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Anion-Induced Increases in the Rate of Colchicine Binding to Tubulin†

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ABSTRACT: The rate of binding of colchicine to tubulin is enhanced by certain anions. Among the inorganic anions tested, only sulfate was effective. The organic anions include mostly dicarboxylic acids, among which tartrate was the most effective. This effect occurs only at low concentrations of colchicine ($<0.6 \times 10^{-5}$ M). The rate increase for sulfate and L-(+)-tartrate is ca. 2.5-fold at 1.0 mM and plateaus at a limiting value of ca. 4-fold at 100 mM. The overall dissociation rate of the colchicine from the complex, which includes both the true rate of dissociation and the rate of irreversible denaturation of tubulin, is not influenced by 1.0 mM tartrate. The affinity constants for colchicine determined from the rate constants are 8.7×10^6 and 2.1×10^7 M^{-1} in the absence and the presence of 1.0 mM L-(+)-tartrate. The limiting value is 3.2×10^7 M^{-1}. The affinity constant calculated from steady-state measurements is 3.2×10^6 M^{-1} with or without anions. The binding of other ligands like podophyllotoxin, vinblastine, and 1-anilino-8-naphthalenesulfonate to tubulin is not affected by tartrate. No major conformational changes resulting from anion treatment could be detected by circular dichroism or intrinsic fluorescence. However, the ability of tubulin to polymerize is inhibited by L-(+)-tartrate at concentrations that increase the rate of colchicine binding. We conclude that anions must have a local effect at or near the binding site which enhances the binding rate of colchicine and which may be related to inhibition of polymerization.

Tubulin, the heterodimeric subunit of microtubules, is characterized by its ability to bind colchicine, podophyllotoxin, vinblastine, GTP, and anilinonaphthalenesulfonic acid (Wilson, 1970; Owellen et al., 1972; Olmsted and Borisy, 1973; Jacobs et al., 1974; Bhattacharyya and Wolff, 1975a). With the exception of colchicine, these ligands appear to bind rapidly to tubulin (Taylor, 1965; Wilson, 1970; Owellen et al., 1972). Colchicine binds slowly with a high temperature coefficient and shows equilibration times of 30–90 min, depending on the colchicine concentration. The reason for this slow binding is obscure, although it has been suggested by Ventilla et al. (1972) that colchicine binding is accompanied by a slow conformational change in the tubulin accompanied by a loss in α-helix content.

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TABLE I: Effects of Inorganic Anions on the Rate of Colchicine Binding.

Salt	Concn (mM)	r_A/r_0[a]
$(NH_4)_2SO_4$	1.0	2.7
Na_2SO_4	1.0	2.5
$MgSO_4$	1.0	2.5
NaCl	1.0, 10, 100	1, 1, 1
NH_4Cl	1.0, 10, 100	1, 1, 1

[a] r_A/r_0 is the ratio of fluorescence intensity of colchicine–tubulin complex incubated for 5 min at 37 °C in presence (r_A) or absence (r_0) of anion. The samples contained 1.7 μM tubulin solution and 0.5 μM colchicine solution in PMG buffer containing 0.25 M sucrose. The excitation wavelength was 350 nm and the emission wavelength was 430 nm in all cases.

TABLE II: Effects of Organic Anions on the Rate of Colchicine Binding.[a]

Anion	Acid Dissoc Constants (pK)	Concn for Twofold Increase (mM)
D-(−)-Tartrate	(2.9, 4.2)	0.8
L-(+)-Tartrate	(2.9, 4.2)	0.8
Mesotartrate	(2.9, 4.2)	0.8
Succinate	(4.2, 5.7)	1.2
Citrate	(3.1, 4.8, 6.4)	1.5
Oxalate	(1.3, 4.3)	1.5
Malonate	(2.9, 5.7)	1.5
Maleate	(2.0, 6.3)	1.8
Fumarate	(3.0, 4.5)	2.0
Phthalate	(3.0, 5.4)	2.5
Isophthalate	(3.6, 4.6)	2.5
Terephthalate	(3.5, 4.5)	2.5
Acetate	(4.7)	3.0

[a] The concentration of anion needed in order to enhance the fluorescence intensity of colchicine–tubulin complex at 430 nm to twofold over the control. The incubation was carried out for 5 min at 37 °C. The samples contained 1.5 μM tubulin solution and 0.5 μM colchicine in PMG buffer containing 0.25 M sucrose. The numbers in the parentheses indicate pK_1 and pK_2, respectively (Kortüm et al., 1961).

