HYPERPHENYLALANINEMIA AND MENTAL RETARDATION
THE EFFECTS OF A HIGH MATERNAL PHENYLALANINE
BLOOD CONCENTRATION ON MOUSE OFFSPRING

APPROVED:

[Signature]
Jack R. Hay
Major Professor

[Signature]
Harold D. Holloway
Minor Professor

[Signature]
Dwane Kingery
Dean of the School of Education

Dean of the Graduate School
HYPERPHENYLALANINEMIA AND MENTAL RETARDATION:
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BLOOD CONCENTRATION ON MOUSE OFFSPRING

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By

Stephen A. Mozara, Jr., B. A.
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CHAPTER I

INTRODUCTION

Researchers have found that the mental deficiency of phenylketonuria in children can be prevented or favorably modified if a special low-phenylalanine diet is started early in life (26). Clinical findings have shown that this restricted diet can be terminated at approximately age five without any adverse effects even though the child continues to have an abnormal metabolism (21, 26). However, recent evidence of intrauterine retardation in offspring of phenylketonurics raises the question of whether or not the restricted diet should be re-established when a phenylketonuric mother becomes pregnant (31, 32). The congenital retardation is not the result of a genetic factor because the damaged children in question are not genotypically phenylketonuric. It is thought by Mabry that the abnormal maternal phenylalanine metabolism has a direct in utero effect on the growing fetus (32). In effect, this can be much worse than the child's inheriting phenylketonuria, because with phenylketonuria he can be put on a therapeutic diet and the retardation arrested. It has been found that with intrauterine retardation the damage is inevitable and the child is born irreversibly retarded (42).

This study was concerned with setting up a similar situation wherein pregnant mice had an abnormally high
phenylalanine metabolism. Through physical and intellectual assessment of their offspring, it would then be possible to determine what effects the abnormal metabolism had during pregnancy and whether or not a restricted diet need be resumed at that time.

In order to better understand the present problems facing these treated phenylketonuric females, a review of the history, dynamics, clinical course, and treatment of phenylketonuria is appropriate. In the early 1930's the Norwegian physician and biochemist Ashborn Folling was confronted with two mentally retarded children who produced a strange odor (7). Upon examining the children, Folling discovered that the urine of both reacted with ferric chloride to produce an unusual green color. He was able to prove that this color was due to the presence of phenylpyruvic acid, which he crystallized in pure form from the urine sample. The strange odor was found by Folling to be linked with phenylacetate. This same ferric chloride test and subsequent modifications paved the way for present-day early detection and treatment of phenylketonuria (7).

As news spread of the newly discovered "Folling's Disease," as it was called, screening programs were initiated in institutions for the mentally retarded. These programs resulted in incidence rates, genetic etiology, clinical course, and eventual mapping out of the metabolic error (22).
The name phenylketonuria was suggested because phenylpyruvic acid, the substance responsible for the green color reaction with ferric chloride in the urine, is a phenylketone (38). The disorder has also been known as phenylpyruvic oligophrenia. Clinical usage, however, has allowed for its rather lengthy name to be abbreviated simply as PKU. This abbreviation will be used throughout the remainder of this text.

Through screening programs it has been found that PKU occurs in one-half to one percent of all institutionalized mental defectives. From these results it has been estimated that the disorder occurs once in every 20,000 to 40,000 live births (22, 1). More extensive testing of blood phenylalanine levels of newborn babies in the United States implies that the incidence approaches one in 10,000 live births (17). Both sexes are equally affected, and all races tend to be involved, although incidence is higher in Europeans (22) and lower in Ashkenazi Jews (8) and Negroes.

It was early recognized that PKU was of genetic origin for the following reasons: It often involved more than one child in the family; the parents were normal, and approximately one in four children in the involved family had the disorder (15). This information led to the conclusion that PKU was inherited by a simple autosomal, recessive gene. Thus, for each pregnancy of a couple heterozygous for the PKU gene, there is a one in four chance that the child will
be PKU and a three in four chance of a phenotypically normal child.

Langdell, a researcher in the area, referred to PKU as a paradigm of the concept of a twisted mind from a twisted molecule. He stated that:

As a recessively inherited disease (not sex-linked), phenylketonuria occurs only if a baby inherits from each parent a "twisted" DNA (deoxynucleic acid) molecule that has one segment (gene) with disruption of the four amino acids that record the genetic code. Because of this defect, the DNA molecule produces an RNA (ribonucleic acid) that lacks the proper code message to form the enzyme, phenylalanine hydroxylase, that normally converts the essential amino acid phenylalanine to tyrosine in the liver. This chemical blockage dams up the normal metabolic flow, causing a rising tide of phenylalanine in the bloodstream that reaches abnormal levels 20 times the normal limit of 2 mg./100 ml. A metabolite, phenylpyruvic acid, "spills over" the kidney threshold into the urine after the serum phenylalanine level reaches 10 to 15 mg./100 ml. (29, p. 49).

