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# Aggregation of Microtubule Subunit Protein. Effects of Divalent Cations, Colchicine and Vinblastine*

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ABSTRACT: The self-association of calf brain microtubule subunit protein (tubulin) has been studied. Divalent cations (Mg or Ca) induce the reversible aggregation of tubulin. Depending upon the divalent ion concentration, aggregation may result in an increase in sedimentation rate, formation of a 30S peak, or formation of a fibrous precipitate. Divalent cation induced precipitation is specific for the active, colchicine binding protein and is a useful step in the purification of tubulin. Colchicine alone has no apparent effect on the aggregation of tubulin, but it *increases* significantly the divalent ion induced aggregation. Vinblastine induces a strong, well-defined aggregation of tubulin and also increases and alters the effect of divalent cations. Low concentrations of vinblastine and divalent cations will precipitate tubulin. The observed differences between the various colchicine- or vinblastine-induced ultrastructural change may be related to the effects of divalent cations on the actions of these drugs.

The isolation from porcine brain of a protein, which is believed to be a subunit of microtubules, has been reported recently (Weisenberg *et al.*, 1968). This protein, tubulin (Mohri, 1968), is characterized by its ability to bind the antimitotic drug colchicine, by its ability to bind guanosine nucleotides and by its physical and chemical properties. Native tubulin has a sedimentation coefficient of 5.8 S and a molecular weight close to 120,000. Its amino acid composition is grossly similar to that of actin (Weisenberg *et al.*, 1968; Shelanski and Taylor, 1968), although marked differences both in the amino acid composition (Stephens and Linck, 1969) and in peptide maps (Stephens, 1970) exist between the two proteins from the same species.

Although identified as a subunit of microtubules (Weisenberg *et al.*, 1968), native tubulin has not yet been successfully repolymerized into microtubules. It has been found, nevertheless, that tubulin can undergo reversible aggregation (Weisenberg *et al.*, 1968; Weisenberg and Timasheff, 1969). The present work describes the results of studies on the effects of divalent cations and the antimitotic drugs, colchicine and vinblastine, on the aggregation of tubulin. Vinblastine, which has been observed to induce the formation of "microtubule crystals" *in vivo* (Bensch and Malawista, 1969) and to precipitate tubulin *in vitro* (Marantz *et al.*, 1969), induces the reversible aggregation of tubulin. Colchicine, which dissociates microtubules *in vivo*, increases the divalent ion-induced aggregation of this protein.

## Materials and Methods

All chemicals (unless otherwise noted) were reagent grade. GTP was grade II-S from Sigma Chemicals; colchicine was from Fisher Chemical Co. Vinblastine sulfate was a gift from Eli Lilly Co.

Protein concentration was determined by absorption at 278 nm using an absorptivity of 1.2 l./g. This value includes the contribution from the tightly bound guanosine nucleotide.

Colchicine binding was determined (unless otherwise noted) by the DEAE filter assay (Weisenberg *et al.*, 1968) using tritiated colchicine obtained from New England Nuclear Corp. Vinblastine binding was determined by gel filtration on Sephadex G-100 and followed by analysis of the fractions for protein and vinblastine; in these experiments, vinblastine concentration was determined spectrophotometrically using a molar extinction coefficient of $6.06 \times 10^3$ at 310 nm, while the protein concentration was determined by the Lowry procedure (Lowry *et al.*, 1951).

Analytical ultracentrifugation was performed in a Spinco Model E instrument equipped with an electronic speed control and the RTIC temperature control unit. All runs were done in 12-mm, 2° sector Kel-F cells. Sedimentation

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coefficients were measured from the displacement of the peak of the schlieren pattern.

*Purification of Tubulin.* The colchicine binding protein was isolated from calf brain by a slightly modified procedure of Weisenberg *et al.* (1968). All solutions (unless otherwise noted) were made in a buffer containing 0.01 M sodium phosphate, $10^{-4}$ M GTP, and 5 mM $MgCl_2$ at pH 7 (PMG).[1] The initial steps of homogenization and ammonium sulfate precipitation were unchanged. Fractionation with DEAE-Sephadex was performed by the batch method. After adsorption of the protein to the DEAE, it was washed twice in 0.4 M KCl (rather than 0.5 M, which had been used previously). The adsorbed protein was eluted with 0.8 M KCl as before and then precipitated with ammonium sulfate (248 g/l.). The protein was used in some experiments after this point, but it was found to be incompletely purified and an additional step, precipitation by $MgCl_2$, was added. The last ammonium sulfate precipitate was resuspended in PMG and the excess ammonium sulfate was removed either by gel filtration with Sephadex G-25 or by overnight dialysis against two changes of PMG (500 ml each). Complete removal of excess ammonium sulfate is essential for successful $MgCl_2$ precipitation. The protein was then precipitated by the slow addition of 0.5 M $MgCl_2$ to a final concentration of 0.05 M. The solution was allowed to stand in the cold for 10 min and the precipitate was collected by centrifugation. The precipitate was generally washed once in 0.05 M $MgCl_2$, resuspended, and dialyzed in the desired buffer (usually 0.01 M sodium phosphate–$10^{-4}$ M GTP, pH 7). The Mg precipitation may be repeated but this does not appear to increase significantly the purity and results in some loss of material.

