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EFFECT OF SOLUTION VARIABLES ON THE BINDING OF VINBLASTINE TO TUBULIN*

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Abstract—Vinblastine binding to tublin was measured in different buffers using tubulin prepared by two different methods and three different binding assay methods. In 100 mM 1,4-piperazinediethanesulfonic acid (Pipes) buffer containing 1 mM $MgSO_4$ and 1 mM ethylene glycol bis (β-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), the data appeared to be consistent with one site with a K_a value of $3.4 \times 10^6\ M^{-1}$ and another site with a K_a value of $2.8 \times 10^5\ M^{-1}$. However, in buffers of lower ionic strengths and without Mg^{2+} the K_a values were lower. The lowest value ($2 \times 10^4\ M^{-1}$) was obtained in 10 mM phosphate buffer, in which only one site was evident under the conditions used. Neither the binding assay used nor the method for tubulin preparation affected the K_a value. Using HPLC, aggregation induced by vinblastine was evident in buffers which gave the largest K_a values. Tubulin aggregation in the presence of vinblastine was also confirmed by analytical ultracentrifugation. The results support the proposal of Na and Timasheff [*Biochemistry* **25**, 6214 (1986)] that the apparent K_a value is influenced by the degree of aggregation induced by vinblastine and that the intrinsic binding constant to the dimer is represented by the lowest value, about $2 \times 10^4\ M^{-1}$.

The anti-mitotic alkaloids from *Catharanthus roseus* (usually called *Vinca* alkaloids) are used as anti-neoplastic drugs and have, as major targets, microtubules and the major protein component of microtubules, tubulin. One difficulty in determining the exact mechanism by which the *Catharanthus* alkaloids act in cells is that there is no general agreement concerning the value of the association constants for the binding of the alkaloids to tubulin. In fact, reported values for vinblastine (VLB)‡ binding vary over a 250-fold range, from a high of $6 \times 10^6\ M^{-1}$ [1, 2] to $2.3 \times 10^4\ M^{-1}$ [3]. A consideration of the mechanism of action of the *Catharanthus* alkaloids, and of their interaction with tubulin and microtubules, requires knowledge of the binding constant to tubulin. Some of the variables which could contribute to the wide discrepancy in reported values include different methods for tubulin isolation, different methods for measuring binding, and different solution conditions in the binding assays. We sought to determine which of these variables, if any, is responsible for the differences in reported binding constants.

MATERIALS AND METHODS

Isolation of tubulin. Purified bovine brain tubulin was prepared according to a previously described modification [4] of the assembly–disassembly method of Shelanski *et al.* [5] and phosphocellulose chromatography [4], with final storage in PEM buffer (0.1 M Pipes, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.9). Bovine brain tubulin was also purified by another method [6], which employs PMG buffer (10 mM sodium phosphate, 0.5 mM $MgCl_2$, 0.1 mM GTP, pH 7) and involves $(NH_4)_2SO_4$ fractionation, DEAE-Sephadex batch purification and precipitation with 50 mM $MgCl_2$. In both cases the tubulin solution was drop frozen in liquid N_2 and stored at −70°.

Vinblastine binding. Tubulin solutions were thawed, centrifuged for 10 min at 27,000 *g* and 0° to remove particulate debris, and then exchanged into the appropriate reaction buffer by passage through a Bio Gel P-6DG desalting column. The resultant protein concentration was determined by the method of Bradford [7] or from the A_{275} value using an ε value of 1.13 ml/mg/cm. The binding affinity of [^{3}H]VLB to tubulin was then determined by one of three methods. The first was a gel column centrifugation technique described by Penefsky [8], using Sephadex G-25 (fine). In this method 0.6 ml of Sephadex G-25 slurry was packed into a 1-ml plastic syringe by centrifugation in a clinical centrifuge. A 0.2-ml solution of tubulin and VLB was applied, and the syringe was centrifuged again. The protein concentration and amount of VLB (bound) were determined in the eluate. The free VLB concentration in the original solution was then calculated. A batch gel elution assay [9] was also employed, using Sephadex G-50. Aliquots (60 mg) of the dry gel were placed into test tubes and were incubated overnight with 0.6 ml of buffer. The tubulin–VLB solution (0.4 ml) was added, and the tubes were rotated slowly for 45 min on an orbit shaker. The gel was allowed to settle for 10 min, and the

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‡ Abbreviations: VLB, vinblastine; Pipes, 1,4-piperazinediethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis (β-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; and DMSO, dimethyl sulfoxide.