Assays of colchicine binding during purification of tubulin revealed an apparent yield of binding activity exceeding 100% which appeared to be associated with presence of ammonium sulfate. We have therefore investigated the possibility that the ionic composition of the media might influence the binding kinetics of colchicine and shed light on the unusually long equilibration time required for binding. Since the colchicine-binding site decays fairly rapidly, it was important to attempt to accelerate the binding in order to obtain more reliable equilibrium constants. It was found that certain anions accelerate colchicine binding to tubulin.

Materials and Methods

Materials. Chemicals were obtained as follows: GTP (grade II-S), tartaric acids and maleic acid from Sigma; sodium fumarate from Boehringer; sodium selenate and tellurate from BDH, England; all other chemicals were of reagent grade. Podophyllotoxin was generously supplied by Dr. W. J. Gensler of Boston University. Tritiated colchicine (ring C, [*methoxy*-^{3}H]), a product of New England Nuclear Corp., had a specific activity of 2.5 Ci/mmol. [^{3}H]Vinblastine was prepared as previously described and had a specific activity ~19 Ci/mmol and ~95% radiochemical purity (Bhattacharyya and Wolff, 1975b). [^{3}H]Podophyllotoxin was the generous gift of Dr. Martin Flavin; it had a specific activity of 3.4 Ci/mmol (Flavin and Slaughter, 1974).

Methods. Tubulin was prepared from rat brains using the procedure of Weisenberg et al. (1968) except that DEAE[1]-cellulose was used. The protein yielded a single band in overloaded gels by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Weber and Osborn, 1969). For polymerization experiments, the protein was purified from rat brain extracts by three cycles of polymerization and depolymerization, according to the method of Shelanski et al. (1973). The kinetics of tubule assembly have been studied by turbidimetric measurements at 350 nm as described by Gaskin et al. (1974).

Colchicine binding was determined by the DEAE filter paper method (Williams and Wolff, 1972) or by fluorescence (Bhattacharyya and Wolff, 1974).

The binding of [^{3}H]podophyllotoxin to tubulin was assayed by the DEAE-cellulose filter disc method as used in case of colchicine binding, except that three filter papers are used instead of two. To 1 ml of cold PMG buffer, containing 1×10^{-5} M podophyllotoxin, 100 μl of the sample was added and the mixture was filtered with mild suction over a period of 2–3 min. The filters were then washed three times with cold PMG buffer and were counted. Protein was determined by the method of Lowry et al. (1951). Circular dichroic spectra were measured on a Cary 60 spectropolarimeter.

Results and Discussion

The rate of colchicine binding was linear for at least the first 8 min of the reaction when using 1.0 μM colchicine. This was true whether the binding was measured by fluorescence or by the use of labeled colchicine. A 5-min assay period was used routinely. For convenience, most of the time the rate was measured by the fluorescence method. The increased binding was only observed when the anion was sulfate (Table I). Cations such as NH_4^+, Na^+, or Mg^{2+} did not affect binding at these concentrations. Moreover neither 100 mM NaCl nor $(NH_4)Cl$ affected binding. This makes it unlikely that the enhanced binding is due simply to ionic strength. Other inorganic anions examined and found to have <20% or no effect on the rate of colchicine binding at 1 mM concentration included bromide, iodide, thiocyanate, nitrate, selenate, tellurate, phosphate, pyrophosphate, and arsenate. Among these anions selenate and phosphate showed about 40% increase in the binding rate at a concentration of 15 mM.