The purified calf brain tubulin may be lyophilized for storage but, unless it is performed correctly, lyophilization causes severe aggregation. It is particularly important to keep the protein concentration high relative to the salt concentration and the following procedure has been found to give satisfactory results. The Mg precipitate was resuspended in $10^{-3}$ M sodium phosphate–$10^{-4}$ M GTP (pH 7, no $MgCl_2$) up to a final concentration of about 10 mg/ml. The solution was dialyzed with rapid stirring against two changes of the same buffer for 4–5 hr; it was then frozen *rapidly* in a Dry Ice–acetone bath, lyophilized, and stored below 0°.

## Results

*Comparison of Calf and Porcine Tubulins.* Since in previous work porcine brain had been used as a source material for the purification of tubulin (Weisenberg *et al.*, 1968), while in the present work calf brain was used, it was of interest to determine the similarities and differences between the two proteins. The major difference became evident in the results of purification. The procedure that had been successful with porcine brain in yielding only a single major component produced more than one component when calf brain was used. These other components could be mostly removed, however, by the addition of the final $MgCl_2$ precipitation step. When the purification procedure described above was used, a single component was observed both by gel filtration on Sephadex G-150 and by analytical ultracentrifugation, although a small amount of highly aggregated material was always present. Acrylamide gel electrophoresis of calf brain tubulin denatured in 8 M urea and reduced with mercaptoethanol showed a single major band and two minor bands containing less than 5% of the total protein.

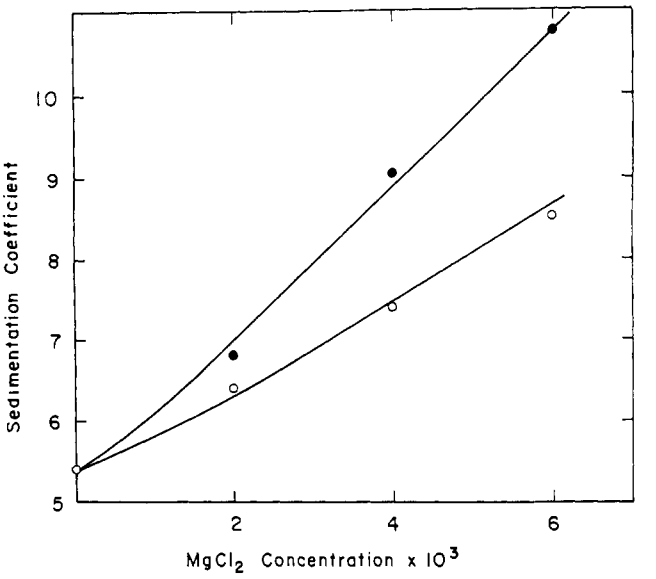


FIGURE 1: Mg-induced aggregation of tubulin in the absence and presence of colchicine. The protein concentration was about 8 mg/ml in 0.009 M sodium phosphate–$10^{-4}$ M GTP (pH 6.5). All runs were done at 60,000 rpm at 22°. The sedimentation coefficients were measured at the apeces of the main boundary and do not include contributions from faster, minor components. (●) Preincubated 30 min at 37° in $5 \times 10^{-4}$ M colchicine. (○) No colchicine added.

The physical properties of calf brain tubulin were very similar to those of the porcine protein. The molecular weight of calf brain tubulin determined by high-speed equilibrium sedimentation (Yphantis, 1964) was essentially the same as that of the porcine brain protein at similar conditions (Weisenberg *et al.*, 1968). After treatment with 5 M guanidine hydrochloride and 0.1 M mercaptoethanol, the apparent molecular weight fell to a value of 46,000, uncorrected for preferential interaction with solvent components (Casassa and Eisenberg, 1964). A diffusion coefficient of $5.1 \times 10^{-7}$ was obtained from the exclusion volume from gel filtration chromatography on Sephadex G-150, using the method of Andrews (1964).

