Weiner, A. M., Platt, T., and Weber, K. (1972), *J. Biol. Chem. 247,* 3242.
Wilson, W. D., and Foster, J. F. (1971), *Biochemistry 10,* 1772.
Woods, K. R., and Wang, K. T. (1967), *Biochim. Biophys. Acta 133,* 369.
Zurawski, V. R., Jr. (1973), Ph.D. Thesis, Purdue University.
Zurawski, V. R., Jr., and Foster, J. F. (1974), *Biochemistry 13,* 3465.

The Mechanism of Action of Vinblastine. Binding of [*acetyl*-^{3}H]Vinblastine to Embryonic Chick Brain Tubulin and Tubulin from Sea Urchin Sperm Tail Outer Doublet Microtubules†

Leslie Wilson,* Karen M. Creswell, and Daniel Chin

ABSTRACT: Tritium-labeled vinblastine, specific activity 107 Ci/mol, was prepared by acetylation of desacetylvinblastine with [^{3}H]acetic anhydride, and has been employed in a study of vinblastine binding to tubulin. There are two high affinity vinblastine-binding sites per mole of embryonic chick brain tubulin (K_A = 3–5 × 10^5 l./mol). Binding to these sites was rapid, and relatively independent of temperature between 37 and 0°C. Vincristine sulfate and desacetylvinblastine sulfate, two other active vinca alkaloid derivatives, competitively inhibited the binding of vinblastine. The inhibition constant for vincristine was 1.7 × 10^{-5} *M*; and for desacetylvinblastine, 2 × 10^{-5} *M*. The vinblastine binding activity of tubulin decayed upon aging, but this property was not studied in detail. Vinblastine did not depolymerize stable sea urchin sperm tail outer doublet microtubules, nor did it bind to these microtubules. However, tubulin solubilized from the B subfiber of the outer doublet microtubules possessed the two high affinity binding sites (K_A = 1–3 × 10^5 l./mol). These data suggest that vinblastine destroys microtubules in cells primarily by inhibition of microtubule polymerization, and does not directly destroy preformed microtubules.

The antimitotic drug, vinblastine, belongs to a class of chemically related dimeric alkaloids known commonly as the vinca alkaloids. Along with vinblastine, other active vinca alkaloids such as vincristine and desacetylvinblastine inhibit mitosis by destroying the microtubules of the mitotic apparatus. This results in accumulation (due to blockage) of cells at the metaphase stage (reviewed in Olmsted and Borisy, 1973; Margulis, 1973; Wilson and Bryan, 1974). In addition to its antimitotic effects, vinblastine and other active vinca alkaloids can destroy microtubules in a wide variety of cells and tissues, thereby disrupting the many biological functions which depend upon this class of subcellular organelles (Olmsted and Borisy, 1973; Margulis, 1973; Wilson and Bryan, 1974). In many cases, dissolution of microtubules within cells and tissues by vinblastine is associated with the formation of highly regular, birefringent crystals (Schochet et al., 1968; Bensch and Malawista, 1969; Nagayama and Dales, 1970; Bryan, 1971). Bryan (1971, 1972a,b) isolated vinblastine-induced crystals from unfertilized sea urchin eggs, and found them to be composed of tubulin complexed to vinblastine, in a molar ratio of 1 mol of vinblastine/mol of tubulin. The finding that vinblastine caused crystal formation in cells in vivo was shortly followed by the demonstration that high concentrations of vinblastine precipitate tubulin in vitro. It has been thought that these two actions of vinblastine are mechanistically related (Bensch et al., 1969; Marantz et al., 1969). However, the ability of high vinblastine concentrations to precipitate tubulin in vitro seems to be a nonspecific effect, since vinblastine can precipitate a large number of other acidic proteins, including muscle actin, as well as nucleic acids (e.g., double-stranded DNA) (Wilson et al., 1970). These data suggested there might be two classes of binding sites for vinblastine on tubulin: a high affinity class (biologically important) and a low affinity class (biologically unimportant). Owellen et al. (1972, 1974) have studied the high affinity binding of vinblastine to purified pig and rat brain tubulin utilizing a DE81 filter paper assay, and obtained a binding constant of 5–6 × 10^6 l./mol at 37°C, and one vinblastine binding site per 2 mol of tubulin.

In this report, we have prepared tritium-labeled vinblastine, and have studied its binding to purified embryonic chick brain tubulin, and tubulin solubilized from sea urchin sperm tail outer doublet microtubules, utilizing a number of different assay procedures. The high affinity binding of vinblastine to brain tubulin has been shown to be biologically specific, and the properties of the binding reaction have been partially characterized. We have also investigated the binding of vinblastine to intact sea urchin sperm tail outer doublet microtubules. Our results suggest that vinca alkaloids disrupt microtubules in cells primarily by preventing

† From the Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305. *Received April 21, 1975.* A portion of this research was carried out at the University of Washington, Friday Harbor Laboratories, Friday Harbor, Wash. A preliminary report was presented at the 14th Annual Meeting of the American Society for Cell Biology (1974), *J. Cell Biol. 63,* 373a. This investigation was supported by Grant CI-95 from the American Cancer Society, and U.S. Public Health Service Grant NS09335 from the Division of Neurological Diseases and Stroke.

microtubule polymerization, and not by directly destroying performed microtubules. In subsequent reports we will describe the characteristics of vinca alkaloid inhibition of in vitro microtubule assembly, and the vinblastine-binding characteristics of purified sea urchin egg vinblastine–tubulin crystals.