Table II lists the organic anions in the order of decreasing effectiveness. The list comprises primarily dicarboxylic acids with tartrate the most active. No appreciable difference was obtained between the D-(−) and L-(+) forms of tartrate. The maximal response to L-(+)-tartrate or sulfate that could be obtained below concentrations where ionic strength effects might be expected, was an approximately fourfold increase in the rate of colchicine binding (Figure 1). The ratio of the rates with and without anion was linear with the logarithm of the anion concentration up to approximately 10 mM and then plateaued near 100 mM anion.

Charge separation appeared to make little difference in enhancing the rate of colchicine binding since oxalate, malonate, and succinate had identical activities and maleate and

[1] Abbreviations used: DEAE, diethylaminoethyl; PMG, 10 mM sodium phosphate (pH 6.5), 10 mM $MgCl_2$, and 0.1 mM GTP solution.

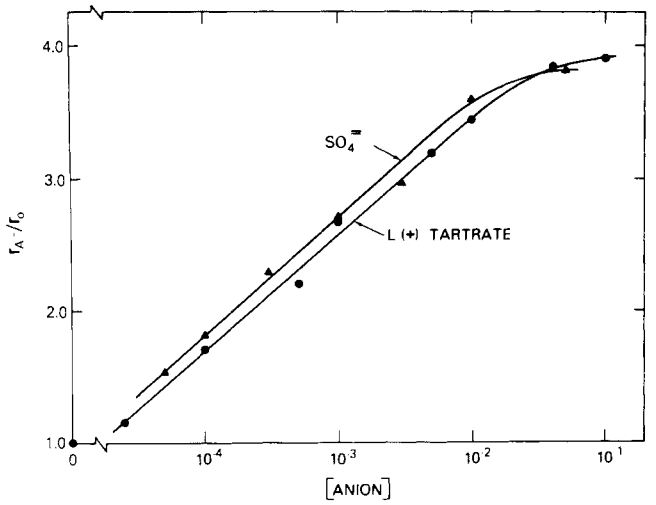

FIGURE 1: Concentration dependency of anion-induced rate enhancement of colchicine binding to tubulin. The protein concentration was 1.8 μM and [^{3}H]colchicine was 0.5 μM in all experiments. Assay was carried out after 5-min incubation at 37 °C by a DEAE filter disc method (Williams and Wolff, 1972).

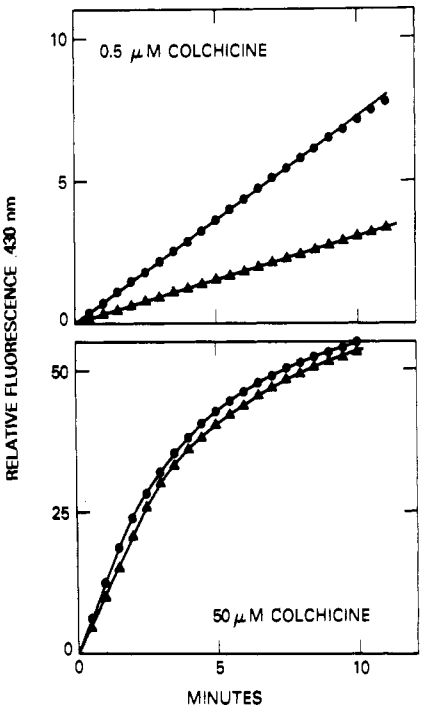

FIGURE 2: Time course of colchicine binding to tubulin with and without L-(+)-tartrate in different colchicine concentrations. In both pictures samples with (●) and without (▲) 1.0 mM L-(+)-tartrate were incubated with 1.95×10^{-6} M tubulin solution at 37 °C. At the indicated time fluorescence at 430 nm was recorded. The excitation wavelength was 350 nm.

