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Promotion of Fluorescence upon Binding of Colchicine to Tubulin

(brain tubulin/tropolones/colchicine analogues)

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ABSTRACT **Colchicine, which does not fluoresce in aqueous media and organic solvents, exhibits marked fluorescence on combination with brain tubulin, with a corrected excitation maximum at 362 nm, an emission maximum at 435 nm, and a quantum yield of about 0.03. From fluorescence measurements it was found that rat brain tubulin binds 0.83 moles of colchicine per dimer (molecular weight 110,000) with an association constant of 3.2 μM^{-1} at pH 7.0 and 37°. These results are in excellent agreement with those obtained with the binding of [^{3}H]-colchicine. The enthalpy of binding is 10 kcal/mole, with an entropy change of 62 entropy units. The fluorescence can be ascribed to the tropolone moiety. However, the A ring of colchicine is also involved in binding. Denaturing agents abolish fluorescence, whereas podophyllotoxin, another antimitotic agent, decreases fluorescence competitively. Fluorescence is a convenient method for determining the binding of colchicine to tubulin that does not require the separation of free colchicine from bound colchicine and yields values for physical and biochemical parameters that are in excellent agreement with those obtained from the binding of [^{3}H]colchicine.**

A large body of evidence supports the view that microtubules from various sources are largely, if not completely, composed of tubulin, a dimeric protein of molecular weight 110,000–120,000 composed of two similar, but not identical, monomers (1). One of the most critical characteristics of tubulin is its ability to bind colchicine, an agent that leads to disaggregation of the polymerized form of this protein. The binding reaction of colchicine to tubulin has generally been studied by the use of labeled colchicine and subsequent separation of bound from free ligand by Sephadex gel filtration or ion exchange separation on DEAE–cellulose impregnated filter disc (2). Arai and Okuyama (3) recently pointed out that colchicine becomes fluorescent when bound to tubulin. We report here that fluorescence measurements offer a convenient method for measuring the colchicine tubulin interaction, which is based on the fact that colchicine fluoresces only in the bound form and not in the free state so that separation is not required. Thus, kinetic and thermodynamic parameters are easily obtained under equilibrium conditions.

METHODS AND MATERIALS

Tubulin was prepared from rat brains in 10 mM Na phosphate (pH 7.0), 10 mM $MgCl_2$, and by the procedure of Weisenberg *et al.* (2) except that DEAE–cellulose was used in place of DEAE–Sephadex. The purified tubulin solution was rapidly frozen in small aliquots and stored at −20°. The protein used gave a single band in sodium dodecyl sulfate–polyacrylamide gels (4), and its amino-acid analysis agreed well with that of Weisenberg *et al.* (2). The concentration of protein was determined by the method of Lowry *et al.* (5), with albumin as a standard. The analogues used were gifts of Dr. Colin Chignell, National Institutes of Health. Tropolone methyl ether was synthesized with diazomethane according to published procedures (6, 7). Podophyllotoxin was the generous gift of Dr. W. J. Gensler of Boston University. All other chemicals were reagent grade. For routine analysis, tubes containing increasing quantities of the cleared protein in phosphate–$MgCl_2$–GTP buffer (pH 7.0), containing 0.25 M sucrose and 1.02 μM colchicine, were incubated at 37° (unless otherwise indicated). Fluorescence was measured in the thermostated chamber of a Hitachi-Perkin-Elmer MPF-3 spectrofluorometer with excitation and emission wavelengths of 353 and 430 nm, respectively. The absorbance of the tubulin–colchicine complex was maintained below 0.05 at 353 nm for fluorescence measurements. The instrument was frequently standardized with quinine sulfate in 0.05 M H_2SO_4. Corrected emission and excitation spectra were measured with a Turner model 210 spectrofluorometer, which gives corrected spectra in quanta band width. The quantum yields of colchicine–tubulin complexes were calculated by comparing the absorbances, at the exciting wavelength and the area of the emission spectra, of the colchicine–tubulin complex with quinine sulfate in 1.0 M H_2SO_4 whose quantum yield was taken as 0.546 at 25° (8). For convenience, routine analyses of tubulin–colchicine fluorescence were done with the Perkin-Elmer fluorometer.

