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Effects of Nocodazole on Structures of Calf Brain Tubulin†

James C. Lee,* Deborah J. Field, and Lucy L. Y. Lee

ABSTRACT: The interaction of nocodazole with calf brain tubulin was studied to determine the effect of such interaction on the structure of tubulin. The effect of nocodazole on the self-association of tubulin was monitored by turbidity measurements and velocity sedimentation. Sedimentation patterns indicate that nocodazole neither induces tubulin to undergo self-association to form higher orders of aggregate nor does it perturb the equilibrium of the reaction leading to the formation of 42S double-ring structures although nocodazole binds to both the tubulin dimers and the polymeric form. Nocodazole does, however, inhibit the in vitro reconstitution of microtubules, and the presence of microtubule-associated proteins does not amplify the inhibitory effect of the drug. The conformational changes in tubulin upon binding of nocodazole were monitored by differential spectroscopy, circular dichroism, fluorescence, and chemical modification of sulfhydryl residues. Results from these studies show that the sulfhydryl residues become more accessible to chemical modification. In contrast, the binding of nocodazole does not significantly alter the net environment of tryptophan chromophores. These residues are apparently not all located on the surface of the tubulin molecule and at least some are partially buried.

Nocodazole (methyl [5-(2-thienylcarbonyl)-1*H*-benzimidazol-2-yl]carbamate, R17934) is a new synthetic drug which has been shown to have antimitotic (DeBrabander et al., 1976) and antitumoral activity (DeBrabander et al., 1975; Atassi & Tagnon, 1975). The action of this agent is readily reversible and relatively rapid. Within 20 min of exposure to nocodazole, cells show gross morphological changes which include gradual disappearance of microtubules and rounding of cells with a loss of directional movement (DeBrabander et al., 1976). After 2–5 h of exposure, cell morphology becomes further modified as cell organelles lose their perinuclear organization. In addition to these gross morphological changes, cells treated with nocodazole undergo a form of abortive mitosis which terminates with the production of polyploid cells. Preliminary investigations of experimental animals show that malignant cells are more susceptible to the antitumoral activity of the drug than are nonmalignant cells (DeBrabander et al., 1976). Nocodazole, therefore, is a potentially useful drug with specificity directed toward malignant cells.

The target site of the drug has been investigated on several levels. At the ultrastructural level treatment with 10^{-7}–3×10^{-5} M nocodazole results in the loss of most of the cytoplasmic microtubules within 10 min (DeBrabander et al., 1975). At the molecular level, nocodazole inhibits the polymerization of brain tubulin in vitro (Hoebeke et al., 1976; Friedman & Platzer, 1978; Ireland et al., 1979) and probably binds to microtubule protein. On the basis of the results of a ligand binding study, Hoebeke et al. (1976) suggested that nocodazole competitively inhibits colchicine binding to microtubule protein even though there is no structural similarity between these two drugs. Brodie et al. (1979) arrived at a similar conclusion in their report. The molecular mechanism(s) through which nocodazole exerts its effect is still unknown. In view of the possible clinical significance of nocodazole–tubulin interactions, a study was initiated to study the effects of the drug on the structure of tubulin. The results of in vitro reconstitution and perturbation of the protein structure by nocodazole are described in this paper. A preliminary report of this work has been presented earlier (Lee & Lee, 1979a).

Materials and Methods

The disodium salt of GTP[1] was obtained from Boehringer Mannheim Biochemicals. Dimethyl sulfoxide, 5,5′-dithiobis(2-nitrobenzoic acid), and *p*-mercuric benzoate were purchased from Sigma Chemical Co. Gel electrophoretic grade acrylamide (Lot 1639) and nocodazole were purchased from Bethesda Research Laboratories, Inc. and Aldrich Chemical Co., Inc., respectively. Extreme purity grade guanidine hydrochloride, from Heico, Inc., was used after filtration through a sintered-glass filter. Phosphocellulose was obtained from Whatman Ltd.

Calf brain tubulin was purified by both the modified Weisenberg procedure (Weisenberg et al., 1968; Weisenberg & Timasheff, 1970; Lee et al., 1973; Lee, 1980) and the polymerization and depolymerization method of Shelanski et al. (1973), as modified by Runge et al. (1979) with 90-min periods of centrifugation at 106000*g* and 4 °C. Cycle tubulin was further purified by phosphocellulose chromatography as described previously (Lee et al., 1978a). These will be referred to as W-tubulin, c-tubulin, and PC-tubulin, respectively. Tubulin concentrations were determined spectrophotometrically in 6 M Gdn-HCl by using an absorptivity of 1.03 L/(g·cm) at 275 nm.[2]

