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Carbon-13 Nuclear Magnetic Resonance Study of Microtubule Protein: Evidence for a Second Colchicine Site Involved in the Inhibition of Microtubule Assembly†

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ABSTRACT: A ^{13}C nuclear magnetic resonance study of bovine microtubule protein was carried out at 43 kG in the presence and absence of colchicine ^{13}C labeled at the tropolone methoxy. Analysis indicated that tubulin has at least two colchicine binding sites: a quasi-irreversibly bound, high-affinity site (i.e., the $K_D < 5$ μM site generally accepted as the site of colchicine action) as well as a low-affinity site(s) ($K_D \sim 650$ μM) with which free colchicine rapidly exchanges (>100 s^{-1}). The methoxy resonance is broadened to different apparent extents as a result of binding at these two sites (50- vs. 150-Hz broadening for the high- and low-affinity sites, respectively) but undergoes no change in chemical shift upon binding. The low-affinity sites are interpreted to be analogous to the sites deduced by Schmitt and Atlas [Schmitt, H., & Atlas, D. (1976) *J. Mol. Biol. 102*, 743-758] from labeling studies using bromocolchicine. These sites are likely to be the sites responsible for the abrupt halt in microtubule assembly ("capping") observed at high colchicine concentrations (>20 μM)—a qualitatively different behavior from that observed at low colchicine concentrations [Sternlicht, H., Ringel, I., & Szasz, J. (1983) *Biophys. J. 42*, 255-267]. Carbon-13 spectra from the aliphatic carbons of microtubule protein consist of narrow resonances—many with line widths < 30 Hz—superimposed on a broad background. The narrow resonances were assigned to flexible regions in nontubulin proteins [microtubule-associated proteins (MAPs)], in accord with an earlier ^{1}H nuclear magnetic resonance study of microtubule protein [Woody, R. W., Clark, D. C., Roberts, G. C. K., Martin, S. R., & Bayley, P. M. (1983) *Biochemistry 22*, 2186-2192]. This assignment was supported by ^{13}C NMR analysis of phosphocellulose-purified (MAP-depleted) tubulin as well as heat-stable MAPs. Aliphatic carbons in the MAP preparations were characterized by narrow resonances indicative of carbons with considerable motional freedom whereas the aliphatic regions of phosphocellulose-purified tubulin were, for the most part, characterized by broad resonances indicative of carbons with restricted mobility. However, a moderately narrow resonance (≲50-Hz line width) coincident with the C_γ resonance of glutamate was detected in ^{13}C NMR spectra of tubulin which indicated that a fraction of the glutamic acid residues is relatively mobile. These mobile residues are likely to be at the carboxy-terminus ends, regions rich in glutamates, where secondary structure considerations suggest flexibility.

Colchicine, a potent inhibitor of microtubule assembly, disrupts a variety of microtubule-dependent cellular processes (Dustin, 1978; Oppenhein et al., 1973; Reaven & Reaven, 1975). Inhibition is generally attributed to a 1:1 tight-binding complex of tubulin and colchicine (TC)[1] with an apparent dissociation constant (K_D) of ~0.1-3 μM (McClure & Paulson, 1977)[2] and a dissociation half-life of ~12 h at 37 °C (Wilson, 1970). Furthermore, it is apparent at substoichiometric concentrations of TC relative to tubulin (Olmsted

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[1] Abbreviations: TC, 1:1 tight-binding complex of tubulin and colchicine; tc, low-affinity form of tubulin–colchicine complex(es); MAPs, microtubule-associated proteins; MTP, microtubule protein consisting of ~85% tubulin and ~15% MAPs; PC-tubulin, MAP-depleted tubulin preparations obtained by phosphocellulose chromatography; [^{13}C]-colchicine, colchicine whose tropolone methoxy is ^{13}C labeled; T_1, spin-lattice relaxation time; NOE, nuclear Overhauser enhancement factor; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MS-2.5 M glycerol, microtubule protein stabilizing buffer (pH 6.7) consisting of 0.1 M MES, 2 mM EGTA, 0.1 mM EDTA, 2 mM mercaptoethanol, 0.5 mM $MgCl_2$, and 2.5 M glycerol; HMPA, hexamethylphosphoramide; TLC, thin-layer chromatography; Me_4Si, tetramethylsilane; BSA, bovine serum albumin.

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& Borisy, 1973; Wilson et al., 1975). Various mechanisms such as copolymerization of TC with tubulin (Sternlicht & Ringel, 1979; Farrell & Wilson, 1980; Sternlicht et al., 1983), "capping" of microtubule ends by TC (Margolis & Wilson, 1978; Margolis et al., 1980), and interference with oligomeric additions to microtubule ends (Deery & Weisenberg, 1981) have been proposed to rationalize "substoichiometric" inhibition. All models presume that tubulin bound to colchicine has an altered conformation which by some chain of events alters assembly. Affinity labeling of tubulin with bromocolchicine (Schmitt & Atlas, 1976) and with an arylazido photoaffinity analogue of colchicine (Barnes et al., 1982) implicates the α subunit as the site of colchicine binding, while immunological (Morgan & Spooner, 1983) and stopped-flow (Garland, 1978) studies, as well as a study of the effects of colchicine on the reversible dissociation of tubulin dimer (Detrich et al., 1982), support the hypothesis that tubulin undergoes a conformational change upon binding colchicine. Circular dichroism (CD) measurements (Detrich et al., 1981) suggest that the conformation of colchicine is also altered in TC.

Nuclear magnetic resonance spectroscopy has been used to study ligand–macromolecular interactions in solution and potentially can provide both structural and dynamic data [cf. Dwek (1973) and Wuthrich (1976)]. We undertook a ^{13}C nuclear magnetic resonance study using [^{13}C]colchicine labeled at the tropolone methoxy moiety to probe colchicine–tubulin association. Our investigation complements a recent ^{1}H nuclear magnetic resonance study of microtubule protein (Woody et al., 1982, 1983) and is an extension of an earlier study (Ringel & Sternlicht, 1981). ^{13}C labeling of the tropolone methoxy was chosen, in part, because the tropolone methoxy is known to be essential for full colchicine activity [derivatives which lack the methoxy (e.g., colchiceine) are considerably diminished in activity (Dustin, 1978)]. The ^{13}C NMR study was also undertaken to reevaluate whether tubulin has more than one colchicine binding site. Schmitt & Atlas (1976) proposed on the basis of bromocolchicine affinity labeling experiments that tubulin has several low-affinity sites which bind colchicine reversibly. However, bromocolchicine is a potent alkylating agent, and the analysis of Schmitt and Atlas, which required distinguishing specific from nonspecific labeling, has been questioned (Luduena, 1979). A resolution of the question of secondary binding sites was of interest since certain paradoxes concerning colchicine inhibition of microtubule assembly (Sternlicht et al., 1983), evident when the effects of colchicine at low (<1 μM) and high (>20 μM) concentrations are compared, could be best understood if tubulin had multiple binding sites with qualitatively different effects on assembly.

Materials and Methods

Protein Preparation. Microtubule protein (MTP) was isolated by repetitive cycles of assembly–disassembly from bovine brains, following a procedure (Sternlicht & Ringel, 1979) modified from Gaskin et al. (1974). Twice-cycled preparations were used immediately for NMR studies or stored at −20 °C in MS–5 M glycerol buffer (pH 6.7) for subsequent use within 2 weeks. Typically, one bovine brain provided sufficient MTP for one ^{13}C NMR experiment. MTP was ~85% tubulin and ~15% MAPs and nontubulin proteins as determined by gel electrophoresis (Laemmli, 1970). Protein concentrations were determined by the Lowry method (1951) using bovine serum albumin as standard.