VLB concentration in the supernatant fraction was determined. Calculations of bound and free VLB were done as described by Na and Timasheff [10]. The third method was a micropartition assay using the Amicon MPS1 system containing YMT membrane filters [11]. Tubulin–VLB solutions (0.8 ml) were centrifuged through the membrane in a clinical centrifuge until about 25% of the solution had passed through. The VLB concentration (free VLB) in the solution which had passed through the membrane was determined. The association constant for the tubulin–VLB interaction was calculated according to the method of Scatchard [12] in all three cases.

Tubulin aggregation. Tubulin aggregation was monitored by gel exclusion HPLC. Purified tubulin was polymerized for 30 min at 37° at a concentration of 2 mg/ml in PEM buffer, 10% (v/v) DMSO, and 0.5 mM GTP. The resultant microtubules were pelleted by ultracentrifugation, resuspended and cold-depolymerized in the appropriate reaction buffer, and passed through a Bio Gel P-6DG desalting column equilibrated with the resuspension buffer. Tubulin–VLB mixtures were then prepared and allowed to incubate for 20 min at room temperature. The relative degree of tubulin aggregation was determined by employing a TSK 3000 SW column equilibrated with the reaction buffer. The elution times for the standards (BioRad, Richmond, CA) in PEM buffer using a flow rate of 1 ml/min were: aggregate (void volume), 6.1 min; thyroglobulin (670 kD), 6.1 min; gamma globulin (158 kD), 9.4 min; ovalbumin (44 kD), 11.0 min; myoglobin (17 kD), 13.2 min; and Vit B12 (1.35 kD), 15.6 min. To achieve good resolution of standards in 10 mM phosphate and in PM buffer (10 mM sodium phosphate, 0.5 mM $MgCl_2$, pH 7.0), a flow rate of 0.5 ml/min was used. The corresponding elution times for the standards were: 12.5, 12.5, 16.3, 28.0, 31.8 and 32.5 min. VLB eluted with the same elution times as Vit B12 in all buffers. The separated aggregate and free tubulin dimer were monitored by measuring the absorbance at 280 nm.

Analytical ultracentrifugation was also used to monitor the aggregation process. A Beckman model E ultracentrifuge equipped with a photoelectric scanner was used. Sedimentation was done at 42,040 rpm, and scans at 280 nm were taken every 16 min.

[^{3}H]Vinblastine sulfate (13 Ci/mmol; >96% radiopurity) was purchased from Moravek Biochemical Inc., Brea, CA. Vinblastine sulfate was obtained from the Sigma Chemical Co., St. Louis, MO.

RESULTS

Binding of VLB to tubulin prepared by assembly–disassembly and phosphocellulose chromatography was measured in PEM buffer, using the batch gel elution binding assay method. The data we obtained could be fit by assuming one binding site with an apparent K_a of $3.4 \times 10^6\ M^{-1}$ and another site with an apparent K_a of $2.8 \times 10^5\ M^{-1}$ (Fig. 1). The higher value is close to that obtained by several groups using similar buffer conditions but different binding assay methods [1, 2, 13]. K_a values for the apparent high-affinity site were not dependent on the binding assay used (Table 1). Table 1 summarizes data obtained using the three assay methods as well as different buffers, temperatures and tubulin concentrations. The K_a values determined at 5° were about 50% of the values determined at room temperature in the same buffer. The most pronounced differences appear to be due to differences in ionic strength and the presence or absence of Mg^{2+}.

