Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622-627.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70-77.
Fellous, A., Francon, J., Lennon, A. M., & Nunez, J. (1977) *Eur. J. Biochem. 78*, 167-174.
Gross, H. (1967) *Methods Enzymol. 11*, 238-255.
Gundlach, H. G., Stein, W. H., & Moore, S. (1959a) *J. Biol. Chem. 234*, 1754-1760.
Gundlach, H. G., Moore, S., & Stein, W. H. (1959b) *J. Biol. Chem. 234*, 1761-1764.
Gurd, F. R. N. (1967) *Methods Enzymol. 11*, 532-541.
Ikeda, Y., & Steiner, M. (1978) *Biochemistry 17*, 3454-3459.
Kuriyama, R. (1976) *J. Biochem. (Tokyo) 80*, 153-165.
Kuriyama, R. (1977) *J. Biochem.* (Tokyo) *81*, 1115-1125.
Kuriyama, R., & Sakai, H (1974) *J. Biochem. (Tokyo) 76*, 651-654.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Lee, J. C., Frigon, R. P., & Timasheff, S. N. (1973) *J. Biol. Chem. 248*, 7253-7262.
Little, M. (1979) *FEBS Lett. 108*, 283-286.
Lowry, D. H., Rosebrough, N. H., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Ludueña, R. F. (1977) *Fed. Proc.*, 36, *Fed. Am. Soc. Exp. Biol.* 899.
Ludueña, R. F., & Woodward, D. O. (1975) *Ann. N.Y. Acad. Sci. 253*, 272-283.
Ludueña, R. F., & Roach, M. C. (1981) *Biochemistry* (following paper in this issue).
Ludueña, R. F., Shooter, E. M., & Wilson, L. (1977a) *J. Biol. Chem. 252*, 7006-7014.
Ludueña, R. F., Roach, M. C., & Garrison, P. (1977b) *J. Cell Biol. 75*, 272a.
Mellon, M. G., & Rebhun, L. I. (1976) *J. Cell Biol. 70*, 226-238.
Nelles, L. P., & Bamburg, J. R. (1976) *Anal. Biochem. 73*, 522-531.
Nishida, E., & Kobayashi, T. (1977) *J. Biochem. (Tokyo) 81*, 343-347.
Ozawa, H. (1967) *J. Biochem. (Tokyo) 62*, 531-536.
Porter, K. R. (1966) in *Principles of Biomolecular Organization* (Wolstenholme, G. E. W., & O&Connor, M., Eds.) pp 308-345, Little, Brown and Co., Boston, MA.
Riordan, J. F., & Vallee, B. L. (1967) *Methods Enzymol. 11*, 541-548.
Rosen, H. (1957) *Arch. Biochem. Biophys. 67*, 10-15.
Scheele, R. B., & Borisy, G. G. (1979) in *Microtubules* (Roberts, K., & Hyams, J. S., Eds.) pp 175-254, Academic Press, London.
Schmitt, H., & Kram, R. (1978) *Exp. Cell Res. 115*, 408-411.
Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem. 39*, 462-477.
Vallee, R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3206-3210.
Weast, R. C., & Astle, M. J., Eds. (1980) in *CRC Handbook of Chemistry and Physics*, 61st ed., pp F218-F220, CRC Press, Cleveland, OH.
Wilson, L., Anderson, K., & Chin, D. (1976) *Cold Spring Harbor Conf. Cell Proliferation* 3, 1051-1064.
Wold, F. (1972) *Methods Enzymol. 25*, 623-651.
Yang, S., & Criddle, R. S. (1970) *Biochemistry 9*, 3063-3072.

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

† From the Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284. *Received December 18, 1980.* Supported by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Robert A. Welch Foundation (AQ726), and the National Institutes of Health (GM 23476).

0006-2960/81/0420-4444$01.25/0

their binding sites are presumed to overlap, while vinblastine binds at two sites distinct from either of the former (Cortese et al., 1977; Wilson et al., 1975). Nevertheless, it is not clear which parts of the tubulin molecule, or even which of the two polypeptide chains, form the drug binding sites.

Both colchicine and vinblastine affect the conformation of the tubulin molecule at regions distinct from their binding sites. The colchicine-binding reaction is accompanied by a conformational change in the tubulin molecule detectable by fluorescence (Garland, 1978). Colchicine activates the intrinsic guanosine 5′-triphosphate activity of tubulin (David-Pfeuty et al., 1979). In addition, it slows down nonspecific aggregation and stabilizes its own binding site as well as one or both of the vinblastine binding sites and the exchangeable GTP binding site (Ludueña et al., 1977; Becker et al., 1975; Bhattacharyya & Wolff, 1976; Weisenberg et al., 1968). Vinblastine enhances the fluorescence of tubulin bound to 8-anilino-1-naphthalenesulfonate (Ans),[1] indicating an effect of the drug on the hydrophobic region where Ans binds (Bhattacharyya & Wolff, 1975). Vinblastine inhibits nonspecific aggregation but induces a specific aggregation of tubulin into dimers, rings, spirals, and macrotubules (Lee et al., 1975; Erickson, 1975; Fujiwara & Tilney, 1975). Vinblastine also inhibits the intrinsic GTPase activity of tubulin (David-Pfeuty et al., 1979). In addition, vinblastine stabilizes colchicine and GTP binding and increases the rate of colchicine binding (Garland & Teller, 1975; Tan & Lagnado, 1975; Wilson, 1970).

