The Fetal Hydantoin Syndrome:

An Animal Model

by

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A DISSERTATION

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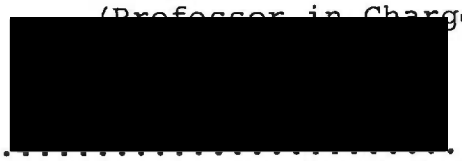

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(Chairman, Graduate Council)

DEDICATION

I would like to dedicate this thesis to the memory of Dr. C.W. 'Bob' Roberts, who passed away early this year. Dr. Roberts was a wonderful teacher, an inspiring scientist, and as fine a man as I'll ever hope to know.

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INTRODUCTION

> "Lord, have pity, sir, on my son, for he is an epileptic and has bad fits, and he keeps falling about, often into the fire, often into the water. I brought him to your disciples, but they could not heal him . . . Jesus then spoke sternly to the boy; the devil left him and from that moment he was cured" (Matthew 17:15-18).

Historical Perspective

Epilepsy, a heterogeneous collection of nervous system disorders, is characterized by paroxysmal discharges in the brain and by recurrent seizures. Man has long been aware of this disorder; our prehistoric legacy of trephined human skulls provides evidence of very early, albeit unsuccessful attempts to eliminate the disease that the Christians would call the "Falling Sickness" (Lennox, 1960), and to which the Greeks even earlier referred as the "Sacred Disease" (Temkin, 1945).

In spite of the fact that great military and political leaders such as Alexander the Great, Napoleon, and Julius Caesar, religious leaders such as Buddha, Mohammed, and St. Paul the Apostle, and literary giants such as Socrates, Pascal, Lord Byron, Dostoevsky, and Flaubert were all believed to have suffered from epilepsy (Gastaut, 1979), the disorder even today evokes stigma, a problem which may have its origins in a pre-Christian period when epilepsy was linked with dark mystical philosophies (Temkin, 1945).

For example, in the period of the Greeks, many illnesses were thought to be caused by demonic possession, and certainly the manifestations of epilepsy such as involuntary jerking, choreatic-type movements, and hallucinations would fit well with our popular conceptions of what possession by an evil spirit might look like (Glaser, 1978).

Some of the earliest treatments for epilepsy were described by Hippocrates and his followers, and later by Galen. Both of these scholars dismissed the commonly held notion that epilepsy was a form of demonic possession. Therefore, they prescribed secular rather than spiritual cures. Their treatment for epilepsy was based on dietary restraint (Temkin, 1945). They felt it was of vital importance to keep digestion in good order, often by the consumption of a concoction of honey and vinegar several times each day (Lennox, 1960). Indeed, from the fifth century B.C. until the early twentieth century, every treatise on epilepsy specified articles of food that should or should not be eaten (Lennox, 1960). Galen's use of powdered human skull bones started a practice that was continued for a thousand years in Europe. Another common prescription was to drink warm, human blood, which was usually obtained in Roman times from a fallen gladiator, and blood from other sources continued to be prescribed in Europe up to the Middle Ages (Lennox, 1960). By the fourth century A.D., the methods used to treat epilepsy included

dietetic, surgical, and pharmacological techniques (Temkin, 1945).

Despite the teachings of the Hippocratic school, which theorized the cause of seizures to be due to 'excessive phlegm' that obstructed the passage of air to the brain (Lennox, 1960), the "demonic possession" theory regained acceptance with the growing role of the Catholic church in the socio-political framework of the Middle Ages. The gospel writers recounted the tale of Christ's curing of the epileptics by casting out demons with his words (Temkin, 1945). St. Valentine became known as the patron saint of the epileptics, as were St. Vitus and St. Michael; those afflicted with epilepsy made pilgrimages to their priories, where hospitals were built (Temkin, 1945). The Catholic church listed epilepsy as one of the cardinal, contagious diseases which included such disorders as bubonic plague, tuberculosis, scabies, anthrax, and leprosy (Temkin, 1945). As Albich, a fifteenth century scholar, proclaimed: "Therefore, neither talk nor bathe with them, since by their mere breath they infect people" (Lennox, 1960).

Not only were the generalized convulsive forms of epilepsy known and chronicled by the dawn of the Dark Ages, but so were also the non-convulsive, 'petit mal', forms of epilepsy. This latter category was first described by the fourteenth century physician Bernard of Gordon. In the

year 1305 he wrote:

> Sometimes the paroxysm is very long and violent, sometimes short. I have seen it so short that the patient had only to lean against a wall or the like, rub his face and it ceased. Sometimes he did not need a support but there came to him a dizziness in the head and blindness in the eyes, and he himself, sensing it, recited the Hail Mary and before he finished it, the paroxysm had passed off and he spat once and the whole thing had passed off, and it used to come often during the day. There are some who, after a paroxysm remember absolutely nothing about the attack or their affliction, and there are some who remember and feel ashamed (Lennox, 1941).

The majority of physicians during the last half of the seventeenth century, and a great many in the early part of the eighteenth century, did not exclude demons or witches as the primary cause of epilepsy (Temkin, 1945). For those physicians who did employ secular cures, the range of pharmacological remedies was only as limited as the imagination of the physician. Often named herbs in epileptic cures included elder, garlic, mistletoe, and peony (Lennox, 1960). The treatment preferred by Thomas Willis, a seventeenth century professor of natural philosophy at Oxford who had written the most complete account of seizures and hysteria to date, required such exotics as powdered human skull bones, dragon's blood, wolf liver, and gall of boar with dried urine (Lennox, 1960). While the efficacy of these treatments is, for the most part, unknown, they likely were preferable to the more radical treatments of cautery and brain surgery, which, more often than not, had secondary effects that were worse than the disease itself.

The nineteenth century brought many advances in the treatment of epilepsy. For the first time epileptics were no longer confined to insane asylums. They were provided with humane hospitalization that permitted the first systematic, controlled studies of the disorder. Drawing on his experience in French hospitals for epileptics, Esquirol described the importance of the patient's confidence in the doctor's cure. This psychological factor was more important than any other aspect of his treatment (Temkin, 1945).

In the early nineteenth century, the prevailing medical opinion regarding epilepsy was that it involved reflex action and cerebral angiospasm caused by changes in the molecular state of the brain. These changes were thought to be mediated by malnutrition or poisoning (Temkin, 1945). Kussmaul and Tenner described convulsions in animal studies and attributed their cause to "sudden interruption in the nutrition of the brain" (Temkin, 1945). In the years between 1861 and 1902, Dr. Hughlings Jackson advanced for the first time the theory of the etiology of epilepsy, namely that seizures developed from an abnormal focus of excessive, electrical discharge in the grey matter of the brain (Jackson, 1931).

The first truly effective anticonvulsant drug, sodium bromide, was introduced by Charles Locock in 1857. It soon replaced the ineffectual zinc oxide treatment of that time. Although sodium bromide was an effective treatment for

epilepsy, it was not without side effects. Patients chronically treated with this drug complained of being sedated, psychic disturbances, skin rashes, and gastric distress (Vida and Gerry, 1977). The search for a safe, effective treatment for epilepsy continued into the twentieth century, when the first practical synthesis of barbiturates was achieved by Fischer and Von Mering in 1903. A second barbiturate drug, phenobarbital (5-ethyl-5 phenylbarbituric acid) was introduced into medical practice in 1912 by three independent researchers: Loewe, Juliusburger, and Impens. In the same year, Alfred Hauptmann recognized the therapeutic value of phenobarbital in the treatment of epilepsy. Phenobarbital remains still an important drug in anticonvulsant therapy, but its hypnotic property prevents physicians from prescribing doses high enough to ensure full protection against seizures.

Currently the drug of choice in the treatment of tonic-clonic (grand mal) seizures and of partial seizures, whether focal or not, is 5, 5-diphenylhydantoin (phenytoin; PHT; Dilantin, the registered trademark for Parke, Davis). The first synthesis of PHT was achieved by Blitz in 1908. Its anticonvulsant properties went unnoticed until Merritt and Putnam (1938b) demonstrated its efficacy against electrically induced seizures in cats. Later that year they reported on the anticonvulsant properties of PHT in humans (Merritt and Putnam, 1938a). This is the drug of choice because it

is less of a sedative than phenobarbital.

In the hundred years of modern anticonvulsant chemotherapy, starting with sodium bromide and on into the era of phenytoin, the negative connotations associated with epilepsy have been steadily eroded. One result of societal acceptance of epilepsy is the prospect of marriage and childbearing for the epileptic woman. More epileptic women than ever, especially in the last thirty years, have married and raised families (Dansky, 1978). At the onset of the twentieth century the fertility rate for epileptic women was 70% of the expected number of liveborn children, a figure that is significantly lower than that of the general population. Presently, the reproductive rates are equivalent for epileptic and non-epileptic women, although the rates for epileptic men are still somewhat depressed. This equivalency, however, is raising a new issue.

The number of malformed children born to epileptic mothers has led many investigators to speculate that a relationship exists between epilepsy, anticonvulsant drugs, and congenital malformations. Although there are fetal risk factors associated with epilepsy (such an anoxia, advanced maternal age, lower socio-economic status, and increased incidence of hydramnios) that could be contributing to the rate of congenital malformations (Fedrick, 1973; Monson, Rosenberg, Hartz, Shapiro, Heinonen, and Slone, 1973; Janz, 1975), many suspect that the malformations are instead the

result of the teratogenic action of anticonvulsant drugs. The following section will examine the results of the studies investigating these possibilities.

Survey of Epidemiological Reports Up to 1980

Less than thirty years after the clinical introduction of phenytoin, the first warning appeared in the literature of possible deleterious effects of the drug when taken during pregnancy (Mueller-Kuppers, 1963). This was followed by the first English language report of the possibility of embryopathy caused by this drug with two German physicians noting the association between epilepsy, anticonvulsant drugs, and congenital malformations (Janz and Fuchs, 1964). A number of case reports followed, most notably that of Meadow (1968), who described six patients whose mothers had received PHT while pregnant. All six of these patients had orofacial clefts and four of them had congenital heart defects.

All of these studies of epileptic pregnancies had been retrospective and had included different control groups, which hindered comparisons between studies. Table 1, drawn heavily from similar tables in reports by Annegers, Elveback, Houser, and Kurland (1974) and by Janz (1975), includes the results of twenty retrospective surveys. The data have been transformed to facilitate comparisons. In a number of these studies, the incidence of malformations was compared between epileptic mothers who had received anticonvulsants

TABLE 1. Epidemiologic studies on anticonvulsants administered to epileptic women and the frequency of malformations as compared with the occurrence of malformations in untreated epileptics and in non-epileptic controls.

		Livebirths to treated mothers with epilepsy			Livebirths to untreated mothers with epilepsy		
Reference		Total	Malfn.	%	Total	Malfn.	%
Janz & Fuchs	(1964)	225	5	2.2	133	0	0
Maroni & Markoff	(1969)	21	1	4.8	14	0	0
Elshove & Van Eck	(1971)	65	10	15.4	-	-	-
Watson & Spellacy	(1971)	51	3	5.9	-	-	-
South	(1972)	22	2	9.1	9	0	0
Speidel & Meadow	(1972)	329	17	5.2	59	0	0
Bjerkedal & Bahna	(1973)	-	-	-	-	-	-
Fedrick	(1973)	217	30	13.8	19	2	10.5
Koppe et al.	(1973)	125	11	8.8	66	2	3.0
Kuenssberg & Knox	(1973)	48	3	6.2	-	-	-
Lowe	(1973)	134	9	6.7	111	3	2.7
Meyer	(1973)	199	17	8.5	124	14	11.3
Millar & Nevins	(1973)	110	7	6.4	-	-	-
Monson et al.	(1973)	205	11	5.5	101	3	3.0
Niswander & Wertelecki	(1973)	-	-	-	-	-	-
Starresveld-Zimmerman et al.	(1973)	279	20	7.2	18	2	11.1
Baile et al.	(1975)	51	5	9.8	-	-	-
Knight & Rhind	(1975)	96	4	4.1	45	1	2.2
Annegers et al.	(1978)	177	19	10.7	82	2	2.4
Okuma et al.	(1978)	478	55	11.5	129	3	2.3
Totals		2832	229	8.1	910	32	3.5

Livebirths to all epileptic mothers			Livebirths to non-epileptic control mothers			Livebirths total population		
Total	Malfn.	%	Total	Malfn.	%	Total	Malfn.	%
358	5	1.4	-	-	-	-	-	-
35	1	2.8	-	-	-	-	-	-
-	-	-	65	0	0	12051	231	1.9
-	-	-	50	0	0	-	-	-
31	2	6.4	-	-	-	7865	190	2.4
388	17	4.4	448	7	1.6	-	-	-
378	17	4.5	-	-	-	112328	2471	2.2
236	32	13.6	649	36	5.5	-	-	-
192	13	6.8	-	-	-	12300	426	3.5
-	-	-	-	-	-	14620	477	3.0
245	12	5.0	-	-	-	31877	877	2.8
323	31	9.6	-	-	-	-	-	-
-	-	-	-	-	-	32227	1235	3.8
306	14	4.6	50591	1240	2.5	-	-	-
413	17	4.1	-	-	-	347097	9372	2.7
297	22	7.4	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
607	58	9.6	-	-	-	-	-	-
259	21	8.1	784	28	3.5	-	-	-
140	5	3.6	-	-	-	69000	2484	3.6
4208	267	6.3	52587	1311	2.5	639365	17733	2.7

and those who had not (Janz and Fuchs, 1964; Maroni and Markoff, 1969; South, 1972; Speidel and Meadow, 1972; Fedrick, 1973; Koppe, Bosman, Oppers, Spaans, and Kloskrman, 1973; Lowe, 1973; Meyer, 1973; Starresveld-Zimmerman, van der Kolk, Meinardi, and Elshove, 1973; Knight, 1975; Annegers, Hauser, Elveback, Anderson, and Kurland, 1978; Okuma, 1978). In these studies the incidence of congenital anomalies among children born to the treated mothers ranged from a low of 2.2% (Janz and Fuchs, 1964) to a high of 13.8% (Fedrick, 1973), the average incidence being 8.2%. This was greater than twice the frequency (3.5%) recorded for epileptic women who were not treated with anticonvulsants during their pregnancies. Two other studies show that the frequency of abnormality in babies of untreated mothers was greater than in babies of treated mothers (Meyer, 1973; Starresveld-Zimmerman et al., 1973). In six studies that had non-epileptics for controls, anomalies in children born to treated epileptics were three times greater than in the control population (Elshove and Von Eck, 1971; Watson and Spellacy, 1971; Meadow, 1972; Monson et al., 1973; Annegers et al., 1978; Fedrick, 1973).

In many of these studies it was common practice to utilize non-epileptics as case-controls. In most instances, the control mother was selected on the basis of similarities with the experimental patient in terms of age, race, hospital, year of birth, and socio-economic status (Fedrick,

1973; Monson et al., 1973). Other studies used as controls the hospital birth subsequent to a birth involving an epileptic mother (Watson and Spellacy, 1971; Speidel and Meadow, 1972).

Not all epidemiologists, however, used case-controlled subjects in their studies. Several investigators chose to use all hospital births during a given interval of time (Elshove and Van Eck, 1971; South, 1972; Monson et al., 1973; Shapiro, Slone, Hartz, Rosenberg, Siskind, Monson, Mitchell, Heinonen, Indänpään-Keikkila, Haro, and Saxen, 1976; Annegers et al., 1978) or all births reported by general practitioners (Kuenssberg and Knox, 1973). Still others chose geographically defined regions as control populations (Fedrick, 1973; Lowe, 1973; Millar and Nevins, 1973; Knight and Rhind, 1975). The Japanese study was conducted on a multi-institutional level, depending primarily on patients attending out-patient clinics throughout Japan (Okuma, Takahasi, Wada, Sato, and Nakane, 1978).

There are two large retrospective studies not mentioned so far. Niswander and Wertelecki (1973) reported all births in a military hospital in the years 1965 through 1971 and Bjerkedal and Bhana (1973) recorded all births in Norway during 1967 and 1968. In these two studies, the incidence of malformations among children born to epileptic women was 4.1% and 4.5%, respectively, whereas the comparable figures in the control populations were 2.7% and 2.8%. The

percentage of reported malformations in these studies is lower than that of some of the other surveys cited in Table 1. This may be due to the fact that these reports did not take into account differences in drug therapy status and included epileptic mothers who did not receive any drugs at all.

The combined results of all twenty surveys reported in the literature up to 1980 indicate that the risk of producing children with malformations is three times greater for the epileptic population (8.1%) than for the general population (2.7%). These figures are based upon 2,832 livebirths to drug-treated epileptic mothers and upon over 600,000 livebirths to mothers in the general population. Yet the malformation rate in 910 livebirths to non-treated epileptic women was 3.5%, a figure only slightly higher than that for the general population. These two comparisons relate to a point of contention noted in many studies, for several authors claim that epileptic women have higher risks regardless of their drug therapy (Fedrick, 1973; Monson et al., 1973; Janz, 1975; Shapiro et al., 1976; Friis, 1979) and several maintain that they do not (Lowe, 1973; Speidel and Meadow, 1974). Therefore, to more precisely separate the effects of anticonvulsive drugs from those of maternal epilepsy in the etiology of the malformations, it would be desirable to obtain data on a large population of epileptic women who are not receiving anticonvulsant drug therapy. It

is unfortunate that most authors did not segregate epileptic mothers who are on anticonvulsants from those who were not receiving any drugs.

Although the rate of malformation in drug-treated epileptics is low compared to the rate of malformations ascribed to other known human teratogens, such as Trimethadione (German, Kowal, and Ehlers, 1970) or Thalidomide (Lenz, 1961; McBride, 1961), it is clearly higher than that in the remainder of the population. With respect to the offspring of women receiving anticonvulsant therapy while pregnant, the weight of evidence strongly favors some causal relationship of anticonvulsant drugs to the production of congenital malformations. The few specific defects that are responsible for this increase are orofacial clefts and congenital heart defects (Table 2). The 2.2% incidence of cleft lip with or without cleft palate recorded for the children of treated epileptic mothers is 8-20 times greater than the frequencies recorded for non-epileptic control mothers in Birmingham (0.14%; Speidel and Meadow, 1972), Cardiff (0.16%; Lowe, 1973), London (0.2%; South, 1972), Groningen (0.27%; Elshove and Van Eck, 1971), Sheffield (0.11%; Knight and Rhind, 1975), and Rochester County (0.19%; Annegers et al., 1978). Furthermore, the average incidence of congenital heart defects (2.7%) among children exposed prenatally to anticonvulsants is four times greater than it is for a heterogeneous Birmingham population (Speidel and Meadow,

Table 2. Frequency of different malformations in children of mothers on anticonvulsants during pregnancy.

Reference		No. of Pregnancies	MALFORMATIONS Orofacial Clefts	Cardiac Anomalies	Anencephaly
Janz & Fuchs	(1964)	225	3	1	-
Maroni & Markoff	(1969)	21	-	-	-
Elshove & Van Eck	(1971)	65	5	2	1
Watson & Spellacy	(1971)	51	0	1	-
South	(1972)	22	2	-	-
Speidel & Meadow	(1972)	329	3	6	-
Fedrick	(1973)	217	1	2	-
Koppe et al.	(1973)	125	1	4	-
Kuenssberg & Knox	(1973)	48	-	-	-
Lowe	(1973)	134	1	1	1
Meyer	(1973)	199	5	5	-
Millar & Nevin	(1973)	110	2	-	-
Mirkin	(1973)	7	2	-	-
Monson et al.	(1973)	205	2	3	1
Starresveld-Zimmerman et al.	(1973)	279	9	7	1
Biale et al.	(1975)	51	1	2	-
Knight & Rhind	(1975)	96	2	3	-
Hanson & Smith	(1976)	35	2	3	-
Smith	(1977)	27	2	7	-
Annegers et al.	(1978)	177	4	8	1
Okuma et al.	(1978)	478	18	15	-
Dansky et al.	(1979)	31	1	3	-
TOTAL		2979	67	79	4
PERCENTAGE			2.2	2.7	0.2

* As combinations of malformations occur, the total numbers of cases can be lower than the sum of the different malformations stated.

MALFORMATIONS							
Micro-cephaly	Neural Defects	Hydro-cephaly	Hypo-spadias	Skeletal Anomalies	G.I. Defects	Misc.	# Cases* malfns.
-	-	-	-	-	1	-	5
-	-	-	1	-	-	-	1
-	1	-	-	1	-	1	10
-	-	1	-	-	-	1	3
-	-	-	-	-	-	-	2
3	-	-	2	1	2	2	17
-	1	-	2	6	1	8	20
-	-	-	-	-	-	6	11
-	-	1	1	-	1	-	3
-	1	-	-	1	-	3	9
-	1	-	-	7	-	-	17
-	1	-	-	-	-	4	7
1	-	-	-	-	-	-	3
1	-	2	-	3	-	-	11
-	1	-	-	1	-	1	20
-	-	-	-	-	-	-	3
-	-	-	-	-	15	-	4
10	-	-	-	19	-	27	14
-	-	-	-	13	-	5	13
-	1	-	-	-	-	1	10
-	3	-	-	11	3	11	55
-	-	-	-	-	-	3	7
15	11	4	8	64	24	82	275
0.5	0.37	.13	0.27	2.1	0.80	2.8	9.2

1972) or populations found in Sheffield (0.55%; Knight and Rhind, 1975), and Rochester County (0.57%; Annegers et al., 1978).

The aforementioned largely retrospective epidemiologic studies are extremely valuable resources, as they point out the need for further prospective studies or laboratory related investigations. However, retrospective studies are not a reliable method to answer specific questions, such as the cause of the increase in malformation among the children of epileptic mothers, nor have any major human teratogens ever been initially detected by this method (Miller, 1970). For the purpose of definition, retrospective studies are those that start with selected cases and controls, and these are compared for the presence or absence of a certain factor, in this case the presence or absence of a congenital defect. Prospective studies are those that start with a cohort of people with a factor and people without a factor (maternal epilepsy) and compare them for the existence of cases or non-cases (malformed or normal offspring) (Pearson, 1979).

In the twenty surveys mentioned above, the overriding criticisms relate either to a less than adequate control group (Elshove and Van Eck, 1971; Watson and Spellacy, 1971; Bjerkedal and Bahna, 1973; Niswander and Wertelecki, 1973; Shapiro et al., 1976) or to the small size of the sample population (Maroni and Markoff, 1969; Elshove and Van Eck,

1971; Watson and Spellacy, 1971; South, 1972; Kuenssberg and Knox, 1973; Millar and Nevins, 1973; Biale, Lewenthal, and Aderet, 1975). To clearly separate the teratogenic effect of the anticonvulsant drugs from that of the disorder, a large study would be required for it is necessary to have more than 800 births to be 90% confident of detecting a two-fold increase in congenital anomalies. This study would have to have control groups of epileptic women who are not receiving anticonvulsant drugs as well as non-epileptic women, a methodology followed by few surveys (Speidel and Meadow, 1972; Fedrick, 1973; Monson et al., 1973; Annegers et al., 1978). Unfortunately, the group of epileptic women who are not on anticonvulsant drug therapy is likely to differ in the severity of the disorder, not only from the group of epileptic women who are receiving anticonvulsants, but also from each other, thereby rendering comparative judgments increasingly difficult to make. In addition, these studies fail to stratify risk factors such as low socio-economic class and differences in maternal age that may constitute additional risks to the unborn fetus.

In summary, while the retrospective studies are not without shortcomings, they help to indicate the direction for future work. In this instance the cited studies point to an increased rate of malformation among offspring of treated epileptic mothers (Table 1), and they also reveal a certain consistency in the type of malformations produced

(Table 2). As all known major teratogens in man are also teratogenic in other, non-human species it is logical to assess the teratogenicity of PHT in lower animals. The following section explores the various animal studies that have been performed and the types and frequencies of the malformations that have been produced.

