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THE EFFECT OF MATERNAL BLOOD PHENYLALANINE LEVEL ON MOUSE MATERNAL PHENYLKETONURIA OFFSPRING

by

Sangbun Chung

A Dissertation Submitted to the Faculty of the COMMITTEE ON NUTRITIONAL SCIENCES (GRADUATE) In Partial Fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA 1999
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Sangbun Chung entitled The Effects of Maternal Blood Phenylalanine Level on Mouse Maternal Phenylketonuria Offspring and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

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ABSTRACT

Maternal phenylketonuria is a disease process caused by the adverse effects of high maternal blood phenylalanine (PHE) on the fetus and it has become an important public health problem that must be solved to prevent a second-generation rebound in the incidence of pathology associated with phenylketonuria (PKU). Unless treated, maternal PKU results in teratogenic effects on the fetus that can lead to mental retardation, microcephaly, intrauterine growth retardation, congenital cardiovascular defects, low birth weight, spontaneous abortion and fetal death. PKU has been recognized as a major challenge for a number of years and has therefore been highly studied. Nevertheless, surprisingly little is known about the pathophysiologic mechanism(s) of PHE toward the fetus. This is in large part due to the technical and ethical limitations associated with clinical research.

To more thoroughly investigate the pathogenesis of this heritable disease and to explore potential therapeutic actions, mouse PKU models were generated. Among these, the genetic mouse model \( Pah^{enu2} \) displays genetic, biochemical and physiological characteristics that are strikingly similar to human PKU and therefore seems appropriate to function as a model of this disease. The overall goals of this project were to use the \( Pah^{enu2} \) mouse to examine the effect of maternal blood PHE level on: 1) The pregnancy outcome of maternal PKU offspring as measured by the incidence of spontaneous abortion and certain key measures of development at birth (i.e., head circumference, weight, and crown-rump length of offspring); and 2) The fetal nutritional status of
maternal PKU offspring as assessed by the levels of PHE, tyrosine (TYR), and other
essential amino acids (EAA) at birth.

In this study, we clearly observed that elevated maternal blood PHE levels, whether they
were caused by the maternal diet or maternal genotype, were responsible for the fetal
abnormalities in maternal PKU. With regard to fetal developmental outcomes, significant
reductions in birth weight, crown-rump length, and head circumference were seen in
offspring gestated under high maternal blood PHE conditions. The incidence of fetal loss
was significantly different between treatment and control groups. Reductions in the levels
of alanine, glutamine, and glutamic acid were observed in fetal blood among offspring
born to mutant mothers with high blood PHE levels. None of the branched chain amino
acids were reduced in maternal PKU offspring.

These findings strongly suggest that there are important maternal genotype and
dietary components but no fetal genotype component to this maternal PKU model. Given
that these maternal factors also appear to be the most important components of human
maternal PKU, this model seems certain to provide a valid animal model to overcome the
difficulties of human studies.
Phenylketonuria (PKU, McKusick no. 261600) is a hereditary disease characterized by severe mental retardation due to lack of the hepatic enzyme phenylalanine hydroxylase (PAH, E.C. 1.14.16.1). Without this enzyme, conversion of phenylalanine (PHE) to tyrosine (TYR) is blocked and PHE accumulates in the tissues. This syndrome is inherited as an autosomal recessive trait. Unless treated, PKU leads to severe mental retardation, neuropsychiatric symptoms and defects in pigmentation (Trefz et al., 1985).

In neonatal PKU, the sole determining factors for pathology are fetal genotype and postnatal fetal diet. A related syndrome, called maternal PKU, is a disease process caused by the adverse effects of high maternal PHE on the fetus. In maternal PKU, the sole determining factors seem to be maternal genotype and maternal diet during gestation. If a pregnant female has PKU and consumes a diet with normal amounts of protein, the fetus is at high risk for birth defects.

Maternal PKU has become a significant public health problem (Schuett et al., 1980; Waisbren et al., 1995). If untreated, maternal PKU results in teratogenic effects on the fetus; typically microcephaly, mental retardation, intrauterine growth retardation, congenital heart defects, low birthweight, spontaneous abortion, and stillbirth (Smith et al., 1990; Lipson et al., 1984; Platt et al., 1992; Naughten and Saul, 1990). In an international survey, 73-92% of offspring from untreated classical maternal PKU
pregnancies were microcephalic at birth and continued to be microcephalic after birth (Lenke and Levy, 1980; Ghavami et al., 1986). Mental retardation is a virtually constant feature among offspring of untreated maternal PKU. Ninety two percent of offspring of untreated maternal PKU have been classified as being mentally retarded (Lenke and Levy, 1980; Ghavami et al., 1986). Congenital heart disease (CHD) occurs in 12-15% of untreated maternal PKU pregnancies (Ghavami et al., 1986), while its prevalence is 1% in the general population. Intrauterine growth retardation has been a relatively constant feature of untreated maternal PKU. Birth weights less than 2,500 g occur in 40-50% of maternal PKU offspring (average birth weight is 3,750 g). Further, a number of groups have reported an increased frequency of spontaneous abortion when maternal blood PHE levels are elevated. Human retrospective studies have indicated that high maternal blood PHE levels (>900 μM) are associated with a high incidence of spontaneous abortion (Lenke and Levy, 1980; Koch et al., 1994). Lenke and Levy (1980) reported that, at a blood PHE concentration of 960 μM or greater, of 25% of total females examined, more than 30% of their pregnancies had miscarriages, while there were no spontaneous abortions among women with PHE concentrations below that level. These rates are approximately twice the frequency of the normal population. The presence of spontaneous abortion in about 20% of total pregnancies has also been observed in Pahenu2 PKU mouse females on normal mouse food (McDonald, unpublished data). Changes in amino acid homeostasis in untreated and incompletely-treated PKU children have been observed (Hjelm et al., 1994). Lower than normal blood concentrations of
most essential amino acids were observed among the \( Pah^{em2} \) PKU mice on a normal
chow diet compared to heterozygous mice (McDonald, unpublished data).

Among humans, high blood PHE levels cause depletions of most of the essential amino
acids, with branched chain amino acids (BCAA’s; valine, leucine, lysine) in brain being
the most affected (McKean et al., 1968). A high ratio of PHE to these BCAA’s impedes
transport of these amino acids by competing for the transport carrier protein (Oldendorf,
1973).

Like the blood-brain barrier, the placenta is sensitive to changes in plasma amino
acid concentrations in the physiologic range (Kennedy, 1971). It is well known that
disturbances in amino acid homeostasis impair protein synthesis leading to retarded fetal
growth (Tonkiss et al., 1993). It is possible, therefore, that the reduced bioavailability of
essential amino acids for the growing fetus, caused by PHE-induced interference, plays a
part in the pathophysiologic effects on fetal development. Therefore, the effect of
maternal PHE dose on the bioavailability of essential amino acids for a fetus has also
been incorporated into this study.

The overall goals of this project were to examine the effect of maternal blood
PHE level on:

1) The pregnancy outcome of mouse maternal PKU as measured by the
   incidence of spontaneous abortion and certain key measures of development at
   birth (i.e., head circumference, birth weight, and crown-rump length);

2) The fetal nutritional status of maternal PKU offspring as assessed by the
   levels of PHE, TYR and other essential amino acids (EAA) at birth.
CHAPTER II

LITERATURE REVIEW

1. Phenylketonuria (PKU)
   a. PKU symptomatology

   The inability to catabolize dietary PHE was one of the first inborn errors of metabolism described (Fölling, 1934). Fölling described ten mentally retarded children who excreted a phenylketone known as phenylpyruvic acid. Since he noticed that phenylpyruvic acid was structurally similar to PHE, he postulated that these children suffered from a disorder of PHE metabolism and that this was the ultimate cause of their mental retardation. This disorder was initially given the designation of oligophrenia phenylpyruvica, but was later renamed PKU. Subsequently, other retarded individuals who excreted phenylpyruvic acid were found to accumulate PHE in their blood and spinal fluid (Jervis et al., 1940). Ultimately, the biochemical blockage by which excess PHE accumulated was identified as the lack of activity of the hepatic enzyme phenylalanine hydroxylase (PAH) (Jervis, 1953). The metabolic phenotype of PKU is comprised of elevated blood PHE and its metabolites, accompanied by relative TYR deficiency (Udenfriend and Cooper, 1952).

   PKU is caused by mutations in the PAH gene (Woo et al., 1983; DiLella et al., 1986). Mutations in this gene can also cause milder forms of HPA, contributing a high degree of biochemical and clinical heterogeneity (Levy et al., 1971). It has therefore been
subdivided into different categories on the basis of pretreatment blood PHE levels:
classical PKU (>1,200 μM), mild PKU 600-1,200 μM ) and HPA (240-600 μM) (Hanley et al., 1987). Normal blood PHE concentrations are 58±15 μM in adults, 60±13 μM in teenagers, and 62±18 μM in childhood (mean±SD). In the newborn, the upper limit of normal is 120 μM (Scriver et al., 1995; Gregory et al., 1986). In untreated classical PKU, blood levels as high as 2,400 μM can be found.

If not treated early enough, classical PKU leads to severe irreversible mental retardation, as well as neuropsychiatric symptoms and defects in pigmentation (Trefz et al., 1985). Individuals with untreated classical PKU, in which the blood PHE concentration is increased ≥20-fold, almost invariably become mentally retarded, often severely so. Those with mild PKU, having a 10- to 15-fold increase in the concentration of blood PHE, also suffer cognitive loss when untreated, but the loss is less severe than in those with classical PKU. Those with HPA, with blood PHE concentration increased 2- to 8-fold, appear to remain cognitively normal and may not require treatment (Scriver et al., 1995). Patients with HPA have levels of PAH activity about 5% of normal, while those with classical PKU have a complete deficiency of PAH activity.