The formation of microtubules was followed by the turbidity method introduced by Gaskin et al. (1974). Three assembly buffers were used. The first consisted of 10^{-2} M Mes, 5×10^{-4} M GTP, 1.0×10^{-3} M $MgCl_2$, 2×10^{-3} M EGTA, and 1% (v/v) Me_2SO at pH 6.9; it will be referred to as buffer A. The second consisted of 10^{-2} M sodium phosphate, 10^{-4} M GTP, 1.6×10^{-2} M $MgCl_2$, 3.4 M glycerol, and 1% (v/v) Me_2SO at pH 7.0; it will be referred to as buffer B. The third is similar to buffer A except it consisted of 10^{-2} M Pipes and 12% (v/v) Me_2SO at pH 7.5; it will be referred to as buffer C. The low solubility of nocodazole in aqueous solution necessitates the incorporation of Me_2SO in the buffer system. Nocodazole at concentrations of up to 10^{-4} M is soluble in 1% Me_2SO; however, in the course of 1–2 h the drug slowly precipitates

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[1] Abbreviations used: Me_2SO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PMB, *p*-mercuric benzoate; Gdn-HCl, guanidine hydrochloride; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; GTP, guanosine 5′-triphosphate; Mes, 4-morpholineethanesulfonic acid.

[2] Private communication from C. Na and S. N. Timasheff.

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out of solution. Me_2SO (12%) was employed so as to maintain drug solubility for experiments lasting more than 2 h in order to circumvent this problem. Turbidity was measured at 350 nm with a Cary 118 or 14 recording spectrophotometer as described previously (Lee & Timasheff, 1975, 1977). Extrapolation of turbidity values obtained at different protein concentrations to zero turbidity gives the critical concentration, C_r. Oosawa & Higashi (1967) have shown that, for helical polymerization such as that of G-actin to F-actin and tubulin to microtubules, the critical concentration is equal, within close approximation, to the inverse of the apparent association constant, K_{app}, for the addition of each subunit to a growing helix. Gaskin et al. (1974) has demonstrated elegantly that indeed the reconstitution of microtubules conforms to the polymerization theory of Oosawa and co-workers (Oosawa & Higashi, 1967; Oosawa & Kasai, 1971) and the analysis according to the theory is valid.

Difference spectra of tubulin in the presence of nocodazole were recorded on a Cary 118 spectrophotometer with 1-cm square tandem cells. The spectra were obtained with protein concentrations of ~1.0 mg/mL at room temperature. A base line was recorded before each experiment, and the spectra were measured from 350 to 240 nm. Repeated scans were normally performed with no observable differences between scans.

The conformation of tubulin was also monitored by circular dichroism using a Cary Model 60 spectropolarimeter equipped with a Model 6001 attachment. The spectra were routinely recorded from 350 to 240 nm. Overlapping spectra were obtained with 0.2-, 0.1-, and 0.01-cm fused silica cells. A value of 109 was used for the mean residue weight of tubulin in the calculation of ellipticities, [θ]. All runs were performed at ~23 °C.

Sulfhydryl titrations were monitored at room temperature by following the formation of the mercaptide complex spectrophotometrically as described previously (Lee & Lee, 1979b). Tubulin solutions (1 mL) of ~1.0 mg/mL in 10^{-2} M sodium phosphate, 10^{-4} M GTP, and 1% (v/v) Me_2SO, pH 7.0, were employed. Stock solutions of PMB and DTNB were prepared in the above buffer at 4×10^{-4} and 4×10^{-3} M, respectively. Values of 7.6×10^3 M^{-1} cm^{-1} at 250 nm and 1.36×10^4 M^{-1} cm^{-1} at 412 nm were used for estimating the formation of complexes in the presence of PMB (Boyer, 1954) and DTNB (Ellman, 1959), respectively. The increase in absorbance with time was analyzed in terms of pseudo-first-order reaction according to

$$\ln \frac{A_\infty - A_t}{A_\infty - A_0} = -kt \qquad (1)$$

where A_0 and A_∞ represent the initial and final absorbance and A_t represents the absorbance at time t. The semilogarithmic plots of the data can be analyzed for distinguishable classes of sulfhydryl groups by extrapolating the linear portion of the plot to $t = 0$. The slope and intercept of the line yield rate constants and the initial concentration of the slow reactive class, respectively (Frost & Pearson, 1961).

The "exposure" of tryptophan residues in tubulin was monitored by the quenching of the tryptophan fluorescence by acrylamide (Eftink & Ghiron, 1976). A rate constant for quenching can be obtained by using the Stern–Volmer equation (Stern & Volmer, 1919):

$$F_0/F = 1 + K_Q[Q] \qquad (2)$$

where F_0 and F are the fluorescence intensities of the fluorophore in the absence and presence of acrylamide, [Q] is the concentration of acrylamide, and K_Q is the collisional quenching constant. Since tubulin contains more than one tryptophan residue with each residue probably fluorescing independently, eq 2 can be written as (Bandyopadhyay & Wu, 1979)

$$F_0/F = \sum \frac{1 + K_{Qi}[Q]}{f_i} \qquad (2a)$$

where f_i is the fraction of the total fluorescence corresponding to the ith tryptophan residue and K_{Qi} is the respective collisional quenching constant. The initial slope of the plot of F_0/F vs. [Q] is approximately equal to the weighted average of the individual quenching constants.