Survey of Animal Experiments Up to 1980

A number of studies have attempted to determine the teratogenicity of PHT in non-human species. Massey (1966) subcutaneously injected A/J mice daily with PHT on gestational days 9-15 (inclusive). Using PHT doses of 12.5, 25, and 50 mg/kg body weight, she reported that 42.8% of the offspring of treated mice had orofacial clefts. This rate was significantly higher than the 8-10% spontaneous rate of occurrence in this strain (Kalter, 1979).

Gibson and Becker (1968) carried out similar investigations with the mouse strains Swiss Webster and A/J, two strains that have been inbred for 90 and 168 generations, respectively. Although they found orofacial clefts and reduced size in fetuses when dams were injected subcutaneously with PHT on days 11-13 of gestation, these fetal changes were not observed when dams were treated on days 9-11. Furthermore, when dams received PHT on days 11-13 of gestation, the A/J strain showed an increased degree of resorption (35.4%) as compared with the controls (14.3%). This was the first embryolethal report of phenytoin in

rodents. Elshove (1969) found lower body weights and an increased incidence (15.3%) of cleft palate in the offspring of Swiss Webster mice mothers treated with intraperitoneal injections of 2.5, 1.9, or 1.75 mg of PHT when compared to untreated controls. Interestingly, when the mice were treated on days 10-13, there was 100% resorption as compared with a 15% incidence of resorption for mice injected on days 11-14. All of the aforementioned studies indicate that PHT is most teratogenic when injected during gestational days 10-14.

Harbison and Becker (1969) administered various doses of PHT to Swiss Webster mice at different gestational times. The dams were treated either with a single intraperitoneal injection of 150 mg/kg on days 8 through 15, or with three subcutaneous injections, in lower doses, on days 8-10 or on days 12-14. Again, the teratogenic insult occurs during the period of organogenesis. Among the dams receiving single injections of PHT, those treated on days 10 or 14 displayed an 80% resorption rate, while those receiving PHT at the other times displayed no increase in the resorption rate. All of the term fetuses from PHT treated dams had lower weights and reduced crown-rump lengths. The reduction in these two parameters resulted primarily from the shortening of the long bones (Harbison and Becker, 1969).

Harbison and Becker (1969) were first to report defects other than cleft palate in the offspring of dams treated

with PHT. At the two higher dose levels (75 and 100 mg/kg) used in their multiple injection study they reported cleft lip, cleft palate, hydronephrosis, renal and intraperitoneal hemorrhage, and delayed ossification and/or unfused sternebrae in over 20% of the progeny of treated dams. Other less commonly observed malformations included open eye, ectrodactyly, internal hydrocephalus, and split cervical centra. Offspring of the mice that were injected on gestational days 12-14 had elevated frequencies of orofacial clefts, while no malformations were induced by treating the mothers on gestational days 8-10. Subsequent work by these investigators, using both a stimulator and an inhibitor of phenytoin biotransformation, indicated that the rapid metabolism of PHT reduced its teratogenicity (Harbison and Becker, 1970). Conversely, agents that inhibited phenytoin metabolism did enhance the teratogenicity of the drug.

In 1974, Harbison and Becker investigated the teratogenicity of three phenytoin metabolites: HPPH (5-hydroxyphenyl-5-phenylhydantoin), diphenylhydantoic acid, and α-aminodiphenylacetic acid. These metabolites were administered to pregnant Swiss Webster mice by intraperitoneal injections on gestational days 11, 12, and 13, or by a single injection on either day 8 or day 10. Equimolar dosages of metabolites failed to increase the incidence of resorbed fetuses or to produce an increase in the incidence of orofacial abnormalities, as compared to PHT. Only

diphenylhydantoic acid, when given in dosages four times the equimolar dosage of PHT, produced a rate of malformation approaching 10%, which is still well below that of the parent compound (Harbison and Becker, 1974). While these results support the contention that phenytoin is the only active teratogen, it must be considered that the addition of exogenous metabolites may not be distributed in the same fashion as are metabolites produced in vivo, and that these subtle differences may produce significant effects during early embryonic development.

Investigators at Guy's Hospital in London (Sullivan and McElhatton, 1975) studied the teratogenicity of most of the commonly prescribed anticonvulsants either by intubating ICI mice (inbred 117 generations) with 40 or 120 mg/kg dosages or by mixing 250 mg/kg dosages into their feed on gestational days 6-16. They found that the incidence of cleft palate was elevated when the mice received the two higher dosages (Sullivan and McElhatton, 1975). They also noted a decrease in fetal weights, but they were reluctant to attribute this to the action of the drug. Comparable results were obtained by Miller and Becker (1975) who chose similar dosages, but administered the drug orally by means of gastric intubation.

In a subsequent study (Sullivan and McElhatton, 1977) 15, 45, and 90 mg/kg dosages of PHT were administered by intubation of CDI dams on gestational days 6-16. Although

PHT administration caused no increase in the number of implants per litter or in the rate of resorptions, malformations frequently were observed in the offspring. Open eye defects were seen in five of the seventeen litters at the 45 mg/kg dosage level. At the highest treatment level (90 mg/kg) reduced fetal weights, delayed ossification of the hands and feet, enlarged cerebral ventricles, and separated basisphenoid bones were the most commonly observed defects. In this study, 42% of the litters showed defects at the highest dosage level. Of the untreated control litters, only 0.62% had fetuses with malformations.

An explanation for the apparent lack of increase in resorption rates in treated mothers was advanced by Fritz and his co-workers (1976). They stated that PHT causes early embryonic loss, but that the drug is not embryolethal when administered after gestational day 14. In their study using the mouse strain TiF/MAG, they placed the uteri in a 20% aqueous solution of ammonium sulphide to visualize deciduomata which would represent resorption of early embryos. In four of the dams (13%) treated with 100 mg/kg and in nine of the dams (53%) on 170 mg/kg of PHT only deciduomata were visible. But after day 14 of gestation only 3% embryonic loss was seen in the highest phenytoin treatment group.

The most consistently cited explanation for the teratogenic action of PHT is a disturbance in the metabolism of

serum folate (Massey, 1966; Gibson and Becker, 1968; Harbison and Becker, 1969, 1972; Schardein, Dresner, Hentz, Petrere, Fitzgerald, and Kurtz, 1973; DeVore and Woodbury, 1977; Netzloff, Streiff, Frias, and Rennert, 1979). It is well established that PHT affects folate metabolism in humans (Hoffbrand and Necheles, 1965; Jensen and Olesen, 1969; Reynolds, Preece, and Chanarin, 1969; Snaith, Mehta, and Raby, 1970; Davis and Woodliff, 1971; Kariks, Perry, and Wood, 1971). It is also well established that comparable anomalies have been reported among the offspring of mothers on hydantoin anticonvulsants (Meadow, 1968, 1970; Speidel and Meadow, 1972, 1974; Annegers et al., 1974, 1978) and among the offspring of mothers exposed to folic acid antagonists during pregnancy (Thiersch, 1952; Goetsch, 1962; Milunsky, Graef, and Gaynor, 1968). Investigators have been able to produce orofacial clefts and other defects in offspring by feeding experimental animals with diets either deficient in folate or diets containing folate antagonists (Richardson and Hogan, 1946; Asling and Nelson, 1950; Hogan, O'Dell, and Whitley, 1950; Giroud and Lefebvres, 1951; Tuchmann-Duplessis and Lefebvres-Boisselot, 1951; Nelson, Asling, and Evans, 1952; Runner, 1954; Trasler, 1958; Monie, Armstrong, and Nelson, 1961; Wilson and Fradkin, 1967). Whether the observed defects are due to abnormal maternal folate metabolism, as believed by some investigators (Fraser and Watt, 1964; Hibbard and Smithells, 1965), is generally

considered unresolved (Kitay, 1968; Scott, Whalley, and Pritchard, 1970; Pritchard, Scott, and Whalley, 1971).

Schardein et al. (1973) tried to modify the effects of phenytoin on the developing mouse embryo by supplementing injections of phenytoin on days 11-13 with varying amounts of folinic acid (5-formyl-5, 6, 7, 8-tetrahydrofolic acid). They found a significant reduction in the number of cleft palates produced when Swiss Webster dams received 20 or 30 mg/kg folinic acid with the injections of 50 mg/kg PHT. At the same phenytoin dosage, however, the addition of 40 or 100 mg/kg folinic acid dramatically increased the incidence of cleft palate in these Swiss Webster mice (Schardein et al., 1973). Based on their data and the work of Mercier-Parot and Tuchmann-Duplessis (1974) on Wistar rats, it appears that supplementation with either folic or folinic acid provides little or no protection against phenytoin induced malformations.

The first published report in which maternal serum PHT levels were monitored was that of DeVore and Woodbury (1977). They treated maternal Sprague-Dawley rats with subcutaneous injections of PHT starting on gestational day 1 and continuing daily until day of sacrifice. This study verified an earlier report (Westmoreland and Bass, 1971) that maternal serum phenytoin levels increase throughout gestation in the rat. In humans, however, serum PHT concentrations tend to decrease during pregnancy (Mygind, Dam, and Christiansen,

1976; Lander, Edwards, Eadie, and Tyrer, 1977; Ramsay, Strauss, Wilder, and Willmore, 1978; Bruni and Willmore, 1979; Dansky, Andermann, Sherwin, Andermann, and Kinch, 1979; Landon and Kirkley, 1979). This is believed to be the result of decreased activity in the P^{450} microsomal enzyme, the site of metabolism of PHT. Another possible explanation is the increase in circulating steroid hormones during pregnancy. These hormones are known to interfere with PHT metabolism (Kutt and Verebly, 1970). After chronic exposure of rats to PHT, DeVore and Woodbury (1977) found an increased incidence of orofacial clefts in the offspring and they attributed the findings either to elevated maternal PHT levels or to a depression in serum folate which they noted to occur on day 14, a critical period in rat embryogenesis. A recent study by Paulson, Paulson, and Jreissaty (1979) also measured maternal PHT concentrations and found a positive relationship between high plasma PHT levels and an increased incidence of cleft palate and neural defects.

In a recent study, Sulik and Johnston (1979) used both scanning and transmission electron micrography to support their contention that phenytoin-induced cleft lip in A/J mice is due to a reduction in the size and number of cellular processes found at the lateral nasal process. They injected their dams on day 10 with 75 mg/kg PHT and found nearly 100% orofacial clefts in this genetically sensitive

strain.* Their micrographs show a failure to achieve a confluent mesenchymal cell process meshwork (CPM) which normally interacts with surface epithelium in the induction of morphogenetic changes (Kelley and Fallon, 1978). It has also been postulated that phenytoin may act more directly to interfere with CPM formation (MacKinney, Vyas, and Walker, 1978). Inasmuch as the lateral nasal processes undergo rapid growth on days 10-11, it is interesting to speculate on another mechanism for the failure to obtain a complete CPM, that of alteration in aerobic oxidative pathways. This idea is supported by the in vitro studies of Mackler, Grace, Tippit, Lemire, Shepard, and Kelley (1975) that have shown phenytoin's ability to block DPNH oxidase activity. Furthermore, it is well established that interference with oxidative metabolism by other drugs can produce cleft palates in chickens and rodents (Landauer, 1954, 1957; Trasler and Leong, 1974).

The most promising of the theories on the mechanism of phenytoin induced teratogenesis implicates a metabolite rather than the parent compound, PHT. It is now thought

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*These mice are sensitive in the sense that in the absence of teratogens, A/J embryos close their palates at a later gestational age than other inbred mouse strains (Walker and Fraser, 1956). Therefore, in the presence of a teratogen that delays palate closure resulting in a cleft palate, there would be a greater frequency of clefts in the sensitive A/J strain than in other mouse strains (Walker and Fraser, 1957).

that the formation of the vicinal trans dihydrodiol metabolite (5-(3,4-dihydroxy))-5-cyclohexadien-1-yl)-5-phenylhydantoin), in adult rodents as well as in humans (Chang, Savory, and Glazko, 1970), must proceed from an intermediary arene epoxide metabolite (Martz, Failinger III, and Blake, 1977). These latter compounds are highly reactive metabolites that bind covalently to macromolecules (Jerina and Daly, 1974). Consequently, phenytoin teratogenesis could result from the formation of these reactive metabolites and their binding to fetal macromolecules during critical periods of embryonic development (Martz et al., 1977). Martz et al. administered 1,2-epoxy-3,3,3-trichloropropane (TCPO; 100 mg/kg), an epoxide hydratase inhibitor along with teratogenic dosages of PHT to random bred Swiss ICR mice. This compound could inhibit the detoxification of epoxide metabolites into less reactive molecules, thereby enhancing the teratogenic effects of PHT. Their treatment did achieve an increase in PHT teratogenesis, doubling the incidence of cleft palate when TCPO is administered along with 50 mg/kg PHT, and nearly tripling the frequency when 75 mg/kg PHT is given to pregnant dams. The ultimate test for this theory of the mechanism of action of PHT would be to test the teratogenicity of the phenytoin-epoxide metabolite, but unfortunately this compound is too unstable to synthesize.

Summarizing the animal studies, it seems then that

orofacial clefts are the most consistently reported defect (Massey, 1966; Gison and Becker, 1968; Harbison and Becker, 1969, 1970, 1972; Mercier-Parot and Tuchmann-Duplessis, 1974; Miller and Becker, 1975; Sullivan and McElhatton, 1975, 1977; Fritz, Muller, and Hess, 1976; Devore and Woodbury, 1977; Martz et al., 1977; Netzloff, 1979), but such anomalies as internal hydrocephalus, hydronephrosis, and ocular defects (Harbison and Becker, 1969, 1972; Miller and Becker, 1975; Sullivan and McElhatton, 1977; Finnell, 1978, 1980) have also been observed. Generally, these malformations reported would not be considered abnormal if the developmental age of the fetus is taken into consideration. Most often, these defects are signs of intrauterine growth deficiency, the obvious exception being observations of cleft palates.

The Fetal Hydantoin Syndrome: 1975-1980

The epidemiologic studies described previously were designed primarily to investigate the association between maternal hydantoin consumption and single congenital defects, such as heart defects or facial clefts. However, some reports indicated a broader range of malformations associated with prenatal exposure to hydantoins (Meadow, 1970; Speidel and Meadow, 1972; Loughnan, Gold, and Vance, 1973; Barr, 1974; Hill, Verniaud, Horning, McCulley, and Morgan, 1974, 1979). In 1975, Hanson and Smith observed five unrelated children at the Dysmorphology Unit, University of

Washington, with similar dysmorphic features; all of their mothers had been exposed in utero to hydantoin anticonvulsants. This pattern of malformations was referred to as the "fetal hydantoin syndrome" (Hanson and Smith, 1975). Previous anecdotal studies focusing on orofacial clefts not withstanding, individuals considered to have the human fetal hydantoin syndrome must exhibit a pattern of abnormalities including at least three of the four major features related to growth and developmental performance: prenatal growth deficiency, postnatal growth deficiency, microcephaly, and mental deficiency (Hanson, Myrianathopoulos, Sedgwick-Harvey, and Smith, 1976). This is not to be confused with the mouse fetal hydantoin syndrome, which will be examined more thoroughly in the following chapters and consists of a similar pattern of abnormalities, including at least four of the following features: prenatal growth deficiency; skeletal, neural, ocular, orofacial, renal, and cardiac defects.

These original five cases of the fetal hydantoin syndrome showed a pattern of malformations that included characteristic craniofacial abnormalities such as a broad, low nasal bridge, epicanthic folds, short and upturned nose, ocular hypertelorism, ptosis or strabismus, prominent and low-set ears, wide mouth with thick, fleshy lips, trigonocephaly with metopic sutural ridging and wide fontanels. The skeletal defects included hypoplasia of the distal

phalanges and nails, a finger-like thumb, and dermatoglyphic patterns characterized by low-arch ridge patterns. Anomalies of the ribs, sternum, and positional limb deformities (calcaneovalgus deformity and pes cavis) were also observed. A definite prenatal growth deficiency was also characteristic and postnatal linear growth was 75% of normal. Developmental performance was usually deficient, with mild to moderate mental retardation often associated with microcephaly. Other less numerous characteristics included: umbilical and inguinal hernias; genital anomalies, including hypospadias; pilonidal sinus; orofacial clefts; renal defects; and cardiac anomalies such as atrial septal defects, ventricular septal defects, and pulmonary stenosis.

Because retrospective studies published prior to the time of formulation of the fetal hydantoin syndrome could project risk figures for drug-treated epileptic mothers based upon only a small portion of the anomalies comprising the fetal hydantoin syndrome, this risk figure would require updating. Moreover, retrospective studies generally have relied upon a clinical evaluation of an infant at the time of its birth or shortly thereafter, but some anomalies, especially cardiac defects, often go undiagnosed until the child is one year of age or older, leading to a significant underreporting of such defects.

The reporting of single malformations has failed in the past to detect the major teratogens in man (Hanson et al.,

1976). Because most teratogens give rise to a pattern of multiple defects, it is far better to search for such a pattern of malformations than for a single defect. The most consistent features of teratogens in humans are pre- and postnatal growth deficiencies, features which are often overlooked. Even in the most thorough of the recently completed major epidemiologic studies, that of the Collaborative Perinatal Project of the National Institute of Neurological and Communicative Disorders and Stroke, these more subtle anomalies are not reported (Heinonen, Slone, and Shapiro, 1977). Fortunately, however, prospective studies undertaken by investigators specifically interested in the teratogenicity of anticonvulsant drugs have reported a frequency of malformations as high as 35.7% in the offspring of drug-treated epileptics (Mirkin, 1971; Hill et al., 1974; 1979; Hanson et al., 1976; Smith, 1977, 1979). This percentage is a fourfold increase over the frequency provided by the retrospective studies. Admittedly, some of this marked difference might be attributed to the bias of the selection by the diagnostician.

The differences between techniques employed in epidemiologic studies can lead to conflicting conclusions, even though the conclusions were drawn from the same data source. For example, the study of Shapiro and colleagues (1976) and that of Hanson et al. (1976) were based upon case histories collected in the Collaborative Perinatal Project,

a prospective study. Shapiro et al. (1976) found no evidence that the major malformations among offspring of drug-treated epileptic mothers were associated with anticonvulsant therapy. They concluded that parental epilepsy, either maternal or paternal, was responsible, and not the medication. Supporting studies are those of Janz (1978) and Friis (1979). This conclusion has been repudiated (Hanson et al., 1976; Annegers et al., 1978; Dansky, 1979). Hanson et al. (1976) also used the resources of the Collaborative Perinatal Project to obtain 104 matched-control mothers in their study of features of the fetal hydantoin syndrome. Although many of the dysmorphic features characteristic of the fetal hydantoin syndrome were not evaluated in the data of the Collaborative Perinatal Project, enough evidence was available for confident diagnoses of the syndrome in 11 of the 104 children (11%) born to hydantoin-treated mothers.

Hanson et al. (1976) also followed 23 Seattle-area epileptic women on hydantoin drug therapy through 35 pregnancies. They based their diagnosis of the fetal hydantoin syndrome on the presence of at least three of the four features that they consider to be important indicators of the syndrome. These four features are: prenatal growth deficiency, postnatal growth deficiency, microcephaly, and mental retardation. On this basis, they found four children (11%) with the fetal hydantoin syndrome and an additional

11 children (31%) with anomalies consistent with prenatal exposure to hydantoins (Hanson et al., 1976). The diagnosis of the fetal hydantoin syndrome in the offspring of women receiving hydantoins during pregnancy in the Collaborative Perinatal Project was comparable to the results of the Seattle area study; both risk figures were 11%.

Since the initial description of the fetal hydantoin syndrome, clinicians have reported on additional cases that fit the general description of this syndrome. From these reports, new information is being accumulated concerning the spectrum of defects associated with the usage of hydantoins. Wilson, Smead, and Char (1978) concluded that the most commonly observed ocular defects in these children are hypertelorism, ptosis, strabismus, colobomata, and glaucoma. Optic nerve hypoplasia, a disorder caused by defective differentiation of the ganglion cell layer of the retina, has been associated with maternal hydantoin consumption by two ophthalmologists (Hoyt and Billings, 1978). There have now been reports of multiple affected siblings (Goodman, Katznelson, Hertz, Katznelson, and Rotem, 1976) and even members of multiple births who express the fetal hydantoin phenotype (Bustamante and Stumpff, 1978).

Because hydantoins have been available to clinicians for over forty years, the late recognition of their teratogenic potential has fostered skepticism regarding the validity of these reports of teratogenicity. In the past

four years, six publications have refuted the teratogenic potential of hydantoin anticonvulsants (Shapiro et al., 1976; Janz, 1978, 1979; Stumpf and Frost, 1978; Friis, 1979; Meadow, 1979). If phenytoin had been introduced only recently and if its teratogenic properties had been quickly recognized, the drug likely would have been removed from the market. However, this was not the case, and therefore many physicians are reluctant to acknowledge the danger that this drug poses for the developing fetus.

To clearly separate the teratogenic effect of the drug from that of the epileptic disorder, a large prospective study would be required. Such a study must include epileptic women and non-epileptic women, because the increased risk associated with epilepsy can only be determined by comparing the malformation rates in children born to untreated epileptic women with those of the non-epileptic population. To withhold anticonvulsants from epileptic mothers would raise ethical questions. Consequently it would be highly desirable to test these questions in an animal model that closely parallels the human situation.

Purpose and Rationale of the Present Study

Epileptic women comprise approximately 0.3 to 0.5% of all pregnancies (Janz, 1975). Data from the Collaborative Perinatal Project suggest that 2 of 1000 births are infants that have been exposed to hydantoins (Hanson et al., 1976). Yet, despite nearly half a century of experience with the

anticonvulsant PHT, its teratogenic potential in man is still uncertain. The human studies have been hampered by the innate heterogeneity of the disease, by the variety of types, combinations and dosages of anticonvulsant drugs, and by the methodology of patient selection. For the most part, the animal studies have been equally unrewarding in the solution of this question of teratogenicity. In the past, the drug usually has been administered to animals in single doses well in excess of the maximal human therapeutic dose (Massey, 1966; Gibson and Becker, 1968; Harbison and Becker, 1969, 1972; Schardein et al., 1973; Mercier-Parot and Tuchmann-Duplessis, 1974; Miller and Becker, 1975; Sullivan and McElhatton, 1975, 1977; Fritz et al., 1976; DeVore and Woodbury, 1977). Although this pattern of drug administration did produce malformation, it may have traumatized the pregnant animal sufficiently so as to confuse any interpretations and, furthermore, it hardly paralleled the human situation.

The inherent inadequacies of both the animal and human studies and the complexity of the relation between the drug effect and the maternal metabolism justify the development of an animal model. Should this model produce the same spectrum of malformations defined as the fetal hydantoin syndrome, it would be invaluable in answering questions as fundamental as the actual etiology of the defects, as well as the important clinical problem of delineating those

segments of the epileptic population who are at an increased risk of producing a malformed child.

In addition to testing the malforming capabilities of PHT, this research protocol will employ ethotoin (5-ethyl-5-phenylhydantoin), another of the hydantoin anticonvulsants. This test of the teratogenicity of ethotoin is especially important due to the current controversy regarding the mechanisms of hydantoin-induced teratogenicity. It is thought that phenytoin is metabolized to arene oxide metabolites, which are the primary teratogens and not the parent compound, phenytoin. Ethotoin is hydroxylated differently than is phenytoin; it never forms epoxide metabolites in the course of its biotransformation.

As proposed by Finnell (1978; 1980), a valid animal model of the fetal hydantoin syndrome would include the following criteria:

1. The animal must have spontaneous seizures in order to approximate those biochemical and physiologic peculiarities specific to the epileptic seizures that could influence the route and rate of PHT metabolism, and fetal oxygenation and nutrition. Although the presence of seizures alone need not constitute or be confused with that heterogeneous collection of disorders called epilepsy, it is important that the origin and clinical manifestations of the seizures be consistent in those animals who express the trait. Further, a control population consisting of genetically defined animals

who differ at the loci responsible for the seizure disorder in the affected animals should be included in the study.