The initial and rate-limiting step in the catabolism of PHE is the irreversible hydroxylation of the aromatic ring, a reaction which is catalyzed by PAH and which yields TYR (Kaufman, 1976; Scriver et al., 1995) (Figure 1). PAH deficiency, therefore, results in increased levels of PHE in the blood stream with concomitant reduced TYR levels (Krause et al., 1985). Some of the excess PHE can be broken down into harmful metabolites such as phenylpyruvic, phenyllactic, phenylethyamine, and phenylacetic
acids, which are called phenylketones. These are abnormal metabolites of PHE metabolism because the minor reactions which yield phenylketones do not occur at all with normal levels of PHE. Even with high levels of PHE, they are not useful for reducing the burden of the high PHE.

It has been reported that these harmful metabolites can cause defects in myelin formation that damage a child's developing nervous system and lead to severe mental retardation (Hsia, 1970; Cowie, 1971). At the same time, the low levels of TYR in affected children result in a reduced amount of skin and hair pigment because the pigment molecule melanin is ultimately produced from TYR. The reduced pigmentation leads to a light complexion and hair color in untreated PKU children (Udenfriend and Cooper, 1952). Reductions in TYR also cause depressed dopamine synthesis, which has been shown to lead to cognitive deficits in a PKU model (Diamond et al., 1994). Other features of untreated PKU include a musty odor, peculiarities of gait, stance, and sitting posture, eczema, and epilepsy (Paine, 1957).

b. Inheritance and population frequency of PKU

PKU is a common inborn error of metabolism occurring in a child that is homozygous for a debilitating PAH gene mutation. The prevalence of PKU offspring is one in 10,000 live births among Caucasian and Oriental populations (Guthrie and Susi, 1963; Scriver et al., 1995). Given this observed frequency of homozygous recessives, the incidence of carriers in the general population can be estimated by the Hardy-Weinberg equation, $p^2 + 2pq + q^2 = 1$. If the chance of a homozygous recessive individual is $q^2$, where $q$ is the frequency of a recessive allele, then the frequency of carriers, $2pq$, is one
in 50 people for a population in Hardy-Weinberg equilibrium. The birth frequency of PKU, at one out of 10,000, is about as frequent as galactosemia and homocystinuria, about 10-fold more frequent than Huntington’s disease, and 10-fold less frequent than cystic fibrosis.

c. Clinical management of PKU

When begun early and maintained consistently, treatment with a diet low in PHE eliminates or ameliorates most PKU symptoms (Woolf et al., 1955; Armstrong and Tyler, 1955). Children born with even the most severe form of PKU can have normal cognitive development when dietary treatment begins in early infancy and blood PHE concentrations are maintained at normal or near normal levels. The mental retardation, though irreversible, can be prevented by dietary treatment, which justifies the early diagnosis of PKU. The diagnosis of PKU is done by a simple blood test. Because of the very positive outcome when children are treated early with a PHE restricted diet, great importance is placed on newborn PKU screening, especially in the countries where the incidence of PKU is high. Newborn screening was begun in the United States in the 1960’s (Guthrie and Susi, 1963). In the first 2 years of screening, 400,000 infants were tested in 29 states and 39 cases of PKU were found.

When PKU is diagnosed in children, they can be placed on a specially formulated low PHE diet. A PHE-restricted diet with casein hydrolysate as the main protein source was shown to significantly reduce blood PHE levels (Bickel et al., 1953). The current diet for classical PKU eliminates all high protein foods. This special diet is very strict in this regard because PHE typically constitutes 5% by weight of dietary protein. Therefore, it
excludes meat, poultry, fish, eggs, milk and milk products, legumes, nuts, and many products containing regular flour. The goal in dietary treatment is to supply only as much PHE as is needed for the synthesis of proteins, so excess PHE does not accumulate. A synthetic formula is used as a nutritional substitute for the eliminated foods. The diet is supplemented with special low protein foods and measured amounts of fruits, vegetables and some grain products.

With the special diet, the detrimental effects of excess PHE on mental development can be largely avoided. In the early days of treating PKU, it was believed that the diet could be stopped at an early age (i.e., 6 to 10 years). However, it is now known that stopping the diet can result in a variety of serious problems. This includes drops in IQ, learning disabilities, behavior problems such as hyperactivity and irritability, neurological problems such as tremors, eczema (a skin disorder) and personality disorders including schizophrenia, panic attacks and agoraphobia (Paine, 1957). Because of the problems that have developed in people who have discontinued the diet, it is now recommended that dietary treatment continue for life. It is generally believed that keeping blood PHE levels in the range of 121-363 μM (normal to three times normal) is the safest, especially in infancy and early childhood. Frequent blood monitoring is essential to achieve this goal.
Figure 1. The metabolic conversion pathways for outflow of L-phenylalanine in the normal steady state: (1) the hydroxylation reaction catalyzed by PAH (major pathway); (2) the transamination reaction (minor pathway); (3) the decarboxylation reaction (minor pathway).

(From Scriver et al., 1995) (Compounds in bold are abnormal metabolites of PHE metabolism as mentioned in the text)
2. Maternal PKU

a. History of maternal PKU

Thirty years after the discovery of classical PKU, a diet therapy was developed that could prevent mental retardation in this disorder (Bickel et al., 1953; Woolf et al., 1955; Armstrong and Tyler, 1955). Shortly thereafter, with the advent of routine newborn screening for PKU (Guthrie and Susie, 1963), it seemed possible to prevent mental retardation in all individuals born with PKU. Indeed, the combined success of these two actions has resulted in a large number of PKU individuals who have been spared the severe mental retardation of PKU. Most such individuals are mentally normal and are now of child bearing age. Unfortunately, most of them have discontinued the PHE-restricted diet since childhood and have high blood PHE levels (Koch et al., 1996; Hanley et al., 1996). It is now clear that women with PKU not treated prior to conception are at increased risk of having a pregnancy that results in serious fetal damage. The severe metabolic imbalance in the mother creates a toxic environment in her uterus, a syndrome called “maternal PKU”. As early as 1969, teratogenic birth defects were reported in two sisters with untreated PKU who had total of 28 pregnancies. Sixteen of 28 pregnancies ended in spontaneous first-trimester abortion and 12 pregnancies were carried to term. The fetus in each of the 12 pregnancies carried to term had intrauterine growth retardation, microcephaly and 9 out of the 12 term infants had cardiac malformations as well (Huntley and Stevenson, 1969). More recently, a number of groups have reported the birth defects of maternal PKU (Smith et al., 1990; Lipson et al., 1984; Platt et al., 1992; Naughten and Saul, 1990; Levy and Waisbren, 1983; Rouse et
In maternal PKU, children born to such mothers are at high risk for a range of birth defects including microcephaly with concomitant mental retardation, congenital heart defects, intrauterine growth retardation, and increased rates of spontaneous abortion and stillbirth (Koch et al., 1996; Lenke and Levy, 1980; Naughten and Saul, 1990).

The syndrome of maternal PKU has become a significant public health problem (Schuett et al., 1980; Waisbren et al., 1995). It is estimated that, at any given time, there are at least 3,000 PKU women of child bearing age who are not currently on dietary treatment and who are thereby at risk for producing offspring with these fetal abnormalities (Waisbren et al., 1988). Given the fact that each PKU woman may produce several offspring with birth defects, the healthcare gains produced by newborn screening and dietary treatment for classical PKU could be rather quickly overshadowed by birth defects resulting from the maternal PKU syndrome, a situation referred to as a second generation rebound.

b. Teratogenic effects of maternal PKU

1) Microcephaly

The most severe changes produced by maternal PKU are brain defects (microcephaly and mental retardation), heart defects (congenital heart disease), and low-birth weight. One of the most frequent teratogenic features of maternal PKU is microcephaly. It is defined by head circumference of more than two standard deviations below the mean. In an international survey, 73-92% of offspring from pregnancies with blood PHE levels greater than 1,200 μM were microcephalic at birth and continued to be microcephalic after birth (Lenke and Levy, 1980; Ghavami et al., 1986). There is a dose
relationship with lower frequencies of microcephaly in maternal PKU offspring at lower maternal PHE level. The frequency is 35% among offspring from untreated pregnancies with blood PHE level lower than 1,000 μM and 24% from pregnancies with blood PHE level lower than 600 μM (Lenke and Levy, 1980). This frequency of 24% is nearly ten times higher than the 2.5% frequency of the general population.

2) Mental retardation

Mental retardation is a virtually constant feature among the offspring of untreated maternal PKU. Ninety two percent of offspring of PKU mothers with blood PHE level of greater than 1,200 μM and 73% of offspring from mothers with blood PHE level lower than 1,200 μM have been classified as being mentally retarded (Lenke and Levy, 1980; Ghavami et al., 1986). An animal model study provides evidence of impaired cognitive functions from even mild elevations of PHE in plasma (Diamond et al., 1994).

3) Congenital heart defects

Congenital heart disease (CHD) occurs in 12-15% of offspring from mothers with blood PHE level of greater than 1,200 μM pregnancies (Ghavami et al., 1986). This is compared with a frequency of less than 1% for CHD among births in the general population (Mitchell et al., 1971). There is a dose-related effect of PHE in the etiology of CHD in the fetus. Among mothers with blood PHE of less than 600 μM, the frequency of CHD in offspring is 6%, about half the rate seen when blood PHE levels are at or above 1,200 μM (Ghavami et al., 1986).
4) Intrauterine growth retardation

Intrauterine growth retardation has been a constant feature of untreated maternal PKU. Birth weight is less than 2,500 g in 40-50% of offspring from maternal PKU pregnancies. Here again, there is a dose response relationship, with progressively lower birth weights at higher maternal PHE levels (Table 1) (Lenke and Levy, 1980). The incidence of premature birth is not increased by maternal PKU, so these low birth weights are more consistent with intrauterine growth retardation.