*Effect of Divalent Cations.* As has been reported previously (Weisenberg *et al.*, 1968), divalent cations can induce the reversible aggregation of tubulin. In the present work, $MgCl_2$ was used as the divalent cation, although in a few experiments using $CaCl_2$, qualitatively similar effects were observed. The effect of Mg is a function of its concentration. At low Mg concentrations, a single sedimentation boundary is observed in the PMG buffer and aggregation is indicated by an increase in the sedimentation coefficient (Figure 1) with an increase in the Mg concentration. This is accompanied by a broadening of the sedimentation boundary and the appearance of a broad trailing edge. At the higher Mg

[1] Abbreviations used which are not listed in *Biochemistry 5*, 1445 (1966), are: PMG, buffer consisting of 0.01 M sodium phosphate, $10^{-4}$ M GTP, and 5 mM $MgCl_2$ (pH 7); VB, vinblastine; Tu, tubulin.

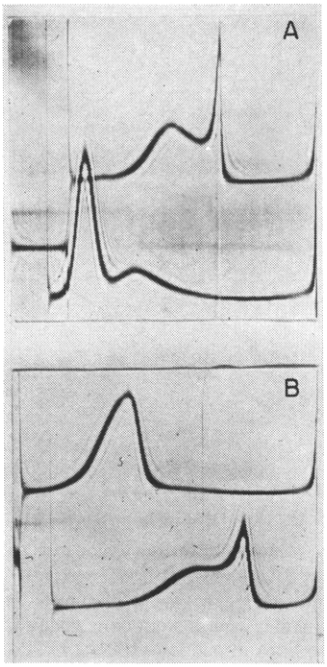


FIGURE 2: Sedimentation of tubulin in the presence of vinblastine and $MgCl_2$. The solvent was 0.01 M sodium phosphate–$10^{-4}$ M GTP (pH 7) with the indicated additions. (A) The upper run contained $10^{-4}$ M vinblastine and $5 \times 10^{-3}$ M $MgCl_2$, the lower run $5 \times 10^{-3}$ M $MgCl_2$. (B) The upper run contained $10^{-4}$ M vinblastine, the lower run $10^{-4}$ M vinblastine and $5 \times 10^{-3}$ M $MgCl_2$ (this was a repeat of the upper run in A at a later time and indicates the variation observed). The speed was 56,000 rpm, the temperature was 22° and the bar angle was 55°. The time after reaching speed was 24 min in part A, and 32 min in part B.

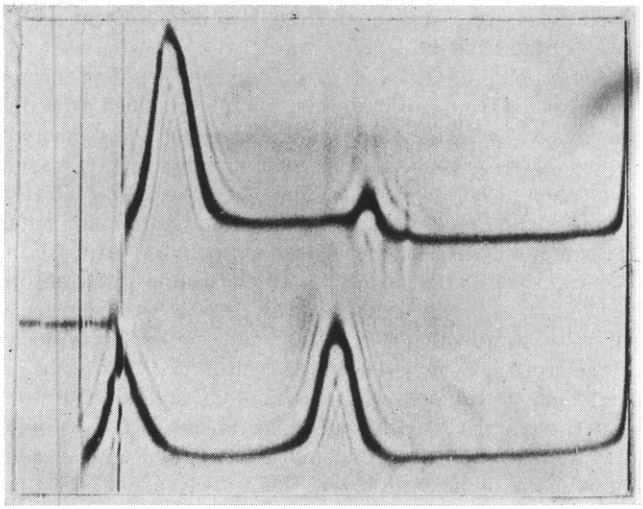

FIGURE 3: Effect of magnesium and colchicine on the formation of the 30S component The protein concentration was about 7 mg/ml in 0.01 M sodium phosphate–$10^{-4}$ M GTP–0.01 M $MgCl_2$ (pH 7). The upper run (1° wedge cell) contained no colchicine. The lower run was preincubated at 37° for 30 min in $5 \times 10^{-4}$ M colchicine. The speed was 56,000 rpm, the bar angle was 55°, the temperature was 23° and the time after reaching speed was 18 min.

concentrations, a broad, more rapid peak appears as seen in the lower pattern of Figure 2A. As the Mg concentration is further increased to the level of 0.01 M, a distinct 30–35S peak is formed as shown in the upper pattern of Figure 3. The schlieren pattern between the slow and rapid peak, however, does not return to the base line. At concentrations of Mg slightly greater than that at which the 30S component becomes pronounced (>0.01 M), tubulin precipitates.