Heat-stable MAPs were prepared by a procedure (Sternlicht et al., 1980) modified from Herzog & Weber (1978). Concentrated MTP preparations (>18 mg/mL) in 25 mM phosphate (pH 6.7) buffer supplemented with NaCl (0.7 M) and dithiothreitol (2 mM) were boiled for 5 min and then spun for 15 min at 3000*g*. Supernatants, which were enriched in heat-stable MAPs (HMW2 and τ as indicated by gel electrophoresis), were used in the ^{13}C NMR studies.

Synthesis of [^{13}C]Colchicine. [^{13}C]Colchicine, enriched >95-fold at the tropolone methoxy group, was synthesized following a procedure modified from that used by New England Nuclear. The synthesis is summarized in eq 1:

$$\underset{\text{colchicine}}{R\text{-}OCH_3} \xrightarrow[5\text{ h, }70\text{ °C}]{H^+,\ H_2O} \underset{\text{colchiceine}}{R\text{-}OH} \xrightarrow[\text{HMPA, 25 °C}]{^{13}CH_3I} \underset{[^{13}\text{C}]\text{colchicine}}{R\text{-}O^{13}CH_3} \qquad (1)$$

One millimole of colchicine (Sigma) was dissolved in aqueous 0.4 N HCl (24 mL) and reacted for 5 h at 70 °C, yielding a white-yellow precipitate which was collected by centrifugation. Chloroform was added to the precipitate. A bilayer suspension was formed, and the colchiceine-enriched chloroform layer was collected, dried over anhydrous $MgSO_4$, and filtered. Chloroform was removed by evaporation, leaving a yellow solid which was recrystallized from dioxane–ether and dried over P_2O_5 under vacuum. The solid (mp 170 °C, yield ~45%) was identified as colchiceine by ^{1}H and ^{13}C NMR (data not shown).

Colchiceine (1 mmol) was dissolved under N_2 in hexamethylphosphoramide (HMPA) (distilled over BaO); 1.1 mmol of Na_2CO_3 and 1.0 mmol of $^{13}CH_3I$ (99% ^{13}C, Stohler Isotopes Co.) were added, and the suspension was stirred overnight at room temperature. HMPA was removed by vacuum distillation, and the residue was dissolved in chloroform, applied to a silica column, and eluted with a chloroform to chloroform–ethanol (9:1) gradient. Eluents were analyzed by TLC (Analtec, 250-μm silica plates, 95:5 chloroform:methanol solvent), and fractions containing colchicine were combined, concentrated, and eluted on preparative 1-mm silica TLC plates (Analtec, 92:8 chloroform:methanol, R_f ~0.5). The colchicine-containing bands were scraped off, dispersed in chloroform and ethanol, sonicated, boiled gently, and filtered. The extracted material was dried over anhydrous $MgSO_4$, filtered, and then distilled to remove chloroform and ethanol, yielding a residual white solid (mp 155 °C, yield ~30%) which was confirmed to be [^{13}C]colchicine >95% labeled at the tropolone methoxy as indicated by NMR [an aliquot dissolved in $CDCl_3$ gave an intense carbon resonance at the characteristic position of the tropolone methoxy carbon (i.e., at 56.2 ppm relative to Me_4Si) [see Blade-Font et al. (1979) and Singh et al. (1977)] while the residual *unsplit* proton resonance at 4.05 ppm characteristic of the methoxy protons was less than 5% of the intensity of the 4.05 ppm singlet from an equivalent concentration of unlabeled colchicine]. The synthesis and extent of labeling were also confirmed by mass spectrometry analysis (data not shown). A 100 mM [^{13}C]colchicine stock solution in water was prepared and supplemented with a small amount of [^{3}H]colchicine (New England Nuclear) to facilitate estimation of colchicine content in the various experiments described below.

Preparation of TC. MTP (50–150 μM) in MS–2.5 M glycerol buffer or 25 mM phosphate buffer (pH 6.7) was incubated with a 50% molar excess of colchicine or [^{13}C]-colchicine (supplemented with [^{3}H]colchicine) for 40–60 min

[2] The lower K_D value represents a kinetic estimate whereas the higher value represents a "binding" estimate. Large uncertainties in the "K_D" value exist in the literature, in part because the colchicine–tubulin system (i.e., the nonexchanging site) is not in equilibrium with free colchicine.

at 37 °C. Free colchicine was removed by extensive dialysis (4 °C) or by treatment with activated charcoal (1 g of HCl-washed Darco G-60 suspended in 10 mL of H_2O and 50 μL of charcoal suspension per 1 mL of protein solution, 5-min incubation at room temperature by centrifugation). At least 95% of the colchicine was protein bound when analyzed by gel filtration (Bio-Rad, P-10 column, 4 °C). TC is relatively stable with little dissociation occurring over several hours at 37 °C (Wilson, 1970) and several days at −20 °C.

NMR Spectroscopy. Twenty-five milliliters of MTP stock (10–15 mg/mL) was diluted with an equal volume of phosphate buffer (25 mM, pH 6.7), supplemented with 1 mM GTP and assembled at 37 °C. Microtubules were collected by centrifugation (200000*g*, 60 min), resuspended in 6 mL of phosphate buffer, depolymerized at 4 °C, and extensively dialyzed against phosphate buffer (4 °C). One milliliters of D_2O was added just prior to the start of the NMR measurements for magnetic field stabilization. Final protein concentrations were typically 14–19 mg/mL except in the case of denatured samples (1 g of urea/mL of protein solution) where protein concentrations were 8–12 mg/mL and PC-tubulin where concentrations were ~5–7 mg/mL. TC was prepared as described above. MTP in these preparations was 12–15 mg/mL with approximately 55–65% of the tubulin complexed to colchicine.

All spectra were recorded on a Bruker WH 270/180 Fourier transform NMR spectrometer equipped with a Nicolet 1180 computer and a deuterium lock channel. ^{13}C NMR spectra were obtained at 45.284 MHz by using 20-mm diameter 7-mL sample tubes. Five watts decoupling power (180 MHz) in the gated broad-band mode was used to decouple protons from carbons without sample heating. Sample temperature was maintained at 18 °C unless indicated otherwise. Usable ^{13}C NMR spectra were obtained after several hours of accumulation (0.9–4-s repetition times, a 1.3-rad magnetization flip angle, and a 35-μs $\pi/2$ pulse width) with no loss of assembly competence in the case of MTP or of the capacity to inhibit tubulin assembly in the case of TC.

Reconstruction of Tubulin ^{13}C NMR Spectra. Simulated spectra for tubulin (Figure 6C) were constructed (Hewlett Packard HP985 computer system) by summing over the carbon resonances from each of the amino acid residues, weighted by the extent to which the various residues are present [as determined from the amino acid composition (Lu & Elzinga, 1977)]. Each resonance was assumed to have a Lorentzian line shape, $g(\nu)$ (Pople et al., 1959), of the form

$$g(\nu) = (2/\pi)\Delta\nu_{1/2}/[\Delta\nu_{1/2}^2 + 90.6^2(\nu_0 - \nu)^2] \quad (2)$$

with ν_0 denoting resonance position in ppm and $\Delta\nu_{1/2}$ the line width at half-height in hertz. The constant, 90.6, denotes twice the radio frequency (in megahertz) at which the study was done. Resonance positions of the carbon atoms were taken from an amino acid study by Horsley et al. (1970) corrected for peptide linkages (Wuthrich, 1976).

