Interaction of Tubulin with Drugs and Alkylating Agents. 1. Alkylation of Tubulin by Iodo[^{14}C]acetamide and *N,N'*-Ethylenebis(iodoacetamide)†

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ABSTRACT: The sulfhydryl groups of tubulin are reported to play a role in regulating microtubule assembly and colchicine binding to tubulin. The alkylating agents iodo[^{14}C]acetamide and its bifunctional analogue *N,N'*-ethylenebis(iodoacetamide) are used as probes for the sulfhydryl groups of tubulin. In the presence of 8 M urea, α- and β-tubulin have 10–11 and 8 alkylatable sulfhydryls, respectively, and one of the high molecular weight proteins (HMW 2) has 5 sulfhydryls/M_r 271 000. In the absence of urea, the rates of alkylation of α- and β-tubulin are significantly lower but that of HMW 2 is unaffected. The sulfhydryls of tubulin are masked in intact microtubules. *N,N'*-Ethylenebis(iodoacetamide) reacts with free tubulin to generate a band, designated β*, which migrates ahead of β on polyacrylamide gels. β* appears to represent a form of β-tubulin containing at least one intrachain cross-link between sulfhydryl groups. Formation of β* is inhibited in intact microtubules and is abolished if tubulin is denatured by 8 M urea, 1% sodium dodecyl sulfate, or boiling. *N,N'*-Ethylenebis(iodoacetamide) may thus be used as a probe for the native conformation of free tubulin.

Microtubules are cylindrical organelles about 250–300 Å in diameter which have critical roles in certain processes in eukaryotic cells, such as mitosis, secretion, and flagellar and ciliary motion (Porter, 1966). The basic structural component of microtubules is the protein tubulin, a heterodimer of two 55 000-dalton polypeptides, α and β (Bryan & Wilson, 1971; Ludueña et al, 1977a). Recently, several laboratories, using mainly preparations from mammalian brain, have been studying microtubule assembly in vitro in an attempt to identify parameters which could regulate this process in vivo (Scheele & Borisy, 1979). One such parameter is the state of oxidation of the sulfhydryl groups of tubulin. Several groups have reported that oxidation of either one, two, or three sulfhydryl groups per tubulin monomer is sufficient to block microtubule assembly (Kuriyama, 1976; Mellon & Rebhun, 1976; Ikeda & Steiner, 1978). With the exception of the work on platelet tubulin by Ikeda & Steiner (1978), the published estimates of the critical sulfhydryl titers were determined entirely spectrophotometrically. This kind of measurement, however, does not distinguish among the contributions of the sulfhydryl groups on α- or β-tubulin or the microtubule-associated proteins (MAPs),[1] nor does it lend itself to subsequent identification of the peptides containing critical sulfhydryl groups following partial hydrolysis of the proteins. In addition, spectrophotometric sulfhydryl assays do not distinguish between the contributions of polymerized and unpolymerized tubulin.

Microtubule assembly in vitro and in vivo is inhibited by low concentrations of drugs such as colchicine, podophyllotoxin, or vinblastine (Wilson et al., 1976). It has been shown in several laboratories that the binding of colchicine to tubulin is inhibited by oxidation of tubulin sulfhydryls, though not as strongly as is the ability to polymerize (Kuriyama & Sakai, 1974). Also, colchicine and vinblastine have been shown to diminish the sulfhydryl titer of tubulin (Schmitt & Kram, 1978). These findings raise the interesting possibility of a relationship between the drug binding sites on the tubulin molecule and those sulfhydryl groups which are critical for microtubule assembly.

In the work described in this paper, we have used iodo[^{14}C]acetamide as a probe for the sulfhydryl groups of α- and β-tubulin and the MAPs in both polymerized and unpolymerized microtubule protein from bovine brains. We have found that under the gentle conditions used here, iodo[^{14}C]-acetamide is specific for sulfhydryl groups and that in intact microtubules the sulfhydryl groups of tubulin are sterically prevented from undergoing alkylation. We have also synthesized a series of bifunctional analogues of iodoacetamide and have found that one of them, *N,N'*-ethylenebis(iodoacetamide) (EBI),[1] can generate, in high yield, at least one intramolecular cross-link between sulfhydryl groups in β-tubulin, suggesting the possibility that this series of compounds could be used in the future to map the location of important sulfhydryl groups in tubulin. In the following paper in this issue (Ludueña & Roach, 1981), we show that the interactions of tubulin with both iodo[^{14}C]acetamide and *N,N'*-ethylenebis(iodoacetamide) are strongly and distinctively influenced by the antimitotic drugs colchicine, podophyllotoxin, and vinblastine.

Parts of this work have been reported in preliminary form elsewhere (Ludueña, 1977; Ludueña et al., 1977b).

Experimental Procedures

Alkylating Agents. Iodo[1-^{14}C]acetamide (Amersham) was diluted with cold iodoacetamide (Sigma) and the specific activity determined by reacting it with reduced glutathione (Sigma) as follows. First, the purity of the reduced glutathione was measured by reacting it with DTNB[1] and reading the A_{412} of the solution (Ellman, 1959). The reduced glutathione was then dissolved in 0.01% NH_4OH and mixed with an excess

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[1] Abbreviations used: MAPs, microtubule-associated proteins; EBI, *N,N'*-ethylenebis(iodoacetamide); buffer A, 100 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.4, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 0.1 mM ethylenediaminetetraacetic acid, 0.5 mM $MgCl_2$, and 1 mM guanosine 5'-triphosphate; buffer B, 20 mM sodium phosphate, pH 6.75, 100 mM sodium glutamate, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 0.5 mM $MgCl_2$, and 2.5 mM guanosine 5'-triphosphate; $NaDodSO_4$, sodium dodecyl sulfate; HMW, the high molecular weight MAP; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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of iodo[^{14}C]acetamide. The reaction proceeded for 2 h at 25 °C in the dark. An aliquot of the reaction mixture was then spotted on Whatman 3MM paper and subjected to electrophoresis at 2000 V for 2 h in a solution of water–pyridine–acetic acid (900:100:4), pH 6.5 (Bennett, 1967). After the run, the paper was dried, stained with ninhydrin, and cut into strips. The radioactive spots were identified with a strip scanner and were eluted from the paper. The eluates were dried, taken up in toluene scintillation fluid, and counted. The specific activity of the iodo[^{14}C]acetamide was taken as equal to that of the carboxamidomethyl derivative of glutathione.