Recently, the sulfhydryl groups of tubulin have emerged as potential probes for the drug binding sites on the tubulin molecule and for the regions which the drugs affect. Schmitt & Kram (1978) have found that colchicine and vinblastine each inhibit the reaction of tubulin with the thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB),[1] from which they conclude that some sulfhydryl groups are probably located at the binding sites for these drugs. Schmitt & Kram, however, used a preparation of tubulin that contained microtubule-associated proteins (MAPS).[1] Since the DTNB reaction is measured in the spectrophotometer, one cannot be certain that the effects they see are due entirely to the sulfhydryls of tubulin rather than those of the MAPs. In the preceding paper (Ludueña & Roach, 1981), we have shown that two of the MAPs (HMW 1 and HMW 2) contain alkylatable groups that are likely to be sulfhydryls. Schmitt & Atlas (1976) have also shown that the covalent binding to tubulin of the alkylating agent bromo[^{3}H]colchicine is inhibited by colchicine and that this inhibition is most marked in α; they suggest, therefore, that the high affinity colchicine binding site is on α.

Since the ability of tubulin to polymerize into microtubules can be inhibited by oxidation of a few of its sulfhydryl groups as well as by binding to drugs, the nature of the possible interactions between these drugs and the sulfhydryl groups is an important area to elucidate. In the work described in this paper, we have examined the alkylation of the sulfhydryl groups in tubulin in the presence of colchicine, podophyllotoxin, and vinblastine, using as probes the alkylating agent iodo[^{14}C]acetamide and the bifunctional reagent *N,N′*-ethylenebis(iodoacetamide) (EBI).[1] These methods allow us to separately examine the sulfhydryls of both α- and β-tubulin and the microtubule-associated proteins. In the preceding paper (Ludueña & Roach, 1981), we have shown that iodo[^{14}C]-acetamide is a useful probe for the sulfhydryl groups of tubulin and that EBI makes a covalent intrachain cross-link in β-tubulin, thereby transforming β into a form, designated β*, which migrates faster on discontinuous polyacrylamide gels containing sodium dodecyl sulfate (Laemmli, 1970). We have found that each of the drugs is a potent inhibitor of alkylation and that the inhibition affects both the α and β chains. We have also found that the effect of vinblastine on alkylation is quite distinct from that of either colchicine or podophyllotoxin, the most striking difference being that colchicine and podophyllotoxin strongly inhibit, whereas vinblastine enhances, the EBI-induced formation of β*. Some of this material has been reported in preliminary form elsewhere (Morgan et al., 1978).

Experimental Procedures

Materials. Colchicine was from Sigma, podophyllotoxin and nocodazole from Aldrich, and vinblastine sulfate was a kind gift from Eli Lilly. Lumicolchcine was synthesized by ultraviolet irradiation of a solution of colchicine in 95% ethanol (Wilson & Friedkin, 1966). Maytansine (NSC 153858) was the kind gift of Dr. John Douros of the Developmental Therapeutics Program, Chemotherapy, National Cancer Institute. *N*-Ethyl[2,3-$^{14}C_2$]maleimide (New England Nuclear) was diluted with cold *N*-ethylmaleimide (Sigma).

Alkylation Reactions. In most experiments, the reaction of tubulin with iodo[^{14}C]acetamide was measured by subjecting the alkylated protein to polyacrylamide gel electrophoresis and then staining, scanning, and slicing the gel and counting the slices as described in the preceding paper (Ludueña & Roach, 1981). In some experiments, however, a different method was used in which the alkylated protein was precipitated by dilution with an equal volume of cold 10% trichloroacetic acid. The precipitate was collected by filtration on Celotate filters. Each filter was then placed in a scintillation vial to which was added 0.5 mL of NCS and 10 mL of toluene scintillation fluid with Omnifluor (New England Nuclear); the vials were then counted in a Beckman LS7000 liquid scintillation counter.

Other Procedures. All other materials and procedures used are described in the preceding paper in this issue (Ludueña & Roach, 1981).

Results

Effect of Drugs on the Alkylation of Tubulin by Iodo[^{14}C]acetamide. When tubulin was incubated with 100 μM concentrations of podophyllotoxin, colchicine, or vinblastine prior to alkylation, the rate of reaction of tubulin with iodo[^{14}C]acetamide was significantly decreased (Figure 1). Vinblastine was most effective at suppressing alkylation, and podophyllotoxin was least effective. Podophyllotoxin, colchicine, and vinblastine suppressed the extent of alkylation of tubulin after 2 h of reaction with iodo[^{14}C]acetamide by 32%, 47%, and 62%, respectively. Colchicine and vinblastine, in combination, were most effective at suppressing alkylation; after 2 h of reaction, the combination suppressed the alkylation of tubulin by 82% (Figure 1).

Very similar results were obtained when the extent of alkylation was measured by precipitating the alkylated tubulin with trichloroacetic acid, filtering the solutions, and counting the filters (Table I). Here, podophyllotoxin, colchicine, and vinblastine suppressed alkylation with iodo[^{14}C]acetamide by 19%, 33%, and 66%, respectively, while colchicine and vinblastine in combination suppressed alkylation by 78%. Table

[1] Abbreviations used: MAPs, microtubule-associate 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,N′,N′*-tetraacetic acid, 0.1 mM ethylenediaminetetraacetic acid, 0.5 mM $MgCl_2$, and 1 mM guanosine 5′-triphosphate; Ans (ansyl), 8-anilino-1-naphthalenesulfonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); $NaDodSO_4$, sodium dodecyl sulfate.