Calculation of Binding Data. Binding constants were obtained from fluorescence data by standard Scatchard analysis: $r = (F_c/F_o)\ (C_o/P_o)$ where r = moles of colchicine bound per mole of tubulin, F_c is the fluorescence of a given solution of colchicine–tubulin complex, and F_o is the fluorescence of an equal concentration of colchicine in excess tubulin, such that all the colchicine is bound, C_o = the total colchicine concentration, and P_o = the total protein concentration. From the known total concentration of colchicine and tubulin and the measured value of r, the free colchicine concentration, C, is calculated from the relation, $C = C_o - rP_o$. For determination of the stoichiometry by the filter disc method, the protein concentration was 100 μg/ml, and colchicine concentrations ranged from 10^{-7} to 10^{-5} M. The mixtures were incubated for 1.5 hr at 37° and assayed according to Williams and Wolff (9).

Absorption spectra were obtained on a Cary model 14 recording spectrophotometer, using 1-cm light path. Tryptophan was determined by the method of Edelhoch (10).

Thin-layer chromatography on silica gel plates was done in four different solvent systems to determine the R_F value of colchicine before and after complex formation: (*1*) $CHCl_3$–CH_3OH (95:5), R_F = 0.59; (*2*) $CHCl_3$–$(CH_3)_2CO$–$(C_2H_5)_2NH$

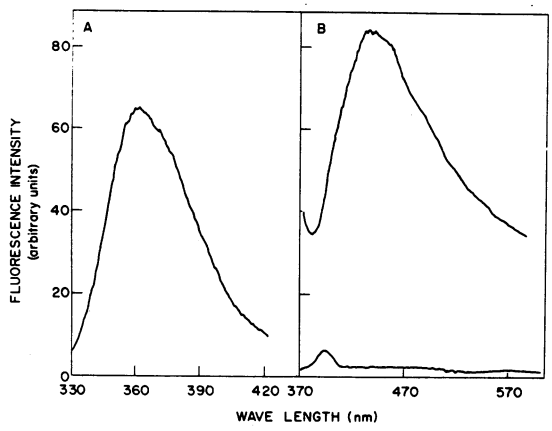


FIG. 1. Fluorescence spectrum of colchicine–tubulin complex. (*A*) Fluorescence excitation spectrum in phosphate–$MgCl_2$–GTP–sucrose buffer (pH 7.0). The reaction mixture contained 2 μM colchicine and 12.1 μM tubulin in the buffer and was incubated for 1.5 hr at 37° before fluorescence was measured. (*B*) (*Top curve*) Fluorescence emission spectrum in phosphate–$MgCl_2$–GTP–sucrose buffer (pH 7.0). *Lower curve* is that resulting from colchicine alone in the same buffer. The complex was prepared by incubating the mixture containing 2.5 μM colchicine and 30 μM tubulin in the buffer for 1.5 hr at 37°.

(50:40:10), R_F = 0.77; (*3*) $CHCl_3$–$(C_2H_5)_2NH$ (90:10), R_F = 0.65; and (*4*) $CHCl_3$–$(CH_3)_2CO$ (50:50), R_F = 0.31. Carriers were identified by fluorescence quenching, and ^3H was determined on 1-cm sections of the gel scraped into 10 ml of Aquasol.

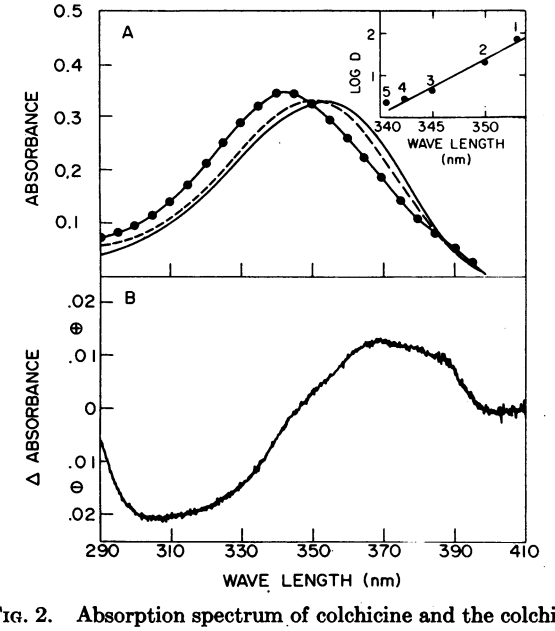


FIG. 2. Absorption spectrum of colchicine and the colchicine–tubulin complex. (*A*) Absorption spectra of 20 μM colchicine in water (*solid line*), in methanol (*dashed line*), and in carbon tetrachloride (●). The *inset* shows the logarithm of dielectric constant of different solvents against wavelength maxima: *1*, water; *2*, ethanol; *3*, benzene; *4*, carbon tetrachloride; and *5*, dioxane. (*B*) Difference spectrum of tubulin–colchicine complex in phosphate–$MgCl_2$–GTP–sucrose buffer. Colchicine (20 μM) and tubulin (51 μM) were incubated at 37° for 1.5 hr, and the spectrum was measured against the same concentration of colchicine and tubulin in separate reference cells.

