MINIREVIEW

The Embryotoxicity of Phenytoin: An Update on Possible Mechanisms (43267)

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Phenytoin (PHT; diphenylhydantoin; Dilantin) is quite effective in the management of epilepsy and is the most commonly prescribed anticonvulsant medication. There is considerable evidence that the drug is embryotoxic in animals, particularly producing orofacial clefting in mice (1–3), and it is also generally considered to be embryotoxic in humans. Epidemiological evidence has suggested that epileptic women have a greater risk of bearing a child with a congenital anomaly than do nonepileptic women and that this risk is even greater when anticonvulsants are ingested during pregnancy (4–8). It was not possible to stratify these epidemiological data to implicate any one anticonvulsant drug. However, a pattern of malformations referred to as the fetal hydantoin syndrome has been described among offspring of patients on PHT monotherapy as well as polytherapy; features of this syndrome include pre- and postnatal growth deficiency, mild to moderate mental retardation, congenital heart defects, and skeletal defects, especially hypoplasia of the distal portions of the digits (9–12). In an animal model, it has been shown that PHT therapy, and not the epileptic disease state nor seizure frequency, is associated with malformations (13–16).

A number of possible mechanisms have been suggested to explain PHT embryotoxicity, including: (i) drug-induced folate deficiency; (ii) a glucocorticoid-mediated mechanism; and (iii) metabolism to a reactive intermediate and binding to embryonic macromolecules. This review will focus on these three possibilities.

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Folate Deficiency

During a normal pregnancy, there is an increased demand for folates that is due, in part, to the rapid growth of fetal, placental, and some maternal tissues. Patients on long-term anticonvulsant therapy have been observed to develop folate deficiency (17, 18), and anticonvulsant therapy during pregnancy could exacerbate a borderline folate deficiency in pregnant patients (19). An inverse relationship between serum folate levels and PHT concentrations has been noted in pregnant epileptics (20, 21).

Dietary deficiency of folate is embryotoxic in rats *in vivo* (22, 23) and *in vitro* (24). In humans, epidemiological studies have suggested that periconceptual multivitamin supplementation, particularly folate supplementation, decreases the incidence of neural tube defects (25–27).

The mechanism(s) responsible for PHT-induced folate deficiency is unknown. Hypotheses have included impaired folate absorption (28), hepatic enzyme induction (29, 30), and increased folate catabolism (31). More recent studies have suggested that neither folate absorption (32) nor folate catabolism (33) are adversely affected by PHT. An alternative mechanism was suggested by Billings (34). It was observed that in mice, chronic PHT treatment decreased plasma folate levels and activity of hepatic 5,10-methylenetetrahydrofolate reductase, a key enzyme in folate metabolism. However, further work is necessary to clarify the relationship of this enzyme inhibition and decreased plasma folate levels to PHT-induced toxicity.

Conflicting results were obtained when PHT and folate supplements were given concurrently to pregnant mice. Folic acid had no effect on the frequencies of resorptions and malformations produced by PHT treatment (35, 36). Folinic acid, a stable folate derivative, decreased the incidence of cleft palate (36) or offered

no significant protection against PHT-induced clefting (37). In another study (38), folinic acid had no effect on the clefting incidence when PHT was administered by gastric intubation, and increased the incidence when the drug was administered in the diet. Dietary administration of folic acid decreased PHT-induced embryotoxicity in rats as quantitated by fetal weight and length, as well as by the number of ossification centers in sternebrae (39). It is difficult to resolve these differences; however, overall there does not appear to be a significant beneficial effect of concurrent administration of folate supplements with PHT.

It has been reported that PHT decreased embryonic folate concentrations in Gestation Day 12 mouse embryos following intraperitoneal administration of PHT (40). A later study reported no difference in folate levels between PHT and vehicle control-treated embryos on Day 10, 12, or 14 of gestation, when PHT was administered chronically via the diet (41). Methodological differences between these two studies could account for the disparity in results. Overall, there does not appear to be a major effect of PHT on embryonic folate levels.

Additional evidence from *in vitro* studies (42) indicates that some PHT-induced embryotoxicity is not a result of folate deficiency. In these experiments, rat embryos were cultured on human serum from epileptic patients on PHT monotherapy; abnormal embryonic development occurred on approximately 70% of these samples. When the sera were supplemented with vitamins (including folate) and amino acids, development improved on several sera samples, but abnormal development still occurred on 26% of the samples. These data suggest that some teratogenic effects may be related to drug-induced nutritional deficits, but such deficits are not solely responsible for PHT-induced embryotoxicity.

