Precipitation of Proteins by Vinblastine and Calcium Ions*

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Abstract. We have found that vinblastine sulfate can precipitate a number of proteins in addition to microtubule protein. Those proteins which precipitate with vinblastine sulfate, all of which were derived from structure, also precipitate with Ca^{2+} ions. Our results suggest that vinblastine sulfate, presumably acting as a cation, precipitates proteins by combining with sites which can also combine with Ca^{2+} ions.

Introduction. The vinca alkaloid vinblastine (VBL) is considered to exert its antimitotic effects through an action on microtubules. The disruptive effects of VBL on microtubules from various sources have been described in a number of cytological investigations.[1-7] Evidence that it interacts directly with microtubule proteins in physiological concentrations provides additional strong support for the hypothesis.[8-10] Furthermore, high concentrations of VBL are capable of precipitating at least one kind of microtubule protein, colchicine-binding protein, from cell-free extracts, and VBL has been employed as a tool for the purification of microtubule proteins.[11,12] However, several biochemical effects of the vinca alkaloids have been reported which may not be related to the ability of these agents to disrupt microtubule structures. One such effect is aggregation of polyribosomes in eucaryotic cells[13] and also in a procaryotic organism, *E. coli*,[14] which is considered to contain no microtubules. Other effects include inhibition of DNA and RNA synthesis.[15-18]

It will be shown that precipitation by VBL is not restricted to microtubule proteins, but is a property of a number of proteins, all of which are derived from cell structure. It will also be shown that the same proteins are precipitated by calcium ions.

Materials. Vinblastine sulfate was a gift from the Eli Lilly Co. The preparation of purified colchicine labeled with tritium in its acetyl moiety has been described previously[19] (spec. act. = 240 mCi/mmole). ^{45}Ca was obtained from Tracerlab (spec. act. = 0.224 Ci/mole).

Supernatant extracts (100,000 × *g*) of chick embryo brains served as the source of microtubule subunit protein (mol wt = 115,000 ± 5000). Embryos at stages between 12 and 18 days of development were used. The biochemical properties of the subunit protein and the characteristics of its colchicine-binding activity will be described in a separate communication.[10]

Erythrocyte membrane proteins were prepared according to the method of Mazia and Ruby.[20] This preparation represents about 70% of the proteins of beef erythrocyte ghosts dissolved in water.

Native ADP actin was a gift from Dr. Betsy Clark. Denatured actin was prepared from native actin by exhaustive dialysis to remove nucleotide.

The "crystalline protein" from fertilization membranes of the sea urchin *Strongylocentrotus purpuratus* was isolated as described by Bryan.[21] This protein forms sheets of tubular elements after ejection from the cortical granules at the time of fertilization.

Hyaline layer protein from *S. purpuratus* eggs was prepared according to the techniques of Yazaki.[22] This is a protein of the hyaline layer which invests the fertilized sea urchin eggs.

Lysozyme was obtained from Nutritional Biochemicals Corporation; bovine plasma albumin, from Pentex; bovine serum albumin (Grade A), from Calbiochem; and bovine β-lactoglobulin, from Armour.

Methods. Colchicine (acetyl-³H) binding and assay: The colchicine-binding reaction occurred in 20 mM sodium phosphate buffer, pH 6.7–6.8, containing 100 mM sodium glutamate, or in 10 mM Tris-HCl pH 7.5. Tubes containing the protein extract were incubated with 2.5×10^{-6} M colchicine (acetyl-³H) for 2 hr at 37°C. Binding was determined by a modification of the method of Weisenberg *et al.*[23] Aliquots of incubation mixture (100 μl) containing free and bound colchicine were applied directly onto slightly moistened 2.5-cm discs of Whatman DE81 Chromedia paper at 0°C. After 10 min the paper discs were washed by immersion in five successive 30–40 ml changes of 10 mM sodium phosphate buffer (5 min each wash, pH 6.8, 0°C) to remove all unbound labeled colchicine. The paper with adhering protein-bound colchicine was counted in a scintillation vial containing 5 ml of Bray's counting fluid.[24] A description of the optimal conditions for the colchicine-binding reaction and assay will appear elsewhere.[10]

Protein: Protein was determined by the method of Lowry *et al.*[25] or by measurement of optical density at 280 nm in a Cary model 15 recording spectrophotometer.