RESULTS AND DISCUSSION

Fluorescence and Absorption Spectra. Although neither colchicine nor tubulin exhibited any fluorescence in phosphate–$MgCl_2$–GTP–sucrose when excited at the absorption peak of colchicine, there is a striking induction of fluorescence when these two compounds are incubated together. The corrected excitation and emission maxima of the complex are at 362 and 453 nm, respectively (Fig. 1). The quantum yield under these conditions was 0.030. The corresponding maxima obtained on the Perkin-Elmer (uncorrected) fluorometer are 353 nm and 430 nm, respectively. Tubulin has an emission peak at 330 nm when excited at 290 nm. Solutions of tubulin (6.5 μM) that contained up to 4.0 μM colchicine had identical fluorescence spectra when excited at 290 nm. There was no quenching of tubulin fluorescence by colchicine nor sensitization of colchicine fluorescence by tubulin. This finding suggests that the 3–4 tryptophan residues per 55,000 molecular weight are probably not near the colchicine-binding site or are of unfavorable orientation, since the emission spectrum of colchicine overlaps sufficiently for energy transfer to occur. In order to investigate the nature of the excitation process, we studied solvent effects. No fluorescence of colchicine could be observed in ethanol, *n*-propanol, *n*-butanol, benzene, carbon tetrachloride, and dioxane. Increasing the solvent viscosity by bringing the phosphate–$MgCl_2$–GTP–sucrose buffer to 50% glycerol or 2.5 M sucrose also failed to promote colchicine fluorescence.

We therefore examined the effect of solvent on colchicine absorption. As shown in Fig. 2*A*, as the polarity of the solvent decreases, there is a considerable hypsochromic shift from the maximum at 353 nm in water to 340 nm in dioxane*. A marked dependence on the dielectric constant of the solvents is depicted in the *inset* of Fig. 2*A*. This is all the more striking since solvents of different chemical classes were used. Binding of colchicine to tubulin produced a small red shift in the absorbance, which was best demonstrated by the difference spectrum (Fig. 2*B*). At no time did we observe a shoulder at 388 nm as reported by Arai and Okuyama (3); however, such a shoulder was seen in the difference spectrum (Fig. 2*B*).

Promotion of colchicine fluorescence is a highly specific property of tubulin and the following other proteins, measured at 2.5 mg/ml with 1 μM colchicine, failed to promote fluorescence at 430 nm when excited at 353 nm: bovine-serum albumin, trypsin, rabbit muscle lactate dehydrogenase, bovine liver glutamate dehydrogenase, ribonuclease, chicken lysozyme, human globin, thyroglobulin, and rabbit muscle actin.

Because of the failure to observe colchicine fluorescence under any conditions other than when bound to tubulin, it was important to ascertain whether there was any chemical change occurring in colchicine upon complex formation. The protein was incubated 1 hr with labeled colchicine and then separated

* According to Kasha (11), a n–π^* mechanism would require a blue shift in the absorption spectra of colchicine with increasing polarity of solvent, whereas the opposite was observed (Fig. 2*A*). n–π^* States can also be promoted to higher energy levels in acid. However, there was no colchicine fluorescence at pH 2 in HCl. Moreover, the log ϵ_{353} would be expected to be ≤ 2, whereas we determined a value of 4.24. Thus, while an n–π^* transition is unlikely, the data on the red shift and the extinction coefficient do not permit us to distinguish between an intramolecular charge transfer and a π–π^* mechanism.

by the DEAE–filter paper disc method. The complexed colchicine was then released from tubulin by heating the DEAE–paper at 60° for 15 min in water. The solution was concentrated and chromatographed on silica plates in four different solvents (see *Methods and Materials*). In all solvents, more than 98% of the total radioactivity moved with authentic colchicine.