A correction factor was applied for the attenuation of the excitation light intensity by the added acrylamide by using the procedure outlined by Parker (1968). The fluorescence experiments were conducted at 22–23 °C with a Perkin-Elmer spectrofluorometer, Model 512. The excitation and emission wavelengths were 292 and 329 nm, respectively.

The sedimentation experiments were conducted at 20 °C and 60000 rpm in a Spinco Model E analytical ultracentrifuge equipped with an electronic speed control, RTIC temperature control, and a photoelectric scanner. For all experiments a Kel-F coated aluminum double-sector centerpiece was used.

Results and Discussion

Earlier reports indicated that nocodazole inhibits the polymerization of brain tubulin in vitro; however, they do not clearly demonstrate the site of interaction. It is conceivable that the microtubule-associated proteins (MAP) may be the primary site of interaction with the drug and in turn influence the reconstitution of microtubule. A detailed study was, therefore, initiated to quantitate the inhibition of microtubule reconstitution by the drug and the effect of microtubule-associated proteins on this reaction. Initial results of the turbidimetric measurements of c-tubulin as a function of nocodazole concentration showed that the turbidity values in the plateau regions are linearly related to the drug concentrations and decrease with increasing drug concentration; nocodazole must be exerting an inhibitory effect on in vitro reconstitution of microtubule as reported (Hoebeke et al., 1976; Friedman & Platzer, 1978; Ireland et al., 1979). For quantitation of the effect of nocodazole on the thermodynamics of microtubule reconstitution, the perturbation of the apparent microtubule association constant, K_{app}, by the drug was monitored by turbidity measurements. These experiments consisted of measurements of C_r in the presence of varying concentrations of nocodazole. Figure 1 presents the typical results of changes in turbidity when a c-tubulin solution in buffer A is heated to 37 °C in the presence of 5×10^{-6} M nocodazole. The turbidity values in the plateau regions are linearly related to the tubulin concentrations, as shown in the inset of Figure 1. Extrapolation to zero turbidity gives a value for C_r of 2 mg/mL which is much higher than that of 0.2 mg/mL determined in the absence of nocodazole. Values of C_r in the presence of varying concentrations of nocodazole are presented in Table I. They show that the presence of nocodazole decreases the apparent association constant and the decrease is a function of nocodazole concentration. The effects of nocodazole on microtubule reconstitution in the absence of MAP were also monitored by turbidity measurements of W-tubulin in assembly buffer B. Results of this investigation are presented in Table I, showing that even in the absence of MAP the presence of nocodazole decreases the apparent association constant although the values of K_{app} for c-tubulin are much higher than that for W-tubulin. The results of these experiments indicate that the presence of MAP does not alter the basic effect of the drug, namely, inhibition of in vitro microtubule recon-

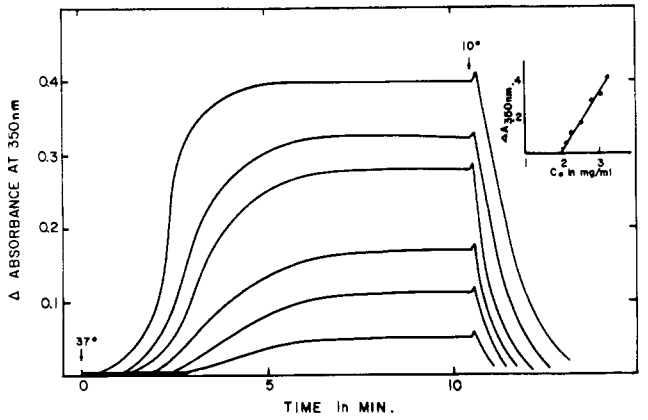

FIGURE 1: Effect of c-tubulin concentration on development of turbidity in the presence of 5×10^{-6} M nocodazole. The solvent was 10^{-2} M Mes, 5×10^{-4} M GTP, 1.0×10^{-3} M $MgCl_2$, 2×10^{-3} M EGTA, and 1% (v/v) Me_2SO at pH 6.9.

Table I: Dependence of Microtubule Growth on Nocodazole Concentration

nocodazole concn ($M \times 10^{-7}$)	K_{app} [(L/mol) $\times 10^4$]		
	c-tubulin[a]	c-tubulin[b]	W-tubulin[c]
1.0	24.3	12.4	12.7
1.5		11.6	
3.0		11.3	8.9
5.0		7.5	8.1
8.0		7.9	
10.0	10.9	6.8	6.0
20.0		5.3	
50.0	6.6		
85.0	6.0		
100.0	6.1		

[a] In the presence of assembly buffer A. [b] In the presence of assembly buffer C. [c] In the presence of assembly buffer B.

