Interaction of a fluorescent analog of *N*-deacetyl-*N*-methyl-colchicine (colcemid) with liver alcohol dehydrogenase

Suparna SENGUPTA[1], Pradip K. MAHAPATRA[1], Gopal CHAKRABARTI[1], Siddhartha ROY[2] and Bhabatarak BHATTACHARYYA[1]

[1] Department of Biochemistry, Bose Institute, Calcutta, India
[2] Department of Biophysics, Bose Institute, Calcutta, India

The evidence for specific binding of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)−colcemid (NBD-colcemid), a fluorescent analog of colcemid (*N*-deacetyl-*N*-methyl-colchicine), to liver alcohol dehydrogenase is presented. Alcohol dehydrogenase bound NBD-colcemid in a time-dependent manner, enhanced the fluorescence intensity, and caused a large blue shift of the emission maximum of the free drug. The specificity of binding was determined for both the colchicine nucleus and the NBD moiety. The binding was not affected by the presence of alcohol or NAD in the reaction mixture. Preincubation of horse liver alcohol dehydrogenase with colcemid inhibited the binding to a considerable extent. NBD-colcemid inhibited the enzymic activity of alcohol dehydrogenase in a mixed-type noncompetitive mode with a K_i value of 32 μM, whereas colcemid showed noncompetitive inhibition with a K_i of 100 μM. The association rate constant of NBD-colcemid binding with liver alcohol dehydrogenase was 587 M^{-1} s^{-1} at 25°C. The stoichiometry and dissociation constant of the binding reaction were 0.62/dimer and 12 μM, respectively. Donor quenching experiments showed that both tryptophans of alcohol dehydrogenase transferred energy to the bound NBD-colcemid. Thus, this study reports the binding of a colchicine analog to a protein other than tubulin with high affinity. It is concluded that NBD-colcemid binding to dehydrogenases is a general phenomenon, but the common structural element(s) that is responsible for the binding activity, and which exists among tubulin and dehydrogenases, has yet to be determined.

Keywords: alcohol dehydrogenase; *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)−colcemid; fluorescence; enzymic inhibition; antimitotic drugs.

Colcemid (*N*-deacetyl-*N*-methyl-colchicine), a structural analog of colchicine, lacks the carbonyl group at the C7 positions of its B-ring. This causes dramatic differences in the interaction of colcemid with tubulin when compared to colchicine. For colchicine, the tubulin interaction is slow, has high activation energy, and is essentially irreversible, whereas for colcemid, the tubulin interaction is relatively fast and reversible, and has similar activation energy [1−3]. Nevertheless, colcemid also interferes with microtubule-dependent cell functions and possesses therapeutic properties like colchicine [4, 5].

We are interested in studying the role of the B-rings of colchicine and colcemid in their binding with tubulin. Unfortunately, the colcemid-tubulin complex is non-fluorescent unlike the colchicine-tubulin complex [6]. A fluorescent-labeled colcemid, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)−colcemid has been synthesized by Hiratsuka and Kato [7], which has a favorably high quantum yield in environments of low polarity and a very low quantum yield in water [7]. These authors reported that NBD-colcemid reversibly disrupted the metaphase spindles of sea urchin eggs [7]. Their results indicate that the NBD-colcemid retains the biological activity of unlabeled col-

cemid, fluorescing strongly when bound to tubulin with excitation and emission maxima at 465 nm and 530 nm, respectively. Assembled microtubules were directly visualized after mixing with NBD-colcemid using a fluorescence microscope. It was reported from this laboratory that NBD-colcemid, like colcemid, binds tubulin at the colchicine site and inhibits tubulin self-assembly [8]. To our surprise, it was observed that NBD-colcemid binds liver alcohol dehydrogenase with spectroscopic features similar to that of the tubulin-NBD-colcemid complex. As colchicine or its analogs are not known to bind to any other protein with high affinity, it would be of interest to investigate the nature of binding of a colchicine analog with liver alcohol dehydrogenase. The results of such a study are presented in this paper.