Analysis of the Rapidly Exchanging Colchicine Binding Site. Broadening of the methoxy resonance in the presence of MTP and PC-tubulin ([^{13}C]colchicine in stoichiometric excess) revealed that tubulin had a second colchicine binding site(s) (see Results). The broadening was dependent on protein and colchicine concentration and was analyzed by following the reformulation by Marshall & Carruthers (1981) of the Swift and Connick equations. For a single site in the fast exchange limit

$$I_0 = \{E_0/[\Delta(\Delta\nu^F)]\}\Delta\nu^B - K_D \quad (3)$$

K_D denotes the dissociation constant of the rapid-exchanging

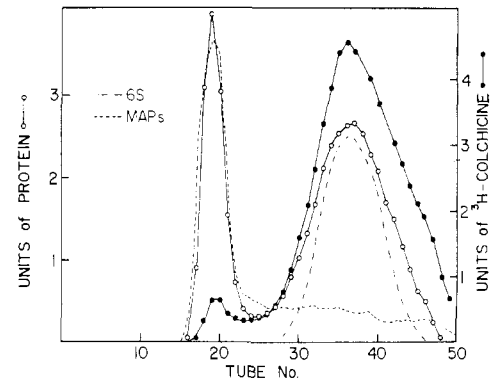

FIGURE 1: Colchicine binds to 6S tubulin. 50 μM MTP in MS–2.5 M glycerol buffer was incubated with 75 μM [^{13}C]colchicine (supplemented with ^3H-labeled colchicine) and dialyzed extensively against MS to remove unbound colchicine (Materials and Methods). A 250-μL aliquot of the incubation mixture was chromatographed at 4 °C on Bio-Gel A15m equilibrated in MS buffer (20-mL bed volume; 420-μL fractions). Elution profiles [protein (○); colchicine (●)] were compared with protein elution profiles from monomeric (6S) tubulin and HMW MAPs obtained by phosphocellulose chromatography (Sternlicht et al., 1980; Cleveland et al., 1977). MTP eluted as two peaks, and at least 85% of the colchicine eluted with the major protein peak (~70% of total protein) which ran coincident with 6S tubulin (–··–). Of the colchicine, 5% eluted with the minor protein peak which was assigned to oligomeric structures [30–36S tubulin formed from approximaatly equal mass amounts of tubulin and MAPs (Scheele & Borisy, 1979)] since it ran coincident with HMW MAPs (- - -) and was observed to be rich in MAPs when aliquots were analyzed by gel electrophoresis (data not shown). Less than 10% of the colchicine was free in solution as indicated by exclusion chromatography on Bio-Rad P10 (data not shown) and by the small shoulder corresponding to free colchicine centered at fraction position 45.

complex, $\Delta\nu^B$, the line width at half-height of colchicine when bound, and $\Delta(\Delta\nu^F)$ the *apparent* change in the free colchicine line width when protein and colchicine concentrations are E_0 and I_0, respectively, *relative* to the protein-free case. That is, $\Delta(\Delta\nu^F) = \Delta\nu_{obsd} - \Delta\nu_{free}$ where $\Delta\nu_{obsd}$ denotes the observed line width and $\Delta\nu_{free}$ the colchicine line width when $E_0 = 0$.

Results

^{13}C NMR Studies of Tightly Bound Colchicine. ^{13}C NMR studies of TC required specifically labeled colchicine as well as high protein concentrations (>10 mg/mL) and large sample volumes (7 mL) to overcome signal to noise limitations. As a result, TC studies were done primarily with microtubule protein preparations (~85% tubulin and 15% MAPs by gel electrophoresis) as opposed to MAP-depleted preparations (e.g., PC-tubulin) since the latter were difficult to prepare at high concentrations and volumes. When high protein concentrations were not required, as in studies of the weak affinity sites (below), both MTP and MAP-depleted preparations were used. [^{13}C]Colchicine, specifically labeled at the 10-methoxy position of the tropolone ring, was synthesized by a procedure modified from that used by New England Nuclear and characterized by NMR to be >95% labeled at the methoxy carbon (Materials and Methods). MTP was incubated with either labeled or unlabeled colchicine; unbound colchicine was removed (Materials and Methods), and the preparations were characterized by column chromatography (Figure 1). MTP consists of monomeric tubulin (6 S) and oligomeric tubulin (high molecular weight complexes stabilized by MAPs) in the approximate mass ratio of 7:3 (Scheele & Borisy, 1979). However, more than 90% of all bound colchicine was bound to 6S tubulin (Figure 1).

^{13}C NMR studies of native MTP (Figure 2) required computer accumulation of spectra over a 4–6-h period (Materials

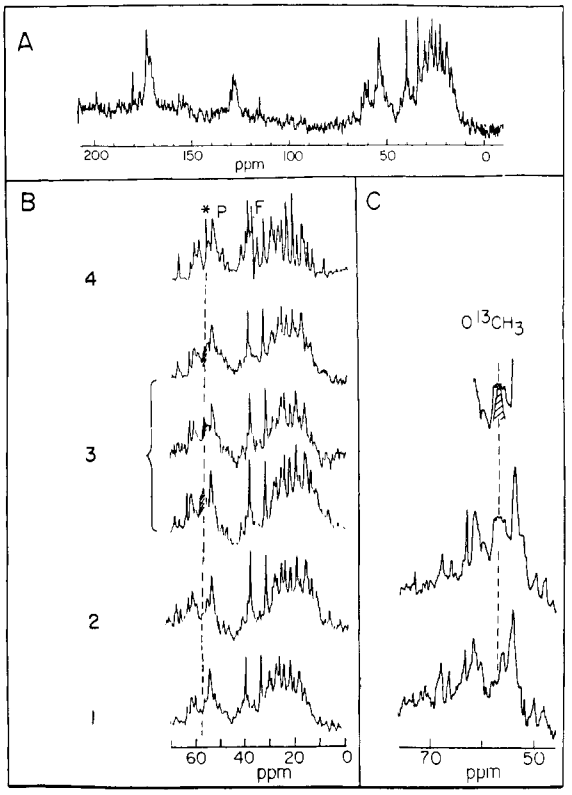

FIGURE 2: Tropolone methoxy resonance broadens (but does not shift) in the TC complex. (A) Spectrum of native MTP (no colchicine) displaying the carbonyl (165–185 ppm), aromatic (110–135 ppm), α (45–62 ppm), and aliphatic (0–70 ppm) carbon regions. The majority of the aliphatic carbon resonances fall in the 0–45 ppm region. (B) Comparison of aliphatic carbon regions. Native MTP preparations incubated with [^{13}C]colchicine (row 3) display a broadened resonance (hatched area) at the 10-methoxy position (dashed line at 56 ppm) which is absent in native preparations lacking colchicine (row 1) or lacking ^{13}C label (row 2). The three spectra in row 3 were derived from three *independent* bovine brain preparations. One of the preparations was denatured with urea (8 M final concentration) and reexamined (row 4). (C) Expanded display comparing the 56 ppm region of TC (middle spectrum) with that of a colchicine-free control (bottom spectrum). An additional resonance from the tropolone methoxy carbon is evident in the middle spectrum. Decomposition of the 56 ppm region in TC into protein and methoxy resonances (the latter indicated as a hatched area) is shown in the top spectrum. TC preparations contained ~0.55–0.65 colchicine/tubulin with >95% of the colchicine (ca. 50–60 μM total concentration) bound to tubulin as indicated by chromatography. MTP concentration was ~18 mg/mL in (A) and ranged from ~12 to 15 mg/mL in (B). A total of 4000 free induction decays were accumulated (4-s repetition time) and Fourier transformed to give (A), while 9000–15000 free inductions were accumulated (1.6-s repetition time) in (B) and (C). All spectra were filtered with a 10-Hz window. Resonance P in (B) at ca. 53 ppm (observed as a peak on a broad shoulder in the α-carbon region) was a useful internal calibration as it is equivalent in intensity to ~100 α-carbons per tubulin molecule. The sharp resonance (*) at 56 ppm in the upper spectrum of (B) originates from free colchicine released by denaturation, whereas the sharp, out of phase signal (F) at ~37 ppm is a "fold-in", an artifact caused by the intense urea signal at ca. 162 ppm.