stitution. A condition was chosen so that the values of K_{app} for both c-tubulin and W-tubulin would be approximately identical in the absence of nocodazole in order to facilitate a quantitative evaluation of the effect of MAP. It was found that in assembly buffer C the value of K_{app} for c-tubulin in the absence of nocodazole was very similar to that of W-tubulin in assembly buffer B. The effects of nocodazole on K_{app} of c-tubulin in assembly buffer C were monitored, and the results are shown in Table I. The apparent association constants of W- and c-tubulins in the presence of the same concentration of nocodazole are identical within the experimental uncertainties. Figure 2 shows the dependence of the apparent association constant on nocodazole concentration. It is evident that the apparent association constants of the different tubulins were affected by nocodazole to a similar extent, both quantitatively and qualitatively. The presence of MAP does not influence the inhibitory effect of nocodazole on microtubule reconstitution. The difference in K_{app} for c-tubulin in the presence of assembly buffer A and W-tubulin in assembly buffer B is most likely a reflection of the presence of MAP which act to enhance the self-assembly reaction without altering the basic properties of tubulin. Such interpretation is strengthened by the observation (Figure 2) made with PC-tubulin, i.e., c-tubulin passed through a phosphocellulose column to remove MAP, which demonstrates that the K_{app} determined in the presence of 5×10^{-7} M nocodazole for c-tubulin without MAP is identical with that for W-tubulin under the same experimental conditions. It may, therefore, be concluded that MAP do not influence the interaction between nocodazole and tubulin. The effective site(s) of drug–

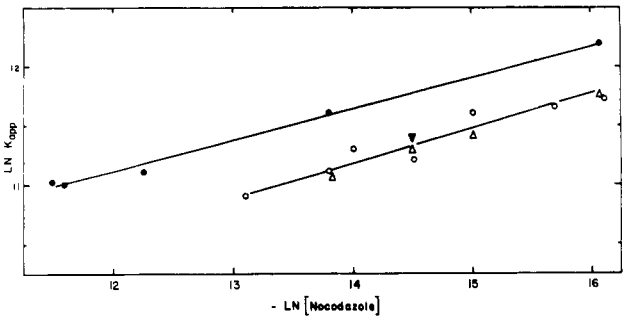

FIGURE 2: Dependence of the apparent propagation constant on nocodazole. The symbols and experimental conditions are (●) c-tubulin in assembly buffer A, (△) W-tubulin in assembly buffer B, (○) c-tubulin in assembly buffer C, and (▼) PC-tubulin in assembly buffer B.

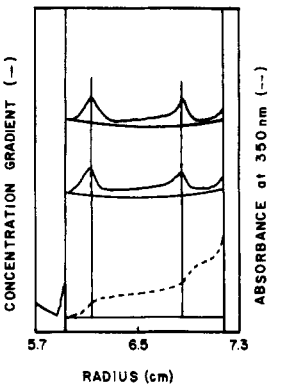

FIGURE 3: Velocity sedimentation patterns of W-tubulin (A) in 10^{-2} M phosphate, 10^{-4} M GTP, and 1.6×10^{-2} M $MgCl_2$ at pH 7.0 and 20 °C and (B) in the same buffer as (A) in the presence of 5×10^{-5} M nocodazole. (C) is a scanner tracing at 350 nm taken simultaneously with the Schlieren pattern. The protein concentration was 9.3 mg/mL, and the pictures were taken 12 min after reaching a speed of 60 000 rpm.

protein interaction is the tubulin molecule.

Having established that nocodazole inhibits in vitro microtubule reconstitution, it is desirable to elucidate the mechanism through which nocodazole exerts its effect. One of the possibilities is that nocodazole may induce formation of alternate polymeric forms as does vinblastine (Bensch & Malawista, 1969; Weisenberg & Timasheff, 1970; Lee et al., 1975; Na & Timasheff, 1980a,b). The sedimentation behavior of W-tubulin in 10^{-2} M phosphate, 10^{-4} M GTP, and 5×10^{-5} M nocodazole at pH 7.0 and 23 °C was monitored to search for tubulin aggregates induced by nocodazole. In the absence of $MgCl_2$, W-tubulin sediments as a single component with a $s_{20,w}$ of 4.95 S at 9.3 mg/mL in the presence or absence of nocodazole. This observation is in good agreement with the literature (Frigon & Timasheff, 1975). The presence of nocodazole, therefore, under the present experimental conditions does not induce W-tubulin to form higher orders of aggrgates. The presence of $MgCl_2$ may, however, exert a synergistic effect. The sedimentation behavior of W-tubulin in 1.6×10^{-2} M $MgCl_2$ was studied, and the results are shown in Figure 3. Under these conditions, it has been shown that tubulin will undergo self-association with the formation of double-ring structures of $s_{20,w} = 42$ S. It is evident from Figure 3 that the presence of nocodazole does not influence the sedimentation pattern, indicating that 5×10^{-5} M nocodazole does not perturb the formation of these double-ring structures, although the formation of microtubule is markedly inhibited at the concentration. This may also imply that the formation of double rings and microtubules represents products of two