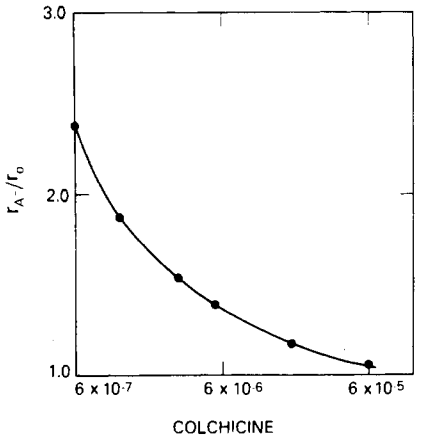

FIGURE 3: Dependency of anion-induced rate enhancement of colchicine–tubulin binding on colchicine concentration. The rate was monitored fluorometrically after 5-min incubation at 37 °C with (r_A-) and without (r_0) 1 mM L-(+)-tartrate. The tubulin was 1.95×10^{-6} M in all cases.

fumarate showed only trivial differences (Table II). Moreover, the three phthalic acid isomers increased the rate to the same extent. Benzoic acid was ineffective at 3 mM, again showing the preference for dicarboxylic acids. The effect did not correlate with the dissociation constants of the acids. The binding assay was carried out at pH 6.5; hence all carboxyl groups except for maleate and the third carboxyl of citrate were essentially fully dissociated. Moreover, no correlation exists between the stimulatory potency of these anions and their stability constants with divalent metals (Sillén and Martell, 1964).

Nature of the Effect. The enhancement in the rate of colchicine binding by anions appeared to be due to an increase in the *rate* of the reaction. Since the rate also varies with colchicine concentrations (Borisy and Taylor, 1967; Williams and Wolff, 1972), we studied the effect of tartrate on colchicine binding at different colchicine concentrations. Typical experiments for low and high colchicine concentrations are shown in Figure 2. The tartrate effect was maximal at low concentration of colchicine. At 0.5 μM colchicine, the rate at 5 min in the presence of 1.0 mM tartrate was approximately 2.7-fold higher than the binding without tartrate. On the other hand, at 5.0×10^{-5} M colchicine the tartrate effect was virtually abolished. The relation between the tartrate effect and the colchicine concentration (ratio of binding rate with tartrate to the rate of binding without tartrate) is depicted in Figure 3. As the concentration of colchicine increased, the difference in the rate of binding with and without anions disappeared and the ratio approached unity. Similar results were obtained with ammonium sulfate and the other effective anions listed in Table II.

As shown in Figure 2, there was no increase in the number of binding sites in the presence of anions. This is shown more clearly in the Scatchard plot (Figure 4) performed under steady-state conditions. Experiments carried out in the presence of 1 mM L-(+)-tartrate yielded binding data that superimpose on control data. Under these conditions there was no change in the apparent number of binding sites (~0.85 mol of colchicine per mol of tubulin) nor in the apparent affinity constant (3.2×10^6 M^{-1}).

Apparent Affinity Constant (K_A). Since tubulin undergoes rapid decay during prolonged incubation, the K_A for colchicine binding in the presence and absence of anions was determined by measuring independently the association and dissociation rate constants of the reactions. To determine k_1, the initial rate of association of colchicine to tubulin has been measured. The reaction between colchicine and tubulin is assumed to be bimolecular, and the rate constant (k_1) is given by the expression:

$$k_1 = (d[CT]/dt)/[C][T]$$

where $d[CT]/dt$ is the instantaneous rate of formation of the complex and [C] and [T] represent the concentrations of free colchicine and unoccupied tubulin binding sites, respectively. As shown in Figure 5, the rate of colchicine binding was linear for at least the first 5 min for the concentrations of colchicine (0.1–2.8 μM) used. The value of k_1 calculated from these data at 5 min is 0.43×10^6 M^{-1} h^{-1}. A similar set of experiments

was performed in the presence of 1.0 mM tartrate and in that case k_1 is 1.1×10^6 M^{-1} h^{-1}. When 10 mM L-(+)-tartrate was used, the value for k_1 increased to 1.4×10^6 M^{-1} h^{-1}. Because of the short duration of the incubation, the tubulin decay would not be expected to be a significant factor in these studies. Using somewhat longer incubation times, Garland and Teller (1975) obtained k_1 for porcine brain tubulin of 0.10×10^6 M^{-1} h^{-1}. Interestingly, this value could be increased by 4.3 mM $CaCl_2$ to 0.54×10^6 M^{-1} h^{-1}.

