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Inhibition of Cell Division and of Microtubule Assembly by Elatone, a Halogenated Sesquiterpene

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SUMMARY

Elatone, the 9-keto derivative of the marine natural product elatol, produces an irreversible dose-dependent inhibition of cell cleavage in synchronously dividing *Strongylocentrotus purpuratus* sea urchin embryos. When added early in the cell cycle, elatone produces a significant and progressive inhibition of [^{3}H]thymidine uptake within 45 min after fertilization, and nearly complete inhibition of [^{3}H]thymidine incorporation during both the S1 and S2 replicative periods. Incorporation studies using eggs preloaded with [^{3}H]thymidine demonstrate that elatone blocks incorporation during S2, a DNA replication period which partially overlaps mitosis. By utilizing the inherent synchrony of this cell-culture system we examined the time course of elatone inhibition. We found that inhibition of cell division and S2-DNA synthesis dropped precipitously if exposure to elatone was withheld until the onset of mitosis, suggesting that the events immediately prior to and/or at the beginning of the M-phase are critical to elatone's mode of action. By using purified beef brain microtubules we have further demonstrated that elatone inhibits the rate of initiation of microtubule assembly *in vitro*, with consequent reduction in the steady-state levels of polymerized microtubules. Although the possibility for other modes of action certainly exists, these results are consistent with the hypothesis that elatone inhibits egg cleavage through inhibition of mitosis.

INTRODUCTION

The study of control mechanisms regulating cell cycle progression is often aided by the use of compounds which inhibit cell division. The use of translational inhibitors to study the synthesis of possible "S-phase-initiating proteins" (1, 2), and the utilization of transcriptional inhibitors to study appearance and stability of cell cycle-dependent mRNAs (3–5) are examples of this approach. Cell division inhibitors are also used as biochemical probes to study the structure and function of cytoplasmic organelles. Colchicine and podophyllotoxin, for example, have become important aids in the study of microtubule assembly (6, 7), superstructure (8), and function (9–11). The development of new biochemical probes has often dramatically extended our understanding of basic processes in cellular physiology. An appreciation of the involvement of microfilaments in various cellular processes was facilitated by the discovery of cytochalasin B, while our understanding of the translational process was extended considerably by puromycin. In this study we report the results of investigations into the processes by which the cell division inhibitor elatone affects progression of naturally synchronous cells through the cell cycle.

Elatone is an oxidation product of the marine natural product elatol, a halogenated sesquiterpene originally isolated by Sims *et al.* (12) from the red algae *Laurencia elata*. By utilizing the inherent synchrony of an invertebrate embryo cell culture system, we have examined how elatone affects certain phases of the cell cycle,[1] the initiation and completion of various biochemical processes associated with those phases, and whether or not any particular periods of susceptibility to elatone occur within the cell cycle. The results of these investigations suggested inhibition of microtubule polymerization as a possible mechanism of action for elatone.

MATERIALS AND METHODS

Cell cleavage study. Sexually mature sea urchins, *Strongylocentrotus purpuratus*, were collected from coastal waters off Santa Barbara, Calif. Ripened gametes were obtained by intercoelomic injection of 0.5 M KCl (13). The eggs were collected into cold (14.5–15°) filtered seawater while spermatozoa were collected undiluted and stored on ice (14). Eggs were washed three times in cold filtered seawater by allowing the eggs to settle into beakers and aspirating off the seawater. Eggs were fertilized

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[1] A preliminary report of this work has been published [*Pharmacologist* 20:210 (1978)].

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by using approximately 1.0 ml of a freshly diluted sperm slurry (three drops of sperm suspension per 50 ml of seawater)/200 ml of a 1% (v/v) suspension of eggs in seawater. The egg suspension was discarded unless 95% fertilization was observed.

Elatone solutions or PG[2] (as vehicle control) were added to identical fertilized egg aliquots approximately 5 min after fertilization. In all cases the final concentration of PG in each aliquot was 0.5%. Eggs were incubated at 14.5–15° with gentle stirring until the time of the first division (approximately 120–135 min after fertilization), and the percentage inhibition of egg cleavage relative to controls was determined microscopically.

Cell cycle study. A freshly fertilized egg culture was split into aliquots and incubated at 14.5–15°. Elatone (4.8×10^{-5} M) was added to these aliquots progressively later in the cell cycle at 15-min intervals. The percentage inhibition of egg cleavage in each aliquot was determined microscopically at the end of the first cycle.

