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Inhibition of Bovine Brain Microtubule Assembly *in Vitro* by Stypoldione

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SUMMARY

Stypoldione, an orthoquinone derived from the brown seaweed *Stypopodium zonale*, inhibited the polymerization of three-cycle-purified bovine brain microtubule protein *in vitro* in a concentration-dependent manner. Fifty per cent inhibition of the extent of polymerization beginning under initiating conditions occurred at a stypoldione concentration of approximately 25 μM, and 50% inhibition of tubulin addition to the assembly ends of microtubules at steady state occurred at a concentration of approximately 8 μM. Only slight structural abnormalities could be detected by negative stain electron microscopy in some of the microtubules that did assemble in the presence of the drug, and no aberrant structural forms of microtubule protein were detected. Stypoldione inhibited the binding of [^{3}H]colchicine to tubulin, with 50% inhibition of colchicine binding activity occurring at a stypoldione concentration of 12–15 μM. Inhibition of colchicine binding activity appeared noncompetitive and was at least partially reversible, suggesting that stypoldione and colchicine bind at separate sites. By assuming that the inhibition constant for the ability of stypoldione to prevent the binding of colchicine to tubulin was equivalent to the dissociation constant for the binding of stypoldione to tubulin, we calculated that approximately 62% of the tubulin present free in solution under initiating conditions and 35–37% of the soluble tubulin under steady-state conditions was complexed with stypoldione when polymerization was inhibited by 50%. These data are consistent with a mechanism in which stypoldione interacts with soluble tubulin and inactivates the tubulin so that it is unable to add to microtubule ends, although a colchicine-like mechanism involving an action of stypoldione at microtubule ends has not been eliminated.

INTRODUCTION

Stypotriol, an *o*-hydroquinone secreted by the marine brown alga *Stypopodium zonale*, and its oxidation product stypoldione, the *o*-quinone (Fig. 1), have been shown to be responsible for the toxic effects of the alga on the reef-dwelling fish *Eupomacentrus leucostictus* (1). Pure stypoldione in sea water is lethal to fish (minimal lethal dose = 1.0 μg/ml), and, because it is considerably more stable than the parent compound, it has been used as the prototype in studies on the mechanism of action of this class of compounds (1, 2).

Initial studies on the mechanism of action of stypoldione revealed that the agent was a potent inhibitor of the first cleavage of fertilized sea urchin embryos (2). These studies also revealed that stypoldione could prevent the polymerization of bovine brain microtubule protein *in vitro*, suggesting the possibility that stypoldione, like the classical mitotic inhibitor, colchicine, might be producing its growth-inhibitory effects by the inhibition of microtubule polymerization. However, recent studies have revealed that the action of stypoldione is more complicated than initially suspected (2a). Stypoldione may not be inhibiting cell growth exclusively by inhibition of microtubule assembly; furthermore, it is possible that another mechanism or mechanisms may be playing a more prominent role(s) in the inhibition.

In the present study, we have characterized the ability of stypoldione to inhibit the polymerization of bovine brain microtubule protein *in vitro* both under initiating conditions and at steady state, and we have investigated the mechanism of inhibition. We have found that stypoldione is a strong inhibitor of microtubule polymerization *in vitro*. Stypoldione inhibits microtubule polymerization at concentrations approximately stoichiometric to the soluble tubulin concentration, and our data suggest that a large proportion of the soluble tubulin pool is bound by drug at concentrations of stypoldione that inhibit polymerization by 50%. These results are in contrast to those obtained with a number of well-studied drugs that inhibit microtubule polymerization at drug concentrations that

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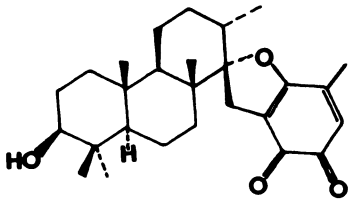

FIG. 1. *Structure of stypoldione*

would allow only a small fraction of the available drug binding sites to be occupied by drug (3–9). One simple interpretation of the data obtained with stypoldione is that the compound may act by binding to and inactivating soluble tubulin so that it cannot participate in the assembly reaction.

MATERIALS AND METHODS

Purification of bovine brain microtubule protein. Bovine brain microtubule protein consisting of approximately 75% tubulin and 25% microtubule-associated proteins was purified without glycerol by three cycles of temperature-dependent polymerization and depolymerization by the method of Asnes and Wilson (10) with slight modification. The purification buffer consisted of 20 mM sodium phosphate, 100 mM sodium L-glutamate, 1.0 mM EGTA,[1] 0.5 mM $MgSO_4$, and 0.02% NaN_3 (phosphate-glutamate buffer). Initial homogenization of the brain tissue was carried out in phosphate-glutamate buffer containing 1 mM dithiothreitol at pH 6.9. All subsequent assembly steps were carried out at pH 6.5 without dithiothreitol. Solubilized microtubule protein was centrifuged for 40 min at 4° at 150,000 × *g* (Beckman Ti50 rotor) prior to the third assembly step to remove a contaminating GDPase activity that interfered with the ability to maintain steady-state GTP levels. After the third assembly step, microtubules were collected by centrifugation through cushions of 50% sucrose in assembly buffer (11) and stored frozen in liquid nitrogen. Microtubule pellets were thawed, resuspended in the appropriate buffer at 0° with Dounce homogenization, and incubated for an additional 10 min at 0°. The microtubule protein solution was then clarified by centrifugation at 33,000 × *g* at 4° (Sorvall, SS-34 rotor), as a final purification step prior to use in an experiment. A molecular weight of 110,000 for the dimeric subunit of tubulin was used in all calculations.

