THE BINDING OF VINCRISTINE, VINBLASTINE AND COLCHICINE TO TUBULIN

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SUMMARY

Preparations of tubulin were examined for their ability to bind vincristine, vinblastine, and colchicine, as measured by adsorption on DEAE impregnated filter paper. Vincristine and vinblastine were found to bind very rapidly with tubulin (<5 min), while colchicine took considerably longer (>4 hr). When varying concentrations of the alkaloids were employed, and the data examined on a Scatchard plot, it was found that colchicine had an association constant of 1.8×10^6 liters/mole, while vinblastine and vincristine had constants of 6.0×10^6 liters/mole and 8.0×10^6 liters/mole respectively. In addition, it was found that the ratio of molar binding of colchicine was always twice that of vinblastine or vincristine.

INTRODUCTION

Microtubule rich structures are found in many types of cells.[2] By appropriate treatment, it is possible to isolate a protein component or population of components, tubulin, purported to be the subunit of microtubular structures.[2, 3] Tubulin preparations have a molecular weight of about 120,000 and are reported to bind one mole of colchicine (CLC) per 120,000 grams of tubulin.[2, 3] At present the precise composition of these protein preparations is not clear.[4]

This class of protein is also known to interact with the antitumor agents vincristine (VCR) and vinblastine (VLB).[5-7] Depending upon concentration, these

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drugs cause both disruption and aggregation *in vivo* of microtubular structures.[8-11] In particular, we are working under the hypothesis that disruption of mitotic spindles and neurotubular components is related to the oncolytic and neurotoxic properties of these drugs. *In vitro*, they may cause stabilization and/or precipitation of isolated tubulin.[12-14]

We have undertaken to characterize more precisely the nature of the interaction of CLC, VLB, and VCR with tubulin using porcine brain as a protein source. We have examined the *in vitro* binding of tritium labeled drugs using adsorption of protein-alkaloid complex to DEAE filter paper as an assay. This technique allowed us to determine both the association constant and the molar ratio of binding sites for each alkaloid.

MATERIALS AND METHODS

Microtubular protein was prepared from porcine brain by the batch technique of Taylor *et al.*[3] except for the substitution of DEAE microgranular cellulose (20 grams of Whatman preswollen cellulose per 500 grams of brain) for DEAE Sephadex. The final purified tubulin was dissolved in 0.01 M Na_2HPO_4/NaH_2PO_4, 0.1 M sodium glutamate, 0.1 mM GTP buffer at pH 6.5, and clarified just prior to use by centrifugation at 100,000 rcf for 30 min. Protein concentration was measured by the method of Lowry.[15]

^{3}H-VLB (169 mCi/mM) was prepared by the method of Beer *et al.*,[16] and ^{3}H-VCR (32.4 mCi/mM) was prepared by the method of Owellen *et al.*[17] ^{3}H-CLC (159 mCi/mM) was purchased from New England Nuclear.

To 25 µl of protein solution was added 10 to 200 µl of radioactive alkaloid solution and sufficient buffer (0.01 M NaH_2PO_4/Na_2HPO_4, 10^{-4} M guanosine triphosphat and 0.1 M sodium glutamate, pH 6.5) to give a final volume of 1.0 ml. Protein concentration was constant within any experiment and was usually about 50 µg/ml. Final alkaloid concentration ranged from 10^{-7} to 10^{-5} M. This mixture was incubated 30 min at 37° C for VLB and VCR experiments and 4 hr for CLC experiments. The extent of binding was then determined by the filter assay technique,[3] whereby the incubate was filtered through DEAE impregnated filter paper at 4° C, followed

by a 10 ml rinse with the above buffer under mild suction. Blanks were determined at each concentration level by using labeled alkaloid and age-denatured protein. The filter paper with adsorbed tubulin-alkaloid complex was then counted directly in 5 ml of Bray's scintillation fluid.

RESULTS

The first parameter investigated was the time required to achieve maximum binding of each alkaloid to the tubulin, and this data is displayed in Figure 1.