Tubulin has been observed to precipitate in two distinct forms in the presence of divalent cations. Direct addition of excess Mg results in a plain milky white precipitate with no apparent structure. However, if the precipitation is carried out slowly by dialysis, the precipitate assumes the form of fibers which can be readily observed by phase-contrast microscopy. A suspension of these fibers is highly viscous and displays a pronounced "silky" turbidity. Preliminary electron microscopic observations have revealed the presence of bundles of fibers, but these do not appear to be microtubular in nature. The fibrous form may also be produced by resuspending the product of rapid precipitation in a small volume of Mg-free buffer, indicating slow nucleation of the ordered structure.

The effect of Mg is highly dependent on solvent conditions. Moderate concentrations of other cations greatly decrease the Mg-induced aggregation. For example, 0.05 M $NaCl_2$ or traces of ammonium sulfate remaining from the purification can completely eliminate the Mg-induced precipitation. The effect of Mg is enhanced by a decrease in pH or a lowering of the temperature. The protein is soluble at 25° at concentrations of Mg and protein which induce precipitation at 4°; furthermore, the fibrous aggregate can be formed reversibly and resolubilized by cooling and warming.

The active colchicine binding form of tubulin is much more easily precipitated by Mg than the "denatured" aggregate of the protein which is constantly being formed. The Mg-precipitable protein has a specific colchicine binding activity significantly greater than that of the nonprecipitable material. The increase in this activity is variable from preparation to preparation but it has been observed to be as high as fourfold. For this reason, Mg precipitation has proven to be a useful final step in the purification procedure. Even at the rather high $MgCl_2$ concentrations used (0.05 M is unnecessarily high, if the ammonium sulfate is totally removed), the precipitation is specific enough to yield a single component. After storage in solution for several days, tubulin can still be precipitated by high concentrations of Mg, but the precipitation is not as rapid, nor as readily reversible as with fresh protein, and the fibrous form does not occur. That the Mg aggregation is a specific property of the native protein is also suggested by the experiments in the presence of colchicine, described below.

*Effect of Colchicine.* The binding of colchicine alone has no apparent effects on the sedimentation of tubulin. It does, however, have a pronounced effect in the presence of Mg ions. At a given Mg concentration, the sedimentation coefficient of tubulin preincubated with colchicine is considerably greater than that of tubulin without colchicine as shown in Figure 1. The increase in $s$ is accompanied by an increase in the width and asymmetry of the boundary, characteristic of an increase in the association constant of a reversibly associating species (Gilbert, 1959). At higher concentrations of Mg, preincubation with colchicine results in an increase in the amount of the 30S component, as shown in Figure 3 for 0.01 M $MgCl_2$. This effect is not specific for Mg since colchicine also increases the aggre-

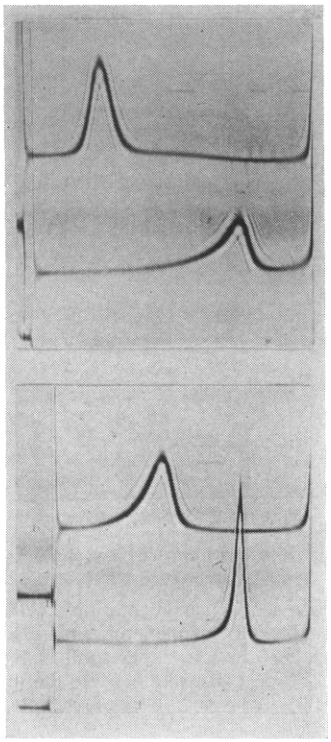

FIGURE 4: Effect of vinblastine on the sedimentation of tubulin. The vinblastine concentrations, from top to bottom, were 0, $10^{-4}$, $5 \times 10^{-5}$, and $10^{-3}$ M. The speed was 56,000 rpm, the temperature was 23° and the bar angle was 55°. The protein concentration was 8 mg/ml. The top photograph was taken at 40 min and the lower one at 32 min after reaching speed.

