0026-895X/89/050708-08$02.00/0

MOLECULAR PHARMACOLOGY, 36:708-715

Role of Microtubule Assembly in Phenytoin Teratogenic Action in the Sea Urchin (*Arbacia punctulata*) Embryo

STEVEN ESTUS and JEFFREY L. BLUMER

Division of Pediatric Pharmacology and Critical Care Rainbow Babies and Childrens Hospital and the Departments of Pediatrics and Pharmacology Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

Received February 16, 1989; Accepted August 23, 1989

SUMMARY

We evaluated the role of microtubule assembly in phenytoin (5-5-diphenylhydantoin) teratogenic activity in the sea urchin embryo. Zygotes were exposed to phenytoin or one of several phenytoin analogs within 15 min of fertilization and the frequency of the resultant malformations was assessed at the cleavage and late gastrula (prism) stages. Concomitant studies of drug uptake into zygotes and drug effects on both microtubule assembly *in vitro* and spindle morphology *in situ* were also performed. Phenytoin, 5-*p*-methylphenyl-5-phenylhydantoin, and 5-*p*-methoxyphenyl-5-phenylhydantoin were teratogenic (approaching 100% affected embryos) at both developmental stages were concentrated rapidly by the zygotes, and induced a shortened mitotic spindle *in situ*. In a separate *in vitro* system using porcine brain microtubular protein, these analogs were shown to inhibit microtubule assembly directly. The major human metabolite of phenytoin, 5-*p*-hydroxyphenyl-5-phenylhydantoin was teratogenic at the prism stage but induced only a 20% incidence of abnormal embryos at the first cleavage. This was attributed to the slow rate of uptake of this analog. This compound inhibited microtubule assembly in the *in vitro* assay and also shortened the mitotic spindle to an extent proportional to its observed weak effect on the first cleavage. Another analog, 5-*p*-hydroxyphenyl-5-*p'*-methylphenylhydantoin was not teratogenic at concentrations up to the limit of its solubility (285 μM). If this analog were as potent inside the cell as either phenyltoin or 5-*p*-hydroxyphenyl-5-phenylhydantoin, the intracellular concentrations achieved should have been sufficient to induce abnormal cleavage. Thus, the lack of teratogenic efficacy of this analog was correlated with its observed lack of effects on either microtubule assembly *in vitro* or spindle formation *in situ*. The anticonvulsant drug ethotoin was not teratogenic at concentrations up to 2.93 mM, apparently due to either poor uptake or inability to inhibit microtubule assembly or both. Overall, these studies are consistent with a hypothesis that phenytoin may induce abnormal development in this system by a direct inhibition of microtubule assembly.

The anticonvulsant drug DPH is one of the most commonly used agents for the chronic treatment of epilepsy. However, DPH use by women during pregnancy has been associated with an increased incidence of certain congenital malformations in their offspring (1). Due to the lack of efficacious and accepted nonteratogenic alternatives, the drug is still prescribed during pregnancy, representing a continuing teratogenic hazard. Because a clear understanding of the basis for DPH teratogenicity is lacking (2) and elucidation of this mechanism could aid in the identification of nonteratogenic anticonvulsant compounds, we have studied DPH teratogenicity in an *in vitro* model based on sea urchin embryogenesis (3, 4).

The sea urchin embryo has several properties that recommend it for teratology studies. Gravid females are inexpensive, are readily accessible, and produce large numbers of eggs (~500,000/female). The gametes fertilize *in vitro* with high efficiency (approaching 100%) and the embryos can be cultured easily in sea water. Study of developmental toxicity is facilitated because embryos develop rapidly with a low frequency of background abnormalities and exposure to various chemicals can be controlled directly. Indeed, sea urchin embryogenesis has been used to examine the developmental toxicity of a multitude of compounds ranging from industrial chemicals such as diphenyl (5) to pharmaceutical agents of many types (5, 6).

DPH manifests several characteristics of a classic teratogen (8) in this model system. The drug induces malformations in a dose-dependent manner (3) and it appears to act uniquely

This work supported in part by Training Grant 5 T32 GM07382-08 0021 from the National Institute of General Medical Sciences and by the Children's Research Foundation of Cleveland. Preliminary data were presented at the Federation of American Societies for Experimental Biology Annual Meeting, Washington, D. C., 1987. Submitted in partial fulfillment of requirements for a Doctor of Philosophy Degree (S.E.), Department of Pharmacology, Case Western Reserve University.

ABBREVIATIONS: DPH, phenytoin; *p*-Me-DPH, 5-*p*-methylphenyl-5-phenylhydantoin; *p*-MeO-DPH, 5-*p*-methoxyphenyl-5-phenylhydantoin; *p*-OH,*p'*-Me-DPH, 5-*p*-methylphenyl-5-*p'*-hydroxyphenylhydantoin; *p*-oH-DPH,5-*p*-hydroxyphenyl-5-phenylhydantoin; FITC, fluorescein isothiocyanate; ASW, artificial sea water; DMSO, dimethyl sulfoxide; HPLC, high pressure liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PBS, phosphate-buffered saline.

during the cleavage stage, demonstrating the presence of a critical period (4).

In examining the mechanism of DPH teratogenicity, we have focused our attention on drug effects during this critical period. DPH induced asymmetric, incomplete, and arrested cleavage (4), malformations that involved abnormal induction of the cleavage furrow. Because the spatial and temporal orientation of the cleavage furrow is dependent on the metaphase mitotic spindle (9, 10), these malformations suggested that DPH might act via alterations in spindle formation and/or function. This suggestion was supported by a cell cycle study that revealed that drug exposure during M phase was required to induce these malformations (4). DPH could alter the mitotic spindle directly via an action on the proteins requisite to spindle formation or indirectly via several mechanisms, including changes in cellular Ca^{2+}concentrations or sulfhydryl content (11).

In *in vitro* studies involving purified microtubular protein, DPH inhibited microtubule assembly (12), a process required for formation of the mitotic spindle *in vivo* (10, 11). Therefore, we postulated that DPH teratogenicity might result from a direct inhibition of microtubule assembly, which would induce, in turn, a faulty spindle, abnormal cleavage, and dysmorphogenesis. This has been evaluated in this study by examining DPH and several analogs for their ability to induce abnormal development, to inhibit microtubule assembly *in vitro*, and to induce an aberrant mitotic spindle *in situ*.

