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Amino acid and biogenic amine studies in experimental hyperphenylalaninemia

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AMINO ACID AND BIOGENIC AMINE STUDIES IN EXPERIMENTAL HYPERPHENYLALANINEMIA

by

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ABSTRACT

AMINO ACID AND BIOGENIC AMINE STUDIES IN EXPERIMENTAL HYPERPHENYLALANINEMIA

by

Ruth E. McChesney

ADVISER: Dr. O. Greengard

The developmental profile of phenylalanine uptake into the brain was defined and its implications for amino acid levels and biogenic amine metabolism in experimental animal models of phenylketonuria (PKU) was investigated.

The technique of rapid intracarotid injection used to study cerebral amino acid influx at physiological blood level was rendered applicable to neonatal rats. The brain uptake index of phenylalanine, thus measured for the first time during the suckling period, was found to be significantly higher at the age of 4 than at 7 or 24 days, with no further decrease occurring after weaning. This developmental change in the blood brain barrier appears to be a factor in the age dependence of cerebral amino acid uptake from pathological blood levels. At the same sustained elevation of plasma phenylalanine concentration, the cerebral accumulation of phenylalanine was found to be greater during the first week of life than at any subsequent age.

Chronic experimental hyperphenylalaninemia during gestation or infancy was associated with elevations of the glycine concentration of the maturing brain, which

correlated quantitatively to increases in the activity of the serine biosynthetic enzyme phosphoserine phosphatase. Analysis of cerebral biogenic amines and metabolites showed that hyperphenylalaninemia maintained by administration of α-methylphenylalanine plus phenylalanine decreased the concentration of cerebral serotonin, 5-hydroxyindoleacetic acid, dopamine, homovanillic acid and norepinephrine. The increase of tryptophan found in the gastric contents (accompanied by elevated phenylalanine) with unchanged levels in peripheral tissues indicates that inhibition of gastrointestinal absorption may contribute to the diminished plasma tryptophan levels. However, inhibition by excess phenylalanine of the cerebral influx of tryptophan (rather than the low plasma levels) was responsible for the diminished brain concentration of tryptophan. This diminution underlies the indoleamine depletion of the brain, whereas the cerebral catecholamine depletion resulting from the action of phenylalanine on biosynthetic enzymes, can arise even at normal tyrosine levels. The methods found for the selective depletion and restoration of the biogenic amine deficits, the identification of a new suppressor of phenylalanine hydroxylase (β-methylphenylalanine), and study of the consequences of gestational and postnatal hyperphenylalaninemia in two different rat strains, provide additional experimental systems for study of the pathogenesis of cerebral maldevelopment in PKU.

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LIST OF ABBREVIATIONS

a-mephe.....alpha-methyl-DL-phenylalanine
b-mephe.....beta-methyl-DL-phenylalanine
BBB.....blood brain barrier
BUI.....brain uptake index
B.Wt.....body weight
cc.....cubic centimeter
cDNA.....complementary deoxyribonucleic acid
CNS.....central nervous system
CSF.....cerebrospinal fluid
DA.....dopamine
DBH.....dopamine-B-hydroxylase
DHBA.....dihydroxybenzylamine
DHPR.....dihydropteridine reductase
dl.....deciliter
DOPA.....3,4-dihydroxyphenylalanine
EC.....electrochemical detection
g.....gram
HPLC.....high performance liquid chromatography
HVA.....homovanillic acid
5HIAA.....5-hydroxyindole-3-acetic acid
5HT.....5-hydroxytryptamine or serotonin
Km .....reaction rate constant
MHPG.....3-methoxy,4-hydroxyphenylglycol
M.....molar
mCi.....milliCurie
mg.....milligram
min.....minute
ml.....milliliter
mm.....millimeter
mM.....millimolar
3MT.....3-methoxytyramine
NE.....norepinephrine
nmol.....nanomole
PAH.....phenylalanine hydroxylase
PCA.....perchloric acid
p-chlphe.....para-chlorophenylalanine
PE.....phenylethylamine
phe.....phenylalanine
PKU.....phenylketonuria
pmol.....picomole
s.c. .....subcutaneous
TCA.....trichloroacetic acid
trp .....tryptophan
tyr .....tyrosine
tyrME.....tyrosine methyl ester
ug .....microgram
ul.....microliter
um.....micrometer
umol.....micromole
v .....volume
w .....weight