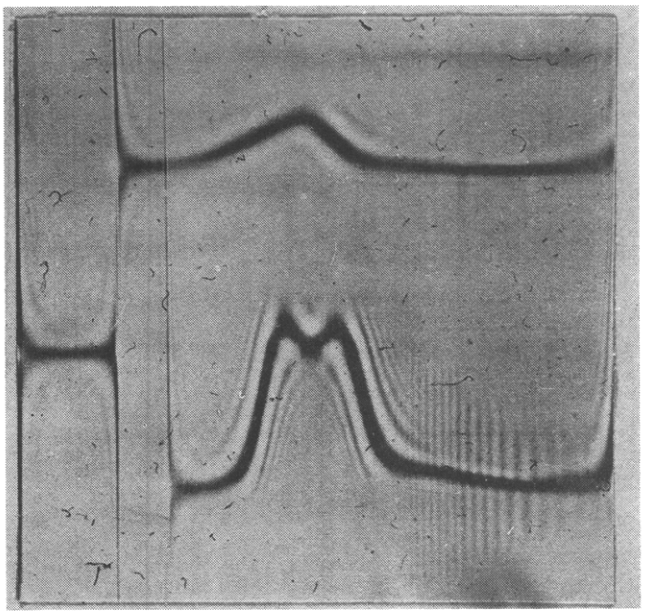

FIGURE 5: Sedimentation of tubulin in low concentrations of vinblastine. The vinblastine concentration in both cells was $2 \times 10^{-5}$ M. The protein concentrations were 2 mg/ml in the upper run and 8 mg/ml in the lower run. The conditions were the same as in Figure 4. The time after reaching speed was 34 min.

gation induced by Ca. There is no change in aggregation if the colchicine is added without preincubation at 37° or if the protein is preincubated without colchicine; therefore, the actual binding of colchicine, which is a slow process (Borisy and Taylor, 1967), is required for the increase in aggregation to occur. Colchicine is bound to both the 30S and 6S "components" as demonstrated directly in experiments using the scanning optical system of the analytical ultracentrifuge. In these experiments it was found that the optical density at 350 nm, which is a measure of colchicine concentration increases throughout the cell with two distinct steps, corresponding to the positions of the 6S and 30S peaks. Careful examination of the patterns of Figure 3 reveal that the bar image does not return to the base line between the peaks, but is slightly lifted off it. This is paralleled by a gradual increase in colchicine concentration between the two steps in the scanning experiments. Such a continuous concentration gradient between peaks is characteristic of a "reaction boundary" (Longsworth, 1959), which would be present if the protein existed in the state of a rapidly reversible association equilibrium (Gilbert, 1955, 1959; Nichol *et al.*, 1964; Townend *et al.*, 1960). In such a rapidly reversible associating system, the entire "reaction boundary" contains a mixture of all the associated species, their amounts being determined by the association constants of successive steps. The Mg-induced aggregation of tubulin, which results in sharply defined well-separated peaks at 6 and 30 S, is typical of a system in which monomer is in equilibrium with a high molecular weight polymer. Using the relationship between the sedimentation coefficient, $s$, and the molecular weight, $M$, valid for spherical symmetry, $s = kM^{2/3}$, the 30S "component" must contain at least 10–13 monomer subunits. For such a degree of association, the two peaks of the "reaction boundary" should be well separated; the area under the slow peak should remain essentially constant with a change in protein concentration, while that under the rapid peak should decrease strongly with a decrease in total protein concentration (Gilbert, 1955, 1959; Josephs and Harrington, 1968); both of these features are observed in the Mg-induced aggregation. When the association equilibrium is between monomer and a large polymer, the areas under the slow and rapid peaks are close approximations of the true distribution of the two molecular species in the system (Josephs and Harrington, 1968). Therefore, it was possible to estimate the extent of binding of colchicine to the 6S and 30S "components" by integrating the areas under the two schlieren peaks, measuring the increase in colchicine concentration over the same radical interval and taking the ratio of the colchicine concentration increase to that of the protein concentration increase across the two peaks. At an initial colchicine concentration of $10^{-4}$ M and protein concentration of 8 mg/ml, this resulted in 1 mole of colchicine bound per mole of tublin (120,000 g) in the 30S "component" (essentially polymer) and about 0.5 mole of colchicine bound to 1 mole of tubulin in the 6S "component" (essentially monomer). This increase in binding of colchicine when tubulin is polymerized suggests that the association constant of the colchicine–tubulin complex increases when tubulin associates. There appears to be, thus, a cooperative effect between the binding of the ligand to the protein, on one hand, and the Mg-induced association of the protein, on the other hand.

