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**HYPERPHENYLALANINEMIA IN THE DEVELOPING RAT: BIOCHEMICAL AND
BEHAVIORAL ALTERATIONS**

City University of New York

Ph.D. 1983

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Clifford A. Brass

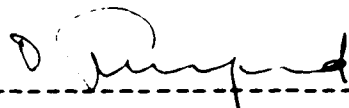
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Graduate Faculty in Biomedical Sciences
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy,
The City University of New York

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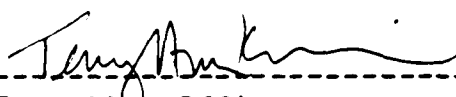
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This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

HYPERPHENYLALANINEMIA IN THE DEVELOPING RAT:
BIOCHEMICAL AND BEHAVIORAL ALTERATIONS

by

Clifford A. Brass

Advisor: Professor Olga Greengard

Hyperphenylalaninemia was produced in neonatal rats through the use of injections of alpha-methylphenylalanine (AMPHE), an in vivo inhibitor of hepatic phenylalanine hydroxylase (PAH), and phenylalanine in order to mimic the human disease state phenylketonuria (PKU). This treatment caused cerebral catecholamine depletion in young animals which could be overcome by increased levels of tyrosine in the brain due to administered or endogenously formed tyrosine. Additionally, the first permanent biochemical (enzyme) change observed in any animal model of PKU, increased striatal dopa decarboxylase activity, is described in these same animals. Other changes observed in adult rats, who had been made hyperphenylalaninemic only during the suckling period of life, include hyperactivity, and permanent brain weight deficits, decreased T-maze performance and increased lateralization in spontaneous rotational activity in female animals. Also, normal male and female rats were found to rotate in a quantitatively different manner in response to amphetamine and apomorphine injections. The basis of these sexually dimorphic responses was explored through gonadal hormone manipulation. These studies were extended to study maternal PKU with the introduction of a new model of this disease based on the dietary introduction of AMPHE and phenylalanine to rat dams. Their fetuses had more than ten-fold elevations in plasma phenylalanine and incurred many of the same cerebral changes (decreased serotonin and branched chain amino acid content, increased phosphoserine phosphatase activity, decreased body and brain weights) as noted in the postnatal model of the disease. Also, developmental changes in the net transport of phenylalanine across the 'blood-brain barrier' are detailed. The clinical relevance of these findings for alternate modes of treatment of PKU, as well as their importance in understanding normal and pathologic brain development are discussed.

This thesis is dedicated to my parents,
HARRY AND IDA BRASS, for their love and
support at all times and their continued
belief in me.

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Of course, I must thank Laura for her support, understanding, encouragement and love during this entire endeavor. Preparation of this manuscript would have been a monumental task without her yeoman-like work in typing all of the tables and figure legends as well as some of the text. Furthermore, she did proof-reading and kept me in shape to get things done.

Most importantly I want to thank Olga for her continual support and instruction from almost the time I first heard the word biochemistry. She has been invaluable to me as both an instructor and role model and has really taught me what science is about and how to 'think like a scientist'. For this, and her friendship, I will always be grateful. She presented me with an interesting problem along with the environment and equipment to probe it. I cannot imagine an environment more conducive to the pursuit of doctoral study, nor a more complete adviser.

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ABBREVIATIONS

a-methylphenylalanine	alpha-methylphenylalanine
AMPHE	alpha-methylphenylalanine
ChAT	choline acetyltransferase
CNS	central nervous system
DDC	dopa decarboxylase
g	gram
K _i	inhibition rate constant
K _m	reaction rate constant
MAO	monoamine oxidase
min	minute
ml	milliliter
mol	mole
ngm	nanogram
nmol	nanomole
PAH	phenylalanine hydroxylase
PCPA	para-chlorophenylalanine
PKU	phenylketonuria
PKUic	phenylketonuric
SAM	S-adenosyl methionine
TH	tyrosine hydroxylase
ug	microgram
ul	microliter
umol	micromole
w	weight

CHAPTER ONE

INTRODUCTION

Although much effort has been devoted to the study of abnormal brain development and mental retardation very little is understood about the links between early pathogenic changes in the brain and later intellectual deficits. This is due both to the complexity of the problem and to a lack of suitable animal models to mimic any of the human disease states which lead to mental retardation. The use of injections of phenylalanine plus α -methylphenylalanine, an *in vivo* inhibitor of liver phenylalanine hydroxylase (PAH), to neonatal rats to mimic the human disease state of phenylketonuria (PKU), as developed by Greengard (Greengard et al., 1976), offers a significant methodological advance in this area of study for several reasons. The treatment imitates the human disease and does so in a generally non-toxic and specific manner (Greengard et al., 1979); PKU represents the best example of a genetic lesion in one organ system (liver PAH deficiency) creating a particular chemical imbalance (hyperphenylalaninemia) whose presence in early development leads to a profound intellectual deficit in later life. Phenylketonuria, one of the most common genetic diseases of metabolic derangement impairing neural development, is also representative of a number of less common aminoacidopathies which also interfere with the development of normal human brain function.

Phenylketonuria is a classic 'developmental disease' in that exposure to the detrimental effects of the disease in the first few years of life alone has been found to cause irreversible mental retardation (Knox et al., 1972), whereas dietary treatment of phenylketonurics during the first several years of life has obviated this effect (Holtzman et al., 1975; Koff et al., 1979). These observations have focused attention on the importance of studying not only the early derangements in development caused by 'experimental phenylketonuria' but also on linking these early changes to later more permanent deficits. Thus, the objective of this thesis project is to elucidate the biochemical and behavioral changes created by the imposition of experimental hyperphenylalaninemia in rats during their early development and to establish connections between early biochemical changes during development and later behavioral and biochemical abnormalities. Achievement of these goals should add to our understanding of both normal and pathologic brain development as well as aid in the development of treatment modalities for phenylketonuria.

HUMAN PHENYLKETONURIA

HISTORICAL PERSPECTIVES - Phenylketonuria is a classic inborn error of metabolism as defined by Garrod (1909). That is, it is a genetic disorder in which the inherited gene defect leads to a specific protein defect (abnormal hepatic phenylalanine hydroxylase) and secondary biochemical consequences (hyperphenylalaninemia) leading to physical and intellectual impairment. Furthermore, this disease demonstrates the relationship of 'nurture and nature' as first described by Galton in that it is the interaction of the genotype (mutant allele for phenylalanine hydroxylase) and environment (adequate intake of the essential amino acid phenylalanine) that determines the phenotype (hyperphenylalaninemia and secondary consequences).

It was in 1934 that the Norwegian physician Asbjorn Folling gave the first description of the disease phenylketonuria (Folling, 1934). A Norwegian mother with two children who were barely able to learn to walk or talk noticed that they also had an intolerable "mousy" odor. After repeated unsuccessful attempts at obtaining medical help for them she contacted Folling. He tested the children's urine with ferric chloride, apparently expecting detection of uri-

nary acetoacetate, and found an unexpected green color which he soon proved to be due to the presence of phenylpyruvic acid. Folling then went to institutions for the retarded where he discovered eight other patients with urinary phenylpyruvate and mental retardation. Some of these were siblings. In reporting these findings he proposed that an inherited defect in phenylalanine metabolism, imbecillitas phenylpyruvica, was responsible for the biochemical abnormalities and mental deficiency. In 1938, Folling further reported elevated plasma and urinary phenylalanine levels in these patients and the excretion of phenyllactate and phenylacetate (the actual cause of the "mousy" odor) were later described. During this time the disease was also referred to as Folling's disease, phenylpyruvic oligophrenia and the now widely adapted name, phenylketonuria (Penrose & Quastel, 1937).

Further investigation of phenylketonuria, advanced by the use of the ferric chloride test on patients in institutions for the retarded, led to Jervis' (1939) recognition that phenylketonuria is inherited as an autosomal recessive trait according to the laws of Mendel. It was not until the mid-1950s, however, that detection and treatment of the disease was significantly advanced.

Two groups (Bickel et al., 1954; Armstrong & Tyler, 1955) introduced the restriction of phenylalanine intake as a treatment for PKU in the 1950s. This treatment was effec-

tive but did not enjoy widespread use at first due to problems in the detection of PKU. A step forward was taken, however, with the introduction by Hsia and colleagues (1956) of a method of heterozygote detection. The greatest advances, though, came in the early 1960s when Guthrie's introduction of a simple assay for phenylalanine (Guthrie, 1961; Guthrie & Susi, 1963) made mass screening programs for PKU possible. In 1963 voluntary screening of children was begun but only 20% of the newborn population was being tested in the United States by 1965. Beginning in 1968 legislative action made the screening of newborns mandatory and by 1975 forty-seven of the fifty states required screening in the neonatal period (Justice & Smith) so that up to 95% of newborn children are now screened and treated.

CLINICAL FEATURES - Infants with phenylketonuria appear normal at birth and subsequently develop clinical abnormalities. However, even the late(r) appearing signs and symptoms are not specific enough to make a definitive diagnosis without the aid of a laboratory examination (Knox, 1972). The earliest symptoms include vomiting (sometimes projectile in nature) and irritability (Partington, 1961) but there are no neonatal problems invariably noted in the first few months of life except for a delay in reaching normal developmental milestones. During the first year or so of life

learning deficits and alterations in motor behavior become quite striking in these children.

Physical changes in phenylketonuric children include microcephaly (Holm & Knox, 1979) and alterations in skin, bone and teeth (Knox, 1972). Enlarged maxillae with widened interdental spaces as well as calcified spicules extending into the long bones have been reported. Eczema and a rough dry skin have been frequently observed and light skin accompanied by blond hair and blue eyes is characteristic of the disease. Although the latter observation may be partially explained by the higher prevalence of PKU in lighter skinned peoples instances of hypopigmented PKUics in darkly pigmented families have been reported. From a review of many cases Knox (1972) has concluded that 'each patient has a relatively lighter complexion than other members of his family'. Coloration changes are thought to be related to decreased melanin synthesis secondary to relative tyrosine deficiency. There are presently no theories to explain the bone abnormalities although it has been noted that skin, teeth and brain are all of ectodermal origin, the latter two undergoing simultaneous postnatal development (Knox, 1972).

The loss of IQ accompanying PKU is severe with a mean intelligent quotient for untreated children of 40. The majority of untreated phenylketonurics are institutionalized and their IQs are generally lower than other institutionalized mental retardates (Kleinman, 1964). Two thirds of them

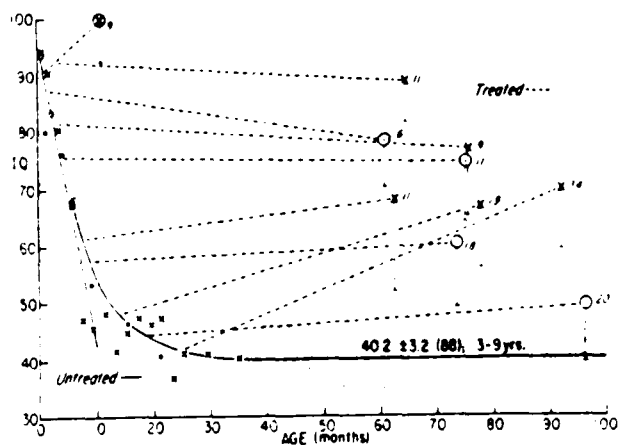
cannot talk and another one third have poor control of sphincter function and cannot walk. There are few that are trainable (IQ>60) although there are some reports of untreated phenylketonurics with normal intelligence (Gaulle et al., 1975). The IQ of phenylketonurics has been found to fall rapidly in the first two years of life and to stabilize thereafter (see Figure I-1). This drop may be due to the accumulative effects of the insult (hyperphenylalaninemia) on the still developing brain, as well as the difficulty in documenting abnormal IQs in very young infants.

Besides the deficits in intelligence there are many other central nervous system abnormalities associated with PKU. These problems tend to parallel the degree of mental deficit of the patient. They include seizures, tremors, hyperkinesia, hypertonicity, hyperactive deep tendon reflexes and a pattern of repetitive movements. Convulsive as well as petit mal seizures often begin in infancy (6-18 months) and stop spontaneously in adulthood. The majority of PKUics are restless and hyperactive and most have electroencephalogram (EEG) abnormalities consistent with deep midline brain damage. Furthermore, a great deal of stereotyped behavior and choreiform movements have been noted as well as infantile reflex patterns, clonus and at times cog-wheel rigidity (Knox, 1972). A summary of the most common clinical findings seen in PKU and their incidence is listed in Table I-1.

FIGURE I-1

IQ LOSS IN PHENYLKETONURICS*

Loss of IQ with age in 392 untreated phenylketonuric patients (—), and preservation of IQ in 158 patients treated with a low-phenylalanine diet (---). Points represent mean IQ and age more than six patients of similar ages who were grouped together. Patients who received dietary treatments were grouped according to age at the start of the treatment (at left origin of dashed line) and final mean IQ and age (at right end of the dashed line).



*From Knox, 1972

PATHOLOGIC CHANGES ASSOCIATED WITH PKU - The only pathologic changes that have been described in PKUics relate to the central nervous system. Although normal myelination in a PKUic was described by Alvord in 1950, others have variously described insufficient myelination (Bennet, 1952; Crome & Pare, 1960; Crome, 1962) as well as other white matter alterations including spongy degeneration in older phenylketonurics (Malamud, 1966; Salguero et al., 1968). The changes do not appear to be invariable or specific although some authors have pointed to a generalized pattern of defective myelination with progressive demyelination in later life. This defect in myelin formation is to be contrasted with primary demyelination as seen in Schilder's disease (Crome, 1962). This dysmyelination has been associated with findings of decreased myelin (Shah et al., 1972), galactolipids (Crome et al., 1962; Agrawal & Davison, 1973) and proteolipids (Menkes, 1968) as well as altered fatty acid composition of individual lipid classes (Foote et al., 1965; Cumings et al., 1968). However, on the whole the changes in cerebral white matter found by various investigators appear to be small compared to the severity of the mental deficits.

BIOCHEMICAL CHANGES - The primary biochemical abnormality in phenylketonuria is the lack of hepatic phenylalanine hydroxylase activity. This block in the normal metabolism of phe-

TABLE I-1

MAJOR CLINICAL FINDINGS IN PHENYLKETONURIA*

<u>Finding</u>	<u>Incidence Percent</u>	<u>Occurrence in Low-Grade Patients</u>
Agitated Behavior	90, 32	+
EEG Abnormalities	80	+/-
Muscular Hypertonicity	75	-
Microcephaly	68	+
Hyperactive Reflexes	66	+
Blond Hair, Blue Eyes	62	-
Inability to Talk	63	+
Hyperkinesis	50	-
Inability to Walk (and Usually Incontinence)	35	+
Tremors	30	+
Eczema	19, 34	-
Seizures	26	+

* From Knox, 1972

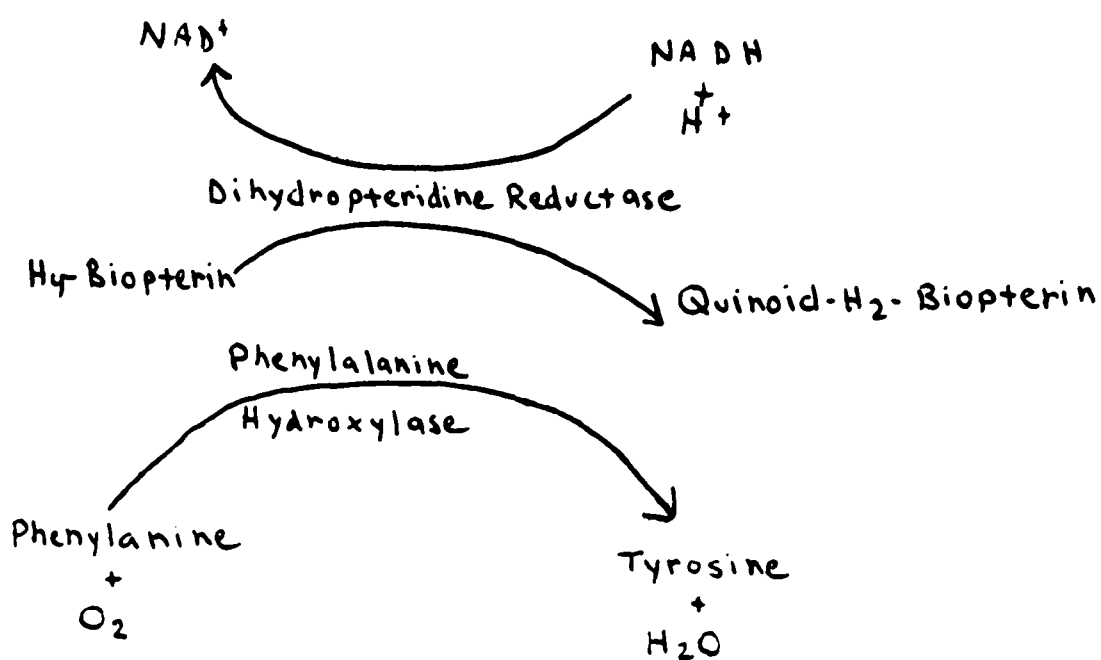
nylalanine was first demonstrated by Jervis (1947) who administered phenylalanine to phenylketonurics and controls and found that only the controls produced an increased amounts of tyrosine. Jervis (1953) later used the newly established tyrosine assay of Udenfriend and Cooper (1952) to show that there was no phenylalanine hydroxylating activity in the liver extracts of phenylketonurics. However, Udenfriend and Bessman (1953) found that the block was not complete since some ^{14}C -phenylalanine administered to phenylketonuric children was converted to labeled tyrosine. Subsequently, Kaufman and his colleagues (Friedman et al., 1972) demonstrated 0.27% of normal hepatic phenylalanine hydroxylase activity in the liver extract of a phenylketonuric patient. Since the assay used by Kaufman was 20-40 times more sensitive than immunological tests that had failed to detect any hepatic phenylalanine hydroxylase in phenylketonuric patients (Friedman et al., 1972) this seemed to indicate that PKU is not due to a deletion of the structural gene for PAH but is caused by low amounts of a normal hydroxylase or by the presence of an abnormal hydroxylase with altered antigenicity and reduced enzymatic activity (Kaufman, 1976). Further analysis of the kinetic activity of the PAH from a phenylketonuric compared to a normal indicated that a structurally altered hydroxylase with low activity was present in the phenylketonuric. Thus, PKU seems to be due to a 'mutation in the gene coding for the structure of phenylalanine hydroxylase' (Kaufman, 1976).

Although knowledge of the relationship between phenylalanine and tyrosine dates back to the work of Embden & Baldes in 1913 and Womack and Rose in 1934 it was not until 1940 that this interconversion was first formally demonstrated (Moss & Schopenheimer, 1940). The complexity of the hydroxylating system was then worked out in detail in the late 1950s and 1960s by Kaufman and others (see Kaufman & Fischer, 1974). Phenylalanine hydroxylase is an iron containing enzyme (two atoms of iron per molecule) which acts as a mixed function oxidase. It uses tetrahydrobiopterin in the reduction of molecular oxygen. One atom of oxygen is added to the phenol group of phenylalanine, forming tyrosine, and the other becomes water. The tetrahydrobiopterin, now oxidized to the dihydroquinone form, is then reduced back to the tetraquinoid form by the enzyme dihydropteridine reductase using NADPH or preferably NADH as a cofactor (see Figure I-2). Another enzyme, the phenylalanine hydroxylase stimulating protein (PHS) is also required for normal phenylalanine hydroxylase activity. It is thought to be involved in the breakdown of intermediates of the hydroxylase reaction.

Phenylalanine is predominately incorporated into proteins or converted to tyrosine. Tyrosine is also incorporated into proteins or converted to catecholamines, melanin and thyroxin. Although phenylalanine may be metabolized by several pathways other than its hydroxylation to tyrosine this

FIGURE I-2

REACTION SCHEME FOR THE ENZYMATIC CONVERSION OF PHENYLALANINE
TO TYROSINE

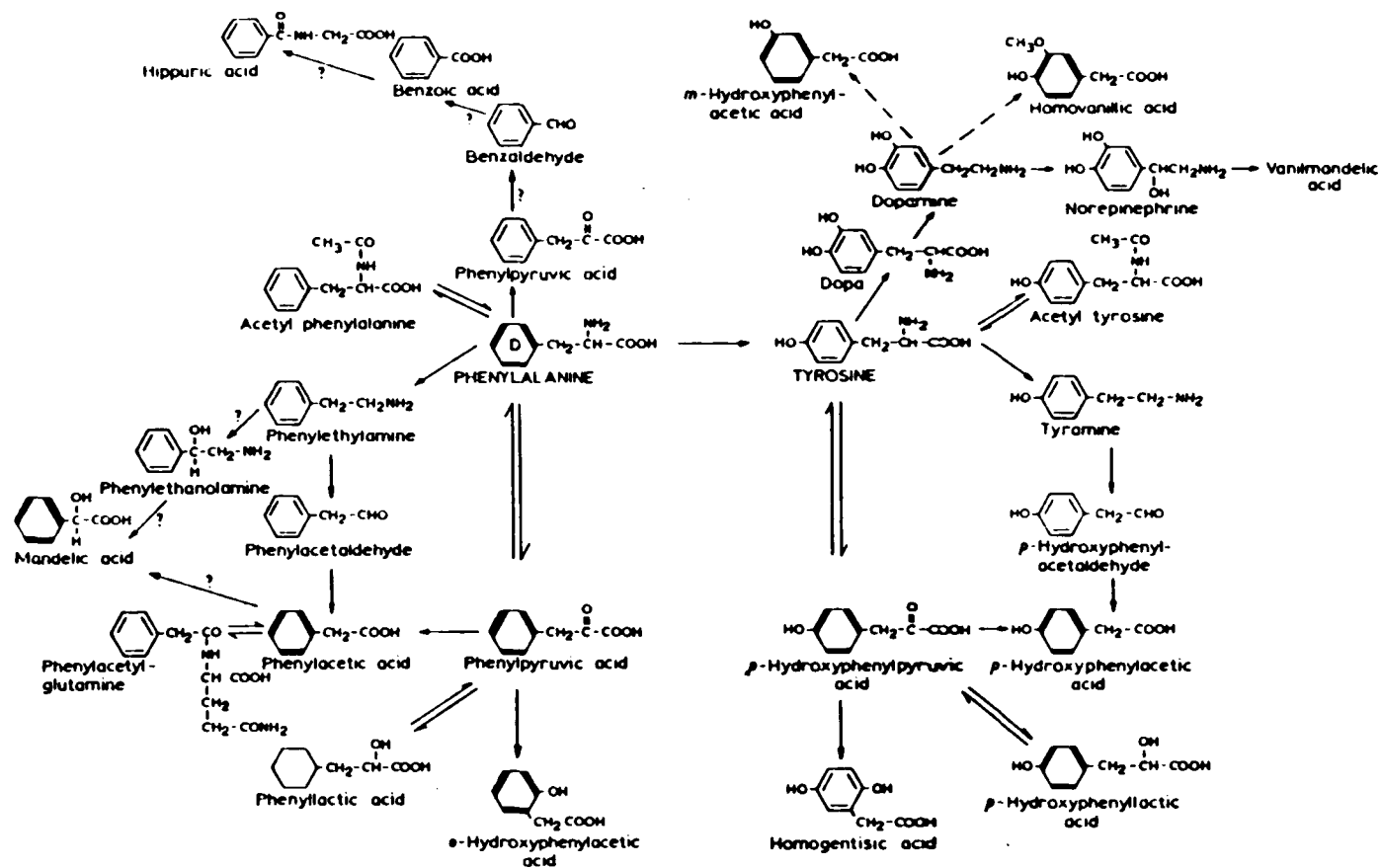


is the only pathway leading to complete oxidation to CO₂ (Kaufman, 1976). Since other phenylalanine metabolites are normally found in only small quantities in the urine and blood, these other pathways must be relatively inactive under normal conditions. Even with a large phenylalanine load nearly 63% is metabolized to tyrosine (Brad & Harper, 1974). However, in the absence of significant PAH activity, as seen in PKU, the alternate pathways of phenylalanine metabolism become significant. Predominant among these is transamination to phenylpyruvate (Brad & Harper, 1974) and decarboxylation to phenylethylamine (David et al., 1974). The various pathways for phenylalanine metabolism are detailed in Figure I-3.

Block of the main pathway of phenylalanine metabolism leads to the accumulation and urinary excretion of many phenylalanine metabolites normally present in only small amounts. Jervis (1952) was able to measure phenylpyruvate in the blood of PKUic patients but not normal controls. Other metabolites have been found to be elevated only in the urine. They include phenyllactate, phenylacetate, phenylethylamine, mandelic acid, o-hydroxyphenylacetic acid and phenylacetylglutamine (Knox, 1972).

Also noteworthy are the decreased levels of certain metabolites in phenylketonurics. McKean (1972) has found decreased levels of cerebral catecholamines in phenylketonuric retardates compared to non-phenylketonuric retardates.

FIGURE 1-3
PATHWAYS FOR PHENYLALANINE METABOLISM*



*From Curtius et al., 1972

Alterations in catecholamine metabolism after a tyrosine load have also been reported in phenylketonurics (see Discussion, Chapter V for details). Decreased plasma and brain serotonin content (McKean, 1972) and increased excretion of serotonin metabolites (Knox, 1972) have also been reported. Thus, interference with four putative neurotransmitters (dopamine, norepinephrine, epinephrine, serotonin) have been implicated in PKU. Furthermore, decreased cerebral levels of branched chain amino acids (McKean, 1972) have been found in PKUics. These changes are thought to be due to competition by phenylalanine with other amino acids for transport into the brain via the L-system of amino acid transport located in the blood-brain barrier. Alterations in the plasma concentrations of several amino acids have also been noted in PKU (Efron et al., 1969) as well as alterations in the cerebrospinal fluid content of many amino acids (Van Sande et al., 1970; Quentin et al., 1974).

PKU: INCIDENCE AND SCREENING - Since Jervis' first observations (Jervis, 1934) it has been clearly shown that phenylketonuria is inherited as an autosomal recessive trait. In 1954 Jervis found that 40% of the siblings of phenylketonurics were also affected. When this figure was corrected for uncounted families of heterozygous parents with only normal children it was found that 27% of the children of two heter-

ozygous parents are affected. This is very close to the 25% expected for the transmission of an autosomal recessive condition. The disease has been found in all parts of the world although the incidence varies widely between different racial groups. Frequency in the United States has been calculated as between 1 in 14,000 (Berman et al., 1969) and 1 in 18,500 (Knox, 1972). Among whites the frequency is higher (1 in 11,000; Scrivner & Clow, 1980b) with lower frequencies among blacks, Orientals and Ashkenazic Jews. On the other hand higher frequencies have been found in Northern European countries, among gypsies (1 in 40) and among the Celtic population with an incidence of 1 in 4,000 found in Ireland (Tourian & Sidman, 1978). There are no sex differences in either the incidence or severity of clinical manifestations (Knox, 1972) although more males than females have been initially detected with elevated levels of plasma phenylalanine. This was found to be due to the earlier postpartum rise in plasma phenylalanine levels in males.

The original laboratory examination for PKU, testing of urine for phenylpyruvate with ferric chloride, was unreliable and mass screening for PKU was not established until the 1960s when the introduction of Guthrie's simplified assay for phenylalanine (Guthrie, 1961; Guthrie & Susi, 1963) made more reliable screening possible. The collection of a drop of blood from a neonate onto a filter paper and subsequent phenylalanine analysis is now a routine procedure in most

developed countries where PKU is of concern. Problems still exist in screening programs with too early sampling of blood. Blood should be sampled at least 24 hours, and preferably 72 hours, after birth to increase the sensitivity of testing. Once elevated plasma phenylalanine levels have been detected confirmation of the diagnosis of PKU can be made by: 1) confirmation of the elevated plasma phenylalanine level (>20 mg/100ml), 2) a positive urine ferric chloride test for phenylpyruvic acid after 3 days on a 1:2 evaporated milk formula, 3) measurement of plasma tyrosine after a phenylalanine load (it should not increase) and 4) measurement of plasma phenylalanine after dietary phenylalanine restriction (it should decrease markedly) (Rosenberg & Scrivner, 1974; Tourian & Sidbury, 1978). Both parents may also be tested for heterozygosity (heterozygotes have normal intelligence; Blumenthal, 1967) by administering a phenylalanine load and measuring serial plasma phenylalanine and tyrosine concentrations (Tourian & Sidbury, 1978) or measuring phenylalanine/tyrosine ratios before and after meals (Griffin & Elsab, 1975). Overall, mass screening of neonates for PKU has been shown to be cost effective (see Scrivner & Clow, 1980b for detailed review) although much confusion still exists concerning the less frequently occurring (1 in 43,000) hyperphenylalaninemias.

OTHER TYPES OF HYPERPHENYLALANINEMIAS - Besides phenylketonuria (classic hyperphenylalaninemia) there are several other conditions manifested by abnormally high plasma phenylalanine levels but without the clinical symptomatology of PKU. With the advent of mass screening for PKU these conditions have become increasingly detected and confused with classical PKU. However, these other conditions are marked by different primary lesions, lower plasma phenylalanine levels and different clinical problems than PKU. Clinically, the most important point is that the damage accompanying PKU is seen only with persistent high levels of plasma phenylalanine (>20 mg/100 ml; Berman et al., 1969; Knox et al., 1974). Although these other genetic abnormalities are manifested by elevated phenylalanine levels, these milder phenylalanine elevations per se are not the cause of any major clinical damage but are merely harmless (relatively) accompaniments of some other primary problem. Thus, phenylalanine restriction, the present treatment for PKU, is unnecessary or undesirable in patients with these atypical hyperphenylalaninemias (Tourian & Sidbury, 1978).

There are presently nine classes of hyperphenylalaninemia including PKU (Type I) (see Sidman & Tourian, 1983 for a detailed review). Types II, III and IV are clinically silent and due to decreased PAH activity, maturational delay of PAH activity and phenylalanine transaminase deficiency respectively. Types V and VI are due to deficient

and abnormal dihydropteridine reductases respectively. Although some of the clinical symptoms in these two diseases (especially Type V) are similar to those seen in PKU (seizures, abnormal development) the symptoms will not be reversed by phenylalanine restriction since the primary CNS defect in these diseases is probably neurotransmitter (serotonin and catecholamine) deficiencies caused by a lack of dihydropteridine reductase activity which is necessary for the cerebral synthesis of these substances. Thus, Butler (Butler et al., 1978) has found some success in treating these patients with hydroxylated precursors of serotonin and the catecholamines and bypassing this defective hydroxylation step. Type VII hyperphenylalaninemia is a rare, poorly characterized disease, and Types VIII and IX are primarily hypertyrosinemias with secondary increases in plasma phenylalanine levels.

TREATMENT - The standard treatment for PKU is dietary phenylalanine restriction through the use of the phenylalanine-free protein hydrolysates Lofenalac or PKUaid. Other foods are given in measured amounts so that phenylalanine intake is between 30 to 35 mg per kg body weight per day (The normal range is 50-200 mg/kg/day; Koch et al., 1976 or more; Partington & Lewis, 1963). The goal of most present treatments is blood phenylalanine levels less than 3-10 mg/100

ml. The diet is instituted as soon as the diagnosis of PKU is confirmed since it has been shown that there is an inverse relationship between the time of institution of dietary treatment and eventual IQ (see Figure I-1; Knox, 1972; Smith & Wolf, 1974). Decrease in plasma phenylalanine levels, even when instituted later in childhood, have been shown to reverse several other clinical symptoms including hypopigmentation, seizures and other motor abnormalities (Knox, 1972). In fact phenylalanine restriction has been shown to be useful in decreasing some of these symptoms in adult patients (Marholin et al., 1978). Other treatment regimens such as the use of phenylalanine ammonia lyase in vivo (Ambrus et al., 1978) for stabilized enzyme replacement (Weiss et al., 1977) have not yet proven to be successful.

Dietary treatment for PKU is not without complications as it is difficult to balance phenylalanine restriction with adequate nutrition. Several complications of dietary treatment have been reported. They include osteoporosis, hypoglycemia, megaloblastic anemia and cutaneous lesions (Menkes & Koch, 1976). All of these problems are secondary to insufficient phenylalanine intake. Adequate supply of tyrosine, an essential amino acid in phenylketonurics, is also important. Further problems arise from the psychological and social stresses that arise from compliance with such a restrictive diet (Pueschel et al., 1977). Peer pressures on school-aged children add to the emotional problems of treated phenylketonurics and reduce dietary compliance.

It is unclear at what age the dietary therapy can be terminated (see Chapter VII). Although it is known that treatment during the first few years of life is most important there are no objective scientific criteria to decide when phenylalanine restriction is no longer needed. This is because the basis of the particular vulnerability of the immature brain is unknown. Six years of age is presently a popular time for diet termination but several recent studies have not agreed on whether subsequent intellectual damage does (Smith et al., 1978; Williams et al., 1979) or does not (Holtzman et al., 1975; Koff et al., 1979) occur when a regular diet is reinstituted at that time. Although treatment has been very successful and treated phenylketonurics are not retarded, such individuals may not reach their full intellectual potential (Berry et al., 1979).

MATERNAL PKU - With the advent of early screening and dietary therapy an increasing number of phenylketonuric women are bearing children. Since previously most phenylketonuric women were institutionalized and did not have many children, these new developments have led to a dramatic increase in prenatal exposure to high phenylalanine levels. It has been estimated that 'given average reproductive rates, the frequency of new cases of PKU-related mental retardation could return to its former level after only one

generation if no treatment is available to protect their offspring' (Lenke & Levy, 1980).

Dent (1957) was the first to draw attention to the mental retardation of children of phenylketonuric women. Several years later Denniston (1963) and Mabry (Mabry et al., 1963) reported cases of mental retardation in the heterozygous offspring of phenylketonuric women. Since then congenital heart disease, intrauterine growth retardation and microcephaly have also been observed in these children (Frankenburg et al., 1968; Fisch et al., 1969; MacCready & Levy, 1972). Lenke and Levy (1980) have recently reviewed data on 524 pregnancies in 155 phenylketonuric women and found increased frequencies of mental retardation, microcephaly and congenital heart disease correlating with the mothers blood phenylalanine levels. These authors found that 81% of the women with plasma phenylalanine levels greater than 20 mg/dl to have retarded offspring whereas only 43% and 20% of those mothers with phenylalanine levels in the 16-19 and 11-15 mg/dl ranges respectively had retarded offspring.

Due to a lack of data it is presently unclear whether phenylalanine restriction of phenylketonuric mothers is effective in preventing damage to their offspring (Lenke & Levy, 1980). Differences in the time of gestation when dietary treatment was begun and problems with patient compliance may have complicated interpretation of the few stud-

ies done. However, there is a general trend of improved outcome with earlier initiated low phenylalanine diets (Lenke & Levy, 1980). Some recent clinical reports though have emphasized the importance of early treatment of pregnant phenylketonurics (Nielson & Wamberg, 1979; Smith et al., 1979). Development of rational guidelines for the treatment of pregnant phenylketonuric women must await further clinical studies and information from animal models of the disease.

ANIMAL MODELS OF PKU

Phenylketonuria has been a popular area for study with animal models of the disease for several reasons. It is the most common of the aminoacidemias causing mental retardation and one of the first whose primary cause was clearly outlined. The existence of a primary hepatic defect (lack of phenylalanine hydroxylase activity) which underlies tertiary brain damage (mental retardation, seizures etc.) as mediated through easily studied secondary effects (hyperphenylalaninemia) has presented a clear system with which to study the causes of mental retardation.

Phenylketonuria can be seen as representative of the many less common disease states which are marked by an heritable metabolic error resulting in elevated plasma levels of an amino acid and concomitant cerebral damage. Some of these have obvious similarities to PKU in either known or postulated pathogenic mechanisms. For example, non-ketotic hyperglycinemia is noted by increased cerebral glycine levels (Perry et al., 1975; de Groot et al., 1978), a finding in rodent models of PKU (Isaacs & Greengard; Diemel & Greengard, 1977). Also it is easy to postulate that increased plasma levels of other L-system amino acids such as tyrosine, histidine or branched chain amino acids would cause cerebral damage similar to that seen in PKU by also blocking

transport of other L-system amino acids into the brain. Alterations in the cerebral content of various amino acids may cause important alterations in protein synthesis. However, such theorizing may be misleading since the amino acid elevation seen in PKU is much greater than that observed in the other aminoacidopathies such as maple syrup urine disease, hypervalinemia (Dancus & Levitz, 1978), hypertyrosinemia (LaDu, 1978) or hyperlysinemia. Many of the other aminoacidemias are rare and poorly characterized which increases the difficulty of describing them and in seeking common pathogenic mechanisms among them, if any do exist. Among the other aminoacidemias causing or accompanied by CNS damage are: hyperargininemia (Cederbaum et al., 1975), citrullinemia, hyperornithemia, hydroxylysuria, hyperprolinemia, hypervalinemia and hyperlysinemia (Standbury & Wynngarden, 1978; Shih, 1973). Furthermore, new metabolic disorders are being described. At present most of the active research on aminoacidurias and cerebral damage is focused on PKU.

EXPERIMENTAL HYPERPHENYLALANINEMIA - Since the late 1950s many investigators have designed models to mimic human phenylketonuria in laboratory animals (for detailed reviews see Karrer & Cahilly, 1965; Vorhees et al., 1980; Lane & Neuhoff, 1980). These attempts have revolved around the use of

genetic (breeding of animals with reduced hepatic PAH activity) and metabolic (use of phenylalanine loads and/or inhibitors of phenylalanine hydroxylase) techniques. Unfortunately most of these efforts have been unsuccessful in meeting all the criteria of a good animal model of PKU. An adequate experimental system should include the following characteristics:

I) MODEL

elevation of plasma phenylalanine levels to at least
1000 nmol/ml for most of the day
elevation of secondary phenylalanine metabolites (phenylacetate, phenyllactate etc.) similar to what is
seen in human phenylketonurics
maintenance of high plasma phenylalanine to tyrosine
ratios (8-20:1) preferably without elevation in
plasma tyrosine levels above normal
imposition of the above changes during the early period
of brain maturation in the animal used

II) SECONDARY CHANGES

demonstration of neurochemical changes similar to those
seen in the brains of human phenylketonurics
demonstration of a variety of behavioral (learning,
activity) deficits in adult animals whose treatment
has been discontinued with maturity

III) CONTROLS

evidence that the drugs used to inhibit hepatic PAH are not in themselves highly toxic in the quantities used and responsible for any of the secondary changes observed

adequate controls in the handling, rearing (e.g use of split litters) etc. for behavioral work

successful use in more than one animal strain or species

Early attempts had been made to use a genetic mutant, the dilute lethal mouse (Searle, 1952), as a model for PKU. Although this animal had neurologic abnormalities, claims that it was deficient in phenylalanine hydroxylase were found to be untrue. Similarly, other breeding attempts have failed to develop a genetic animal model for PKU (Sidman & Tourian, 1978).

The first attempt at creating hyperphenylalaninemia in an animal by feeding large amounts of phenylalanine was made by Auerbach, Waisman and Wykoff in 1958. Other investigators utilized the same concept of high dose phenylalanine loading with intravenous or intraperitoneal injections (Prensky, Fischman & Daftari, 1974) or intubation feedings (Schalock, 1969). Similarly, Waisman (Waisman et al., 1960; Waisman & Harlow, 1965) adopted this same model to primates.

Although a large literature has been derived from these studies they all failed to mimic phenylketonuria in the most basic ways. Administration of phenylalanine alone does not lead to high elevations of plasma phenylalanine for more than a few hours since most of the administered load is metabolized to tyrosine. Thus, neither phenylalanine to tyrosine ratios nor absolute plasma levels of phenylalanine approached the levels seen in PKU. Furthermore, these studies highlight the fact that many investigators do not even measure plasma phenylalanine levels (especially at multiple time points) despite making claims of mimicking the disease.

A significant advance in animal models of PKU was made in 1966 with the demonstration by Koe and Weissman that the phenylalanine analogue, para-chlorophenylalanine (PCPA), decreases the activity of hepatic phenylalanine hydroxylase in vivo as well as that of cerebral tryptophan hydroxylase. This then led to the introduction of a number of models utilizing PCPA alone or in conjunction with phenylalanine administration. Watt and Martin (1969) introduced the use of PCPA injections alone to rats to mimic PKU. However, despite the efficacy of the drug the remaining PAH activity was sufficient to maintain near normal plasma phenylalanine levels. Wapnir (Wapnir et al., 1970) was able to overcome this problem somewhat by giving increased doses of PCPA, but this treatment was highly toxic. Chamove (Chamove et al., 1973) was able to take advantage of the fact that the mon-

key's hepatic PAH activity, like man's, is 5 times less than the rats and obtained moderate phenylalanine elevations using lower doses of PCPA alone. Some other authors (Gimenez et al., 1974) have also attempted to produce hyperphenylalaninemia with the low to normal plasma tyrosine levels seen in PKU by administering more than one metabolic blocker of phenylalanine metabolism. For example, Dhondt (Dhondt et al., 1977) used PCPA plus the dihydropteridine reductase inhibitor cotrimoxazole, without adding phenylalanine, to produce high plasma phenylalanine levels. Such models are poor imitators of classic PKU since dihydropteridine reductase inhibition itself has more serious consequences than hyperphenylalaninemia as seen in the disease dihydropteridine reductase deficiency which is clearly a different disease entity than PKU.

The first real advance in obtaining high phenylalanine elevations (>1000 nmol/ml) with increased plasma phenylalanine to tyrosine ratios (8-10:1 versus 1:2 without the use of inhibitors) came from the use of PCPA in conjunction with phenylalanine loads (Lipton et al., 1967). Subsequently, Butcher (1970) made use of PCPA plus phenylalanine in a diet to model gestational PKU and Copenhaver (Copenhaver et al., 1970) developed a similar model using this PAH inhibitor. Use of combinations of PCPA and phenylalanine have been the major model of experimental PKU in the 1970s with much behavioral (Anderson & Guroff, 1970; Anderson et al., 1974)

as well as biochemical (Lane & Neuhoﬀ, 1980) data coming from these studies.

Although the PCPA model offered significant advances from previous endeavors at producing hyperphenylalaninemia it was not without its problems. Para-chlorophenylalanine is itself a highly toxic substance and animals treated with PCPA plus phenylalanine become very ill. They suffer severe body weight loss (25-50%; DelValle et al., 1978; Lane et al., 1979) with high mortality rates. It is not surprising that 35% brain weight losses are also inflicted since PCPA has been shown to be toxic to neuroblastoma cells in culture (Kelly & Johnson, 1978). Furthermore, the fact that PCPA is also a potent inhibitor of tryptophan hydroxylase presents many serious complications. For example, brain serotonin depletion is greater in PCPA plus phenylalanine treated animals (87%; Berry et al., 1975; Lane et al., 1979) than in human phenylketonurics (McKean, 1972) and this depletion can be caused by PCPA alone (Koe & Weissman, 1966) which is often used as a tool to study serotonin metabolism alone (Aprison & Hingsten, 1972). Many authors have ignored these complications and omitted necessary controls while using this model of PKU.

In 1976 a significant new advance was made in animal models of PKU with the introduction of a new less toxic hepatic phenylalanine hydroxylase inhibitor, alpha-methyl-

phenylalanine, by Greengard (Greengard et al., 1976).

Although a less potent PAH inhibitor than PCPA, appropriate amounts of α -methylphenylalanine could be administered to rats to obtain similar PAH inhibition (75%; Greengard et al., 1979) without serious side effects. The time course of PAH inhibition throughout suckling life and changes in plasma phenylalanine elevations over 24 hours with this treatment have been detailed (Dienel, 1977; DelValle et al., 1979) and treatment of suckling rats with daily subcutaneous injections of α -methylphenylalanine plus phenylalanine has become the best present model of experimental PKU. It has recently been adopted by a growing number of laboratory groups (see below).

The α -methylphenylalanine model has been successful in meeting the following criteria for a good PKU paradigm: 1) It raises plasma phenylalanine levels to 10-30 times normal (>1000 nmol/ml) for most of the day and maintains high plasma to tyrosine ratios in the plasma (Greengard et al., 1976; DelValle et al., 1978; Lane et al., 1980). 2) Treatment in the suckling period causes a 10-fold rise in serum phenylpyruvate and large increases in urinary phenylpyruvate (30-40 fold), phenyllactate (50-60 fold) and other rare phenylalanine metabolites whose concentrations rise to detectable levels only in phenylketonurics (Lane et al., 1979). 3) The cerebral serotonin (Isaacs & Greengard, 1980; Lane et al., 1980) and catecholamine depletion (Chapter V), and

decrease in branched chain amino acids (Greengard et al., 1979; ref) seen in PKU is reproduced. 4) Treatment during the early developing period only causes behavioral deficits later in life (Greengard et al., 1979; Lane et al., 1979; Luttges & Gerren, 1979). 5) Changes in myelin composition and deposition similar to those observed in the brains of autopsied phenylketonurics have been observed (Lane et al., 1979). This data has been collected from experiments with several different strains of rats as well as mice. In fact all of the major changes noted in the plasma and brain of phenylketonurics (except for the cerebral catecholamine depletion) have been demonstrated in this rodent model of the disease. With the validity of the model confirmed investigations have more recently focused on delineating additional pathologic changes accompanying hyperphenylalaninemia.

Among the other observations recently noted using the α -methylphenylalanine model have been a rise in cerebral glycine content during the early treatment period (Isaacs & Greengard, 1980; Diemel, 1981). Nigam and Labar (1979) have reported a decreased density of cortical synaptic terminals, especially the smaller terminals in superficial layers of the cortex, and Figlewicz and Druse (1980) as well as Johnson and Shah (1980) have discovered myelin deficits in the brains of model phenylketonuric rodents. Additionally, Binek and co-workers (1981) have observed polyribosome

disaggregation and decreased rates of polypeptide elongation in experimental mice. Both Spero (1982) and Huether (Huether et al., 1982a; Huether et al., 1982b) have reported developmental changes in cerebral cytoarchitecture in 'phenylketonuric rats'. It must be noted, however, in reviewing these studies that the minor alterations in treatment regimen used by many of these investigators do not always have the simple effect expected (see Chapter III) by them so the effectiveness of these new regimens using α -methylphenylalanine plus phenylalanine must be demonstrated before these new models are accepted.

MECHANISM OF THE CEREBRAL DEFECT

With the advent of improved models of PKU attention has turned to investigating the mechanism underlying the observed neurochemical changes, ascertaining the importance of these changes in causing the permanent mental deficits and defining previously unobserved changes accompanying hyperphenylalaninemia. The decrease in cerebral branched chain amino acids is the only well understood neurochemical change in PKU. Clarification of the role of high phenylalanine levels in blocking the transport of other L-system amino acids into the brain (Oldendorf, 1973) has led to proposals for the treatment of PKU with large doses of other neutral amino acids to both restore their cerebral levels and to decrease phenylalanine uptake into the brain. Further understanding of the mechanism of transmitter depletion in the brain of phenylketonurics may also lead to additional treatment protocols. Presently, both substrate depletion (i.e. lowered cerebral tryptophan and tyrosine levels) and enzyme inhibition (lowered tyrosine hydroxylase, tryptophan hydroxylase or dopa decarboxylase activity due to enzyme inhibition by phenylalanine or its metabolites) have been implicated as the primary causes of cerebral serotonin and catecholamine depletion in PKU.

Despite the great number of studies undertaken to define the pathologic changes in animal models of PKU many theories but few facts have surfaced to explain the relationship of early neurochemical changes to permanent behavioral deficits. Animal models provide a unique system to demonstrate such relationships because the acute neurochemical changes accompanying treatment in the neonatal period can be separated from the permanent cerebral changes that exist in the adult non-hyperphenylalaninemic animal. Although little evidence for such changes exist, it is these long-lasting changes caused by hyperphenylalaninemia in the early growth period that must underlie the later behavioral changes in rats and presumably the permanent mental defect in human phenylketonurics.

In focusing in on proposed mechanisms of long-lasting cerebral damage different investigators have concentrated on different aspects of cerebral damage seen in phenylketonurics or in animal models of the disease. For example, some theorize that cerebral serotonin or catecholamine depletion in early life may lead to permanent brain damage in some yet unspecified way. Others feel that dysmyelination is the root cause of the permanent defect despite the fact that such changes are relatively minor and not invariable in retarded phenylketonurics. Another area of interest is in decreased cerebral protein synthesis during development. Although the known perturbations of cerebral amino acid con-

tent in hyperphenylalaninemic animals and man may cause such changes, most of the evidence in favor of this theory has come from in vitro studies or questionable animal models. There is no evidence of 'impressive' net protein deficit in the newer animal models of the disease in which branched chain amino acid depletion has been reproduced. A great number of papers also point to interference with cerebral energy metabolism (Barbato & Barbato, 1969; Swaiman & Lemieux, 1969; Gaull et al., 1978) as being the central defect in PKU. However, most of this work also relies on in vitro studies. More recently investigators have focused in on the role of cerebral glycine elevations (Isaacs & Greengard, 1980) and other changes which have been found in animal models of PKU but whose significance to the human disease state is unknown.

Another issue in PKU research is whether it is phenylalanine itself or elevations in its metabolites that are the primary toxin in the disease. Although much work has been done on the neurochemical (Loo et al., 1972; Loo et al., 1980) and behavioral (Fulton et al., 1980) effects of the major phenylalanine metabolites, the concentration of these substances needed to produce significant effects are usually an order of magnitude or more greater than the levels found in phenylketonurics. Still, it is not clear which factor or combination of factors underlie the creation of a 'permanent mental defect' in PKU.

In terms of basic science the basis of the particular vulnerability of the immature brain to permanent damage is of special interest. Although neuroscientists have often invoked the vague notion of a critical period of brain growth (Sands et al., 1979; Dobbing, 1980) the nature and basis of this time of increased susceptibility to damage has never been fully explained. Animal models of PKU provide a perfect setting to investigate this question since PKU is a 'developmental disease' with a known period of early vulnerability.

The goal of this dissertation is to investigate the above issues by studying the biochemical and behavioral alterations accompanying hyperphenylalaninemia in the developing rat. Changes in the growth and development of model PKUic animals are described (Chapter III) as well as alterations in several behavioral parameters of adult animals made hyperphenylalaninemic early on in life (Chapter IV). A sex difference in the effects of hyperphenylalaninemia was found in both physical and behavioral measurements and further investigated. Furthermore, an attempt is made to elucidate the biochemical mechanism underlying permanent behavioral changes by utilizing different treatment protocols in early life and correlating early biochemical changes with the subsequent behavior of the mature animals. Particular emphasis is placed on the catecholamines where the first demonstration of dopamine and norepinephrine depletion in an animal

model of PKU is made as well as a description of the first long-term protein change (elevation of striatal dopa decarboxylase activity) related to neonatal hyperphenylalaninemia (Chapter V). These investigations are extended into the fetal period with a new model of gestational PKU (Chapter VI) in which the above issues are further addressed as well as those issues peculiar to maternal PKU. Some issues on the nature of the critical period of brain development, in terms of the development of brain amino acid transport systems, are broached in Chapter VII. It is hoped that these studies will significantly add to the present understanding of cerebral development, the pathogenesis of mental retardation and the mechanism underlying the permanent cerebral defect in PKU.

CHAPTER TWO

EXPERIMENTAL METHODS

ANIMAL PROCEDURES

CDF Fischer rats (Charles River Breeding Laboratories, Wilmington, Mass.) given food and water ad libitum and raised on a 12:12 hr light:dark cycle were used in the majority of studies described in this text. In two groups of experiments, where indicated (Chapter IV, Appendix), litters of similarly raised Sprague-Dawley rats were used.

INJECTION SOLUTIONS - Solutions of l-phenylalanine (152 umoles/ml), α -methylphenylalanine (70 umoles/ml; Sigma, St. Louis, Mo.) and phenylalanine (152 umoles/ml) plus α -methylphenylalanine (70 umoles/ml) were prepared by dissolving these compounds in 0.9% NaCl with heating. L-tyrosine (152 umoles/ml) was suspended in 0.9% NaCl and this suspension was sonicated to reduce particle size. Glycine (200 mg/ml) and serine (1200 mg/ml) were dissolved in 0.9% saline. Carboxymethylcellulose (10 or 7.5 mg/ml; CM32, Biorad Laboratories, Richmond, Ca.) was added to the phenylalanine or phenylalanine plus α -methylphenylalanine solutions. Para-chlorophenylalanine (26 umoles/ml; Sigma, St Louis, Mo.) was suspended in the phenylalanine solution and sonicated. All solutions used for chronic treatments were adjusted to pH

7.2. Aliquots were taken from the above solutions after shaking, and injected subcutaneously (DelValle & Greengard, 1976) .

CHRONIC TREATMENTS - The standard chronic treatment to produce hyperphenylalaninemia throughout suckling life consisted of daily subcutaneous injections of phenylalanine (5.2 umoles/g body weight) plus α -methylphenylalanine (2.4 umoles/g body weight) from age 2 to 21 days. This protocol was used for various studies described in Chapters III, IV and V. Several treatment groups of varying duration (e.g. 2-10, 7-15, 7-21 days) received the same injections during the days indicated. Other groups were injected with phenylalanine (5.2 umoles/g) daily and p-chlorophenylalanine (0.9 umoles/g) every other day or glycine (0.7 mg/g) plus serine (2 mg/g) daily on days seven through twenty-one of life. Chronic α -methylphenylalanine treatment consisted of daily injections of 2.4 umoles/g of α -methylphenylalanine only on days 2 to 21. 'Control' animals received daily injections of 0.9% NaCl. In all experiments animals were separated within each litter into control and experimental groups so that interlitter variation could be minimized.