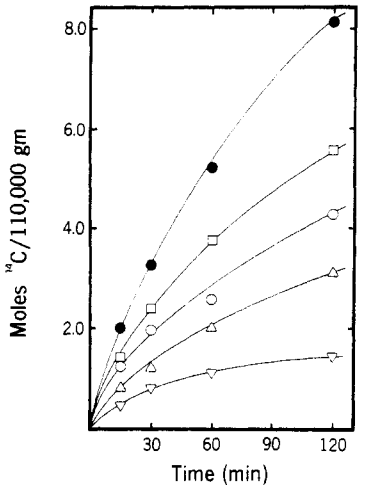

FIGURE 1: Effects of colchicine, podophyllotoxin, and vinblastine on the rate of alkylation of tubulin by iodo[^{14}C]acetamide. Aliquots (205 μL) of phosphocellulose-purified tubulin (0.71 mg/mL) were preincubated for 2 h at 37 °C in the absence (●) or presence of 100 μM concentrations of either colchicine (○), podophyllotoxin (□), or vinblastine (△) or in the presence of 100 μM concentrations of both colchicine and vinblastine (▽). At the end of 2 h, 5 μL of iodo[^{14}C]acetamide (5.25 Ci/mol) was added to each sample to a final concentration of 1.26 mM and the incubations were continued at 37 °C for the indicated lengths of time. At the end of that time, samples were processed and analyzed as described in the previous paper (Ludueña & Roach, 1981). Each point represents a separate incubation.

Table I: Effect of Drugs on the Incorporation of ^{14}C into Tubulin and Aldolase[a]

treatment	tubulin[b] (mol of ^{14}C/ 110 000 g)	% of control	aldolase[c] (mol of ^{14}C/ 40 000 g)	% of control
control	5.03 ± 0.28	100 ± 6	0.61 ± 0.07	100 ± 11
podophyllotoxin	4.05 ± 0.18	81 ± 6	0.53 ± 0.06	88 ± 14
colchicine	3.35 ± 0.06	67 ± 4	0.55 ± 0.03	91 ± 11
vinblastine	1.70 ± 0.14	34 ± 3	0.52 ± 0.01	85 ± 10
vinblastine + colchicine	1.10 ± 0.14	22 ± 3	0.72 ± 0.06	118 ± 16

[a] Aliquots (260 μL) of each protein were incubated for 2 h at 37 °C in buffer A in the presence of 100 μM concentrations of the indicated drug. Then 5 μL of iodo[^{14}C]acetamide was added to each solution to a final concentration of 1.29 mM. The incubation was continued for 1 h at 37 °C, and then each solution was diluted with an equal volume of cold 10% trichloroacetic acid. The protein was then precipitated and collected on filters where radioactivity was determined as described under Experimental Procedures. Each figure represents the average of three separate incubations. The standard deviations are given. [b] Tubulin concentration 0.72 mg/mL. Specific radioactivity of the iodo[^{14}C]acetamide was 1.31 Ci/mol. [c] Aldolase concentration 0.47 mg/mL. Specific radioactivity of the iodo[^{14}C]acetamide was 5.25 Ci/mol.

I also shows that the suppressive effect of the drugs on alkylation was a specific one since none of the drugs had a significant effect on the alkylation of aldolase.

In other experiments in which the tubulin contained MAPs, the drugs also suppressed alkylation. In one such experiment, 100 μM concentrations of podophyllotoxin, colchicine, and vinblastine suppressed the extent of the alkylation of tubulin after 2 h of reaction with iodo[^{14}C]acetamide by 25%, 47%, and 72%, respectively, while colchicine and vinblastine in combination suppressed the extent of alkylation of tubulin by 84% (results not shown).

The effects of the drugs on the alkylation of tubulin were dependent on their concentrations, as shown in Figure 2. In this experiment, in which the tubulin concentration was 6.5

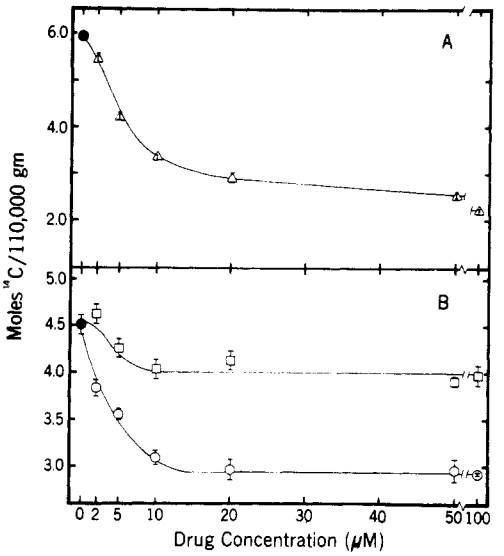

FIGURE 2: Effects of drug concentrations on the alkylation of tubulin with iodo[^{14}C]acetamide. (A) Aliquots (260 μL) of phosphocellulose-purified tubulin (0.72 mg/mL) were incubated for 2 h at 37 °C in the prescence (△) or absence (●) of the indicated concentrations of vinblastine. At the end of 2 h, 5 μL of iodo[^{14}C]acetamide (5.25 Ci/mol) was added to each sample to a final concentration of 1.29 mM. The incubations were continued for 1 h, and then the samples were processed and analyzed as described in the preceding paper (Ludueña & Roach, 1981). Each point represents the average of two or three separate incubations. The standard deviations are shown. (B) Conditions were identical with those in (A), but the drugs tested were colchicine (○) and podophyllotoxin (□).

Table II: Effects of 10 μM Colchicine and 10 μM Vinblastine on the Relative Alkylation of α- and β-Tubulin with Iodo[^{14}C]acetamide[a]

treatment	mol of ^{14}C/ (110 000 g)	% suppression by drug[b]	sp act. of β/ sp act. of α[c]
control	4.23 ± 0.28	0 ± 6.5	0.877 ± 0.122
10 μM colchicine	2.85 ± 0.02	32.6 ± 6.9	0.768 ± 0.102
10 μM vinblastine	2.54 ± 0.18	40.0 ± 8.1	0.821 ± 0.141
10 μM colchicine + 10 μM vinblastine	1.25 ± 0.18	70.4 ± 9.0	0.847 ± 0.172

[a] Aliquots (260 μL) of phosphocellulose-purified tubulin were incubated for 2 h at 37 °C in the presence of 10 μM concentrations of either colchicine or vinblastine or of both drugs together. At the end of 2 h, 5 μL of iodo[^{14}C]acetamide (4.42 Ci/mol) was added to each sample to a final concentration of 1.29 mM. The incubation at 37 °C was continued for 1 h, and the samples were then dialyzed against buffer A, reduced, and carboxamidomethylated. Each figure represents the average of three separate incubations. The standard deviations are given. [b] The values in the first column are divided by 4.23 and then subtracted from 1 and multiplied by 100%. [c] Sp act. = specific radioactivity.