5) Spontaneous abortion

A number of groups have reported an increased frequency of spontaneous abortion when maternal blood PHE levels are elevated during pregnancy. Human retrospective studies have indicated that high maternal blood PHE levels (>900 µM) are associated with a high incidence of spontaneous abortion (Lenke and Levy, 1980; Koch et al., 1994). Lenke and Levy (1980) reported that, at a blood PHE concentration of 960 µM or greater, of 25 % of total females examined, more than 30% of their pregnancies had miscarriages, while there were no spontaneous abortions among women with PHE concentrations below that level. Huntley and Stevenson (1969) reported that 16 of 28 (57%) pregnancies ended in spontaneous first-trimester abortion. This rate is approximately four times the frequency in the normal population, which is 15%. Platt et al., (1992) reported that fetal loss due to spontaneous abortion among women with HPA comprised 17% of total pregnancies. The presence of spontaneous abortion has also been observed in the Pah<sup>enu</sup> PKU model on a normal mouse chow diet, comprising about 20% of total pregnancies (McDonald, unpublished data).
It is clear that offspring from mothers who have a mild form of PKU or HPA are much less damaged than those from classic maternal PKU pregnancies. This suggests a dose-dependent relationship between the maternal PHE level and the fetopathy in maternal PKU. Whether this dose-response in maternal PKU is of a continuous or threshold type is as yet unclear. It has been suggested that there is a threshold level for teratogenesis at a maternal PHE level of approximately 600 μM (Levy and Waisbren, 1983). A continuous teratogenic effect of dose response in maternal PKU has been observed by others (Drogari et al., 1987). These authors suggested that there were continuous reductions in both birth weight and birth head circumference for each 200 μM increase in the maternal blood PHE level. More recently, it was concluded that, for offspring cognitive development, there seems to be a maternal blood PHE threshold of 400 μM. The differences between maternal PHE below and above 400 μM for offspring birth length, birth head circumference and IQ were significant with P values 0.05, 0.005, and 0.02, respectively (Levy et al., 1994).
Table 1. Teratogenic effects and their frequencies relative to the degree of maternal HPA in offspring from untreated maternal PKU and HPA (Lenke and Levy, 1980)

<table>
<thead>
<tr>
<th>Birth defects (%)</th>
<th>Maternal PHE (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;1,200</td>
</tr>
<tr>
<td>Mental retardation</td>
<td>92</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>73</td>
</tr>
<tr>
<td>CHD</td>
<td>12</td>
</tr>
<tr>
<td>Low birth weight</td>
<td>40</td>
</tr>
</tbody>
</table>
c. Pathogenesis of maternal PKU

Maternal PKU is capable of causing embryopathy and fetopathy even in non-phenylketonuric offspring. In contrast to neonatal PKU, the sole determining factors for maternal PKU seem to be maternal diet and maternal genotype, regardless of fetal genotype. The pathogenesis of this syndrome is unknown. But, it has been widely acknowledged that the increased concentration of PHE and/or its metabolites are the primary suspects for the teratogenic factor(s) in maternal PKU (Michals et al., 1986). It may be related to direct toxicity in certain fetal organs aggravated by excess PHE derived from a positive transplacental gradient. Due to this positive transplacental PHE concentration gradient across the placental barrier into the fetal compartment, the fetus is exposed to 1.5-2 times the PHE concentration in the maternal blood stream, yielding an even greater risk for the developing fetus than anticipated on the basis of the maternal value alone (Schoonheyt et al., 1994).

Hypotheses of pathogenesis in maternal PKU have mainly centered on the placental transport of amino acids. These hypotheses rely upon data indicating that PHE shares an active placental transport system with several other neutral amino acids, including TYR, BCAA’s and methionine (Boyd and Yudilevich, 1987; Kudo and Boyd, 1990). PHE and TYR compete for the same transporter proteins to cross the blood-brain barrier. Increases in the ratio of PHE to TYR in plasma result in less TYR crossing into the brain, especially since the transporters have a higher affinity for PHE than for TYR (Padridge and Oldendorf, 1977; Herrero et al., 1983). Inhibition by PHE of the transport
of several large neutral amino acids across the placenta causes reduction of neurotransmitter production. Elevated levels of PHE reduce the amount of TYR available for dopamine production (Diamond et al., 1994) and tryptophan available for serotonin production (Woolley and van der Hoeven, 1965). Reduced uptake of leucine in fetal plasma and tissues (Okano et al., 1986) and in fetal brain (Takada et al., 1974) was observed by loading pregnant rats with PHE. Under such conditions, the fetus lacks sufficient amino acids for optimal protein synthesis. It is therefore possible that the reduced bioavailability of essential amino acids for the growing fetus, caused by PHE-induced interference, further results in pathophysiologic effects on fetal development.

Changes in amino acid homeostasis in untreated and incompletely treated PKU children have been observed (Hjelm et al., 1994). Among humans, high blood PHE levels caused depletions of most of the essential amino acids, with BCAA’s in brain being the most affected (McKean et al., 1968). These BCAA’s are transported across the blood-brain barrier via a saturable transport mechanism and a high ratio of PHE to BCAA’s impedes transport of these amino acids by competing for the transport carrier protein (Oldendorf, 1973). Like the blood-brain barrier, the placenta is sensitive to changes in plasma amino acids concentrations in the physiologic range (Kennedy et al., 1971).

Exposure of animals to PHE metabolites also produced embryopathies. Phenylpyruvic acid (PPA), one of the major metabolites found in PKU, is believed to play an important role in the etiology of the mental retardation found in PKU. PPA inhibits pyruvate decarboxylase in the brain, which leads to a reduced production of acetyl-CoA for energy production through the tricarboxylic acid cycle. Furthermore, in
PKU an inhibition at the site of pyruvate decarboxylation depresses the amount of acetyl CoA available for the synthesis of fatty acid and cholesterol, which are primary precursors of myelin (Bowden and McArthur, 1972). Depression in the synthesis of myelin and myelin precursors coupled with energy deficiency explain the reduced myelin deposition found concomitant with the mental retardation in PKU. Growth retardation in animals exposed to PPA acid has also been reported (Hamers et al., 1989).

Phenylethylamine was found to be the most toxic agent, followed by 2-hydroxy phenylacetic acid. PHE was among the least toxic of the agents tested (Denno and Sadler, 1990). While PHE metabolites may contribute to pathology in PKU, their contribution to maternal PKU pathology is very controversial; they do not seem to cross the placental barrier and it seems unlikely that fetal metabolism will produce them in high concentrations.

d. Clinical management of maternal PKU

Since PKU is treatable by a low PHE diet and intrauterine PHE excess is a primary suspect for the teratogenic factor in maternal PKU, controlling the level of maternal PHE is the most promising therapy for preventing fetal pathogenesis. A low PHE diet is essential for the treatment of maternal PKU. Studies in pregnant PKU women reveal that fetal damage from maternal PKU can be largely prevented by dietary therapy. However, the therapy must begin before conception and maintained throughout the pregnancy to maximize the possibility of a normal pregnancy outcome (Rohr et al., 1987; Drogari et al., 1987).
The gestational age of the fetus at the onset of dietary treatment, as well as the ability to maintain the maternal blood PHE at near normal levels during pregnancy, are very important factors in fetal development outcome. The longer the delay to normalize maternal blood PHE levels, the higher the risk of producing offspring with birth defects (Cipcic-Schmidt et al., 1996; Koch et al., 1993; Brenton and Lilburn, 1996). For minimizing the risks of maternal PKU as much as possible, it is strongly recommended that PKU women need to start the PHE-restricted diet preconceptionally, maintain blood PHE levels between 120 μM and 360 μM throughout the pregnancy, and receive good prenatal care and nutritional guidance (Koch et al., 1994). Maintaining maternal blood PHE levels between 120 μM and 360 μM has also been shown to protect later intellectual development (Hanley et al., 1996).

**Earlier efforts to produce animal models for human PKU**

For the past few decades, considerable research on PKU has been undertaken to characterize its pathophysiologic mechanisms. However, it has proven difficult to define the important aspects of its pathophysiology using human subjects. The relatively small numbers of PKU patients available to individual researchers, the confounding incidence of dietary noncompliance and the genetic heterogeneity of humans are some of the important difficulties in human PKU research. These shortcomings have stimulated a number of researchers to develop valid animal models for human PKU.

In an effort to investigate the pathophysiology of PKU, many attempts have been made to introduce a "PKU-like" state in genotypically normal animals by
overadministration of PHE to TYR. The early PKU models were produced with the fact that PAH is made at low levels (only 30-50% of normal adult values) in early postnatal life (Waisman, 1962) and albino rats (Perez and Schmidt, 1963; Adelman et al., 1973). Due to low levels of PAH, these models exhibited elevated blood PHE levels. This interruption causes accumulation of PHE and induces symptoms of PKU. But, they also exhibited elevated levels of blood TYR far in excess of PHE, which is not a characteristic of PKU. Noted limitations of these animal models are that they do not in general display an adequate number of the biochemical or behavioral symptoms found in human phenylketonuria.

Soon after, drug-induced animal models treated with chemical inhibitors of the PHE hydroxylase were produced. Genetically normal animals (mice and rats) were administered PHE along with the irreversible PAH inhibitors of $p$-chlophenylalanine (Lipton et al., 1967; Butcher et al., 1976; Loo et al., 1984), $\alpha$-methylphenylalanine (Delvalle et al., 1978; Lane et al., 1980), and L-ethionine (Gehrmann et al., 1989). However, despite success in these procedures, there are widely-recognized drawbacks that prevent the clear conclusions needed to define the cause-effect relationships of PKU pathophysiology. The enzyme inhibitors used to produce the PKU-like state have known side effects that can confound data interpretation such that 1) PAH retained some residual activity with these models, unlike human PKU. Thus, the TYR level in these animals was increased whereas TYR is maintained at a relatively low level in human PKU; 2) the inhibitors also inhibit TYR and tryptophan hydroxylases, thus introducing the possibility
of directly reducing brain neurotransmitter levels, perhaps to a much greater extent than in PKU.