MATERIALS AND METHODS

Lyophilised horse liver alcohol dehydrogenase (LADH), porcine heart isocitrate dehydrogenase, porcine heart malate dehydrogenase, bovine liver glutamate dehydrogenase, yeast glyceraldehyde-3-phosphate dehydrogenase, NAD, colchicine, and colcemid were from Sigma. 4-(*N*-Iodo-acetoxyethyl-*N*-methyl)-amino-7-nitrobenz-2-oxa-1,3-diazole and NBD-colcemid were purchased from Molecular Probes. All other chemicals used were reagent grade and succinimide was recrystallized from water before use.

The dehydrogenase solutions were dialyzed for 24 h at 4°C against 0.1 M potassium phosphate, pH 7.5, and centrifuged at 5854 *g* for 15 min at 4°C to remove any precipitable material.

The concentration of LADH was calculated using a molar absorption coefficient of $\varepsilon_{280} = 3.53 \times 10^4$ M^{-1} cm^{-1} [9].

Enzyme assay and inhibition study. Enzymic activities were determined spectrophotometrically [10] in a Beckman DU-70 spectrophotometer at 25°C by measuring absorbance at 340 nm. The activities were determined in 0.1 M glycine/NaOH, pH 9.5. Different concentrations of NAD and 6.5% ethanol were added to the reaction mixture. The enzyme previously diluted in ice-cold buffer, was added and the kinetics were followed at 340 nm. The enzyme was diluted so that the changes in absorbance were linear. The rate of the reaction was calculated from the absorbance change and the kinetic data were plotted as reciprocal rates against reciprocal NAD concentrations according to Lineweaver and Burk [11].

In the case of the inhibition study, the required concentration of NBD-colcemid was added to the reaction mixture containing NAD and alcohol, the enzyme was added, and the absorbance was followed at 340 nm. From the Lineweaver-Burk plots, K_i values were calculated using the appropriate equations [12].

Fluorescence methods. All fluorescence experiments were carried out in a Hitachi F-3000 spectrofluorometer at 25°C. The excitation and emission wavelengths for NBD-colcemid were at 465 nm and 530 nm, respectively, with bandpasses of 5 nm each unless mentioned otherwise. For the tryptophan residues of LADH, the excitation and emission wavelengths were at 295 nm and 340 nm, respectively. The rate constant of the LADH-NBD-colcemid interaction was measured under pseudo-first-order conditions at 25°C. The binding parameters of the LADH-NBD-colcemid interaction were determined according to Klotz [13] and Scatchard [14]. CD spectra were obtained at 20°C in a Jasco-600 spectropolarimeter with 1 cm pathlength.

Donor quenching of tryptophan-NBD-colcemid energy transfer. The donor quenching experiment was performed at 1 µM LADH and 5 µM NBD-colcemid according to the method of Bhattacharyya et al. [15]. The energy-transfer efficiency in the presence of the quencher was measured according to the equation

$$\frac{F_{D+A}}{F_A^\circ} = 1 + \frac{\varepsilon_D C_D}{\varepsilon_A C_A} E,$$

where F_{D+A} is the fluorescence of the donor-acceptor complex in presence of the quencher and F_A° is the fluorescence of the acceptor only, which remains unaltered in the presence or absence of quencher. ε_D and ε_A are the absorption coefficients of the donor and the acceptor, respectively, at the wavelength where the fluorescence of the donor is measured. C_D and C_A are the concentrations of the donor and acceptor in the complex and E is the energy-transfer efficiency. An equation can be constructed combining the energy-transfer efficiencies in the presence and absence of quencher:

$$\frac{F^\circ}{F} = \frac{F_{D+A}^\circ - F_A^\circ}{F_{D+A} - F_A^\circ} = \frac{E^\circ}{E} = 1 + \tau_{D,A} k_q [Q],$$

where $\tau_{D,A}$ is the lifetime of the donor in the presence of acceptor but in the absence of quencher. This equation can be rearranged in the form of a Lehrer equation [16]:

$$F^\circ/(F^\circ - F) = 1/f_a + 1/(f_a k_{sv}[Q]).$$

A Lehrer-type plot of $F^\circ/(F^\circ - F)$ versus $1/Q$ where F° is the fluorescence of the LADH-NBD-colcemid complex in the absence of quencher and F is the fluorescence of the complex in the presence of the quencher, was constructed to determine the proportion of energy transfer from quenchable and non-quenchable donors. The emission and excitation wavelengths were 540 nm and 295 nm, respectively. The emission wavelength for
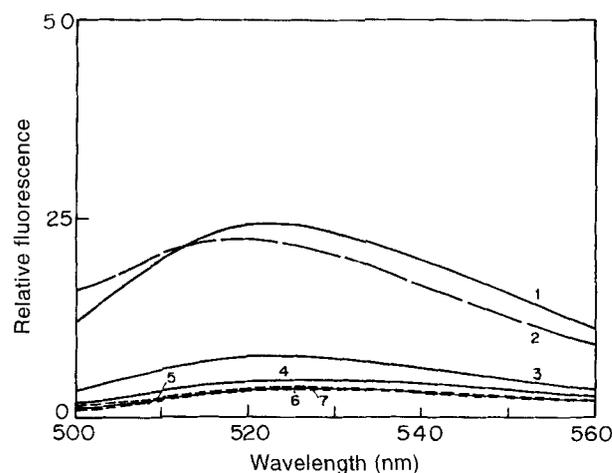

Fig. 1. **The fluorescence emission spectra of different proteins with NBD-colcemid.** 5 µM NBD-colcemid was mixed with 5 µM of the followng proteins: (1) tubulin; (2) LADH; (3) hexokinase; (4) BSA; (5) papain; (6) lysozyme (all in 10 mM potassium phosphate, pH 6.95, containing 0.5 mM EGTA and 0.25 mM MgCl$_2$ at 25°C). The fluorescence spectra were recorded after 5 min incubation at 25°C. The excitation wavelength was at 465 nm in all cases and the emission spectrum of NBD-colcemid alone is shown in [8].

energy transfer measurements was at 540 nm, as NBD-colcemid has a large emission at this wavelength and no significant emission from direct excitation of tryptophan residues exists at 540 nm. Recrystallised succinimide had no absorbance at 295, 340 or 540 nm. The fluorescence values were corrected for the inner-filter effect where necessary. The R_o value was calculated according to the equation.

$$R_o = 8.79 \times 10^{-5} (J\chi^2 n^{-4} \phi_D)^{1/6} A,$$

where J is the overlap integral, χ^2 is the orientation factor, n is the refractive index of the medium between donor and acceptor, and ϕ_D is the quantum yield of the donor in the absence of the acceptor. The distances between the tryptophans of LADH and NBD-colcemid were calculated by the following equation:

$$R = R_o[(1-E)/E]^{1/6}.$$

All these calculations were performed according to Cantor and Schimmel and references therein [17].

RESULTS

Fig. 1 shows the fluorescence emission spectra of NBD-colcemid in the presence of different proteins. It is observed that, like tubulin, horse liver alcohol dehydrogenase enhanced the NBD-colcemid fluorescence intensity with a large blue shift of the emission maximum. The emission maximum of free NBD-colcemid shifted 5 nm upon binding to tubulin and the shift was even more (9 nm) upon binding to LADH. This indicated the interaction of NBD-colcemid with LADH. Pretreatment of LADH with colcemid strongly inhibited enhancement of NBD-colcemid fluorescence at colcemid concentrations of approximately 1 mM (data not shown). The fluorescence of NBD-colcemid decreased sharply when LADH was preincubated with increasing concentrations of urea indicating that the integrity of the protein tertiary structure was required for binding. The addition of LADH to alkyl NBD analogs produced no change in the NBD-fluorescence indicating that the high affinity of NBD-colcemid to LADH and the enhanced fluorescence is a combined function of both the NBD group and the colchicine nucleus. Un-
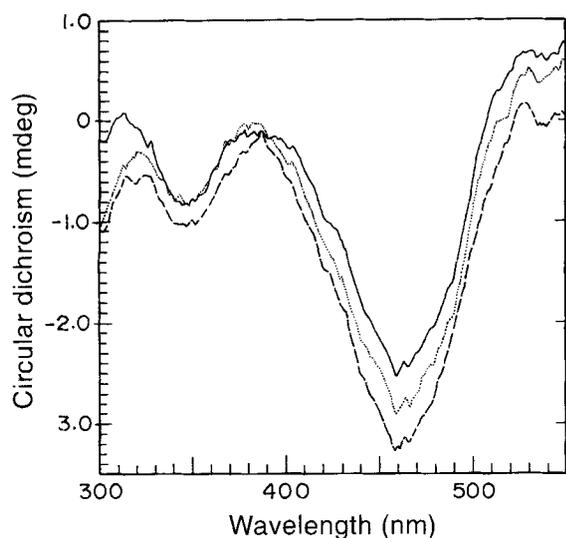
Fig. 2. Circular dichroic spectra of NBD-colcemid in the presence or absence of LADH. 7 µM NBD-colcemid was mixed with 50 µM LADH at 20°C in 0.1 M potassium phosphate, pH 7.5, containing 0.5 mM MgCl₂, and the spectra were followed with time. The spectra are as follows: NBD-colcemid (——); NBD-colcemid in the presence of LADH immediately after mixing (·····); NBD-colcemid with LADH after 20 min (– – –).