μM, podophyllotoxin, at concentrations of 10 μM or higher, suppressed alkylation by 16% and was half-maximally effective at approximately 5 μM. Colchicine suppressed the alkylation of tubulin by 35% at drug concentrations above 20 μM and was half-maximally effective at 3–4 μM. Vinblastine had the most pronounced effect: 100 μM vinblastine suppressed alkylation by 62% and was half-maximally effective at approximately 4 μM. The concentrations at which these drugs were half-maximally effective are all less than the tubulin concentrations, indicating that these effects require that the drugs bind at their high-affinity binding sites.

The suppressive effects of the drugs on alkylation were observed in both the α and β chains. Table II shows that the presence of the drug had no significant effect on the ratio of the specific radioactivities of the α and β chains, implying that

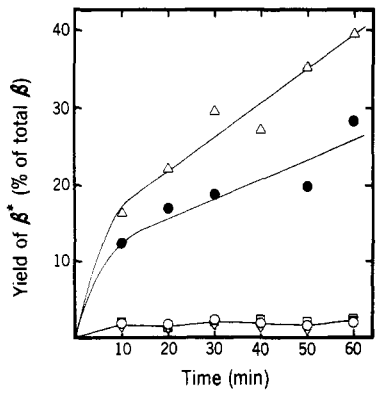

FIGURE 3: Effect of drugs on the reaction of tubulin with EBI. Aliquots (265 μL) of phosphocellulose-purified tubulin (0.71 mg/mL) in buffer A containing reduced and carboxymethylated conalbumin (0.19 mg/mL) were incubated for 1 h at 37 °C in the absence (●) or presence of 100 μM concentrations of either colchicine (○), podophyllotoxin (□), vinblastine (△), or colchicine and vinblastine together (▽). The samples were then reacted at 30 °C for the indicated times with 0.315 mM EBI. The samples were then cooled to 1 °C, processed, and analyzed on gels as in Figure 1. The amount of β* was determined, using conalbumin as the internal standard as previously described (Ludueña & Roach, 1981). Each point represents a separate incubation.

the two chains were affected equally by any given drug. Table II also shows that the suppressive effects of colchicine and vinblastine were additive. In other words, colchicine and vinblastine individually suppressed alkylation by 32.8 ± 6.9% and 40.0 ± 8.1%, respectively. The sum of these two numbers is 72.8 ± 10.6%, statistically indistinguishable from the suppressive effects of the two drugs in combination, which is 70.4 ± 9.0% (Table II). This observation is consistent with the two drugs binding to different high-affinity sites on the tubulin molecule. It is interesting that at 100 μM concentrations, colchicine and vinblastine no longer have additive effects, as seen in Figure 1. If they did, one would expect the two drugs in combination to completely suppress alkylation after 2 h of reaction, since the sum of their individual suppressive effects is 109%. Instead, they suppress alkylation by only 82%. Similarly, when the inhibition of alkylation is measured by the Cl_3CCOOH-precipitation assay (Table I), the sum of the colchicine inhibition (33%) and the vinblastine inhibition (66%) is 99%, and yet the two drugs in combination suppressed alkylation by only 78%. These observations probably reflect the presence of vinblastine binding sites of low affinity, which may overlap with the colchicine site (Wilson, 1975).

Effect of Drugs on the Alkylation of Tubulin by N,N'-Ethylenebis(iodoacetamide). In the preceding paper in this issue, we have shown that the bifunctional alkylating agent, EBI, when reacted with nondenatured tubulin, converts β into a form, β*, which migrates significantly faster on polyacrylamide gels (Laemmli, 1970), presumably due to the introduction of an intrachain cross-link in β. The effects of drugs on this reaction were examined. Figure 3 shows that colchicine, podophyllotoxin, and vinblastine had pronounced and different effects on the production of β* by EBI. Preincubation of phosphocellulose-purified tubulin with 100 μM concentrations of either colchicine or podophyllotoxin almost completely inhibited the production of β* by EBI. After 1 h of reaction with EBI, the yields of β* in the colchicine- and podophyllotoxin-treated samples were respectively 7% and 8% that of the untreated control. A similar effect was observed with 100 μM colchicine and 100 μM vinblastine in combination. In contrast, however, 100 μM vinblastine increased the yield of β* by EBI; after 1 h of reaction with EBI, 40% of β in the vinblastine-

Table III: Effect of Drugs on the Production of Aggregated Tubulin by EBI[a]

drug addition	yield of aggregated tubulin (% of total tubulin)	% suppression
none	21.6 ± 3.2 (5)	0 ± 15.0
vinblastine	7.1 ± 5.6 (6)	67.1 ± 31.6
podophyllotoxin	10.8 ± 2.5 (6)	50.0 ± 20.2
colchicine	9.0 ± 5.0 (6)	58.3 ± 28.8
vinblastine + colchicine	3.6 ± 4.8 (6)	83.3 ± 29.7

[a] Aliquots (265 μL) of phosphocellulose-purified tubulin (0.71 mg/mL) in buffer A containing reduced and carboxymethylated conalbumin (0.19 mg/mL) were incubated for 1 h at 37 °C in the presence of 100 μM concentrations of the indicated drugs. Samples were then reacted at 30 °C for 10–60 min with 0.315 mM EBI. Samples were then dialyzed, reduced, carboxymethylated, and analyzed on gels (Laemmli, 1970). The yield of high molecular weight aggregated tubulin was estimated in the previous paper (Ludueña & Roach, 1981). The table averages the yields of aggregated tubulin over the different time points. Only the samples incubated without drugs showed a significant time dependence of the yield of aggregate, ranging from 16% at 10 min to 25% at 50 min. The number of samples averaged is given in parentheses.