and Methods). Aliquots taken at the beginning and end of the ^{13}C NMR measurements differed no more than 15% in their ability to assemble into microtubules (colchicine-free studies) or substoichiometrically inhibit microtubule assembly (colchicine studies), indicating that the preparations underwent little denaturation during the ^{13}C NMR study (data now shown). The aliphatic carbon region of MTP (Figure 2A) displayed a number of relatively narrow resonances superimposed on a broad background, which were attributed to microtubule-associated proteins (see below). Spectra from MTP incubated with labeled colchicine and free of unbound colchicine displayed an additional resonance (hatched area, row 3 of Figure 2B) that was attributed to [^{13}C]colchicine complexed to 6S tubulin since (i) the resonance was absent in preparations lacking colchicine (row 1 of Figure 2B) or lacking label (row 2, Figure 2B) while (ii) chromatography analysis indicated that >90% of the colchicine in these samples was tightly bound to 6S tubulin (Figure 1). This resonance, evident as a weak shoulder at the *unshifted* 10-methoxy position [dashed line at 56 ppm (Figure 2B)], had an ~40–60-Hz line width (Figure 2C) which appeared to be independent of temperature (4–25 °C) (data not shown). As expected, the 56 ppm shoulder was lost upon protein denaturation and was replaced by a sharp resonance (line width <2 Hz) characteristic of free [^{13}C]colchicine (row 4, Figure 2B). The finding that the 10-methoxy resonance was unshifted in TC places certain constraints on the binding site (see Discussion). We could not determine spin–lattice relaxation times (T_1) nor NOE[3] values for the methoxy resonance of bound colchicine because of interferences from protein resonances and small amounts of free colchicine, which were in rapid exchange with secondary sites on tubulin, liberated during the course (10–24 h) of the T_1 and NOE measurements.

Table I: Comparison of Dissociation Constants and Methoxy Line Widths

	$\Delta\nu_{1/2}$ (Hz)	K_D (μM)
colchicine in presence of denatured protein	~2[a]	
tight-binding (TC) complex		
MTP	55 ± 15	0.1–3[b]
fast exchanging complex[c]		
MTP	150	740
PC-tubulin	131	560
	140 ± 10[d]	650 ± 90[d]

[a] Estimated line width after correction for 10-Hz filter broadening [cf. Figures 2B (top spectrum) and 3A]. [b] From McClure & Paulson (1977). [c] Analyzed by using eq 3 (Materials and Methods) and assuming only one rapidly exchanging site (cf. Figures 3 and 4). This assumption may have led to an overestimation of methoxy broadening (see Discussion). [d] Average values (±SD) for the fast exchanging complex.

^{13}C NMR Studies of the Low-Affinity Binding Site. If tubulin has only a high-affinity, *tight-binding* site (Wilson et al., 1974; Bhattacharyya & Wolff, 1974, 1976; Sherline et al., 1975; McClure & Paulson, 1977), then ^{13}C NMR spectra of MTP or PC-tubulin samples containing a stoichiometric excess of labeled colchicine should be a sum of a narrow resonance at 56 ppm (from free colchicine) and a broad resonance at 56 ppm (from bound colchicine) (Figure 2) superimposed on the ^{13}C NMR profile of the protein, with signal intensities (i.e., areas) proportional to the amounts of free and bound colchicine present. If [^{13}C]colchicine were present in a large stoichiometric excess, then for all practical purposes one should observe a single narrow resonance centered at 56 ppm with line widths *independent* of protein concentration. On the other hand, if

[3] Line widths, T_1, and NOE parameters reflect the dipolar interaction between carbons and bonded protons and are sensitive to the "tumbling" motion of the protein (i.e., its rotational correlation time) and other motions, such as rotations about internal bonds, which modulate that interaction. Consequently, these parameters provide a direct measure of "flexibility" (Wilbur et al., 1976) at ligand binding sites which can be related to protein structure and conformation (Wuthrich, 1976). For example, the NOE is 3.0 for proton-bonded carbons when tumbling and segmental motions are rapid compared with the proton frequency ($2\pi \times$ 180 MHz) but approaches 1 as these motions slow to rates less than the carbon resonance frequency ($2\pi \times$ 45 MHz) (resonances from "flexible" carbons with NOEs of 3 are 3-fold more intense than those from carbons with NOEs of 1, all other factors being the same).

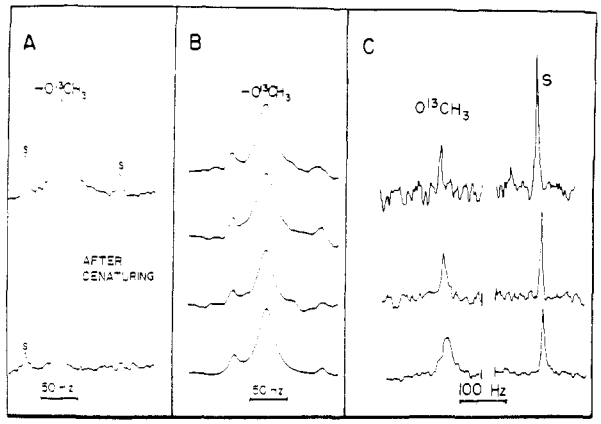

FIGURE 3: ^{13}C NMR study of the rapidly exchanging colchicine binding site (i.e., colchicine present in stoichiometric excess). (A) [^{13}C]Colchicine (1.1 mM) and native MTP (~20 mg/mL) (room temperature study): (top spectrum) 10-methoxy resonance is broadened ~16 Hz relative to residual MES buffer (s); (bottom spectrum) broadening is eliminated when the protein is denatured (100 °C, 5 min in the presence of 1% SDS and dithiothreitol). (B) Changing the temperature had little effect on the [^{13}C]colchicine line width; temperatures of 4, 12, 22, and 26 °C were used from top to bottom. Colchicine and MTP concentrations were as in (A). Resonances were 10-Hz filter-broadened. (C) Changing the protein concentration affected the [^{13}C]colchicine line width. Concentrated MTP (~21 mg/mL) was prepared in 20 mM MES buffer, and [^{13}C]colchicine was added to give an ~6-fold molar excess relative to tubulin [as in (A)]. The mixture was diluted stepwise with 20 mM MES buffer, with MES buffer resonance (peak S) serving as an internal control. Tubulin and [^{13}C]colchicine concentrations (micromolar) were, respectively, 167 and 1100 (bottom spectrum), 90 and 570 (middle spectrum), and 7 and 43 (top spectrum).

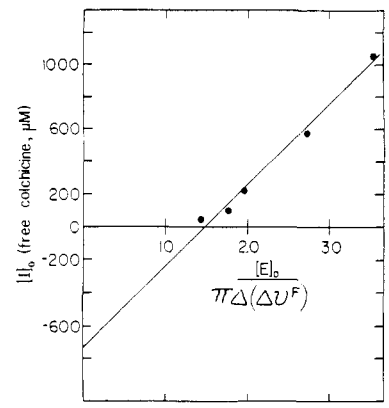

FIGURE 4: Determination of K_D and $\Delta\nu^B$ for [^{13}C]colchicine bound at the rapidly exchanging site. Broadening of the 10-methoxy resonance, $\Delta(\Delta\nu^F)$, was determined for MTP preparations in which total colchicine (I_0) and tubulin (E_0) concentrations were varied while holding I_0/E_0 constant ($I_0/E_0 \sim 6$ as in Figure 3). When I_0 was plotted against $E_0/[\pi\Delta(\Delta\nu^F)]$ (eq 3 under Materials and Methods), a straight line was obtained as shown, and $\pi\Delta\nu^B$ and K_D were determined from the slope and intercept, respectively.