To determine the dissociation rate for the tubulin–colchicine complex, tubulin was incubated with 50 μM colchicine for 1.5 h after which the remaining free colchicine was removed by passing the sample through a Sephadex G-50 column (24 × 1.4 cm) equilibrated with PMG buffer and then eluted with the same buffer. The colchicine–tubulin complex, eluted in the void volume, was incubated at 37 °C with or without 1.0 mM tartrate and the total dissociation of the colchicine from the colchicine–tubulin complex was monitored fluorometrically. It was assumed that the rate of reassociation of unbound colchicine was negligible since the concentration of unbound colchicine and tubulin were extremely low. The apparent dissociation rate is composed of at least two components, true dissociation of the complex and decay of the colchicine binding site. The relative contribution of these two rates to the total dissociation is unknown at present. Similar experiments were carried out in the presence of 1.0 mM tartrate. The half-life for the colchicine–tubulin complex was 14 h in the absence *or presence* of 1 mM tartrate. Therefore, the presence of tartrate exerted no direct effect on the off-rate of the colchicine–tubulin complex.[2] This implies that both the true dissociation and that resulting from decay of the binding site were unaffected by tartrate, although it is not possible to rule out compensating changes in the two dissociation processes. The dissociation rate constant $k_{off} = (k_{-1} + k_{decay})$ was determined from the equation

$$0.693/t_{1/2} = k_{off}$$

where k_{off} is the rate constant for loss of colchicine from tubulin, k_{-1} is the true first-order dissociation constant, and k_{decay} describes the rate of loss of colchicine binding ability. Substituting 14 h for $t_{1/2}$ gives $k_{off} = 0.049$ h^{-1}. The apparent K_A calculated by dividing k_1 by k_{off} is 8.7×10^6 M^{-1} in the absence of anion, 2.1×10^7 M^{-1} in the presence of 1.0 mM L-(+)-tartrate, and 2.9×10^7 M^{-1} with 10 mM L-(+)-tartrate, the differences all arising from differences in k_1. These values should be compared with the steady-state value of 3.2×10^6 M^{-1} obtained at 90 min (Bhattacharyya and Wolff, 1974). The true association constant, i.e., k_1/k_{-1}, may be substantially greater if the k_{decay} constitutes a large proportion of k_{off}. Using an exchange method, Garland and Teller (1975) have determined these two rate constants independently and found 0.017 h^{-1} for k_{-1} and 0.019 h^{-1} for k_{decay}. The sum of these is $k_{off} = 0.036$ h^{-1}, a value in good agreement with that obtained in the present investigation using different methods. If k_{-1} constitutes ~½ of k_{off} in our studies as well, then the K_A values would be doubled.

Recently, an affinity constant (corrected for decay) of 4.2 × 10^7 M^{-1} for colchicine–tubulin binding has been reported by Sherline et al. (1975). In their studies, the affinity constant was calculated from the initial rate and the k_{off} was corrected for denaturation of colchicine binding sites.

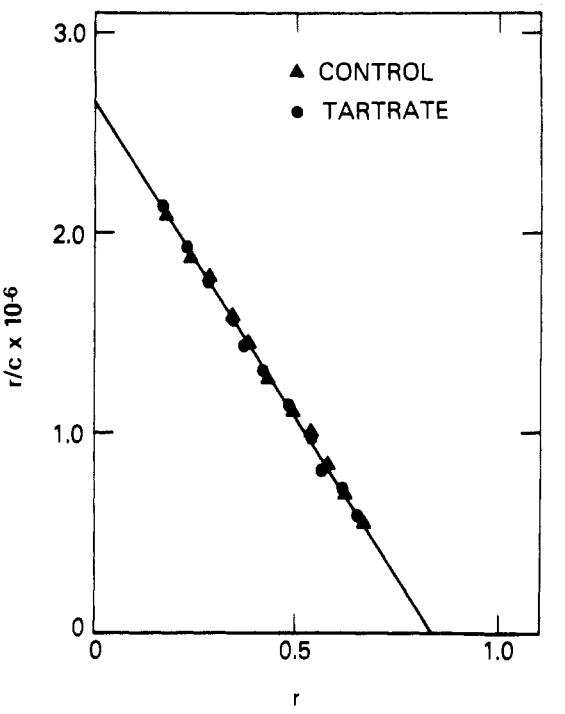

FIGURE 4: Scatchard plot of colchicine binding to tubulin. Binding was assayed by the fluorescence method in the presence (●) and the absence (▲) of 1.0 mM L-(+)-tartrate. The colchicine concentration was 1.05 μM in all cases and the protein concentration was varied from 0.2×10^{-6} to 8×10^{-6} M. Incubation was carried out for 1.5 h at 37 °C.