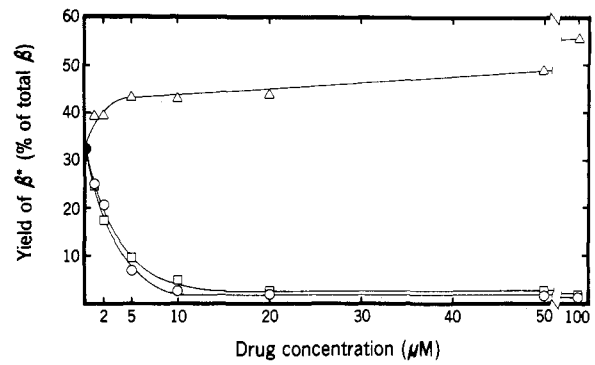

FIGURE 4: Effect of drug concentration on the reaction of tubulin with EBI. Aliquots (260 μL) of phosphocellulose-purified tubulin (0.72 mg/mL) containing reduced and carboxymethylated conalbumin (0.19 mg/mL) were incubated for 2 h at 37 °C in the absence (●) or presence of the indicated concentrations of colchicine (○), podophyllotoxin (□), or vinblastine (△). At the end of 2 h, 5 μL of EBI was added to a final concentration of 0.64 mM, and the incubations were continued for 1 h at 30 °C. The samples were processed and analyzed as described in the preceding paper (Ludueña & Roach, 1981).

treated sampled had been converted to β*, an increase of 40% over the untreated control. Maytansine (100 μM), a competitive inhibitor of vinblastine for binding to tubulin (Bhattacharyya & Wolff, 1977), also increased the relative yield of β* (R. F. Ludueña & M. C. Roach, unpublished results).

In addition to generating β*, EBI also caused tubulin to polymerize into a high molecular weight aggregate unable to penetrate into a 6% gel (Ludueña & Roach, 1981). Colchicine, podophyllotoxin, and vinblastine each inhibited the formation of this aggregate (Table III). The relative effectiveness of the drugs in inhibiting EBI-induced aggregate formation was similar to their relative effectiveness at suppression of the alkylation of tubulin with iodo[^{14}C]acetamide, with podophyllotoxin being the least effective inhibitor and vinblastine and colchicine in combination being most effective.

The effects on the relative yield of β* were dependent on the drug concentrations (Figure 4). Treatment of phosphocellulose-purified tubulin with podophyllotoxin at concentrations of 20 μM or higher caused 93% inhibition of β* formation, with the drug reaching half-maximal effectiveness at

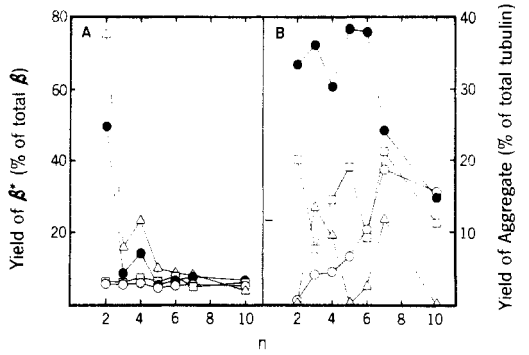

FIGURE 5: Effect of drugs on the formation of β^* (A) and of cross-linked aggregate (B) by *N,N'*-polymethylenebis(iodoacetamide) derivatives of different chain lengths. Aliquots (265 μL) of phosphocellulose-purified tubulin (0.72 mg/mL) containing reduced and carboxymethylated conalbumin (0.19 mg/mL) were incubated for 2 h at 37 °C in the absence (●) or presence of 98 μM concentrations of either colchicine (○), podophyllotoxin (□), or vinblastine (△). At the end of 2 h, 5 μL of one of the *N,N'*-polymethylenebis(iodoacetamide) derivatives was added to a final concentration of 0.64 mM, and the incubations were continued for 1 h at 30 °C. At the end of this time, the samples were processed and analyzed as described in the preceding paper (Ludueña & Roach, 1981). The relative yields of β^* (A) and cross-linked aggregate (B) are plotted as a function of *n*, the number of methylene groups in compounds of the general formula $ICH_2CONH(CH_2)_nNHCOCH_2I$.

a concentration of 2–3 μM. Similarly, colchicine, at concentrations of 10 μM or higher, caused 94% inhibition of β^* formation and was half-maximally effective at about 3 μM. In contrast, vinblastine, at concentrations of 5 μM or higher, increased β^* formation by 34–78% and was half-maximally effective at 5–10 μM. Since the tubulin concentration in this experiment was 6.5 μM, the drug-induced suppression or enhancement of β^* formation is likely to require that the drugs bind to high-affinity sites on the tubulin molecule. Almost identical results were obtained when samples of unfractionated microtubule protein were incubated with EBI in the presence of a series of concentrations of colchicine, podophyllotoxin, and vinblastine (not shown).

The drug nocodazole, a competitive inhibitor of colchicine binding to tubulin (Hoebeke et al., 1976), also inhibited the formation of β^* by EBI, but a 30 μM concentration of the drug was required for half-maximal inhibition (not shown). Lumicolchicine (20 μM) had no effect on the production of β^* by EBI.

