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Immobilization-dependent Fluorescence of Colchicine*

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B. Bhattacharyya‡ and J. Wolff

From the National Institute of Arthritis, Metabolism, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

Colchicine fluoresces when bound to tubulin but not in water, dioxane, or benzene. The basis of the fluorescence has now been investigated. Colchicine fluoresces in higher alcohols and shows a blue shift as a function of chain length. Glycerol produces a higher fluorescence efficiency and a further blue shift. Plots of 1/fluorescence *versus* T/η yield straight lines for both alcohols and glycerol/water mixtures. Fluorescence in glycerol/dimethyl sulfoxide mixtures, in which the dielectric constant remains unchanged, varies as a function of solvent viscosity. Even highly nonpolar solvents such as dioxane require a threshold viscosity for fluorescence to occur. When solvent polarity was decreased at constant viscosity, there was also an enhancement of colchicine fluorescence, but this effect appeared to be smaller than that obtained with increasing viscosity.

Immobilization by covalent attachment of desacetylcolchicine to thyroglobulin, serum albumin, or lysozyme also promotes fluorescence from the drug. By contrast, the highly rigid analogue of colchicine, imerubrine, fluoresces in water and is unaffected by viscosity changes.

We conclude that a major contribution to colchicine fluorescence stems from immobilization of colchicine in the site and that this response to immobilization depends, in part, on the partially flexible nature of the drug. Since certain other flexible molecules such as auramine O, reduced flavines, and diarylalkanes also require increased viscosity or binding to macromolecules to fluoresce at room temperature, we propose that immobilization-enhanced fluorescence may be more common than heretofore believed.

Colchicine is the most important of the drugs that have been used to study microtubule assembly. It binds to tubulin with dissociation constants in the low micromolar range depending on the conditions of the assay and of the protein. There is one site per heterodimer. Kinetically, binding is a two-step process—a rapid step is followed by a slow step that is pseudoirreversible and has been ascribed to a conformational change in tubulin which is highly temperature-dependent (Ventilla *et al.* 1972; Garland, 1978; Lambeir and Engelborghs, 1981; Andreu and Timasheff, 1982b). The tubulin-colchicine complex so formed is relatively stable, adds to polymerizing microtubules, and thereby retards subsequent dimer addition. The details for this inhibition are still a matter of dispute (Margolis and Wilson, 1977; Sternlicht and Ringel, 1978; Zackroff *et al.*, 1980; Zeeberg *et al.*, 1980) but the end result is a shift to the left in the dimer → polymer equilibrium. Binding of colchicine at these affinities is specific for tubulin and has not been observed with numerous other proteins.

When colchicine binds to the tubulin dimer, it fluoresces with an emission maximum of 435 nm and a quantum yield of 0.03 whereas there is virtually no fluorescence in aqueous media. The fluorescence can be ascribed to the tropolone moiety (C ring) of the drug and has the properties of a $\pi^* \rightarrow \pi$ transition with fluorescence lifetimes (1.14 ± 0.02 ns) characteristic of the singlet state (Bhattacharyya and Wolff, 1974; Arai and Okuyama, 1975; Letterier and Rieger, 1975; Ide and Engelborghs, 1981). Since there is virtually no fluorescence in the absence of the protein, fluorescence is a useful measurement of colchicine binding that does not require the separation of free and bound ligand. The method has since been used in a number of laboratories (Garland, 1978; Lambeir and Engelborghs, 1981; Andreu and Timasheff, 1982a).

The basis of the promotion of fluorescence from the tropolone fluorophore of colchicine upon binding to tubulin was not readily explained. Enhancement of fluorescence on binding of a small ligand to a protein is generally explained as due to the hydrophobic environment provided by the binding site, thus lowering the probability of mobile dipole-dependent internal conversion reactions. The fact that solvents of low dielectric constant mimic such enhancement is used as support for such interpretations. Although such solvents produced the appropriate blue shifts in the absorption spectrum of colchicine, no fluorescence could be detected at colchicine concentrations that readily produced fluorescence when bound to tubulin (Bhattacharyya and Wolff, 1974; Arai and Okuyama, 1975). We considered the possibility that binding produced a more nearly planar conformation in colchicine which would enhance extended conjugation and hence fluorescence. This would not be possible in the nonplanar conformer of the unliganded drug. However, removal of the constraints imposed on the molecule by the B ring, as in the analogue 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone failed to induce substantial increases in the fluorescence of the unbound ligand (Choudhury *et al.* 1983). It was thus not clear to what extent increased planarity would contribute to fluorescence. We were also not able to obtain evidence for Schiff base formation through the tropolone carbonyl group and all bound colchicine could be extracted intact from the complex.

It is, however, also possible to diminish competition with fluorescence by internal conversion reactions by the use of solvents of high viscosity that restrict rotational relaxations. In the present study, we show that at high viscosities free colchicine will fluoresce and it is the *immobilization* of the colchicine and/or the solvent (rather than its hydrophobic environment) that accounts for the bulk of the observed fluorescence.

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‡ On leave from the Bose Institute, Calcutta, India.

