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Effect of Stypoldione on Cell Cycle Progression, DNA and Protein Synthesis, and Cell Division in Cultured Sea Urchin Embryos

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SUMMARY

We have found that stypoldione, a bright red *o*-quinone isolated from the brown alga *Stypopodium zonale*, inhibits the division of sea urchin embryos in a concentration-dependent manner (IC_{50} approximately 2.5×10^{-6} M). Although previous studies have shown this marine natural product to inhibit beef brain microtubule assembly *in vitro* [*Fed. Proc.* **39**:26–29 (1980); *Mol. Pharmacol.* **24**:493–499 (1983)], we have found that stypoldione does *not* accumulate sea urchin embryos in mitosis and hence does *not* act like a mitotic spindle poison. We have also shown this marine natural product to inhibit both amino acid and nucleoside uptake. By preloading sea urchin embryos with nucleoside (i.e., [^{3}H]thymidine) in order to dissociate effects on uptake from those on incorporation, we found that stypoldione in fact produces no significant inhibition of the *M* phase-independent S1 period of DNA synthesis, a result which suggests that stypoldione has no direct effect on DNA synthesis. In contrast, stypoldione *did* reduce the extent of amino acid incorporation in embryos preloaded with [^{3}H]leucine. An inhibition of incorporation was apparent as early as 20 min after fertilization, and incorporation was reduced to 50% of control by 40 min postfertilization. This result suggests that stypoldione might inhibit cleavage via an inhibition of translation, although the existence of other inhibitory mechanisms cannot yet be ruled out. Cytological examination revealed that sea urchin embryos did not progress beyond-interphase or very early prophase when incubated in the presence of 1.0×10^{-5} M stypoldione. The nuclear membranes remained intact, and chromatin did not condense into chromosomes in these arrested embryos. These results indicate that embryos exposed to stypoldione early in the cell cycle initiate and complete the M phase-independent S1 period of DNA synthesis, but stop cell cycle progression prior to the start of prophase of mitosis. The period between S phase and mitosis is referred to, by definition, as the "G2" phase of the cell cycle. The result therefore suggest that stypoldione blocks cell cycle progression (and, ultimately, cell division) by inhibiting progression through G2. This compound may represent a new class of G2-accumulating agents.

INTRODUCTION

Stypoldione, the *o*-quinone oxidation product of stypotriol (an *o*-hydroquinone), was originally isolated by Gerwick *et al.* (1) at the Scripps Institution of Oceanography. Both compounds were found to be present in seawater conditioned by exposure to the marine brown alga *Stypopodium zonale*, and such seawater was found to exhibit a potent ichthyotoxicity against the reef-dwelling herbivorous fish, *Eupomacentrus leucostictus* (2). Although pure stypotriol was found to be substantially more toxic than stypoldione (reported minimal lethal doses against *E. leocostictus* are 0.2 and 1.0 μg/ml, respectively), stypotriol was also found to undergo an extremely rapid oxidation to its quinone derivative (2). The fact that pure stypoldione is also ichthyotoxic, that it is the major toxic metabolite of *S. zonale*, and that it is considerably more stable and was available in greater quantity, led us to adopt stypoldione as the prototype in studies on the mechanism of action of this pharmacologically novel class of compounds.

Our initial work with this marine natural product revealed it to be both a potent inhibitor of sea urchin egg cleavage and an inhibitor of microtobule assembly *in vitro* (3). Preliminary work at the National Cancer Institute has since shown that stypoldione increases the survival time in P388 leukemic mice (T/C of 142 at 200 mg/kg[1]), indicating that the ability of this compound to

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[1] W. Fenical, personal communication.

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inhibit cell division is not limited to marine organisms. The mechanisms responsible for producing the cytotoxicity, ichthyotoxicity, and anti-tumor activity exhibited by stypoldione have yet to be elucidated. While O'Brien *et al.* (4) have examined in detail the effects of stypoldione on microtubule assembly *in vitro*, we have continued our characterization of the mechanism(s) of action of stypoldione using the sea urchin embryo test system. Our results indicate that stypoldione does *not* produce its activity solely through the inhibition of microtubule assembly, but in fact can inhibit a number of biochemical processes. Data accumulated to this point suggest that stypoldione might inhibit cell division via translational inhibition, although other mechanism(s) of action certainly cannot be ruled out. The unusual spectrum of activity exhibited by stypoldione is examined, and certain implications concerning the possible use of this compound as a probe of the G2 phase of the cell cycle are discussed.

MATERIALS AND METHODS

Cell cleavage study. The effect of stypoldione on sea urchin embryo cleavage was determined by methods described previously (5). Stypoldione or vehicle control was added to *Strongylocentrotus purpuratus* or *Lytechinus pictus* egg slurries at approximately 5–10 min after fertilization unless otherwise noted. Incubation temperatures for the two species were 15.5° and 18.5°, respectively. At the time of first division in controls, the percentage of stypoldione-treated embryos which had completed division was determined microscopically. Results were then expressed as percentage inhibition of egg cleavage, relative to controls. For cytological studies, 2-ml aliquots of embryos were removed at various times after fertilization and fixed in ethanol/glacial acetic acid (3:1) for 8 hr, resuspended in 50% (v/v) acetic acid overnight, and then stained in 2% (w/v) aceto-orcein in 50% (v/v) acetic acid for approximately 24 hr prior to examination by phase or Nomarski microscopy (×100–400).

Cumulative incorporation of [^{3}H]thymidine into DNA. Incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material was measured by the method of Hinegardner *et al.* (6), as modified by White and Jacobs (5).