Experimental Procedures

Materials. DPH, poly-L-lysine, and affinity-purified FITC-labeled goat anti-rat immunoglobulin G antibody were obtained from Sigma Chemical Company (St. Louis, MO). Podophyllotoxin, DPH, *p*-Me-DPH, *p*-OH-DPH. *p*-OH,*p'*-Me-DPH, and Hoechst DNA stain 33,258 were purchased from Aldrich Chemical Company (Milwaukee, WI). Ethotoin was a generous gift of Abbott Laboratories (Abbott Park, IL). Monoclonal antitubulin antibody (YL½ ascites fluid) was purchased from Accurate Chemical and Scientific Company (Westbury, NY). Hyper-Tech film was obtained from Microfluor Limited (Stoneybrook, NY).

The *p*-MeO-DPH analog was synthesized from *p*-methoxy benzil (Pfaltz and Bauer, Inc., Stamford, CO) and urea by the method of Biltz and Slotta (13). Identity was confirmed by mass spectrometry. The molecular ion had a mass of 282.0983, accurate within analytical error to a calculated mass of 282.1004, and the spectrum was otherwise very similar to that of DPH. Purity was enhanced by recrystallization and verified by HPLC and melting point (210–211°).

Animal maintenance and embryo preparation. ASW was made by adding Instant Ocean sea salts (Aquarium Systems, Mentor, OH) to distilled water to a specific gravity of 1.021 g/ml and adjusting to pH 8.0 with 1.0 N HCl. Sea urchins (*Arbacia punctulata*) are gravid according to the season and geographic location; from November to February they were obtained from Gulf Specimen Company (Panacea, FL) and in July from The Marine Biology Laboratory (Woods Hole, MA). They were kept in a laboratory aquarium where they remained fertile for as long as 6 months.

Gametes were obtained by applying 9 V electrical stimulation to the shells of adult animals to induce spawning (7). Eggs were allowed to settle in ASW before fertilization. Sperm were collected undiluted. Eggs were fertilized by adding freshly diluted sperm to an egg suspension (~15,000 eggs/ml), followed by gentle agitation for 2–3 min. Fertilization efficiency was determined by microscopic inspection of an aliquot of eggs for the presence of fertilization membranes. Only batches of eggs exhibiting a fertilization efficiency ≥95% were used for these studies.

Teratology assays. The compounds to be tested were dissolved in minimal amounts of DMSO. Zygote exposure to varying drug concentrations (total volume of 1.0 ml) was initiated within 15 min of fertilization. Samples were incubated in 5-ml covered dishes at 20–21°. The spontaneous malformation rate was minimized by culturing zygotes at a density that allowed them to settle as a monolayer (≤15,000 embryos/ml). The final concentration of DMSO was maintained at 1%, which was shown independently not to alter development as defined by our criteria (see below).

The frequency of abnormal morphogenesis was determined by scoring 100 embryos from each aliquot during the cleavage stage (about 60 min after fertilization) and again at the late gastrula/prism stage (24 hr after fertilization) via light microscopy. Criteria for scoring a cleavage stage zygote as abnormal included incomplete, asymmetric, or arrested cleavage (Fig. 1). Criteria for scoring a prism stage embryo as abnormal included failure to shed the fertilization membrane, lack of motility, bleb formation, or dissolution (Fig. 2).

The malformation frequency in solvent-treated control aliquots was subtracted from that in drug-treated aliquots. If the former exceeded 10%, the assay was discarded. Dose-response curves were evaluated by probit analysis using the data points of the 5–95% response range (14).

Drug uptake assays. Zygotes (15,000/ml) were exposed to the individual drugs in ASW containing 1% DMSO (final volume, 1 ml). Samples were incubated at 20° with frequent agitation for intervals ranging from 10 to 90 min. At the end of the exposure period, 4 ml of 20° ASW were added and the zygotes were sedimented immediately in a clinical centrifuge at top speed for 15 sec. The supernatant was aspirated and the pellet was washed twice with 5 ml of 4° ASW. For both a teratogen (DPH) and a nonteratogen (*p*-OH,*p'*-Me-DPH) (see below), a greater number of washes was shown not to wash away additional drug (data not shown).

The final pellet was solubilized by the addition of 2 ml of methanol followed by a 10-sec sonication by a Sonifier Cell Disrupter (Heat Systems model W185 equipped with a microtip). Samples were extracted further by vortexing for 10 sec and were then centrifuged for 10 min at top speed in a clinical centrifuge. The supernatant was removed and evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in methanol and injected onto a MicroPak MCH-10 C-18 reverse phase HPLC column.

Analogs were eluted at a constant flow rate of 1.0 ml/min, with an approximately exponential acetonitrile gradient in water (16 to 40% over 23 min). Compounds were detected by absorbance at 258 nm. Peak areas were normalized relative to the recovery of an internal standard and converted into drug quantities by reference to standard curves constructed for each compound. Preliminary experiments revealed that the percentage of recovery of the different analogs was equivalent when methanol was used to extract the compounds from the embryos. Therefore, DPH was included as an internal standard in the methanol used for extraction for samples examining the uptake of *p*-OH-DPH, ethotoin, *p*-OH,*p'*-Me-DPH, and *p*-Me-DPH. *p*-Me-DPH eluted separately from *p*-MeO-DPH and DPH and served as their internal standard.

Uptake was defined as the amount of drug that increased with exposure duration, in order to correct for drug adsorption by the zygotes. Therefore, the values reported have been corrected for the amount of drug associated with the zygote pellet after a "zero-min" exposure interval, i.e., zygotes were washed as soon as drug was added. The extent of this correction as a percentage of the total drug in the pellet after incubation ranged from 2 to 13% (*p*-OH-DPH to DPH).

Uptake is reported as the apparent intracellular drug concentration, which was calculated as the amount of drug taken up/net embryo volume/sample. This volume was determined by counting the number of embryos/10 μl of stock solution using a microscope and applying a conversion factor of 3.10×10^{-10} liter/embryo (7).

***In vitro* microtubule assembly assays.** For convenience, microtubular protein was purified from porcine brain because sufficient quantities of zygote protein were not readily available. This alternative was justified on the basis of the high evolutionary conservation of tubulin, in terms of both primary sequence (11) and pharmacological

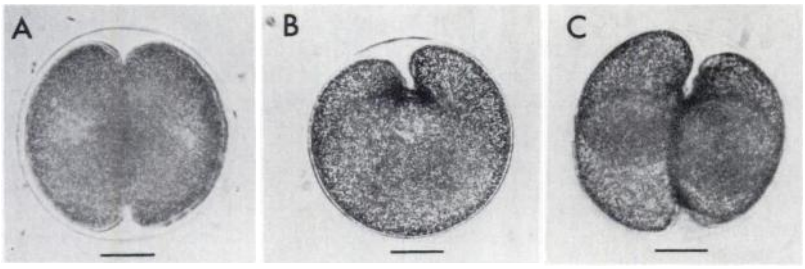


Fig. 1. Normal and DPH-induced abnormal cleavage stage zygotes. These photographs depict normal cleavage occurring in a zygote from a control sample (A) and incomplete (B) and asymmetric cleavage (C) occurring in zygotes that were exposed to DPH (65 μM). Drug exposures were initiated within 15 min after fertilization and the first cleavage occurred 55–60 min after fertilization. Zygotes were scored as abnormal if they underwent asymmetric or incomplete cleavage or if they had not initiated a cleavage furrow when they were examined. Each *scale bar* represents 20 μm.