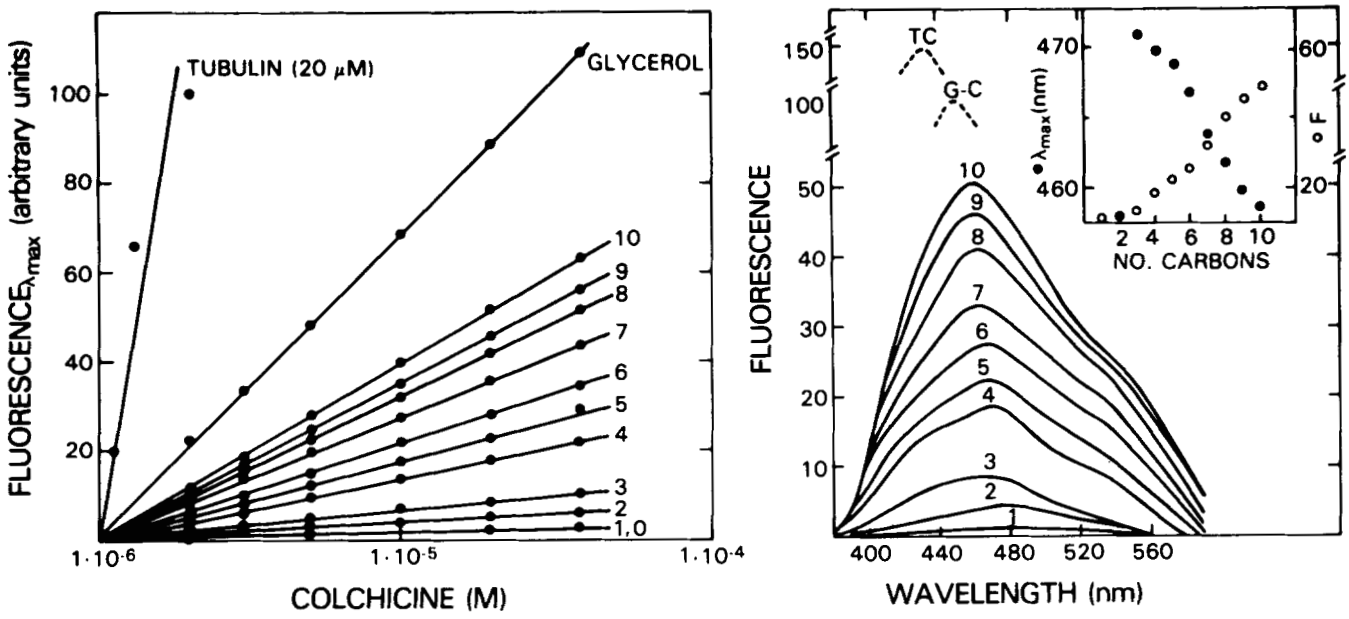

FIG. 1. **Colchicine fluorescence in different solvents.** *Left panel*, fluorescence as a function of colchicine concentration in different normal alcohols, glycerol, and tubulin. Fluorescence intensities were measured at the respective emission maxima and were different for alcohols, glycerol, and tubulin (see *right panel*). Excitation was at 380 nm and the fluorescence was measured using a 390 nm emission filter. The *numbers* on the *curves* represent the carbon atoms of the normal alcohols; thus, *O* = H_2O, *1* = methanol, etc. Samples containing tubulin (2.4×10^{-5} M) were preincubated at 37 °C for 1 h with colchicine before the fluorescence was measured at 30 °C. *Right panel*, fluorescence emission spectra of colchicine in alcohols, glycerol, and tubulin. The colchicine concentration is 1.8×10^{-6} M. In all cases, the excitation wavelength was 380 nm and the scans were performed using a 390 nm emission filter. *Numbers* are as in *left panel*; *TC* is the tubulin-colchicine complex; *G-C* is colchicine in 100% glycerol. The *inset* shows the fluorescence intensity and the emission maxima as a function of the number of carbon atoms of normal alcohols.

MATERIALS AND METHODS

Equine liver alcohol dehydrogenase, bovine serum albumin, rabbit muscle aldolase, and lysozyme were obtained from Sigma. 19 S thyroglobulin was a kind gift from Roland Lippoldt of our Institute. Glycerol (Gold Label), Me_2SO[1] (Gold Label), colchicine, auramine O, and polyethylene glycols of different molecular weights were products of Aldrich. Normal alcohols were obtained either from Sigma or Aldrich. Dioxane was obtained from J. T. Baker and freed from peroxides using activated alumina columns. Imerubrine was the kind gift of Drs. M. Cava (University of Pennsylvania) and J. V. Silverton (National Institutes of Health).

Viscometry—Ostwald capillary viscometers were immersed in a large water bath regulated at 30 °C and outflow times were measured to 0.2 s using a stopwatch. The viscosity was then determined from the relation

$$\eta/\eta_0 = (t/t_0)(\rho/\rho_0)$$

where η_0, t_0, and ρ_0 are the viscosity, outflow time, and density for the reference solvents, water or glycerol. The viscosity of pure glycerol was taken as 612 centipoise at 30 °C. Viscosities of other solvents used agreed closely with literature values when available. Densities were determined with Gay-Lussac pycnometers of 1.0-ml capacity. All per cent values listed are as grams of glycerol/100 ml.

Spectrofluorometry—Fluorescence measurements were carried out in thermostated cuvettes at 30 °C in a Perkin-Elmer MPF-3L instrument. The excitation wavelength was 380 nm for colchicine and isocolchicine and emission was generally measured at the emission maximum. The excitation wavelength was chosen at 380 nm to avoid inner filter effects from solutions where high colchicine concentrations were used and also to minimize interference from pure glycerol. Under these conditions, we did not observe any significant inner filter effect.

Dielectric Constant—The dielectric constants of different solutions were determined with a Sargent chemical oscillometer, Model 5, at 26–28 °C operating at 5 MHz. We thank Dr. Normal Sharpless of our Institute for helping us with these determinations.

Covalent Coupling with Proteins—The protein to be coupled (8–10 mg) dissolved in 1 ml of 0.1 M NaCl was neutralized to pH 7.0 and was added to 7.5 mg of desacetyl colchicine. 125 mg of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide dissolved in 0.5 ml of H_2O was then added to the above mixture at room temperature in the dark. The mixture was allowed to stand at room temperature for 30 min and was then extensively dialyzed in the dark against 0.1 M NaCl and then H_2O for 24 h. The absorption spectrum of the resulting mixture showed the characteristic tropolone peak at 353 nm. The different preparations yielded a 353:280 nm ratio of 0.63 to 0.85. We thank Dr. Arnold Brossi of this Institute for the generous supply of desacetylcolchicine and isocolchicine.