Turbidometric assay of microtubule polymerization. Polymerization of microtubule protein beginning with soluble protein (initiation conditions) was monitored by light scattering at 350 nm with a Gilford Model 2400 spectrophotometer using 1.4-ml cuvettes with 1-cm light paths contained in a water-jacketed chamber (10). The turbidity is directly proportional to the mass of microtubules formed and is insensitive to the lengths of the microtubules per se (12). Assembly was carried out at 30° in 100 mM MES/1.0 mM EGTA/1.0 mM $MgSO_4$ (pH 6.75) (MEM buffer) at approximately 2 mg/ml total microtubule protein in the presence of 100 μM GTP and a GTP-regenerating system consisting of 10 mM acetyl phosphate and acetate kinase (0.1 IU/ml) (13). The regenerating system is required for maintaining constant levels of GTP throughout the course of the experiments.

Stypoldione, dissolved in 100% Me_2SO, or Me_2SO alone was added to the microtubule protein solution prior to initiation of assembly at 4° as 1% of the final sample volume. Samples were pipetted gently with a Pasteur pipette, then assembled by placing at 30°.

Assessment of steady-state [^{3}H]GTP incorporation into microtubules. [^{3}H]GTP was used to measure the net addition of tubulin to the assembly ends of steady-state microtubules as previously described (11), and a rapid filtration procedure modified from one described by Maccioni and Seeds (14) was used to collect microtubules and to quantitate net tubulin addition at the microtubule assembly ends (15). Microtubule protein (usually 2 mg/ml total microtubule protein) was polymerized at 30° for 45 min, sheared to mean microtubule lengths of approximately 1.5 μm by passage three times through a 25-gauge needle/syringe assembly, and incubated for at least an additional 10 min to ensure a stable steady state (11). [^{3}H]GTP, 40 μl/ml of final suspension (400 μCi/ml), was placed in a dry test tube, the ethanol/water vehicle was evaporated, and the tube was warmed to 30° before the microtubule suspension was added. The mixture was then gently vortexed to disperse the label evenly, and immediately pipetted into warmed tubes containing either stypoldione in 100% Me_2SO or Me_2SO alone. The solvent was uniformly present as 1% (v/v) of the final reaction mixture. Timing was begun as soon as mixing had been completed.

At the times desired for assay, 50-μl aliquots of the control or drug-treated microtubule mixture were diluted into 4 ml of microtubule stabilizing buffer at 30°, consisting of 25% glycerol (v/v), 10% Me_2SO (v/v), and 5 mM ATP in 65 mM MES, 0.65 mM EGTA, and 0.65 mM $MgSO_4$ (pH 6.75, 30°). This served to stop all ^{3}H-labeled nucleotide-tubulin uptake and loss. ATP (Sigma, Type II) was included in the stabilizing buffer to minimize nonspecific nucleotide adherence to the filters. Diluted suspensions of stabilized microtubules were then vortexed and filtered immediately through Whatman GF/F 2.4-cm glass-fiber filters on a Millipore 25-mm glass microanalysis S.S. support equipped with a stainless steel screen filter support. The labeled microtubules, retained on the filters, were subsequently washed four times with 5-ml volumes of stabilizing buffer at 30°. Finally, retained radioactivity was solubilized and counted in 10 ml of Cytoscint liquid scintillation fluid (Westchem, Inc.) using a Beckman LS8000 liquid scintillation counter. The rate of incorporation of tubulin at the net assembly ends of the microtubules was determined by measuring the mean microtubule lengths of the microtubules in suspension and calculating the molecules of tubulin incorporated per microtubule using a value of 1675 molecules of tubulin per micrometer of microtubule length (11). The rate of tubulin addition can be expressed in terms of the rate of tubulin flux from one end of the microtubule to the other; for this preparation of microtubule protein, it was between 0.4 and 0.7 μm/hr.

Colchicine binding assays. Incubation of [^{3}H]colchicine (diluted to a final specific activity of 0.1 Ci/mmole) with tubulin was carried out in 20 mM sodium phosphate/100 mM sodium L-glutamate/0.5 mM $MgSO_4$ (pH 6.75, 30°) at a final microtubule protein concentration of 0.2 mg/ml [1.4 μM tubulin (6)] as described previously (16). Stypoldione in 100% Me_2SO, or Me_2SO alone, was added to incubation mixtures yielding a final Me_2SO concentration of 1% (v/v). Bound colchicine was separated from free colchicine by gel filtration on columns (1 × 18 cm) of Bio-Gel P10 (Bio-Rad Laboratories, Richmond, Calif.). The binding reaction between colchicine and tubulin has been very well characterized. There is one colchicine binding site per mole of tubulin with tubulins from a number of higher eukaryotic species, and the binding constant for brain tubulin at 37° is approximately 1–2 μM (17–21).