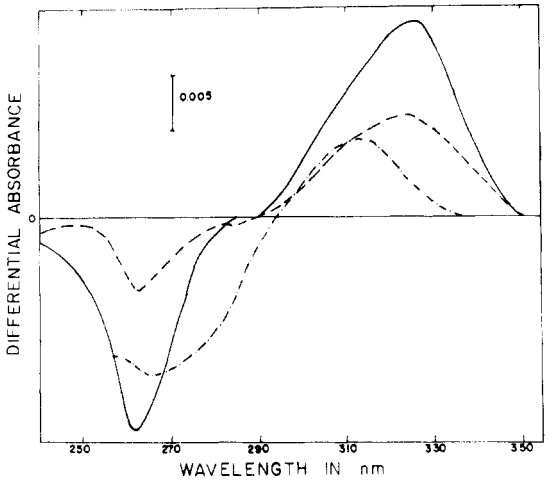

FIGURE 4: Difference spectra of nocodazole–tubulin complex and of nocodazole in organic solvents. The nocodazole–tubulin complex was in 10^{-2} M phosphate, 10^{-4} M GTP, and 1% Me_2SO at pH 7.0 and 22–23 °C. The protein concentration was 1 mg/mL, and the nocodazole concentrations were (---) 1×10^{-5} M and (—) 5×10^{-5} M. (-·-) is the difference spectrum of 5×10^{-5} M nocodazole in ethanol (D = 24) vs. that in methanol (D = 33) where D is the dielectric constant of the solvent at 25 °C.

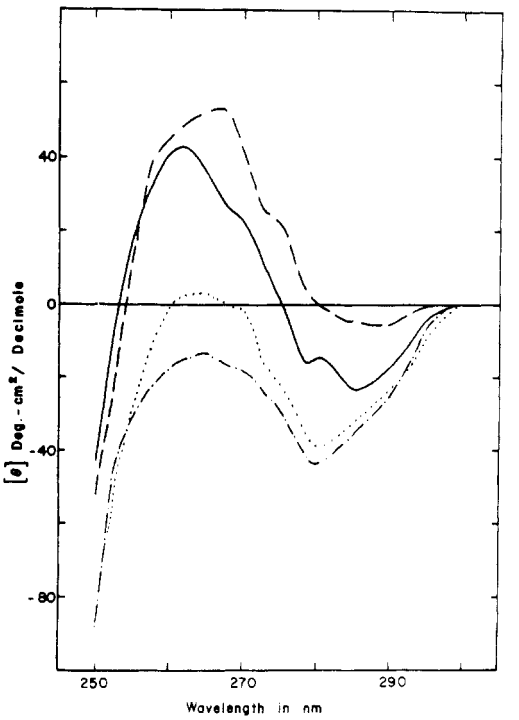

FIGURE 5: Circular dichroism of tubulin and nocodazole–tubulin complexes. The symbols and experimental conditions are as follows: (—) W-tubulin in 10^{-2} M phosphate, 10^{-4} M GTP, and 12% (v/v) Me_2SO at pH 7.0; (---) W-tubulin and 5×10^{-5} M nocodazole in the same buffer; (-·-) c-tubulin in 0.1 M Pipes, 10^{-4} M GTP, and 12% (v/v) Me_2SO at pH 7.0; (···) c-tubulin and 5×10^{-5} M nocodazole in the same buffer. Tubulin concentration ranges from 1.3–1.4 mg/mL, and all experiments were performed at 22–23 °C.

independent, parallel, self-association reactions of tubulin, an interpretation in agreement with the previous proposal of Lee & Timasheff (1975, 1977) and Weisenberg & Rosenfeld (1975).

Figure 3 also presents the concentration distribution of nocodazole in addition to the protein gradient of tubulin in 1.6×10^{-2} M $MgCl_2$. Stepwise changes of nocodazole concentration appear at both the slow and fast sedimenting peaks indicating that nocodazole binds to both the monomeric and polymeric forms of tubulin in this reaction without perturbing its equilibrium.

These experiments suggest that nocodazole does not induce the formation of aberrant tubulin aggregates which is one possible mechanism of inhibiting the formation of microtubules. An alternative mode of inhibition is conformational change. The drug might exert its effect by inducing a conformational change in tubulin which would render it incapable of incorporation into the microtubule while not significantly influencing its capability to form double-ring structures. Differential spectroscopy, circular dichroism, fluorescence, and chemical modification were employed to monitor the structure of tubulin in the presence of nocodazole.