Auramine O was coupled to proteins by a similar procedure except that 0.2 M $NaHCO_3$ was used in place of NaCl. In some experiments, a suspension of the dye in H_2O was used, in others a saturated solution. The absorbance of the extensively dialyzed product was 0.34–0.38 at 450 nm.

RESULTS

Our initial attempts to elicit fluorescence from colchicine in solvents of low polarity were unsuccessful at concentrations of the drug that gave maximal fluorescence when bound to tubulin, even though sizable blue shifts occurred in the absorption spectra (Bhattacharyya and Wolff, 1974). However, we have now found that much larger concentrations of colchicine *will* fluoresce in such solvents. In Fig. 1 (*left panel*), the fluorescence of colchicine in a series of normal alcohols is depicted as a function of drug concentration. Fluorescence was a linear function of colchicine concentration over the 50-fold range tested. At all colchicine concentrations, glycerol (discussed below) yielded more fluorescence than the alcohols but this was always less than could be attained by binding to tubulin. At the same time, there was a pronounced blue shift in the emission upon changing solvents from methanol to octanol as shown in the *right panel* of Fig. 1. These changes are compared as a function of the number of carbon atoms of

[1] The abbreviation used is: Me_2SO, dimethyl sulfoxide.

the alcohols in the *inset*. The blue shift was to be expected from the decreasing polarity of the higher alcohols. We are at a loss to explain the very large red shifts observed by Arai and Okuyama (1975) with lower alcohols. The fact that glycerol and tubulin caused further blue shifts led us to examine the contribution that viscosity might make to the fluorescence efficiency.

Viscosity Effects—When the reciprocal of the colchicine fluorescence in normal alcohols was plotted as a function of the reciprocal of the viscosity (according to Oster and Nishijima, 1956), a straight line relation was obtained as depicted in Fig. 2. This suggested that the major solvent contribution to the observed fluorescence appeared to be the restriction of dipole mobility or fluorophore mobility resulting from increased viscosity. The contribution from the decreasing dielectric constant that occurs with increasing chain length was not readily visible in this plot but is discussed below.

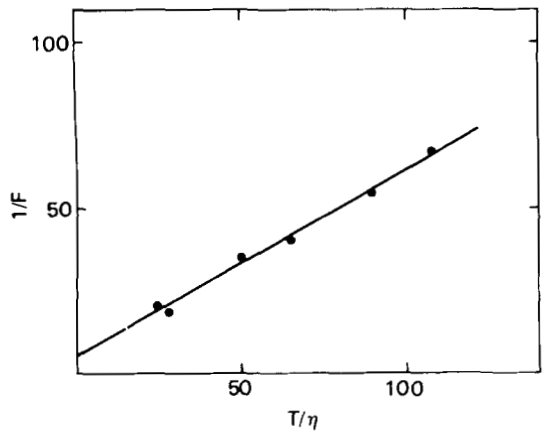

FIG. 2. **Relation of colchicine fluorescence to the viscosity in different normal alcohols.** Reciprocals of the fluorescence intensity at the respective emission maxima were taken from Fig. 1 (*right panel*) and plotted against T/η, where T is the absolute temperature, fixed at 30 °C, η is the viscosity in centipoise, and F is the fluorescence at the λ_{max} in arbitrary units. Other conditions were as described in the legend to Fig. 1.

In order to assess in more detail the role of viscosity in abating nonradiative energy loss from the excited state of colchicine, the role of glycerol in the fluorescence was examined further. We have already shown that pure glycerol increased the fluorescence yield and led to a blue shift (Fig. 1). Increasing concentrations of glycerol in water increased the fluorescence yield from colchicine (Fig. 3). To reduce the contribution of the dielectric constant, mixtures of glycerol and dimethyl sulfoxide were prepared; the dielectric constants are, respectively, 42.2 and 44.1 (measured at 26 °C), hence permitting the assessment of an almost pure viscosity effect without changes in the polarity of the solvent. Although there was some upward displacement from the glycerol/water curve at lower viscosities, the fluorescence response to viscosity in the glycerol/Me_2SO solvent closely resembled that in glycerol/water (Fig. 3), indicating that in glycerol/water there is only a small effect from changes in the dielectric constant and that viscosity is the major determinant promoting fluorescence in colchicine. As demonstrated in the *right-hand panel* of Fig. 3, the glycerol-induced enhancement of fluorescence yield was accompanied by a significant blue shift (from 460 nm at 82% glycerol to 447 at 100% glycerol) in the emission from colchicine.