2. The spontaneous seizures must be controlled or eliminated by PHT treatment.

3. The drug must be administered orally.

4. As the central question is whether the maternal epilepsy or the anticonvulsant drug therapy is responsible for the occurrence of congenital anomalies, a valid animal model must control both variables. The second requirement calls for seizure control, which should develop at doses within the normal therapeutic range for the species in question. Further, the plasma drug levels must be monitored and adjusted to fall within the mouse equivalent of the human therapeutic range, or 2.5-10 µg/ml plasma. As the percent of free drug is greater in the mouse than it is in man (Woodbury and Swinyard, 1972), the mouse therapeutic range should be scaled downwards from the 5-20 µg/ml plasma for humans to 2.5-10 µg/ml plasma for the mouse. This theoretical range is further supported by studies on seizure control in quaking mice where efficacious dosages (20-60 mg/kg) correlate well with plasma concentrations of 2-10 µg/ml (Sidman, Green, and Appel, 1965; Finnell, 1978).

5. PHT must be administered to the animal before mating and treatment must be continued throughout gestation. Studies on human epileptics have shown that when daily therapeutic doses are given orally, the serum concentration

increases slowly until a stable level is reached (Buchthal and Lennox-Buchthal, 1972). It usually takes 5 to 7 days at low doses, and somewhat longer at higher doses, to achieve the optimal therapeutic range (Harbison and Becker, 1969; Plaa, 1974). This time period is necessary to allow for the induction of enzyme systems associated with PHT metabolism that cause the characteristic fall in serum IgA and folic acid levels reported to result from chronic PHT use (Janz, 1975).

6. The offspring of the treated animals must exhibit those malformations observed in the offspring of epileptic women on hydantoin therapy. Furthermore, the frequency of the malformations must be significantly higher than that by spontaneous occurrence.

7. A dose-response curve that yields from 0 to 100% affected offspring should be observed (Wilson, 1965). Such a curve is characteristic of teratogenic agents.

Pharmacology of the Hydantoins

Biotransformation

Diphenylhydantoin is a lipid soluble weak acid (pKa 8.6) that is largely undissociated at physiological pH. As the drug is poorly soluble in aqueous solutions, salts of PHT can be formed by involvement of the imidic hydrogen through the occurrence of lactam-lactim tautomerism (Vida and Gerry, 1977). The lipoid nature of PHT allows it readily to penetrate cell membranes by nonionic diffusion. For this

reason it is almost completely reabsorbed from the glomerular filtrate and very little undissociated PHT is excreted in the urine.

Phenytoin is metabolized by the hepatic hydroxylase complex in the endoplasmic reticulum of hepatocytes (Alvin and Bush, 1977). The hydroxymetabolites are converted to very polar glucuronic acid conjugates that are both filtered and secreted into kidney tubules and secreted into the bile. The hydroxylation reactions occur at one of the 5-phenyl groups via an arene oxide intermediate to phenols, 5-(3- or 4-hydroxyphenyl)-5-phenyl-hydantoin (HPPH) which is the primary metabolite in both humans (50-83%) and the mouse (81%) and a dihydrodiol 5-(3, 4-dihydroxy-1, 5-cyclohexadien-1-yl)-5-phenylhydantoin (3 - 12% in humans, 19% in mouse). Arene oxide intermediates which are thought to be a feature common to all aromatic hydroxylations are enzymatically hydrated to dihydrodiols by epoxide hydrase (Boyland and Sims, 1960; Jerina, Daly, Zaltman-Nirenberg, and Udenfriend, 1970; Fouts and Kutt, 1972) (Figure 1). As previously mentioned, these metabolites are highly reactive compounds capable of denaturing biomolecules, and thus are prime candidates for directly injuring fetal tissues. The rate of the hydroxylation reactions is concentration dependent. At high serum PHT concentrations (>15 µg/ml) the rate of hydroxylation is independent of serum concentration, while below 15 µg/ml the rate declines with declining serum

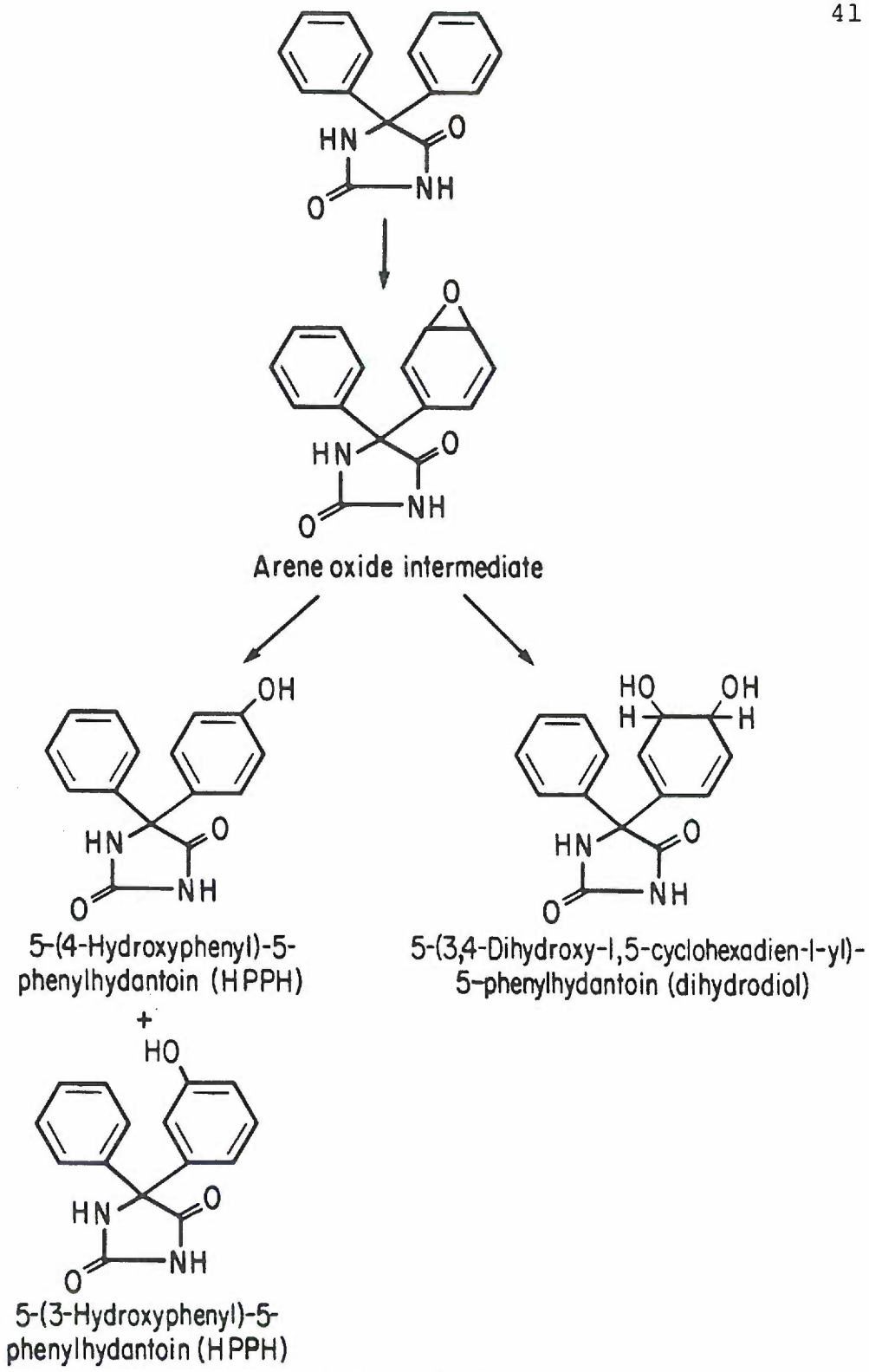

Figure 1 **Route of Metabolism of Phenytoin**

concentrations, typically first order kinetics (Gerber and Arnold, 1969).

There are additional phenytoin metabolites that have been discovered. Chang, Okerholm, and Glazko (1972a, b) report the presence of both 3, 4-(5-(3, 4-dihydroxyphenyl)-5-phenylhydantoin, and 3-0-methylcatechol metabolites. They are most likely a product of any dihydrodiol that is not conjugated and secreted (Glazko, 1975). Diphenylhydantoic acid and α-aminodiphenylacetic acid are two minor metabolites that appear when the hydantoin ring itself is ruptured enzymatically (Kozelka and Hine, 1943).

Two pathways have been discovered for the biotransformation of ethotoin. As seen in Figure 2, in the first pathway, hydroxylation of the phenyl rings is followed by conjugation with glucuronic acid while in the other pathway, N-demethylation is followed by enzymatic ring opening, catalyzed by the enzyme dihydropyrimidinase, forming the metabolite 2-phenylhydantoic acid (Dudley, Bius, and Butler, 1970; Alvin and Bush, 1977).

Plasma Binding

In plasma, phenytoin is tightly but reversibly bound to albumin and to α-globulins probably at the same sites that bind free fatty acids or thyroxine (Alvin and Bush, 1977). Ten percent (range 4-31%) of phenytoin is unbound in human plasma; however, this binding profile may be altered in pathological conditions that change the concentration or

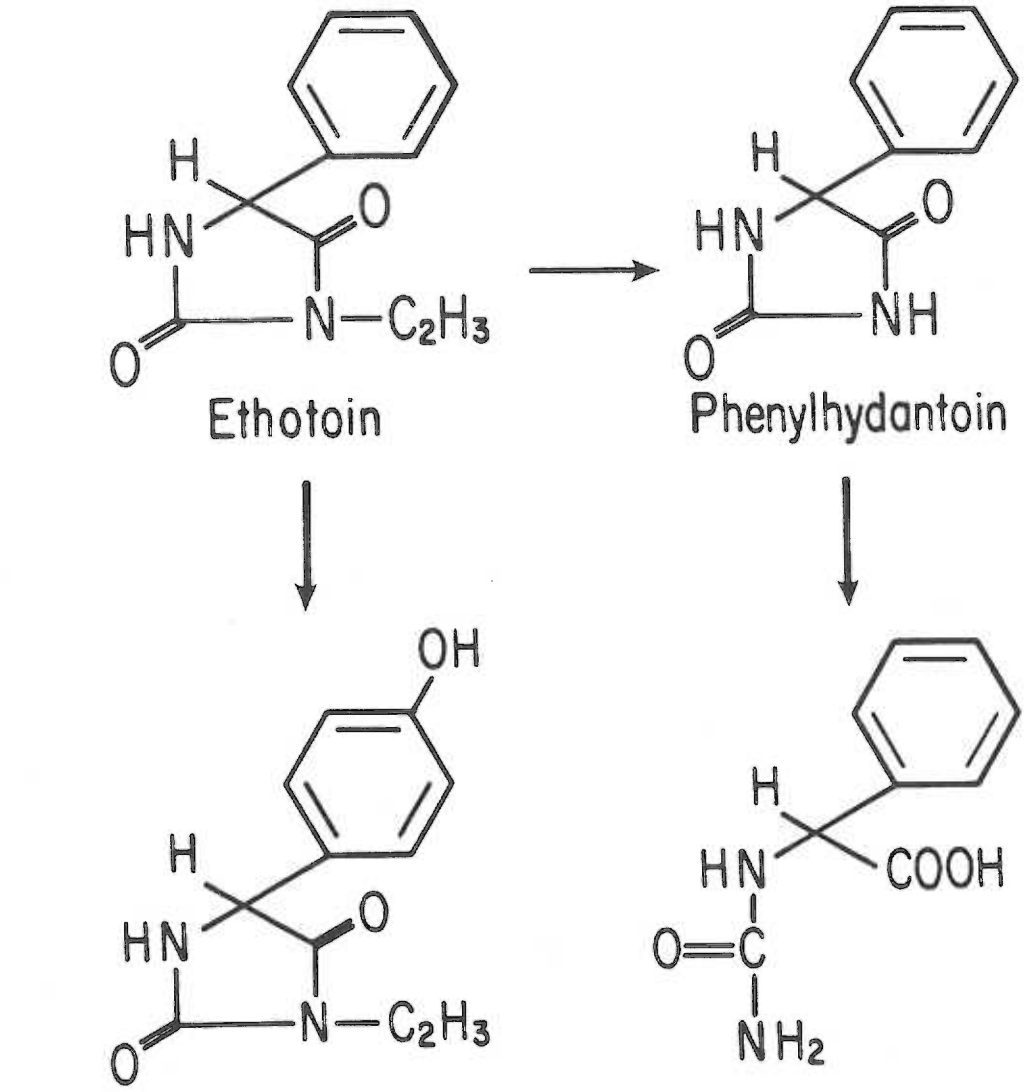

Fig. 2 Route of metabolism of ethotoin.

molecular form of blood proteins (Lunde, Anders, Yaffe, Lund, and Sjoqvist, 1970; Ehrnebo, Agurell, Jalling, and Boreus, 1971; Woodbury and Swinyard, 1972; Blum, Riegelman, and Becker, 1972; Glazko and Chang, 1972; Booker and Darcey, 1973; Olsen, Bennett, and Porter, 1975; Porter and Layzer, 1975; Löscher, 1979). The percent bound varies only slightly with plasma concentrations and the shift is towards more free drug at the higher plasma concentrations (Lunde et al., 1970).

There are considerable species differences in the amount of bound phenytoin in the plasma. In dogs, 64% of PHT is albumin-bound, 76% in cats, 80-90% in rats, and roughly 80% bound in the mouse (Woodbury and Swinyard, 1972). Thus, when making statements of equivalences in terms of total plasma concentrations in two different species, it is important to compensate for differences in binding affinities and to compare the two on the basis of free drug, which is the pharmacologically active portion. As mice have 10% more free drug than humans, they do not require as high a total plasma concentration to receive the same pharmacological effects as would a human. To this end, the plasma concentrations considered to parallel the human therapeutic range would be reduced to between 2.5 and 10 µg/ml plasma for the mouse.

The protein bound fraction of ethotoin in humans represents one-half of the total drug concentration in the

plasma (Troupin, Friel, Lovely, and Wilensky, 1979). Comparable protein binding studies have not been investigated in the mouse.

Distribution Studies

As a consequence of remaining nonionized at physiological pH, PHT rapidly crosses all membranes and it initially accumulates in the more highly perfused tissues, especially the liver, kidney, and brain. These organs then act as a reservoir from which phenytoin is distributed to the less perfused tissues (Alvin and Bush, 1977). Within fifteen minutes the drug has reached its maximum volume of distribution, which is 1.6-2.5 times the concentration of free PHT in the plasma, or 0.6 L/kg (Woodbury and Swinyard, 1972). The concentration of phenytoin in the brain is 1-3 times the total plasma concentration and 6-10 times greater than the free PHT plasma level as a result of binding to subcellular brain cell fractions (Dill, Kazenko, Wolf, and Glazko, 1956; Noach, Woodbury, and Goodman, 1958; Firemark, Barlow, and Roth, 1963; Nakamura, Masuda, Nakatsuji, and Hiroka, 1966; Kemp and Woodbury, 1971; Westmoreland and Bass, 1971). It is also found in higher concentrations within cells than in the extracellular matrix although it will distribute freely to extracellular fluids including saliva and bile. As the concentration of free PHT is the same in all tissues of the body as that of plasma, any accumulation of the drug must be the result of binding affinities (Woodbury and Swinyard,

1972). It will also cross the placenta and reach equilibrium between mother and fetus in a variety of rodents, rabbits, and in humans one hour after the injection of radiolabeled phenytoin into the maternal system (Mirkin, 1971; Westmoreland and Bass, 1971; Waddell and Mirkin, 1972; Harbison, 1978).

The kinetics of ethotoin more closely resemble the single compartment model indicative of a drug that reaches instantaneous equilibrium with the tissue it enters. There is a monophasic decline in plasma concentration over time, with the biological half-life of the drug being two hours (Troupin et al., 1979).

Physiological Changes During Pregnancy

The effect of pregnancy on epilepsy in an individual is quite unpredictable (Dimsdale, 1959). In a large survey by Knight and Rhind (1975) half the women reported no change in the frequency of seizures during pregnancy, while 45% indicated an increased frequency. Only 5% had fewer seizures after conceiving. Other surveys in the literature report either an increase or no change in seizure frequency (Baptisti, 1938; Burnett, 1946; Suter and Klingman, 1957; Maroni and Markoff, 1969; Ramsay et al., 1978). Only two small studies, one by Sabin and Oxorn (1956) and the other by Mygind et al. (1976) indicate a decreased frequency of seizures.

Several theories attempt to explain variations in

effect of pregnancy on epileptic women. It has been proposed that increased fluid retention along with the increased blood volume, up 25 to 50% from the non-pregnant volume (Caton, Roby, Reid, and Gibson, 1949), and the volume of fetal tissues may contribute to increasing the volume of distribution of PHT, thereby lowering plasma PHT concentrations (Lander et al., 1977). As a direct relationship exists between plasma drug levels and clinical efficacy, this lowering of plasma concentration may be responsible for increases in the number of seizures (Burnett, 1946; Goodwin and Lawson, 1947; Klein, Goodfriend, and Shey, 1956).

Another possibility was developed in a rat model. It is based on an increased plasma binding of phenytoin to albumin during pregnancy, leaving less free drug available for immediate transport to the liver. Thus, there is a decreased rate of biotransformation leading to an elevation of serum levels in the rat (Westmoreland and Bass, 1971). This is not the case in humans, where not only is plasma phenytoin binding unaltered during pregnancy (Hooper, Bochner, Eadie, and Tyrer, 1974) but plasma PHT concentrations decline up to 40% (Mygind et al., 1976; Lander et al., 1977; Ramsay et al., 1978; Dansky et al., 1979; Landon and Kirkley, 1979). In all probability the decline in human plasma PHT level results from a combination of increased metabolic activity and capacity of the maternal liver, fetal and placental drug metabolism, dilution effects, and the effect of

dietary folic acid supplements.

A possible explanation for the discrepancy between rats and humans concerning the effect of pregnancy on plasma PHT levels may be the influence of folic acid. This vitamin is usually prescribed during pregnancy, and high dose folic acid therapy has been observed to lower plasma phenytoin concentrations in both pregnant (Strauss and Bernstein, 1974) and non-pregnant (Jensen and Olesen, 1969; Baylis, Crowley, Preece, Sylvester, and Marks, 1971) women. During pregnancy there is a reduction in microsomal enzyme activity in both rodents and humans (Guarino, Gram, Call, and Gillette, 1969; Dean and Stock, 1975; Gut, Becker, and Gutova, 1976). Folic acid is believed to enhance the biotransformation of phenytoin by maintaining the non-pregnant levels of the hepatic enzyme phenytoin hydroxylase, even during pregnancy (Blake, Collins, Miyasaki, and Cohen, 1978).

It is well established that chronic hydantoin anti-convulsant therapy can result in depressed levels of circulating folates (Klipstein, 1969) which in turn can result in several major pregnancy complications. These include placental abruptions, abortion, perinatal mortality, and congenital malformations (Fraser and Watt, 1964; Hibbard, 1964; Hibbard, Hibbard, and Jeffcoate, 1965; Hibbard and Smithels, 1965; Martin, Harper, and Kelso, 1965; Streiffe and Little, 1967; Stone, 1968). Such observations strongly support the theory linking a metabolic interaction between

folic acid and phenytoin (Blake et al., 1978). It is currently thought that phenytoin is inhibiting the enzyme folate conjugase, which normally splits dietary folate into simpler monoglutamate forms. By so doing, absorption of the folate polyglutamates is impaired, leading to a deficiency state that is no doubt exaggerated by the demands of pregnancy (Hoffbrand and Necheles, 1968).

Direct vs. Indirect Effects on the Fetus

The mechanism by which phenytoin exerts its deleterious fetal effects is as yet unresolved. As mentioned in the section on animal experiments, two widely held theories are maternal folic acid deficiency exerting an indirect effect on the fetus by altering the maternal metabolism in such a way as to have deleterious effects on the fetus and an arene oxide metabolite exerting a direct effect on fetal macromolecules, where the purported teratogen is introduced to the maternal system and subsequently passes through the placenta, ultimately binding to fetal tissues where it affects one of a variety of possible mechanisms. As the folate deficiency theory has been thoroughly discussed under animal experiments, the focus here will be on studies pertaining to the direct action of phenytoin on the fetus.

Early work by Mirkin (1971) and Waddell and Mirkin (1972) investigating the placental and fetal localization of phenytoin and its metabolite clearly indicates the presence of these compounds in fetal rats, mice, and humans.

Phenytoin administered to pregnant rats either by intravenous or intraperitoneal injection readily crossed the placenta and reached peak concentrations in fetal liver and brain (Mirkin, 1971). Using ^{14}C-labelled phenytoin and injecting pregnant mice just prior to delivery, a greater quantity of the drug was found in fetal liver, brain, kidney, heart, adrenal cortex, and corpora lutea (Waddell and Mirkin, 1972). These same tissues have the highest concentration in both rat and human fetuses (Mirkin, 1971). It is interesting that the kidney, brain, and heart should be target organs, for very often these organs are the most severely affected by the teratogenicity of phenytoin (Harbison and Becker, 1969; Sullivan and McElhatton, 1977; Finnell, 1978, 1980).

Harbison (1978) has investigated variations in placental transport of phenytoin between animal species during gestation. He found a difference in the rate of placental transfer at the different developmental stages. In rats, more PHT was found in fetal tissues on day 8, the period of implantation, and on day 11, the start of organogenesis, than on days 14-17, the period of fetal maturation. Similar gestational dependent differences were found not only in the mouse but in humans as well, without corresponding maternal PHT concentrations (Harbison, 1978).

Of the various experimental animals commonly used, the fetal and placental concentrations of PHT are higher in the

mouse than in rats, hamsters, or rabbits (Stevens and Harbison, 1974; Harbison, 1978). Although plasma concentrations of PHT were comparable in rat and mouse dams, the concentration of the drug in fetal mice was almost twice that found in fetal rats. Rabbit and hamster maternal plasma levels of PHT were twice those found in mice, but the fetal concentration in those two species was less than in the mouse. Perhaps the differences between the various species could be related to maternal protein binding, with those species having the most unbound drug being those with the highest fetal PHT concentrations. While the percent bound has not been reported for the rabbit or hamster, the protein binding profile of the rat is more bound than that of the mouse (Woodbury and Swinyard, 1972). This would be concordant with the bound versus unbound theory, and this may even account for the rat's resistance to phenytoin induced embryopathy, while the mouse appears to be relatively sensitive to the drug.

Another possible reason for species' variation would be differences in the ability to metabolize the drug by the placenta or by the fetal livers in the different species. While numerous aromatic oxidation reactions have been demonstrated in placental tissue extracts, the metabolic capacity of the placenta is certainly less on a unit weight basis than either the maternal or fetal liver (Mirkin, 1975). Therefore, it is questionable whether differences in

placental metabolism of PHT between species contributes greatly to the observed differences in fetal concentrations. While the levels of microsomal enzymes present in fetal livers are uniformly low, they are present in rat, rabbit, hamster, mouse, and human fetuses just prior to parturition (Eling, Harbison, Becker, and Fouts, 1970; Mirkin, 1975) and thus it is unlikely that quantitative differences in fetal drug metabolism alone can account for the observed differences in fetal PHT binding.

Phenytoin, as with most all compounds having molecular weights under 600, will pass the placental barrier (Mirkin, 1971; Waddell and Mirkin, 1972) and is incorporated into fetal tissues (Stevens and Harbison, 1974; Harbison, 1978). This does little to resolve the question of direct versus indirect effects on the fetus. If anything, conflicting reports serve to bolster the arguments of those who still refuse to acknowledge the adverse effects of these drugs on developing embryos (Shapiro et al., 1976; Apt and Gaffney, 1977; Janz, 1978, 1979; Stumpf and Frost, 1978; Friis, 1979; Meadow, 1979). Janz (1978) claims the experimental animal evidence to date has not convincingly shown the existence of a fetal hydantoin syndrome in non-human species. He based this judgment on the fact that no cardiac anomalies have been reported in animals treated with hydantoins. However, very recent work has shown such anomalies (Finnell, 1978, 1980; Sulik et al., 1979).