OTHER TREATMENTS - The acute experiments described in Chapter III lasted for one to seven days. The standard amounts

of phenylalanine and α -methylphenylalanine (or less where indicated) were used but these substances were injected a) twice a day, b) along with carboxymethylcellulose or c) in various combinations of phenylalanine plus α -methylphenylalanine, phenylalanine alone or α -methylphenylalanine alone. The standard amounts of α -methylphenylalanine and phenylalanine (or 2.5 times as much) were used in the acute experiments described in Chapter V; α -methylphenylalanine alone was given on day 6 to inhibit hepatic phenylalanine hydroxylase for several acute experiments completed on day 7. Tyrosine or phenylalanine alone given in 3 closely timed injections were also given as indicated in the text. For the studies of phenylalanine transport into the brain (Chapter VII) α -methylphenylalanine (2.4 μ moles/g) was given one day before each experiment was begun and was followed the next day with multiple injections of phenylalanine.

GONADAL HORMONE MANIPULATIONS

NEONATAL CASTRATION - Two day old male rats were anesthetized by cold shock; animals were buried in ice for 3-5 minutes or until they no longer responded to manipulation of their limbs. A vertical incision was then made at the midline distal to the umbilicus and proximal to the genital tubercle. Testes were located on each side with straight tip forceps and they were removed. The incision was closed

with 1-2 sutures of chromic 5-0 gut (Ethicon) and several drops of collodion (Fischer) placed over the stitches. Female control rats underwent sham operations that were identical except no tissues were excised. All animals were warmed to body temperature in an oven (37°-40° C) and rubbed in cage shavings before being returned to their dams.

TESTOSTERONE INJECTIONS - Two day old female rats were injected with 1.25 mg of testosterone proprionate (4-androsten-17 β -ol-3-one proprionate, Sigma, St. Louis, Mo.) dissolved in mineral oil (12.5 mg/ml). Control animals received the vehicle only.

ESTROGEN INJECTIONS - Previously castrated adult males (mean body weight = 180 grams) received daily injections of 30 ug of estradiol (B-estradiol 3-benzoate; Sigma, St. Louis, Mo.) dissolved in sesame oil (300 ug/ml) for two weeks.

GESTATIONAL HYPERPHENYLALANINEMIA - Adult female Fischer rats were time mated with males of the same strain. Vaginal smears were taken daily and upon finding a sperm positive smear, the female was designated as one day pregnant. Dams were allowed food and water "ad libitum". Except where noted otherwise food consisted of Purina Lab Chow until the 12th day of gestation, at which time the experimental rats

were switched to pellets of the same chow supplemented with 0.5% d,l-a-methylphenylalanine and 3% or 7% phenylalanine. Rats were maintained on a twelve hour light (0700 to 1900 hours)/dark cycle. Dams were always sacrificed between 10 and 11 A.M. on the appropriate gestational day.

OTHER PHENYLALANINE DIETS - Weanlings were placed on diets consisting of crushed pellets of Purina Lab Chow to which 0.5% d,l-a-methylphenylalanine plus various amounts (1-7%) phenylalanine were added (Chapter VII). Food was allowed "ad libitum" in these studies.

BLOOD SAMPLING PROCEDURES - Blood for plasma phenylalanine or tyrosine analysis was collected in heparinized tubes from the neck veins of decapitated animals with the following two exceptions. Blood was taken from pregnant female rats at various times of the day by sequentially cutting the tail vein. Repeated sampling of blood for over 24 hours in a pregnant female rat on a diet of 0.5% a-methylphenylalanine plus 7% phenylalanine was accomplished by sampling through a catheterized neck vein. The catheter was placed in the right jugular vein as described by Weeks (1972), and the cannula was heparanized between each blood sampling.

SACRIFICE AND BRAIN DISSECTION - All animals were sacrificed by decapitation, either by guillotine or with a pair of scissors. Brains were immediately removed, except for the olfactory bulbs, and weighed. Striata were removed by the method of Glowinski and Iverson (1966). Frontal cortex refers to that area of the cortex anterior to the optic chiasm. Cortex refers to the remaining cortex of the brain or the entire cortex (frontal cortex included). Midbrain, hippocampus, brainstem (medulla oblongata plus pons) and hypothalamus were removed as described by Glowinski and Iverson (1966). When dissected, fetal brains were separated into cerebellum, brain stem and the remaining forebrain.

BEHAVIORAL METHODS

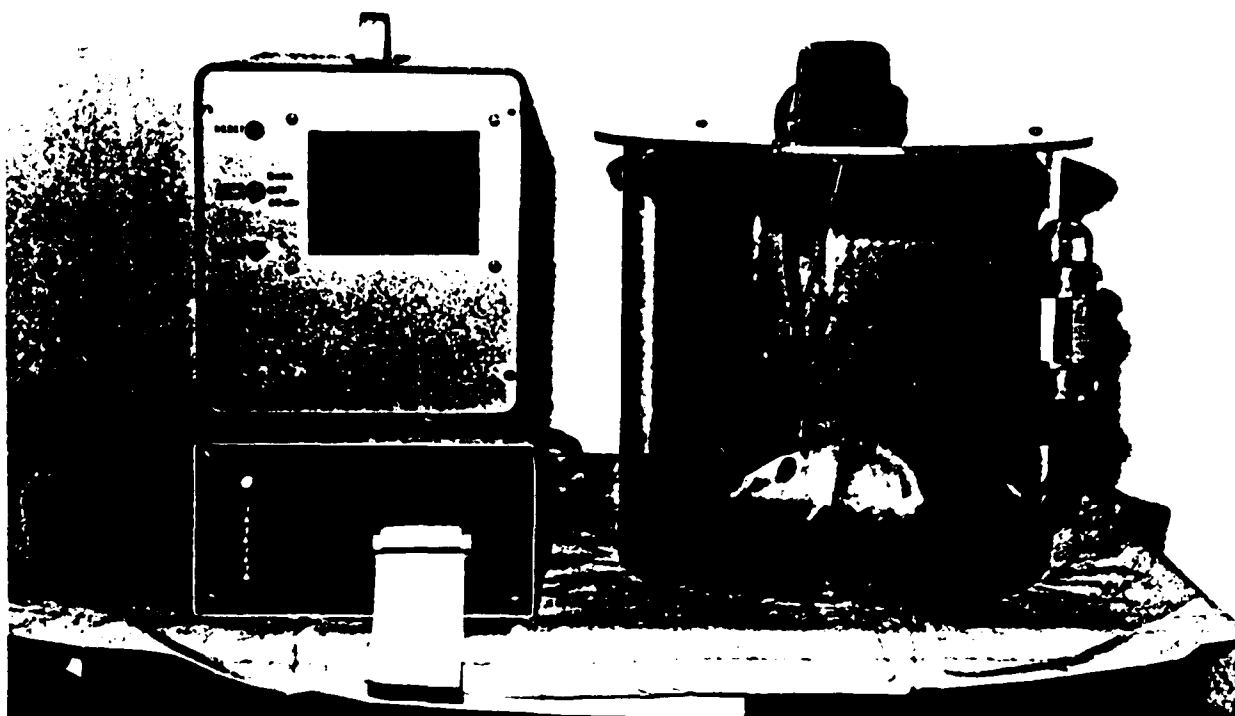
Behavioral studies were conducted in environmentally controlled rooms (temperature=22° C, 60% humidity, 12 hrs light:12 hrs dark cycle) using adult (> 90 days old) animals.

TWENTY-FOUR HOUR SPONTANEOUS ROTATION - Intact animals were individually tested for spontaneous 24 hour rotation. Both food and water were provided "ad libitum" during the test period which comprised 12 hours of light and 12 hours of dark. Each rat was placed in one of twelve "rotometers" (Greenstein & Glick, 1975; see Figure II-1), a modified version of the original apparatus described by Ungerstedt and Arbuthnott (1970). The apparatus consisted of a clear plexiglass sphere (30.5 cm in diameter) placed on a flat wire mesh floor on which the rat rotated. A flexible wire harness was tightened around the abdomen of each animal. This wire was then connected to a shaft leading to a photoelectric position sensing device which could differentiate between full (360°) and quarter (90°) rotations to the left or right. Rotations were automatically recorded on a print-out counter at one or two hour intervals for 24 hours.

FIGURE II-1

ROTOMETER APPARATUS

The apparatus pictured below is the rotometer (see text) used for measurement of both 24 hour spontaneous rotation and one hour drug-induced rotation.



Full rotations to the left or right, their sum (total full rotations) and their difference (net rotations) were separately totaled for each rat. The direction of rotational preference, i.e. the direction in which more full rotations were recorded, as well as the percent preference ((net rotations/total full rotations) x 100) were noted for each animal. Random activity as represented by extra quarter turns, the number of quarter turns recorded minus those quarter turns contributing to full turns (i.e. 4 x full turns), was determined for each direction (left, right) as well as the sum (total extra quarter turns) for each rat tested. Statistics for these parameters of rotation were calculated for the different groups of animals tested. Since most groups tested had control and "experimental" animals within each litter, interlitter comparisons could be made and the effect of interlitter variations in rotational behavior was thereby minimized.

DRUG-INDUCED ROTATION - Animals were individually tested for drug-induced rotation in the middle of their light cycle. Each rat was given an intraperitoneal injection of the test drug or its diluent, harnessed and placed in a rotometer. Rotations were automatically recorded on a print-out counter at 5-minute intervals for 1 hour after injection. Each animal was tested by the various drug dose

regimens described with a period of not less than 3 days between drug injections.

T-MAZE TESTING - To examine learning ability rats were trained to perform a spatially-elicited escape response. Each animal was placed in the long arm of a black plexiglass T-maze (stem=30 cm, arms=15 cm each) with a wire grid floor, and a scrambled foot shock (0.6 mA AC) was administered until the animal reached the "correct" side of the maze. After each trial the animal was removed from the arm of the maze and placed in the stem once again for the next trial. Testing was terminated when the criteria of 5 correct initial choices out of 6 trials was reached. In the first series of experiments on rats treated with phenylalanine plus α -methylphenylalanine on days 2 to 21 of life and their saline treated littermates, half of each set of rats were trained to go to the side ipsilateral to and the other half to the side contralateral to their preferred direction of rotation (24 hr). In later experiments all animals were trained to go to the side opposite their initial choice during their first trial. Some groups of animals were also tested for retention; three days after initial training each animal was re-tested with the same side of the maze designated the correct side. Reversal training, four days after the second test date, consisted of teaching the animal to go to the side opposite of previous training.

LOCOMOTOR ACTIVITY - To measure locomotor activity each animal was placed in a photocell cage (Lehigh Valley No. 145-03) where each interruption of a light beam was recorded for 30 minutes on a Sodeco counter. Alternatively, locomotor activity was measured over a 24 hour period by placing each rat in a Whamann Activity Wheel (running wheel). The phase of the menstrual cycle of each female rat entering the running wheel paradigm was determined by microscopically examining the vaginal smear of each rat before placing it into the running wheel apparatus. Food and water were provided in each running wheel apparatus.

AMINO ACID ANALYSIS

Brains used for phenylalanine and tyrosine analysis were homogenized in 2 or 4 volumes of trichloroacetic acid (final concentration 0.3 M) and centrifuged for 20 minutes at 105,000 x g. Blood was centrifuged for five minutes and the plasma deproteinized with 2 or 4 volumes of trichloroacetic acid (TCA, final concentration 0.3 M). Phenylalanine was measured in deproteinized plasma and brain supernatants fluorometrically by the method of McCaman and Robins (1962) as modified by Faulkner (1965). Tyrosine was assayed using a modification (Wong et. al., 1964) of the spectrophotometric assay of Udenfriend and Cooper (1952). When high tyrosine levels were expected the less sensitive colorimetric assay for tyrosine of Udenfriend and Cooper (1952) as modified by McGee et al., (1972) was used. Neither the phenylalanine nor the tyrosine assays were altered by the addition of high levels (500 nmoles) of α -methylphenylalanine. The concentrations of other amino acids were measured in a Beckman 120 C Automatic Analyzer. For this purpose brain samples were homogenized in 5 volumes of 0.6M TCA and centrifuged; plasma samples were deproteinized with 5 volumes of 3% sulfosalicylic acid.

Brain tissue for glycine analysis was prepared by the addition of TCA to water homogenates (final concentration 0.3M). Glycine content was estimated by a micromodification of the method of Goodwin and Stampwala (1973). A 200 microliter incubation mixture containing 0.1M H₂SO₄, 0.2% chloramine-T, 0.15M TCA (final concentrations), and 100 microliters of deproteinized supernatant was heated at 100°C for 10 minutes, rapidly cooled and then heated at 100°C for 30 minutes after the addition of 1.8 ml of chromotropic acid reagent in H₂SO₄. Absorbance at 570 nm was determined using a Zeiss spectrophotometer PM6. Recovery of glycine was 95-100%, and was not altered by the addition of α -methylphenylalanine or phenylalanine to a final concentration of 2.5 mM or 5 mM. This concentration of phenylalanine is greater than the highest one found in the brains of experimental rats.

NEUROTRANSMITTER ASSAYS

RADIOENZYMATIC ASSAY (REA) FOR CATECHOLAMINES - Dopamine and norepinephrine were measured in the brains of fetal and suckling rats with a highly sensitive radioenzymatic assay. Brains were homogenized in 1.5-2 volumes of 0.2 N perchloric acid, centrifuged at 7000 x g for 10 minutes at 4° C and the supernatant stored under nitrogen gas at -20° C for less than six weeks. Assays were performed by a modification of the method of Coyle and Henry (1973) using the technique of Saller and Zigmond (1978) to separate the methylated catecholamines. Catechol-O-methyltransferase (COMT) was prepared by the method of Axelrod and Tomchick (1958) as described by Coyle and Henry (1973). Each reaction tube (.50ml conical polypropylene tube) contained 9-12 microliters of brain homogenate, 10 ul COMT and a total volume of 40 microliters with final concentrations of the following: 50 mM MgCl₂, 1.5 mM DIT, 0.3 M Tris-HCl Buffer (pH 9.6), 6.5 mM EGTA, 1.5 mM pargyline HCl, 4 uM SAM with 1.25 mM sulfuric acid/ethanol (9:1 v:v), 0.5-1.0 uCi ³H-SAM, 0.13 M NaOH and 0.08-0.10 M perchloric acid. Blanks were prepared by substituting 40 mM CaCl₂ for the MgCl₂ to inhibit the COMT. Internal standards were prepared by adding 1-1.5

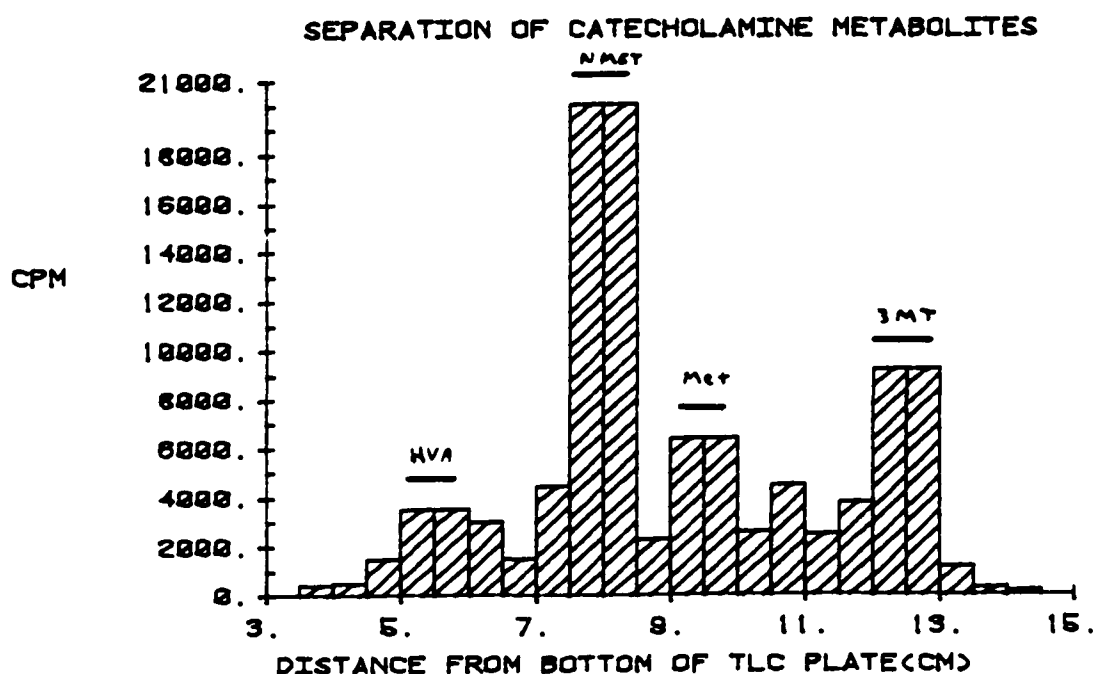
nanograms of dopamine and norepinephrine to tubes containing the usual reaction mixture including homogenate. (All the above chemicals were purchased from Sigma Chemical Co., St Louis, Mo.) Reactions were run for 45 minutes at 30° C, terminated by the addition of 12 ul of a 20% phosphotungstic acid:1.2 N HCl solution (w/v), and spun at 7000 x g for 10 minutes at 4° C. The entire supernatant was then spotted on the absorbent layer of an LK6DF TLC plate (20 x 20 cm, 0.250 mm silica; Whatman Inc., Clifton, N.J.) with non-radioactive tracer substances (3-methoxytyramine, homovanillic acid, nor-metanephrine and metanephrine) and developed in the dark at room temperature for 110-140 minutes in a chloroform:methanol:ethylamine solution (16:3:2). After the plates were dried the spots corresponding to nor-metanephrine and 3-methoxytyramine were visualized under an ultra-violet light, removed from the plate using strip mix (Applied Sciences, State Park, Pa.), eluted with 0.2 ml of 0.5 M borate buffer (pH 9.0) and counted overnight in 5 ml of Econofluor (New England Nuclear, Boston, Mass.) containing 2.5% di-(2-ethyl-hexyl) phosphoric acid (Sigma, St. Louis, Mo.) (v/v). Dopamine and norepinephrine were clearly separated from each other and the radioactive precursor by this procedure (Figure II-2).

Each brain homogenate was assayed three times and the average value used. Calculations of dopamine and norepinephrine content were based on the calculation of the cpm

FIGURE II-2

SEPARATION OF CATECHOLAMINE METABOLITES BY THIN LAYER
CHROMATOGRAPHY

Three nanograms of dopamine and norepinephrine each and 1.5 nanograms of epinephrine and dopac (3,4-dihydroxyphenylacetic acid) each were mixed together and radioenzymatically methylated. The products were then spotted and run on a fluorescent TLC plate. Each half cm section from the bottom of the plate was then scraped off into vials and the radioactivity in each measured in a scintillation counter and plotted as cpm vs distance from the bottom of the plate. Darkened lines correspond to the localization of the methylated products (homovanillic acid, HVA; normetanephrine, NMET; metanephrine, MET; 3-methoxytyramine, 3MT) by fluorescent visualization of non-radioactive compounds run on the TLC plate in parallel with the radioactive compounds.



recovered from known internal standards from several reaction tubes. This 'recovery' or variation in methylation of catecholamine standards did not vary with the different brain homogenates used in this study. The maximal sensitivity (twice blank) using this system was 0.34 picomoles (65 picograms) dopamine and 0.48 picomoles (82 picograms) norepinephrine. The assay was linear for up to 14 nanograms dopamine per sample (Figure II-3).

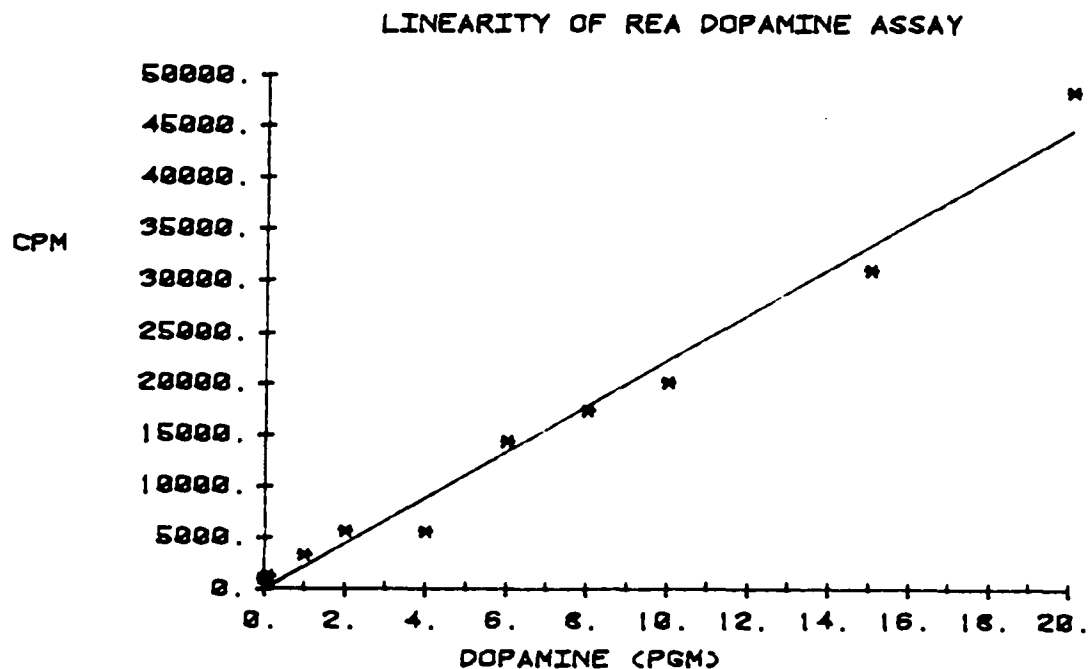
FLUOROMETRIC CATECHOLAMINE ASSAY - Dopamine and norepinephrine were quantified in the brains of adult rats using a modification (Ciarlone, 1976; Ciarlone, 1978) of the hydroxyindole technique of Ansell and Beeson (1968). This method avoided the necessity of an alumina column purification step (Chanq, 1964). The assay was reproducible as long as the pH of the final solution to be spectrofluorometrically measured was strictly controlled (see Lavery & Taylor, 1968) and yielded values consistent with those obtained using the radioenzymatic assay (see Appendix).

SEROTONIN ASSAY - Serotonin and 5-hydroxyindole acetic acid (5-HIAA) were measured by the method of Curzon and Green (1970). Samples were prepared by homogenizing brain in cold (-20° C) acidified butanol and stored at -20° C until use.

FIGURE II-3

LINEARITY OF RADIOENZYMATIC ASSAY OF CATECHOLAMINES

Various amounts of dopamine (0-20 nanograms) were radioenzymatically methylated (see text) and the product, 3-methoxytyramine, separated by thin layer chromatography from non-specific radioactivity. The amount of product isolated (measured as counts per minute) was then plotted against the amount of dopamine in the assay. The resultant curve (line fitted by linear regression with correlation coefficient of $R = 0.99$, $R^2 = 0.98$; significance of regression $p < 0.0001$) is linear up to 20 nanograms.



ENZYME ASSAYS

IMPROVED ASSAY FOR TYROSINE HYDROXYLASE - Many authors have found difficulties in reproducibly assaying tyrosine hydroxylase (E.C. 1.14.16.2) activity in the central nervous system. The literature contains reports of a wide range of activities in the same tissues (e.g. Coyle, 1972; Mann, 1978; Mann, 1980). Some of these problems may relate to the common use of subsaturating amounts of substrate (e.g. Coyle & Axelrod, 1972; Mann, 1980). Another difficulty encountered, the lack of a linear response with increasing concentrations of tissue (see Kuczenski et al., 1972; Nagatsu et al., 1971), has only been fully overcome with the cumbersome use of acetone powders of the tissues studied (Mann, 1978a, Mann, 1978b). Although Mann (1980) has subsequently improved the assay to avoid the use of these powders, this new assay was linear only up to 3 mg of caudate nucleus. Through the use of high concentrations of NaCl, Fe and Triton X-100 as suggested by Mann (1980) we have modified the original assay of Nagatsu et al., (1964) so that maximal enzyme activity can be measured in whole homogenates of rat striate with a linear response with up to 10 mg of tissue.

PREPARATION OF SUBSTRATE - Tyrosine (1-ring-3,5- ^3H 50 Ci/mmol; New England Nuclear, Boston, Mass.) was purified in 0.1 ml portions by passing the acidified substrate (0.1 ml tyrosine, 0.05 ml glacial acetic acid, 1.0 ml H_2O) through a column (0.5 x 3 cm) of cation exchange resin (BioRad Ag 50W-x8, H^+ form, 200-400 mesh; BioRad Laboratories, Richmond, Ca.) and washing with 500-1000 ml of distilled water. The tyrosine was then eluted with 15 ml of 2N HCl, aliquoted into 0.5 and 1.0 ml portions, lyophilized and frozen at -20°C until use. Substrate prepared in this manner was stable for more than one month. Blank (reaction run without DMPH₄ cofactor) to standard (cpm ^3H -tyrosine used in assay) ratios varied between 0.39% to 0.52% during that period. Although the blank value (due to the exchange of tritium between tyrosine and H_2O) rose thereafter, this substrate could be successfully used up to 3 to 4 months after preparation.

ASSAY PROCEDURE - Assay was by a modification of the method of Nagatsu et al., (1964). Tubes (0.30 ml total volume) contained final concentrations of the following: 200 mM NaAcetate buffer (pH 6.0), 280 mM NaCl, 200 units catalase (E.C. 1.11.1.6 from bovine liver; Sigma, St Louis, Mo.), 5 mM 6,7-dimethyl-5,6,7,8 tetrahydropteridine (DMPH₄) HCl (Sigma, St. Louis, Mo.), 10 mM DTT, 10 mM $\text{Fe}(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 0.5mM tyrosine containing 0.04-0.08 uCi of purified

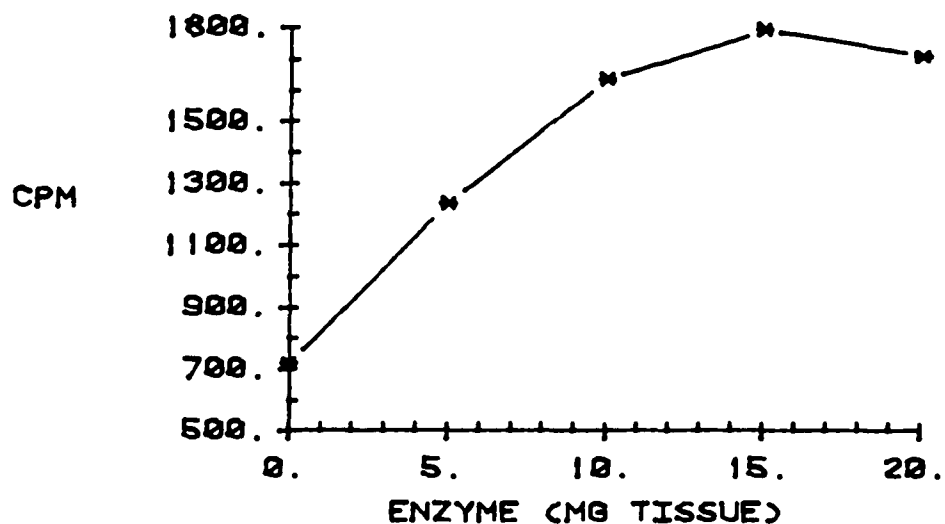
^3H -tyrosine (see above) and 0-0.10 ml of striatal homogenate with 0.2% Triton X-100. Rat striatum was homogenized in either 0.32 M sucrose or 0.25 M NaAcetate buffer (pH 6.0) containing 0.6% Triton X-100. The reaction, run at 37° C, was linear for over 10 minutes with either homogenate. Enzyme activity measured in the whole homogenate was 86% and 93% of the activities found in the 6000g x 10 min supernatants of sucrose and NaAcetate homogenates respectively. The assay was linear using up to 10 mg of whole homogenate of the NaAcetate buffer (Figure II-4) or 8.5 mg using sucrose homogenization medium. Three types of blanks were tested. Reactions run without enzyme still yielded 348 cpm (using 140,000 cpm ^3H -tyrosine standard) whereas reaction tubes missing the synthetic cofactor DMPH₄ had 284 cpm and zero time blanks 231 cpm. Subsequently, zero cofactor (-DMPH₄) blanks were used in all experiments. Although enzyme activity was highest in NaAcetate homogenates, sucrose homogenates of rat striate were used for the experiments described in Chapter V so that other enzymes (dopa decarboxylase, choline acetyltransferase) could be assayed in the same homogenates. Tyrosine hydroxylase can also be successfully measured in 1M NaPhosphate (pH 7.0) homogenates.

All reactions described in Chapter V were initiated by the addition of the tyrosine substrate, incubated for 8 minutes at 37° C in a shaking water bath and terminated with 200 microliters of glacial acetic acid. Each reaction ves-

FIGURE II-4

LINEARITY OF TYROSINE HYDROXYLASE ASSAY

Assays were performed as described in text except that the amount of rat striatal tissue extract used in each incubation varied. Under modified conditions used (see text) the assay was linear to 10 mg of brain tissue.



sel was then centrifuged at 7,000 x g for 10 minutes and the supernatant poured on a column (0.5 x 3 cm) of BioRad 50W-x8 cation exchange resin. After the column was washed twice with 0.5 ml distilled water the total effluent was counted in 10 ml of Instagel (Packard Instrument Co., Downers Grove, Illinois). The efficiency of recovery of tritiated water from these columns was 87.9%. For the experiments described in section II of Chapter V the same striatal homogenate was used for each set (phenylalanine, α -methylphenylalanine) of experiments, although the maximal activity of both striatal preparations from several rats each was similar. In these experiments phenylalanine or α -methylphenylalanine was added to the reaction vessels as indicated and the tyrosine content varied over the 0.5 to 0.05 mM range.

OTHER ENZYME ASSAYS - Enzyme activities, assayed under optimal conditions, are reported in units per gram. One unit represents the metabolism of one nanomole of substrate per minute. All assays were performed on fresh tissue. Striata were homogenized in 9 volumes of 0.32 M sucrose containing 0.6% Triton X-100, and all enzymes measured in any one experiment were assayed from this whole homogenate preparation from each individual rat. Choline acetyltransferase (E.C. 2.3.1.6) was measured by the method of Fonnum (1975). Monoamine oxidase (E.C. 1.4.3.4) was measured by

the method of Wurtman and Axelrod (1963) as described by Naqatsu (1972) using tyramine as a substrate (MAO A + B) or using serotonin as a substrate (MAO A) where specifically indicated. Dopa decarboxylase (aromatic L-amino acid decarboxylase, E.C. 4.1.1.26) was assayed by a modification of the method of Lamprecht and Coyle (1972) using 0.1M NaPhosphate (pH 7.2) buffer without the addition of EDTA or ascorbic acid. The substrate, C^{14} carboxyl d,l,3-4 dihydroxyphenylalanine (specific activity = 49.4 mCi/mM) was purchased from New England Nuclear (Boston, Mass.). For proper extraction of the trapped radioactive CO_2 product it was necessary to add 10% methanol to the Econofluor scintillation fluid used. Tryptophan hydroxylase (E.C. 1.14.16.4) was assayed in 0.01 M Tris-HCl, 0.2 mM DTT homogenates of rat midbrain by the method described by Ichiyama et al., (1970).

For the assay of phosphoserine phosphatase (E.C. 3.1.3.3) fetal brain was homogenized in ice-cold water and (after removing a sample for glycine analysis) centrifuged at 105,000 x g for 30 minutes; the undialyzed supernatant was assayed by the method of Knox et al., (1969). For the assay of all the other enzymes a pool of fetal brains, a pool of fetal brain parts, individual brains or brain parts of postnatal rats, a pool of fetal livers or a section of liver from a postnatal rat were homogenized in 9 volumes of 0.15 M KCl and centrifuged for 30 minutes at 105,000 x g.

The resulting supernatant fluids were used for the determination of the following enzymes: hexokinase (E.C. 2.7.1.1), glutamate dehydrogenase (E.C. 1.4.1.2) and aspartate aminotransferase (E.C. 2.6.1.1) were measured by methods described by MacDonnel and Greengard (1974). Also assayed were malate dehydrogenase (E.C. 1.1.1.37; Schonk & Boxer, 1964), glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49; Krebs & Eggleston, 1965), alpha-glycerophosphate dehydrogenase (E.C. 1.1.99.5; Schonk & Boxer, 1964), glutamate decarboxylase (E.C. 4.1.1.15; MacDonnel & Greengard, 1975), aldolase (E.C. 4.1.2.13; Schapira et al., 1963), thymidine kinase (E.C. 2.7.1.75; Machovich & Greengard, 1972), adenyate kinase (E.C. 2.7.4.3; Cayanis, 1978), cytochrome oxidase (E.C. 1.9.3.1; Cooperstein & Lazcrow, 1954; Margoliash, 1954), glycine synthase (E.C. 2.1.2.0; Balinsky et al., 1973) and creatine kinase (E.C. 2.7.3.2; Bruin et al., 1973). Peptidyl proline hydroxylase (PPH) was assayed by the method of Zimmerberg et al., (1975) with separation of the tritiated water product as described by Peterkofsky and DiBlasio (1975). beta-galactosidase (E.C. 3.2.1.23) and alpha-glucosidase (E.C. 3.2.1.20) were assayed by the method of Peters et al., (1975) as modified by Greengard et al., (1980). Cathepsin C (E.C. 3.4.14.1), gamma-glutamyl transpeptidase (E.C. 2.3.2.2) and acid phosphatase (E.C. 3.1.13.2) were measured by the methods of Seymour and Peters (1977) and 5'nucleotidase (E.C. 3.1.3.5) by the method of

Avruch and Wallach (1971). The assay of hepatic phenylalanine hydroxylase (E.C. 1.14.16.1) was as described by Greengard and DelValle (1976).

Protein was measured by the method of Lowry et al., (1951) and DNA by the procedure of Kissane and Robins (1958) as modified by Hinegardner (1971).

CHAPTER THREE

MODEL PHENYLKETONURIA:

ALTERATIONS IN GROWTH AND METABOLISM

(I) GROWTH OF HYPERPHENYLALANINEMIC RATS

Since the introduction of daily injections of phenylalanine plus α -methylphenylalanine to induce hyperphenylalaninemia in experimental rats (Greengard et al., 1976), many laboratories have adopted this animal model of PKU (e.g. Lane et al., 1980; Nigam & Labar, 1979; Luttges & Gerren 1979; Binek et al., 1981). Still, basic information on how this treatment affects the growth of these animals is relatively meagre. Greengard and co-workers (1976) as well as others (DelValle et al., 1978; Lane et al., 1980; Diemel, 1979) have described small decreases in the body and brain weights of hyperphenylalaninemic rats during their treatment period. However, none of these studies extended their observations past the 40th day of the animal's life, or considered the sex of the animals studied. Since PKU is a developmental disease in which early insults create permanent changes, it is important to know the long term effects of the model PKU on the growth of experimental animals. Also, in light of the different behavioral responses of male and female rats given phenylalanine plus α -methylphenylalanine early in life (Chapter IV), the importance of examining the growth effects in each sex separately cannot be underestimated.

Head circumference of untreated PKUic adults has been found to be 1.4 standard deviations below the mean circumference of the general population (Holm & Knox, 1979). This deficit in head size is noted soon after birth but is small and non-significant until 4 months to 2 years of age at which time PKUics are 1.6 standard deviations below the mean. It is not clear whether microcephaly progresses with maturity. The relationship between PKU and stature is even less clear. Home-reared PKUics appear to have 'normal for age' heights (Fisch et al., 1966; Holm & Knox, 1979), whereas, those institutionalized with the disease are shorter than the general population (Nellhaus, 1968; Paine, 1957; Holm & Knox, 1979). Whether the reduced height in institutionalized PKUics is due to greater severity of their disease or non-specific factors that depress growth in institutionalized populations (Mosier et al., 1965) is not known.

The first section of this chapter describes in detail the deficits in body and brain weight of male and female rats given phenylalanine plus α -methylphenylalanine during their 2nd to 21st days of life. Body weight and brain weight are used as gross equivalents to the body height (reflecting general metabolic effects) and head circumference (effect on brain size) measurements taken in humans. Although both males and females recover from early body weight deficits, only male rats fully catch up in respect to

brain weight. Changes in the weights of particular brain areas are also investigated, but no brain region studied seemed to be inordinately responsible for the loss in brain mass. The effects of gonadal hormone manipulation (neonatal castration, neonatal testosterone treatment) on body and brain weight are also reported.

RESULTS

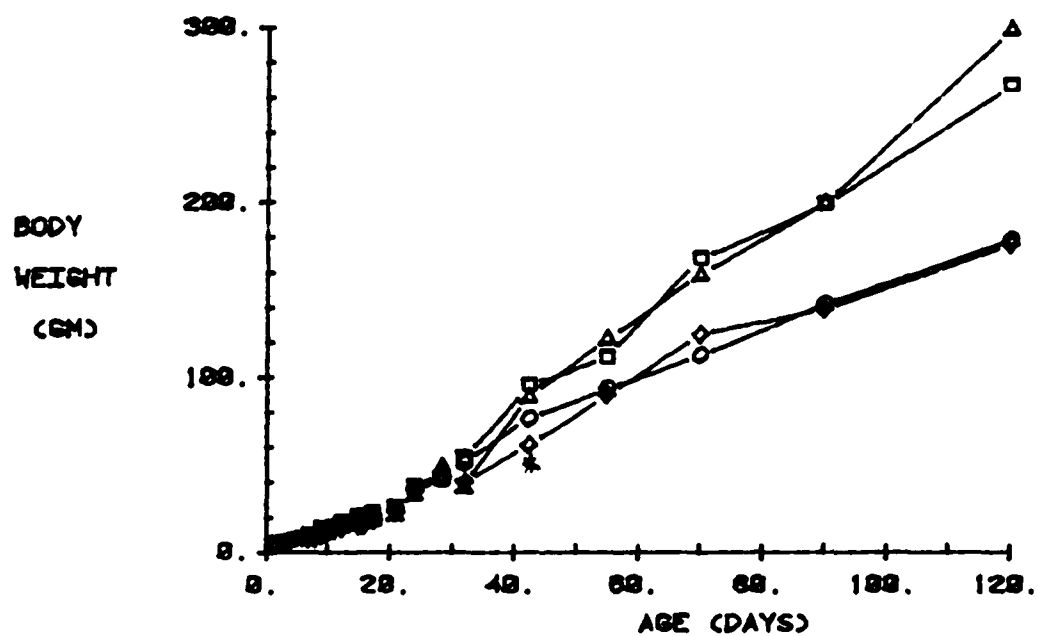
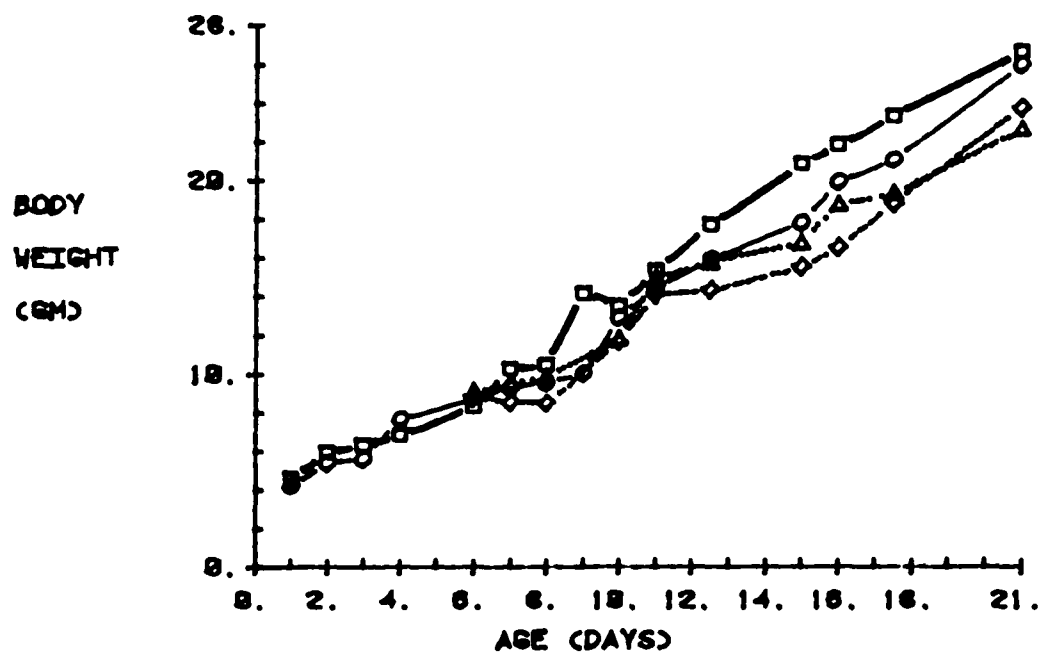
Both male and female rats given daily injections of phenylalanine plus α -methylphenylalanine on days 2 to 21 of life showed small reductions in their body weights (Figure III-1). Although both groups eventually recovered from this weight loss, female rats suffered more severe and longer lasting deficits. Males fully caught up to their saline injected littermates by day 40, whereas experimental females exhibited their largest weight deficit at this age. By day 70 the female weight loss was only 10% and no longer statistically significant, and on day 90 no body weight differences between control and experimental rats were seen at all in either sex.

Similarly, neonatal hyperphenylalaninemia resulted in more severe and prolonged brain weight deficits in female rats (Figure III-2) than in their similarly treated male littermates (Figure III-3). Brain weight loss was progressive in male rats during the treatment period and subsequently declined. Significant weight deficit was absent by day 70 and essentially equivalent brain weights were found in control and experimental adult (120-150 days old) rats. In contrast, female PKUic rats had a significant brain

FIGURE III-1

BODY GROWTH IN RATS EXPOSED TO NEONATAL
HYPERPHENYLALANINEMIA AND CONTROLS

Rats were given daily injections of phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w), (Experimental), or an equivalent volume of saline diluent (Control) on the 2nd to 21st days of life. Each point represents the mean body weight of 2-38 animals from several different litters. The standard deviation is within the range of the symbols themselves in all cases.
* indicates significant difference ($p < 0.01$) from control value.



—□— CONTROL MALE
 —○— CONTROL FEMALE
 —△— EXPERIMENTAL MALE
 —◇— EXPERIMENTAL FEMALE

FIGURE III-2

BRAIN GROWTH IN FEMALE RATS EXPOSED TO NEONATAL
HYPERPHENYLALANEMIA AND CONTROLS

Rats were given daily injections of phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w), (Experimental) or an equivalent volume of saline diluent (Control) on the 2nd to 21st days of life. Each point represents the mean body weight of 2-38 animals from several different litters. Where standard deviation bars are not discernible they are within the physical confines of the symbols themselves.

	0-21	0-Adult
Control Female		
Experimental Female		

* indicates significant difference ($p < 0.01$)
from control value.

BRWTS: FEMALE CONTR VS EXPER

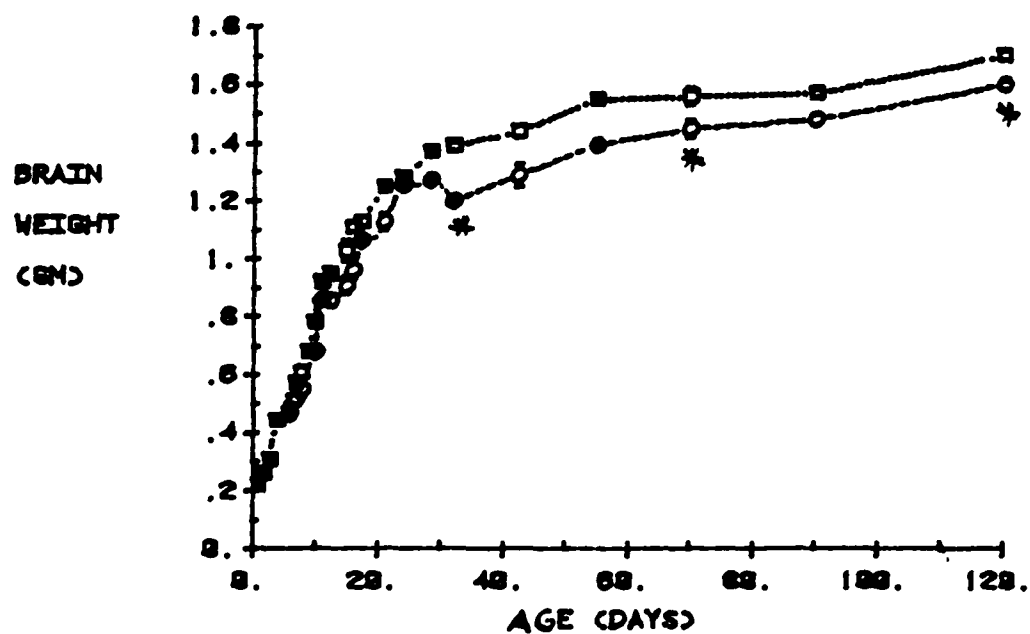
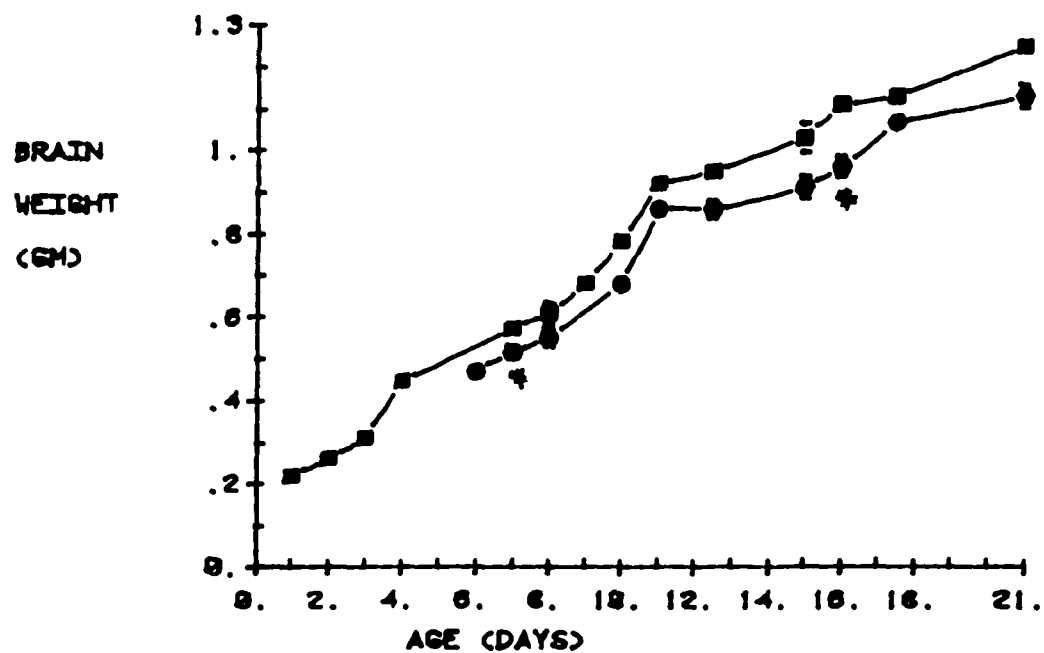
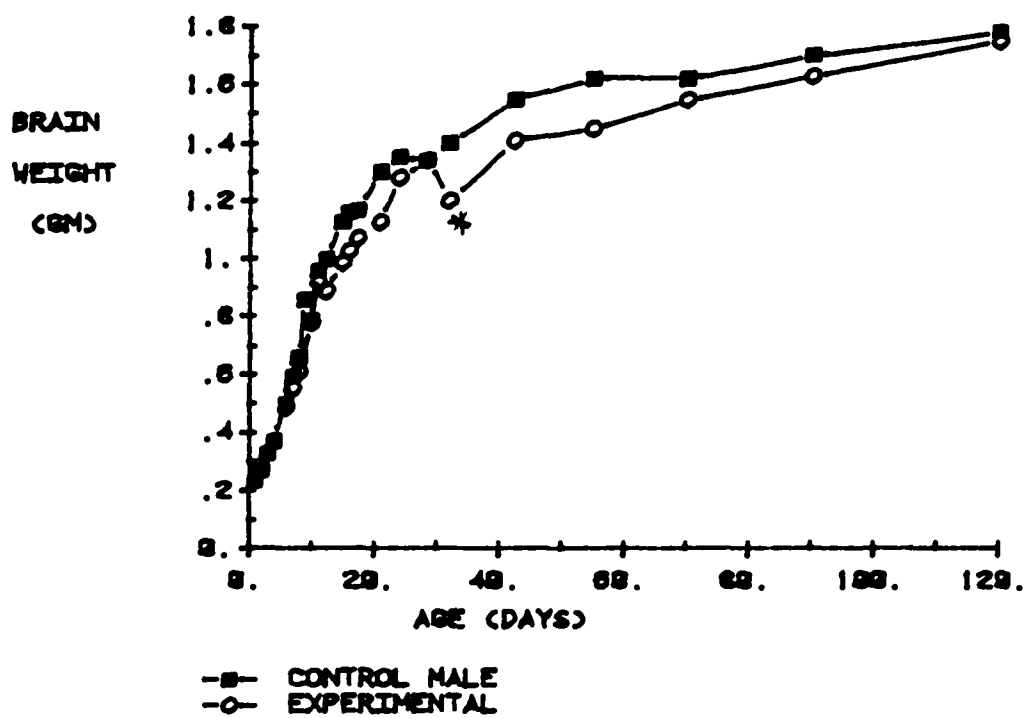
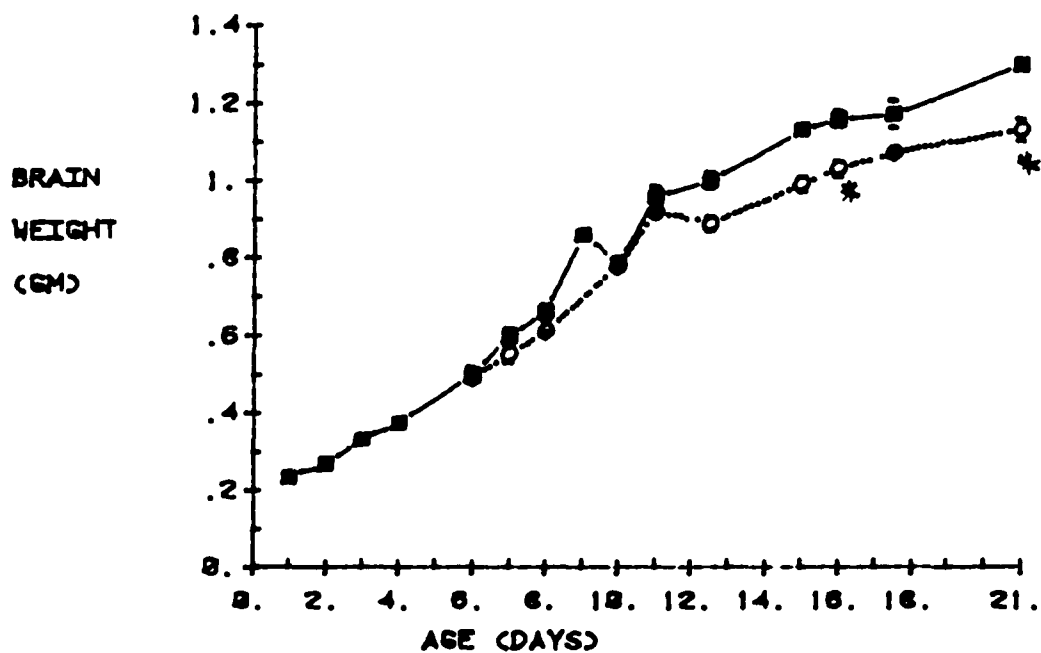


FIGURE III-3

BRAIN GROWTH IN MALE RATS EXPOSED TO NEONATAL
HYPERPHENYLALANEMIA AND CONTROLS

Rats were given daily injections of phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w), (Experimental) or an equivalent volume of saline diluent (Control) on the 2nd to 21st days of life. Each point represents the mean body weight of 2-38 animals from several different litters. Where standard deviation bars are not discernible they are within the physical confines of the symbols themselves.
* indicates significant difference ($p < 0.01$) from control value,

BRVTS: MALE EXP VS CONTROL



weight deficit by day 7 as opposed to day 16 in the males. The insult was most pronounced in the females on day 16 (15% below control) and persisted throughout life. Adult PKUic females still had brains that were significantly lighter (6% reduction) than their control littermates. Thus, hyperphenylalaninemia in the suckling period resulted in a permanent physical defect in female but not male rats.

Table III-1 shows that the 6% decrease in the brain weight of adult PKUic female rats was not borne primarily by any one of the brain areas studied. This table also shows that PKUic female rats injected with testosterone propionate on the second day of life did not show the brain weight deficit seen in hormonally normal PKUic females. (These rats had body weights intermediate between those of male and female adults.) Furthermore, neonatally castrated PKUic males had significantly reduced brain weights compared to control male but not control female adults. Normal adult male rats had not only greater body weights but significantly greater brain weights than control females. (Differences in the growth of normal male and female rats and the growth of animals given alternate experimental treatments are described in detail in the Appendix.)