like tubulin, the incubation of colchicine or colcemid with LADH did not produce any fluorescence. Based on these results reported above, it is likely that both colchicine and colcemid bind to LADH without the promotion of fluorescence.

The enhancement of NBD fluorescence that occurs upon binding to alcohol dehydrogenase suggests the involvement of NBD group of NBD-colcemid in the interaction with the protein. The involvement of the NBD group in the binding process was further confirmed by the circular dichroic spectra of NBD-colcemid in the presence of LADH. As shown in Fig. 2, the CD band corresponding to the NBD group of NBD-colcemid (380 – 520 nm) changed significantly upon the addition of LADH and the ellipticity further changed with time.

Many colchicine analogs are well known pharmacological agents that are believed to act primarily through their effects on tubulin. If colchicine analogs can interact with other cellular targets, new mechanisms of action producing desirable pharmacological or undesirable side effects may be revealed. To test whether this binding may have any biological significance, we tested the effect of NBD-colcemid and colcemid on the enzyme activity of LADH. The rates were measured with different NAD concentrations using ethanol as a substrate in the presence of either NBD-colcemid or colcemid. The Lineweaver-Burk plots suggested that NBD-colcemid is a mixed-type noncompetitive inhibitor with K_i of approximately 32 µM (Fig. 3A), whereas colcemid showed noncompetitive inhibition with K_i of 100 µM (Fig. 3B). The mode of inhibition was also consistent with the observation that the presence of a saturating amount of NAD (1 mM) or ethanol (0.2 M) had no effect on NBD-colcemid binding to LADH (data not shown). This indicated that the NBD–colcemid-binding site was different from the coenzyme or substrate-binding site.