This "spilled over" phenylpyruvic acid is the same metabolite that Polling discovered in the urine samples of the two mentally retarded Norwegian children, and it is the same metabolite which led to the mapping out of the metabolic error.

A point to be emphasized is that phenylalanine is an essential amino acid. Amino acids make up proteins and nearly all foods supply protein containing five percent phenylalanine; hence, a child who inherits PKU, although normal at birth, will rapidly begin to build up a high phenylalanine blood level due to the ingestion of milk, the basic diet of all infants, which is high in protein.
Researchers believed that the continued high level of phenylalanine or its related metabolites was responsible directly or indirectly for the mental retardation. In 1953 dietary treatments low in phenylalanine were first described (4, 45, 2). These diets have since proven to be adequate in controlling the phenylalanine blood concentration and result in a physically and mentally normal child (9, 21, 26).

Although the exact mechanism responsible for the adverse effects of elevated levels of phenylalanine or abnormal metabolites of phenylalanine metabolism has not been clarified, a number of facts have emerged. Experimental evidence (18, 13, 35, 39) reveals an inhibitory effect on enzyme systems active in significant metabolic and transport processes. Serotonin, a most important neurohormone, and its products are known to be inhibited in untreated PKU patients and can be normalized under therapeutic dietary control (37). In addition, there has been shown to be a failure or loss of myelination in about a third of PKU patients examined histologically (11). On the other hand, there has been an occasional case of untreated PKU reported without adverse effects. This has served to point out the many yet unanswered questions concerning the biochemical dynamic of this abnormality.

The untreated PKU patient is born apparently normal; then he begins to show retardation early in life. A subtle
change may be noticed at about three or four months of age. Apathy is evident and development is slowed down or arrested until by two or three years of age most are in the severely retarded range of intelligence. Ninety per cent of the known untreated cases have fallen into this bracket (22, 36). Irritable behavior, convulsion seizures, and abnormal electroencephalograms have been frequent. Other signs have included a positive Babinski, ankle clonus, eczema, the musty odor of phenylacetate, and the characteristic pale skin, bleached hair, and blue eyes resulting from disturbed tyrosine metabolism that interferes with the formation of melanin pigmentation.

For untreated PKU children the average age for sitting alone is from twelve to fifteen months; the average age for walking is two and one-half years, for talking three and one-half years. Some never learn to walk, and many never learn to talk.

Two basic tests are used for screening infants, urine tests and blood tests. Urine tests include the Ferric Chloride Test Tube Test (15), Ferric Chloride Diaper Test (6), Phenistic (R) Test (40), and the Dinitrophenylhydrazine Test Tube Test (39). The Ferric Chloride Test Tube Test is the oldest, best known, and most widely used of any of the urine tests for PKU. The Dinitrophenylhydrazine Test Tube Test is the most sensitive and reliable of the urine tests but is prohibitively expensive and difficult. The Guthrie
Inhibition Assay Test is the major blood test used (16). It is a simple and inexpensive test and can be performed in large volume. Blood tests are preferred over urine tests for one major reason. For phenylpyruvic acid to be present in the urine and result in a positive ferric chloride test, the phenylalanine blood level must exceed renal threshold. By the time this level is attained, neural damage can already have taken place. On the other hand, the blood test can detect an abnormal phenylalanine level in the blood before it builds up to renal threshold and is capable of beginning neural damage.

If screening tests show positive three consecutive times, then quantitative serum phenylalanine determinations are used for final confirmation and clinical care (16). These various methods (3, 19, 20, 28, 33) are too complex for description here.

With final confirmation of PKU it is recommended that the child be put on the therapeutic diet as early as possible, because the greatest damage tends to take place in the first months of life (32). Knox (26) has calculated that an average of half an I.Q. point is lost each week that treatment is delayed within the first three years of life. Older patients have to be considered individually, but it has been found that the diet can lead to limited intellectual, emotional, and physical improvement even in these progressed cases (10).
All low-phenylalanine diets are based on synthetic foods which provide all the essential amino acids excluding phenylalanine. This material is commercially produced by hydrolysing milk casein and removing phenylalanine by filtration. Other amino acids removed in the processing must be replaced. It is then fortified through activated charcoal.

The aim of this diet is to lower the blood phenylalanine from the abnormally high levels caused by the disease to near-normal levels while promoting normal body growth by providing the essentials for good nutrition. However, this diet is so low in phenylalanine that it must be supplemented with carbohydrates, oil, minerals, and vitamins. Otherwise, there would be poor growth and a paradoxical rise in serum phenylalanine due to catabolism of body protein.