N,N'-Ethylenebis(iodoacetamide) (EBI)[1] was synthesized from ethylenediamine (Aldrich) and iodoacetyl chloride (K & K Biochemicals) by the method of Ozawa (1967). The synthetic material was dissolved in dimethyl-d_6 sulfoxide, and its structure was analyzed by nuclear magnetic resonance spectroscopy on a Varian XL-100, using residual proton signals in the dimethyl sulfoxide as a reference. The spectrum showed that the iodoacetylation of ethylenediamine had gone 75% of the way to completion, and thus, in the experiments described here, the functional concentration of EBI was assumed to be equal to 75% of the total material. By similar methods, *N,N'*-propylenebis(iodoacetamide), *N,N'*-tetramethylenebis(iodoacetamide), *N,N'*-pentamethylenebis(iodoacetamide), and *N,N'*-hexamethylenebis(iodoacetamide) were synthesized and analyzed by nuclear magnetic resonance spectroscopy to determine their activity. *N,N'*-Heptamethylenebis(iodoacetamide) and *N,N'*-decamethylenebis(iodoacetamide) were also synthesized, but their structures were not analyzed by nuclear magnetic resonance spectroscopy. The structures of all the *N,N'*-polymethylenebis(iodoacetamide) derivatives were confirmed by mass spectrometry, using a Hewlett–Packard 5982 mass spectrometer (S. Weintraub and R. F. Ludueña, unpublished results). 4,4'-Bis(iodoacetamido)stilbene-2,2'-disulfonic acid was synthesized from iodoacetyl chloride and stilbenediamine-2,2'-disulfonic acid by the method of Ozawa (1967); this derivative was used in experiments, but its structure was not analyzed.

Other Materials. Aldolase, ovalbumin, bovine serum albumin, conalbumin, myoglobin, and GTP were from Sigma. Sodium iodoacetate (Sigma) was purified by reprecipitation from acetone. Acrylamide and *N,N'*-methylenebis(acrylamide) were from Eastman. The acrylamide solutions were filtered through charcoal prior to use. *S*-(Carboxymethyl)-cysteine was from Sigma; τ-(carboxymethyl)histidine, π-(carboxymethyl)histidine, and π,τ-bis(carboxymethyl)histidine were from Calbiochem. N^{ϵ}-(Carboxymethyl)lysine and the *S*-(carboxymethyl)sulfonium salt of methionine were prepared by the methods of Gundlach et al. (1959a,b). All these carboxymethylated amino acids were the kind gifts of Drs. J. Nishimura and M. Matula. *S*-(Carboxymethyl)homocysteine was prepared from the *S*-(carboxymethyl)sulfonium salt of methionine by acid hydrolysis (Gundlach et al., 1959b).

Tubulin Preparation. Bovine brains were purchased from the All-State Packing Company, Inc., and from the Roegelein Co., San Antonio, TX. Microtubule protein was prepared from the cerebra of freshly killed animals by the method of Fellous et al. (1977).

The protein thus obtained was stored as a pellet at −80 °C. Prior to each alkylation experiment, a pellet was resuspended in buffer A[1] and incubated for 45 min at 1 °C. The suspension was then centrifuged (27000*g*, 30 min, 1 °C) and the supernatant placed on a column of phosphocellulose (Whatman, P11) equilibrated in buffer A (Fellous et al., 1977). Pure tubulin eluting from this column was used in most alkylation experiments. For experiments in which the alkylation of whole microtubules or of the HMW[1] proteins was studied, microtubule protein was prepared from bovine cerebra by the procedure of Asnes & Wilson (1979), a method which gives a higher yield of HMW, relative to tubulin, than does that of Fellous et al. (1977). For experiments, a pellet was thawed, resuspended in buffer B[1], incubated for 30 min at 1 °C, and centrifuged at 12500*g* for 45 min at 4 °C. The protein in the resulting supernatant, which was used for experiments, consisted of 77% tubulin by weight, 16% the high molecular weight (HMW) proteins, and 17% other proteins, as determined by electrophoresis on polyacrylamide gels (Laemmli, 1970). Of the HMW proteins, 26% was HMW 1 (M_r 285 000) and 74% was HMW 2 (M_r 271 000).

Alkylation Reactions. In experiments in which assembly-competent microtubule protein was alkylated, samples of protein were made 0.1 mM in griseofulvin prior to addition of the alkylating agent in order to prevent microtubule assembly since assembly itself strongly inhibits alkylation. Griseofulvin was omitted from experiments in which phosphocellulose-purified tubulin was used since this tubulin does not polymerize under the conditions of these experiments. The alkylating agent was then added, and the reaction was allowed to proceed for 0.25–3 h. Samples were then treated with equimolar β-mercaptoethanol, dialyzed against either A or B at 1 °C, reduced and alkylated (Crestfield et al., 1963), and analyzed on 6% discontinuous acrylamide gels containing sodium dodecyl sulfate by using either the system of Laemmli (1970) or that of Yang & Criddle (1970). The gels were stained with fast green and scanned at 640 nm in a Gilford 250 spectrophotometer equipped with a linear transport. The gels were sliced, and the slices were soaked in NCS Solubilizer and then in toluene scintillation fluid and counted in a Beckman scintillation counter.

The amount of protein placed on the gel was determined by the method of Lowry et al. (1951). Preliminary experiments showed that the intensity of staining of tubulin bands with fast green was directly proportional to the amount of tubulin placed on the gel in the tubulin concentration range used in these experiments. Molecular weights were determined by using conalbumin, bovine serum albumin, ovalbumin, and aldolase as standards.

In certain experiments in which the tubulin samples had been treated with *N,N'*-ethylenebis(iodoacetamide), the following method was used to determine the amount of cross-linked tubulin that did not migrate into polyacrylamide gels. Freshly purified tubulin was mixed with reduced and carboxymethylated conalbumin prior to incubation and alkylation. The resulting samples were analyzed by polyacrylamide gel electrophoresis, and the gels were stained and scanned as described.

The percentage of aggregated tubulin (% Ta) in a sample *n* treated with EBI was derived from

$$\% \text{ Ta} = [1 - (R_n/R_0)] \times 100\%$$

where R_n represents the ratio, in sample *n*, of the total area of the tubulin peaks to the area of the conalbumin peak on the gel scan of sample *n* and R_0 represents the ratio of tubulin to conalbumin in a control sample that had not been treated with EBI. Analysis of the experimental results showed that reduced and carboxymethylated conalbumin did not react with EBI and was, therefore, an appropriate internal standard to estimate the amount of tubulin that was unable to migrate onto gels. In some experiments, small amounts of cross-linked dimer, trimer, and tetramer were generated. Unless otherwise indicated, these were included with the high molecular weight

cross-linked aggregate for the purpose of calculation.