Overall, there is no definitive data to support a role for folate deficiency in PHT-induced embryotoxicity.

PHT and Glucocorticoids

It has been suggested that PHT and glucocorticoids disrupt normal palatal development by the same or a very similar mechanism (43–45). With respect to their embryotoxicity, these compounds share several features. Mice of the C57BL/6J strain are resistant to both PHT and glucocorticoid-induced orofacial clefting, while A/J mice are sensitive to both compounds (46–50). Additionally, both compounds decrease DNA and protein synthesis in the palate (51–53).

Details of the relationship between PHT and glucocorticoids is unclear. A teratogenic dose of PHT significantly increased maternal glucocorticoid levels in A/J mice for 48 hr after dosing, while a nonteratogenic dose elevated these levels for only 1–2 hr (54). Removal of the maternal adrenal glands, the primary source of the endogenous hormone, did not alter PHT-induced clefting (55). However, results from a more recent investigation demonstrated that PHT significantly increased the incidence of cleft palate in offspring of adrenalectomized mice (56).

The mechanism(s) for the disruption in normal palatal development by glucocorticoids or PHT is unknown. Glucocorticoids have a number of physiological effects that may enhance an organism's resistance to stress. Several of these effects may be modulated by intracellular mediators, such as arachidonic acid metabolites (57). A good deal of experimental work has focused on the role of these metabolites in PHT-induced orofacial clefting.

It has been postulated that PHT can act as an alternative ligand for the glucocorticoid receptor (58). Interaction between a glucocorticoid and the receptor may trigger the release of phospholipase inhibitory proteins, which act as second messengers to inhibit activity of phospholipase A_2 (59). This lipolytic enzyme catalyzes the hydrolysis of phospholipids, resulting in the release of arachidonic acid as a free fatty acid (60). Inhibition of phospholipase A_2 results in a decrease in intracellular arachidonic acid, which acts as a precursor in the synthesis of leukotrienes, prostaglandins, and thromboxanes (Fig. 1).

When palatal shelves were cultured *in vitro*, cortisol and PHT both inhibited the breakdown of the medial edge epithelium of the palate, a necessary step in normal palatal fusion (61). This effect of PHT was decreased by addition of cortexolone, an antiglucocorticoid that

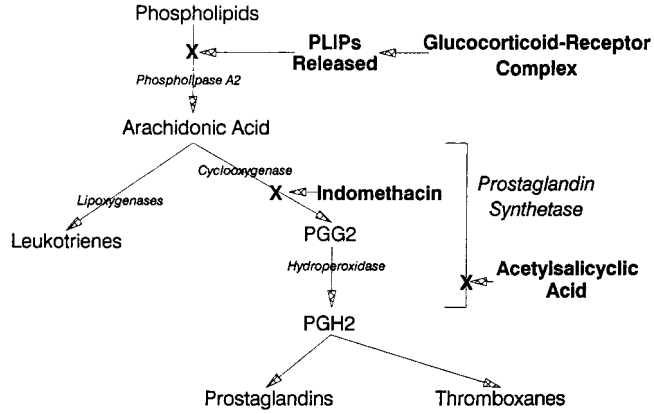

Figure 1. A generalized pathway of prostaglandin biosynthesis is depicted. Phospholipase A_2 is the enzyme that catalyzes the release of arachidonic acid from membrane phospholipids, and this enzyme can be inhibited by phospholipase inhibitory proteins (PLIPs). The binding of a glucocorticoid to its receptor is responsible for the release of PLIPs. Lipoxygenases are involved in the synthesis of leukotrienes from arachidonic acid. Cyclooxygenase, which can be inhibited by indomethacin, catalyzes the conversion of arachidonic acid to PGG_2, and hydroperoxidase catalyzes the synthesis of PGH_2. These two enzyme activities, cyclooxygenase and hydroperoxidase, make up prostaglandin synthetase; this activity can be inhibited by acetylsalicyclic acid. It has been speculated that binding of PHT to the glucocorticoid receptor might also stimulate the release of PLIPs, and that this could be involved in the embryotoxic effects of the drug.