The binding of NBD-colcemid with LADH quenches the tryptophan fluorescence of the enzyme. The quenching of the tryptophan fluorescence after mixing LADH with NBD-colcemid was followed as a function of time. Quenching increased with time attaining saturation at about 2 h (Fig. 4A) indicating that the bound drug was near the tryptophan(s) of LADH. The
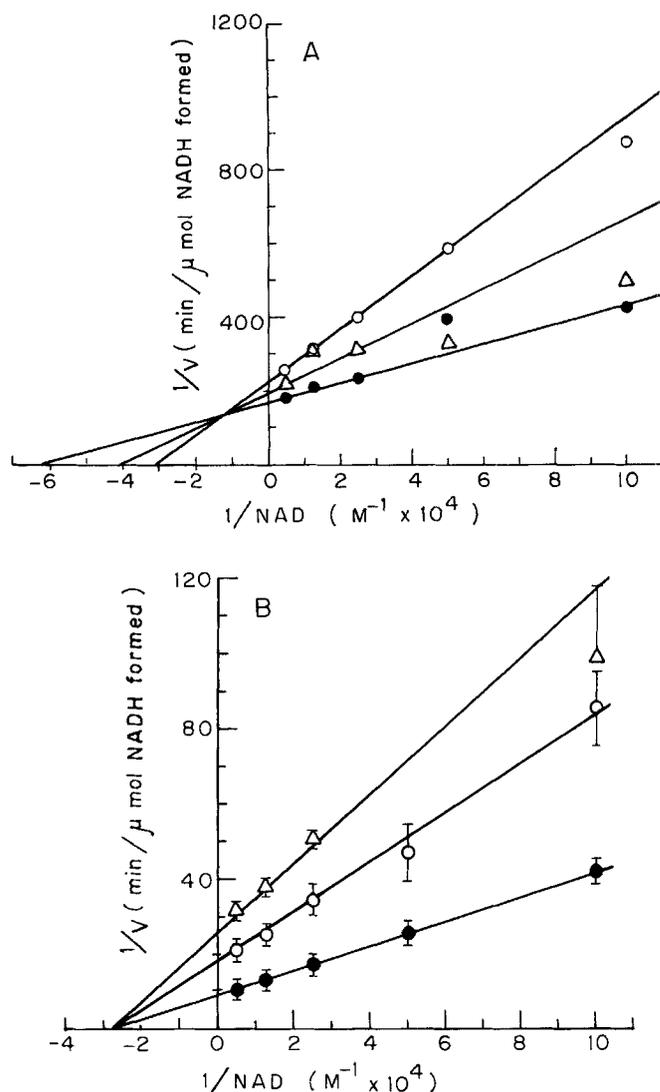

Fig. 3. Lineweaver-Burk plot of inhibition of LADH enzymic activity by NBD-colcemid (A) and colcemid (B). The formation of NADH was followed by observing the absorbance at 340 nm. The reaction mixtures contained 1.43 M ethanol, 10−200 µM NAD and the following: (A) no NBD-colcemid (●); 25 µM NBD-colcemid (△); 50 µM NBD colcemid (○); (B) no colcemid (●); 100 µM colcemid (△); 180 µM colcemid in 0.1 M glycine/NaOH, pH 9.5 (○).

time dependence of binding was also evident from the kinetic profile in Fig. 4B. The kinetics of the association reaction of NBD-colcemid with LADH were studied under pseudo-first-order conditions by monitoring the increase in ligand emission intensity as a function of time. The NBD-colcemid data were then fitted to a sum of exponentials. A single exponential function with a zero-time offset was enough to fit the data satisfactorily. A typical kinetic profile is shown in Fig. 4B. The association rate constant for the LADH and NBD-colcemid interaction was 587 M⁻¹ s⁻¹.

The fluorescence of free NBD-colcemid is enhanced 11-fold upon binding to LADH. A titration of NBD-colcemid with LADH at 25°C is shown in the inset of Fig. 5. The data were analysed by the method of Klotz, yielding a dissociation constant of 10.4 µM for the NBD-colcemid-LADH interaction. The dissociation constant and stoichiometry were also measured by Scatchard analysis (Fig. 5) and were 12.5 µM and 0.62 mol/mol LADH dimer, respectively.
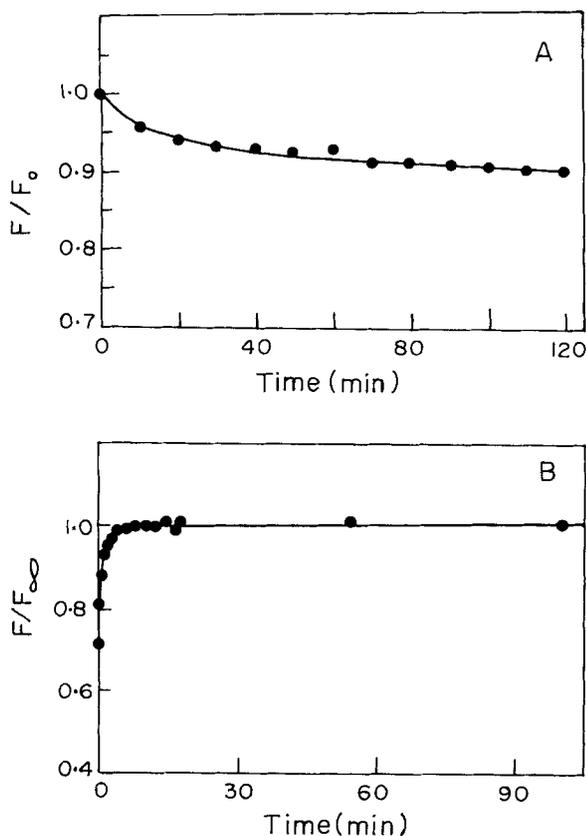
Fig. 5. Scatchard plot of LADH binding to NBD-colcemid. 2 μM LADH was titrated with 0.4−20 μM NBD-colcemid. Inset, titration of NBD-colcemid with LADH. 0.5 μM NBD colcemid was titrated with 1−10 μM LADH. The excitation and emission wavelengths were at 500 nm and 530 nm, respectively. The solution conditions are the same as in Fig. 2.