Similarly, in order to determine the percentage of β-tubulin that had been converted to the faster moving β^* (% β^*) by EBI, the following equation was used:

$$\% \beta^* = [\beta_n^*/(C_n R_b)] \times 100\%$$

where β_n^* and C_n are the areas on the gel scan of sample n of the β^* and conalbumin peaks, respectively, and R_b is the ratio of β-tubulin to conalbumin in a control sample that had not been treated with EBI. The small amount of β_2-tubulin was included with β for the purpose of these calculations.

Identification of Alkylated Residues. Protein samples were reacted with 1.31–1.34 mM iodo[^{14}C]acetamide for 2 h at 37 °C. The samples were then reduced and carboxymethylated (Crestfield et al., 1963), dialyzed against water, lyophilized, and hydrolyzed in 6 N HCl for 24 h at 110 °C. The hydrolysates were dried, dissolved in 3.8 M sodium acetate, pH 5.3 (Rosen, 1957), and spotted on Whatman 3MM paper, which was then subjected to electrophoresis in pyridine–acetate at 2000 V for 2 h. The carboxymethylated derivatives of cysteine, lysine, histidine, and homocysteine were used as standards. The unlabeled spots were identified with ninhydrin; the labeled spots were located with a Packard 7201 radiochromatogram scanner and then eluted for counting.

Peptide Analysis. Samples of reduced and carboxymethylated tubulin were run on gels (Laemmli, 1970), and the α, β, and β^* bands were visualized by the method of Nelles & Bamburg (1976). The protein bands were then cut out of the gels, eluted from the gel slices, and dialyzed extensively at 1 °C against chloroform–saturated water. They were then lyophilized and subsequently dissolved at a protein concentration of 5 mg/mL in 70% formic acid containing 0.945 M CNBr (Gross, 1967). The samples were then incubated 24 h at 25 °C in the dark, then diluted with water, lyophilized, and analyzed on 12.5% polyacrylamide gels containing 0.17% sodium dodecyl sulfate and 8 M urea (Swank & Munkres, 1971). Myoglobin and its CNBr peptides were used as molecular weight standards.

Results

Reactions of Tubulin and HMW with Iodo[^{14}C]acetamide. For determination of the number of groups in tubulin that would react with iodo[^{14}C]acetamide, samples of phosphocellulose-purified tubulin were incubated with a series of iodo[^{14}C]acetamide concentrations in the presence or absence of 8 M urea (Figure 1). In the presence of 8 M urea, the incorporation of ^{14}C into α- and β-tubulin was concentration dependent below approximately 2–3 mM iodo[^{14}C]acetamide. At this level, plateau values of incorporation were observed of about 10–11 mol/mol of α and 8 mol/mol of β. In the absence of urea, incorporation of label into both α and β was diminished; at 2.73 mM iodo[^{14}C]acetamide, for example, incorporation of label into α and β after 2 h of reaction was 34% and 47%, respectively, that of the urea-treated samples. When microtubule protein prepared by the method of Asnes & Wilson (1979) was reacted with iodo[^{14}C]acetamide in the presence of either 8 M urea or 6 M guanidine hydrochloride, the tubulin in this preparation was alkylated to the same extent as was phosphocellulose-purified tubulin (results not shown). The extent of alkylation of tubulin was not dependent on the presence or absence of glycerol in the isolation procedure. In order to obtain the maximal stoichiometry, however, it was necessary to add iodo[^{14}C]acetamide and urea to the tubulin sample at the same time; preincubation of tubulin with 8 M urea for 2 h prior to alkylation caused the incorporation of ^{14}C into tubulin to decrease by 57% (R. F. Luduena and M. C. Roach, unpublished results).

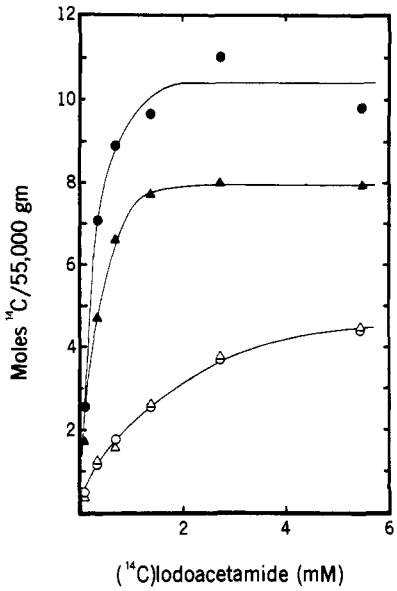

FIGURE 1: Effect of iodo[^{14}C]acetamide concentration on the alkylation of α- and β-tubulin in the presence and absence of 8 M urea. Aliquots (250 μL) of phosphocellulose-purified tubulin (0.75 mg/mL) were incubated for 2 h at 37 °C in buffer A in the presence of the indicated concentrations of iodo[^{14}C]acetamide (4.42 Ci/mol) in the presence (●, ▲) or absence (○, △) of 8 M urea. At the end of 2 h, the samples were placed on ice and made 5.35 mM in β-mercaptoethanol. They were then dialyzed against buffer A, and reduced, carboxamidomethylated, and analyzed on gels (Laemmli, 1970). The gels were stained, scanned, and sliced, and the slices were counted as described under Experimental Procedures. The specific radioactivities of the α (●, ○) and β (▲, △) chains are shown.

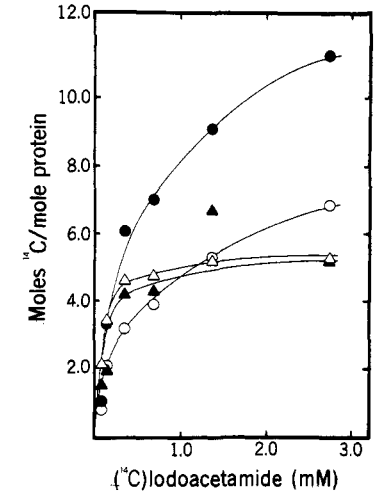

FIGURE 2: Effect of iodo[^{14}C]acetamide concentration on the alkylation of HMW 1 and HMW 2 in the presence and absence of 8 M urea. Aliquots (250 μL) of microtubule protein (0.93 mg/mL), prepared according to Asnes & Wilson (1979), containing 100 μM griseofulvin, were incubated for 2 h at 37 °C in buffer B in the presence of the indicated concentrations of iodo[^{14}C]acetamide (3.77 Ci/mol) and in the presence (●, ▲) or absence (○, △) of 8 M urea. After 2 h, the samples were reduced, carboxymethylated, processed, and analyzed on gels (Laemmli, 1970), as in Figure 1. The specific radioactivities of HMW 1 (●, ○) and HMW 2 (▲, △) are given.

