cooperativity between sites), eq A-24 reduces to

$$I(t) = \beta e^{-k_2 t} + \gamma e^{-k_1 t} \quad \text{(A-25)}$$

and the reaction follows double-exponential kinetics with observed rate constants corresponding to the real k_{off} values, k_1 and k_2.

When the competing metal ion is Eu(III) or Yb(III), the tryptophan fluorescence is quenched as the bound metal ion dissociates and Eu(III) or Yb(III) binds. In this case, the observed signal will behave temporally according to eq A-23 and A-25. If the competing metal ion is Tb(III), however, and the reaction is monitored by the appearance of the sensitized Tb(III) signal, the intensity will follow eq A-26, where

$$I(t) = (\theta + \phi)D(t) + \theta C(t) + \phi B(t) \quad \text{(A-26)}$$

θ and ϕ represent the degree of sensitization of Tb(III) in the respective sites. Since $k_1 = k_4$ and $k_2 = k_3$, this leads to

$$I(t) = \theta + \phi - \theta e^{-k_2 t} - \phi e^{-k_1 t} \quad \text{(A-27)}$$

Registry No. Ca, 7440-70-2; Cd, 7440-43-9; Pr, 7440-10-0; Nd, 7440-00-8; Sm, 7440-19-9; Eu, 7440-53-1; Gd, 7440-54-2; Tb, 7440-27-9; Dy, 7429-91-6; Ho, 7440-60-0; Er, 7440-52-0; Yb, 7440-64-4; Lu, 7439-94-3.

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Vincristine-Induced Self-Association of Calf Brain Tubulin†

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ABSTRACT: The vincristine-induced self-association of tubulin has been examined in a sedimentation velocity study as a function of free drug concentration in PG buffer (0.01 M NaP_i and 10^{-4} M GTP, pH 7.0) at 20 °C. Analysis of the weight-average sedimentation coefficient ($\bar{s}_{20,w}$) as a function of protein concentration showed a good fit with the model of an indefinite, isodesmic self-association mechanism. Analysis of the apparent association constants in terms of the Wyman linkage relations showed a good fit to mediation of the self-association by the binding of one ligand molecule. The intrinsic association constant for dimerization of the vincristine-liganded tubulin was found to be 3.8×10^5 M^{-1}, and the intrinsic equilibrium constant for the binding of the self-association-linked vincristine molecule had a value of 3.5×10^4 M^{-1}, consistent with that measured by fluorescence in our laboratory [Prakash, V., & Timasheff, S. N. (1983) *J. Biol. Chem. 258*, 1689–1697]. Both reactions are stronger in the presence of vincristine than of vinblastine, reflecting the oxidation of a $-CH_3$ group to $-CHO$ when going from the latter drug to the former one.

Vincristine (VCR)[1] is a vinca alkaloid derived from the periwinkle plant (*Catharanthus rosea* Linn). At very small concentrations, it is known to be an effective mitosis-arresting agent. It also has important applications in the treatment of certain neoplastic diseases (Palmer et al., 1960; Cutts, 1961; Johnson et al., 1963; Wilson et al., 1976; Owellen et al., 1976; Dustin, 1978; Skovsgaard, 1978; Redmond & Tuffery, 1979; Grush & Morgan, 1979; Zeller et al., 1979; Newlands & Bagshawe, 1979; Massa et al., 1979; Morgan & Crossen, 1980; Klein et al., 1980). Early morphological studies have indicated the destruction of spindle microtubules by VCR, leading to arrested mitosis (Cutts et al., 1960; Cutts, 1961; Cardinali et al., 1963; Johnson et al., 1963; Frei et al., 1964; George et al., 1965). Vincristine induces the aggregation of tubulin molecules both in the cytoplasm and in vitro with the formation of paracrystalline structures (Donoso et al., 1979; Dustin, 1978; Na & Timasheff, 1982). The specific binding of VCR to tubulin was first demonstrated by Owellen et al. (1972), while Lee et al. (1975) have shown that VCR binds strongly to tubulin dimers. Wilson (1970) has reported that nearly complete stabilization of the colchicine binding activity of

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[1] Abbreviations: VCR, vincristine; PG buffer, 10^{-2} M sodium phosphate and 1×10^{-4} M GTP, pH 7.0; PMG buffer, PG buffer containing 5×10^{-4} M $MgCl_2$; Gdn·HCl, guanidine hydrochloride.

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tubulin is obtained at high concentrations of vincristine. All these reports strongly imply that the destruction of mitotic spindles is the consequence of specific interactions between VCR and the microtubule protein. Recently, we have shown that VCR inhibits microtubule assembly and binds to the tubulin dimer with a binding constant of 3.5×10^4 L/mol at 25 °C (Prakash & Timasheff, 1980a,b, 1983a). Furthermore, an examination of the sedimentation behavior of tubulin as a function of the total amount of VCR added to the system (Prakash & Timasheff, 1983a) led to the hypothesis that VCR induces tubulin self-association into indefinite polymers, in a manner analogous to vinblastine (Na & Timasheff, 1980a,b). In view of these findings, we have carried out a detailed investigation of the mechanism of tubulin self-association induced by VCR, and the results are reported in this paper.