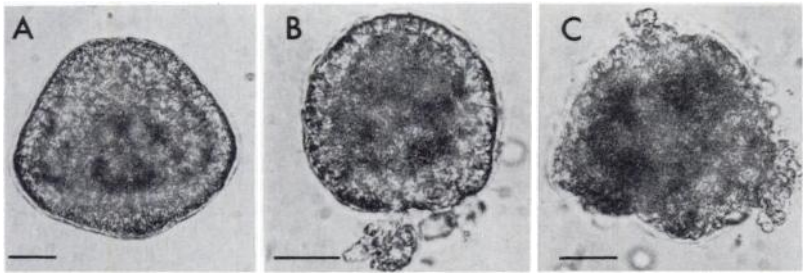


Fig. 2. Normal and DPH-induced abnormal prism stage embryos. These photographs depict prism stage embryos from control (A) and DPH-treated (B and C) samples approximately 24 hr after fertilization. The abnormal embryos display bleb formation (B) and dissolution (C) induced by DPH at concentrations of 65 and 95 μM, respectively. Each *scale bar* represents 20 μm.

sensitivity; many compounds that alter mammalian brain microtubule assembly *in vitro* (11) have been shown to alter microtubule assembly and/or microtubule-dependent processes in the sea urchin embryo. These modulators include inhibitors of microtubule assembly, such as colchicine (15), podophyllotoxin (16), and maytansine (17), and compounds that stabilize microtubules, such as taxol (18) and deuterated water (15). Porcine brain microtubular protein was purified, stored in aliquots, and prepared fresh daily, essentially as described by MacKinney *et al.* (12) except that the fresh brains were subjected to Waring blender as opposed to Dounce homogenization. The final reaction volume of 350 μl contained 1.7 mg/ml microtubular protein, 1 mM GTP, 0.1 mM $MgCl_2$, 100 mM PIPES (pH 6.94 at 21°), and 0–1.60 mM DPH. DPH was dissolved in minimal volumes of 0.1 N NaOH, which were then diluted such that a constant volume of this diluent was added to each sample. Assembly was initiated by transferring the solutions at 4° into a quartz cuvette at 33°. Microtubules scatter light, allowing microtubule assembly to be measured as changes in absorbance at 350 nm (19).

The assembly activity of microtubular protein stock solutions declined over the course of a day. For this reason, each assay consisted of five samples run simultaneously, of which up to four were experimental samples while the fifth was a control or solvent control sample for standardization. Drug effects were then quantitated as a percentage of concurrent control samples.

The analogs, except for ethotoin, were examined in a manner similar to DPH at a drug concentration at which DPH had a maximal effect (1.60 mM). Due to the limited alkaline solubility of ethotoin, it was examined using 1% ethanol (final concentration) as a solvent. The effects of DPH on microtubule assembly in the presence of 1% ethanol were examined in parallel, to control for a possible effect of ethanol on microtubule assembly. The final assembly buffer for each drug included both the alkaline and 1% ethanol diluents and drug effects were determined relative to similarly treated solvent control samples.

***In vitro* microtubule assembly data analysis.** Drug effects are presented in terms of relative apparent association rate constants (20). The elongation stage of assembly can be modeled as

$$A_t = A_{\text{maximum}} - A_{\text{maximum}}e^{-kt}$$

where A_t is the absorbance at time t, A_{maximum} is the absorbance at assembly equilibrium, and k is the apparent assembly rate constant. This equation can be rewritten as

$$\phi = -kt$$

where

$$\phi = \ln\{(A_{\text{maximum}} - A_t)/A_{\text{maximum}}\}$$

Thus, the apparent assembly rate constant was defined as the absolute value of the slope of the line of ϕ versus time. These plots were linear for several minutes following the nucleation phase (20) and were analyzed by linear regression. A minimum of five data points were used for these analyses; the correlation coefficients of the vast majority of samples were >0.990 and the minimum correlation coefficient was 0.987.

***In situ* microtubule assembly assays.** The indirect immunofluorescent staining procedure of Balczon and Schatten (21) was used with slight modifications. Zygotes stripped of fertilization membranes were incubated with the various drugs, within 20 min of fertilization, in 1% DMSO in Ca^{2+}-free ASW (21). About 55 min after fertilization, the incubation solutions were diluted 1/50 (v/v) into a solution containing 10 mM EGTA, 1 mM PIPES, 0.55 mM $MgCl_2$, 25% glycerol, 1% Nonidet P-40, and 1 mM Na-*p*-tosyl-L-arginine methyl ester (pH 6.7 at 21°). This solution permeabilized the zygotes and extracted yolk under conditions that stabilized microtubule structures. After 45 min, the translucent zygotes were allowed to settle onto polylysine-coated slides and were then fixed in −10° methanol for at least 6 min.

After rehydration in PBS, the zygotes were stained sequentially with a monoclonal antitubulin primary antibody (YL½ ascites fluid) and an FITC-labeled secondary antibody for at least 45 min each. Samples were stained at room temperature at antibody dilutions of 1/20 in PBS and each exposure was followed by a 30-min PBS rinse. Samples were then stained with 3 μg/ml Hoechst DNA stain 33,258 in PBS for 10 min. After a 10-min PBS rinse, the slides were rinsed with distilled water and mounted in a solution of 60 mg/ml 1,4-diazabicyclo-(2.2.2)-octane dissolved in Elvanol mountant (22), (generously provided by Dr. Jeffrey Salisbury, Department of Developmental Genetics and Anatomy, Case Western Reserve University).

Fluorescent staining was observed with a Nikon Optiphot microscope with epifluorescent illumination and a 40× objective. Photomicrographs were taken with Hyper-Tech film at an ASA of 1600.

***In situ* microtubule assembly data analysis.** The effects of the drugs on the metaphase mitotic spindle were quantitated by measuring spindle lengths. Metaphase spindles were identified by a single linear DNA stain located at the metaphase plate. Spindle lengths were measured directly from stained zygotes using a calibrated reticule in the microscope eyepiece; a photomicrograph record of each measurement was kept. Only spindles where both asters were in focus within a single focal plane were measured, to minimize artifactual shortening of the spindle due to an oblique viewing angle. The interassay variation in metaphase spindle lengths was slight, i.e., spindle lengths of control

samples in the three DPH dose-response experiments were [mean ± SD (n)] 42.5 ± 3.4 (14), 42.8 ± 6.8 (5), and 46.8 ± 1.8 (5) μm. The effects of the various treatments on spindle length were compared using ANOVA and *a posteriori* Sheffé t tests (23).