Results. Precipitation of colchicine binding protein with vinblastine: The colchicine-binding protein found in supernatant extracts of brain and other tissues is thought to be a microtubule protein.[10,23,26,27] The addition of VBL to a 100,000 × *g* supernatant extract of chick embryo brain at 37°C containing previously formed colchicine-protein complex resulted in the rapid precipitation of the complex (Fig. 1). The amount of complex that precipitated depended
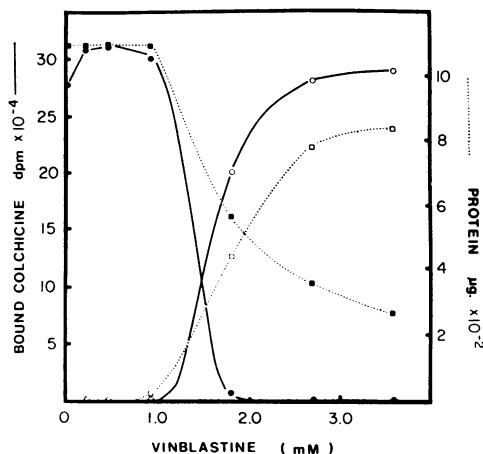

FIG. 1.—Precipitation of bound colchicine complex by vinblastine. A 100,000 × *g* supernatant extract of chick embryo brain (3.6 ml, 2.2 mg of protein/ml in 20 mM phosphate buffer, pH 6.8, containing 100 mM glutamate) was incubated with 2.5×10^{-6} M colchicine (acetyl-³H) for 2 hr at 37°C and the amount of bound colchicine complex was determined (see *Methods*). Increasing concentrations of VBL were then added to 500-μl aliquots of the supernatant extract, each containing 1.1 mg of protein and 277,400 dpm of bound colchicine in a total volume of 1.10 ml. After complete precipitation (37°C, 30 min) samples were centrifuged at 5000 × *g* for 10 min. The isolated precipitates slowly redissolved upon resuspension in fresh VBL-free buffer (1 hr, 0°.). Bound colchicine was determined, both in the redissolved precipitate and in the remaining supernatant extract, for each concentration of VBL. The increase in bound colchicine complex between 0 and 0.2 mM VBL was due to stabilization of the microtubule protein.[10] Protein was determined, after precipitation in 5% trichloroacetic acid, washing each protein residue twice with anhydrous methanol, and dissolving in 1 N NaOH.[28] ●, bound colchicine, VBL soluble; O, bound colchicine, VBL precipitable; ■, protein, VBL soluble; □, protein, VBL precipitable.

upon the concentration of VBL added to the extract; all of the previously formed complex precipitated with concentrations of this drug of more than 2×10^{-3} M. The VBL-precipitated material slowly redissolved when resuspended with stirring in fresh VBL-free buffer at 0°C. As seen in Figure 1, more than 95% of the bound colchicine complex originally present in the 100,000 $\times$ g supernatant extract was recovered in the redissolved precipitate and the colchicine was still bound to protein. In another experiment, precipitation of microtubule protein with 3×10^{-3} M VBL was performed prior to incubation of the extract with labeled colchicine. No colchine-binding activity was detected in the supernatant extract after the removal of the VBL precipitated material, and all of the expected colchicine-binding activity was found in the redissolved vinblastine sulfate precipitate. Therefore, in high concentrations, VBL can quantitatively precipitate colchicine binding protein from solution, whether or not colchicine is bound to it.

Maximum precipitation occurred near pH 7 and decreased markedly as the pH was lowered. Protein precipitation was favored by elevated temperatures. With 3.3×10^{-3} M VBL, VBL-precipitated material from brain supernatant extracts could be reversibly redissolved and precipitated by alternating the temperature between 0° and 37°C.