Materials and Methods

All chemicals (unless stated otherwise) were of reagent grade. Sucrose and ammonium sulfate were of ultrapure grade, obtained from Schwarz/Mann. Guanosine 5′-triphosphate (GTP), type II-S from Sigma Chemical Co., was used in the routine buffer and also in the buffer in which tubulin was stored. Otherwise, GTP from Boeringher Mannheim was used in all the buffers in the preparation of tubulin. Guanidine hydrochloride was purchased from Heico Co. It was treated with activated charcoal, clarified, passed through a 40-μm Millipore filter twice, and stored at room temperature. Vincristine sulfate was a gift from Eli-Lilly and Co. through the courtesy of Dr. K. Gerzon and was used without further purification. Calf brains were brought in ice from the local slaughterhouse and were used within 1 h after the animals were sacrificed.

Preparation of Tubulin. Tubulin was isolated from calf brains by the method developed by Weisenberg et al. (1968) and Weisenberg & Timasheff (1970) as modified by Lee et al. (1973) and Na & Timasheff (1982). The $MgCl_2$ concentration was kept at 0.5 mM throughout the preparation. The tubulin prepared in this manner was stored in 1 M sucrose–PMG buffer (10^{-2} M sodium phosphate, 5×10^{-4} M $MgCl_2$, and 1×10^{-4} M GTP, pH 7.0) under liquid nitrogen until needed (Frigon & Lee, 1972).

Preparation of Tubulin Solutions for Sedimentation Velocity Experiments. The stored tubulin was removed from liquid nitrogen and brought to equilibration at ice temperature. Generally, 0.5–0.7 mL of tubulin solution was handled for each equilibration. After the tubulin had been thawed, the concentration of sucrose in it was reduced by loading it into a short column of Sephadex G-25 medium, 6 × 0.9 cm in dimension, previously equilibrated with PG buffer. The protein-containing column was then spun at 3000g for 10 min at 4 °C, and the eluate was collected. This solution, which is not clear, was then centrifuged at 12000*g* for 30 min at 4 °C to obtain a clear solution of tubulin. The concentration of tubulin in this solution was determined spectrophotometrically. Vincristine was added from a stock solution to a final volume of 0.5 mL and a ratio of VCR to tubulin of 2.0 on a mole to mole basis. This solution of the tubulin–VCR mixture was equilibrated at 20 °C for 20 min. To equilibrate the tubulin with a known concentration of free VCR (Wyman, 1964; Lee & Timasheff, 1977; Na & Timasheff, 1980a,b), the protein solution was carefully layered on top of a column of Sephadex G-25 medium, 30 × 0.9 cm in dimension, which had been equilibrated with the desired concentration of VCR in PG buffer and maintained at 20 °C by circulating water through a Forma Scientific water bath and circulator. The protein was allowed to penetrate the column slowly. Once all of the protein had entered the column, small aliquots of PG buffer containing VCR at the same concentration as that equilibrated with the column were layered on the column which was allowed to run at a flow rate of 0.2 mL/min. Fractions of 0.4 mL of eluate were collected, and the protein concentration was determined in each. Either dilution of the fractions or intermixing of the fractions was avoided in order to keep the perturbation of the free VCR concentration to a minimum. The fractions were allowed to sit at 20 °C, and the samples were loaded into the analytical ultracentrifuge cell for the velocity sedimentation experiments. To prevent interference by the formation of the 9S aggregate of tubulin (Prakash & Timasheff, 1982), most of the experiments were finished within 3 h from the time that the protein was taken out of liquid nitrogen.

Protein Concentration Determination. The protein concentration in the presence of VCR was determined spectrophotometrically at low VCR concentrations, and by the measurement of the schlieren peak area at high VCR concentrations. In the absorbance method, a small aliquot of the protein solution was diluted in 6 M Gdn·HCl, and the absorbance of the solution was measured at 275 nm with a Cary 118 spectrophotometer, using a tubulin extinction coefficient of 1.03 mL mg^{-1} cm^{-1} at 275 nm in the presence of 6 M Gdn·HCl (Na & Timasheff, 1981). At higher VCR concentrations, $>8 \times 10^{-5}$ M, the contribution of VCR to the absorbance at 275 nm interfered seriously with the protein concentration determination. In such cases, the protein concentration was determined by measuring the area under the schlieren peak in the ultracentrifuge with proper correction for radial dilution and extrapolation to zero time. At the borderline VCR concentration of 8×10^{-5} M, the protein concentration was determined by both methods and found to be identical within experimental error.