The importance of viscosity is further demonstrated by the

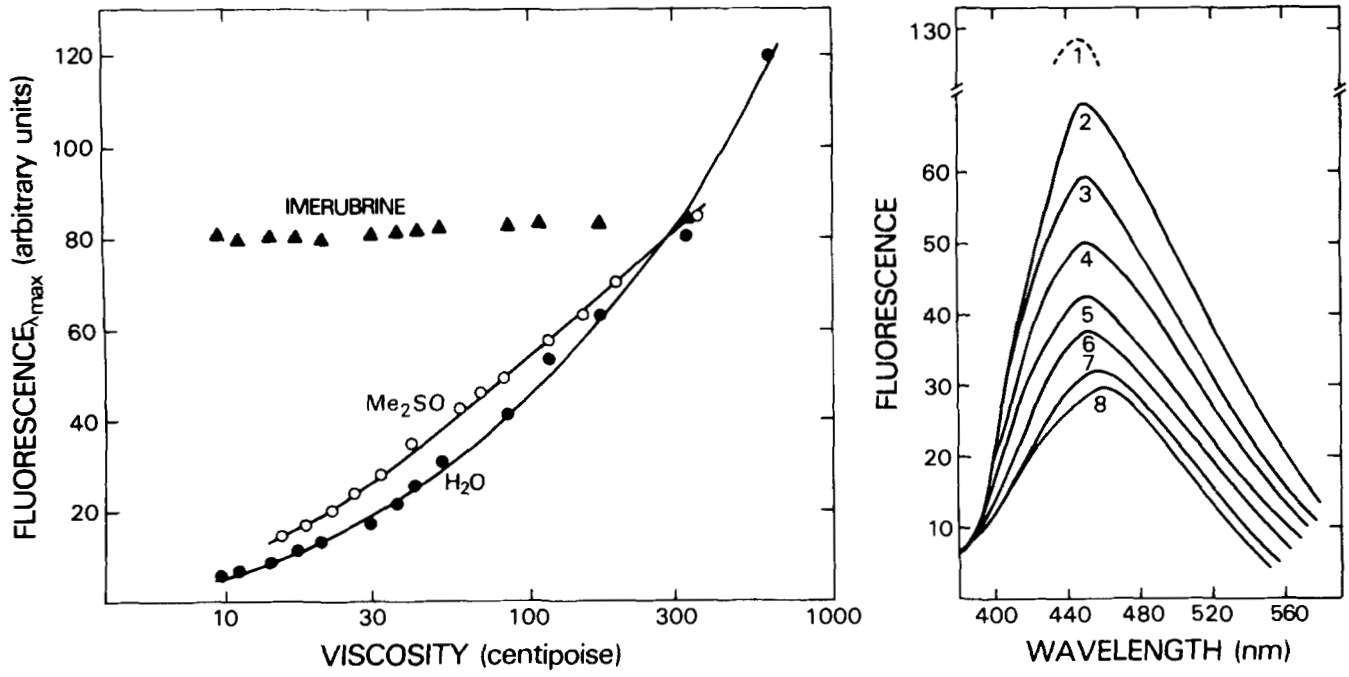

FIG. 3. **Fluorescence of colchicine and imerubrine in solutions of different glycerol concentrations.** The fluorescence of colchicine (1.8×10^{-5} M) and imerubrine in different glycerol concentrations is plotted against the viscosity. *Left panel*, glycerol concentrations were varied by adding either H_2O or Me_2SO to pure glycerol. Viscosities were measured at 30 °C. The excitation wavelengths were 380 and 450 nm for colchicine and imerubrine, respectively. Fluorescence emission maxima shifted in different glycerol concentrations (see *right panel*) and ranged from 450–460 nm for colchicine and 560–568 nm for imerubrine (not shown). *Right panel*, fluorescence emission spectra of colchicine (1.8×10^{-5} M) in aqueous solutions containing different glycerol concentrations. Glycerol concentrations are as follows: *1*, 100%, *2*, 92%; *3*, 90%; *4*, 87%; *5*, 86%; *6*, 85%; *7*, 83%; and *8*, 82%. Excitation was at 380 nm and the scans were performed using a 390 nm emission filter.

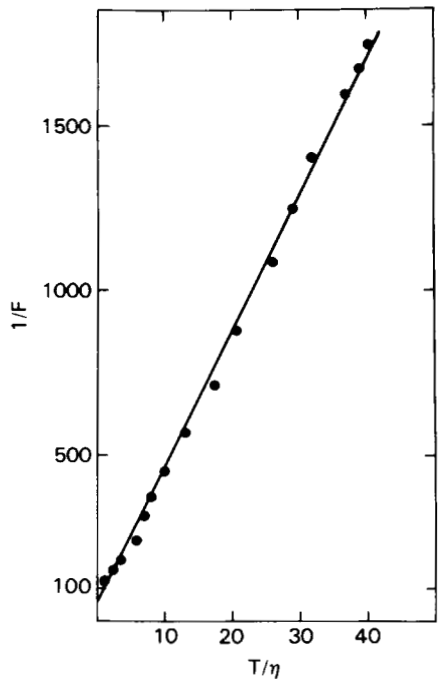

FIG. 4. **The relation of colchicine fluorescence to the viscosity at different glycerol concentrations.** The reciprocal of the fluorescence intensity of colchicine in different glycerol solutions is plotted against T/η. Data were taken from Fig. 3. Other conditions were as described in the legend to Fig. 3. This plot is based on the empirical equations:

$$\text{fluorescence intensity} = \frac{\eta/T}{\alpha + \beta(\eta/T)}$$

where $\alpha + \beta$ are constants (Nishijima and Oster, 1956). The probability for occurrence of fluorescence, vibrational, and rotational dissipations of the excited state is a function of the sum of their rate constants and the quantum yield, ϕ, can be written:

$$1/\phi = 1 + \frac{\tau_f}{\tau_v} + \frac{\tau_f}{\tau_r}$$

where τ_f, τ_v, and τ_r are the reciprocals of the rate constants (lifetimes) for the above three processes, respectively. To the extent that τ_r represents a diffusion-controlled process, it is proportional to the diffusion constant and hence to T/η (Stokes-Einstein). The equation can thus be written in linear form:

$$1/\phi = 1 + \frac{\tau_f}{\tau_v} + a\tau_f\,(T/\eta)$$

where a is a proportionality constant.

linear plot of $1/F$ *versus* T/η in Fig. 4. The intercept at infinite viscosity allows an estimate of the maximum contribution likely to be made by the viscosity effect. The fluorescence value so obtained was 200 (arbitrary units). Under otherwise similar conditions, the fluorescence of the tubulin-colchicine complex in aqueous buffers was 348 (arbitrary units). Whether these viscosity effects are mainly on the mobility of the solvent dipoles or on the fluorophore is not specified by the above results. However, a rigid analogue of colchicine called imerubrine (Silverton *et al.*, 1977) (see Fig. 5), which fluoresces in polar solvents of low viscosity, exhibited no fluorescence enhancement as a function of viscosity changes of the solvent, as shown in Fig. 3 (*solid triangles*).[2] This suggests that colchicine may dissipate some of its excited state energy by intramolecular rotation and that solvents of high viscosity reduce this form of internal conversion (see "Discussion").