CHAPTER 1

INTRODUCTION

In 1934 Folling described the excess urinary excretion of phenylpyruvic acid among a group of patients, several of them siblings, and noted the associated mental retardation, (Folling, 1934). Thus named phenylketonuria (Penrose & Quastel, 1937), PKU has come to be recognized as an inborn error of metabolism (Garrod, 1908), the consequence of which is severe mental and neurological disability. The studies of Jervis established an autosomal recessive mode of inheritance (Jervis, 1939) and that a deficiency of the enzyme phenylalanine hydroxylase was the underlying biochemical abnormality (Jervis, 1953). The introduction of a diet low in phenylalanine (phe) demonstrated that it was possible to reverse many of the adverse effects of PKU (Bickel et al, 1954). This dietary intervention together with the advent of the Guthrie microbiological inhibition assay of phe, (Guthrie & Susi, 1963) meant that once detected by routine neonatal screening, affected infants could receive diet therapy during childhood to prevent the mental retardation.

In the intervening years however, since initiation of neonatal screening programs and dietary therapy, a number of additional features have became apparent. The heterogeneity of hyperphenylalaninemia is now recognized (Kaufman et al, 1975), some of the variant forms showing severe neurological and mental deterioration despite the

low phe diet (Butler et al, 1978). With the attainment of childbearing age by treated phenylketonurics, yet another consequence has become apparent, in that offspring born to PKU mothers show a greatly increased risk of mental retardation (Dent, 1957) as well as other abnormalities (Lenke & Levy, 1980). This potentially teratogenic action of hyperphenylalaninemia during gestation is now a significant health problem.

PKU is found worldwide, however it is highest in Causasian populations of Celtic or Western European origin (Scriver & Clow, 1980). In addition to PKU being the most common heritable metabolic disorder in which the central nervous system (CNS) is susceptible to damage (Brady, 1976), it has been predicted that although the increase in PKU gene frequency from reproduction will have a negligible influence on the occurrence of mental retardation from PKU itself, the increased phenotypic effect from maternal PKU could lead to a rebound in the frequency of mental retardation (Kirkman, 1982). Thus despite the accumulation of biochemical and genetic information about PKU, the cause of the mental disability associated with PKU remains unknown and there are now new issues to be addressed in the current management of PKU such as the prevention of teratogenicity associated with maternal hyperphenylalaninemia and determination of the postnatal age at which to safely discontinue the phe-restricted diet.

Phenylalanine Hydroxylation: The Diagnosis and Classification of Hyperphenylalaninemia.