The basic arguments proposed by those who question the existence of a human fetal hydantoin syndrome is that there is no drug specificity, with many anticonvulsants producing similar effects (Janz, 1978). Although a fetal primidone embryopathy has been reported (Seip, 1976; Berkowitz, 1979; Rudd and Freedom, 1979) the pattern of characteristic features was not the same as that found in children exposed in utero to hydantoins (Hanson et al., 1976). A second argument against the concept of a fetal hydantoin syndrome is based upon a genetic relationship between epilepsy and orofacial clefts. In one study, 17% of the patients examined for corrective treatment of cleft lip with or without cleft palate had first or second degree relatives with epilepsy (Dronamraju, 1970). This rationale is then extended to other malformations such as congenital heart defects, which are found in 5% of children with cleft lip with or without cleft palate (Meadow, 1977). While these arguments, especially the latter one, suggest a multifactorial etiology for many of the malformations commonly observed among the offspring of epileptic women, the fact remains that it is the maternal history of hydantoin consumption that is the common link with the excessively high rates of congenital defects, not only orofacial or heart anomalies, but also skeletal changes and certain miscellaneous defects found in the infants of epileptic mothers.

The following experiments are not designed in such a

way as to categorically define the effect as direct or indirect. That is work for the future. An attempt was made to define an animal model for the fetal hydantoin syndrome that would demonstrate the teratogenicity of this widely used anticonvulsant, and also establish a model system that would be amenable to experimental applications addressing specific questions on the pathogenesis of the fetal hydantoin syndrome. Further, ethotoin was included in this system so that the hypothesis of a toxic intermediary metabolite acting as the primary teratogen could be investigated.

MATERIAL AND METHODS

Animals

C57BL/5J, +/qk mice (Mus musculus) were obtained in 1975 from the Jackson Laboratories, Bar Harbor, Maine and housed with the Medical Genetics breeding colony in the animal care facilities of the Neurological Sciences Institute of Good Samaritan Hospital. From these animals two breeding lines were maintained. One line, C57BL/6J, +/+ (the control mice), were in their eighth generation in our facilities at the commencement of this project. The other line consisted of C57BL/6J, +/qk and C57BL/6J, qk/qk mice, also in their eighth generation. Two other inbred mouse strains, Swiss-Vancouver (SWV) and C_3H/Ml were obtained from the Medical Genetics breeding colony at the University of British Columbia. The SWV mice were in their fifty-eighth generation and the C_3H mice in their thirtieth generation at the commencement of this project. All animals were housed in standard clear polycarbonate cages, females in pairs and males with sibs, and maintained on a 12-hour light cycle (0600-1800 hr). They were allowed ad libitum access to Wayne Lab-Blox F6 and tap water unless otherwise indicated.

Hydantoin Treatment Levels

Drug treatment with 5,5-Diphenylhydantoin sodium (PHT, Sigma Chemicals, St. Louis, Mo.) was administered in four

dose levels, 0, 20, 40, and 60 mg/kg body weight. While these levels may seem excessive when compared to the human therapeutic dose of 3.5-10 mg/kg/day (Buchthal and Lennox-Buchthal, 1972), they are actually comparable when metabolic activity and body surface area are taken into consideration (see Appendix E) (Brodie, Maikel, and Jondorf, 1958). When PHT is given to mice in human dosages, it is eliminated very rapidly and serum PHT levels fall well below the desired mouse therapeutic range.

The treatment levels were selected in such a way as to bracket the desired therapeutic range, with one treatment group on a dosage regime that would yield plasma concentrations on the lower limit of the therapeutic range. Two other treatment groups were put on dosages that would represent the mid and upper limits of the therapeutic range. The highest dose level that could be given to the dams without causing severe toxicity was 60 mg/kg. At this dosage the majority of females were free of any clinical manifestations of PHT intoxication, though some were lethargic, indicating a mild to moderate degree of toxicity. An intermediate dose of 40 mg/kg was included to comply with the World Health Organization guidelines for teratogen testing (Wilson, 1967).

Treatment with ethotoin (ETH; Peganone, a registered trademark; Abbott Laboratories, N. Chicago, Ill.), another hydantoin-type anticonvulsant drug, required different considerations. In humans, ETH has a very short biological

half life (<6 hrs) and frequent, relatively high dosages of this drug are required to keep plasma concentrations within the suggested human therapeutic range of 15-50 µg per ml (Larsen and Naestoft, 1974). The usual dose of ethotoin is, therefore, five to six times the usual dose for phenytoin (Troupin et al., 1979). For the purposes of this animal study a fivefold increase over that of phenytoin was chosen, making four treatment levels of 0, 100, 200, and 300 mg/kg.

Calculation of Drug Doses

A stock solution of PHT consisting of 1 mg PHT sodium to 1 ml distilled water, pH 10.2, was made fresh daily. The drug was measured out to one ten-thousandth of a gram in a Mettler single beam analytical balance and was then transferred to Erlenmeyer Flasks. After the addition of a small quantity of 2M NaOH, an appropriate quantity of distilled water was added and the pH adjusted to 10.2 with a Radiometer Copenhagen Model 26 pH meter. The contents of the flask were repeatedly aspirated by a Pasteur pipette to ensure that the drug had completely dissolved.

The desired quantity of stock solution was measured out with a 10 ml glass serological pipette (Kimball; Toledo, Ohio), and was then diluted with distilled water, pH 10.2. The daily PHT-water solution was placed in Falcon 50 ml conical graduated centrifuge tubes with a standard metal drinking tube in a number 6 rubber stopper (Fisher Scientific Co., Pittsburg, Pa.). In the preparation of the

ethotoin stock solution, 2 mg of ethotoin to each ml of pH adjusted distilled water was used as the higher dosages of this drug required a stronger concentration of the stock solution. Otherwise the preparation of the solution was identical to that of phenytoin. The control mice (treatment level 0 mg/kg) for both the ethotoin and phenytoin experiments received distilled water adjusted to pH 10.2 in the same 50 ml conical graduated centrifuge tubes as did those animals receiving hydantoin anticonvulsants.

The amount of stock solution required for each cage was determined by calculating the animal's weight, average water consumption, and the treatment level it was to receive. Conversion tables were established to facilitate rapid calculations. The first conversion table (Appendix A) gives the amount of PHT a mouse of a given weight must consume to be receiving the desired dosage. The second conversion table (Appendix B) corrects for the animal's failure to consume 50 ml of liquid daily. The product of the values obtained from these tables is the amount of stock solution required for a given cage. A detailed explanation of the usage of these tables can be found in Appendix C. The same procedure was used to arrive at the daily stock solution requirements for those animals receiving ethotoin instead of phenytoin. The only difference here was that the product of the values obtained from Appendix A and Appendix B was multiplied by 5 to give stock solutions corresponding to

100, 200, and 300 mg dosages.

Diet Administration

Virgin females 60 to 90 days old were used in both the phenytoin and ethotoin teratological studies. Although the pharmacological properties of PHT have been well described in both man and mouse (Frey and Kampmann, 1965; Gerber and Arnold, 1968; Kutt and Verebely, 1970; Kutt, 1971; Gerber and Lynn, 1972; Glazko, 1975), the animal literature on ethotoin was non-existent. For all studies, the mice were gradually introduced to the drug to avoid weight loss, sickness, and refusal to drink, which have been reported in mice given large doses (0.3 mg/ml) of PHT (Frey and Kampmann, 1965).

In order to construct a dose response curve, the various doses of PHT and ETH were administered to virgin mice over a 20 day period. This allowed ample time for them to become tolerant to the drugs (Frey and Kampmann, 1965) and for the plasma drug concentrations to reach a plateau (Plaa, 1974). The observed initial accumulation followed by maintenance of a stable blood level is an important characteristic of phenytoin and other clinically proven anticonvulsant drugs (Kutt, 1971).

The dams were randomly assigned to one of four treatment levels. At five day intervals for fifteen days, blood samples were drawn from the retroorbital sinus using heparinized microhematocrit tubes (Fisher Scientific Co.,

Pittsburg, Pa.). To maximize possible variability in blood PHT and ETH levels due to circadian fluctuations, the samples were collected over a fourteen-hour period, 9 am to 11 pm on the day of the blood sampling. The blood was collected in 500 µl plastic microcentrifuge tubes (West Coast Scientific, Berkeley, Calif.) and spun down in a model CL clinical centrifuge (International Equipment Co., Needham Hts., Mass.) for ten minutes at 3000 g. At least 50 µl of plasma were removed with the aid of a gas-tight syringe (Hamilton Co., Reno, Nev.) and transferred to clean microcentrifuge tubes. These were frozen until the day of the assay.

In addition to the three blood samples drawn prior to mating, a final blood sample was taken on the day the dam was to be sacrificed. It was therefore possible to ensure that the animals were at a steady state in terms of the drug level in their plasma prior to any attempted mating. Further, comparisons could be made between pre-pregnancy plasma concentrations and those obtained at the time of the hysterectomy.

Seizure Control Study

A study of 12 C57 BL/6J, qk/qk female mice was conducted to determine the effectiveness of orally administered ETH in controlling their seizures. The mice were housed in pairs and allowed free access to water containing their dose of ETH. Starting on level 1 (0 mg/kg), a background

frequency of seizures recorded in terms of seizures per mouse day was ascertained for each mouse (Sidman et al., 1965). Dosages were then elevated in a stepwise fashion through the remaining three treatment levels for seven-day periods during which the mice were observed daily for one hour. At fifteen minute intervals during the observation period, the animals were gently lifted by their tails and slowly turned 180° (Goldstein, 1973). Those animals that exhibited clonic-tonic seizures from the time the wire cage lid had been removed until the time the lid had been replaced after turning the mice, were recorded as having had a seizure. Similar studies on the effectiveness of PHT in controlling seizures in quaking (qk) mice have been reported (Sidman et al., 1965; Finnell, 1978, 1980).

Determination of Plasma Hydantoin Concentrations

The Assay

The assay procedure used to determine plasma hydantoin concentrations was a modification of the high pressure liquid chromatographic technique of Kabra, Stafford, and Marton (1977). The primary advantages of this method include the use of microvolumes of plasma (50 µl), the lack of solvent extraction steps, the short sample preparation time, and the relative speed of the analysis. The method is also highly reproducible and within day runs yielded a coefficient of variability of 2.4% while the coefficient of variability between days was 5.6%. The samples were analyzed

within an hour after thawing. Contaminating peaks, presumably the decomposition products from the plasma, appear if samples are allowed to stand for more than five hours.

The Instrumentation

This assay was performed on an Altex Model 332 Gradient Liquid Chromatograph (Altex Corp., Berkeley, Calif.) which features two Altex Model 110A single piston reciprocating pumps and a temperature controlled oven. A Model 210 sample injection valve with a 200 µl sample coil was the port of entry on this machine. The absorbance detector was a Hitachi Model 100-30 UV-VIS Spectrophotometer (Hitachi Ltd., Tokyo, Japan) with a variable wavelength detector. The recorder was a Hewlett-Packard Model 18850A (Hewlett-Packard, Corvallis, Ore.). The column used for this assay was a reverse-phase column Bondapak C_{18}, measuring 30 cm x 4 mm (Waters Associates, Inc., Milford, Mass.).

The Standard Curve

Stock solutions of phenytoin (phenytoin sodium; Sigma Chemicals, St. Louis, Mo.) and ethotoin (Abbott Laboratories, N. Chicago, Ill.) 1 mg/ml in absolute ethanol (200 grade) were freshly prepared for each assay. One in ten dilutions of these solutions were then sequentially prepared with distilled water to 100, 10, and 1 µg/ml concentrations. Portions of these solutions were utilized for the preparation of the standard curve, which was prepared in the following manner.

In tightly stoppered 5 ml reactivials (Pierce, Rockford, Ill.) fifty microliter aliquots of plasma were added to various quantities of stock solutions and distilled water was added to a final aqueous volume of 150 µl (Table 3). Fifty microliters of a solution containing acetonitrile (CH_3CN; Nanograde, Burdeck & Jackson Laboratories, Muskegon, Mich.) and 50 µg/ml of sodium hexabarbital (Sigma Chemicals, St. Louis, Mo.) was added followed by an additional 150 µl of acetonitrile. The acetonitrile was used to precipitate plasma proteins; the hexabarbital functioned as the internal standard.

This mixture was then vortexed for approximately fifteen seconds and centrifuged at 2000 rpm for five minutes (International Equipment Co., Needham Hts., Mass.) to separate the proteins from the supernatant. A 100 µl sample of the supernatant was then removed with a gas-tight syringe (Hamilton Co., Reno, Nev.) and injected through the sample valve onto the column. The column was continuously eluted with an acetonitrile-acetate buffer (27% CH_3CN by volume). This buffer (0.002M, pH 3.65) was prepared fresh as needed by adding 1.5 ml of 1 M sodium acetate to 730 ml of distilled water. To complete the mobile phase, 270 ml of acetonitrile were added to this mixture and the buffer was filtered through a Millipore Type GS (0.22 µm), and Type FH filters (Millipore, Bedford, Mass.). The buffer was pumped through the column at a flow rate of 2.0 ml/minute.

TABLE 3. Standard curve preparation.

Total in Vial (ng)	Plasma Vol. (µl)	CH_3CN (µl)	ISTD (ml)	PHT Vol. (µl)	H_2O (µl)
0	50	150	50	0	100
50	50	150	50	50 (1 µg/ml)	50
100	50	150	50	100 (1 µg/ml)	0
250	50	150	50	25 (10 µg/ml)	75
500	50	150	50	50 (10 µg/ml)	50
1000	50	150	50	100 (10 µg/ml)	0

The room temperature was 22°C and the column effluent monitored at 195 nm.

The standard curve contained 50 to 1000 ng of phenytoin, which covered the range of test samples. Correlation coefficients of the standard curve were always in excess of 0.99 for the specified working range. For a set of six curves $r = 0.999 \pm .0014$ and the composite regression equation was $y = a + bx$. A composite standard curve can be seen in Figure 3, while sample tracing can be seen in Figures 4-6.

To calculate the final concentration of PHT in the test plasma, the areas under the curve ratios were taken and the following equation applied:

$$(\text{PHT}) = \frac{y - a}{b} \left(\frac{1000}{20}\right) \mu g/ml$$

It should also be noted that there were never any interfering peaks co-eluting with PHT in the plasma samples observed in these experiments.

Experimental Design

Experimental Animals

The experimental protocol was designed to comply with the criteria established for an animal model of the fetal hydantoin syndrome. The first requirement called for the use of an organism with a spontaneous seizure disorder. For this reason the mouse mutant quaking (*qk*), characterized by spontaneously recurring convulsions in homozygous (*qk/qk*) animals, was selected (Sidman et al., 1965). Female mice of

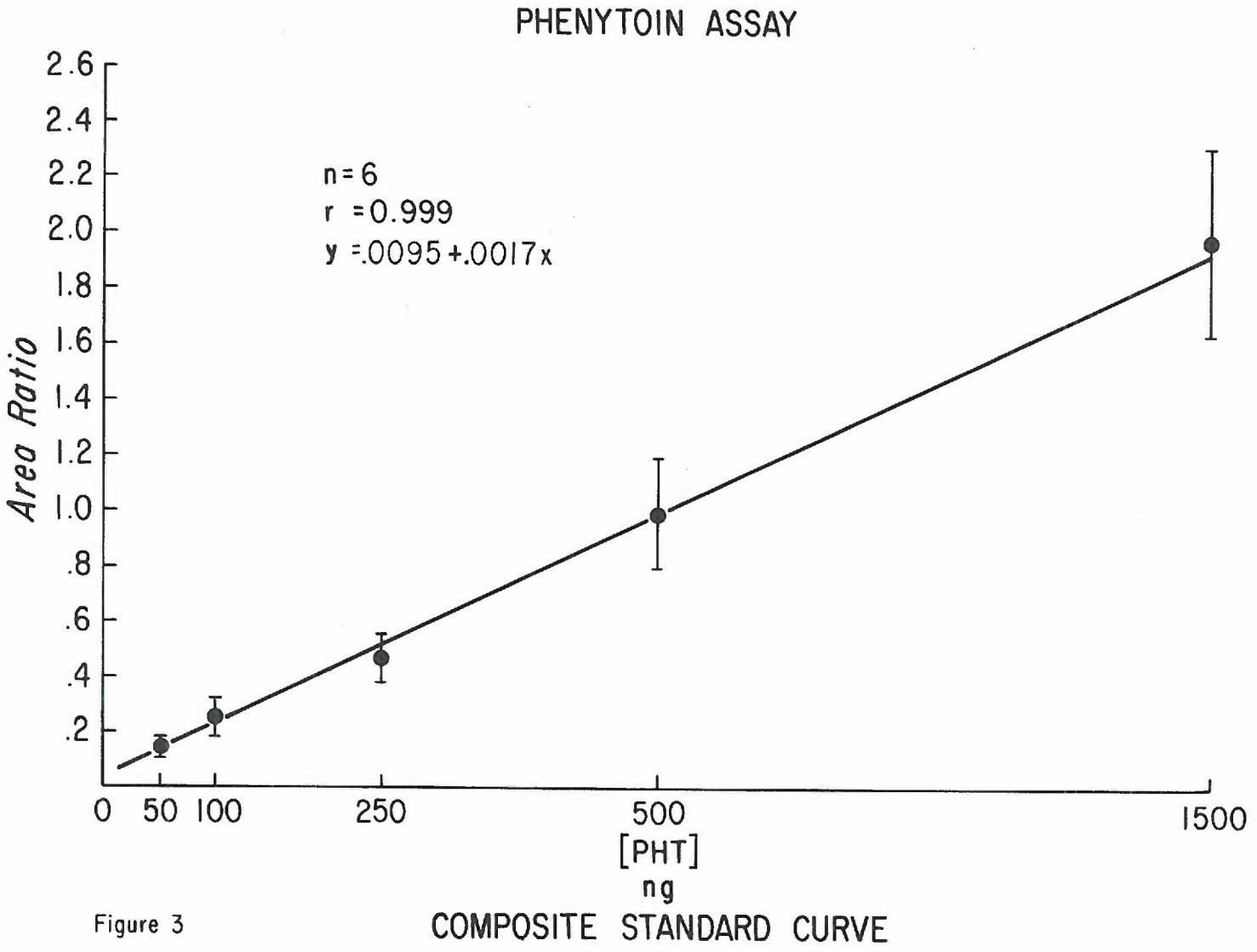
PHENYTOIN ASSAY
n = 6
r = 0.999
y = .0095 + .0017x
Area Ratio
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
50
100
250
500
1500
[PHT]
ng
COMPOSITE STANDARD CURVE

Figure 3

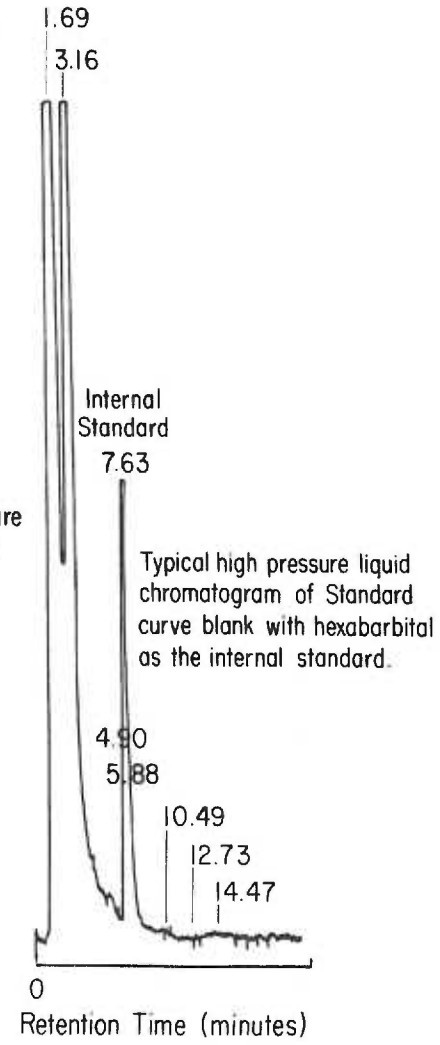

Figure 4 Typical high pressure liquid chromatogram of Standard curve blank with hexabarbital as the internal standard.

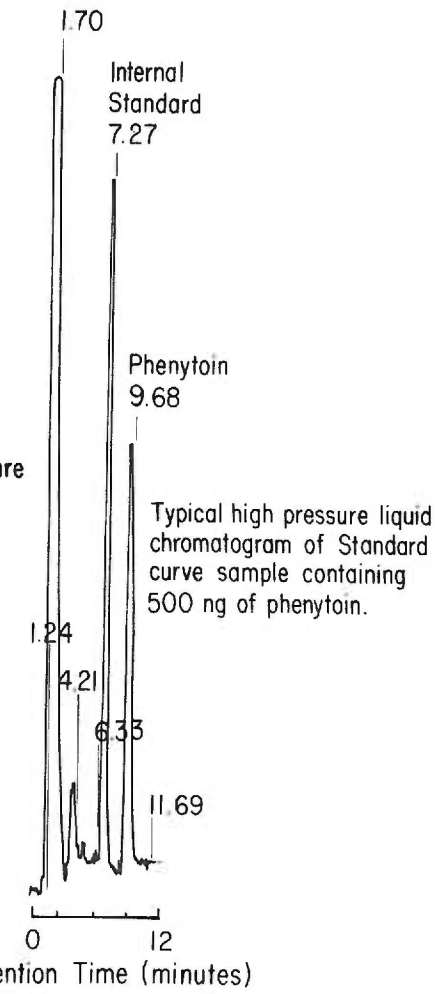

Figure 5 Typical high pressure liquid chromatogram of Standard curve sample containing 500 ng of phenytoin.

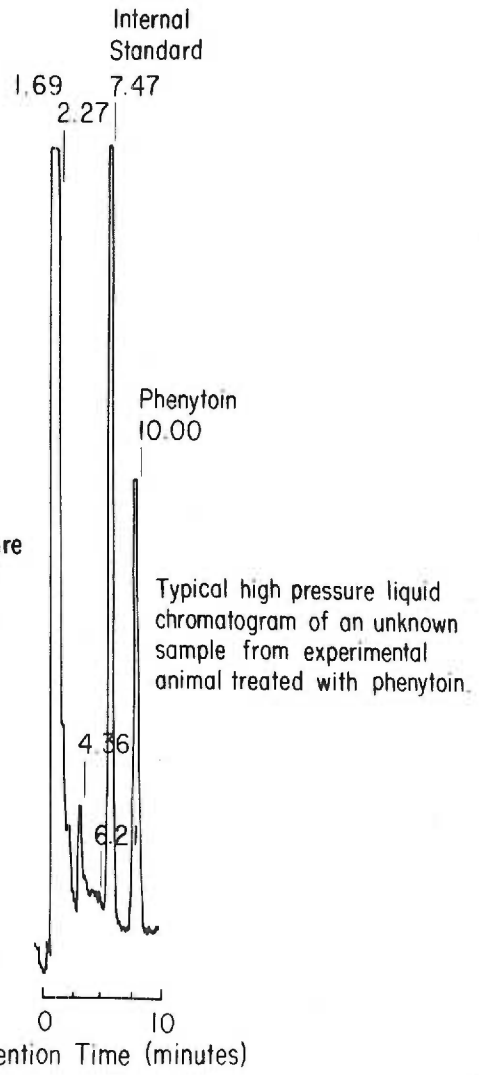

Figure 6 Typical high pressure liquid chromatogram of an unknown sample from experimental animal treated with phenytoin.

three genotypes were randomly assigned to one of four PTH treatment groups (0, 20, 40, and 60 mg/kg body weight). To define the role of the quaking gene (qk) in the etiology of the malformations, dams that were heterozygous (+/qk) for the mutant gene and homozygous non-quaking (+/+) dams were also included in the study.

To determine if genotypic differences exist between inbred mouse strains to their susceptibility to PHT induced teratogenicity, the inbred lines SWV and C_3H were included in this study. These animals have no neurological defects and were selected due to their excellent reproductive capabilities. They were treated in a similar manner as the C57BL/6J genotypes.