Cumulative incorporation of [^{3}H]phenylalanine into protein. Aliquots of a 2% (v/v) slurry of fertilized *L. pictus* embryos were added to beakers containing [^{3}H]phenylalanine (specific activity 20 Ci/mmole, New England Nuclear Corporation, Boston, Mass.), yielding a final activity of 0.333 μCi/ml and a final concentration of 1.66×10^{-8} M. Stypoldione (1.18×10^{-5} M) or vehicle control was added at approximately 5–10 min postfertilization. Duplicate 1.0-ml samples were precipitated in 4 volumes of 10% trichloroacetic acid at 10-min intervals, followed by heating to 60° for 20 min in order to release any [^{3}H]phenylalanine that may have been bound to phenylalanine/tRNA. This precipitated material was then collected on GF/C glass-fiber filters, washed repeatedly in 10% trichloroacetic acid, air-dried, solubilized in 0.5 ml of NCS (Amersham/Searle Corporation, Arlington Heights, Ill.), and counted using a liquid scintillation mixture containing 3 g of PPO (2,5-diphenyloxazole) and 200 mg of POPOP (1, 4-bis[2-(5-phenyloxazolyl)]benzene) per liter of toluene, as described for [^{3}H]thymidine.

[^{3}H]Thymidine uptake rate as a function of time after fertilization. Uptake of [^{3}H]thymidine was measured using a modification of the method described previously by White and Jacobs (5). Briefly stated, stypoldione (1.18×10^{-5} M) or vehicle control was added at 5–10 min after fertilization. Duplicate 1.0-ml samples were removed from each aliquot at 15-min intervals after fertilization and transferred to tubes containing [^{3}H]thymidine (6.0 Ci/mmole) in seawater. The final activity of the labeled seawater was 10 μCi/ml at a final thymidine concentration of 1.0×10^{-5} M. After a 5-min "pulse," eggs were sedimented, washed repeatedly with seawater, and killed by the addition of 5 ml of 10% trichloroacetic acid. Aliquots of the trichloroacetic acid-soluble extract were then counted in Aquasol (New England Nuclear Corporation) as described previously (5).

[^{3}H]Phenylalanine uptake and incorporation rates as a function of time after fertilization. The experimental protocol used for determining the rate of uptake of [^{3}H]phenylalanine was essentially the same as that described for [^{3}H]thymidine uptake. The final activity of the [^{3}H]phenyalanine used during the 5-min pulse was 2 μCi/ml at a final concentration of 1.0×10^{-5} M. In addition, the trichloroacetic acid-insoluble material was collected on GF/C glass-fiber filters, washed with 25 ml of ice-cold 5% trichloroacetic acid, and processed for incorporated radioactivity to obtain the [^{3}H]phenylalanine incorporation rate.

Cumulative incorporation in embryos preloaded with [^{3}H]thymidine. The procedure followed was essentially identical with that described previously (5). Briefly stated, [^{3}H]thymidine was added to embryos at approximately 5 min after fertilization, and embryos were incubated in this medium until 17 min after fertilization. Embryos were then washed repeatedly in seawater to remove extracellular [^{3}H]thymidine and resuspended in normal seawater; the culture was split into two equal aliquots. At 22 min after fertilization, one aliquot received 1.0×10^{-5} M stypoldione and the other received the appropriate solvent control. Samples were then removed from each aliquot at 5-min intervals thereafter and processed for incorporated [^{3}H]thymidine as described (5).

Cumulative incorporation in embryos preloaded with [^{3}H]leucine. An unfertilized 4% (v/v) *S. purpuratus* egg slurry was preloaded with [^{3}H]leucine for 1 hr prior to fertilization. Eggs were incubated in 0.22-μm-filtered seawater containing streptomycin (50 μg/ml), penicillin G (100 IU/ml), and [^{3}H]leucine (specific activity 5 Ci/mmole at 1 mCi/ml) at a final activity of 2 μCi/ml and a final concentration of 4.0×10^{-7} M. After 1 hr the eggs were washed four times to remove extracellular [^{3}H]leucine. Eggs were then diluted to a 1% (v/v) slurry and fertilized, and stypoldione (1.0×10^{-5} M) or an equal volume of dimethyl sulfoxide (solvent control) was added at 4 min after fertilization. At 5-min intervals after fertilization, duplicate 1.0-ml samples were withdrawn from each aliquot and transferred to tubes containing 1.0 ml of 20% trichloroacetic acid and unlabeled leucine (1 mg/ml). The tubes were incubated for 30 min at 75° and allowed to incubate overnight prior to centrifugation (2000 × *g*) to sediment the insoluble material. The amount of [^{3}H]leucine in the trichloroacetic acid-soluble fraction was determined by transferring duplicate 0.50-ml supernatant samples to vials containing 10 ml of Aquasol and counting by liquid scintillation. The amount of [^{3}H]leucine incorporated into protein was determined as described above for incorporation of [^{3}H]phenylalanine.

Chemicals. Stypoldione was supplied by Dr. William Fenical (Scripps Institution of Oceanography, La Jolla, Calif.). Purified stypoldione (as brick-red crystals) was stored at −70°. Stypoldione solutions (stored at −20°) were prepared fresh each week, using ethanol or dimethyl sulfoxide as solvent. Care was taken to note the color of the stypoldione solutions prior to use in each experiment; those solutions in which the color exhibited any noticeable shift to brown were discarded. All other chemicals were supplied by Sigma Chemical Company (St. Louis, Mo.) except where otherwise noted.

RESULTS

Effects on sea urchin embryo cleavage. As shown in Fig. 1, stypoldine produced a concentration-dependent inhibition of the first cleavage in *Strongylocentrotus purpuratus* sea urchin embryos; the IC_{50} for the inhibition of cleavage was approximately 2.5×10^{-6} M. The log concentration-response curve was remarkably steep, since the concentrations producing from 10% to 90% inhibition spanned only a 3-fold concentration range. An essentially identical dose-response curve was also obtained using *Lytechinus pictus* embryos (data not