The effect of ionic strength was dramatic. At an ionic strength of close to 0.2 M (0.1 M Pipes and 0.1 M phosphate buffers), the K_a was about $1 \times 10^6\ M^{-1}$. A 10-fold reduction in the phosphate concentration resulted in a 50-fold reduction in the binding constant. The range of VLB concentration used at the lower ionic strengths permitted the detection of only one binding site. The value we obtained in 10 mM phosphate is very close to that obtained by others who used this buffer [3, 10]. A large decrease in the K_a value was also observed when the Pipes concentration in PEM buffer was reduced 10-fold. The presence of Mg^{2+} increased binding at low and high ionic strengths. For example, the binding constant increased 16-fold when 0.5 mM Mg^{2+} was included in 10 mM phosphate buffer and 6- to 8-fold when 1 mM Mg^{2+} was present with 0.1 M Pipes. Other experiments done to show the effect of ionic strength are summarized in Table 2. In these experiments the ionic strength was increased to about 0.2 M by adding various salts to 10 mM Pipes, and the results were compared to those obtained in PEM buffer. It is clear that increasing ionic strength with a variety of salts increased the binding of VLB by tubulin and that the presence of Mg^{2+} had an additional effect. It is also seen that a small further increase in binding was obtained when the Pipes concentration in PEM was raised from 0.1 M to 0.5 M.

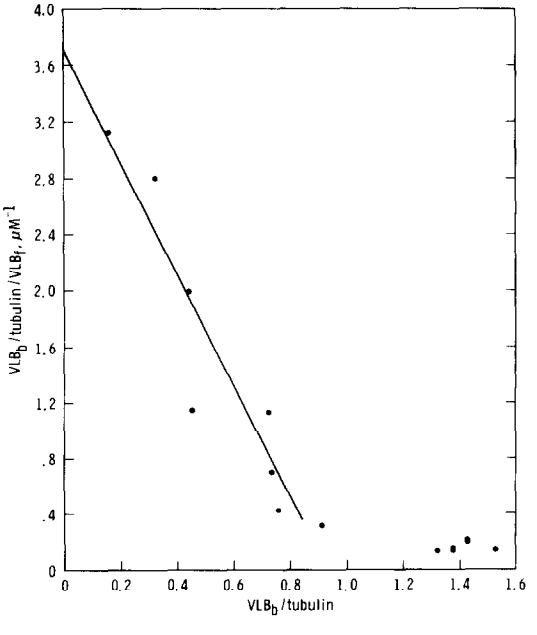

Fig. 1. Binding of VLB to tubulin in PEM buffer determined by the batch gel elution method. The tubulin concentration was 2 μM and the VLB concentration ranged from 0.5 to 13 μM. By linear regression the K_a value was calculated to be $3.36 \times 10^6\ M^{-1}$ ($r = -0.94$).

Table 1. High-affinity association constants for vinblastine

Buffer*	Ionic strength (mM)	Tubulin (μM)	T (°C)	Method†	K_a ($M^{-1} \times 10^{-6}$)
PEM, 0.1 M	219	2	5	1	2.5
PEM, 0.1 M	219	2	22	2	3.4
PEM, 0.1 M	219	0.5	22	3	5.0
PEM, 0.01 M	28	10	22	1	0.2
Pipes, 0.1 M	212	2	22	1	0.7
Phosphate, 0.1 M	180	10	22	1	1.0
PM	20	10	5	1	0.16
PM	20	10	22	2	0.32
PM (10 mM $MgCl_2$)	48	2	22	1	1.4
PMG	20	2	22	2	0.5
PMG (20 mM $MgCl_2$)	78	2	5	1	1.6
Phosphate, 0.01 M	18	10	22	1	0.02

* Buffers: 0.1 M PEM, 0.1 M Pipes, pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$; 0.01 M PEM, PEM with 0.01 M Pipes; phosphate, sodium phosphate, pH 7.0; PM, 10 mM sodium phosphate, pH 7.0, 0.5 mM $MgCl_2$; PM (10 mM $MgCl_2$), PM with 10 mM $MgCl_2$; PMG, PM with 0.1 mM GTP; PMG (20 mM $MgCl_2$), PMG with 20 mM $MgCl_2$.