Low-phenylalanine products available in Europe include Cymogren (12) and Minafen (34). In the United States Lofenalac (30) has replaced Ketonil (25). Dietary tables accompanying these products enable maintenance of near-normal phenylalanine levels. In addition, repeated blood tests are needed to guide adjustment of this diet.

Presently, scattered experiences have indicated that children over three have maintained their I.Q.'s upon termination of the diet. Although the majority of researchers feel that age four is a safe time for discontinuing the diet, some believe that termination should not occur until adolescence (27, 5).
In addition, recent evidence (31) suggests that treated PKU females who are desirous of pregnancy may have to return to their low-phenylalanine diet during the entire pregnancy. This particular idea is beginning to receive added attention and is felt to have been associated with many unexplained cases of mental retardation which might have been prevented.

In support of this concept Mabry (32, p. 5) reported the incidence of three PKU mothers giving birth to children who were not genetically PKU, but who were, nevertheless, mentally retarded. He felt that "this observation supports the concept that a high blood phenylalanine level in the pregnant woman causes irreparable damage to the otherwise normal brain of her unborn child."

Stevenson (42) gave an account of two PKU sisters on non-restricted diets whose pregnancies had resulted in sixteen spontaneous abortions, six offspring who died in infancy, and four presently surviving children. He stated that all live births to these females had varying combinations of premature birth weights, microcephaly, cardiac defect, mental and physical retardation, dislocated hips, and strabismus. He emphasized that none of these children had PKU.

Numerous observations (14, 21, 24, 26) have shown that before birth and the early months of life are the most vulnerable period for the brain. These observations aid in understanding why the PKU child's brain is not damaged when he is taken off the therapeutic diet at approximately age
five. That is to say that even though the child's phenylalanine blood concentration returns to its previously harmful levels upon termination of the diet, there is no neurological damage evidenced because the brain has passed its vulnerable growth period.

Kerr and his colleagues (24) have recently demonstrated that amino acids are actively transported across the mammalian placenta. In addition, he found that the concentrations in fetal blood are higher than the maternal concentrations.

Kang and Paine (23) found that pregnant women heterozygous for PKU had a higher blood phenylalanine concentration than both normal pregnant and non-pregnant PKU heterozygote controls. Mabry (32) felt this evidence enough to suggest the possibility that the normal placental process might magnify the maternal biochemical error and produce an even more profound disturbance in the fetus.

Mabry emphasized that all these observations supported the concept of transplacental hyperphenylalaninemia-induced brain damage. He stressed this by stating:

When family studies, experimental data and genetic information described above are considered as a whole, we are led to believe that the phenylketonuric mother's hyperphenylalaninemia and related plasma alterations damage the brain of her otherwise normal fetus and that maternal phenylketonuria is causing a clinically significant number of retarded children. An immediate implication is that this form of mental retardation is avoidable, either by prevention of the pregnancy or by induction of a lowered blood phenylalanine concentration during gestation (32, p. 1336).
Although a number of researchers (41, 44) have simulated PKU in animals through dietary manipulation and injection of phenylalanine, nearly all of this was induced after birth. There has been little work in the area of simulating a situation wherein intrauterine effects could be studied.

The one related study, to date, was performed by Thompson and Kano (43). Through dietary manipulation a hyperphenylalaninemia situation was induced in pregnant female rats, and offspring were evaluated. No significant physical or intellectual deficits were observed; however, the authors noted a temperament or emotional difference between the control and experimental groups. They concluded that there was an emotional change but not an intellectual one.

Considering the lack of research in this area and its importance in the field of mental retardation and learning, it was the purpose of this study to simulate a situation of PKU in pregnant mice in order to study the physical and intellectual difference between offspring of pregnant mice intubated with a solution of phenylalanine and offspring of pregnant mice intubated with a placebo. Fetal and postpartum deaths were utilized as physical measures while learning ability in a maze was used as an intellectual measure.
The assumption was made that a high maternal phenylalanine blood concentration in mice would lead to significantly more physical and intellectual abnormalities than in the offspring of mice having a normal maternal phenylalanine blood concentration. Having made the assumptions that phenylalanine is in a solution, that such a solution can cross the placental barrier to cause intrauterine growth retardation, and that the earlier in pregnancy that the transport takes place, the greater the retardation effect, the following hypotheses were assumed.

1. Offspring of pregnant mice intubated with a phenylalanine solution will show a significantly higher incidence of morbidity and/or mortality than those offspring of pregnant mice intubated with a placebo.

2. Offspring of pregnant mice intubated with a phenylalanine solution will perform significantly less adequately in a learning situation than will offspring of pregnant mice intubated with a placebo.