TABLE III-1

THE EFFECTS OF NEONATAL HYPERPHENYLALANINEMIA ON ADULT BODY WEIGHT AND REGIONAL BRAIN WEIGHT

Rat pups were injected with phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w); (experimental) or an equivalent volume of saline diluent (controls) daily during the 2nd to 21st days of life (see Methods). One group of female rats were additionally given one injection of testosterone propionate (1.25 mg) on the second day of life (Eq-TP) and the group of male rats were castrated on the 2nd day of life (Eq-castrate). All animals were sacrificed as adults (> 120 days). Brains were excised and dissected as described in Methods. Values represent one mean $\pm$ SD (n) with * denoting significant differences ($p < 0.01$) from control values.

Treatment	Body Weight g	Brain Weight g	Cerebellum mg	Striate mg	Frontal Cortex mg	Brain Stem mg	Midbrain mg
Control Male	267 $\pm$ 39(36)	1.78 $\pm$ 10(38)	258 $\pm$ 33(20)	74 $\pm$ 13(18)	310 $\pm$ 73(3)	373 $\pm$ 38(10)	139 $\pm$ 21(5)
Experimental Male	299 $\pm$ 57(47)	1.75 $\pm$ 11(47)	263 $\pm$ 27(22)	73 $\pm$ 12(22)	277 $\pm$ 58(16)	378 $\pm$ 38(7)	158 $\pm$ 10(4)
Control Female	179 $\pm$ 26(38)	1.70 $\pm$.08(40)*	225 $\pm$ 29(12)	82 $\pm$ 11(8)	308 $\pm$ 48(6)	353 $\pm$ 50(6)	134 $\pm$ 14(4)
Experimental Female	176 $\pm$ 24(38)	1.60 $\pm$.10(42)*	208 $\pm$ 37(13)	70 $\pm$ 18(10)	306 $\pm$ 22(5)	360 $\pm$ 36(5)	126 $\pm$ 16(4)
Experimental Female TP	280 $\pm$ 29(9)	1.71 $\pm$.07(9)	247 $\pm$ 20(9)	66 $\pm$ 6(9)	282 $\pm$ 34(9)		
Experimental Male Castrate	194 $\pm$ 24(6)	1.67 $\pm$.08(5)*					

DISCUSSION

Female rats given phenylalanine plus α -methylphenylalanine during days 2 to 21 of life sustained both quantitatively and qualitatively greater growth deficits than similarly treated males did. They incurred more severe and longer lasting delays in general body growth (body weight, Figure III-1). Their lag in brain growth was not only earlier in occurrence and greater than that seen in their male littermates, but qualitatively different as well, in that male experimental rats fully recovered from cerebral weight losses (Figure III-3) whereas experimental female rats suffered permanent brain weight losses (Figure III-2). Qualitative differences between the growth retardation of male and female PKUics have not been reported. However, since no large compilation of growth data on untreated PKUics has analyzed the data by sex (e.g. Holm & Knox, 1979), it is unknown whether smaller quantitative differences between the growth deficits in male and female PKUics exist. The growth of treated phenylketonurics differs subtly between the two sexes. Treated female, but not male, PKUics show a tendency toward greater weight gain and larger head circumferences than 'normal' children (Holm et al., 1979).

Giving testosterone to 2 day old PKUic females caused an increase in their body and brain weights to levels comparable to control females but less than control or experimental males. Concordantly, removal of testosterone influences, through neonatal castration, resulted in a decrease in the body and brain weight of male PKUic rats. Their adult brain weights were less than that of intact males or even control females (but not significantly so) but greater than that of experimental females. Since no hormonal manipulations were executed in control animals it is not possible to fully determine to what extent these brain weight changes were due to 'masculinization' or 'feminization' of the rat or to an interaction between testosterone and the damage caused by high phenylalanine content. Thus, it is not known whether one injection of testosterone fully masculinized the growth capacity of female PKUic rats and the hyperphenylalaninemia still limited the growth potential of these rats, or if perhaps the testosterone counteracted the growth inhibiting effect of high phenylalanine but only partially increased the female brain growth potential (toward male levels). Since each treatment was only partially effective in masculinizing or feminizing the body weights of these animals, the same may be true in terms of the brain weight effects. This suggests that the difference between the brain weights of PKUic females and PKUic castrate males, and the difference in weights between PKUic males and PKUic testosterone treated females, may be almost entirely due to the

partial effectiveness of the hormonal manipulations. This would imply that testosterone plays an important role in protecting the developing brain from the long term effects of hyperphenylalaninemia. This hypothesis is accentuated by the fact that males rapidly and fully recover their brain mass at the time of increased testosterone production. It is of interest that the group of animals (testosterone treated females) whose brain weights more closely approached those of the opposite sex, also exhibited behaviors more like them (Chapter IV).

The decrease in overall brain weight of female PKUic rats was not particularly manifested in any one of the specific brain areas studied. One might expect that the growth in later developing areas of the brain, for example the cerebellum, would be particularly affected by neonatally imposed hyperphenylalaninemia since these areas are still differentiating at the time of the insult. Huther and Neuhoff (1981) have detailed the weight loss and changes in patterns of differentiation in the rat cerebellum caused by phenylalanine plus α -methylphenylalanine treatments. Our present analysis indicates a more generalized growth retardation in all the brain areas studied. However, the greater deviations in weighing small dissected brain parts, as opposed to whole brain, does necessitate that larger samples be obtained before small changes in the brain weights of these areas are noted.

(II) ALTERNATIVE MANIPULATIONS OF PLASMA AND
CEREBRAL PHENYLALANINE LEVELS IN SUCKLING RATS

Although daily injections of suckling rats with phenylalanine plus α -methylphenylalanine may represent the best imitation of PKU in an animal model yet reported, this model is not without its limitations. While peak plasma and brain phenylalanine elevations correspond to those seen in phenylketonuric children, these elevations are transient; they do not last throughout the entire day (Dienel, 1977) as they presumably do in the human disease state. Furthermore, data presented in the Appendix indicate that the present treatment may cause only a borderline insult which is easily perturbed by presently unknown and therefore uncontrollable factors. For these reasons the establishment of an improved rat model of PKU, one in which plasma and brain phenylalanine elevations are longer lasting, yet animal toxicity is not increased, was sought.

Section II describes several generally unsuccessful approaches to improving the present model. Longer lasting plasma elevations of phenylalanine were sought through multiple injections of phenylalanine and/or α -methylphenylalanine during each day, injection of phenylalanine plus α -me-

thylphenylalanine with a carboxymethylcellulose vehicle to try to slow the absorption of phenylalanine into the circulatory system, and transfer of phenylalanine to suckling rats through their dams' milk. Although none of these treatments lead to a new treatment model they may have been helpful in furthering our understanding of the complex mechanisms of phenylalanine homeostasis.

RESULTS

Although we have been able to confirm earlier work demonstrating the success of daily phenylalanine plus α -methylphenylalanine injections in substantially raising the plasma phenylalanine levels in young suckling (2-17 days old) rats (e.g. see lines 9 and 10, Table III-4), the effect of such treatments of male and female rats had not been previously investigated. We now report that there are no apparent sex differences in the hyperphenylalaninemia or cerebral phenylalanine elevations that follow such treatments. Seven hours after the last daily injection of phenylalanine plus α -methylphenylalanine six day old male rats had essentially the same elevations in plasma phenylalanine (male= 2.66 ± 0.4 (6), female= 2.84 ± 0.6 (6) μ moles/ml plasma) and brain phenylalanine (male= 2.04 ± 0.02 (6), female= 2.05 ± 0.51 (6) μ moles/g brain) as their female littermates. Control plasma (male= 0.07 ± 0.03 (6), female= 0.06 ± 0.01 (6) μ moles/ml plasma) and brain (male= 0.07 ± 0.01 (6), female= 0.07 ± 0.02 (6) μ moles/g brain) values were also similar for both sexes.

Despite the very high peak elevations in plasma phenylalanine levels achieved, it is not possible to maintain phenylalanine levels similar to those present in PKUic chil-

dren over a 24 hour period with the present protocol (Dienel, 1977). Therefore, efforts were made to devise new treatments, still utilizing phenylalanine and α -methylphenylalanine injections, that would extend the amount of time each day during which 'PKU-like' elevations in plasma phenylalanine could be maintained. One such attempt took advantage of the high plasma phenylalanine elevations lasting throughout each day found in adult female rats fed a diet supplemented with 0.5% α -methylphenylalanine and 3% or 7% phenylalanine (see Chapter VI). It was hoped that transfer of maternal phenylalanine through the dams' milk to suckling pups who feed constantly throughout the day might aid in achieving similarly long-lasting hyperphenylalaninemia in their pups. However, 6-7 day old suckling pups of dams on the above diet failed to show large rises in plasma or brain phenylalanine when sampled in the morning (11am-noon plasma phenylalanine = 275 ± 95 nmoles/ml plasma, brain phenylalanine = 202 ± 58 nmoles/g brain) or in the evening (8 pm plasma phenylalanine = 107, 131 nmoles/ml, brain phenylalanine = 238, 282 nmoles/g).

Another effort to lengthen the hours of each day of severe hyperphenylalaninemia involved the use of multiple injections of phenylalanine plus α -methylphenylalanine or phenylalanine alone during each day. None of the various treatments (Table III-2) were effective in raising plasma or brain phenylalanine to high levels 16-24 hours after the

TABLE III- 2

PLASMA AND BRAIN PHENYLALANINE LEVELS IN RATS FOLLOWING MULTIPLE DAILY INJECTIONS WITH α -METHYLPHENYLALANINE AND PHENYLALANINE

Suckling animals were injected with α -methylphenylalanine (2.4 umoles/g body w) plus phenylalanine (5.2 umoles/g body w) for the number of days listed under 'Pre-treatment'. Beginning at 0 hours (24 hours after last injection of pre-treatment schedule) they then received 2-4 injections of various doses of phenylalanine (phe) and/or α -methylphenylalanine (amphe). The dose in umoles/g body weight for each injection with time of treatment in parenthesis (0 hours is the first injection) is listed under 'Injection Schedule'. Animals were then sacrificed and plasma and brain phenylalanine levels determined. All rats were killed between the 14th and 16th days of life.

<u>Pretreatment</u> <u>Type</u>	<u># of</u> <u>days</u>	<u>Treatment Schedule</u> <u>umoles/g body w(hrs)</u>	<u>Time of</u> <u>Sacrifice</u>	<u>Hrs Since</u> <u>Last Injection</u>	<u>Plasma</u> <u>phe</u> <u>nmol/ml</u>	<u>Brain</u> <u>phe</u> <u>nmol/gm</u>
None		Saline (0 hr)	24	24	135, 174	184, 240
amphe & phe	2	2.4 amphe & 5.2 phe (0 hr), 2.6 phe (3.5 hr)	17	14.5	192	250
amphe & phe	2	2.4 amphe & 5.2 phe (0 hr), 5.2 phe (3.5 hr)	17	14.5	168, 172	136, 146
amphe & phe	2	2.4 amphe & 5.2 phe (0 hr), 2.6 phe (5 hr)	16	11	118, 222	206, 149
amphe & phe	2	2.4 amphe & 5.2 phe (0 hr), 5.2 phe (5 hr)	16	11	138	225
amphe & phe	3	2.4 amphe & 5.2 phe (0 hr), 5.2 phe (6 hr)	20	14	117+0(3)	169+70(3)
amphe & phe	1	1.2 amphe & 2.6 phe (0 hr), 1.2 amphe & 2.6 phe (8 hr)	24	16	79, 136	202, 126
amphe & phe	15	2.4 amphe & 5.2 phe (0 hr), 2.6 phe (8 hr)	22	14	122, 179	126, 120
amphe & phe	15	2.4 amphe & 5.2 phe (0 hr), 2.6 phe (8 hr)	24	16	158, 110	167, 133
amphe & phe	15	2.4 amphe & 5.2 phe (0 hr), 5.2 phe (8 hr)	24	16	196, 126	128, 133
amphe & phe	15	2.4 amphe & 5.2 phe (0 hr), 2.6 phe (8 hr), 5.2 phe (24hr)	32	8	1263, 695	515, 358
amphe & phe	15	2.4 amphe & 5.2 phe (0 hr), 5.2 phe (8 hr), 5.2 phe (24 hr)	32	8	357, 2274	212, 878
amphe & phe	15	1.2 amphe & 2.6 phe (0 hr), 2.6 phe (8 hr)	48	16	2210	230
amphe & phe	15	2.4 amphe & 5.2 phe (0 hr), 5.2 phe (8 hr), 5.2 phe (24 hr), 5.2 phe (32 hr)	48	16	657	146

initial daily injection. In fact, plasma phenylalanine content was lower in 14-16 day old rats given multiple injections (Table III-2) than in rats receiving only the initial daily injection. Thus, administration of additional phenylalanine with a 2nd or 3rd daily injection was counterproductive to achieving a longer lasting hyperphenylalaninemia.

A third approach toward improving our current experimental treatment involved the use of carboxymethylcellulose (CMC), an inert polymer, in conjunction with phenylalanine and α -methylphenylalanine, to slow the release of phenylalanine into the plasma and thus hopefully, lengthen the period of plasma phenylalanine elevation. Such treatments, whether utilizing a single (lines 2 + 4, Table III-3) or multiple daily injections (lines 3, 5-18, Table III-3) were largely unsuccessful. Twice a day injections of phenylalanine plus α -methylphenylalanine separated by eight hours and utilizing CMC in the second injection (lines 5-8, Table III-3) were much more successful in maintaining high plasma phenylalanine levels than similar treatments (lines 6-9, Table III-2) without CMC. This suggests some utility in pursuing the use of this agent. However, no clear pattern of reproducible and long-lasting elevations of plasma phenylalanine were found using CMC with phenylalanine and α -methylphenylalanine.

The complexities encountered in attempting to circumvent the animal's mechanism of phenylalanine homeostasis is

TABLE III- 3

PLASMA AND BRAIN PHENYLALANINE LEVELS IN RATS INJECTED WITH α -METHYLPHENYLALANINE, PHENYLALANINE
AND CARBOXYMETHYLCELLULOSE

Suckling rats were injected once with α -methylphenylalanine (2.4 umoles/g body w) and/or phenylalanine (2.4 umoles/g body w) and/or carboxymethylcellulose (0.2 mg/g body w) as indicated under 'Pre-treatment'. Beginning at 0 hrs (24 hrs after pre-treatment) they received 1-4 injections of various doses of phenylalanine (phe), α -methylphenylalanine (amphe) and/or carboxymethylcellulose (cmc). The dose (umoles/g body w for phenylalanine and α -methylphenylalanine; mg/ml for carboxymethylcellulose) with time of injection in parenthesis (0 hrs is the time of 1st injection) is listed for each treatment under 'Injection Schedule'. The time of sacrifice and total time from last injection to sacrifice are also recorded. All animals were sacrificed between the ages of 11 and 17 days.

<u>Pretreatment</u>	<u>Injection Schedule</u> umoles/g body w (hrs)	<u>Time of</u> <u>Sacrifice</u>	<u>Hrs Since</u> <u>Last Injection</u>	<u>Plasma</u> <u>phe</u> nmol/ml	<u>Brain</u> <u>phe</u> nmol/g
None	Saline (0 hr)	24	24	116, 101	175, 123
amphe & phe & cmc	2.4 amphe & 5.2 phe & 0.2 cmc (0 hr), 2.4 amphe & 5.2 phe & 0.2 cmc (8 hr)	21	13	3201, 4917	1209, 2105
amphe & phe & cmc	2.4 amphe & 5.2 phe & 0.2 cmc (0 hr)	21	21	107, 111	92, 90
amphe	2.4 amphe & 5.2 phe & 0.2 cmc (0 hr)	18	18	129	305
amphe	2.4 amphe & 5.2 phe & 0.2 cmc (0 hr), 2.4 amphe & 5.2 phe & 0.2 cmc (8 hr)	18	10	5068, 5218	3408, 3896
amphe	2.4 amphe & 5.2 phe & 0.2 cmc (0 hr), 5.2 phe & 0.2 cmc (8 hr)	24	16	3116	3611
amphe	2.4 amphe & 5.2 phe (0 hr), 2.4 amphe & 5.2 phe (8 hr)	20	12	4579	1244
amphe	2.4 amphe & 5.2 phe & 0.2 cmc (0 hr) 2.4 amphe & 5.2 phe & 0.2 cmc (8 hr)	20	12	1099	556
amphe	2.4 amphe & 5.2 phe & 0.2 cmc (0 hr), 6.9 phe & 0.27 cmc (8 hr)	20	12	293	155
amphe	1.2 amphe & 5.2 phe & 0.2 cmc (0 hr), 1.2 amphe & 2.2 phe & 0.1 cmc (8 hr)	20	12	130	119
amphe	1.8 amphe & 3.9 phe & 0.15 cmc (0 hr), 3.5 phe & 0.13 cmc (8 hr)	20	12	110	
amphe & phe	2.4 amphe & 5.2 phe (0 hr), 5.2 phe & 0.15 cmc (19 hr)	25	16	92, 96	132, 125
amphe & phe	2.4 amphe & 5.2 phe & 0.15 cmc (0 hr), 5.2 phe & 0.15 cmc (9 hr)	25	16	106, 118	130, 123
amphe & phe	1.2 amphe & 2.6 phe & 0.08 cmc (0 hr), 2.6 phe & 0.08 cmc (6 hr), 1.2 amphe & 2.6 phe & 0.08 cmc (22 hr), 2.6 phe & 0.08 cmc (30 hr)	46	16	72, 154	114, 91
amphe & phe	1.2 amphe & 5.2 phe & 0.15 cmc (0 hr), 1.2 amphe & 5.2 phe & 0.15 cmc (6 hr), 1.2 amphe & 5.2 phe & 0.15 cmc (22 hr), 1.2 amphe & 5.2 phe & 0.15 cmc (30 hr)	46	16	118, 108	73, 100
amphe & phe	2.4 amphe & 5.2 phe & 0.15 cmc (0 hr), 2.4 amphe & 5.2 phe & 0.15 cmc (6 hr), 2.4 amphe & 5.2 phe & 0.15 cmc (22 hr), 2.5 amphe & 5.2 phe & 0.15 cmc (30 hr)	46	16	77, 94	109, 91
amphe & phe	2.4 amphe & 5.2 phe (0 hr), 2.4 amphe & 5.2 phe (9 hr)	25	16	115	96

further demonstrated in Table III-4. Although plasma phenylalanine returns to normal by 24 hours after the last injection of phenylalanine plus α -methylphenylalanine (Dienel, 1977), we see that prior treatment with phenylalanine in addition to the PAH inhibitor α -methylphenylalanine is important in maintaining high plasma phenylalanine after injection of phenylalanine alone (lines 4 + 7, Table III-4). Pre-treatment with α -methylphenylalanine plus phenylalanine for 5 days rather than 1 also serves to heighten the response to phenylalanine (plus α -methylphenylalanine) 6 hours after injection (lines 8 + 9, Table III-4). The combination of these two factors may underlie the observation that in rats given α -methylphenylalanine only on day 6 it takes 2.5 times as much phenylalanine alone on day 7 to get an equivalent elevation of plasma phenylalanine as that seen when phenylalanine plus α -methylphenylalanine is injected into rats pre-treated with α -methylphenylalanine plus phenylalanine for five days (lines 5 + 9, Table III-4). Furthermore, α -methylphenylalanine when administered with phenylalanine causes a higher rise in plasma phenylalanine than does phenylalanine alone (Lines 7 + 9, Table III-4). However, α -methylphenylalanine alone only raises plasma phenylalanine levels minimally (line 2, Table III-4). Even more surprisingly, injection of 5.2 μ moles/g of phenylalanine led to a hyperphenylalaninemia equivalent to that seen when 13 μ moles/g phenylalanine alone was administered to rats which

TABLE III-4

PLASMA PHENYLALANINE LEVELS WITH DIFFERENT ALPHA-METHYLPHENYLALANINE PLUS
PHENYLALANINE TREATMENT PROTOCOLS

Suckling rats were injected with α -methylphenylalanine (2.4 umoles/g body w), phenylalanine (5.2 umoles/g body w) or both on the day(s) of life indicated under 'Pretreatment'. On postnatal day seven they were injected with these same substances, saline vehicle (saline), 12.5 umoles/g body w of phenylalanine (2.5 x phe), or tyrosine (5.2 umoles/g body w) as indicated under 'Treatment'. At the number of hours indicated after injection, they were decapitated, cervical trunk blood obtained and phenylalanine measured. Values represent the mean $\pm$ SD(n).

<u>Pretreatment</u>		<u>Treatment</u>		<u>Plasma Phenylalanine</u> (umole/ml)
<u>Type</u>	<u>Days</u>	<u>Type</u>	<u>Hours post injection</u>	
-	-	saline	6	143 $\pm$ 53 (5)
-	-	amphe	6	269, 247
amphe	6	phe	3	1453 $\pm$ 469 (4)
amphe	6	phe	6	1268, 1668
amphe	6	2.5 x phe	6	3194 $\pm$ 323 (4)
amphe	6	phe & tyr	6	3672 $\pm$ 830 (7)
amphe & phe	2-6	phe	6	896 $\pm$ 96 (4)
amphe & phe	6	amphe & phe	6	2430, 2807
amphe & phe	2-6	amphe & phe	6	2750 $\pm$ 540 (12)
amphe & phe	2-6	amphe & phe	12	1250 $\pm$ 200 (4)

had been given α -methylphenylalanine the day before (lines 6
+ 5, Table III-4).

DISCUSSION

Section II of this chapter demonstrates the difficulties encountered in attempting to further circumvent the rats control of phenylalanine homeostasis. Phenylalanine and/or α -methylphenylalanine do not appear to pass through the mothers milk or to be absorbed in the gut of the weanling in sufficient quantities to affect the suckling rats plasma phenylalanine levels a great deal. Furthermore, injecting phenylalanine into a rat more than once a day with or without additional α -methylphenylalanine results in a decrement in the long term (16-24 hour post-injection) hyperphenylalaninemia achieved (Table III-2). There are two obvious explanations for this paradoxical result. Additional daily injections may serve to stress the rat with subsequent induction of hepatic PAH and increased phenylalanine catabolism. This PAH induction requires only a few hours to take effect (DelValle et al., 1978). Another possible explanation may be understood in terms of the previous data which showed that high levels of phenylalanine may serve to disinhibit the effect of α -methylphenylalanine on PAH. Specifically, a second injection of phenylalanine a few hours after α -methylphenylalanine plus phenylalanine

injection causes a lowering of the plasma phenylalanine levels a few hours later (Dienel, 1977). Thus, our second phenylalanine injection may serve to increase PAH activity.

(The variation in time between the 1st and 2nd injections was aimed at circumventing this effect.) The mechanism of this action is unknown, but such effects call attention to our ignorance of amino acid homeostasis and the importance of documenting the actual phenylalanine fluctuations which accompany any experimental manipulation attempted. Paradigms which include multiple daily injections are not necessarily better models for maintaining longer lasting hyperphenylalaninemia.

Although plasma phenylalanine elevations were higher when carboxymethylcellulose was added to the phenylalanine used in multiple daily injection protocols, the addition of this compound did not seem to significantly aid in achieving high plasma phenylalanine levels for 24 hours a day when only a single injection of phenylalanine plus α -methylphenylalanine is given. And its use does not overcome the drawbacks of multiple daily injection treatments enough to present a significant improvement in methodology at this time. It is possible, though, that this approach with modifications in the times of injection, pH of the injection solution, type of polymer used or charging of the diluent may eventually lead to an improvement of the present model (Greengard et al., 1977) for experimental PKU.

The last table (Table III-4) further reflects our present ignorance of phenylalanine homeostasis. An effect of 'phenylalanine loading' may be seen; phenylalanine disappears from plasma 24 hours after injection, but it still may accumulate in some tissues (although not in brain). Thus, a second injection of phenylalanine may elevate tissue (including brain) levels to a greater extent than in an animal with no previous injections or previous phenylalanine buildup since the distribution bulk is less. The present studies supply no direct evidence for this theory, and it is possible some unknown metabolic effect may underlie this phenomena. The interaction between α -methylphenylalanine and phenylalanine is less well understood. It appears that the two may compete with each other for transport sites (see Chapter V) and therefore addition of α -methylphenylalanine to phenylalanine injections may aid in slowing down phenylalanine excretion and transport into peripheral tissues. This process would contribute to the higher plasma phenylalanine levels found when α -methylphenylalanine is injected along with phenylalanine (as opposed to phenylalanine alone) into a rat with already inhibited PAH. Much more detailed study, including assay of phenylalanine tissue levels and excretion rates, are required before we reach any real understanding of the in vivo interaction of these two compounds. The present studies are of practical importance in adding to our knowledge of how to manipulate phenylalanine

levels in the rat. Gaining equivalent plasma phenylalanine levels using phenylalanine with or without additional α -methylphenylalanine is important for many studies such as those conducted in Chapter V.

CHAPTER FOUR

BEHAVIORAL MEASUREMENTS IN NORMAL AND MODEL PHENYLKETONURIC RATS

(I) BEHAVIORAL ABNORMALITIES
IN MODEL PHENYLKETONURIC RATS

Although there is a clear causal connection between the hyperphenylalaninemia in infancy accompanying PKU and the subsequent mental retardation in maturity, the neurochemical basis of this cerebral maldevelopment is obscure. By studying the behavior of adult rats exposed in early life to our treatment mimicking PKU we hope to discover behavioral abnormalities which may confirm the appropriateness of this treatment as a model of phenylketonuria. Furthermore, by using the presence of such abnormalities as markers for the behavioral response to PKU in the rat, we may investigate the neurochemical basis of such changes by subjecting young developing rats to various chemical manipulations that selectively and independently mimic specific biochemical changes seen with our model treatment.

The first section of this chapter describes the deficits in T-maze learning and increases in locomotor activity found in adult (female) rats given phenylalanine plus α -methylphenylalanine during their 2nd to 21st days of life. These changes may be rodent equivalents to the mental retardation and hyperactivity seen in PKUics. Additionally,

abnormalities in cerebral lateralization, a behavioral measure not studied in PKUics but possibly related to learning disabilities, are detailed. We also present behavioral results from rats treated to reproduce selectively a) the glycine elevation and b) the catecholamine depletion (see Chapter V) which accompanies early exposure to our model PKU treatment.

RESULTS

Fischer rats treated with phenylalanine plus alpha-methylphenylalanine from the 2nd to 21st days of life exhibited several behavioral abnormalities compared to their saline injected littermates when tested at 90-120 days of life (Table IV-1). Female, but not, male experimental rats showed a significant increase in the lateralization of their rotational activity (A). Their '% preference', a measure of the rats relative preference (50-100%) to turn in one direction (left or right) versus the other, increased from 68% to 81%. This change was due to an increase in the number of rotations in the preferred direction and not a decrease in the number of full turns in the less favored direction. Net rotations, the difference between full rotations in each direction, increased from 51 to 126 in these rats. This increase was not due to a generalized enhancement in all activity since total extra quarter turns, a measure of random movement, was not raised. Male experimental rats showed similar, but smaller and statistically non-significant increases in these same measures.

Both male and female experimental rats showed significant increases in their locomotor activity as measured in

TABLE IV-1

BEHAVIORAL RESULTS FROM ADULT RATS HAVING UNDERGONE NEONATAL
HYPERPHENYLALANINEMIA

Rats were given daily subcutaneous injections with phenylalanine (5.2 $\mu\text{m/g}$ body w) plus alphanmethylphenylalanine (2.4 $\mu\text{m/g}$ body w); (experimental) or saline diluent (control) on their 2nd to 21st days of life. They were then raised to 90-120 days of age and tested for rotational behavior, locomotor activity and T-maze learning ability. All values are means $\pm$ SD of results from 12 control males, 15 control females, 11 experimental males and 14 experimental females. Significant differences from control ($p < 0.01$) are denoted by *.

		<u>Control</u>	<u>Experimental</u>
<u>Rotometer tests (24 hour data)</u>			
% Preference	Male	66 $\pm$ 7	68 $\pm$ 8
	Female	68 $\pm$ 11	81 $\pm$ 9
Net Rotations	Male	30 $\pm$ 18	41 $\pm$ 30
	Female	51 $\pm$ 39	126 $\pm$ 72*
Extra $\frac{1}{2}$ Turns	Male	449 $\pm$ 181	521 $\pm$ 226
	Female	560 $\pm$ 244	610 $\pm$ 141
<u>Locomotor Activity</u>			
Photocell counts 30/min	Male	1554 $\pm$ 162	1807 $\pm$ 144*
	Female	1857 $\pm$ 166*	2124 $\pm$ 208*
<u>T-Maze Learning</u>			
Trials to Criterion	Male	7.2 $\pm$ 1.3	7.8 $\pm$ 1.5
	Female	7.2 $\pm$ 1.3	12.2 $\pm$ 3.3*

photocell cages (B). However, only female rats exhibited a significant impairment in T-maze performance. In fact, the number of trials to criteria (5 out of 6 correct choices) was artificially held down in this group of animals by the adoption of a 15-trial ceiling. This was necessitated by the fact that female experimental rats frequently rotated in the stem of the maze in response to shock, so that entry into either the left or right arms of the maze became a chance occurrence. This observation seemed to link the exaggerated side preference of these animals with interference in this learning process. This connection, however, was not convincing since some of the many litters subsequently tested showed the same exaggeration of lateralization but no deficit in T-maze learning. Moreover, there was no strong correlation between T-maze performance and rotational asymmetry in individuals.

In an effort to ascertain which specific early biochemical abnormalities are linked to the later behavioral changes noted above, young developing Fischer rats were subject to chemical manipulations to mimic independently specific biochemical changes seen with hyperphenylalaninemia. Table IV-2 shows the behavioral results from rats given glycine plus serine injections from the 7th to 21st days of life to imitate the cerebral glycine elevations found in hyperphenylalaninemic rats at this age (Isaacs & Greengard, 1980). Although this treatment was successful in raising

TABLE IV-2

BEHAVIORAL RESULTS FROM RATS WITH EXPERIMENTAL HYPERGLYCINEMIA

Rats were given daily subcutaneous injections with glycine (9.3 $\mu\text{m/g}$ body w) plus serine (19 $\mu\text{m/g}$ body w); (experimental), or saline vehicle only (control) on days 7-21 of life. At 90-120 days of age, they were tested for 24 hour rotational behavior, locomotor activity and T-maze learning ability. Results are from several different litters, raised at different times with control and experimental animals in each litter.

<u>Rotometer Tests (24 Hours)</u>		<u>Control</u>	<u>Experimental</u>
% Preference	Male	69+3 (22)	70+4 (11)
	Female	68+3 (19)	81+5 (6) ^a
Net Rotations	Male	45+14	54+15
	Female	66+19	54+16
Total Full Rotations	Male	97+15	126+25
	Female	131+19	78+15
Total 1/4 Turns	Male	395+53	395+99
	Female	383+56	430+166
<u>Locomotor Activity</u>			
Photocell Counts/ 30 min.	Male	1503+85	1486+113
	Female	1604+97	1673+116
<u>T-Maze Learning</u>			
Trials to Criteria 1st Test	Male	10.0+3.32	7.67+1.15
	Female	6.8+1.50	6.67+1.58
Retest	Male	7.0+1.73	5.7+0.6
	Female	11.3+7.57	8.0+3.0
<u>Direction of Preference</u>			
1st Trial	Male	2R-3L	1R-2L
	Female	1R-4L	2R-1L

^a debotes significant difference from control, $P < 0.05$.

cerebral glycine levels in a manner similar to our model PKU treatment (Isaacs & Greengard, 1980), these animals did not show the same behavioral changes as the 'PKUic' rats. The only significant change noted, an increase in '% preference' in female experimental rats from 68% to 81% (A), was not due to an increase in full turns in the preferred direction but a decrease in turns in the non-preferred direction. Net rotations fell from 66 to 54, a non-significant change. Thus, these animals did not show the same increase in lateralization of rotational activity as 'PKUic' rats.

Another group of rats was injected with α -methylphenylalanine alone to mimic the catecholamine depletion which accompanies hyperphenylalaninemia in young suckling rats (see Chapter V). These animals did not exhibit the same behavioral abnormalities as rats with experimental PKU (Table IV-3). Female rats did not have increased rotational asymmetry and neither male nor female rats showed increased locomotor activity. It is possible, however, that the decreased body size of these animals (see Appendix) may have affected this latter result. Surprisingly, male rats given α -methylphenylalanine alone from the 2nd to 21st days of life showed a significant increase in the number of rotations (47 to 128) as well as an increase in '% Preference' (70% to 78%).

TABLE IV-3

BEHAVIORAL RESULTS FROM RATS NEONATALLY TREATED WITH ALPHA-METHYLPHENYLALANINE

Rats were given daily subcutaneous injections with alpha-methylphenylalanine (2.4um/g body w) on days 2-15 or days days 2-21 of life, or with saline vehicle only (control). At 90-120 days of age they were tested for 24 hour rotational behavior, locomotor activity and T-maze learning ability. Values are mean $\pm$ SD(n) with * denoting significant differences ($p < 0.01$) from control.

		2-15		2-21	
		<u>Control</u>	<u>Experimental</u>	<u>Control</u>	<u>Experimental</u>
<u>Rotometer Tests (24 Hours)</u>					
% Preference	Male	68 $\pm$ 2 (18)	57 $\pm$ 2 (3)	70 $\pm$ 1 (197)	78 $\pm$ 6 (8)
	Female	70 $\pm$ 3 (23)	72 $\pm$ 6 (4)	71 $\pm$ 1 (193)	63 $\pm$ 1 (11)
Net Rotations	Male	38 $\pm$ 16	11 $\pm$ 5	47 $\pm$ 4	128 $\pm$ 46*
	Female	57 $\pm$ 21	84 $\pm$ 37	77 $\pm$ 7	23 $\pm$ 4
Total Full Rotations	Male	87 $\pm$ 17	73 $\pm$ 19	103 $\pm$ 5	190 $\pm$ 46**
	Female	132 $\pm$ 21	170 $\pm$ 42	151 $\pm$ 10	96 $\pm$ 19
Total $\frac{1}{4}$ Turns	Male	461 $\pm$ 73	435 $\pm$ 12	377 $\pm$ 17	400 $\pm$ 98
	Female	414 $\pm$ 70	277 $\pm$ 31	433 $\pm$ 26	424 $\pm$ 70
<u>Locomotor Activity</u>					
Photocell Counts/30 min.	Male	1280 $\pm$ 138	1622 $\pm$ 167	1508 $\pm$ 31	1391 $\pm$ 149
	Female	1752 $\pm$ 54	1827 $\pm$ 84	1664 $\pm$ 33	1530 $\pm$ 171
<u>T-Maze Learning</u>					
Trials to Criteria	Male	6.75 $\pm$.96 (4)	7.17 $\pm$ 1.17 (6)	-	-
	Female	8.60 $\pm$ 1.67 (5)	8.00 $\pm$ 2.10 (6)	-	-
Direction of Preference	Male	5R-4L	1R-5L	-	-
	Female	5R-5L	4R-2L	-	-

(II) SEX DIFFERENCES IN CEREBRAL LATERALIZATION

Having noted the sexual dimorphism in the behavioral response of rats to hyperphenylalaninemia in suckling life (section I), an investigation into the basis of this sex difference was initiated. One factor which may play a role in the different responses to PKU of each sex in rotometer tests may be the difference in cerebral asymmetry between normal (saline injected) males and females. This difference is explored in this section. Also the interaction between sex and experimental treatment is investigated through manipulation of the hormonal status of hyperphenylalaninemic rats.

This section details the increased turning behavior of normal female rats as compared to males. It also shows that female rats given testosterone on the second day of life do not show the typical exaggeration of cerebral lateralization seen in other PKUic females. However, the interaction between neonatal male castration and early hyperphenylalaninemia in regards to cerebral asymmetry is unclear.

RESULTS

Table IV-4 shows the results from 24 hour testing in rotometers of 97 male and 88 female rats previously injected with saline. Although normal male and female rats did not differ significantly in their '% Preference' or relative frequency of rotation, female rats made significantly greater numbers of net rotations (77 versus 43 for males) as a consequence of increased numbers of full rotations (151 versus 93 for males). There were no significant differences between males and females in the amount of random activity, as measured by total extra 1/4 turns, recorded; but, saline injected females did show a significant increase in their locomotor activity (photocell) when compared to their male littermates.

As a means of testing the contribution of gonadal hormones in determining the sexually dimorphic response of rats to model PKU two experiments were conducted. In the first one the organizational role of testosterone on cerebral lateralization was studied. Rats from several litters were separated into groups: males, females receiving 1.25 mg testosterone propionate and females receiving the sesame oil vehicle only. These substances were injected subcutaneously

TABLE IV-4

ROTATIONAL BEHAVIOR IN SALINE INJECTED FISCHER RATS

Behavioral data from experiments on adult animals (90-120 days) previously injected with saline for different time periods of suckling life (2-21, 2-15, 2-10, 7-21, 7-15) were grouped together for comparison of sex differences in all "control" animals. Values are mean $\pm$ SEM from 97 male and 88 female animals. Significant differences between males and females ($p < 0.01$) are denoted by *.

<u>Rotometer Tests</u>	<u>Male</u>	<u>Female</u>
% Preference	70 $\pm$ 1	71 $\pm$ 1
Net Rotations	43 $\pm$ 6	77 $\pm$ 71*
Total Full Rotations	93 $\pm$ 7	151 $\pm$ 10*
Total $\frac{1}{2}$ Turns	377 $\pm$ 17	433 $\pm$ 26
<u>Locomotor Activity</u>		
Photocell Counts/30 min	1508 $\pm$ 31	1664 $\pm$ 33*

on the 2nd day of life and all three groups of rats received daily injections of phenylalanine plus α -methylphenylalanine during the 2nd to 21st days of life. Twenty-four hour rotometer testing of these rats at maturity (90-120 days old, Table IV-5) shows that the female PKUic rats injected with the sesame oil vehicle had an increase in '% Preference' and a significant increase in the number of net rotations when compared to the other two groups of PKUic rats or non-PKUic females previously tested (Tables IV-1, IV-4). In contrast, the female rats neonatally injected with testosterone had rotational responses nearly identical to their male littermates. Thus, the early imposition of hyperphenylalaninemia caused an exaggeration of cerebral asymmetry in female rats, but not in their testosterone treated female littermates nor their male siblings. No significant changes in total extra quarter turns or locomotor activity (photo-cell) were found.

The effectiveness of the neonatal testosterone treatment was confirmed by testing these rats for another sexually dimorphic behavior. Figure IV-1 shows that female rats in mid-estrus or di-estrus have 24 hour running wheel activities similar to male rats but significantly lower (303 versus 504 in controls) than females in the pro-estrus or estrus phases of their menstrual cycle. The female rats in the above study given testosterone on day two failed to show any cyclical changes in running wheel activity when tested

TABLE IV-5

24 HOUR ROTATIONAL ACTIVITY IN HORMONALLY MANIPULATED RATS EXPOSED TO HYPERPHENYLALANINEMIA
ON DAYS 2-21 OF LIFE

Experiment A - Female rats were given 1.25 mg testosterone proprionate in sesame oil (TP) or vehicle only. Both groups and their male littermates were made hyperphenylalaninemic on days 2-21 of life, and tested for 24 hour rotational activity on the 90-120th day of life.

Experiment B - Male rats were castrated on the 2nd day of life and their female littermates received sham operations. Both groups were made hyperphenylalaninemic on days 2-21 of life, and tested for rotational activity on the 90-120th day of life. Castrated males were retested after 2 weeks of daily subcutaneous injections with 15 ug/kg estradiol benzoate (EB) in sesame oil.

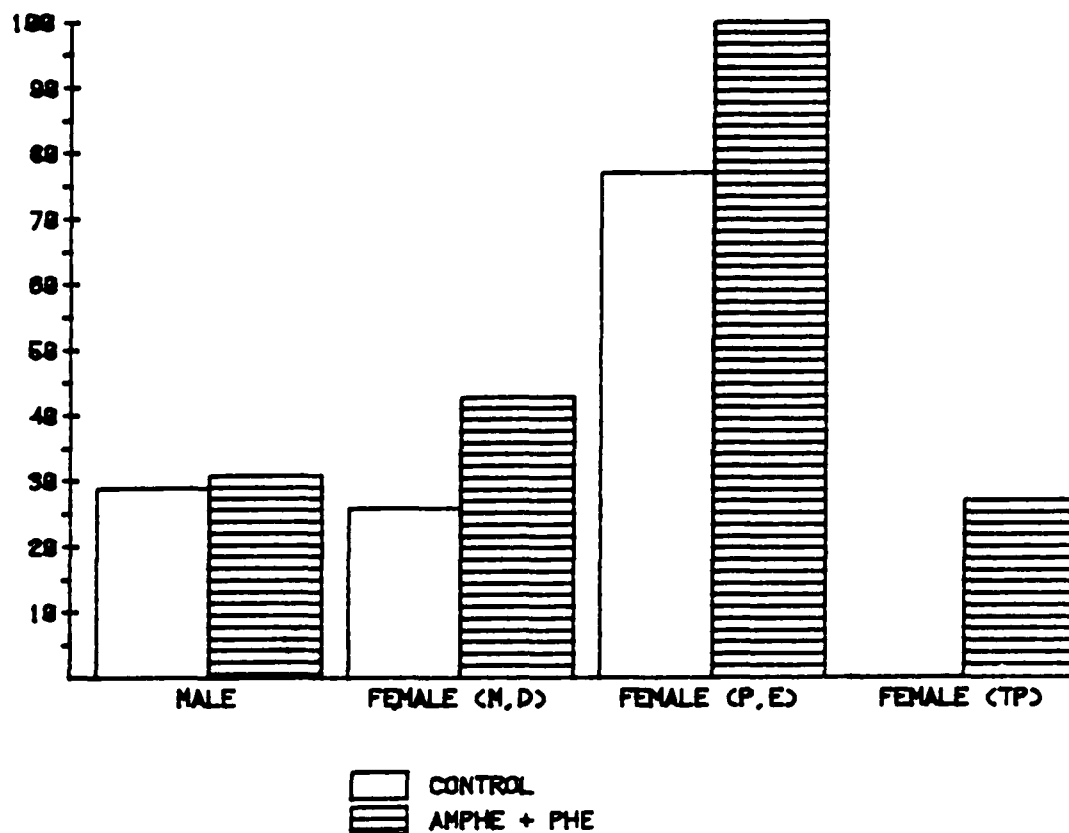
Significant differences from control ($p < 0.01$) are denoted by *.

<u>Group</u>	<u>Number of Animals</u>	<u>% Preference</u>	<u>Net Rotations in Dominant Direction</u>	<u>Total Full Rotations</u>	<u>Total 1/4 Turns</u>	<u>Locomotor Activity</u>
<u>A</u>						
Female	5	87+11	139*+161	-	356+186	1457+243
Females with TP	10	76+11	61+59	-	327+141	1496+425
Male	8	80+14	69+36	-	376+320	1537+268
<u>B</u>						
Sham Castrated Female	20	69+3	60+25	118+32	552+219	1163+178
Castrated Male	17	71+3	61+17	121+26	326+55	1204+201
Castrated Male with EB	8	76+7	45+17	95+20	255+81	-

FIGURE IV-1

TWENTY-FOUR HOUR RUNNING WHEEL ACTIVITY IN CONTROL AND EXPERIMENTAL RATS

Rat pups, including those females injected with 1.25 mg of testosterone propionate on the second day of life (TP), were injected with phenylalanine (5.2 μ moles/g body w) plus α -methylphenylalanine (2.4 μ moles/g body w) during the 2nd to 21st days of life. Each adult (>90 days old) rat was then placed in the running wheel apparatus for 24 hours and the number of wheel rotations recorded. The menstrual phase of each female rat was determined by microscopically examining its vaginal smear before placement into the apparatus. Each female rat was tested in both mid-estrus/diestrus (M, D) and pro-estrus/estrus (P, E) phases. Female rats that received testosterone propionate (TP) were found to be acyclic.



as adults. Measurements from each rat for six consecutive days failed to show any large day to day variation within that period as would be expected of rats with a normal 4-5 days estrus cycle. In fact an average of the highest (running wheel) responses on any two consecutive days from each of these rats yields a value (320) similar to that of male rats (341) or females in mid or di-estrus phase (303) (Fig. IV-1). Thus, the testosterone treatment clearly affected brain areas controlling a known sexually dimorphic behavior (Beatty, 1979; Gentry and Wade, 1976). Additionally, this data helps clarify the long-debated issue of the nature of hormonal influences in modulating running wheel activity (see Beatty, 1979) by demonstrating the organizational effects of testosterone in determining this sexually dimorphic behavior. Figure IV-1 also shows that adult female rats treated with phenylalanine only on days 2 through 15 of life had greater running wheel activities in all estrus phases than their saline injected littermates. However, this increase was not significant with the small number of animals tested.

In the second experiment conducted to study the role of gonadal hormones mediating the sexually dimorphic response to model PKU, the male rats in several litters were castrated on day 2 and their female littermates underwent a sham operation at the same time. Both males and females received daily injections of phenylalanine plus α -methylphe-

nylalanine on the 3rd through 21st days of life. Table IV-5 shows that neither the males nor females showed an exaggeration of rotational asymmetry when compared to previous groups of saline injected animals tested at maturity (90-120 days). Similarly, a subgroup of these castrated males retested after daily injections with estradiol benzoate (15 ug/kg in sesame oil) for two weeks did not have significantly different 24 hour rotational responses. The effectiveness of neonatal castration was confirmed by anatomic examination at sacrifice. Also, the hepatic monoamine oxidase activity, which differs between males and females (see Chapter V) of these rats ($415 \pm 34(6)$) was nearer to that of females ($391 \pm 53(5)$) than males ($454 \pm 26(4)$).

(III) SEX DIFFERENCES IN DRUG-INDUCED
ROTATIONAL BEHAVIOR

Although measurement of 24 hour rotational activity has been successfully used to study differences in laterality between male and female PKUic rats (Sections I & II), another paradigm, that of drug induced rotation, has also proved to be of great value in studying cerebral asymmetry (Glick et al., 1977; Jerussi & Glick, 1976). Studies of drug-induced rotation of previously hyperphenylalaninemic rats was thought to be of value for two reasons. First, the more vigorous rotational responses of animals given drugs which interact with catecholaminergic systems (amphetamine, apomorphine) may unmask treatment differences not seen clearly in the non-drug-induced 24 hour rotation test. Thus, if neonatal hyperphenylalaninemia affected the cerebral asymmetry of male rats in a quantitatively but not qualitatively different way than females, then this may be brought out by this study. Secondly, by using drugs which primarily act at pre-synaptic (amphetamine) or post-synaptic (apomorphine) sites, the mechanism underlying sex related difference in rotational asymmetry may be studied.

This section demonstrates large sex difference in rotational responses to amphetamine, but not apomorphine. No clear treatment effects are seen. Additionally, the hormonal basis of the sex difference is investigated. Castration of male rats at birth produces a more female like response to amphetamine, but this "female-like" behavior is more fully achieved by also treating these rats with estradiol.

RESULTS

Figure IV-2 shows the dose response curve to amphetamine for male and female Fischer rats. As previously reported for female Sprague-Dawley rats (Jerussi & Glick, 1976), female Fischer rats greatly increase their net rotations in response to amphetamine. However, male rats maintain low levels of rotation in response to amphetamine in the 0.25-1.0 mg/kg dose range. Analysis of variance for the unbalanced data set was calculated using a general linear models (GLM) procedure (SAS Institute 1979) for the model: net rotations = function (sex, dose, sex x dose). Utilizing the type IV results derived, net rotations varied with sex ($P < 0.0005$) and dose ($P < 0.05$) of amphetamine administered. Although the difference between male and female net rotations per hour seems to increase with drug dose there was no significant sex x dose interaction ($P = 0.176$). Not only are the net rotations of female rats significantly greater than their male littermates at 0.25, 0.50 and 1.0 mg/kg amphetamine, but the female response at 0.25 and 0.50 mg/kg amphetamine is greater than that of males at 1.0 mg/kg ($P < 0.05$ for one tailed test). In contrast, male and female Fischer rats respond nearly identically to intra-

FIGURE IV-2

DOSE RESPONSE CURVE FOR AMPHETAMINE-INDUCED ROTATION
IN MALE AND FEMALE RATS

Adult rats (90 days old) were given an intraperitoneal injection of D-amphetamine sulfate dissolved in physiological saline (0.9%); they were then placed in rotometers and rotations were recorded for 60 minutes. Each point represents the mean net rotations $\pm$ SE with $n \geq 9$ at each dose for both males (+) and females (*).

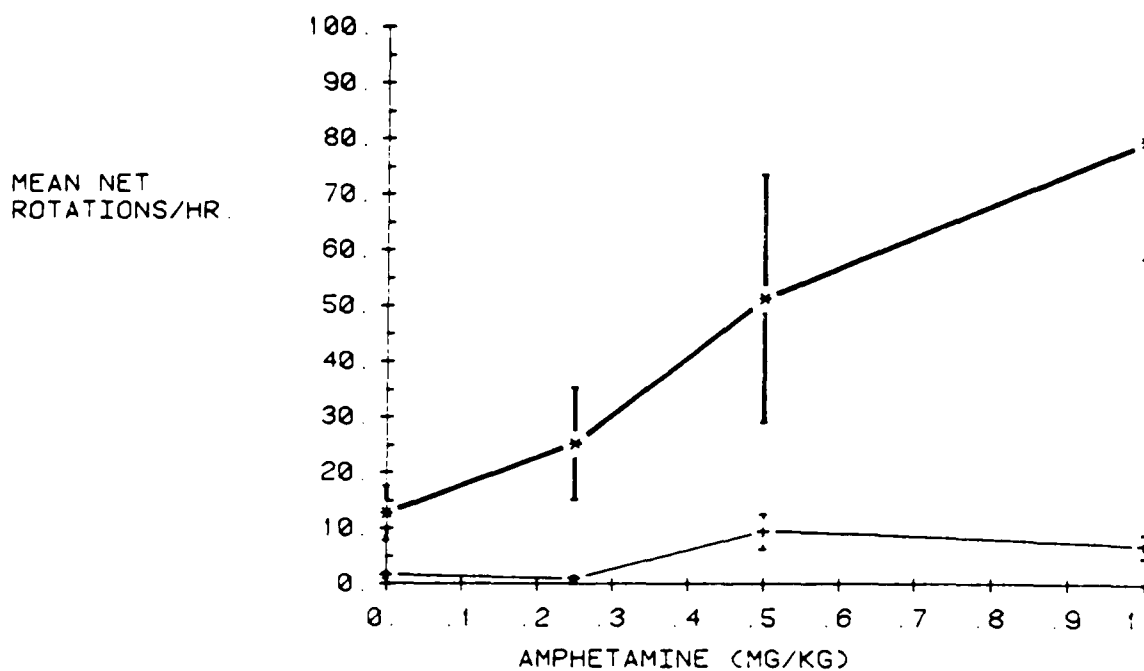
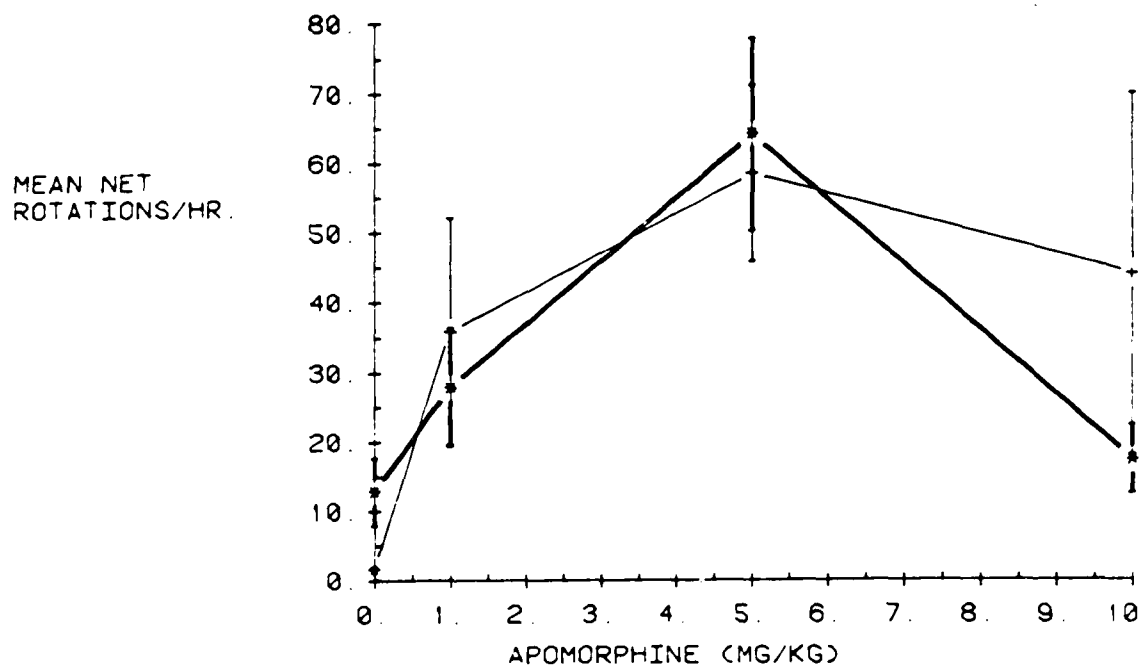


FIGURE IV-3

DOSE RESPONSE CURVE FOR APOMORPHINE-INDUCED ROTATION IN
MALE AND FEMALE RATS

Adult rats (90 days old) were given an intraperitoneal injection of apomorphine hydrochloride dissolved in physiological saline (0.9%); they were then placed in rotometers and rotations were recorded for 60 minutes. Each point represents the mean net rotations $\pm$ SE with $n \geq 9$ at each dose for both males (+) and females (*).



peritoneal apomorphine injections (see Fig. IV-3, using GLM $P = 0.599$), although net rotations are clearly dose-dependent (using GLM, $P < 0.001$).