Stoichiometry. The tubulin-induced fluorescence of colchicine was used to determine the number of colchicine-binding sites per dimer of tubulin. The number of binding sites was determined from the fluorescence curves (Fig. 3*A*) from a plot of r/C against r (Fig. 3*B*). The number of colchicine-binding sites per tubulin dimer (110,000 g) was 0.83 by fluorescence and 0.90 by the DEAE–filter disc method with [^{3}H]colchicine (9) (Fig. 3*C*). This is in agreement with published values, which vary between 0.7 and 1.0 (12, 13). This variation is probably due to the known tendency of tubulin to lose colchicine-binding activity during storage. The possibility that there are additional weaker binding sites discernible at high colchicine concentrations cannot be excluded by the data.

The affinity constant for the tubulin–colchicine complex in phosphate–$MgCl_2$–GTP–sucrose buffer (pH 7.0) at 37°, calculated from the Scatchard plot in Fig. 3, is 3.2 μM^{-1}. The value obtained by the isotopic method was 1.8 μM^{-1}. Both constants are in good agreement with previously published values (12, 14, 15).

Temperature Dependence of colchicine binding by tubulin was studied at pH 7.0 in order to evaluate the thermodynamic parameters. The results (Fig. 4) indicate that the affinity constant increases about 3-fold as temperature is increased from 20° to 37°. At 45° a marked reduction of binding was observed. A plot of log K against $1/T$ was linear in the region of 20–37° (*inset*, Fig. 4) and permitted an estimate of 10 kcal/mole for the enthalpy of binding and −9.2 kcal/mole for the free energy of reaction at 37°; $\Delta S = 62$ entropy units. A large entropy change (79.5 entropy units) for colchicine binding to vinblastine-induced paracrystals of tubulin has been reported by Bryan (13). A similarly large entropy change occurs during microtubule assembly (16), which may be considered as the binding of tubulin to tubulin. Although these data might favor the view (17) that the binding and polymerization sites are the same, the fact that Ca^{2+} has no effect on colchicine binding (unpublished observations), whereas it blocks polymerization (18), does not support the identity of these two sites.

Effect of Protein Stabilizers. Microtubule stabilizers like hexylene glycol (2.5%, v/v) and D_2O (50%, v/v) enhance the fluorescence of the tubulin–colchicine complex only slightly (about 6%). No enhancement in binding is detected with the DEAE–filter disc method of labeled colchicine. Vinblastine is thought to stabilize the native configuration of the protein and preserve colchicine-binding activity without directly affecting the binding site (19–22). When the decay of colchicine-binding activity was monitored at 37° by fluorescence, it was observed that the protein lost only 18% of its initial binding in the presence of 0.2 mM vinblastine sulfate, whereas without vinblastine, 76% of binding activity was lost over the same 7-hr interval. This agrees with earlier findings with [^{3}H]colchicine (22), and again shows that these two methods appear to measure the same binding process. When vinblastine is added just before the fluorescence measurement, no change is observed in the fluorescence spectrum.

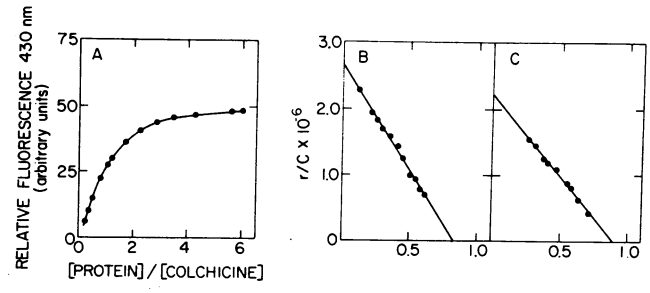


FIG. 3. Binding parameters of the colchicine–tubulin complex. (*A*) Colchicine–tubulin fluorescence at 430 nm as a function of increasing amounts of tubulin; 1.02 μM colchicine, incubated in phosphate–$MgCl_2$–GTP–sucrose buffer (pH 7.0) at 37° for 1.5 hr. (*B*) Scatchard plot of colchicine-binding by tubulin, calculated from Fig. 3*A*, as described under *Methods and Materials*. (*C*) Scatchard plot of [^{3}H]colchicine binding by tubulin assayed by the DEAE–filter disc method (2, 9). The reaction mixtures contained 100 μg/ml of tubulin with increasing concentrations of colchicine in phosphate–$MgCl_2$–GTP–sucrose buffer (pH 7.0). Incubations were carried out at 37° for 1.5 hr. Free [^{3}H]colchicine concentrations were determined from the difference between total and bound ligand.