Results

Teratology assays. Several compounds closely related to DPH were screened for their ability to induce malformations. This initiated examination of the possible correlation between drug-induced developmental toxicity and effects on microtubule assembly. Zygote exposure to the drugs began within 15 min of fertilization and continued throughout embryogenesis. The frequency of resultant malformations was determined during both the DPH critical period (cleavage stage) (4) and at a relative developmental endpoint (prism stage).

Malformations induced by the analogs were identical to those induced by DPH. Several compounds were efficacious during both the critical cleavage stage and, as would be expected, the prism stage (Fig. 3). Although the compounds tended to be more potent at the prism stage, these differences were not statistically significant ($p > 0.05$) and were attributed to the compounds acting at each of the first few cleavages of the cleavage stage so that the net effect was a slightly greater potency as assessed at the prism stage. *p*-Me-DPH was as potent as DPH (average ED_{50} = 60 and 61 μM, respectively), whereas *p*-MeO-DPH (average ED_{50} = 159 μM) was significantly less potent at the level of 95% confidence, as determined by probit analysis (14).

The dose-dependent toxicity of *p*-OH-DPH was significantly different at the two developmental stages. Zygote exposure to *p*-OH-DPH at the limit of its solubility under these conditions (430 μM) resulted in a 20% incidence of abnormal first cleavage (Fig. 3D). At the prism stage, the drug was strikingly more potent (ED_{50} = 100 μM) and efficacious. These results suggested that this compound must act with greater potency and efficacy at some point between first cleavage and the prism stage.

Neither *p*-OH,*p'*-Me-DPH nor ethotoin altered development at the cleavage or prism stages at concentrations up to 285 μM (solubility limit) and 2.93 mM, respectively.

Uptake assays. Because limited drug uptake might limit teratogenic efficacy, the analogs were screened to identify those undergoing limited uptake. For DPH, uptake has been shown previously to be linearly dependent on the extracellular drug concentration and to reach equilibrium within 20 min of the initiation of exposure (4).

Uptake of DPH, *p*-Me-DPH, and *p*-MeO-DPH was examined at extracellular concentrations equal to their teratogenic ED_{50} values. The latter two compounds were concentrated readily by the zygotes, achieving intracellular concentrations 2.4 and 4.0 times greater than that of DPH, respectively (Table 1). Because

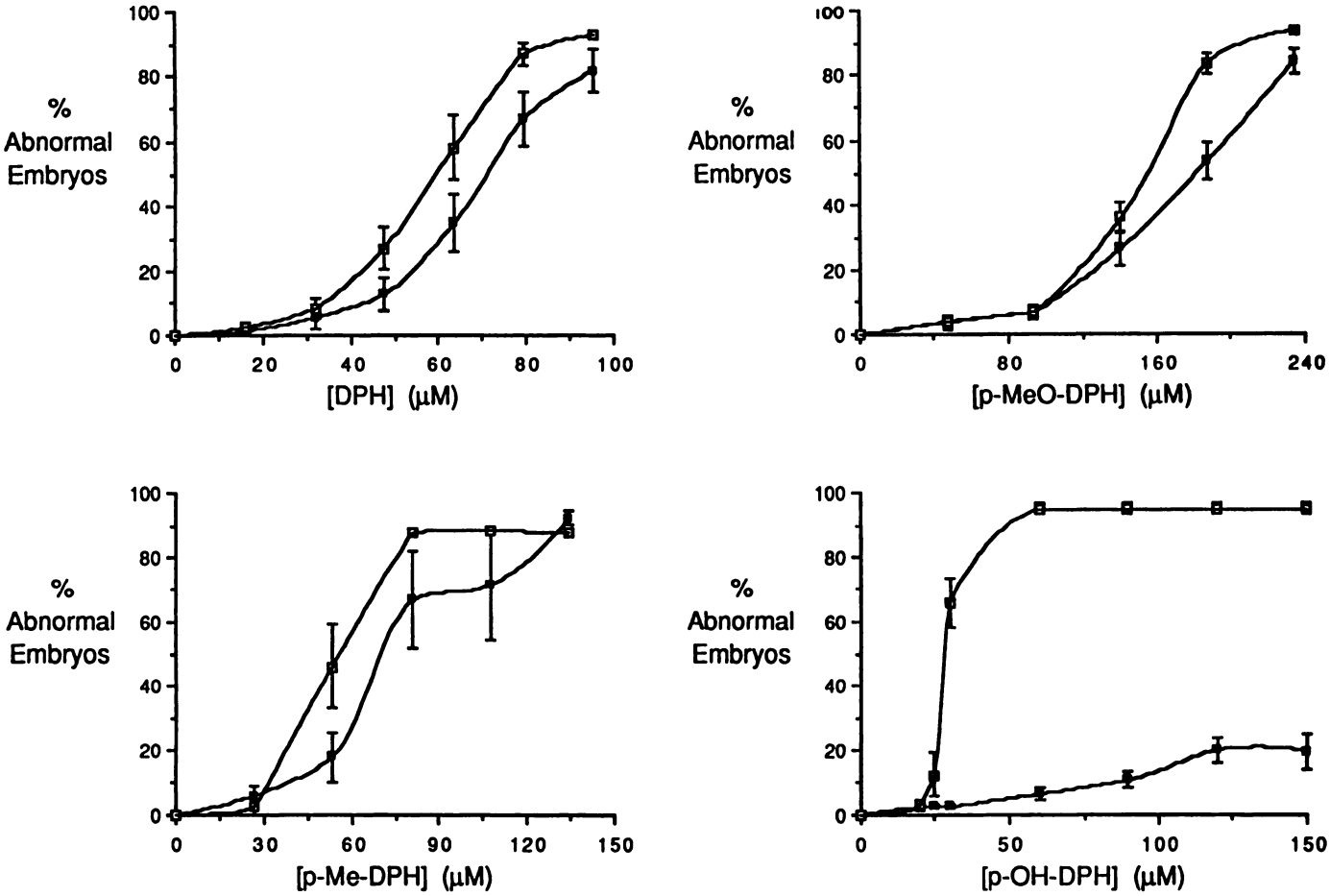


Fig. 3. Dose dependence of malformations induced by DPH (A), *p*-Me-DPH (B), *p*-MeO-DPH (C), and *p*-OH-DPH (D). Zygote exposure to the drugs began within 15 min of fertilization and continued throughout embryogenesis. The frequency of abnormal development was scored at the cleavage (■) and prism (□) stages. The concentration dependence of developmental toxicity varied significantly between these stages for only *p*-OH-DPN. These results represent the mean ± standard error of at least three separate experiments.