Miscellaneous procedures and materials. Protein was determined by the method of Lowry *et al.* (22), with bovine serum albumin as a standard. Negative stain electron microscopy was carried out by the procedure of Olmsted *et al.* (23), and electron microscope grids were examined with a Philips EM elecron microscope operating at 80 kV. Tritium-labeled colchicine was obtained from New England Nuclear Corporation (Boston, Mass.) (specific activity, 23.2 Ci/mmole), and [^{3}H]GTP was obtained from ICN Pharmaceuticals (Cleveland, Ohio) (23.0 Ci/mmole). Me_2SO and glycerol were obtained from Mallinckrodt, Inc. (St. Louis, Mo.). All other chemicals were obtained from Sigma Chemical Company (St. Louis, Mo.) and, unless indicated otherwise, were grade I. Stypoldione was obtained from Dr. William Fenical (Scripps Institute of Oceanography, La Jolla, Calif.).

[1] The abbreviations used are: EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Me_2SO, dimethyl sulfoxide; MES, 2-(*N*-morpholino)-ethanesulfonic acid.

RESULTS

Inhibition of microtubule polymerization by stypoldione. Stypoldione inhibited the polymerization of bovine brain microtubule protein under initiating conditions and at steady state in a concentration-dependent manner. Under initiating conditions at a microtubule protein concentration of 2 mg/ml, 15 μM and 28 μM stypoldione caused a lag in the initiation of assembly, and inhibited the initial rate and final extent of polymer formation (Fig. 2A). The concentration-dependence for the ability of stypoldione to inhibit the final extent of assembly after 60 min of incubation at 30° is shown in Fig. 2B. The log concentration-effect curve was remarkably steep. Fifty per cent inhibition occurred at 20 μM–25 μM stypoldione, and complete inhibition was attained at approximately 30 μM drug.

The ability of stypoldione to inhibit the net addition of tubulin to the assembly ends of steady-state bovine brain microtubules *in vitro* was determined using [^{3}H] guanine nucleotide incorporation as a measure of tubulin addition (see Materials and Methods). The kinetics of [^{3}H]guanine nucleotide incorporation at the assembly ends of steady-state microtubules in the absence and in the presence of 20 μM stypoldione are shown in Fig. 3. Both control and stypoldione-treated samples displayed an initial burst of incorporation followed by a slower, approximately linear incorporation rate as described previously (15). The reason for the initial burst of nucleotide incorporation is not understood and is presently under investigation in this and in other laboratories (24). The linear incorporation of labeled nucleotide observed between 10 min and 60 min of incorporation reflects the steady state rate of growth at the microtubule assembly ends (15).

Stypoldione reduced the assembly end growth rate by approximately the same extent (60–70% at the concentration used) throughout the 60 min tested. The concen-