When the alkylation of the HMW proteins was studied (Figure 2), the incorporation of ^{14}C into HMW 2 exhibited a concentration dependence below approximately 1.0–1.5 mM iodo[^{14}C]acetamide, up to a plateau value of about 5 mol/mol of protein. The presence of 8 M urea did not affect the results. In contrast, the incorporation of label into HMW 1 did not reach a plateau below 2.73 mM iodo[^{14}C]acetamide and was strongly enhanced by urea.

For identification of the residues in the proteins which reacted with iodo[^{14}C]acetamide, the following experiments were done. Phosphocellulose-purified tubulin (0.98 mg/mL) was reacted with 1.34 mM iodo[^{14}C]acetamide for 2 h at 37 °C and then hydrolyzed, and the hydrolysate was subjected to high-voltage paper electrophoresis, with a set of carboxymethylated amino acids as standards, as described under Experimental Procedures. The results showed that all the ^{14}C label was contained in a ninhydrin-positive spot that comigrated with *S*-(carboxymethyl)cysteine. There were no ninhydrin-positive spots that corresponded to τ-(carboxymethyl)histidine, π-(carboxymethyl)histidine, π,τ-bis(carboxymethyl)histidine, N^{ϵ}-(carboxymethyl)lysine, or *S*-(carboxymethyl)homocysteine. No radioactivity was detectable at these positions. The sensitivity of the method used was such that carboxymethylation of residues other than cysteine would have been detectable at levels of incorporation greater than 0.026 mol/mol of tubulin.

In another experiment, samples of microtubule protein prepared by the method of Asnes & Wilson (1979) (0.87 mg/mL) were incubated with 1.31 mM iodo[^{14}C]acetamide for 1 h at 37 °C in buffer B in the presence or absence of 8 M urea. The samples were hydrolyzed and analyzed as above. There were very few differences between the samples. Again, the only ninhydrin-positive spot which could be identified with a carboxymethylated amino acid comigrated with *S*-(carboxymethyl)cysteine. This spot also contained the only radioactive peak detectable by the strip scanner. When the region corresponding to *S*-(carboxymethyl)homocysteine and τ,π-bis(carboxymethyl)histidine was eluted and counted, about 1–2% of the total label was found there. If this did not represent diffusion from the large and immediately adjacent *S*-(carboxymethyl)cysteine peak, then this could imply incorporation of ^{14}C label into methionine or histidine at levels no greater than 0.10 mol/mol of tubulin or 2.0 mol/mol of HMW.

Reaction of Tubulin with N,N'-Ethylenebis(iodoacetamide). When samples of freshly prepared tubulin or microtubule protein were reacted with EBI at 30 °C under nondenaturing conditions, then reduced, carboxymethylated, and analyzed on discontinuous polyacrylamide gels in the presence of sodium dodecyl sulfate (Laemmli, 1970), it was observed that the β-tubulin band had diminished greatly in intensity as compared with an unreacted control and that a faster moving band, here designated as β*, had appeared (Figure 3, sample b). The slightly slower moving band, β_2, identified as a form of β by Little (1979), was not significantly affected by EBI under these conditions. On this gel system, when tubulin was electrophoresed together with protein standards of known molecular weight, β* migrated with an apparent molecular weight of 56 000, as compared to 60 000 for β and 65 000 for α. The appearance of β* as a band distinct from β was a phenomenon restricted to gels run in the system of Laemmli (1970). When samples of EBI-treated tubulin were analyzed on the similar electrophoretic system of Yang & Criddle (1970), β and β* comigrated.

The relative yield of β* on Laemmli gels increased and that of β decreased, with increasing durations of reaction and with increasing concentrations of EBI. The ratio of the intensity of α to the sum of the intensities of β and β* was unaffected under these conditions and was the same as the ratio of the intensity of α to that of β in unreacted controls. The ratio of α to the sum of β and β* was also unaffected by the presence of 100 μM concentrations of vinblastine, colchicine, or podophyllotoxin (Ludueña & Roach, 1981).

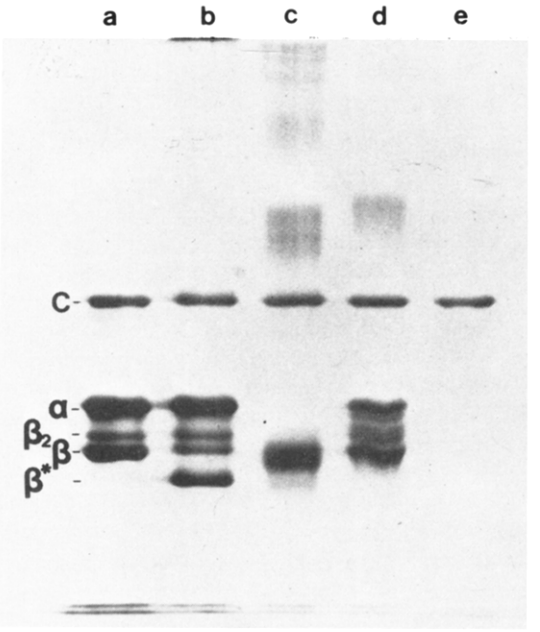

FIGURE 3: Effect of EBI treatment on the electrophoretic mobility of tubulin. Aliquots (255 μL) of phosphocellulose-purified tublin (0.76 mg/mL) containing reduced and carboxymethylated conalbumin (0.20 mg/mL) were incubated at 30 °C for 60 min in the presence of either 1.34 mM iodoacetamide (a) or 0.67 mM EBI (b–e). Sample c contained 8 M urea, and sample d contained 1% sodium dodecyl sulfate. Sample e was incubated for 2 min at 100 °C prior to incubation with EBI. Samples were reduced, carboxymethylated, and subjected to electrophoresis on a slab gel of 6% polyacrylamide (Laemmli, 1970). The gel was stained with Coomassie Brilliant Blue G-250. The direction of electrophoresis was from top to bottom.