Incorporation of [^{3}H]dThd into DNA. Incorporation of [^{3}H]dThd into TCA-insoluble material was measured, with the following modifications, by the method of Hinegardner *et al.* (15). Portions (100 ml) of a 2% (v/v) slurry of unfertilized eggs were added to beakers containing [*methyl*-^{3}H]dThd (6.0 Ci/mmole, New England Nuclear Corporation, Boston, Mass.), yielding a final activity of 2.5 μCi/ml and a final concentration of 4.16×10^{-7} M [^{3}H]dThd. Eggs were then fertilized, elatone (4.8×10^{-5} M) or PG was added (5 min after fertilization), and embryos were stirred gently throughout the incubation period. Duplicate 1.0-ml samples were removed at 10-min intervals and precipitated in three volumes of ice-cold 10% TCA containing a 10^3-fold excess of nonradioactive thymidine. The TCA-insoluble fraction was washed several times by centrifugation, resuspended in cold 5% TCA, and collected on Whatman GF/A glass fiber filters using a Millipore filtration manifold. The samples were then washed with 20 ml of cold 5% TCA, air-dried, and solubilized in NCS (Amersham/Searle Corporation, Arlington Heights, Ill.) for 6 hr prior to addition of 7 ml of liquid scintillation mixture containing 3 g of 2,5-diphenyloxazole and 200 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene. Samples were allowed to sit overnight to allow for dissipation of any chemiluminescence; they were then counted on a Beckman liquid scintillation counter.

Determination of thymidine kinase activity. Thymidine kinase activity was measured by a modification of the method of Kitt and Dubbs (16). Immediately after fertilization, embryos were hand-centrifuged (at approximately 100 × *g*), the seawater was aspirated off, and the eggs were washed once in 10 volumes of ice-cold homogenization buffer (0.25 M KCl, 0.5 M glycine, 0.05 M $MgCl_2$, and 0.005 M ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid pH 7.2), then resuspended in 5 volumes of buffer and homogenized for 45 sec at 4° using a motor-driven Teflon pestle (Bellco Glass, Inc., Vineland, N. J.). Aliquots (450 μl) of homogenate were added to tubes containing 500 μl of reaction mixture (5 mM ATP, 2 mM $MgCl_2$, 14 mM Tris [pH 7.4], and 1 mM α-glycerolphosphate) plus [^{3}H]dThd (specific activity 6.0 Ci/mmole; final activity 47.6 μCi/ml; final concentration 7.93×10^{-6} M[^{3}H]dThd) and either PG or elatone (4.8×10^{-5} M). At 3-min intervals until 21 min after fertilization, 20-μl samples were removed and spotted onto separate 2.5-cm Whatman DE-81 cellulose discs which had been presaturated with 10 mM nonradioactive thymidine. Discs were air-dried, washed first with 1 mM ammonium formate (pH 7.5) and then with 100% ethanol, and air-dried again. The discs were then placed in tubes containing 1.5 ml of 0.5 N HCl and incubated at 60° for 20 min; 1.0 ml of eluate from each tube was added to vials containing 7.0 ml Aquasol (New England Nuclear Corporation) and counted by liquid scintillation.

Uptake of [^{3}H]dThd. Uptake of [^{3}H]dThd was measured using the method of Vacquier and Brandriff (17). A 1% (v/v) egg suspension was fertilized and immediately divided into two equal aliquots followed by addition of elatone (4.8×10^{-5} M) or PG (0.5% v/v) 5 min after fertilization. Duplicate 1.0-ml samples were removed from each aliquot at 15-min intervals after fertilization and added to tubes containing [^{3}H]dThd in seawater (specific activity 6.0 Ci/ml; final activity 1 μCi/ml; final concentration 1.66×10^{-7} M). After a 5-min "pulse," eggs were sedimented by hand centrifugation (at approximately 100 × *g*) and the supernatant was aspirated off. Eggs were then washed twice with 10 ml of ice-cold seawater and killed by the addition of 1 ml of ice-cold 5% TCA. The soluble unincorporated [^{3}H]dThd was then extracted in the TCA overnight, followed by centrifugation to sediment the insoluble material. Aliquots (0.8 ml) of the supernatants were then added to vials containing 7 ml of Aquasol and counted by liquid scintillation.