tubulin had a bound colchicine that was in *rapid* equilibrium with free colchicine, one would observe a single resonance centered at 56 ppm with a weighted average line width *dependent* on the relative amounts of bound and free colchicine (Swift & Connick, 1965; Wuthrich, 1976). Tropolone methoxy line widths were measured over a range of colchicine (45–1100 μM) and protein (1–21 mg/mL) concentrations, with colchicine present in a 5-fold or greater stoichiometric excess relative to tubulin, and found to vary in a manner indicating that free colchicine exchanges rapidly with bound colchicine(s) (Figures 3 and 4, Table I). We observed a 16-Hz line broadening relative to buffer when colchicine and MTP concentrations were ~1 mM and ~20 mg/mL, respectively (Figure 3A), while a line broadening of ~1.5–2 Hz was observed when colchicine and MTP concentrations were as low as 45 μM and ~1 mg/mL, respectively (Figure 3C). The broadening was apparently temperature independent (Figure 3B) and was specific to native 6S tubulin since PC-tubulin gave comparable levels of broadening (cf. Table I) and no broadening was observed with denatured tubulin (Figure 3A, lower spectrum) or with BSA (data not shown). Line widths were analyzed (Figure 4) in accord with the reformulation by Marshall & Carruthers (1981) of the Swift and Connick equations (eq 3 under Materials and Methods), and estimates for the dissociation constant (K_D) and line width of the bound complex ($\Delta\nu^B$) were obtained by assuming a single rapidly exchanging site on tubulin (Figure 4, Table I). Although colchicine binds weakly to the exchanging site (apparent $K_D \sim 650 \pm 90$ μM), the line width of the 10-methoxy resonance in the complex was estimated to be factor of 2 or 3 larger than that of TC (Table I), suggesting either that the low-affinity site is more restricted in mobility than the tight-binding site or that the assumption of just one low-affinity site in tubulin is not correct (see Discussion). In another study (Figure 7A, below) 105 μM colchicine was incubated with ~17 mg/mL MTP (~100 μM total active tubulin) to give ~60 μM TC and 40 μM free colchicine, and the ^{13}C NMR spectrum was recorded. Using the parameters of Table I ($K_D \sim 650$ μM, $\Delta\nu^B \sim 150$ Hz) and assuming colchicine binds noncompetitively at the high- and low-affinity sites, we calculate that 5.5% of the total colchicine should be reversibly bound under these conditions and that free colchicine should display a 32-Hz-wide line [10-Hz filter broadening + ~22-Hz broadening as a result of rapid exchange with the weak binding site(s)]. This predicted value is in good agreement with the observed free colchicine line width of 36 Hz (Figure 7A).

MAPs Are "Flexible" Proteins While Tubulin Is Much More "Rigid". ^{13}C NMR of the aliphatic carbons of MTP [Figures 2 and 5 (bottom spectrum)] displayed narrow resonances superimposed on a broad background indicative of carbons in flexible or highly mobile regions (Wilbur et al., 1976; Wuthrich, 1976; Jardetzky & Roberts, 1981). Furthermore, several resonances such as the 34.0 ppm resonance, which we assign to C_γ of glutamic acid (Wuthrich, 1976), and the 39.7 ppm resonance, which we assign to C_β of aspartic acid and/or to C_ϵ of lysine and C_β of leucine (Wuthrich, 1976), had apparent line widths of the order of 20 Hz similar to that observed in denatured spectra (Figure 5, middle spectrum). Colchicine binding had no detectable effect on these resonances (compare rows 2 and 3 with row 1 in Figure 2B) and also had no apparent effect on the aromatic and carbonyl resonances (cf. Figure 2A and 7A). Narrow resonances in the aliphatic region arose from assembly-competent protein, as indicated by their presence in microtubule *polymer* preparations assembled from MTP (Figure 6C), and not from denatured/assembly-incompetent tubulin present in MTP preparations, although the latter may have made a small contribution. Preparations enriched in denatured/assembly-incompetent forms of tubulin (<20% of total protein) can be collected partially free of MAPs by inducing MTP to assemble and pelleting out the microtubules (MAPs preferentially associate with microtubules). The aliphatic region from such supernatants consists of resolvable but weak resonances superimposed on a broad background (Figure 6B) similar to the broad background in native MTP. There was no indication of an increase in segmental motion and an accompanying pattern of intense, narrow resonances that might account for the narrow resonances in the MTP preparations. Denaturation of native MTP with urea (Figure 5, middle spectrum), on the

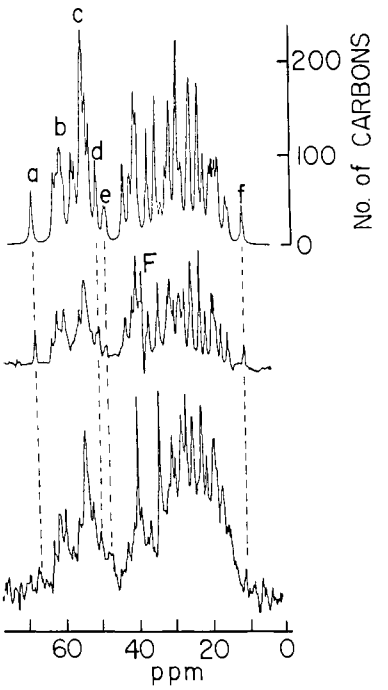

FIGURE 5: Comparisons of MTP spectra with simulated spectra for tubulin. Spectra were accumulated at a 0.9-s repetition rate and filtered with a 10-Hz window. The sharp out of phase resonance (F) in the middle spectrum is a "fold-in" artifact. (Bottom spectrum) Aliphatic carbon region of native MTP (15 000 accumulations); (middle spectrum) same region after denaturation with 8 M urea (68 000 accumulations); (top spectrum) simulated aliphatic region for tubulin as a superposition of 30-Hz-wide Lorentzian lines. Several of the signals arose from just one type of amino acid residue (e.g., peaks a, d, and e from the C_α's of Thr, Ala, and Pro, respectively, and peak f from the C_δ of Ile); most are composites representing the superposition of resonances from more than one type of amino acid residue (e.g., peak b from the C_α's of Val, Thr, and Ile; peak c mainly C_α's of Leu and Asp). The intense resonances at 34.0 and 39.7 ppm in native MTP (bottom spectrum) were assigned to glutamic acids (34.0 ppm signal) and to aspartic acid, lysines, and leucines (39.7 ppm signal) (see text).

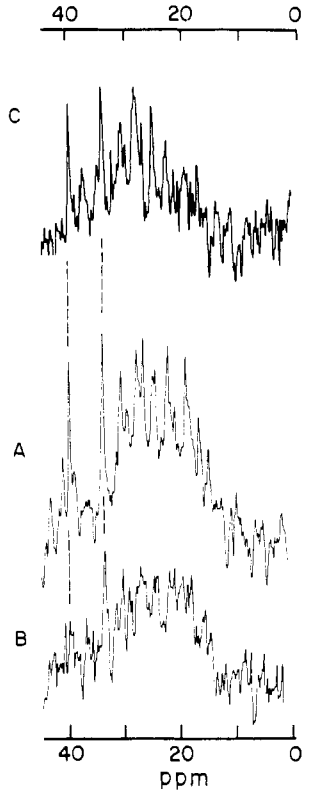

FIGURE 6: Comparison of native MTP spectra with that of microtubule polymer and supernatant. Two samples of MTP (~17 mg/mL) (one supplemented with 0.5 mM GTP) were prepared in 25 mM phosphate buffer (pH 6.7), incubated at 37 °C for 60 min, and centrifuged. ^{13}C NMR spectra from the supernatants were recorded and compared (~20 000 accumulations at 18 °C). Supernatant from the control (no GTP) contained assembly-competent tubulin and MAPs, while supernatant from the GTP-supplemented sample, which had polymerized into microtubules, contained primarily assembly-incompetent and denatured tubulin, as well as residual amounts of assembly-competent tubulin and MAPs (the major portion being in the microtubule fraction). (A) Supernatant (~11.5 mg/mL) from the control sample (no GTP). Narrow resonances of nearly equal intensity were observed at 39.7 and 34.0 ppm. (B) Supernatant (~5 mg/mL) from the GTP-supplemented sample. The 39.7 ppm resonance is diminished in intensity relative to the 34.0 ppm resonance. In a further study, ~17 mg/mL MTP was supplemented with 0.5 mM GTP and polymerized at 37 °C, and the spectrum of the microtubule suspension was recorded (14 000 scans at 30 °C). The sample was then centrifuged, and the spectrum of the supernatant (~20% of total protein) was similarly recorded and subtracted from that of the suspension. The difference spectrum, which represents the ^{13}C NMR of microtubule polymer (~80% of total protein), is displayed in (C). Narrow resonances of nearly equal intensity are observed at 39.7 and 34.0 ppm.

other hand, reduced the broad background and caused a narrowing indicative of an increase in segmental motion, and also altered the relative intensities of the resonances (e.g., the strong resonances at 34.0 and 39.7 ppm observed in native MTP preparations were relatively diminished).