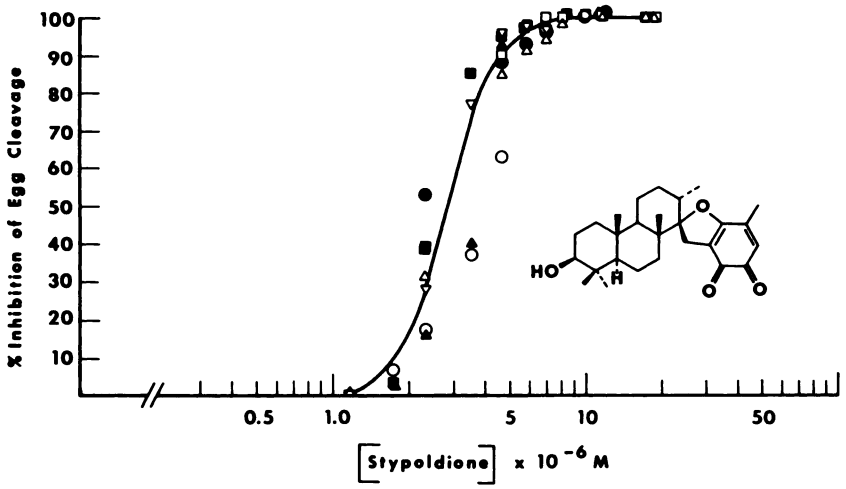

FIG. 1. *Log concentration-response curve for inhibition of Strongylocentrotus purpuratus embryo cleavage*
Results from seven representative experiments are shown. Stypoldione was added 5–10 min after fertilization.

shown), a result which indicated that the inhibitory activity of stypoldione was not restricted to a particular species of echinoderm embryo. Finally, the ability of stypoldione to block division was apparently irreversible in both species, as evidenced by the fact that inhibition of egg cleavage could not be overcome by washing the arrested embryos three times with 500 volumes of seawater and reincubating them in seawater for an additional 2 hr (i.e., one complete cell cycle time).

Effect of stypoldione on cumulative [^{3}H]phenylalanine incorporation. Initiation of the fertilization program results in a dramatic increase in metabolic activity, one aspect of this activation being a rapid increase in protein synthesis after fertilization. It has been previously reported that translational inhibitors will block cleavage of the sea urchin embryo if added early in the cell cycle and in sufficiently high concentrations (7, 8). To investigate the possibility that stypoldione inhibited translation (and, as a consequence, egg cleavage), we measured the extent of cumulative [^{3}H]phenylalanine incorporation in embryos exposed to 1.18×10^{-5} M stypoldione at 5 min after fertilization. As shown in Fig. 2, a reduction in cumulative [^{3}H]phenylalanine incorporation was observed in stypoldione-treated embryos as early as 20 min after fertilization. This inhibition reached approximately 50% within 30 min after fertilization, and then continued at that level of inhibition for the remainder of the cell cycle. Cleavage was completely inhibited at this concentration of stypoldione. These results suggest that if stypoldione were to act solely by the mechanism of translational inhibition, protein synthesis need only be inhibited by 50% in order to suppress division of the sea urchin embryo completely.

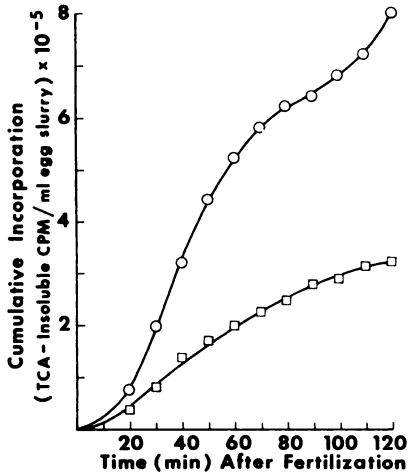

FIG. 2. *Effect of stypoldione on cumulative [^{3}H]thymidine incorporation during the first cell cycle in Lytechnius pictus sea urchin embryos*
Stypoldione (1.18×10^{-5} M) (□) or vehicle control (○) was added at 8 min after fertilization. [^{3}H]Phenylalanine was added at 3 min after fertilization. Similar results were obtained in *Stronglylocentrotus purpuratus*. *TCA*, Trichloracetic acid.

Effects of stypoldione on [^{3}H]phenylalanine uptake and incorporation rates. The reduced incorporation of [^{3}H]phenylalanine observed in Fig. 2 might be accounted for by a stypoldione-mediated inhibition of amino acid uptake. The effects of stypoldione on [^{3}H]phenylalanine uptake rates were therefore examined throughout the first cell cycle in *S. purpuratus* sea urchin embryos. In addition, we simultaneously examined the effect of stypoldione on [^{3}H]phenylalanine incorporation rates.

Preliminary studies were first undertaken to determine the optimal experimental conditions for measuring phenylalanine uptake rates in sea urchin embryos. Maximal uptake rates were observed by using a pulse duration of 5 min at an extracellular phenylalanine concentration of $2.0–3.0 \times 10^{-5}$ M or greater (data not shown). When incorporation rates were measured as a function of pulse duration (using a saturating extracellular phenylalanine concentration), linear incorporation rates were observed when the pulse duration ranged from 1 to 10 min (data not shown). This result indicated that the measurement of incorporation rates could be carried out under the same conditions as those used to measure uptake rates (i.e., a pulse duration of 5 min and an extracellular phenylalanine concentration of 5.0×10^{-5} M).

As illustrated in Fig. 3, the [^{3}H]phenylalanine uptake and incorporation rates increased rapidly after fertiliza-

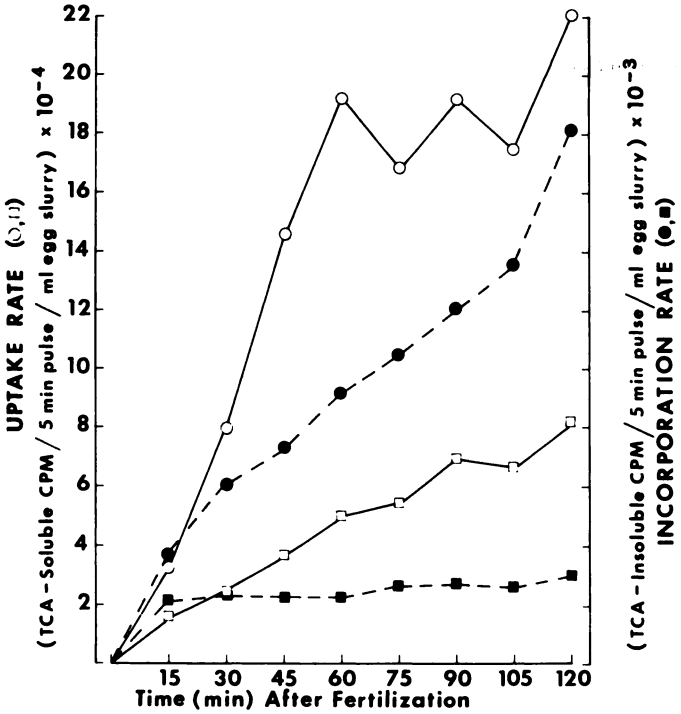

FIG. 3. *Effect of stypoldione on [³H]phenylalanine uptake (○, □) and incorporation (●, ■) rate during the first division cycle of Strongylocentrotus purpuratus*