Determination of effect on microtubule assembly. Beef brain microtubules were isolated without the use of glycerol from crude brain homogenate using three cycles of disassembly/assembly followed by ultracentrifugation of the cold-labile microtubules through buffered 50% sucrose as described by Asnes and Wilson (18). Pellets of purified microtubular protein were stored in liquid N_2 until used.

Thawed pellets of microtubules were resuspended via Dounce homogenization at 4° in L-GNP buffer, depolymerized on ice for 20 min, and centrifuged at 39,100 × *g* for 10 min at 4°. The pelleted material was discarded, and to the supernatant was added 0.25 volume of a solution containing 25 mM GTP, 5 mM $MgCl_2$, and 10 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid in L-GNP, pH 6.75. Aliquots (500 μl) of this tubulin solution (4 mg/ml) were then added to ice-cold cuvettes containing 495 μl of L-GNP buffer and 5 μl of elatone solution or PG. Cuvettes were then warmed to 30° in a water-jacketed Gilford spectrophotometer equipped with an automatic recorder and microtubule assembly followed by measurement of light scattering every 20 sec at 350 nm after the method of Gaskin *et al.* (19). The protein concentration was determined by the method of Lowry *et al.* (20).

Chemicals. Elatone and various derivatives were supplied by Dr. William Fenical (Scripps Institution of

[2] The abbreviations used are: PG, propylene glycol; TCA, trichloroacetic acid; L-GNP buffer, 100 mM sodium glutamate-20 mM sodium phosphate buffer, pH 6.75; [^{3}H]dThd, [^{3}H]thymidine.

Oceanography). Purified elatone was stored in a −70 ° Revco freezer. Elatone solutions (stored at 0°) were prepared fresh each week using 100% PG as vehicle. All other reagents were supplied through Sigma Chemical Company (St. Louis, Mo.) except where otherwise noted.

RESULTS

Drug effects on egg cleavage. S. purpuratus eggs undergo highly synchronous divisions to the two-cell stage when incubated at 15° in either seawater or a 1% (v/v) solution of PG in seawater. As shown in Fig. 1, elatone, when added approximately 5 min after fertilization, produces a dose-dependent inhibition of egg cleavage. This inhibition was not reversed by washing with seawater. The ED_{50} for inhibition of egg cleavage is 1.1×10^{-5} M as determined from the log dose-response curve.

Cell cycle study. At the end of the first division in control cultures the percentage inhibition of egg cleavage in cultures exposed to elatone at increasingly later points in the cell cycle was determined, and the resultant value was plotted as a function of time of elatone addition as shown in Fig. 2. We found that the inhibition of egg cleavage dropped precipitously if elatone was added at the onset of mitosis, suggesting that events immediately prior to and/or at the beginning of the M-phase are critical to elatone's mode of action. Eggs exposed to elatone later in the cell cycle (i.e., approximately 90 min after fertilization) complete the first cleavage but stop development at the two-cell stage.

Effects on S-phase (DNA replicative period). Elatone produced maximal inhibition of cleavage when added early in the cell cycle. To examine the possibility that DNA replication is blocked or altered, we studied the effects of elatone on [^{3}H]dThd incorporation into DNA throughout the first division cycle. The results of these experiments are shown in Fig. 3. In control embryos, two periods of incorporation are observed; the S1 period occurring 30–50 min after fertilization and the S2 period occurring 110–130 min after fertilization. In contrast to the pattern to incorporation observed in untreated embryos, the elatone-treated embryos demonstrate nearly complete inhibition of [^{3}H]dThd incorporation during the S1 period. Subsequently, although there is a gradual rise in incorporation approaching the S1 level, the elatone-treated eggs still exhibit 85% inhibition relative to control values measured at the end of S2. This aberrant incorporation spans the duration of both mitosis and S2 (cf. Fig. 2) and represents a departure from the cell cycle controls operating in untreated embryos.

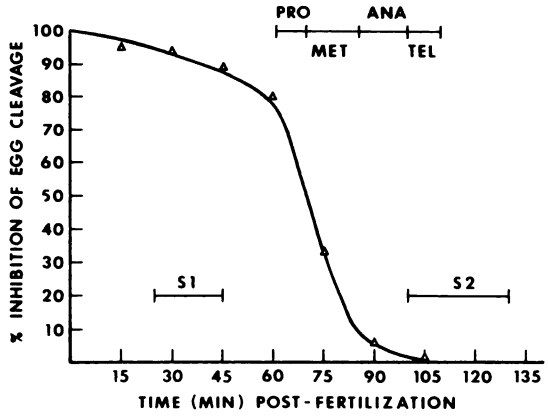

FIG. 2. *Cell cycle study*
Elatone (4.8×10^{-5} M) was added to separate egg aliquots progressively later in the cell cycle. S1 and S2 are the first and second periods of DNA synthesis, respectively. PRO, MET, ANA, and *TEL* are prophase, metaphase, anaphase, and telophase of mitosis, respectively.