The aliphatic region of tubulin was simulated (Materials and Methods) by using amino acid composition data from Lu & Elzinga (1977) and amino acid chemical shifts from Wuthrich (1976). The simulation (Figure 5, top spectrum) was in good agreement with that of denatured MTP (Figure 5, middle spectrum) but differed from that of native MTP (Figure 5, bottom spectrum). Native MTP spectra display a broad background—which comprises the major portion of the ^{13}C NMR resonances—absent in denatured and simulated spectra. In addition, the intense narrow resonances at 39.7 and 34.0 ppm present in native MTP spectra are much less prominent in denatured and simulated spectra. These differences indicate that a major fraction of the tubulin aliphatic carbons in native MTP is "immobile" with dipolar broadened resonances, which overlap to give the broad background in native MTP, and suggest either that tubulin also has highly mobile regions whose carbons give rise to the narrow resonances observed in native MTP spectra or that nontubulin proteins, i.e., MAPs, contribute significantly to the spectra despite their relatively low mass percentage (~15%). Woody et al. (1983) observed narrow resonances in the ^{1}H NMR spectra of MTP and microtubule polymer which they ascribed to protons in highly flexible domains from MAP proteins.[4] A comparative ^{13}C NMR study of MAPs and PC-tubulin (Figure 7) confirmed the Woody et al. assignment. Spectra from the aliphatic carbons of the heat-stable MAP fraction (Figure 7B) consisted almost entirely of narrow resonances similar to that in Figure 2. On the other hand, ^{13}C NMR spectra from the aliphatic carbons of PC-tubulin (0–45 ppm region in Figure 7C) were broad and largely unresolved except for a weak, fine structure whose most prominent component was a moderately narrow (~50 Hz) resonance at 34.0 ppm. Further comparisons indicated that while MAPs made a significant contri-

[4] MAPs have nonglobular conformations which apparently allow for considerable segmental motion in the native state. One of the MAPs (HMW2) is observed in electron microscopy as a threadlike protrusion extending from microtubules and 30S tubulin oligomers (Scheele & Borisy, 1979).

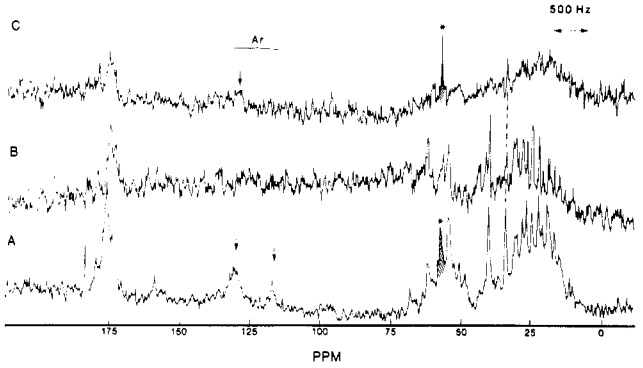

FIGURE 7: MAP carbons give rise to the narrow resonances in the aliphatic region of MTP. (A) ^{13}C NMR of ~17 mg/mL native MTP (39 000 accumulations) supplemented with 105 μM [^{13}C]colchicine (60% bound, 40% free). (B) ^{13}C NMR of heat-stable MAPs (Materials and Methods) prepared from a ca. 23 mg/mL MTP solution (126 000 accumulations). (C) ^{13}C NMR of ~5 mg/mL PC-tubulin supplemented with [^{13}C]colchicine (82 000 accumulations). PC-tubulin spectra lack the narrow resonance at 39.7 ppm observed in (A) and (B) but display the 34.0 ppm resonance. Spectra were obtained at 18 °C with a 1.6-s repetition rate and are 10-Hz filter broadened. "Ar" delineates the aromatic region where specific resonances (↓) were observed. The hatched areas (*) at 56 ppm correspond to the 10-methoxy resonance of [^{13}C]colchicine and are composites formed from both bound and free colchicine with free colchicine making the dominant contribution to the signal intensity. Free colchicine resonances, which are ~36 and 17 Hz wide in (A) and (C), respectively, are broadened by exchange with weak binding sites (cf. Figures 3 and 4 and the text).

bution to the aliphatic region, they made less of a contribution to other portions of the MTP spectra. MAPs have a low percentage of aromatic residues [<3.5% of total residues (Cleveland et al., 1977)]. Accordingly, the signal intensity of the aromatic region in MAP spectra should be much less than those of the carbonyl and aliphatic carbon regions. In the example shown (Figure 7B), aromatic resonances were not evident above the noise although strong signals were obtained from the carbonyl and aliphatic carbons. On the other hand, aromatic resonances are prominently present in MTP spectra (Figure 2A) and were also seen in PC-tubulin spectra, despite the low signal to noise characteristic of our dilute MAP-depleted preparations (Figure 7C). These observations suggest that aromatic resonances in the MTP studies arose mainly from tubulin. The relative contributions that MAPs and tubulin make to the carbonyl region of MTP were more difficult to assess, although it appears, after correcting for concentration differences in the MAP and PC-tubulin study (Figure 7), that tubulin carbons make the major contribution.

A Fraction of the Glutamic Acid Residues of Tubulin Is in Flexible Regions. Native MTP spectra displayed two strong resonances of nearly equal intensity at 34.0 and 39.7 ppm (Figure 2). However, MAPs displayed a 34.0 ppm resonance that was less intense than that of the 39.7 ppm resonance (Figure 7B), while the ^{13}C NMR of PC-tubulin displayed a resolved peak (line width ~40–60 Hz) at 34.0 ppm and none at 39.7 ppm (Figure 7C). These observations suggest that the 34.0 ppm resonance in MTP, which is coincident with the resonance position of the C_γ carbon of glutamic acid (see above), is a composite of resonances from *both MAPs and 6S tubulin.* Colchicine binding did not appear to affect this composite signal (cf. Figures 2A,B and 7A). Furthermore, the 34.0 ppm peak observed in 6S tubulin (Figure 7C) *was also present in supernatants partially depleted of MAPs which were derived from microtubule suspensions* (Figure 6B) and contributes to the narrow 34.0 ppm resonance in microtubule polymer (Figure 6C), implying that mobility is retained in the polymer. We estimate that ~16–33 mobile glutamates out of a total of 76 glutamate residues in tubulin (Ponstingl et al., 1981) contribute to the narrow 34.0 ppm resonance. This range of values was obtained by assuming mobile C_γ's have an NOE of ~2 while rigid aliphatics have an NOE of ~1 and involved comparing areas of the 34.0 ppm peak in PC-tubulin (Figure 7C) and in supernatants depleted of MAPs (Figure 6B) with the total area of the resonance falling between 10 and 42 ppm [the latter region arises from ~1600 carbons (Ponstingl et al., 1981; Horsley et al., 1970)]. PC-tubulin data alone (Figure 7C) suggested ~16 highly mobile C_γ glutamates. We tentatively assign the mobile C_γ glutamate resonances to the glutamate-rich carboxy termini of tubulin.