Stypoldione (□, ■) or vehicle control (○, ●) was added approximately 5 min after fertilization. At 15-min intervals after fertilization, duplicate egg slurry samples were pulsed with [³H]phenylalinse for 5 min and washed three times by centrifugation; the pelleted eggs were killed by the addition of trichloroacetic acid (*TCA*). Uptake rate is defined as the amount of [³H]phenylalanine accumulated into the trichloroacetic acid-soluble pool by the end of the 5-min pulse. Incorporation rate is defined as the amount of [³H]phenylalanine accumulated into trichloroacetic acid-insoluble material by the end of the pulse.

tion in the control embryos. The pattern of amino acid uptake rates we observed during the first cell cycle in sea urchin embryos are in excellent agreement with results reported by Mitchison and Cummins (9) and Epel (10). The pattern of amino acid incorporation rates also appears to be quite similar to those reported by Sofer *et al.* (11) and Fry and Gross (12). Embryos incubated in the continuous presence of 1.0×10^{-5} M stypoldione, however, exhibited 69% and 61% reductions in the amino acid uptake and incorporation rates (respectively) at 30 min after fertilization. By 120 min after fertilization (i.e., approximately the time of first cleavage in control embryos) the uptake and incorporation rates had been reduced to approximately 37% and 16.5% of control (respectively).

Effect of stypoldione on embryos preloaded with [³H] leucine. The inhibition of [³H]phenylalanine incorporation into protein produced by stypoldione was difficult to interpret in light of the fact that stypoldione also significantly inhibited amino acid uptake. It remained possible that the reduced amino acid incorporation we observed was simply a reflection of the fact that less [³H]phenylalanine was entering the stypoldione-treated embryos, thus giving rise to an intracellular precursor pool of lower specific activity and, hence, a trichloroacetic acid-insoluble fraction of lower activity. In order to circumvent this problem we chose to preload embryos with [³H] leucine for 1 hr prior to fertilization, thus dissociating stypoldione's effects on incorporation from those on uptake. After loading, the eggs were washed free of extracellular labeled amino acid and fertilized, and stypoldione was added at approximately 5 min after fertilization. The amount of labeled amino acid present in the trichloroacetic acid-soluble and insoluble fractions were then measured as a function of time after fertilization.

The effect of stypoldione on [³H]leucine incorporation in these preloaded embryos is illustrated in Fig. 4A. Inhibition of incorporation was apparent by 20 min postfertilization in stypoldione-treated embryos. This inhibition progressed throughout the remainder of the experiment, such that by 60 min postfertilization the stypoldione-treated embryos exhibited only 43% of the control incorporation.

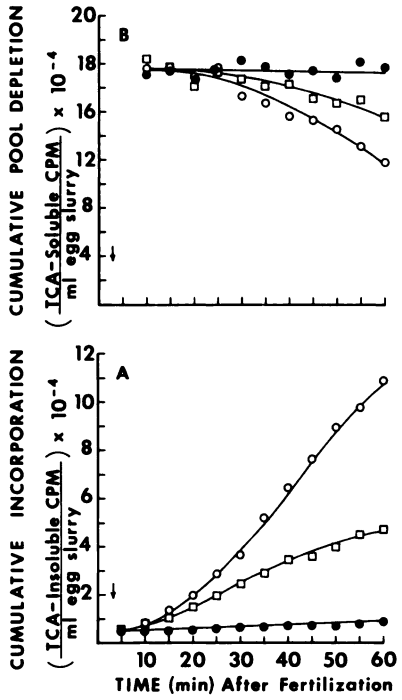

FIG. 4. *Effect of stypoldione on cumulative [³H]leucine incorporation and cumulative loss in embryos preloaded with [³H]leucine*

A. Effect of stypoldione on cumulative [³H]leucine incorporation into *Strongylocentrotus purpuratus* embryos preloaded with [³H]leucine for 1 hr prior to fertilization. After loading, eggs were washed and fertilized, and stypoldione (1.0×10^{-5} M) (□) or vehicle control (○) was added at 3–4 min after fertilization. Cumulative [³H]leucine incorporation in an identical preloaded unfertilized egg aliquot (●) was also determined. Duplicate 1.0-ml egg or embryo aliquots were transferred at the times indicated to tubes containing 1.0 ml of 20% trichloroacetic acid (*TCA*). [³H]Leucine incorporation into the trichloroacetic acid-precipitatable material was determined as given under Materials and Methods. Trichloroacetic acid-soluble material was saved for analysis as given in B.

B. Cumulative loss of [³H]leucine from the trichloroacetic acid-soluble fraction of preloaded embryos. Aliquots of the trichloroacetic acid-soluble extracts (saved from the experiment depicted in A) were counted to show the decrease in the free intracellular [³H]leucine pool as a function of the time after fertilization. ○, Fertilized controls; □, fertilized stypoldione-treated embryos; ●, unfertilized eggs.