Effect on thymidine kinase. Thymidine kinase activity plays an essential role in the utilization of [^{3}H]dThd. Inhibition of thymidine kinase by elatone would result in a decreased amount of intracellular [^{3}H]thymidine triphosphate which in turn would reduce the amount of radionucleotide incorporated into DNA.

Determinations of thymidine kinase activity (data not shown) indicate that elatone produces no significant inhibition of this enzyme. The phosphorylation reactions followed approximately linear kinetics throughout the first 18 min, during which time and elatone-treated egg homogenates (4.8×10^{-5} M elatone) exhibited between 85% and 100% of control activity.

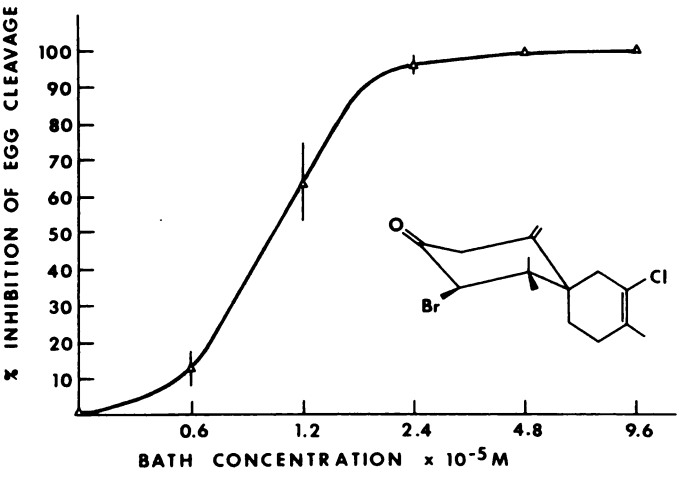

FIG. 1. *Log dose-response curve for inhibition of egg cleavage*
Elatone in all cases was added approximately 5 min after fertilization.

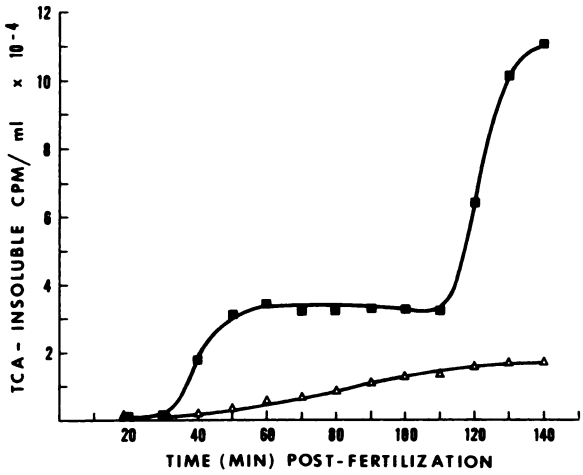

FIG. 3. *[^{3}H]dThd incorporation into fertilized eggs during the first division cycle*
Elatone (4.8×10^{-5} M; △) or PG (as control; ■) was added approximately 5 min after fertilization. [^{3}H]dTHd was added immediately after fertilization.

Effect on [^{3}H]dThd uptake. Inhibition of [^{3}H]dThd uptake by elatone might also explain the reduced incorporation. To examine this possibility, fertilized eggs were incubated in the presence of either elatone or PG and the rate of [^{3}H]dThd uptake (5-min pulse duration) was measured at 15-min intervals throughout the first cell cycle. As depicted in Fig. 4, elatone produced a significant inhibition of [^{3}H]dThd uptake within 45 min after fertilization, suggesting the possibility that the inhibition of [^{3}H]dThd incorporation could be accounted for by inhibition of [^{3}H]dThd uptake.