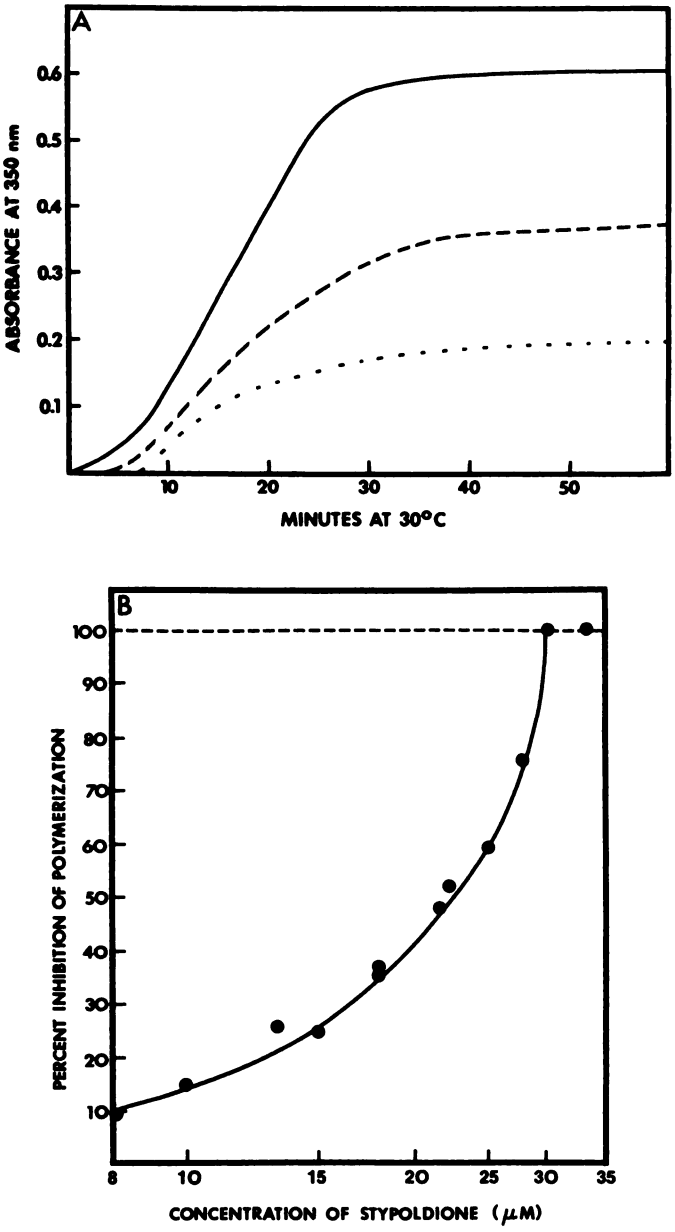

FIG. 2. *Inhibition of microtubule polymerization by stypoldione*

Bovine brain microtubule protein (2 mg/ml total protein, 13.6 μM tubulin) was polymerized at 30° in the presence of stypoldione or a solvent control. Assembly was monitored spectrophotometrically as described under Materials and Methods. A. Polymerization kinetics of samples containing 1% (v/v) Me_2SO (*solid line*), 15 μM stypoldione (*long dashes*), or 28 μM stypoldione (*short dashes*). B. Inhibition of the extent of microtubule polymerization by stypoldione: concentration dependence. After 60 min of polymerization, the absorbance at each stypoldione concentration was determined relative to control values. The reduction in final absorbance due to stypoldione is expressed as percent inhibition of polymerization.

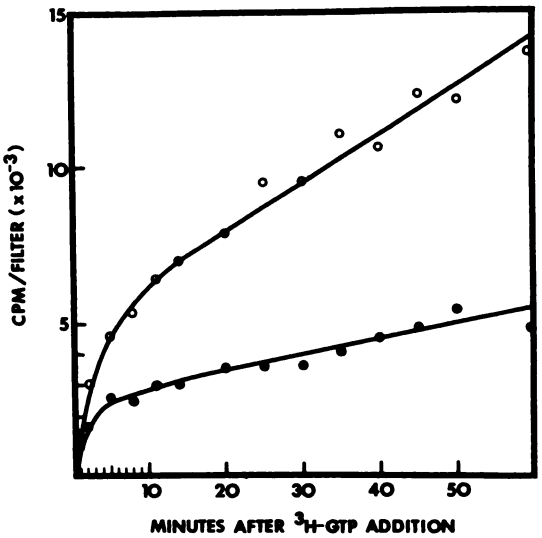

FIG. 3. *Kinetics of [^{3}H]GTP incorporation into steady-state microtubules: inhibiton of assembly by stypoldione*

A solution of bovine brain microtubule protein (2 mg/ml total protein) was assembled at 30° in the presence of a GTP-regenerating system. After 45 min of incubation at 30°, the microtubules were sheared, and 15 min later were exposed to [^{3}H]GTP and 20 μM stypoldione or Me_2SO, as described under Materials and Methods. At the times indicated after drug addition, 50-μl aliquots were analyzed for label incorporation by filter assay. Kinetics of tubulin addition for control (○) and for samples treated with 20 μM stypoldione (●) are expressed as counts per minute per filter.

tration dependence for the ability of stypoldione to inhibit the net tubulin addition rate at the assembly ends of the microtubules was determined after 60 min of drug incubation, and the results are shown in Fig. 4 in the form of a log concentration-effect curve. The inhibition curve is considerably more shallow than that obtained under initiating conditions, with 50% inhibition of tubulin addition occurring at approximately 8 μM stypoldione. Under initiating conditions, the ratio of total added stypoldione to total tubulin at the stypoldione concentration that produced 50% inhibition of assembly was approximately 1.5:1, whereas at steady state the ratio of total added stypoldione to soluble tubulin was approximately 4.5:1 (1.8 μM soluble tubulin).

A microtubule protein preparation that was assembled in the presence of 40 μM stypoldione was examined by negative stain electron microscopy to determine whether the drug produced any aberrant structural forms of microtubules or microtubule proteins, such as protofilament arrays similar to those produced by the *Vinca* alkaloids (e.g., see ref. 9). This concentration of stypoldione prevented assembly almost completely under the conditions used. No aberrant forms of microtubule protein were detected, and the microtubules that did form in the presence of the drug appeared to have normal morphology, except that there was occasional evidence of slight variation in the diameters of some of the microtubules (data not shown).

Effect of stypoldione on the binding of [^{3}H]colchicine to tubulin. There is a single high-affinity binding site on the tubulin dimer for colchicine (17–21), and we found that we could utilize the colchicine binding activity of tubulin to study several aspects of the interaction of stypoldione with tubulin. The concentration dependence for the ability of stypoldione to inhibit the binding of [^{3}H]colchicine to tubulin is shown in Fig. 5. Half-maximal inhibition of colchicine binding to tubulin occurred at approximately 12–15 μM stypoldione.