It remained remotely possible, however, that the decreased incorporation we observed was due to a stypoldione-mediated alteration in membrane permeability. If stypoldione altered the permeability of the embryos such that [^{3}H]leucine diffused out of the cell, then there would be less intracellular [^{3}H]leucine available for incorporation. To test this possibility, we measured the intracellular [^{3}H]leucine pool size in both control and stypoldione-treated embryos. Figure 4B shows the changes in the amount of [^{3}H]leucine present in the trichloroacetic acid-soluble fractions as a function of time after fertilization. The amount of intracellular [^{3}H]leucine remaining in the stypoldione-treated embryos was clearly greater than that present in the fertilized controls. This result argues against a stypoldione-induced efflux of the [^{3}H] leucine. This result also argues against a general disruption of the plasma membrane in the presence of stypoldione. Agents which grossly disorganize the plasma membrane (i.e., detergents, bile salts, large unsaturated fatty acids) will usually produce a rapid cell lysis (which was not observed) and a simultaneous loss of normal permeability barriers. If stypoldione acted by this mechanism, an efflux of [^{3}H]leucine (down its concentration gradient) should have been observed.

Effects of stypoldione on cumulative [^{3}H]thymidine incorporation. Translational inhibitors might well be expected to inhibit DNA synthesis, since chromosomal replication has been shown to be dependent upon continued protein synthesis in many types of cells (13, 14). In order to determine whether DNA synthesis was inhibited in arrested embryos, we next examined the effects of stypoldione on [^{3}H]thymidine incorporation during both the S1 and S2 DNA replicative periods of the first division cycle.

Two periods of incorporation were observed in the control embryos, as shown in Fig. 5. The first period of DNA synthesis (occurring at approximately 30–50 min after fertilization) was initiated and completed well before the onset of mitosis, and is referred to as the M phase-independent S1 period. The second period of DNA synthesis (occurring between 100 and 120 min after fertilization) was initiated immediately after anaphase of mitosis and was completed within a few minutes after cleavage furrow formation. This second period of DNA synthesis was apparently dependent upon the completion of mitosis for its initiation (5, 6), and is hence referred to as the M phase-associated S2 period.

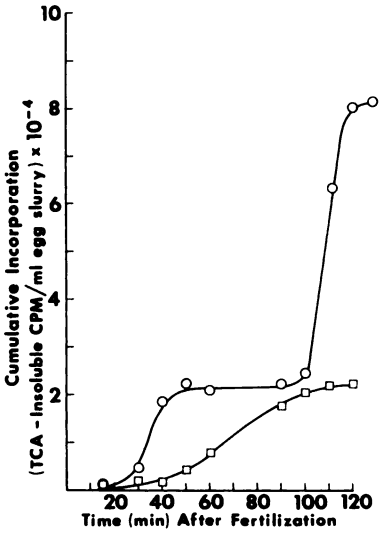

FIG. 5. *Effect of stypodione on cumulative [^{3}H]thymidine incorporation during the first cell cycle in Stronglylocentrotus purpuratus*

Fertilized embryos were incubated in the presence (□) or absence (○) of 1.0×10^{-5} M stypoldione. Stypoldione was added at approximately 5 min after fertilization. [^{3}H]Thymidine was added immediately after fertilization. *TCA*, Trichloroacetic acid.

As shown in Fig. 5, both the S1 and S2 periods of DNA replication were altered in the presence of 1.0×10^{-5} M stypoldione (i.e., a concentration producing a complete inhibition of cleavage). The extent of [^{3}H]thymidine incorporation in stypoldine-treated embryos was only 21% of the control value at 50 min after fertilization (i.e., the end of the S1 period in control embryos). There subsequently followed a gradual increase in incorporation in the stypoldione-treated embryos which, by approximately 120 min after fertilization, usually attained the control S1 level. This depressed and prolonged pattern of incorporation in stypoldione-arrested embryos is suggestive of an aberrant S1 period (i.e., an attempt by the cell to initiate and complete the first round of DNA replication, even though the process seemed to occur at an extremely slow rate relative to controls). In contrast to the effect of stypoldione on S1, [^{3}H]thymidine incorporation during S2 was completely and permanently blocked. Cumulative [^{3}H]thymidine incorporation never rose beyond the S1 level in stypoldione-treated embryos, even when examined at 3 hr after fertilization. These results suggested that (a) the S1 period, although aberrant, could apparently be initiated and ultimately completed in stypoldione-arrested embryos, and (b) initiation of the S2 period of DNA synthesis was apparently completely inhibited in the presence of stypoldione.

Effects of stypoldione on [^{3}H]thymidine uptake rates. As explained previously, accurate determination of the extent of macromolecular synthesis using the "labeled precursor technique" generally requires that entry of the radiolabeled precursor molecule into the cell be unimpeded. Inhibition of [^{3}H]thymidine uptake by stypoldione would act to decrease the specific activity of the intracellular thymidine pool, which in turn might explain the reduced incorporation illustrated in Fig. 5. To examine this possibility, we measured the average rate of accumulation of [^{3}H]thymidine into the free intracellular (trichloroacetic acid-soluble) pool at 15-min intervals throughout the first cell cycle in *S. purpuratus* sea urchin embryos.

Prior to examining the effects of stypoldione on thymidine uptake, however, the echinoderm thymidine transport system itself had to be characterized. Optimization procedures similar to those described for the development of phenylalanine uptake rates were also developed for thymidine uptake. We found (data not shown) that thymidine uptake rates were maximal when measured using a pulse duration of 5 min and an extracellular thymidine concentration of 5.0×10^{-5} M. This initial characterization of the echinoderm thymidine transport system then permitted the determination of initial uptake rates.

As shown in Fig. 6, stypoldione produced a 64% inhi-

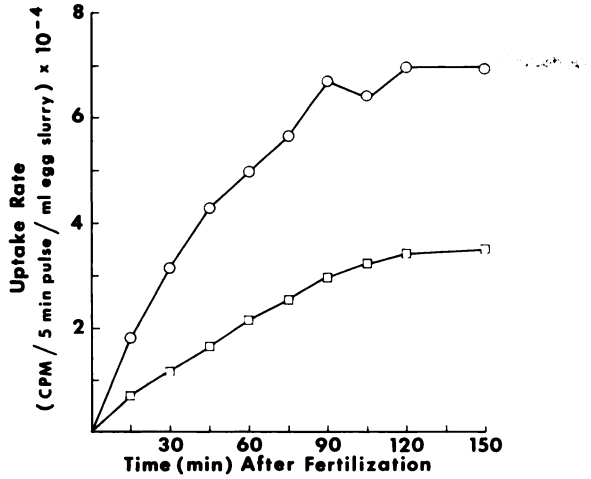

FIG. 6. *Effect of stypodione on [³H]thymidine uptake rate during the first cell cycle in Strongylocentrotus purpuratus*