3. Offspring of pregnant mice intubated with a phenylalanine solution early in pregnancy will show a significantly greater physical and intellectual deficit than offspring of pregnant mice intubated with a phenylalanine solution late in pregnancy.
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18. Hanson, A., "Inhibition of Brain Glutamic Acid Decarboxylase by Phenylalanine Metabolites," Naturwissenschaft, VI (October, 1958), 423.


CHAPTER II

METHOD

Subjects

Breeding stock consisted of sixty naive mice of the colony C 57 BL/6J from the Jackson Memorial Laboratories. All mice were four months old at breeding. The resulting offspring, which made up the experimental and control groups, numbered one hundred and forty. These were tested at six months of age. This particular strain was selected because it has been found to be generally resistant to the effects of phenylalanine and therefore does not bias the results in studies utilizing this amino acid. All subjects were maintained on ad libitum food and water.

Apparatus

A four-unit, water-filled, T maze was employed. The maze was similar in design to one used by Biel (1) for rats but was scaled down to accommodate mice (see Appendix).

Construction of the maze was designed to conform to the dimensions of an unpainted stainless steel tank, 18" long, 14" wide, and 14" deep. The 14"-high walls of the maze proper were constructed of eighteen-gauge galvanized sheet metal and were left unpainted. The goal consisted of an 8"-long wire ramp which ascended at a 45° angle to a wire platform 2" wide.
Water depth was 7", preventing subjects from touching the bottom, and the walls were slippery enough to prevent resting. Water temperature was held at a constant 15° centi-grade as a means of motivation. Also held constant were room temperature, lighting and sound. A standard stopwatch was used to time runs.

Procedure

Breeding was commenced by placing two females with one male per cage. Daily pregnancy tests were made at 9:00 A.M. This was found by a pilot study to be the optimal time for detection of vaginal or coital plugs. The vaginal plug method, one of the most reliable, according to Rugh (2), consists of observing for the occurrence of dried mucous formation at the opening of the vagina, in addition to local swelling and reddening. The plug must be sought within a few hours of formation because it soon dried out and crumbles and can be torn down by urination.

Upon detection of pregnancy, each female was randomly assigned to an Early, Middle or Late Pregnancy Experimental or an Early, Middle or Late Pregnancy Control group and placed in an individual cage to await daily intubation. Subjects remained in these individual cages until the pregnancy came to term.

At parturition the offspring were counted and observed for morbidity, then left with their mothers until weaning at
three weeks of age. All efforts were exercised to avoid excessive handling of the pups since this could have led to their destruction by the mother.

At four weeks of age the pups were isolated to prevent mating. Males were caged individually, and females were placed two to a cage.

All subjects were checked daily for physical progress and activity level until maze testing began at six months of age. A pilot study showed this age to possess sufficient strength for the swimming task.

The first day of maze activity began with each mouse receiving three training trials in the 18" straight channel. This consisted of placing the mouse at the end of the straight channel opposite the exit ramp and timing him to assess swimming ability. An additional purpose was to familiarize the subjects with swimming in a straight channel. A guillotine door closed off the remaining channels.

Actual maze transit was begun on the second day and continued for a total of eight days. A maze transit run consisted of placing an individual subject in the end of the start channel with his head facing in the correct direction. Timing began the moment he touched the water and was stopped the moment his forepaws touched the exit ramp. Each mouse was allowed three minutes to reach the goal. In the event the subject could not complete the maze in three minutes, he was guided through to the goal with a glass pestle. As
soon as he reached the ramp and climbed to the platform, each subject was dried with a clean laboratory towel and placed in his individual cage. All subjects received three trials per day for eight days. With each trial, maze transit time and number of errors were recorded. An error was the entry of the complete body into a cul. Throughout testing only one mouse at a time was allowed in the maze.

Intubations

Intubations were performed with a one-cubic-centimeter tuberculin syringe fitted with an 18-gauge needle. Fastened over the tip of the needle was a three-inch section of #190 Intramedic Polyethylene Tubing.

A table-mounted wire triangle with a one-inch base and five-inch sides was employed to assist in keeping the mouse's mouth open as the tube was inserted into its esophagus. The phenylalanine was then introduced into the stomach for absorption, by depressing the plunger in the syringe.

All intubation dosages of the experimental amino acids, DL phenylalanine, were made according to the ratio suggested by Schalosk and Klopfer (3). This suggested ratio of three grams DL phenylalanine per kilogram of body weight resulted in an intubation dosage of .18 milliliter of phenylalanine solution. The phenylalanine solution was prepared as follows: five grams of DL phenylalanine were added to 100 milliliters of sterile physiological saline (isotonic sodium chloride.
the solutions were clear. Intubations were administered at 40° centigrade.