As shown in Figure 1, a considerable amount of the soluble protein present in the 100,000 $\times$ g brain extract precipitated along with the colchicine complex, and at high VBL concentrations (3×10^{-3} M), this was approximately 75% of the total protein originally present in the extract. The specific activity of the precipitated colchicine-complex was increased less than twofold even at the most selective concentration of VBL (Table 1). Therefore, vinblastine sulfate precipitation appears to be unsuitable for the purification of microtubule protein from chick embryo brain extracts. However, this is not necessarily true of other systems, since large quantities of other vinblastine-precipitable proteins may not be present in these systems.

Vinblastine precipitation of other proteins: A variety of other proteins was tested for VBL precipitability in order to determine the specificity of the VBL-

TABLE 1. *Precipitation of colchicine-complex by vinblastine from a 100,000 $\times$ g supernatant extract of chick embryo brain.**

Vinblastine concentration (M)	Total bound colchicine precipitated (dpm)	Total protein precipitated (μg)	Specific activity of precipitated bound-complex (dpm/μg protein)
0	0	0	...
1.8×10^{-4}	0	0	...
4.5×10^{-4}	0	0	...
9.0×10^{-4}	1,000	20	~50
1.8×10^{-3}	200,000	450	444
2.7×10^{-3}	279,100	790	353
3.6×10^{-3}	288,800	850	340

* See legend of Fig. 1 for details of the procedure. The original soluble brain extract contained 1100 μg of protein and 277,400 dpm of bound colchicine. The initial specific activity, defined as the dpm of bound colchicine/μg of protein present in the original extract, was 252 dpm/μg protein. The specific activity of the precipitated bound colchicine complex (dpm bound/μg of protein in the redissolved precipitate) appears in the last column.

TABLE 2. *Precipitation of proteins by vinblastine.**

Protein	Total protein before precipitation (mg or (dpm bound))	Total protein recovered after precipitation (mg or (dpm bound))		Percentage of recovered protein (or bound colchicine) precipitated
		Soluble	Precipitated	
Protein-bound colchicine (^{3}H)†	(300,000)	(0)	(289,000)	(97)
Chick brain protein†	1.10	0.27	0.85	76
Erythrocyte membrane proteins	1.45	0.16	1.37	90
Erythrocyte membrane proteins	3.02	...	3.20	100
Actin (native)	1.04	0.03	1.18	98
Actin (denatured)	1.02	0.04	1.07	97
"Crystalline protein"	1.02	0.05	1.03	96
"Crystalline protein"	...	0.07	1.71	96
Hyaline layer protein	0.57	0.03	0.59	95
Bovine serum albumin	3.00	2.11	0.82	28
Bovine plasma albumin	4.28	3.20	0.90	22
Lysozyme	2.00	1.40	0.05	<1
β-lactoglobulin	4.00	2.9	0.07	<3

* VBL, 3.3×10^{-3} M, was added to tubes containing the proteins to be tested in 10 or 20 mM sodium phosphate buffer, pH 6.8–7.1, in a total volume of 1.2 ml. Samples were incubated for 1 hr at 37°C, and the resulting precipitates were separated from the supernatant fractions after centrifugation at $5000 \times g$ for 10 min. The total protein in each supernatant fraction and in each precipitate was determined by one of the following methods:

Method 1: Precipitates were dissolved in 0.1 N NaOH. The supernatant fractions and redissolved precipitates were dialyzed a minimum of 36 hr to remove all associated VBL. The protein content of each was then determined.

Method 2: Supernatant fractions were precipitated in 5% perchloric acid. The original precipitate, and the perchloric acid precipitate obtained from the supernatant fraction, were extracted twice with absolute methanol. Protein residues were then assayed.

† Precipitation and analysis of protein and protein-bound colchicine from $100,000 \times g$ supernatant extracts of chick embryo brain were performed as described in the legend of Fig. 1.

precipitation reaction. The results are summarized in Table 2. Colchicine-binding protein, erythrocyte membrane proteins, actin, *S. purpuratus* "crystalline protein," and hyaline-layer protein were precipitated quantitatively by VBL. Only partial precipitation occurred with albumin, while the precipitation of lysozyme and β-lactoglobulin was negligible.

