

The Teratogenic Effect of Dilantin on Preimplantation  
Mouse Embryo Development

An Honors Thesis (HONRS 499)

by

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### Abstract

Dilantin, diphenylhydantoin (DPH), is an anticonvulsant drug used to control the disorder known as epilepsy. Dilantin, however, is a known human teratogen and pregnant females taking this drug have an increased risk of spontaneous miscarriages and of giving birth to babies with fetal hydantoin syndrome. To study the effects of Dilantin at preimplantation stages, varying concentrations of the drug were added directly to cultures of 2-cell mouse embryos. After two days of culture, the embryos were analyzed for percent development beyond the two cell stage. Although effects of Dilantin were observed, no true dosage response curve was found in the therapeutic range in the cultures where Dilantin was added directly. Cultures containing 5  $\mu\text{g}/\text{ml}$  DPH promoted normal development to morula and blastocyst stages (46%). DPH at 10 and 20  $\mu\text{g}/\text{ml}$  concentrations promoted development to the 4-8 cell stage but morula and blastocyst stages were reduced (40.5% and 53%). This effect became more severe at 50 and 100  $\mu\text{g}/\text{ml}$  levels. Further studies that investigate whether the teratogenic agent is the parent drug or actually a metabolic intermediate of DPH were performed by addition of a microsomal  $S_9$  fraction to cultures of two cell mouse embryos. The  $S_9$  fraction alone was found to not be teratogenic to the embryos itself, however, the addition of DPH and  $S_9$  fraction increased the toxic effect of the drug.

## Introduction

Epilepsy is a disorder which can be controlled through the use of anticonvulsant drugs. However, up to 30% of epileptic mothers on anticonvulsant medication give birth to babies with fetal hydantoin syndrome (Hanson, 1986). This syndrome is characterized by various morphological deformities (Hanson, 1986, Hill, 1974). Considering that 0.3-0.5% of the population are female epileptics, the teratogenesis of anticonvulsants is an extremely important problem (Finnell, 1981). Many studies at postimplantation stages have yielded conflicting results. Pregnant epileptics also have an increased risk of early spontaneous miscarriages which may suggest an effect before implantation into the uterus (Annegers, et al., 1974, Biale, et al. 1975, and Hill, et al., 1974). To study the preimplantation effects of an anticonvulsant drug, Dilantin (DPH), in vitro, different concentrations of the drug were added directly to cultures of two cell mouse embryos. After two days of culture, the embryos were analyzed for the percent development to each of the preimplantation stages. The results of this study will hopefully lead to a better understanding of how anticonvulsants cause birth defects and miscarriages. In order to fully comprehend this problem background information on epilepsy and anticonvulsants, the metabolism of Dilantin, mouse embryonic development, and embryo culture are required.

### Epilepsy and Anticonvulsants:

Dilantin, diphenylhydantoin (DPH), is an anticonvulsant drug used to control epilepsy (Hanson, 1986). Dilantin, however, is a known human teratogen and pregnant females taking this drug have an increased risk of giving birth to children with fetal hydantoin syndrome (Annegers et al., 1974, Biale et al., 1975, Chatot et al., 1984). Fetal hydantoin syndrome is characterized by cleft lip and/or palate, craniofacial malformations, hypoplasia of the digits, mental retardation, urogenital defects, ventricular septal defects and other morphological defects (Hanson, 1986, Hill et al., 1974). Attempts to identify causes of this teratogenicity in humans have resulted in conflicting reports. Shapiro et al. (1976) reported that the epileptic disorder itself was the main cause of malformations. In 1981, Richard Finnell developed a mouse model to study the effects of anticonvulsant drugs. Finnell developed a test animal that closely approximated the human epileptic disorder which had spontaneous seizures that could be controlled or eliminated with the oral administration of anticonvulsants. The plasma drug concentrations of these mice fell within the human therapeutic level, and the offspring of these mice exhibited defects similar to human fetal hydantoin syndrome (1981). The experimental data showed that the incidence of animals born with malformations increased with an increased level of the drug (Finnell,

1981). Finnell's model demonstrated that the anticonvulsant drug was the cause of the increased risk of birth defects, not the epilepsy (1981). Karolyi found a difference in the susceptibility of different strains of mice to anticonvulsant drug teratogenicity for example C57BL/6J strain is more susceptible, whereas A/J is less susceptible. Chatot et al. (1984) found that when anticonvulsants were added directly to rat embryo cultures, there was no direct effect of the drug on the embryos, suggesting that the active teratogen could be a metabolite and not the parent drug.