The discrepant results in the affinity constants determined at steady-state conditions (3×10^6 M^{-1}) and from rate constants (9×10^6 to 3×10^7 M^{-1} depending on tartrate concentrations) are not fully explained by loss of the binding sites due to denaturation as proposed by Sherline et al. (1975). In the worst case, in which tubulin is assumed to denature at the faster rate of uncomplexed tubulin ($t_{1/2}$ = 3 h), 30% of the colchicine binding sites would be lost during a 90-min assay. Yet the binding rate will have fallen off by more than threefold as judged from the differences in K_A. This difficulty is seen also in the data of Sherline et al. (1975) where sucrose did not increase the apparent K_A despite a fourfold reduction in the rate of denaturation of uncomplexed tubulin. The reason for the discrepancy in the two sets of equilibrium constants thus remains to be explained. It is possible that denaturation of the site proceeds gradually through a series of steps involving decreasing affinity for colchicine.

Conformational Changes. To see if the anion effect was due to a conformational change, we measured the circular dichroic spectra of tubulin in the absence and presence of 1.0 mM tartrate. This anion did not change the circular dichroic spectra over a range of 200–280 nm. Moreover, the tartrate failed to produce any change in the intrinsic tryptophan fluorescence either in the intensity or in the emission maximum of tubulin. Finally, the anions tested had no effect on the colchicine emission spectrum at the concentrations that elicited rate increases.

In order to determine whether the tartrate effect on binding was restricted to the domain of the colchicine site, we studied the effect of this anion on the binding of two ligands known to bind to tubulin at different sites. Anilinonaphthalenesulfonic

[2] Since tubulin is more labile in the absence of colchicine, it was possible that tartrate might protect free tubulin even though it had no effect on the colchicine–tubulin complex. To test this, tubulin (2 mg/ml) was incubated at 37 °C with 1.0 mM L-(+)-tartrate and sampled at different times for [^{3}H]colchicine binding after a 1:10 dilution. The half-life of colchicine binding activity (3.0 h) was not altered by preincubation with tartrate, nor was there any difference in the response to L-(+)-tartrate after preincubation of tubulin in its absence.