In other efforts, mouse embryos (Denno and Sadler, 1990) and rat embryos (Roux et al., 1991) were cultured in the presence of PHE and/or its phenylketone metabolites. These two types of procedures either produced or simulated high maternal blood PHE levels and appeared to produce neural tube defects and some other PKU embryopathies. In these procedures the cultured embryos were placed in artificial physiologic conditions without a placental barrier, which is very unlike a normal pregnancy. Owing to these and other limitations, interest grew to create a genetic model. It was reasoned that, because such a model would be much closer to the actual cause of human maternal PKU, unrelated and artifactual effects would be minimized.

**Genetic mouse models for human PKU**

The genetics, biochemistry, and physiology of mice are well characterized and quite similar to humans (Fisher, 1997; Bedell et al., 1997). Mouse mutants with defined biochemical and genetic abnormalities have many potential applications in the study of human inborn errors of metabolism. For investigating the pathogenesis of genetic disease and potential therapies, mouse models of human genetic disorders can be valuable resources. Such models provide numerous experimental advantages; 1) they can have an identical or very similar mutation to known human PKU cases; 2) since the mutations are isolated in an inbred genetic background, the test and control animals are genetically similar in every respect except for the presence of the PKU mutations; 3) relatively large
numbers of affected progeny can be produced with ease; 4) more invasive assessments can be done; and 5) mice are easy to handle and manipulate for a well-controlled study. Therefore, mouse mutants can be excellent substitutes to overcome the shortcomings of human studies, including lack of important control groups due to ethical considerations. Further, the use of mice deals effectively with the issues of dietary noncompliance and genetic heterogeneity of human study populations.

Mouse genetic PKU models have been successfully generated by germline mutagenesis with $N$-ethyl-$N$-nitrosourea (McDonald et al., 1990a; Shedlovsky et al., 1993). Three distinct mouse PKU lines were created: $\text{Pah}^{\text{enu1}}$, $\text{Pah}^{\text{enu2}}$ and $\text{Pah}^{\text{enu3}}$ (reviewed in McDonald et al., 1990b; McDonald et al., 1990c; McDonald, 1995a; and McDonald, 1995b). The $\text{Pah}^{\text{enu1}}$ line has a similar metabolic phenotype to human mild HPA. The mouse lines $\text{Pah}^{\text{enu2}}$ and $\text{Pah}^{\text{enu3}}$ demonstrate the diagnostic features of human classical PKU: having greater than 1,200 μM of blood PHE with normal diet, less than 5% of normal hepatic PAH activity, and hypopigmentation while on normal diet. $\text{Pah}^{\text{enu2}}$ has proven especially desirable for such modeling studies because it reproduces better than $\text{Pah}^{\text{enu3}}$. Further, it has a mutation that is very similar to a known human PKU mutation (McDonald and Charlton, 1997).

These and other defects seem to arise in the offspring of mouse PKU mothers on normal diet because, as in human PKU mothers, a fetal-to-maternal PHE concentration gradient of 1.5-2.0 exists in favor of the fetus across the placenta (McDonald et al., 1997). Because of these similarities, the $\text{Pah}^{\text{enu2}}$ genetic mouse PKU model has been repeatedly mentioned by workers in the PKU field as holding great potential for studying
the pathogenesis of maternal PKU and to understand the relationship between dietary
PHE intake and fetal pathophysiology (Matalon et al., 1994; Scriven et al., 1995; Hanley
et al., 1996; Levy et al., 1996). For example, these models can be used to examine both
varying levels and types of dietary constituents with the goal of examining the mental
effects in exposed animals. Mutant females can be fed diets of differing compositions to
examine the effect on gestational outcome.

Recently, a completely defined synthetic diet was formulated for this mouse
model in consultation with Harlan-Teklad, Inc. (Madison, WI). This diet permits the
facile manipulation of the dietary PHE exposure level because it contains no protein,
provides all amino acids separately, and completely lacks PHE. A level of PHE in the
drinking water has been discovered that normalizes the blood PHE levels of homozygous
mutants. Homozygous mutant animals appear healthy while on this diet, get pregnant at
normal frequencies, and raise their litters to adulthood.
CHAPTER III

METHODS

1. Experimental design

Crosses: Test Cross: \( \Phi Pah^{enu2}/ Pah^{enu2} \times \sigma Pah^{enu2}/+ (BTBR) \)
Control Cross: \( \Phi Pah^{enu2}/+ \times \sigma Pah^{enu2}/ Pah^{enu2} (BTBR) \)

Table 2. Experimental design

<table>
<thead>
<tr>
<th>Dietary treatment group</th>
<th>T_0</th>
<th>T_3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal dietary condition</td>
<td>Amino acid diet + 0.625 g/l PHE</td>
<td>Amino acid diet + 6 g/l PHE</td>
</tr>
<tr>
<td>Maternal blood PHE range</td>
<td>90-120 (\mu)M</td>
<td>&gt;1200 (\mu)M</td>
</tr>
<tr>
<td>Spontaneous abortion incidence</td>
<td>Rare</td>
<td>Unknown</td>
</tr>
<tr>
<td>Other fetal pregnancy indicators</td>
<td>Birth weight Head circumference Crown-rump length</td>
<td>Birth weight Head circumference Crown-rump length</td>
</tr>
<tr>
<td>Fetal nutritional indices</td>
<td>PHE, Normal TYR, Normal Other EAA, Normal</td>
<td>PHE, High TYR, Low Other EAA, unknown</td>
</tr>
</tbody>
</table>
2. Mouse strains and diets

a. Mouse strains

This experiment was approved by the Wichita State University Animal Care and Use Committee. *BTBR-Pah* \textsuperscript{enu2} mutant and *BTBR* control mice were obtained from a colony maintained by Dr. McDonald in the animal holding facilities of Wichita State University and from the Jackson Laboratory (Bar Harbor, ME). The mice were housed in polycarbonate microisolator cages (Lab Products, Inc, Maywood, NJ). Their environment was maintained at a constant temperature of 22-24 °C, 50% relative humidity and a 12-hour light cycle (0600-1800h). The mice had free access to a normal mouse chow diet and water while they were mating. Females were bred individually with male mice. Homozygous (*Pah* \textsuperscript{enu2}/*Pah* \textsuperscript{enu2}) mutant females were crossed to heterozygous (*Pah* \textsuperscript{enu2}/+) males to produce litters that were roughly half homozygotes and half heterozygotes to provide homozygous and heterozygous mutants gestated in the same uterus. Control litters were produced by crossing heterozygous (*Pah* \textsuperscript{enu2}/+) females with homozygous (*Pah* \textsuperscript{enu2}/*Pah* \textsuperscript{enu2}) mutant males. Females in mating cages were checked every morning for the presence of a copulatory plug. Noon on the day of the plug was designated 0.5 days postcoitum (dpc). Upon plug detection, females were assigned to one of the two experimental diets (i.e., T\textsubscript{0} vs. T\textsubscript{3}). Females were provided with drinking water *ad libitum* with an appropriate PHE concentration (0.625 g/l:T\textsubscript{0}, 6 g/l:T\textsubscript{3}) to produce the desired maternal blood PHE concentration. A synthetic amino acid defined diet devoid of PHE was given to provide for other nutritional needs (Table 2).
At 18.5 dpc, the female mice were euthanized by cervical dislocation and their abdominal cavity was opened to expose the internal organs. Maternal blood was collected with a microhematocrit capillary tube from the dorsal aorta for the determination of PHE and other amino acids. PHE concentrations were determined by a fluorometric assay, described in Sigma Diagnostics Procedure No. 60-F (McCaman and Robin, 1962). The fetuses were collected by grasping the cervix with blunt-ended forceps and cutting it free from the vagina. Beginning at the ovary end of left uterine horn, the fetuses were dissected individually and left connected to the maternal blood supply for as long as possible (Figure 2). Blood at the neck of fetus was collected with a microhematocrit capillary tube and was used for fetal amino acid analysis. Tissues (fetal head) were flash frozen in liquid nitrogen and stored at −20 °C. DNA extracted from fetal tissues was used for sexing the fetuses.
Figure 2. Dissection for removing fetuses from the uterus. (A,B) The uterus is removed by cutting across the cervix. (C) The membrane (mesometrium) holding the uterus to the body wall is trimmed away, and the uterus is cut below the junction with the oviduct.
b. Diet

The synthetic amino acid (AA) diet, Teklad Research Diet #TD97152, (Harlan-Teklad, Madison, WI), contains all essential amino acids except PHE including 1.5% TYR; L-alanine (3.5 g/kg); L-arginine HCl (12.1 g/kg); L-asparagine (6.0 g/kg); L-aspartic acid (3.5 g/kg); L-cystine (3.5 g/kg); L-glutamic acid (40.0 g/kg); glycine (23.3 g/kg); L-histidine HCl·H2O (4.5 g/kg); L-isoleucine (8.2 g/kg); L-leucine (11.1 g/kg); L-lysine HCl (18 g/kg); L-methionine (8.2 g/kg); L-proline (3.5 g/kg); L-serine (3.5 g/kg); L-threonine (8.2 g/kg); L-tryptophan (1.8 g/kg); L-tyrosine (15 g/kg); L-valine (8.2 g/kg). The desired amount of PHE (0.0625 g/l or 6 g/l) was provided in drinking water. The synthetic AA diet also contains sucrose (488.38 g/kg); corn starch (150 g/kg); corn oil (100 g/kg); cellulose fiber (30 g/kg); mineral mix, AIN-76 (170915) (35 g/kg); calcium phosphate, dibasic (4.5 g/kg); vitamin mix, Teklad 40060 (10 g/kg); and ethoxyquin as an antioxidant (0.02 g/kg).