† Binding assay methods: (1) column centrifugation; (2) batch gel elution; and (3) micropartition.

In the studies reported above, tubulin was purified by the assembly–disassembly and phosphocellulose procedure. The fact that VLB binding could be drastically affected by the nature of the solution conditions implies that the protein isolation procedure is not the reason for the large variation in K_a values reported in the literature. However, we also investigated this possibility by isolating tubulin by another method [6]. This protein was then exchanged into either PM or PEM buffers and binding assays done in these same buffers. VLB binding was essentially equal for both tubulin preparations when compared in the same buffer.

Because the *Catharanthus* alkaloids are known to induce aggregation of tubulin, we determined whether aggregation occurred under conditions used in our binding studies. Gel exclusion HPLC was one method used for this purpose, and some of the data are summarized in Fig. 2. In the experiments in Fig. 2, tubulin was incubated with different concentrations of VLB in PEM and passed through a size exclusion column in buffer containing VLB. Thus, equilibrium conditions were maintained. Control experiments were also done in the absence of VLB. Some tubulin preparations contained a small amount of aggregated protein which eluted with the void volume.

It is clear from these results that increasing the concentration of VLB caused the dimer elution peak to broaden and to elute at an earlier time than expected for the dimer. Also evident was a peak which eluted immediately following the void volume. The latter peak appears to be a high molecular weight aggregate not in equilibrium with the remainder of the protein. The broader peak probably represents several protein species in equilibrium. The data do not permit an assignment of the degree of VLB binding to the various species. The degree of effect on the elution time and profile was correlated with the amount of VLB bound to tubulin. Even at a ratio of VLB to tubulin of 0.1, there was evidence of a small amount of higher molecular weight species eluting before the dimer (Fig. 2b). In Fig. 2f, it is apparent that the aggregation phenomenon occurs at a protein concentration as low as 0.5 μM.

Experiments similar to those reported in Fig. 2

Table 2. VLB binding in the presence of different salts

Buffer	VLB bound/tubulin		
	2 μM VLB*	5 μM VLB*	10 μM VLB*
10 mM Pipes	0.021	0.043	0.14
10 mM Pipes + 180 mM sodium glutamate	0.079	0.16	0.25
10 mM Pipes + 110 mM sodium phosphate	0.11	0.17	0.27
10 mM Pipes + 210 mM Mes	0.13	0.23	0.54
10 mM Pipes + 200 mM NaCl	0.16	0.29	0.37
100 mM Pipes	0.12	0.24	0.32
PEM	0.40	0.59	
500 mM PEM†	0.55	0.70	0.77

* Refers to total VLB concentration. The tubulin concentration was 2 μM.

† PEM with 500 mM Pipes.