Experimental Section

Methods

Preparation of [acetyl-^3H]Vinblastine. Acetylation of Desacetylvinblastine. Vinblastine sulfate (specific activity 107 Ci/mol) was prepared by acetylation of desacetylvinblastine with tritium-labeled acetic anhydride by a modification of the method described by Greenius et al. (1968). Desacetylvinblastine sulfate, 10 mg, was dissolved in 10 ml of distilled water, and converted to the free base by adjustment of the pH to 7.8 with NaOH.[1] Desacetylvinblastine base was extracted from the aqueous solution with approximately 40 ml of benzene, and the benzene extract was evaporated to dryness in vacuo at 40°C. The desacetylvinblastine was then dissolved in 500 μl of pyridine, and the solution was transferred to a 10 mm × 90 mm tapered glass (Kimax) tube, and evaporated to dryness in vacuo at 40°C. The desacetylvinblastine was maintained in vacuo for 2 hr just prior to acetylation. Acetylation was carried out by adding a 5 molar excess of tritium-labeled acetic anhydride in benzene (reported specific activity of 400 Ci/mol) and heating at 50°C (oil bath) for 2 hr. A small reaction volume (i.e., 50 μl) was found to be critical for maximum acetylation.

Purification of Vinblastine by Thin-Layer Chromatography. The reaction product was dissolved in 500 μl of pyridine, and evaporated to dryness in vacuo at 55°C. The product was redissolved in 500 μl of redistilled methanol, and streaked along an origin line on an Eastman thin-layer chromatogram sheet. The chromatogram was developed with acetone as described by Creswell (1972) (R_f of vinblastine 0.45; R_f of desacetylvinblastine 0.25; R_f of photodegradation products 0.63). The vinblastine-containing band of silica gel was scraped from the chromatogram sheet into a scintered glass funnel; and the vinblastine was collected by washing with seven 2-ml portions of methanol. The methanol extracts were evaporated to dryness in vacuo, and the [*acetyl*-^{3}H]vinblastine was converted to the salt form by addition of 4 ml of 40 m*M* sodium phosphate buffer (pH 3.7). In the salt form, the vinca alkaloids appear to be stable to light. The labeled vinblastine was further purified by carboxymethylcellulose column chromatography as described by Creswell (1972). This step serves to remove any photodegradation product formed during the thin-layer chromatographic separation.

Criteria for Purity. The final product was judged to be pure by a number of criteria. The labeled product comigrated with unlabeled vinblastine by thin-layer chromatography as described above. The labeled product also comigrated with unlabeled vinblastine by column chromatography on carboxymethylcellulose, with the ratio of tritium to absorbancy at 270 nm remaining constant throughout the vinblastine peak. Two biological assays were also employed to verify purity. The labeled product became bound in vivo to [*acetyl*-^{3}H]vinblastine induced sea urchin egg tubulin crystals with a molar ratio of vinblastine to tubulin of 0.9 mol of vinblastine/mol of tubulin (see Bryan, 1972). Finally, serial dilution of the labeled product with unlabeled vinblastine resulted in a linear decrease in the specific activity of the [*acetyl*-^{3}H]vinblastine bound to chick embryonic brain tubulin.

Purification of Chick Embryo Brain Tubulin. Purification of tubulin from freshly dissected brains of 13- to 18-day-old chick embryos was accomplished by the single-step procedure described previously (Bryan and Wilson, 1971), except that sodium pyrophosphate buffer at pH 7.0 was employed rather than sodium phosphate buffer and DEAE-cellulose (Whatman DE 52) was employed rather than DEAE-Sephadex according to the procedure of Eipper (1972). The tubulin eluted at an NaCl concentration of 0.27 *M*. Purity was greater than 95% as determined by discontinuous polyacrylamide gel electrophoresis in sodium dodecyl sulfate as described by Luduena and Woodward (1973). The purified tubulin bound 0.72 mol of colchicine/mol of tubulin (uncorrected for loss of colchicine binding activity which occurred during purification). A mass of 110000 daltons was utilized for all calculations of drug binding stoichiometry and binding constants.

Sea Urchin Sperm Tail Outer Doublet Microtubules. Outer doublet microtubules from sperm tails of the west coast (USA) sea urchin *Stronglyocentrotus purpuratus* were purified as described previously (Wilson and Meza, 1973). Outer doublets were stored as a suspension (approximately 10 mg of tubulin/ml) in 10 m*M* Tris-HCl (pH 8.0) at 0°C (ice bath). The B subfibers of the outer doublet microtubules were solubilized by resuspending the microtubules in 20 m*M* sodium phosphate buffer (pH 6.8) and warming at 37°C for 2 hr. Between 13 and 20% of the total outer doublet tubulin was solubilized under these conditions. The solubilized tubulin was approximately 85% pure, as judged by polyacrylamide gel electrophoresis (Wilson and Meza, 1973).

Vinblastine–Tubulin Complex Formation: Incubation Procedures. Solutions of purified chick embryo brain tubulin in 20 m*M* sodium pyrophosphate (pH 7.0)–270 m*M* NaCl–2.5 m*M* $MgCl_2$ were routinely diluted with 20 m*M* sodium pyrophosphate buffer at pH 7.0 to reduce the NaCl concentration to 150 m*M*. Desired concentrations of labeled vinblastine were added, then solutions were incubated in a total volume of 500 μl. Usually, triplicate 100-μl aliquots of each incubation mixture were assayed for vinblastine–tubulin complex formation as described below. Tubulin solubilized from sea urchin outer doublet microtubules in 20 m*M* sodium phosphate (pH 6.8) was incubated with labeled vinblastine and assayed for vinblastine–tubulin complex formation as described below.