Isocolchicine—One of the remarkable properties of the colchicine binding site of tubulin is the very high structural specificity for the tropolone moiety such that isocolchicine (Fig. 5) neither binds to tubulin (Zweig and Chignell, 1973) nor fluoresces (Bhattacharyya and Wolff, 1974). It was, therefore, important to know whether this inversion in the tropolone ring had any effect on the intrinsic fluorescence properties of the methyl tropolone fluorophore. As shown in Fig. 6, isocolchicine showed intense fluorescence in glycerol and the lack of isocolchicine fluorescence in the presence of tubulin can be ascribed solely to its inability to bind to the site.

Dielectric Effects—To gain information on the dielectric contribution to the fluorescence emission of colchicine, solvents of constant viscosity were devised that allowed us to vary the dielectric constant, ϵ, over a substantial range. The effect of mixtures of polyethylene glycol (400)/ethanol mixtures with decanol on the fluorescence of colchicine is shown in Fig. 7. It is apparent that when ϵ falls below 13–14, there is a sharp rise in fluorescence efficiency. Above this value, there was little effect of changing dielectric constant which may account, in part, for the linearity of the glycerol/water curve of Fig. 4.

The relation between η and ϵ in the fluorescence of colchicine is examined in Fig. 8, where we compare the effect of polyethylene glycol (600) when added to water, Me_2SO, or dioxane such that both viscosity and the dielectric constant can vary. The data show that the lower the ϵ, the lower the viscosity required to promote fluorescence, but a threshold viscosity is required at *all* values of the dielectric constant. This is depicted in Fig. 8 as a narrow valley (shaded) along the dielectric axis even near the origin. These requirements were unknown in our earlier study and probably account for the failure to observe fluorescence in nonpolar solvents (Bhattacharyya and Wolff, 1974). It is also clear that both viscosity and polarity determine the ability of colchicine to fluoresce in the absence of tubulin. The relative importance of the two effects cannot be directly gauged from Fig. 8 because the choice of scalar units is somewhat arbitrary.

Covalent Immobilization—If immobilization of colchicine in the binding site of tubulin accounts for a major portion of the observed fluorescence (as indicated by the above viscosity-

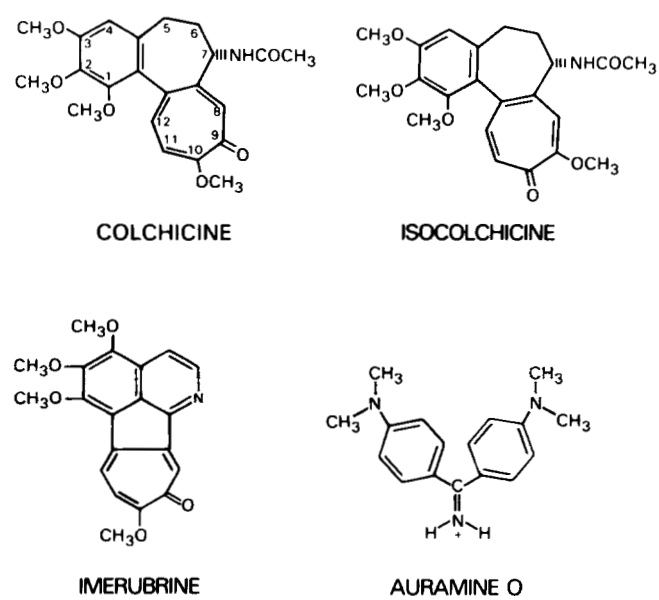

FIG. 5. **The structures of colchicine, isocolchicine, imerubrine, and auramine O.**

[2] This analogue does not bind to tubulin as judged by displacement of [^{3}H]colchicine.

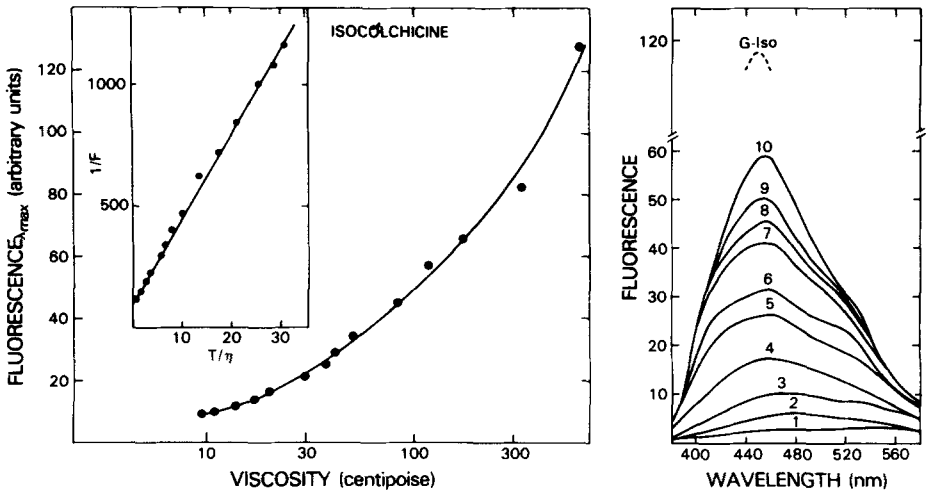

FIG. 6. **The relation of isocolchicine fluorescence to the viscosity in different glycerol solutions.** *Left panel*, the fluorescence intensity of isocolchicine (2×10^{-5} M) in different glycerol concentrations was plotted against viscosity. The excitation wavelength was 380 nm and the fluorescence was measured at the respective emission maxima. Viscosity and fluorescence intensity were measured at 30 °C as described under "Materials and Methods." In the *inset*, the reciprocals of the fluorescence intensity of isocolchicine in different glycerol solutions were plotted against T/η. *Right panel*, fluorescence of isocolchicine in different normal alcohols. The isocolchicine concentration is 2×10^{-5} M. In all cases, the excitation wavelength was 380 nm and the scans were performed using a 390-nm emission filter. The *numbers* on the *curves* represent the number of carbon atoms of the normal alcohols.