In order to see if the inhibition of β^* formation induced by colchicine and podophyllotoxin was due to a drug-induced conformational change which moved sulfhydryls apart so that they become unable to cross-link with each other, the effects of the drugs on the reaction of tubulin with longer cross-linkers were examined (Figure 5). Samples of tubulin were incubated in the presence or absence of 98 μM concentrations of the drugs and then reacted with a series of *N,N'*-polymethylenebis(iodoacetamide) derivatives of increasing chain length. In the absence of drugs, EBI generated a 50% yield of β^*, and *N,N'*-tetramethylenebis(iodoacetamide) generated a 14% yield. The other cross-linkers generated β^* in yields ranging from 5% to 9%. For most of these derivatives, colchicine and podophyllotoxin suppressed β^* formation; in no case was the yield of β^* in the presence of these drugs higher than 8%. In contrast, 100 μM vinblastine enhanced β^* formation by each of the cross-linkers except for *N,N'*-decamethylenebis(iodoacetamide). The extent of this enhancement ranged from 9% to 81%, averaging 45 ± 26% (Figure 5A). When the yield of cross-linked aggregate was measured, it was found that each of the drugs inhibited aggregate formation by most of the cross-linkers (Figure 5B). When the suppressive effects of the drugs were averaged for each drug, it appeared that their relative effects on aggregate formation were similar to their relative effects on alkylation of tubulin with iodo[^{14}C]acetamide. In other words, podophyllotoxin inhibited aggregate formation by 52 ± 34%, colchicine by 72 ± 38%, and vinblastine by 83 ± 38%.

Effect of Drugs on the Reaction of Microtubule Protein with N-Ethyl[2,3-$^{14}C_2$]maleimide. In a preliminary experiment, samples of microtubule protein (1.0 mg/mL) were incubated for 2 h at 37 °C in the presence of 100 μM concentrations of either colchicine, podophyllotoxin, or vinblastine and then reacted for 30 min at 37 °C with 3.0 mM *N*-ethyl[2,3-$^{14}C_2$]maleimide (6.83 Ci/mol). After the reaction, the samples were processed and analyzed as described above. The results showed that colchicine suppressed the alkylation of tubulin by 55% relative to a control sample that had not been treated with drugs; podophyllotoxin and vinblastine each suppressed the alkylation of tubulin by 64%. It was observed that the α- and β-tubulin bands from samples that had been alkylated with *N*-ethylmaleimide were poorly resolved in gels. Therefore, the studies with *N*-ethylmaleimide were not pursued further.

Discussion

Effect of Drugs on the Alkylation of Tubulin. Colchicine, podophyllotoxin, and vinblastine each affected the interaction of tubulin with alkylating agents. Each of these drugs inhibited the incorporation of either iodo[^{14}C]acetamide or *N*-ethyl-[2,3-$^{14}C_2$]maleimide into tubulin and the formation of a high molecular weight tubulin aggregate by EBI. In addition, vinblastine enhanced the formation of β^* from β-tubulin by EBI. These effects were observed to approach half-maximal levels at drug concentrations lower than the tubulin concentration, suggesting that the effects required that the drugs bind to tubulin at high-affinity sites or, in other words, that these are specific effects rather than a nonspecific inhibition of alkylation by these drugs. This conclusion is corroborated by the fact that colchicine and vinblastine do not affect the alkylation of aldolase by iodo[^{14}C]acetamide. In short, these drugs affected the interaction of alkylating agents with the sulfhydryl groups of tubulin. These findings are consistent with those reported by Schmitt & Kram (1978), who observed that incubation of tubulin with ether colchicine or vinblastine causes a decrease in the rate at which tubulin reacts with DTNB.

On the basis of the results reported here, it appears that these drugs may be classified according to their effects on the alkylation of tubulin, one class consisting of colchicine and podophyllotoxin and the other of vinblastine. In every experiment reported here, both colchicine and podophyllotoxin inhibited the alkylation of both α- and β-tubulin with iodo-[^{14}C]acetamide and were very effective at inhibiting the formation of β^* by EBI. All of these effects were independent of the presence of MAPs. There was one major difference observed between the effects of colchicine and podophyllotoxin. Colchicine was more effective than podophyllotoxin at inhibiting alkylation. It is not clear whether this was due to podophyllotoxin affecting fewer sulfhydryl groups than colchicine or whether the fact that podophyllotoxin's binding to tubulin, unlike colchicine's, is reversible (Wilson, 1975) may make alkylation easier.

Vinblastine's effects on alkylation were markedly different from those of the other two drugs. Vinblastine suppressed alkylation of tubulin with iodo[^{14}C]acetamide more strongly than did either colchicine or podophyllotoxin. It also inhibited

EBI-induced formation of a cross-linked aggregate more effectively than did the other two drugs. Another difference between vinblastine and the other two drugs was that vinblastine enhanced, rather than inhibited, the formation of β^* by EBI. Since vinblastine is known to induce tubulin aggregation under certain conditions (Lee et al., 1975; Erickson, 1975; Fujiwara & Tilney, 1975) and since microtubules react very poorly with alkylating agents, one might argue that the unique effects of vinblastine on the alkylation of tubulin are not due directly to its binding to tubulin, but rather to the induction of a tubulin aggregate in which many otherwise accessible sulfhydryls are blocked. This alternative explantation is unlikely, however, since no aggregation was observed in tubulin samples at vinblastine concentrations lower than 100 μM.

In conclusion, it seems that there is a correlation between the location of the drug binding sites on the tubulin molecule and the nature of the effects of the drugs on alkylation. Colchicine and podophyllotoxin, which bind to overlapping sites on the tubulin molecule, inhibit the formation of β^*. The simplest explanation for this is that the presence of the drugs at their binding sites somehow affects the area on β which is involved with the formation of the intrachain cross-link. This model is corroborated by the observation that nocodazole, which apparently binds to the colchicine site (Hoebeke et al., 1976), also inhibited β^* formation by EBI. Vinblastine binds to tubulin at sites distinct from the colchicine/podophyllotoxin site (Wilson, 1975), and its effect is to enhance the formation of β^* by EBI. One possibility is that the presence of the drug at its binding site may somehow enhance the alkylation of those sulfhydryls on β which are involved in the formation of β^*. Another possibility is that vinblastine, although it suppresses the alkylation of the other sulfhydryls, leaves these particular sulfhydryls unaffected, thereby permitting them to react with EBI more readily. By this model, the enhancement of β^* formation caused by vinblastine could be an indirect result of vinblastine's inhibiting other alkylation reactions by EBI which could compete with β^* formation. The fact that maytansine, which binds at the vinblastine site (Bhattacharyya & Wolff, 1977), also increases β^* formation is consistent with either of these models.

