 1978). Our data suggest that there may be different species of tubulin that differ from each other in the extent of their participation in this type of regulation. Thus, $\alpha\beta_1$ may be a form of tubulin that responds to a sulfhydryl-directed regulatory mechanism, whereas the assembly of $\alpha\beta_2$ may be regulated by some other mechanism. The experiments shown in Tables I and II and in Figure 6 imply that the drug-binding properties of $\alpha\beta_1$ and $\alpha\beta_2$ are similar. It thus appears that the major differences between these two forms of tubulin are their tissue and organismal distributions and their response to a sulfhydryl-directed inhibitor of microtubule assembly in vitro.

Acknowledgments

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