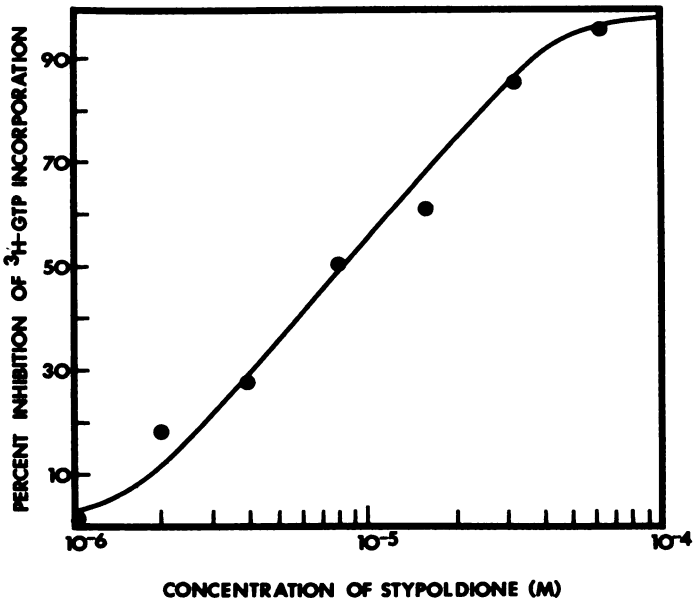

FIG. 4. *Concentration effect relationship for inhibition of steady-state microtubule assembly by stypoldione*

The effect of various concentrations of stypoldione upon bovine brain microtubule assembly at steady state was assessed using the procedure outlined in the legend to Fig. 3. The diminution of incorporation of [^{3}H]GTP relative to control values after 60 min of exposure to the label is expressed as percent inhibition for each drug concentration.

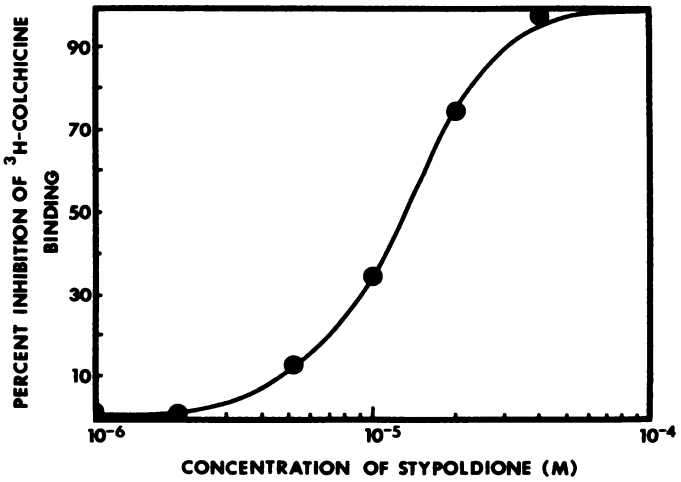

FIG. 5. *Inhibition of colchicine binding to tubulin by stypoldione: concentration dependence*

Bovine brain microtubule protein (0.2 mg/ml total protein) was incubated for 3 hr at 30° with 4 μM [^{3}H]colchicine, along with various concentrations of stypoldione or a solvent control. After incubation, the [^{3}H]colchicine-tubulin complex was separated from free [^{3}H]colchicine by gel filtration, and the bound radioactivity was determined (see Materials and Methods). The reduction in bound radioactivity is displayed as percent inhibition of control binding for each concentration of stypoldione.

Because the colchicine binding activity of tubulin in solution is unstable and spontaneously decays according to first-order kinetics (e.g., see ref. 16), it was of interest to determine whether the inhibition of colchicine binding activity by stypoldione was due to an increase in the rate of decay of the tubulin. Microtubule protein was incubated at 30° in the presence and absence of 10 μM or 20 μM stypoldione, and, at various times, aliquots of the reaction mixture were incubated with [^{3}H]colchicine to determine the amount of colchicine binding activity remaining. The half-times for spontaneous decay of the colchicine binding activity determined from the slopes of semilogarithmic plots of the data (16) (Fig. 6) were 12.5 and 13.5 hr in two control experiments, and approximately the same at 12.7 hr in the presence of 20 μM stypoldione, and 11.1 hr in the presence of 10 μM stypoldione. Thus, stypoldione does not appear to increase the rate of spontaneous decay of the colchicine binding activity of tubulin.