forms an inactive receptor complex (62), suggesting that binding of PHT to the glucocorticoid receptor is involved in breakdown of the medial edge epithelium. Treatment of mouse embryos *in vitro* with PHT produced abnormalities in embryonic head folds and visceral arches (63, 64). These defects consisted of misshapen or missing folds and/or arches. Addition of arachidonic acid decreased the frequency of PHT-induced defects, suggesting that the anticonvulsant somehow caused a deficiency of arachidonic acid. Addition of cortexolone with PHT (which might compete with PHT for binding to the glucocorticoid receptor) also decreased the number of abnormal embryos, suggesting involvement of the glucocorticoid receptor. Treatment of embryos with indomethacin, an inhibitor of cyclooxygenase, resulted in the same types and frequencies of defects as did PHT alone. The combination of PHT, arachidonic acid, and indomethacin was as embryotoxic as either PHT or indomethacin alone. The authors interpreted these data as evidence that PHT was embryotoxic through its effects on prostaglandin synthesis, which were mediated by the drug's interaction with the glucocorticoid receptor.

It has been suggested that the prostaglandin synthetic pathway might also be involved with metabolism of PHT. Kubow and Wells (65) found that the anticonvulsant could be metabolized *in vitro* by prostaglandin synthetase to a reactive intermediate that bound macromolecules. *In vivo* pretreatment with either acetylsalicylic acid (which inhibits cyclooxygenase), the antioxidant caffeic acid, or the free-radical spin-trapping agent, α-phenyl-*N*-*t*-butylnitrone, decreased the incidence of cleft palate in CD-1 mice, and this was associated with a decrease in covalent binding of radioactivity derived from PHT (66). These data were interpreted as evidence that a reactive intermediate formed during the prostaglandin synthetase-mediated metabolism of PHT was involved in the drug's embryotoxic effects. However, it is possible that the compounds given to dams may have had multiple effects that would make interpretation of the data very difficult. Further work needs to be done to clarify the role of this metabolic pathway on the embryotoxic effects of PHT.

Determination of the role of glucocorticoids and the glucocorticoid receptor in PHT-induced embryotoxicity needs further characterization. Although much work has focused on the prostaglandin synthetic pathway, it is necessary to keep in mind the myriad of physiological effects of glucocorticoids. The hypothesis of PHT binding to the glucocorticoid receptor and altering arachidonic acid release and prostaglandin synthesis is intriguing; however, the work to support this hypothesis has been done almost entirely *in vitro* and its relevance to *in vivo* embryotoxicity is unclear. Additionally, it has been reported that PHT did not bind *in vitro* to the glucocorticoid receptor even when the drug was present in 1000-fold excess (67). The role of PHT metabolism by the prostaglandin synthetic pathway also needs further work; the balance between metabolism of PHT by this pathway and the hepatic P-450-mediated pathway is unknown.

Metabolism

Phenytoin is extensively metabolized with 10% or less of an ingested dose of the drug excreted as the parent compound in urine (68, 69). Diphenylhydantoic acid and α-aminodiphenylacetic acid, metabolites in which the hydantoin ring structure is opened, constitute only minor urinary components, suggesting that ring hydrolysis is not a primary metabolic pathway (69). The major route of PHT metabolism is mediated by cytochrome P-450 (Fig. 2). The predominant urinary metabolite is 5-(4-hydroxyphenyl)-5-phenylhydantoin (pHPPH) (70), which is present in human urine as 50–75% of an ingested dose (68) and is primarily excreted as a glucuronide conjugate (69). A dihydrodiol metabolite, 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (PHT-DHD) was identified in rat urine (71) and may constitute 3–13% of the human urinary excretion of an ingested dose (72). Production of PHT-DHD is taken as evidence of formation of an epoxide intermediate (73). The catechol or its methoxylated derivative has also been observed in the urine of humans (74), mice (75), and rats (76), as well as in incubation mixtures using isolated rat hepatocytes or mouse hepatic 9000*g* supernatant (77). The catechol can be formed either by aromatization of PHT-DHD or by hydroxylation of pHPPH (74, 77, 78).