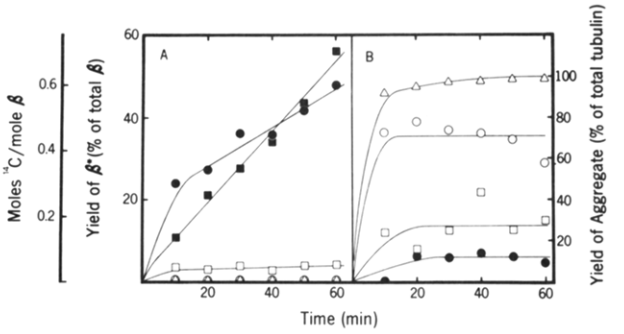

FIGURE 4: Effects of denaturing agents on the rate of reaction of tubulin with EBI. Aliquots (255 μL) of phosphocellulose-purified tubulin (0.76 mg/mL) containing reduced and carboxymethylated conalbumin (0.20 mg/mL) were incubated in buffer A at 30 °C for the indicated times with 0.67 mM EBI in the absence (●) or presence of either 8 M urea (○) or 1% $NaDodSO_4$ (□). One batch of tubulin (△) was preincubated at 100 °C for 2 min and then cooled on ice prior to alkylation with EBI. Another set of samples (■) contained 1.34 mM iodo[^{14}C]acetamide (4.42 Ci/mol) instead of EBI and contained no denaturing agent. The samples were processed and analyzed as described under Experimental Procedures. The figure shows the rate of production of β* (A) and of cross-linked aggregate (B). "Aggregate" includes all cross-linked forms of tubulin heavier than the monomeric α and β subunits. The yield of aggregate was calculated as described under Experimental Procedures. Part A also shows the incorporation of ^{14}C label into β-tubulin in the samples treated with iodo[^{14}C]acetamide. Each point represents a separate incubation.

The CNBr peptide map of β* was similar to, but not identical with, that of β. Out of 16 peptides in the map of β, 14 were also present in β*. EBI treatment did not generate new peptides, but appeared to alter their relative amounts. All peptides in β larger than M_r 25 000 were present in β*, but in decreased quantities, relative to two major peptides of M_r

22000 and M_r 8000, suggesting that digestion was more complete in β^* than in β.

Figure 4A compares the rates of reaction of β-tubulin at 30 °C with 0.67 mM EBI and 1.34 mM iodo[^{14}C]acetamide. At 10 min, the sample treated with EBI attained a yield of β^* of 24%. At the same time, in an identical sample treated with iodo[^{14}C]acetamide, β-tubulin incorporated 0.134 mol of label/mol of protein. If β^* represents an intramolecularly cross-linked form of β, then we can estimate that at least 0.48 group of β reacted with EBI in 10 min, while only 0.134 reacted with a functionally equivalent concentration of iodo-[^{14}C]acetamide. In other words, in this experiment, EBI may have reacted with β at least 3.6 times as fast as did iodo-[^{14}C]acetamide.

EBI also reacted with α, but did not affect its electrophoretic mobility on either the gel system of Laemmli (1970) or that of Yang & Criddle (1970). Examination of the CNBr peptide maps of α extracted from a sample that had been reacted with EBI revealed the presence of 11 out of the 12 peptides observed in α from unreacted samples. The map of the EBI-treated α-tubulin lacked a peptide of M_r 41000 and had a greatly decreased yield of a M_r 22000 peptide. In addition, the EBI-treated α-tubulin contained two peptides of M_r 45000 and 26000 not found in untreated α.

In addition to producing β^*, EBI also caused tubulin to form aggregates of very high molecular weight which were unable to migrate into a 6% polyacrylamide gel and whose yield was accordingly estimated indirectly by measuring the loss of material from the tubulin bands on the gel. When reduced and carboxymethylated conalbumin was used as an internal standard, the loss of tubulin could be measured with accuracy. It was found that after 10 min of reaction at 30 °C with 0.67 mM EBI, 12% of the tubulin formed the high molecular weight aggregate. Increasing the duration of the reaction appeared to have little effect on aggregate formation. Under these same conditions, EBI caused no increase in the yield of cross-linked dimer above the 1–4% level of "natural" dimer that was present even in untreated tubulin (Ludueña et al., 1977a,b).

The relative yields of β^* and high molecular weight aggregate were highly dependent on the temperature of the reaction, the nature of the tubulin sample, and the length of the bifunctional alkylating agent used. If tubulin was reacted with 0.64 mM EBI for 2 h at 37 °C, only 8% of β was transformed to β^*, but 93% of α and 84% of β aggregated. When a sample containing microtubules was reacted with EBI and then centrifuged to separate the microtubules from unpolymerized tubulin, the yield of β^* in the unpolymerized tubulin was 71%, whereas in the intact microtubule, the yield was 4.5%.

β^* could not be generated in tubulin whose native conformation had been abolished. When tubulin was incubated at 100 °C for 2 min prior to EBI treatment, no β^* was produced and over 93% of the tubulin aggregated (Figure 4). In these samples, the cross-linked aggregate was unable to migrate into the 2.5% polyacrylamide stacking gel. No dimer was generated in this sample (Figure 3, sample e). Similarly, when the reaction with EBI was carried out in the presence of 8 M urea, no β^* was produced, and at least 58% of the tubulin in these samples formed a cross-linked aggregate (Figure 4). In the presence of 8 M urea, 10–18% of the tubulin in these samples migrated as a cross-linked dimer (Figure 3, sample c). When tubulin was denatured in 1% $NaDodSO_4$,[1] the yield of β^* was 3–4%, but the electrophoretic mobility of β^* was altered: it migrated as a small shoulder on the leading edge of the β band. Six to fifteen percent of the tubulin in the $NaDodSO_4$-treated