The ability of stypoldione to inhibit the binding of [^{3}H] colchicine to tubulin appeared to be at least partially reversible. Recovery of approximately one-half of the colchicine binding activity lost during a 1-hr exposure to 100 μM stypoldione occurred immediately after passage of the drug-treated protein through columns (1 × 18 cm) of Bio-Gel P10. A similar degree of reversibility occurred upon a 1:4 dilution of the drug-treated protein solution into buffer. The degree of dilution used was approximately equal to that which occurred by passage of the treated protein through the gel filtration columns. However, dialysis for 3 hr after passage of the treated protein through the gel filtration columns did not result in the

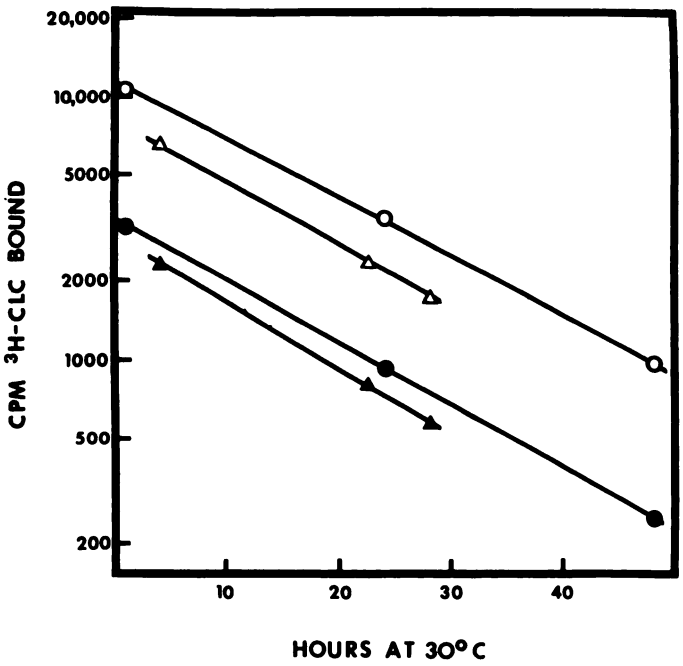

FIG. 6. *Decay of colchicine binding sites on tubulin at 30°: effect of stypoldione*

Bovine brain microtubule protein (0.2 mg/ml total protein) was incubated with (●, ▲) or without (○, △) stypoldione at 30°. At the times shown, aliquots were incubated with [³H]colchicine (*³H-CLC*) for 2 hr (●, ○) or for 3 hr (▲, △) at 30°. Following incubation with [³H] colchicine, bound radioactivity was determined by gel filtration as previously described (16). Two separate experiments are shown: *circles* represent data from one experiment; *triangles*, the second. Controls in these experiments showed half-times for loss of colchicine binding activity of 13.5 and 12.5 hr in experiments 1 and 2, respectively; the decay half-time for tubulin treated with 20 μM stypoldione (○) was 12.7 hr, and with 10 μM stypoldione (△) it was 11.1 hr.

recovery of any additional colchicine binding activity. Further, we were unable to reassemble the drug-treated microtubule protein after gel filtration. The significance of these results is not clear, but they may indicate the presence of two classes of stypoldione binding sites.

The influence of stypoldione on the affinity and stoichiometry of colchicine binding to tubulin was investigated by Scatchard analysis (25). As shown in Fig. 7, the affinity of colchicine for tubulin was essentially unaltered by the presence of 20 μM stypoldione. However, this concentration of stypoldione did reduce the number of available colchicine sites per tubulin dimer from 0.75[2] to 0.32. This degree of inhibition at 20 μM stypoldione agrees well with results obtained in the experiment of Fig. 5, and the results indicate that stypoldione prevents the binding of colchicine to tubulin by a noncompetitive mechanism.

DISCUSSION

Inhibition of microtubule polymerization by stypoldione. The assembly of microtubules *in vitro* has many of the properties of a nucleated helical polymerization reaction of the type described by Oosawa and Kasai (27) for the polymerization of actin, with growth and shortening of the microtubule occurring by addition and loss of tubulin at the ends of the polymer (see refs. 28–30 for recent reviews). Equilibrium reactions for tubulin addition and loss exist at both microtubule ends, and the kinetics of tubulin addition and loss at the two ends are different (e.g., see refs. 31–33). Upon initiation of polymerization beginning with soluble microtubule protein, nucleation (formation of short microtubules) is followed by elongation, with growth occurring at both microtubule ends. A steady-state plateau is ultimately attained, which reflects the summation of the equilibrium reactions at the two microtubule ends. At steady state in the presence of GTP, net addition of tubulin occurs at one end of the microtubule (the steady state assembly end, or A-end), and an equivalent net loss of tubulin occurs at the opposite end of the microtubule (the disassembly end, or D-end). Thus, a unidirectional flux of tubulin from one end of the microtubule to the other occurs (11, 30, 33–36). The apparent kinetic constants and flux rate for the microtubule protein preparation used in this study have been determined recently (11, 33).