The AIN-76 mineral mix contains calcium phosphate, dibasic (500 g/kg); sodium chloride (74 g/kg); potassium citrate, monohydrate (220 g/kg); potassium sulfate (52 g/kg); magnesium oxide (24 g/kg); manganese carbonate (3.5 g/kg); ferric citrate (6 g/kg); zinc carbonate (1.6 g/kg); cupric carbonate (0.3 g/kg); potassium iodate (0.01 g/kg); sodium selenite (0.01 g/kg); chromium potassium sulfate (0.55 g/kg); and sucrose (118.03 g/kg).

The Teklad 40060 vitamin mix contains p-aminobenzoic acid (11.0132 g/kg); ascorbic acid, coated (97.5%, 101.6604 g/kg); biotin (0.0441 g/kg); vitamin B12 (0.1% trituration, 2.9736 g/kg); calcium pantothenate (6.6079 g/kg); choline dihydrogen citrate
(349.6916 g/kg); folic acid (0.1982 g/kg); inositol (11.0132 g/kg); menadione (4.9559 g/kg); niacin (9.9119 g/kg); pyridoxine HCl (2.2026 g/kg); riboflavin (2.2026 g/kg); thiamine HCl (2.2026 g/kg); dry vitamin A palmitate (500,000 U/g, 3.9648 g/kg); dry vitamin D3 (500,000 U/g, 0.4405 g/kg); dry vitamin E acetate (500 U/g, 24.2291 g/kg); and corn starch (diluent) (466.6878 g/kg).

3. Pregnancy outcomes

a. Spontaneous abortion incidence

Previous work showed that when fetuses are gestated on the amino acid diet with the Tₐ level of PHE in the water, the spontaneous abortion rate seems low. However, the spontaneous abortion rate seen when PKU mothers were maintained on normal mouse chow was 30-40%, leading us to suspect that the incidence would be common when extreme hyperphenylalaninemia was produced with the defined diet. Females were checked daily for evidence of complete or partial litter loss and scored accordingly. A positive score resulted from either complete or partial litter loss before term.

b. Head circumference, crown-rump length and birth weight

Size and weight data were grouped according to sex so as to avoid possible sex-related differences in these parameters and instead to focus on differences that arise due to maternal dietary condition or maternal genotype. Shortly before birth at 18.5 dpc, offspring were collected by hysterectomy, cleaned of any remaining membranes or placenta, bled for PHE determination, and weighed to determine their birth weight. Crown-rump length, a widely-used measure of fetal mouse development, was determined using an Iwanson Spring Caliper as described in Saad, 1990 (Figure 3). Maximum head
circumference was measured by wrapping a thread around the pup's head (Saad, 1990). Head circumference measurements were done in triplicate for each fetus and the mean value of the three measurements was used (Figure 3).
Figure 3. Crown-rump length and head circumference measurement
4. Fetal essential amino acids

It has already been established that PHE, TYR, and other essential amino acids are in the normal range for animals maintained on the amino acid diet. This study was designed to examine the effect of high maternal dietary PHE on the concentration of TYR and the other essential amino acids in fetuses gestated under these circumstances. Essential amino acids in small volumes of whole blood were quantified by the commercial testing service, Neogen, Inc. (Pittsburgh, PA), using isotope-dilution tandem mass spectrometry (Chace et al., 1993).

5. Spectrofluorometric Assay

A spectrofluorometric assay allowed the quantitative determination of PHE in plasma. A fluorescent compound is formed when ninhydrin reacts with PHE under conditions that minimize the contribution of other serum constituents that also fluoresce with this reagent. The fluorometric response from PHE is greatly enhanced by the presence of the dipeptide L-leucyl-L-alanine. The pH during the reaction was strictly controlled at 5.8 ±0.1 by means of a succinate buffer, in order to optimize fluorescence and maximize specificity. Blood was collected in a microhematocrit tube containing heparin, transferred to a 250 μl centrifuge tube, and centrifuged at 16,250 x g for 10 minutes to obtain plasma. An equal volume of 0.4 M TCA was added to samples containing at least 20 μl plasma. For plasma samples of less than 10 μl volume, 0.25 M TCA was added to give enough protein-free supernatant, which was used to incubate with ninhydrin-peptide solution. Dilution factors of 2 and 5 were taken into account for the
samples of 0.4 M TCA and 0.25 M TCA, respectively. Samples containing TCA were centrifuged for 5 minutes at 16,250 x g. Clear protein-free supernatant (20 μl) was added into a tube containing 300 μl ninhydrin-peptide solution. Ninhydrin-peptide solution was prepared by mixing succinate buffer (1.5 mM), L-leucyl-L-alanine (0.5 mg), and ninhydrin (5.4 mg). 20 μl of water for blank and 20 μl of a PHE standard solution were added into tubes containing 300 μl ninhydrin-peptide solution for a PHE standard curve. All standard and test samples were incubated in a 60 ±1°C water bath for 2 hours. After 2 hours of incubation, tubes were cooled down in cold tap water for about 3 minutes. Diluted copper solution (1.5 ml) (0.6 g/dl) was added to each tube and the tube was mixed by inversion several times. Samples were transferred to cuvettes for fluorescence determination using a Model 450 Digital Fluorometer (Barnstead/Thermolyne, Dubuque, IA). The fluorometer was adjusted to zero with a blank and the relative fluorescence of the standards and each test sample was recorded within 45 minutes at the excitation and emission wavelengths of 360 nm and 415 nm, respectively. The standard samples (1.25, 2.5, 5.0, 10, 20, 30, 40 mg PHE/dl) were used to calculate a dose response curve. The concentration of PHE in each test sample was calculated from the slope of that curve.

6. Isolation of genomic DNA from fetal tissues

a. DNA extraction from fetal tissues

Tissues were crushed to produce a fine powder amenable to proteinase K digestion. Approximately 0.2 - 0.3 g of tissues were ground in liquid nitrogen with a prechilled mortar and pestle. The finely powdered tissue was resuspended in 1.2 ml digestion buffer (100 mM NaCl, 10 mM Tris.Cl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% (w/v) sodium
dodecyl sulfate, 0.1 mg/ml proteinase K) per 100 mg tissue. The powdered tissue was lysed and digested by incubating the samples in digestion buffer overnight with shaking at 50°C. Afterwards, the samples were a viscous and indiscernible sludge. The DNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1), centrifuged at ambient temperature at 200 x g for 10 minutes. This step was repeated three times for pure DNA extraction. Finally, the top aqueous layer was transferred to a new tube, ½ volume of 7.5 M ammonium acetate was added, followed by 2 original volumes of 100% prechilled ethanol. The DNA immediately formed a stringy precipitate. The precipitated DNA was recovered by centrifuging at 200 x g for 2 minutes at 4°C. The DNA pellet was then rinsed with prechilled 70% ethanol and resuspended in TE buffer (10 mM TrisCl, pH 7.4, 1 mM EDTA, pH 8.0). A UV absorption DU-70 spectrophotometer (Beckman, Fullerton, CA) was used to estimate the concentration of DNA in a sample. The absorbance of nucleic acids at a wavelength of 260 nm is quantitative for a relatively pure nucleic acid preparation in at least microgram quantities. Proteins have peak absorption at 280 nm. The ratio of absorbance at 260 nm and 280 nm indicates the degree to which the nucleic acid has been purified from protein. A 260/280 ratio of 1.8 or higher is indicative of relatively pure DNA.

b. Elution of DNA from S&S Isocode ® stix PCR template preparation dipsticks

Using a microhematocrit capillary tube, blood was obtained and dispensed onto an Isocode ® dipstick (Schleicher & Schuell, Keene, NH, USA). The dipstick was then placed in a clean, dry container with desiccant for a minimum of 3 hours or baked at 80°C for 15 to 20 minutes. The dipstick was placed over an open sterile microcentrifuge
tube, while closing the cover, the end of the stick was pulled so that a special triangular section detached and fell directly into the tube. The triangle was rinsed with 500 \( \mu l \) of dH\(_2\)O by pulse-vortexing 3 times for at least 5 seconds each. After washing, water was removed with a sterile pipet, the tube was centrifuged at 16,250 \( \times \) g for 5 seconds, and residual droplets of water were pipetted off. Sterile water (50 \( \mu l \)) was added to the microcentrifuge tube and the tube was heated to 85°C for 30 minutes. After incubation, the tube was gently tapped 20 times, the supernatant was removed to a new microcentrifuge tube, and 5 \( \mu l \) template was used for 50 \( \mu l \) of a PCR reaction volume.

7. PCR-based genotyping of fetuses

The identity of the induced mutation of \textit{Pah}\textsuperscript{enu2} was characterized by DNA sequence analysis (McDonald and Charlton, 1997). The mutation in the \textit{Pah}\textsuperscript{enu2} line was predicted to create a new recognition site for the restriction enzyme either \textit{Alw} 26I (Promega Co., Madison, WI) or \textit{Bsm}A1 (New England Biolabs Inc., Beverly, MA) by altering the sequence from nucleotides 831 through 835 from GTCTT to GTCTC. To detect this restriction fragment length polymorphism, approximately 100ng of genomic DNA was used as a template for PCR amplification and gene-specific oligonucleotide primer pairs (Genosys Biotechnologies, Inc., Woodlands, TX) were used to amplify the exon 7 of mouse \textit{Pah} of 132bp. The upstream primer was 5'-

\begin{verbatim}
ACTTGTACTGGTTTCCGCCT-3'
\end{verbatim}

and the downstream primer was 5'-

\begin{verbatim}
AGGTGTTGTACATGGGCTTAG-3'
\end{verbatim}

PCR amplification was conducted with 50 \( \mu l \) reaction volume containing 320 \( \mu M \) dNTP (Boehringer Mannheim, GmbH, Germany) 1 \( \mu M \) each primer, 100 ng template, 0.5 units \textit{Tag} DNA polymerase (Promega, Madison,
2.5 mM MgCl₂. Mineral oil (Perkin Elmer, Branchburg, NJ) was added into reaction
tubes in order to prevent evaporation of water. Thermocycle parameters were as follows:
30 iterations of denaturation for 1 minute at 94°C, annealing for 1 minute at 60°C, and
extension for 1 minute at 72°C using Thermolyn Temptronic Thermocycler
(Barnstead/Thermolyne, Chicago, IL). Presterilized Aerosol resistant tips were used for
PCR amplification which are DNAse and RNAse free (Molecular Bio-Products, San
Diego, CA). Thin-Walled Gene Amp Reaction Tubes (Perkin Elmer Cetus, Branchburg,
NJ) were used. Digestion with either of the isoschizomers $Alw\ 26I$ or $Bsm\ Al$ yielded a
distribution of fragments with a distinct banding pattern for each of the three possible
genotypes. Wild type (+/+), generated restriction fragments of 82 and 50 bp,
heterozygotes ($Pah\ enu^2/+)$ generated fragments of 82, 50, 48, and 34 bp, and homozygous
mutants ($Pah\ enu^2/Pah\ enu^2$) generated fragments of 50, 48, and 34 bp.