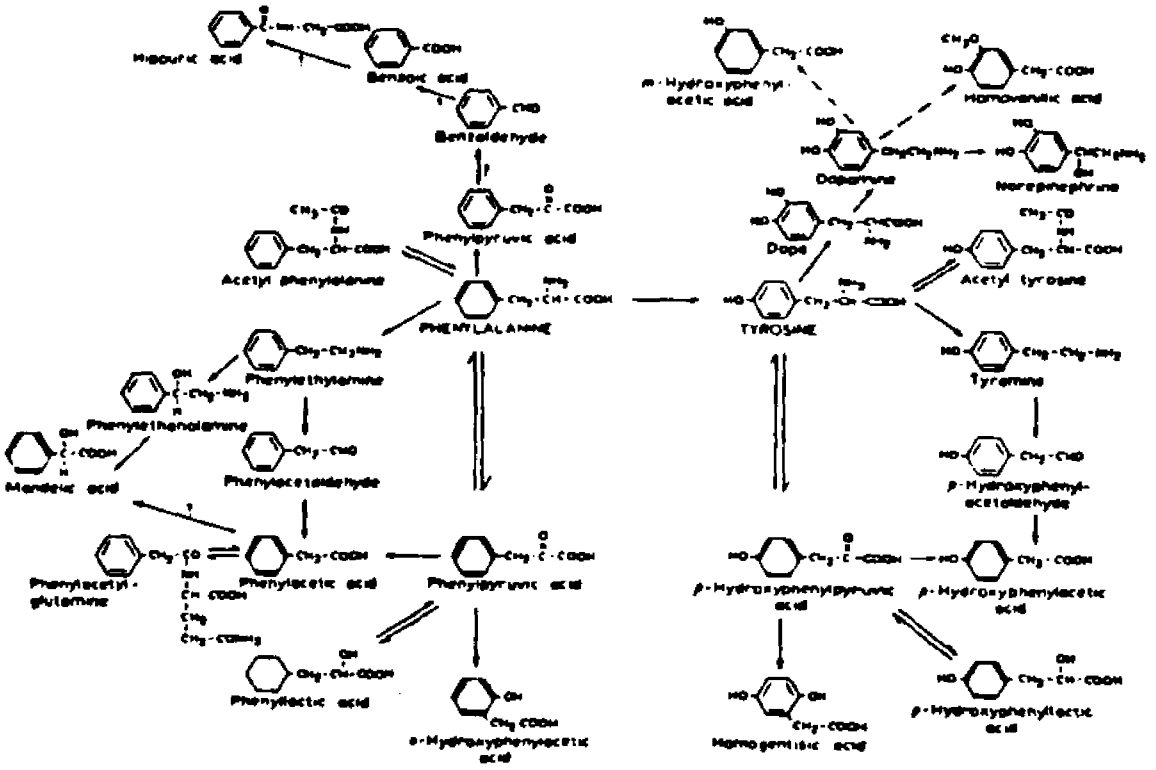
Phenylalanine hydroxylase (EC 1.14.16.1) (PAH) has been shown to be associated with a number of components among which are its natural coenzyme 5,6,7,8,-L-erythro-tetrahydrobiopterin and the enzyme dihydropteridine reductase (EC 1.6.99.7)(DHPR). The para-hydroxylation reaction utilizes stoichiometric amounts of L-phenylalanine (phe), tetrahydrobiopterin and oxygen, with formation of the products L-tyrosine, quinonoid dihydrobiopterin and water. This irreversible reaction also represents the first step in the major catabolic pathway of the benzene ring of phe (Embden & Baldes, 1913) and when blocked, normally minor pathways of side chain modification and conjugation become more pronounced giving rise to detectable levels of phe metabolites (Knox, 1972). As depicted in Figure I-1, these compounds can arise by transamination (phenylpyruvic acid) or decarboxylation (phenylethylamine) followed by oxidation (phenylacetic acid) and conjugation to form the more polar compounds phenylacetylglutamine (primates and humans) or phenylacetylglycine (e.g. rodents) (Meister, 1965; James et al, 1972).

Deficiencies of PAH activity can range from virtual absence of enzyme activity to a maturational delay in enzyme production, normally present in human fetal liver by the eleventh week of gestation and rising to adult

FIGURE I-1

PHENYLALANINE METABOLISM

As adapted from Curtuis et al, (1972), the conversion of phe to tyrosine by PAH is shown as well as other pathways of phe metabolism.



levels thereafter (DelValle & Greengard, 1977). DHPR plays a primary role in the regeneration of tetrahydrobiopterin from dihydrobiopterin and hence the PAH hydroxylation reaction is dependent on a functional biopterin synthetic pathway. Thus defects in a number of these components or reactions can prevent phe hydroxylation and lead to the phenotype of hyperphenylalaninemia. The understanding and hence classification of the heterogeneity of hyperphenylalaninemia has continuously undergone much revision since the introduction of the screening programs. The three principal categories (Scriver & Clow, 1980; Tourian & Sidbury, 1983), are due to deficiencies of PAH (Classical PKU), DHPR, and biopterin synthesis.

Unless the PKU infant is the offspring of a PKU mother and has been exposed to gestational hyperphenylalaninemia, the levels of phe are normal at birth and the PKU infant appears asymptomatic. Very shortly thereafter a steady increase in blood phe occurs reaching a maximum at 6 to 7 days (Holtzman et al, 1974). In addition to the increased levels of phenylketones, other biochemical abnormalities include anomalous levels of amino acids in the blood (Linnewah & Ehrlich, 1962), CSF (Van Sande et al; 1970; Quentin et al, 1974) and brain (McKean, 1972) and decreased indices of biogenic amine metabolism (Weil-Malherbe 1955; Pare et al, 1957). While PAH is present in the liver and also to a minor degree in

kidney, the other components of the hydroxylation system show a different tissue distribution from PAH. DHPR is present in several tissues including the brain and deficiency of this enzyme or a block in biopterin synthesis is associated with loss of cofactor essential also for the other aromatic amino acid hydroxylases; tyrosine hydroxylase (EC 1.14.16.2.) and tryptophan hydroxylase (EC 1.14.16.4.). The resulting diminished hydroxylation of tyrosine and tryptophan is thus associated with decreased levels of biogenic amines in biopterin deficiency (Butler et al, 1978).