There are many other antiepileptic drugs used to control seizures, not just Dilantin. It is possible that phenobarbital and carbamazepine (other frequently used anticonvulsant drugs) work in the same way as DPH. All of these compounds contain an aromatic ring and use aromatic hydroxylation as a major metabolic pathway (Spielberg et al., 1981). These reactions may have active arene oxides as intermediates which can be toxic (Jerina and Daly, 1974). Spielberg et al. (1981) found that four anticonvulsants were toxic only with the addition of a microsomal system (phenobarbital and DPH were included in that study).

Dilantin is often used in combinations with phenobarbital and/or carbamazepine, although monotherapy is the hoped for method of seizure control. These epileptic patients must continue taking the medication even during

pregnancy. The damage to the fetus from anoxia if a seizure were to occur could be greater than the defects that can result from anticonvulsant drugs.

#### Metabolism of Dilantin:

Metabolism of drugs and other foreign agents is a detoxification mechanism of biological systems. Cytochrome P-450 and its subsequent isozymes are found in the liver (highest concentration), the intestine and the kidney of rats, mice, and humans and are used to metabolize drugs to a phenol for final excretion from the body (de Waziers et al., 1986, Jerina and Daly, 1974). However, during the conversion of anticonvulsants to their phenolic metabolites, there are arene oxide intermediates which are formed (Jerina and Daly, 1974, Martz et al., 1977, Spielberg et al., 1981). Arene oxides are extremely reactive and can bind covalently to cellular macromolecules including DNA, RNA, and protein (Jerina and Daly, 1974, Martz et al., 1977, Oesch, 1976, Shanks et al., 1989, Spielberg et al., 1981). This binding can result in mutations of genetic material, cell death, and birth defects (Spielberg, 1982).

The oxidative metabolites of anticonvulsant drugs created by cytochrome P450 are usually removed through the use of epoxide hydrolase (Buehler et al., 1990) (See Figure 1). Microsomal epoxide hydrolase catalyzes the hydrolysis of arene oxides to diols. Epoxide hydrolase is found in the endoplasmic reticulum and the nuclear envelope of cells

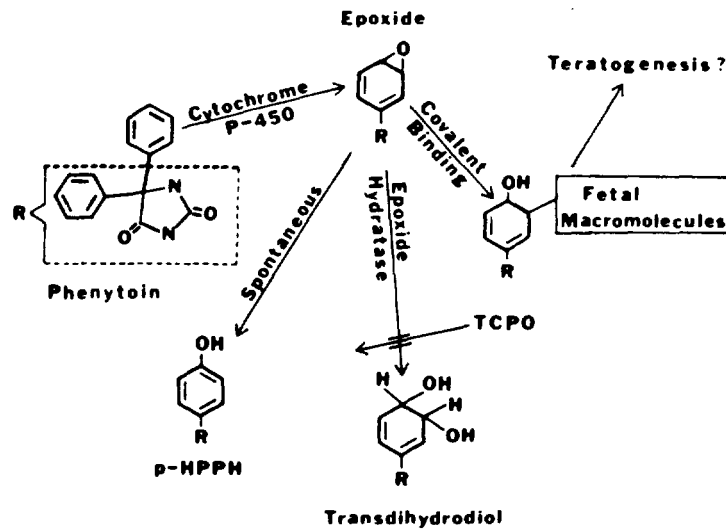


Figure 1: Metabolism of Dilantin in tissues (Mukhtar, 1979).

where it is associated with the cytochrome P450 system. The cytochrome P450 enzymes react with the DPH to form the arene oxide, then the epoxide hydrolase enzyme catalyzes the reaction which converts the arene oxide to the final phenolic metabolite which can then be excreted from the body (Mukhtar et al., 1979). The cytochrome P450 enzymes can further metabolize that phenol to a highly electrophilic diol epoxide that is not a good substrate for epoxide hydrolase but can form covalent bonds with cell macromolecules (Jerina and Daly, 1974). These reactions occur on the membranes of cells and not only detoxify the drug but may also activate it to form one or more teratogenic intermediates in the reaction (de Montellano, 1986, Mukhtar et al., 1979). In support of this suggestion Shanks et al. (1989) found that the addition of a cytochrome P450 microsomal system to preimplantation mouse embryo

cultures increased the toxicity of Dilantin.