TABLE 1
Survey examination of uptake of DPH and several analogs

Analog	Extracellular concentration[a]	Exposure duration[b]	Apparent intracellular concentration
	μM	min	μM
DPH	61	10	252 ± 24[c]
p-Me-DPH	60	10	606 ± 85[c]
p-MeO-DPH	159	10	1019 ± 71[c]
p-OH-DPH	266	45	117 ± 11[d]
p-OH-DPH	266	90	215 ± 21[d]
p-OH,*p'*-Me-DPH	266	45	276 ± 46[c]
p-OH,*p'*-Me-DPH	266	90	316 ± 79[c]
Ethotoin	2440	45, 90	Not detected[e]

[a] For the first three compounds, exposure concentrations for each compound were their teratogenic ED_{50} concentrations. Uptake of *p*-OH,*p'*-Me-DPH was maximized by examining it at a concentration (266 μM) near saturation (285 μM). Uptake of *p*-OH-DPH was examined at the same extracellular concentration for comparative purposes.

[b] An exposure duration of 10 min was chosen for initial experiments in order to compare the results with a large quantity of data that were available examining DPH uptake after the same period (4). For DPH, the results obtained using the HPLC assay described here were very similar to those reported elsewhere, wherein uptake was determined using [^{14}C]DPH (4). For the remaining compounds, exposure intervals were based on the exposure duration before first cleavage in the teratology studies (~45 min). These compounds were also examined after 90 min to examine whether they had achieved equilibrium.

[c] Mean ± range of two determinations, each done in duplicate.

[d] Mean ± standard error of four determinations, each done in duplicate.

[e] Due to a relatively low extinction coefficient, the limit of detection for ethotoin was 300 μM.

the intracellular concentrations attained by these compounds in 10 min were greater than that attained by DPH at equilibrium [430 μM (4)], uptake of these compounds was not examined further.

Uptake of the remaining analogs was not as readily detectable. When examined following an exposure interval approximately equal to the duration of drug exposure before first cleavage in the teratology studies (45 min), uptake of *p*-OH-DPH was less than that of DPH (Table 1). This suggests that the limited teratogenic efficacy of this analog at the first cleavage might have been due to this low rate of uptake. However, in contrast to DPH, intracellular concentrations of *p*-OH-DPH were still increasing at an approximately linear rate 90 min after exposure was initiated (Table 1). Because the critical period of DPH action included the first 5 hr after fertilization and several cleavages (4), it is possible that *p*-OH-DPH attained intracellular concentrations during this critical period that were sufficient for it to act with greater efficacy and thus induce the observed incidence of prism stage malformations (Fig. 3D).

Uptake of *p*-OH,*p'*-Me-DPH was also examined (Table 1). Uptake appeared to reach equilibrium within 45 min and, therefore, intracellular concentrations were maximal by the time of first cleavage. To determine whether limited uptake might explain the lack of developmental toxicity of this compound, the toxicity of similar intracellular concentrations of the other analogs was considered. An intracellular DPH concentration equal to that attained by *p*-OH,*p'*-Me-DPH in 45 min would be achieved at equilibrium in zygotes exposed to 38 μM DPH (4). In contrast to the lack of toxicity of *p*-OH,*p'*-Me-DPH, zygote exposure to 38 μM DPH resulted in ~10% incidence of abnormal cleavage (Fig. 3A). Compared with *p*-OH-DPH and assuming that drug uptake was proportional to the extracellular concentration, uptake of *p*-OH,*p'*-Me-DPH would have been greater even when both drugs were at the limit of their solubility (430 and 285 μM, respectively). However, only *p*-OH-DPH induced abnormal cleavage (Fig. 3D). Thus, uptake of *p*-OH,*p'*-Me-DPH was sufficient to induce malformations if it was as potent inside the cell as DPH or *p*-OH-DPH. However, teratogenic intracellular concentrations of *p*-Me-DPH and *p*-MeO-DPH were greater than those attainable by *p*-OH,*p'*-Me-DPH (Table 1). This suggests that *p*-OH,*p'*-Me-DPH uptake might have been insufficient to induce malformations if it had an intracellular teratogenic potency approaching that of these compounds.

Uptake of ethotoin was not detectable under these conditions (Table 1), suggesting that uptake might limit the teratogenicity of this drug.

***In vitro* microtubule assembly assays.** The ability of DPH and the analogs to inhibit microtubule assembly directly was examined in an *in vitro* turbidimetric assay involving purified microtubular protein. The primary effect of DPH was to decrease the apparent rate of microtubule assembly (Fig. 4). DPH decreased the apparent assembly rate constant, acting over a narrow dose range, with an ED_{50} value of approximately 910 μM (Fig. 5). This value is about twice the intracellular DPH concentration (430 μM) attained in zygotes exposed to the teratogenic ED_{50} concentration (61 μM) until equilibrium (4).

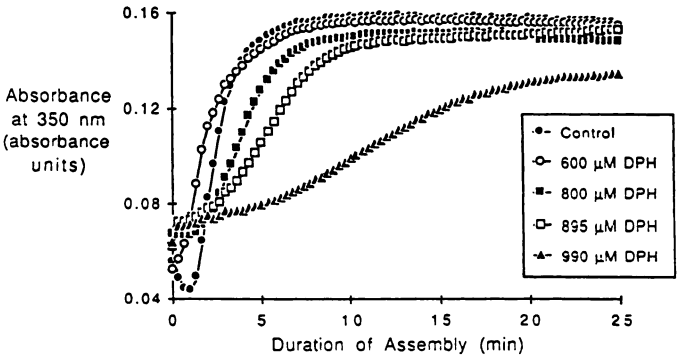


Fig. 4. Effect of DPH on microtubule assembly *in vitro*. Microtubule assembly was monitored as changes in absorbance at 350 nm. These changes reflected alterations in turbidity arising from microtubule formation. Solutions containing microtubular protein (1.7 mg/ml) and various concentrations of DPH were prepared at 4° and then placed in a cuvette chamber at 33° to initiate assembly. This figure depicts typical data, demonstrating the effects of DPH. The drug appears to be a more potent inhibitor of the rate of assembly than the total amount of assembly.