[³H]Thymidine uptake rates were measured in the presence (□) or absence (○) of 1.0×10^{-5} M stypoldione. Stypoldione was added approximately 5 min after fertilization. Duplicate egg samples were removed at 15-min intervals after fertilization and pulsed with [³H] thymidine for 5 min. Eggs were then washed three times by centrifugation, and the pelleted eggs were killed by addition of trichloroacetic acid. Uptake rate is defined as the amount of [³H]thymidine accumulated into the trichloroacetic acid-soluble pool at the end of the 5-min pulse.

bition of [³H]thymidine uptake within 30 min after fertilization, suggesting the possibility that the inhibition of [³H]thymidine incorporation we observed in cumulative labeling experiments might be accounted for, at least in part, by the inhibition of thymidine uptake.

Effect of stypoldione on embryos preloaded with [³H] thymidine. In order to dissociate the effect of stypoldione on [³H]thymidine uptake from an effect on incorporation, fertilized eggs were preloaded with [³H]thymidine in drug-free seawater for the first 17 min after fertilization. The embryos were then washed and resuspended in normal seawater, and stypoldione (10 μM) or vehicle control was added at 22 min postfertilization. Cumulative [³H]thymidine incorporation during the S1 and S2 DNA replicative periods was then determined as described previously. This experimental design precluses any drug-mediated effect on uptake, since the embryos had already taken up the [³H]thymidine and were already washed free of the extracellular nucleoside well before the addition of stypoldione.

As shown in Fig. 7 stypoldione completely inhibited S2 while exerting only minimal inhibition of [³H]thymidine incorporation during the M phase-independent S1 period of DNA replication. It is important to note that cleavage was completely suppressed in these embryos. In light of the previous results showing an inhibition of thymidine uptake by stypoldione, these observations suggest (a) that inhibition of S1 is not a prerequisite for the inhibition of cleavage produced by stypoldione, (b) that the reduced S1 [³H]thymidine incorporation produced by stypoldione under continuous labeling conditions (cf. Fig. 5) might indeed be due to an inhibition of [³H]thymidine uptake, and (c) stypoldione-treated embryos probably do not complete transit through mitosis and hence do not initiate the S2 period of DNA replication.

Effects on [³H]thymidine incorporation in preloaded embryos: a comparative study between stypoldione and podophyllotoxin. Stypoldione has been previous shown to inhibit beef brain microtubule assembly *in vitro* (3, 4). This results suggested that stypoldione might block cleavage of the sea urchin embryo via inhibition of microtuble assembly. Mitotic spindle poisons would in fact be expected to produce effects on [³H]thymidine incorporation quite similar to those produced by stypoldione. Mizel and Wilson (15) have shown that the substiochiometric microtubule assembly inhibitors podophyllotoxin and colchicine inhibit nucleoside uptake in cultured mammalian cells, with K_i values of 6.0×10^{-6} M and 4.0×10^{-5} M, respectively. Inhibition of nucleoside uptake would thus be expected to produce an inhibition of [³H] thymidine incorporation into DNA during cumulative labeling experiments, but not in embryos which had been preloaded with the nucleoside. However, mitotic spindle poisons would be expected to block the S2 period of DNA replication in sea urchin embryos, since initiation of S2 is dependent upon the completion of mitosis.

Podophyllotoxin (1.0×10^{-6} M) was found to produce [³H]thymidine incorporation pattern in preloaded embryos essentially identical with that obtained with stypoldione (data not shown). Incorporation during the S1 period occurred normally in podophyllotoxin-treated em-

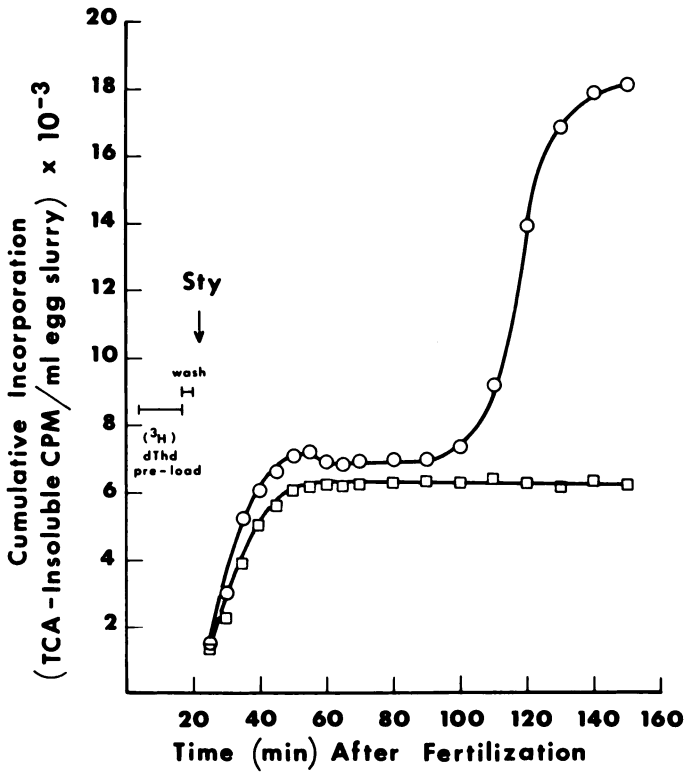

FIG. 7. *Effect of stypoldione on [³H]thymidine incorporation into embryos preloaded during the first cell cycle*

Stronglylocentrotus purpuratus embryos were loaded with [³H]thymidine for the first 17 min after fertilization and quickly washed (to remove extracellular [³H]thymidine, followed by the addition (*arrow*) of 1.0×10^{-5} M stypoldione. The [³H]thymidine incorporated into trichloroacetic acid (*TCA*)-insoluble material from control or stypoldione-treated embryos was then determined at various times after fertilization.

bryos (as would be expected, since microtubule assembly inhibitors reportedly have no effect on DNA synthesis), whereas the S2 period was completely inhibited. The fact that this [^{3}H]thymidine incorporation pattern was essentially identical with that obtained with stypoldione provided additional support for the hypothesis that stypoldione (via inhibition of microtuble assembly) might inhibit cleavage by blocking cells in mitosis.