A standard intubation procedure was carried out for all subjects, in that all experimental subjects received .18 milliliters of DL phenylalanine solution and all control subjects received .18 milliliters of sterile physiological saline. Extreme caution was exercised in seeing that all subjects were handled in the same way throughout intubation and that the only treatment differential was what was being intubated and not how, or the amount.

The intubation schedule according to groups was as follows:

The Early pregnancy groups (both experimental and control) received their first intubation on the seventh day of pregnancy. These constituted the 1?E and 1?C groups respectively. They then received one intubation per day every day thereafter until parturition.

The Middle pregnancy groups (both experimental and control) received their first intubation on the twelfth day of pregnancy. These constituted the 12E and 12C groups respectively. They then received one intubation per day every day thereafter until parturition.

The Late pregnancy groups (both experimental and control) received their first intubation on the seventeenth day of pregnancy. These constituted the 7E and 7C groups,
respectively. They then received one intubation per day every day thereafter until partuition.

Attempt was made to hold the variables of time, temperature, place, and sound constant throughout intubations.
CHAPTER BIBLIOGRAPHY


CHAPTER III

RESULTS

In an attempt to measure the effects of certain maternal amino acid levels on the developing mouse fetus, thirty-six female mice were administered DL phenylalanine at various stages of pregnancy.

Table I represents the mortality rates of all offspring of the thirty-six subjects as observed from birth to ten days following birth.

<p>| TABLE I |
|---|---|---|---|---|---|
| TOTAL PERCENT OF SURVIVING OFFSPRING IN EACH GROUP |</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>17C</th>
<th>17E</th>
<th>12C</th>
<th>12E</th>
<th>7C</th>
<th>7E</th>
</tr>
</thead>
<tbody>
<tr>
<td>93%</td>
<td>100%</td>
<td>100%</td>
<td>50%</td>
<td>97%</td>
<td>27%</td>
<td></td>
</tr>
</tbody>
</table>

The similarity of all control groups indicates no significant effects owing to intubation of a placebo. In addition, the similarity of the Late Pregnancy Experimental Group (17E) to its matched Control Group (17C) indicates no significant effects owing to the experimental treatment. However, it is clearly shown that the Middle Pregnancy Experimental Group (12E) and Early Pregnancy Experimental Group (7E) experienced a treatment effect.
A $t$ test for differences between proportions was used to determine the significance of differences between the control and experimental groups at each level. Table II contains the results of these tests.

**TABLE II**

PERCENTAGES AND LEVELS OF SIGNIFICANCE BETWEEN C AND E GROUPS

<table>
<thead>
<tr>
<th>Groups</th>
<th>Percent Survivors</th>
<th>N</th>
<th>t</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>17C</td>
<td>93</td>
<td>28</td>
<td>1.36</td>
<td>56</td>
<td>P=.1</td>
</tr>
<tr>
<td>17E</td>
<td>100</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12C</td>
<td>100</td>
<td>30</td>
<td>4.42</td>
<td>43</td>
<td>P=.001</td>
</tr>
<tr>
<td>12E</td>
<td>50</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7C</td>
<td>97</td>
<td>29</td>
<td>5.51</td>
<td>35</td>
<td>P=.001</td>
</tr>
<tr>
<td>7E</td>
<td>27</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It can be seen in Table II that no significant difference was found between 17E and 17C groups. On the other hand, a high degree of significance is found between both 12E and 12C and between 7E and 7C, thus indicating a treatment differential for both these groups.

In addition, $t$ tests for proportions were calculated between all E groups. Table III contains the results of these tests.

The high degree of significance illustrated in Table III between 17E and 12E and between 17E and 7E supports a relation
### Table III

**Percentages and Levels of Significance Between E and E Groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Percent Survivors</th>
<th>N</th>
<th>t</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>17E</td>
<td>100</td>
<td>30</td>
<td>4.42</td>
<td>43</td>
<td>P=.001</td>
</tr>
<tr>
<td>12E</td>
<td>50</td>
<td>15</td>
<td>3.12</td>
<td>36</td>
<td>P=.001</td>
</tr>
<tr>
<td>17E</td>
<td>100</td>
<td>30</td>
<td>5.90</td>
<td>36</td>
<td>P=.001</td>
</tr>
<tr>
<td>12E</td>
<td>50</td>
<td>15</td>
<td>1.80</td>
<td>21</td>
<td>P=.1</td>
</tr>
<tr>
<td>7E</td>
<td>27</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Between the stages of pregnancy in which phenylalanine intubation took place and the resulting rate of mortality. However, no such significant difference was found to exist between 12E and 7E as concerning this variable.

Table IV contains the results of eight days of maze training and testing as expressed in mean errors for each group per day.