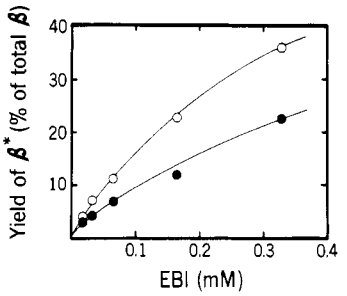

FIGURE 5: Effect of iodoacetamide on the production of β^* by EBI. Aliquots (255 μL) of phosphocellulose-purified tubulin (0.74 mg/mL) containing reduced and carboxymethylated conalbumin (0.20 mg/mL) were incubated in buffer A in the presence (●) or absence (○) of 0.138 mM iodo[^{14}C]acetamide (4.06 Ci/mol) for 1 h at 37 °C. At the end of 1 h, 5 μL of EBI was added to each sample to give the indicated final concentrations and the incubation continued for 1 h at 30 °C. The samples were then cooled to 1 °C, made 1.29 mM in β-mercaptoethanol, dialyzed against buffer A, and reduced, carboxymethylated, and analyzed on gels (Laemmli, 1970). The relative yield of β^* was determined by using conalbumin as the internal standard as described under Experimental procedures.

samples migrated as a cross-linked dimer (Figure 3, sample d). β^* was also generated, although in decreased quantities, when tubulin was reacted with other *N,N'*-polymethylenebis(iodoacetamide) derivatives. In a representative experiment in which microtubule protein was reacted with 0.64 mM concentrations of these derivatives for 1 h at 30 °C, EBI generated a 50% yield of β^*, and the other derivatives generated the following yields: *N,N'*-tetramethylenebis(iodoacetamide), 14%; *N,N'*-propylenebis(iodoacetamide), 9%; *N,N'*-pentamethylenebis(iodoacetamide), 5%; *N,N'*-hexamethylenebis(iodoacetamide), 7%; *N,N'*-heptamethylenebis-(iodoacetamide), 8%; *N,N'*-decamethylenebis(iodoacetamide), 6%. In another experiment, reaction of tubulin with 4,4'-bis(iodoacetamido)stilbene-2,2'-disulfonic acid generated no β^*. All of these derivatives were reactive as shown by the fact that they cross-linked HMW to generate an aggregate unable to migrate into a 6% gel. Iodoacetamide was unable to generate β^* under any set of conditions. Also, there were no conditions in which bands intermediate in mobility between β and β^* were generated.

EBI and iodo[^{14}C]acetamide inhibited each other's reactions with tubulin. When samples of tubulin were incubated with 1.34 mM iodo[^{14}C]acetamide for 1 h at 37 °C and then reacted with a series of EBI concentrations, the relative yield of β^* was decreased by 23–48% (Figure 5). Iodoacetamide pretreatment also decreased the production of high molecular weight aggregate by EBI by 18–100% (not shown). The extent of incorporation of ^{14}C label into tubulin diminished with increasing EBI concentration ranging from 3.07 mol of label/mol of tubulin in the presence of 6.54 μM EBI to 1.78 mol of label/mol of tubulin in the presence of 327 μM EBI. The specific radioactivities in the tubulin bands were generally highest in β and lowest in β^*. In the sample treated with 327 μM EBI, for example, the specific radioactivities of the α, β, and β^* bands were, respectively, 1.55, 2.86, and 0.44 mol of label/mol of protein.

Analogous results were generated in the converse experiment (Figure 6) in which samples of tubulin were incubated for 15 min at 30 °C in the presence or absence of 0.67 mM EBI and then reacted with a series of concentrations of iodo[^{14}C]-acetamide. The incorporation of ^{14}C label into tubulin was greatly decreased in the EBI-treated sample. The inhibition was observed in both α and β, but was most marked in β (Figure 6). EBI pretreatment inhibited the reaction of α- and β-tubulin with 1.31 mM iodo[^{14}C]acetamide by 68% and 79%,

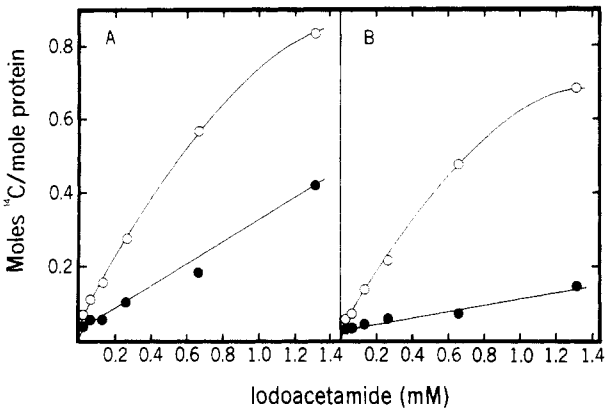

FIGURE 6: Effect of EBI on the reaction of α- and β-tubulin with iodo[^{14}C]acetamide. Aliquots (255 μL) of phosphocellulose-purified tubulin (0.74 mg/mL) were incubated for 15 min at 30 °C in buffer A in the presence (●) or absence (○) of 0.67 mM EBI. Iodo-[^{14}C]acetamide (5 μL) (4.06 Ci/mol) was then added to each sample to give the indicated final concentrations and the incubation continued for 60 min. Samples were processed and analyzed on gels (Yang & Criddle, 1970) as described under Experimental Procedures. The figures show the specific radioactivities of α-tubulin (A) and β-tubulin (B). On this gel system, β and β* comigrate.

respectively. When analyzed on the system of Laemmli (1970), the distribution of label among α, β, and β* was similar to that of the experiment shown in Figure 5, with β having the highest specific radioactivity and β* the lowest. In the sample treated with 0.66 mM iodo[^{14}C]acetamide, for instance, the specific radioactivities of α, β, and β* were respectively 0.19, 0.25, and 0.051 mol of ^{14}C label/mol of protein.

Discussion

Reaction of Microtubule Protein with Iodo[^{14}C]acetamide. Although alkyl halides such as iodoacetamide generally react most efficiently with sulfhydryl groups in proteins, they are known to react to lesser extents with other amino acids such as methionine, lysine, and histidine (Gurd, 1967). For this reason, in order to demonstrate that iodo[^{14}C]acetamide is a useful probe for the sulfhydryl groups of tubulin, it is necessary to show that it is indeed specific for these groups on the tubulin molecule. Analysis of the radioactive amino acids generated when tubulin treated with iodo[^{14}C]acetamide was hydrolyzed indicated that the only radioactive ninhydrin-positive spot was *S*-(carboxymethyl) cysteine. The sensitivity of the analysis was such that, at most, only 0.026 mol of an amino acid other than cysteine per mol of tubulin could have reacted with iodo[^{14}C]acetamide. When the sample analyzed contained HMW as well as tubulin, a small amount of radioactivity was found in the region corresponding to *S*-(carboxymethyl)-homocysteine and τ,π-bis(carboxymethyl)histidine. No ninhydrin-positive spots were seen here, and it is therefore likely that this radioactivity could be explained by diffusion from the adjacent and strongly labeled *S*-(carboxymethyl)cysteine spot. Nevertheless, the possibility cannot be completely eliminated that up to 2 mol of either methionine or histidine in HMW per mol of protein can react with iodo[^{14}C]acetamide. It does appear, however, that iodo[^{14}C]acetamide is an excellent probe to characterize the sulfhydryl groups of tubulin. Although *N*-ethylmaleimide is known to be more specific for sulfhydryls than is iodoacetamide (Riordan & Vallee, 1967), preliminary experiments with *N*-ethyl[2,3-^{14}C$_2$]maleimide indicated that the reaction product with tubulin exhibited very little resolution between the α and β bands, so this reagent was not used further.