Sedimentation Velocity. Sedimentation velocity experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with a rotor temperature indicator and control (RTIC) unit, an electronic speed control, and a photoelectric scanner. Routine experiments were carried out in a Kel-F-coated aluminum double-sector centerpiece with quartz windows and with sapphire windows for runs using the scanner. The optics of the system were aligned according to the procedures of Dyson (1970) and Richards et al. (1971a,b). Generally, the experiments were performed at 20 °C at 60000 rpm, unless stated otherwise. The plates were read on a Nikon Model 6C microcomparator equipped with an Elk Model 9200 precision digital positioner and an electronic digital display. The $s_{20,w}$ values were calculated according to the standard procedure (Schachman, 1959). Schlieren patterns were recorded on Kodak metallographic or Kodak type II-G spectroscopic plates.

It is absolutely essential that, for a rapidly equilibrating ligand-induced self-association system, there should exist no concentration gradient of either the macromolecule or the ligand in the plateau region (Na & Timasheff, 1980a). In practice, there is frequently a ligand gradient across the reaction boundary leading to the development of bimodal patterns. In such a case, the displacement of the second moment corresponds rigorously to the weight-average sedimentation at the total ligand and macromolecule concentrations found in the plateau region (Goldberg, 1953).

Results

The tubulin isolated by the modified Weisenberg procedure is a well-characterized system (Lee et al., 1973; Frigon & Timasheff, 1975a,b; Lee & Timasheff, 1975; Na & Timasheff, 1980a; Prakash & Timasheff, 1980a,b, 1983a). From the

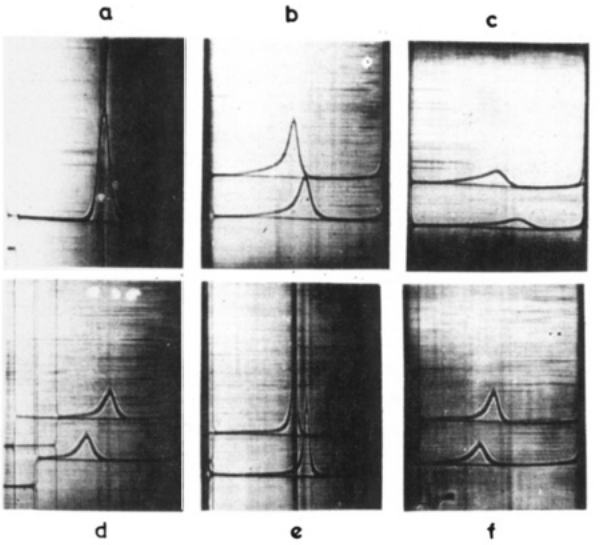


FIGURE 1: Representative sedimentation velocity profiles of tubulin in PG buffer with various concentrations of free VCR. All the sedimentation experiments were performed at 20 °C and at 60 000 rpm, and photographs were obtained at a bar angle of 70°, unless stated otherwise. The free VCR concentration, the protein concentration, and the time of the photograph after reaching two-thirds of full speed, respectively, are the following: (a) tubulin in PG buffer, 15 mg/mL, 53 min; (b) 5×10^{-6} M free VCR (upper), 10.5 mg/mL tubulin (lower), 9.6 mg/mL tubulin, 40 min; (c) 1.56×10^{-5} M free VCR (upper), 5 mg/mL tubulin (lower), 3.55 mg/mL tubulin, 40 min; (d) 4.74×10^{-5} M free VCR (upper), 4.2 mg/mL tubulin (lower), 3.9 mg/mL tubulin, 16 min; (e) 8.2×10^{-5} M free VCR (upper), 10.43 mg/mL tubulin (lower), 9.2 mg/mL tubulin, 24 min; (f) 1.5×10^{-4} M free VCR (upper), 7.36 mg/mL tubulin (lower), 5.77 mg/mL tubulin, 20 min.

hydrodynamic point of view, the protein has been shown to exist as a stable dimer of 110 000 molecular weight and sediments in a single symmetrical peak (frame a of Figure 1) with $s^0_{20,w} = 5.8$ S. The concentration dependence of the sedimentation coefficient below 10.5 mg/mL, shown in Figure 2, indicates that the behavior of tubulin conforms to the standard equation

$$s = s^0(1 - gC) \tag{1}$$

with a value for the hydrodynamic nonideality constant, g, of 0.018 mL/mg (Frigon & Timasheff, 1975a) where C is protein concentration in milligrams per milliliter. The effect of VCR on the sedimentation behavior of tubulin was examined in PG buffer at free VCR concentrations between 5.0×10^{-6} and 1.5×10^{-4} M and tubulin concentrations from 0.6 to 10 mg/mL, and typical results are shown in Figure 1. At these conditions, the sedimentation pattern of tubulin assumed the shape of a single peak skewed forward with a sharp leading edge and a sloping trailing edge extending toward the meniscus. As free [VCR] increased, the area under the trailing edge decreased, as seen in frames b–f of Figure 1. These results indicate clearly that VCR induces tubulin to undergo a polymerization reaction.