Cytological examination of stypoldione-arrested sea urchin embryos. Microtubule assembly inhibitors would be expected to block cells in metaphase (more precisely, prometaphase) of mitosis. Sea urchin embryos were therefore incubated in the presence of 1.0×10^{-5} M stypoldione, 1.0×10^{-6} M podophyllotoxin (as a "positive drug control"), or an equivalent volume of ethanol (as solvent control) throughout the first cell cycle. At 60 min postfertilization and at 10-min intervals thereafter until completion of the first cleavage in controls, aliquots from each of the three embryo slurries were transferred to centrifuge tubes, pelleted, fixed, and stained with acetoorcein as described under Materials and Methods. Microscopic examination of the embryos revealed that podophyllotoxin did block embryos in mitosis (as expected), whereas stypoldione did not. As shown in Fig. 8, the nuclear membranes were clearly broken down, and fully condensed chromosomes were formed in podophyllotoxin-treated embryos. Although the absence of a functional mitotic spindle disorganized the alignment of chromosomes along the metaphase plate, embryos were clearly blocked in "prometaphase" of mitosis by podophyllotoxin. In contrast, stypoldione-treated embryos did not appear to progress into prophase, as indicated by the fact that the nuclear membranes remained intact and the chromatin did not condense in these arrested embryos.

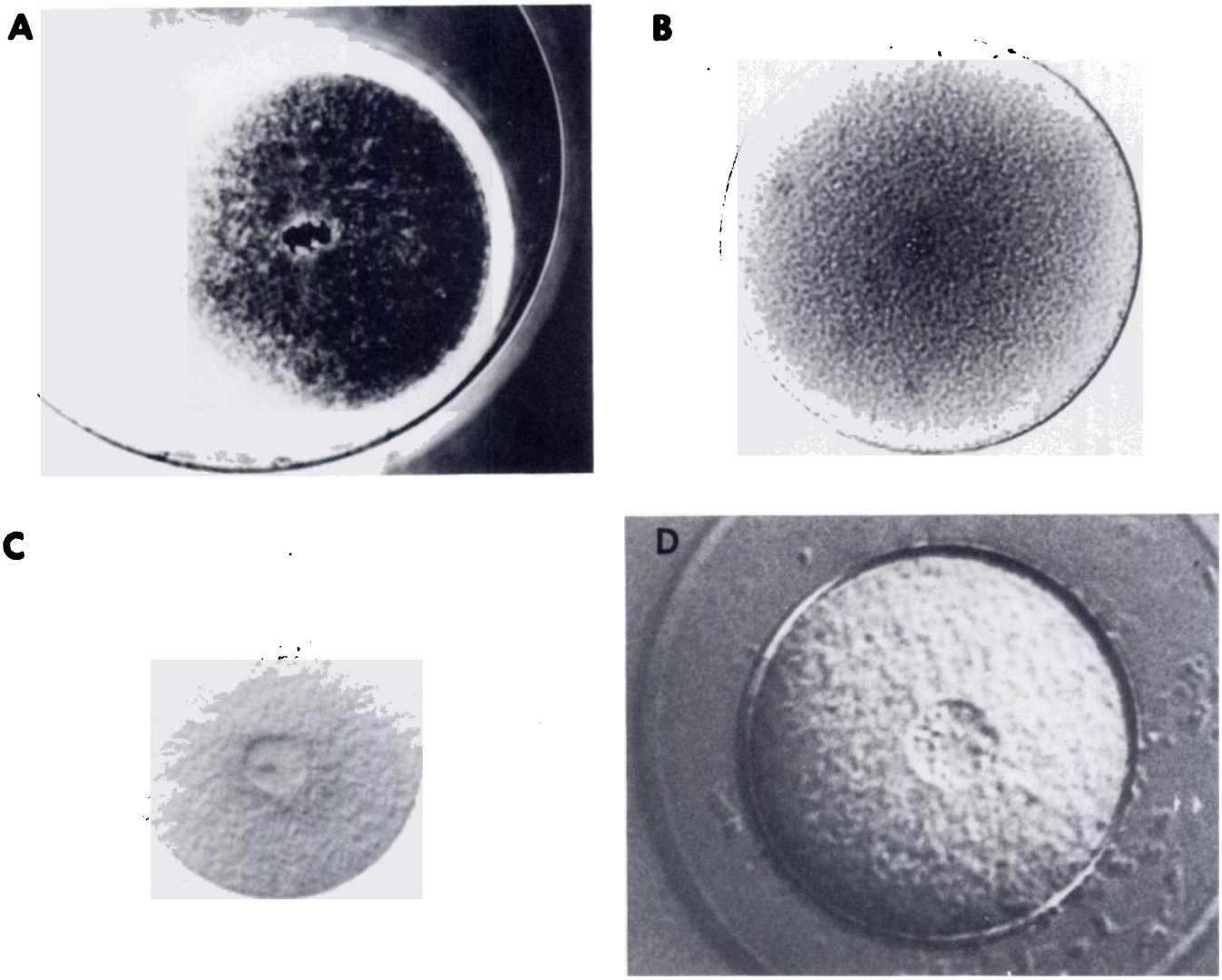

FIG. 8. *Stronglylocentrotus purpuratus embryos following incubation in various drugs or ethanol solvent control*

A, Control embryo fixed at 90 min after fertilization to show normal metaphase; B, embryos incubated in the presence of 1.0×10^{-6} M podophyllotoxin (embryos fixed at 130 min after fertilization, when all control embryos had already completed the first division); C, embryo incubated in the continuous presence of 1.0×10^{-5} M stypoldione (embryo fixed at 130 min after fertilization, when all control embryos had already completed division); D, control embryo fixed at 60 min after fertilization to show late interphase/very early prophase nucleus (note beginning of chromosomal condensation).