Discussion

^{13}C NMR studies of MTP and PC-tubulin preparations containing colchicine ^{13}C labeled at the tropolone methoxy indicated that tubulin has at least two colchicine binding sites: a quasi-irreversibly binding, high-affinity site (Figures 1 and 2B) generally accepted as the site of colchicine action and alow-affinity site(s) ($K_{D,apparent}$ ~ 650 ± 90 μM) with which free colchicine rapidly exchanges (Figures 3, 4, and 7) whose significance remains to be established. In both cases, the tropolone methoxy resonance broadened but did not shift upon binding. The apparent line widths of the methoxy resonance in the high- and low-affinity complexes were ~50 and 150 Hz, respectively (Table I), and agree well with the calculated value of ~100 Hz for a dipolar line-broadening mechanism obtained by neglecting internal rotation of the methoxy about its symmetry axis and assuming a rotational correlation time for tubulin (3×10^{-8} s) characteristic of ca. M_r 100 000 globular proteins [cf. Browne et al. (1973), Wilbur et al. (1976), and Dwek (1973)]. In contrast, the methoxy line width in uncomplexed colchicine was ~2 Hz (corrected for filter broadening) when measured in the presence of denatured tubulin (Figures 2B and 3A). Furthermore, methoxy broadening in the weak binding complex appeared to be temperature independent (4–26 °C) (Figure 3B) consistent with a rapid exchange rate which we estimate[5] to be >300 s^{-1}. In contrast, the residence time of colchicine in TC is at least several hours (Wilson, 1970; McClure & Paulson, 1977).

The broadening attributed to binding at the low-affinity site(s) appeared to be specific to native tubulin since preparations depleted of MAPs gave similar levels of broadening as MTP (Table I) while broadening was not observed with denatured tubulin (Figure 3A, lower spectrum) or with control protein (BSA) (data not shown). In addition, low- and high-affinity sites appeared to bind colchicine noncompetitively. For example, similar levels of broadening were deduced by using MTP or MTP preincubated with colchicine to form TC (data not shown). Furthermore, the broadening observed upon addition of a stoichiometric excess of colchicine to MTP preparations did not change with time. If binding were competitive, we should have observed a decrease in the free colchicine line width as the more slowly equilibrating TC complex formed. We do not know, however, whether both binding sites are present on the same molecule. Tubulin from a variety of source, including brain, is a heterogeneous and complex mixture of α- and β-isotubulins [cf. Stephens (1982) and George et al. (1981)] which reflect genomic differences as well

[5] If changes in chemical shift with binding are small relative to line broadening (this study), rapid exchange on the NMR time scale implies that the exchange rate is at least 2π times larger than the line broadening (in hertz) that accompanies binding (Swift & Connick, 1965).

as posttranslational modification. These isotubulins can also differ conformationally (Gozes & Littauer, 1978; Barnstable & Gozes, 1980). Furthermore, heterogeneity may account for the observation that assembly-competent tubulin does not bind colchicine in a strict 1:1 ratio (TC preparations typically contain <0.6–0.7 colchicine/tubulin). In view of these considerations, we cannot exclude the possibility that the weak binding site(s) exists(s) only in isotubulins which lack the tight-binding site. Clarification of these subtle aspects of colchicine binding to tubulin requires extensive studies beyond the scope of the present paper.

Barnes et al. (1981) presented a preliminary analysis of modified Dixon plots of colchicine binding data obtained in the absence and presence of competitive inhibitors which suggested that tubulin has both high- and low-affinity binding sites. They proposed that colchicine binding to the low-affinity sites increases ($K_D \sim 35$ μM) following binding at the high-affinity site. We were unable to confirm their model by ^{13}C NMR which indicates either that ~35 μM low-affinity sites do not exist under our experimental conditions or that these sites are in slow exchange with free colchicine. [Slowly exchanging colchicine would have been obscured by tight-binding, nonexchanging colchicine (Figure 2B) and by rapidly exchanging colchicine (Figures 3 and 4).]

In a related study, Schmitt and Atlas examined binding competition between colchicine and its affinity label analogue bromo[^{3}H]colchicine and reported that bromo[^{3}H]colchicine bound specifically to two types of sites (I and II). Type I sites (~0.4 site/tubulin) were located on the α chain, and binding to these sites occurred with an apparent K_D of ~6 μM which is in the range of values estimated for the K_D of TC. Type II sites (~3 sites/tubulin) were located principally on the β chain, and binding to these sites occurred with an apparent K_D of ~250 μM (corrected for inactive stereoisomer). Schmitt and Atlas noted type I sites were competitively occupied by colchicines that remained tightly bound to tubulin while type II sites were competitively occupied by colchicines that were reversibly associated with tubulin. Their labeling experiments further suggested that colchicine binding at the reversible sites induced conformational changes in both α and β chains. However, bromocolchicine is a potent alkylating agent (Luduena, 1979), and conclusions based on affinity labeling with this agent must be treated cautiously. In this connection, recent affinity labeling studies with an arylazido photoaffinity analogue of colchicine (Barnes et al., 1982) support the assignment of the tight-binding colchicine site to the α chain.[6] Our ^{13}C NMR study also supports the study of Schmitt and Atlas and suggests that the rapidly exchanging colchicine detected spectroscopically (Figures 4 and 5, Table I) are analogous to the reversibly binding colchicines hypothesized by Schmitt and Atlas. We obtained a K_D value of ~650 ± 90 μM for colchicine binding at the weak affinity site which, allowing for the different experimental approaches, is in reasonable agreement with the apparent value of ~250 μM deduced by Schmitt and Atlas for type II bromo[^{3}H]colchicine binding and is consistent with the prediction by Schmitt and Atlas of similar affinities for reversibly bound colchicine and bromocolchicine [cf. Figure 8 in Schmitt & Atlas (1976)]. Furthermore, had we assumed three "equivalent" low-affinity sites for colchicine in our line-width analysis (Table I), so as to be consistent with the number of type II bromocolchicine sites deduced by Schmitt and Atlas, the estimated methoxy line width for weakly bound colchicine would have been close to that of tightly bound colchicine (i.e., ~50 Hz) and it would not be necessary to hypothesize ad hoc mechanisms such as differences in "local flexibility" or in the extent to which the methoxys undergo internal rotation about their symmetry axis to account for apparent differences in line width.

We suspect that secondary sites for colchicine binding play an important role in assembly inhibition at high colchicine concentrations (>20 μM), the neglect of which underlies many of the current paradoxes concerning colchicine inhibition of microtuble assembly. For example, at low colchicine concentrations (<2 μM), assembly inhibition conforms to the copolymerization model (Sternlicht & Ringel, 1979; Farrell & Wilson, 1980; Bergen et al., 1983; Sternlicht et al., 1983) whereas at high colchicine concentrations (>20 μM) a qualitatively different behavior is observed and inhibition appears to conform to the "capping" model (Margolis & Wilson, 1978). In both cases, inhibition has been attributed to TC [see Sternlicht et al. (1983) for a detailed review]. McClure & Paulson (1977) reported that the apparent rate constant for the second-order association of colchicine and microtubule protein is ~130 M^{-1} s^{-1}. One consequence of the small association rate is that microtubule protein and colchicine must be incubated for at least 0.5–1 h at 37 °C to obtain significant amounts of bound colchicine (0.2–0.6 mol of colchine/mol of tubulin) at the nonexchanging site (Wilson, 1970). If the nonexchanging site is the only functionally important site in tubulin, we find it difficult on kinetic grounds to understand why a stoichiometric excess of colchicine, e.g., 50 μM, added to several milligrams per milliliter of microtubule protein while it is assembling *abruptly halts* assembly within seconds or less (Margolis & Wilson, 1978; Sternlicht et al., 1983). This abrupt cessation of assembly [rationalized as a capping of microtubule ends by TC complexes (Margolis & Wilson, 1978)] occurs despite the fact that only a very small fraction of tubulin (<0.1% of total tubulin) could have bound colchicine at the nonexchanging site during the few seconds of incubation preceding inhibition. Furthermore, at these low levels of concentration, TC should inhibit assembly by copolymerization, i.e., by inducing a *monotonic decrease* in the assembly rates similar to that which is observed when TC is added to assemblying microtubule protein (Sternlicht & Ringel, 1979). However, under copolymerization conditions, the changes in assembly rates would be small and cannot account for the abrupt halt observed experimentally. We can more readily understand abrupt inhibition if binding at the reversible sites plays a role since these rapidly exchanging sites should equilibrate within fractions of a second upon addition of colchicine. The extent of colchicine binding at these reversible sites is not insignificant. Continuing with the above illustration [i.e., ~50 μM colchicine, 2 mg/mL MTP (~18 μM tubulin)], we estimate for one rapidly exchanging site per tubulin ($K_D \sim 650$ μM) that at any instant of time ~8% of the tubulins will have bound colchicine. The inhibition model we consequently favor is one where copolymerization of TC with tubulin is the principal mechanism of inhibition at low colchicine concentration, with an alternate mechanism involving binding at the weak-affinity site(s) important at high colchicine concentrations (>20 μM). The qualitatively different effects that tight and weakly bound colchicine have on assembly presumably arise because colchicine complexed to the α chain in TC (Schmitt & Atlas, 1976; Barnes et al., 1982)