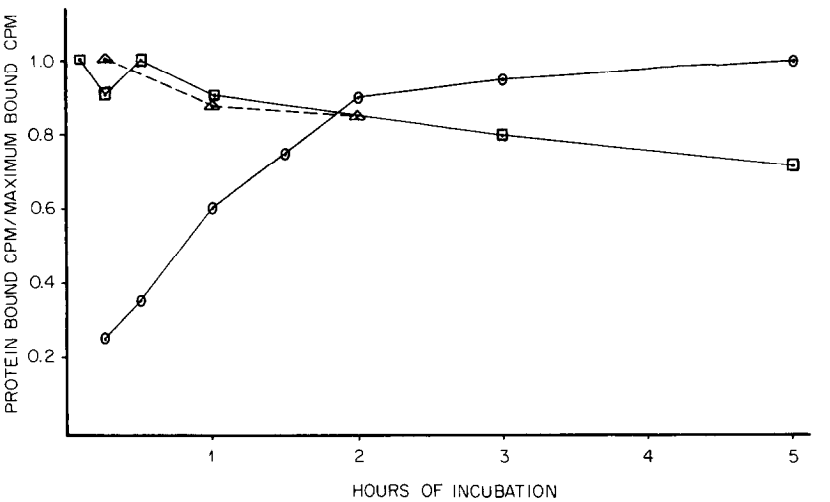

Figure 1. Binding of VLB, VCR and CLC to tubulin.
□ = VLB, Δ = VCR, 0 = CLC

The binding of ^{3}H-CLC was found to approach a maximum value in 4 hr,[cf. 18] while VLB and VCR rapdily reached their maximum values in <5 min. In fact, in one experiment, VLB was found to be maximally bound after 15 min of incubation at 0° C. At 4 hr, the amount of bound CLC radioactivity was still slowly rising, but longer incubation times (in separate experiments) resulted only in very small further increments. It was from this data that we derived our incubation times of 4 hr for CLC and 30 min for VLB and VCR.

It has been found that tubulin denatures fairly rapidly at 37° C, and that this loss is deterred by the presence of CLC, VLB, or VCR.[3, 18] Also, it has been shown that VLB and CLC have different binding sites.[6, 7, 12-13] We, therefore,

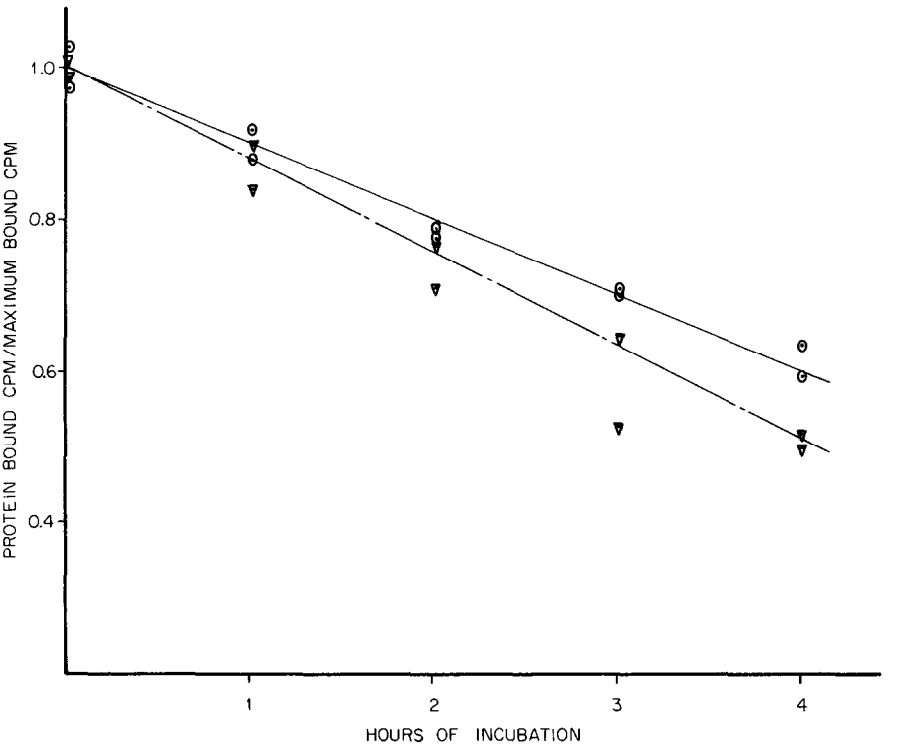

Figure 2. Decay of VLB binding to tubulin - CLC complex. O = 3 X 10^{-6} M CLC, ∇ = 3 X 10^{-7} M CLC

used ^{3}H-VLB to estimate the rate of denaturation of protein in the presence of CLC. In Figure 2, we see the rate of decay of VLB binding activity of protein that has been incubated in the presence of unlabeled CLC. This data is in agreement with observations of Wilson on the decay rate of CLC binding activity in the presence of CLC.[7] This information allowed us to correct all CLC binding data to zero time for the 4 hr incubations with ^{3}H-CLC.