The differential spectra of tubulin in the presence of two concentrations of nocodazole are shown in Figure 4. The differential peak at 263 nm indicates the possibility of perturbation of aromatic chromophores, although the peak at 325 nm does not correspond to any readily identifiable amino acid chromophores. It actually may represent a contribution from the perturbation of nocodazole transitions by transfer to a less polar environment. Figure 4 also shows the differential spectrum of nocodazole in solvents of different dielectric constants. It shows that a decrease in the polarity of the environment results in a nocodazole difference spectrum which has the general features of the spectrum observed for a nocodazole–tubulin complex. The peak at 263 nm which is observed only in the presence of nocodazole and tubulin might represent a perturbation of tyrosyl chromophores (Donovan, 1969). The peaks between 270 and 290 nm, usually observed for perturbation of tyrosyl chromophores, are probably obscured by the nocodazole differential spectrum in that same wavelength range resulting in a net spectrum of no apparent perturbation in the range. It may be concluded that during the formation of a nocodazole–tubulin complex both the nocodazole transitions and some aromtic chromophores, most probably tyrosine, in tubulin are perturbed. The exact nature of the nocodazole transitions affected by binding to tubulin is not known.

Circular dichroism was employed as a means to monitor the tertiary structure of tubulin in order to gain more insights into the nature of conformational changes induced by the formation of a nocodazole–tubulin complex. Figure 5 presents the results of this study which includes CD spectra of both c- and W-tubulins. The near-UV CD spectrum of W-tubulin shows the same transitions due to aromatic chromophores as reported previously (Lee et al., 1978b). The peaks at 263 and 278 nm are most probably due to tyrosine chromophores. The presence of 12% (v/v) Me_2SO does not alter the CD spectrum, indicating no significant structural changes are induced by Me_2SO. In the presence of 5×10^{-5} M nocodazole, however, a significant change in the near-UV CD spectrum is observed as shown in Figure 5. The peak at 263 nm becomes more positive with a concomitant red shift to 267 nm. Apparently as a consequence of such changes, the absorption between 275 and 295 nm becomes less negative and is accompanied by a loss of fine structures. Such spectral changes may be analyzed as a consequence of perturbation of tyrosine chromophores with little or no change in the environment of the tryptophan chromophores. The near-UV CD spectrum of the tubulin–nocodazole complex, however, may also be due to an extrinsic Cotton effect of nocodazole upon binding to tubulin. At present there are no experimental observations to differentiate these possibilities.

An identical CD experiment was conducted with c-tubulin. It is evident that the general features of the spectrum resemble that of W-tubulin, as shown in Figure 5; however, the mag-

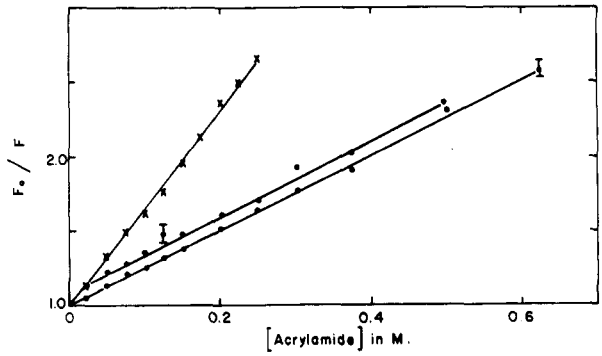

FIGURE 6: Effect of acrylamide on Stern–Volmer plots of W-tubulin. The protein concentration was 0.100 mg/mL. The symbols and experimental conditions are as follows: (●) W-tubulin in 0.1 M Pipes, 10^{-4} M GTP, and 12% (v/v) Me_2SO at pH 7.0; (○) W-tubulin and 5×10^{-5} M nocodazole in the same buffer; (X) W-tubulin in 6 M Gdn-HCl with and without β-mercaptoethanol.

nitudes of ellipticity have changed. The peak at 263 nm assumed a negative value with the peak at 280 nm becoming more negative and with a loss of fine structures at 278 and 286 nm. Such changes may reflect the change in tubulin structure in the presence of MAP or they may be due to the contribution of optical activity of chromophores in MAP. The difference in the CD spectra observed for W- and c-tubulins cannot be attributed to the difference in buffer systems employed. It has been shown previously that there are no observable changes in CD spectra for W-tubulin in either phosphate buffer or Pipes buffer (Lee & Timasheff, 1977). In the presence of nocodazole, however, the CD spectrum of c-tubulin is altered in a fashion parallel to that of W-tubulin, as shown by the dotted line in Figure 5. The absorptivity of the peak at 263 nm becomes more positive, and it may be caused by a similar kind of structural transition observed in W-tubulin. On the basis of the results of CD study, it may be concluded that the interaction of nocodazole with tubulin most probably leads to a perturbation of tyrosine chromophores.

Although the changes in the near-UV CD spectra can be analyzed as perturbation of the tyrosine residues, other chromophores may also be perturbed. A study, therefore, was initiated to probe the environment of tryptophan residues in the presence and absence of nocodazole. The method chosen involves the quenching of tryptophan fluorescence by acrylamide (Eftink & Ghiron, 1976), an agent which decreases the fluorescence intensity via physical contact with the excited indole ring.