#### Genetics of Metabolism:

Although not much is known about the enzymes and genetics surrounding metabolism of anticonvulsant drugs in animals, there have been studies conducted concerning humans. Spielberg et al. (1981) investigated the metabolites of DPH through an in vitro assay using human lymphocytes. He used lymphocytes because they are easily obtained and they contain defenses against metabolites (i.e. epoxide hydrolase) (Spielberg et al., 1981). A microsomal system was prepared by injecting mice with phenobarbital in order to induce cytochrome P450 microsomes (Speilberg et al., 1981). This microsomal protein fraction was then incubated with DPH and the lymphocytes which were obtained from a human blood sample (Spielberg et al., 1981). Spielberg et al. (1981) also added TCPO, an inhibitor of epoxide hydrolase, to one culture. The dose-response curves for anticonvulsant toxicity with a microsomal system show that at high concentrations of DPH, there was a direct toxic effect of the drug on the cells, however, within actual therapeutic plasma levels, there was no or minimal toxicity (Spielberg et al., 1981). When the epoxide hydrolase activity was inhibited by the addition of the TCPO, the percentage of dead cells after sixteen hours of incubation increased from 10-20% (without TCPO) to 40-50% within the



therapeutic range (Spielberg et al., 1981). Spielberg's results suggest that an intermediate in the metabolism of Dilantin which is usually removed by a reaction catalyzed by epoxide hydrolase may be the actual teratogen (Spielberg et al., 1981).

Buehler et al. in 1990 analyzed a random sample of pregnant women for their level of epoxide hydrolase activity. He then ran a chromatographic assay which revealed a "trimodal distribution" of the levels of activity, one high, an intermediate activity, and one low were observed (Buehler et al., 1990). This distribution is characteristic of an enzyme that is regulated by a single gene with two allelic forms (Buehler et al., 1990). The low microsomal epoxide hydrolase activity ranged from 0-50% of the standard with a mean of 31%. This low activity could be of the genotype "ee" or the homozygous recessive. The intermediate activity would then be the heterozygous condition, "Ee," and was represented by the range from 50-75% of the standard with a mean of 59%. Finally the high enzyme activity which ranged from 75-100% of the standard with a mean of 94% represented the homozygous dominant, "EE" (Buehler et al., 1990) (see figure two).

To determine patient risk for teratogenicity, the genotype of both the mother and the fetus must be considered; some metabolism can occur in maternal tissue, placenta and fetal tissues. Those patients that would be at

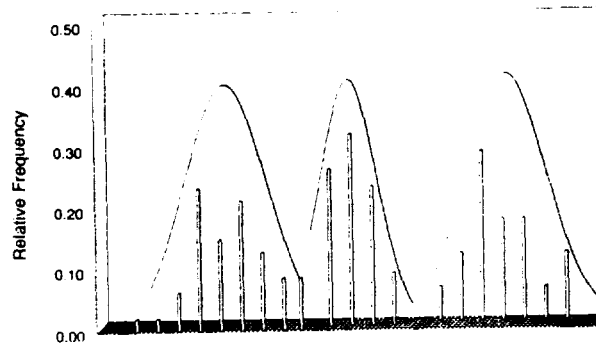


Figure 2: Trimodal distribution of epoxide hydrolase (Buehler, 1990).

risk for giving birth to babies with fetal hydantoin syndrome would be those with epoxide hydrolase activity at the low end of the activity range who were exposed to DPH during the pregnancy and also those fetuses that are the product of two heterozygotes (Buehler et al., 1990). These infants that would have the "at risk" genotype when exposed to the anticonvulsant in utero would not readily metabolize the intermediate arene oxide to the final phenolic metabolite. This would, in turn, make those fetuses more susceptible to the teratogenic effects of the anticonvulsant (Buehler et al., 1990).