Model of Self-Association. The shape of the sedimentation patterns observed at VCR concentrations between 5×10^{-6} and 1.5×10^{-4} M, i.e., a single forward-skewed peak with a trailing edge (see Figure 1, frames b–f), conforms in shape to that predicted by Holloway & Cox (1974) in stimulation calculations for the model of a stepwise indefinite self-association of the type

$$\begin{aligned} A + A &\rightleftharpoons A_2 & K_2 &= [A_2]/[A]^2 \\ A_2 + A &\rightleftharpoons A_3 & K_3 &= [A_3]/[A_2][A] \\ A_{n-1} + A &\rightleftharpoons A_n & K_n &= [A_n]/[A_{n-1}][A] \end{aligned} \tag{2}$$

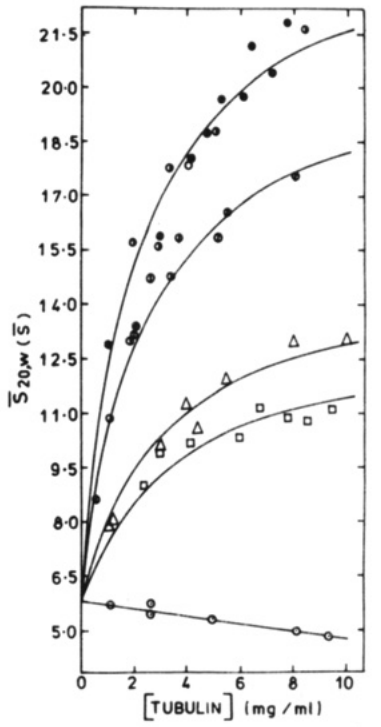


FIGURE 2: Weight-average sedimentation coefficients ($\bar{s}_{20,w}$) of tubulin determined as a function of total protein concentration. Tubulin was equilibrated with different concentrations of VCR [(○) PG only; (□) 5×10^{-6} M VCR; (△) 1.56×10^{-5} M VCR; (◐) 4.74×10^{-5} M VCR; (◑) 8.2×10^{-5} M VCR; (●) 1.5×10^{-4} M VCR] in PG buffer using a flow column of Sephadex G-25 gel to equilibrate, as described under Materials and Methods; 0.4-mL aliquots were collected from the column and used directly for ultracentrifugation without dilution to avoid changes in free VCR concentration. The solid lines are least-squares fittings of the experimental data by the isodesmic, indefinite, self-association model described in the text.

where i and $[A_i]$ denote the ith aggregate and its molar concentration and K_i's are the association constants for the formation of successive bonds between monomer and $(i - 1)$-mer expressed in molar units. The above kind of self-association is referred to as an isodesmic self-association if all the K_i's are equal. (Chun et al., 1969; Chun & Kim, 1969; Reisler et al., 1970). Since the tubulin association pattern in the presence of VCR appears to be very similar to that described by Na & Timasheff (1980a,b) for the vinblastine-induced association of this protein, the method of analysis developed by these authors was adopted for the present system as well.

If the concentration of the ith aggregate, C_i, is expressed in units of milligrams per milliliter, the formation constant of the ith aggregate, k_i, in milliliters per milligram units, for an isodesmic system becomes

$$k_i = C_i/C_1^i = i(k_2/2)^{i-1} \tag{3}$$

$$k_2 = C_2/C_1^2 = 2K_2/M_1 \tag{4}$$

where M_1 is the molecular weight of the monomeric unit in the self-association reaction. For the purpose of calculation, M_1 was taken as 110 000 since the α–β tubulin dimer is the kinetically stable unit in the polymerization reaction (Detrich & Williams, 1978). The total weight concentration of the protein, C_0, in an isodesmic self-association can be expressed as

$$C_0 = \sum_{i=1}^{n} C_i = \sum_{i=1}^{n} i(k_2/2)^{i-1} C_1^i \tag{5}$$

When the self-association is indefinite, the infinite series converges, satisfying the condition $(k_2C_1/2) < 1$, and reduces

itself to (Reisler et al., 1970)

$$C_0 = C_1[1 - (k_2C_i/2)]^2 \quad (6)$$

These equations permit one to calculate the concentration of the monomer and that of each ith polymer for any value of the total protein concentration, C_0, and the dimer formation constant, k_2.

In a velocity sedimentation experiment, where the principle of molecular transport is involved, the mass distribution of species in a reversibly self-associating system can be related to the weight-average sedimentation coefficient by

$$\bar{s} = \sum_{i=1}^{n} s_iC_i / \sum_{i=1}^{n} C_i \quad (7)$$

where s_i is the sedimentation coefficient of the ith-associated species. A combination of eq 1, 6, and 7 leads to an expression for the weight-average sedimentation coefficient, given by

$$\bar{s} = \sum_{i=1}^{n} s_i^0(1 - g_iC_0)i(k_2/2)^{i-1}C_1^i / \sum_{i=1}^{n} (k_2/2)^{i-1}C_1^i \quad (8)$$

where g_i is the hydrodynamic nonideality constant of the ith species.