Inhibition of microtubule polymerization by colchicine and by the *Vinca* alkaloids occurs at drug concentrations far less than the concentration of tubulin (3–9). This phenomenon has been termed "substoichiometric inhibition." For example, half-maximal inhibition of tubulin addition to the A-ends of steady-state bovine brain microtubules at 30° occurs at approximately 0.13 μM colchicine. Under the conditions used, the ratio of total added colchicine to total tubulin present in solution is 1:14, and only 2.1% of the tubulin in solution is complexed with the drug (6). These results indicate that colchicine is not acting by binding to and inactivating the soluble

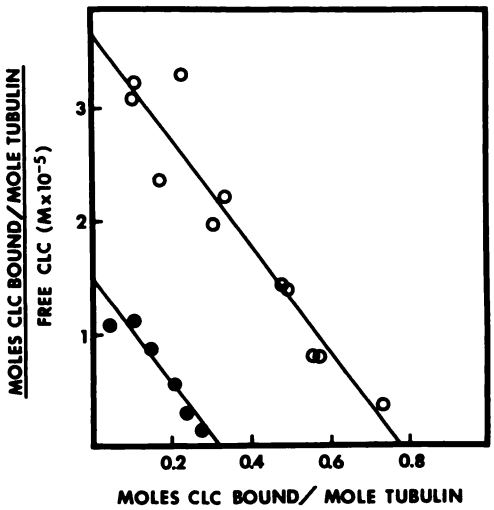

FIG. 7. *Effect of stypoldione upon colchicine binding to tubulin: Scatchard analysis*

The binding of [³H]colchicine (*CLC*) (concentration range, 0.5 μM–20 μM) to tubulin was determined as described in the legend to Fig. 5. The least-squares regression lines of colchicine binding data are shown for the binding of colchicine to tubulin in the presence of a solvent control (○) or 20 μM stypoldione (●). A semilogarithmic plot of the control binding data was *s*-shaped, exhibited an inflection point at 2–4 μM colchicine, and showed clear indication of saturation (26).

[2] The maximal stoichiometry of colchicine binding to tubulin obtained on Scatchard plots is usually between 0.6 and 0.8 mole of colchicine/mole of tubulin, mostly due to the decay of the binding activity (see refs. 18–21).

tubulin so that it cannot participate in the polymerization reaction. Vinblastine inhibits microtubule polymerization in a similar fashion, and both colchicine and vinblastine appear to inhibit assembly by decreasing the rate of drug-free tubulin addition to microtubule ends by binding or becoming incorporated, either as free drug or complexed with tubulin, at the ends of microtubules (3–9).

Stypoldione inhibited the rate and extent of bovine brain microtubule protein polymerization *in vitro* during initation of polymerization, and it inhibited the rate of net tubulin addition to the A-ends of the microtubules at steady state (Figs. 2 and 4). However, in contrast to the low ratio of drug-bound tubulin to uncomplexed tubulin that occurs upon inhibition of assembly by colchicine or vinblastine, 50% inhibition of assembly by stypoldione under initiation conditions occurred at approximately 22 μM drug, and the ratio of added stypoldione to tubulin was approximately 1.6:1. Similarly, 50% inhibition of tubulin addition to the A-ends of microtubules at steady state occurred at approximately 8 μM stypoldione, and a total stypoldione to soluble tubulin ratio of approximately 4.5:1 (Figs. 2B and 4).

It is necessary to determine the binding constant for the binding of stypoldione to tubulin in order to calculate the quantities of tubulin bound by the drug at the drug concentration that inhibits assembly by 50%. We have used the ability of stypoldione to inhibit the colchicine binding activity of tubulin to estimate the binding constant for stypoldione. An indirect method was required because of the unavailability of radioactive stypoldione. For our analysis, we have assumed that stypoldione binds rapidly and reversibly to tubulin at a specific site (see below) and that half-maximal inhibition of colchicine binding activity occurs at a stypoldione concentration that half-maximally saturates the site. We have also assumed that the inhibition is brought about by an interaction with tubulin, and does not involve any of the microtubule-associated proteins.

Stypoldione inhibited the binding of [^{3}H]colchicine to tubulin in a concentration-dependent manner, with 50% inhibition occurring between 12 and 15 μM stypoldione (Fig. 5). Inhibition of colchicine binding to tubulin by stypoldione appeared noncompetitive (Fig. 7) and was at least partially reversible. Using these results, we estimate an apparent inhibition constant for the ability of stypoldione to inhibit the binding of colchicine to tubulin by 50% to be approximately 13.5 μM.

The quantity of tubulin bound by stypoldione at 50% inhibition of polymerization beginning at initiation of assembly or at steady-state can be estimated using the value of 13.5 μM as the binding constant for stypoldione. We calculate that approximately 62% of the tubulin present in solution under initiation conditions was complexed with stypoldione at the drug concentration that produced 50% inhibition of assembly, and that approximately 37% of the soluble tubulin was bound by drug at the stypoldione concentration that inhibited net tubulin addition to the A-ends of steady-state microtubules by 50%. This analysis indicates that the degree of tubulin binding by stypoldione under initiation conditions and at steady state is similar, with approximately 37–62% of the soluble tubulin bound by drug at a drug concentration that inhibits microtubule polymerization by 50%. One simple interpretation of these results is that stypoldione inhibits microtubule polymerization by binding to and inactivating soluble tubulin so that it is incapable of adding to microtubule ends. Another possibility is that the compound may act by binding to soluble tubulin and lowering the affinity of the tubulin for the microtubule ends. A more detailed analysis of the mechanism of action of this compound must await the availability of radioactively labeled stypoldione.