Effect on [^{3}H]dThd incorporation into preloaded embryos. In order to dissociate the effect of elatone on [^{3}H]dThd uptake from its effect on incorporation, fertilized eggs were incubated in seawater containing [^{3}H]dThd for 75 min during the first cell cycle to load the eggs. The eggs were then washed and resuspended in normal seawater. Backflux of the intracellular [^{3}H]dThd is probably minimal, since thymidine kinase rapidly phosphorylates this nucleoside. These embryos were then incubated in normal seawater for the remainder of the first cell cycle, followed by addition of elatone (4.8×10^{-5} M) or PG immediately after the first cleavage. Podophyllotoxin (1.9×10^{-5} M) was added to a separate beaker of preloaded embryos for purposes of comparison. Duplicate samples were removed from elatone-, podophyllotoxin-, or PG-treated embryos throughout the remainder of the second cell cycle, and the [^{3}H]dThd incorporated into DNA in each sample was determined. This experimental design precludes any effect elatone may have on uptake, since embryos had already taken up the [^{3}H]dThd and were washed free of this extracellular nucleoside well before the addition of elatone.

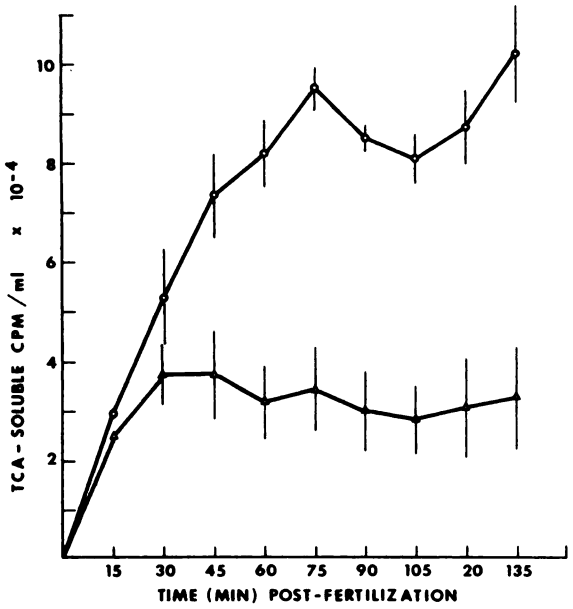

FIG. 4. *[^{3}H]dThd uptake into fertilized eggs during the first division cycle*

Elatone (4.8×10^{-5} M; △) or PG (as control; ○) was added 5 min after fertilization. At 15-min intervals after fertilization, duplicate egg aliquots were "pulsed" with [^{3}H]dThd for 5 min, washed three times (total wash time under 90 sec), and the unincorporated TCA-soluble [^{3}H]dThd was measured. Bars represent standard error of the mean; n = 3 experiments.

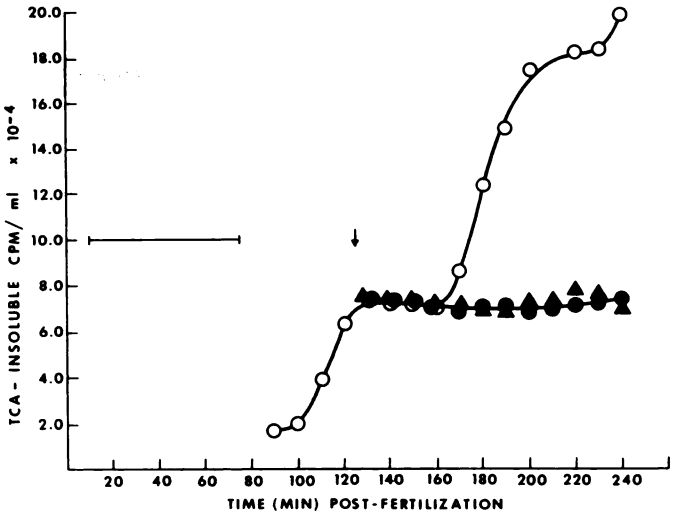

FIG. 5. *Effect of elatone and podophyllotoxin on [^{3}H]dThd incorporation during the second cell cycle*

Fertilized eggs were incubated in seawater containing [^{3}H]dThd until 75 min after fertilization (indicated by the *bar*), after which time the eggs were washed and resuspended in normal seawater. Immediately after the first division, elatone (4.8×10^{-5} M; ●), podophyllotoxin (1.9×10^{-5} M; ▲) or PG control (○) was added (addition time indicated by *arrow*) and the [^{3}H]dThd incorporated during the second cell cycle was measured.