Denaturation of tubulin with 8 M urea increased the extent to which both α- and β-tubulin were able to react with iodo-[^{14}C]acetamide (Figure 1). We can estimate that α has 10–11 alkylatable sulfhydryls and that β has 8. These figures correlate well with the reported values, based on amino acid analysis, of 10 cysteines for chick brain α and 8 for chick brain β (Bryan & Wilson, 1971). Lee et al. (1973) found 11 cysteines per monomer of bovine tubulin of which 2 were apparently present as a disulfide. This adds up to 18 free sulfhydryls per dimer, similar to the total observed here of 18–19. These figures are higher than those reported by others for tubulin prepared by cycles of assembly and disassembly; using spectrophotmetric techniques, several laboratories have reported the presence of, at most, seven free sulfhydryls per tubulin monomer (Kuriyama, 1976, 1977; Kuriyama & Sakai, 1974; Mellon & Rebhun, 1976; Nishida & Kobayashi, 1977). One reason for this apparent contradiction may be that there are other proteins in preparations of recycled microtubule protein. Furthermore, we have observed that the sulfhydryl groups in tubulin that are exposed by denaturation with urea oxidize very rapidly if they are preincubated at 37 °C (R. F. Ludueña and M. C. Roach, unpublished results).

Figure 2 shows that both HMW 1 and HMW 2 contained significant numbers of sulfhydryl groups. This implies that estimates of the sulfhydryl titers of tubulin obtained spectrophotometrically should be reevaluated, in light of the possibility that the preparations used may have contained significant quantities of reactive proteins other than tubulin. This, in turn, raises the interesting possibility that the sulfhydryl groups of HMW may play a significant role in the regulation of microtubule assembly. Further experiments using HMW alkylated to different extents will be necessary in order to elucidate this problem. It is interesting that treatment with 8 M urea increased the alkylation of HMW 1 but did not affect the alkylation of HMW 2 (Figure 2). This finding is consistent with the hypothesis that HMW 2 is a long filamentous protein (Vallee, 1980) which is likely to be altered relatively little by denaturation, and it is also consistent with the observation that HMW 1 precipitates upon boiling whereas HMW 2 does not precipitate and also retains its polymerization-promoting activity (Fellous et al., 1977).

Reaction of Tubulin with N,N'-Ethylenebis(iodoacetamide). The bifunctional cross-linking reagent, EBI, reacted with tubulin to generate a variety of products. The most striking of these was a protein, here designated as β*, which on discontinuous polyacrylamide gels containing sodium dodecyl sulfate (Laemmli, 1970) migrated with an apparent molecular weight of 56 000 ahead of the slower moving α and β bands whose apparent molecular weights on this sytem were 65 000 and 60 000, respectively. In addition, EBI decreased the yield of β-tubulin and also diminshed its reactivity with iodo[^{14}C]-acetamide. EBI also lowered the reactivity of α-tubulin and altered its CNBr peptide map. The alterations in α, in which two normally occurring peptides appeared in greatly diminished yields and were replaced by two slightly heavier peptides, suggest that EBI may be forming intrachain covalent cross-links in α. EBI also generated a high molecular weight aggregate which was unable to migrate into a 6% gel. Interestingly, EBI did not generate any covalently cross-linked dimer or larger oligomers, except when the tubulin was reacted with EBI in the presence of either 8 M urea or 1% NaDodSO$_4$. This suggests that the reactive groups in the tubulin dimer are not well-placed for intradimeric cross-linking by EBI.

Several pieces of evidence indicate that the β* band was derived from β. First, the yields of β and β* were always inversely related to one another; when one increased, the other decreased. In contrast, the relative yields of α and β* were

not correlated with each other. Second, the ratio of α to the sum of β and β^* was not significantly altered in nondenatured tubulin and was the same as the ratio of α to β in tubulin that was not treated with EBI. Third, the sum of β and β^* in EBI-treated samples never exceeded the amount of β in untreated samples whereas the sum of α and β^* often exceeded the amount of α in untreated samples. Finally, the CNBr peptide map of β^* was very similar to that of β; although the relative amounts of the peptides were altered in the map of β^*, all but two of the 16 peptides observed in the map of β were present in that of β^*.

It is likely that β^* represents an intrachain cross-linked form of β for the following reasons: First, iodoacetamide, the monofunctional analogue of EBI, did not, under any circumstances, generate β^*. According to Wold (1972), the fact that a derivative generated by a bifunctional compound is not produced by its monofunctional analogue is a necessary criterion for interpreting the derivative as the product of a cross-link rather than of a chemical modification. Second, the formation of β^* was critically dependent on the tubulin molecule retaining its native conformation. Denaturation of tubulin induced by boiling or by incubation with 8 M urea or 1% $NaDodSO_4$ strongly inhibited the formation of β^* (Figure 4). In contrast, denaturation enhanced the ability of tubulin to react with iodo[^{14}C]acetamide (Figure 1). The most reasonable explanation for these findings is that β^* formation requires that the reactive groups on the tubulin molecule be at a certain distance from one another in order to be cross-linked. Third, the yield of β^* is markedly dependent on the length of the cross-linking reagent used in the alkylation reaction, with EBI and *N,N'*-tetramethylenebis(iodoacetamide) being the most effective, again suggesting that the distance between the reactive groups is important. If β^*, instead of being formed by intrachain cross-linking, were produced by reaction of a group on the molecule with only one of the iodoacetamide moieties on the cross-linker, than its formation should be independent of the length of the cross-linker. Fourth, the fact that EBI reacts more rapidly with β-tubulin than does a 2-fold molar excess of iodo[^{14}C]acetamide (Figure 4) is consistent with a cross-linking reaction in which the second iodoacetamide moiety of EBI reacts very rapidly with the tubulin molecule as a result of being very near to a reactive group once the first iodoacetamide moiety has reacted. Finally, the higher mobility of β^* on polyacrylamide gels in the presence of $NaDodSO_4$ is consistent with its interaction, with $NaDodSO_4$ being constrained by the presence of an intrachain bond; intrachain disulfide bridges are known to increase the electrophoretic mobility of certain proteins on $NaDodSO_4$ gels, and one could expect an intrachain cross-link to do the same. The fact that the electrophoretic difference between β and β^* was not observable in the very similar system of Yang & Criddle (1970) suggests that there may be subtle and as yet poorly understood factors which govern the interaction of tubulin with $NaDodSO_4$ if certain intrachain bonds are present in the tubulin molecule; α-tubulin, for example, is known to behave anomalously on certain $NaDodSO_4$ gel systems (Ludueña & Woodward, 1975).