Fig. 4. Tryptophan fluorescence quenching (A) and time-dependent binding (B) of NBD-colcemid binding to tubulin. (A) 2 μM LADH was mixed with 4 μM NBD-colcemid and the tryptophan fluorescence was followed for 2 h at 336 nm exciting at 295 nm. (B) 1 μM LADH was mixed with 25 μM NBD-colcemid at 15 °C. The fluorescence intensity at 530 nm with time was recorded as many times as possible. The excitation wavelength was at 465 nm. For both A and B, the solution conditions are as in Fig. 2.

Fig. 6. Lehrer plot of succinimide quenching of LADH tryptophan fluorescence during energy transfer with NBD-colcemid. 1 μM LADH was complexed with 5 μM NBD-colcemid and the complex was titrated with succinimide (0.017−0.5 M). The emission wavelength was at 540 nm and after each addition of succinimide, the excitation intensity was recorded at 295 nm. Inset, Lehrer plot of succinimide quenching of tryptophan fluorescence of LADH. 1 μM LADH was titrated with 0.017−0.5 M succinimide. The emission wavelength was at 340 nm and after each addition of succinimide, the excitation intensity was recorded at 295 nm. The solution conditions were the same as in Fig. 2.

It is known that LADH contains two tryptophan residues: Trp15 and Trp314. Trp15 is exposed to the solvent and can be quenched by external quenchers whereas Trp314 is buried and inaccessible to quenchers [18]. To obtain information about the position of the NBD−colcemid-binding site on LADH, energy transfer studies of NBD-colcemid with the tryptophan residues of LADH were performed; the absorption spectra of NBD-colcemid overlap well with the emission spectra of tryptophan. As only one tryptophan molecule of LADH could be quenched by collisional quenchers, the donor quenching of fluorescence energy transfer may give an idea about the distance of the NBD−colcemid-binding site from the tryptophan residues. In this case, succinimide does not quench the acceptor NBD-colcemid (data not shown). The inset of Fig. 6 represents the Lehrer plot of succinimide quenching of tryptophan fluorescence of LADH in the absence of NBD-colcemid. The y-axis intercept of 1.9 indicates that approximately 50% of the tryptophan molecules could be quenched, which is expected from the previously known data, and probably indicates that Trp15 is quenched. Fig. 6 shows a Lehrer-type plot of donor quenching of fluorescence energy transfer of the LADH-NBD-colcemid complex. In this case, the intercept on the y-axis is 1.85 indicating that 45% of the energy transfer occurred from tryptophan that could not be quenched by succinimide and the remaining 55% was from the tryptophan residue that could be quenched. The estimated energy-transfer efficiency value was 1.5 indicating significant energy transfer from both the tryptophans. The energy transfer from the quench-
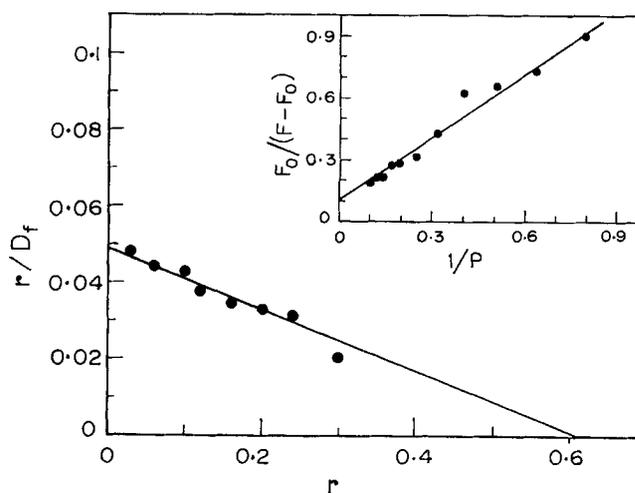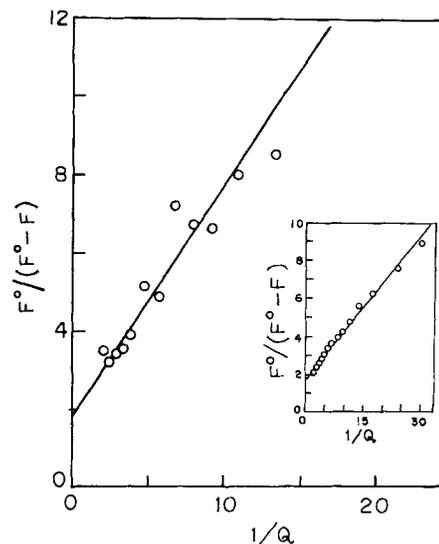
able tryptophan is therefore 1.5×0.55, i.e. 0.825. This suggests a distance of 2.3−3.0 nm between NBD-colcemid and the quenchable Trp15. Similarly, the estimated distances from Trp314 may be approximately 2.6−3.5 nm. These estimates are significantly shorter than the NADH-Trp15 distances, which are 3.6 nm and 5.2 nm, and significantly longer than the NADH-Trp314 distances, which are 1.7 nm and 2.4 nm (from X-ray crystal structure data of the NADH-dimethyl sulphoxide-LADH ternary complex; [14]) supporting the idea of a binding site distinct from the coenzyme-binding site.