*Effect of Vinblastine.* Addition of vinblastine (VB) induces tubulin to undergo a characteristic aggregation, as shown in Figure 4. At a moderate protein concentration (about 10

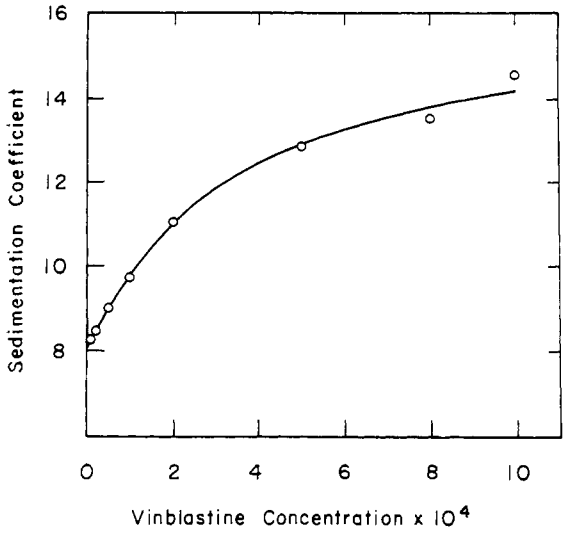


FIGURE 6: Effect of vinblastine on the sedimentation coefficient. The conditions were the same as in Figure 4. Below $2 \times 10^{-5}$ M vinblastine, the sedimentation rate refers to the faster of the two observed peaks.

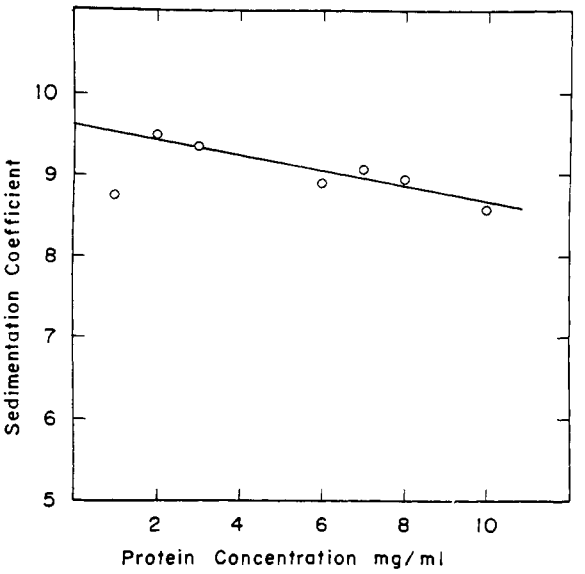


FIGURE 8: Dependence of sedimentation coefficient on protein concentration at low vinblastine concentration. The vinblastine concentration was $2 \times 10^{-5}$ M. Other conditions were the same as in Figure 4. Where two peaks were present, the $s$ refers to the faster peak.

mg/ml) and at a low VB concentration ($2 \times 10^{-5}$ M or less), two peaks at about 6 and 9 S are observed during centrifugation (lower pattern of Figure 5). At higher concentrations of VB or at lower protein concentrations, only a single peak with a broad trailing edge is observed (upper pattern of Figure 5). As the VB concentration is increased, the sedimentation coefficient at the apex of the pattern increases (Figures 4 and 6) and the sedimentation boundary sharpens (Figure 4). The increase in $s$ is most rapid at low VB concentrations, and at the highest concentrations examined ($10^{-3}$ M) the sedimentation rate is still increasing slowly.

If the VB concentration is held constant and the protein concentration is varied, the sedimentation rate increases initially with increasing protein concentration, but, at higher concentrations, it begins to decrease (Figure 7). The general shapes of the plots of $s$ as a function of protein concentration are similar at different VB concentrations but the slopes of the curves and the maximum value of $s$ reached increase with increasing concentrations of VB. The increase of the sedimentation coefficient with increasing protein concentration is consistent with a system undergoing a rapidly reversible aggregation induced by VB (Gilbert, 1955, 1959). The asymmetry of the sedimentation boundary in the presence of VB would then be a direct consequence of the reversibility of the aggregation (Gilbert, 1959). It should be noted that the molecular patterns of the VB-induced aggregation are clearly different from those of the Mg-induced aggregation. In the presence of VB, the dependence of the sedimentation properties on VB concentration strongly suggests a progressive association of the type (Gilbert, 1959) $A + A \rightleftharpoons A_2$; $A_2 + A \rightleftharpoons A_3$; . . . ; $A_n + A \rightleftharpoons A_{n+1}$; the long-trailing edge indicates that intermediate aggregates must be present. The Mg-induced aggregation, however, appears to be of the type $nA \rightleftharpoons A_n$ with intermediate aggregates present in extremely low concentrations. Both processes, however, are more complicated than indicated, since they involve the reversible binding of Mg and VB as well as the aggregation reaction itself. The resulting patterns, however, should not differ significantly from those predicted by the Gilbert (1955, 1959) theory, if Mg and VB are at concentrations well in excess over the protein concentration (Goad and Cann, 1969).