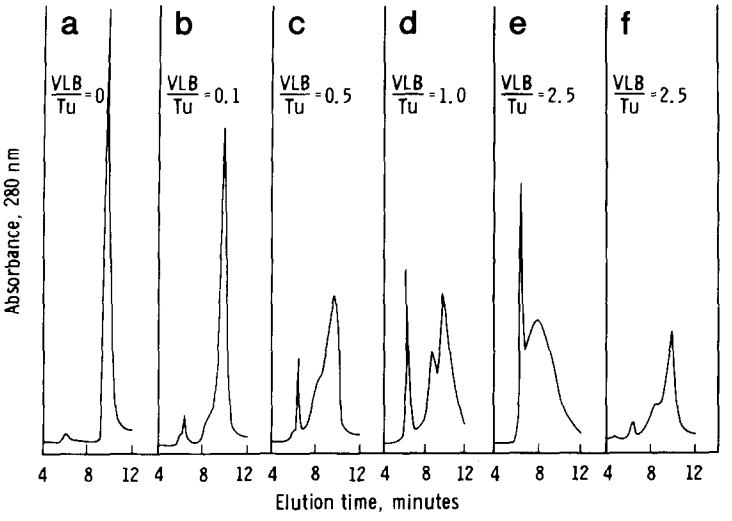

Fig. 2. VLB-induced aggregation of tubulin. Tubulin, either in the absence or presence of VLB, was applied to a 300 mm × 7.5 mm TSK 3000 SW column in PEM at room temperature. Elution was done with buffer containing or lacking VLB. In panels a–e, the concentration of tubulin was 2 μM. The VLB concentrations in panels b, c, d, and e were 0.2, 1, 2 and 5 μM respectively. In panel f, tubulin was 0.5 μM and VLB was 1.25 μM. The calculated VLB binding (VLB/tubulin) values were: b, 0.09; c, 0.41; d, 0.70; e, 0.93; and f, 0.78. The presence of VLB in the buffer had no effect on the elution profile of the standard proteins. The VLB/tubulin initial ratios are also given.

were done with other buffer systems, and some of the results are presented in Fig. 3. These results demonstrate that, in PM, aggregation was evident with two peaks present, both eluting before the time expected for the dimer. In phosphate buffer without Mg^{2+} the effect was less, although a shoulder on the leading edge of the main peak was evident (Fig. 3d). When Figs. 3b and d are compared to Fig. 2e, it is clear that at the same VLB/tubulin ratio of 2.5 the greatest degree of aggregation occurred in PEM and the least in phosphate, which correlates with the differences in binding constants in the different buffers.

The difference in the state of VLB-induced tubulin aggregation in different buffers was also demonstrated by analytical ultracentrifugation (Fig. 4). In PEM buffer the sedimentation coefficient of tubulin increased dramatically as the VLB concentration was increased. The sedimentation coefficient at the midpoint of the concentration profile increased from 5.7S in the absence of VLB to 20.2S in the presence of 12.5 μM VLB. In addition, the boundary became broadened at the higher VLB concentrations. The apparent sedimentation coefficients for the 25% and 75% points of the distribution profile in the presence of 12.5 μM VLB were 14.8S and 22.8S respectively. This spread substantiates the existence of a polydisperse mixture of species. A comparison of the sedimentation coefficients in PEM (20.2S) and 10 mM phosphate (7.3S) under identical concentrations of tubulin (5 μM) and VLB (12.5 μM) clearly demonstrates the difference in VLB-induced aggregation in these two buffer systems.

The HPLC experiments to demonstrate VLB-induced aggregation described above were done under equilibrium conditions, i.e. VLB was included in the elution buffer. We examined the reversibility

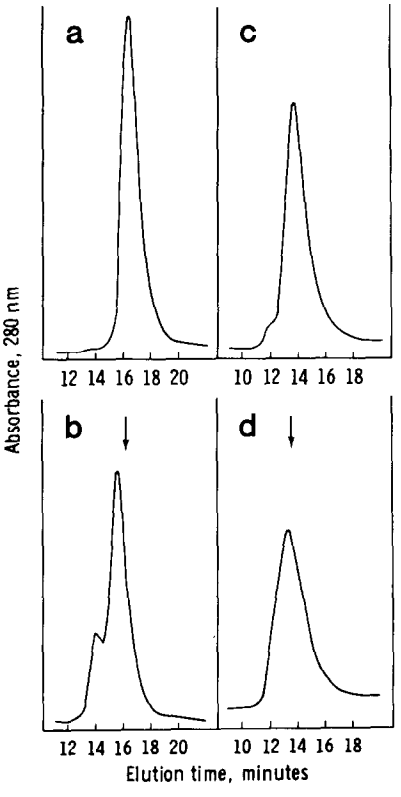

Fig. 3. VLB-induced aggregation in phosphate buffers. The experiments were done as described in Fig. 2 and under Materials and Methods. The tubulin concentration was 2 μM. Panels a and b, PM buffer. Panels c and d, 10 mM phosphate buffer. In panels b and d, the VLB concentration was 5 μM. The calculated VLB binding (VLB/tubulin) was 0.26 in b and 0.12 in d. The arrows in b and d refer to the expected position of the dimer.