The Molecular Genetics of PKU and Hyperphenylalaninemia:

PAH has been purified from rodent and primate livers (Kaufman & Fisher, 1970; Woo et al, 1974, Kaufman, 1983). Isolation of a rat liver complementary DNA (cDNA) for PAH was reported in 1982 (Robson et al, 1982). Subsequently the cloning and nucleotide sequence determination of a full-length human PAH cDNA was described (Kwok et al, 1985). Based on the deduced amino acid sequence, a 96% sequence homology of the human protein with the corresponding rat enzyme was predicted. The human PAH gene has now been assigned to chromosome 12 (Lidsky et al, 1984) and extensive nucleotide homology demonstrated for the three aromatic amino acid hydroxylases, PAH, tryptophan hydroxylase and tyrosine hydroxylase (Grennet et al, 1987). A number of restriction fragment length polymorphisms have been identified, mapped at the human

PAH locus and used to identify haplotypes of normal and PKU alleles (Lidsky et al, 1985). Some of the PKU alleles have now been sequenced and the mutations characterized (DiLella et al, 1986, 1987). A cDNA for DHPR has now also been isolated (Lockyer et al, 1987).

Postnatal Dietary Intervention in the Management of PKU:

Despite the continued efforts to improve screening, diagnosis can be missed in the early postnatal period and the consequent delays of treatment increase the risk of mental impairment. Treatment of PKU infants commenced within the very early postnatal period has led to an increase in IQ scores compared with later treatment (Guttler & Wamberg, 1977; Bickel,1987). Accurate diagnosis is also essential in order to initiate appropriate therapy and management, particularly in biopterin deficiency, where the low phe diet alone does not reverse the amine deficits. Therapy may include supplementation with the precursors 5-hydroxytryptophan and L-3,4-dihydroxyphenylalanine (L-DOPA) or more recently with purified tetrahydrobiopterin (Niederweiser et al, 1982). The dramatic clinical improvement demonstrated by the introduction of the low phe diet to PKU children has tended to overshadow exploration of whether or not some precursor supplementation should be also considered.

An increasingly important issue in the management of PKU is when to terminate the diet (i.e. during the first decade of life or later during the teenage years).

Conflicting reports in the literature show, on the one hand, attainment of normal IQ levels (Bickel & Grubel-Kaiser, 1982) or, conversely, a fall in IQ scores (Cabalska et al, 1977; Melnick et al, 1981). Only relatively recently have any studies attempted to correlate specific neuropsychological and social functioning tests with either plasma phe concentration and/or indices of monoamine metabolism in PKU children both prior to and following diet termination. Blood phe concentration at the time of testing in 27 PKU children was shown to predict performance in a battery of tests developed to detect subtle brain dysfunction; the tests included measurement of IQ, steadiness, concept formation and tactile-motor problem solution (Brunner et al, 1983). In 10 older treated PKU's also given neuropsychological tests in a cross-over design, a blood phe of greater than 1.3 mM correlated with a deterioration in performance in tests of higher integrative function (Krause et al, 1985). This impairment was also associated with diminished urinary dopamine (DA) and serotonin (5HT). In the only study where monoamine metabolites were measured, discontinuation of the diet led to an increase in plasma and cerebrospinal fluid (CSF) phe and was associated with reductions in the DA metabolite, homovanillic acid (HVA) and the principal metabolite of 5HT, 5-hydroxyindole acetic acid (5HIAA) (Lou et al, 1985). A linear relationship was found between tests of reaction time

variability and CSF 5HIAA, but the number of children in the study was only 4. Skills related to social functioning have also been shown to undergo deterioration upon diet discontinuation and found to be inversely correlated with phe levels in the blood (Matthews et al, 1986). In the large collaborative study reported recently of 119 10 year old PKU children who had commenced the restricted phe diet before the age of 65 days, the age at which the control of blood phe was lost and persistently exceeded 15 mg/ml (0.9 mM) was the best and often only predictor of both IQ at age 8 or 10 years and the deficit in the respective IQ when compared with siblings or parents (Holtzman et al, 1986). This age of dietary control loss was the best predictor of the difference in scores from unaffected siblings on the Wide Range Achievement Test of PKU children at 8 years and also of any behavior problems. The authors also commented on the fact that for spelling and arithmetic subscores, children with a very recent termination of dietary control demonstrated as great a deficit as those with loss at an earlier age, indicating that the effects of phe elevation may be more immediately apparent on achievement tests than on IQ scores. A difficulty with mathematics has been observed previously (Berry et al, 1979) and is further supported by a study of school-age early treated PKU children, most of whom had discontinued the diet (Pennington et al, 1985). Although there was not a