### Table IV

**Group Mean Maze Errors Per Day**

<table>
<thead>
<tr>
<th>Group</th>
<th>17C</th>
<th>17E</th>
<th>12C</th>
<th>12E</th>
<th>7C</th>
<th>7E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>2</td>
<td>3</td>
<td>1.86</td>
<td>2.80</td>
<td>2.30</td>
<td>4</td>
</tr>
<tr>
<td>Day 2</td>
<td>1.80</td>
<td>1.78</td>
<td>1.36</td>
<td>1.80</td>
<td>1.70</td>
<td>1.75</td>
</tr>
<tr>
<td>Day 3</td>
<td>.97</td>
<td>1.15</td>
<td>1.36</td>
<td>.88</td>
<td>1.30</td>
<td>1.70</td>
</tr>
<tr>
<td>Day 4</td>
<td>.82</td>
<td>.78</td>
<td>.83</td>
<td>.38</td>
<td>1.75</td>
<td>1.12</td>
</tr>
<tr>
<td>Day 5</td>
<td>.50</td>
<td>.65</td>
<td>.33</td>
<td>.70</td>
<td>.87</td>
<td>.68</td>
</tr>
</tbody>
</table>
Due to the lack of variability illustrated in Table IV, no further statistics were computed for maze errors. It was then accepted that no intellectual deficit was exhibited in the form of maze error.

Table V represents the mean maze transit time for each group per day. The mean for the three trials per day is expressed in seconds.

TABLE IV--Continued

<table>
<thead>
<tr>
<th>Group</th>
<th>17C</th>
<th>17E</th>
<th>12C</th>
<th>12E</th>
<th>7C</th>
<th>7E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 6</td>
<td>.44</td>
<td>.45</td>
<td>.40</td>
<td>.83</td>
<td>.87</td>
<td>.68</td>
</tr>
<tr>
<td>Day 7</td>
<td>.33</td>
<td>.23</td>
<td>.07</td>
<td>.39</td>
<td>.54</td>
<td>.58</td>
</tr>
<tr>
<td>Day 8</td>
<td>.40</td>
<td>.14</td>
<td>.10</td>
<td>.33</td>
<td>.12</td>
<td>.08</td>
</tr>
</tbody>
</table>

TABLE V

GROUP MEAN MAZE TRANSIT TIME PER DAY IN SECONDS

<table>
<thead>
<tr>
<th>Group</th>
<th>17C</th>
<th>17E</th>
<th>12C</th>
<th>12E</th>
<th>7C</th>
<th>7E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>63.5</td>
<td>72</td>
<td>40</td>
<td>38</td>
<td>48</td>
<td>66.8</td>
</tr>
<tr>
<td>Day 2</td>
<td>46</td>
<td>35.6</td>
<td>53.5</td>
<td>35</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>Day 3</td>
<td>44</td>
<td>26.6</td>
<td>36</td>
<td>35</td>
<td>21.7</td>
<td>13</td>
</tr>
<tr>
<td>Day 4</td>
<td>33</td>
<td>21.6</td>
<td>36</td>
<td>31</td>
<td>30</td>
<td>12.3</td>
</tr>
<tr>
<td>Day 5</td>
<td>32.5</td>
<td>28</td>
<td>33.6</td>
<td>40.7</td>
<td>25.7</td>
<td>22.6</td>
</tr>
<tr>
<td>Day 6</td>
<td>27.4</td>
<td>17.3</td>
<td>23</td>
<td>30</td>
<td>38</td>
<td>10.5</td>
</tr>
<tr>
<td>Day 7</td>
<td>34.5</td>
<td>16.6</td>
<td>28.5</td>
<td>42.6</td>
<td>52.7</td>
<td>20.6</td>
</tr>
<tr>
<td>Day 8</td>
<td>21</td>
<td>11.6</td>
<td>13.9</td>
<td>37.9</td>
<td>49.2</td>
<td>14</td>
</tr>
</tbody>
</table>
Again, owing to the erratic spread of variability, no further statistics were computed for maze transit time as a measure of intelligence. It was then accepted that there was no intellectual deficit owing to experimental treatment as measured by maze transit time.
CHAPTER IV

DISCUSSION

The results in Table II revealed that there was a significant difference between the two earliest experimental groups and their respective control groups. The significant differences illustrated that the conditions of intubation of phenylalanine and intubation of a placebo were significantly different from each other at a level greater than .001 for the 12E and 12C and 7E and 7C pregnancy groups. However, no significant difference was found to exist between the 7E group and the 7C group.