In the calculation $\bar{s}$ for an indefinite self-association, consecutive terms corresponding to species of higher degrees of association in eq 8 are counted continuously until practically all of the protein (nearly 99.9%) is accounted for, following the procedure of Holloway & Cox (1974). For the evaluation of $\bar{s}$ according to the above procedure, values of $s_1^0 = 5.8$ S and $g_1 = 0.018$ mL/mg were used (Frigon & Timasheff, 1975a). For the higher associated species, since the corresponding values of s_i^0 and g_i are not available, it was assumed that the sedimentation coefficient of the ith aggregate, s_i^0, is related to s_1^0 by

$$s_i^0 = i^{2/3}/s_1^0 \quad (9)$$

and the hydrodynamic nonideality constants, g_i, for all aggregated species are equal to 0.018 mL/mg (Na & Timasheff, 1980a).

Equation 9 contains the assumptions that all the polymeric species have the same partial specific volume as the monomer, as well as spherical symmetry. While this is not rigorously correct, introduction of additional parameters into the calculation would only complicate it without yielding any new insight into the mechanism of the reaction. Simulated sedimentation patterns for the present simple mechanism of polymerization of the tubulin–vinblastine system have given excellent agreement with the experimental results (Na & Timasheff, 1980a). One important characteristic of this model of self-association is that there is no energetically favored end product. The attainment of a plateau in the values of $\bar{s}_{20,w}$ is the result solely of statistical self-limiting, caused by a limit in protein concentration, when ligand remains in excess.

Model Fitting. To model fit the velocity sedimentation data, the weight-average sedimentation coefficient of tubulin was calculated by numerical integration of the sedimentation boundaries, as described by Na & Timasheff (1980a), as a function of tubulin concentration in the presence of VCR at several concentrations of the free ligand, namely, 5.6×10^{-6}, 1.56×10^{-5}, 4.74×10^{-5}, 8.2×10^{-5}, and 1.5×10^{-4} M VCR. Typical results of the dependence of the weight-average sedimentation coefficient on tubulin concentration up to 10 mg/mL are shown in Figure 2. Also shown is the effect of protein concentration on the weight-average sedimentation coefficient of tubulin alone in PG buffer up to a protein concentration of 9.5 mg/mL. As shown in Figure 2, in the presence of VCR, the weight-average sedimentation coefficient of tubulin increases hyperbolically with increasing protein concentration, just as in the presence of vinblastine (Na & Timasheff, 1980a). Thus, at 10 mg/mL protein, the $\bar{s}_{20,w}$ value is 11.0 S in the presence of 5×10^{-6} M free VCR, and it attains 21.5 S in the presence of 1.5×10^{-4} M free VCR, indicating a strong dependence of the polymerization on VCR concentration. At the two highest VCR concentrations used, 8.2×10^{-5} and 1.5×10^{-4} M, the $\bar{s}_{20,w}$ values are essentially identical over the entire protein concentration range studied, indicating saturation of the effect of VCR concentration on tubulin polymerization. The data points were curve fitted by least-squares using the method of Na & Timasheff (1980a), and the results are represented by the solid lines shown in Figure 2 for each free VCR concentration. It is evident that the model of an isodesmic, indefinite self-association accommodates the data quite well. The apparent dimerization constants derived from the above calculations are listed in Table I, along with the apparent standard free energy changes, $\Delta G°_{app}$, for the respective VCR concentrations.

Since the least-squares fitting satisfied all the experimental data points, an attempt was made to calculate as a function of the total protein concentration the mass distribution of tubulin present as different polymeric species. Figure 3 shows such a distribution of polymers for a value of $k_2 = 1.1$ mL/mg, which corresponds to 1.5×10^{-5} M free VCR. At low protein concentration, the 110000 molecular weight tubulin dimer is the predominant species. For higher species, e.g., dimers, M_r 220000, the weight percentage increases from 0 to 30% at 1.3

Table I: Dependence of the Self-Association of Tubulin on the Concentration of Free Vincristine

$[VCR]_{free}$ (mol/L)	k_2^{app} (mL/mg)	K_2^{app} [a] (L/mol)	$\Delta G°_{app}$ (kcal/mol)
5.0×10^{-6}	0.75	4.1×10^4 (8.8×10^3)	−6.17
1.56×10^{-5}	1.1	6.1×10^4	−6.39
4.74×10^{-5}	3.2	1.8×10^5 (5.4×10^4)	−7.01
8.20×10^{-5}	5.5	3.0×10^5	−7.32
1.50×10^{-4}	5.2	2.9×10^5 (1.2×10^5)	−7.29

[a] The values in parentheses are the corresponding equilibrium constants for tubulin self-association in the presence of vinblastine (Na & Timasheff, 1980b).