consistent pattern of cerebral lateralization, deficits were consistently observed in two neuropsychological domains which could be related to conceptual and visuospatial skills. Diet termination at 7 to 8 years was not found to alter pre-existing EEG patterns which were markedly abnormal when treatment was delayed and less so in early treated children (Behbehani, 1985). This rapidly accumulating evidence pointing to mental deterioration has led to recommendations that patients resume dietary phe restriction. However reinstitution has been described as a "struggle" (Barabas et al, 1984; Barabas & Matthews, 1986) or difficult and often unsuccessful (Michals et al, 1985). The need to identify children who are particularly vulnerable to the effect of phe has been emphasized (Barabas & Matthews, 1986). It has been recommended to keep children on a strict diet until the age of 10 or older, followed then by a somewhat higher phe intake on a relaxed but still protein-restricted diet to keep the blood of phe less than 20 mg/dl (Bickel,1985).

Very little is known about the effect of supplementing the diet with amino acids which may be at reduced levels in the brain, especially tyrosine and tryptophan, or other biosynthetic precursors of the catecholamines and 5HT as L-DOPA and 5-hydroxytryptophan. One study has demonstrated the feasibility of this therapy but because no age-matched control values were given for comparison, it cannot be concluded that the basal levels

of biogenic amines, although raised, were in fact restored to normal (Lou, 1985).

Maternal PKU:

The deleterious consequences of maternal PKU which have been demonstrated in non-PKU offspring are mental retardation, congenital abnormalities, intrauterine growth retardation and microcephaly (Levy et al, 1980). The range and extent of the teratogenic effect of hyperphenylalaninemia appears similar to that of other teratogens with a spectrum of abnormalities, the most severe being embryolethality (Wilson, 1977). A correlation between maternal blood phe levels and the abnormalities observed in her offspring has been noted (Lenke & Levy, 1980), particularly the occurrence of mental retardation (Levy & Waisbren, 1983). Reduction of phe intake during pregnancy results in some diminution in the chance of intrauterine damage but diet therapy initiated before conception has proven to be a more efficacious means of reducing the risk (Lenke & Levy,1980; Levy, 1981; Drogari et al, 1987; Levy,1987).

Animal Models of PKU and Hyperphenylalaninemia:

In the absence of an animal strain carrying a characterized genetic defect in PAH , efforts have been directed toward the establishment of an experimental model of PKU (Karrer & Cahilly, 1965; Vorhees et al, 1980). Criteria for the establishment of such a model include a

sustained hyperphenylalaninemia with blood phe levels equal to or greater than 15 mg/dl and a concomitant high blood phe:tyrosine ratio. In order to mimic the early postnatal onset of PKU, induction of hyperphenylalaninemia during the period of neonatal development is considered appropriate, and the study of any enduring effects, particularly behavioral anomalies, is performed after termination of the treatment. Finally the specific effects of hyperphenylalaninemia 'per se' should be assessed to preclude possible nonspecific effects on parameters such as growth and weight.

Studies of hyperphenylalaninemia in animal models have included administration of phe itself, (Auerbach et al, 1958) or phe given along with various analogs of phe known to inhibit PAH. The introduction of para-chlorophenylalanine (p-chlphe) as an effective inhibitor of the enzyme (Lipton et al, 1967), paved the way for a number of studies using this chemical but problems of toxicity and decreased body growth have hampered interpretation of many of the biochemical and behavioral results. In 1976 α-methylphenylalanine (a-mephe) was introduced as an effective 'in vivo' suppressor of PAH activity (Greengard et al, 1976). Its non-toxicity and feasibility of use has led to the establishment of a regimen of a-mephe supplemented with phe for the induction of hyperphenylalaninemia during the postnatal (DelValle et al, 1978; Greengard et al,1979; Lane & Neuhoff, 1980) and

gestational periods (Brass et al,1982; Spero & Yu, 1983; Sadava & Sutcliffe, 1988). Using this model, treatment during the first three weeks of life has been shown to lead to permanent behavioral anomalies including learning ability, locomotor activity and an abnormality in lateralization of circling activity (Luttges & Gerren, 1979; Glick & Greengard, 1980; Lane et al, 1980; Brass, 1983; Strupp et al,1984).