Vinblastine–Tubulin Complex Formation: Assay Procedures. Assay of vinblastine–tubulin complex formation presented several significant technical problems. Equilibrium dialysis was not suitable for determination of the biologically specific binding of vinblastine to tubulin for two reasons. First, more than 36 hr (usually 48 hr) were required to reach equilibrium, and, during this length of time, there was considerable aggregation of the tubulin, and an apparent 10–20-fold decrease in the binding constant. Secondly,

[1] The free base forms of vinblastine derivatives are unstable to light (Greenius et al., 1968; Creswell, 1972) and, upon exposure, rapidly became photodegraded. Photodegraded derivatives, which are biologically inactive (i.e., do not inhibit cell growth) can be separated from nondegraded derivatives by thin-layer chromatography as described in the text, and have an altered ultraviolet spectrum (Creswell, 1972). Thus, while working with the vinca alkaloids in their free base forms it was necessary to carry out all procedures in the dark.

vinblastine became bound to the inside walls of the dialysis chambers and to the dialysis membrane, resulting in highly variable binding data. Gel filtration procedures previously used for the assay of colchicine to tubulin (Wilson, 1970) also were not suitable for vinblastine. Because of the moderately weak binding constant, much of the previously bound vinblastine dissociated from the complex as it filtered down the column. A DEAE-impregnated filter paper disc assay procedure previously employed by Owellen et al. for assay of tubulin–vinblastine complex formation could be employed for investigating the kinetics of the binding reaction, for estimating the apparent binding affinity, and to study the influence of other chemical agents and reaction conditions on vinblastine–tubulin complex formation. The equilibrium Sephadex gel procedure of Hirose and Kano (1971) was employed to determine the binding affinity and stoichiometry of the binding reaction. Stoichiometry was also verified by equilibrium gel filtration.

DE81 Paper Disc Assay. This method, employed previously for determination of colchicine binding to tubulin (Wilson, 1970; Borisy, 1972), takes advantage of the high affinity of tubulin for DEAE-cellulose impregnated filter paper discs. Aliquots of incubation mixture (usually 100 μl) containing free and tubulin-bound [*acetyl*-^{3}H]vinblastine were applied directly to slightly moistened 2.5-cm discs of Whatman DE81 Chromedia paper (placed on parafilm) and incubated for 10 min at 0°C. Paper discs were washed to remove free [*acetyl*-^{3}H]vinblastine by immersion in six successive 100-ml changes of 10 m*M* sodium phosphate buffer (pH 6.8) (5 min/wash, 0°C). Approximately 65% of the originally applied tubulin remained bound to the disc after the washing steps. The paper discs with adhering protein-bound [*acetyl*-^{3}H]vinblastine were then counted directly in a scintillation vial containing 10 ml of Bray's solution (Bray, 1960). In all cases, controls were run in the absence of tubulin, and this background was subtracted from the quantity of vinblastine bound to the discs in the presence of tubulin. Binding results of duplicate experiments agreed to within 10%. With 2.5×10^{-5} *M* vinblastine, increasing the tubulin concentration between 0.5×10^{-9} and 3×10^{-9} mol of tubulin/assay resulted in a linear increase in binding activity. Moreover, the specific activity (mol of vinblastine bound/mol of tubulin) remained constant throughout this protein concentration range (data not shown). All subsequent experiments were carried out within this tubulin concentration range.

Equilibrium gel filtration was carried out on 1 × 18 cm columns of Bio-Gel P10 as described by Hummel and Dreyer (1962). *Brain tubulin:* columns were equilibrated with 50 ml of 150 m*M* NaCl, 20 m*M* sodium pyrophosphate, and 1 m*M* $MgCl_2$ (pH 7.0) containing 7.50×10^{-5} *M* labeled vinblastine. *Sea urchin sperm tail outer-doublet tubulin:* columns were equilibrated with 20 m*M* sodium phosphate (pH 6.8) containing 7.50×10^{-5} *M* labeled vinblastine. Chick brain tubulin (1.030 mg) or solubilized outer-doublet tubulin (1.010 mg) in the appropriate equilibration buffer was passed through the column, and 1.0-ml fractions were collected. Bound vinblastine (mol/mol of tubulin) in the tubulin-containing fractions (fractions 16 and 17) was calculated after subtracting the baseline value of free vinblastine from the total quantity of vinblastine present in the tubulin-containing fractions.

Equilibrium Sephadex Gel Procedure. This procedure, developed by Hirose and Kano (1971), involves measurement of bound and free vinblastine in the supernatant volume of a swollen Sephadex G-50 bead suspension. Beads are permeable to the vinblastine, but not to the tubulin. The concentrations of bound and free vinblastine can be calculated after determining the distribution coefficient of vinblastine in the presence and absence of tubulin. The advantage of this procedure is that it permits determination of vinblastine binding to tubulin under equilibrium conditions.

Miscellaneous Procedures. Protein concentrations were determined by the method of Lowry et al. (1951) with standard solutions of purified chick embryo brain tubulin. Tritium was determined with a Packard TriCarb liquid scintillation spectrometer. Internal standards of tritiated water were used to determine counting efficiency.

Materials

Chick Eggs. Fertilized chick eggs were obtained from Kimber Farms, Fremont, Calif., and incubated at 38°C in a humidified Jamesway incubator, equipped with an egg-turning device which rotated the eggs every 2 hr.