This significant difference between the 12E and 12C and 7E and 7C pregnancy groups indicated that an increased mortality rate could result due to a high maternal level of phenylalanine during the middle and early stages of pregnancy. Although the levels of significance were the same, it can be noted in Table II that the percentage of mortality was approximately twice as great with each earlier level of pregnancy. This indicates that the earlier in pregnancy the intubation took place, the greater the rate of mortality in offspring.

The results in Table III showed a significant difference between the 7E group and 12E group, and between the 17E group
and the 7E group. However, no significant difference was found to exist between the 12E group and 7E group. This lack of significance is felt to arise from the low number of subjects in each of these seven-and twelve-day experimental groups due to the higher mortality rates experienced. However, the significance of the other experimental groups warrants acceptance that the earlier in pregnancy the intubation takes place, the greater the mortality rate.

This evidence lends some support to the theory that elevated maternal levels of phenylalanine during pregnancy can lead to an increased mortality rate in offspring, and in turn confirms the hypothesis that offspring intubated with a phenylalanine solution will show a significantly higher incidence of morbidity and/or mortality than those offspring intubated with a placebo.

This evidence also partially confirms the additional hypothesis that offspring of pregnant mice intubated with a phenylalanine solution early in pregnancy will show a significantly greater physical (mortality) and intellectual deficit than offspring of pregnant mice intubated with a phenylalanine solution late in pregnancy. By partial confirmation is meant that the physical deficit was confirmed but that the intellectual deficit was not. This lack of intellectual deficit will be discussed later.

On the basis of the assumption that phenylalanine is in a solution and of relatively simple molecular structure
and that such a solution can cross the placental barrier to cause intrauterine damage to the developing fetus, the results of this study are compatible with the observations of Stevenson (3) who gave an account of two PKU sisters on non-restricted diets whose pregnancies had resulted in sixteen spontaneous abortions, six offspring who died in infancy, and four surviving children. He stressed that all surviving children had varying combinations of physical and mental retardation and that none of these children were genetically PKU. This evidence would definitely indicate intrauterine retardation.

Although the results of maze testing shown in Tables IV and V indicated no intellectual damage, it is felt that the remaining offspring in the earlier groups should be classified as a survival of the fit. That is, when an animal experiences prenatal damage or is in some way defective, it is generally reabsorbed or dies soon after birth. Hence, it is felt that if the aborted animals had survived, they would have shown an intellectual deficit and that the surviving animals were stronger and less susceptible to being damaged by the adverse intrauterine environment. In support of this concept is the observation that the mice in the earliest experimental group were faster and more hyperactive than any of the others. Nevertheless, the evidence of increased fetal and congenital mortality, as revealed in the two earlier experimental groups, lends much support to the various
theories which suggest adverse fetal effect due to increased maternal phenylalanine levels during pregnancy.

A leading researcher in the area, Mabry (2), emphasized that the treated PKU mother's hyperphenylalaninemia and related plasma alterations were causing a significant amount of intrauterine brain damage and consequent mental retardation. He stated that "this form of mental retardation is avoidable, either by prevention of the pregnancy or by induction of a lowered blood phenylalanine concentration during gestation."

Again, although no intellectual deficit was observed in this study, it is felt that the increased mortality rate is sufficient evidence to warrant support of this same theory of hyperphenylalaninemia-induced brain damage.

What happens in hyperphenylalaninemia, in essence, is that the PKU mother's phenylalanine blood concentration is at a dangerously high level. This level is not dangerous to the mother because her brain has already passed the vulnerable stages of development at approximately age five. However, since Kerr (1) has demonstrated that amino acids are actively transported across the mammalian placenta, it is feasible to assume that the elevated maternal levels of phenylalanine would have a definite effect on the developing fetus. In addition, Kerr (1) found that phenylalanine concentrations in fetal blood were higher than those in maternal blood; therefore, this magnified effect would almost assure
fetal damage. Since this elevated phenylalanine level has been found to be damaging, this situation is actually much worse than a child's merely inheriting PKU, because with PKU the child can be placed on a low-phenylalanine diet and prevent brain damage, whereas in the case of hyperphenylalaninemia-induced brain damage, the child is irreversibly retarded before birth.

A solution, as suggested by Mabry (2), would be to return the mother in question to the low-phenylalanine diet immediately upon confirmation of pregnancy. Actually, considering that the greatest amount of damage (in this study) took place early in pregnancy, it would be advisable for these PKU mothers to plan each child and to make attempts to resume the low-phenylalanine diet before conception, thereby avoiding the profound damage which could occur in the first trimester. Likewise, since little or no damage occurred in the late groups (in this study), then resumption of a normal diet before parturition could be considered.

However, more research is needed in this area before any definite conclusions can be drawn. The limitations of this study must also be taken into consideration.