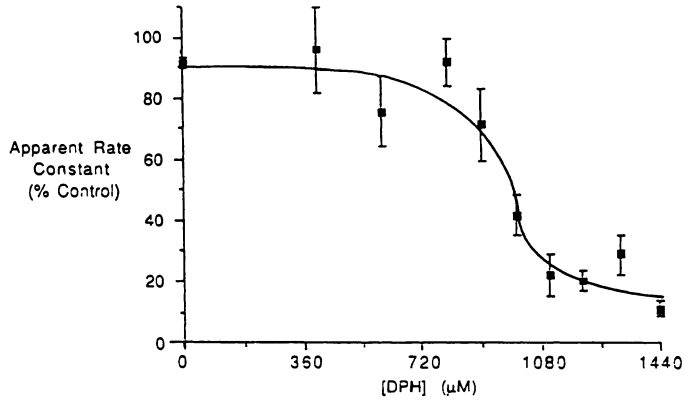


Fig. 5. Dose dependence of DPH reduction in the apparent rate constant for microtubule assembly. Relative apparent rate constants were determined as described in the text. These data were obtained using a single preparation of microtubular protein and represent the mean ± standard error of at least four assays.

This 2-fold difference in DPH potency seems minor, considering that one assay quantitates microtubule assembly *in vitro* and the other cleavage success *in vivo*. Multiple factors could account for such a difference including that microtubule assembly may be influenced by modulators present in one system and not the other, such as various microtubule-associated proteins (11), and that the percentage of inhibition of microtubule assembly may not correlate directly with the frequency of abnormal cleavage.

The effects of the analogs (except ethotoin) on assembly were examined at a drug concentration at which DPH markedly decreased the rate of microtubule assembly (1.60 mM). Those analogs that were inhibitory were similar to DPH in that they decreased the rate of assembly more dramatically than the extent of assembly. All four of the teratogenic compounds inhibited microtubule assembly (Table 2).

p-OH,*p'*-Me-DPH did not alter assembly (Table 2). Because it was concentrated inside the zygotes to an extent such that similar concentrations of DPH or *p*-OH-DPH would have induced malformations, this lack of inhibitory activity is consistent with its lack of developmental toxicity.

When examined in parallel with DPH, ethotoin did not alter assembly even at concentrations twice that of DPH (Table 1). Thus, the absence of teratogenic activity of ethotoin may have been due to poor uptake or inability to inhibit assembly or both.

***In situ* microtubule assembly assays.** The ability of DPH and the analogs to alter spindle morphology was addressed directly via immunofluorescence. Spindle morphology in DPH-treated zygotes was abnormal (Fig. 6; Table 3). Metaphase spindles in zygotes exposed to a DPH concentration below the teratogenic threshold (16 μM) appeared normal, although they were shorter than those in control or solvent-treated control zygotes. At ED_{50} (61 μM) and maximally teratogenic concentrations (122 μM), metaphase spindles were progressively shorter due to a decrease in interpolar distances and, at the latter concentration, smaller asters (Fig. 6). These results indicate that DPH alters spindle length in a concentration-dependent manner that is similar to that observed for DPH effects on development. Differences in the threshold concentrations suggest that relatively small changes in spindle length are compatible with normal cleavage.

To examine whether a correlation might exist between the ability of a drug to alter the mitotic spindle and to induce abnormal cleavage, the effects of the DPH analogs were determined. Zygote exposure to *p*-Me-DPH and *p*-MeO-DPH at maximally teratogenic extracellular concentrations (Fig. 3) decreased spindle length to an extent similar to that induced by DPH (Table 3).

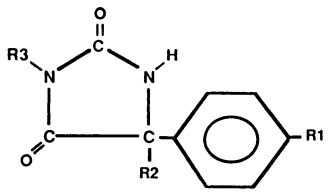
Zygote exposure to *p*-OH-DPH at the limit of its solubility induced a significant decrease in the length of the spindle relative to that observed in the solvent control samples (Table 3). However, a further comparison of the effects of this analog revealed that it was not significantly more effective than the nonteratogenic DPH concentration (Table 3), a result consistent with the limited effects of this analog on the first cleavage.

Zygote exposure to *p*-OH,*p'*-Me-DPH at a concentration near saturation decreased the spindle length less than that of the nonteratogenic DPH concentration (Table 3). This is consistent with the lack of *p*-OH,*p'*-Me-DPH effects on assembly *in vitro* or on cleavage. The effects of the anticonvulsant drug ethotoin on spindle length were also less than that of the lowest DPH concentration.

TABLE 2

Effect of DPH and analogs on microtubule assembly *in vitro*

Values are mean ± standard error (*n*). The first five compounds were examined at a single drug concentration of 1.60 mM. In contrast to the teratology studies, the compounds did not precipitate at these concentrations, presumably because their solubility was enhanced by protein binding. The results are reported as a percentage of the apparent rate constant of concurrent alkaline solvent control samples, which was 0.495 ± 0.090 min^{-1} (mean ± SE, n = 7). This apparent rate constant represents the product of the actual assembly rate constant and the concentration number of nucleation sites in the solution (16). The number of nucleation sites was not determined in this study. In the second set of experiments, the effects of DPH and ethotoin were examined at concentrations of 1.20 mM and 2.40 mM, respectively. The solvent control sample for this assay, as well as the DPH and ethotoin samples, contained both the alkaline and 1% ethanol (final concentration) diluents. The apparent rate constants in solvent control samples in these studies was 0.604 ± 0.035 min^{-1} (mean ± SE, n = 3).



Substituents				Apparent rate constant
R1	R2	R3		
				% of concurrent control
H	Phenyl	H	(DPH)	19.8 ± 4.0 (3)
CH_3	Phenyl	H		9.5 ± 1.0 (2)
CH_3O	Phenyl	H		68.4 ± 10.3 (5)
OH	Phenyl	H		51.8 ± 7.7 (2)
CH_3	*p*-OH phenyl	H		98.0 ± 12.7 (5)
H	Phenyl	H (DPH)		18.9 ± 9.6 (3)
H	H	CH_2 CH_3	(Ethotoin)	103.8 ± 4.4 (3)

Discussion

Elucidation of a teratogenic mechanism(s) for DPH is complicated by the diverse biochemical actions of the drug. DPH could conceivably alter developmental processes by several mechanisms including inhibition of Ca^{2+} ion flux (24) or inhibition of microtubule assembly (12). Also, conflicting evidence has been presented regarding a hypothesis that DPH teratogenicity might be mediated by a reactive epoxide metabolic intermediate (2).