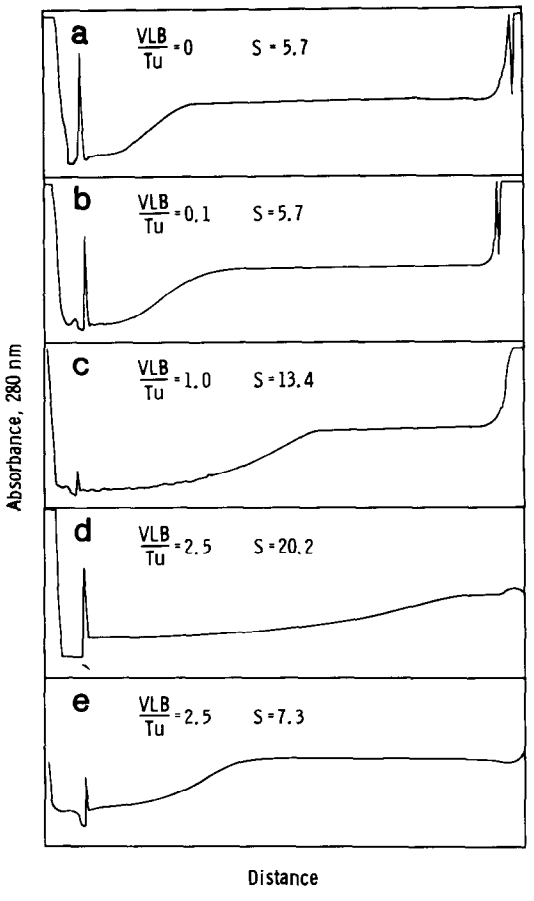

Fig. 4. VLB-induced aggregation of tubulin as detected by analytical ultracentrifugation. Samples of VLB and tubulin (5 μM) in PEM or 10 mM phosphate buffer were centrifuged at 42,040 rpm. Sedimentation progress was observed with a UV-scanner. The profiles shown are after 48 min of centrifugation after speed was reached. The sedimentation constants given in the figure are calculated from the midpoint of the curve. The VLB/tubulin initial ratios are also given. Panels a–d, PEM; panel e, 10 mM phosphate buffer. The calculated VLB binding (VLB/tubulin) values in these experiments were: b, 0.09; c, 0.8; d, 0.97; and e, 0.19.

of the binding and aggregation phenomenon by passing a tubulin–[^{3}H]VLB mixture through a column in the absence of VLB in the buffer. The elution profile shown in Fig. 5 demonstrates some reversibility during the elution from the HPLC column (compare Fig. 5 with Fig. 2e). It appeared as if radioactivity was associated with the fastest eluting protein fraction as well as in an area where VLB is known to elute.

DISCUSSION

In this work we sought an explanation for the fact that values for the association constant of VLB binding to tubulin in the literature vary over a 250-fold range. Three possible explanations were considered: the method of tubulin preparation, the method used for the binding assay, and the solution conditions used in the binding assay. Our results clearly show that different solution conditions in the binding assay are responsible for the discrepancies in the reported values for the association constants. Using the same solution components, no differences in association constants were found when tubulin obtained by two different and commonly used preparative methods was compared. In addition, there were no noticeable differences in K_a values when three different binding assay methods were compared.