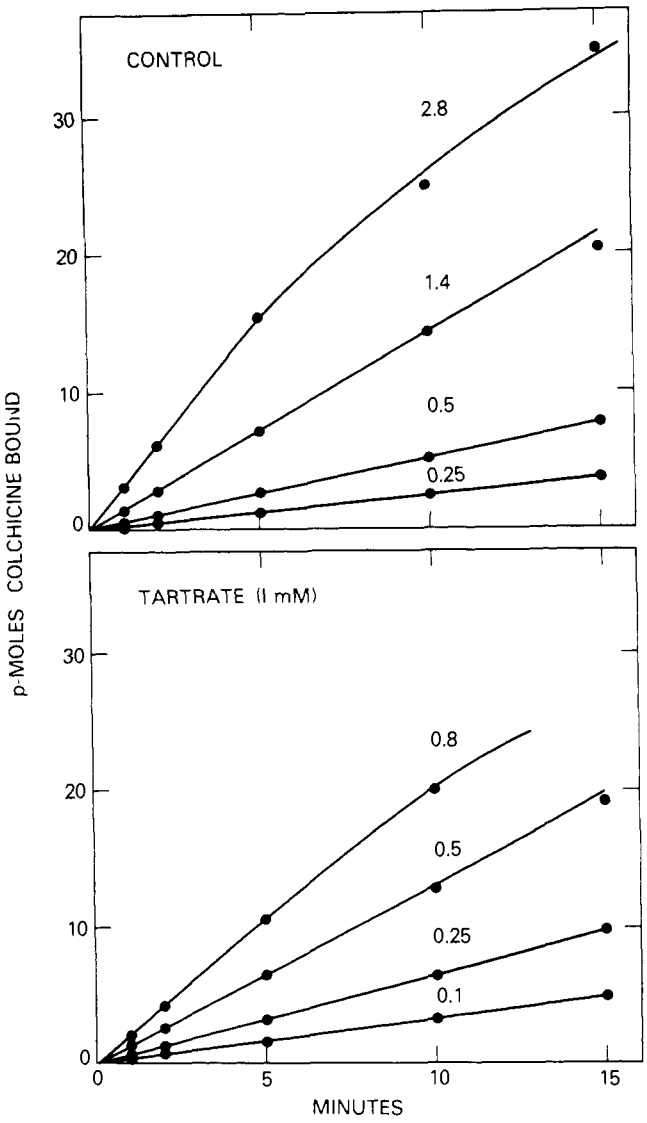

FIGURE 5: Effect of initial colchicine concentration on the time course of colchicine binding to tubulin at 37 °C. The colchicine concentration in μM is indicated to the right of the appropriate curve. The tubulin concentration was 1.6×10^{-6} M and 100 μl of the sample was used in the assay, done by the DEAE filter disc method. The binding was expressed as the amount of colchicine bound per 160 pmol of tubulin in all cases.

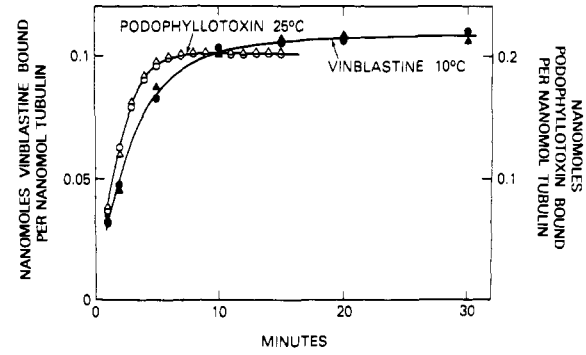

FIGURE 6: Time dependence for the binding of [^{3}H]vinblastine and [^{3}H]podophyllotoxin to tubulin. The vinblastine binding was carried out at 10 °C. A solution of tubulin (1.0×10^{-6} M) and vinblastine (0.5×10^{-6} M) was incubated at 10 °C. At the times indicated, an aliquot was removed and assayed for bound vinblastine by the DEAE filter disc method exactly as employed for the determination of bound colchicine. Podophyllotoxin binding was carried out at 25 °C. A solution of tubulin (1.0×10^{-6} M) and podophyllotoxin (0.5×10^{-6} M) was incubated at 25 °C. At the indicated time, an aliquot was removed and bound podophyllotoxin was determined by DEAE filter disc method mentioned in Methods section. In both the ligands, the binding was expressed as the amount of ligand bound per nmol of tubulin.

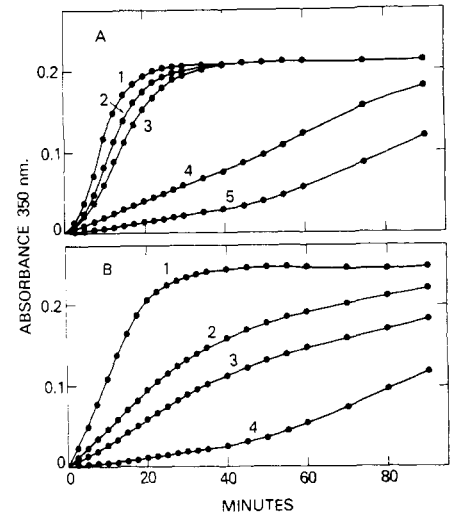

FIGURE 7: Effect of L-(+)-tartrate on the assembly of tubulin at 37 °C. Absorbance was measured in a thermostated controlled chamber of the spectrophotometer at the indicated times. (A) Tubulin concentration was 1.5 mg/ml in all cases: (1) protein only; (2) protein with 1.0 mM L-(+)-tartrate in 4 M glycerol; (3) protein with 10 mM L-(+)-tartrate in 4 M glycerol; (4) protein with 1 mM L-(+)-tartrate; and (5) protein with 10 mM L-(+)-tartrate. (B) The tartrate concentration was 10 mM in all the cases. The protein concentration was 3.5 mg/ml in 1; 3.0 mg/ml in 2; 2.6 mg/ml in 3; and 1.5 mg/ml in 4.