When additional DEAE filter paper was employed, or when the volume of rinse was widely varied, no significant change in protein bound radioactive counts was detected. Recovery of 83% of the bound radioactivity from the filter paper was achieved using a dilute H_2SO_4 rinse. Subsequent thin layer chromatography of this material revealed unchanged VLB.

The data from incubation with varying concentrations of the alkaloids is displayed in Figure 3 as a Scatchard plot. A linear relationship indicates a

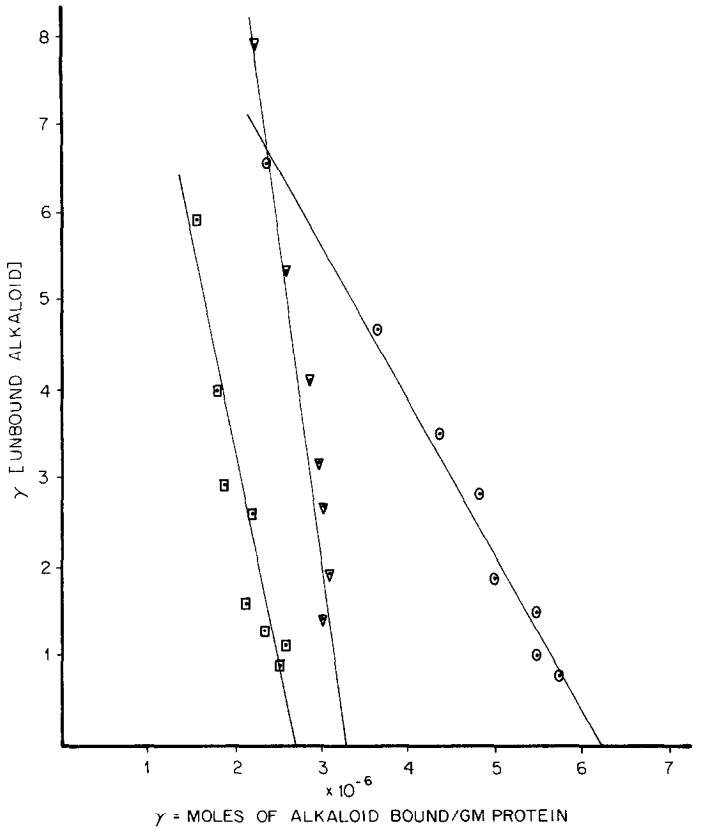

Figure 3. Scatchard plot of VLB, VCR and CLC tubulin binding. □ = VLB, ▽ = VCR, O = CLC

single association constant over the range measured, but per se does not preclude more than a single site of binding. Though in this experiment there is some scatter, when the data from many individual experiments were analyzed, it was obvious that a straight line relationship was present for all three alkaloids. The slope of this line is the negative of the association constant and the intercept on the abscissa is a measure of the number of binding sites per gram of protein. The CLC intercept, therefore, provides a measure of the specific activity of the tubulin preparation. The specific activity of our protein preparations, as assayed by CLC binding capability, is similar to those reported in the literature. We have not independently measured the molecular weight of this protein or determined the electrophoretic mobility of its subunit(s), but if

we assume that the molecular weight is 120,000 gm/mole,[3] we can estimate that 0.75 moles of CLC are bound per mole of protein. In many other experiments, slightly higher and lower tubulin specific activities were obtained, but in no case were the association constants or binding ratios between CLC and VLC-VCR significantly altered.

The association constant of CLC is calculated to be 1.8 X 10^6 liter/mole and is in rough agreement with prior *in vivo* data.[19] The association constants for VLB and VCR are significantly higher than that of CLC with values of 6.0 X 10^6 liter/mole and 8.0 X 10^6 liter/mole respectively. We find it most interesting that these values correlate with the relative ability of the alkaloids to produce clinical neurotoxicity, in the order VCR>VLB>CLC.

When the molar extent of binding is examined, we see that VLB and VCR bind to almost exactly 1/2 the number of sites per gram of tubulin as CLC. This may be related to Weisenberg's observation of dimerization of a 120,000 MW preparation wherein one molecule of VLB was associated with a 240,000 molecular weight species.[14]

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