Table 1. The enhancement of NBD-colcemid fluorescence in the presence of different dehydrogenases. 5 μM NBD-colcemid was mixed with 5 μM of the different dehydrogenases in 0.1 M potassium phosphate, pH 7.5, containing 0.5 mM $MgCl_2$ at 25°C. The fluorescence was measured at 530 nm upon excitation at 465 nm after 5 min incubation at 25°C. The fluorescence increase for tubulin was considered as 100%.

Proteins	Fluorescence enhancement
	%
Tubulin	100
Isocitrate dehydrogenase	83
Alcohol dehydrogenase	80
Glyceraldehyde-3-phosphate dehydrogenase	72
Malate dehydrogenase	50
Glutamate dehydrogenase	50

Although the specificities of the interaction of colchicine and colcemid with tubulin are compared with that of the enzyme-substrate interaction, it was reported that colchicine and colcemid bind glyceraldehyde-3-phosphate dehydrogenase with K_d values of 2×10^{-5} M and 6×10^{-5} M [18], respectively. Thus, it would be interesting to see whether dehydrogenases possess the general property of binding colchicine and its analogs. Table 1 shows that the enhancements of NBD-colcemid fluorescence with different dehydrogenases decrease in the following order: porcine heart isocitrate dehydrogenase ≈ LADH > yeast glyceraldehyde-3-phosphate dehydrogenase > porcine heart malate dehydrogenase = bovine liver glutamate dehydrogenase.

DISCUSSION

The pharmacological importance of colchicine is mainly due to its unique property of arresting eukaryotic cell division. Colchicine and its analogs exert this unique effect upon binding to the major microtubular protein, tubulin. Besides cell division, there are other cellular functions such as secretion of hormones, axoplasmic transport, maintainance of cell shape, and cellular motility that are affected by colchicine at very low concentrations. Moreover, it has not been proved unequivocally that all these effects of colchicine are exerted through binding to tubulin. There is a report where colchicine and colcemid are shown to bind to yeast glyceraldehyde-3-phosphate dehydrogenase [19].