The largest association constants were obtained in buffers that had the highest ionic strengths and contained Mg^{2+}. The highest values reported in the literature were also obtained with buffers of high ionic strength or with a high Mg^{2+} concentration present: 10 mM phosphate with 10 mM $MgCl_2$ [1], 10 mM phosphate with 100 mM glutamate [2], 50 mM phosphate with 5 mM $MgCl_2$ and 150 mM NaCl [13]. The lowest values were obtained in a low ionic strength buffer, 10 mM phosphate [3]. A binding constant comparable to the highest values obtained in our studies was found in the binding of vincristine (VCR) to cell extracts when 10 mM phosphate with 10 mM $MgCl_2$ was used as the buffer [14]. The differences in values (250-fold) seem large for simple ionic effects on the association of VLB with tubulin. The results are better explained by the proposal of Na and Timasheff [10], that the large discrepancy in reported *Catharanthus* alkaloid binding constants is due to a ligand-mediated plus-facilitated protein aggregation process which is more pronounced under certain solution conditions. Na and Timasheff [10] have proposed that the lower binding constant observed in 10 mM phosphate buffer represents the intrinsic binding constant to the dimer and that the higher values are apparent values due to an indefinite protein aggregation phenomenon which shifts the binding equilibrium. Indeed, these workers did show VLB-induced aggre-

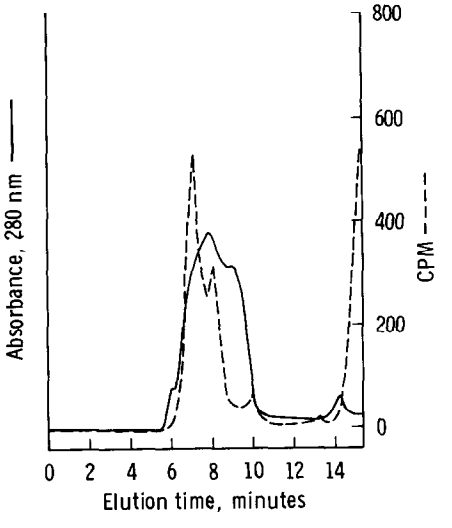

Fig. 5. Elution of the tubulin–VLB complex under non-equilibrium conditions. Tubulin (2 μM) and [^{3}H]VLB (5 μM) were incubated for 20 min at room temperature in PEM before HPLC separation in PEM. Samples of 300 μl were collected, and aliquots were counted.

gation of tubulin which was more prevalent when Mg^{2+} was present at tubulin concentrations of 16 μM and higher [10, 15]. Our HPLC experiments, although qualitative, definitely showed a large degree of aggregation under conditions where there was efficient VLB binding, even at tubulin concentrations as low as 0.5 μM. The aggregation under some conditions resulted in a sharp peak which eluted much earlier than the dimer and does not appear to be in equilibrium with the dimer, and a broader peak indicative of a protein aggregation system in rapid equilibrium which also eluted earlier than the dimer. In some cases, a shoulder was apparent on the leading edge of the main peak. The aggregation phenomenon and the influence of buffer were substantiated by ultracentrifugal analysis. It appears that factors which are known to stimulate tubulin assembly in the absence of VLB (e.g. Mg^{2+} and optimum ionic strength) increase the extent of the VLB-promoted aggregation process, thereby shifting the equilibrium and increasing the apparent K_a value. In none of our experiments did the solutions become turbid; thus, the type of aggregate produced is not the same as that produced at high tubulin, Mg^{2+} and VLB concentrations [15]. Examination with the electron microscope revealed only the presence of amorphous aggregates.

In addition to disagreements in the literature on the binding constant, there exists some disagreement over the number and equivalency of binding sites. One laboratory has interpreted binding data in terms of one specific high-affinity site and one specific lower affinity site [1]. Some investigators have reported two sites with equal affinity [3, 13]. Others have proposed the existence of a single specific site and two or more non-specific sites [10]. Our results in PEM appear to be consistent with two sites of different affinity; however, this is probably a result of the aggregation phenomenon and, as Na and Timasheff have pointed out [10, 15], the shape and slope of the Scatchard plot vary with the extent of aggregation of the protein. Because extensive aggregation occurred under conditions where less than one VLB was bound per tubulin dimer (Fig. 2), the binding of the first molecule of VLB must be associated with the aggregation process.