The addition of denaturing agents such as 1% sodium dodecyl sulfate, 4 M guanidine hydrochloride, and 4 M urea completely abolished the fluorescence of tubulin–colchicine complex, indicating the need for the native conformation of tubulin for colchicine binding. When these agents were added to the preformed colchicine–tubulin complex, fluorescence was abolished.

Colchicine Analogues. Derivatives of colchicine can be divided into three structural types (Table 1). Structure *I* has the ring structure of colchicine itself; structure *II* is isomeric with structure *I* on ring *C;* and structure *III* has a six-membered *C*-ring replacing the tropolone ring. Colchicine, colcemid, and trimethylcolchicinic acid, which belong to group *I*, bind to tubulin and fluoresce. However, when a bulky sub-

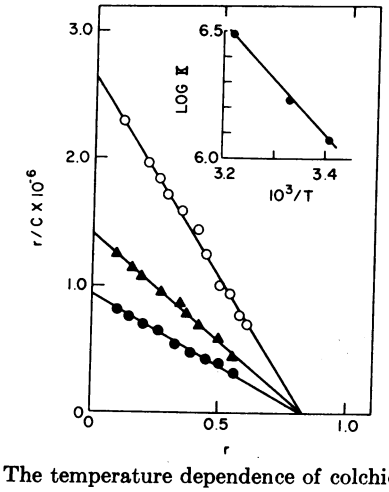
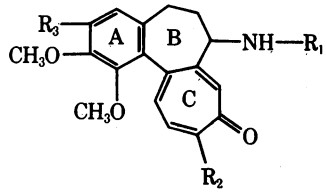
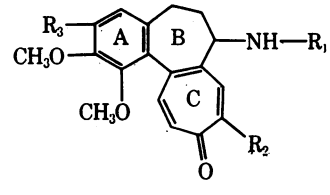


FIG. 4. The temperature dependence of colchicine binding to tubulin. The reaction mixtures contained 1.02 μM colchicine in phosphate–$MgCl_2$–GTP–sucrose buffer (pH 7.0) and varying concentrations of tubulin. They were incubated for 1.5 hr at the indicated temperatures before fluorescence was measured. Free and bound colchicine concentrations were determined as described in *Methods and Materials*. Scatchard plots are for 20° (●); 27° (▲), and 37° (○). The *inset* shows log K against $1/T$.

TABLE 1. *Fluorescence properties of colchicine and its analogues*



I



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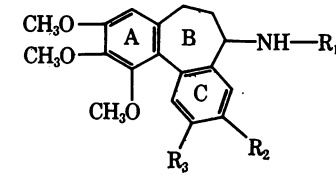

III

Compound	R_1	R_2	R_3	Fluorescence maxima (nm)† in H_2O	in benzene	with tubulin
Structure I						
Colchicine	$COCH_3$	OCH_3	OCH_3	—	—	430, 435 (corrected)
Colcemid	CH_3	OCH_3	OCH_3	—	—	428
TMCA*	H	OH	OCH_3	425	425	425
Colchicoside	$COCH_3$	OCH_3	$C_6H_{11}O_7$	—	—	—
Structure II						
Colchiceine	$COCH_3$	OH	OCH_3	425	425	425
Isocolchicine	$COCH_3$	OCH_3	OCH_3	—	—	428 (slight fluorescence)
Structure III						
Colchinol	H	OH	H	—	—	—
Other analogues						
Tropolone				410	410	410
Methyl ether of tropolone				—	—	—

* TMCA, trimethylcolchicinic acid. (—), No fluorescence.
† Excitation at 353 nm.

stituent is present, as in colchicoside, no fluorescence or binding (9) occurs. The fluorescence results are in agreement with those on the displacement of labeled colchicine in thyroid tubulin (23). This finding suggests that the *A* ring is a determinant for colchicine binding. The importance of the *A* ring is further indicated in studies with podophyllotoxin. This antimitotic agent has been reported to affect the colchicine-binding site of tubulin (13, 22). We studied its effect on colchicine binding fluorometrically at different concentrations of podophyllotoxin (Fig. 5). Podophyllotoxin inhibits competitively with a K_i of 2 μM, and presumably competes for the same binding site. Since the inhibitor has a benzene ring substituted by three vicinal OCH_3 groups, it is possible that podophyllotoxin interacts with that portion of the binding site that recognizes the *A* ring of colchicine.