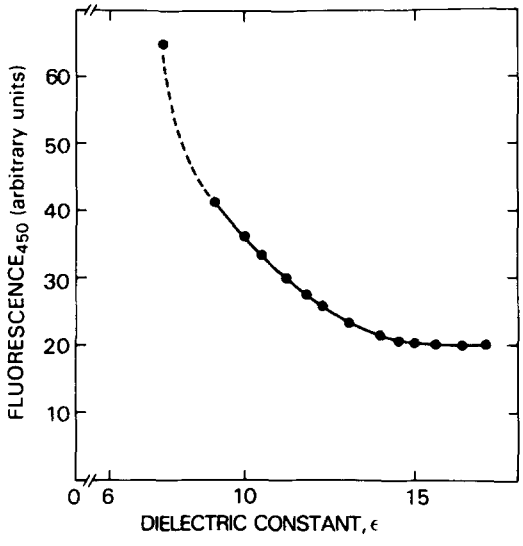

FIG. 7. **Solvent dielectric effects on colchicine fluorescence.** Two volumes of polyethylene glycol (M_r = 400) were mixed with 1 volume of absolute ethanol to give a mixture having the same viscosity as that of decanol (11 centipoises at 30 °C). Solutions of different dielectric values of decreasing order were prepared by mixing a fixed amount of polyethylene glycol/ethanol mixture with different amounts of decanol. The fluorescence of colchicine (1.8×10^{-5} M) was measured at 30 °C, with excitation at 380 nm and emission at 450 nm using a 390 nm emission filter.

dielectric comparisons), then nonspecific immobilization of the drug by covalent attachment to proteins that do not have a binding site should also promote fluorescence. This indeed proved to be the case as shown in Fig. 9. Coupling of desacetyl colchicine to bovine serum albumin, bovine 19 S thyroglobulin, or chicken lysozyme by means of a water-soluble carbodiimide led to adducts containing covalently bound drug that exhibit emission spectra characteristic of the tropolone chromophore, although the quantum efficiency was less than for colchicine when noncovalently bound to the binding site of tubulin. The small fluorescence exhibited by colchicine in the presence of serum albumin may be due to the low affinity binding sometimes reported for serum (Donigian and Owellen, 1973). The comparative fluorescence intensities under these different solvent conditions are summarized in Table I. The reversal in efficiencies in glycerol or tubulin for the two drug isomers that is dependent on binding should be noted as should the disproportionate blue shift resulting from binding to tubulin.

In view of the apparent nonspecificity of the immobilization effect on colchicine fluorescence, it is quite surprising that the high affinity binding of the drug to antibodies (prepared against serum albumin-desacetylcolchicine covalent adducts (Wolff *et al.*, 1980)) was not accompanied by colchicine fluorescence even though there is some diminution of the negative circular dichroic band at 340 nm (Detrich *et al.*, 1981). The binding domains for colchicine in this protein had different specificities than in tubulin and it seems possible that the antibody site quenches the fluorescence expected from the immobilization.

A similar example of immobilization is found in the interaction of auramine O (Fig. 5) (Conrad *et al.*, 1970) with horse liver alcohol dehydrogenase. This cationic diphenylmethane dye is a noncompetitive inhibitor with respect to both NAD^+ and ethanol but did not bind to 16 other proteins. Binding of auramine O to the dehydrogenase promotes fluorescence with an emission maximum of 523 nm, a quantum yield of 0.05, and an excited state lifetime of 3 ± 1 ns. Such fluorescence is not observed in water, ethanol, dioxane, benzene, or hexane but is observed in viscous solutions (Oster and Nishijima, 1956). Chen (1977) has, however, observed weak fluorescence of auramine O in water and lower alcohols (quantum yields 4×10^{-5} to 5×10^{-4}) and low affinity binding for one site on bovine serum albumin that would not be readily detected under our conditions of measurement. Moreover, adsorption of the dye to polyanions (DNA) (Oster and Nishijima, 1964) or chromatophores of *Rhodospirillum rubrum* (Kobayashi and Nishimura, 1972) also promotes fluorescence. When auramine O was coupled to several proteins previously shown to bind this dye poorly or not at all (Conrad *et al.*, 1970; Chen, 1977), fluorescence could readily be demonstrated (Table II).