Several observations support the hypothesis that, in the sea urchin embryo, DPH inhibits microtubule assembly directly, leading to abnormal spindle formation, aberrant cleavage, and ultimately dysmorphogenesis. First, DPH inhibited microtubule assembly in *in vitro* assays involving purified microtubular protein (Fig. 4) (12), suggesting that the drug may interact directly with the proteins involved in assembly. Second, microtubule assembly is required for the formation and function of the mitotic apparatus (10, 11), which in turn is responsible for induction of the cleavage furrow (9, 10); DPH altered the morphology of both the mitotic apparatus (Table 3; Fig. 6) and the cleavage furrow (Figs. 1 and 3) and induced these changes at similar drug concentrations, suggesting these effects may be related. Further, other inhibitors of microtubule assembly such as podophyllotoxin (16) and maytansine (17) induced dose-dependent cleavage stage malformations identical to those of DPH, suggesting that the ability to inhibit microtubule assembly is sufficient to account for the effects of DPH on cleavage.

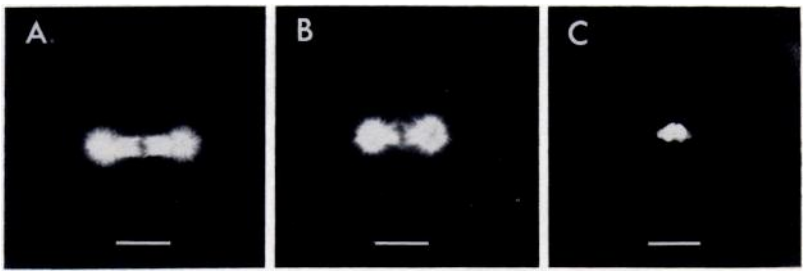


Fig. 6. Effects of DPH on metaphase mitotic apparatuses. Zygotes were exposed to DPH within 20 min of fertilization and processed for indirect immunofluorescence and DNA staining at the time of first cleavage. Metaphase spindles were identified as those with a linear DNA stain located between the spindle poles. Depicted here are the microtubule staining patterns of metaphase spindles from zygotes exposed to either ASW alone (A), or DPH at a concentration of 61 μM (B) or 122 μM (C). DPH exposure is associated with decrease in interpolar spindle length (B and C) and astral size (C). Each *scale bar* represents 20 μm.

TABLE 3

Drug effects on metaphase spindle length

Condition	Extracellular [Drug]	Metaphase Spindle Length[a]
	μM	μm
Control		41.9 ± 0.7 (45)
Solvent control		40.1 ± 0.6 (29)
DPH	16	35.1 ± 0.9 (26)[b]
DPH	61	27.5 ± 1.0 (13)[b]
DPH	122	17.9 ± 0.8 (28)[b]
p-Me-DPH	120	15.9 ± 1.2 (9)[b]
p-MeO-DPH	248	19.6 ± 0.5 (27)[b]
p-OH-DPH	450	33.3 ± 1.4 (16)[b]
p-OH,*p*′-Me-DPH	260	38.3 ± 0.9 (21)
Ethotoin	2440	36.6 ± 1.0 (13)
Podophyllotoxin[c]	0.145	14.9 ± 0.5 (7)[b]

[a] Mean ± SE (number of spindles measured). Spindle lengths were measured from zygotes prepared in at least two separate experiments.

[b] Spindle lengths in all of the DPH-treated groups were found to be significantly less than those in the solvent control group ($p < 0.001$). Similarly, comparison of the effects of the various other compounds relative to the solvent control sample revealed that the average spindle lengths in all except the *p*-OH,*p*′-Me-DPH and ethotoin samples were significantly decreased ($p < 0.001$). The spindles in these two groups were not significantly less ($p > 0.05$). Another ANOVA comparison of the effects of 16 μM DPH and the various analogs revealed that *p*-OH-DPH, *p*-OH,*p*′-MeDPH, and ethotoin did not significantly alter spindle length relative to this treatment ($p < 0.05$). The effects of the other compounds were highly significant ($p < 0.001$).

[c] The effects of podophyllotoxin were determined at a drug concentration that caused 85–100% arrested cleavage (data not shown) (20) to examine whether a recognized inhibitor of microtubule assembly would shorten the spindle to an extent similar to that of DPH.

TABLE 4

Relationship of teratogenicity to analog uptake, microtubule assembly, and spindle shortening

Compound	Teratogen	Uptake	Assembly	Spindle
DPH[a]	+	+	+	+
p-Me-DPH[a]	+	+	+	+
p-MeO-DPH[a]	+	+	+	+
p-OH-DPH[b]	+/−	+	+	+/−
p-OH,*p*′-Me-DPH[c]	−	+/−	−	−
Ethotoin[d]	−	−	−	−

[a] The ability to inhibit microtubule assembly and to shorten the spindle was associated with teratogenic efficacy in the first three compounds.

[b] Slow uptake of *p*-OH-DPH might have limited the effects of this drug on the spindle and the first cleavage.

[c] Because uptake of *p*-OH,*p*′-Me-DPH produced intracellular concentrations sufficient for DPH and *p*-OH-DPH to alter cleavage, the lack of teratogenic activity of *p*-OH,*p*′-Me-DPH might have been due to its lack of effect on microtubule assembly.

[d] The lack of teratogenicity of ethotoin might have been due to either poor uptake or inability to inhibit microtubule assembly or both.

Third, critical period studies have indicated that DPH actions during the cleavage stage initiate malformations that became apparent later in development (4), suggesting that DPH effects on cell division result in subsequent dysmorphogenesis. Fourth, we found a positive correlation between analog teratogenicity, uptake, ability to inhibit microtubule assembly *in vitro*, and ability to shorten the mitotic spindle (Table 4), linking critical aspects of the proposed mechanism. Overall, these results are consistent with the hypothesis that the DPH teratogenicity is the result of an inhibition of microtubule assembly *in situ*. Because the parent drug itself inhibits microtubule assembly directly, this suggests the parent drug may be the proximate teratogen.

Although DPH could inhibit microtubule assembly directly to induce the observed malformations, it is conceivable that the drug might act indirectly. For example, DPH alters Ca^{2+} flux (24) and Ca^{2+} at micromolar concentrations inhibits microtubule assembly both *in vitro* and *in situ* (11). However, in the *in vitro* assay examining microtubule assembly directly, there was little possibility for a drug action via Ca^{2+} because the microtubular protein was purified in the presence of the Ca^{2+} chelator EGTA. Further, inhibition of Ca^{2+} influx *in vivo* is unlikely to explain the effects of DPH because zygotes cleave normally in Ca^{2+}-free ASW, i.e., in the immunofluorescent studies.

DPH could inhibit microtubule assembly *in vivo* by a second indirect mechanism. Others have postulated that DPH is metabolized to a reactive intermediate that covalently binds to cellular macromolecules, inhibiting their function and thus causing abnormal development (25). However, the teratogenic effects of zygote exposure to DPH from fertilization until the beginning of M phase were negligible, especially relative to the frequency of malformations induced by a shorter exposure during M phase alone (4). This suggests that the effects of DPH are rapid, reversible, and do not accumulate, characteristics of a direct effect of a parent drug.