In the present study, we show that a biologically active structural analog of colchicine, NBD-colcemid, binds to horse liver alcohol dehydrogenase with high affinity. The interaction of LADH with NBD-colcemid enhances the intrinsic fluorescence of the drug and has been used for the determination of binding parameters. The stoichiometry of 0.62 for the binding of NBD-colcemid to LADH might indicate that one molecule of the drug binds/dimer LADH. Unlike tubulin, the incubation of colchicine with LADH does not show any drug fluorescence. However, both colchicine and colcemid bind LADH and inhibit enzymic activity. The preincubation of LADH with colcemid decreases the extent of enhanced fluorescence. It is possible that this in-

teraction does not lead to a fluorescent complex like that observed in the case of the interaction of colchicine with its antibody [20]. The K_i values for colcemid and NBD-colcemid are 100 μM and 32 μM, respectively, indicating that the substitution of the NBD group in the side chain of colcemid enhances its affinity for the enzyme about three times. The NBD group has considerable hydrophobic character and may provide excess binding energy through hydrophobic interactions, thus strengthening the binding. The covalently linked NBD group may also result in energetically favorable entropy contributions (like a chelate effect) towards binding, compared to the colchicine nucleus alone. NBD-colcemid also participates in energy transfer with the two tryptophans of LADH.

Finally, the binding of NBD-colcemid with several dehydrogenases appears to be very interesting. A common structural element among dehydrogenases is the Rossmann fold, which binds NAD [21]. However, NAD does not compete with the NBD-colcemid binding indicating that the drug-binding site is different from the coenzyme-binding site. The common structural element(s) that exists among tubulin and dehydrogenases and which accounts for their colchicine-binding and analog-binding activities remains to be established.

REFERENCES

1. Banerjee, A. C. & Bhattacharyya, B. (1979) FEBS Lett. 99, 333–336.
2. Ray, K., Bhattacharyya, B. & Biswas, B. B. (1984) Eur. J. Biochem. 142, 577–581.
3. Pyles, E. A. & Bane Hastie, S. (1993) Biochemistry 32, 2329–2336.
4. Inoue, S. & Sato, H. (1967) J. Gen. Physiol. 50, 259–292.
5. Zweig, M. H. & Chignell, C. F. (1973) Biochem. Pharmacol. 22, 214.
6. Ray, K., Bhattacharyya, B. & Biswas, B. (1981) J. Biol. Chem. 256, 6241–6244.
7. Hiratsuka, T. & Kata, T. (1987) J. Biol. Chem. 262, 6318–6322.
8. Sengupta, S., Puri, K. D., Surolia, A., Roy, S. & Bhattacharyya, B. (1993) Eur. J. Biochem. 212, 387–393.
9. Strambini, G. B. & Gonnelli, M. (1990) Biochemistry 29, 196–203.
10. Algar, E. M., Seeley, T. L. & Holmes, R. S. (1983) Eur. J. Biochem. 137, 139–147.
11. Lineweaver, H. & Burk, D. (1934) J. Am. Chem. Soc. 56, 658.
12. Segel, I. H. (1975) Enzyme kinetics, John Wiley & Sons, Inc., USA.
13. Klotz, I. M. (1986) Introduction to biomolecular energetics, Academic Press Inc., Orlando, FL, USA.
14. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. USA 51, 660–672.
15. Bhattacharyya, A., Bhattacharyya, B. & Roy, S. (1993) Eur. J. Biochem. 216, 757–761.
16. Lehrer, S. S. (1971) Biochemistry 10, 3254–3263.
17. Cantor, C. R. & Schimmel, P. R. (1980) Biophysical chemistry, Part 2, W. H. Freeman and Co., San Francisco.
18. Abdallah, M. A., Biellmann, J. F., Wiget, P., Joppich-Kuher, R. & Luisi, P. L. (1978) Eur. J. Biochem. 89, 397–405.
19. Brownlee, A. G. & Polya, G. M. (1980) Eur. J. Biochem. 109, 51–59.
20. Wolff, J., Capraro, H. G. & Cook, G. H. (1980) J. Biol. Chem. 225, 7144–7148.
21. Rao, S. T. & Rossmann, M. G. (1973) J. Mol. Biol. 76, 235–250.