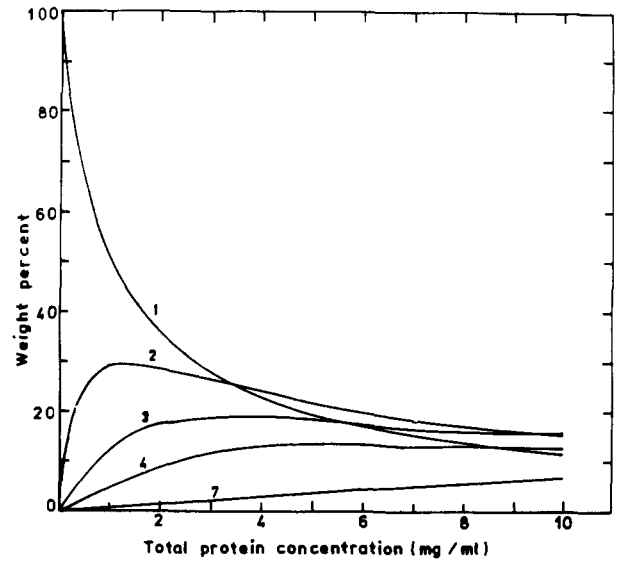


FIGURE 3: Mass distribution of tubulin among different size polymers for an isodesmic, indefinite self-association and its dependence on the total protein concentration; $K_2^{app} = 6.1 \times 10^4$ M^{-1}. The number next to each curve is the corresponding degree of polymerization.

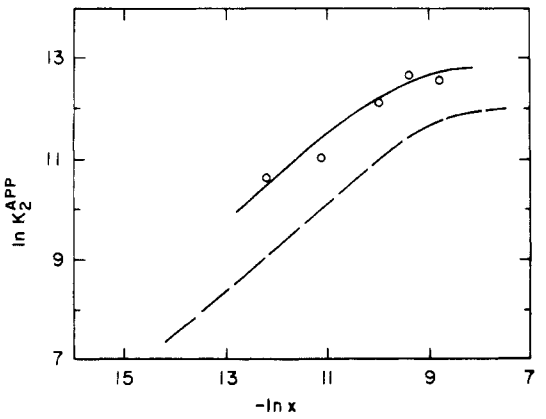
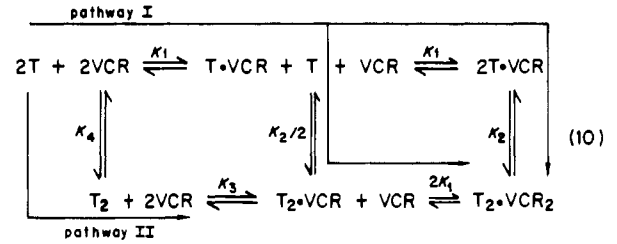


FIGURE 4: Wyman plot of the experimental apparent dimerization constants for the VCR-induced self-association of tubulin (O) and its least-squares fitting according to the model described in the text. The dashed line is the data taken from Na & Timasheff (1980b) for the tubulin–vinblastine system.

mg/mL, where it reaches a maximum before decreasing slowly. Below 10 mg/mL total protein concentration, no aggregate with a degree of polymerization greater than 7 (M_r 1.1×10^6) is present in an amount exceeding 5% of the total protein by weight. However, the tendency is for protein to become more evenly distributed among different size polymers with increasing protein concentration. This pattern of polymer distribution is similar to that of the vinblastine-induced tubulin association system (Na & Timasheff, 1980a). The characteristic difference is that the relative amounts of the higher polymers induced by VCR are higher than those induced by vinblastine at identical concentrations. This is the source of the observed higher weight-average sedimentation coefficients of tubulin in the presence of VCR.

Analysis of the Linkage between Vincristine Binding and Tubulin Self-Association. The data of Figure 2 and Table I were analyzed in terms of the linkage functions described by Na & Timasheff (1980b) in order to elucidate the exact stoichiometry and the linkage mechanism between VCR binding and tubulin self-association. This is based on the Wyman (1964) linkage theory applied to sedimenting systems in rapid reaction equilibrium (Gilbert, 1955, 1959, 1963; Gilbert & Gilbert, 1973). The Wyman (1964) plot of ln K_2^{app} as a function of ln [x], where [x] is the concentration of free ligand, shown in Figure 4, indicates that the equilibrium constant increases with increasing ligand concentration in a manner similar to the tubulin–vinblastine system. At high free VCR concentrations, this effect appears to become saturated since the values of the association constants at 8.2×10^{-5} and 1.5×10^{-4} M VCR are essentially identical. This suggestion that a plateau is being attained in the dependence of the apparent association constants on free VCR concentration is consistent with the ligand-mediated pathway being kinetically open. This does not exclude polymerization via the ligand-facilitated mechanism. Figure 4 brings out an important different between the VCR and the vinblastine systems. For any given concentration of ligand, the value of K_2^{app} is higher for VCR than for vinblastine, indicating that the effect of VCR on tubulin polymerization is stronger (see Table I).