The epoxide intermediate can undergo isomerization to pHPPH, be converted to PHT-DHD by action of epoxide hydrase, or bind to tissue macromolecules (79). Hydroxylation of pHPPH would be expected to generate a phenol epoxide intermediate which could also bind to macromolecules, and greater levels of covalent binding were observed *in vitro* with pHPPH as substrate than with PHT as substrate (80). Additionally, there appears to be a sex difference in rats in the metabolism of pHPPH to the catechol, with hepatocytes from male rats forming approximately 10 times more catechol from pHPPH than is formed by hepatocytes from female rats (77, 81).

It is not clear if PHT or a metabolite is the teratogen. In early work with mice, PHT was implicated as the teratogenic agent. It was observed that pretreatment with phenobarbital increased maternal PHT metabolism, leading to a decrease in plasma PHT concentration; when compared to PHT treatment only, there were fewer orofacial clefts, with no difference in resorption frequency in the combination treatment (82). The inverse was true when mice were pretreated with SKF 525A, an inhibitor of PHT metabolism. This combination treatment elevated plasma PHT concentration,

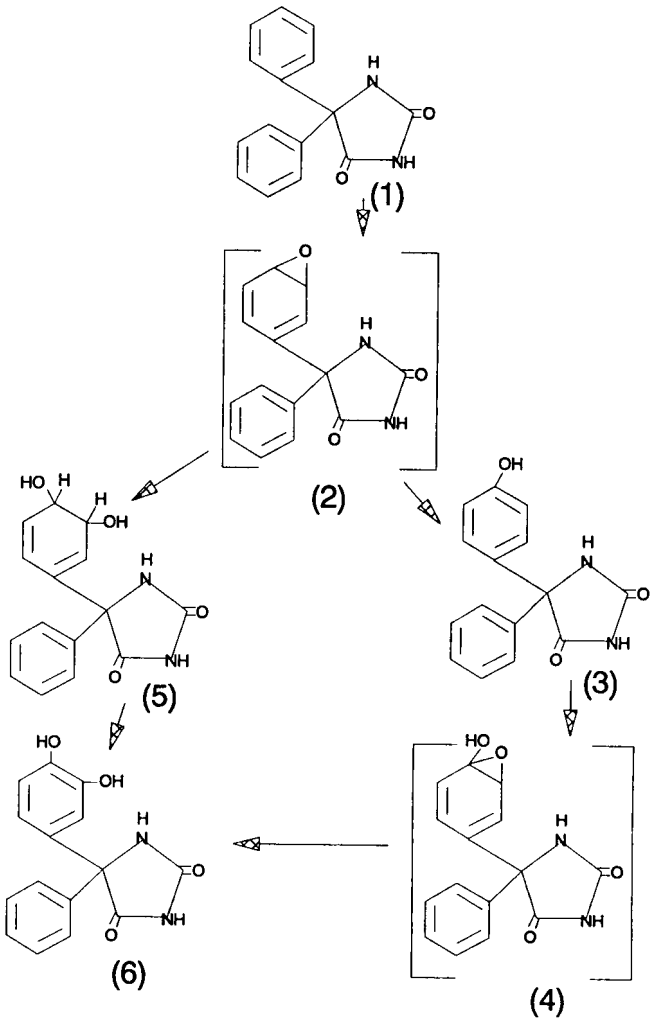

Figure 2. Cytochrome P-450-mediated metabolism of PHT is depicted. Structures shown are: (1) PHT; (2) postulated PHT-epoxide; (3) pHPPH; (4) postulated pHPPH-epoxide; (5) PHT-DHD; and (6) PHT-catechol. Epoxide hydrase catalyzes the conversion of PHT-epoxide to PHT-DHD; the catechol may subsequently be formed by the action of dihydrodiol dehydrogenase. A second P-450-mediated step may be involved in formation of the pHPPH-epoxide from the phenol metabolite. The catechol can be produced by isomerization of the phenol-epoxide. It has been suggested that PHT-catechol can be further metabolized to a quinone or semiquinone.

fetal resorption frequency, and the incidence of cleft palate. Pretreatment with either phenobarbital or SKF 525A was found to alter PHT distribution and metabolism, but did not affect absorption of the drug (83). Interpretation of these data is difficult due to the multiple effects of the pretreatments used. SKF 525A is a weak inducer of P-450 metabolism, as well as an inhibitor. Phenobarbital induces not only P-450, but also other enzyme activities, such as glutathione transferase, epoxide hydrase, and glucuronosyltransferase, which may act to detoxify the epoxide intermediate.