The results of this experiment, illustrated in Fig. 5, reveal a complete inhibition of [^{3}H]dThd incorporation. From these data we concluded that the inhibition of intracellular [^{3}H]dThd incorporation was due to uninitiated and/or interrupted DNA synthesis. Similar results were obtained with podophyllotoxin (an inhibitor of microtubular polymerization and nucleoside uptake), indicating that inhibition of M-phase-associated (S2-type) DNA synthesis in the sea urchin embryo can be produced indirectly by inhibition of mitosis.

Effect on microtubule polymerization in vitro. The observation that both podophyllotoxin and elatone produced remarkably similar patterns of inhibition of [^{3}H]dThd incorporation suggested the possibility that the ability of elatone to inhibit DNA synthesis might be secondary to mitotic inhibition. To examine this possibility further we studied the effect of elatone on microtubule assembly *in vitro*.

The microtubule purification method of Asnes and Wilson (18) utilizes the fact that cold-labile microtubules will depolymerize to free tubulin upon cooling to 4° and repolymerize upon warming to 30° in the presence of GTP. Differences in the sedimentation properties of free and polymerized tubulin relative to other contaminating cellular material allows rapid purification by repeated cycles of assembly and disassembly. Protein purified by this method is composed almost exclusively of tubulin and small amounts of microtubule-associated proteins as determined by gel electrophoretic analysis.

Since the turbidity developed during microtubule assembly is relatively insensitive to the length or over-all shape of the microtubules formed and is principally a function of the total mass of supramolecular structures present (19), samples were fixed and negatively stained for electron microscopy according to the method of Mar-

golis and Wilson (6) in order to confirm the presence of assembled microtubules after light scattering measurements. In the samples examined, microtubules were the predominant polymeric structures; neither tubulin aggregates nor abnormal-looking microtubules (i.e., ribbons or circles) were observed.

The results of a typical beef brain microtubule assembly experiment are illustrated in Fig. 6. In this *in vitro* polymerization system elatone produced a dose-dependent inhibition of both the rate of initiation (i.e., the rate at which microtubular polymerization is initiated from "nucleation centers") and the steady-state levels (i.e., the final concentration of assembled microtubules existing when the assembly and disassembly processes have reached steady state). The data illustrated in Fig. 6 further show that increasing concentrations of elatone prolonged the assembly lag time (i.e., the time period occurring between the temperature shift to 30° and the start of the assembly reaction), suggesting that elatone may increase the time required for the spontaneous formation of functional nucleation centers.

A dose-response curve, representing the ability of elatone to inhibit the rate of initiation, was developed using data pooled from 10 separate microtubule assembly experiments. These data were then utilized in the construction of a double reciprocal plot yielding an apparent inhibition constant (K_i) of 5.5×10^{-5} M, as shown in Fig. 7.

DISCUSSION

The class of halogenated sesquiterpenes represented by elatone has not previously been examined for effects on eukaryotic cell division or cell cycle progression. We have found elatone to irreversibly inhibit cleavage of the fertilized sea urchin egg in a dose-dependent manner. Utilizing the fact that fertilized eggs progress synchronously through the cell cycle, we further observed that elatone's ability to inhibit cell division is rapidly reduced

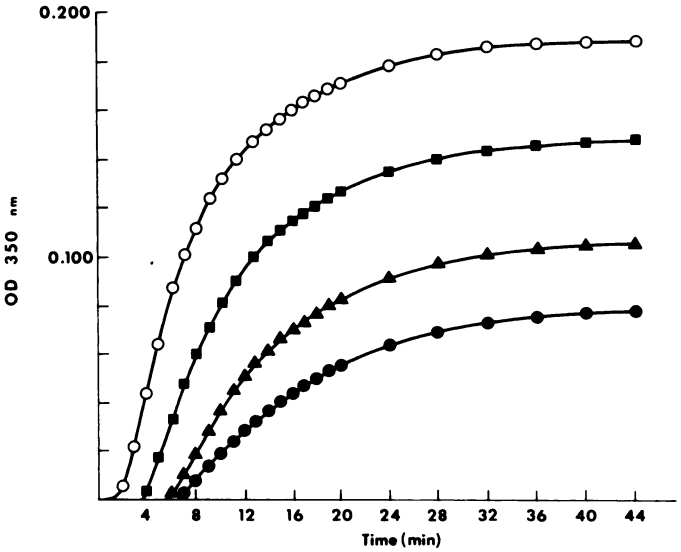

FIG. 6. *Effect of elatone on microtubule assembly in vitro*
Beef brain tubulin was assembled upon warming to 30° in the presence of PG [0.5% (v/v); ○] or elatone at 3.6×10^{-5} M (■), 4.8×10^{-5} M (▲), or 6.0×10^{-5} M (●).