Buehler et al. (1990) then concluded that if the level of epoxide hydrolase activity was a major factor in the severity of the final malformations, it would be possible to use that level as a preindication of whether a patient would give birth to a baby with fetal hydantoin syndrome. Buehler et al. (1990) tested his theory by investigating the pregnancies of 19 epileptic women who were treated only with DPH. Amniocentesis and examination of epoxide hydrolase activity in the cells of the fetuses demonstrated that cells

from four of the fetuses were below 30% of the standard epoxide hydrolase activity and the other fifteen were above 30% of the standard epoxide hydrolase activity (Buehler et al., 1990, Finnell et al., 1990). At birth all four of the infants with epoxide hydrolase activity below 30% of the standard had morphological defects classified as fetal hydantoin syndrome and the other fifteen infants were considered normal (Buehler et al., 1990, Finnell et al., 1990).

The results of these studies suggest that the genotype of the developing fetuses may be used for determination of at risk fetuses (Buehler et al., 1990 and Finnell et al., 1990). These experiments also support the theory that the intermediate which is removed by epoxide hydrolase is the active teratogen under actual therapeutic conditions.

#### Preimplantation Mouse Embryonic Development:

Mouse embryonic development begins after fertilization with the zygote. Directly after this union of the male and female gametes, the fertilized egg (Figure 3A) begins a series of mitotic cell divisions through the 2-cell (Figure 3B), 4-cell (Figure 3C), and 8-cell stages. Then the embryo forms tight junctions and compacts upon itself to become a morula with 8-16 cells (Figure 3D). Further divisions and cavitation by pumping fluid into the center of the embryo to form a blastocoel is referred to as the blastocyst stage

Figure 3E and F). The blastocyst which is composed of the inner cell mass (ICM) and the trophoblast is capable of being implanted into the uterine wall (see Figure 3).

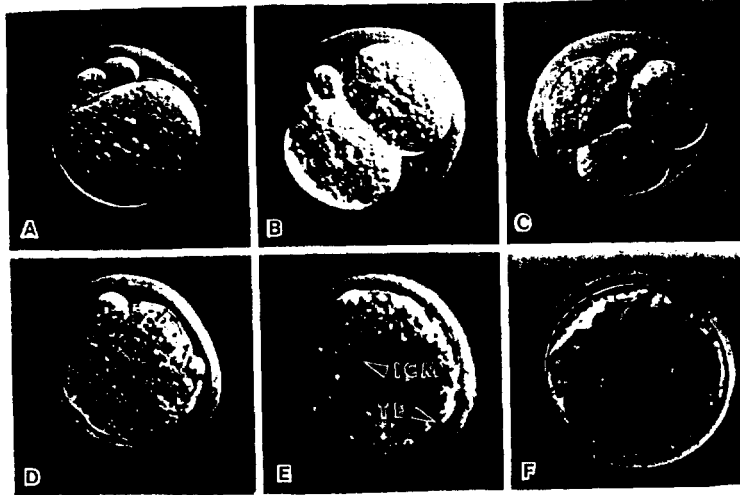


Figure 3: Preimplantation stages of mouse embryo development: (a) zygote; (B) 2-cell stage; (C) 4-cell stage; (D) morula stage; (E) early blastocyst; (F) late blastocyst. (Rossant and Pedersen ed., 1986).

#### Embryo Culture:

To be able to observe continual development of the preimplantation embryos, an in vitro culture system comparable to in vivo conditions needed to be established. In the past it has been difficult to derive in vitro grown cells which are comparable to those grown in vivo. Chatot et al., (1989) developed a modified mouse embryo culture medium, CZB medium, which promotes the development of 1-cell embryos to the blastocyst stage in strains of mice that show a 2-cell "block" (development is arrested at the 2-cell

stage) in vitro in other media. Strains which do not normally block can also be cultured using CZB medium (Chatot et al, 1990a). CZB differs from the other commonly used media because it contains glutamine and EDTA. This medium also lacks glucose and has a high lactate to pyruvate ratio. Embryos cultured in CZB medium are comparable to controls in expression of alkaline phosphatase (Chatot and Ziomek, unpublished observations), in general protein synthetic patterns (Poueymirou et al., 1989), in use of glutamine as an energy substrate (Chatot et al., 1990b) and in expression of the glutamine cycling enzyme glutaminase (Chatot et al., 1990c; Chatot and Kamper, 1992) but are slightly slower (approximately 1/2 day) in overall cell numbers compared to in vivo (Chatot et al., 1990a). This media provides close to normal in vitro development and can be used to study teratogenic effects on preimplantation embryos in vitro.