In view of these arguments, it is probable that β^* is an intrachain cross-linked form of β. We cannot, however, state how many intrachain cross-links are present in β^* nor can we rule out the possibility that certain intrachain cross-links could be present in β and not alter its mobility on $NaDodSO_4$ gels. This is likely to be the case in α, where the peptide maps suggest that EBI induces intrachain cross-links, and yet the mobility of α on gels is unaltered. An analogous explanation is likely to account for the presence, in EBI-treated samples, of a residual band migrating at the same rate as does the major β band in untreated samples. Since more than two sulfhydryl groups are available in β, it is possible that EBI can form, probably in lesser yield, other intrachain cross-links in β besides the ones responsible for β^*. If some of these other cross-links involve a sulfhydryl group that would otherwise participate in a different cross-link to form β^*, then those molecules of β that contain these other cross-links would be unable to become β^*. Hence, it is conceivable that these β molecules would migrate as a residual β band. The fact that the yield of residual β is lowest in vinblastine-treated samples is consistent with evidence presented in the following paper in this issue (Ludueña & Roach, 1981) that vinblastine enhances β^* formation and suppresses competing reactions involving EBI.

Although, in view of the results described here, it is likely that iodo[^{14}C]acetamide reacts only with the sulfhydryl residues of tubulin, it is somewhat more difficult to be certain about which amino acid residues reacted with EBI. The fact that preincubation with EBI markedly diminished the ability of both α- and β-tubulin to react with iodo[^{14}C]acetamide (Figure 6) strongly indicates that EBI reacts with sulfhydryl groups, but does not prove that it cannot react with other groups. The fact that iodoacetamide preincubation inhibited β^* formation suggests that EBI must react with sulfhydryl groups in order to form β^*. Similarly, iodoacetamide preincubation diminished the formation of high molecular weight aggregate by EBI, which, by the same argument, suggests that EBI must react with sulfhydryl groups in order for this aggregate to form. Perhaps the most telling observation is that the specific radioactivity of β^* in tubulin that was treated with iodo[^{14}C]acetamide as well as with EBI was much lower than the specific radioactivity of β in the same sample. This indicates that β^* formation must require EBI reacting with sulfhydryl groups since if the reactive groups involved in β^* formation were not sulfhydryls, then iodo[^{14}C]acetamide should react equally as well with β^* as it does with β. In summary, although we cannot eliminate the possibility that EBI reacts with more than one kind of amino acid residue in tubulin, it seems likely that the formation of β^* by EBI requires that EBI react with sulfhydryl groups and that these groups be, at most, 11.7 Å apart.[2]

In conclusion, therefore, it appears that EBI, like iodo[^{14}C]acetamide, may also be used to study the sulfhydryl groups of tubulin. EBI appears to generate an intrachain bond in nondenatured β-tubulin, an observation that raises the possibility for future work using bifunctional alkylating agents to map the important sulfhydryl groups of tubulin. In the following paper (Ludueña & Roach, 1981), we shall present evidence that the reaction of tubulin with EBI is a very sensitive indicator of the interaction of tubulin with antimitotic drugs.

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[2] Estimates of cross-linker lengths were calculated from data on bond lengths and angles given by Weast & Astle (1980).

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Interaction of Tubulin with Drugs and Alkylating Agents. 2. Effects of Colchicine, Podophyllotoxin, and Vinblastine on the Alkylation of Tubulin†

Richard F. Ludueña* and Mary Carmen Roach

ABSTRACT: The antimitotic drugs colchicine, podophyllotoxin, and vinblastine are known to be potent inhibitors of microtubule polymerization, but little is known about how they affect the chemical properties of the tubulin molecule. In the preceding paper [Ludueña, R. F., & Roach, M. C. (1981) *Biochemistry* (preceding paper in this issue)], we have shown that the alkylating agent iodo[^{14}C]acetamide reacts specifically with the sulfhydryl groups of tubulin and that its bifunctional analogue, *N,N'*-ethylenebis(iodoacetamide) (EBI), reacts with native tubulin to convert β-tubulin into a form, designated β*, which appears to represent an intrachain cross-linked form of β. In this paper, we have incubated tubulin with the drugs prior to alkylation and measured their effects on the alkylation reactions. We have found that at 100 μM concentrations, podophyllotoxin, colchicine, and vinblastine inhibited the reaction of tubulin with iodo[^{14}C]acetamide by 19-32%, 33-47%, and 62-72%, respectively; each drug was half-maximally effective at 3-5 μM, indicating that the suppressive effects of the drugs were mediated by their high-affinity binding sites. Similarly, β* formation induced by EBI was suppressed by 92-94% in the presence of either colchicine or podophyllotoxin. In contrast, vinblastine enhanced β* formation by 40%. Alkylation with longer chain analogues of EBI revealed no evidence that the reactive sulfhydryls were being pushed apart by the drugs. These results indicate that each of the drugs has potent effects on the accessibility of the sulfhydryl groups of tubulin and that the effects of vinblastine are very different from those of either colchicine or podophyllotoxin.

Tubulin, the basic structural component of microtubules, interacts strongly with three structurally dissimilar compounds: colchicine, podophyllotoxin, and vinblastine. These drugs have been very useful probes in studies of the tubulin molecule and of microtubule assembly and function (Wilson, 1975; Wilson et al., 1976). Although these compounds are known to be potent inhibitors of microtubule assembly and to bind strongly to the tubulin molecule, very little detailed information is available about the drug binding sites on the tubulin molecule and of the areas on the molecule whose conformation is affected by the drugs. Colchicine and podophyllotoxin are competitive inhibitors of each other's binding to tubulin, and

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