Chemicals. Vinblastine sulfate, vincristine sulfate, desacetylvinblastine sulfate, and leurosidine were all generous gifts from Dr. Koert Gerzon, of the Eli Lilly Co., Indianapolis, Ind. Tritiated acetic anhydride (400 Ci/mol) was obtained from New England Nuclear Corporation, Boston, Mass. [*acetyl*-^{3}H]Colchicine (155 Ci/mol) was prepared as described previously (Wilson and Friedkin, 1966). Benzene and pyridine utilized in the preparation of tritium-labeled vinblastine were both of spectrophotometric grade. Carboxymethylcellulose (Cellex-CM) and Bio-Gel P10 were obtained from the Bio-Rad Laboratories. Sephadex G-50 was obtained from Pharmacia, Inc., Piscataway, N.J. All thin-layer chromatography was performed on commercially prepared silica gel plates (chromatogram sheets 6060, Eastman Organic Chemicals). Squares (side = 2.5 cm) of Whatman DE81 Chromedia paper were cut from 46 × 57 cm sheets obtained from the H. Reeve Angel and Co., Inc., Clifton, N.J. All other chemicals were of analytical grade.

Results

Characterization of Vinblastine-Binding Sites on Chick Embryo Brain Tubulin

Dependence on Time, Temperature, and Ionic Strength. The binding of low concentrations of vinblastine (between 0.1 and 10 μM) was rapid at all temperatures between 0 and 37°C. Binding increased to a plateau within 5–10 min both at 0 and at 37°C. In addition, binding was not strongly dependent upon temperature. At 0°C, maximum binding values after incubation for 30–60 min were only 10–20% lower than those obtained at 37°C (data not shown). Vinblastine binding to chick embryo brain tubulin was not very dependent upon ionic strength. Vinblastine-binding activity was depressed slightly as the concentration of NaCl was increased between 25 and 250 m*M* (data not shown). Since a salt concentration of 150 m*M* is optimal for stabilization of the colchicine binding activity of purified chick embryo brain tubulin (Wilson, 1970, and unpublished data), this concentration of NaCl was used in most binding experiments.

Competitive Inhibition of Vinblastine Binding to Chick Embryo Brain Tubulin by Other Active Vinca Alkaloids. The binding of vinblastine was inhibited in a competitive manner by other biologically active vinca alkaloids. Results of an experiment demonstrating the competitive inhibition of vinblastine binding activity by vincristine are shown in

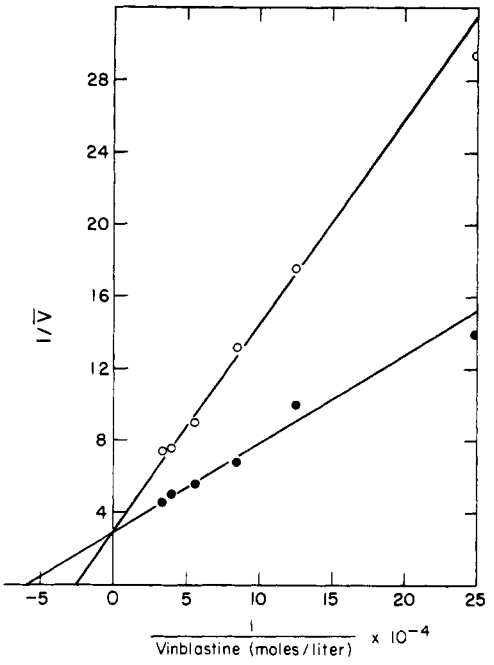

FIGURE 1: Inhibition of vinblastine binding by vincristine to freshly purified chick embryonic brain tubulin. Aliquots containing 570 μg of freshly purified tubulin in 20 mM sodium pyrophosphate–150 mM NaCl (pH 7.0) were incubated with different concentrations of labeled vinblastine in the absence (●) and in the presence (○) of 1.0×10^{-5} M vincristine sulfate for 30 min at 37°C (total volume = 500 μl). Bound vinblastine was determined by the paper disc assay procedure (see Methods). The inhibition constant (K_i) for vincristine was 1.7×10^{-5} M.

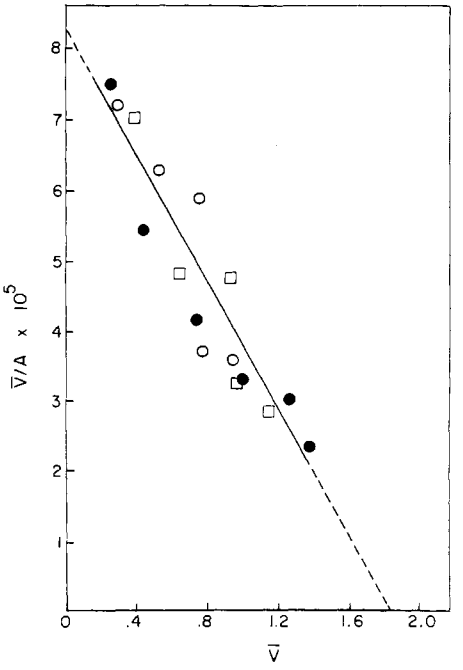

FIGURE 2: Affinity and number of binding sites for vinblastine from freshly purified embryonic chick brain. Aliquots, 80 mg of Sephadex G-50 beads, were placed in 13 × 100 mm tubes and swollen by the addition of 0.5 ml of 150 mM NaCl–5 mM $MgCl_2$–50 mM sodium pyrophosphate (pH 7.0). To each bead suspension was added 0.25 ml of the above buffer containing desired quantities of labeled vinblastine, and the suspensions were equilibrated at 4°C for up to 20 hr before use. Tubulin solutions in the above buffer, 0.25 ml, were added to each tube, and the suspensions were incubated with intermittent agitation for 2 hr at 4°C. Aliquots of the supernatant fraction, 50 μl, were removed for determination of labeled vinblastine. Tubes containing no tubulin were employed for determination of the control distribution coefficient of each vinblastine concentration. The different symbols represent three different tubulin preparations, purified in identical fashion. One tubulin sample (●) contained prebound colchicine. Data were plotted in the form described by Scatchard (1949). $\bar{V}$ = mol of vinblastine bound per mol of tubulin. [A] = free vinblastine (mol/l.). The equation for the regression line is $\bar{V}/[A] = 8.19 \times 10^5 - 4.49 \times 10^5 \bar{V}$. The coefficient of determination (r^2) = 0.8165, n = 16.