The general significance of sex differences in drug-induced rotation was confirmed in experimentation with another rat strain. Sprague-Dawley female rats rotated significantly more (net rotations/hr: mean $\pm$ SE (n) = 50.2 ± 10.7 (20), $p < 0.001$) than their male littermates (14.9 ± 3.6 (21)) in response to 1 mg/kg of intraperitoneally injected amphetamine. As with Fischer rats this difference was due to the failure of male rats to respond to amphetamine. The sex difference in response to apomorphine is seen in the opposite direction; male rats rotated significantly more (54.3 ± 13.3 (21), $p < 0.001$) than female rats (14.0 ± 2.1 (20)) when administered 10 mg/kg apomorphine.

We also found that Fischer rats treated with phenylalanine plus α -methylphenylalanine on their 2nd to 21st days of life did not vary significantly from their saline injected littermates in net rotations in response to amphetamine (Figure IV-4) or apomorphine (Figure IV-5). Still, the difference in response to amphetamine between control and experimental female animals increased with the drug dosage. Thus, the possibility remains that experimental female rats may rotate significantly more than control animals if given more than the 1 mg/kg amphetamine used in this study. As with control animals female experimental rats rotated

FIGURE IV-4

DOSE RESPONSE CURVE FOR AMPHETAMINE-INDUCED ROTATION
IN RATS EXPOSED TO NEONATAL HYPERPHENYLALANINEMIA AND
CONTROLS

Rats were injected with phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w) (Experimental) or saline diluent (control) on days 2 to 21 of life. At maturity (90 days old) they were given an intraperitoneal injection of D-amphetamine sulfate dissolved in physiological saline (0.9%); they were then placed in rotometers and rotations recorded for 60 minutes. Each point represents the mean net rotations $\pm$ SE with $n \geq 9$ at each dose.

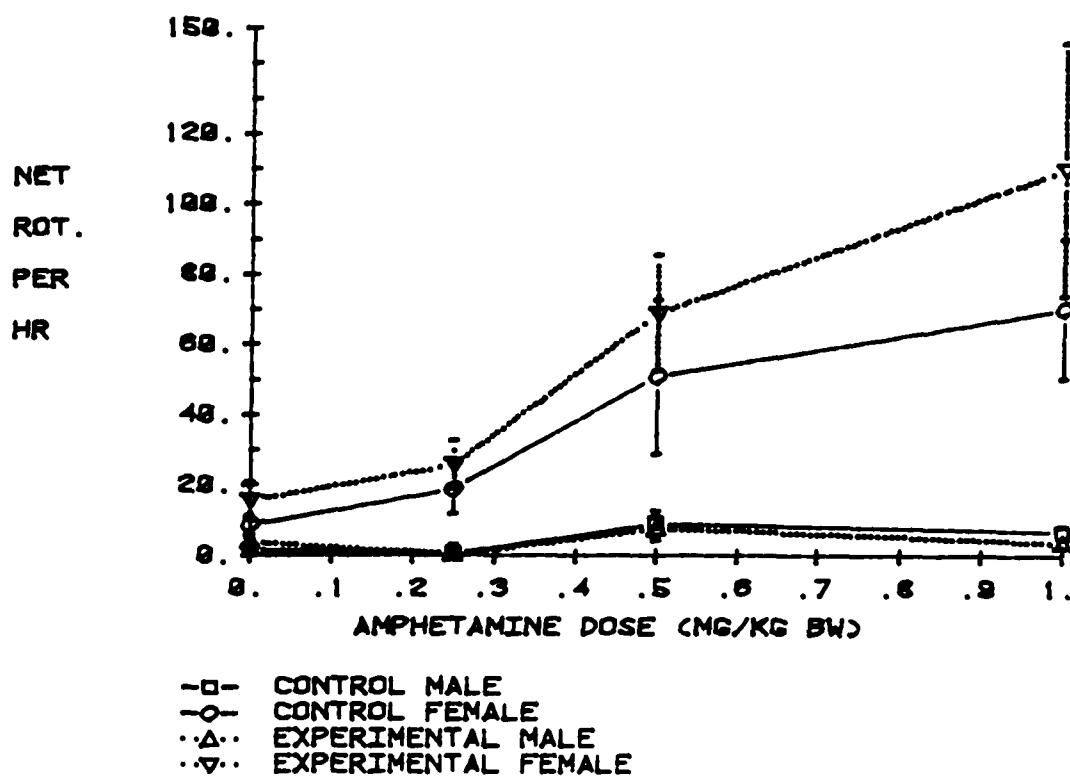
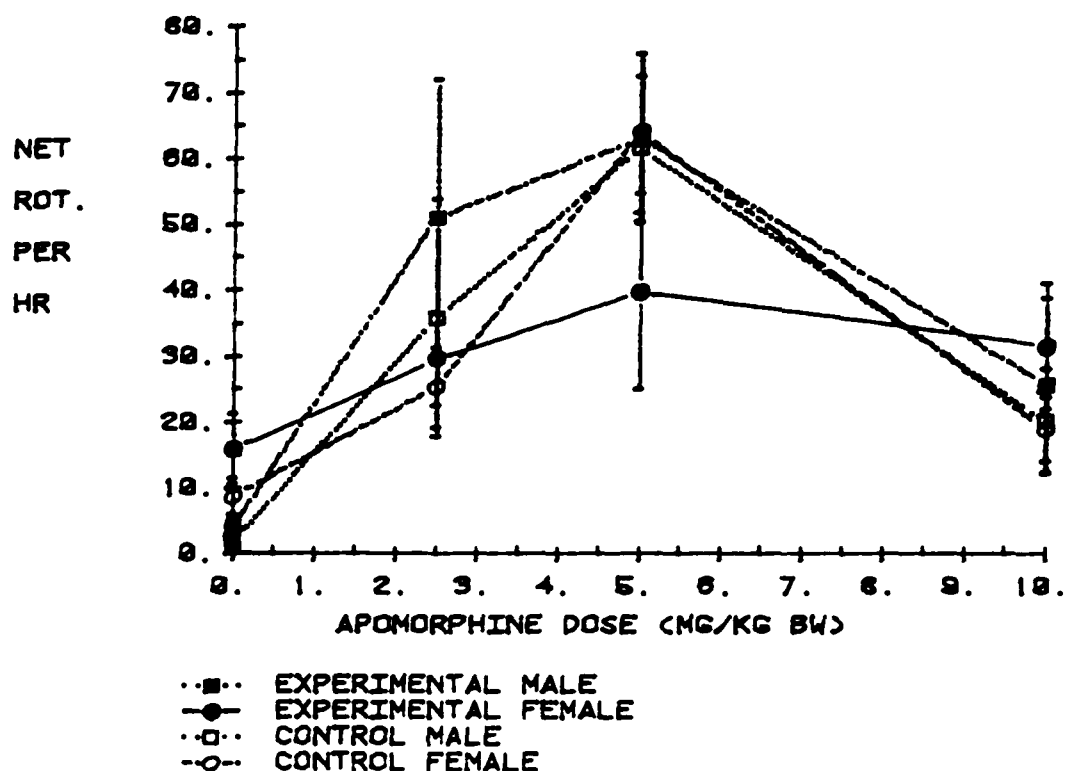


FIGURE IV-5

DOSE RESPONSE CURVE FOR APOMORPHINE-INDUCED ROTATION IN RATS EXPOSED TO NEONATAL HYPERPHENYLALANEMIC AND CONTROLS

Rats were injected with phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w) (Experimental) or saline diluent (Control) on days 2 to 21 of life. At maturity (90 days old) they were given an intraperitoneal injection of apomorphine hydrochloride dissolved in physiological saline (0.9%); they were then placed in rotometers and rotations recorded for 60 minutes. Each point represents the mean net rotation $\pm$ SE with N>9 at each dose.



significantly more than experimental males when injected with amphetamine.

Male and female control rats responded nearly identically to apomorphine, but male experimental rats appeared to rotate more than their female littermates with this drug. Although this difference was not statistically significant, it was striking in comparison to the nearly identical responses of male and female control rats. Since all of the rats tended to rotate primarily in one direction (left or right) in response to apomorphine or amphetamine, comparison of '% Preference' values between the different groups tested was of little value. Group means for this variable only varied between 72% and 89%. Total extra quarter turns increased within each group with drug dosage, but the variation between different treatment groups also proved to be non-significant.

The basis of the sexually dimorphic response to amphetamine was investigated by studying the drug induced rotation of male rats castrated at 2 days of age before and after estradiol treatment. Table IV-6 shows that castrated males rotated significantly more in response to 1 mg/kg amphetamine than intact males, but that this response increases even more after these same rats are injected with the female gonadal hormone estrogen. In fact, castrate males treated with estradiol benzoate for two weeks performed more net rotations than intact females. The '% Pref-

TABLE IV-6

AMPHETAMINE-INDUCED ROTATION IN HORMONALLY MANIPULATED RATS

Adult rats (90-120 days old) were given intraperitoneal injections of d-amphetamine sulfate (1 mg/kg body w), placed in rotometers and their rotational activity recorded for the subsequent hour. Castrated males refer to a group of animals whose testes were removed on the 2nd postnatal day of life. These same animals were retested at a later date after receiving daily subcutaneous injections of estradiol benzoate (15 μ gm/kg body w) dissolved in sesame oil for two weeks (castrate male w/EB). Sham females are those female littermates of the male castrate rats who received sham operations on the 2nd day of life. Animals in every group received daily injections of phenylalanine (5.2 μ moles/g body w) plus α -methylphenylalanine (2.4 μ moles/g body w) on the 2nd to 21st days of life. All values are means $\pm$ SD with the superscripts ^a and ^b denoting significant differences ($p < 0.01$) from females and males respectively.

<u>Experimental Group</u>	<u>N</u>	<u>% Preference</u>	<u>Net Rotations</u>	<u>Total Full Turns</u>	<u>Extra 1/2 Turns</u>
Male	10	78 $\pm$ 6	4 $\pm$ 2 ^a	11 $\pm$ 4 ^a	77 $\pm$ 25
Castrate Male	13	82 $\pm$ 5	46 $\pm$ 22 ^b	55 $\pm$ 20 ^{a, b}	107 $\pm$ 23
Castrate Male w/EB	8	100 $\pm$ 0 ^{a, b}	144 $\pm$ 43 ^b	146 $\pm$ 44 ^b	20 $\pm$ 6
Sham Operated Female	13	85 $\pm$ 4	75 $\pm$ 30	87 $\pm$ 30	85 $\pm$ 17
Female	10	83 $\pm$ 6	110 $\pm$ 36	145 $\pm$ 31	79 $\pm$ 25

erence' and total full turns both increased in a similar manner in response to these hormonal manipulations (Table IV-6).

In another experiment we found that female rats given testosterone on the 2nd day of life made fewer net rotations when given 0.5 mg/kg amphetamine than their female littermates. Although this difference was not significant, the response of the testosterone treated animals was significantly reduced in comparison to the earlier groups of female rats tested. All of the animals in the above studies had been injected with phenylalanine plus α -methylphenylalanine during the 2nd to 21st days of life, and the success of the hormonal manipulations were confirmed as described in section II of this chapter.

DISCUSSION

The hyperactivity and reduced learning capacity found in adult rats treated with phenylalanine plus α -methylphenylalanine early in life are consistent with observations of the most striking behavioral abnormalities, hyperactivity and mental retardation, seen in phenylketonurics. These findings along with similar observations in rats (Lane et al., 1980) and mice (Luttges & Gerren, 1979) treated in the same manner confirm the appropriateness of this regimen as a model of the disease. Although enduring behavioral abnormalities also accompany treatment with phenylalanine plus *p*-chlorophenylalanine (PCPA; Anderson et al., 1973, Anderson et al., 1974; Butcher, 1970), an alternate suppressor of hepatic phenylalanine hydroxylase activity, the toxicity accompanying this treatment casts doubt on the specificity of these effects. PCPA is a potent depletor of cerebral serotonin (Koe & Weissman, 1966) and in combination with phenylalanine causes severe weight loss (over 50%) and mortality (DelValle et al., 1978; Lane et al., 1980), rendering animals unsuitable for behavioral study.

A new observation related to early hyperphenylalaninemia is the exaggeration of cerebral asymmetry as measured by

rotational activity in mature 'PKUic' rats. Although cerebral lateralization has not been studied in PKUics there is evidence that the degree of lateralization, present in humans at infancy (Turkewicz, 1977; Wade et al., 1975), may affect learning ability in children (Kershner, 1978). This chapter presents some evidence supporting this link in rodents. Table IV-1 demonstrates that the increase in relative frequency of rotation in the preferred direction ('% Preference') was accompanied by an increase in the number of full turns. Thus, this increase in '% Preference' was due to an increase in the number of turns in the dominant direction, i.e. an increment in rotational activity. Experimental female rats also had a concomitant increase in locomotor activity and an impairment in T-maze performance. Since circling, due to an intrinsic nigrostriatal asymmetry, is a common mode of movement in rats any increase in this asymmetry may affect the overall motor activity (locomotor activity, number of full turns) as well as spatially dependent tasks ('% Preference', T-maze learning).

This connection between altered cerebral asymmetry and behavioral abnormalities in learning and locomotor activity is not absolutely clear since male 'PKUic' rats also showed significant hyperactivity but only small non-significant increases in rotational activity and no deficits in T-maze learning. Furthermore, some litters of female 'PKUic' rats exhibited exaggerated rotational activity and increased

locomotor activity but no learning deficiencies. These data may reflect not only on the relative importance (or unimportance) of alterations in cerebral asymmetry in causing changes in these other behaviors, but may also indicate that we are viewing the variable expression of a suboptimal insult. Variation in the effectiveness (see Appendix) of treatments may be reflected in the selective appearance of various behavioral findings. Those abnormalities measured by the more sensitive tests (e.g. rotational activity) may be detected first followed by the appearance of the more subtle findings (e.g. T-maze learning deficits) in litters receiving more effective treatments in suckling life. Thus, the dissociation (in some groups of animals) of the 3 behavioral abnormalities does not necessarily reflect on their mechanistic connection, but may only be a measure of the relative sensitivity of the tests involved. The possible biochemical basis for such behavioral variations in experimentally treated animals is discussed in Chapters V and VII.

Neither selective imitation of the cerebral glycine elevation nor the catecholamine depletion which accompanies hyperphenylalaninemia in suckling rats was successful in reproducing the same behavioral abnormalities noted in rats given phenylalanine plus α -methylphenylalanine. This result seems to discredit either glycine elevation or catecholamine depletion alone as the sole or primary factor in the exaggeration of cerebral asymmetry and raising of locomotor

activity in 'PKUic' rats. Still, neither alternate treatment exactly duplicated the specific neurochemical changes seen with our regimen for experimental PKU. Injection of rats with glycine and serine does elevate glycine to levels similar to those seen in our hyperphenylalaninemic rats (de Groot et al., 1979) but this is accompanied by an even higher elevation in serine (from 0.87 to 7.90 $\mu\text{mol/g}$) not seen in our 'PKUic' rats. The injection of α -methylphenylalanine alone produces a depletion of cerebral dopamine which is more severe and follows a different time course than that seen when phenylalanine plus α -methylphenylalanine is given. (Chapter V).

The fact that α -methylphenylalanine does alter rotational asymmetry, albeit in male but not female rats, is further evidence that interference with the development of the nigro-striatal catecholaminergic system may underlie changes in cerebral lateralization. The different behavioral responses to α -methylphenylalanine and phenylalanine plus α -methylphenylalanine treatments, the former affecting male animals and the latter primarily affecting females, may involve an interaction between differences in the time course of the development of cerebral catecholamine systems between the two sexes (see Appendix) and differences in the magnitude and time course of the dopamine depletion accompanying each treatment (See Chapter V). The details of such differences are not well understood, but further work using

these two treatments may aid in our understanding of the development of cerebral asymmetry in male and female rats.

The discovery of a sexually dimorphic reaction by rats to early hyperphenylalaninemia was unexpected since no sex differences have been noted in the incidence of PKU or the severity of its sequelae (Knox, 1972). Previous studies of the behavior of rats with model PKU have not reported separate results for the two sexes, so the universality of our findings is unclear. However, there are basic differences between man and rodent in the sexual maturation of their brains that may partially explain the incongruity of these findings.

Sex differences in brain function, in both man and other animals, have been attributed to both organizational and activational influences (Ehrhardt & Meyer-Bahlburg, 1981; MacLusky & Naftolin, 1981; McEwen et al., 1979). That is, the organization or imprinting of the brain that occurs early in life in response to plasma testosterone (acting as either testosterone or converted estrogen in the brain) and the activational or direct response at maturity to gonadal hormones, especially estrogens, control the expression of sexually dimorphic characteristics. Many behavioral (Baum, 1979; Beatty, 1979; Pfaff, 1970), biochemical (Arnold & Sattiel, 1979; Crowley et al., 1978; Lieberburg et al., 1977; Luine et al., 1975; Miller & Quay, 1979) and histological (Ratner & Adamo, 1971) sex differences found in the brains

of male and female animals are due to one of these two types of effects or a combination thereof. A key difference between man and rat in the development of sex dependent brain attributes is that sexual organization of the human brain is thought to be primarily a pre-natal event (Ehrhardt & Meyer-Bahlburg, 1981), whereas sexual imprinting of the rat brain occurs mainly (MacLusky & Naftolin, 1981; McEwen, 1978), although not exclusively (Ward & Weisz, 1980), after birth. Thus hyperphenylalaninemia in our suckling animal is imposed during a period of sexual maturation of the brain and an interaction between these two events may occur in the rat but not in man.

Although our observations comprise the first report of an excess of a natural substance contributing to altered rotational behavior, if hyperphenylalaninemia is viewed as a toxic state, then its exaggeration of cerebral asymmetry in rats may not be surprising. It is consistent with reports that brain injury caused by cerebral infarction affects left and right cerebral hemispheres differently (Robinson et al., 1975, Robinson & Coyle, 1979; Robinson, 1979). Robinson has demonstrated that the gross tissue injury following right or left middle cerebral artery ligation in adult rats is equal, but right sided injury is followed by a temporary (20-40 days) reduction of brain catecholamines including a 20% depletion of dopamine in the substantia nigra and a transient increase in locomotor activity not found with left

sided injury. Thus, imposition of our model PKU may be an analogous event, with longer lasting behavioral and biochemical (Chapter V, Section II) consequences due to its occurrence during a critical period of the young animals cerebral development.

That brain insults may not only cause asymmetric damage, but also sexually dimorphic responses is not without precedent in animal or human studies. Recovery from neurologic injury has been noted to occur at different rates in males and females. For example, testosterone is thought to be responsible for the accelerated recovery in males from peripheral nerve damage. Thus equivalent injury, but sexually dimorphic recovery responses, could explain the greater behavioral effects of our treatment seen in female than male rats. This possibility is highlighted by the fact that both male and female rats suffer equivalent brain weight losses in response to hyperphenylalaninemia early in life, but only male rats fully recover from this deficit later in life (Chapter III). These observations on experimental animals are not without human relevance since a recent review of the literature concerning human brain damage suggests that there are sex differences in the effects of unilateral brain damage on intelligence (Inglis & Lawson, 1981).

Another, simpler explanation for the sex difference in the behavioral response to phenylalanine plus α -methylpheny-

lalanine treatment lies in the possibility that male and female brains were exposed to different degrees of hyperphenylalaninemia, or different amounts of metabolic products of phenylalanine, during infancy. Chapter III documents the equivalent rise of plasma and brain phenylalanine in 7 day old male and female rats undergoing our usual treatment. However, an appreciation of the importance of phenylalanine to tyrosine ratios in determining cerebral dopamine depletion (Chapter V) suggests that this analysis may be too simple to fully dismiss the possibility of physiologically significant sex differences in phenylalanine and tyrosine homeostasis. Adult male rats have higher hepatic PAH activity than mature females (McGee & Greengard, 1972). Since even small differences in the amount of tyrosine produced from the excess phenylalanine in our model may have important neurochemical implications, sex differences in the age development curves of hepatic PAH may be important. Although the equivalent percentage catecholamine depletion in 7 day male and female rats implies equivalent cerebral phenylalanine/tyrosine ratios at 6 hours post injection (Chapter V) this information is not known for other ages or times. Thus, possible sex differences in the amino acid homeostasis of suckling rats may play a role in determining the sexually dimorphic behavioral response to model PKU. In contrast, PAH not only develops earlier in man (50% by the 10th week of gestation: Raiha, 1973; DelValle & Greengard,

1977), but more importantly PKUics have nearly undetectable (0.27%, Friedman et al., 1973) hepatic PAH activities due to the genetic defect and males and females have equivalent plasma phenylalanine elevations (Andrews et al., 1973).

Preliminary work (Section II) investigating the role of gonadal hormones in determining 'PKU' induced increases in the cerebral asymmetry of female rats showed that neonatal testosterone obliterates this effect. Thus, the organizational effect of testosterone seems to have an important imprinting effect on rat cerebral asymmetry. Unfortunately experiments with neonatal castration of male rats, which was expected to possibly feminize the brains of these animals by removing the early influence of gonadal testosterone, were neither confirmatory with nor contradictory to the earlier study. Since even the intact hyperphenylalaninemic female rats in this study failed to show the expected increase in rotational activity as adults, the fact that castrated 'PKUic' males showed no changes in rotational behavior could not be clearly interpreted. The failure of these rats to behaviorally respond to early hyperphenylalaninemia may be related to the anoxia of cold anesthesia and stress of surgery or to some unknown change of conditions in early life (see Appendix).

Since the mature female rats given testosterone at age two days appeared to be acyclic, as judged by the running wheel studies (Figure IV-1), one cannot neglect the possible

importance of rising estrogens in adult life contributing to the expression of altered cerebral asymmetry in a manner similar to their role in regulating amphetamine-induced rotation (See below). Further experimentation, including the injection of male 'PKUic' rats with estradiol and the oophorectomy of adult female 'PKUic' rats, must be conducted in order to fully understand the organizational and activation influences of gonadal hormones in the expression of this anomalous behavior.

However, section II also demonstrates that normal male and female rats differ significantly in rates of rotation. Thus, it is difficult to distinguish between the role of gonadal hormones in directly altering the effects of our experimental treatment, from their possible role in establishing sexually dimorphic systems (see below) controlling lateralization in males and females, which may respond differently to hyperphenylalaninemia. As an example of the first conjecture, testosterone may contribute to a quicker recovery of cerebral damage caused by hyperphenylalaninemia in male rats and testosterone treated female rats. This effect of testosterone has been demonstrated in the peripheral nervous system of rats and this suggestion is further legitimized by the observation that both male and female hyperphenylalaninemic rats suffer equivalent brain weight deficits early in life but only the males fully recover from this (Chapter III). The existence of different mechanisms

regulating rotational asymmetry in male and female rats is discussed below in reference to drug-induced rotation.

If hyperphenylalaninemia had qualitatively the same but quantitatively different effects on cerebral lateralization in the two sexes then we might expect that the first of the above alternatives, high phenylalanine interfering with the same system of lateralization to different degrees, to be true. However, an extension of our studies to include drug-induced rotation failed to find any differences between control and experimental male rats in their responses to apomorphine or amphetamine. Even the differences in net rotation between control and experimental females were small (using amphetamine) with the doses used. But, discovery of the sexually dimorphic response to amphetamine contributes to our understanding of the possibly different mechanisms controlling cerebral asymmetry in the two sexes. And it is these data (section III) that support the notion that similar insults imposed on qualitatively different systems controlling rotational asymmetry (see below) determine the sexually dimorphic behavioral response of rats to neonatal hyperphenylalaninemia. The discovery that an excess of a normal physiologic substance (phenylalanine) can significantly alter cerebral asymmetry indicates that neonatal hyperphenylalaninemia may also be a superior model to study the development of cerebral lateralization, as well as the effects of this abnormality on cognitive function in the

adult. Previous models used to study rotational asymmetry have depended on the use of surgical lesions or toxic drug treatments.

Results in section III agree with Robinson's independent finding that amphetamine elicits more lateralized (net) rotations in female than male Sprague-Dawley rats (Robinson et al., 1980), and extends this finding to another strain (Fischer). Since this sex difference has now been shown using a more moderate and reasonable drug dose, and is found in 3 independently raised populations, it appears to be a meaningful biological phenomenon and not a strain- or dose-specific peculiarity. The importance of sex in relation to apomorphine-induced rotation, being different in the two strains tested, is less clear.

The greater behavioral response by females injected with amphetamine reported here might be attributed to the slower metabolism of this drug. Female rats concentrate more amphetamine in their brains than male rats given a comparable drug dose per body weight (Groppetti & Costa, 1969; Meyer & Lytle, 1978). However, the sex difference in brain amphetamine accumulation in response to a 1 mg/kg intraperitoneal injection of amphetamine was found to be less than 2-fold during the first hour after drug administration (Meyer & Lytle, 1978). Thus, males given 1 mg/kg of amphetamine should have a greater brain concentration of this

drug than females injected with only half that dose. Since Fischer females given 0.25 mg/kg amphetamine rotate significantly more than their male littermates receiving 4 times that drug dose, it would appear that it is not differential drug accumulation, but differences in brain sensitivity to amphetamine that underlie this phenomenon. This reasoning has been directly confirmed in a preliminary report by Becker and colleagues (1981). They have shown that equivalent brain amphetamine levels are reached with the administration of 1.56 mg/kg and 1.25 mg/kg amphetamine to male and female rats respectively. Females made significantly more net rotations than males when given these respective doses. Additionally, this same group (Robinson et al., 1981) has demonstrated that sex differences in a related behavior, 1/4 turns made in response to electrical stimulation of the nigro-striatal dopaminergic bundle, are due to the direct central action of gonadal hormones, and not to differential drug metabolism.

Previous work has provided evidence that circling in intact rats is a reasonably sensitive assay of nigrostriatal dopaminergic activity (Glick et al., 1977). Thus, the sexually dimorphic behavioral response to amphetamine seen here may reflect underlying biochemical differences in this system. Sex differences have been reported in striatal dopamine content (Crowley et al., 1978). Furthermore, there are several indications that female gonadal steroids may modu-

late striatal dopaminergic activity including amphetamine stimulated striatal dopamine release (Becker & Ramirez, 1979; Becker & Ramirez, 1981), striatal dopamine content (Crowley et al., 1978), dopamine metabolism (Jori et al., 1976) and post-synaptic dopamine sensitivity (Gordon et al., 1980; Hruska & Silbergeld, 1980; Nausieda et al., 1979). Sex differences in many non-reproductive behaviors have been observed and attributed to the early organizational effects of gonadal hormones on the central nervous system, or to their activational role in the adult animal (Beatty, 1979). Although estrogen levels in the adult seem to modulate amphetamine-induced stereotypy, a behavior dependent on nigrostriatal dopamine release, its importance in amphetamine induced rotation of adult rats with unilateral nigrostriatal lesions seems minimal (Hruska & Silbergeld, 1980). However, preliminary work reported by Becker et al., (1981) implicates estrogenic determination of sex differences in rotational responses to amphetamine in intact rats. They report that female rats in their estrus phase exhibit significantly more net rotations in response to amphetamine than females in mid-diestrus, estrus or pro-estrus phases of their estrus cycle. Furthermore, male rats did not rotate significantly differently than female rats in diestrus. The importance of the activational influences of estrogen in modulating amphetamine-induced rotation is also seen in our finding that estrogen treatment significantly raises the number of net

rotations in response to amphetamine performed by neonatally castrated male rats. (In light of the above observations of Becker it might be expected that these rats would make even more net full rotations than intact female rats since the female rats tested had different levels of estrogen depending on their estrus cycle when tested, whereas the treated male rats presumably all had high levels of estradiol. This trend was noted, but not at a statistically significant level.) Since the neonatally castrated males rotated more than intact males, the organizational influences of gonadal hormones (testosterone) also seem to be important in determining rotational responses to amphetamine. The amphetamine-elicited responses of intact adult males given estradiol benzoate, and of adult females given testosterone at birth, must be studied in order to clarify the relative significance of the organizational and activational roles of gonadal hormones in regulating this behavior. Past work and the evidence presented here indicates that both roles are essential to the full establishment of the sexually dimorphic response.

Based on several lines of evidence (e.g. rats tend to rotate in opposite directions in response to amphetamine and apomorphine; Glick et al., 1977b) it had been suggested that rotational asymmetry may be due to both pre-synaptic (dopamine stores) and post-synaptic (number or sensitivity of dopamine receptors) asymmetries, and that these intrinsic

asymmetries are inversely related (Glick et al., 1977; Jerussi et al., 1977). That is, receptor response is greater on the side where dopamine release is less. If this is so, our present findings in Sprague-Dawley rats of a sexually dimorphic response to amphetamine and apomorphine suggests a different basis for rotational asymmetry in the two sexes. Although both types of asymmetry may exist in both sexes, a pre-synaptic asymmetry seems to predominate in females and a post-synaptic mechanism appears to be more important in Sprague-Dawley males. Even though male and female Fischer rats respond similarly to apomorphine, the finding that female rats rotate more in response to amphetamine than apomorphine and that male rats respond more to apomorphine than amphetamine suggests a predominance of different mechanisms underlying rotational asymmetry for the two sexes in Fischer rats as well.

The studies in section III are also of more general importance. The elucidation of sex differences in striatal function has clinical relevance. It may relate to the many reports linking estrogen status to the clinical state seen in patients with disorders of dopamine metabolism of the basal ganglia. Choreiform disorders have been associated with the elevated estrogen levels accompanying pregnancy or the use of oral contraceptives (Donaldson, 1978), and estrogen may precipitate Parkinsonian symptoms in males or females receiving neuroleptics (Gratton, 1960) or improve

the symptoms of tardive dyskinesia (Bedard et al., 1977). A greater understanding of the organizational and activational roles of gonadal hormones in determining basal ganglia function and drug sensitivity may aid in the treatment of many of these diseases and other disorders of striatal dopamine function (Chase, 1982).

CHAPTER FIVE

ALTERATIONS IN CEREBRAL CATECHOLAMINERGIC SYSTEMS

WITH EXPERIMENTAL PHENYLKETONURIA

(I) MODULATION OF CEREBRAL CATECHOLAMINE
LEVELS DURING HYPERPHENYLALANINEMIA

The behavioral abnormalities found in adult animals previously subjected to hyperphenylalaninemia early in life suggests altered catecholamine metabolism in these animals. Adult rats which had been subjected to our experimental treatment during the first three postnatal weeks, have shown an anomaly in the lateralization of their circling activity (Chapter IV), a behavioral pattern known to be regulated by dopaminergic, nigrostriatal pathways (Glick et al., 1977; Pycock, 1980). Furthermore, studies on this and other models of PKU have revealed abnormalities in additional behavioral parameters, such as maze learning ability and locomotor activity (Chapter IV; Luttges & Gerren, 1979; Glick & Greenqard, 1980; Lane et al., 1980) which are also influenced by dopaminergic systems (McLean et al., 1980; Studelska & Beatty, 1978). Catecholamine depletion has also been found in the brains of human phenylketonuric retardates (McKean, 1972). Although the role of this decrease in both dopamine and norepinephrine in contributing to the profound mental retardation found in PKU is unknown, animal experimentation (Kempf et al., 1974), including work with monkeys

(Brozoski et al., 1979; Simon et al., 1980), has directly linked higher mental function with cerebral catecholamine metabolism.

This chapter describes changes in cerebral catecholamine levels in suckling rats during hyperphenylalaninemia produced by our model PKU treatment (see Chapter II). Animal models of PKU have reproduced many of the changes that occur in humans with this disease including drastic elevations of phenylalanine content (DelValle et al., 1978; Dhondt et al., 1977; Greengard et al., 1976), decreases in cerebral branched chain amino acid levels (Greengard et al., 1979; Isaacs & Greengard, 1980; Lane et al., 1980; Lowden & LaRamee 1969) and cerebral serotonin depletion (Isaacs & Greengard 1980; Lane et al., 1980; McKean et al., 1968). However, the catecholamine depletion seen in the brains of human PKUics (McKean, 1972) is the one abnormality that has never been reported in any animal model of the disease. The first section of this chapter shows that there is, in fact, a deficit in cerebral catecholamines in a reasonably good rat model of PKU, and explains why this has not been noted previously.

RESULTS

Changes in the brain catecholamine levels of suckling rats exposed to experimental phenylketonuria (Greengard et al., 1976; DelValle et al., 1978) were studied. The animals given this treatment from 2 days of age showed decreased levels of cerebral catecholamines when killed 5-7 hours after their last injection. Cerebral dopamine was significantly decreased by 27% and 34% in 7 and 11 day old rats respectively, but showed no significant deficit in 16 day old animals. Norepinephrine was decreased by 37% of control values in day 7, and showed a 27% decrease on day 16 (Table V-1).

Cerebral catecholamine concentrations were also analyzed according to sex. At 11 and 16 days no sex differences were seen. However, at 7 days of age male rats had a higher concentration of both dopamine (22% higher, $p < 0.01$) and norepinephrine (27 % higher, $p < 0.01$; Table V-1). Relative catecholamine depletion was equivalent in both sexes; male and female pups had 27% depletions of their basal (control) dopamine levels, and 34% and 39% decreases respectively in their norepinephrine content. But the absolute catecholamine concentrations in the female hyperphenylalani-

TABLE V-1

EFFECT OF CHRONIC HYPERPHENYLALANINEMIA ON CEREBRAL CATECHOLAMINES

Animals were injected daily (subcutaneously) with α -methylphenylalanine (2.4 umoles/g body w) plus phenylalanine (5.2 umoles/g body w) or saline (controls) from postnatal day two until the day of sacrifice. Five to seven hours after the last injection animals were decapitated and cerebral dopamine and norepinephrine measured as described in the Methods' section. All values are expressed as nanograms catecholamine per gram (ng/g) brain weight. Means followed by SD, refer to the number of animals in parenthesis. a: $P < .01$ different from control female, b: $P < .01$ from corresponding females, c: $P < .01$ from controls, d: $P < .025$ from control.

Age (days)	Cat	Control		Experimental	
		Male	Female	Male	Female
7	DA	403 $\pm$ 113 (6) ^a	330 $\pm$ 47 (6)	293 $\pm$ 30 (7) ^{b,c}	242 $\pm$ 65 (6) ^c
		366 $\pm$ 12 (12)		269 $\pm$ 84 ^c (13)	
	NE	501 $\pm$ 58 (3) ^b	393 $\pm$ 24 (4)	306 $\pm$ 120 (4) ^{b,c}	261 $\pm$ 71 (4) ^c
		439 $\pm$ 78 (7)		284 $\pm$ 142 ^c (8)	
9	DA	660	464	317	439
11		746 $\pm$ 151 (3)	897 $\pm$ 203 (3)	632 $\pm$ 55 (4)	597 $\pm$ 26 (2)
		822 $\pm$ 180 (6)		611 $\pm$ 52 (6) ^c	
16	DA	592 $\pm$ 135 (4)	667 $\pm$ 78 (6)	597 $\pm$ 200 (5)	611 $\pm$ 122 (5)
		637 $\pm$ 104 (9)		604 $\pm$ 157 (10)	
	NE	506 $\pm$ 149 (4)	607 $\pm$ 149 (6)	386 $\pm$ 202 (4)	446 $\pm$ 66 (5)
		567 $\pm$ 150 (10)		416 $\pm$ 145 (10) ^d	
18	DA	543	699	574	444

nemic rats, owing to their lower basal values, were lower than in their male littermates. To simplify the interpretation of data and decrease the number of variables all subsequent experiments described in this paper were conducted on seven day old female rats.

The time course of cerebral catecholamine depletion was studied by measurements at 6, 12 and 26 hours after the last injection of phenylalanine plus AMPHE. Dopamine levels were lowest at 6 hours, and by 12 hours had almost returned to control values (Figure V-1). This contrasts markedly with the dopamine depletion observed when these rats were injected with AMPHE alone. At 6 hours after the last injection cerebral dopamine levels were only 50% of control values, and at 18 hours they were still only 77% of normal. With a single injection of AMPHE the dopamine content was reduced to 64%, 50% and 66% of control values at 3, 6 and 12 hours after injection. Thus, AMPHE itself caused a more severe and longer-lasting dopamine depletion than did injection of AMPHE plus phenylalanine. However, the effect of AMPHE itself also appears to dissipate by 24 hours. The fact that the depletion (at 6 hours) is not greater in the chronic experiments than in the acute experiments (i.e. 6 hours after an injection to previously untreated rats) also indicates a lack of cumulative effect.

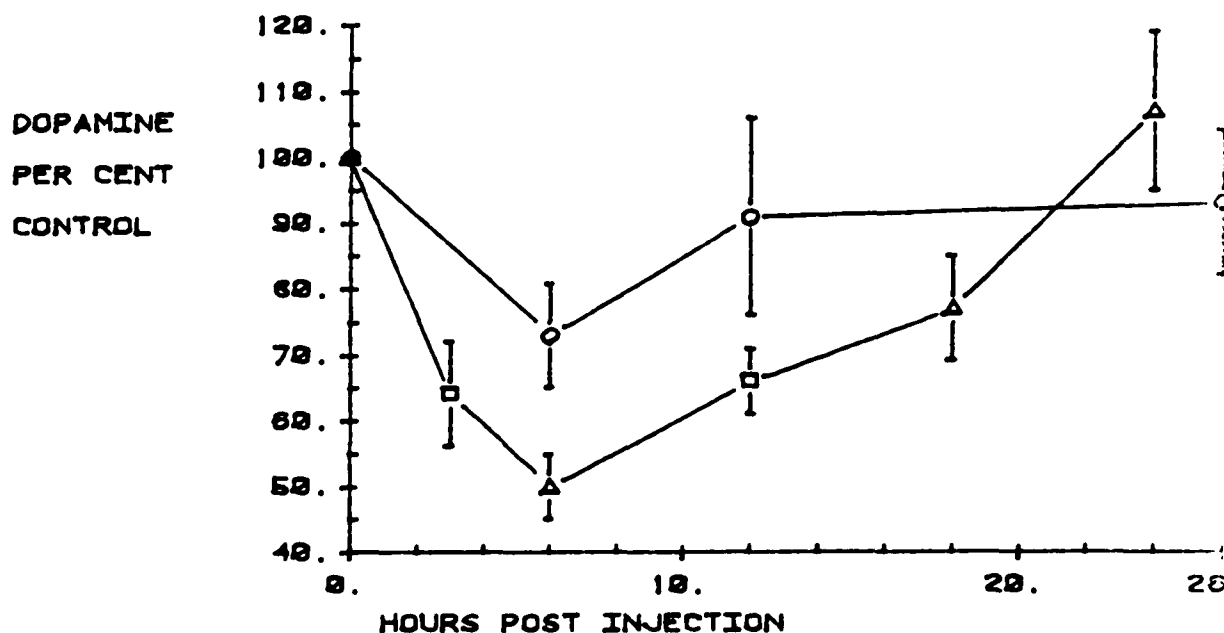
Since our model of phenylketonuria includes the use of AMPHE, itself a potent depletor of cerebral catecholamines,

FIGURE V-1

TIME COURSE OF DOPAMINE DEPLETION IN RESPONSE TO
 PHENYLALANINE PLUS α -METHYLPHENYLALANINE OR α -
 METHYLPHENYLALANINE TREATMENTS

Female rats were injected with phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g) or α -methylphenylalanine only (2.4 umoles/g) daily from the second day of life (chronic) or on day 7 only (acute). Animals were killed on day seven at the indicated hour after their last injection. Cerebral dopamine was measured as described in Methods, and is expressed as a per cent $\pm$ S.E.M. of dopamine levels of control (saline injected) littermates sacrificed at the same time. Each point represents the mean value of 4-6 rats.

- α -methylphenylalanine plus phenylalanine (chronic)
- △ α -methylphenylalanine only (chronic)
- α -methylphenylalanine only (acute)



the relationship between increases in phenylalanine (as seen in phenylketonuria) and catecholamine loss was unclear. Thus, we decided to investigate the role of high phenylalanine itself in depleting catecholamines in our model system. However, one injection of phenylalanine alone will not sustain high increases in plasma or brain phenylalanine for many hours since it is rapidly metabolized by the hepatic phenylalanine hydroxylase. Therefore, we injected AMPHE alone, and one day later, when the hepatic phenylalanine hydroxylase was suppressed (DelValle et al., 1978) but the cerebral dopamine was no longer depleted (Figure V-1), we injected 5 umoles/gram of phenylalanine. This resulted in an increase in the brain phenylalanine to 1400 umol/g at 6 hours, but no depletion of cerebral dopamine (Table V-2). However, injection of 12.5 umol/g of phenylalanine into animals with similarly inhibited hepatic phenylalanine hydroxylase produced an elevation of brain phenylalanine to 2900 umol/g at 6 hours and a 24% deficit in cerebral dopamine. This increase in brain phenylalanine at 6 hours was similar to that (2600 umol/g) seen in the brains of rats chronically injected with AMPHE and phenylalanine, and the cerebral dopamine decrease (27%) was also similar (see Table V-3).

When three doses of phenylalanine (enough to increase cerebral phenylalanine to 2788 umol/g at 6 hours) were given without a prior injection of AMPHE (line 5, Table V-2) no catecholamine depletion occurred. However, since the

TABLE V-2

CATECHOLAMINE DEPLETION IN RESPONSE TO VARIOUS CEREBRAL LEVELS OF PHENYLALANINE AND TYROSINE

Rats in group two (second line) and three (third line) received 5.0 and 12.5 umoles/g body weight phenylalanine, respectively. The fourth group of animals (line four) was injected with 5 umoles/g phenylalanine plus 5 umoles/g tyrosine. All of these animals (groups 1-4) had their liver phenylalanine hydroxylase activity reduced by prior treatment with α -methylphenylalanine. The fifth group of rats received 13 umoles/g of phenylalanine administered in three doses of 5 umoles/g, followed one hour later by 3.9 umoles/g and another 3.9 umoles/g four hours after that. All rats were 7 day old females which were sacrificed 6-7 hours after their first injection of phenylalanine. Catecholamine levels are expressed as a per cent $\pm$ S.D. (n) of the catecholamine levels of control (saline injected) littermates treated and assayed at the same time. Phenylalanine and tyrosine levels (nanomoles/gram brain $\pm$ S.D. (n) were determined in the brains of a separate group of animals treated similarly to those in which cerebral catecholamines were assayed.

	TREATMENT (umoles/g)		BRAIN AMINO ACIDS (umoles/g)		BRAIN CATECHOLAMINES (per cent control)	
	<u>Phenylalanine</u>	<u>Tyrosine</u>	<u>Phenylalanine</u>	<u>Tyrosine</u>	<u>Dopamine</u>	<u>Norepinephrine</u>
<u>Hepatic PAH</u>						
Inhibited	-	-	85 $\pm$ 12 (4)	63 $\pm$ 17 (3)	110	-
Inhibited	5	-	1400	87 $\pm$ 23 (4)	100 $\pm$ 15 (4)	86 $\pm$ 18 (4)
Inhibited	12.5	-	2900 $\pm$ 303 (6)	94 $\pm$ 34 (3)	76 $\pm$ 14 (3)	90 $\pm$ 19 (3)
Inhibited	5	5	3835 $\pm$ 930 (4)	472 $\pm$ 219 (4)	104 $\pm$ 11 (3)	116 $\pm$ 16 (3)
Normal	13	-	2788 $\pm$ 236 (4)	400 $\pm$ 135 (4)	113 $\pm$ 27 (6)	86 $\pm$ 21 (6)

TABLE V-3

COMPARISON OF THE DOPAMINE DEPLETING EFFECTS OF α -METHYLPHENYLALANINE
WITH THAT OF PHENYLALANINE, AND OF α -METHYLPHENYLALANINE PLUS
PHENYLALANINE

Seven day old female rats with reduced phenylalanine hydroxylase activity were injected with saline (line 1), phenylalanine alone (12.5 umoles/g, line 2), α -methylphenylalanine (2.4 umoles/g, line 3), or α -methylphenylalanine (2.4 umoles/g, line 3), or α -methylphenylalanine (2.4 umoles/g) plus phenylalanine (5.2 umoles/g, line 4). Five to seven hours after injection their brains were removed and cerebral dopamine levels determined in relation to those of saline injected animals (per cent control). Similarly treated animals were used to determine the cerebral phenylalanine and tyrosine content of animals undergoing these treatments. Values represent the mean $\pm$ S.D. (n).

<u>Treatment</u>	<u>Phenylalanine</u>	<u>Tyrosine</u>	<u>Dopamine (% control)</u>
Saline	80 $\pm$ 17(5)	77 $\pm$ 13(3)	100 $\pm$ 14(6)
Phenylalanine	3194 $\pm$ 323(4)	94 $\pm$ 34(3)	76 $\pm$ 14(3)
α -methylphenylalanine	269 $\pm$ 32(3)	82 $\pm$ 11(3)	50 $\pm$ 12(6)
α -methylphenylalanine plus phenylalanine	2620 $\pm$ 420(6)	120 $\pm$ 27(4)	73 $\pm$ 20(6)

hepatic phenylalanine hydroxylase was not inhibited in these animals, much of the phenylalanine was metabolized to tyrosine, causing an increase in the cerebral tyrosine (from a control value of 65 $\mu\text{mol/g}$) to 400 $\mu\text{mol/g}$. To obtain similarly high cerebral tyrosine concentrations in rats with inhibited phenylalanine hydroxylase, rats were given 5 $\mu\text{mol/g}$ of tyrosine along with 5 $\mu\text{mol/g}$ of phenylalanine one day after treatment with AMPHE. Brain phenylalanine was markedly increased in these animals, as was cerebral tyrosine, and once again cerebral dopamine concentrations remained unchanged (line 4, Table V-2).

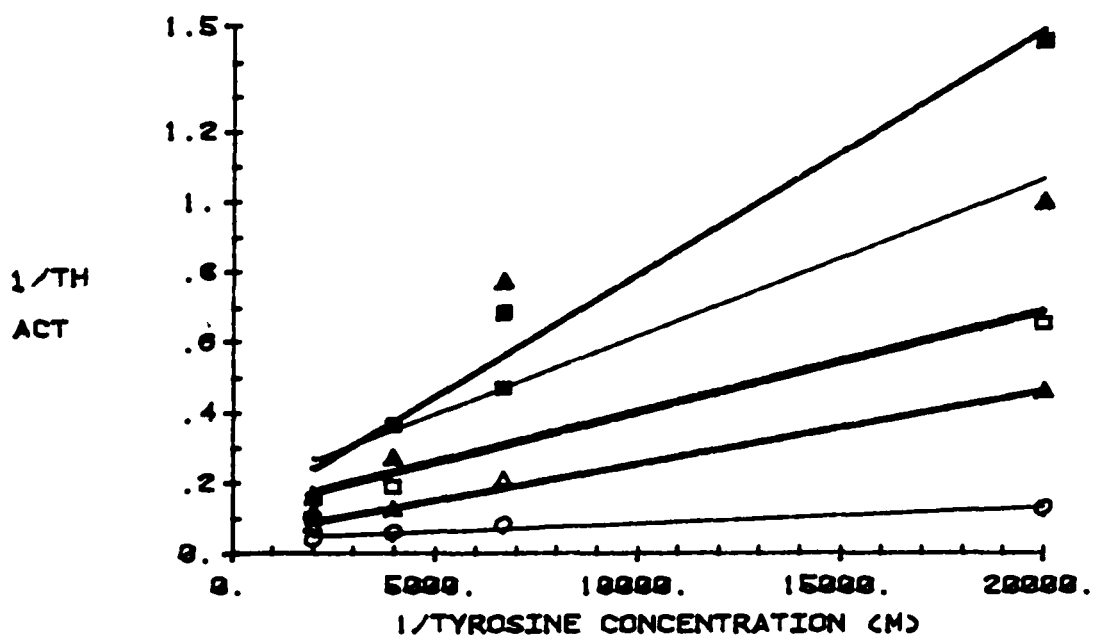
One possible explanation for the apparent reversal by tyrosine of the cerebral catecholamine depletion accompanying hyperphenylalaninemia may be that the phenylalanine acts as a competitive inhibitor of tyrosine hydroxylase, and additional tyrosine overcomes this inhibition. To approach this question, tyrosine hydroxylase activity in striatal homogenates was measured in vitro with varying concentrations of phenylalanine and tyrosine. A double-reciprocal plot of the data for these experiments (Figure V-2) shows that phenylalanine does act as a competitive inhibitor ($K_i = 2.19 \times 10^{-4} \text{ M}$) of striatal tyrosine hydroxylase. It is clear that at least in vitro one can overcome phenylalanine inhibition of tyrosine hydroxylase by raising the tyrosine concentrations above the value in vivo. For example, tyrosine hydroxylase activity was 7.59 $\mu\text{mol/min/g}$ with a tyrosine concentration of 50 μM (the approximate value in vivo)

FIGURE V-2

DOUBLE RECIPROCAL PLOT OF TYROSINE CONCENTRATION
AGAINST THE RATE OF TYROSINE HYDROXYLATION WITH
VARIOUS LEVELS OF PHENYLALANINE

The standard incubation mixture plus various concentrations of phenylalanine was used. Enzyme was from a pool of the striatum of several adult rats. Assays were performed in triplicate for each point and the average value used. The K_i , as calculated from the curves whose slope and intercept values were significant (with $P < .05$), was 2.19×10^{-4} M.

- , 0 Phenylalanine
- △, 0.6 mM Phenylalanine
- , 1.2 mM Phenylalanine
- ▲, 2.4 mM Phenylalanine
- , 3.6 mM Phenylalanine



but only 1.00 when 2.4 mM phenylalanine (the level seen in PKU and in our model of the disease) was added to the incubation. However, when the tyrosine concentration was raised to 5 uM, tyrosine hydroxylase activity in the presence of 2.4 mM phenylalanine was 8.47 umol/min/g. This value is similar to the tyrosine hydroxylase activity in vitro measured with tyrosine concentrations found in vivo (in the absence of excess phenylalanine).

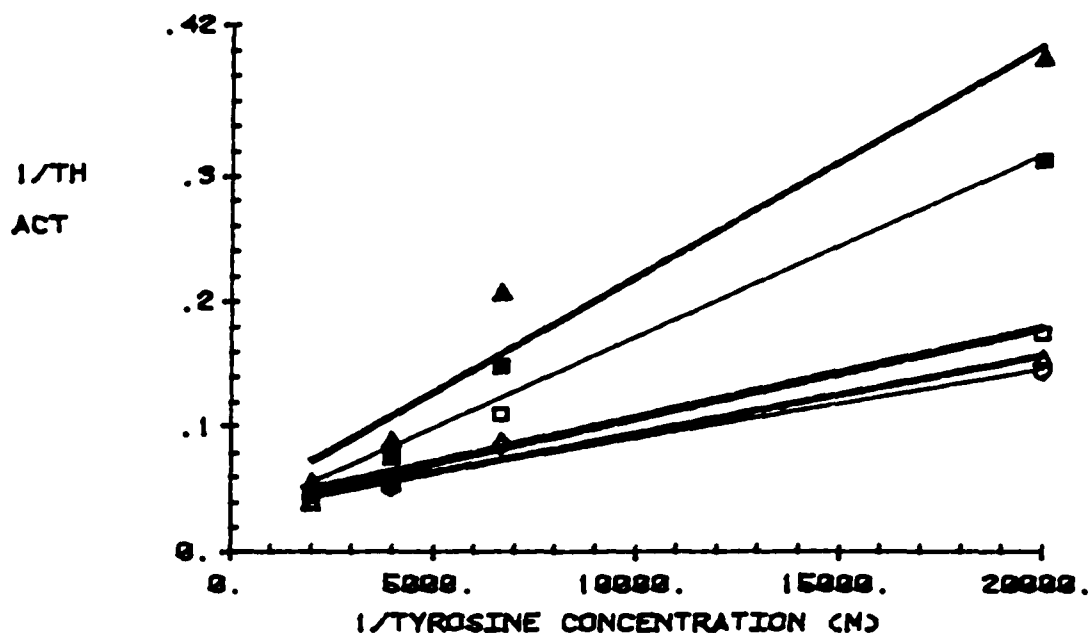
As previously reported by Udenfriend et al., (1965) for the adrenal enzyme, AMPHE was found to be a competitive inhibitor of (striatal) tyrosine hydroxylase. From the present double-reciprocal plots of activity against the various tyrosine and AMPHE concentrations used (Figure V-3), the K_i of AMPHE (1.46×10^{-3} M) is an order of magnitude greater than that for phenylalanine (2.19×10^{-4} M). Nonetheless, AMPHE was found to be a more potent depletor of cerebral catecholamines than was phenylalanine. Moreover, their effects were not additive in respect to catecholamine depletion in vivo; the depletion seen 6 hours after the injection of AMPHE plus phenylalanine was less, rather than more, than that seen 6 hours after injection of AMPHE alone (Table V-3, lines 3 & 4). They also differed with respect to cerebral tyrosine content, which was higher with the dual treatment than with AMPHE alone (see column 2, Table V-3). The dopamine depletion accompanying the injection of AMPHE plus phenylalanine was similar to that seen in rats (with

FIGURE V-3

DOUBLE RECIPROCAL PLOT OF TYROSINE CONCENTRATION
AGAINST THE RATE OF TYROSINE HYDROXYLATION WITH
VARIOUS LEVELS OF α -METHYLPHENYLALANINE

The standard incubation mixture plus various concentrations of α -methylphenylalanine was used. Enzyme was from a pool of the striatum of several adult rats. Assays were performed in triplicate for each point and the average value used. The K_i , as calculated from the curves whose slope and intercept values were significant (with $p < 0.05$), was 1.46×10^{-3} .