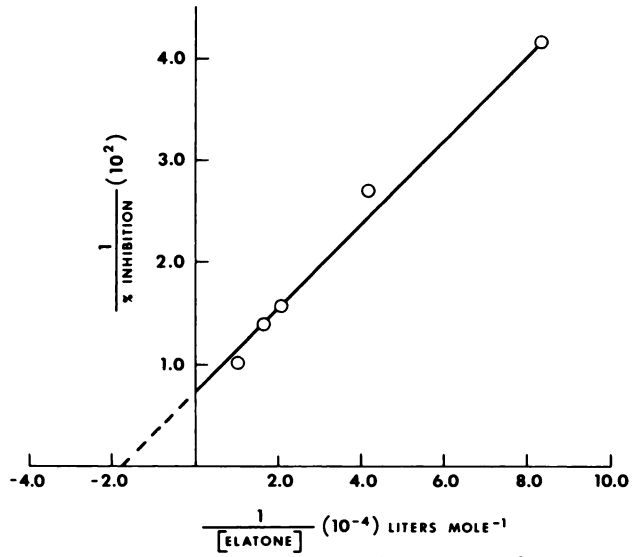

FIG. 7. *Double reciprocal plot of log-dose response data representing the effect of elatone on the rate of initiation of microtubule assembly*
The rate of initiation is defined by the slope of the initial linear region of the assembly curve. *% Inhibition* refers to the inhibition of the initiation rate relative to that of controls.

as eggs progress into mitosis, suggesting that events immediately prior to and/or at the beginning of the M-phase are critical to the mode of action of elatone, since it is the ordered completion of such processes as DNA synthesis and microtubule assembly/disassembly which delineate, respectively, the S- and M-phases of the cell cycle, we chose to examine the effect of elatone on these processes in greater detail.

We have shown that the addition of 4.8×10^{-5} M elatone early in the cell cycle reduces [^{3}H]dThd uptake to approximately 50% of control within 45 min after fertilization, progressing gradually to approximately 65–70% inhibition within 120 min after fertilization. This concentration of elatone also produces nearly complete inhibition of incorporation during both the S1 and S2 replicative periods. This distinction between S1 and S2 replicative periods is extremely important and as such requires some elaboration. Hinegardner *et al.* (15) demonstrated that, with the exception of the first DNA replication period of the first cell cycle (i.e., the S1 period), DNA synthesis occurs simultaneously with telophase of mitosis during early development of the *S. purpuratus* embryo. In other words, the S2 replication period shares with all subsequent replication periods the common feature of partially overlapping mitosis. In contrast, the S1 period occurs only during the first cell cycle, precedes mitosis, and is considered M-phase-independent. These distinctions must be kept in mind when interpreting [^{3}H]dThd incorporation studies in the sea urchin embryo.

The reduced [^{3}H]dThd incorporation we observe during S1 remains difficult to interpret because of the inhibition of uptake produced by elatone and the inability to preload unfertilized eggs adequately with [^{3}H]dThd. These problems complicate an examination of the effects of elatone on DNA synthesis during S1. In some experiments we found that [^{3}H]dThd incorporation eventually

rose to the S1 level (in none of our experiments did incorporation rise above the S1 level), suggesting that the rate of M-phase-independent (S1-type) DNA synthesis may be only retarded by elatone rather than actually blocked. In contrast to these problems with S1, it is relatively easy to study S2-type DNA replication without the added complication of altered [^{3}H]dThd uptake. By using embryos preloaded with [^{3}H]dThd we have demonstrated that the inhibition of incorporation during the second cell cycle is independent of elatone's effect on [^{3}H]dThd uptake. These results show conclusively that M-phase-associated S2-type DNA synthesis does not occur in the presence of elatone.

Since M- and S-phases overlap in the cell cycle, it seemed logical that the inhibitors of microtubule polymerization should indirectly block the initiation of (S2-type) DNA synthesis as a consequence of their ability to disrupt mitosis. In support of this hypothesis, we have shown that podophyllotoxin completely blocks S2-type DNA synthesis, producing a pattern of [^{3}H]dThd incorporation nearly identical with that observed with elatone. This observation suggested that the ability of elatone to inhibit DNA synthesis might be a consequence of, and secondary to, mitotic inhibition.