The effects of solution components on the apparent K_a value of VLB binding and the state of aggregation of tubulin are relevant to studies of the effects of drugs on the steady-state addition of the tubulin dimer to the ends of microtubules. These studies are usually done in PEM or a similar buffer. The steady-state concentration of unpolymerized tubulin in such experiments is usually about 2 μM. Concentrations of VLB at $\geq$2 μM would then be expected to produce a significant degree of aggregation of free tubulin. Fifty percent inhibition of the addition of tubulin to the ends of microtubules at steady state is usually found at concentrations of about 2×10^{-7} M [16, 17], the approximate K_d value in PEM buffer found in this study. Data have been presented which indicate that this substoichiometric inhibition is due to the binding of VLB to one or two molecules of tubulin at the microtubule ends [16]. When we examined the effect of 2×10^{-7} M VLB on the state of aggregation of 2 μM tubulin, we found a small amount of the protein to be in a form which eluted in front of the dimer peak (Fig. 2b). The VLB-induced tubulin aggregation may contribute to the substoichiometric inhibition found with the *Catharanthus* alkaloids, but it is difficult to evaluate the magnitude of this contribution. An interesting suggestion relating the VLB-induced aggregation to assembly inhibition is that the VLB-induced association of tubulin occurs at microtubule ends thus blocking normal tubulin addition [17].

Is the VLB-induced aggregation related to the cellular effects of the *Vinca* drugs? This question is not possible to answer at this time. Houghton *et al.* [14] treated a Rh12 tumor homogenate supernatant fraction with [^{3}H]VCR and found by size exclusion HPLC that the [^{3}H] eluted with a single binding species equivalent to the molecular weight of the tubulin dimer. This would imply that the aggregation phenomenon does not take place in cell extracts. However, the HPLC column was run under non-equilibrium conditions and, as we have shown, some reversibility of the aggregation occurs under these conditions. In addition, only 10 nM VCR was used in these studies. Without knowing the tubulin concentration in the supernatant it is impossible to know the VCR/tubulin ratio. In the reported experiments no free VCR eluted from the HPLC column, indicating that an excess of tubulin was present. As mentioned previously, our experiments at a VLB/tubulin ratio of 0.1 in PEM showed only a small degree of aggregation.

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REFERENCES

1. B. Bhattacharya and J. Wolff, *Proc. natn. Acad. Sci. U.S.A.* **73**, 2375 (1976).
2. R. J. Owellen, A. H. Owens Jr. and D. W. Donigian, *Biochem. biophys. Res. Commun.* **47**, 685 (1972).
3. J. C. Lee, D. Harrison and S. N. Timasheff, *J. biol. Chem.* **250**, 9276 (1975).
4. J. P. Nath, G. R. Eagle and R. H. Himes, *Biochemistry* **24**, 1555 (1985).
5. M. L. Shelanski, F. Gaskin and C. R. Cantor, *Proc. natn. Acad. Sci. U.S.A.* **70**, 765 (1973).
6. J. C. Lee, *Meth. Cell Biol.* **24A**, 9 (1982).
7. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
8. F. Penefsky, *J. biol. Chem.* **252**, 2891 (1977).
9. P. Fasella, G. G. Hammes and P. R. Schimmel, *Biochim. biophys. Acta* 103, 708 (1965).
10. G. C. Na and S. N. Timasheff, *Biochemistry* **25**, 6214 (1986).
11. J. A. Sophianopoulas, S. J. Durham, A. J. Sophianopoulas, H. R. Ragsdale and W. P. Cropper, *Archs Biochem. Biophys.* **187**, 132 (1978).
12. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
13. L. Wilson, K. M. Creswell and D. Chin, *Biochemistry* **14**, 5586 (1975).
14. J. A. Houghton, L. G. Williams, R. K. Dodge, S. L. George, B. J. Hazelton and P. J. Houghton, *Biochem. Pharmac.* **36**, 81 (1987).
15. G. C. Na and S. N. Timasheff, *Biochemistry* **25**, 6222 (1986).
16. L. Wilson, M. A. Jordan, A. Morse and R. L. Margolis, *J. molec. Biol.* **159**, 125 (1982).
17. M. A. Jordan, R. H. Himes and L. Wilson, *Cancer Res.* **45**, 2741 (1985).