Among the group *II* analogues, isocolchicine shows only very feeble fluorescence in the presence of tubulin. The possibility that this was due to contamination by colchicine could not be ruled out. It is, nevertheless, clear from the low fluorescence of this analogue that the configuration of the tropolone ring is critical for the induction of fluorescence by tubulin. While colchiceine fluoresces strongly when complexed to tubulin, the structural implications of this are not clear, since tautomeric forms are highly probable (6).

The absence of fluorescence when colchinol (group *III*) is added to tubulin confirms the view that the fluorescence is due to the tropolone ring. This conclusion is further supported by the finding that tropolone, which fluoresces in aqueous and organic solvents, shows a 3-fold enhancement of fluorescence upon interaction with tubulin. Whenever the tropolone ring has a free OH group, as in trimethyl colchicinic acid, colchiceine, or tropolone, fluorescence occurs in the absence of tubulin in either aqueous or organic solvents. Nevertheless, there is a 2- to 3-fold enhancement of fluorescence in the presence of tubulin. This is in agreement with the findings of Williams (24) that *ortho para* directing groups tend to enhance fluorescence and to counteract the quenching effect of electron-withdrawing groups such as carbonyl groups. The OCH_3 group is apparently much weaker in this respect, and the methyl ether of tropolone did not fluoresce in aqueous or nonpolar solvents. To our surprise, this compound did not fluoresce in the presence of tubulin. To investigate whether or not the methyl ether of tropolone was bound, we compared the abilities of tropolone and its methyl ether to displace [^{3}H]colchicine from its tubulin complex. The bindings of these two analogues, as expressed by the K_i obtained from modified Dixon plots, were 58 μM for tropolone and 50 μM for the methyl ether. Since both of these tropolones are bound equally, these findings suggest that the *A* ring contributes significantly to fluorescence.

CONCLUSIONS

The tubulin-induced fluorescence of colchicine provides a convenient method for measurement of either colchicine or tubulin. A major advantage is the fact that separation of free colchicine is unnecessary, since it does not contribute to the fluorescence. As far as we have been able to compare, results obtained with fluorescence are identical to those obtained by the use of [^{3}H]colchicine followed by Sephadex or ion exchange separation. This is true for the affinity constant (compare Fig. 3 with refs. 12, 14, and 15), the stoichiometry (12, 13), the pH optimum (22, 25), and the effect of analogues

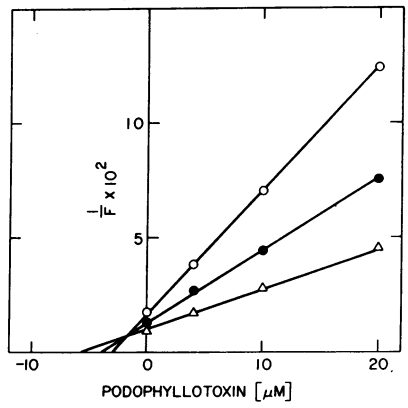

FIG. 5. Modified Dixon plot demonstrating the effect of podophyllotoxin on the colchicine-binding reaction. The concentration of podophyllotoxin present is plotted against the reciprocal of the fluorescence, F, at several concentrations of colchicine: (○) 1.0 μM; (●) 2.1 μM; (△) 3.57 μM. The reaction mixtures contained 200 μg/ml of tubulin in phosphate–$MgCl_2$–GTP–sucrose (pH 7.0). Fluorescence was measured after 1.5 hr of incubation at 37°.

(22, 23). Because of the relatively low quantum efficiency of fluorescence (0.03), it is probable that the radioactive method is more sensitive, although it, in turn, is limited by the flow rate at high protein concentrations.

Analogue studies reveal that there are at least two sites on the colchicine molecule involved in binding to tubulin. The site at which fluorescence is induced is most probably the tropolone moiety, as shown by the absence of fluorescence in colchinol. It requires correct positioning of the carbonyl moiety of the tropolone ring, as shown by the very low fluorescence exhibited by isocolchicine. A second site, which contributes to binding, involves the *A* ring, since substitution of one of its OCH_3 groups by a hexose completely abolished fluorescence and its ability to displace colchicine from tubulin (23). This is supported by the fact that podophyllotoxin, which shares the trimethoxy benzene moiety with colchicine, is a competitive inhibitor. Nevertheless, the *A* ring also contributes to the fluorescence, as shown by a comparison of colchicine with tropolone methyl ether.

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