- 0 mM α -methylphenylalanine
- △ 0.01 mM α -methylphenylalanine
- 0.10 mM α -methylphenylalanine
- 0.50 mM α -methylphenylalanine
- ▲ 1.00 mM α -methylphenylalanine



reduced phenylalanine hydroxylase activity) given enough phenylalanine to achieve similar elevations in cerebral phenylalanine (Table V-3, lines 2 & 4).

DISCUSSION

The finding that experimental hyperphenylalaninemia induced by the daily injection of phenylalanine plus α -methylphenylalanine causes a depletion of cerebral catecholamines in suckling rats confirms the appropriateness of this treatment as a model for human phenylketonuria. Both direct and indirect evidence suggests that catecholamine metabolism is altered in phenylketonuria. McKean (1972) has found in autopsy studies a significant decrease in caudate dopamine and norepinephrine levels in the brains of phenylketonuric retardates as compared to non-PKUic retardates. Curtius (Curtius et al., 1972) has demonstrated that after an oral tyrosine load the urinary excretion of the catecholamine metabolites vanillyl mandelic acid (VMA), homovanillic acid and 3-methoxy-4-hydroxyphenylene glycol is much lower in hyperphenylalaninemic patients than in those with normal blood phenylalanine levels. Others have reported lower urinary VMA (Cession-Fossion et al., 1966), dopamine, epinephrine and norepinephrine (Nadler & Hsia, 1961) excretion, and low blood epinephrine and norepinephrine levels (Nadler & Hsia, 1961; Weil-Malherbe, 1955) in subjects with elevated plasma phenylalanine.

That severe hyperphenylalaninemia would lead to a depletion of cerebral catecholamines is not surprising in view of the fact that phenylalanine was shown to inhibit the activity of tyrosine hydroxylase (Nagastu et al., 1964), which is thought to be the rate limiting enzyme in catecholamine synthesis (Levitt et al., 1965). However, this corollary of the human condition could apparently not be imitated in animal models of PKU, so that no experimental system was available to explore the various reasons put forward to explain this catecholamine depletion. Among these were: a) high levels of phenylalanine inhibiting tyrosine hydroxylase, b) subnormal tyrosine levels limiting the rate of the tyrosine hydroxylase reaction, and c) metabolites of phenylalanine (arising from the transamination pathway) compromising catecholamine synthesis in some unspecified way.

Although theory a) was based on evidence for the inhibition by phenylalanine of the tyrosine hydroxylase activity in vitro, further studies were required to determine whether or not this inhibition could entirely account for the hyperphenylalaninemia induced dopamine depletion in vivo. The data presented in this section appears to provide an affirmative answer. Alternative b) can definitely be excluded since the tyrosine levels in our hyperphenylalaninemic rats were above rather than below normal. The assumption (c) that metabolites of phenylalanine are responsible for the catecholamine depletion also appears to be superfluous.

Tyrosine supplementation of the severely hyperphenylalaninemic rats is unlikely to have prevented the formation of these metabolites and yet it abolished the catecholamine deficit. It did so at tyrosine levels similar to those needed to overcome the effect of excess phenylalanine on the tyrosine hydroxylase activity in vitro. The observations described here thus provide some of the missing evidence in favor of the tyrosine hydroxylase inhibition theory of catecholamine depletion of PKU.

Rat models of phenylketonuria showing no cerebral catecholamine deficits include Wistar rats rendered hyperphenylalaninemic with essentially the same methods as used here (Lane et al., 1980). One obvious explanation for this observation may be that brains were sampled at such late times after the daily injection of phenylalanine plus α -methylphenylalanine when the catecholamines were at near normal levels (see Figure V-1). Another explanation, of more general importance, lies in the ease with which a relatively small increase in tyrosine can counteract the catecholamine depleting effect of hyperphenylalaninemia (Table V-2, lines 3 & 4). Thus, a less efficacious treatment, that is, one in which the brain phenylalanine levels are lower or the cerebral tyrosine levels are somewhat higher than in our model of phenylketonuria, may not produce noticeable catecholamine depletion.

The failure of our treatment to reduce dopamine levels in the brains of 16 day old rats may be rooted in this same phenomenon. The small rise in phenylalanine hydroxylase activity during the suckling period (McGee et al., 1972) despite α -methylphenylalanine treatment (DelValle et al., 1978), may result in a cerebral phenylalanine/tyrosine ratio in the 16-day-old rat too low to decrease significantly the rate of tyrosine hydroxylation. A more complex phenomenon which would contribute to the preservation of cerebral dopamine in older animals is the evolving capacity for its storage. The number of dopamine and norepinephrine storage vesicles increases during development, particularly during the second week of life in the rat (Coyle and Axelrod, 1971; Kirksey et al., 1978; Deskin et al., 1981). As the capacity to store catecholamines and protect them from enzymatic destruction increases, the brain may become more capable of withstanding the temporary loss in synthetic potential imposed by each daily injection of phenylalanine plus α -methylphenylalanine.

RELEVANCE TO THE TREATMENT OF PHENYLKETONURIA

In view of indications that cerebral catecholamine deficiency compromises higher intellectual functions (see Introduction) this new evidence for the importance of tyrosine as well as phenylalanine levels in modulating the dopa-

mine content of the hyperphenylalaninemic brain may be pertinent to the management of phenylketonuria. For it suggests that the severe cerebral catecholamine depletion experienced by phenylketonurics may be reversed by a dietary regimen including tyrosine supplementation. Since the dietary addition of an appropriate excess of tyrosine would be effective with only a moderate decrease in phenylalanine intake, this could be accomplished more easily than the present treatment of phenylketonuria where minimal phenylalanine intake requires severe food limitations which involves social trauma, frequent failures to comply with treatment, and difficulty in balancing phenylalanine restriction against the amount required for optimal growth. A further advantage is that the administration of tyrosine may have physiologically significant effects only in those areas with existing catecholamine imbalance (Sved et al., 1979; Wurtman et al., 1980). (Tyrosine supplementation may restore catecholamine levels when tyrosine hydroxylation is occurring at a sub-optimal rate (Table V-2; Gibson & Wurtman, 1977; Malamud et al., 1980), but it appears that tyrosine hydroxylase is saturated in the normal state and the addition of tyrosine will not raise catecholamine levels above normal in this condition (Mandel, 1978; Sved & Fernstrom, 1981)). It has recently been reported (Butler et al., 1981) that children deficient in dihydropteridine reductase, and thus in catecholamines and serotonin, benefit from treatment

with precursors of these neuroactive amines. Thus, the concept of treating a cerebral amine deficiency with precursor administration is not new, although this is the first suggestion of the possible usefulness of such an approach to correcting some of cerebral abnormalities associated with phenylketonuria.

ALPHA-METHYLPHENYLALANINE AS AN EXPERIMENTAL TOOL

Suppression of phenylalanine hydroxylase in vivo, with an injection of α -methylphenylalanine (with or without phenylalanine) is a slow irreversible process. Minimal activity is attained in 20 hours, and only after an interval (about 2 days) required for the resynthesis of the enzyme does the activity return to normal (DelValle et al., 1978). Thus, an advantage of using this agent to imitate phenylketonuria is that administration once in two days is sufficient to maintain steadily low phenylalanine hydroxylase activity in vivo.

The inhibition by α -methylphenylalanine of tyrosine hydroxylase, on the other hand, is rapid and reversible in vitro, and the catecholamine depletion in vivo begins to be restored within a few hours (Figure V-1). There was a time interval following α -mphe administration when the dopamine levels had returned to normal but the hepatic phenylalanine hydroxylase, and thus the elimination of injected phenylala-

nine, was still suppressed. Thus, the catecholamine deficit ensuing this injection is attributable to the phenylalanine itself. These results are in accord with the fact that phenylalanine as well as its alpha-methyl analogue are effective inhibitors of tyrosine hydroxylase in vitro. However, the magnitude of the dopamine depletion was greater in response to AMPHE alone than to AMPHE plus phenylalanine. One explanation for this is that the small elevation of tyrosine associated with the combined treatment is enough to preclude the inhibition of tyrosine hydroxylase by AMPHE, and thus mask the depleting effect of AMPHE. Another possibility is that excess phenylalanine blocks the transport of AMPHE into the brain. In either case, a general, practical implication of the observations summarized in Table V-3 is that, since elevated concentrations of an amino acid can minimize the side effects of synthetic analogues, study of the organism treated with such an analogue alone does not predict the extent to which these side effects will in fact be operative in animals treated with both substances.

Rats given alpha-methylphenylalanine alone, therefore, cannot be considered as "controls" for those, which, injected with phenylalanine as well, were meant to imitate the human disease of phenylketonuria. Instead, they can be looked upon as a new experimental system for research on aberrant dopaminergic functions associated with several conditions, including the minimal brain damage syndrome (i.e. attention

deficit disorder) in children. The functional consequences of the reversible dopamine depletion or of prolonged depletion maintained with chronic AMPHE treatment are not well known (see Chapter IV). The obvious potential of this system to provide new insights comes from the fact that the mechanism underlying the experimental abnormality in catecholamine metabolism is clearly different from that in currently used models where this is achieved through irreversible destruction of particular brain structures by surgical lesion or by administration of dopamine analogues (e.g. 5-hydroxydopamine) which destroy catecholaminergic neurons.

(II) LONG LASTING ENZYMATIC CHANGES IN RATS
EXPOSED TO EARLY POSTNATAL HYPERPHENYLALANINEMIA

Since changes in catecholamine levels in the brains of hyperphenylalaninemic rats did not extend into maturity, at which time behavioral abnormalities were noted (Chapter IV), it was difficult to see how these early changes were linked to behavioral aberrations. To further investigate the possible role of changes in cerebral catecholaminergic systems in mediating behavioral changes in PKU, the effects of our model PKU on the development of enzymes involved in catecholamine synthesis (tyrosine hydroxylase, aromatic l-amino acid decarboxylase) and degradation (monoamine oxidase) were examined.

Of the multitude of cerebral enzymes studied in rats with experimental PKU (DelValle et al., 1978; Diemel, 1977; Greengard et al., 1977; Greengard, unpublished), only phosphoserine phosphatase (DelValle et al., 1978; Isaacs & Greengard 1980) and glycine synthetase (Isaacs & Greengard, 1980), two enzymes involved in glycine regulation, have been found to change. Both enzymes return to normal activity by the 25th day of life (Greengard, unpublished).

Treatments which may alter brain catecholamine levels can also effect the cerebral activities of catecholamine synthesizing enzymes. For example, perinatal undernutrition is followed by a decrease in cerebral norepinephrine and dopamine and an increase in tyrosine hydroxylase activity (Shoemaker & Wurtman, 1971), whereas administration of the dopamine precursor l-dopa results in a decrease in the cerebral activity of l-amino acid decarboxylase (Dairman et al., 1971), the enzyme which catalyzes dopamine synthesis from l-dopa. Based on this evidence it seemed reasonable to investigate the effect of early postnatal hyperphenylalaninemia, which alters cerebral catecholamine levels (section I, Chapter V), on the activities of catecholamine regulating enzymes.

This section of Chapter V describes the first permanent biochemical (protein) change accompanying hyperphenylalaninemia in any experimental model of PKU. A significant increase in striatal aromatic l-amino acid decarboxylase (also referred to as dopa decarboxylase (DDC)) activity is noted in 70 and 120 day old, but not younger, animals. Striatal monoamine oxidase also appears to rise in response to model PKU.

RESULTS

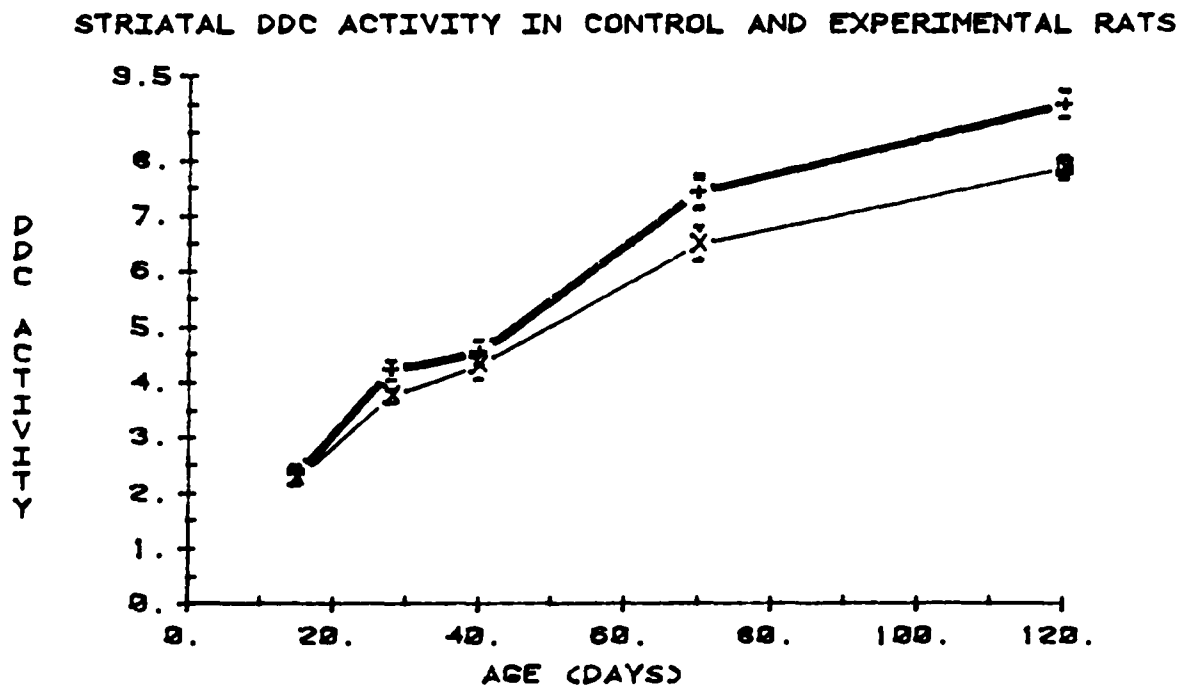
Fischer rats subjected to our usual protocol to mimic PKU during the 2nd to 21st days of life and their saline injected littermates were studied for striatal enzyme activities at 15, 28, 40, 70 and 120-140 (adult) days of life. Striata were the brain region chosen for examination due to the importance of this structure in the mediation of rotational behavior in rats (Glick et al., 1977; Pycock, 1980), the pre-eminence of dopamine in this area and the fact that both tyrosine hydroxylase (Coyle, 1972; Porcher & Heller, 1972) and dopa decarboxylase (Porcher & Heller, 1972; Sims et al., 1973) are highly concentrated in this brain area. At each age studied the striatal dopa decarboxylase (DDC) activity was higher in the experimental animals than in their control littermates (Figure V-4). DDC was only slightly elevated in young experimental animals, being 5% higher than controls at 40 days, but rose to be 14-15% higher than control values at 70 and 120 days. This elevation was statistically significant in 70 day old ($p < 0.05$) and adult ($p < 0.01$) animals.

In contrast, there were no differences between control and experimental animals in the striatal activities of the

FIGURE V-4

DOPA DECARBOXYLASE ACTIVITY IN DEVELOPING CONTROL AND
HYPERPHENYLALANEMIC RATS

Suckling rats were injected with phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w) (+), α -methylphenylalanine alone (2.4 umoles/g body w) (□) or saline diluent (X) on days 2 to 21 of life. They were then sacrificed on the indicated day, striates removed and dopa decarboxylase activity determined. Each value represents the mean $\pm$ S.D. in units/g striate with 15 $\geq$ 5 for each point.



enzyme controlling the rate limiting step in catecholamine synthesis (Levitt et al., 1965), tyrosine hydroxylase, or of another neurotransmitter synthesizing enzyme, choline acetyltransferase (ChAT) (Table V-4). Striatal monoamine oxidase (total A & B subtype activity), responsible for the enzymatic destruction of catecholamines and serotonin, was elevated 5% in 15 day old hyperphenylalaninemic rats and 12% ($p < 0.05$) in 70 day old experimental animals. This enzyme was not measured at other ages in this group of animals. Additionally, no significant sex differences were found in striatal DDC, TH, ChAT or MAO activities in either the control or experimental group (Table V-4; Appendix).

The studies described above were extended, in some instances, to include measurement of hepatic DDC and MAO activities. Table V-4 shows that at 15 and 70 days no changes in the hepatic activity of either enzyme was found in rats with elevated striatal activities. It was noted, however, that there was a sex difference in hepatic MAO activity. Male rats had a 23% greater activity than females at 70 days (Table V-4), and a 35% higher activity ($597 \pm 75(5)$ versus $442 \pm 86(6)$, $p < 0.01$) at 120 days. This was the only clear sex difference found in enzyme activities in this study. However, preliminary data suggests possible sex differences in frontal cortex DDC and MAO activities of adult animals (MAO: female = 122,124 male = 113,119; DDC: female = 2.35, 2.40 male = 1.86, 1.95).

TABLE V-4

STRIATAL AND HEPATIC ENZYME ACTIVITIES IN CONTROL AND HYPERPHENYLALANINEMIC RATS

Rat pups were injected with phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w) (E) or an equivalent volume of saline (C) during the 2nd to 21st days of life. Males (M) and females (F) were then sacrificed on the indicated day, their striates and/or livers removed and enzyme activities determined. All values represent the mean $\pm$ SD(n) with the superscripts * ($p < 0.01$) and ** ($p < 0.05$) indicating significant differences from control.

Age (Days)	Sex	Striatal Dopamine Decarboxylase units/g		Striatal Tyrosine Hydroxylase units/g		Striatal Choline Acetyltransferase units/g	
		C	E	C	E	C	E
15	M	2.40 $\pm$ 0.68 (6)	2.44 $\pm$ 0.68 (6)	24.70, 21.82	21.73 $\pm$ 3.9 (3)	208, 189	192 $\pm$ 21 (3)
	F	2.16 $\pm$ 0.26 (6)	2.15 $\pm$ 0.57 (6)	-	23.08	-	198
	M&F	2.28 $\pm$ 0.51 (12)	2.33 $\pm$ 0.60 (12)	24.70, 21.82	22.07 $\pm$ 3.26 (4)	208, 189	194 $\pm$ 17 (4)
28	M	3.58 $\pm$ 0.30 (5)	4.40 $\pm$ 0.42 (4)	26.59 $\pm$ 1.49 (4)	26.27 $\pm$ 4.54 (3)	488 $\pm$ 43 (4)	547 $\pm$ 14 (3)
	F	4.05 $\pm$ 0.19 (3)	4.00 $\pm$ 0.54 (3)	24.26	24.38	447	501
	M&F	3.76 $\pm$ 0.34 (8)	4.23 $\pm$ 0.48 (7)	26.12 $\pm$ 1.65 (5)	26.83 $\pm$ 3.87 (4)	480 $\pm$ 41 (5)	536 $\pm$ 26 (4)
40	M	4.39 $\pm$ 1.13 (6)	4.83 $\pm$ 0.66 (7)	34.59 $\pm$ 7.27 (5)	33.16 $\pm$ 4.24 (5)	621 $\pm$ 85 (4)	708 $\pm$ 52 (4)
	F	4.25 $\pm$ 0.83 (9)	4.14 $\pm$ 0.72 (5)	32.83 $\pm$ 3.54 (7)	37.59 $\pm$ 3.78 (4)	567 $\pm$ 37 (5)	626 $\pm$ 75 (4)
	M&F	4.31 $\pm$ 0.92 (15)	4.54 $\pm$ 0.75 (12)	33.56 $\pm$ 5.18 (12)	35.13 $\pm$ 4.45 (9)	591 $\pm$ 65 (9)	667 $\pm$ 74 (8)
70	M	6.47 $\pm$ 1.00 (6)	7.50 $\pm$ 0.92 (5)	-	-	725 $\pm$ 127 (3)	763 $\pm$ 35 (3)
	F	6.57 $\pm$ 0.85 (3)	7.35 $\pm$ 1.09 (4)	-	-	763, 732	736, 769
	M&F	6.50 $\pm$ 0.90 (9)	7.43 $\pm$ 0.93 (9) **	-	-	734 $\pm$ 91 (5)	759 $\pm$ 28 (5)
120+	M	7.76 $\pm$ 0.61 (3)	9.00 $\pm$ 0.52 (3)	36.05, 42.97	35.36 $\pm$ 4.37 (3)	765 $\pm$ 72 (3)	816 $\pm$ 28 (3)
	F	7.90, 8.11	9.02 $\pm$ 0.82 (5)	32.60, 38.62	40.59 $\pm$ 3.79 (5)	751, 810	847 $\pm$ 60 (5)
	M&F	7.86 $\pm$ 0.46 (5)	9.01 $\pm$ 0.68 (8) *	37.56 $\pm$ 4.37 (4)	38.63 $\pm$ 4.58 (8)	771 $\pm$ 56 (5)	836 $\pm$ 50 (8)

Age (Days)	Sex	Striatal Monoamine Oxidase units/g		Hepatic Monoamine Oxidase units/g		Hepatic Dopa Decarboxylase units/g	
		C	E	C	E	C	E
15	M	155+25 (3)	162+3 (3)	681+63 (3)	657+56 (3)	11.1+1.0 (3)	10.1+0.9 (3)
	F	135+10 (3)	141+19 (3)	653+40 (4)	649+46 (4)	12.4+1.0 (4)	10.8+1.5 (4)
	M&F	154+21 (6)	152+16 (6)	670+49 (7)	652+46 (7)	11.8+1.2 (7)	10.5+1.2 (7)
70	M	130+13 (3)	140+11 (3)	520+52 (3)	560+44 (3)	25.9+0.17 (3)	25.4, 21.2
	F	120, 122	145, 138	480, 488	580, 552	13.0	22.6, 17.4
	M&F	126+10 (5)	141+8 (5) **	504+40 (5)	564+32 (5) **	22.7+6.4 (4)	21.7+3.3 (4)
120+	M	-	-	576, 620	596+104 (3)	19.8, 17.2	21.9+2.1 (3)
	F	-	-	433+131 (5)	451+36 (3)	26.2+2.9 (3)	26.5+6.2 (3)
	M&F	-	-	499+130 (5)	524+105 (6)	23.1+4.8 (5)	24.2+4.8 (6)
all M				597+75 (5) *			
all F				442+86 (2)			

DISCUSSION

The significant increase in striatal DDC activity found in 70 and 120-140 day old rats previously hyperphenylalaninemic during the 2nd to 21st days of life is the first permanent metabolic change found in any animal model of PKU. The increase was seen at all ages in experimental rats compared to controls within the same litters in ten different groups of animals. This discovery provides an important inroad to direct further study on permanent changes caused by hyperphenylalaninemia early in life and their relationship to the pathogenesis of the permanent mental deficits which accompany PKU.

There are several biochemical changes found in the brains of PKUic children and adults (for review see: Dodge et al., 1974; Kaufman, 1976; Knox, 1972; Menkes & Koch, 1976) which have been replicated in animal models of PKU during the early treatment period when hyperphenylalaninemia is imposed, but are not seen in these animals when they mature. These transient changes include serotonin (Isaacs & Greengard, 1980; Lane et al., 1980; McKean et al., 1976) and catecholamine (see section (I)) depletion and decreases in branched chain amino acids (Greengard et al., 1976; Lane et

al., 1980; Lowden & LaRamee, 1969). Since these animals still exhibit behavioral anomalies as adults, when these cerebral deficits have been corrected, these temporary changes themselves (which persist in mature PKUics who unlike our animals are still exposed to high levels of phenylalanine) cannot be directly responsible for the abnormal mental state following early hyperphenylalaninemia. It is more likely that a permanent biochemical change existing in the adult animal is responsible for its altered behavior at that time. By analogy, those changes which exist in mature humans in response to continual elevation of plasma phenylalanine, but which cannot be replicated in adult animals in successful models of the disease (see Chapter VII), may not be the critical metabolic abnormalities underlying altered behavior and retardation.

Although the early biochemical changes seen in both hyperphenylalaninemic animals and man may trigger later appearing metabolic changes, we must focus on these secondary effects which persist into adult life irrespective of the animals later plasma phenylalanine levels in order to understand the pathophysiology of the cerebral defect. Treatment of PKUics after a certain early period of life (first few years) may correct some of the biochemical consequences of hyperphenylalaninemia (e.g. decrease in catecholamine synthesis (Curtius et al., 1972)) but will not improve their prognosis in terms of mental function (Bruhl

et al., 1946; Hsia et al., 1958). Thus, any change which is responsive to correction of the hyperphenylalaninemic state during maturity, at which time the mental state no longer changes, cannot be the direct causative factor underlying the retardation. Only those changes independent of the phenylalanine levels in later life can be causes of the permanent mental deficit. In animal models of PKU, where phenylalanine levels are effectively raised only in early life, any biochemical change found in the mature animal is a candidate for the metabolic change underlying the altered mental state. The elevation in striatal DDC described here is the first metabolic change noted in mature animals exposed to early hyperphenylalaninemia. The importance of the cerebral catecholamine depletion observed in phenylketonurics (McKean, 1972) and only immature rats (section (I)) may lie in its role in establishing long-term metabolic changes such as the rise in striatal DDC activity seen in the adult.

Although it is tempting to link the deficits in cerebral catecholamine levels in early life to the subsequent increases in DDC and behavioral abnormalities, this association is not so clear. Others have described perturbations in catecholamine systems early in life which cause changes in enzymes controlling catecholamine metabolism which are seen only at a more mature stage (Lydiard et al., 1975). Furthermore, this late increase in tyrosine hydroxylase activity was accompanied by behavioral changes but no abnormali-

ties in catecholamine levels (Lydiard & Sparber, 1977). This type of effect has been explained by the pendulum theory (Eiduson, 1966) which asserts that the organism in trying to overcome a reduction of catecholamines shifts its homeostatic setpoint to produce more synthetic enzyme. However, like a pendulum, the organism in moving to correct a deficit overshoots the desired setpoint and thus creates an excess (of TH or possibly DDC as applied to the present case). This change occurs slowly and is irreversible, being reflected in permanent enzyme increases in later life. This rationale cannot be simply applied to our PKUic rats since α -methylphenylalanine alone causes a greater depletion of catecholamines but no increase in striatal DDC. Furthermore, TH not DDC is thought to be the rate controlling enzyme in catecholamine biosynthesis and therefore the logical candidate for homeostatic control. Since DDC (aromatic l-amino acid decarboxylase) is also important in serotonin synthesis it is possible that its increase in PKUic animals may be related to the serotonin depletion seen in hyperphenylalaninemic suckling rats (Isaacs & Greengard, 1980; Lane et al., 1980; McKean et al., 1968). Measurement of tryptophan hydroxylase, the enzyme catalyzing the rate limiting step in serotonin synthesis (Jequier et al., 1967), in the brains of adult Fischer rats with increased striatal DDC may help clarify this issue since tryptophan hydroxylase, unlike DDC, is specific for the serotonergic system. Our present

data concerning this enzyme is insufficient to address this point (see Appendix).

The lack of sex differences in the enzyme activities of any of the cerebral enzymes measured presents a difficulty in trying to relate these changes to the behavioral abnormalities seen primarily in female experimental rats. The only clear sex difference noted in this study was the higher hepatic MAO activity in male animals. It is unlikely that this difference could contribute to the altered cerebral catecholamine metabolism. The possibility that regional sex differences in the enzymes studied or other proteins may underlie the sex differences in behavior of PKUic rats remains. (In this light the possible sex differences in cortical DDC and MAO are of interest). However, such differences are not necessary to explain the sexual dimorphism in behavior. Behavioral differences could be due not only to different biochemical responses to PKU by male and female rats, but also may be the result of the same biochemical changes in both sexes interacting with some other sexually dimorphic system. For example, a change in catecholamine metabolism may have different effects in male and female rats if these animals have 'different' dopamine receptors.

The change in striatal monoamine oxidase seen in 70 day old Fischer rats previously made hyperphenylalaninemic is consistent with an overall altered catecholamine metabolism in these animals. However, it is also possibly a reflection

of altered serotonin metabolism. That the MAO elevation was found in animals with altered DDC activity, but not in the rats with normal DDC activity and normal behavior (see Appendix), suggests a connection between elevations in the synthetic enzyme and the catabolic MAO. Since the synthetic and catabolic rates for any substance must be equal in the steady-state, one might expect an increase in the amount of a synthetic enzyme (such as DDC) to be accompanied by an increase in the content of a degradative one (such as MAO). Since tryptamine, a good substrate for MAO A and MAO B, was used in our assay it is possible that either or both of these isomers were elevated. However, MAO A which preferably utilizes norepinephrine and serotonin as well as dopamine (Neff et al., 1973) is the predominant form (80%) found in rat striate (Garrick et al., 1979; Schoepp & Azzaro, 1981). It should be noted though that the affinity of the different MAO subtypes for dopamine may differ in man (Glaser et al., 1977).

CHAPTER SIX

HYPERPHENYLALANINEMIA IN THE DEVELOPING FETUS

INTRODUCTION

Several studies have indicated that mental retardation is associated not only with phenylketonuria but is also exhibited by the majority of heterozygous infants of phenylketonuric mothers (Crome & Stern, 1975; Fisch et al., 1969; Frankenburg et al., 1968; Lenke & Levy, 1980; MacCready & Levy, 1972). This indicates that permanent damage of cerebral functions can occur if severe hyperphenylalaninemia is restricted to intrauterine life. The common end result of mental retardation does not necessarily imply a common pathogenic pathway; the immediate impact of the same metabolic abnormality may well be different at different stages of ontogeny. Studies on phenylketonurics and on rats subjected to experimental hyperphenylalaninemia have provided some insights into the postnatal biochemical changes, however, little is known about those occurring in utero. The unavailability of suitable animal models has been one difficulty. Among previously explored methods, only the injection to pregnant rats of phenylalanine together with p-chlorophenylalanine was reported to cause significant hyperphenylalaninemia. Studies on this experimental system, however, were severely limited by the toxic side effects and

lethality associated with this treatment (Anderson, 1976; Copenhaver et al., 1974;). An alternate suppressor of phenylalanine hydroxylase, *o*-methylphenylalanine (Greengard et al., 1976), at least in young postnatal rats, was found to be less toxic than *p*-chlorophenylalanine (DelValle et al., 1978; Lane et al., 1980). Animals rendered hyperphenylalaninemic with phenylalanine plus *o*-methylphenylalanine during infancy, therefore, are now extensively used for the study of neurochemical and behavioral abnormalities (Figlewicz & Druze, 1980; Glick & Greengard, 1980; Huther & Neuhoff, 1981; Isaacs & Greengard, 1980; Johnson & Shah, 1980; Lane et al., 1980; Luttges & Gerren, 1979; Nigam & Labar, 1979; Chapters III-V). Whether this treatment is suitable for inducing severe hyperphenylalaninemia in pregnant rats and their fetuses has not yet been tested. This was one purpose of the present chapter. The other was to compare some of the prenatal effects of excess phenylalanine with those effects that it is known to cause after birth. The results describe a regimen of *o*-methylphenylalanine plus phenylalanine which induces severe and prolonged hyperphenylalaninemia but does not interfere with the pre- or postnatal survival of the rats. Observations on chemical abnormalities in the developing brain are also presented. The present fetal insult was similar to the neonatally induced PKU in that the cerebral serotonin and branched chain amino acids were depleted and glycine content and phosphoserine phosphat-

tase activity rose. However, in contrast to the results from our postnatal model of PKU (Chapter V) cerebral dopamine levels were unaffected by gestational hyperphenylalaninemia.

Most of the results from 'experimental' animals described in this chapter derive from rats fed a diet of 3% phenylalanine plus 0.5% α -methylphenylalanine and their fetuses (See Chapter II, Material and Methods). However, data from experiments in which animals were given a diet with a higher phenylalanine content, 7% phenylalanine plus 0.5% α -methylphenylalanine, are included in a second section. This latter diet proved to be more potent than the 3% phenylalanine diet in terms of fetal brain phenylalanine elevations, increases in cerebral glycine levels and phosphoserine phosphatase activity and prolonged postnatal deficits in body and brain growth.

RESULTS

While subcutaneous or intraperitoneal injections of phenylalanine plus α -methylphenylalanine have been our usual method of inducing severe hyperphenylalaninemia (Greengard & DelValle, 1976; Greengard et al., 1976; Isaacs & Greengard, 1980) some observations on 22-33 day old rats indicated that similar results could be obtained by the incorporation of these substances into solid food (DelValle et al., 1978). To avoid daily injections, this diet (see Methods) was given to pregnant rats. It reduced their hepatic phenylalanine hydroxylase activity by 83%, i.e. from 601 to 104 units/g. The inhibition was thus similar to that seen in immature rats (Greengard et al., 1976). Since rats are nocturnal eaters, the degree of hyperphenylalaninemia induced by the experimental diet might be expected to be higher during the night than during the daytime. Observations on blood sampled at different times throughout the day indicated that this indeed was the case. The plasma phenylalanine levels of the pregnant rats on the experimental diet were highest during the dark period (i.e. at midnight, 2 A.M. or 6 A.M.). They were 30-50% and 60% lower at 10 PM and 6PM, respectively.

In all subsequent experiments the animals were killed between 10 and 11 AM; therefore, the 10-20 fold elevations

in maternal plasma phenylalanine (see Table VI-1) represent low estimates, that is, lower than those prevailing during two-thirds of each day. This should also be true for elevations in the phenylalanine content of fetal plasma since these correlate ($r = 0.92$ $p < .0001$) with the levels seen in the corresponding dam (see Figure VI-1). However, the absolute levels were nearly three times higher in the fetuses. This is apparent from the values averaged for the different dams (1086 ± 359 nmol/ml as opposed to 2922 ± 801 in the fetuses) as well as from individual comparisons (see Table VI-1).

The results of experiments in which we also determined the phenylalanine concentration of the fetal brains are illustrated in Table VI-1. There was a 4-10 fold (mean $\pm$ SD = 6.5 ± 1.7) elevation in the brain phenylalanine concentration of experimental fetuses compared with controls. However, only a 2-5 fold elevation (3.7 ± 1.1) was seen in the maternal brain. This is clear not only from the absolute values (third and last columns of Table VI-1) but also from the brain/plasma phenylalanine ratios, which were 0.10-0.43 (0.29 ± 0.13) in the dams but no less than 0.41 (0.67 ± 0.18) in their fetuses analyzed at the same time.

The elevations in the phenylalanine content of fetal plasma or brain showed no consistent variation with gestational age. Nor, as illustrated for the last prenatal day, did they differ between male and female fetuses of the same

FIGURE VI-1

RELATIONSHIP OF FETAL TO MATERNAL PLASMA PHENYLALANINE CONCENTRATIONS

Blood was sampled between 10-11 AM on the 20th, 21st or 22nd day of gestation in normal (unfilled circle) and hyperphenylalaninemic (solid circles) dams. Each point refers to the plasma of one dam (ordinate) and to a pool of plasma collected from its fetuses (abscissa). These points determine a line fitted by linear regression ($p < 0.001$) with a correlation coefficient of $R = 0.92$.

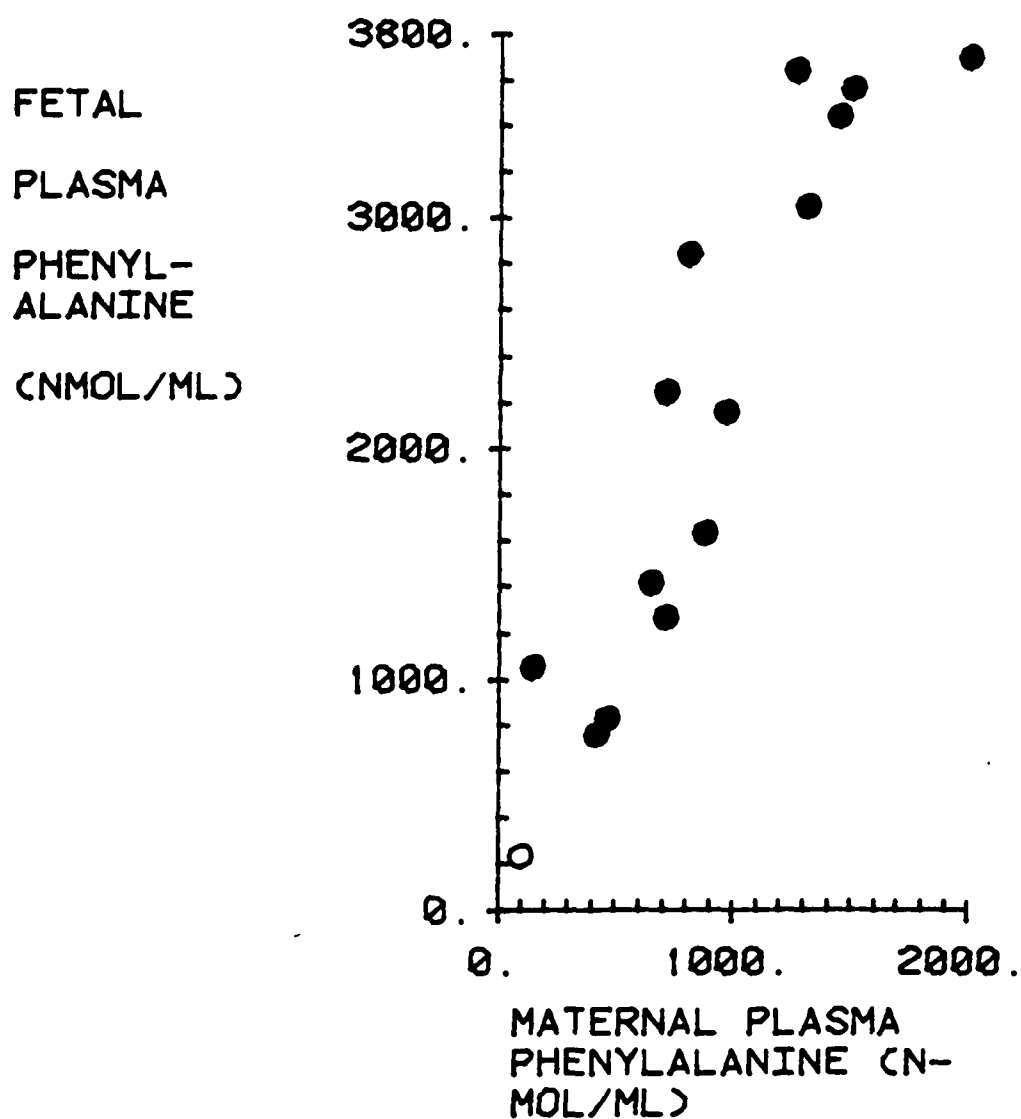


TABLE VI-1

THE PHENYLALANINE CONTENT OF MATERNAL AND FETAL PLASMA AND BRAIN

Rats were started on the experimental diet (E1=3% phe, E2=7% phe) on the twelfth day of gestation. They were killed, as were the dams on the control diet, between 10-11 AM on the gestational day indicated. The control values are means $\pm$ SD of results on the indicated number of dams, or of results on one fetal brain and pooled fetal blood from six different litters. Under "Experimental", data on each horizontal line refer to the same dam, and to one brain and pooled plasma of fetuses born to that dam. Values on brain or pooled plasma followed by M and F refer to male and female fetuses, respectively. NB refers to pups of experimental dams assayed 4-6 hours after birth. Significant differences from control is denoted by ^a, $p < 0.01$; significant differences from E₁ is denoted by ^b, $p < 0.01$.

	Gestational day	Dam		Fetus	
		nmole/ml plasma	nmole/g brain	nmole/ml plasma	nmole/g brain
Control	19-22	96 $\pm$ 31 (6)	80 $\pm$ 17 (5)	243 $\pm$ 62 (6)	292 $\pm$ 52 (6)
Experimental-1	17-22	1080 $\pm$ 344 (10) ^a	296 $\pm$ 86 (7) ^a	2922 $\pm$ 801 (8) ^a	1893 $\pm$ 520 (10) ^a
E2	17-22	1743 $\pm$ 1485 (6) ^a	314 $\pm$ 66 (3) ^a	3300 $\pm$ 383 (3) ^a	3315 $\pm$ 1303 (4) ^{a,b}
E1	17	965	-	-	2935
	18	378	375	-	2173
	19	1166	372	-	1521
	20	895	-	-	2400
		810	177	2850	1176
	21	1440	-	3447	1894
		1822	191	3660	1606
	22	885	382	1647	1414
		1254	291	3843 F; 3414 M	2056 F, 1981 M
		690	282	2454 F; 2064 M	1751
NB		472	-	822	726
		120	82	936	588

dam. However, the degree of hyperphenylalaninemia decreased rapidly during neonatal life. Three to five hours after birth fetal brain and plasma phenylalanine levels were only about four times that of controls (last two lines of Table VI-1). This may be due, in part, to the daily trough in plasma phenylalanine levels at this time of day (See Figure VI-4).

The experimental interference caused small decreases in body and brain weight, but not in the crown-to-rump length of the fetuses (Table VI-2). The weight deficits were apparent by the 20th, and they were of the same magnitude (10-11%) on the 22nd day of gestation. The litter sizes of the experimental (9.5 ± 1.8) and control (8.9 ± 2.1) dams were not significantly different. Also, all of the dams appeared to be healthy, and to sustain normal weight gains during pregnancy. Table VI-2 also shows that, in the experimental fetuses, the amount of DNA and protein per gram brain was normal.

Several dams were allowed to give birth and their offspring were followed for growth and survival. None of the dams on the experimental diet delivered before term; their litter sizes were normal, and no obvious anatomical malformations could be detected. Of the offspring followed until 170 days of age, one whole control litter (out of 5) and 2 of 6 experimental litters died for unknown reasons before the 60th day of life. In the remaining litters 36 of the 37

TABLE VI-2

EFFECT OF HYPERPHENYLALANINEMIA ON FETAL BODY AND BRAIN GROWTH

Dams were placed on the experimental diet during the twelfth day of gestation and sacrificed on the indicated day. Values without SD refer to the average of measurements (of length, body or brain weight) on individual fetuses within a litter, or to measurements of (DNA and protein) on pooled brains of fetuses of the same litter. Values with SD refer to the means of such results on three to four different litters. The asterisks indicate significant differences ($p < 0.01$) between the experimental (E) and control (C) results.

Days of Gestation	Body Length		Body Weight		Brain Weight		Brain DNA Conc		Brain Protein Conc	
	C	(cm) E	C	(g) E	C	(mg) E	C	(mg/g) E	C	(mg/g) E
16	1.08	1.06	.23 .24	.24	44.4 34.1	34.3	5.7	6.49	67 57	64
17	1.28 $\pm$.14	1.25 $\pm$.13	.41 $\pm$.02	.38 $\pm$.02	52.4 $\pm$ 3.7	46.8 $\pm$ 4.5			67 $\pm$ (3)	70 $\pm$ 1(3)
18	1.76	1.53	.82	.75	85	71			-	70
19	2.05	2.02	1.10	1.14	90.5	91	4.57	4.70	69 61	71 58
20	2.67 2.61	2.73 2.69	1.98 1.86	1.78 1.80	122.6 113.8	110.7 104.1			59 66	70
21	3.29 3.10	2.98 3.11	3.06 3.21	2.81 2.83	153 150	129.7 139			59 $\pm$ 6(3)	-
22	3.65 $\pm$.18	3.52 $\pm$.18	4.46 $\pm$.12	3.95 $\pm$.26*	183.9 $\pm$ 4.6	165.5 $\pm$ 9.3*	3.74 $\pm$.39	3.952 $\pm$ 0.36	54 $\pm$ 2	61 $\pm$ 8

control offspring reached weaning age (22nd postnatal day) and 34 adulthood. Forty-one of 44 experimental animals reached weaning age and 34 of 34 (one whole litter lost) attained adulthood (lines 6 and 7 of Table VI-3).

The experimental offspring showed a small but significant body weight deficit at birth, but this was made up by the time of weaning (Table VI-3). The decrease in brain growth seen in the offspring is also recovered by females in adulthood. However, male rats continue to show a very small but significant brain weight deficit, even though body weight had recovered from the early insult (lines 4 and 5 of Table VI-3).

The offspring of rats on the experimental diet from conception exhibited even greater deficits in body weight at birth, but had normal weights at the time of weaning. Survival at the time of weaning in this group was only 79%.

The results on the measurements of amino acids in brain and plasma are shown in Table VI-4. The cerebral concentrations of methionine and the branched chain amino acids valine, leucine and isoleucine were all decreased in both the dam and fetus exposed to hyperphenylalaninemia. There was no consistent relationship between the cerebral level of these amino acids in the fetus, and the fetal plasma values. This contrasts with increases in the cerebral concentrations of phenylalanine and tyrosine which reflect changes in the plasma. The concentration of the other amino acids measured

TABLE VI-3

THE POSTNATAL GROWTH AND SURVIVAL OF RATS HYPERPHENYLALANINEMIC DURING GESTATION

Offspring of control dams (C) and those on the experimental diet from the twelfth to the last gestational day (E_1) were counted and weighed at birth, on the 22nd day after birth (just before weaning), and at the age of 170 days. Rats exposed to hyperphenylalaninemia from the first day of gestation (E_2) were weighed at birth and at 22 days of age. The adult brain weights refer to the same rats while the newborn brain weights refer to a separate group which received the same treatment during gestation but were sacrificed on the first postnatal day. The values for brain and body weights are mean $\pm$ SD of results on the number of individuals in parentheses. Survival is expressed in percent born, with the numbers at birth (see in parentheses under newborn body weight) taken as 100. a: $p < 0.01$ from control, b: $p < 0.025$ from control, c: $p < 0.01$ from E_1 .

	<u>NEWBORN</u>		<u>ADULT</u>	
			<u>MALE</u>	<u>FEMALE</u>
<u>BODY WEIGHT, g</u>				
C	4.59 $\pm$ 0.25 (37)		260 $\pm$ 20 (14)	153 $\pm$ 12 (19)
E_1	4.25 $\pm$ 0.32 ^a (44)		240 $\pm$ 36 (16)	159 $\pm$ 14 (18)
E_2	3.90 $\pm$ 0.44 ^{a, c} (37)			
<u>BRAIN WEIGHT, mg</u>				
	<u>MALE</u>	<u>FEMALE</u>		
C	219 $\pm$ 13 (11)	211 $\pm$ 13 (14)	1790 $\pm$ 30 (14)	1680 $\pm$ 60 (18)
E_1	197 $\pm$ 10 (6) ^a	200 $\pm$ 11 (10) ^b	1720 $\pm$ 60 (16) ^a	1660 $\pm$ 60 (18)
<u>SURVIVAL</u>				
	<u>From Birth to 21 Days</u>		<u>From 22 Days to 170 Days</u>	
C	97%		92%	
E_1	91%		100%	
E_2	79%		-	

TABLE VI-4

THE EFFECT OF GESTATIONAL HYPERPHENYLALANINEMIA ON BRANCHED CHAIN AMINO ACIDS IN BRAIN AND PLASMA

Experimental rats (E) were placed on the phenylalanine plus α -methylphenylalanine diet twelve days after conception. The results shown refer to one such rat, and to a control (C) sacrificed on the 22nd day of gestation. The maternal tissues, and the pooled plasma and brains of all fetuses from the same dam, were assayed in the automatic amino acid analyzer. Values are expressed as nanamoles per milliliter of plasma or gram brain.

AMINO ACID	MATERNAL PLASMA		MATERNAL BRAIN		FETAL PLASMA		FETAL BRAIN	
	C	E	C	E	C	E	C	E
Phenylalanine	36	1095	32	188	136	1572	80	1065
Tyrosine	59	178	30	30	274	384	148	178
Valine	104	227	48	28	426	418	193	130
Leucine	76	155	40	28	279	310	148	123
Isoleucine	53	115	20	12	161	152	80	60
Methionine	25	45	8	5	92	65	28	20

did not change in the fetal brain in response to hyperphenylalaninemia.

Changes in the fetal cerebral concentration of another amino acid, the putative inhibitory neurotransmitter, glycine, are described in detail in Table VI-5. In normal fetal brain it decreased between the 16th and last day of gestation. This is in general agreement with the observations of Davis and Himwich (1973). Hyperphenylalaninemia prevented this decrease; every experimental fetus had higher brain glycine concentrations than age matched controls on gestational days 16-22 and this increase was seen in forebrain, cerebellum and brainstem on day 22. However, the level of another putative neurotransmitter, serotonin, showed a 20-30% deficit in the brains of hyperphenylalaninemic 20-, 21-, and 22-day fetuses (Table VI-5). The concentration of the major serotonin metabolite, 5-HIAA, decreased by 71-77%; control values on fetal days 20, 21 and 22 were 0.81, 0.46 and 1.33 ± 0.14 (3) micromoles per gram respectively, whereas fetuses (of the same age) exposed to high phenylalanine had only 0.19, 0.11 and 0.39 ± 0.05 (3) $\mu\text{mol/g}$ respectively. During the first postnatal day, cerebral levels of serotonin (Table VI-5) and 5-HIAA (control = 1.04, 1.14; experimental = 1.08) returned to normal.

Surprisingly, there was no change in the dopamine or norepinephrine levels in the brains of experimental fetal rats (Table VI-6). This contrasts with the decreases in

TABLE VI-5

THE CEREBRAL GLYCINE AND SEROTONIN CONTENT OF HYPERPHENYLALANINEMIC FETUSES

Dams placed on the experimental diet on the twelfth day of gestation (E) or controls (C) were killed on the indicated days. The attainment of elevated fetal brain phenylalanine levels (as represented in Table VI-1) was verified in each case. A single fetal brain was used for each glycine determination, whereas a pool of two fetal brains from each litter was used for the measurement of serotonin and 5-HIAA. The values refer to a single litter or to the mean $\pm$ SD of result on the number of litters in parentheses. Values for brain parts refer to the 22nd gestational day. NB: 10-24 hours after birth.

<u>GESTATIONAL DAY</u>	<u>GLYCINE</u>		<u>SEROTONIN</u>		<u>5-HIAA</u>	
	<u>C</u>	<u>E</u>	<u>C</u>	<u>E</u>	<u>C</u>	<u>E</u>
	<u>umole/g brain</u>		<u>umole/g brain</u>		<u>umole/g brain</u>	
16	1.83, 2.02	2.38				
17	1.73 $\pm$ 0.07 (3)	1.84 $\pm$ 0.02 (3)				
18	1.35					
19	1.34, 1.46	1.60				
20	1.42	2.14	0.94	0.75	0.81	0.19
21	1.39, 1.31	1.82 $\pm$ 0.10 (3)	1.11	0.78	0.46	0.11
22	1.37, 1.19	2.01 $\pm$ 0.32 (3)	1.29 $\pm$ 0.10 (3)	1.01 $\pm$ 0.09 (3)	1.33 $\pm$ 0.14 (3)	0.39 $\pm$ 0.05 (3)
New Born	1.89 $\pm$ 0.14 (8)	2.01 $\pm$ 0.21 (3)	1.26, 1.10	1.33, 1.11	1.04, 1.14	1.08
	<u>Glycine</u>	<u>Glycine</u>	<u>Glycine</u>			
	<u>Forebrain</u>	<u>Cerebellum</u>	<u>Brain Stem</u>			
C	1.34	1.17	2.56			
E	2.12	2.17	3.18			

TABLE VI-6

CATECHOLAMINE LEVELS IN FETAL RATS EXPOSED TO GESTATIONAL
HYPERPHENYLALANINEMIA

Dams placed on the experimental diet on the twelfth day of gestation (experimental) and controls were killed on the indicated days. The attainment of elevated fetal brain phenylalanine levels (as represented in Table VI-1) was verified in each case using the brains of littermates. A single fetal brain was used for each dopamine and norepinephrine determination. Each value (pg/mg brain w) refers to a single litter or to the mean $\pm$ SD of results on the number of litters in parenthesis.

<u>Age (Fetal Days)</u>	<u>Dopamine</u>		<u>Norepinephrine</u>	
	<u>Control</u> pg/mg	<u>Experimental</u> pg/mg	<u>Control</u> pg/mg	<u>Experimental</u> pg/mg
20	140	95	72	158
21	81,72	94	161,93	136
22	162 $\pm$ 18 (5)	180 $\pm$ 31 (6)	195 $\pm$ 86 (5)	159 $\pm$ 51 (6)

catecholamines which follow hyperphenylalaninemia in the suckling rats (Chapter V). In order to determine if this difference was due to physiological differences between the fetal and neonatal animals or to the method and extent of hyperphenylalaninemia induced in each group, a second group of experiments was done. Pregnant dams were given α -methylphenylalanine to inhibit their hepatic PAH activities and then injected with phenylalanine or α -methylphenylalanine the next day. Figure VI-2 shows that injection of 6 μ moles/g of phenylalanine into a pregnant rat on its 22nd day of gestation caused no decrease in cerebral dopamine 6 hrs later, although this treatment causes large elevations in fetal brain phenylalanine. However, injection of 2.4 μ moles/g of α -methylphenylalanine caused a severe dopamine depletion. Both treatments lowered cerebral norepinephrine.