In the ethotoin study, only C57BL/6J (+/+) females were used and they were randomly assigned to one of four treatment groups (0, 100, 200, 300 mg/kg). The reasons for only treating C57BL/6J (+/+) were varied but essentially the study was limited to this group of animals since the criteria established for a valid animal model of the fetal hydantoin syndrome could not be satisfied using this drug. The second criterion established the need to control seizures in the experimental animals, and as shown in the seizure control study, there was no effective seizure control in the quaking mice even at the highest dosage. Therefore, in light of the poor reproduction rates of homozygous quaking (qk/qk) mice, the abundance of non-quaking (+/+) mice, and

the limited supply of ethotoin available, it was decided to only use the (+/+) dams for this study.

Route of Drug Administration

The PHT and ETH were administered orally as a salt suspension in the animals' drinking water. The water intake of each cage was checked daily to maintain the proper dosage. The drug therapy started at least two weeks prior to the first attempt at mating and was continued throughout gestation. Serum concentrations of the drugs were determined to ensure serum levels that were within the desired experimental range.

Matings

The matings were initiated by placing single drug naive C57BL/6J +/+ males in the cages of paired females of all three C57BL/6J genotypes and single males of SWV and C_3H into cages of paired females of their respective strains. The mice were allowed three hours to mate, during which time the drinking water was removed so as to prevent exposing the males to the drugs, which might thereby confound the results. At the end of the mating period the males were returned to their own cages and the females were examined. Twelve hours after the discovery of a vaginal plug was taken to indicate gestational day 1. The dams continued to be treated with PHT throughout gestation. On day 18, the mice were weighed, bled from the retroorbital sinus and killed by cervical dislocation.

Fetal Examination

Immediately after cervical dislocation, the maternal abdominal cavity was opened and the uterine contents removed. The location and position of all viable fetuses and resorption sites were noted. The fetuses were removed by severing the umbilical cords and were checked for limb or mouth movements. Those fetuses that failed to show any spontaneous or elicited movements, or that were pale in color were regarded as dead and included among the resorptions for the statistical analysis. The viable fetuses were weighed on the Sartorious top loading balance (Model 1106, Westbury, N.Y.) to one hundredth of a gram. With the aid of a magnifier-illuminator the fetuses were sexed and immediately examined for external malformations of the head, palate, trunk, limbs, and digits. Randomly, one-third of the fetuses were placed in 95% ETOH for alizarin red staining (Crary, 1962) and the remaining two-thirds placed in Bouin's solution. This latter group was examined for internal malformations using the free-hand razor blade technique of Wilson (1965) and its modification (Barrow and Taylor, 1969). Those fetuses that had been stained for skeletal examination were placed in Petri dishes filled with gelatin and examined under a dissecting microscope. The soft tissue sections also were examined with a dissecting microscope by placing the 1-2 mm cross section slices in 70% ETOH in white porcelain spot plates.

Statistical Analysis

The 0.05 level of significance was set for all statistical analysis. The mean differences between measurements were tested using a one-way analysis of variance (Sokal and Rohlf, 1969). Where differences were found to be significant a Student-Newman-Kuels range test was utilized to discern the source of the significance (Sokal and Rohlf, 1969). A Chi-square "goodness of fit" test was used as indicated for testing the sex ratio of viable fetuses and as part of the Zar method (1974) to compare correlation coefficients. All calculations including the descriptive statistics used in the seizure control study and throughout the test were performed on a Hewlitt-Packard Model 65 programmable calculator (Hewlitt-Packard, Corvallis, Ore.).

Photography

The photographs of the various skeletal and soft tissue sections were taken with a Nikon F2A camera (Nikon, Tokyo, Japan) through a Nikormat 80 mm enlarging lens on the end of the Nikon Pb-5 bellows.

RESULTS

Experiment I

Plasma Concentrations of PHT

Phenytoin was administered orally for a fifteen-day period to female mice of all three strains. Blood samples were drawn at five-day intervals and plasma PHT concentrations were determined. A final blood sample was drawn at the time of autopsy and plasma PHT levels were compared for the four measurements. No significant differences were found in PHT plasma levels in samples drawn at different times in any strains or genotypes (see Appendix D for Tables 1a-1o). The plasma PHT concentrations were found to increase significantly in all three strains with increasing amounts of drug administered, as shown in Table 4 (Appendix D, Tables 12, 18, 24, 30, 36). There were no significant differences in the plasma PHT concentrations among the SWV, C_3H, and C57BL/6J (+/+, +/qk, qk/qk) mouse strains at the 20 mg/kg, 40 mg/kg, or 60 mg/kg dosage level (Appendix D, Tables 2a, 2b, 2c). The plasma concentrations of phenytoin ranged from a mean of 2.69 µg/ml for C57BL/6J (+/qk) females given the 20 mg/kg dosage to 12.73 µg/ml for the C57Bl/6J (qk/qk) females given the 60 mg/kg dosage.

Daily Water Consumption

Because the phenytoin was administered in the dam's

TABLE 4. Effects of PHT treatments on water consumption, plasma PHT concentration, and maternal liver weight in pregnant female mice.

Strains (Genotypes)	PHT Treatment (mg/kg body wt.)	Daily Water Consumption (ml) Mean ± SEM	Plasma PHT Concentrations (μg/ml) Mean ± SEM	Maternal Liver Wt. per 100 gm. (gm) Mean ± SEM
SWV (N=10 litters for each treatment)	0	8.32 ± .46	0	5.49 ± .14
	20	8.21 ± .85	4.13 ± .51	6.20 ± .24
	40	8.22 ± .71	7.77 ± .58	6.13 ± .29
	60	7.72 ± .58	11.03 ± 1.48	6.63 ± .19
C_3H (N=10 litters for each treatment)	0	9.09 ± .51	0	5.13 ± .13
	20	8.95 ± .56	3.49 ± .42	5.57 ± .20
	40	8.34 ± .47	7.13 ± .55	6.01 ± .22
	60	7.83 ± .65	9.54 ± .42	5.93 ± .14
C57 (+/+) (N=5 litters for each treatment)	0	5.76 ± .45	0	4.69 ± .22
	20	5.51 ± .51	3.22 ± .17	5.30 ± .15
	40	6.03 ± .46	7.05 ± .81	5.49 ± .22
	60	5.07 ± .74	12.10 ± 1.04	5.45 ± .22
C57 (+/qk) (N=5 litters for each treatment)	0	6.40 ± .28	0	4.67 ± .17
	20	5.64 ± .62	2.69 ± .23	5.03 ± .14
	40	6.35 ± .45	5.77 ± .70	4.97 ± .13
	60	5.52 ± .58	10.96 ± .90	4.99 ± .28
C57 (qk/qk) (N=5 litters for each treatment)	0	5.60 ± .40	0	4.27 ± .18
	20	5.88 ± .54	4.12 ± .54	5.29 ± .14
	40	5.18 ± .25	7.40 ± .60	5.68 ± .13
	60	5.13 ± .61	12.43 ± 1.90	6.29 ± .28

drinking water it was important to monitor day to day fluctuations in water consumption (Table 4). There were no significant differences in the amount of water consumed daily by the mice as the dosage level increased, regardless of their strain (Appendix D, Tables 13, 19, 25, 31, 37). There was, however, a significant difference in the amount of water consumed daily among the strains at all four dosage levels (Appendix D, Tables 6a, 6b, 6c, and 6d). A Student-Newman-Keuls procedure indicated that the water consumption patterns of the SWV and C_3H differed significantly from the C57 genotypes, which themselves overlapped indicating equality in their daily water intake (Sokal and Rohlf, 1969).

Maternal Liver Weights

Maternal liver weights at the time of autopsy are presented in Table 4 as grams per 100 grams of body weight. This was done in order to standardize differences among the strains in maternal weights, thereby allowing direct comparisons of the three strains. With the exception of the C57BL/6J (+/qk) genotype, all mouse strains showed significant differences in maternal liver weights with increasing amounts of PHT administered (Appendix D, Tables 11, 17, 23, 29, 35). These weights were also significantly different among the mouse strains at all PHT dosage levels (Appendix D, Tables 4a, 4b, 4c, and 4d). Student-Newman-Keuls tests (Sokal and Rohlf, 1969) were performed on maternal liver weights at dosage level 1 (0 mg/kg) and determined that the

significant difference between strains was due to large differences between the SWV and all C57BL/6J genotypes. At dosage level 2 (20 mg/kg) the SWV livers were heavier than the C_3H and all of the C57BL/6J genotypes. At the 40 mg/kg dosage SWV differed significantly from the C57BL/6J genotypes and partially overlapped the C_3H maternal liver weights. At the highest drug dosage, the Student-Newman-Keuls procedure determined that all strains and genotypes differed from those weights ascribed to dams of C57BL/6J (+/qk) genotype.

Implantation and Resorption

The effects of PHT treatment on implantation and resorption sites are shown in Table 5. The figure for number of implantation sites is the sum of live births, still births, and resorption sites. Average implantations per litter did not differ significantly within any of the strains (Appendix D, Tables 8, 14, 20, 26, 32); however, there were significantly more implants per litter in the SWV and the C_3H strains as compared to any of the C57BL/6J genotypes as indicated by a Student-Newman-Keuls test and this was true at all PHT dosage levels (Appendix D, Tables 5a, 5b, 5c, and 5d). Similarly, the average number of resorptions per litter did not differ significantly within the strains as the dosage levels increased, with the sole exception of the C57BL/6J (+/qk) females (Appendix D, Tables 9, 15, 21, 27, 33). Nor were there significant differences

TABLE 5. Effect of PHT treatment on implantation and resorption.

Strain (Genotype)	PHT Treatment (mg/kg body wt.)	Number of Implants	Average Implants (Mean ± SEM)	Number of Resorptions (Mean ± SEM)	Resorption (%)
SWV (N=10 litters for each treatment)	0	121	12.10 ± .64	1.30 ± .33	11
	20	119	11.90 ± 1.36	1.80 ± .57	15
	40	118	11.80 ± .36	2.10 ± .94	18
	60	115	11.50 ± 1.11	1.60 ± .65	14
C_3H (N=10 litters for each treatment)	0	102	10.20 ± .57	0.40 ± .22	4
	20	102	10.20 ± .44	1.30 ± .60	13
	40	92	9.20 ± .61	1.80 ± .80	20
	60	94	9.40 ± .40	1.40 ± .97	15
C57 (+/+) (N=5 litters for each treatment)	0	44	8.80 ± .49	0.20 ± .20	2
	20	36	7.20 ± .73	0.60 ± .40	8
	40	39	7.80 ± .49	1.20 ± .58	15
	60	38	7.60 ± .81	1.60 ± .93	21
C57 (+/qk) (N=5 litters for each treatment)	0	44	8.80 ± .37	0.60 ± .40	7
	20	40	8.00 ± .45	0.20 ± .20	2
	40	37	7.40 ± .60	1.00 ± .45	14
	60	44	8.80 ± .49	2.40 ± .68	25
C57 (qk/qk) (N=5 litters for each treatment)	0	39	7.80 ± .58	0.60 ± .40	8
	20	37	7.40 ± .60	0.60 ± .40	8
	40	40	8.00 ± .32	0.60 ± .40	5
	60	32	6.40 ± .40	1.20 ± .80	19

among the mouse strains in terms of mean number of resorptions (Appendix D, Tables 7a, 7b, 7c, and 7d). By using the method of Zar (1974) the correlation coefficients of resorptions per strain at a given plasma concentration were tested and found to be equal ($X^2_{0.05,\ 4} = 9.488$, $p = 0.975$), thereby allowing that data to be consolidated and examined irrespective of strain. By combining data from all strains, Figure 7 illustrates the statistically significant increase in the percentage of resorption with increasing PHT dosage (Appendix D, Table 7e).

Fetal Measurements

The results of observations on the number of live births, the sex, and the number of abnormalities (expressed as percentages) are shown in Table 6; this table also includes measurements of fetal weights, expressed as averages. The sex ratios were not significantly different from the expected 1:1 ratio for all of the litters examined ($X^2 = 0.24$ and $0.75 < P < 0.50$). Fetal weights were significantly decreased within all strains as the dosage of phenytoin increased (Appendix D, Tables 10, 16, 22, 28, 34). The Student-Newman-Keuls mathematical procedure (Sokal and Rohlf, 1969) confirmed that, regardless of the treatment, SWV fetuses weighed significantly less than those of the C_3H strain with the C57Bl/6J genotypes being intermediate. There were genotypic differences in mean fetal weights among the C57BL/6J genotypes (Appendix D, Tables 38a, 38b, 38c, and 38d). A Student-Newman-Keuls test showed that the (+/gk)

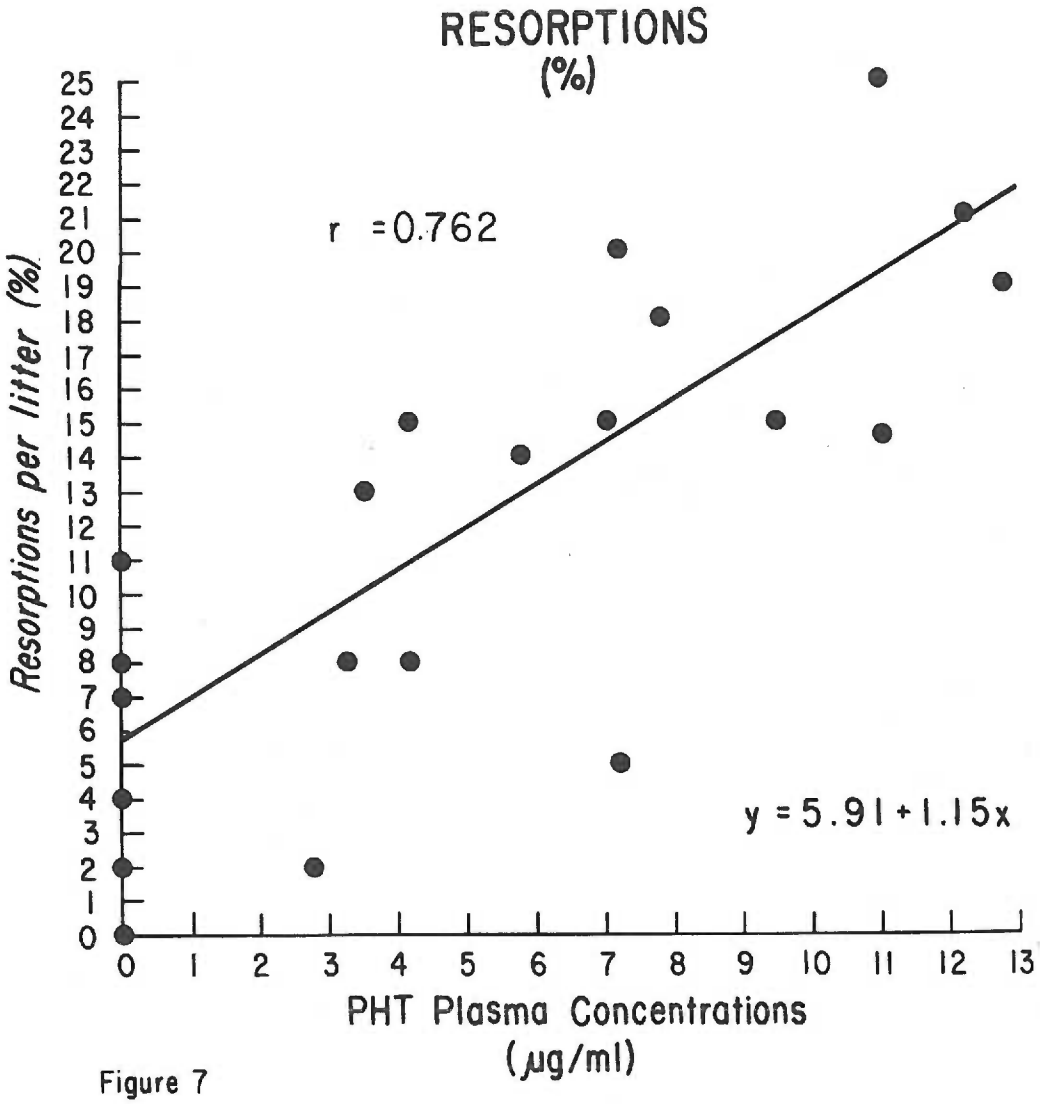
RESORPTIONS
(%)
r =0.762
y = 5.91 + 1.15x
Resorptions per litter (%)
PHT Plasma Concentrations
(µg/ml)
0 1 2 3 4 5 6 7 8 9 10 11 12 13
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

Figure 7

TABLE 6. Effect of PHT treatment on live births, sex, fetal weights, and abnormalities.

Strain (Genotype)	PHT Treatment (mg/kg body wt.)	Number of Live Births	Sex F	Sex M	Fetal Weights (gm.) (Mean ± SEM)	Abnormal (%)
SWV (N=10 litters for each treatment)	0	108	58	50	0.81 ± .01	6
	20	101	49	52	0.73 ± .01	41
	40	97	45	52	0.68 ± .02	53
	60	99	50	49	0.64 ± .01	85
C_3H (N=10 litters for each treatment)	0	98	50	48	0.99 ± .01	4
	20	89	43	46	0.86 ± .01	40
	40	75	40	35	0.84 ± .01	40
	60	80	46	34	0.82 ± .01	56
C57 (+/+) (N=5 litters for each treatment)	0	43	20	23	0.98 ± .02	2
	20	33	15	18	0.80 ± .02	42
	40	33	15	18	0.69 ± .02	73
	60	30	17	13	0.73 ± .02	70
C57 (+/qk) (N=5 litters for each treatment)	0	41	19	22	1.03 ± .02	5
	20	39	17	22	0.77 ± .02	44
	40	32	15	17	0.85 ± .03	44
	60	32	17	15	0.72 ± .01	78
C57 (qk/qk) (N=5 litters for each treatment)	0	36	21	15	1.05 ± .03	0
	20	34	17	17	0.85 ± .02	29
	40	38	21	17	0.72 ± .02	61
	60	26	17	9	0.75 ± .02	77

differed significantly from the homozygous (qk/qk) quaking dam category at dosage level 2, while at dosage level 3 the heterozygous (+/qk) dams' mean fetal weight differed significantly from those of the (+/+) mothers. There were significant differences among the three strains for average fetal weights at all of the dosage levels (Appendix D, Tables 3a, 3b, 3c, and 3d).

Fetal Anomalies

The incidence of fetuses born with one or more skeletal or soft tissue abnormalities increased with increasing dosage of PHT (Table 6). Again, the Zar method (1974) indicated there were no significant differences in the percent abnormal at a given plasma PHT concentration among the three strains ($X^2_{0.05,\ 4} = 9.4880$, $P = 0.975$). This relationship, which was true for all strains, is graphically displayed in Figure 8. It is noteworthy that the homozygous quaking (qk/qk) dams who did not receive PHT produced normal offspring. The most commonly observed skeletal abnormalities were an incomplete or apparently missing occipital bone (Figure 9) and incomplete ossification of the distal phalanges (Figure 10). These kinds of abnormalities were observed in all strains subjected to the lowest phenytoin dosage (Table 7). Furthermore, with greater dosages of PHT sternebrae were sometimes missing or misaligned and vertebral centra were sometimes shaped like triangles. A slight reduction in the size and extent of ossification of the

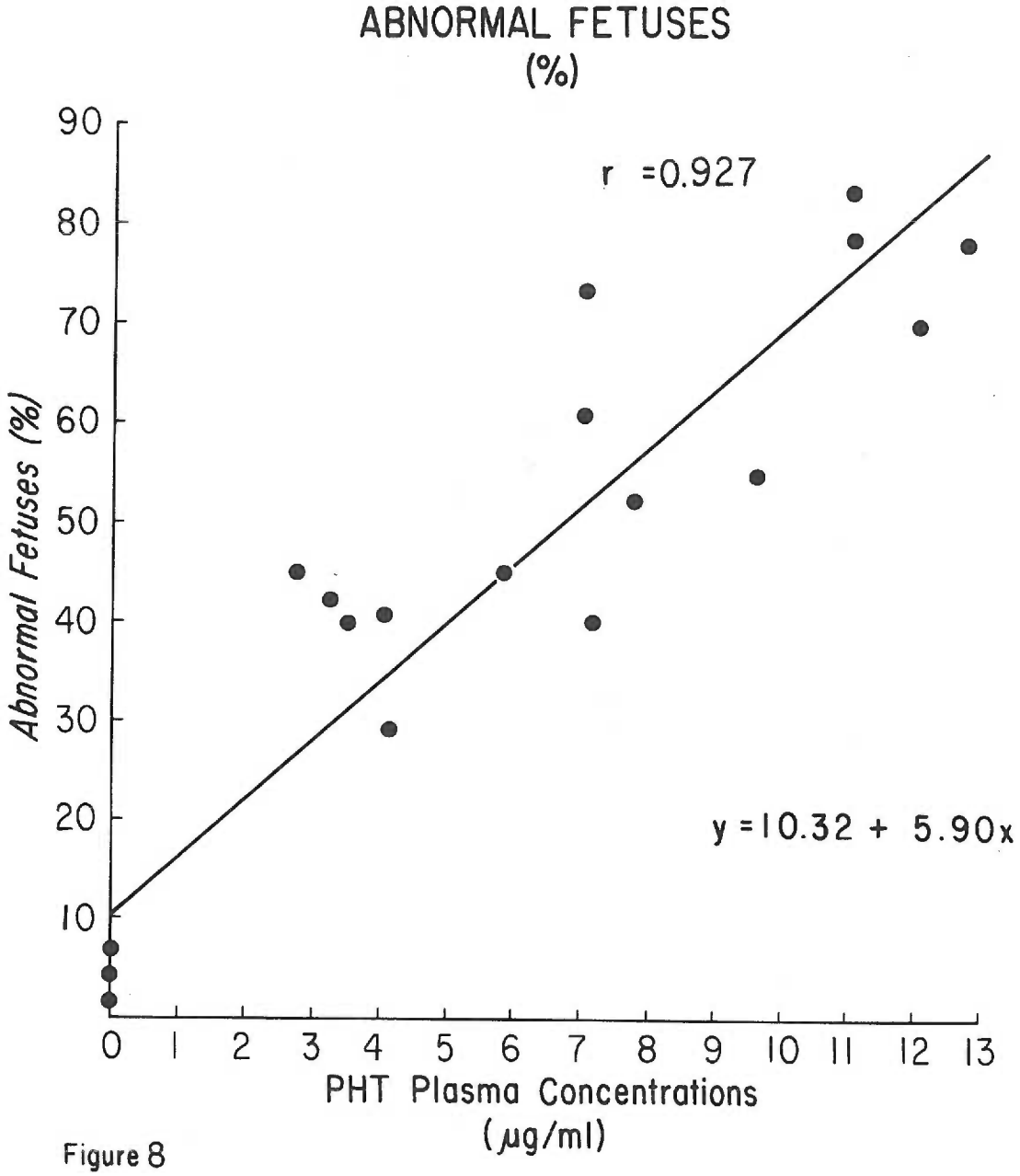
ABNORMAL FETUSES
(%)
r =0.927
y = 10.32 + 5.90x
Abnormal Fetuses (%)
90
80
70
60
50
40
30
20
10
0 1 2 3 4 5 6 7 8 9 10 11 12 13
PHT Plasma Concentrations
(µg/ml)