Several conclusions can be drawn from this work. First, the sea urchin embryo appears to be a simple and useful model for the evaluation of teratogenic mechanisms. Its extensive history as a model system in developmental biology provides a wealth of information concerning basic processes and the effects of xenobiotics, thus facilitating mechanistic studies. Second, DPH teratogenicity in this model may be the result of an inhibition of microtubule assembly leading to abnormal cleavage. Because tubulin, microtubule assembly, and the role of microtubules in cell division are highly conserved in evolution (11), this conclusion could have ramifications extending beyond this animal model. Indeed, decreased cell proliferation has been postulated to be a general teratogenic mechanism (26). Third, it appears that DPH acts directly to inhibit assembly and, therefore, the parent drug may be the proximate teratogen.

References

1. Hanson, J. W., and D. W. Smith. The fetal hydantoin syndrome. *J. Pediatr.* **87**:285–290 (1975).
2. Brown, L. P., O. P. Flint, T. C. Orton, and G. G. Gibson. Chemical teratogenesis: testing methods and the role of metabolism. *Drug Metab. Rev.* **17**:221–260 (1986).
3. Czinn, S., T. Kahn, D. Frank, W. Speck, and J. Blumer. Teratogenic effects of phenytoin (PHT), valproic acid (VPA), carbamazepine (CBZ) and phe-

nobarbital (PBT) in the developing American sea urchin (ASU). *Pediatr. Res.* **17**:147A (1983).
4. Estus, S., and J. L. Blumer. Critical period and cell cycle phase of phenytoin teratogenic action in the sea urchin (*Arbacia punctulata*) embryo. J. Pharmacol. Exp. Ther. **251**:782–789 (1989).
5. Pagano, G., A. Esposito, G. G. Giordano, E. Vamvakinos, I. Quinto, G. Bronzetti, C. Bauer, C. Corsi, R. Nieri, and A. Ciajolo. Genotoxicity and teratogenicity of diphenyl and diphenyl ether: a study of sea urchins, yeast and *Salmonella typhimurium*. *Teratogen. Carcinogen. Mutagen.* **3**:377–393 (1983).
6. Gustafson, T., and M. Toneby. On the role of serotonin and acetylcholine in sea urchin morphogenesis. *Exp. Cell Res.* **62**:102–117 (1970).
7. Harvey, E. B. *The American Arbacia and other sea urchins.* Princeton University Press, Princeton, NJ (1956).
8. Wilson, J. G. Current status of teratology, in *Handbook of Teratology*, Vol. 2. *Mechanisms and Pathogenesis*, (J. G. Wilson and F. C. Fraser, eds.). Plenum Press, New York, 47–74 (1979).
9. Rappaport, R. The biophysics of cleavage and cleavage of geometrically altered cells, in *The Sea Urchin Embryo* (G. Czihak, ed.). Springer-Verlag, New York, 308–332 (1975).
10. Inoue, S. Cell division and the mitotic spindle. *J. Cell Biol.* **91**:131s–147s (1981).
11. Dustin, P. *Microtubules.* Springer-Verlag, New York (1982).
12. MacKinney, A. A., Jr., R. Vyas, and D. Walker. Hydantoin drugs inhibit polymerization of pure microtubular protein. *J. Pharmacol. Exp. Ther.* **204**:189–194 (1978).
13. Biltz, H., and K. Slotta. Mitteilung aus dem chemischen institute der universitat zu Breslau uber die herstellung von hydantoinen. *J. Prakt. Chemie,* **113**:233–267 (1926).
14. Litchfield, J. T., Jr., and F. Wilcoxon. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.* **96**:99–113 (1948).
15. Marsland, D., and R. Hecht. Cell division: combined anti-mitotic effects of colchicine and heavy water on first cleavage in the eggs of *Arbacia punctulata*. *Exp. Cell Res.* **51**:602–608 (1968).
16. Comman, I., and M. E. Cornman. The actions of podophyllin and its fractions on marine eggs. *Ann. N. Y. Acad. Sci.* **51**:1443–1481 (1951).
17. Remillard, S., L. I. Rebhun, G. A. Howie, and S. M. Kupchan. Antimitotic activity of the potent tumor inhibitor maytansine. *Science* (*Wash. D. C.*) **189**:1002–1005 (1975).
18. Schatten, G., H. Schatten, T. H. Bestor, and R. Balczon. Taxol inhibits the nuclear movements during fertilization and induces asters in unfertilized eggs. *J. Cell Biol.* **94**:45–465 (1982).
19. Gaskin, F., C. R. Cantor, and M. L. Shelanski. Turbidimetric studies of the *in vitro* assembly and disassembly of porcine neurotubules. *J. Mol. Biol.* **89**:737–758 (1974).
20. Sternlicht, H., and I. Ringel. Colchicine inhibition of microtubule assembly via copolymer formation. *J. Biol. Chem.* **254**:10540–10550 (1979).
21. Balczon, R., and G. Schatten. Microtubule-containing detergent-extracted cytoskeletons in sea urchin eggs from fertilization through cell division: antitubulin immunofluorescence microscopy. *Cell Motil.* **3**:213–228 (1983).
22. Rodriguez, J., and F. Deinhardt. Preparation of a semipermanent mounting medium for fluorescent antibody studies. *Virology* **12**:316–317 (1960).
23. Kleinbaum, D. G., and L. L. Kupper. *Applied Regression Analysis and Other Multivariable Methods.* Duxbury Press, North Scituate, MA (1978).
24. Messing, R. O., C. L. Carpenter, and D. A. Greenberg. Mechanism of calcium channel inhibition by phenytoin: comparison with classical calcium channel antagonists. *J. Pharmacol. Exp. Ther.* **235**:407–411 (1985).
25. Martz, F., C. Failinger III, and D. A. Blake. Phenytoin teratogenesis: correlation between embryopathic effect and covalent binding of putative arene oxide metabolite in gestational tissue. *J. Pharmacol. Exp. Ther.* **203**:231–239 (1977).
26. Scott, W. J. Cell death and reduced cell proliferation, in *Handbook of Teratology*, Vol. 2. *Mechanisms and Pathogenesis* (J. G. Wilson and F. C. Fraser, eds.). Plenum Press, New York, 81–98 (1979).

Send reprint requests to: J. L. Blumer, Division of Pediatric Pharmacology and Critical Care, Department of Pediatrics, Box 386, University of Virginia, Children's Medical Center, Charlottesville, VA 22908