Calcium precipitation of proteins: Those proteins which completely precipitated with VBL exhibit certain other common features (see *Discussion*). Of special interest to us was the finding that all of them could be precipitated by calcium ions. The precipitation of proteins by Ca^{2+} was investigated in some detail with erythrocyte membrane proteins and with microtubule protein from chick embryo brains. The addition of Ca^{2+} to a solution of erythrocyte membrane proteins caused precipitation of the protein in a concentration-dependent manner. About 80–85% of the total protein precipitated at Ca^{2+} concentrations above 2×10^{-2} M. The concentration of Ca^{2+} required for maximum precipitation of the erythrocyte membrane proteins was about 10 times higher than was the concentration of VBL, and precipitation by Ca^{2+} was less complete than with this drug. Similar results were obtained with colchicine-binding protein from chick embryo brain extracts (Fig. 2). The addition of Ca^{2+} to a $100,000 \times g$ supernatant extract of chick embryo brain containing previously formed colchicine-protein complex resulted in a rapid precipitation of the complex. The amount of complex precipitated depended upon the Ca^{2+} concentration. About 65% of the total colchicine complex originally present in the extract precipi-

FIG. 2.—Calcium precipitation of bound colchicine complex. A $100,000 \times g$ supernatant extract of 12-day-old chick embryo brain (5.0 ml, 7.5 mg of protein/ml in 10 mM Tris-HCl, pH 7.5) was incubated with 2.5×10^{-6} M colchicine (acetyl-^{3}H) for 90 min at 37°C. This was then diluted with 10 mM Tris-HCl, pH 7.5 to a final protein concentration of 2.5 mg/ml. Increasing concentrations of $CaCl_2$ were added to 2.0 ml aliquots of the diluted supernatant extract which contained 100,000 cpm of bound colchicine. The final volume after Ca^{2+} addition was 3.0 ml. After complete precipitation (0°C, 60 min) samples were centrifuged at $10,001 \times g$ for 15 min.

The pellets were redissolved by mild sonication in 1.0 ml of 20 mM potassium phosphate buffer containing 10 mM EDTA, 5 mM GTP at pH 7.0. Bound colchicine and total protein were determined in both the redissolved pellet and supernatant fractions. ●, bound CLC, Ca^{2+} soluble; O, bound colchicine, Ca^{2+} precipitable; ■, protein, Ca^{2+} soluble; □, protein, Ca^{2+} precipitable.
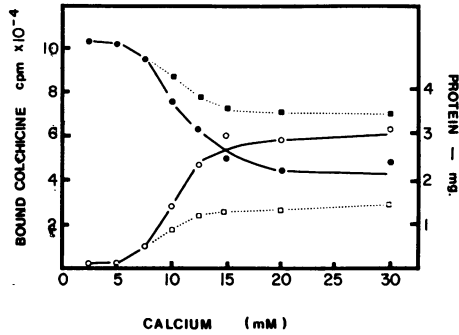
tated from solution with calcium concentrations higher than 20 mM. The Ca^{2+}-precipitated material immediately redissolved when resuspended in fresh Ca^{2+}-free buffer at 0°C. As was the case in the VBL experiment (Fig. 1), all of the colchicine complex that was lost from the supernatant extract was recovered in the redissolved precipitate, and the colchicine was still bound to protein. In another experiment, precipitation by Ca^{2+} was performed prior to binding colchicine to the protein, with similar results. Therefore, as with precipitation by VBL, Ca^{2+} precipitates microtubule protein whether or not colchicine is bound to the protein. Much of the soluble protein originally present in the $100,000 \times g$ extract coprecipitated with the colchicine-complex (28%), but this was less than that precipitated by VBL. Precipitation of colchicine