After the PCR phase of the genotyping, gel electrophoresis was used in two
different ways to accomplish the overall assessment. First, a portion of the PCR reaction
was used for minigel analysis to assess the performance and specificity of each primer
pair. Small DNA fragments were separated by polyacrylamide gel electrophoresis using
The Sturdier Vertical Slab Gel Unit Model SE400 (Hoefer Scientific Instruments, San
Francisco, CA). The glass plates were assembled with spacers for casting the gel. Gel
solution was prepared for 7.5% of 29:1 acrylamide:bisacrylamide concentrations. 7.5% of
polyacrylamide gel gave a maximum resolution of DNA fragments from 60 to 400 bp. 25
μl of TEMED and 250 μl of 10% ammonium persulfate were mixed into gel solution. 1X
TBE (Tris/acetate/EDTA) electrophoresis buffer (89 mM Tris base, 89 mM boric acid, 2
mM ethylenediaminetetraacetic acid, disodium salt) was used to fill the lower reservoir of gel tank. After polymerization of the gel had completed, the comb was removed and the sample wells cleaned with buffer. The restriction fragments were loaded with loading buffer (0.05% (w/v) bromophenol blue, 0.05% Xylene cyanol (w/v), 20mM EDTA).

Either pGEM DNA (Promega, Madison, WI) or φX174DNA/HinfI marker (Promega, Madison, WI) was loaded with sample to determine the progress of DNA movement. Using a DC power supply, the gel was ran at about 5 V/cm, taking care to avoid excessive heating. Electrophoresis was conducted at 80 V for 3 hours. Gel plates were detached from electrophoresis apparatus and stained for 15 minutes in 0.5 μg/ml ethidium bromide and destained for 15 minutes in water. The DNA fragments were visualized under transilluminator Gel Doc1000 (Bio-Rad Laboratories, Hercules, CA) and photographed. After confirmation of desired PCR amplification product, 30 μl of the PCR amplification was digested with 2 units of either Alw26I or BsmAI adding it to reaction buffer and incubating for overnight. The DNA fragments digested by restriction enzyme were electrophoresed through a 20% nondenaturing polyacrylamide gel with 1xTBE buffer. 20% polyacrylamide gave maximum resolution of DNA fragments from 5 to 100 bp. Then the fragments were visualized by ethidium bromide staining (0.5 μg/ml) and ultraviolet illumination (Figure 4).
Figure 4. PCR-based genotyping method of fetuses.

A) Restriction enzyme sites of *Pah* exon 7
B) Lane 1, 6 (φX174DNA/*Hinf*I marker) DNA, lane 2 (wild type), lane 3 (heterozygotes), lane 4 (homozygotes), lane 5 (uncut DNA).
8. **PCR-based sexing of fetuses**

The genomic DNA isolated from tissues was used for the sexual identification of each fetus. Primers were designed to amplify the SMC gene, which gives a Y-band that is distinct in size from the X-band; females gave a single band and males gave two bands because of an SMC intron difference between the X and Y genes. SMC oligonucleotide primers were SMCX-1, 5'-CCGCTGCCAAATTCTTTGG-3' and SMC4-1, 5'-TGAAGCTTTTGGCTTTGAG-3' (Genosys Biotechnologies, Woodlands, TX). PCR amplification was conducted in a 50 μl reaction volume containing 800 μM dNTP (Boehringer Mannheim, Germany), 1μM each primer, 10 ng DNA template, 0.5 units Taq polymerase (Promega, Madison, WI), 1.5 mM MgCl₂. For a positive reaction control, λ DNA was used. For a negative reaction control, no DNA template was used. Thermocycle parameters were as follows: 35 iterations of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 58°C, and extension for 30 seconds at 72°C using a Thermolyne Temptronic Thermocycler (Barnstead-Thermolyne, Chicago, IL). Mineral oil (Perkin Elmer Cetus, Branchburg, NJ) was added into each PCR tube in order to prevent water evaporation and reflux during thermocycling. Presterilized aerosol-resistant pipette tips (Molecular Bio-Products, San Diego, CA) and thin-walled Gene Amp reaction tubes (Perkin Elmer Cetus, Branchburg, NJ) were used for PCR amplification. A portion of the resulting 132 bp-amplification product was used for minigel analysis to assess the performance and specificity of each primer pair. Small DNA fragments were separated by polyacrylamide gel electrophoresis using the Vertical Slab Gel Unit Model SE400 (Hoefer Scientific Instruments, San Francisco, CA). The glass plates were assembled
with 160 mm x 1.5 mm spacers for casting the gel. The gel solution was a 7.5% (v/v) solution of a 29:1 mixture of acrylamide:bisacrylamide in 1X TBE buffer (Tris/borate/EDTA, 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0). Such a gel provides maximum resolution of DNA fragments from 60 bp to 400 bp. 25μl of TEMED and 250 μl of 10% (w/v) ammonium persulfate were mixed into the gel solution to initiate polymerization. TBE (1X) electrophoresis buffer was used to fill the lower reservoir of the gel tank. After gel polymerization was completed, the comb was removed and the wells were cleaned with buffer. The PCR reactions (10 μl) were loaded after adding 1.1 μl loading buffer (0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 20 mM EDTA). Either pGEM (Promega, Madison, WI) or φX174DNA/Hind I marker DNA (Promega, Madison, WI) was also loaded on the gel with the PCR samples to determine the relationship between migration distance and molecular weight. Using a DC power supply, the gel was electrophoresed at a field strength of about 5 V/cm, taking care to avoid excessive heating. Electrophoresis was typically conducted at 80 V for 3 hours. Afterwards, the gel plates were detached from the electrophoresis apparatus and the gel was stained for 15 min in 0.5 μg/ml ethidium bromide and destained for 15 min in water. The DNA fragments were visualized under a Gel Doc1000 transilluminator (Bio-Rad Laboratories, Hercules, CA) and photographed (Figure 5).

9. **Statistical analysis**

Determinations providing continuous data (fetal developmental parameters and fetal nutritional indices) were analyzed by Student t-test. With this method, variance was analyzed by taking into account both test PHE level ($T_3$) vs. control PHE level ($T_0$) and
test cross offspring vs. control cross offspring. Two-by-two factorial analysis of variance (ANOVA) consisting of two types of maternal genotype and maternal diet was used to test for the interaction between maternal genotype and maternal diet.

Determination of discrete data (i.e., spontaneous abortion incidence, mole, stillbirth) and fetal genotyping results were assembled into contingency tables and analyzed by the chi-square analysis. In this case the contingency table contained the incidence of the particular defect under the control condition compared to the incidence of the same defect under the test condition.
CHAPTER IV

RESULTS

1. Pregnancy outcomes

a. Spontaneous abortion incidence and fetal loss

Spontaneous abortion was noted in a test T_3 treatment female who had already started to deliver three aborted fetuses at 18 dpc. An emergency hysterectomy was performed and, when the abdominal cavity was opened, one fetus was left in the right uterine horn and was found to be dead. Another spontaneous abortion was noted in a control T_0 treatment female. When the abdominal cavity was opened at 18.5 dpc, the right uterine horn was greatly dilated, indicating possible previous fetal occupancy. One fetus remained in the right uterine horn and, although dead, was developed to late developmental term.

Other forms of fetal loss, including moles and stillbirths were also noted. Moles are defined as early fetal death, which is in the process of being absorbed. On the other hand, stillbirth is defined as the fetus surviving to term but being born dead or late fetal death. In order to determine whether any classes are significantly different from others in the frequency of fetal loss, the data for moles, spontaneous abortion, and stillbirth were grouped together and compared by the chi-square test. Approximately 5 times greater fetal losses were observed in test females than in control females. It was noted that there was a significant difference in fetal loss between test females and control females.
(P<0.001). Noticeable phenomena were observed in the test T_3 treatment female. In three fetuses born to mothers in the T_3 test condition, the internal organs were not internalized and the skin was fragile and sticky. A deciduoma was noted in one fetus.

b. Head circumference, crown-rump length, and birth weight

Developmental parameters of pregnancy such as head circumference, crown-rump length, and birth weight showed a significant maternal diet effect in test females. All of these three developmental parameters were significantly reduced in T_3 test females compared to T_0 test females (P<0.0001). However, no significant maternal diet effects were observed in control females (P>0.05) for T_3 control vs. T_0 control. Further, there were significant maternal genotype effects on these developmental parameters of pregnancy in T_3 test females compared to T_3 control females. Significant reductions in birth weight, crown-rump length, and head circumference were observed (P=0.0019, P<0.0001, and P=0.05, respectively), but no significant maternal genotype effect in control females was observed (P>0.05) for T_0 test vs. T_0 control (Table 3).