Gestational hyperphenylalaninemia had no effect on the activity per gram brain of hexokinase, malate dehydrogenase or several other enzymes measured (Table VI-7). Of the twenty enzymes quantified in the brain only phosphoserine phosphatase, which is involved in glycine synthesis, responded to hyperphenylalaninemia (Figure VI-3). In the brain's of control animals its activity rose during the 16th to 18th days of gestation. The phosphoserine phosphatase activity was clearly above normal in the brain's of hyperphenylalaninemic fetuses from the 19th to the 22nd day of gestation. On the 22nd gestational day, the increase was

FIGURE VI-2

FETAL CEREBRAL CATECHOLAMINE DEPLETION ACCOMPANYING MATERNAL
INJECTION WITH PHENYLALANINE OR α -METHYLPHENYLALANINE

Pregnant dams were injected on their 21st gestational day with 2.4 μ moles/g body w of α -methylphenylalanine (phe group) or saline (amphe, control groups). On the 22nd day of gestation dams were injected with 6 μ moles/g body w of phenylalanine (phe), 2.4 μ moles/g body w of α -methylphenylalanine (amphe) or an equivalent volume of saline (control). All animals were sacrificed 6 hours after injection and the brains of their fetuses (8-10) pooled for the radioenzymatic determination of dopamine and norepinephrine content. Results are expressed as % of control values.

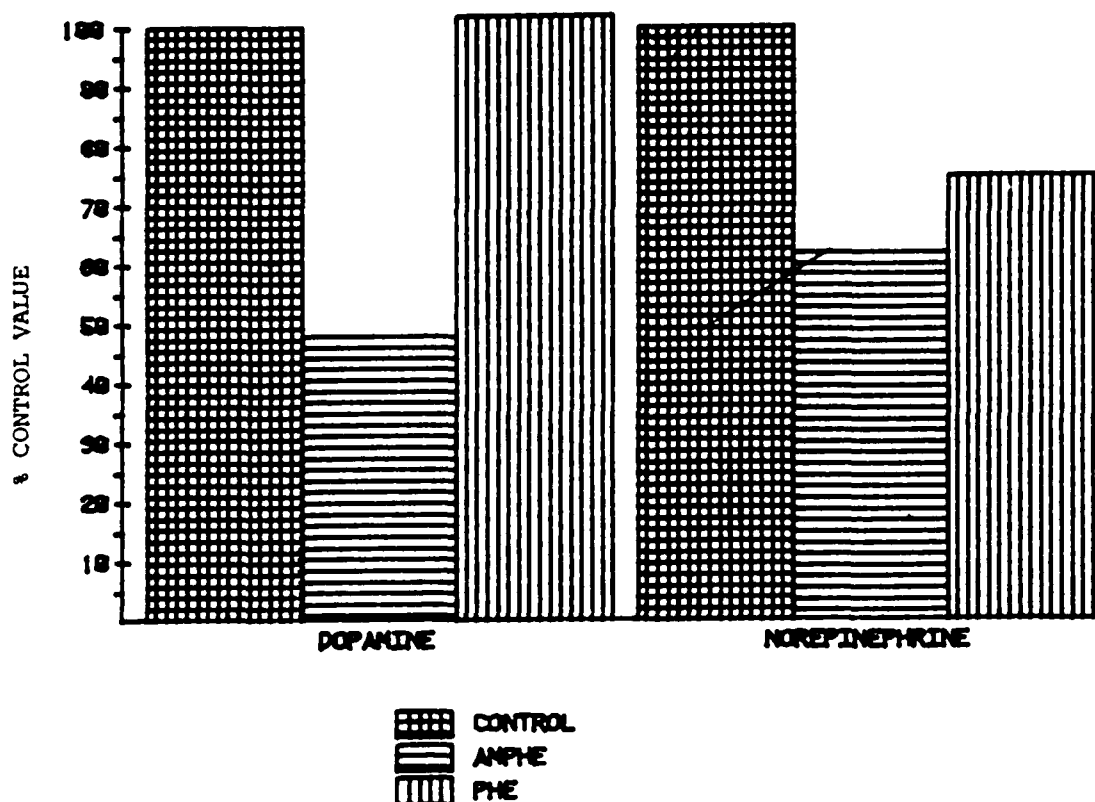


TABLE VI-7

ENZYME ACTIVITIES IN FETAL BRAINS DURING HYPERPHENYLALANINEMIA

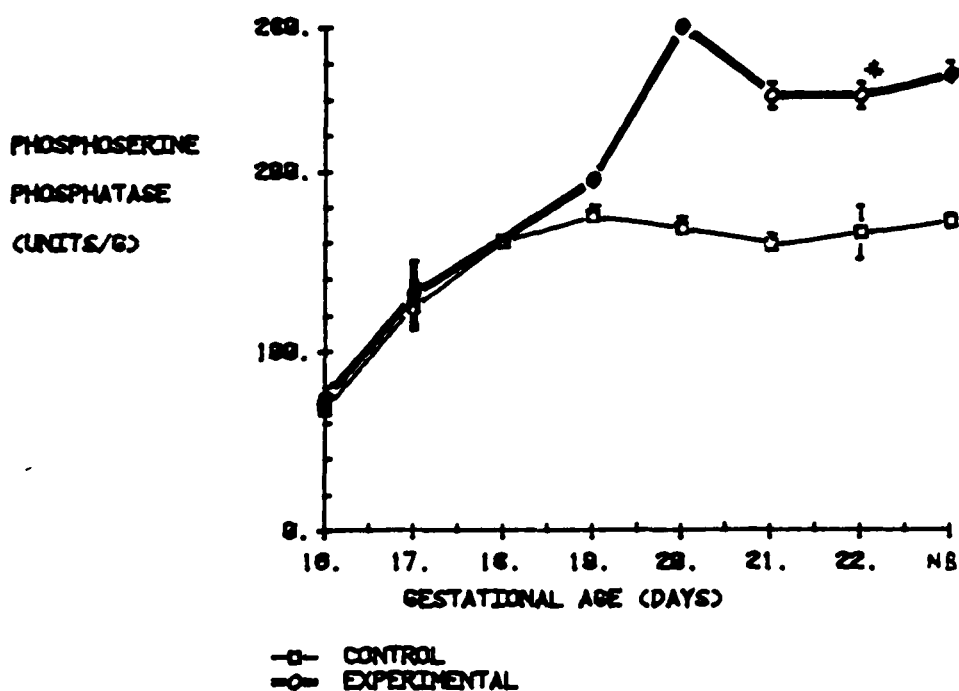
Dams on the experimental diet from the twelfth day of gestation (E) and controls (C) were killed on the indicated gestational day. The enzyme activities (units/g fetal brain) refer to pools of fetal brains from one litter, or are means $\pm$ SD of results on three different litters. ND: not detectable.

Enzyme	<u>21 Days</u>		<u>22 Days</u>	
	<u>C</u>	<u>E</u>	<u>C</u>	<u>E</u>
Hexokinase	2.70, 2.74	2.50 $\pm$ 0.36	2.90, 2.91	2.90 $\pm$ 0.35
Glutamate dehydrogenase	2.59, 2.95	2.91	2.95	2.91, 3.04
Aspartate amino- transferase	12.2, 13.6	13.4	13.1	11.1, 12.8
Malate Dehydrogenase	36.2	32.2, 37.0	49.1	48.8, 36.0
Glucose-6-phosphate dehydrogenase	1.21	1.20, 1.30	1.20	1.55, 1.13
α -glycerophosphate dehydrogenase	ND	ND	ND	ND
5'nucleotidase	0.30	-	-	0.19
Adenylate kinase	16.05	11.67, 15.45	16.25	18.58, 16.71
Thymidine kinase	-	4.84	3.63	3.22, 3.62
Aldolase	1.20	1.12, 1.30	1.75	1.80, 1.22
β -galactosidase	-	80	77	81, 90
α -glucosidase	-	53.4	67.8	66.3, 62.4
γ -glutamyl trans- peptidase	-	109	115	83, 106
Pyruvate kinase	-	37.8	50.1	-
Cytochrome oxidase	45.7	44.9	47.9	46.8
Cathepsin-C	-	31.0	33.0	31.7, 31.1
Monoamine oxidase	230	262	232	-
Glutamate decarboxylase	82.1, 64.6	80.3	92.6	89.8, 57.2
Peptidyl proline hydroxylase	1.90	1.80	-	-

FIGURE VI-3

EFFECT OF GESTATIONAL HYPERPHENYLALANINEMIA
ON FETAL CEREBRAL PHOSPHOSERINE PHOSPHATASE ACTIVITY

Dams placed on a diet supplemented with 0.5% α -methylphenylalanine plus 3% phenylalanine (experimental) from the twelfth day of gestation and controls were killed on the indicated days. Each point represents the mean enzyme activity $\pm$ SD (units/g fetal brain) from a pool of fetal brains from one to three litters. The experimental value at 22 days is significantly less ($p < 0.01$) than the control value. NB: newborn



seen in the brain stem (C= 143,131; E= 202) and cerebellum (C= 115,99; E= 203) as well as in the forebrain (C= 176,179; E= 345). No changes were found in these brain areas in the activities of any of the other 15 enzymes measured (Table VI-8). Also, none of the 17 hepatic enzymes assayed responded to the experimental treatment. (Table VI-9).

SEVEN PER CENT PHENYLALANINE DIET

The phenylalanine content was somewhat higher in both the plasma and brain dams on the 7% phenylalanine diet and their fetuses than on the corresponding animal given 3% phenylalanine enriched food; this increase was greatest and of statistical significance only for fetal brain phenylalanine levels. (Table VI-1) Sequential sampling of blood from the jugular vein of a single pregnant rat on the 7% phenylalanine diet showed plasma phenylalanine levels were greatly elevated for most of the day (Figure VI-4). This is in agreement with the more scanty data obtained from several different animals on the 3% phenylalanine diet. Fetuses exposed to this more severe hyperphenylalaninemia showed larger decreases in both body and brain weight than rats on the 3% phenylalanine diet (Table VI-10). There did not appear to be any sex difference in the fetal response to elevated phenylalanine levels; male control and experimental

TABLE VI-8

ENZYME ACTIVITIES IN FETAL RAT BRAIN PARTS OF CONTROL AND HYPERPHENYLALANEMIC RATS

Dams on the experimental diet from the twelfth day of gestation (E) and controls (C) were killed on the indicated gestational day. The enzyme activities (units/g fetal brain) refer to pools of brain parts from one litter. Dissections were done as described in Methods.

Gestational Day-Treatment	MALATE DEHYDROGENASE			GLUCOSE-6-PHOSPHATE DEHYDROGENASE			ADENYLATE KINASE		
	Fore- brain	Cere- bellum	Brain Stem	Fore- brain	Cere- bellum	Brain Stem	Fore- brain	Cere- bellum	Brain Stem
21-C	35.7	36.2	38.3	1.29	1.18	0.84	15.4	17.7	17.8
21-E	32.9	30.2	33.5	1.26	1.1	1.04	11.6	10.4	13.4
	36.2	37.2	39.7	1.35	1.2	1.20	15.7	17.6	13.0
22-C	48.4	51.1	50.3	1.2	1.2	1.2	16.6	15.4	15.5
22-E	36.4	32.8	37.2	1.16	1.08	1.03	17.5	11.5	18.1
	48.3	50.7	49.5	1.45	1.74	1.81	19.3	16.3	17.5
	HEXOKINASE			ALDOLASE			γ -GLUTANYL TRANSPEP- TIDASE		
21-C	-	-	-	1.12	1.39	1.43	-	-	-
21-E	2.41	1.33	2.2	1.0	1.25	1.50	113	96	104
	2.36	2.2	2.9	1.21	1.23	1.71			
22-C	2.6	3.14	4.04	1.68	1.68	2.10	111	136	107
22-E	2.43	2.3	3.3	1.52	2.32	2.56	79	92	92
				1.11	1.45	1.51	107	113	94
	β -GALACTOSIDASE			α -GLUCOSIDASE			CYTOCHROME OXIDASE		
21-C	-	-	-	-	-	-	44.8	45.8	49.5
21-E	74.6	73.9	104.4	43.7	73	75.8	43.6	41.0	54.7
22-C	64	82	121	52	90	109.8	43.4	52.9	59.9
22-E	73.5	73	121	57.2	77.7	95.6	43.7	59.4	49.0
	83	103	107	52.4	88	83			
	GLUTANATE DEHYDROGENASE			ASPARTATE AMINOTRANSFERASE			PHOSPHOSERINE PHOSPHETASE		
21-C	2.26	2.54	3.47	13.6	18.4	13.9	179	99	131
21-E	2.12	1.90	2.63	10.9	13.2	15.8	-	-	-
	2.25	2.44	4.58	11.9	12.1	14.1			
22-C	2.71	3.6	4.90	14.4	19.3	22.5	176	115	143
22-E	1.74	3.94	3.63	14.7	18.2	20.0	345	203	202
	2.1	2.54	3.50	11.1	13.7	15.4			
	CATHEPSIN C			PEPTIDYL-PROLINE HYDROXYLASE			5'-NUCLEOTIDASE		
21-C	-	-	-	1.81	1.83	-	0.25	0.32	0.54
21-E	29.7	23.2	41.3	1.65	1.91	2.32	-	-	-
22-C	29.7	30	47.6	-	-	-	-	-	-
22-E	31	30	36.2	-	-	-	0.11	0.23	0.50
	29.7	34	34.8						
	PYRUVATE KINASE								
21-C	-	-	-						
21-E	46.1	44.6	43.0						
22-C	49.5	58.8	50.2						
22-E	-	-	-						

TABLE VI-9

HEPTATIC ENZYME ACTIVITY IN CONTROL AND HYPERPHENYLALANINEMIC FETAL RATS

Dams on the experimental diet from the twelfth day of gestation (E) and controls (C) were killed on the indicated gestational day. The enzyme activities (units/g fetal liver) refer to a pool of fetal livers from one litter. DNA content is expressed as mg per g.

<u>Enzyme</u>	<u>21 Days</u>		<u>22 Days</u>	
	<u>C</u>	<u>E</u>	<u>C</u>	<u>E</u>
Hexokinase	-	0.99, 1.23	1.30	1.36
Aldolase	1.84	2.75	1.84, 4.90	5.15, 1.60
β -galactosidase	-	207	344	289, 295
α -glucosidase	-	46	70	54, 41
γ -glutamyl trans-peptidase	-	3478, 4141	4739	5196
Pyruvate kinase	-	34.4	39.1	-
Cytochrome oxidase	58.3	55.6	69.6	63.4
Cathepsin-C	-	752	1474	771, 656
Peptidyl proline hydroxylase	1.75	2.02	-	-
Aspartate amino-transferase	47.6	35.0, 53.6	-	50.1
Glutamate dehydrogenase	-	50.1	78.7	63.5
Malate dehydrogenase	61.3	53.4, 72.4	138.0	120.0, 83.4
Glucose-6-phosphate dehydrogenase	1.88	1.93, 1.90	1.80	1.97, 1.84
α -glycerophosphate dehydrogenase	5.00	5.60, 5.20	5.50	5.31, 4.50
Adenylate kinase	12.40	7.47, 10.20	13.90	12.38, 14.40
5'nucleotidase	2.83	-	-	1.93
Phenylethanolamine N-methyltransferase	0.165, 0.138	0.160	0.195	0.270
DNA	7.49	4.90, 6.60	4.75	2.06, 4.06

FIGURE VI-4

24 HOUR FLUCTUATION OF PLASMA PHENYLALANINE LEVELS
IN A PREGNANT RAT ON A 7% PHENYLALANINE PLUS 0.5%
 α -METHYLPHENYLALANINE DIET

A pregnant dam was placed on a high phenylalanine diet (7% phenylalanine plus 0.5% α -methylphenylalanine) 12 days after conception. A catheter was then placed in its internal jugular vein and blood was sampled frequently over a continuous 24 hour period. Comparable plasma phenylalanine levels for a control dam are $96 \pm 31(6)$ (dashed line). The time scale is over 24 hours with 0-12 hours representing the AM and 12-24 the PM.

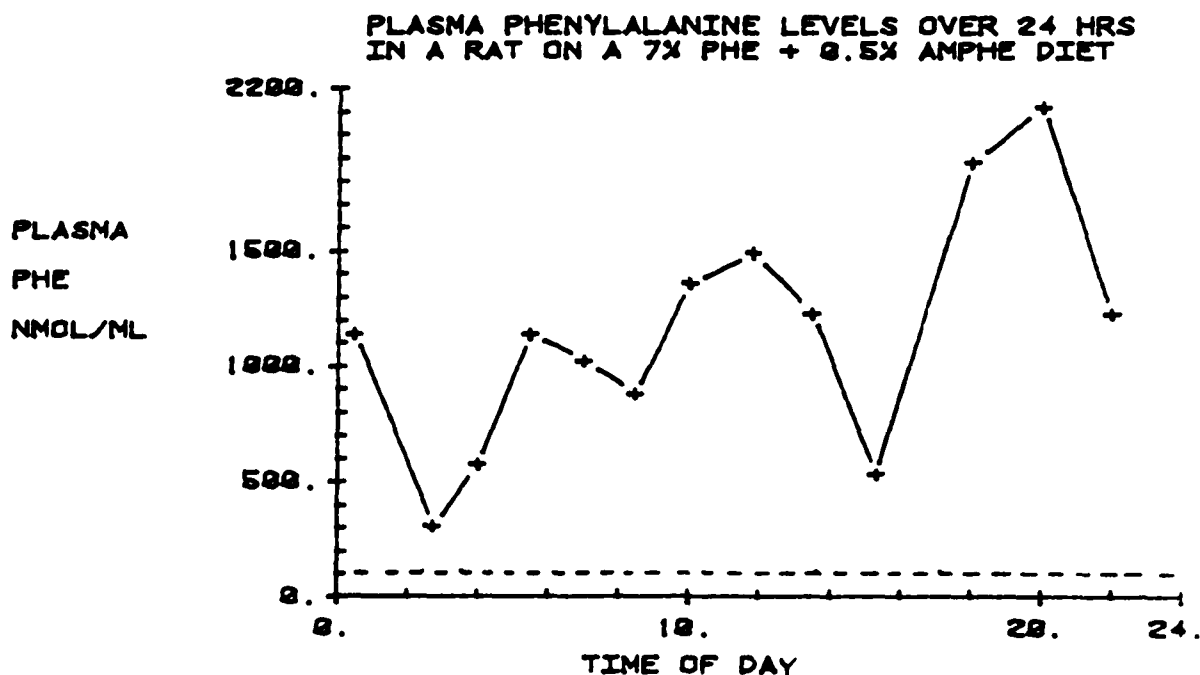


TABLE VI-10

THE EFFECT OF HIGH LEVEL GESTATIONAL HYPERPHENYLALANINEMIA ON FETAL AND POSTNATAL BODY AND BRAIN GROWTH

Dams placed on the 7% phenylalanine (plus 0.5% alpha-methylphenylalanine) diet from the twelfth day of gestation (E) and controls (C) were tested on the indicated prenatal (F#) or postnatal day. Values refer to individual pups or the mean $\pm$ SD(n) of several rats from one or more litters. The typescript ^a in the table indicates significant differences ($p < 0.01$) between experimental and control results. NB: newborn

Age	Treatment	Body Weight (g)		Body Length (cm)		Brain Weight (mg)	
		Male	Female	Male	Female	Male	Female
F17	C	.41 $\pm$.02 (3)		1.28 $\pm$.14 (3)		52.4 $\pm$ 3.7 (3)	
	E	.33		1.28		31.4	
F20	C	1.98, 1.86		2.67, 2.61		122.6, 113.8	
	E	1.64 $\pm$.09 (6)		2.54 $\pm$.10 (6)		95.5 $\pm$ 9 (6)	
F22	C	4.57 $\pm$.08 (4)	4.35 $\pm$.08 (4)	3.72 $\pm$.20 (3)	3.67 $\pm$.21 (3)	181.8 $\pm$ 5.71 (4)	182.2 $\pm$ 3.93 (4)
	E	3.71, 3.41	3.43, 3.16	3.65, 3.63	3.62, 3.50	141.2, 140.3	135.6, 135.4
NB	C	4.6	4.25	-	-	219 $\pm$ 13 (11)	211 $\pm$ 13 (14)
	E	3.30 $\pm$.20 (17)	3.08 $\pm$.16 (9)	3.20 $\pm$.11 (11)	3.15 $\pm$.11 (5)	143 $\pm$ 12 (10) ^a	134 $\pm$ 14 (6) ^a
48	C	95.6 $\pm$ 13.1 (6)	77.3 $\pm$ 12.5 (9)	-	-	1.55 $\pm$.07 (6)	1.44 $\pm$.05 (9)
	E	83 $\pm$ 11 (7)	61 $\pm$ 13 (11) ^a	-	-	1.37 $\pm$.15 (7) ^a	1.26 $\pm$.10 (11) ^a
70	C	168 $\pm$ 28 (6)	-	-	-	1.62 $\pm$.09 (6)	-
	E	121 $\pm$ 6 (7) ^a	-	-	-	1.47 $\pm$.03 (7) ^a	-

animals appeared to be somewhat larger than their female counterparts. Although litter size (8.1 ± 3.4) did not vary significantly from the control value (8.9 ± 2.1), this high phenylalanine treatment appeared to be quite toxic since none of the ten litters given this 7% phenylalanine diet from conception gave birth. In fact, no fetuses were noted in these rats at 17 days gestation. Cerebral glycine content (3.00, 3.07 μ moles/g) and phosphoserine phosphatase activity (346, 382 nmoles/min/g) were elevated more in 22 day old fetuses of dams on the 7% diet, than those of rats given the 3% phenylalanine diet.

Both male and female rats had equivalent reductions of body (28% decreased) and brain mass (35%) at birth. Brain weights were also equivalently decreases at 48 days of age (12%). At 70 days body and brain growth had still not fully recovered from the fetal insult (Table VI-10).

BEHAVIORAL RESULTS

The behavior of adult rats exposed to high phenylalanine in utero (3% phenylalanine diet on days 11-22 of gestation) were studied. Unlike Fischer rats made hyperphenylalaninemic during the suckling period (Chapter IV), these rats showed no changes in T-maze learning, locomotor activity or cerebral lateralization (Table VI-11).

TABLE VI-11

BEHAVIORAL RESULTS FROM RATS EXPOSED TO GESTATIONAL HYPERPHENYLALANINEMIA

Pregnant rats were maintained on a diet of Purina Lab Chow with 0.5% ~~α~~-methylphe plus 0.3% phe added from the 11th day of pregnancy until the day of parturition. The offspring of these animals were raised until 90-120 days of age, at which time they were tested for 24 hour rotational activity, locometer activity and T-maze learning ability. Control animals are from separate litters raised at the same time, but whose dams received a diet of Purina Lab Chow only. Values represent mean $\pm$ SEM(n).

	<u>Control</u>	<u>Experimental</u>
<u>Rotometer Tests</u>		
% Preference		
Male	69 $\pm$ 11 (5)	73 $\pm$ 5 (11)
Female	71 $\pm$ 10 (4)	71 $\pm$ 3 (15)
Net Rotations		
Male	65 $\pm$ 18	89 $\pm$ 33
Female	132 $\pm$ 112	53 $\pm$ 11
<u>Locomotor Activity</u>		
Photocell Counts/30 min.		
Male	1716 $\pm$ 263	1622 $\pm$ 100
Female	2285 $\pm$ 62	2139 $\pm$ 82
<u>T-Maze Learning</u>		
Trials to Criteria		
Male	8.54 $\pm$ 1.45 (4)	8.42 $\pm$ 2.03 (12)
Female	8.53 $\pm$ 2.48 (4)	7.44 $\pm$ 1.92 (15)
Direction of Turning Preference-		
First Trial		
Male	9R-4L	13R-4L
Female	9R-10L	5R-13L

DISCUSSION

This study shows that by incorporating α -methylphenylalanine and excess phenylalanine into the solid diet of pregnant rats it is possible to sustain high levels of phenylalanine in the fetal blood and brain. In light of clinical reports implicating even modest hyperphenylalaninemia in the subsequent damage to offspring, (MacCready & Levy 1972) the ten to twenty-fold elevation in rat fetal plasma phenylalanine seems to constitute an appropriate model for human gestational phenylketonuria. The maternal plasma phenylalanine is elevated seven to fifteen-fold for two thirds of the day, but the degree and persistence of the fetal hyperphenylalaninemia may be even greater (see Table VI-1 and text). This follows from our demonstration of a 2.5 to 3 fold concentration ratio of fetal to maternal plasma phenylalanine, and from studies which indicate that the placenta acts as a reservoir and lengthens the time during which elevations of phenylalanine in the fetal plasma (in response to those in the maternal plasma) persist (Lines & Waisman, 1971; Wapnir & Dierks-Vertling, 1971).

The enduring consequences of hyperphenylalaninemia may be due to the accumulation of excess phenylalanine in the

brain. In our model system, we see the preferential accumulation of phenylalanine in the fetal brain. There is a 4-10 fold elevation in fetal brain, in contrast to a 2-5 fold increase in the brain of the dam. Also, the brain to plasma phenylalanine ratios are much higher in the fetus (0.67) than in the dam (0.29). Thus, the brain phenylalanine concentrations in the fetus were raised up to 2900 nmol/g (Table VI-1, rats on 3% phenylalanine diet). The fetal/maternal plasma ratio, and the brain/ plasma phenylalanine ratios in the dam and fetus are all in agreement with previous work using Fischer rats (Anderson, 1976). These differences, seen at two age extremes (fetal and adult) point to the importance of brain transport processes in the age dependent vulnerability of the central nervous system to hyperphenylalaninemia (See Chapter VII). Similarly, genetic differences in transport processes between individuals with similar plasma phenylalanine elevations (Andrews et al., 1973), may lead to variation in central nervous system phenylalanine content, possibly explaining the relatively normal brain development of some hyperphenylalaninemic individuals.

Several workers have used dietary phenylalanine or a daily maternal injection of phenylalanine to mimic maternal phenylketonuria, (Boqqs & Waisman, 1964; Luse et al., 1970), but in doing so monitored plasma phenylalanine levels at one time only. Such treatments are ineffective in raising phe-

nylalanine concentrations for any length of time (Lipton et al., 1967). Other workers have administered phenylalanine plus the phenylalanine hydroxylase inhibitor p-chlorophenylalanine to the dam to induce fetal hyperphenylalaninemia (Anderson, 1973; Butcher, 1970). This treatment was more successful in elevating plasma phenylalanine levels than previous models of maternal phenylketonuria. However, it was accompanied by pup mortality, and has not been clearly shown to drastically elevate fetal plasma phenylalanine levels for long periods of each day. Our present treatment (3% phenylalanine plus 0.5% α -methylphenylalanine) maintained a 10-20 fold elevation in fetal plasma phenylalanine for over 16 hours per day. Rats on the 7% phenylalanine appeared to have similar increases in plasma phenylalanine for two-thirds of the day, and 5 fold elevations the remainder of the time (Figure VI-4). Even the fluctuations in phenylalanine levels in our model mimic the human condition, where plasma phenylalanine levels also vary during the day in response to food intake.

The quantitative alterations in amino acid levels, of course, are not identical to the human disease state. In particular, the plasma tyrosine levels of adult phenylketonuric women (or men) are normal, (Menkes & Koch, 1972) whereas in our experimental dams, owing to the incomplete suppression by α -methylphenylalanine of the hepatic PAH, (Greenqard et al., 1976) the tyrosine levels are elevated.

With respect to fetal tyrosine content, however, the discrepancy should be less pronounced. The human fetal liver, by about the tenth week, attains at least 50% of its adult phenylalanine hydroxylase activity (DelValle & Greengard, 1977; Raiha, 1973). Thus, while the phenylketonuric mother is devoid of the enzyme, its heterozygous fetus (with at least 25% of the normal adult phenylalanine hydroxylase activity) should be able to convert some of the excess circulating phenylalanine to raise its own tyrosine levels to even above normal. This may explain the 'high normal' tyrosine content of the amniotic fluid of phenylketonuric women (Thomas et al., 1971). Furthermore, since the plasma tyrosine levels of these women do not correlate with the I.Q. of their children (Lenke & Levy, 1980) (whereas phenylalanine levels do) it does not appear that low maternal plasma tyrosine is central to the pathogenesis of maternal phenylketonuria. Among the inevitable shortcomings of an animal model of gestational phenylketonuria, the deviation here with respect to maternal tyrosine levels is thus not a crucial one.

Neither α -methylphenylalanine nor the hyperphenylalaninemia produced by its dietary introduction to the pregnant rat with 3% phenylalanine is lethal to the developing fetus. Rats maintained on this diet from the twelfth day of gestation showed no changes in litter size or in the postnatal survival of offspring until adulthood (Table VI-3). Thus, the diet used in these studies is not accompanied by any

gross toxic side effects and allows animals be raised for behavioral studies. The high rate of mortality (Anderson, 1976) or maternal muricide (Copenhaver et al., 1974) resulting from treatment with p-chlorophenylalanine (plus phenylalanine) must be specific to this analogue since these effects are not seen when a more severe hyperphenylalaninemia over a longer period of gestation is induced using α -methylphenylalanine (plus phenylalanine). Similarly, high mortality is observed when suckling rats are made hyperphenylalaninemic with injections of p-chlorophenylalanine plus phenylalanine but not when α -methylphenylalanine plus phenylalanine is used (DelValle et al., 1978, Lane et al., 1980).

However, hyperphenylalaninemia itself can be lethal to fetal development but only at levels higher than those achieved with our usual treatment. Although all five dams placed on our standard experimental diet from day one of conception gave birth to normal size litters, 79% of which survived to 22 days, none of the ten dams placed on a diet containing 7% phenylalanine (plus 0.5% α -methylphenylalanine) gave birth at all. Neither fetuses nor placentae were found in two dams on this latter diet when examined at 17 days of gestational age. This suggests that very high phenylalanine may block implantation of the embryo or cause very early fetal resorption.

The treatment caused a deficit in two specific growth parameters, fetal body and brain weight (Tables VI-2, VI-10), and not a delay in all aspects of maturation. Other indices of gestational age, such as crown-to-rump length (Table VI-2) and several enzyme activities (Tables VI-7, VI-8, VI-9) were unchanged in the same experimental fetuses which showed reduced body and brain weights. This reduction is in accord with clinical observations on the children of phenylketonuric mothers (Fisch et al., 1969, Frankenburg et al., 1968, Lenke et al., 1980, MacCready & Levy, 1977). Since the concentration (mg per g) of cerebral protein or DNA (Table VI-2) remained normal, the decrease in total brain weight in the hyperphenylalaninemic fetuses must be due to a reduction in the total number of cells and not a decrease in cell size.

Fetuses of dams given the 7% phenylalanine diet from the 11th day of gestation had even greater lags in brain and body growth than those fetuses exposed to the 3% phenylalanine diet. Since the former animals had significantly higher cerebral phenylalanine levels, this suggests that phenylalanine is toxic to the developing animal in a dose dependent manner. Although fetal and postnatal experimental males were generally somewhat larger than their female littermates, this was also true for control animals (Table VI-10). Thus, in contrast to the sexually dimorphic response of rats exposed to hyperphenylalaninemia in the

suckling period (Chapter III), gestational PKU inhibited the growth of male and female animals equivalently. Male and female brain weight deficits (Table VI-10) were still similar at 48 days of age. These results suggest that it is the state of the male or female animal at the time of insult, and not later occurring differences (e.g. testosterone exposure), that determines its response. Since males and females respond similarly before birth, sex dependent changes that occur in the suckling period, the time of sexual imprinting of the rat brain, must determine the quantitative differences in the weight losses of males and females with postnatal PKU. If exposure to later physiological differences, for instance the higher male levels of testosterone, accounted for the faster recovery by males of brain and body weight losses seen in rats given phenylalanine plus α -methylphenylalanine on days 2 to 21 of life (Chapter III), then similar sex differences would be seen at 50 days of age in rats exposed to fetal PKU.

Chemical interferences during ontogeny are usually seen to effect systems which develop particularly rapidly at the time of insult. Thus, it was not surprising that hyperphenylalaninemia during the 12th to 22nd days of gestation, a period during which more than 95% of the fetal increment in body weight occurs, resulted in deficits in this parameter. However, it was unexpected that extending our experimental treatment back from day 12 to day 1 of gestation would cause

a significantly increased deficit in the neonatal weight of gestationally hyperphenylalaninemic rats (line 3 in Table VI-3). This deficit was overcome by the 22nd postnatal day, but only 79% of them survived to this age. Additionally, initiating the 7% phenylalanine diet on the day of conception instead of the 11th gestational day resulted in early fetal demise. These results have important clinical implications. They indicate that very early hyperphenylalaninemia can interfere with fetal growth and gestational or postnatal survival. These effects emphasize the vulnerability of the young fetus to hyperphenylalaninemia and thus the necessity of immediate dietary treatment of identified pregnant phenylketonurics.

The only well understood mechanism underlying chemical alterations in the brains of phenylketonurics, and of animal models of the disease, is the inhibition by excess phenylalanine of the cerebral uptake of amino acids sharing a common transport system (Antonias & Colson, 1975; Carver, 1965; Lowden & La Rance, 1969; McKean et al., 1968). The present results show that this mechanism is operative in the 22-day fetus as well (see methionine, leucine, isoleucine and valine in Table VI-4). We also observed a decrease in the cerebral concentration of the putative neurotransmitter serotonin and its major metabolite, 5-HIAA. This is similar to decreases seen in phenylketonuric children (McKean, 1972) and in neonatal rats made hyperphenylalaninemic (Isaacs &

Greenqard, 1980; Lane et al., 1980). The fact that cerebral serotonin (and 5-HIAA) are not lowered in the experimental neonate, whose plasma phenylalanine levels rapidly return toward normal, is consistent with observations on suckling rats. The reduction in serotonin concentration appears to be a transient response directly related to several-fold elevations in phenylalanine within the past several hours (Isaacs & Greenqard, 1980). This contrasts with the change in cerebral glycine in suckling rats which lasts several days after the cessation of hyperphenylalaninemia.

The only major qualitative difference between the responses of fetal animals and suckling animals exposed to high phenylalanine levels was the lack of cerebral catecholamine depletion in the former group. Since the time course of phenylalanine elevation was so important in (eliciting) the dopamine depletion in 7 day old rats (Chapter V), injection of phenylalanine into the dam was utilized to mimic these same temporal conditions of phenylalanine exposure in the fetus. Despite the greater plasma phenylalanine levels achieved in the fetal rats, the analagous treatment still failed to deplete cerebral catecholamines in these animals. Thus, the resistance of cerebral dopamine stores in the fetus to change in response to hyperphenylalaninemia appears to be an important difference between gestational and postnatal PKU.

An explanation of these different responses may be found in the observation that fetal animals maintain tyrosine levels several-fold higher than those in the dam (Table VI-4 and text). In Chapter V it was demonstrated that elevating cerebral tyrosine levels prevented the dopamine depletion that usually accompanied high phenylalanine levels in 7 day old rats. Therefore, the high cerebral tyrosine levels maintained by the fetal rat, which are further elevated following a high phenylalanine load, may protect it from the experimental insult. This suggests that the maintenance of high levels of many amino acids in the fetus may serve not only to meet the needs of an actively growing organism, but also act as a protective mechanism for the anomaly during a period of great vulnerability. Humans also maintain higher amino acid levels during fetal life. Since the heterozygous fetus of a PKUic mother still has significant PAH activity (see above), this same protective mechanism may be active in gestational PKU.

The observation that injection of α -methylphenylalanine alone does cause cerebral dopamine depletion of the fetus, whereas dietary exposure to α -methylphenylalanine plus phenylalanine or injection of phenylalanine alone does not, further supports the contention made in Chapter V that α -methylphenylalanine alone is not a control for the hyperphenylalaninemia produced by phenylalanine plus α -methylphenylalanine. Once again we see that α -methylphenylala-

nine can cause rapid (within 6 hours) dopamine depletion. Therefore, its mode of action (tyrosine hydroxylase inhibition) must be different in the brain than in the liver since it takes 18 hours to take full effect there (DelValle & Greenqard 1978).

As previously demonstrated in suckling rats (Isaacs & Greenqard 1980), the imposition of hyperphenylalaninemia in the fetus is also associated with increased cerebral glycine content as well as an elevation in the activity of phosphoserine phosphatase, the enzyme catalyzing the conversion of phosphoserine to serine, the principle precursor of cerebral glycine (Daly et al., 1976). Since both glycine levels and PSP activity are further elevated in the brains of fetuses exposed to even greater cerebral phenylalanine elevations (i.e. those exposed to 7% phenylalanine diet), these increases appear to be directly related to the cerebral phenylalanine content. That altered glycine may play a central role in the neuropathogenesis of phenylketonuria is suggested by the fact that another inborn error of metabolism, non-ketotic hyperglycinemia, is hallmarked by a 2-3 fold elevation in cerebral glycine as well as by severe neurologic abnormalities and mental retardation (Perry et al., 1975). While the cerebral glycine content of phenylketonurics is unknown and their cerebrospinal fluid levels of glycine are normal, the elevated serine concentration in their cerebrospinal fluid (Von Sande et al., 1970) may possibly

suggest some abnormalities in the intra-cerebral regulation of this pathway.

Although many similar biochemical changes are seen in our gestational and postnatal models of hyperphenylalaninemia, the immediate and long term effects of these changes on the organism are not necessarily the same. For example, adult rats exposed to gestational hyperphenylalaninemia did not show any of the behavioral abnormalities (Table VI-11) which follow the postnatal treatments (Chapter IV). Also, heterozygous infants of untreated PKUic mothers exhibit not only the microcephaly and intellectual deficits seen in PKUic children, but also congenital heart disease and anatomical malformation (Lenke & Levy, 1980; Fisch et al., 1969). The concept that the vulnerability to various biochemical insults may vary with the maturational state of the organism is central to the definition of PKU as a developmental disease. This view, that early exposure to hyperphenylalaninemia irreversibly interferes with neural maturation, has been well established clinically. PKUic children whose plasma phenylalanine levels are controlled during their first several years of life only attain average intelligence, (Koff et al. 1979, Williamson et al., 1981), whereas neglect of treatment during this period leads to severe mental retardation.

This new model should facilitate the study of the effects of hyperphenylalaninemia at different stages of

neural development. Such studies are important for the information they may yield on normal neural development, on the selective effects of early hyperphenylalaninemia, and more generally, on the neural changes that may underlie 'mental retardation' states. Chemical interferences applied to intact systems have often proved useful in gaining insight into 'normal' processes. Hyperphenylalaninemia has been shown to have dramatic effects postnatally on the cytoarchitectural development of the rat cerebellum (Huther & Neuuhoff, 1981), a brain area maturing relatively late. Gestational hyperphenylalaninemia might have an impact on other, earlier developing brain areas (Spero & Yu, 1982). Because information on the timing of cell differentiation and migration of various cell groups in the rodent brain now exists (Rodier, 1980), application of our experimental treatment during restricted periods of gestation may elucidate the effects of hyperphenylalaninemia on different brain areas or particular cell groups. Secondary effects of temporally selective interferences may also provide insight into the unfolding of normal ontogeny.

CHAPTER SEVEN

DEVELOPMENTAL CHANGES
IN THE TRANSPORT OF PHENYLALANINE
ACROSS THE BLOOD-BRAIN BARRIER

INTRODUCTION

Phenylketonuria has been labeled a 'developmental disease' because exposure to high plasma phenylalanine levels in the first few years of life alone has been found to cause irreversible mental retardation (Knox et al., 1972) whereas control of the plasma phenylalanine levels (through diet) during the first several years of life has obviated this effect (Holtzman et al., 1975; Koff et al., 1979) despite a lack of subsequent plasma phenylalanine control. The classical explanation of these findings invoked the 'critical growth period' of the brain. According to this theory insults during the first few years of life, when the brain is growing at its maximal rate and establishing new connections causes irreversible damage that is qualitatively different than the damage incurred during any later period. Thus, the high plasma phenylalanine damages the immature growing brain in an irreversible manner that cannot be mimicked in later life.

Another explanation exists, however, to account for the importance of the early period of life in the pathophysiology of this disease. It may be not only variation with age in the vulnerability of the brain but differences in the

degree of chemical insult to which it is exposed that changes with maturation (Partington & Lewis, 1963). High levels of cerebral phenylalanine may be the primary factor underlying the biochemical and behavioral deficits accompanying PKU (see Chapter V). Therefore, age related changes in the regulation of brain phenylalanine during hyperphenylalaninemia may determine the time period during which the brain receives its maximal insult. It is this possibility, that the brain phenylalanine content in the hyperphenylalaninemic animal may vary with maturity, that is investigated in this chapter. The confirmation of this theory would not only aid in our understanding of the developmental nature of the damage incurred in PKU but it may also lead to the advancement of a hypothesis explaining the observation that a few genetic PKUics suffer no measurable mental retardation despite a lack of treatment. This could be due to a precocious or maldevelopment of the blood-brain transport system for phenylalanine.

Although transport of amino acids and other substances into the brain has been a subject of much recent study (e.g. Pardridge, 1977) little is known about the regulation of high levels of amino acids in the brain during different ages. All substances that enter the brain, except for small uncharged molecules, must be primarily transported through cerebral capillaries in what is referred to as the 'blood-brain barrier'. Several separate transport systems have

been defined in the blood-brain barrier (Pardridge, 1977; Pratt, 1979). One is the L-amino acid system which transports several amino acids including phenylalanine. This transport system has been well studied and the kinetics of transport for the various L-system amino acids has been determined, but no one has studied this system in relation to the unusually high amino acid levels seen with phenylketonuria. Developmentally, the system has been studied not only in terms of the formation of tight junctions and the exclusionary properties of the barrier which form quite early (Purdy & Bondy, 1976; Saunders, 1977), but also in respect to physiologic changes in the transport functions of the blood-brain barrier. Cornford and colleagues (1982) recently demonstrated developmental changes in the 'brain uptake index' of several substances including the L-system amino acid tryptophan. However, this index only measured differences in the rate of net transport into the brain with normal physiologic concentrations of substances over a few second period. Developmental changes in net transport at non-physiologic levels has never been studied in detail before the present endeavor.

In this chapter we demonstrate that the brain, by virtue of its blood-brain barrier system, will maintain only a certain cerebral phenylalanine concentration despite higher plasma phenylalanine levels. And this maximum net transport of phenylalanine into the brain decreases with age so that

the young rat brain is exposed to much higher levels of phenylalanine than that of the more mature animal despite equivalent plasma phenylalanine elevations. This developmental change occurs primarily during the first few weeks of life and thus coincides with the time period during which the rat is most vulnerable to both the biochemical and behavioral effects of experimental phenylketonuria.

RESULTS

Studies on pregnant rats placed on phenylalanine plus α -methylphenylalanine supplemented diets and their fetuses (see Chapter VI) first revealed the large differences in brain phenylalanine accumulation from the plasma between mature and immature animals. Table VII-1 shows that the hyperphenylalaninemic dams were able to concentrate phenylalanine into the brain at only 314 nmol/g brain whereas their fetuses with 2-3 times higher levels of plasma phenylalanine accumulated 6-10 times higher brain concentrations (1893-3315 nmol/g brain) with plasma/brain phenylalanine ratios 2-5 times lower than the adult animals. The development of this difference in the ability to transport and accumulate phenylalanine into the brain was further studied by placing young post-weanling rats on diets containing 0.5% α -methylphenylalanine plus 1-7% phenylalanine and then determining their plasma and brain phenylalanine levels. This work showed that young (23-32 day old) post-weanling rats were intermediate between the fetal and adult animals in their ability to accumulate brain phenylalanine. For example, 23 day old rats on a 3% phenylalanine diet had brain phenylalanine levels of only 848 nmol/g brain compared

TABLE VII-1

PLASMA AND BRAIN PHENYLALANINE LEVELS OF RATS MAINTAINED ON HIGH
PHENYLALANINE DIETS

Animals were placed on diets of Purina Lab Chow with 0.5% α -methylphenylalanine plus 1-7% phenylalanine added as indicated under '% phe in Diet'. Adult rats (120+) were on these diets for 5-10 days before sacrifice and their fetuses (gestational age 17-22 days) were exposed to hyperphenylalanemia for the same period. All other rats were begun on their diets on the 20th day of life. 0% phenylalanine in diet indicates control animals receiving only Purina Lab chow. Values represent the mean $\pm$ SD (n).

<u>Age</u> <u>(days)</u>	<u>% phe</u> <u>in Diet</u>	<u>Plasma</u> <u>Phenylalanine</u> <u>nmol/ml</u>	<u>Cerebral</u> <u>Phenylalanine</u> <u>nmol/g</u>	<u>Plasma/Brain</u> <u>Phenylalanine Ratio</u>
Fetal	0%	243 $\pm$ 62 (6)	292 $\pm$ 52 (6)	0.83
Fetal	3%	2922 $\pm$ 801 (8)	1893 $\pm$ 520 (10)	1.54
Fetal	7%	3300 $\pm$ 383 (3)	3315 $\pm$ 1301 (4)	1.15
23	1.5%	667 $\pm$ 408 (5)	271 $\pm$ 106 (5)	2.46
23	3%	3332 $\pm$ 803 (7)	848 $\pm$ 365 (7)	3.93
23	7%	2112, 5654	814, 1542	2.59, 3.67
25	3%	1400 $\pm$ 131 (5)	750 $\pm$ 54 (5)	1.81
25	7%	4779, 4904	1416, 1257	3.38, 3.90
27	7%	4835 $\pm$ 427 (3)	615 $\pm$ 49 (3)	7.86
29	7%	8903, 5223	2163, 1818	4.11, 2.88
30	3%	1175 $\pm$ 702 (8)	364 $\pm$ 182 (10)	3.23
32	7%	2268, 4718	1370, 1393	1.65, 3.89
120+	0%	96 $\pm$ 31 (7)	80 $\pm$ 17 (5)	1.20
120+	3%	1080 $\pm$ 345 (10)	296 $\pm$ 87 (7)	3.65
120+	7%	1743 $\pm$ 1485 (6)	314 $\pm$ 66 (3)	5.55

to levels of 3315 nmol/g brain in fetal animals with similar plasma phenylalanine levels. Twenty-five day old rats on the 3% phenylalanine diet had brain phenylalanine concentrations (750 nmol/g) much higher than adult rats on the 7% phenylalanine diet (314 nmol/g) who had even higher plasma phenylalanine levels (Table VII-1). Unfortunately, this dietary approach to the study of the development of phenylalanine transport into the brain proved limited in that it could not be used to study the important suckling period of life nor produce equally high levels of plasma phenylalanine in all age groups.

To circumvent the above difficulties and further define the development of quantitative changes in brain phenylalanine transport a second approach was taken. Rats of various ages were given subcutaneous injections with α -methylphenylalanine to inhibit their hepatic phenylalanine hydroxylase activity and 18-24 hours later were given multiple subcutaneous injections of phenylalanine. By varying the amount of phenylalanine and time(s) of injection in each age group one could then attempt to produce equivalent plasma phenylalanine elevations in all animals. This would then provide a standard plasma phenylalanine level and enable direct comparison of brain phenylalanine levels in animals at different ages.

Although the approach outlined above may appear straightforward its practical achievement proved to be quite

difficult. Using the V_{max} for phenylalanine transport into the brain (30 nmol/min/g) determined by Pardridge and Oldendorf (1975) one can estimate that it would take almost two hours for the rat brain to accumulate 3000 nanamoles of phenylalanine per gram of brain. In actuality this time was even greater (Figure VII-1). Thus, one must be able to elevate plasma phenylalanine levels for several hours before determining the maximum level of brain concentration. However, the rat has an excellent homeostatic mechanism which resists prolonged elevations of plasma phenylalanine levels (e.g. see Chapter III). Although α -methylphenylalanine can be used to try to circumvent this control, the hepatic phenylalanine hydroxylase activity rises (McGee et al., 1972) and the ability to suppress this activity decreases as the animal matures (DeValle et al., 1978). Thus, the metabolism of any given phenylalanine load increases with age so that greater doses and more frequent injections of phenylalanine must be given to older animals in order to achieve the same plasma phenylalanine elevation as in younger animals for several hours.

Because of the difficulties outlined above many different treatment protocols were utilized at different ages in order to produce approximately equivalent plasma phenylalanine levels in the range seen in phenylketonuria. Figures VII-1, 2 and 3 display some representative results at three important ages: 4, 16, 70 days. As seen in Figure VII-1

FIGURE VII-1

TIME COURSE OF ELEVATION OF PLASMA AND CEREBRAL
 PHENYLALANINE CONCENTRATIONS IN FOUR DAY OLD RATS

Three day old rats were injected with α -methylphenylalanine (2.4 μ moles/g body w) and on day 4 of life were given multiple injections of phenylalanine. (see Methods) Each solid circle represents the plasma phenylalanine concentration and each unfilled circle the corresponding brain phenylalanine concentration of one rat sacrificed on the indicated hour(s) after its first phenylalanine injection.

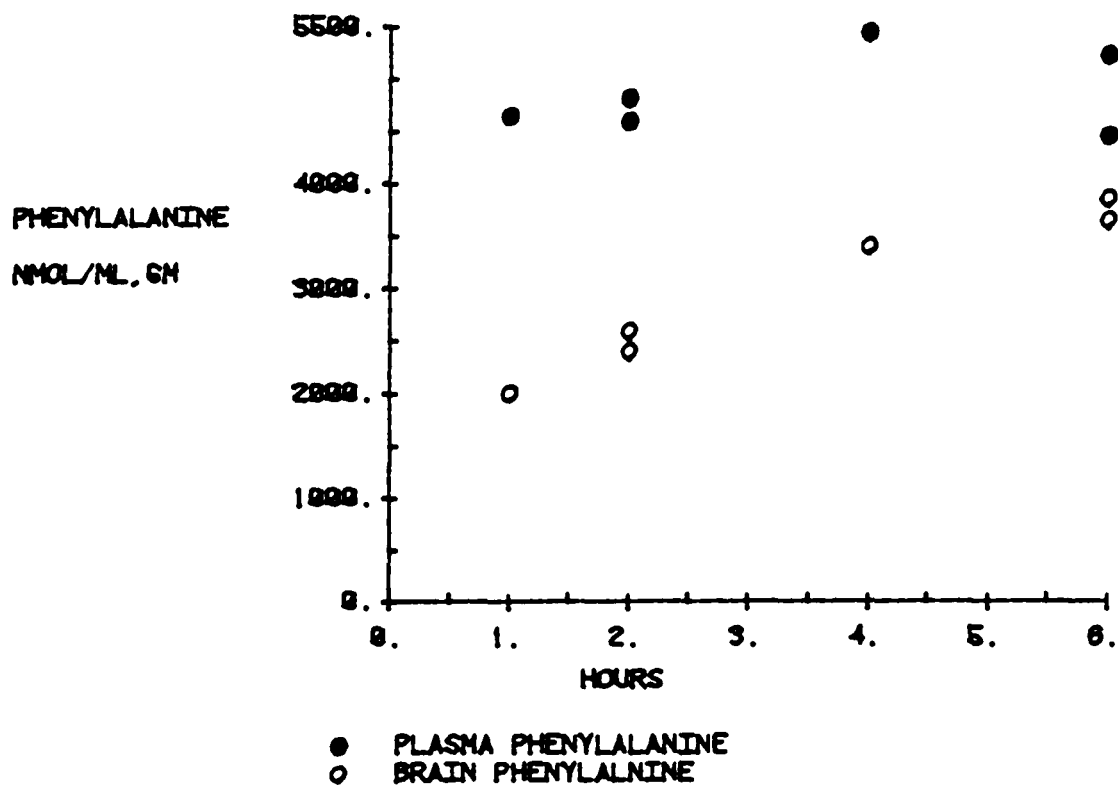


FIGURE VII-2

TIME COURSE OF ELEVATION OF PLASMA AND CEREBRAL
PHENYLALANINE CONCENTRATIONS IN SIXTEEN DAY OLD RATS

Fifteen day old rats were injected with α -methyl-phenylalanine (2.4 umoles/g body w) and on day 16 of life were given multiple injections of phenylalanine. (see Methods). Each solid circle represents the mean plasma phenylalanine concentration and each unfilled circle the mean brain phenylalanine concentration of 3-5 rats sacrificed on the indicated hour(s) after their first phenylalanine injection. Bars indicate the standard error of the mean at each point.