TABLE 3. *Precipitation of protein by calcium ions.**

Protein	Protein concentration mg/ml or (dpm bound)	Ca^{2+} concentration (mM)	Percentage of protein or precipitated (dpm bound)
Protein-bound colchicine (acetyl-^{3}H)[†]	(103,000)	30	(65)
Chick brain protein[†]	5.0	30	28
Erythrocyte membrane protein	1.4	20	82
Actin[‡]	5–10	1–2	100
"Crystalline protein"	1.0–1.5	20	80–85
Hyaline layer protein	1.0–1.5	20	80–85
Bovine serum albumin	1.0	50	<2
Bovine plasma albumin	1.0	50	<2
Lysozyme	1.0	50	<2
β-lactoglobulin	1.0	50	<2

* Calcium chloride was added to tubes containing the proteins to be tested in 10 mM Tris-HCl pH 7.5, in a total volume of 0.5 ml. Samples were incubated for 1–2 hr at 0°C, and the resulting precipitates were separated from the supernatant fractions after centrifugation at $5000 \times g$ for 10 min. Total protein in each supernatant fraction and in each precipitate was determined.

† Precipitation of protein and protein-bound colchicine from $100,000 \times g$ supernatant extracts of chick embryo brain was performed as described in the legend of Fig. 2.
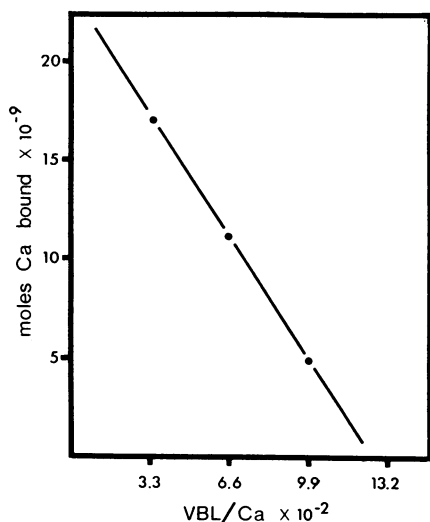
‡ Data of Martonosi.[3]

FIG. 3.—Effect of VBL concentration on binding of ^{45}Ca to erythrocyte membrane protein. Increasing concentrations of VBL were added to tubes containing 250 mg of erythrocyte membrane protein and 8.35 mM ^{45}Ca in 50 mM Tris-HCl, pH 7.6. After 60 min at 37°C, the precipitates were collected on glass fiber filters and washed several times with 10 ml of 0.5 M KCl-10 mM Tris-HCl, pH 7.6.

complex by Ca^{2+} did not vary with temperature between 0° and 37°C, but did exhibit a marked pH dependence with a maximum at pH 7.4.

All of the proteins surveyed for precipitation with VBL were tested for their ability to precipitate with Ca^{2+} ions. These results are summarized in Table 3. Precipitation of proteins by Ca^{2+} was generally not as complete as with VBL, but the specificity for precipitation of the various proteins tested was remarkably similar for both the drug and Ca^{2+}. For example, colchicine-binding protein was quantitatively precipitated by VBL, and only 65% precipitated by Ca^{2+}. Similarly, albumin was about 25% precipitated with VBL, while no precipitation was observed with Ca^{2+}, even though albumin binds Ca^{2+} ions.[29]

It is conceivable that Ca^{2+} and VBL precipitate proteins by a common mechanism. One possibility is that the drug acts as a cation. The results of the following experiments suggest that calcium and VBL are acting at the same sites.

Erythrocyte membrane protein was incubated with 8.4×10^{-7} M ^{45}Ca. The amount of ^{45}Ca bound to protein was then determined after precipitation of the protein with increasing concentrations of VBL (Fig. 3). A linear decrease in
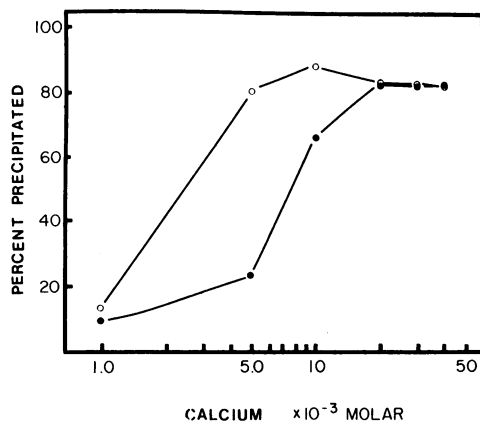