To begin with, the subjects used in this study were mice, as opposed to humans, thus presenting problems of extra-polation over such a wide range of the phylo-genetic scale. Secondly, although the dosage level was validated in a
it had on each individual mouse; that is, it was not precisely known how much each individual mouse's phenylalanine blood level was increased, but it could only be assumed that each subject experienced the same effect from having received the same dose.

In the one related study to date, Thompson and Kano (3) found no intellectual or physical damage, but they did conclude that there was a tempermental or emotional change. Their study differed from this one in that they used rats rather than mice, and diet was used rather than intubation. Also the fact that they employed urine tests to assess phenylalanine blood concentrations might lend more validity to their study. However, their phenylalanine dosage level as expressed in relation to kilogram body weight was not stated; hence it is assumed that the difference in dosage levels was an influencing factor in the conflicting results of these two studies.

The intubation method of administering phenylalanine solutions was selected because it presented less danger of puncturing vital organs, as compared to interperitoneal injection, and there was also less danger of aseptic needle conditions, as compared to the injection method. On the other hand, there was a danger that the subjects would regurgitate the solution, but this occurred only a few times in the course of hundreds of intubations.
Another variable to be considered is age of offspring at testing. It might be argued that the testing should have occurred before six months of age. Taking this into consideration, a pilot study was conducted, and it was found that the subjects did not possess sufficient strength to perform the swimming task before five to six months of age.

Another suggested variable concerned the effects of the high maternal phenylalanine blood concentration on lactation, which if reduced could lead to a high infant mortality rate. Again, a pilot study was conducted in which three litters of 7E pups were nursed by three 7C mothers, and the three 7C pups were nursed by three 7E mothers. This resulted in a seventy percent mortality rate among the 7E offspring (who were nursed by a normal mother), and only one death among the 7C offspring (who were nursed by a high phenylalanine mother). It was concluded that there was no adverse lactation effect due to a high maternal phenylalanine concentration.

On the basis of the experimental evidence and the assumptions that were made, it was concluded that a high maternal phenylalanine blood concentration in mice would result in a high rate of fetal and congenital mortality in offspring, and that the earlier in pregnancy that this phenylalanine blood concentration was elevated, the more profound the effect. However, it was not concluded that a high maternal phenylalanine blood concentration would result in an intellectual
deficit in the offspring, as hypothesized. It is to be recognized that these conclusions are justified only if the limitations of this study are taken into consideration.
CHAPTER BIBLIOGRAPHY


CHAPTER V

SUMMARY AND RECOMMENDATIONS

Summary

Two hundred black, naive mice were used as subjects in the present study. Sixty subjects were mated and the resulting thirty-six pregnant females were matched at three different levels of pregnancy in a high maternal phenylalanine blood concentration-normal maternal phenylalanine blood concentration relationship, and were intubated with either DL phenylalanine or placebo. The offspring were physically and intellectually assessed in an attempt to measure the effects of a high maternal phenylalanine blood concentration on offspring. This study also attempted to test the theory that the state of hyperphenylalaninemia in PKU mothers is causing a clinically significant number of retarded children which could be prevented.

The following hypotheses were tested:

1. Offspring of pregnant mice intubated with a phenylalanine solution will show a significantly higher incidence of morbidity and/or mortality than those offspring of pregnant mice intubated with a placebo.

2. Offspring of pregnant mice intubated with a phenylalanine solution will perform significantly less adequately
in a learning situation than will offspring of pregnant mice intubated with a placebo.

c. Offspring of pregnant mice intubated with a phenylalanine solution early in pregnancy will show a significantly greater physical and intellectual deficit than offspring of pregnant mice intubated with a phenylalanine solution late in pregnancy.

Significant differences were found to exist between some but not all treatment conditions. Statistical analysis of the data revealed a physical deficit which increased with intubations that took place in the early stages of pregnancy, but the analysis did not support an intellectual difference.

These results indicated that the high maternal phenylalanine blood concentration during pregnancy can lead to a high mortality rate in offspring and that the earlier in pregnancy that this high level exists, the more profound the damage.

Recommendations

On the basis of the results and conclusions of this investigation, it is evident that there are a number of related conditions and refinements that are in need of presentation and application.

1. Additional research should be done utilizing animals higher on the phylo-genetic scale (e.g., monkeys).
This might allow for a more accurate assessment of intellectual damage.

2. Future investigations should utilize a method of accurately assessing and rigidly controlling the prevailing biochemistry of each subject.

3. Future research should introduce a number of different levels of phenylalanine blood concentrations in an attempt to establish a critical range during pregnancy.

4. PKU females desirous of pregnancy should return to their low-phenylalanine diet before conception or soon thereafter until further research demonstrates otherwise.
APPENDIX

MAZE DESIGN

Scale: 3/8"
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Books


Articles


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