Figure 8

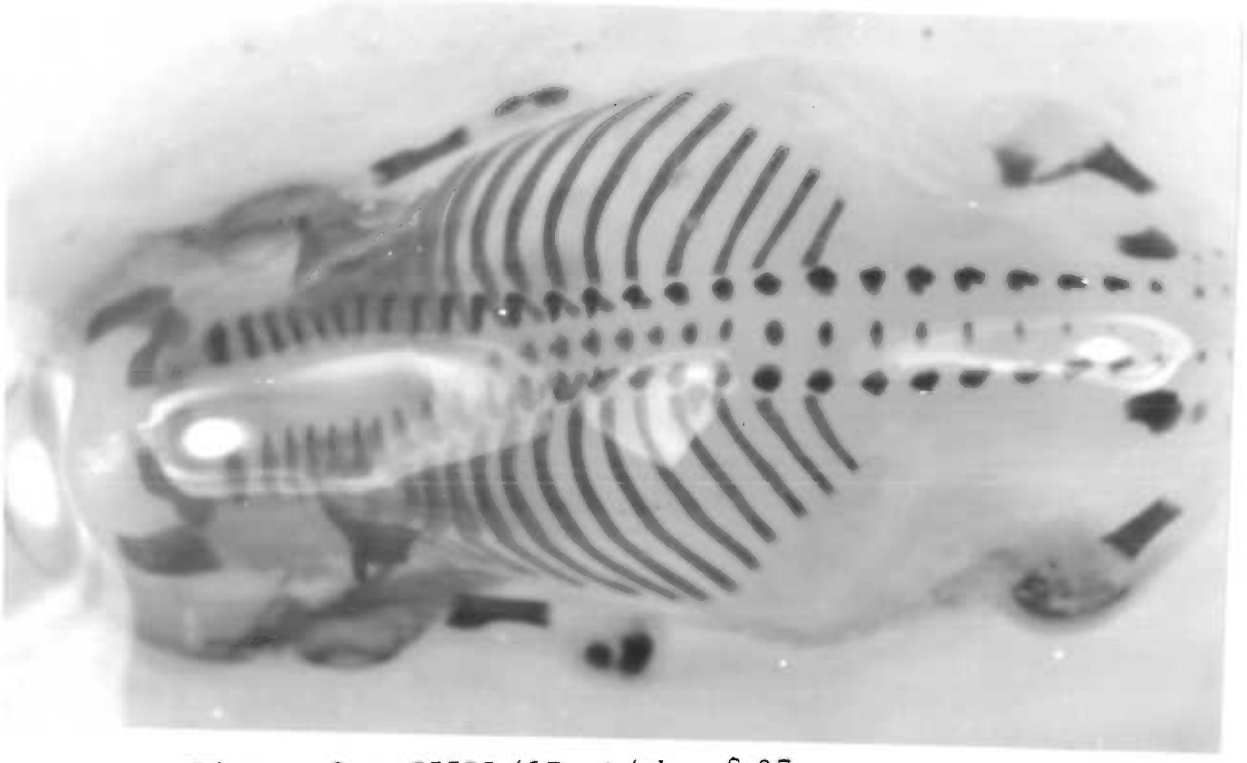

Figure 9. C57BL/6J +/qk ♀ 07
20 mg/kg PHT
Delayed ossification of supraocciptal bone
Note triangular shaped vertebral centra
(10 X)

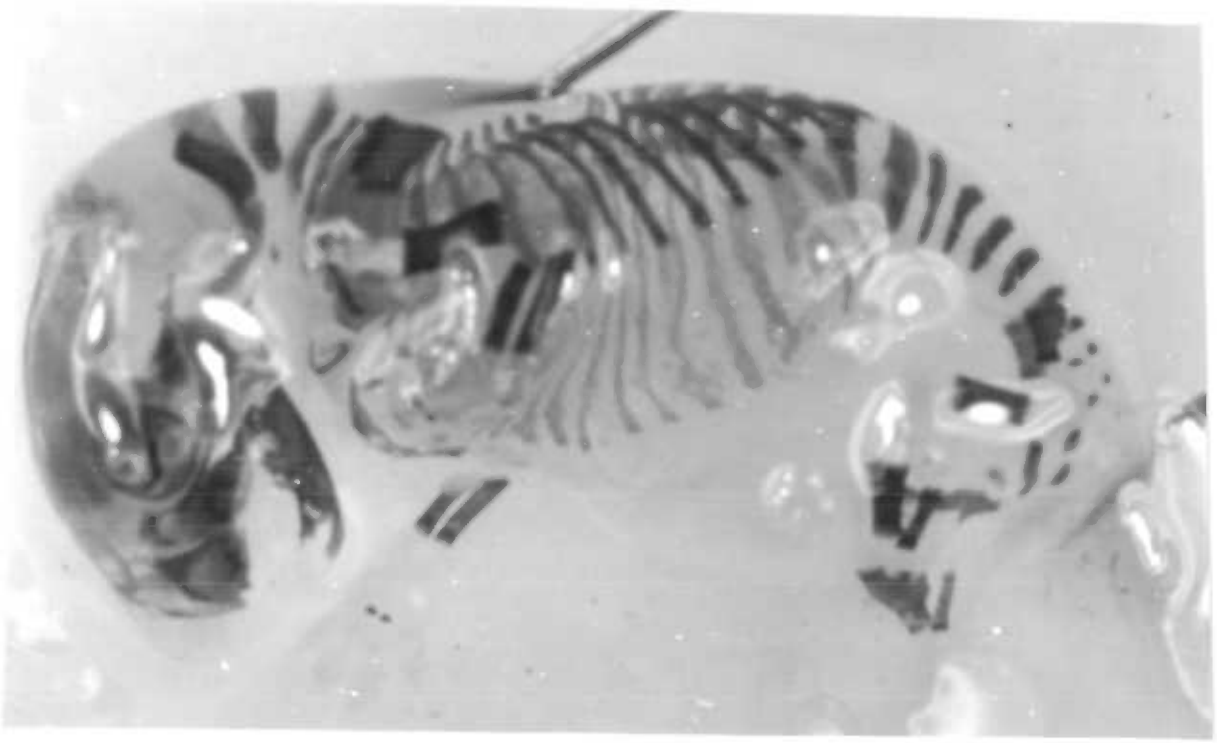

Figure 10. SWV ♀ 27
40 mg/kg PHT
Missing ossification of phalanges and
metacarpals and metatarsals. (10 X)

TABLE 7. Types and frequencies of skeletal anomalies.

Strain (Genotype)	PHT Treatment (mg/kg body wt.)	Fetuses Examined	Occipital Bone	Sternebrae	Distal Phalanges	Vertebral Centra	Facial Bones	Misc.*	Abnormal (%)
SWV (N=10	0	31	0	1	1	1	0	0	3
litters	20	31	1	7	6	2	1	0	23
for each	40	34	4	13	12	1	2	0	38
treatment)	60	32	12	17	21	10	6	0	69
C_3H (N=10	0	34	3	2	3	0	0	0	9
litters	20	30	13	1	12	0	2	0	43
for each	40	24	14	2	10	3	3	0	42
treatment)	60	27	16	2	16	2	3	0	59
C57 (+/+)	0	11	0	0	0	0	0	0	0
(N=5 litters	20	11	6	4	6	2	1	0	54
for each	40	10	9	4	9	3	5	3	90
treatment)	60	9	6	1	6	0	3	0	67
C57 (+/qk)	0	13	0	0	0	0	0	0	0
(N=5 litters	20	12	5	2	1	1	2	0	42
for each	40	10	3	0	1	2	0	0	30
treatment)	60	10	8	3	8	1	4	1	80
C57 (qk/qk)	0	13	0	0	0	0	0	0	0
(N=5 litters	20	11	2	0	2	0	2	0	27
for each	40	13	10	2	10	3	2	0	77
treatment)	60	8	7	2	4	2	3	0	88

*Miscellaneous anomalies include: missing pubis and ischium; crooked ribs.

TABLE 8. Types and frequencies of soft tissue anomalies.

Strain (Genotype)	PHT Treatment (mg/kg body wt.)	Fetuses Examined	Dilated Cerebral Ventricles	Heart & Great Vessels	Ocular	Renal	Cleft Palate	Hypo. Digit	Misc.*	Abn. (%)
SWV (N=10	0	74	3	2	0	0	0	0	0	7
litters	20	70	19	18	0	9	2	2	0	49
for each	40	62	25	9	6	15	4	9	1	61
treatment)	60	65	44	19	11	36	2	34	1	92
C_3H (N=10	0	64	1	0	0	0	0	0	0	2
litters	20	59	12	5	0	10	0	4	0	39
for each	40	51	13	4	1	8	0	4	2	39
treatment)	60	53	17	8	0	15	1	9	0	55
C57 (+/+)	0	30	1	0	0	0	0	0	0	3
(N=5 litters	20	22	0	4	0	5	0	0	0	36
for each	40	23	7	6	5	12	0	6	2	65
treatment)	60	21	5	8	1	5	0	7	0	71
C57 (+/qk)	0	28	0	2	0	0	0	0	0	7
(N=5 litters	20	27	5	7	0	3	0	0	1	44
for each	40	22	1	7	4	3	0	2	1	50
treatment)	60	22	7	4	4	9	0	8	1	77
C57 (qk/qk)	0	23	0	0	0	0	0	0	0	0
(N=5 litters	20	23	1	6	0	2	0	0	0	30
for each	40	25	9	5	2	8	1	7	0	52
treatment)	60	18	6	5	7	3	0	6	1	72

*Miscellaneous anomalies include: subdural hematoma, malpositioned testes, hypoplastic adrenals, gastroschisis, and tracheoesophageal fistula.

facial bones (usually the nasal, frontal, maxillary, premaxillary, and mandibular) was occasionally noticed in treated fetuses of all strains, but never in the offspring of any untreated animals. The remaining skeletal defects were observed in two fetuses, which displayed two instances of incomplete ossification of the pubis and ischium and one instance of fusion and misalignment of the ribs.

The results of the soft tissue examinations are seen in Table 8. One of the two most common kinds of defects involved the cerebral ventricles, which were either dilated or immaturely developed (Figure 11). The other most common kind involved the heart and great vessels. Specific defects of the latter category were as follows: interventricular septal defects (Figure 12), interatrial septal defects, hypoplastic atria (Figure 13), tricuspid atresia, aortic valvular atresia, pulmonary stenosis, persistent truncus arteriosus, preductal coarctation of the aorta, and transposition of the great vessels. These defects were observed in fetuses belonging to the lowest PHT dosage level, and the percentage of these defects, both ventricular and cardiac, increased with increasing PHT dosage. The ocular abnormalities included anophthalmia (Figure 14), microophthalmia, and lidgaps (Figure 15). The ocular anomalies were more often observed in SWV or C57BL/6J fetuses, as lidgaps could not be evaluated in the C_3H mice, who are genetically open-lidded at birth. The renal defects were either hydronephrosis

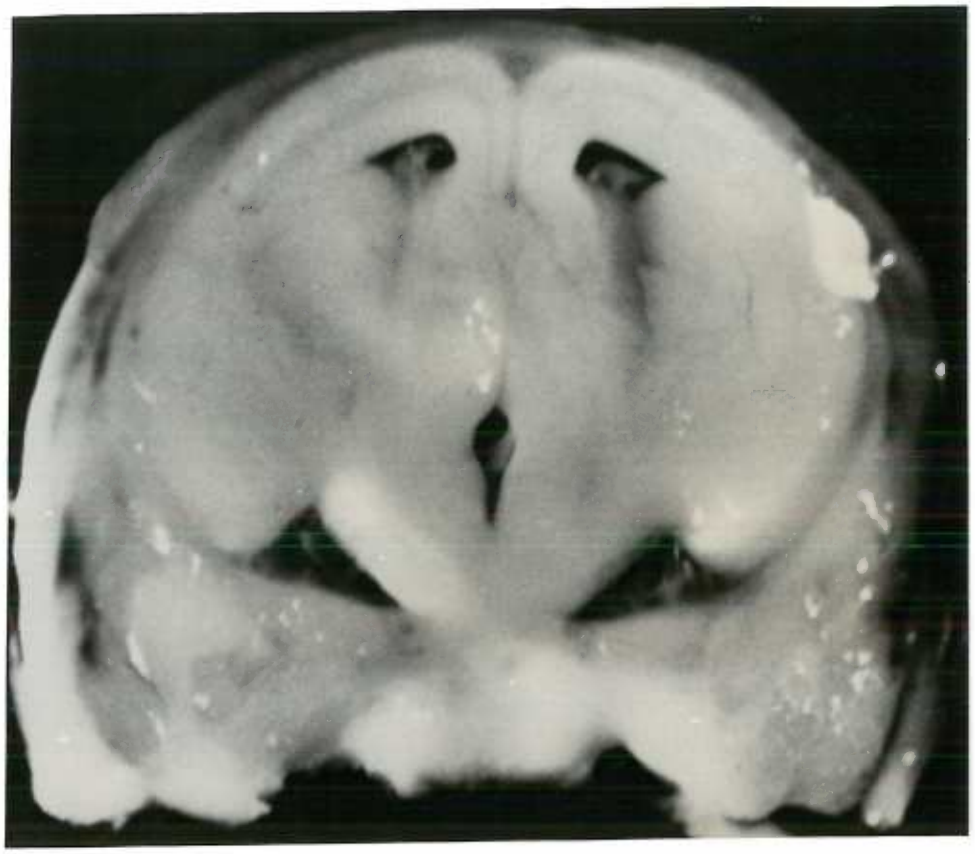

Figure 11. C57BL/6J +/+ ♀ 72
60 mg/kg PHT
Dilated lateral and third ventricles
(12 X)

Figure 12. C_3H ♀ 61
20 mg/kg PHT
Interventricular septal defect
(12 X)

Figure 13. C57BL/6J +/+ ♀ 72
60 mg/kg PHT
Hypoplastic atria

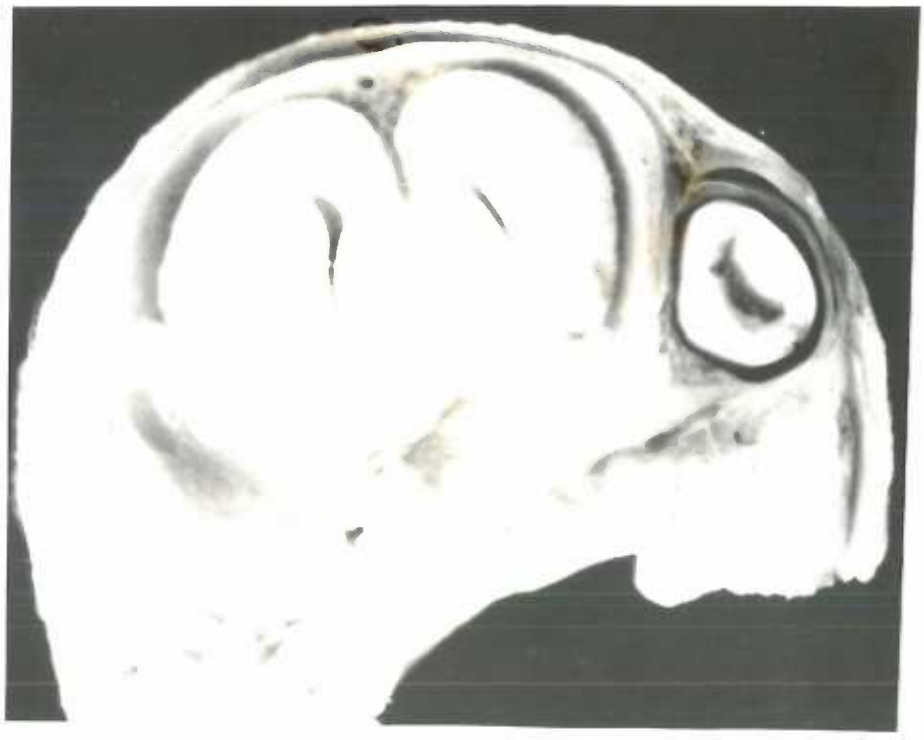

Figure 14. C57BL/6J +/qk ♀ 21
40 mg/kg PHT
Unilateral anophthalmia

Figure 15. C57BL/6J qk/qk ♀ 80
40 mg/kg PHT
Lidgap

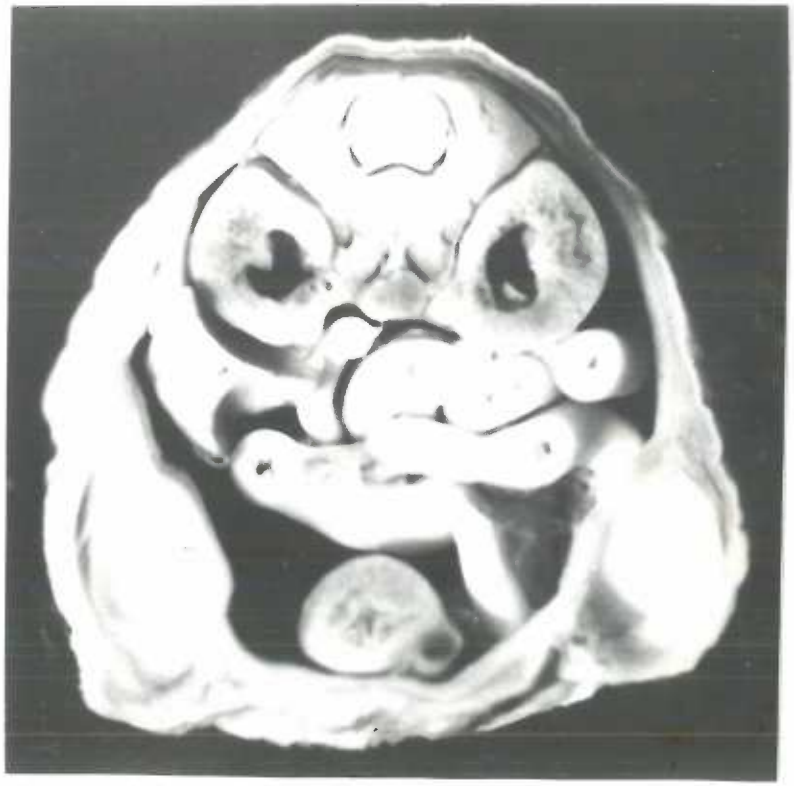

Figure 16. C_3H ♀ 14
60 mg/kg PHT
Bilateral hydronephrosis

(Figure 16) or hypoplastic kidneys, except for a single case of duplex ureters. Digital hypoplasia was most commonly observed at the intermediate and high dosages in all three strains. Cleft palates (Figure 17) were almost entirely restricted to SWV fetuses, with only one instance each in the C_3H and C57BL/6J strains. Other abnormalities observed included malpositioned testes, subdural hematoma, tracheo-esophogeal fistula, missing or hypoplastic adrenals, and gastroschisis (Figure 18).

To summarize the results of Experiment 1, maternal plasma PHT concentrations and liver weights, and the percent of abnormal offspring increased with increasing phenytoin dosage, while the average weight per fetus decreased. Further, significant differences were noted in the daily water consumption and in the number of implantation sites among the mouse strains on a given dosage level. The percent of resorptions per litter showed a gradual trend towards elevation at the higher drug dosages. However, this trend was not statistically significant.

EXPERIMENT 2

Seizure Control

The efficacy of orally administering ethotoin to homozygous quaking (qk/qk) mice was examined in order to meet the second condition outlined on the idealized criteria for an animal model for the fetal hydantoin syndrome. This condition requires that the seizures be controlled or

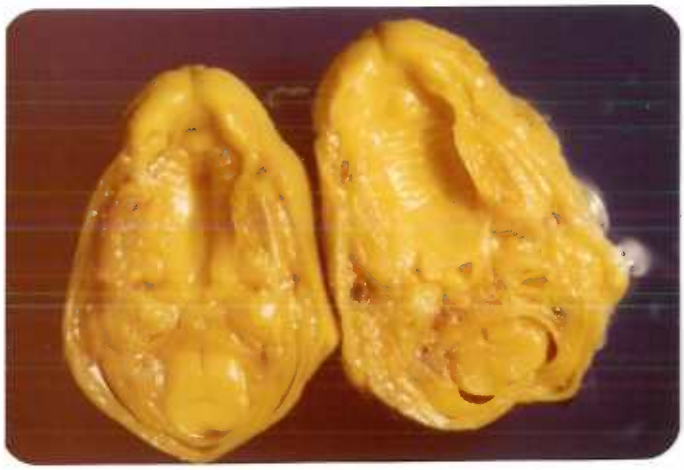

Figure 17. C57BL/6J qk/qk ♀ 80
40 mg/kg PHT
Cleft palate (on left) and control palate (rt)

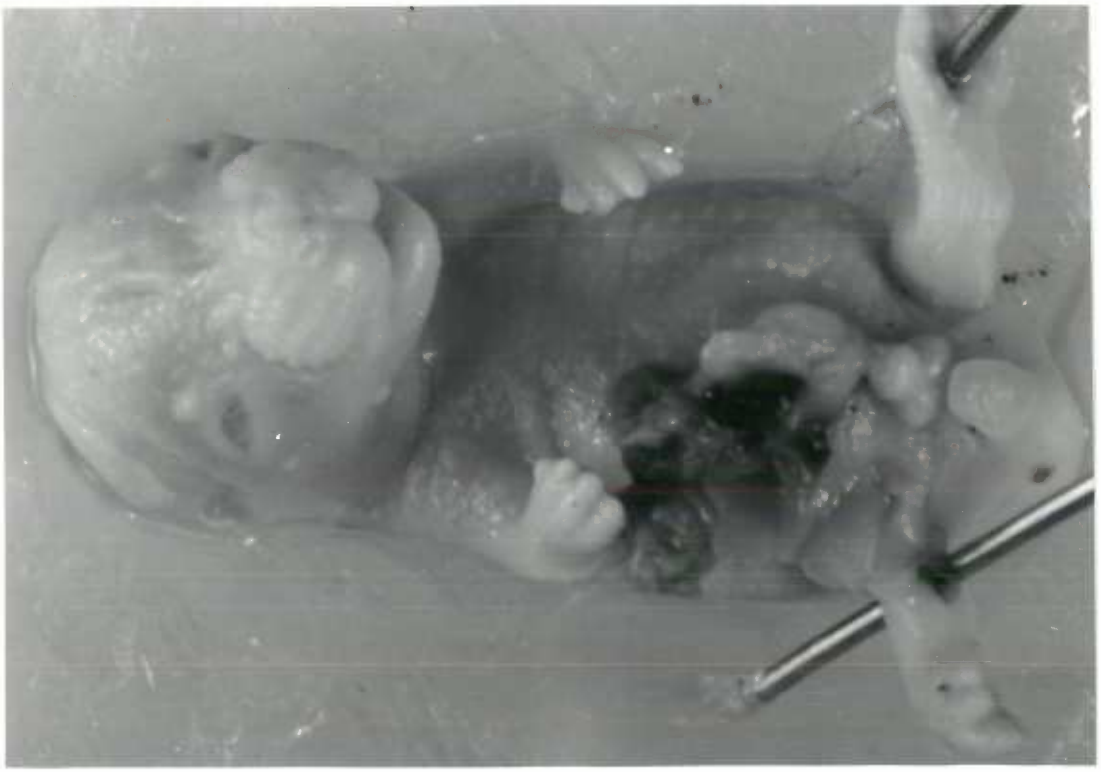

Figure 18. SWV ♀ 05
40 mg/kg PHT
Gastroschisis and bilateral lidgaps

eliminated. As seen in Table 9, the average frequency of nearly two seizures per mouse day was not significantly altered even by the administration of the highest dosage, 300 mg/kg body weight (Appendix D, Table 44).

Plasma ETH Concentrations

Because there is no consensus as to the optimal therapeutic plasma concentration of ethotoin in human clinical studies, no attempt was made to develop an assay of plasma ETH concentrations in the mice.

Maternal Effects

The amount of water consumed daily by the dams was not significantly decreased by increasing the concentration of ethotoin in the drinking water (Appendix D, Table 40). Similarly, maternal liver weights, as shown in Table 10, were not significantly different at the various dosage levels (Appendix D, Table 39).

Implantation and Resorptions

The effects of ETH treatment on implantation and resorption are shown in Table 11. As in the previous experiment, the figure for number of implantation sites is the sum of live births, still births, and resorption sites. While the mean number of implants declined as the dosage level increased, it was not statistically significant (Appendix D, Table 41). Similarly, there were no significant differences in the number of resorptions per litter (Appendix D, Table 42), although at the intermediate dosage the actual percentage

Table 9. Efficacy of ETH administered orally to quaking (qk/qk) mice. (N=12 females for each treatment)

ETH Treatment (mg/kg body wt.)	Seizures* (Mean ± SEM)
0	1.93 ± .37
100	2.30 ± .22
200	1.87 ± .23
300	1.50 ± .30

*Expressed as seizures per mouse day.