Figure 1. The inhibition constant for vincristine in this experiment was 1.7×10^{-5} M. Another active derivative, desacetylvinblastine, also competitively inhibited the binding of vinblastine, with a similar inhibition constant of 2×10^{-5} M (data not shown).

Affinity Constant and Number of Binding Sites. The affinity of vinblastine for freshly purified chick embryo brain tubulin was determined with the use of the equilibrium Sephadex gel procedure and a Scatchard analysis (Scatchard, 1949), as shown in Figure 2. A straight line was obtained indicating a single affinity class of sites, and the binding constant calculated from the slope of the line was 4.5×10^5 l./mol (4°C). Considerable variation in the apparent binding constant occurred depending upon the age of the purified tubulin. Storage of the tubulin frozen (in liquid nitrogen) for as little as 12 hr reduced the affinity for vinblastine to 2.4×10^4 l./mol (37°C). Storage under these conditions in the presence of 0.5 mM GTP or 0.72 mol of colchicine bound per mol of tubulin did not affect the loss of affinity for vinblastine. A slightly lower affinity of $1\text{–}3 \times 10^5$ l./mol was obtained utilizing the DE81 paper disc assay procedure (data not shown). Temperature between 0 and 37°C had very little effect on the binding constant. In one experiment utilizing the DE81 filter disc assay, identical binding constants of 1.7×10^5 l./mol were obtained both at 0 and at 37°C with freshly purified tubulin.

There appear to be two vinblastine sites per mole of tubulin, as determined by the equilibrium Sephadex gel procedure (Figure 2). The number of vinblastine-binding sites per mole of tubulin was also determined independently by the equilibrium gel filtration procedure of Hummel and Dreyer (1962). The mean value for mole of vinblastine bound per mole of tubulin in two experiments (two peak protein fractions per experiment; see Figure 5) was 1.9. Thus, there seem to be two vinblastine sites per dimer on embryonic chick brain tubulin.

The value obtained for the number of binding sites per mole of tubulin with the DE81 filter disc assay was 0.45 (data not shown). This low value is similar to that obtained by Owellen et al. (1972). The major disadvantage of the filter disc assay is that the free vinblastine is separated from the vinblastine–tubulin complex. Since the vinblastine is not very tightly bound to the tubulin, a considerable quantity of the previously bound vinblastine dissociates from the complex during the assay, thus giving rise to the low stoichiometry. The use of this procedure to obtain an apparent binding constant does seem to be valid, at least within a factor of 2 as compared with the equilibrium method, and depends upon the assumption that there is a single dissociation rate constant for loss of vinblastine at all tubulin–vinblastine complex concentrations.

Relationship between Vinblastine Binding Sites, and Colchicine and Guanine Nucleotide Binding Sites. Incubation of freshly purified tubulin with 2 mM GTP did not influence the binding of vinblastine. Similarly, vinblastine binding to a tubulin colchicine complex (0.7 mol of colchicine bound/mol of tubulin) was identical with that of tubulin without bound colchicine. The inclusion of 1 mM $CaCl_2$

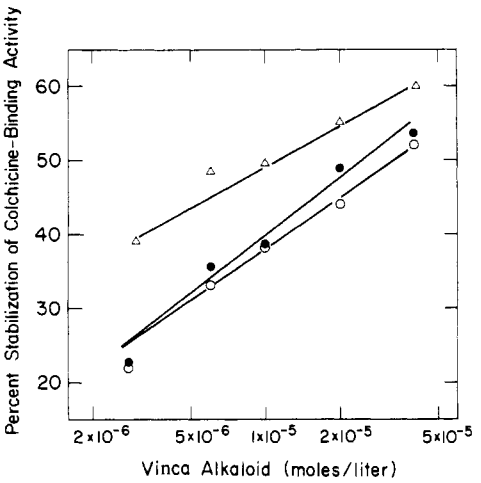

FIGURE 3: Stabilization of colchicine binding activity of purified embryonic chick brain tubulin by vinca alkaloids. Tubes containing freshly purified embryonic chick brain tubulin (271 μg) in 20 mM sodium pyrophosphate–140 mM NaCl (pH 7.0) were incubated for 3 hr at 37° with increasing concentrations of vinblastine, vincristine, or desacetylvinblastine in a total volume of 525 μl. The colchicine binding activity in each tube was determined after an additional incubation with [*acetyl*-^{3}H]colchicine for 1 hr at 37°C. A control aging experiment was performed without any vinca alkaloid to determine the initial colchicine binding activity and the decay rate was described previously (Bamburg et al., 1973). The percent of binding activity remaining after 3-hr incubation in the presence of the vinca alkaloids was compared with the unstabilized control, and the initial binding activity of the tubulin. The half-time for loss of colchicine binding activity of the unstabilized tubulin was 114 min. Bound colchicine was assayed by the Bio-Gel P10 gel filtration assay procedure (Bamburg et al., 1973). (○) Desacetylvinblastine; (●) vinblastine; (△) vincristine.