Interaction of stypoldione with tubulin. Scatchard analysis of colchicine binding to tubulin in the presence of stypoldione indicated that stypoldione did not affect the affinity of colchicine for tubulin, but did reduce the number of available sites (Fig. 7). Such data would be obtained if (a) stypoldione bound to tubulin reversibly at a site other than the colchicine binding site and allosterically inactivated the colchicine site, or (b) stypoldione bound irreversibly, either at the colchicine site or at some other site on the tubulin molecule.

The possibility that stypoldione reacts with tubulin irreversibly appears to be unlikely for several reasons. First, approximately 50% reversal of the inhibition of colchicine binding activity occurred upon passage of stypoldione-treated microtubule protein through gel filtration columns to remove unbound ligand, or by dilution of stypoldione-treated microtubule protein samples. Second, the extent of assembly inhibition at any specific stypoldione concentration was attained quickly, and the extent did not change upon further incubation (Figs. 3 and 6). For example, in the experiment described in Fig. 6, there was approximately 7.4 times as much stypoldione as tubulin present at 10 μM stypoldione, and a 15-fold excess at 20 μM stypoldione. If an irreversible complex were being formed between stypoldione and tubulin, one would have expected that, at both stypoldione concentrations used, the binding sites for stypoldione would eventually become fully saturated.

However, it is important to emphasize that although approximately 50% regain of colchicine binding activity was obtained from passing stypoldione-treated microtubule protein through a gel filtration column, no further regain of colchicine binding activity occurred upon the subsequent dialysis of the protein against drug-free buffer during a 3-hr time period. The significance of this result is not clear and may be indicative of a multistep binding reaction or the presence of more than a single class of stypoldione binding sites on tubulin. Taken together, our results indicate that stypoldione interacts reversibly with tubulin at a site other than the colchicine binding site. However, an unequivocal analysis of the interaction of stypoldione with tubulin must await the availability of more direct methods of analysis.

On the mechanism of inhibition of cell growth by stypoldione. Stypoldione inhibits the first cleavage division in *Strongylocentrotus purpuratus* (sea urchin) embryos in a concentration-dependent manner, with 50% inhibition occurring at approximately 2.5 μM drug (2a). Initial studies on the ability of stypoldione to inhibit the polymerization of bovine brain microtubule protein *in vitro* suggested the possibility that stypoldione, like many of the drugs that inhibit the first division of newly fertilized sea urchin gametes, was inhibiting cell division by the disruption of microtubules (2). However, recent evidence of White and Jacobs (2a) indicates that the mech-

anism of inhibition of cell growth and division by stypoldione may be more complicated than initially suspected. For example, stypoldione inhibits both the rates of amino acid uptake and incorporation in fertilized sea urchin gametes; and in fertilized sea urchin eggs, cultured mouse 3T3 cells, and Chinese Hamster Ovary cells, mitotic index studies did not reveal an accumulation of M-phase cells, characteristic of exposure to drugs such as colchicine and vinblastine, which appear to act predominantly by disrupting microtubules.

It is clear that stypoldione can inhibit the polymerization of microtubules *in vitro*, and the mechanism of the inhibition appears to involve an impairment of the ability of soluble tubulin to participate in the polymerization reaction. Such impairment requires stoichiometric binding of stypoldione with tubulin in soluble pools, and, therefore, would require sufficiently high stypoldione concentrations to saturate an appreciable proportion of the "active" soluble tubulin pool.

In the sea urchin egg, for example, the concentration of the soluble tubulin pool is approximately 29 μM (37). Fifty per cent inhibition of the first division and cleavage in the sea urchin occurs at approximately 2.5 μM stypoldione (2a). If (a) the binding constant between stypoldione and sea urchin tubulin is similar to that of stypoldione and brain tubulin (14.5 μM), and (b) the concentration of free stypoldione in the cells is the same as the extracellular concentration, and (c) 50% inhibition of microtubule assembly in the cells results in a 50% inhibition of mitotic spindle function, then inhibition of microtubule polymerization would not be the primary mechanism responsible for the inhibition of cell division in this organism. If all assumptions were valid, the quantity of tubulin bound by stypoldione at 50% inhibition of cell division would be less than one-sixth of the total tubulin pool. However, only a 5- to 6-fold higher affinity of stypoldione for sea urchin egg tubulin would be sufficient to produce a 50% inhibition of microtubule assembly in the sea urchin egg at 2.5 μM stypoldione. Thus, it is possible that inhibition of microtubule assembly by stypoldione plays a prominent role in the mechanism of action of the drug. Studies in which the degree of inhibition of microtubule polymerization produced by stypoldione in eggs is correlated with inhibition of cell division by stypoldione may help to answer this question.

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