DISCUSSION

Stypoldione, the *o*-quinone derived from the brown alga *Stypodium zonale*, represents a new structural class of cell division inhibitors. As far as we are aware, there have been no reported pharmacological investigations of structurally similar *o*-quinones.

In preliminary studies with this marine natural product (3), we found that stypoldione produces an irreversible concentration-dependent inhibition of sea urchin embryo cleavage and inhibits beef brain microtubule assembly *in vitro*: Stypoldione has been shown to inhibit beef brain mircotubule assembly *in vitro* (3, 4). It is tempting to speculate that this single mechanism might be responsible for the activity of stypoldione. However, several lines of evidence argue strongly against such a restricted hypothesis. First, inhibitors of microtubule assembly will generally block cells in metaphase by virtue of disrupting the mitotic spindle apparatus. In contrast, stypoldione proved incapable of accumulating sea urchin embryos in mitotis, even when assayed over a wide concentration range. Identical results have also been obtained using CHO (Chinese hamster ovary), 3T3 (Swiss mouse), and CEM (human lymphoblast) cells,[2] indicating that our results are not peculiar to echinoderm embryos. Second, Sofer *et al.* (11) and, later, Fry and Gross (12) have shown that the microtubule assembly inhibitors colchicine and colcemid have no significant effect on labeled amino acid uptake or incorporation rates in sea urchin embryos, even when assayed at inhibitor concentrations which completely block syngamy and formation of the mitotic spindle apparatus. These results demonstrate an obvious qualitative difference between stypoldione and other inhibitors of microtubule assembly and suggest that stypoldione may be interacting with receptors other than (or at least in addition to) tubulin. Third, it has been demonstrated previously that microtubule assembly inhibitors do not interfere with nuclear membrane breakdown or chromosomal condensation (16), yet the cytological evidence reported here indicates that both processes are either greatly retarded or blocked in stypoldione-treated embryos.

In pursuing the nature of this inhibition of cell division, we examined the effect of stypoldione on DNA synthesis as measured by [^{3}H]thymidine incorporation. In cumulative labeling experiments (in which [^{3}H]thymidine and stypoldione were added to freshly fertilized eggs at essentially the same time), we observed a depressed incorporation during S1 and a complete inhibition of incorporation during S2. Interpretation of these results was complicated, however, by the fact that stypoldione also significantly inhibited [^{3}H]thymidine uptake. In experiments in which embryos were preloaded with [^{3}H]thymidine prior to the addition of stypoldione, we found that the M phase-independent S1 period of DNA replication proceeded in a relatively normal fashion, whereas the M phase-associated S2 period was again completely inhibited. These results suggested (a) that the process of DNA synthesis per se was not directly inhibited by stypoldione (since incorportion during the M phase-independent S1 period occurred normally), and (b) that the inhibition of S2 was probably a result of an inhibition of initiation of DNA synthesis (i.e., if mitosis could not be completed, then the S phase would not be initiated). The fact that stypoldione could be added well after the completion of S1 yet still produce a complete inhibition of cleavage (data not shown) also indicated that S1 inhibition played no crucial role in the ability of stypoldione to inhibit division.

An examination of the rates of amino acid uptake and incorporation into protein revealed that these processes were inhibited by stypoldione. By preloading embryos with [^{3}H]leucine to dissociate effects on uptake from those on incorporation, we have further shown in a more direct fashion that stypoldione produces a significant inhibition of protein synthesis in sea urchin embryos. Interestingly, inhibitors of protein synthesis have been shown (a) to inhibit completely [^{3}H]thymidine incorporation during S2 in echinoderm embryos while producing no inhibition of S1 (8, 17) and (b) to prevent the breakdown on the nuclear membrane and chromosomal condensation in *S. purpuratus* sea urchin embryos (8). These results are obviously very similar to those we have observed for stypoldione. These similarities (i.e., inhibition of amino acid incorporation, inhibition of thymidine incorporation during S2 but not S1, inhibition of nuclear membrane breakdown and chromosomal condensation) suggest that stypoldione might inhibit cleavage by inhibiting translation.

However, there are a few considerations which serve to complicate the simple hypothesis that all of the effects produced by stypoldione are the result of translational inhibition. Inhibition of protein synthesis by such agents as emetine or puromycin, for example, will produce a profound inhibition of cell division in cultured mammalian cells (13, 14, 18), yet this is the case in sea urchin embryos only if these translational inhibitors are added early in the cell cycle (7, 8). At a concentration of 1.00×10^{-4} M, emetine will produce a complete inhibition of protein synthesis within 1 min of its addition (8, 19), yet Wagenaar and Mazia (8) have found this potent translational inhibitor effective in blocking the first cleavage only when added prior to 35 min after fertilization. In contrast, we have found that stypoldione is able to inhibit cleavage completely even when added as late as 80 min. post-fertilization (data not shown). These differences in inhibitory kinetics suggest that translational inhibition is probably not the sole mechanism by which stypoldione inhibits cell division. It may be possible that when stypoldione is added after 35 min postfertilization, some combination of inhibitory effects on translation and microtubule assembly is responsible for the observed inhibition of cell division. Finally, results obtained using flow cytofluorimetric analysis on cultured mammalian cells indicates that stypodione completely blocks cell division and accumulates cells with a 4*n* content of DNA without producing a significant increase in the mitotic index.[2] Translational inhibitors have been previously shown to inhibit cell cycle progression essentially everywhere in the cell cycle (i.e., they are cell cycle-independent), so that DNA synthesis will generally be inhibited in mammalian cells cultured in the presence of these cytotoxins (13, 14, 18). These results thus suggest that stypoldione

[2] S. J. White, G. Boder, and R. S. Jacobs, manuscript in preparation.

is in fact a true "G2-accumulating agent," exhibiting a cell cycle specificity not usually associated with translational inhibitors. G2 is still a relatively uncharacterized portion of the cell cycle. If stypodione does prove to block cells in G2, this compound might become an extremely useful pharmacological probe with which to examine those biochemical processes occurring in G2.

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