## **Materials and Methods**

### Superovulation and Mating

NSA females (Harlan Sprague Dawley, Indianapolis, Indiana) were superovulated by intraperitoneal injections of Pregnant Mare Serum Gonadotropin (PMS, 10 IU Sigma) followed 48 hours later by an intraperitoneal injection of human Chorionic Gonadotropin (hCG, 5 IU Sigma). Females were mated immediately following hCG injection with B6SJLF1/J males (Jackson Laboratory, Bar Harbor, ME).

### Embryo Isolation:

Females were sacrificed 47-53 hours post-hCG injection, the oviducts with a portion of the uterine horn were removed and placed into Hank's Balanced Salt solution (HBSS+BSA). The embryos were then flushed from the oviducts and washed in HBSS+BSA to remove debris and placed in holding drops of CZB medium. After being placed in holding drops, randomly distributed 2-cell embryos were washed through a drop of fresh CZB medium and placed in a final culture drop: 15 embryos were cultured in a 50 $\mu$ l drop. Cultures were gassed in a sealed culture chamber with 5% CO<sub>2</sub>/5% O<sub>2</sub>/90% N<sub>2</sub> and incubated at 37°C for 48 hours. The embryos were cultured in the presence and absence of Dilantin (5, 10, 20, 50, and 100  $\mu$ g/ml added to cultures in a total volume of 2  $\mu$ l). Controls were run with no Dilantin and vehicle controls

included 2 $\mu$ l of 0.01N sodium hydroxide (NaOH).

#### Embryo Analysis:

The embryos were collected after 48 hours of culture and were fixed in 4% paraformaldehyde solution in PBS. The embryos were scored for percent development to each preimplantation stage (2C, 4-8C, M, and B). The cultures were run in triplicate. In analyzing the data, the results were averaged across the samples for each concentration and the SEM was determined.

#### Microsome Preparation and Titration:

2 NSA females were injected with 0.1 ml  $\beta$ -naphthoflavone (24mg/ml) in chloroform/0.9% saline (diluted 1:10). Forty-eight hours later, the mice were sacrificed and the livers were removed. The liver tissue slices were incubated for 15 min in ice-cold 1.15% KCl and then homogenized on ice with 2 volumes of ice-cold 1.15% KCl. The homogenate was centrifuged at 9000 x g for 20 minutes at 4°C. The supernatant was aliquoted and stored at -70°C. Total protein was determined using the Lowry Assay with BSA as the standard. Two cell embryo cultures were performed using an S<sub>9</sub> fraction (10, 20, 40, 80, 100, and 160 $\mu$ g/ml) for titration  $\pm$  DPH (10  $\mu$ g/ml).

### **Results**

Dilantin was added directly to cultures of two cell

mouse embryos both within and above the therapeutic range. Development of embryos to stages beyond two cell were evaluated after two days. Table 1 lists direct embryo results from 3-4 replicates of the DPH dose response curve. These results are represented as percent development to each stage in Figure 4 and 5. When DPH was added within the therapeutic range, development was retarded although this was not dose related (Figure 4). Cultures containing  $5\mu\text{g/ml}$  DPH showed an increase in the number of embryos which only developed to the four-eight-cell stage. This was due to a reduction in the number of morula, but the number of blastocysts remained consistent with controls. At the 10 and  $20\mu\text{g/ml}$  levels a more pronounced effect was observed with overall reduced development, 3% ( $10\mu\text{g/ml}$ ) and 13% ( $20\mu\text{g/ml}$ ) of the embryos did not develop beyond the two-cell stage and only 3% and 5% respectively of the two-cell embryos developed to the blastocyst stage at this concentration. This effect became more severe at the 50 and  $100\mu\text{g/ml}$  levels (58% and 97% of the embryos stayed at either the two or four-eight-cell stage and there were no blastocysts formed (Figure 5)), however these levels are 2.5-5 times the normal therapeutic level of the drug. Although a direct dose related response was absent, DPH did have an effect on development. These results suggested that the embryos may be capable of forming a teratogenic intermediate.