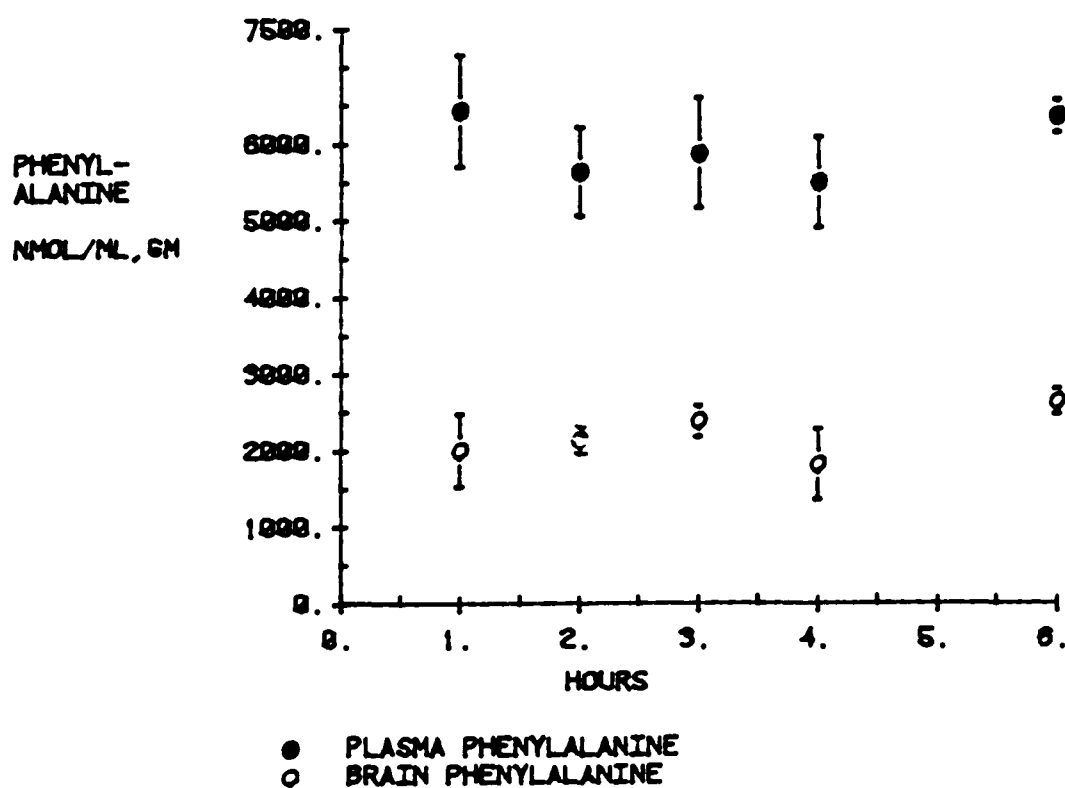
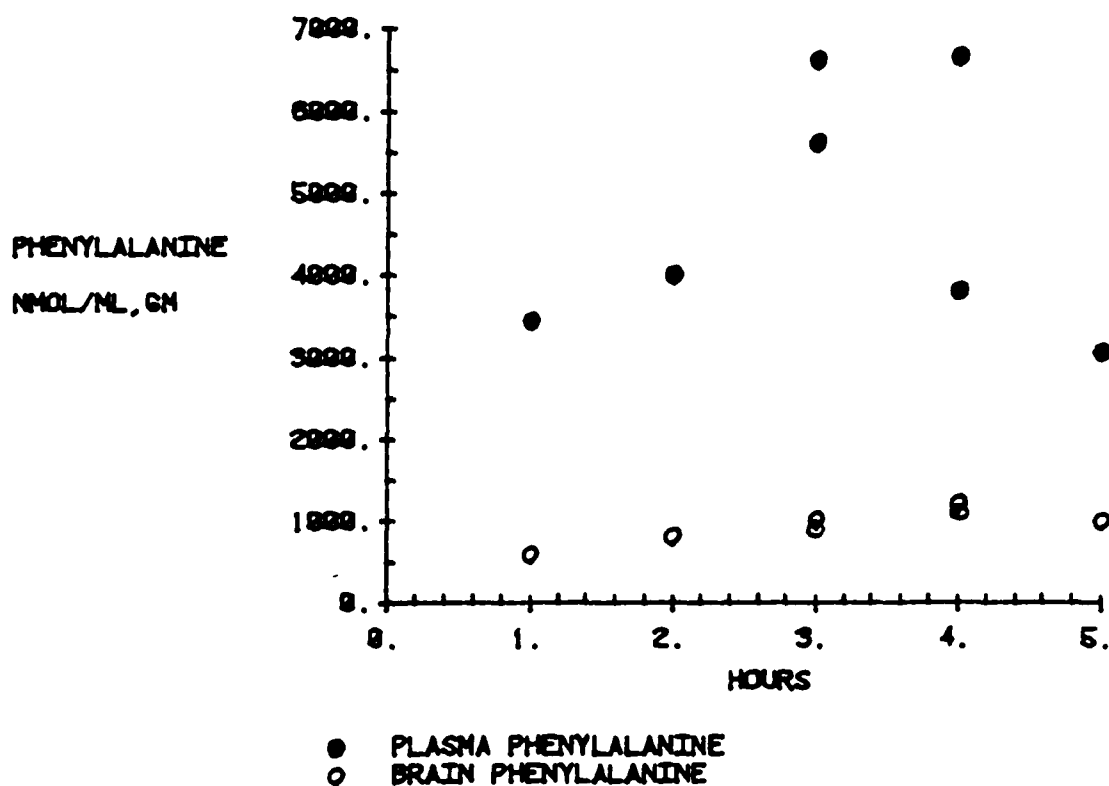


FIGURE VII-3

TIME COURSE OF ELEVATION OF PLASMA AND CEREBRAL
PHENYLALANINE CONCENTRATIONS IN SEVENTY DAY OLD RATS

Sixty-nine day old rats were injected with α -methylphenylalanine (2.4 μ moles/g body w) and on day seventy of life were given multiple injections of phenylalanine (see Methods). Each solid circle represents the plasma phenylalanine concentration and each unfilled circle the corresponding brain phenylalanine concentration of one rat sacrificed on the indicated hour(s) after its first phenylalanine injection.



the four day old rat attained brain phenylalanine levels greater than 3000 nmol/g when plasma phenylalanine levels were raised to the 5000 nmol/ml range. In contrast 16 day old animals with comparable plasma phenylalanine levels had brain phenylalanine concentrations below 3000 nmol/g (Figure VII-3). These levels at 16 days were notably higher than those achieved in 70 day old rats (Figure VII-3). Thus, a progressive decrease in net phenylalanine transport into the brain was noted with increasing age in animals with plasma phenylalanine levels in the phenylketonuric range.

The developmental increase in the ability to restrict phenylalanine transport into the brain can be seen in more detail in Figure VII-4. Here the mean brain phenylalanine levels (plotted as % of the 4 day value) achieved after three hours or more of plasma phenylalanine elevation in the phenylketonuric range (between 4000-7000 nmol/ml) is displayed for the different age groups studied. We see that the most rapid decrease in brain phenylalanine content occurs between days 4 and 16 with a more gradual decrease to adult levels occurring after that.

To further define the changes in the early suckling period (between ages 4 and 16) a similar study was done in which more age groups were studied and plasma phenylalanine levels were raised to greater than 10,000 nmol/ml for more than three hours (Figure VII-5). Although this plasma phenylalanine level is higher than that observed in PKU it was

FIGURE VII-4

DEVELOPMENTAL CHANGE IN NET CEREBRAL PHENYLALANINE
UPTAKE ACCOMPANYING PLASMA PHENYLALANINE ELEVATIONS
OF 4-7,000 NANOMOL/ML

Rats were injected with α -methylphenylalanine (2.4 μ moles/g body w) and 18 to 24 hours later were given multiple injections of phenylalanine. Each bar represents the mean brain phenylalanine concentration, expressed as per cent of the 4-day value, of 5-11 rats whose plasma phenylalanine concentrations were raised to 4,000-7,000 nmol/ml plasma for more than 3 hours on the indicated postnatal day of life.

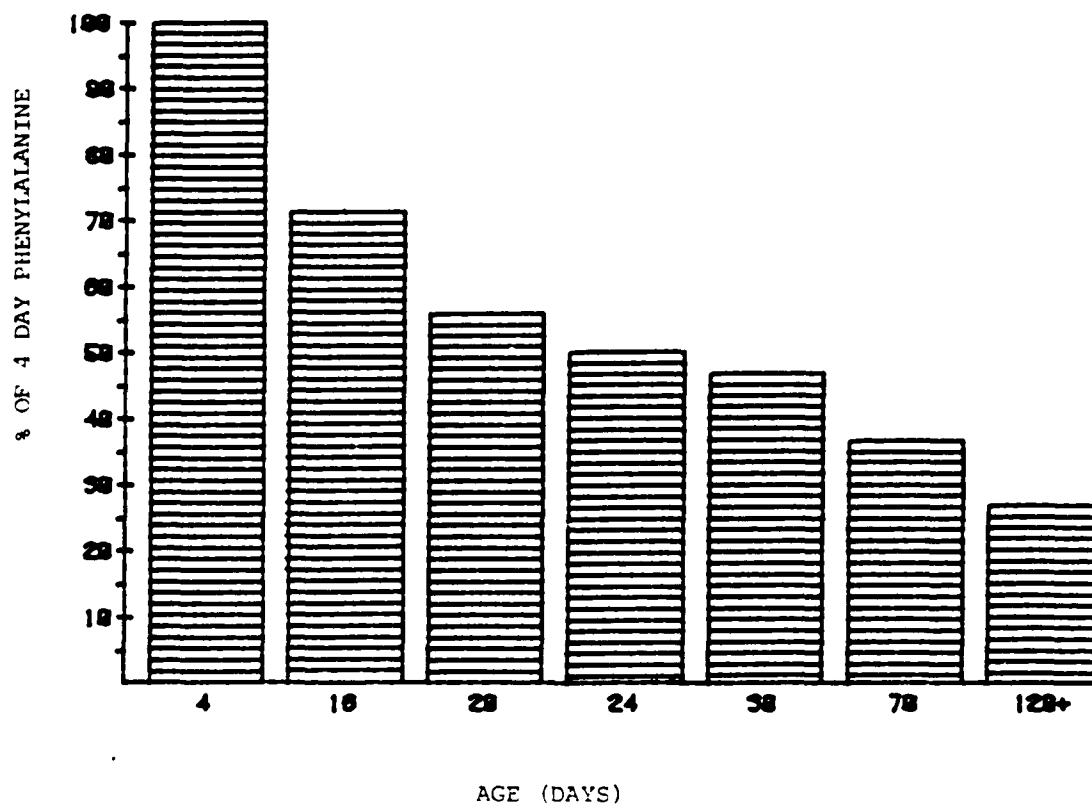
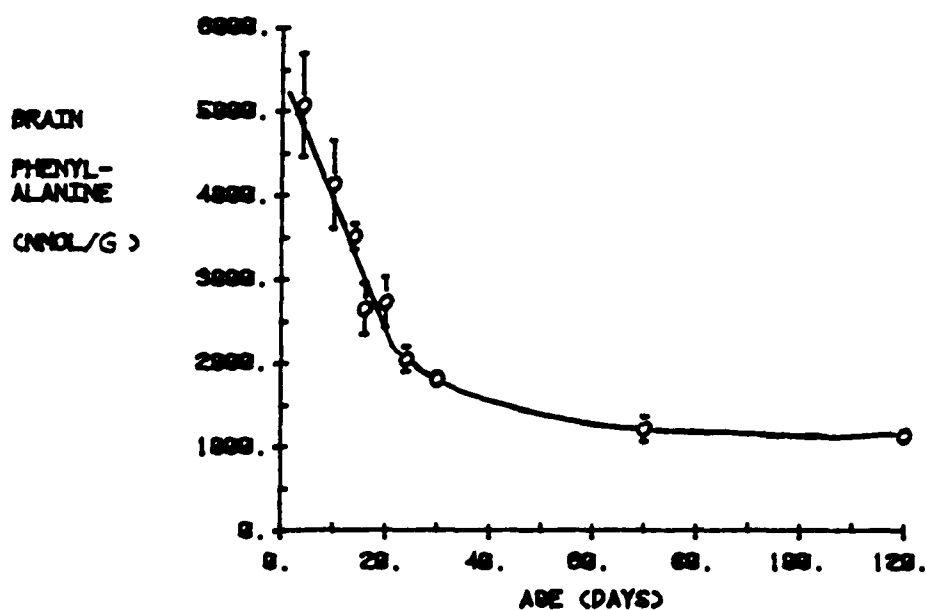


FIGURE VII-5

DEVELOPMENTAL CHANGE IN NET CEREBRAL PHENYLALANINE
UPTAKE ACCOMPANYING PLASMA PHENYLALANINE ELEVATIONS
OF MORE THAN 10,000 NANOMOL/ML

Rats were injected with α -methylphenylalanine (2.4 μ moles/g body w) and 18-24 hours later were given multiple injections of phenylalanine. Each point represents the mean brain phenylalanine concentration of 4-8 rats whose plasma phenylalanine levels were raised to more than 10,000 nmol/ml plasma for more than 3 hours on the indicated day of life. Bars represent the standard error of the mean at each point.



thought that the use of such high levels would even more accurately define developmental changes in the rat brain transport system by 1) minimizing errors secondary to the lack of exactly equivalent plasma phenylalanine curves for rats at different ages and 2) accentuating differences in the early period by raising the absolute brain phenylalanine levels. Figure VII-5 largely confirms the previous results and delineates the rapid decrease in net transport of phenylalanine into the brain during the suckling period which is followed by a more gradual decline from day 24 to the adult level. No sex differences in the brain accumulation of phenylalanine were noted in any of the experiments described here.

Having found the largest part of the change in brain phenylalanine transport to occur during the weanling period, the question arose whether this developmental change was mediated by the late suckling rise in cortisol which has been shown to regulate the development of a cluster of hepatic enzymes in the rat (Greenward, 1971; Greenward, 1975). When rats were injected with cortisol (0.25 mg/10 g body w) on days 7, 10 and 14 of life their brain phenylalanine levels at age 16, after more than 3 hours of hyperphenylalaninemia in the range of 4,000-7,000 nmol/ml was $2817 \pm 195(5)$ nmol/g whereas that of control animals (no previous injections) was $2385 \pm 176(11)$ nmol/g. Similarly, rats injected with an equivalent dose of cortisol on days 7, 11

and 15 of life had equivalent brain phenylalanine accumulations at 20 days ($1979 \pm 101(4)$ nmol/g) as controls ($1896 \pm 77(6)$ nmol/g). Thus, exogenous cortisol administration during the fetal period did not seem to affect the development of the rats' ability to restrict phenylalanine from the brain.

DISCUSSION

The data presented in this chapter clearly demonstrates the increased capacity of the mature rat to maintain lower brain than plasma phenylalanine levels when compared to younger animals. More than half of this developmental change occurs during the first three weeks of life, with a more gradual transition to adult function thereafter. There are, however, two complications which may bias the data. First, the increased difficulty with age in sustaining large increases in plasma phenylalanine levels for many hours biases the brain phenylalanine data toward somewhat lower levels in the older animals. This problem is limited because the time required for the brain to reach its final 'equilibrium' levels of phenylalanine is less in the older animal whose net accumulation of brain phenylalanine is less. Thus, at the theoretical V_{max} rate of 30 nmol/min/g (Pardridge & Oldendorf, 1975) it may take a few hours for brain phenylalanine content to rise to the equilibrium level of 3-5,000 nmol/g in a young animal (see Figure VII-1), whereas much less time is required to reach the lower equilibrium levels (e.g. 1200 nmol/g; Figure VII-3) in those animals in which it is more difficult to sustain prolonged

plasma phenylalanine elevations. The second complication arises from the presumably greater plasma tyrosine levels in the older animals. As the hepatic phenylalanine hydroxylase increases in these animals much of the phenylalanine load is converted to tyrosine which then competes with phenylalanine for transport by the L-amino acid transport system into the brain. Theoretically a large problem, this factor appears to be of little importance in limiting total net transport of phenylalanine into the brain since the actual cerebral phenylalanine content was seen to vary little in older animals after the first few hours of hyperphenylalaninemia (Figure VII-3). This is probably due to the fact that the rat has good homeostatic control of plasma tyrosine levels and the K_m for phenylalanine transport is somewhat lower than that for tyrosine (Pardridge, 1977). Thus, despite these relatively minor experimental problems the results tabulated in this chapter do demonstrate a very real developmental change in the ability of the rat brain to maintain lower brain than plasma phenylalanine levels.

No work comparable to the present effort has been done to study developmental changes in the net transport of amino acids into the brain at high plasma amino acid levels. The uptake of several L-system amino acids in vivo has been shown to be lower in adult than neonate mice (Seta et al., 1972) but these studies were done at only 2 ages and with much smaller plasma amino acid elevations. Others have

studied changes in the transport of L-system amino acids into the brain using the intracarotid injection technique (Oldendorf, 1970) in which the transport of physiologic amounts of substrate are measured over a period of seconds. Using this method Sershen and Lajtha (1976) have shown that the 'brain uptake index' (BUI) of phenylalanine is greater in the newborn than the adult rat. Oldendorf and his colleagues (Cornford et al., 1982) have recently established a curve for the developmental change in the 'brain uptake index' of the L-system amino acid tryptophan in rabbits that resembles the temporal change in net phenylalanine transport into the brain (Figure VII-5). Both show rapid declines during the suckling period (35 days for rabbit) beginning in the first few days of life with the major difference being that the BUI for tryptophan increases slightly from day 28 to the adult period. However, the 'brain uptake index' measures something different than that which we are measuring in the present studies. According to some it 'reflect(s) the nutritional requirements of the developing brain' (Cornford et al., 1982), or may measure the velocity of transport into the brain. In summary, there have been no similar studies to compare our present data with although the general thrust of previous work (less transport into more mature animals) is in agreement with the data presented in this chapter.

The pattern of the developmental change in the transport of phenylalanine detailed in this chapter (see Figures VII-4,5) is similar to the developmental pattern of some cerebral enzymes (e.g. glutamate dehydrogenase, MacDonnel & Greengard, 1974; malate NADP-dehydrogenase, Greengard, 1975) but unlike that of the majority of enzymes (in both brain and liver) whose activity changes toward adult levels primarily in the early postnatal period (MacDonnel & Greengard, 1974; Greengard, 1975). Greengard (1971) has described several clusters of enzymes in the liver, each of which develop with particular temporal characteristics and respond to various hormonal stimuli. The developmental pattern of net phenylalanine transport into the brain most resembles that of the 'late suckling' cluster of enzymes since the majority of change in each occurs in the first three weeks (suckling period). However, the 'late suckling' pattern of development, seen in hepatic and cerebral enzymes, differs significantly from the developmental changes in net phenylalanine transport into the brain in that the increase toward adult values occurs primarily between the 14th and 21st days of life in response to rising levels of plasma cortisol. Thus, in retrospect it was not surprising that the earlier occurring changes in the net transport of phenylalanine into the brain was not affected by early cortisol treatment (see Results). Furthermore, although hormonal manipulation of cerebral enzymes has been accomplished (e.g. α -glycerophosp-

hate dehydrogenase; DeVellis & Inglish, 1968) evidence for hormonal control of cerebral enzyme development has been difficult to obtain and the magnitude of hormonally induced changes have been small when compared to the better detailed hepatic effects (Greenqard, 1971).

Although there is little information on the hormonal control of the blood-brain barrier transport systems, many studies have been done on the hormonal modulation of transport across membranes in other organs. Insulin, growth hormone and estradiol have all been found to affect the transport of A-system neutral amino acids in various tissues. (Riggs, 1970). These effects, however, have been quite small. For example, insulin treatment has been shown to increase the transport of alanine into the rat diaphragm by only 20% (Riggs & McKirahan, 1973). Modification of L-system neutral amino acid transport has been achieved primarily with thyroid hormone (Riggs, 1970). Therefore, future studies on the possible hormonal regulation of amino acid transport in the brain should concentrate on thyroid hormone since the A-system of amino acid transport is not present in the brain.

The mechanism of this developmental increase in the capacity to maintain lower brain than plasma phenylalanine levels is unclear. Documentation of the early formation of the tight junctions in endothelial cells which constitute the primary site of the 'barrier' in the 'blood brain barri-

er' system (Pardridge, 1977; Pratt, 1979) make the further development of barrier function an unlikely cause of the observed changes in net brain phenylalanine uptake. Furthermore, even at 4 days of age (Figure VII-1) brain phenylalanine levels did not reach plasma levels indicating an intact barrier to simple diffusion at this early age.

In studying the transport of amino acids into the brain many experiments have been done using short-term infusions in vivo (e.g. Oldendorf, 1973; Sershen & Lajtha, 1976; Oldendorf et al., 1976; Pardridge, 1977) as well as incubation of isolated preparations such as brain slices (e.g. Levi et al., 1967; Lahdesmaki & Hannus, 1977; Laasko, 1978). The former studies are purported to reflect the function of the actual 'blood brain barrier' since the rate-limiting transport across cerebral capillaries is measured whereas the latter experiments quantify the less restrictive transport from the extra-cellular spaces into the brain cells themselves. In measuring long term net transport in vivo, in an attempt to study equilibrium conditions at pathologically high plasma amino acid levels, we may be no longer studying short term rate limiting processes (i.e. capillary transport alone) but we may be observing developmental changes dependent on both capillary and cellular transport instead. Therefore, it may be misleading to refer to either type of study alone in seeking explanations for the present observations (Levi, 1977).

Since brain phenylalanine content is determined by both influx and efflux, an increased efflux capacity in mature animals could be responsible for the observed decrease in net uptake into the brain. Unfortunately, there is a relative dearth of information on the transport of substances out of the brain. Lajtha and Toth (1961) have demonstrated that the adult rat will transport leucine out of the brain against a concentration gradient, whereas the newborn brain did not demonstrate similar capabilities. Furthermore, these same investigators (Lajtha & Toth, 1962) have demonstrated the efflux of phenylalanine down a concentration gradient to the plasma. From the data presented a maximal efflux rate of 22 nmol/min/g can be estimated. This rate approaches that of the calculated V_{max} of phenylalanine influx through the 'blood brain barrier' (30 nmol/min/g; Pardridge & Oldendorf, 1975). Such an active efflux system, coupled with the known pial vessel transport of phenylalanine from the cerebrospinal fluid into the blood (Levin et al., 1974), could provide the adult animal with adequate control of brain phenylalanine content. That is, the efflux rate could be raised to the influx rate at a certain cerebral phenylalanine level so that net uptake is zero and the brain phenylalanine content remains below that of the plasma level. However, Lajtha (Lajtha & Toth, 1961) did not observe efflux of cerebral phenylalanine against a concentration gradient. This is surprising in that one might

expect one transport system (L-amino acid system) to control efflux as well as influx of both leucine and phenylalanine. But there is precedent for different transport systems controlling transport in different directions across cell membranes (Horecker et al., 1960), and the lack of 'net' efflux of phenylalanine against a concentration gradient observed by Lajtha does not contradict our theory that efflux in the adult animal can be raised to prevent a 'net' change in brain phenylalanine content. Therefore, it is unclear if the relatively few studies on brain efflux mechanisms support the possibility of an increased adult capacity for amino acid efflux as being the major factor determining the developmental increase in ability to maintain lower brain than plasma phenylalanine levels in the rat.

The possibility that alterations in brain phenylalanine metabolism contribute to the developmental differences in brain phenylalanine levels observed seems remote. Although cerebral phenylalanine hydroxylase activity has been observed in the brain (Baqchi & Smith, 1977) it is quite low and it has been shown that the major phenylalanine metabolites, phenylactate and phenylpyruvate, make up less than 1% of phenylalanine derived radioactivity in the brains of rats with markedly increased cerebral phenylalanine levels (Lajtha & Toth, 1962). Furthermore, changes in the availability of energy for transport systems has been found not to play an important role in developmental alterations in

'blood brain barrier' transport systems (Laasko, 1978) and the relatively minor changes in cerebral vasculature and extra-cellular volume accompanying development are not nearly large enough to account for the observed differences in cerebral phenylalanine elevations. In summary, an increase in the control of amino acid efflux with development appears to be the most likely, yet undemonstrated, mechanism for decreased net phenylalanine transport into the brain during hyperphenylalaninemia.

One can only speculate on the advantages of increased cerebral amino acid uptake in young animals. Cerebral fluctuations of certain amino acids (tryptophan, tyrosine) may serve as developmental signals for differentiating neurotransmitter systems in the brain. Alterations in both cerebral tryptophan (Green et al., 1962; Colmenares et al., 1975) and tyrosine (Gibson & Wurtman, 1972; Mandel, 1978) levels have been shown to alter neurotransmitter levels (serotonin and catecholamines respectively) and serotonin has been implicated as a hormonal regulator of early brain differentiation (Lauder & Krebs, 1978). Whereas the fully differentiated adult brain may require protection from shifts in plasma amino acid levels which accompany feeding such alterations may serve as useful developmental signals for the developing brain. On the other hand, the inability of the young animal to maintain low brain phenylalanine levels may be a simple reflection of its immaturity. Like many

other developmental changes, it may simply reflect the vulnerability of the young animal which is overcome with the accomplishment of the mature state. The demonstrated vulnerability of the suckling rat to the harmful effects of hyperphenylalaninemia (Chapters III, IV and V) certainly supports this latter point of view.

RELEVANCE TO THE TREATMENT OF PHENYLKETONURIA

The evidence presented in this chapter is consistent with the theory that the increased capacity to maintain lower brain than plasma phenylalanine levels that is acquired with maturity may be a major factor in the decreasing vulnerability of the mature animal to hyperphenylalaninemia. As the hyperphenylalaninemic rat matures, less phenylalanine enters its brain and the actual insult to the brain is thereby limited. This is in agreement with experimental evidence in these same animals that both biochemical (Chapter V) and behavioral (Chapter IV) damage is sustained only in the early period when brain phenylalanine levels are at their peak.

Extension of the present rodent findings to man may lead to the development of a scientific rationale for deciding when the dietary treatment of phenylketonuric children can be safely terminated. At present there is no universal agreement on when phenylalanine restriction is no longer

necessary. Since several studies indicate that treatment until 4-5 years of age is adequate (Holtzman et al., 1975; Koff et al., 1979) and several other reports contradict this evidence (Smith et al., 1978; Williams et al., 1979) a recent review of phenylketonuria has advised continuation of the ' . . . dietary treatment of the patient with PKU as long as possible . . . ' (Scrivner & Clow, 1980b). Comparisons of rat and human brain development can be made so that an equivalent period of decreased human brain phenylalanine uptake can be calculated. This would lead to a proposed age for therapeutic diet termination based on some other objective criteria.

Changes in the brain transport of phenylalanine may not only determine the period of vulnerability to hyperphenylalaninemia but may also provide a basis to explain the normal mental state of some untreated phenylketonurics (Gaull et al., 1975). Andrews and his colleagues (1973) have reported a significant correlation between granulocyte phenylalanine content and the degree of learning disability in non-treated phenylketonurics. Learning disability was not related to plasma phenylalanine levels in the reviewed cases. Although it is unknown whether cerebral and granulocyte transport is related, the Andrews data suggest that they are and that it is the level of brain phenylalanine as determined by individual variations in cerebral transport that determines the degree of brain damage caused by hyperphenylalaninemia. The

irrelevance of variations in plasma phenylalanine levels further underlies the importance of regulation by transport mechanisms. This work is consistent with the data presented in this chapter which also implicates the importance of the cerebral transport system in determining the vulnerability of the brain to hyperphenylalaninemia. It is a small step to extend this hypothesis to explain non-retarded untreated phenylketonurics as being individuals with genetically aberrant or precociously developed cerebral transport systems, that is, individuals whose brains can maintain low cerebral phenylalanine levels from early on in life despite elevated plasma levels.

Discovery of developmental changes in net cerebral phenylalanine transport also has important implications in terms of animal models of PKU. It is clear from Figure VII-5 that in order to maintain a consistent brain phenylalanine elevation throughout suckling life an increased degree of hyperphenylalaninemia must be achieved with increasing age. Achievement of this goal with more frequent phenylalanine injections (see Chapter III) may significantly improve the present model of the disease. The data presented in this chapter also draws our attention to the fact that adult levels of cerebral phenylalanine are most often quoted from the human literature and that these values may be significantly less than those found in young PKUics. Furthermore, Partington and Lewis (1963) have demonstrated

that phenylketonurics maintain significantly higher plasma phenylalanine levels during their first ten months of life than later on. This may further magnify the transport effect and in itself be a major factor in determining the greater degree of biochemical abnormality to which the immature brain is exposed. Thus a higher degree of both plasma (50 fold) and cerebral phenylalanine elevation than previously calculated should be obtained in order to accurately mimic the human disease state.

CHAPTER EIGHT

CONCLUSIONS

The data gathered in this dissertation address many of the active issues in PKU research today, as well as some additional areas of neuroscience investigation. New neurochemical findings in animals subjected to our experimental phenylketonuria are reported as well as behavioral findings on adult animals having undergone neonatal hyperphenylalaninemia. The exact nature of the chemical insult in PKU (phenylalanine or its metabolites) was probed, and an approach to studying the mechanism of the irreversible cerebral defect was opened up with the description of the first permanent enzymatic changes found in any animal model of PKU. Additionally, a new animal model of maternal phenylketonuria is presented, and the nature of the 'critical period' of brain vulnerability to hyperphenylalaninemia is explored. The hormonal regulation of sexually dimorphic behavior patterns, and their relationship to PKU, are also examined.

In Chapter V the first description of cerebral catecholamine depletion in an animal model of PKU is made (Section I). By reproducing this characteristic of human PKU, as well as demonstrating behavioral changes in adult animals who were treated earlier in life (Section I, Chapter IV), the appropriateness of the α -methylphenylalanine plus phenylalanine model of PKU is confirmed. Furthermore, these

studies and corroborating in vitro evidence revealed the importance of elevated cerebral phenylalanine to tyrosine ratios in causing the catecholamine depletion, presumably by inhibiting cerebral tyrosine hydroxylase activity. Thus, increased plasma levels of usually rare phenylalanine metabolites and decreased cerebral tyrosine levels were seen to be unimportant in the pathogenesis of at least this particular PKU-related cerebral abnormality. The elucidation of this probable mechanism of catecholamine depletion accompanying hyperphenylalaninemia has also added to our understanding of some of the differences between classic postnatal PKU and gestational PKU. Hyperphenylalaninemic fetal animals did not demonstrate cerebral dopamine depletion (Chapter VI). This was presumably due to the placental maintenance of increased plasma tyrosine levels in the fetus. Such elevations were shown to counteract the catecholamine depleting effect of phenylalanine elevations in suckling rats and also to overcome phenylalanine inhibition of striatal tyrosine hydroxylase in vitro.

Although cerebral catecholamine depletion did not extend beyond the early treatment period a permanent change in catecholamine metabolism was discovered (Section II, Chapter V). The increase in striatal dopa decarboxylase and monoamine oxidase activities in the adult animals represent the first description of lasting enzymatic changes following the imposition of hyperphenylalaninemia in the early period

of the animal's life. These changes, although small, take on added significance when contrasted with the negative results gleaned from the measurement of many other enzymes. Together with the associated discovery of abnormalities exhibited in adult life in a behavior (spontaneous circling activity) known to be regulated by dopaminergic nigrostriatal activity, these findings present us with the first real insight into the relationship of early biochemical damage (catecholamine depletion in response to neonatal hyperphenylalaninemia) and later, more permanent changes. It should be noted that dopa decarboxylase and monoamine oxidase are involved in the synthesis and degradation of serotonin, as well as the catecholamines, so that the turnover of both may be affected by the altered enzyme activities.

Further study of catecholamine and serotonin metabolism in adult rats subjected to neonatal hyperphenylalaninemia should significantly increase our understanding of the links between early neurochemical changes during development and more permanent effects, as well as aid in our understanding of the relationship between neurochemical effects and behavioral changes. The discovery of sexually dimorphic behavioral responses to our treatment was quite surprising in light of the equivalent neurochemical changes in males and females. Although the role of organisational (neonatal testosterone) and activational (primarily adult estrogens) hormonal influences on these behavioral changes have been par-

tially delineated (Section III, Chapter IV), the neurochemical correlates of these effects are unknown. The discovery of sex differences in rotation in response to amphetamine and apomorphine (Section II, Chapter IV), as well as in spontaneous rotational activity, suggests that differences in pre- and post-synaptic receptor mechanisms between males and females may exist. However, the interaction between the experimental insult (hyperphenylalaninemia and subsequent catecholaminergic effects) and the sexually dimorphic neurotransmission systems are yet to be described. Neurochemical analysis of this interaction will aid in our understanding of the normal development of these systems and help to further delineate the neurochemical basis of permanent cerebral damage in PKU.

In Chapter VI a new animal model of gestational phenylketonuria is presented. By giving pregnant dams a diet supplemented with α -methylphenylalanine and phenylalanine the plasma phenylalanine levels of their fetuses were elevated to more than ten times normal for more than two thirds of each day. The introduction of this model is timely in that a large number of previously treated phenylketonuric women are now reaching reproductive age, making maternal PKU a growing problem and one about which relatively little is known. Similar to postnatal hyperphenylalaninemia we found that hyperphenylalaninemic fetuses exhibited decreases in cerebral serotonin and branched chain amino acids as well as

increases in cerebral glycine and phosphoserine phosphatase. The two conditions are not identical, however, since no dopamine depletion was observed with the prenatal insult. Discovery of the increased brain weight deficits with the earlier imposition of our treatment has important implications for the human disease in that it suggests that the treatment of pregnant phenylketonurics should begin at the time of conception. Furthermore, this new paradigm, when used in conjunction with the postnatal model, provides a new tool to study the mechanism of hyperphenylalaninemia-induced cerebral damage.

In Chapter VII a complete development curve for the transport of phenylalanine into the brain during hyperphenylalaninemia is presented. The discovery that the mature animal has a greater capacity to restrict large amounts of plasma phenylalanine from entering the brain suggests that the development of cerebral transport systems may play a role in defining the time period of increased vulnerability of the brain to hyperphenylalaninemia-induced damage. Thus, the immature brain may be subject to a greater degree of chemical insult (more phenylalanine) than the relatively damage-resistant mature brain. The creation of a pathologic state, hyperphenylalaninemia, provides an excellent paradigm to study the development of cerebral amino acid transport systems and the mechanisms of amino acid homeostasis in the brain. Overall, it is hoped that the findings presented in

this thesis will aid in the continuing effort to describe cerebral development in normal and pathologic states.

APPENDIX

(I) NORMAL PARAMETERS

During the course of studies on hyperphenylalaninemic animals conducted for this thesis many values of normal developing rats were obtained. This data concerning enzyme development and distribution in the brain, cerebral catecholamine development and fetal growth may not be cogent to the main exposition of this dissertation but nonetheless comprises an important accumulation of data.

A) ENZYME DEVELOPMENT AND DISTRIBUTION

Table A-1 shows the distribution in different brain areas, as well as hepatic values, of the activity of the enzymes cytochrome oxidase and gamma-glutamyl transpeptidase for neonate and adult animals. DNA content (mg per g) is also listed. Figure A-1 shows the complete development of cerebral monoamine oxidase activity.

B) CATECHOLAMINE DEVELOPMENT AND REGIONAL DISTRIBUTION

Table A-2 shows the regional distribution of dopamine and norepinephrine in the adult brain as determined by the radioenzymatic assay described in Chapter II. Using the fluorometric method it was shown that adult animals who had

TABLE A-1

ENZYME AND DNA CONTENT IN NEWBORN AND ADULT RAT BRAIN PARTS

Control rats were sacrificed at birth (newborn=NB) or as adults (120 days old). Enzyme activities (units/g tissue) and DNA content (mg per g) were determined as described in Chapter II.

		<u>Whole Brain</u>	<u>Cortex</u>	<u>Cerebellum</u>	<u>Brainstem</u>	<u>Liver</u>
Cytochrome Oxidase	NB	50 $\pm$ 4	46, 48	50, 55	56, 61	106 $\pm$ 11
	Adult	175	226 $\pm$ 14	216 $\pm$ 46	191 $\pm$ 18	260 $\pm$ 44
Glutamyl Transpeptidase	NB	104 $\pm$ 18	95, 96	101, 104	109, 122	-
	Adult	785 $\pm$ 119	779 $\pm$ 64	719 $\pm$ 88	787 $\pm$ 38	-
DNA	NB	4.38 $\pm$.33	3.39, 3.64	3.30, 3.54	3.06, 2.81	6.25, 5.87
	Adult	1.60	1.02 $\pm$ 0.16	4.98 $\pm$ 1.28	0.84 $\pm$ 0.06	2.02 $\pm$ 0.41

FIGURE A-1

DEVELOPMENT OF CEREBRAL MONOAMINE OXIDASE
ACTIVITY

Control rats were killed at the indicated age with negative numbers indicating the number of days before birth (i.e. - 5days represents the 17th fetal day of life). Monoamine oxidase activity, types A and B combined, is expressed as per cent of the adult level.

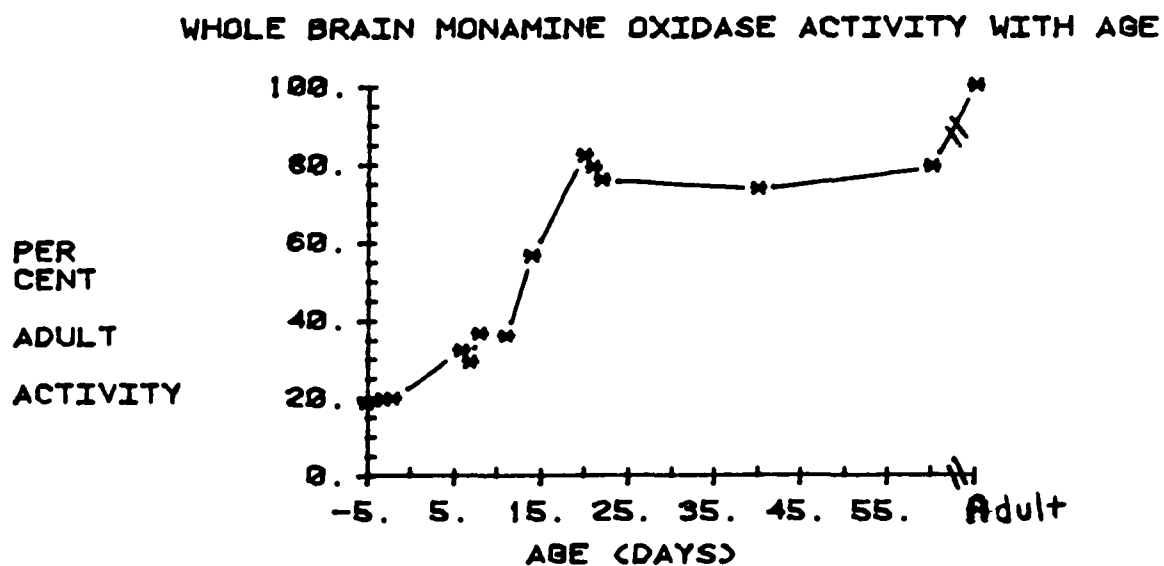


TABLE A-2

REGIONAL BRAIN DISTRIBUTION OF DOPAMINE AND NOREPINEPHRINE

Control adult Fischer rats were sacrificed and their brains dissected into several regions as indicated. Dopamine and norepinephrine levels were determined using the radioenzymatic method described in Chapter II.

	<u>Dopamine</u> (ug/g)	<u>Norepinephrine</u> (ug/g)
Frontal Cortex	2.01±0.92 (9)	0.88±0.30 (10)
Striate	12.10±2.96 (6)	0.62±0.14 (9)
Thalamus	0.33±0.10 (6)	0.79±0.43 (6)
Hypothalamus	0.43±0.16	3.24±0.89 (7)
Brainstem	0.16	0.63, 0.57

been rendered hyperphenylalaninemic during the 2nd to 21st days of life did not exhibit striatal (control= 10.46 ± 3.01 (3), experimental= 12.10 ± 2.96 (3)) or whole brain (control= 1.15 ± 0.20 (3), experimental= 1.12 ± 2.96 (3)) dopamine depletion. Although this latter method is not highly sensitive, and thus unsuitable for neonatal work, it was found to be accurate in that the results obtained (adult striatal dopamine= 11.57 ± 5.83) were similar to those derived from the more sensitive radioenzymatic method (12.10 ± 2.96). Figure A-2 shows a curve of the development of dopamine in the brain.

C) FETAL GROWTH

In the course of studying gestational hyperphenylalaninemia (Chapter VI) much data was collected on the growth of normal fetal Fischer rats. Figure A-3 shows the linear increase in fetal body length with age. Linear regression of this data determined a correlation coefficient of 0.985 with the drawn slope significant at the level of $p < 0.0001$. Similarly, fetal brain weight increased with age in a linear manner (Figure A-4) with the same high correlation coefficients ($r = 0.985$) and significance levels ($p < 0.0001$) for the slope. A plot of fetal body weight versus brain weight (Figure A-5) also resulted in a straight line with a high correlation coefficient ($r = 0.979$) and fitted slope with a

Figure A-2

DEVELOPMENT OF CEREBRAL DOPAMINE CONTENT

Fischer rats were sacrificed on the indicated day and their cerebral dopamine content determined. Fetal age is denoted by the number of days before birth (B). Each point represents the mean determined from 3 to 12 animals of both sexes.

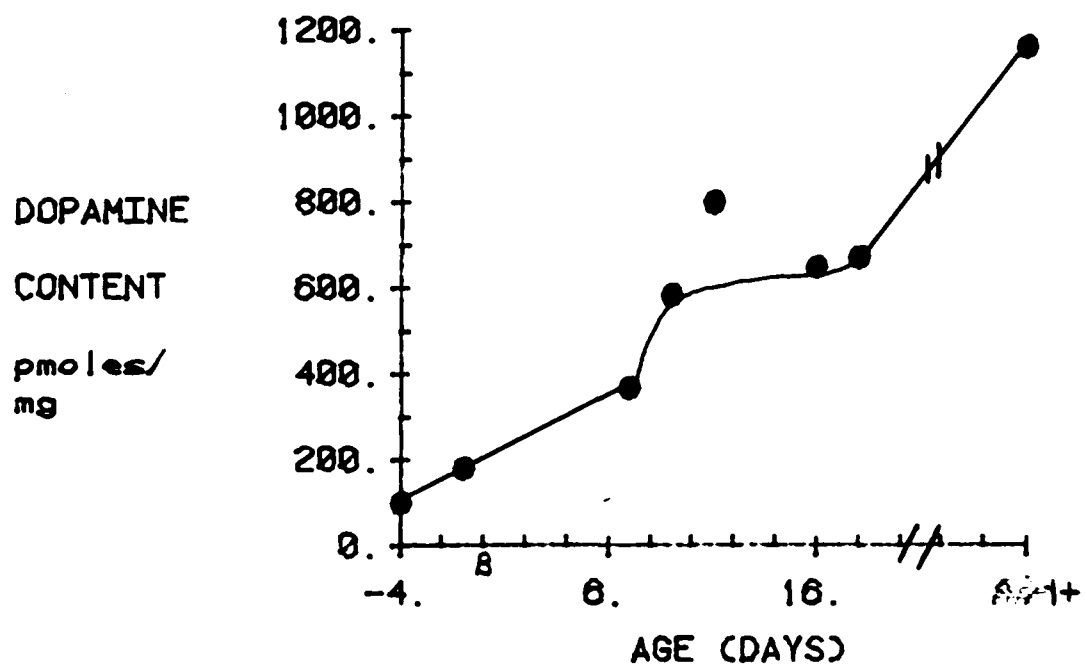


FIGURE A-3

CROWN-TO-RUMP GROWTH IN FETAL FISCHER RATS

Rats were time-mated (conception=day 0, birth=22.5) and then sacrificed on the indicated day of gestation. Each point represents the mean of the body lengths (crown-to-rump) of all the fetuses from 2 to twelve litters.

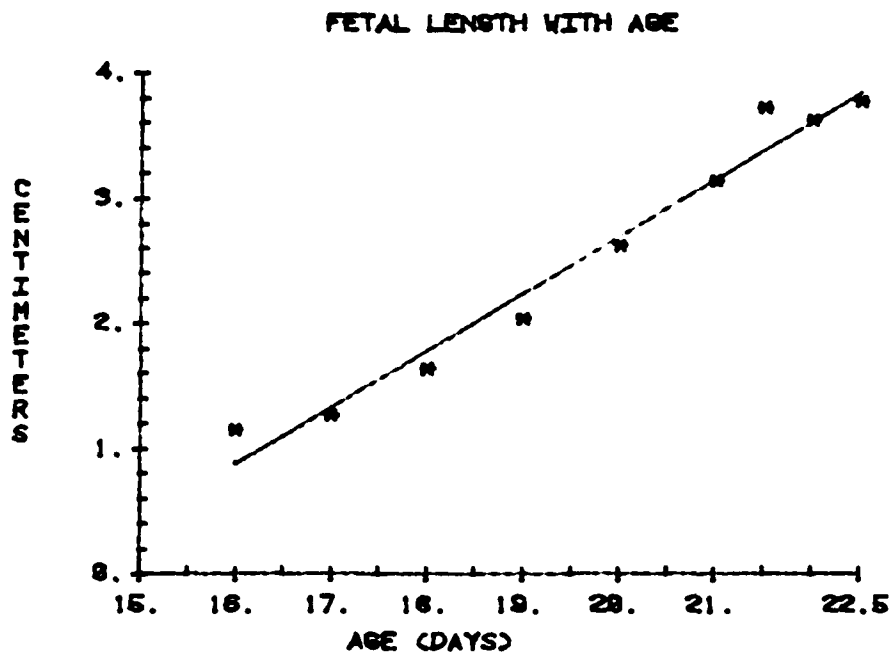


FIGURE A-4

BRAIN GROWTH IN FETAL FISCHER RATS

Rats were time-mated (conception=day 0, birth=22.5) and then sacrificed on the indicated day of gestation. Each point represents the mean of the brain weights of all the fetuses from one to eight litters.

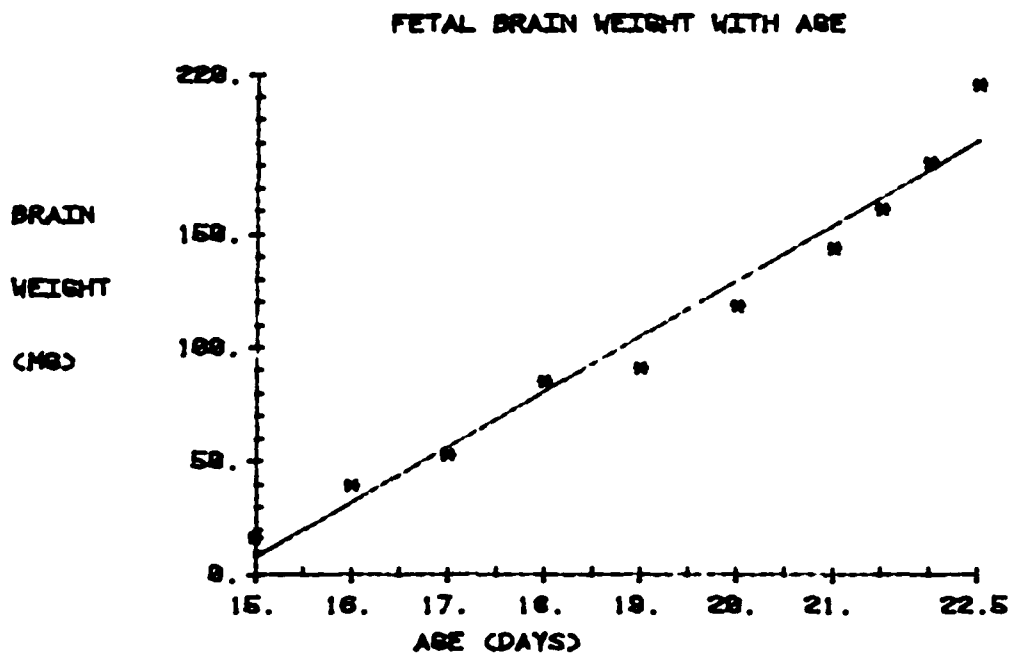
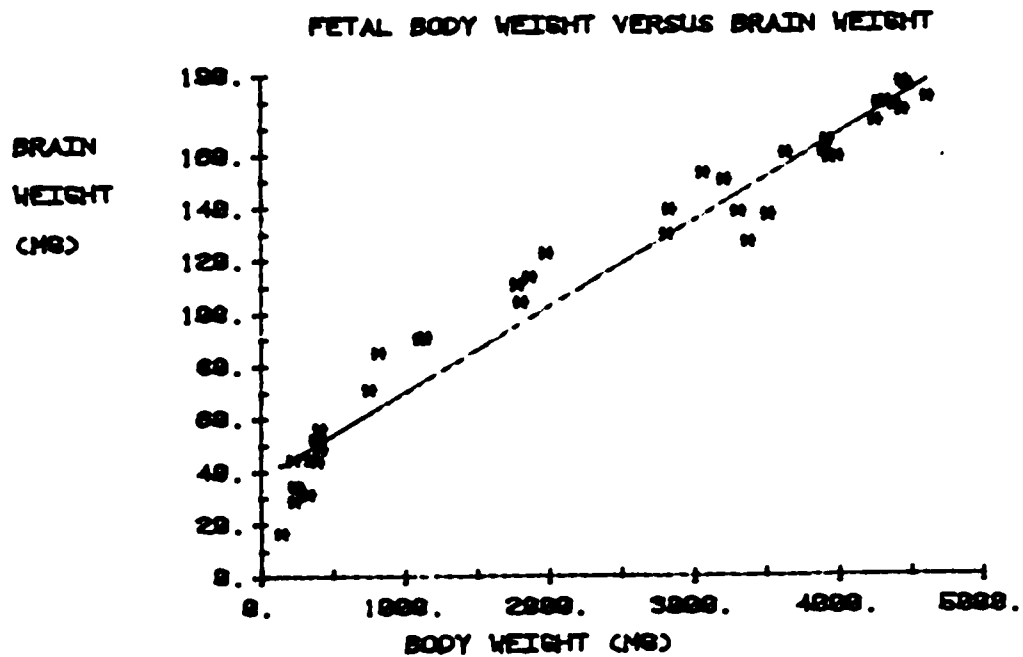


FIGURE A-5

BODY WEIGHT VERSUS BRAIN WEIGHT IN FETAL FISCHER RATS

Each point represents the body weight and corresponding brain weight of individual fetal rats (control or hyper-phenylalaninemic) sacrificed between the 15th and 22nd days of gestation.

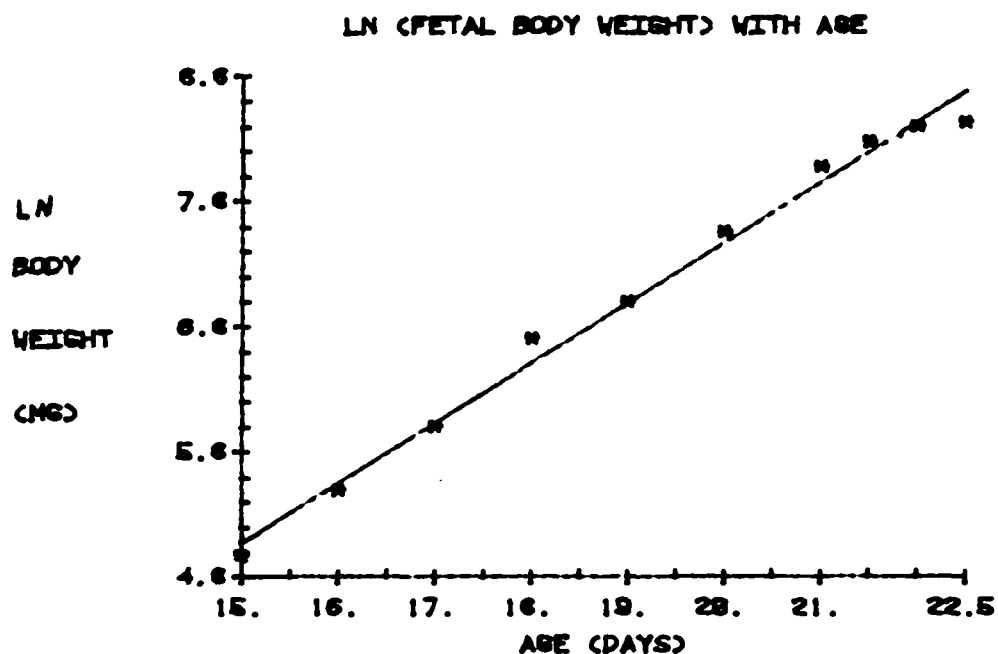


significance level of $p < 0.0001$). However, fetal body weight did not increase linearly with age but increased as a logarithmic function (Figure A-6; $r = 0.995$, $p < 0.001$ for slope). These curves should be valuable for other investigators working with Fischer rats during fetal life.

FIGURE A-6

BODY GROWTH IN FETAL FISCHER RATS

Rats were time-mated (conception=day 0, birth=day 22.5) and then sacrificed on the indicated day of gestation. Each point represents the mean of the natural logarithm of the body weights of all the fetuses from two to seven litters.



(II) NEGATIVE RESULTS

In this section the negative results from experiments on several different topics related to experimental PKU are presented.

4) HEXOKINASE AND CREATINE KINASE IN THE PKUic RAT

Many cerebral parameters have been studied in young rats during their exposure to hyperphenylalaninemia induced by injections of phenylalanine plus α -methylphenylalanine or other means. In the course of collecting data on 'PKUic' rats many of the well established changes which accompany hyperphenylalaninemia (decrease in branched chain amino acids, glycine elevation, serotonin depletion etc.) have been confirmed. However, in several instances it seemed important to further investigate the negative claims from earlier investigations.