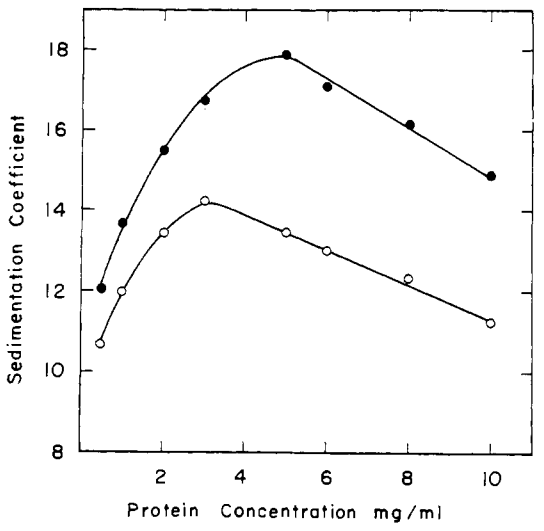


FIGURE 7: Dependence of the sedimentation coefficient upon protein concentration at constant vinblastine concentration. The conditions were the same as in Figure 4. The vinblastine concentrations were $10^{-3}$ M (●) and $10^{-4}$ M (○).

The results shown in Figure 7, as well as those of a similar experiment performed at a lower VB concentration (Figure 8) indicate an extrapolated sedimentation coefficient of about 9.5 S at zero protein concentration. This value is consistent with a dimer of the 5.8S tubulin monomer and indicates that the first step of the aggregation is very strong. The presence of two peaks at low VB concentrations (Figure 5) further sup-

TABLE I: Aggregation of Tubulin under Various Conditions.

| Type of Aggregate | Sedimentation Properties | Conditions | Comments |
|---|---|---|---|
| Irreversible, "denatured" | Extremely variable | Storage at high concentration or in an aggregated form (Mg or VB induced), poor lyophilization | Not prevented by SH reagents; partially prevented by GTP |
| Divalent cation induced (soluble) | 30–35 S at optimum conditions. Increase in S and width of peak at less than optimum | Up to 0.01 M Mg or Ca. Increased by colchicine; decreased by NaCl, KCl, and $(NH_4)_2SO_4$ | 30S form probably intermediate in the formation of the fibrous precipitate |
| Divalent cation precipitate | Fibrous precipitate | Greater than about 0.01 M Mg or Ca. Increased aggregation at low temperature | Sensitive to ionic strength and protein concentration Specific for native protein |
| Vinblastine induced | Up to about 18 S depends on VB and protein concentration. Asymmetric boundary | Not greatly dependent on ionic strength or pH | High association constant for first step |
| Vinblastine plus divalent cation | Up to about 25 S, depending on VB and Mg or Ca concentration. Sharp or hypersharp boundary. Precipitation at higher VB or divalent cation concentrations | Divalent cation effect dependent on same conditions as above | |

ports a very strong first step, since theory indicates that, in the absence of strong ligand binding there should be only a single peak for a readily reversible dimerization (Gilbert, 1959). Two peaks could be produced if VB were bound much more strongly to the aggregated protein than to the monomer. In such a case, at low VB concentration, separation of the aggregate from the monomer would also remove the VB from the nonassociated protein, which, deprived of the association mediating small molecule, would remain behind as a second nonassociating peak. In fact, the patterns of Figure 5 are strikingly similar to those predicted theoretically (see Cann, 1970, Figure 83) for the rapidly reequilibrating dimerization of a protein mediated by the strong binding of a single ligand molecule.[2] At the concentrations of tubulin (Tu) and VB used, the lower pattern of Figure 5 would correspond to an equal mass distribution of the protein between monomer and dimer in the reaction $2Tu + VB \rightleftharpoons (Tu)_2VB$, with very little free VB remaining behind the rapidly sedimenting peak. The upper pattern is also predictable in terms of the same theory and stoichiometry. In this case, the excess of free ligand results in the almost complete reduction of the sedimentation pattern to that predicted for dimerization by the Gilbert (1959) theory.