Except for the above quantitative differences, the results with VCR are qualitatively similar to the vinblastine-induced polymerization of tubulin described by Na & Timasheff (1980a,b). By analogy with vinblastine, one can fit the data to two models of ligand-induced mechanisms of association. First, if only one VCR binding site is pertinent to the self-association, the mechanism requires the binding of one VCR molecule for the formation (ligand-mediated mechanism) or stabilization (ligand-facilitated mechanism) of each intertubulin bond. For this stoichiometry and with the assumption of a ligand-mediated pathway, the linkage can be expressed by pathway I of eq 10; namely, the polymerization can proceed



(10)

by addition of unliganded tubulin to the T·VCR complex with subsequent binding of a second VCR molecule and by the direct dimerization of two T·VCR complexes. In eq 10, K_i's are association equilibrium constants, T is tubulin, and VCR is vincristine. Pathway II is the ligand-facilitated pathway. The pertinent relation between the intrinsic dimerization constant, K_2, and the experimentally observable apparent dimerization constant, K_2^{app}, is given by eq 11 for pathway I of eq 10.

$$K_2^{app} = \frac{[T_2\cdot VCR] + [T_2\cdot VCR_2]}{([T] + [T\cdot VCR])^2} = \frac{K_1K_2[VCR](1 + 2K_1[VCR])}{2(1 + K_1[VCR])^2}\left(\frac{\partial \ln K_2^{app}}{\partial \ln [VCR]}\right)_{T,P,a_{j\neq VCR}} = \frac{1 + 3K_1[VCR]}{(1 + K_1[VCR])(1 + 2K_1[VCR])} \quad (11)$$

Fitting of the data to pathway I of eq 10 by eq 11 results in values of the intrinsic equilibrium constants, $K_1 = 3.5 \times 10^4$ L/mol and $K_2 = 3.8 \times 10^5$ L/mol. The stoichiometry assumed in the second mechanism has two VCR binding sites on each tubulin dimer, the binding of one VCR molecule to any one of the two sites enabling the protein to self-associate. This results in the same value of the intrinsic polymerization equilibrium constant, K_2, but in a value of the intrinsic ligand binding constant, K_1, equal to half of that found in the first mechanism (Na & Timasheff, 1980b). In this mechanism, the binding of the second VCR molecule to a tubulin dimer does not enhance any further the self-association of the protein. While the data over the free VCR concentration range studied are consistent with a VCR-mediated mode of polymerization, they do not exclude the participation also of the VCR-facilitated mode (pathway II of eq 10). As shown by Na & Timasheff (1980b), a contribution from this pathway would become significant only at ligand concentrations [VCR] $<<$ K_1^{-1}, i.e., [VCR] $< 1 \times 10^{-6}$ M. The estimated maximal value of K_4 for the tubulin–VCR system is 1×10^4 M^{-1}.

Discussion

The self-association of tubulin in the presence of VCR, described in this paper, indicates a strong interaction between vincristine and tubulin. Lee et al. (1975), Owellen et al. (1976), and Prakash & Timasheff (1980a,b, 1983a) have shown that vincristine binds to tubulin rather strongly. Lee et al. (1975) and Na & Timasheff (1980a,b) have shown that vinblastine also interacts strongly with tubulin, leading to self-association of the protein. The intrinsic drug binding constants, K_1, in the presence of VCR and vinblastine are 3.5 $\times 10^4$ and 1.8×10^4 L/mol at 20 °C, reflecting a difference of 0.4 kcal/mol in the standard free energy of binding. From Table I and Figure 4, it is apparent that, for the same con-

centrations of ligand, VCR has higher values of K_2^{app} than vinblastine. For example, at 5×10^{-6} M ligand concentration, vincristine has a K_2^{app} value of 4.1×10^4 L/mol and vinblastine 8.8×10^3 L/mol, and at 5×10^{-5} M ligand concentration, vinblastine has a K_2^{app} value of 1.8×10^5 L/mol, whereas vinblastine has a value of 5.4×10^4 L/mol. Thus, at low ligand concentration, essentially 4 times as much protein exists in the associated state in the presence of VCR as in that of vinblastine, reflecting an apparent difference in the standard free energy of self-association of close to 1 kcal/mol. This difference reflects the fact that the intrinsic polymerization constant in the presence of VCR, 3.8×10^5 L/mol, is essentially twice as great as that in the presence of vinblastine. The corresponding values of the standard free energy of dimerization at 20 °C, ΔG°_2, are −7.5 kcal/mol in the presence of VCR and −7.0 kcal/mol in the presence of vinblastine. This results in much higher values of $s_{20,w}$ for the VCR than the vinblastine system. Thus, at identical conditions, e.g., 10 mg/mL tubulin and 1.5×10^{-4} M VCR, the $s_{20,w}$ value is close to 23 S, as compared to 17 S in the presence of 2.0×10^{-4} M vinblastine (Na & Timasheff, 1980b).