TABLE 10. Effect of ETH treatment on water intake and maternal liver weights in pregnant C57 (+/+) females.

Number of Females	ETH Treatment (mg/kg body wt.)	Daily Water Intake (ml)	Liver Wt. Per 100 Gm. (Mean ± SEM) (gm.)
5	0	5.76 ± .45	4.69 ± .22
5	100	5.60 ± .62	4.68 ± .21
5	200	5.88 ± .50	4.99 ± .29
5	300	5.52 ± .48	4.69 ± .30

TABLE 11. Effect of ETH treatment on implantation and resorption in C57 (+/+) females.

Number of Females	ETH Treatment (mg/kg body wt.)	Number of Implants	Average Implants (Mean ± SEM)	Average Resorptions (Mean ± SEM)	Resorptions (%)
5	0	44	8.80 ± .49	0.20 ± .20	2
5	100	43	8.60 ± .40	0.60 ± .24	7
5	200	37	7.40 ± .87	2.60 ± 1.25*	35
5	300	31	6.20 ± 1.20	0.80 ± .80	13

* NOTE: 2 dams completely resorbed.

of resorbed fetuses reached 35% (Table 11), indicating that resorptions tended to increase with an increase in dosage of ETH.

Fetal Measurements

The results of observations on the number of live births, the sex, and the number of abnormal fetuses (expressed as percentages) are recorded in Table 12. This table also includes measurements of fetal weights expressed as averages. The sex ratios were not significantly different from the expected 1:1 ratio for all of the litters ($X^2 = 0.48$ and $0.50 \leq P \leq 0.25$). The fetal weights were significantly decreased as the dosage level of ETH was increased (Appendix D, Table 43), and the percentage of abnormal offspring increased with increasing ETH dosage.

Fetal Anomalies

The incidence of fetuses born with either skeletal (see Table 13) or soft tissue abnormalities (see Table 14) increased with increasing dosages of ETH. The most commonly observed skeletal abnormality was a delay in the ossification of or apparent absence of the distal phalanges and a delay in the ossification of the mid-facial bones. Triangular shaping of vertebral centra was observed only in fetuses belonging to the highest dosage category. The predominant soft tissue defect was hypoplastic atria of the heart. The ocular defects consisted only of lidgaps (Figure 19) and these were seen in fetuses belonging to the highest dosage

TABLE 12. Effect of ETH treatment on live births, sex, fetal weights, and fetal abnormalities in C57 (+/+) females. (N=5 litters for each treatment)

ETH Treatment (mg/kg body wt.)	Live Births	Sex F	Sex M	Fetal Wt. (gm) (Mean ± SEM)	Abnormal Fetuses (%)
0	43	20	23	0.98 ± .02	2
100	40	18	22	0.82 ± .03	40
200	24	10	14	0.84 ± .03	58
300	27	15	12	0.75 ± .03	52

TABLE 13. Types and frequencies of skeletal abnormalities in fetuses of C57 (+/+) females treated with ethotoin. (N=5 litters for each treatment)

ETH Treatment (mg/kg body wt.)	Fetuses Examined	Occipital Bone	Sternebrae	Distal Phalanges	Vertebral Centra	Facial Bones	Abnormal (%)
0	11	0	0	0	0	0	0
100	12	5	0	0	0	2	42
200	9	6	0	2	0	2	67
300	9	8	2	6	3	0	89

TABLE 14. Types and frequencies of soft tissue anomalies in fetuses of C57 (+/+) females treated with ethotoin. (N=5 litters for each treatment)

ETH Treatment (mg/kg body wt.)	Fetuses Examined	Dilated Cerebral Ventricles	Heart & Great Vessels	Ocular	Renal	Abnormal (%)
0	30	1	0	0	0	3
100	28	0	9	0	1	39
200	15	3	3	0	2	53
300	18	3	2	0	1	34

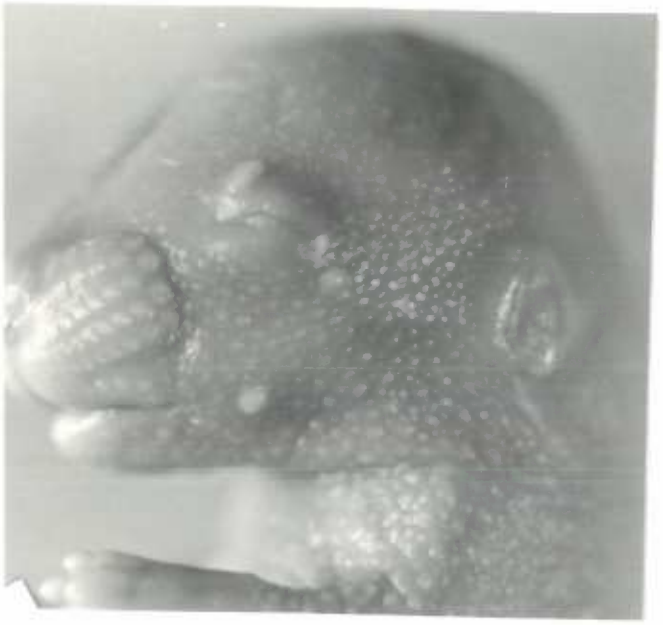

Figure 19. C57BL/6J +/+ ♀12
300 mg/kg ETH
Lidgap

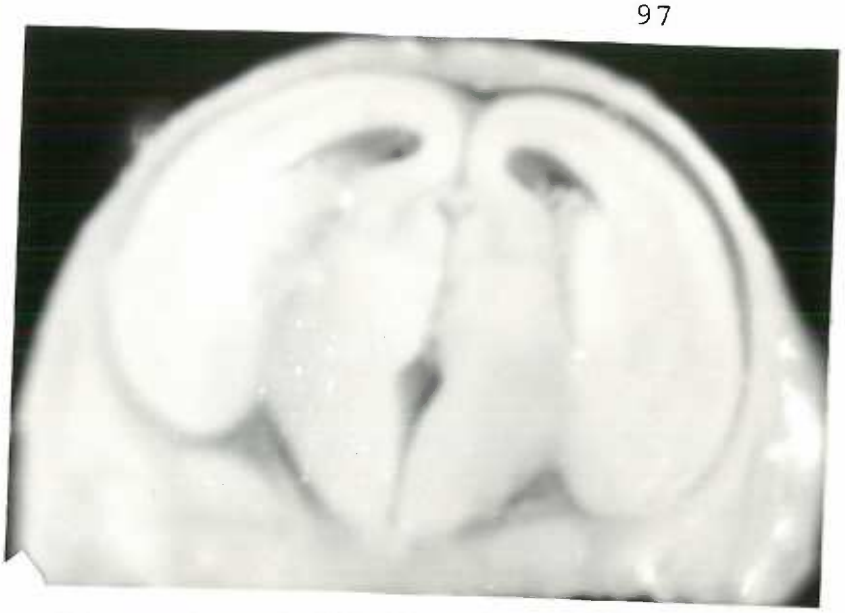

Figure 21. C57BL/6J +/+ ♀17
200 mg/kg ETH
Dilated lateral
Ventricles

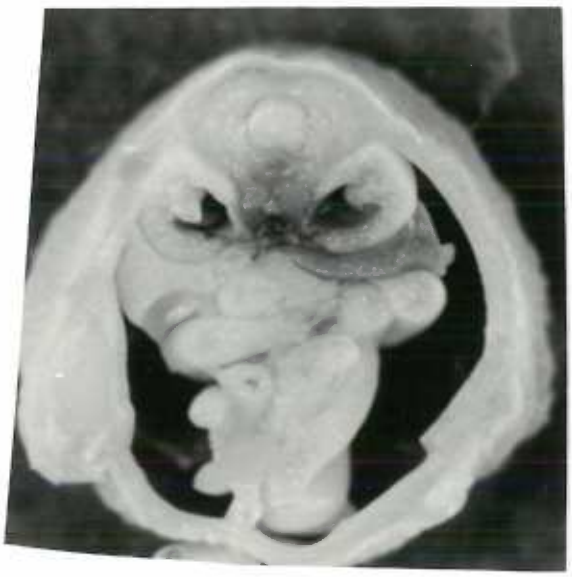

Figure 20. C57BL/6J +/+ ♀06
200 mg/kg ETH
Bilateral hydronephrosis

level. Dilation of cerebral ventricle (Figure 20) was relatively common, but was not as severe as that seen in the offspring of animals treated with phenytoin. The renal anomalies consisted of either bilateral or unilateral (right) hydronephrosis (Figure 21).

In summary, the percent of resorptions and percent of abnormal offspring increased with increasing dosages of ethotoin. Furthermore, the average weight per fetus decreased as ETH dosage increased. Water consumption, maternal liver weight, and the average number of implantation sites did not change significantly with increases in ETH dosage.

DISCUSSION

The most important goal of the present research was to determine whether or not the reported increase in congenital malformations observed in the offspring of epileptic women was the result of maternal epilepsy or of anticonvulsant drug therapy, or both. The clinical data collected over the past seventeen years had suggested a causal relationship between maternal use of anticonvulsant drugs and birth defects. Of the many anticonvulsant drugs commercially available, phenytoin (Dilantin®) has been implicated most often as the teratogenic agent (Speidel and Meadow, 1974; Janz, 1975) so this drug was utilized in the present study.

The results of the present research indicate that phenytoin, and not epilepsy, is causally related to birth defects. Seizure disorders, which are spontaneous in mice homozygous for the quaking gene (qk/qk), were ameliorated by orally administered phenytoin, but this drug therapy produced in these animals a striking increase in the number of defective offspring. Furthermore, heavier dosages of phenytoin produced higher percentages of defective offspring. On the other hand, the pregnancy outcomes of the quaking dams were not different from the pregnancy outcomes of the non-neurologically impaired dams.

The conclusions drawn from experimentation on qk/qk

mice are identical with those on +/+ and +/gk mice of the same strain. As seen in Table 7, the dose-response curve was observed in two other genetically distinct inbred mouse strains, and there seems to be no genetic basis to the etiology of the malformations. Rather, the malformations clearly result from maternal hydantoin consumption during pregnancy.

The failure to find a strain difference in susceptibility to the syndrome was perhaps one of the most interesting findings to come out of this research. In the few compounds that have been studied using multiple inbred mouse strains, namely cortisone, 6-aminonicotinamide (6-AN), and ethanol, there have always been differences in drug tolerance between the strains (Biddle and Fraser, 1976; Biddle, 1977, 1978; Chernoff, 1977). Of these examples, cortisone is the best known. In 1950, it was shown to produce cleft palates in mice (Baxter and Fraser, 1950) and in 1954 differences in the frequencies of cortisone-induced cleft palate were shown between strains (Kalter, 1954). In that study, the A/J strain was found to be more susceptible than mice of the C57BL/6J strain, yet F_1 embryos of A/J dams displayed a higher frequency of cortisone-induced cleft palate than did F_1 embryos of C57BL/6J dams, indicating a maternal effect. Using probit analysis, Biddle (1978) showed that although the mechanism involved in cleft palate production did not differ between the strains, the dosage

tolerance did differ. This was also true for the SWV mouse strain (Biddle, 1978). They differed not in the mechanism involved in cleft palate production but in the tolerance. In his study of the fetal alcohol syndrome Chernoff (1977) compared fetal weights, number of resorptions, and rates of malformations in CBA, C_3H, and C57BL/6J mouse strains maintained on the same diet of alcohol. He found the CBA strain to be more sensitive to alcohol teratogenesis than C_3H, which in turn was more sensitive than C57BL/6J. Because environment and treatment were identical for the three strains, these findings are highly suggestive of a genetic component interacting with alcohol and resulting in the observed strain differences. It is surprising, then, that using the same mouse strains, indeed, mice derived from the same breeding colony (Mouse Genetics Breeding Colony, University of British Columbia) as the one used by Biddle and Chernoff, there were no strain differences observed for phenytoin-induced teratogenicity. This observation was initially noted for the C57BL/6J genotypes in a pilot study conducted in a different laboratory, under slightly varied environmental conditions, thereby enhancing the credibility ity of these current results. Thus, the enzyme systems involved in phenytoin metabolism and likely those also involved in phenytoin-induced teratogenesis appear to be genetically similar among SWV, C_3H, and C57BL/6J mouse strains.

Female mice were treated with orally administered phenytoin without apparent toxicity and without effect on their daily fluid consumption (Table 4). With increasing dosages of PHT, plasma PHT concentrations increased in all of the strains studied. The drug-exposed fetuses weighed significantly less than their controls within all strains and genotypes studied and the between-strains differences also were significant. The SWV and C_3H strains of mice had significantly more implants per litter than the C57BL/6J genotypes. Indeed, these strains were selected as the neurologically unimpaired controls because they were proven breeders with large litters. The percentages of fetuses resorbed were not significantly different between strains as the dosage levels increased (Table 5), but the variations within the individual groups were so great that it could have obscured any differences that may have existed between strains. In the heterozygous (+/qk) dams, there were significantly more resorptions at the 60 mg/kg dosage level than in the untreated heterozygous (+/qk) controls. This was the only difference of statistical significance in resorption rates within a strain or genotype as dosage levels increased.

Malformations, which included both skeletal and soft tissues, exhibited a specific dose-response pattern irrespective of fetal sex (Tables 6-8). Skeletal ossification begins between day 16 and 17 in the mouse, and skeletal maturity can be assessed in fetuses by a three-step approach

(Fritz and Hess, 1970). Initially, skeletal maturity is assessed by noting those bones that are ossified and those in which ossification has yet to occur. Those parts of the skeleton particularly noteworthy are the phalanges, the sternebrae (Lorke, 1965; McColl, Globus, and Robinson, 1965; Schumacher, Blake, Gurian, and Gillette, 1968), and the vertebral centra (Murphy, 1962). Secondly, alizarin-stained skeletons are examined for areas of incomplete ossification. In this study, "bipartite" sternebrae, triangular shaped vertebral centra, and the supraoccipital bone were the most commonly seen anomalies of incomplete ossification and these were apparent at all PHT dosages. Finally, skeletal maturity can be determined by screening for pathological forms of ossification. Abnormally shaped pubis and ischium bones observed at the 40 and 60 mg/kg dosages and retracted or misshaped facial bones, including the maxilla, pre-maxilla, frontal, nasal, and mandibular bones, seen at all treatment levels fall into this final category. The vertebral centra-defect, found in all strains and at all treatment levels, was particularly striking, with the apex of the triangle pointed downwards; it was reminiscent of normal day 16 mouse fetuses (Theiler, 1972). Detecting an increased rate of missing and incompletely ossified bones is indicative of a non-specific drug-induced intrauterine growth retardation (Fritz and Hess, 1970).

Anomalies of the soft tissues included dilated cerebral

ventricles, which occurred in all strains from the lowest treatment levels. It usually involved only a symmetrical enlargement of the lateral ventricles, but often the third ventricles were also dilated. At the highest treatment level, particularly noteworthy in the SWV strain, the severity of the dilation was such that it could be considered a form of internal hydrocephalus. Cardiac anomalies and those involving the great vessels were found in 19% of the treated offspring. While atrial septal defects were difficult to assess due to the techniques involved in fetal heart dissection, defects of interventricular septum, particularly the muscular interventricular septum, were easily scored by this approach. Tricuspid atresia was indicated by the presence of a large right atrium, an interventricular septal defect, and a tricuspid valve that was either barely visible or completely absent from the floor of the right atrium. In aortic valvular atresia, there was marked hypoplasia of the aorta, left atrium, and left ventricle, while a wide open ductus arteriosus fed blood into the aorta. The preponderance of cardiac defects at the 20 mg/kg dosage were due to the presence of bilateral or unilateral hypoplastic atria. This anomaly was seen in all three strains and is likely to represent either an immature but normally developed atria, or they may reflect subtle changes in blood flow in the fetus just prior to sacrifice. In all likelihood the hypoplastic atria are compatible with fetal growth and

development. Orofacial clefts, the predominant defect reported in the early retrospective human epidemiologic surveys of phenytoin-induced teratogenicity, were observed in only 2% of the offspring of PHT-treated dams, with the vast majority of cleft palates found in the SWV fetuses. Interestingly, hypoplastic digits were observed in all three strains. This anomaly is one of the most easily distinguishable clinical features of the human fetal hydantoin syndrome.

The present study was designed to satisfy criteria of how an animal model could closely parallel the human situation involving phenytoin treatment and epilepsy, so as to create a fetal hydantoin syndrome in the mouse. Similarities between the human and mouse fetal hydantoin syndrome are shown in Table 15. Note that the category of growth deficiency refers to low fetal weight and incomplete ossification; the category of perinatal death, although not observed because of the experimental design, might have been approximated by the high frequencies of cardiac and renal anomalies. Because of the experimental protocol, the category of performance could not be evaluated.

This experiment differed from all previous attempts to study the teratogenicity of phenytoin. For instance, the range of PHT dosages used in this study, while superficially appearing to be quite large, is actually comparable to dosages prescribed for human epileptics (Appendix E), and was

TABLE 15. Similarities between the human and mouse fetal hydantoin syndrome

	Human	Mouse
1. Growth Deficiency	X	X
2. Skeletal Anomalies	X	X
3. Neural Anomalies	X	X
4. Cardiac Anomalies	X	X
5. Orofacial Clefting	X	X
6. Ocular Anomalies	X	X
7. Genitourinary Anomalies	X	X
8. Perinatal Death	X	?
9. Low Performance	X	?

generally compatible with those studies administering the drug for a short period during organogenesis (Massey, 1966; Gibson and Becker, 1968; Schardein et al., 1973; Sullivan and McElhatton, 1977) but much lower than the treatment regime of many other studies (Harbison and Becker, 1969, 1972; Mercier-Parot and Tuchmann-Duplessis, 1974; Miller and Becker, 1975; Sullivan and McElhatton, 1975; Fritz et al., 1976; Martz et al., 1977; Paulson et al., 1979). The route of administration was also different. In this study, the animals' drinking water was utilized as the vehicle to administer the drug, a methodology worked out in earlier studies by the author (Finnell, 1978, 1980). Only two other studies used non-invasive routes of drug administration by mixing large quantities of PHT in with powdered animal feed (Fritz et al., 1976; Paulson et al., 1979). Others either intubated the dams (Harbison and Becker, 1969; Miller and Becker, 1975; Sullivan and McElhatton, 1975, 1977; Fritz et al., 1976) or injected PHT subcutaneously (Massey, 1966; Gibson and Becker, 1968; Harbison and Becker, 1969; Martz et al., 1977) or injected it intraperitoneally (Harbison and Becker, 1969; Schardein et al., 1973; Mercier-Parot and Tuchmann-Duplessis, 1974; Miller and Becker, 1975; DeVore and Woodbury, 1977).

The time the phenytoin was administered also differed among studies. In this study, phenytoin was administered two weeks prior to and throughout gestation. Many studies

elected to give the drug for only a short period during organogenesis (Massey, 1966; Gibson and Becker, 1968; Harbison and Becker, 1969, 1972; Schardein et al., 1973; Miller and Becker, 1975; Sullivan and McElhatton, 1975). A few studies used a longer period of exposure spanning days 6-16 (Sullivan and McElhatton, 1975, 1975; Fritz et al., 1976), one study treated dams from the start of gestation (DeVore and Woodbury, 1977) and still others chose to administer the drug on a single day (Martz et al., 1977; Sulik et al., 1979). Although short-term exposure is considered to be more precise in determining acute embryotoxicity, it does not meet the needs of all experimental situations (Wilson, 1973). In a model system designed to closely parallel the human situation, chronic administration of a drug is essential. Because human epileptics have usually received anticonvulsant drug therapy from the time the seizure disorder is diagnosed, they have usually received medication long enough to induce maximally their hepatic, drug-metabolizing enzymes before any pregnancy is initiated. The criticism directed at long-term exposure to the drug is that it provides the experimental organism with enough time for adaptive reactions, such as the induction of liver microsomal enzyme systems (Burns, 1970). But in the present study, this adaptive response was both desirable and planned. With maternal enzyme systems maximally induced, fluctuations in maternal plasma PHT concentrations were minimal thereby

making constant the amount of drug available to the embryo.

In mice, phenytoin has been shown to induce its own metabolism (Gerber and Arnold, 1969). When a drug has this capability, its plasma concentrations can be expected to differ between an animal given a single injection and one given an equivalent dosage over a four-day period (Conney and Burns, 1972). Thus, those studies that describe marked teratogenic effects from a single or short treatment with PHT (Massey, 1966; Gibson and Becker, 1968; Harbison and Becker, 1969, 1972, 1974; Miller and Becker, 1975; Sullivan and McElhatton, 1975; Martz et al., 1977; Paulson et al., 1979) may not have permitted maximal induction of the maternal enzyme systems. This phenomenon was demonstrated by King, Weaver, and Narrod (1965) using the antihistamine chlorcyclizine. They administered 50 mg/kg dosages on gestational days 1-15 which resulted in a significant decrease in the incidence of cleft palate when compared with the results of the experiment where dams were injected on single gestational days. This kind of explanation may account for the low incidence in the present study of cleft palate (2%) and other defects, and for the low incidence of malformations in those investigations using long-term drug administration (Sullivan and McElhatton, 1975, 1977; Fritz et al., 1976) as compared with studies of short-term drug administration (Massey, 1966; Gibson and Becker, 1968; Harbison and Becker, 1969, 1972, 1974; Mercier-Parot and

Tuchmann-Duplessis, 1974; Miller and Becker, 1975; Martz et al., 1977; Paulson et al, 1979; Sulik et al., 1979).

Given the above differences in protocol, it is not surprising that the results presented in the previous chapter are different in certain aspects from published studies previously mentioned. The current study did not find phenytoin to be as embryolethal as it has been found at certain high dosages (Harbison and Becker, 1969), although many studies did have comparable resorption rates (Fritz et al., 1976; Sullivan and McElhatton, 1977). The spectrum of malformations reported in the previous chapter was more extensive than any study yet reviewed. In those instances where internal malformations were recorded, their overall frequency was less than that found in this study. For instance, Harbison and Becker (1969) found hydronephrotic kidneys in 8% of the drug exposed fetuses, while the incidence was closer to 23% in this study, and their 6% incidence of unossified sternebrae was much less than the 22% frequency observed by this author. As cardiac anomalies were observed in 19% of the fetuses exposed to phenytoin in utero, and no such anomalies had been reported previously, it likely reflects differences in techniques utilized to score for fetal malformations and may underscore the general differences between this and previously published studies on phenytoin-induced teratogenesis.

A clear departure from the earlier studies has been the

monitoring of maternal plasma PHT concentrations (Finnell, 1978, 1980; Paulson et al., 1979; Sulik et al., 1979). This is particularly important with phenytoin as this study and that of Paulson (1979) clearly indicate a direct relationship between maternal plasma PHT levels and the percent of abnormal fetuses. Further work is indicated to define what changes, if any, are occurring during gestation in terms of maternal plasma PHT concentrations. This type of question may best be answered by an experiment involving blood samples drawn at five-day intervals both prior to and throughout gestation. Future studies should also be concerned with obtaining fetal blood to determine its concentration of phenytoin.

The present study notes a statistically significant rise in maternal liver weights with increasing drug dosages for both the SWV and C_3H mice and for the C57BL/6J (qk/qk) mice. There was no such increase, however, in the homozygous non-affected (+/+) and heterozygous (+/qk) C57BL/6J genotypes. The observed increase in liver mass is likely due to either a hepatocytic proliferation, especially due to increased synthesis of cytochrome P^{450}-associated enzymes within the rapidly proliferating endoplasmic reticulum (Fingl and Woodbury, 1970) or an increase in fatty depositions within the liver. This is an interesting finding, in as much as the latter two genotypes had disproportionately large increases in plasma PTH concentrations going from the

mid- to the high-dosage level, whereas the SWV and C_3H dams continued their linear increase in serum concentrations. Thus, the failure of the C57BL/6J females to expand their liver mass may have resulted in the saturation of drug-binding sites at the 40 mg/kg dosage level while those of females of the other strains were still unsaturated at the 60 mg/kg dosage level.