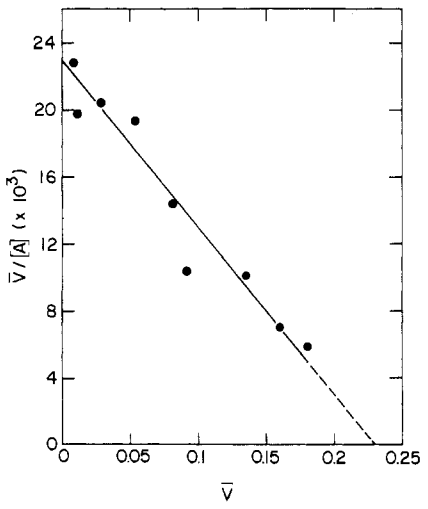

FIGURE 4: Affinity of vinblastine for tubulin solubilized from sea urchin sperm tail outer doublet microtubules. Tubes containing 246 μg of tubulin solubilized from sea urchin sperm tail outer doublet microtubules in 20 mM sodium phosphate buffer–100 mM sodium glutamate (pH 6.8) were incubated with different concentrations of [*acetyl*-^{3}H]vinblastine for 30 min at 22°C (total volume = 300 μl). Bound vinblastine was determined by the paper disc assay procedure (see Methods). $\bar{V}$ = mol of vinblastine bound/mol of tubulin (not corrected for approximately 15% impurity). [A] = free vinblastine concentration (mol/l.). The equation for the regression line is $\bar{V}/[A] = 22.5 \times 10^3 - 98 \times 10^3 \bar{V}$. The coefficient of determination (r^2) = 0.94.

also had no effect on the binding of vinblastine to chick embryo brain tubulin. Thus, the vinblastine binding sites, the colchicine binding site, and the two guanine nucleotide binding sites are all different, as well as any calcium binding sites that may be on the tubulin.

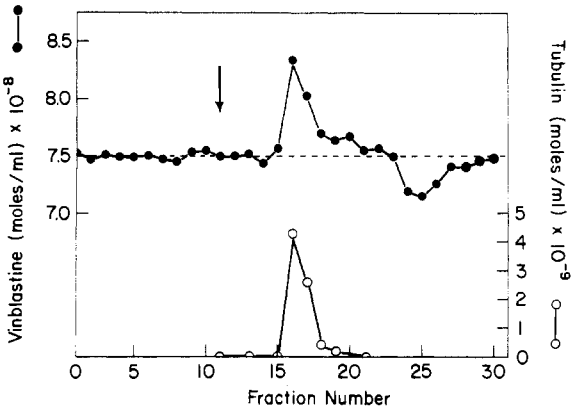

FIGURE 5: Equilibrium gel filtration. Binding of [*acetyl*-^{3}H]vinblastine to tubulin from sea urchin sperm tail outer doublet microtubules. Equilibrium gel filtration was carried out as described under Methods. Solubilized outer doublet tubulin, 1.010 mg, was applied, and the column run at a vinblastine concentration of 7.5×10^{-5} M. The arrow denotes application of the tubulin. (●) Labeled vinblastine; (○) tubulin. Fractions 16 and 17 contained 1.9 and 2.0 mol of vinblastine bound/mol of tubulin. A small correction of binding values to saturating vinblastine concentration (utilizing a binding constant of 1.0×10^5 l./mol; Figure 4) yielded values of 2.2 and 2.3 mol of vinblastine bound per mol of tubulin, for fractions 16 and 17, respectively.

Relationship between Vinca Alkaloid Binding Affinities for Tubulin, and Abilities to Stabilize Colchicine Binding Activity. The colchicine binding activity of tubulin is unstable, and decays according to first-order kinetics with half-times that vary considerably depending upon conditions in solution (e.g., pH, ionic strength, temperature) and the concentration of "active" (colchicine-binding) tubulin (Weisenberg et al., 1968; Wilson, 1970; Bamburg et al., 1973). Addition of vinblastine or vincristine to solutions of tubulin prevent this decay (Wilson, 1970; Wilson and Meza, 1973). The three active vinca alkaloids, vinblastine, vincristine, and desacetylvinblastine, all bind to tubulin with very similar affinities. If this interaction of vinca alkaloids is responsible for the stabilizing effect, then the three derivatives should have approximately the same ability to stabilize the colchicine binding activity of chick embryo brain tubulin. This appears to be the case as shown in Figure 3. Vinblastine, desacetylvinblastine, and vincristine stabilize the colchicine binding activity of purified chick embryo brain tubulin in a concentration-dependent manner. The concentrations of the three derivatives that stabilize by 50% are 1.1×10^{-5} M; 2.5×10^{-5} M; and 3.3×10^{-5} M; for vincristine, vinblastine, and desacetylvinblastine, respectively. These values agree approximately with the apparent affinities of these derivatives for tubulin. Another vinca alkaloid, leurosidine, which is 100-fold less potent than vinblastine in its ability to inhibit mitosis in cultured EHB cells (Creswell, 1972) is 100-fold weaker than vinblastine in stabilizing the colchicine binding activity of tubulin, and catharanthine, a still less active vinca alkaloid derivative, is tenfold weaker than leurosidine (data not shown).

[acetyl-^{3}H]Vinblastine Binding Activity of Tubulin Solubilized from Stable Outer-Doublet Microtubules. Addition of vinblastine to tubulin solubilized from purified sea urchin sperm tail outer-doublet microtubules stabilized the colchicine binding activity of the tubulin (Wilson and Meza, 1973) suggesting that high affinity vinblastine-binding sites were present. That vinblastine can bind to this tubulin with an affinity similar to that for embryonic chick brain tubulin is shown in a Scatchard analysis (Figure 4). A

linear plot was obtained with an association constant of 1.0 $\times 10^5$ l./mol at 37°C. Vinblastine–tubulin stoichiometry was determined by equilibrium gel filtration (Figure 5). The molar ratio in the two tubulin peak fractions, 16 and 17, was 2.2 and 2.3 mol of vinblastine/mol of tubulin. Thus, similar to embryonic chick brain tubulin, there seem to be two vinblastine binding sites/mol of tubulin. Similar to the results with the binding of vinblastine to chick embryo brain tubulin, the binding of 2.9×10^{-6} *M* vinblastine to outer doublet tubulin was rapid, reaching a plateau within 10 min; and was not significantly dependent upon temperature, between 0 and 37°C.