Investigation of the Cerebral Deficit in PKU and in Animal Models

In addition to the mental retardation in PKU, there may also be manifestations of disturbed behavior such as hyperactivity and irritability, convulsions, abnormal EEG patterns and other neurological conditions (Knox, 1972). With the exception of the mental retardation, almost all of these symptoms are reversible upon reduction of the phe intake. Findings of microcephaly and decreased brain weight in human autopsy PKU brain have also been demonstrated in animal studies of chronic hyperphenylalaninemia (Chase & O'Brien, 1970; Lane et al, 1980; Brass et al, 1982). Defective myelinization was the most consistently observed early histological observation in human PKU (Alvord et al, 1950; Crome et al, 1962; Malamud, 1966), but there has been neither consistency nor agreement as to the exact nature of the abnormality (Knox, 1972), and it has been suggested that these changes may be in fact secondary or nonspecific. A number of studies in

animals have also investigated the composition of myelin in the brain in chronic hyperphenylalaninemia (Shah et al, 1972; Berger et al, 1980; Lane et al, 1980; Figlewicz & Druse, 1980), however, unless the hyperphenylalaninemia is coincident with the proliferation of glial cells and the production of myelin, the interpretation of these results may be incorrect. Exposure at a later postnatal age was reported to result in the decrease of total myelin (Hommes et al, 1982).

Many theories have been put forward for the underlying cerebral damage in PKU but to date the reason remains obscure (Rosenberg & Scriver, 1980; Scriver & Clow, 1980; Tourian & Sidbury, 1983). In addition to defective myelinization, other hypotheses include neurotoxic effects of phe metabolites and consequences of altered cerebral amino acid levels affecting protein and biogenic amine (especially neurotransmitter) synthesis or in the level of specific amino acids such as glutamine. It has also been suggested that rather than a single cause, the damage represents the outcome of a number of chemical abnormalities occurring within the brain as a consequence of the deranged internal milieu present during critical phases of development (Menkes, 1967). The concept of a 'vulnerable period' has stressed the susceptibility of the developing brain to insults incurred during a time when the processes of cell division, synaptogenesis and

Alternatively there may be a time during development when the brain is exposed to a greater degree of the insult. A preliminary study in the case of phe has suggested that, as it matures, the developing brain progressively diminishes its capacity to accumulate phe, (Greengard & Brass, 1984) but the underlying mechanism(s) is presently unknown.

The unique control of substance entry into the brain has been shown to be linked to the presence of the 'blood brain barrier'(BBB), the anatomical basis of which is the endothelial cell of the brain capillary (Reese & Karnovsky, 1967). The presence of tight junctions and lack of pinocytotic vesicles are consistent with the presence of exclusionary function. Although considerable maturity of the 'barrier' is already present at birth, as the brain develops the 'barrier' has been shown to become more exclusive. The presence of transport mechanisms via saturable, stereospecific carrier systems is believed to regulate the uptake and hence permeability of amino acids (Christensen, 1953; Oldendorf & Szabo, 1976; Pardridge, 1983). Consistent with a competitive effect of phe on other large neutral amino acids sharing the same transport system, decreased cerebral levels of amino acids of this L system (namely tyrosine, tryptophan, methionine, histidine and the branched chain amino acids leucine, isoleucine and valine) have been demonstrated in human autopsy samples (McKean, 1972) and in animal models of

hyperphenylalaninemia (McKean et al, 1968; Lowden & LaRamee, 1969; Greengard et al, 1979; Isaacs & Greengard, 1980, Lane et al, 1980). In untreated PKU, decreased uptake into the brain of radiolabelled selomethionine has also been shown (Oldendorf, 1973).