After analyzing data by sex, it was noted that there was no significant difference between female and male fetuses regarding developmental parameters and other essential amino acid data. Therefore, all data for each variable from both females and males (Tables 4 and 5) were pooled to increase sample size shown in table 3.
Table 3. Diet and genotype effects in test vs. control offspring

<table>
<thead>
<tr>
<th></th>
<th>Mean (SEM)</th>
<th>Mean (SEM)</th>
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<tbody>
<tr>
<td></td>
<td>$T_3$ test</td>
<td>$T_6$ test</td>
</tr>
<tr>
<td></td>
<td>(n=80)</td>
<td>(n=85)</td>
</tr>
<tr>
<td>BW (g)</td>
<td>0.70 (0.02)</td>
<td>0.88 (0.02)</td>
</tr>
<tr>
<td>CR (cm)</td>
<td>1.77 (0.03)</td>
<td>1.95 (0.02)</td>
</tr>
<tr>
<td>HC (cm)</td>
<td>1.75 (0.02)</td>
<td>1.94 (0.02)</td>
</tr>
<tr>
<td></td>
<td>$T_3$ control</td>
<td>$T_6$ control</td>
</tr>
<tr>
<td></td>
<td>(n=17)</td>
<td>(n=5)</td>
</tr>
<tr>
<td>BW (g)</td>
<td>0.86 (0.03)</td>
<td>0.91 (0.04)</td>
</tr>
<tr>
<td>CR (cm)</td>
<td>2.07 (0.03)</td>
<td>2.18 (0.05)</td>
</tr>
<tr>
<td>HC (cm)</td>
<td>1.86 (0.03)</td>
<td>1.89 (0.02)</td>
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</tbody>
</table>

Table 4. Diet and genotype effects in test vs. control female offspring

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>$T_3$ test</td>
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<tr>
<td></td>
<td>(n=38)</td>
<td>(n=36)</td>
</tr>
<tr>
<td>BW (g)</td>
<td>0.70 (0.04)</td>
<td>0.88 (0.03)</td>
</tr>
<tr>
<td>CR (cm)</td>
<td>1.79 (0.04)</td>
<td>1.95 (0.03)</td>
</tr>
<tr>
<td>HC (cm)</td>
<td>1.77 (0.03)</td>
<td>1.93 (0.03)</td>
</tr>
<tr>
<td></td>
<td>$T_3$ control</td>
<td>$T_6$ control</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>BW (g)</td>
<td>0.87 (0.11)</td>
<td>0.93 (0.01)</td>
</tr>
<tr>
<td>CR (cm)</td>
<td>2.13 (0.09)</td>
<td>2.20 (0.00)</td>
</tr>
<tr>
<td>HC (cm)</td>
<td>1.83 (0.09)</td>
<td>1.88 (0.01)</td>
</tr>
</tbody>
</table>
Table 5. Diet and genotype effects in test vs. control male offspring

<table>
<thead>
<tr>
<th></th>
<th>Mean (SEM)</th>
<th>Mean (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T&lt;sub&gt;3&lt;/sub&gt; test (n=42)</td>
<td>T&lt;sub&gt;4&lt;/sub&gt; test (n=49)</td>
</tr>
<tr>
<td>BW (g)</td>
<td>0.69 (0.03)</td>
<td>0.89 (0.03)</td>
</tr>
<tr>
<td>CR (cm)</td>
<td>1.74 (0.05)</td>
<td>1.96 (0.03)</td>
</tr>
<tr>
<td>HC (cm)</td>
<td>1.74 (0.03)</td>
<td>1.95 (0.02)</td>
</tr>
<tr>
<td></td>
<td>T&lt;sub&gt;3&lt;/sub&gt; control (n=13)</td>
<td>T&lt;sub&gt;4&lt;/sub&gt; control (n=2)</td>
</tr>
<tr>
<td>BW (g)</td>
<td>0.86 (0.25)</td>
<td>0.88 (0.11)</td>
</tr>
<tr>
<td>CR (cm)</td>
<td>2.05 (0.02)</td>
<td>2.15 (0.15)</td>
</tr>
<tr>
<td>HC (cm)</td>
<td>1.87 (0.03)</td>
<td>1.92 (0.12)</td>
</tr>
</tbody>
</table>

2. Fetal essential amino acids

a. Fetal PHE and TYR concentrations

Significant maternal diet and maternal genotype effects were observed on PHE and TYR concentrations (P<0.0001, P<0.0001, respectively). Significantly increased PHE levels were observed in test female groups compared to control female groups (P<0.0001). Maternal blood PHE levels in T<sub>3</sub> test treatment females was increased more than 22 times compared to T<sub>3</sub> control females. This blood PHE level corresponds to classical PKU blood PHE levels of greater than 1,200 μM.

On the other hand, levels of TYR were significantly reduced in T<sub>3</sub> test treatment females compared to T<sub>3</sub> control levels (P<0.0001) (Table 6). Reduced levels of TYR is typical of
classical PKU concomitant with elevated blood PHE level. Interestingly, a two-by-two factorial ANOVA indicated a significant interaction of genotype and diet for TYR and PHE level (P<0.001). The average ratio of fetal-to-maternal PHE and TYR was 2.14 ±0.06, 2.69 ±0.08, respectively.

Table 6. PHE and TYR concentrations in test vs. control offspring

<table>
<thead>
<tr>
<th></th>
<th>Mean (SEM)</th>
<th>Mean (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T&lt;sub&gt;3&lt;/sub&gt; test (n=52)</td>
<td>T&lt;sub&gt;0&lt;/sub&gt; test (n=57)</td>
</tr>
<tr>
<td>PHE (μM)</td>
<td>2525.14 (107.73)</td>
<td>230.15 (60.26)</td>
</tr>
<tr>
<td>TYR (μM)</td>
<td>137.63 (4.97)</td>
<td>108.44 (3.88)</td>
</tr>
<tr>
<td></td>
<td>T&lt;sub&gt;3&lt;/sub&gt; control (n=17)</td>
<td>T&lt;sub&gt;0&lt;/sub&gt; control (n=5)</td>
</tr>
<tr>
<td>PHE (μM)</td>
<td>112.18 (18.60)</td>
<td>92.26 (15.80)</td>
</tr>
<tr>
<td>TYR (μM)</td>
<td>247.07 (17.99)</td>
<td>130.84 (24.75)</td>
</tr>
</tbody>
</table>

Initially, there was concern that PHE in the drinking water might lead to avoidance (i.e., homozygous females might drink less water because of the PHE addition). However, there were no differences in drinking water consumption between homozygous and heterozygous females. Average water consumption was 9-10 ml per day for both T<sub>0</sub> and T<sub>3</sub> levels. Further, there was concern that fetal genotype would contribute to fetopathy. The genotype test of approximately half of the fetuses examined in the study revealed that approximately 38% of fetuses were mutant homozygotes and 62% of fetal genotype were heterozygotes in a test T<sub>3</sub> cross examined. The chi-square test revealed that 38% was different from 62%, indicating the ratio of fetal genotype was not 1:1.
b. Fetal essential amino acids

The nutritional status of maternal PKU offspring was assessed by the levels of PHE, TYR, and other essential amino acids (EAA) at birth. Significant maternal diet effects were revealed for several large neutral amino acids in the offspring of mutant mothers with high blood PHE levels compared to those with low blood PHE levels. Significantly reduced levels of alanine, glutamine, and glutamic acid were observed ($P=0.0061$, $P=0.0033$, $P=0.0033$, respectively). Interestingly, increased levels of glycine, methionine, and histidine were observed ($P<0.0001$, $P=0.0045$, $P<0.0001$). On the other hand, there were no changes in valine, leucine, and aspartic acid ($P=0.30$, $P=0.83$, $P=0.40$).

Significant maternal genotype effects were also observed in the offspring of mutant mothers with high blood PHE level compared to those of heterozygous mothers exposed to high blood PHE levels. Reductions in the levels of alanine, glutamine, glutamic acid, methionine, valine, and histidine were observed ($P<0.0001$, $P=0.045$, $P=0.0001$, $P=0.012$, $P<0.0001$, $P=0.0061$). Interestingly, increased levels of glycine and no changes in leucine and aspartic acid were observed ($P<0.0001$, $P=0.08$, $P=0.28$) (Table 7).
Table 7. Diet and genotype effects on essential amino acids concentration

<table>
<thead>
<tr>
<th></th>
<th>Mean (SEM) T3 test (n=52)</th>
<th>Mean (SEM) T0 test (n=57)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>624.82 (30.26)</td>
<td>380.86 (11.70)</td>
</tr>
<tr>
<td>Alanine</td>
<td>446.55 (16.31)</td>
<td>508.60 (15.09)</td>
</tr>
<tr>
<td>Valine</td>
<td>201.43 (8.01)</td>
<td>190.07 (7.32)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>93.19 (4.02)</td>
<td>110.42 (4.07)</td>
</tr>
<tr>
<td>Leucine</td>
<td>286.15 (9.31)</td>
<td>283.47 (8.23)</td>
</tr>
<tr>
<td>Methionine</td>
<td>108.75 (5.19)</td>
<td>91.62 (3.06)</td>
</tr>
<tr>
<td>Histidine</td>
<td>65.68 (4.80)</td>
<td>34.49 (1.44)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>72.60 (4.46)</td>
<td>68.16 (2.89)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>219.60 (7.95)</td>
<td>250.81 (6.78)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean (SEM) T3 control (n=17)</th>
<th>Mean (SEM) T0 control (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>310.91 (14.67)</td>
<td>352.10 (67.36)</td>
</tr>
<tr>
<td>Alanine</td>
<td>745.28 (54.83)</td>
<td>478.67 (53.36)</td>
</tr>
<tr>
<td>Valine</td>
<td>284.46 (23.58)</td>
<td>211.61 (53.36)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>108.29 (4.01)</td>
<td>122.46 (25.36)</td>
</tr>
<tr>
<td>Leucine</td>
<td>317.02 (11.89)</td>
<td>303.14 (12.01)</td>
</tr>
<tr>
<td>Methionine</td>
<td>135.57 (8.97)</td>
<td>63.19 (50.44)</td>
</tr>
<tr>
<td>Histidine</td>
<td>94.70 (10.31)</td>
<td>57.33 (10.64)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>82.03 (6.89)</td>
<td>46.23 (10.18)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>286.31 (15.01)</td>
<td>214.30 (9.33)</td>
</tr>
</tbody>
</table>
3. PCR-based sexing of fetuses

Lane 1, 10 (pGem DNA marker), lane 2 (λ DNA positive PCR control), lane 3-6 (fetal samples), lane 7-8 (positive control for both female and male), lane 9 (negative control).