The VB-induced aggregation is strongly influenced by divalent cations. At moderate VB ($10^{-4}$ M) and low Mg concentrations ($10^{-4}$–$10^{-3}$ M), the increase in aggregation is reflected by a shift in the sedimentation boundary toward higher $s$ values. At Mg concentrations higher than about $10^{-3}$ M, a second, faster peak appears as shown in Figure 2, although there is not always a clear separation between the two peaks. The faster peak is usually quite sharp and, at high Mg or VB concentrations, may become hypersharp. The sedimentation coefficient of this peak is variable and ranges from about 19 to 25 S depending upon the Mg, VB, and protein concentrations. At sufficiently high concentrations of VB and Mg, tubulin precipitates. The concentrations of VB and Mg at which precipitation begins depends on the extent of VB and Mg binding to the protein. The strength of binding is such that direct addition of $10^{-4}$ M VB and $5 \times 10^{-3}$ M $MgCl_2$ or $5 \times 10^{-4}$ M VB and $2 \times 10^{-3}$ M $MgCl_2$ to protein at a concentration of the order of $10^{-4}$ M is necessary to initiate precipitation. However, precipitation can be induced at lower VB and $MgCl_2$ concentrations ($2 \times 10^{-5}$ M VB and $2 \times 10^{-3}$ M $MgCl_2$) by dialysis, since a large pool of free ligand is available. VB-induced precipitation has never been observed in the absence of divalent cations. The precipitate may be rapidly redissolved by removal of the Mg by dialysis and, more slowly, by removal of the VB. Complete redissolution to the 6S form has not been observed, however. This is probably due to the occurrence during precipitation of irreversible aggregation simultaneously with the reversible VB-induced association. This interpretation is supported by measurements of the stability of colchicine binding activity. There is a more rapid loss of colchicine binding activity of tubulin stored in solution with VB than without it.

Preincubation of tubulin with colchicine has no effect on the VB-induced aggregation, although it does increase the effect of Mg. The addition of a large excess of GTP also has no effect. Preliminary experiments have also indicated that the addition of VB does not affect the kinetics of colchicine binding.

The binding of VB to tubulin can be demonstrated readily by gel filtration or by equilibrium dialysis. In the gel filtration experiments 1 ml of tubulin was preincubated with excess VB

[2] We thank Dr. J. R. Cann for pointing out this similarity to us.

($10^{-5}$ M) and the protein was collected after passage through a 13 × 1 cm Sephadex G-100 column. The number of moles of VB bound per mole of tubulin (120,000 g) varied considerably in different experiments but was always less than 2 moles/mole. Equilibrium dialysis gave similar results.

## Discussion

Although up to now there have been no reports of the successful repolymerization of microtubules *in vitro* from the native subunit, the present work does establish that tubulin can easily undergo self-association, which is either strongly affected or totally dependent on specific agents, such as colchicine and vinblastine. The results of the observations are summarized in Table I. Since both colchicine and VB disrupt the normal microtubule structure *in vivo*, it is rather surprising that they both increase association *in vitro*. One explanation for this observation is that tubulin, as isolated by the present procedure may be in an "inactive" conformation which polymerizes poorly, while colchicine and VB stabilize a conformation similar to but not identical with the normal polymerizing conformation; alternately, the colchicine and VB-induced polymerizations may correspond to other pathways, which involve different sites of self-association. Even if the aggregating effects of colchicine, VB, and Mg are not related to the formation of microtubules *per se*, they do suggest that microtubule polymerization could be controlled by interactions with small molecules.

The ability of divalent cations to induce aggregation suggests that they may be involved in microtubule polymerization. However, the increased aggregation induced *in vitro* by bound colchicine and by lower temperatures is the exact opposite of their effects on microtubules *in vivo* (Roth, 1967). The *in vitro* effects of VB, on the other hand, seem entirely consistent with the various *in vivo* results which have been reported with this drug. The disruption of microtubules (Krishan, 1968), the formation of fibers (Wisniewski *et al.*, 1968) and the formation of "microtubule crystals" (Bensch and Malawista, 1968) which have been observed after VB treatment, may possibly be related to the effects of divalent cations on the action of VB. Likewise, the increase in divalent cation induced aggregation caused by colchicine may explain the occasional observations of colchicine-induced fiber formation *in vivo* (Wisniewski *et al.*, 1968; Robbins and Gonatas, 1964).

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