Another suggested effect of hyperphenylalaninemia through a reduction in amino acids entering the brain, is interference with protein synthesis both as a direct effect on steps of ribosomal protein synthesis, (Taub & Johnson, 1975; Binek et al, 1981) and as an indirect effect through competition by phe with the uptake of other amino acids sharing the same L transport system (Oldendorf, 1973). Treatment with a mixture of large neutral amino acids was shown to prevent the decreased brain protein synthesis despite the continued presence of hyperphenylalaninemia in both acute (Hughes & Johnson, 1978) and chronic studies (Binek-Singer & Johnson, 1982).

Evidence has been slowly accumulating that hyperphenylalaninemia exerts a direct effect on the neuron itself causing delayed development. One human autopsy study has indicated retardation in development of the cortical plate, as shown by increased cell packing density and reduced nerve cell size (Bauman & Kemper, 1982). Among changes reported from postnatal animal studies have been a decreased density of cortical synaptic terminals (Nigam & Labar, 1979), and in one study of gestational hyperphenylalaninemia, reduced thickness of the cortical

plate accompanied by increased packing density and decreased size of post-mitotic neurons (Spero & Yu, 1983).

Concomitantly with the hyperphenylalaninemia, the concentration of many other amino acids in the blood may decrease (Linnewah & Ehrlich, 1962; Efron et al, 1969). This is apparently not due to altered dietary intake or to increased renal excretion but has been postulated to reflect a complex interaction of phe with other amino acids in tissues, some support for which was shown by a recent finding of altered amino acid profiles in periphal tissues in chronically hyperphenylalaninemic rats (Huether et al, 1984). The observation of low plasma glutamine levels in a retarded phenylketonuric, a feature which was not observed in his non-retarded PKU sibling, (Perry et al, 1970) was shortly followed by another study in which plasma levels of glutamine were also decreased (McKean & Peterson, 1970). However the levels of glutamine in the CSF and brain of retarded phenylketonurics in this latter study were found to be increased. In view of the conjugation product phenylacetylglutamine being formed from phenylacetic acid and glutamine in greatly increased amounts in PKU (Meister et al, 1965), these observations taken together have formed the basis for implicating an abnormality in glutamine as a cause of the cerebral abnormality (Blau, 1979; Rosenberg & Scriver,1980). In rats rendered hyperphenylalaninemic by treatment with a-mephe plus phe, the metabolites of phe formed are similar

to those in PKU (Lane et al, 1980) except that the principal conjugate formed is phenylacetylglycine (Meister, 1965; James et al, 1972). Among changes in cerebral amino acids in this animal model is a persistent elevation in glycine (Isaacs & Greengard,1980; Lane et al, 1980) and an increased activity of the enzyme phosphoserine phosphatase (PSP) (DelValle et al, 1978; Issacs & Greengard, 1980). The role of phe metabolites in the pathogenesis of the mental defect remains controversial (Blau, 1979; Rosenberg & Scriver, 1980; Tourian & Sidbury, 1983). Little is known about the exact biochemistry of their formation and tissue levels in PKU (Blau,1979).

Biogenic Amine Metabolism in Hyperphenylalaninemia:

The possibility of altered biogenic amine metabolism in PKU was suggested following findings of decreased blood epinephrine and norepinephrine (Weil-Malherbe, 1955; Nadler & Hsia, 1961), 5HT and 5HIAA (Pare et al, 1957) and decreased urinary vanillyl mandelic acid (Cession et al, 1966). By lowering blood phe it was demonstrated that many of these symptoms could be reversed.

Experimental animal studies during this time also provided evidence that high phe could lead to a reduction in 5HT in the brain (Yuwiler & Loutit, 1961, McKean et al, 1968). This was subsequently postulated to be a result of competitive inhibition of tryptophan (or 5-hydroxytryptophan) transport into the brain, (McKean et

al, 1962; Yuwiler et al, 1965) or prevention of hydroxylation of tryptophan (Yuwiler et al,1965, Lovenberg 1968). Inhibition of tyrosine hydroxylase activity by phe was also shown (Nagatsu et al, 1964; Levitt et al, 1965).

Autopsy studies of human phenylketonuric brain in 1972 provided the first direct evidence of disturbed cerebral biogenic amine levels (McKean, 1972). The concentrations of 5HT, DA and NE were decreased to 30-40% of normal values and the levels of tryptophan and tyrosine in the same tissue showed reductions of 40-50%. A lowering of phe intake was shown to reverse the decreases in metabolite levels. In DHPR deficiency, autopsy studies have also found disturbance of amine levels in the brain (Butler et al, 1978). Studies of biogenic amine metabolism in patients with both PKU and Hyperphenylalaninemia due to biopterin deficiency, have shown that the diminished levels of metabolites such as homovanillic acid (HVA) when the phe levels were high could also be reversed (Curtius et al, 1972; Butler et al, 1981).