[6] Nonexchanging colchicine may bind at or near a α–β interface. Detrich et al. (1982), for example, reported that colchicine stabilizes tubulin against dissociation into monomer subunits, while Luduena & Roach (1981) reported that tightly bound colchicine interfered with the alkylation of β-chain sulfhydryl(s) and affected the extent to which β-sulfhydryls could be cross-linked.

and to the α and β chains in the low-affinity complex (Schmitt & Atlas, 1976) induces different kinds of protein conformational changes. The nature of these putative conformational changes (Garland, 1978; Morgan & Spooner, 1983; Schmitt & Atlas, 1976) remains to be established.

In summary, this study represents the first use of ^{13}C NMR to investigate tubulin conformation and ligand interactions. We identified by ^{13}C NMR secondary colchicine binding site(s) in tubulin which we propose are involved in assembly inhibition at high colchicine concentrations (>20 μM), and we confirmed that MAPs have highly flexible domains. In addition, the ^{13}C NMR study suggested certain constraints on colchicine binding: The finding that the ^{13}C-enriched methoxy resonance did not shift in TC or in the weak binding complex (Figures 2, 3A, and 7A,C), for example, argues that colchicine is not in an aromatic pocket when bound since these pockets induce ring current shifts (Sternlicht & Wilson, 1968; Wuthrich, 1976). This interpretation is consistent with an earlier fluorescence study of TC (Bhattacharya & Wolf, 1974, and 1975) which failed to detect energy transfer from excited tryptophan to colchicine despite the fact that adequate spectral overlap exists. In addition, colchicine binding did not perturb the intense 34.0 ppm resonance in MTP (Figure 2B), a composite resonance arising from mobile C_γ glutamate carbons in tubulin and MAPs. We tentatively assign the mobile glutamates of tubulin to glutamates in the carboxy-termini regions (residues 425–450 in α and β).[7] If this assignment is corrent, the lack of a colchicine effect on the 34.0 ppm peak suggests that colchicine does not bind at the carboxy terminus.

Acknowledgments

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Registry No. Colchicine, 64-86-8.

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[7] Approximately 18 glutamates, or ~25% of the total glutamates in tubulin, cluster in the carboxy-terminus region between residues 425 and 450 (Ponstingl et al., 1981; Krauhs et al., 1981; Valenzuela et al., 1981). We suspect this region is coil, rather than helix as suggested by secondary structure predictions (Ponstingl et al., 1979; M. Glynias, M. Yaffe, and H. Sternlicht, unpublished results). Standard predictive methods are statistical in nature and ignore electrostatic interactions. For example, polyglutamic acid is predicted to be helical over the entire pH range (M. Glynias et al., unpublished results) by using Chou and Fasman rules or the Garnier et al. method, but polyglutamic acid actually undergoes a "helix → coil" transition, attributed to electrostatic repulsion between the negatively charged carboxyls, when the pH is raised above 5.5 (Appel & Yang, 1965).

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Carbon-13 Nuclear Magnetic Resonance Studies of Cobalamins†

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ABSTRACT: The carbon-13 nuclear magnetic resonance spectra of aquocobalamin, adenosylcobalamin, methylcobalamin, and (carboxymethyl)cobalamin have been interpreted. The assignments were made by a comparison of the spectra with that of cyanocobalamin, by a study of the pH dependence of the chemical shifts, by an analysis of the effect of the axial ligands on the carbon atoms of the corrin ring, and by a study of the specific line broadening effect of the paramagnetic ions Mn^{2+} and Gd^{3+}. The chemical shift changes that accompany the "base-on" → "base-off" conversion of the organocobalamins demonstrate that the conformation of the "western" half of the corrin ring and the conformations of the a, b, c, d, f, and g side chains are relatively constant. In contrast, the conformations of the "eastern" half of the corrin ring and the e propionamide side chain are highly variable.

In earlier publications we (Anton et al., 1982; Bratt & Hogenkamp, 1982) presented the complete interpretation of the ^{13}C NMR spectrum of cyanocobalamin. The assignments of the 65 resonances were based on earlier biosynthetic studies (Scott et al., 1974, 1976; Battersby et al., 1976), on our systematic analysis of the ^{13}C NMR spectra of cyanocobalamin and a number of its analogues (Anton et al., 1982), and on a study of the effect of the paramagnetic ions Mn^{2+} and Gd^{3+} on the ^{13}C NMR spectra of three cyanocobalaminmonocarboxylic acids. The complete interpretation of ^{13}C NMR spectrum of heptamethyldicyanocobyrinate has been reported by Ernst (1981) and by Battersby et al. (1982). We have now extended these studies to the interpretation of the ^{13}C NMR spectra of the naturally occurring cobalamins: aquocobalamin, methylcobalamin, adenosylcobalamin, and (carboxymethyl)-cobalamin.

Thus far, the structures of only two cobalamins, cyanocobalamin and adenosylcobalamin, have been established by crystallographic analysis. Furthermore, all the cobinamides and the "base-off" forms of the cobalamins have not been obtained as crystalline preparations. Thus, alternate methods for the determination of their structures have to be explored.

Several years ago Doddrell & Allerhand (1971) determined the ^{13}C NMR spectra of cyanocobalamin, dicyanocobalamin, adenosylcobalamin, and dicyanocobinamide at 15.08 MHz. Several assignments were made by comparing these spectra with those of model compounds, by an analysis of the ^{13}C–H and ^{13}C–^{31}P coupling, and by the determination of the relative T_1 values of selected resonances. Unfortunately, the resolution at 15.08 MHz was not sufficient to resolve all the resonances, and many of the resonances could only be assigned in groups.

At 62.9 MHz all but a few of the resonances of the cobalamins are resolved, and therefore, the complete interpretation of their ^{13}C NMR spectra is now feasible. In the present study we have used several approaches to make the assignments. First, we have compared the spectra of the cobalamins with that of cyanocobalamin. Second, we have analyzed the pH dependence of the chemical shifts of the cobalamins. Third, we have studied the effect of the axial ligands on the chemical shift of the carbon atoms of the corrin ring (cis effect), and finally, we have monitored the specific line broadening effect of the paramagnetic ions Mn^{2+} and Gd^{3+}.

The carbon–cobalt bond of the organocobalamins is quite stable under physiological conditions in the dark, and thus, in the active holoenzymes, the enzyme must promote the cleavage of this bond. It has been generally accepted that this interaction between the cobalamin coenzymes and their re-

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