Harbison and Becker (84) observed that pHPPH, diphenylhydantoic acid, and α-aminodiphenylacetic acid were not teratogenic *in vivo*, but PHT did increase clefting. They suggested that PHT is the teratogen; however, the ability of the metabolites to cross the placenta was not addressed in this study.

Work with hydantoins that are structurally similar to PHT suggested that metabolism through the arene oxide pathway might not be involved in embryotoxicity (85). Resorption frequency was significantly increased, and fetal weight was decreased, following administration of *l*-nirvanol; there were no adverse effects using the *d*-isomer. Both isomers were metabolized through an arene oxide-generating pathway; however, far more phenol, dihydrodiol, and catechol were produced with the *d*-isomer than with the *l*-isomer, suggesting greater production of the arene oxide metabolite from the *d*-isomer. Therefore, there was not the expected correlation between arene oxide metabolism and embryotoxicity. However, placental transport and fetal metabolism of these hydantoins were not examined.

A number of investigators have noted variability among mouse strains in their teratogenic response to PHT (2, 16, 48, 49, 86–88). It was possible to delineate "fast" and "slow" metabolizers among several inbred strains of mice; however, there was no correlation between rate of metabolism and sensitivity of that strain to the embryotoxic effects of the drug (89). Additionally, Hansen and Hodes (90) found that microsomes from pregnant A/J females (a strain sensitive to PHT effects) produced greater levels of pHPPH and PHT-DHD *in vitro* than did microsomes from pregnant C57BL/6J females (a strain resistant to PHT effects). However, this difference disappeared following pretreatment of mice with PHT. This suggests that the differential susceptibility of the two strains was unlikely to be due to differences in PHT metabolism. However, whether there is variation in hepatic metabolism of the drug *in vivo* is unknown.

In rodent whole-embryo culture experiments, PHT produced dose-dependent decreases in the number of somite pairs, crown-rump length, and yolk sac diameter, as well as in DNA and protein contents (91). It also increased the frequency of morphologically abnormal embryos with defects in rotation, neural tube closure, and underdeveloped maxillary processes and/or mandibular arches (91). However, these effects were produced only at concentrations of PHT of 35 μg/ml or higher, which are above the human therapeutic range of 10–20 μg/ml. Yolk sac diameter was decreased at these concentrations, and the number of yolk sac defects was significantly increased. It is not clear whether the increased level of embryotoxicity observed at these high concentrations was a direct effect of PHT on embryonic development or a secondary effect related to the adverse effects of the drug on normal yolk sac function.

Other data suggest that binding of a reactive intermediate to fetal macromolecules may play a role in PHT teratogenesis in mice (92, 93). Martz *et al.* (92)

found that administration of the combination of 1,2-epoxy-3,3,3-trichloropropane, an inhibitor of epoxide hydrase activity, and PHT to mice increased the incidence of orofacial clefting and resorptions; there was also an increase in binding of PHT-derived radioactivity to fetal macromolecules. Further evidence to support a role for a reactive intermediate in PHT-induced embryotoxicity was reported by Shanks *et al.* (94), who used a rodent whole embryo culture system. At a PHT concentration of 20 μg/ml, they observed decreases in yolk sac diameter, crown-rump length, protein content, and development, as measured by the difference in the number of somite pairs from the beginning to the end of the culture period. Addition of a bioactivation system to increase PHT metabolism further decreased yolk sac diameter and protein content, as well as increased the number of embryos with defects in rotation and closure of the anterior neuropore. Although at first glance, it might appear from these data that unmetabolized PHT was embryotoxic, the authors noted covalent binding of radioactivity derived from PHT associated with embryonic proteins in the absence of the exogenous bioactivation system. Cytochrome P-450-mediated metabolism has been observed in embryos; however, the level is generally quite low (95). These data suggest that mouse embryos were capable of metabolizing PHT to an intermediate capable of covalent binding, and that this binding was further increased by the addition of an exogenous bioactivation system. There was also an association between an increase in defects and an increase in covalent binding.