**DPH EFFECTS ON DEVELOPMENT**  
(THERAPEUTIC RANGE)

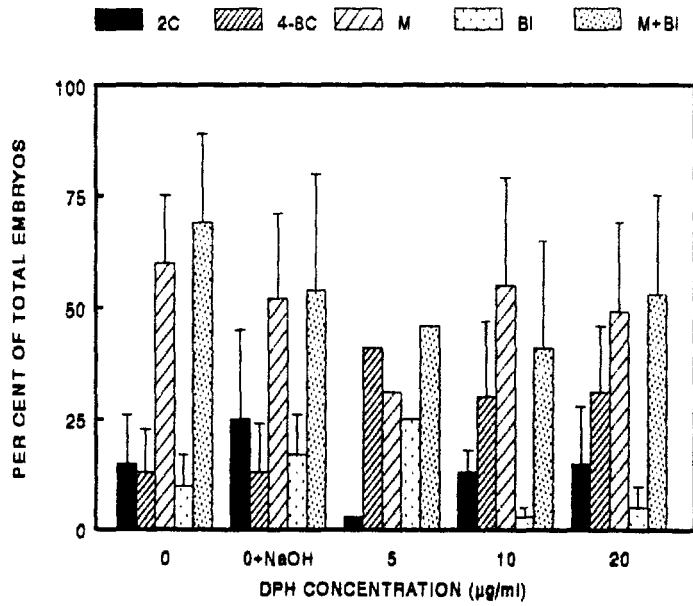


Figure 4: Percent mouse embryo development beyond 2-cell after direct addition of DPH to cultures at concentrations within the therapeutic range.

Table 1:

**Number of Embryos at Each Stage  
of Development After Addition of DPH**

[DPH] µg/ml	2C	4-8C	M	B	M+B	N
0	9	8	38	6	46	61
0+NaOH	9	7	27	9	36	52
5	1	13	10	8	18	32
10	5	12	22	1	23	40
20	9	8	29	3	32	59
50	5	13	13	0	13	31
100	14	19	1	0	1	34

2C, two-cell embryos; 4-8 C, four-eight-cell embryos; M, morula; B, blastocyst; N, total number of embryos cultured.

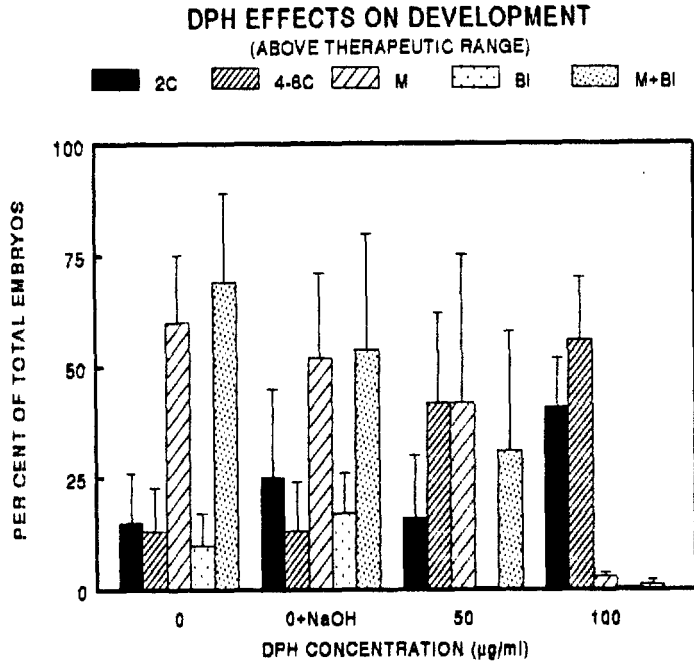


Figure 5: Percent mouse embryo development beyond 2-cell after direct addition of DPH to cultures at concentrations above the therapeutic range.

To explore the question of the effect of a teratogenic intermediate on development, two cell cultures were exposed to an  $S_9$  microsomal fraction capable of metabolizing DPH in the presence and absence of DPH.  $S_9$  fractions were prepared by the method described in Shanks et al. (1989). Lowry protein assays were used to determine the concentration of protein to titrate microsomes in the cultures. BSA was used as a standard (Figure 6). The protein concentration in the  $S_9$  fraction was determined using the 1:1000 dilution from the standard curve to be 89.2mg/ml. The protein concentrations are shown in Table 2.