We have studied the effects of elatone on beef brain microtubule polymerization *in vitro*, demonstrating that elatone both prolongs the assembly lag time and inhibits the rate of initiation of polymerization in a dose-dependent manner (apparent $K_i = 5.5 \times 10^{-5}$ M by double reciprocal plot). The steady-state levels are also reduced, probably as a consequence of the effect of elatone on initiation. Two different experimental approaches, electron microscopy and a podophyllotoxin assembly/aggregation assay (data not shown), revealed that elatone does not inhibit microtubule polymerization by a simple aggregation of tubulin.

Mizel and Wilson (21) have shown that both podophyllotoxin and colchicine inhibit nucleoside uptake in cultured mammalian cells, with K_i values of 6×10^{-6} M and 4×10^{-5} M, respectively. We find it interesting that elatone also significantly inhibits thymidine uptake in cultured sea urchin eggs. The observation that both podophyllotoxin and elatone (a) produce complete inhibition of M-phase associated (S2-type) DNA synthesis in a manner which can be dissociated from their effect on nucleoside uptake, (b) produce a dose-dependent inhibition of egg cleavage (22), and (c) produce a dose-dependent inhibition of microtubule assembly *in vitro* (23) supports the hypothesis that elatone inhibits mitosis via inhibition of microtubule assembly.

Such a hypothesis is still tentative, however, as other inhibitors of microtubule assembly exhibit effects quantitatively and qualitatively different from those of elatone. Using another sea urchin, *Arbacia punctulata*, Zimmerman and Zimmerman (24) have shown that colcemid blocks the pronuclear fusion event immediately preceding S1 but permits some degree of [^{3}H]dThd incorporation as visualized by autoradiography. Although no attempt was made to quantify the rate and extent of [^{3}H]dThd incorporation relative to controls, their results show that at least some degree of DNA synthesis can occur during S1. We do in fact observe [^{3}H]dThd incorporation in elatone-treated embryos which approaches the S1 level, but the period of incorporation often does not coincide with the S1 phase in controls. Whether or not the apparent difference exhibited by elatone and colcemid in the rate of M-phase-independent DNA synthesis (as measured by [^{3}H]dThd incorporation) is qualitative or simply quantitative awaits further experimentation and is unknown at this time.

Mechanistic differences between elatone and other microtubule assembly inhibitors may also exist. Such spindle poisons as colchicine and podophyllotoxin clearly produce substoichiometric poisoning (25) of microtubule assembly *in vitro*, as evidenced by the fact that both compounds inhibit assembly at concentrations in which the molar ratio of drug to tubulin is less than 1, thus accounting for their activity at extremely low concentrations. In contrast, elatone is effective as an inhibitor of assembly at higher concentrations (molar ratios of approximately 1 or higher). This suggests that elatone may not act via a substoichiometric poisoning mechanism or, if it does, the affinity for its receptor is quite low.

The ED_{50} of elatone for inhibition of egg cleavage is approximately five times lower than the K_i for inhibition of microtubule assembly. Initially, we expected elatone to inhibit assembly *in vitro* at a much lower concentration than that required to inhibit cleavage, based on the assumption that drug permeability barriers presented by the cell membrane have been removed, as have organelles which may sequester the drug and/or possess enzyme(s) associated with drug metabolism. Recently, however, Wang *et al.* (26) have demonstrated that the assumption of greater activity *in vitro* does not hold for all microtubule inhibitors. In these studies it was shown that the minimal concentration of podophyllotoxin required to inhibit cleavage completely in *S. purpuratus* embryos was $5 \times ^{-8}$ M, whereas a 100-fold higher concentration (5×10^{-6} M) was required to inhibit completely microtubule assembly *in vitro* ($K_i = 2 \times 10^{-6}$ M). Wang *et al.* (26) reported a similar relationship for colchicine and the tumor inhibitor steganacin. With respect to elatone, whether this paradox in K_i values represents a difference in its affinity for sea urchin tubulins as compared with those from beef brain, whether it reflects a possible accumulation of the highly hydrophobic elatone molecules by the intact sea urchin egg, or whether it reflects the possibility that egg cleavage might be prevented by inhibiting only a small fraction (or an especially sensitive subset) of spindle microtubules is unknown. While the mechanism of action of elatone remains incompletely defined at this time, the evidence we have presented here suggests that elatone blocks the initiation of the S-phase and prevents division of the sea urchin embryo as a consequence of mitotic inhibition.

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