The results of such studies are analyzed by the Stern–Volmer plots shown in Figure 6. In the absence of nocodazole and under the present experimental conditions, the plot is linear. In this experimental system a positive deviation would suggest static quenching or extreme accessibility of the fluorophore, whereas a negative deviation usually suggests that the fluorescence of certain tryptophan residues is selectively quenched before others. In the case of tubulin, the linearity of the plot may be interpreted as a situation in which the tryptophan residues are probably equally "exposed" with a value of 5.0 for K_Q, the weighted average collisional quenching constant. Such a value implies that the tryptophan residues are partially shielded from direct contact with acrylamide (Eftink & Ghiron, 1976). The present experiment, however, does not yield information on the number of residues exposed. Furthermore, the results do not allow one to rule out the possibility that the linear plot is a consequence of compensation of heterogeneity by static quenching.

The slope of the Stern–Volmer plot, as shown in Figure 6, is not altered by the presence of 5×10^{-5} M nocodazole. This implies that the formation of a nocodazole–tubulin complex does not alter the net exposure of tryptophan residues although the identity of individual exposed residues might have changed. The absence of significant structural changes involving tryptophan residues is consistent with the observation obtained from circular dichroism. Having determined the accessibility of tryptophan residues in the native state of tubulin, it was of interest to establish an internal reference in order to compare the relative accessibility of these residues. The completely unfolded state of tubulin was chosen to be the reference state. The quenching of tryptophan fluorescence was, therefore, measured in the presence of 6 M Gdn-HCl with and without the reducing agent β-mercaptoethanol. The result is shown in Figure 6. It is evident from the steeper slope (K_Q = 13) that the residues are more accessible to acrylamide quenching. Identical results were obtained regardless of the intactness of the disulfide linkage in tubulin. Hence, in the present system, values of 0 and 13 for K_Q may represent the two extreme cases of completely buried and exposed tryptophan residues in tubulin. A value of 5.0 for K_Q most likely indicates that the tryptophan residues are positioned away from the surface of the tubulin molecule but yet are still accessible to collisional quenching by acrylamide.

Preliminary reports in the literature indicate that the sulfhydryl groups of tubulin may play a role in polymerization of tubulin (Kuriyama & Sakai, 1974; Mann et al., 1978). Evidently the modification of one to two sulfhydryl residues can completely inhibit the in vitro reconstitution of microtubule. Since these sulfhydryl groups appear to be located in regions of the protein molecule which are important for self-association, it seems reasonable that changes in the chemical reactivity of these residues could serve as an index of alterations in the structure of the protein. It was for this reason that studies were conducted to monitor the accessibility of these residues to chemical modification by sulfhydryl-specific reagents in the presence and absence of nocodazole. The initial studies employing PMB showed that the reaction is quite rapid with ~80% of it completed within 30 s. Although the rate of reaction could not be accurately estimated, the total number of sulfhydryl residues reacted with PMB could be determined. It was shown that the presence of nocodazole at concentrations of up to 10^{-4} M did not alter the total number of these residues accessible to PMB; hence, the formation of a nocodazole–tubulin complex does not alter the structure of tubulin in such a manner that any of the sulfhydryl residues become buried. Other specific reagents were screened in order to quantitatively estimate the effect of nocodazole on the reactivity of tubulin sulfhydryl residues. It was found that DTNB reacts with tubulin sulfhydryl residues at a rate slow enough to be monitored under the present experimental conditions. Curve 1 in Figure 7A represents a typical reaction profile of tubulin with DTNB at pH 7.0. It clearly demonstrates the multiphasic nature of the reaction indicating the presence of a heterogeneous population of sulfhydryl residues; furthermore, it was found that the maximum number of sulfhydryl residues titratable by DTNB is 6.2 per tubulin dimer of 110 000 molecular weight.[3] This is in sharp contrast to a total of 18–19 residues accessible to PMB (Lee et al., 1973;

[3] The maximum number of sulfhydryl residues titratable varied from one tubulin preparation to another. Within a single preparation, however, the results could be reproduced consistently. The averaged value for the maximum number was 7 ± 1 and was apparently related to the age of the tubulin preparation. The freshly prepared protein yielded higher values.