Table 2:

Lowry Assay for Protein Concentration

[BSA] $\mu\text{g/ml}$	$A_{750\text{nm}}$	Dilution of $S_0$	$A_{750\text{nm}}$	[ $S_0$ ] $\text{mg/ml}$
0	0.00	1:100	0.467	480.11
50	0.058	1:1000	0.093	89.23
100	0.102	1:10,000	0.046	39.97
200	0.212	1:100,000	0.030	23.20
400	0.395			

Lowry Protein Standard Curve

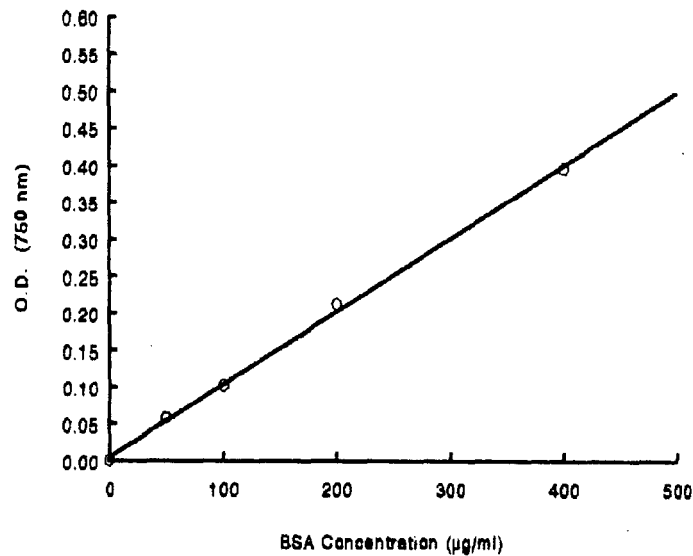


Figure 6: Lowry protein assay standard curve.

As  $S_0$  fractions are often toxic at high concentrations, a dose titration was performed to determine a maximum concentration at which embryos will develop normally in the  $S_0$  with no DPH added to the culture. Cultures of two-cell embryos that were exposed to the  $S_0$  fraction did not show an appreciable toxic effect as compared to the controls in the experiment up to  $80\mu\text{g/ml}$  (Table 3 and Figure 7).

Table 3:

Number of Embryos at Each Stage of Development After the Addition of a Microsomal Fraction

[S <sub>0</sub> ] μg/ml	2C	4-8C	M	B	Abn	N
0	0	13	2	0	0	15
10	0	9	0	0	3	12
20	0	10	4	0	1	15
40	0	0	2	0	13	15
80	0	11	1	0	3	15
100	0	0	0	0	4	4
160	0	1	0	0	6	7

Addition of P450 microsomes, no DPH

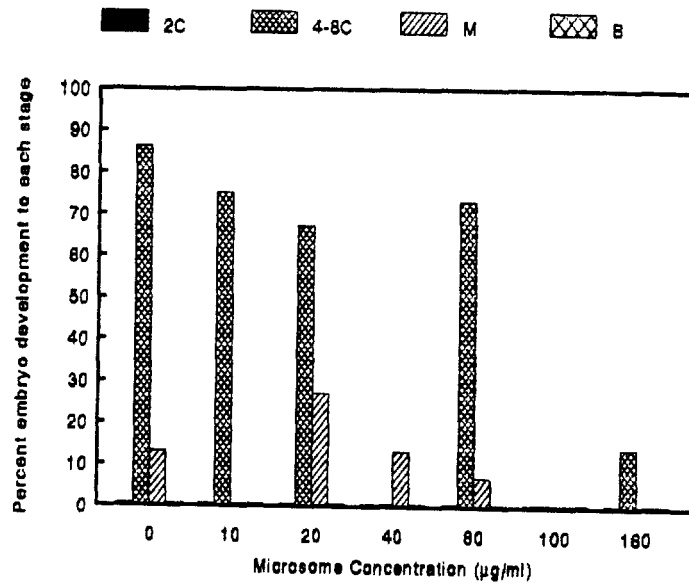


Figure 7: Titration of S<sub>0</sub> microsome toxicity on 2-cell mouse embryos.

When an S<sub>0</sub> fraction was added to the cultures along with 10 μg/ml DPH the embryos did not develop or developed abnormally (Table 4 and Figure 8). This toxic effect

suggests that a metabolite generated by the  $S_9$  system acting on DPH is the actual teratogen.