There are, in principle, three possible mechanisms by which the alkylation of a sulfhydryl group on tubulin could be affected by the binding of a drug to the tubulin molecule. In one mechanism, which we shall call "steric", the sulfhydryl group is located at the binding site of the drug, and its alkylation is inhibited because the presence of the drug molecule makes the sulfhydryl group inaccessible to alkylating agents. By the next mechanism, which we shall designate as "active allosteric", the affected sulfhydryl group is not located at the drug binding site, but the binding of the drug to its binding site causes the tubulin molecule to undergo a conformational change which alters the configuration around the sulfhydryl group in such a way as to make it either more or less accessible. The third mechanism, which we shall call "passive allosteric", is similar to the "active allosteric" model. The only difference is that the binding of the drug to the molecule does not alter the configuration around the affected sulfhydryl but simply makes it more or less rigid, or, in other words, stabilizes or destabilizes it with respect to denaturation. Oxidation of tubulin sulfhydryls is known to alter the tubulin molecule to inhibit microtubule assembly and colchicine and GTP binding (Kuriyama, 1976; Nishida & Kobayashi, 1977; Mellon & Rebhun, 1976; Kuriyama & Sakai, 1974; Solomon et al., 1973). It is conceivable that oxidation of tubulin sulfhydryls is a synergistic process in which oxidation leads to denaturation which exposes more sulfhydryls for oxidation, and so forth. By the "passive allosteric" model, the presence of drugs bound to the tubulin molecule could stabilize it against denaturation induced by alkylating agents, and the result would be an apparent decrease in the rate of alkylation of tubulin.

On the basis of the data presented in this paper, it is not possible to determine which of these three mechanisms is involved in the interactions of any given drug and any given sulfhydryl group. One should note, however, that when tubulin is treated with both 10 μM colchicine and 10 μM vinblastine prior to alkylation with iodo[^{14}C]acetamide, the inhibitory effect of the two drugs in combination is equal to the sum of the effects of the two drugs separately (Table II). This implies that the sulfhydryls affected by colchicine and those affected by vinblastine represent two nonoverlapping sets of sulfhydryls. Since colchicine and vinblastine are known to bind to different high-affinity sites on the tubulin molecule, it is tempting to speculate that at least one sulfhydryl in each set is located at one of the drug binding sites. If this were the case, then one could imagine that the strongest drug-induced suppression of alkylation would be observed with sulfhydryls located at the drug binding sites rather than sulfhydryls affected by drug-induced conformational changes. The fact that the strongest effect reported in this paper is the inhibition by colchicine and podophyllotoxin of β^* formation with EBI suggests that one or both of the sulfhydryls involved in β^* formation may be located at the colchicine/podophyllotoxin binding site. This speculation is consistent with the observation that vinblastine-induced tubulin spirals, in which alkylation in general is greatly inhibited, react very well with [^{3}H]colchicine and generate a high yield of β^* upon treatment with EBI. This implies that the EBI and colchicine binding sites, if not identical, are at least close together on the tubulin molecule (Ludueña et al., 1980; P. Palanivelu and R. F. Ludueña, unpublished results).

Another possibility is that colchicine and podophyllotoxin act to separate those sulfhydryls involved in β^* formation so that they can no longer make an intrachain cross-link. Such ligand-induced changes in intramolecular cross-linking patterns have recently been observed in other proteins, such as (Na^+, K^+)-ATPase (Askari et al., 1980) and myosin (Burke & Reisler, 1977). Figure 5, however, indicates that the suppressive effects of colchicine and podophyllotoxin on β^* formation are not affected by changing the length of the cross-linker from 11.7 to 21.7 Å.[2] It appears, therefore, that the effects of colchicine and podophyllotoxin on these sulfhydryls are due to inhibiting the reactivity or accessibility of one or more of these sulfhydryls rather than merely separating them.

Implication of These Results. The experiments described in this paper indicate that colchicine, podophyllotoxin, and vinblastine have significant effects on the alkylation of the sulfhydryl groups of tubulin. Each of these drugs is also a powerful inhibitor of microtubule assembly in vivo and in vitro (Wilson et al., 1974, 1976). It is interesting that microtubule assembly also may be inhibited by oxidation of tubulin sulfhydryl groups (Kuriyama, 1976; Mellon & Rebhun, 1976). It is conceivable that these two kinds of inhibition are related. Since the ability of tubulin to polymerize may be abolished by oxidation of only 1–3 sulfhydryls/M_r 55000 whereas complete inhibition of colchicine binding requires that all the available sulfhydryls be oxidized (Mellon & Rebhun, 1976;

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

Ikeda & Steiner, 1978), it is likely that the sulfhydryl groups critical for polymerization are not located at the colchicine binding site. In view of these possibilities, it is conceivable that there is a sulfhydryl-containing site or sites on the tubulin molecule which must be in a certain conformation in order for polymerization to occur. This conformation then could be altered, with concomitant loss of the ability to polymerize, either by oxidation of the sulfhydryl group at this site or by an allosteric effect produced by a drug molecule binding elsewhere on the molecule. Since these drugs alter the accessibility of the sulfhydryl groups of tubulin, it is possible that one way the drugs, or any endogenous analogue they may have, function in vivo is to modulate the susceptibility of tubulin sulfhydryls to interact with endogenous thiols, such as glutathione.