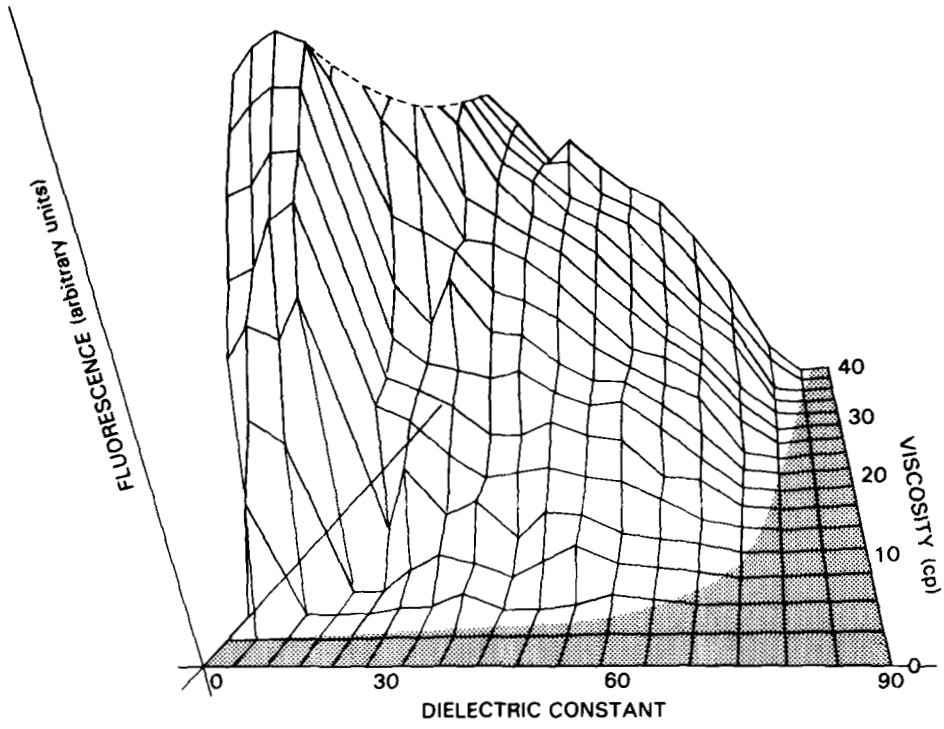

FIG. 8. **The effect of viscosity on the promotion of colchicine fluorescence in solvents of different dielectric constants.** Solutions were made by adding increasing amounts of polyethylene glycol (M_r = 600) to dioxane, dimethyl sulfoxide, or water. The fluorescence of colchicine (1.8×10^{-5} M) was measured at 30 °C. In all cases, the excitation was at 380 nm and the emission intensities were measured at 450 nm using a 390 nm emission filter. Viscosity in centipoise is plotted on the *y* axis, dielectric constants on the *x* axis, and fluorescence in arbitrary units on the *z* axis. The *dashed line* indicates a discontinuity for which data were not available. The *shaded area* indicates the zone of viscosity threshold to obtain fluorescence.

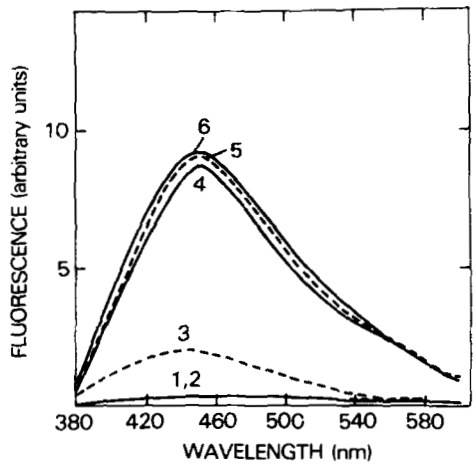

FIG. 9. **Fluorescence emission spectra of desacetylcolchicine covalently coupled to proteins.** Solutions of the coupled proteins were diluted to absorptions of 0.05 at 380 nm and the scan was performed using a 390 nm emission filter. Control solutions contain the same amount of free protein and desacetylcolchicine with absorptions of 0.05 *A* at 380 nm. The *numbers* on the *curves* indicate the following: *1,2*, desacetylcolchicine + thyroglobulin or lysozyme. *3*, desacetylcolchicine + bovine serum albumin. *4*, desacetylcolchicine coupled to lysozyme. *5*, desacetylcolchicine coupled to bovine serum albumin. *6*, desacetylcolchicine coupled to thyroglobulin.

The emission spectra were similar to those in glycerol but were blue-shifted with respect to those seen upon binding to horse liver alcohol dehydrogenase. The fluorescence intensity was less than obtained from binding to the specific site.

TABLE I

Fluorescence of colchicine, desacetylcolchicine, and isocolchicine under different experimental conditions

In all experiments, total fluorescence is expressed per 0.05 *A* unit at 380 nm. The fluorescence was measured (using a 390 nm emission filter) at the respective emission maxima. Under these conditions, no inner filter effect could be observed.

Experimental conditions	Fluorescence (arbitrary units)	Emission maximum
Colchicine		
Water	<1	
Dioxane	<1	
Benzene	<1	
Bovine serum albumin (BSA) (2.4 mg/ml)	2	430–435
Glycerol	100	448
Tubulin (2.4 mg/ml)	150	430
Desacetylcolchicine		
Water	3	
Dioxane	2	
Benzene	3	
Thyroglobulin (0.9 mg/ml)	<1	
Thyroglobulin-*covalent* (0.9 mg/ml)	8.2	451
BSA (1.1 mg/ml)	<1	
BSA-*covalent* (1.1 mg/ml)	8.1	450
Lysozyme (1.3 mg/ml)	<1	
Lysozyme-*covalent* (1.3 mg/ml)	8.0	452
Glycerol	38	448
Tubulin (2.4 mg/ml)	48	430
Isocolchicine		
Water	<1	
Dioxane	<1	
Benzene	<1	
Glycerol	130	448
Tubulin (2.4 mg/ml)	5	430

TABLE II

Comparison of auramine O fluorescence in different experimental conditions

In all experiments, total fluorescence is expressed per 0.068 *A* at 450 nm. The excitation wavelength was 450 nm and emission was measured at 510 nm in all cases except when scans were performed. No inner filter effect was detectable under these conditions.