What gives rise to this difference in behavior of the two drugs with respect to tubulin, both in their strength of binding to the protein and in the strength of the intertubulin bond in the induction polymerization? The similarity of the tubulin self-association patterns in the presence of the two drugs strongly suggests that the nature of the intertubulin contacts in the two reactions is the same. The strengthening of the polymerization most probably reflects subtle changes in the area of contact induced by the stronger binding of VCR to the protein. One possible reason for the stronger binding of VCR can be found in the structures of the two drugs: VCR differs from vinblastine by having a –CHO group in place of a $-CH_3$ in the vindoline moiety of the complex molecule. This change in the oxidation state of one carbon atom should impart a more polar character to VCR. Whether this difference in polar characteristics is the factor responsible for the observed results is difficult to assess. The chemical difference can also lead to a difference in steric fit. The two vinca alkaloids under discussion have complex structures. They consist of vindoline and a catharanthine moieties, both of which interact with tubulin (V. Prakash and S. N. Timasheff, unpublished results). Introduction of the polar group into VCR could lead to a difference in folding of the entire drug and, thus, a perturbation of the individual interactions with tubulin of the different parts of these bidentate ligands. V. Prakash and S. N. Timasheff (unpublished results) have shown that catharanthine is more effective in bringing about the polymerization of tubulin than vindoline, even though neither individually is as effective as either vinblastine or VCR (Na & Timasheff, 1980a; Prakash & Timasheff, 1983a).

The bimodal velocity sedimentation patterns observed at low concentrations of free VCR and reported previously (Prakash & Timasheff, 1983a) can be fully understood now in terms of the polymerization mechanism proposed in this study. They are typical of the theoretical predictions of Cann & Goad (1970a,b, 1972) and Cann & Kegeles (1974) for a ligand-induced self-association in rapid reequilibration, when there is a gradient of unbound ligand across the sedimentation boundary (Prakash & Timasheff, 1983a). In such systems, bimodality is favored by an increase in total protein concentration, an increase in rotor speed, and a decrease in free ligand concentration; i.e., under a given set of conditions, it is favored by an increase in the sedimentation time and the accompanying broadening of the boundary as has been described for the tubulin–VCR system (Prakash & Timasheff, 1983a). Thus, this bimodality conforms fully to the model of an isodesmic indefinite self-association described in this paper. Separation of the boundary into two peaks reflects solely the gradient in free ligand across the reaction boundary (Prakash & Timasheff, 1983b) and in no way signifies the appearance of an intermediate aggregated species along the pathway of self-association.

Registry No. Vincristine, 57-22-7.

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Peptide Repeats in a Mussel Glue Protein: Theme and Variations†

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ABSTRACT: The adhesive protein from *Mytilus edulis* contains 75–80 closely related, repeated peptide sequences in its primary structure. These peptides can be resolved following digestion with trypsin by reversed-phase high-pressure liquid chromatography. The most frequently repeated sequence is the decapeptide Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-Dopa-Lys (peptide E). Variations of this occur in peptides B with Hyp-3 and Dopa-5, C with Dopa-5, and D with Hyp-3, respectively. Lesser amounts of hexapeptides (A and B′) that are lacking residues 4–7 also occur. Peptide A has the sequence Ala-Lys-Pro-Thr-Dopa-Lys, whereas B′ contains Tyr instead of Dopa. 4-Hydroxyproline occurs at positions 3 and 7 and occasionally at position 6 of the decapeptide; 3-hydroxyproline occurs only at position 6. Adhesiveness of the protein may be related to the repetition of Dopa residues, the catecholic moiety of which has strong hydrogen-bonding and metal-liganding capabilities.

The marine mussel *Mytilus edulis* is a sedentary mollusk whose survival depends largely on its capacity to attach expeditiously to solid objects under water. Attachment is mediated by the byssus, a bundle of silky threads that is proximally connected to the animal by a rootlike process and distally connected to a foreign surface by adhesive plaques (Brown, 1952). The plaques contain a glue called the polyphenolic protein (Tamarin et al., 1976; Waite & Tanzer, 1980). This protein is the product of an exocrine gland located in the foot of the mussel and seems to be applied as a foam by the foot to a foreign surface (Waite, 1983a).

Research has recently focused on the chemical nature of the polyphenolic protein as well as the mussel's adhesive delivery system because much needed synthetic underwater adhesives have not yet been successfully formulated by man. Thus far, the polyphenolic protein has been identified as a highly basic protein of intermediate molecular weight (125000). Eight residues account for 90% of the amino acids in the protein, i.e., lysine, hydroxyproline, alanine, serine, threonine, proline, tyrosine, and 3,4-dihydroxyphenylalanine (Dopa) (Waite & Tanzer, 1981). Evidence for repetitive sequences in the polyphenolic protein was obtained by trypsin digestion, which suggested that more than 75 repeats of a decapeptide having the sequence Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-Dopa-Lys (Waite, 1983b) occur in the protein. Although purification of the decapeptide was ostensibly accomplished by methods based on classical ion-exchange

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