The results reported here also are relevant to the search to locate the colchicine binding site on the tubulin molecule. It has been reported by Schmitt & Atlas (1976) that the binding site for colchicine may be located on α-tubulin. In light of these results, this conclusion may need to be reevaluated. Schmitt & Atlas (1976) used as their alkylating agent bromo[^{3}H]colchicine, which they observed could react both specifically and nonspecifically with tubulin. They also found that pretreatment of tubulin with colchicine inhibits the reaction with bromo[^{3}H]colchicine and that the effect of colchicine is significantly greater on α-tubulin than on β-tubulin; hence, they concluded that the binding site probably is located on α. However, as we have seen, colchicine suppresses the reactivity of both α- and β-tubulin with a variety of alkylating agents, and bromocolchicine is in essence an alkylating agent; thus, it is not surprising that colchicine should inhibit the reactivity of tubulin with bromocolchicine. The fact that it may inhibit the reactivity of α somewhat more strongly than that of β does not necessarily mean that colchicine binds to α but also could imply that its effect on α as measured by this assay is more pronounced than its effect on β. Hence, the precise location of the binding site of colchicine or any other drug on the tubulin molecule must still be considered unknown.

Nevertheless, the fact that the antimitotic drugs have strong and distinct effects on the alkylation of tubulin, as we have shown, implies that our methods could be used to identify precisely those areas on the tubulin molecule whose conformation is affected by the drugs and perhaps also to locate the drug binding sites. In addition, the interactions of drugs with the sulfhydryl groups of tubulin reported here may ultimately increase our understanding of the roles of both in regulating microtubule assembly.

Acknowledgments

We thank Larry Barnes and Jonathan Nishimura for their suggestions and Phyllis Trcka for technical assistance.

References

Askari, A., Huang, W. H., & Antieau, J. M. (1980) *Biochemistry 19*, 1132–1140.

Becker, J. S., Oliver, J. M., & Berlin, R. D. (1975) *Nature (London) 254*, 152–154.

Bhattacharyya, B., & Wolff, J. (1975) *Arch. Biochem. Biophys. 167*, 264–269.

Bhattacharyya, B., & Wolf, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2375–2378.

Bhattacharyya, B., & Wolff, J. (1977) *FEBS Lett. 75*, 159–162.

Burke, M., & Reisler, E. (1977) *Biochemistry 16*, 5559–5563.

Cortese, F., Bhattacharyya, B., & Wolff, J. (1977) *J. Biol. Chem. 252*, 1134–1140.

David-Pfeuty, T., Simon, C., & Pantaloni, D. (1979) *J. Biol. Chem. 254*, 11696–11702.

Erickson, H. P. (1975) *Ann. N.Y. Acad. Sci. 253*, 51–52.

Fujiwara, K., & Tilney, L. G. (1975) *Ann. N.Y. Acad. Sci. 253*, 27–50.

Garland, D. L. (1978) *Biochemistry 17*, 4266–4272.

Garland, D., & Teller, D. C. (1975) *Ann. N.Y. Acad. Sci. 253*, 232–238.

Hoebeke, J. Van Nijen, G., & DeBrabander, M. (1976) *Biochem. Biophys. Res. Commun. 69*, 319–324.

Ikeda, Y., & Steiner, M. (1978) *Biochemistry 17*, 3454–3459.

Kuriyama, R. (1976) *J. Biochem. (Tokyo) 80*, 153–165.

Kuriyama, R., & Sakai, H. (1974) *J. Biochem. (Tokyo) 76*, 651–654.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lee, J. C., Harrison, D., & Timasheff, S. N. (1975) *J. Biol. Chem. 250*, 9276–9282.

Ludueña, R. F., & Roach, M. C. (1981) *Biochemistry* (preceding paper in this issue).

Ludueña, R. F., Shooter, E. M., & Wilson, L. (1977) *J. Biol. Chem. 252*, 7006–7014.

Ludueña, R. F., Fellous, A., Francon, J., & Nunez, J. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1818.

Mellon, M. G., & Rebhun, L. I. (1976) *J. Cell Biol. 70*, 226–238.

Morgan, J. L., Ludueña, R. F., & Spooner, B. S. (1978) *J. Cell Biol. 79*, 292a.

Nishida, E., & Kobayashi, T. (1977) *J. Biochem. (Tokyo) 81*, 343–347.

Schmitt, H., & Atlas, D. (1976) *J. Mol. Biol. 102*, 743–758.

Schmitt, H., & Kram, R. (1978) *Exp. Cell Res. 115*, 408–411.

Solomon, F., Monard, D., & Rentsch, M. (1973) *J. Mol. Biol. 78*, 569–573.

Tan, L. P., & Lagnado, J. R. (1975) *Biochem. Soc. Trans. 3*, 121–124.

Weast, R. C., & Astle, M. J. Eds. (1980) in *CRC Handbook of Chemistry and Physics*, 61st ed., pp F218–F220, CRC Press, Cleveland, OH.

Weisenberg, R. C. Borisy, G. G., & Taylor, E. W. (1968) *Biochemistry 7*, 4466–4479.

Wilson, L. (1970) *Biochemistry 9*, 4999–5007.

Wilson, L. (1975) *Ann. N.Y. Acad. Sci. 253*, 213–231.

Wilson, L., & Friedkin, M. (1966) *Biochemistry 5*, 2463–2468.

Wilson, L., Bamburg, J. R., Mizel, S. B., Grisham, L. M., & Creswell, K. M. (1974) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 158–166.

Wilson, L., Creswell, K. M., & Chin, D. (1975) *Biochemistry 14*, 5586–5592.

Wilson, L., Anderson, K., & Chin, D. (1976) *Cold Spring Harbor Conf. Cell Proliferation 3*, 1051–1064.