Lack of Vinblastine Binding to Intact Outer-Doublet Microtubules. Similar to results with colchicine (Wilson and Meza, 1973), vinblastine does not depolymerize outer doublet microtubules. For example, outer-doublet microtubules suspended in 20 m*M* sodium phosphate (pH 6.8) were partially solubilized by warming at 37°C for 2.5 hr in the presence and absence of 8.9×10^{-5} *M* vinblastine. Before dissolution, the quantity of tubulin contained in the microtubules was 463 (±28) μg of protein/ml. After dissolution, control microtubules (no vinblastine) had 370 (±23) μg of protein/ml remaining, while microtubules depolymerized in the presence of vinblastine contained a similar quantity of tubulin (360 ± 22 μg of protein/ml). Thus, vinblastine did not increase the rate of dissolution of the microtubules.

Once it was established that tubulin solubilized from outer doublet microtubules possessed vinblastine binding sites, it became meaningful to determine whether the sites were accessible to vinblastine in assembled microtubules. Six identical 500-μl aliquots containing suspended purified outer-doublet microtubules were incubated with 1.03×10^{-5} *M* [*acetyl*-^{3}H]vinblastine for 30 min at 21°C. The microtubules were then pelleted by centrifugation at 8000*g* for 10 min, and the amount of vinblastine bound to them was determined by measuring the amount of radioactivity remaining in the supernatant fraction. The protein concentration in the experiment was 1.14×10^{-5} mol/l., therefore, there was an approximately equal concentration of vinblastine and tubulin. No detectable vinblastine was removed from the supernatant fraction (pelleted along with the outer doublet microtubules). Therefore there was not detectable binding of vinblastine to the microtubules. Identical results were obtained in a second similar experiment. This argues that either the vinblastine binding sites are not on the surface of the microtubules, or that if they are on the surface, the affinity for vinblastine is greatly decreased when the tubulin is assembled. If the affinity has been decreased, the highest possible binding constant can be estimated by substituting the pipetting error value for the quantity of vinblastine that could have been bound in this experiment. The value comes out to be 5×10^2 l./mol. Therefore, if the binding site is on the surface of the microtubule, its affinity must be reduced at least by a factor of 1000.

Discussion

High Affinity Vinblastine-Binding Sites. There are two biologically specific vinblastine binding sites/tubulin molecule, with apparent binding affinities with freshly prepared embryonic chick brain tubulin of $3\text{–}5 \times 10^5$ l./mol. These sites are on different areas of the tubulin molecule than the two guanine nucleotide sites, and the colchicine binding site. The characteristics of the vinblastine-binding reaction to tubulin differ significantly from those described for the colchicine binding reaction to tubulin (Borisy and Taylor, 1967a,b; Wilson and Friedkin, 1967; Wilson, 1970; Bryan, 1972b). In contrast with the binding of colchicine, the binding of vinblastine to embryonic chick brain tubulin is very rapid, and relatively independent of temperature between 0 and 37°C. Vincristine and desacetylvinblastine, vinca alkaloid derivatives which possess the ability to disrupt microtubules in cells, compete with vinblastine for these sites.

The complex which forms between chick embryo brain tubulin and vinblastine is not very stable. In contrast to colchicine–tubulin complexes, passage of a vinblastine–tubulin complex through a short gel-filtration column (1 × 18 cm, Bio-Gel P10), or assay by the DE81 paper disc assay procedure results in loss of more than half of the originally bound vinblastine. The binding affinity obtained with chick brain tubulin is lower than that reported by Owellen et al. (1972) with porcine brain tubulin. These investigators obtained a binding constant of 6×10^6 l./mol at 37°. However, the vinblastine–porcine brain–tubulin complex is stable during passage through a gel filtration column (Owellen et al., 1974). Thus, the affinities of vinblastine for tubulins from diverse sources might be different. Both with chick embryo brain tubulin and porcine brain tubulin (Owellen et al., 1972), the vinblastine binding activity decays upon aging. With chick embryo brain tubulin, the decay is accompanied by a decrease in the binding affinity. Thus, differences in decay rates could also be responsible for the differences in binding affinities between our results and those of Owellen and his coworkers. In all cases in this work, vinblastine binding characteristics were determined utilizing freshly purified tubulin which was no more than 4 hr old after tissue disruption. The loss of binding affinity for vinblastine upon storage of the tubulin has not yet been characterized further.

There is a large difference between the concentrations of the active vinca alkaloids which half-maximally saturate the tubulin sites ($\sim 3 \times 10^{-6}$ *M*) and the concentrations which cause disruption of microtubules in mammalian cells ($\sim 7\text{–}8 \times 10^{-8}$ *M*). This phenomenon is due to a striking "poisoning effect" of the vinca alkaloids on microtubule polymerization (Wilson et al., 1974) which will be described in detail in a subsequent report (K. Anderson, K. Creswell, and L. Wilson, manuscript in preparation).