In the biotransformation of phenytoin, the formation of vicinal trans-dihydrodiol metabolites in humans and in rodents suggests that epoxide (arene oxide) compounds may be involved in the teratogenic properties of phenytoin. Epoxides are obligatory intermediates in the metabolism of polycyclic aromatic hydrocarbons that bind covalently to macromolecules (Jerina and Daly, 1974). It has been speculated that PHT teratogenicity is due to the adverse effects of arene oxides on fetal nucleic acids during critical periods of embryogenesis (Martz et al., 1977; Harbison, 1978). That theory provided the rationale of Experiment 2, which explored the relationship between phenytoin-induced teratogenesis and hepatic enzyme function.

The compound selected for use in Experiment 2 was another member of the hydantoin family of drugs, ethotoin. This compound is a commercially available, yet seldom prescribed, anticonvulsant. It is not a metabolite of phenytoin as it is metabolized in a different hydroxylation pathway that does not lead to the formation of a dihydrodiol

and therefore, does not form epoxides. It was initially thought that if ethotoin did not produce malformations in the mouse further support would be given to the theory of arene oxides as the primary teratogen. If, however, ethotoin was teratogenic, the theory that the epoxides were the teratogens needs to be revised. Having found ethotoin to be teratogenic in mice, it is now felt that future emphasis should be placed on other possible mechanisms of phenytoin-induced teratogenicity, rather than on the theory of covalent binding by epoxide intermediates as the causal agent for adverse fetal effects.

The administration of ethotoin to pregnant mice resulted in a generalized pattern of intrauterine growth retardation and associated defects in the fetuses. Specific defects associated with the use of phenytoin, such as digital hypoplasia, were not observed as a result of the dosages used in this study, but this is not to say that they would not have occurred at higher dosage levels. Thus, it is not possible to determine from the data whether the mechanism of ethotoin-induced teratogenicity is the same as that of phenytoin-induced teratogenicity. It is possible that PTH and ETH act as folic acid antagonists or act independently, such as by phenytoin being altered to an epoxide, and ethotoin causing entirely different metabolic changes that are detrimental to the developing embryos. Therefore, until a complete dose-response curve is created for

ethotoin-induced teratogenicity, it is not possible to comment on the possibility that the highly reactive epoxide metabolites are the principal teratogens in phenytoin-induced teratogenicity.

In conclusion, the results of this study have provided answers to several basic questions about the fetal hydantoin syndrome. It was demonstrated that the risk for fetal insult was dependent upon the maternal plasma phenytoin concentration and these plasma drug levels were solely determined by the amount of phenytoin administered to the animal. Abnormal fetuses were obtained from dams with plasma PHT concentrations as low as 2.69 µg/ml, but the lowest level compatible with the production of normal offspring has yet to be determined in humans.

All indications gained from this study suggest that phenytoin is the agent responsible for the congenital malformations in mice, and therefore probably in children born to epileptic mothers on hydantoin anticonvulsant drug therapy rather than the presence of maternal or paternal epilepsy. Further support for this conclusion is found in the medical literature. A family was studied in which trizygotic triplets were born to an epileptic mother treated with phenytoin. All offspring showed features compatible with a diagnosis of the fetal hydantoin syndrome. All three of these infants, genetically related but not identical, were probably exposed to equal quantities of the circulating

drug, and all three were similarly affected (Bustamante and Stumpf, 1978).

As yet no acceptable evidence of a dose-response relationship for phenytoin-induced teratogenicity has been shown to exist in humans. Recently, significantly higher maternal PHT plasma levels were found in drug-treated epileptic mothers bearing abnormal offspring than in those epileptic mothers bearing normal children (Dansky et al., 1979). Since this is precisely what was observed in the animal model, it would seem appropriate that continued monitoring of such patients is essential in order to define the lower limits of the teratogenic dose in humans. The current practice is to increase the drug-dosage to correct anticipated declines of maternal plasma phenytoin concentration when pregnancy occurs. If anything can be learned from the results of the animal model, it would be that physicians should strive toward reducing, rather than increasing the drug dosage in pregnancy towards the lowest plasma PHT concentrations that prevent seizures. With careful monitoring of plasma PHT concentrations and with appropriate counselling on the risks involved with continued phenytoin therapy during pregnancy, it should be possible to reduce dramatically the number of infants born with the fetal hydantoin syndrome.

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PHT DOSE

Weight/gm	20 mg/kg Dose #2	40 mg/kg Dose #3	60 mg/kg Dose #4
60	1.20	2.40	3.60
59.5	1.19	2.38	3.57
59	1.18	2.36	3.54
58.5	1.17	2.34	3.51
58	1.16	2.32	3.48
57.5	1.15	2.30	3.45
57	1.14	2.28	3.42
56.5	1.13	2.26	3.39
56	1.12	2.24	3.36
55.5	1.11	2.22	3.33
55	1.10	2.20	3.30
54.5	1.09	2.18	3.27
54	1.08	2.16	3.24
53.5	1.07	2.14	3.21
53	1.06	2.12	3.18
52.5	1.05	2.10	3.15
52	1.04	2.08	3.12
51.5	1.03	2.06	3.09
51	1.02	2.04	3.06
50.5	1.01	2.02	3.03
50	1.00	2.00	3.00
49.5	.99	1.98	2.97
49	.98	1.96	2.94
48.5	.97	1.94	2.91
48	.96	1.92	2.88
47.5	.95	1.90	2.85
47	.94	1.88	2.82
46.5	.93	1.86	2.79
46	.92	1.84	2.76
45.5	.91	1.82	2.73
45	.90	1.80	2.70
44.5	.89	1.78	2.67
44	.88	1.76	2.64
43.5	.87	1.74	2.61
43	.86	1.72	2.58
42.5	.85	1.70	2.55
42	.84	1.68	2.52
41.5	.83	1.66	2.49
41	.82	1.64	2.46
40.5	.81	1.62	2.43
40	.80	1.60	2.40
39.5	.79	1.58	2.37
39	.78	1.56	2.34
38.5	.77	1.54	2.31
38	.76	1.52	2.28
37.5	.75	1.50	2.25
37	.74	1.48	2.22
36.5	.73	1.46	2.19
36	.72	1.44	2.16
35.5	.71	1.42	2.13
35	.70	1.40	2.10

Water Consumption per Cage	1/Fraction of 50 ml
5 ml	10
5.5	9.09
6	8.33
6.5	7.70
7	7.14
7.5	6.67
8	6.25
8.5	5.88
9	5.55
9.5	5.26
10	5.00
10.5	4.76
11	4.54
11.5	4.35
12	4.16
12.5	4.00
13	3.85
13.5	3.70
14	3.57
14.5	3.45
15	3.33
15.5	3.22
16	3.12
16.5	3.03
17	2.94
17.5	2.86
18	2.77
18.5	2.70
19	2.63
19.5	2.56
20	2.50
20.5	2.44
21	2.38
21.5	2.32
22	2.27
22.5	2.22
23	2.17
23.5	2.13
24	2.08
24.5	2.04
25	2.00
25.5	1.96
26	1.92
26.5	1.89
27	1.85
27.5	1.82
28	1.79
28.5	1.75
29	1.71
29.5	1.70
30	1.67

APPENDIX C

For example, a cage containing 50 grams of mice who were to receive a 20 mg/kg dose, averaged 10 ml of drinking water daily. From the first conversion table (Appendix A) looking across from 50 grams to the 20 mg/kg dose, is the value 1.0. On the second conversion table (Appendix B), across from 10 ml of water consumed, is the value 5.0. The product of the two values obtained from the conversion tables (1.0 and 5.0) is the correct amount of stock solution which will then be diluted down to 50 ml total volume which will be the appropriate dose for this cage.

APPENDIX D

ANALYSIS OF VARIANCE TABLES

1. Serum Levels Between Samplings

1a. SWV LEVEL 2

Source	d.f.	SS	MS	F
Treatment	3	19.65	6.55	1.31
Error	28	140.22	5.01	
Total	31	159.87		

1b. SWV LEVEL 3

Source	d.f.	SS	MS	F
Treatment	3	50.48	16.83	1.92
Error	35	307.00	8.77	
Total	38	357.48		

1c. SWV LEVEL 4

Source	d.f.	SS	MS	F
Treatment	3	178.34	59.45	2.57
Error	28	649.17	23.11	
Total	31	825.53		

1d. C_3H LEVEL 2

Source	d.f.	SS	MS	F
Treatment	3	10.10	3.37	0.88
Error	26	99.49	3.83	
Total	19	109.59		

1e. C_3H LEVEL 3

Source	d.f.	SS	MS	F
Treatment	3	27.09	9.03	0.72
Error	23	287.98	12.52	
Total	26	315.06		

1f. C_3H LEVEL 4

Source	d.f.	SS	MS	F
Treatment	3	10.16	3.39	0.68
Error	29	144.76	4.99	
Total	32	154.92		

1g. C57(+/+) LEVEL 2

Source	d.f.	SS	MS	F
Treatment	3	14.38	4.79	3.19
Error	13	19.53	1.50	
Total	16	33.91		

1h. C57(+/+) LEVEL 3

Source	d.f.	SS	MS	F
Treatment	3	4.07	1.36	0.28
Error	12	58.62	4.89	
Total	15	62.69		

1i. C57(+/+) LEVEL 4

Source	d.f.	SS	MS	F
Treatment	3	20.88	6.96	0.57
Error	14	170.55	12.18	
Total	17	191.43		

1j. C57(+/qk) LEVEL 2

Source	d.f.	SS	MS	F
Treatment	3	0.16	0.05	0.10
Error	9	4.76		
Total	12	4.92		

1k. C57(+/qk) LEVEL 3

Source	d.f.	SS	MS	F
Treatment	3	13.98	4.66	0.84
Error	9	50.07	5.56	
Total	12	64.05		

1ℓ. C57(+/qk) LEVEL 4

Source	d.f.	SS	MS	F
Treatment	3	5.84	1.95	0.18
Error	10	107.34	10.73	
Total	13	113.18		

1m. C57(qk/qk) LEVEL 2

Source	d.f.	SS	MS	F
Treatment	3	12.21	4.07	1.17
Error	11	38.14	3.47	
Total	14	50.35		

1n. C57(qk/qk) LEVEL 3

Source	d.f.	SS	MS	F
Treatment	3	10.23	3.41	0.54
Error	10	63.34	6.33	
Total	13	73.57		

1o. C57 (qk/qk) LEVEL 4

Source	d.f.	SS	MS	F
Treatment	3	53.71	17.90	0.74
Error	14	339.65	24.26	
Total	17	393.36		

2. Serum Levels Between Strains

2a. LEVEL 2

Source	d.f.	SS	MS	F
Treatment	4	9.17	2.29	1.47
Error	30	46.77	1.56	
Total	34	55.94		

2b. LEVEL 3

Source	d.f.	SS	MS	F
Treatment	4	13.80	3.45	1.17
Error	30	88.55	2.95	
Total	34	102.35		

2c. LEVEL 4

Source	d.f.	SS	MS	F
Treatment	4	42.53	10.63	0.99
Error	30	322.32	10.74	
Total	34	364.84		

3. Fetal Weights Between Strains

3a. LEVEL 1

Source	d.f.	SS	MS	F
Treatment	4	2.88	0.72	37.42*
Error	321	6.17	0.02	
Total	325	9.04		

3b. LEVEL 2

Source	d.f.	SS	MS	F
Treatment	4	0.85	0.21	13.90*
Error	290	4.42	0.0153	
Total	294	5.27		

3c. LEVEL 3

Source	d.f.	SS	MS	F
Treatment	4	1.59	0.40	21.14*
Error	270	5.09	0.02	
Total	274	6.68		

3d. LEVEL 4

Source	d.f.	SS	MS	F
Treatment	4	1.43	0.36	27.01*
Error	261	3.46	0.01	
Total	265	4.89		

---

*Significant ($p < .05$)

4. Liver Weights Between Strains

4a. LEVEL 1

Source	d.f.	SS	MS	F
Treatment	4	4.29	1.07	6.18*
Error	25	4.34	0.17	
Total	29	8.63		

4b. LEVEL 2

Source	d.f.	SS	MS	F
Treatment	4	6.17	1.54	4.62*
Error	30	10.02	0.33	
Total	34	16.19		

4c. LEVEL 3

Source	d.f.	SS	MS	F
Treatment	4	5.34	1.34	3.11*
Error	28	12.02	0.43	
Total	32	17.36		

4d. LEVEL 4

Source	d.f.	SS	MS	F
Treatment	4	10.01	2.50	9.02*
Error	29	8.04	0.28	
Total	33	18.05		

---

*Significant ($p < .05$)

5. Implants Between Strains

5a. LEVEL 1

Source	d.f.	SS	MS	F
Treatment	4	83.10	20.78	7.70*
Error	30	80.90	2.70	
Total	34	164.00		

5b. LEVEL 2

Source	d.f.	SS	MS	F
Treatment	4	122.19	30.55	37.70*
Error	30	206.50	6.88	
Total	34	328.69		

5c. LEVEL 3

Source	d.f.	SS	MS	F
Treatment	4	100.19	25.05	12.59*
Error	30	59.70	1.99	
Total	34	159.89		

5d. LEVEL 4

Source	d.f.	SS	MS	F
Treatment	4	10.67	26.52	5.45*
Error	30	146.10	4.87	
Total	34	252.17		

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*Significant ($p < .05$)

6. Water Consumption Between Strains

6a. LEVEL 1

Source	d.f.	SS	MS	F
Treatment	4	566.99	141.75	44.27*
Error	61	155.30	3.20	
Total	65	762.29		

6b. LEVEL 2

Source	d.f.	SS	MS	F
Treatment	4	459.12	114.78	27.57*
Error	47	195.66	4.16	
Total	51	654.78		

6c. LEVEL 3

Source	d.f.	SS	MS	F
Treatment	4	375.95	93.99	24.88*
Error	58	219.10	3.78	
Total	62	595.05		

6d. LEVEL 4

Source	d.f.	SS	MS	F
Treatment	4	358.23	89.56	17.30*
Error	47	243.26	5.18	
Total	51	601.49		

*Significant ($p < .05$)

7. Resorptions Between Strains

7a. LEVEL 1

Source	d.f.	SS	MS	F
Treatment	4	5.84	1.46	2.02
Error	30	21.70	0.72	
Total	34	27.54		

7b. LEVEL 2

Source	d.f.	SS	MS	F
Treatment	4	11.84	2.96	1.29
Error	30	68.90	2.30	
Total	34	80.74		

7c. LEVEL 3

Source	d.f.	SS	MS	F
Treatment	4	12.24	3.06	0.62
Error	30	148.50	4.95	
Total	34	160.74		

7d. LEVEL 4

Source	d.f.	SS	MS	F
Treatment	4	4.40	1.10	0.20
Error	30	162.00	5.40	
Total	34	166.40		

7e. RESORPTIONS (STRAINS COMBINED)

Source	d.f.	SS	MS	F
Treatment	3	455.20	157.73	6.63*
Error	16	366.00	22.88	
Total	19	821.20		

ANOVA BETWEEN TREATMENTS WITHIN INDIVIDUAL STRAINS

8. Implants (SWV)

Source	d.f.	SS	MS	F
Treatment	3	1.88	0.62	0.07
Error	36	325.90	9.05	
Total	39	327.78		

9. Resorptions (SWV)

Source	d.f.	SS	MS	F
Treatment	3	3.28	1.09	0.25
Error	36	156.70	4.35	
Total	39	159.98		

10. Fetal Weights (SWV)

Source	d.f.	SS	MS	F
Treatment	3	1.61	0.54	26.91*
Error	401	7.99	0.02	
Total	404	9.60		

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*Significant ($p < .05$)

11. Liver Weights (SWV)

Source	d.f.	SS	MS	F
Treatment	3	5.90	1.97	4.16*
Error	35	16.56	0.47	
Total	38	22.46		

12. Serum Levels (SWV)

Source	d.f.	SS	MS	F
Treatment	2	238.71	119.36	12.88*
Error	27	250.25	9.27	
Total	29	488.96		

13. Water Consumption (SWV)

Source	d.f.	SS	MS	F
Treatment	3	13.31	4.44	0.84
Error	55	291.49	5.30	
Total	58	304.80		

14. Implants (C_3H)

Source	d.f.	SS	MS	F
Treatment	3	8.30	2.77	1.05
Error	36	95.20	2.64	
Total	39	103.50		

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*Significant ($p < .05$)

15. Resorptions (C_3H)

Source	d.f.	SS	MS	F
Treatment	3	10.48	3.49	0.70
Error	36	178.50	4.96	
Total	39	188.98		

16. Fetal Weights (C_3H)

Source	d.f.	SS	MS	F
Treatment	3	1.50	0.50	34.60*
Error	338	4.90	0.01	
Total	341	6.40		

17. Liver Weights (C_3H)

Source	d.f.	SS	MS	F
Treatment	3	4.67	1.56	5.41*
Error	35	10.08	0.29	
Total	38	14.75		

18. Serum Levels (C_3H)

Source	d.f.	SS	MS	F
Treatment	2	185.27	92.64	42.02*
Error	27	59.52	2.20	
Total	29	244.78		

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*Significant ($p < .05$)

19. Water Consumption (C_3H)

Source	d.f.	SS	MS	F
Treatment	3	46.35	15.45	1.72
Error	62	555.90	8.97	
Total	65	602.25		

20. Implants (C57, +/+)

Source	d.f.	SS	MS	F
Treatment	3	6.95	2.32	1.10
Error	16	33.60	2.1	
Total	19	40.55		

21. Resorptions (C57, +/+)

Source	d.f.	SS	MS	F
Treatment	3	5.80	1.93	1.10
Error	16	28.00	1.75	
Total	19	33.80		

22. Fetal Weight (C57, +/+)

Source	d.f.	SS	MS	F
Treatment	3	1.94	0.647	57.45*
Error	135	1.52	0.01	
Total	138	3.46		

---

*Significant ($p < .05$)

23. Liver Weights (C57, +/+)

Source	d.f.	SS	MS	F
Treatment	3	2.02	0.67	3.38*
Error	15	2.98	0.20	
Total	18	5.00		

24. Serum Levels (C57, +/+)

Source	d.f.	SS	MS	F
Treatment	2	198.54	99.27	33.86*
Error	12	35.18	2.93	
Total	14	233.72		

25. Water Consumption (C57, +/+)

Source	d.f.	SS	MS	F
Treatment	3	20.93	6.98	2.36
Error	37	109.34	2.96	
Total	40	130.27		

26. Implants (C57, +/qk)

Source	d.f.	SS	MS	F
Treatment	3	6.96	2.32	1.97
Error	16	18.80	1.18	
Total	19	25.75		

---

*Significant ($p < .05$)

27. Resorptions (C57, +/qk)

Source	d.f.	SS	MS	F
Treatment	3	11.20	3.73	4.04*
Error	16	14.80	0.93	
Total	19	26.00		

28. Fetal Weights (C57, +/qk)

Source	d.f.	SS	MS	F
Treatment	3	6.24	2.08	3.63*
Error	141	80.81	0.57	
Total	144	87.05		

29. Liver Weights (C57, +/qk)

Source	d.f.	SS	MS	F
Treatment	3	0.27	0.09	0.51
Error	14	2.46	0.18	
Total	17	2.73		

30. Serum Levels (C57, +/qk)

Source	d.f.	SS	MS	F
Treatment	2	174.78	87.39	38.74*
Error	12	27.07	2.26	
Total	14	201.85		

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*Significant ($p < .05$)

31. Water Consumption (C57, +/qk)

Source	d.f.	SS	MS	F
Treatment	3	12.73	4.24	3.02
Error	19	26.69	1.40	
Total	22	39.42		

32. Implants (C57, qk/qk)

Source	d.f.	SS	MS	F
Treatment	3	7.60	2.53	2.11
Error	16	19.20	1.20	
Total	19	26.80		

33. Resorptions (C57, qk/qk)

Source	d.f.	SS	MS	F
Treatment	3	1.80	0.60	0.47
Error	16	20.40	1.28	
Total	19	22.20		

34. Fetal Weights (C57, qk/qk)

Source	d.f.	SS	MS	F
Treatment	3	2.35	0.78	49.57*
Error	130	2.06	0.02	
Total	133	4.41		

*Significant ($p < .05$)

35. Liver Weights (C57, qk/qk)

Source	d.f.	SS	MS	F
Treatment	3	6.44	2.15	11.97*
Error	13	2.33	0.18	
Total	16	8.78		

36. Serum Levels (C57, qk/qk)

Source	d.f.	SS	MS	F
Treatment	2	188.83	94.42	13.23*
Error	12	85.62	7.13	
Total	14	274.45		

37. Water Consumption (C57, qk/qk)

Source	d.f.	SS	MS	F
Treatment	3	15.93	5.31	2.49
Error	38	80.94	2.13	
Total	41	96.87		

38. Fetal Weights - 657BL/6J Genotypic Differences

38a. LEVEL 1

Source	d.f.	SS	MS	F
Treatment	2	0.10	0.05	2.92
Error	117	2.01	0.02	
Total	119	2.11		

---

*Significant ($p < .05$)

38b. LEVEL 2

Source	d.f.	SS	MS	F
Treatment	2	0.11	0.05	4.18*
Error	103	1.35	0.01	
Total	105	1.46		

38c. LEVEL 3

Source	d.f.	SS	MS	F
Treatment	2	0.52	0.26	13.52*
Error	100	1.93	0.02	
Total	102	2.45		

38d. LEVEL 4

Source	d.f.	SS	MS	F
Treatment	2	0.01	0.005	0.562
Error	85	0.76	0.0089	
Total	87	0.77		

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39. Liver Weight

Source	d.f.	SS	MS	F
Treatment	3	0.3544	0.118	0.359
Error	16	5.2645	0.329	
Total	19	5.6189		

---

*Significant ($p < .05$)

40. Water Consumption

Source	d.f.	SS	MS	F
Treatment	3	4.727	1.5757	0.7859
Error	29	58.148	2.0051	
Total	32	62.875		

41. Implants

Source	d.f.	SS	MS	F
Treatment	3	21.75	7.25	2.6364
Error	16	44.00	2.75	
Total	19	65.75		

42. Resorptions

Source	d.f.	SS	MS	F
Treatment	3	16.95	5.65	1.965
Error	16	46.00	2.875	
Total	19	62.95		

43. Fetal Weights

Source	d.f.	SS	MS	F
Treatment	3	1.013	0.3376	17.902*
Error	129	2.4327	0.0189	
Total	132	3.4455		

*Significant ($p < .05$)

44. Seizure Control

Source	d.f.	SS	MS	F
Treatment	3	1.90	0.63	1.33
Error	19	9.08	0.48	
Total	22	10.98		

APPENDIX E

RELATIONSHIP OF DRUG DOSAGE TO BODY SURFACE AREA

In attempting to establish a relationship between dosage and body weight in adult animals, it was proposed that dosages vary as a function of body surface area. This is proportional to the two-thirds power of the body weight (Clark, 1937). Therefore, when considering dosage relations between animals of widely varied sizes, it is critical to consider relative metabolic rates, hence the surface area of the organism relative to man's.

The following examples have been worked out using the formula:

$$\frac{\text{dosage}}{(\text{mg/kg})} \times \sqrt[3]{\frac{\text{body wt}}{(\text{kg})}} = \text{constant (K)}$$

1. For an average adult weighing 64 kg and receiving 300 mg of phenytoin per day, the following relationship exists.

$$4.7 \text{ mg/kg} \times \sqrt[3]{64 \text{ kg}} = 18.8$$

2. For a 356 m adult mouse receiving the 60 mg/kg/day dosage of phenytoin, the following relationship exists.

$$60 \text{ mg/kg} \times \sqrt[3]{.035 \text{ kg}} = 19.6$$

Thus, the constants for the two examples are virtually identical.