acid (Bhattacharyya and Wolff, 1975a) binding was studied by fluorescence emission at 465 nm. The fluorescence was not affected by 3.0 mM L-(+)-tartrate. Equilibrium was attained so rapidly that we were not able to measure rate effects even when the temperature was reduced to 10 °C. On the other hand, [^{3}H]vinblastine binding was sufficiently slow at 10 °C that rate measurements were possible. However, 3.0 mM L-(+)-tartrate had no effect on the rate of vinblastine binding (Figure 6) nor on the total amount of this ligand bound at equilibrium, which was attained at 10 min under these conditions. It seems likely, therefore, that the tartrate effect possesses a certain specificity for colchicine binding, and that there is no major conformational change in the tubulin molecule with the treatment of 1 mM tartrate.

Podophyllotoxin is a competitive inhibitor for colchicine binding (Wilson, 1970; Bhattacharyya and Wolff, 1974). To our surprise, the binding of [^{3}H]podophyllotoxin to rat brain tubulin at 25 °C was *not* accelerated by the presence of 3.0 mM L-(+)-tartrate. Equilibrium was reached in 6 min (Figure 6) in agreement with the statement by Flavin and Slaughter (1974). This rapid binding is in marked contrast to colchicine. It is tempting to suggest that the tartrate effect is exerted primarily on the B or C ring portion of the colchicine binding site but further studies are required to substantiate this hypothesis.

The suggestion has been made that the polymerization and colchicine-binding sites are the same (Wilson and Meza, 1973). We, therefore, investigated the effect of tartrate on the rate of polymerization of tubulin isolated by the method of Shelanski et al. (1973). As shown in Figure 7, tartrate (even at low concentration) inhibited the rate of polymerization. This inhibitory effect on the rate of polymerization could be overcome in two ways: (a) by adding 4 M glycerol (Figure 7A); (b) by increasing the concentration of protein in the system (Figure

7B). Additional evidence for the similarity of these two sites stems from the facts that both podophyllotoxin and rotenone bind at the colchicine site and block polymerization (Wilson, 1970; Hoebeke and Nijen, 1975), and both processes show high positive entropies (Inoue and Sato, 1967; Bhattacharyya and Wolff, 1974). On the other hand, neither calcium nor vinblastine interacts at the colchicine binding site (Wilson et al., 1970; Wilson, 1970), and yet both depolymerize microtubules. Thus a decision regarding the identity of the polymerization and colchicine-binding sites cannot be made at present.

Since $CaCl_2$ depolymerizes microtubules, Garland and Teller (1975) ascribed the enhancement of k_1 by $CaCl_2$ to the fact that depolymerization of microtubules is rate limiting. Since we have used column-purified tubulin that does not polymerize under the conditions recommended by Shelanski et al. (1973) (unpublished observations) presumably due to the absence of the τ protein (Weingarten et al., 1975), such an explanation cannot account for our results. Moreover, when our tubulin preparation was chromatographed on a Bio-Gel A-15 column (24 × 1.4 cm) in PMG buffer, only two protein peaks were seen, 6 S and a small peak near the void volume. The proportion near the void volume amounted to 5.8% of the total protein in control samples and 5.6% in the presence of 10 mM L-(+)-tartrate. It thus seems unlikely that a major change in the state of aggregation of tubulin occurs in the presence of anions.

Our current working model for the colchicine binding site is that normally this site, or at least that portion that recognizes the tropolone moiety, is not readily accessible to colchicine. A local change, not seen at the vinblastine site, can be induced by a variety of factors that make this site more accessible. These include the anions studied in the present investigation, Ca^{2+} (Garland and Teller, 1975), temperature (Taylor, 1965) and, perhaps, colchicine itself.

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