Table 4:

Number of Embryos at Each Stage of Development After Addition of  $S_9$  + DPH

[S9] $\mu\text{g/ml}$	2C	4-8C	M	B	Abn	N
0+NaOH	0	9	0	0	3	12
10	0	0	0	0	14	14
20	0	0	0	0	15	15
40	0	0	0	0	15	15
80	0	2	0	0	17	19
100	0	1	0	0	9	10
160	0	0	0	0	13	13

DPH concentration in all dishes =  $10\mu\text{g/ml}$ .

Addition of P450 microsomes + DPH

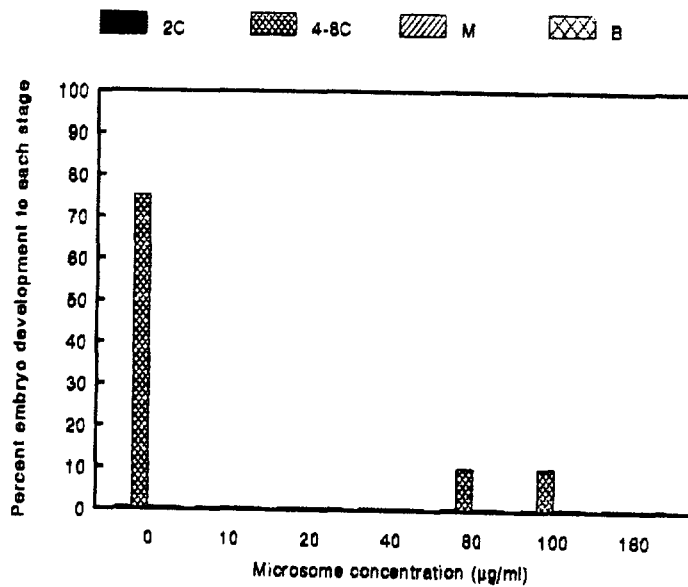


Figure 8: Effect of DPH +  $S_9$  microsomes on cultured 2-cell mouse embryos.

### Discussion

Embryos that were cultured in the presence of various

concentrations of DPH (from 0-100 $\mu$ g/ml) showed an effect of the drug which did not represent a true dosage response. This suggests that an intermediate of Dilantin is the proximal teratogen and that the NSA x B<sub>1</sub>SJLF<sub>1</sub> mouse embryo is capable of synthesizing this intermediate. These results disagree with the observations of Chatot et al., (1984) where the direct addition of Dilantin to post-implantation rat embryo cultures produced no effect, however, Chatot et al. (1984) believe that at that stage in development, the rat is not capable of metabolizing the drug. The results do agree with the susceptibility of the mouse embryos to DPH effects as demonstrated by Finnell (1981) and Shanks et al. (1989) at post-implantation stages. A microsomal fraction (S<sub>0</sub>) was not found to be toxic to the embryos alone, however, when Dilantin (10 $\mu$ g/ml) was added to the cultures along with the S<sub>0</sub> fraction, the toxicity of the system increases. Spielberg et al. (1981) and Strickler et al. (1985) also found that the addition of a microsomal fraction did increase the toxicity of Dilantin to the lymphocytes in culture. Shanks et al. (1989) found that the addition of an S<sub>0</sub> fraction + NADPH to cultures of post-implantation mouse embryos increased the teratogenic effects of Dilantin.

To continue to characterize the effects of this drug on preimplantation mouse embryo development, additional experiments could be suggested. Culture of one cell embryos

± DPH ± S<sub>9</sub>, would allow us to assess the toxicity of Dilantin from the beginning of preimplantation development. Since at 10µg/ml S<sub>9</sub> + 10µg/ml DPH a severe toxic effect was observed relative to controls, perhaps both the concentration of S<sub>9</sub> and DPH should be decreased or titrated downward to demonstrate a more specific retardation of development. Another strain of mouse either more susceptible or less susceptible to the teratogenic effects of Dilantin could be used. The capacity of the embryo to metabolize the drug via endogenous cytochrome P<sub>450</sub> systems could also be investigated and the expression of epoxide hydrolase could be assessed in the embryo by RT-PCR or an enzymatic assay for epoxide hydrolase activity. The molecular mechanisms which cause growth retardation could be studied by examining how the expression of specific genes are affected, such as genes which control the cell cycle (cyclins) or genes for growth factor receptors like: EGF, PDGF, or IGF receptor. This could be performed using RT-PCR to look at RNA expression or antibodies to evaluate protein expression. These types of experiments would help to elucidate the mechanisms of the metabolism of Dilantin in preimplantation mouse embryos.

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