Evidence to support a role for an epoxide intermediate in PHT-induced teratogenesis in humans was presented by Strickler *et al.* (96). Using a lymphocyte cytotoxicity bioassay, they found that 14 of 24 children exposed to PHT throughout pregnancy demonstrated an increase in lymphocyte death produced by PHT metabolites. There was an association between this increased toxicity of PHT metabolites and the presence of one or more major birth defects. There was no association with either the number or pattern of minor defects; however, evidence for the role of an epoxide intermediate in production of the minor defects associated with the fetal hydantoin syndrome was recently presented by Buehler *et al.* (97). They found low activity of epoxide hydrase in amniocytes from four pregnancies which each resulted in the birth of an infant with features of the fetal hydantoin syndrome. An additional 15 pregnancies monitored in this prospective study had intermediate or high epoxide hydrase activity, and none of these infants had characteristics of the fetal hydantoin syndrome. These observations suggest a role for an epoxide intermediate in PHT-induced embryotoxicity.

Although an epoxide intermediate is suspect, other reactive products might also be involved. The identity(ies) of the PHT-derived radioactivity bound to tissue macromolecules has not been determined. It is possible that free radicals produced either from metabolism of PHT-catechol (80) or by the prostaglandin synthetase pathway (66) could be involved. Evidence was presented which suggested that much of the binding of PHT-derived radioactivity could be accounted for by the binding of PHT-catechol and its subsequent metabolism to a quinone and/or semiquinone (80). Modulators such as glutathione, ascorbate, and *N*-acetylcysteine decreased the amount of covalent binding to liver microsomes *in vitro*.

Modulation of glutathione levels has been associated with PHT-induced embryotoxicity. Glutathione is known to bind to reactive intermediates to decrease their reactivity. Pretreatment with acetaminophen, which is reported to decrease hepatic glutathione, increased the frequencies of PHT-induced cleft palates and resorptions, while decreasing fetal weight (98). There was also an increase in covalent binding of radioactivity derived from PHT in embryonic tissue on Day 12 of gestation. Pretreatment with diethyl maleate, another depletor of hepatic glutathione, significantly increased PHT-induced clefting without altering the resorption frequency (99), and has also been reported to increase covalent binding of radioactivity derived from PHT (100). Treatment with buthionine sulfoxime, which inhibits γ-glutamylcysteine synthetase, the enzyme responsible for the first step in glutathione synthesis, significantly increased the incidences of cleft palates and resorptions, as well as decreased fetal weight (99). The chemotherapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea is reported to inhibit glutathione reductase activity, which would be expected to result in a decrease in intracellular glutathione; pretreatment inhibited embryonic glutathione reductase activity for 4 or 24 hr, decreased PHT-induced clefting, increased the resorption frequency, and decreased fetal weight (101). Recent work demonstrated that thiols and thiol-modifying agents decreased *in vitro* binding of PHT metabolites to A/J hepatic microsomal protein and increased metabolic production of PHT-DHD and pHPPH (102). However, the *in vivo* consequences of thiol modulation are unknown, since a glutathione conjugate of PHT has never been identified (103).

Generally, the bulk of the experimental evidence would seem to suggest some role for reactive intermediates in PHT-induced embryotoxicity. The identity of these intermediate(s) is not known. It is also not clear whether this mechanism will explain all of the embryotoxic effects of the drug. Additionally, while the association between covalent binding and embryotoxicity is intriguing, the role that covalent binding to macromolecules plays in abnormal development is unknown.

Summary and Conclusions

PHT has multiple effects in adult humans and animals, and there is no reason to assume that it will

not have multiple effects in embryos and fetuses. Although one of the first associations between anticonvulsant therapy and an adverse development effect in humans was noted in 1964 (104), the mechanism(s) whereby these adverse effects occur has thus far eluded research efforts. In this review, I have focused on three possible mechanisms. Overall, the evidence does not appear to implicate folate deficiency in PHT-induced embryotoxicity. A role for glucocorticoids or interaction between PHT and the glucocorticoid receptor has not been ruled out. However, a significant amount of work remains to be done to examine the involvement of the arachidonic acid cascade in PHT-induced embryotoxicity *in vivo*. The bulk of the experimental evidence would seem to favor a role for the generation of a reactive intermediate and its subsequent binding to embryonic macromolecules. This metabolite(s) has not been identified. Additionally, the association between covalent binding of metabolites and embryotoxicity remains simply an association; a causal relationship has not been established. Much work remains to be done to determine whether any of these possibilities, some other possibility, or a combination of several mechanisms will explain the adverse developmental effects of this very important, therapeutically useful anticonvulsant.

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