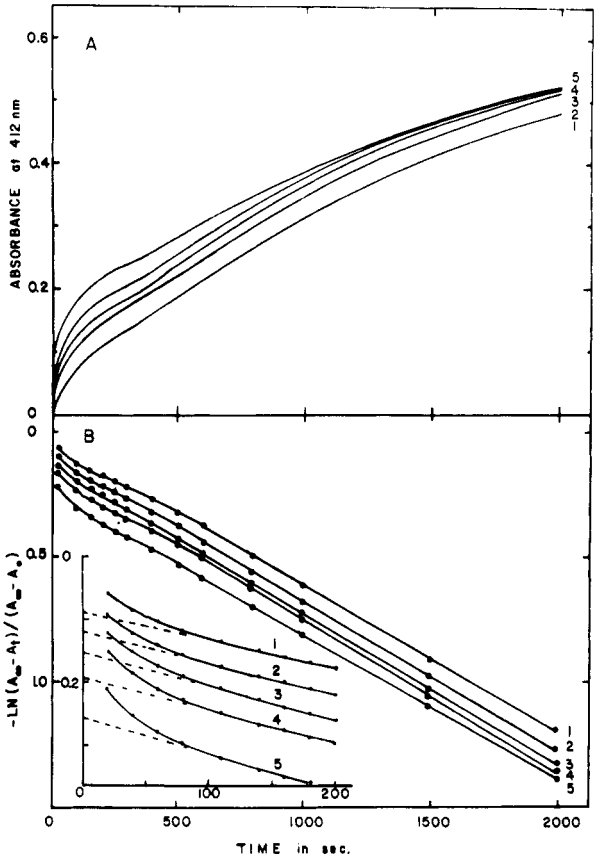

FIGURE 7: Kinetics of chemical modification of W-tubulin sulfhydryl residues by DTNB. The protein concentration was 0.90 mg/mL. The nocodazole concentrations were (1) none, (2) 10^{-7} M, (3) 10^{-6} M, (4) 10^{-5} M, and (5) 10^{-4} M. (A) Relation between absorbance at 412 nm and time as a function of nocodazole concentration. (B) Analysis of the reaction between W-tubulin and DTNB in terms of pseudo-first-order kinetics and the effect of nocodazole on the rate constant.

Lee & Lee, 1979a,b; present study) or DTNB at pH 8.5 (Lee et al., 1973); thus, only a fraction of the titratable sulfhydryl residues is accessible to DTNB at pH 7.0. These data were further analyzed to obtain pseudo-first-order rate constants according to eq 1. Curve 1 in Figure 7B presents a typical plot. It is evident from the nonlinearity of the plot that the population of reactive sulfhydryl residues is heterogeneous. Another interesting observation is the apparent break in the plots at 300 s as shown in Figure 7B. The presence of the break is persistent in all the experiments although the molecular event which leads to the break is presently unknown. One possibility is that tubulin undergoes a structural change as a consequence of the chemical modification of two or more sulfhydryl residues. This structural change could involve either a dissociation of tubulin or a regional unfolding of the polypeptide chain. The exact nature of such changes is now under investigation.

After determination of the basic behavior of sulfhydryl groups in tubulin, the effect of nocodazole on the accessibility of these residues was studied. These results are shown in Figure 7A. It is evident that nocodazole induced a pronounced change in the accessibility of these residues which was manifested as an enhanced rate of reaction; this change was directly related to nocodazole concentration. The total number of residues titrated by DTNB, however, remained the same, with an average value of 6.2 ± 0.2 residues per tubulin dimer of 110000 molecular weight. These data were then analyzed for rate constants of the less reactive form as shown in Figure 7B and the inset. It is evident that the rate constants for the slow reacting group are identical and assumed the same value of $(7 \pm 1) \times 10^{-4}$ s^{-1} as in the absence of nocodazole. The pronounced change in reactivity observed in the presence of nocodazole could be a result of (1) a change in the relative concentration of reactive species, (2) a change in the rate constant of the fast reactive species, or (3) a combination of both. At present there are too few data points to permit an estimate of the faster rate of reaction. Nevertheless, this change is not likely due to a shift in the equilibrium of tubulin self-association to larger aggregates since the sedimentation velocity experiments indicate that nocodazole does not significantly perturb the sedimentation behavior of tubulin (Figure 3), although it is possible that the formation of a tubulin–nocodazole complex perturbs the equilibrium between α and β subunit interaction (Detrich & Williams, 1978). This perturbation, however, is most likely not observable at the high tubulin concentrations used in these sedimentation experiments. In addition, the observed change cannot be simply due to the presence of Me_2SO, since control experiments with Me_2SO indicated no detectable changes in either the reactivity of these groups or in the total number of titratable residues. The observation that nocodazole increases the accessibility of sulfhydryl residues is consistant with the conclusion based on the CD data that tubulin undergoes a structural change upon interacting with nocodazole.

In conclusion, the present study shows that nocodazole is a potent inhibitor of in vitro microtubule assembly, yet it does not significantly alter the self-assembly of tubulin to the 42S ring structure. This observation implies that the formation of rings and microtubules most likely represents two parallel and independent processes of tubulin self-assembly. Furthermore, the observation that the presence of MAP does not qualitatively influence the inhibitory effect of nocodazole implies that the effective site of drug interaction is the tubulin molecule itself. The formation of a nocodazole–tubulin complex apparently induces conformational changes in tubulin resulting in increased exposure of some sulfhydryl and possibly tyrosine residues. There is, however, no detectable net perturbation in the environments of tryptophan residues. The basic thermodynamic parameters which govern the interaction between nocodazole and tubulin have yet to be established, and the mechanism which is responsible for the inhibitory effect of the drug has yet to be elucidated. Furthermore, there is no information to explain the apparent high specificity of the drug against neoplastic cells. Much is, therefore, to be learned about the interaction between nocodazole and tubulin.

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