Experimental conditions	Fluorescence	Emission λ_{max}
		nm
Water + auramine O	~0	
Bovine serum albumin (1.9 mg/ml) + auramine O	~0	
Aldolase (1.6 mg/ml) + auramine O	~0	
Covalently coupled bovine serum albumin to auramine O (1.6 mg/ml)	36	512
Covalently coupled aldolase to auramine O (1.6 mg/ml)	29	512
Pure glycerol + auramine O	81	512
Liver alcohol dehydrogenase (8.1×10^{-6} M) + auramine O	320	518

DISCUSSION

The experiments presented in this study clearly show that colchicine *can* fluoresce in the absence of tubulin and that such fluorescence can be promoted by immobilization either by viscous media or by binding to macromolecules, either specifically or covalently. Moreover, it has been shown that colchicine fluoresces in glasses of ether, isopentane, ethanol (5:5:2) at 77 K and even in ice in a temperature-dependent manner (Letterier and Rieger, 1975). Covalent attachment of colchicine to three different proteins led, in each case, to the promotion of fluorescence—an effect that was not substantially influenced by the size of the protein molecule. The quantum efficiency of these adducts was less than that attainable from binding (noncovalently) to the tubulin site. Since we (Cortese *et al.*, 1977) and others (Wilson, 1970; Detrich *et al.*, 1982) have shown that there are at least two binding domains in the site, one for the tropolone moiety (C ring), the other for the trimethoxybenzene moiety (A ring), it seems probable that such two-point attachment provides greater immobilization and hence greater quantum yields. We are not yet in a position to decide whether mobile solvent dipoles or intramolecular rotations are more important in the "quenching" of colchicine fluorescence. Since the quantum yield of the tubulin-colchicine complex is only 0.03 (Bhattacharyya and Wolff, 1974), various other quenching mechanisms may also occur.

A number of examples exist in the literature that suggest that immobilization-induced enhancement of fluorescence resulting from protein binding may be more prevalent than generally imagined. Thus, reduced flavines, which are often thought not to fluoresce, show good fluorescence upon formation of a covalent adduct with protein (or by increase of viscosity in glassy solvents) (Ghisla *et al.*, 1974). The nonplanarity of the molecule along the N_5-N_{10} axis and the vibrational inversions about this axis (Tauscher *et al.*, 1973) are thought to contribute substantially to the dissipation of energy from the unliganded flavine. Binding to protein promotes fluorescence because of a "freezing of the vibrational inversion processes ... at least in those flavoproteins which show a fluorescence in the reduced state" (Ghisla *et al.*, 1974).

The viscosity- or binding-dependent fluorescence enhancement exhibited by auramine O (Conrad *et al.*, 1970) mentioned and confirmed above has been interpreted (Oster and Nishijima, 1956) as resulting from a restriction of internal rotation of the aromatic groups with respect to one another resulting from highly viscous media or the binding to the surface of macromolecules. It has been pointed out that this quenching of auramine O fluorescence via internal rotational diffusion processes occurs only if the movement of the groups of the dye relative to each other exceeds 2° during the lifetime of the excited state (Oster and Nishijima, 1956). The authors state: "that any chemical linkages connecting these groups which prevent rotation will not allow quenching of this type to take place...." We think that this description equally applies to what happens when colchicine is bound to tubulin or is linked nonspecifically to protein and it applies to imerubrine. It is of interest that the related, and still flexible, triphenylmethane dyes do not fluoresce in water or organic solvents but do fluoresce in glycerol or when bound to methacrylate polymers, again presumably due to diminished internal conversion reactions as internal rotation becomes restricted (Oster and Oster, 1961). The small viscosity effect on the fluorescence of the rigid molecule anthracene, compared to the large viscosity effect on the fluorescence of di-9-anthryl ethane (Bowen and Seaman, 1961) constitutes another case of immobilization of a flexible molecule. Finally, it has been shown that rigidization of phosphatidylcholine micelles by cholesterol increases the quantum yield of retinol fluorescence (without a significant change in the polarity of the environment). A more rigid polarity probe was unaffected by cholesterol (Radda, 1971). From the experiments presented in this study, it appears justified to add colchicine to this list of flexible or partially flexible chromophores.

It seems probable that at least a part of the immobilization-dependent fluorescence enhancement of colchicine may be due to effects on the flexibility of the tropolone ring and/or to internal rotation of the tropolone (C) ring with respect to the trimethoxybenzene moiety (A ring). Although crystallographic findings suggested that colchicine was a quite rigid molecule (Lessinger & Margulis, 1978), the tropolone moiety is *not* planar but is in a shallow boat conformation and the diaryl torsion angle is 51° or 53°. Detrich *et al.* (1981) have proposed the existence of boat-boat isomerism as well as rotation about the diaryl bond to 19° and suggested that such conformational changes occur in the drug upon binding. Conceivably then, internal conversion in the unliganded drug results from rapid isomerism between these conformers and binding to tubulin may restrict such motion. The intense fluorescence of imerubrine, a rigid analogue of colchicine, in the absence of immobilization by viscosity or binding is consistent with such an interpretation as is the lack of effect of viscosity on its fluorescence (Fig. 4). On the other hand, a part of the fluorescence promoted from colchicine upon binding to tubulin may derive from stabilization of one conformation in the tubulin-colchicine complex. Extrapolation of the $1/F$ *versus* T/η curve of Fig. 4 to infinite viscosity yields a quantum efficiency for colchicine that is ~57% of that attainable by binding to tubulin. Thus, more than half of the fluorescence of the tubulin-colchicine complex can, in principle, be ascribed to immobilization. This may be the maximum enhancement attainable by immobilization since the purified colchicine-tubulin complex shows no additional fluorescence in glycerol.[3] Whether the enhancing effect of a low dielectric environment and of any specific binding of a more fluorescent conformer that may occur can account for the remainder of the fluorescence of the complex remains to be determined.

It is commonplace in the interpretation of protein binding-induced enhancement of fluorescence of small ligands to ascribe these effects to the hydrophobic nature of the binding site because similar effects can be seen in nonpolar solvents.

[3] B. Bhattacharyya and J. Wolff, unpublished observations.

As the present results make clear, however, similar enhancement can be achieved by the immobilization of the ligand in the binding site. Thus, it would seem prudent to consider this alternative mechanism for abating the nonradiative dissipation of excited state energy before attributing the fluorescence enhancement to the hydrophobic environment in the binding site especially in the case of flexible molecules. The relative contribution of these two factors is readily estimated by the use of suitable solvents.

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