Of the many enzymes assayed in the brains of hyperphenylalaninemic rats only a few (see Chapter V) have been shown to change in response to elevated phenylalanine levels. Although hexokinase is among the 'non-responding' enzymes (DelValle & Greengard, 1975; Diemel, 1977) previous investigators have not reported soluble and particulate or male and female activities separately. Here we demonstrate

notable but non-significant increases in soluble hexokinase activity in the brains of young hyperphenylalaninemic rats, as well as significant differences between soluble cerebral hexokinase activity in males and females (Table A-3). Additionally, creatine kinase is added to the list of enzymes whose whole brain activity does not change in response to early hyperphenylalaninemia.

Data for rats aged 6 through 11 days and 20 through 22 days were grouped together in this study since no differences were seen in enzyme activities within each of these age groups. No differences were found between hyperphenylalaninemic and saline injected rats in the cerebral activities of creatine kinase or hexokinase (Table A-3). Rats in both the younger (6-11 days) and older (20-22 days) groups were sacrificed 4-8 hours after their last daily injection of phenylalanine plus α -methylphenylalanine. Although total hexokinase activity was similar in control and experimental rats there was a notable (25-36%) although statistically non-significant increase in the soluble hexokinase activity measured in both experimental male and female rats. We also noted that although total hexokinase activity is nearly identical in the brains of the males and females studied, soluble hexokinase activity is significantly higher (50%, $p < 0.01$) in the brains of male rats. The significance of this finding in terms of PKU is not readily apparent, however, this is one of the first reports of a sexual

TABLE A-3

HEXOKINASE AND CREATINE KINASE ACTIVITIES IN THE BRAINS OF
CONTROL AND HYPERPHENYLALANINEMIC NEONATAL RATS

Rats were given daily subcutaneous injections of phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w) beginning on the second day of life. All rats were sacrificed between the 6th and 11th days of life 3 hours after their last injection. All activity is expressed as units/g tissue. The asterisk indicates a significant difference ($p < 0.01$) from the corresponding females.

<u>Hexokinase</u>	<u>Supernatant</u>	<u>Precipitate</u>	<u>Total</u>
<u>Control</u>			
Male	1.41 $\pm$ 0.28 (8)	3.64 $\pm$ 1.15 (5)	3.05 $\pm$ 1.05
Female	0.81 $\pm$ 0.29 (3)	4.04 $\pm$ 0.67 (3)	4.95 $\pm$ 0.95 (3)
Male & Female	1.22 $\pm$ 0.37 (8)	3.79 $\pm$ 0.96 (8)	5.01 $\pm$ 0.95 (8)
<u>Experimental</u>			
Male	1.92 $\pm$ 0.58 (5)	3.39 $\pm$ 0.67 (5)	5.31 $\pm$ 0.77 (5)
Female	1.04 $\pm$ 0.37 (4)	4.29 $\pm$ 0.42 (4)	5.31 $\pm$ 0.23 (4)
Male & Female	1.53 $\pm$ 0.66 (9)	3.78 $\pm$ 0.70 (9)	5.31 $\pm$ 0.56 (9)
<u>Male</u> Control & Experimental	1.67 $\pm$ 0.51 (10) *	3.52 $\pm$ 0.90	5.18 $\pm$ 0.88
<u>Female</u> Control & Experimental	0.99 $\pm$ 0.32 (7)	4.17 $\pm$ 0.49	5.16 $\pm$ 0.60
<u>Creatine Kinase</u>			
Control			2.45, 3.13
Experimental			3.16, 3.16

dimorphism in cerebral enzyme activity at such an early age. Further study of sex-based differences in hexokinase development would be of interest in terms of understanding how gonadal hormones determine sexually dimorphic patterns in the brain.

B) PLASMA TRIGLYCERIDES DURING HYPERPHENYLALANINEMIA

There are many reports of defective myelination in phenylketonurics and in hyperphenylalaninemic rats (see Chapter I) which is accompanied by changes in cholesterol and fatty acid synthesis. These latter changes may represent general metabolic responses to elevated phenylalanine or its metabolites. To examine this hypothesis, as well as verify data from another laboratory (Huther, personal communication), triglycerides and cholesterol levels were determined in the plasma of young hyperphenylalaninemic rats.

Suckling rats given daily injections of phenylalanine plus α -methylphenylalanine had normal plasma triglyceride and cholesterol levels when examined. This was true whether sampling occurred during a time of high plasma phenylalanine (4 or 7 hours post-injection) or at a time when plasma phenylalanine had returned to control levels (24 hours post-injection). Plasma triglyceride levels increased similarly with age in control and experimental rats between the ages of 7 and 22 days (Table A-4).

TABLE A-4

PLASMA TRIGLYCERIDE AND CHOLESTEROL LEVELS IN CONTROL AND HYPER-
 PHENYLALANINEMIC RATS

Rats were given daily subcutaneous injections of phenylalanine (5.2 umoles/g body w) plus -methylphenylalanine (2.4 umoles/g) (experimental:E), or its saline diluent (Control:C) beginning on the second day of life. Animals were then killed at the age indicated with the number of hours between last injection and sacrifice listed under 'Hrs Post-Injection'.

<u>Age</u>	<u>Hrs. Post- Injection</u>	<u>Triglycerides</u>		<u>Cholesterol</u>	
		<u>C</u>	<u>E</u>	<u>C</u>	<u>E</u>
7	7	100	96	102	107
		150	144	100	96
8	10	194	200	-	-
15	24	125	138	129	137
16	4	170	188	147	144

C) ADULT BODY AND BRAIN WEIGHT CHANGES WITH VARIOUS
EXPERIMENTAL TREATMENTS

The effects of various neonatal treatments on the body and brain weights of adult animals are shown in Table A-5. Here the gross toxicity of para-chlorophenylalanine is confirmed. We also note that the cerebral glycine elevating treatment caused a significant brain weight deficit. Furthermore, α -methylphenylalanine alone caused greater body and brain weight losses than the standard hyperphenylalaninemia treatment that used this same amount of α -methylphenylalanine plus phenylalanine. This last observation further underlines the point made in Chapter V that the use of α -methylphenylalanine alone is not a control for the α -methylphenylalanine plus phenylalanine treatment. Use of our standard treatment protocol (phenylalanine plus α -methylphenylalanine) for more limited neonatal periods had effects in the same direction as the complete (days 2-22) treatment (i.e. decreased female but not male brain weights) although this effect was only significant in the day 2-10 treatment group with the limited number of animals tested.

Unlike Fischer rats, Sprague-Dawley males ($C = 2.05 \pm 0.07$ (11), $E = 1.95 \pm 0.07$ (8)) but not females ($C = 1.88 \pm 0.12$ (6), $E = 1.88 \pm 0.08$ (9)) had permanent brain weight deficits due to the imposition of early (days 2-22) hyperphenylalaninemia. The reasons for this strain difference are not readily

TABLE A-5

ADULT BODY AND BRAIN WEIGHTS OF RATS GIVEN NON-STANDARD NEONATAL TREATMENTS

Rats in the first three treatment groups were given the standard treatment for experimental hyperphenylalaninemia (phe 5.2 $\mu\text{m/g}$ body w plus α -mephe 2.4 $\mu\text{m/g}$ body w) but for more limited periods: days 2-10, in the first group and days 2-15 and 7-15 in the second and third groups respectively. Rats in the fourth group (α -mephe) received only α -mephe (2.4 $\mu\text{m/g}$ body w) injections during the second to twenty-first days of life, animals in the fifth group received phe (5.2 $\mu\text{m/g}$ body w) plus p-chlorophenylalanine (0.9 $\mu\text{m/g}$ body w) every other day on days 2-21, and animals in the last group (glycine plus serine) received injections of glycine (9.3 $\mu\text{m/g}$ body w) plus serine (19 $\mu\text{m/g}$ body w) during days 7-21 of life. All animals were weighed and sacrificed at an age greater than 120 days. All values represent the mean $\pm$ SD (n). Significant differences from control are indicated by * with $p < 0.01$.

Treatment			Male		Female	
			C	E	C	E
PHE & AMPHE 2-10	Brain Weight		1.74 $\pm$.13 (3)	1.81 $\pm$.09 (11)	1.73 $\pm$.02 (4)	1.63 $\pm$.06 (6) *
	Body Weight		272 $\pm$ 33 (3)	315 $\pm$ 53 (11)	181 $\pm$ 8 (4)	177 $\pm$ 15 (6)
PHE & AMPHE 2-15	Brain Weight		1.73, 1.79	1.76 $\pm$.09 (4)	1.67 $\pm$.08 (10)	1.62 $\pm$.09 (11)
	Body Weight		245, 286	307 $\pm$ 72 (4)	189 $\pm$ 12 (10)	185 $\pm$ 15 (11)
PHE & AMPHE 7-15	Brain Weight		1.85, 1.96	1.81 $\pm$.06 (3)	1.75, 1.72	1.64, 1.61
	Body Weight		306, 303	326 $\pm$ 24 (3)	173, 173	167, 179
α -MPHE	Brain Weight			1.63 $\pm$.13 (4) *		1.58 $\pm$.08 (14) *
	Body Weight			239 $\pm$ 46 (5)		147 $\pm$ 31 (14) *
PCPA	Brain Weight			1.35, 1.43	1.57, 1.58	1.18, 1.25
	Body Weight			158, 145	138, 115	71, 104
Glycine plus Serine	Brain Weight					1.65 $\pm$.05 (6) *
	Body Weight					184 $\pm$ 20 (6)

apparent. However, it should be noted that Sprague-Dawley rats mature at a faster rate than Fischer rats do and the developmental state of the animal during its exposure to high phenylalanine levels is certainly an important factor in determining the damage incurred in this developmental disease.

D) NEGATIVE BEHAVIORAL RESULTS

The results from behavioral studies on Sprague-Dawley rats given our standard phenylalanine plus α -methylphenylalanine treatment on days 2-22 of life is shown in Table A-6. Female Sprague-Dawley rats, when studied as adults, did not show the same behavioral alterations as similarly treated Fischer rats. In fact male animals seemed to have increased rotational activity, although this small increase in '% Preference' and net rotations was not significant with the number of animals tested. This is interesting in that it was these same males, and not the females, who also exhibited permanent brain weight deficits.

Studies initiated to see if the age at which reliable rotometer testing of rats is conducted could be lowered, showed that Fischer rats have identical responses in 24 hour rotometer testing at 60 and 90 (our usual minimal age for testing) days of age (Table A-7). Thus, it may not be necessary to raise rats to 3 months of age before behavioral

TABLE A-6

BEHAVIORAL RESULTS FROM ADULT SPRAGUE-DAWLEY RATS MADE HYPERPHENYLALANINEMIC
ON DAYS 2-21 OF LIFE

Sprague-Dawley rats were injected with phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w) (experimental) or saline vehicle (control) on the 2nd to 21st days of life. On the 90th to 120th days of life they were tested for 24 hour spontaneous rotational activity, locomotor activity and T-maze learning.

<u>Rotometer tests</u>		<u>Control</u>	<u>Experimental</u>
Preference	Male	67+12 (18)	72+15 (15)
	Female	64+25 (9)	64+14 (14)
Net Rotations	Male	34+35	71+95
	Female	45+37	41+62
Direction of Rotation (R-L)	Male	8-9-1	8-7
	Female	7-2	7-7
<u>Locomotor Activity</u>			
Photocell Counts/ 30 min.	Male	1313+460	1289+386
	Female	1412+676	1697+403
<u>T-Maze Learning</u>			
Trials to Criteria	Male	8.00+1.58 (5)	8.40+.55 (5)
	Female	10.00+2.55 (5)	8.80+3.56 (5)

TABLE A-7

ROTATIONAL BEHAVIOR AT SIXTY AND NINETY DAYS OF FEMALE RATS

Female Fischer rats were injected with phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w) (experimental) or saline vehicle only (control) on days 2-21 of life. They were then tested for rotational and locomotor activity at 60 days of age and again at 90 days.

<u>Rotometer Tests</u>		<u>60 Day</u>	<u>90 Day</u>
% Preference	Control	69 $\pm$ 3 (11)	75 $\pm$ 4 (11)
	Experimental	71 $\pm$ 4 (10)	68 $\pm$ 4 (9)
Net Rotations	Control	43 $\pm$ 25	50 $\pm$ 18
	Experimental	35 $\pm$ 12	36 $\pm$ 18
Total Full Rotations	Control	85 $\pm$ 31	107 $\pm$ 34
	Experimental	74 $\pm$ 20	81 $\pm$ 30
<u>Locomotor Activity</u>			
Photocell Counts/ 30 min.	Control	886 $\pm$ 226 (6)	-
	Experimental	829 $\pm$ 203	-

testing is begun. However, it was first noted in this study that a few litters of Fischer rats given phenylalanine plus α -methylphenylalanine on days 2 to 21 of life failed to show the increased rotational asymmetry and hyperactivity previously seen (Chapter IV). Despite these observations, the vast majority of numerous litters raised over a two year period have shown the behavioral alterations recorded in Table IV-1. Those few litters yielding negative behavioral results were all raised at the same time; so their behavior serves more to point out the borderline nature of our model insult than to cast doubt on the validity of the previous observations.

Furthermore, some litters had alterations in rotational activity but not maze learning deficits. This finding may reflect not only on the relative importance of alterations in cerebral asymmetry in causing changes in these other behaviors, but also on the 'completeness' of our model treatment. Since a few litters of 'PKUic' rats had no measurable behavioral abnormalities in any of our tests we may be viewing the variable expression of a borderline insult. Variation in the effectiveness (see Chapter V) of treatments may be reflected in selective loss of behavioral findings. Those abnormalities measured by less sensitive tests (e.g. T-maze performance) may be lost first followed by the loss of the less subtle findings in litters receiving less effective treatments in suckling life. The possible basis for such treatment failures is discussed in Chapters V and VII.

E) NEGATIVE BIOCHEMICAL RESULTS

Despite the convincing increase in striatal dopa decarboxylase activity described in Chapter V, a few groups of animals studied at a later date failed to demonstrate this change (Table A-8). These animals were raised during a different climatic period, early spring as opposed to winter, than the litters represented in Figure V-4 and comprise those animals which also failed to demonstrate the increased lateralization of rotational behavior previously shown to exist in numerous litters (Chapter IV). Tryptophan hydroxylase was measured in the midbrain, an area of high serotonin concentration, of one group of these animals and like the DDC in this group failed to show any changes in activity in the experimental group (Table A-8).

Sprague-Dawley rats, treated the same way as Fischer animals, did not show any changes in adult (120 day) striatal DDC, TH or ChAT activities (Table A-8). These animals were raised at the same time as Fischer rats which had elevated striatal DDC activity, but the Sprague-Dawley rats did not have similar behavioral abnormalities (see above, Section C). Similarly, Fischer rats injected with α -methylphenylalanine alone (2.4 μ moles/g body w) from the 2nd to 21st days of life had normal striatal DDC (see Chapter V) activity and were without the behavioral aberrations seen in hyperphenylalaninemic animals.

TABLE A-8

STRIATAL AND MIDBRAIN ENZYME ACTIVITIES IN FISCHER AND SPRAGUE-DAWLEY RATS

Fischer and Sprague-Dawley rat pups were injected with phenylalanine (5.2 umoles/g body w) plus α -methylphenylalanine (2.4 umoles/g body w) (E) or an equivalent volume of saline (C) during the 2nd to 21st days of life. Males (M) and females (F) were then sacrificed as adults (120 days old), their striates and/or midbrains removed and enzyme activities determined. All values represent the mean $\pm$ SD(n).

		<u>Fischer Rats</u>		<u>Sprague-Dawley Rats</u>	
		<u>C</u>	<u>E</u>	<u>C</u>	<u>E</u>
Striatal Dopa Decarboxylase	M	8.23, 8.18	7.70 $\pm$ 1.01 (3)	7.75 $\pm$ 1.10 (6)	7.52 $\pm$ 0.30 (6)
	F	7.29 $\pm$ 1.38 (4)	6.72 $\pm$ 0.12 (3)	7.77 $\pm$ 0.64 (6)	7.32 $\pm$ 1.4 (6)
	M&F	7.59 $\pm$ 1.17 (6)	7.21 $\pm$ 0.84 (6)	7.63 $\pm$ 0.86 (12)	7.42 $\pm$ 0.96 (12)
Striatal Tyrosine Hydroxylase	M	-	-	31.38 $\pm$ 8.08 (6)	36.13 $\pm$ 2.91 (4)
	F	-	-	32.38 $\pm$ 5.98 (6)	30.94 $\pm$ 5.96 (6)
	M&F	-	-	31.88 $\pm$ 6.80 (12)	33.02 $\pm$ 5.45 (10)
Striatal Choline Acetyltransferase	M	-	-	6.70 $\pm$ 41 (6)	707 $\pm$ 79 (6)
	F	-	-	661 $\pm$ 48 (6)	632 $\pm$ 91 (6)
	M&F	-	-	665 $\pm$ 42 (12)	670 $\pm$ 90 (12)
Midbrain Trypophan Hydroxylase	M	17.66, 18.46	18.51 $\pm$ 1.72 (3)	-	-
	F	19.65 $\pm$ 0.64 (3)	20.03 $\pm$ 1.86 (3)	-	-
	M&F	19.12 $\pm$ 0.99 (5)	19.27 $\pm$ 1.80 (6)	-	-

This evidence that adult Sprague-Dawley rats treated with phenylalanine plus α -methylphenylalanine and adult Fischer rats given α -methylphenylalanine alone on days 2-21 of life showed neither the same behavioral abnormalities nor the increased striatal DDC activity found in most Fischer rats given phenylalanine plus α -methylphenylalanine during this same period suggests some relationship between the behavioral abnormalities and this biochemical change. This impression is further strengthened by the fact that those litters of experimental Fischer rats which did not have the increased striatal DDC activity also failed to show the increased rotational asymmetry found in a great number of Fischer litters similarly treated. That these anomalous litters were reared, albeit at a later time than those represented in Figure V-4, may reflect on the borderline nature of the insult in this animal model of PKU. This may involve several factors. For instance, in Chapter V the key role of the phenylalanine to tyrosine ratio in determining catecholamine depletion and possibly subsequent changes in cerebral catecholaminergic regulation in this model of PKU is demonstrated. Environmental stress could cause an early induction of enzymes controlling amino acid homeostasis and thereby change the phenylalanine/tyrosine ratio and the degree of dopamine depletion. Also relevant to this discussion is the work of Cools and co-workers (1978) who reported a seasonal variation in the behavioral responses of cats to

the administration of catecholaminergic agents into their caudate nuclei. The fact that all the Fischer litters yielding negative biochemical results were raised during the same period of time suggests that some environmental stress effect of this nature underlies this phenomenon.

Differences between rat strains in amino acid homeostasis could also explain the negative biochemical and behavioral results seen in Sprague Dawley rats. That the initial observation (of increased striatal DDC activity in Fischer rats) is a genuine reflection of the earlier effective hyperphenylalaninemia is confirmed not only by the observation that non-responding rats had no other signs (behavioral abnormalities) of early hyperphenylalaninemia but also by the fact that the increase in striatal DDC was seen at all ages in experimental rats compared to controls within the same litter,. Thus, the many variables which could effect litter variations do not play a role in the initial observation (Chapter V) involving ten different groups of animals.

BIBLIOGRAPHY

Auerbach, V. H., Waisman, H. A. and Wyckoff, L. B., *Nature*, 182: 871 (1958).

Agrawal, H. C. and Davison, A. N. in Myelination and Amino Acid Imbalance in the Developing Brain, Dekker Press, New York: 143 (1973).

Alvord, E. C., Stevenson, L. D., Vogel, F. S. and Engle, R. L., *Exper. Neurol.*, 9: 298 (1950).

Ambrus, C. M., Ambrus, J. L., Horvath, C. et al., *Science*, 201: 837 (1978).

Anderson, A. M., *Develop. Psychobiol.*, 9(2): 157 (1976).

Anderson, A. E., Abramowitz, A. Z. and Guroff, G., , In Proceedings of the Conference on Serotonin and Behavior, ed. Barchas, J. and Usdin, E., Academic Press, New York: 335-349 (1973).

Anderson, A. E. and Guroff, G., *Proc. Nat. Acad. Sci. USA*, 69: 863 (1972).

Anderson, A. E., Rowe, V. and Guroff, G., *Proc. Nat. Acad. Sci. USA*, 71: 21 (1974).

Andrews, T. M., McKeran, R. O., Watts, R. W. E., McPherson, K., and Lax, R., *Q. J. Med.*, 42: 805 (1975).

Ansell, G. B and Beeson, M. F., *Anal. Biochem.*, 23: 196 (1981).

Antonias, K N. and Coulson, W. F, *J. Neurochem.*, 25: 309 (1975).

Aprison, M. H. and Hingten, J. N., *Fed. Proc.*, 3: 121 (1972).

Armstrong, M. D. and Tyler, F. H., *J. Clin. Invest.*, 34: 565 (1955).

Arnold, A. P. and Saltiel, A., *Science*, 205: 702 (1977).

Avruch, J. and Wallach, D. F. H., *Biochimica et Biophysica Acta*, 233: 334 (1971).

- Axelrod, J. and Tomchick, R. , J. Biol. Chem., 233: 702 (1958).
- Baqchi, S. P., Smith, T. M., Biochem. Pharm., 26: 900 (1976).
- Barbato, L. M. and Barbato, I. M., Brain Res., 13: 569 (1969).
- Baum, M. J., Neurosci. Biobehav. Rev., 3: 265 (1979).
- Beatty, W. W., Horm. Behav., 12: 112 (1979).
- Becker, J. B. and Ramirez, V. D., Neurosci. Abstr., 5: 438 (1979).
- Becker, J. B. and Ramirez, V. D., Neuroendocrinology, 32: 168 (1981).
- Bedard, P., Langelier, P. and Villeneuve, A., Lancet, II: 1367 (1977).
- Berman, J. L., Cunningham, G. C., Day, R. W., Ford, R. and Hsia, Y. Y., Am. J. Dis. Child., 117: 54 (1969).
- Berry, H. K., O'Grady, D. J., Perlmutter, L. J. and Bofinger, M. K., Dev. Med. Child Neurol., 21: 311 (1979).
- Berry, H. K., Poncet, I. B., Sutherland, B. S., Burke, R., Biol. Neonate, 26: 102 (1975).
- Bickel, H., Gerrard, J. and Hichmans, E. M., Acta Pediatr., 43: 64 (1959).
- Binek, P. A., Johnson, T. C. and Kelley, C. J., J. Neurochem., 36: 1476 (1976).
- Blumenthal, M. D., J. Psychiat. Res, 5:59 (1967).
- Boqq, D. E. and Waisman, H. A., Proc. Soc. Exp. Biol. Med., 115: 407 (1964).
- Brand, L. M. and Harper, A. E., Biochem J., 142: 231 (1974).
- Brozoski, T. J., Brown, R. M., Rosvold, H. E. and Goldman, P. S., Science, 205: 929 (1979).
- Bruhl, H. H., Arnesen, J. H. and Bruhl, M. G., Amer. J. Ment. Defic., 69: 225 (1964).
- Bruin, W. J., Frantz, R. M. and Sallach, N. J., J. Neurochem., 20: 1649 (1973).

- Butcher, R. E., *Nature*, 22: 555 (1970).
- Butler, I. J., O'Flynn, M. E., Seifert, W. E., Jr. and Howell, R. R., *J. Pediatr.*, 98: 729 (1981).
- Carver, M. J., *J. Neurochem.*, 12: 45 (1965).
- Cayanis, E., *Eur. J. Biochem.*, 9: 617 (1978).
- Cession-Fossion, A., Vanderneuler, R., Dodinval, P. and Chartraine, J. M., *Pathol. Biol.*, 14: 1157 (1966).
- Chamove, A. S., Davenport, J. W., *Develop. Psychobiol.*, 2: 207 (1970).
- Chang, C. C., *Int. J. Neuropharmacol.*, 3: 643 (1964).
- Chase, H. P. and O'Brien, D., *Pediatr. Res.*, 4: 96 (1970).
- Ciarlone, A. E., *Microchem. J.*, 21: 349 (1978).
- Ciarlone, A. E., *Microchem. J.*, 23: 9 (1978).
- Colmaneres, J. L., Wurtman, R. J. and Fernstrom, J. D., *J. Neurochem.*, 25: 825, 1975.
- Cools, A. R., Van Dongen, P., Janssen, H. J. and Megens, A., *Psychopharm.*, 59: 231 (1978).
- Cooperstein, A., and Lazarow, L., *J. Biol. Chem.*, 189: 665 (1951).
- Copenhaver, J. H., Carver, M. J., and Schalock, R. L., *Develop. Psychobiology*, 7(2): 175 (1974).
- Cornford, E. M., Braun, L. D. and Oldendorf, W. H., *Pediatr. Res.*, 16: 324 (1982).
- Coyle, J. T., *Biochem. Pharmacol.*, 21: 1935 (1972).
- Coyle, J. T. and Axelrod, J., *J. Neurochem.*, 18: 2061 (1971).
- Coyle, J. T. and Henry, D., *J. Neurochem.*, 21: 61 (1973).
- Crome, L., *J. Neurol. Neurosurg. Psychiatr.*, 25: 149 (1962).
- Crome, L. and Pare, C. M. B., *J. Ment. Sci.*, 106: 862 (1960).
- Crome, L., Tymms, V. and Wolf, L. I., *J. Neurol. Neurosurg. Psychiatr.*, 25: 143 (1962).

- Crome, L and Stern, J., in Inborn Errors and the Brain: Variability of the Pathogenetic Process ed., F. A. Hommes, C. J. Van den Berg, Academic Press, New York: 229 (1975).
- Crowley, W. R., O'Donohue, T. L. and Jacobowitz, D. M., *Acta Endocr.*, 89: 20 (1978).
- Cummings, S. N., Grundt, I. K. and Yanagihara, T., *J. Neurol. Neurosurg. Psychiat.*, 31: 334 (1968).
- Curtius, H. C., Baerlocher, C. K. and Vollmin, J. A., *Clin. Chim. Acta*, 42: 235 (1972).
- Curzon, G. and Green, A. R., *Brit. J. Pharmacol.*, 39: 653 (1970).
- Dairman, W., Christenson, Udenfriend, S., *Proc. Nat. Acad. Sci.*, 68(9): 2117 (1971).
- David, J. C., Dairman, W. and Udenfriend, P., *Arch. Biochem. and Biophys.*, 160: 561 (1974).
- Davis, J. M. and Himwich, W. A., in Biochemistry of the Developing Brain, ed., W. Himwich, M. Dekker Inc., New York: 55 (1973).
- de Groot, C. J., Bueliverts, V., Touwen, B. C. L., Hommes, F. A., *Proq. Br. Res.*, 48: 199 (1978).
- Dent, C. E. in Etiologic Factors in Mental Retardation, 23rd Ross Pediatric Conference, Ross Laboratories: 32 (1957).
- Deskin, R., Seidler, F. J. Whitmore, W. L. and Slotkin, T. A., *J. Neurochem.*, 36: 1683 (1981).
- DelValle, J. A., Dienel, G. and Greengard, O., *Biochem. J.*, 170: 449 (1978).
- DelValle, J. A. and Greengard, O., *Biochem. J.*, 154: 163 (1976).
- DelValle, J. A. and Greengard, O., *Pediatr. Res.*, 11:2 (1977).
- Denniston, J. C., *J. Pediatr.*, 63: 461 (1963).
- DeVellis, J. and English, D., *J. Neurochem.*, 15: 1061 (1968).
- Dancus, J. and Levitz, M. in The Metabolic Basis of Inherited Disease ed. Stanbury, J. B. et al., Fourth Ed.: 317 (1978).

- Dhondt, J. L., Dautrevaux, M., Biserte, G. and Farriaux, J. P., *Biochimie*, 59: 713 (1977).
- Dienel, G., Doctoral Dissertation, Harvard Univ. (1977).
- Dodge, P. R., Prenskey, A. L., Feign, R. D., in Nutrition and the Developing Nervous System C. V. Mosby Co., St. Louis: 481, (1975).
- Donaldson, J. O., Neurology of Pregnancy Saunders, Philadelphia, 1978.
- Dobbing, J., in Infant and Child Feeding, ed., Thomson, A. M., Bonds, J., Filer, L., Leveille, G. A. and Weil, W., Academic Press, New York (1980).
- Efron, M. L., Kang, E. S., Visaakorpi, J. and Fellers, F. X., *J. Pediatr.*, 74: 125 (1970).
- Ehrhard, A. A., Meyer-Bahlburg, H. F. L., *Science*, 211: 1312 (1981).
- Eiduson, S. J., *J. Neurochem.*, 13: 923 (1966).
- Embsen, G. and Baldes, K., *Biochem J.*, 55: 301 (1913).
- Deskin, R., Seidler, F. J., Whitmore, W. L. and Slotkin, I. A., *J. Neurochem.* 36: 1683 (1981).
- Faulkner, W. R., *Stand. Methods Clin. Chem.*, 5: 199 (1965).
- Figlewicz, D. A. and Druse, M. J., *Exper. Neurol.*, 67: 315 (1980).
- Fisch, R. O., Doeden, D., Lansky, L. L., and Anderson, J. A., *Amer. J. Dis. Child.*, 118: 847 (1969).
- Fonnum, F., *J. Neurochem.*, 24: 407 (1975).
- Foot, J. L., Allen, R. J. and Agranoff, B. W., *J. Lipid Res.*, 6: 518 (1965).
- Frankenburg, W. K., Duncan, B. R., Coffelt, R. W., Koch, R., Coldwell, J. G. and Son, C. D., *J. Pediatr.* 73: 560 (1968).
- Friedman, P. A., Kaufman, S. and Kang, E. S., *Nature*, 240: 157 (1972).
- Friedman, P. A., Fisher, D. B., Kang, E. S. and Kaufman, S., *Proc. Nat. Acad. Sci. USA*, 70: 552 (1973).
- Folling, A., *Z. Physiol. Chem.*, 227: 169 (1934).

Folling, A., Closs, K. and Gammes, T., *Z Physiol. Chem*, 256: 1 (1938).

Fulton, T. R., Triano, T., Rabe, A. and Loo, Y. K., *Life Sciences*, 27: 127 (1980).

Galton, F., Inquiries into Human Faculty and its Development, Everyman Library, London: 128 (1932).

Garrick, N. A., Redmond, D. E., Jr., and Murphy, D. L. in Monoamine Oxidase: Structure, Function and Altered Functions, eds. Singer, T. P. et al., Academic Press, New York: 351 (1979).

Garrod, A., Inborn Errors of Metabolism, Oxford: London (1909).

Gaull, G.E., Tallan, H.H., Lajtha, A. and Rassin, D.K., in Biology of Brain Dysfunction, ed. G.E. Gaull, vol. 3: 47-143, Plenum Press, New York (1975).

Gibson, C. J. and Wurtman, R. J., *Bioch. Pharmac.*, 26: 1137 (1977).

Gibson, C. J. and Wurtman, R. J., *Life Sciences*, 22: 1399 (1978).

Gimenez, C., Valdivieso, F., Mayor, F., *Biochem. Med.*, 11: 81 (1974).

Glick, S. D., and Greengard, O., *Brain Res.*, 202: 243 (1980).

Glick, S. D., Jerussi, T. P. and Zimmerberg, B., Lateralization in the Nervous System Academic Press, New York: 213 (1977).

Glover, V., Sandler, M., Owen, F. et al., *Nature* 265:80 (1977).

Glowinski, J. and Iversen, L., *J. Neurochem.*, 13: 655 (1966).

Goldmann, E. E., *Phys.-Math. Klasse*, 1: 1 (1973).

Goodwin, J. R. and Stampwala, S., *Clin. Chem.*, 19/9: 1010 (1973).

Gordon, J. H., Gorski, R. A., Borison, R. L. and Diamond, B. I., *Pharmacol. Biochem. Behav.*, 12: 515 (1980).

Gratton, L., *Union Med. Can.*, 89: 681 (1960).

- Greenqard, O. in Essays in Biochemistry Volume 7: 159-205, ed. Campbell, P. N. and Dickens, F., Academic Press, N. Y. (1971).
- Greenqard, O., Steroid Biochem., 6: 639 (1975).
- Greenqard, O. and DelValle, J. A., Biochem. J., 154: 619 (1976).
- Greenqard, O., DelValle, J., and Diemel, G., in Models for the Study of Inborn Errors of Metabolism ed. Hommes, F.A., Amsterdam: Elsevier/North Holland: 125-133 (1979).
- Greenqard, O., Head, J. F. and Goldberg, S. L., Cancer Res., 40: 2295 (1980).
- Greenqard, O. and Jamdar, S. C., Biochim. Biophys. Acta., 237: 476 (1971).
- Greenqard, O., Yoss, M. S., and DelValle, J. A., Science, 192: 1007 (1976).
- Greenstein, S. and Glick, S. D., Pharmacol. Biochem. Behav., 3: 507 (1975).
- Green, H., Greenberg, S. M., Ericksen, R. W., Sawyer, J. L. and Ellison, T., J. Pharmacol. Exp. Ther., 136: 174 (1962).
- Griffin, R. F. and Elsas, L. J., J. Pediatr. 86: 512 (1975).
- Groppetti, A. and Costa, E., Int. J. Neuropharmacol., 8: 209 (1969).
- Guthrie, R., J. Am. Med. Assoc., 178: 863 (1961).
- Guthrie, R. and Susi, A., Pediatrics, 32: 338 (1963).
- Hineqardner, R. T., Anal. Biochem., 39: 197 (1971).
- Holm, V. A. and Knox, W. E., Pediatrics, 63: 694 (1979).
- Horecker, B. L., Thomas, J. and Monod, J., J. biol. Chem., 235: 1586 (1960).
- Hruska, R. E. and Silbergeld, E. K., Europ. J. Pharmacol., 61: 397 (1980).
- Hruska, R. E. and Silbergeld, E. K., Science, 208: 1466 (1980).
- Hsia, D. Y., Driscoll, K. W., Troll, W. and Knox, W. E., Nature, 178: 1238 (1956).

- Hsia, D. Y. Y., Know, W. E., Quinn, K. V. and Paine, R. S., *Pediatrics*, 21: 178 (1958).
- Huther, G., Kraus, R. and Neuhoff, V., *Neuroped.*, (1982).
- Huther, G. and Neuhoff, V., *J. Inher. Metabolic Disorders*, In Press.
- Ichiyama, A., Nakamora, S., Nishizuka, Y. and Hayaishi, O., *J. Biol. Chem.*, 245: 1699 (1970).
- Inqlis, J., Lawson, J. S., *Science*, 212: 693 (1981).
- Isaacs, C. and Greengard, O., *Biochem. J.*, 192: 441 (1980).
- Jequier, E., Robinson, D. S., Lovenberg, W., and Sjoerdsma, A., *Biochem. Pharmacol.*, 18: 1071 (1969).
- Jerussi, T. P. and Glick, S. D., *Psychopharmacology*, 47: 249 (1976).
- Jerussi, T. P., Glick, S. D. and Johnson, C. L., *Brain Res.*, 129: 385 (1977).
- Jervis, G. A., *J. Ment. Sci.*, 85: 719 (1939).
- Jervis, G. A., *J. Biol. Chem.*, 169: 651 (1947).
- Jervis, G. A. *J. Neuropath. Exper. Neurol.* 11:4 (1952).
- Jervis, G. A., *Proc. Soc. Exper. Biol and Med.*, 82: 514 (1953).
- Johnson, R. C. and Shah, S. N., *Neurochem. Res.*, 5: 709 (1980).
- Jori, A., Colturani, F., Dolfini, E. and Rutzynski, M., *Neuroendocrinology*, 21: 262 (1976).
- Justice, P. and Smith, G. F., *Am. J. Nursing*, 75: 1303 (1975).
- Karrer, R. and Cahilly, G., *Psychol. Bull.*, 64: 52 (1965).
- Kaufman, S., in Advances in Neurochemistry, ed. B. W. Agranoff and M. H. Abrison, vol. 2: 1:132, Plenum Press, New York (1977).
- Kaufman, S. and Fisher, D. B. in Molecular Mechanisms of Oxygen Activation, ed. O. Hayaishi: 285-369, New York: Academic Press (1974).

- Kelly, C. J. and Johnson, T. C., *Biochem. J.*, 174: 931 (1978).
- Kempf, E., Greilsamer, J., Mack, G. and Mandel, P., *Nature (London)*, 247: 483 (1974).
- Kershner, J. R., *Develop. Psychobiol.*, 11(4): 309 (1978).
- Kirksey, D. F., Seidler, F. J. and Slotkin, T. A., *Brain Res.*, 150: 367 (1978).
- Kissane, J. M. and Robins, E. J., *J. Biol. Chem.*, 233: 184 (1958).
- Kleinman, D. S., *Pediatrics*, 33: 123 (1964).
- Knox, W. E., in The Metabolic Basis of Inherited Disease, ed. J. B. Stanbury, J. B. Wyngaarden, D. S. Frederickson, 3rd ed. : 266, New York: McGraw Hill (1972).
- Knox, W. E., Herzfeld, A. and Hudson, J., *Arch. Biochem. Biophys.*, 132: 397 (1969).
- Koch, R., Schaeffler, G. and Shaw, K. N. F., *J. Tox. Env. Health*, 2: 459 (1976).
- Koe, B. K. and Weissman, A., *J. Pharmacol. Exp. Ther.*, 154: 499 (1966).
- Krebs, H. A. and Eggleston, L. V., *Biochem. J.*, 94: 3C (1965).
- Kuczenski, R. T. and Mandel, A. J., *J. Biol. Chem.*, 247: 3144, (1972).
- Laakso, M. L., *Acta Physiol. Scand.*, 104: 137 (1978).
- LaDu, B. N. in The Metabolic Basis of Inherited Disease ed. Stanbury, J. B. et al., McGraw Hill, New York: 317 (1978).
- Lahdesmaki, P. and Hannus, M. L., *Exp. Brain Res.*, 30: 539 (1977).
- Laittha, A. and Toth, J., *J. Neurochem.*, 8: 216 (1961).
- Laittha, A. and Toth, J., *J. Neurochem.*, 9: 199 (1962).
- Lamprecht, F., Coyle, J. T., *Brain Res.*, 41: 503 (1972).
- Lane, J. D., Schone, B., Langebeck, U., Neuhauff, U. in Models for the Study of Inborn Errors of Metabolism, ed. E. A. Holmes, Elsevier/North Holland, Amsterdam (1979).

- Lane, J. D. and Neuhoff, V., *Naturwissenschaften*, 67: 227 (1980).
- Lane, J. D., Schone, B., Lanzenbeck, U., Neuhoff, V., *Biochim. Biophys. Acta*, 627: 144 (1980).
- Lasala, J. M., Coscia, C. J., *Science*, 203: 283 (1979).
- Lauder, J. M. and Krebs, H., *Dev. Neurosci.*, 1: 15 (1978).
- Laverty, R. and Taylor, K. M., *Anal. Biochem.*, 22: 269 (1968).
- Lenke, R. R. and Levy, H. L., *N. Engl. J. Med.*, 303: 1202 (1980).
- Levi, G., Kandra and Lajtha, A., *Arch. Biochem. Biophys.*, 119: 303 (1967).
- Levin, E., *Exp. Eye Res. Suppl.*, 191: 345 (1977).
- Levitt, M., Soector, S., Sjoerdsma, A. and Udenfriend, S., *J. Pharmacol. Exp. Ther.*, 148: 1 (1965).
- Levitt, M., Gibb, J. W., Daly, J. W., Lipton, M., and Udenfriend, S., *Biochem. Pharm.*, 16: 1313 (1967).
- Lieberburg, I., Wallach, G. and McEwen, B. S., *Brain Res.* 127: 176 (1977).
- Lines, D. R. and Waisman, H. A., *Proc. Soc. Exp. Biol. Med.*, 136: 790 (1971).
- Lipton, M. A., Gordon, R., Guroff, G., and Udenfriend, S., *Science*, 156: 248 (1967).
- Loo, Y. H., Sloho, L. and Horning, M. G., *J. Neurochem.*, 29: 411 (1977).
- Loo, Y. H., Fulton, T., Miller, K. and Wisniewski, H. M., *Life Sciences*, 27: 1283 (1980).
- Lowden, J. A. and LaRamee, M. A., *Can. J. Biochem.*, 47: 883 (1969).
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, 193: 265 (1951).
- Luine, V. N., Khylichevskaya, R. I. and McEwen, B. S., *Brain Res.*, 86: 283 (1975).

- Luse, S. A., Rhys, A., and Lessey, R., *Amer. J. Obstet. Gynecol.*, 108: 387 (1970).
- Luttges, M. W. and Gerren, R. A., *Pharmacol. Biochem. Behav.*, 11: 493 (1979).
- Lydiard, R. B., Fossum, L. H. and Sparber, S. B., *J. Pharm. Exper. Ther.*, 164: 27 (1975).
- Lydiard, R. B. and Sparber, S. B., *Dev. Psychobiol.*, 10: 305 (1977).
- Mabry, C. C., *N. Engl. J. Med.*, 269: 1404 (1963).
- MacCready, R. A. and Levy, H. L., *Amer. J. Obs. Gynecol.*, 113: 121 (1972).
- MacDonnell, P. C. and Greenqard, O., *Arch. Biochem. Biophys.*, 163: 644 (1974).
- MacDonnel, P. C. and Greenqard, O., *J. Neurochem.*, 24: 615 (1975).
- Machovich, R. and Greenqard, O., *Biochim. Biophys. Acta*, 286: 375 (1972).
- MacLusley, N. J., Naftolin, F., *Science*, 211: 1294 (1981).
- Malamud, N., *J. Neuropathol.*, 25: 254 (1966).
- Mandel, A. J., *Am. Rev. Pharmacol. Toxicol.*, 18: 461 (1978).
- Mann, S. P., *Proc. Br. Pharm. Soc.*, 62: 406 (1978a).
- Mann, S. P., *J. Neurochem.*, 34: 747 (1978b).
- Mann, S. P., *Biochem. Pharmac.*, 29: 1593 (1980).
- Marqoliash, E., *Biochem. J.*, 56: 529 (1954).
- Marholin, D., Pohl, R. E., Stewart, R. M. et al., *Pediatr. Res.*, 12: 179 (1978).
- McCaman, M. W. and Robins, E., *J. Lab. Clin. Med.*, 59: 885-890 (1962).
- McEwen, B. S., Davis, P. G., Parsons, B. and Pfaff, D. W., *Ann. Rev. Neurosci.*, 2: 65 (1979).
- McGee, M. M., Greenqard, O. and Knox, W. E., *Biochem. J.*, 127: 669 (1972).
- McKean, C. M., *Brain Res.*, 47: 469 (1972).

- McKean, L. M., Boqqs, D. E. and Peterson, N. A., J. Neurochem., 15: 235 (1968).
- McLean, J. H., Glasser, R. S. and Kostorzewa, R. M., Pharmac. Biochem. Behav., 13: 863 (1980).
- Menkes, J. H., Neurology (Minneap.), 18: 1003 (1968).
- Menkes, J. H. and Koch, R., in Handbook of Clinical Neurology, ed. P. J. Vinken and G. W. Bruyn, Elsevier/North-Holland Biomedical Press, Amsterdam: 29-51 (1977).
- Mosier, H. D., Jr., Grossman, H. J. and Dingman, H. F., Pediatrics, 36: 465 (1965).
- Meyer, E. M. and Lytle, L. D., Proc. West. Pharmacol. Soc., 21: 313 (1978).
- Miller, B. T. and Quay, W. B., Dev. Neurosci, 2: 149 (1979).
- Moss, A. R. and Schoenheimer, R., J. Biol. Chem., 135: 415 (1940).
- Nadler, H. L. and Hsia, D. U., Proc. Soc. Exp. Biol. Med., 107: 721 (1961).
- Naqastu, T., Biochemistry of Catecholamines: The Biochemical Method, University Park Press, Baltimore (1973).
- Naqastu, T., Levitt, M. and Udenfriend, S., J. Biol. Chem., 239: 2910 (1964).
- Naqastu, T., Sudo, Y. and Naqastu, I., J. Neurochem, 18: 2179 (1971).
- Nausieda, P. A., Koller, W. L., Weiner, W. S. and Klawans, H. L., Life Sci., 25: 521 (1979).
- Neff, N. H., Yang, H. Y. and Goridis, C. in Frontiers in Catecholamine Research, eds. Usdin, F. and Snyder, S., Pergamon Press, London: 1973.
- Nellhaus, G., Pediatrics, 41: 106, 1968.
- Neilsen, K. B., Wamberg, E., Weber, J., Lancet, 7: 1245 (1979).
- Niqam, M. P. and Labar, D. R., Brain Res., 179: 195 (1979).
- Oldendorf, W. H., Brain Res., 24: 372 (1970).
- Oldendorf, W. H., Arch Neurol., 28: 45 (1973).

- Oldendorf, W. H. and Szabo, J., *Am. J. Physiol.*, 230: 94 (1976).
- Paine, R. S., *Pediatrics*, 20: 290 (1957).
- Pardridge, W. M. and Oldendorf, W. H., *Biochim. Biophys. Acta*, 401: 128 (1975).
- Partington, M. W., Lewis, E. J. M., *J. of Pediatrics*, 62: 348 (1963).
- Partington, M. W., *Pediatrics*, 27: 465 (1961).
- Penrose, L. and Quastel, J. H., *Biochem. J.*, 31: 266 (1937).
- Perry, T. L., Urquhart, N., MacLean, J., Evans, M. E., Hansen, S. et al., *N. Engl. J. Med.*, 292: 1269 (1975).
- Peters, T. J., Heath, J. R., Jones, P. E. and Peacham, A. D., *Gut*, 16: 826 (1975).
- Peterkofsky, B., DiBlasio, R., *Anal. Biochem.*, 66: 279 (1975).
- Pfaff, D., *J. Comp. Phys. Psych.*, 73: 349 (1970).
- Prensky, A., Fishman, M. A., and Daftari, B., *Brain Res.*, 33: 181 (1971).
- Porcher, W. and Heller, A., *J. Neurochem.*, 19: 1917 (1972).
- Pratt, O. E. in Advances in Perinatal Neurology, vol 1: 21 (1979).
- Pueschel, S. M., Yeatman, S., and Hum, S., *J. Am. Diet. Assoc.*, 70: 506 (1977).
- Purdy, J. L. and Bondy, S. C., *Neuroscience*, 1: 125 (1976).
- Pycock, G.C. J., *Neuroscience*, 5: 461 (1980).
- Quentin, C. D., Behbehani, A. W., Schulte, F. J. and Neuhoff, V., *Neuropadiatrie*, 5: 138 (1974).
- Raiha, N. C. R., *Pediatr. Res.*, 7: 1 (1973).
- Ratner, A. and Adamo, N. J., *Neuroendocrinology*, 8: 26 (1971).
- Riggs, T. R. in Biochemical Actions of Hormones, ed. Litwack, G., vol. 1: 157, New York (1970).

- Riggs, T. R. and McKirahan, K. J., J. Biol. Chem., 248: 6450 (1973).
- Robinson, R. G. Science 205: 707 (1979).
- Robinson, R. G. and Coyle, J. T., Life Sciences, 21: 943 (1979).
- Robinson, R. G., Shoemaker, W. J., Schlumpf, M., Valk, T. and Bloom, F. E., Nature 255: 332 (1975).
- Robinson, T. E., Becker, J. B. and Ramirez, V. D., Brain Res. Bull., 5: 539 (1980).
- Rodier, P. M., Develop. Med. Child. Neurol., 22: 525 (1980).
- Rosenberg, L. E. and Sriver, C. R., in Duncan's Diseases of Metabolism, ed. P.K. Bondy and L.E. Rosenberg, 7th ed., vol 1: 465-653, W.B. Saunders, Philadelphia (1974).
- Salquero, L. F., Itabashi, H. H. and Allen, R. J., Trans. Am. Neurol. Assoc., 93: 274 (1968).
- Saller, C. F. and Zigmond, M. J., Life Sci., 23: 1117 (1964).
- Sands, J., Dobbing, J and Gratrix, C. A., Lancet, Sept. 8, 1979: 503.
- Saunders, N. R., Exp. Eye Res. Suppl., 523 (1977).
- Schalock, R. L., Psychol. Sci., 17: 191 (1969).
- Schapira, F., Dreyfus, J. C. and Schapira, G., Nature(London), 200: 995 (1963).
- Schoepp, D. D., Azzaro, A. J., J. Neurochem., 36(6): 2025 (1981).
- Sriver, C. R. and Clow, C. L., N. Engl. J. Med., 303: 1336 (1980a).
- Sriver, C. R. and Clow, C. L., N. Eng. J. Med., 303: 1394 (1980b).
- Sershen, H. and Lajtha, A., Exp. Neurol., 53: 465 (1976).
- Seta, K., Sershen, H. and Lajtha, A., Brain Res., 47: 415 (1972).
- Seymour, C. A. and Peters, T. J., Clin. Sci. and Mol. Med., 52: 229 (1977).

Shah, S. N., Peterson, N. A., McKean, C. M., J. Neurochem., 19: 2369 (1972).

Shih, V. E., Laboratory Techniques for the Detection of Hereditary Metabolic Disorders, CRC Press (1973).

Shoemaker, W. J. and Wurtman, R. J., Science, 171: 1017 (1971).

Shonk, C. E. and Boxer, G. F., Cancer Res., 24: 709 (1964).

Shonk, C. E., Morris, H. P. and Boxer, G. E., Cancer Res., 25: 671, (1965).

Simon, H., Scatton, B. and LeMoal, M., Nature (London), 286: 150 (1980).

Sims, K. L., Davis, G. A. and Bloom, F. E., J. Neurochem. 20: 449 (1973).

Smith, I., Lobascher, M. E., Stevenson, J. E. et al., Br. Med. J., 2: 723 (1978).

Smith, I., Endohazi, M., Macarthey, F. J. et al., Lancet I: 17 (1979).

Smith, I and Wolff, O. H., Lancet, 2: 540 (1974).

Stanbury, J. B., Wyngaarden, J. B. and Frederickson, D. S., The Metabolic Basis of Inherited Disease Fourth Ed., McGraw Hill, New York (1978).

Studelska, D. R. and Beatty, W. W., J. Comp. Phys. Psych., 92: 297 (1978).

Swaiman, K. F. and Lemieux, B., J. Neurochem, 16: 385 (1967).

Sved, A. and Fernstrom, J., Life Sciences, 29: 743 (1981).

Thomas, G. H., Parmley, T. H., Stevenson, R. E., and Howell, R. R., Am. J. Obstet. Gynecol., 111(1): 38 (1971).

Torchiana, M. L., Porter, C. C., Stone, C. A. and Hansen, H. M., Biochem. Pharmac., 19:1601 (1970).

Tourian, A. Y. and Sidbury, J. B., in The Metabolic Basis of Inherited Diseases, ed. Stanbury, J. B., Wyngaarden, J. B. and Frederickson, D. S., McGraw-Hill, New York:240-255 (1978).

Tourian, A. Y. and Sidbury, J. B. in The Metabolic Basis of Inherited Diseases, ed. Stanbury, J. B., Wyngaarden, J. B. and Fredrickson, D. S., McGraw Hill, New York (1983).

Turkewitz, G. in Lateralization in the Nervous System, ed. Harnad, L. et al., Academic Press, New York: 251 (1977).

Ungerstedt, U. and Arbuthnott, G. W., Br. Res. 24: 485 (1970).

Udenfriend, S. and Bessman, S. P., J. Biol. Chem., 203: 961 (1953).

Udenfriend, S. and Cooper, J. R., J. Biol. Chem., 196: 227 (1952).

Udenfriend, S., Zaltzman-Nirenberg, P. and Nagatsu, T., Biochem. Pharmacol., 14: 837 (1965).

Van Sande, M., Mardens, Y., Adriaenssens, K., and Lowenthal, A., J. Neurochem., 17: 125 (1970).

Vorhees, C. V., Butcher, R. E. and Perry, H. K., Neurosci. and Biobehav. Res., 5: 177 (1981).

Wada, Y. et al., Pediatr. Res., 6: 622 (1972).

Ward, I. L. and Weisz, J., Science, 207: 328 (1980).

Waisman, H. A. and Harlow, H. F., Science, 147: 685 (1965).

Watt, D. D. and Martin, P. R., Life Sci., 8: 1211 (1969).

Wapnir, R. A. and Dierks-Vertling, C., Biol. Neonate., 17: 1373 (1971).

Weeks, J. R. in Methods in Psychobiology, Academic Press, New York, 3: 155 (1972).

Weiss, B., Hui, M. and Lajtha, A., Biochem. Med., 78: 330 (1977).

Weil-Malherbe, H., J. Ment. Sci., 101: 733 (1955).

Williamson, M., Koch, R. and Berlow, S., Pediatrics, 63: 823 (1979).

Womack, M. and Rose, W. C., J. Biol. Chem., 107: 449 (1934).

Wong, P. W. K., O'Flynn, M. and Inouye, T., Clin. Chem., 12: 1098 (1964).

Wurtman, R. J., Scally, M. C., Gibson, C. J. and Hefti, F.
in Catecholamines: Basic and Clinical Frontiers, eds. Usdin,
E., Kopin, I. J., and Barchas, J. D., Pergamon Press, New
York: 64 (1980).

Wurtman, R. J. and Axelrod, J., *Biochem. Pharmac.*, 12: 1439
1963).

Zimmerberg, J., Greengard, O. and Knox, W. E., *Cancer
Research*, 35: 1009 (1975).