Figure 5. PCR-based sexing of fetuses.
Important PKU characteristics in the mouse genetic model

There is little doubt that at least one of the PKU-related biochemical abnormalities in the mother is responsible for the teratogenic effects of maternal PKU, regardless of the precise mechanism of pathogenesis. The toxic agent in maternal PKU is more likely excess PHE itself, rather than metabolites such as phenylacetic acid, phenylethylamine, phenyllactic acid or phenylpyruvate. Several human studies provide supportive lines of evidence. First, PHE itself can readily cross the placenta and create a transplacental concentration gradient (Schoonheyt et al., 1994). Second, after fetal tissues were examined, PHE was increased markedly while its metabolites were present in trace amounts (Levy et al., 1988). Third, a woman with a biochemical defect which produces accumulations of PHE metabolites, but not of PHE, gave birth to a normal child (Dorland et al., 1993).

Given the evidence from human studies, it has been widely acknowledged that the increased concentration of PHE is the primary suspect for the teratogenesis of maternal PKU. In humans, a transplacental PHE gradient in the human female favors the fetus from early pregnancy. A fetal-to-maternal ratio is on the order of 1.5-2.0 in maternal hyperphenylalaninemia, but inter-individual variation in the ratio is high, from 1.13 to 2.91, in clinical reports (Hanley et al., 1987). In this study, like human PKU mothers, mutant mouse mothers concentrated their already greatly elevated levels of PHE across
the placental barrier, yielding average ratios of fetal-to-maternal PHE and TYR of 2.14, 2.69, respectively. Such ratios are consistent with previous findings that a fetal-to-maternal PHE concentration gradient of 1.8 to 2.3 was exhibited among the progeny of mouse PKU females on normal mouse chow diet (McDonald et al., 1997). Furthermore, there was no increase in blood TYR level in this mouse model, rather, blood TYR levels were reduced in test females. Because the maternal blood PHE level exceeds 1,200 μM, which is accepted as an indicator of classical human PKU, it seems likely that the defects arising in this disease model share the same etiology as human maternal PKU. Further, the relatively reduced blood TYR levels in this mouse model signifies the characteristic of PKU.

Pregnancy outcomes of mouse maternal PKU

From what is known so far about maternal PKU, the syndrome results from a combination of maternal genotype and environmental factors. With regard to the genotypic basis of mutant mothers, a mutant maternal genotype is responsible for fetal abnormalities. All offspring are equally affected, indicating that the fetal genotype does not contribute to teratogenicity. With regard to the environmental basis, a treatment which reduces the maternal blood PHE levels during pregnancy can ameliorate the teratogenic effects of maternal PKU. In this study, we clearly see that elevated maternal blood PHE levels, produced by a combination of mutant maternal genotype and high dietary PHE concentration, are responsible for the teratogenic birth defects when examined by several developmental parameters of pregnancy outcome. First,
significantly reduced birth weights in females and males were noted among offspring born to mothers with high blood PHE level. Second, crown-rump length was significantly lower among offspring born to mothers with high blood PHE levels compared those mothers with low blood PHE levels. Third, significantly reduced head circumference was also indicated among offspring born to mothers with high blood PHE levels. The pregnancy endpoints of birth weight, head circumference, and crown-rump length of offspring were inversely affected by increasing maternal blood PHE concentration during gestation. These findings strongly suggest that in mouse PKU, as in human PKU, mutant maternal genotype and dietary PHE combine to produce a maternal PKU situation.

As reported in human studies, spontaneous abortion, moles, and stillbirth are also manifested in this mouse maternal PKU model. The incidence of spontaneous abortion was infrequent in the mouse PKU offspring compared to humans regardless of maternal genotype and maternal diet. This observation is quite different from a previous finding that the presence of spontaneous abortion comprises about 20% of total pregnancies in the Pah<sup>enu2</sup> PKU model on a normal mouse chow diet (McDonald, unpublished data). Although we do not understand the basis of the unexpectedly low frequency of spontaneous abortion on the defined diet, it must be at least one of the many factors that are different between the two diets. This finding is quite contradictory to human reports that the incidence of spontaneous abortion in PKU women is frequent (Lenke and Levy, 1980). Further, in humans it seems likely that there is a threshold level for the incidence of spontaneous abortion and spontaneous abortion is directly proportional to maternal blood PHE level. In humans, maternal blood PHE level is critical not fetal blood PHE
level. However, spontaneous abortion incidence is rare in mice. Further, it does not appear to have a threshold level to cause spontaneous abortion since the fetal blood PHE level was almost twice the level of classical PKU.

Total fetal losses including spontaneous abortion, mole, and stillbirth are significantly different between test and control groups. Significantly increased fetal losses were observed in homozygous mutant females compared to heterozygous females. It does appear that homozygous mutant fetuses are at a selective disadvantage during gestation, at least in the T3 test condition. One possible reason is that the maternal environment with high PHE level is so detrimental that some embryos are absorbed in early pregnancy and therefore are never identified. This finding is consistent with the genotype test of fetuses that made it to term. The offspring were not constituted by half homozygotes and half heterozygotes within the same maternal PKU uterus. This implies an increased selective disadvantage rate of fetal loss in mutant homozygotes.

**The fetal nutritional status of maternal PKU offspring**

Although the exact mechanism of pathogenesis in maternal PKU is unknown, the most compelling hypothesis to date relies upon data indicating that PHE shares active placental transport with several other neutral amino acids, including TYR, the BCAA’s (valine, isoleucine, and leucine) and methionine (Boyd and Yudilevich, 1987; Kudo and Boyd, 1990). Conflicting data have been reported in human studies regarding the level of BCAA’s. Levy et al., in 1988 were not able to find reductions in tissue levels of amino acids in human fetuses from untreated terminated maternal PKU pregnancies. On the
other hand, others have reported that high blood PHE levels caused depletions of most of the essential amino acids, with BCAA's being the most affected (McKean et al., 1968).

There is little experimental evidence for fetal amino acid depletion in the animal maternal PKU models. It was found that leucine uptake in fetal brain (Takeda et al., 1974), plasma and tissues (Okano et al., in 1986) was reduced in hyperphenylalaninemic pregnant rats. However, like in conflicting human data, none of the BCAA's was reduced in maternal PKU offspring. High blood PHE level does not seem to compete with other EAA for several reasons. First, none of BCAA's in fetal plasma were reduced in homozygous mothers. Second, there were significantly increased blood levels of glycine, methionine, and glutamic acid. Third, reductions in the blood level of alanine, glutamine, and glutamic acid were manifested.

From our findings in mouse PKU offspring, the most compelling hypothesis is that pathogenesis in maternal PKU does not seem to be caused by the sharing active transport systems with other EAA. Since reduced EAA was not manifested in mouse PKU offspring, it does not appear that the reduced bioavailability of EAA for the growing fetus caused by PHE-induced interference results in pathophysiologic effects on fetal development. Furthermore, already reduced levels of some EAA were manifested in offspring of homozygous mothers with high blood PHE levels compared to those of heterozygous mothers exposed to high blood PHE levels. Therefore, PKU-related biochemical abnormalities rather than PHE-induced interference seem the most likely cause of reductions in bioavailable EAA for the growing fetus.
These findings in our study strongly suggest that, as in human PKU, an important maternal PKU component has been identified in this mouse model. Therefore, it seems likely that this genetic PKU mouse model will be a valuable substitute to overcome the shortcomings of human studies. Such a model can also override some problems from other types of experimental maternal PKU animal models. For example, those experimental animal models are typically pharmacological models, combining inhibitors of PHE hydroxylation with a PHE load. These models may create side effects, if the inhibitor reaches the fetus. In that case, it will be hard to dissociate its effect from the effect of PHE itself on the fetus (Wooley and van der Hoeven, 1965; Immamura et al., 1993; Diamond et al., 1994).

4. Fetal genotype considerations

Maternal PKU is capable of causing embryopathy and fetopathy in even nonphenylketonuric offspring. Its consequences include microcephaly, mental retardation, and impaired somatic growth in over 80% of exposed fetuses. The sole determining factors seem to be the maternal diet and maternal genotype. By contrast, hyperphenylalaninemia in a father does no harm to offspring (Scriver et al., 1995).

However, these conclusions are so far based on data from human studies which, are in some important respects, uncertain. It is not possible, therefore, to completely exclude the possible contribution of the fetal genotype. Therefore, it is prudent to more rigorously examine the presence or lack of a fetal genotype contribution to fetopathy by producing
half homozygous and half heterozygous offspring within the same maternal PKU uterus. Therefore, the genotype of the crosses were as follows: test cross- $\varnothing Pah^{enu2}/ Pah^{enu2} \times \varnothing Pah^{enu2+/+}$, control cross- $\varnothing Pah^{enu2+/+} X Pah^{enu2+/+}$ (BTBR). These crosses produces a genotypically mixed populations of fetuses, which allow us to investigate any fetal genotype effects.

It is clear from our data that, although genotypically mixed groups of offspring were produced, the genotype differences were inconsequential. All offspring seemed to be equally affected, indicating that the fetal genotype did not contribute to teratogenicity. If there were fetal genotype effects, then there would be two populations and two different population means. The presence of two different means would be revealed by a high variance. Our data is characterized by a very low variance. Such small variances are emblematic of a lack of fetal genotype effect.
REFERENCES