FIG. 4. Calcium precipitation of erythrocyte membrane protein in the presence and absence of vinblastine. Increasing concentrations of $CaCl_2$ were added to tubes containing 1.40 mg each of erythrocyte membrane protein in 10 mM TES buffer at 0°C (the pH of the buffer was adjusted to 7.1 at 37°C). In a second set of tubes, 9.5×10^{-4} M VBL was added immediately prior to the addition of Ca^{2+}. All other conditions were identical to the control. All tubes were then incubated for 1 hr at 37°C. After precipitation, samples were centrifuged at $5000 \times g$ for 10 min. Precipitates were washed twice by centrifugation in 2 ml of anhydrous methanol and dissolved in 1 N NaOH. The percentage of protein precipitated was determined at each concentration of Ca^{2+} for precipitation of erythrocyte membrane proteins in the presence (O) and in the absence (●) of VBL.

the amount of ^{45}Ca bound to the protein was obtained with increasing concentrations of VBL, suggesting a competition for the Ca^{2+} sites by this drug.

In another experiment, precipitation of erythrocyte membrane protein was carried out with increasing concentrations of Ca^{2+} in the presence and absence of 9.5×10^{-4} M VBL. This concentration of VBL was just below the threshold for precipitation of protein by the drug (Fig. 4). The s-shaped log-concentration versus precipitation curve for Ca^{2+} was shifted to lower concentrations of Ca^{2+} in the presence of the VBL, but the maximum amount of precipitable protein was not affected. These data are consistent with the possibility that Ca^{2+} and VBL compete for the same sites on the protein molecules[30] to cause precipitation of the protein from solution.

Discussion. We find that VBL can precipitate a variety of proteins and that the proteins that precipitate with VBL also precipitate with Ca^{2+} ions. The results suggest that VBL is acting as a cation, combining with sites that also combine with Ca^{2+}. The interpretation is further supported in the case of one of the proteins by the evidence of the additivity of VBL and Ca^{2+} as is shown in Figure 4. It is also supported by preliminary experiments with another alkaloid cation, strychnine; in experiments exactly parallel to those with VBL, strychnine at 5×10^{-3} M precipitated 85% of the erythrocyte membrane protein from solution. There is evidence in the literature[31] that a number of alkaloid cations have higher affinities for the ionized groups of proteins than do Ca^{2+} ions.

These results cast no doubt on the efficacy of VBL in precipitating colchicine binding proteins, as described by a number of authors.[12,13] They do indicate that other proteins may be precipitated. It is possible that the conditions of precipitation by VBL will influence the selectivity of the reaction. For example, we have found that the protein of the hyaline layer of fertilized sea urchin eggs is precipitated by VBL at 37°C but not at 0°C, while temperature has little effect on the precipitation of actin or the proteins of erythrocyte membranes. The effects of other variables such as pH and ions other than Ca^{2+} have not been investigated systematically. A further complication is that VBL, presumably acting as a cation, can precipitate DNA and ribosomes. We have found that VBL at a concentration of 3.3×10^{-3} M gave complete precipitation of 0.4 mg of calf thymus DNA (Calbiochem, Grade A) in 1.2 ml of 10 mM sodium phosphate buffer, pH 6.8, at either 0 or 37°C. Similarly, this drug precipitated at least 65% of isolated chick oviduct ribosomes under the same conditions.[32] In summary, precipitation by VBL can provide a valuable procedure in the fractionation of certain proteins, as has already been shown for colchicine-binding proteins, but is not highly specific for the identification of particular proteins, as the colchicine-binding reaction has been shown to be.

Our findings are consistent with the idea that a number of proteins of structure share important common properties. Those proteins which we have precipitated with VBL are all derived from structures and similarities among some of them, especially similarities of amino acid composition and "actin-like" properties, have been discussed in the literature. It will be useful to study the solubility behavior of a great many proteins in the presence of VBL and of Ca^{2+} to determine whether precipitation by these agents is an indicator of interesting and nontrivial common properties.

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