Binding of Vinblastine to Sea Urchin Sperm Tail Outer Doublet Tubulin: Location of Vinblastine-Binding Sites on Intact Microtubules. Sea urchin sperm tail outer doublet microtubules are stable, and are completely resistant to dissolution by colchicine and vinblastine. Yet with colchicine, the tubulin, once solubilized, has been shown to possess a colchicine binding site. These findings permitted us to determine whether the colchicine binding site was free (available to bind colchicine) in intact microtubules. Since no colchicine binding activity could be detected in the intact microtubules, we argued that the tubulin was oriented in the microtubule with the colchicine binding site not exposed on the surface of the microtubule (Wilson and Meza, 1973). An alternative possibility is that the site is on the surface of the microtubule, but when the microtubule is assembled, a conformational change had taken place resulting in a 500–1000-fold decrease in the affinity for colchicine. In either case, it is unlikely that colchicine can directly disrupt preformed microtubules by binding to the microtubules. An identical approach was taken with the high affinity vinblastine-binding sites utilizing solubilized outer-doublet microtubules in this study. Our results have shown that the

outer doublet tubulin, once solubilized, possess two vinblastine binding sites with apparent affinities close to those found for embryonic chick brain tubulin (Figures 4 and 5). Incubation of intact outer doublet microtubules with vinblastine, similar to the situation with colchicine, resulted in no detectable binding of vinblastine. The same interpretations with the vinblastine binding sites apply as in the case of the colchicine binding site. Namely, either a conformational change occurred during assembly of the tubulin resulting in a 1000-fold decrease in affinity, or the vinblastine binding sites are not distributed along the surface of the microtubule. In either case, the results imply that vinblastine does not have a high affinity for assembled microtubules, and probably does not directly destroy the assembled structures. Thus, vinblastine, like colchicine, must disrupt microtubules in cells primarily by blocking the polymerization reaction. This would result in the ultimate depolymerization of labile microtubules, which appear to be in equilibrium with subunit pools (Inoue and Sato, 1967; Olmsted and Borisy, 1973). More stable microtubules such as outer doublet microtubules of cilia and flagella would not be destroyed by this class of drugs.

The method employed in this study is not sensitive enough to detect the possible binding of vinblastine to the ends of these microtubules. Thus, another possible mechanism which cannot be eliminated would involve the binding of vinblastine to an end tubulin molecule. While this mechanism may be very important in inhibition of microtubule assembly, in order for vinblastine to cause that tubulin molecule to dissociate more rapidly than normal from the end of a microtubule, the binding of vinblastine to that tubulin molecule would have to cause a conformational change resulting in a decreased affinity for the remaining polymer. We feel this is unlikely, since in vitro polymerized microtubules in embryonic chick brain extracts are not depolymerized by the addition of 5×10^{-5} *M* vinblastine over a 60-min period (K. Anderson, K. Creswell, and L. Wilson, to be published).

Acknowledgments

We thank Mrs. Leslie Schenker for valuable technical assistance.

References

Bamburg, J. R., Shooter, E. M., and Wilson, L. (1973), *Biochemistry 12,* 1476.

Bensch, K. G., and Malawista, S. E. (1969), *J. Cell Biol. 40,* 95.

Bensch, K. G., Marantz, R., Wisniewski, H., and Shelanski, M. L. (1969), *Science 165,* 495.

Borisy, G. G. (1972), *Anal. Biochem. 50,* 373.

Borisy, G. G., and Taylor, E. W. (1967a), *J. Cell Biol. 34,* 525.

Borisy, G. ., and Taylor, E W. (1967b), *J. Cell Biol. 34,* 535.

Bray, G. A. (1960), *Anal. Biochem. 1,* 279.

Bryan, J. (1971), *Exp. Cell Res. 66,* 129.

Bryan, J. (1972a), *J. Mol. Biol. 66,* 157.

Bryan, J. (1972b), *Biochemistry 11,* 2611.

Bryan, J., and Wilson, L. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1762.

Creswell. K. M. (1972), M.S. Thesis, Stanford University.

Eipper, B. A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2283.

Greenius, H. F., McIntyre, R. W., and Beer, C. T. (1968), *J. Med. Chem. 11,* 254.

Hirose, M., and Kano, Y. (1971), *Biochim. Biophys. Acta 251,* 376.

Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63,* 530.

Inoue, S., and Sato, H. (1967), *J. Gen. Physiol. 50, Suppl.,* 259.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Luduena, R. F., and Woodward, D. O. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3594.

Marantz, R., Ventilla, M., and Shelanski, M. (1969), *Science 165,* 478.

Margulis, L. (1973), *Int. Rev. Cytol. 34,* 333.

Nagayama, A., and Dales, S. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 464.

Olmsted, J. B., and Borisy, G. G. (1973), *Annu. Rev. Biochem. 42,* 507.

Owellen, R. J., Donigian, D. W., and Kuhar, M. J. (1974), *Cancer Res. 34,* 3180.

Owellen, R. J., Owens, A. H., Jr., and Donigian, D. W. (1972), *Biochem. Biophys. Res. Commun. 47* 685.

Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.

Schochet, S. S., Jr., Lambert, P. W., and Earle, K. M. (1968), *J. Neuropathol. Exp. Neurol. 27,* 645.

Weisenberg, R. C., Borisy, G. G., and Taylor, E. W. (1968), *Biochemistry 7,* 4466.

Wilson, L. (1970), *Biochemistry 9,* 4999.

Wilson, L., Anderson, K., and Creswell, K. (1974), *J. Cell Biol. 63,* 373a.

Wilson, L., and Bryan, J. (1974), *Adv. Cell Mol. Biol. 3,* 21.

Wilson, L., Bryan, J., Ruby, A., and Mazia, D. (1970), *Proc. Natl. Acad. Sci. U.S.A. 68,* 807.

Wilson, L., and Friedkin, M. (1966), *Biochemistry 5,* 2463.

Wilson, L., and Friedkin, M. (1967), *Biochemistry 6,* 3126.

Wilson, L., and Meza, I. (1973), *J. Cell Biol. 58,* 709.