Using the regimen of a-mephe plus phe in postnatal animal studies, reductions in cerebral 5HT (Isaacs & Greengard, 1980; Lane et al,1980; Taylor et al,1983;) and catecholamines (Brass & Greengard,1982; Taylor et al, 1983) have also been observed. Although there have been no reports of altered intrauterine indole or catecholamine

metabolism associated with human maternal PKU, preliminary studies in animals have indicated that fetal 5HT (Brass et al, 1982) and catecholamines (Brass, 1983) are also reduced. The relationship between the degree of inhibition of tyrosine hydroxylase and the catecholamine deficit has drawn attention to the importance of phe levels in the brain as a modulator of this effect (Brass & Greengard, 1982), and may explain why in another study where cerebral phe concentrations were not adequate (Lane et al, 1980), the catecholamines failed to show a decrease. The fact that it is still possible to see a decrease in monoamines at an age in hyperphenylalaninemic rats equivalent to the age at which dietary restriction of phe is being relaxed in humans, suggests that the potential vulnerability of the brain may persist longer than originally estimated (Taylor et al, 1983). Although reductions in both DA and NE have been observed in these two studies, at present there is relatively little information available about the NE deficit. Measurement of the activity of the first two enzymes in the biosynthetic pathway of the catecholamines, tyrosine hydroxylase and dopa decarboxylase (Brass & Greengard,1982; Brass, 1983) have been made but no information is available on the effect of chronic hyperphenylalaninemia on dopamine B hydroxylase (DBH) activity, the enzyme responsible for NE biosynthesis.

The 5HT reduction in hyperphenylalaninemic rats has

been shown to be related to the level of tryptophan in the brain (Greengard & Wolfe, 1987). But it is not known whether the reduced brain tryptophan is a result of the decreased blood levels in hyperphenylalaninemia as has been suggested (Huether et al, 1984) rather than the generally accepted mechanism of competition for uptake at the BBB (Oldendorf, 1973). Under various physiological conditions the pool of tryptophan in the brain available for 5HT synthesis apparently depends somewhat on the supply from the blood (Fernstrom, 1983).

It is known that the deficits in biogenic amines in PKU and chronically hyperphenylalaninemic rats are reversible by withdrawal of phe but that the mental disability is permanent. The hypothesis that biogenic amines may serve non-transmitter roles during early brain cell development has been gradually gaining both direct and indirect support (Black, 1982). Altered onset of neuronal differentiation in fetal rat brain as a result of 'in utero' treatment with p-chlphe, a 5HT depletor, was localized to brain regions known to contain 5-HT terminals or to have a high 5HT level in the adult (Lauder & Krebs, 1978; Lauder, 1983). Thus the model has been proposed that 5HT in developing neuronal axons acts as a differentiation signal regulating the time of neuronal genesis in cells later recognized as the appropriate targets for synaptogenesis. One problem with interpretation of this study is that restoration of the 5-HT levels by precursor

in the presence of p-chlphe was not performed in order to eliminate an effect of the analog itself. Nevertheless indirect evidence for some important pre-transmitter neuromodulatory function of various biogenic amines is accumulating (Buznikov, 1984). The appearance of catecholamines early in fetal development in both neuronal and non-neuronal structures is further support for a function during growth and differentiation. The implications of this neuronal effect are of significance in view of the recent morphological studies demonstrating cell changes in PKU and hyperphenylalaninemia in animals and the known alterations in levels of 5HT and catecholamines.

The aim of this research is thus to investigate factors which have been implicated in the relationship of gestational and postnatal hyperphenylalaninemia to the changes in amino acid and biogenic amine metabolism and to the permanent neurobiological impairment. By characterization of mechanisms involved in these abnormalities, further insight may be gained into the pathogenesis of developmental defects associated with other syndromes. The following chapters describe studies using experimental models of hyperphenylalaninemia designed to elucidate mechanisms involved in amino acid and biogenic amine metabolism and to study how uptake and accumulation of phe by the developing brain may play a key role in modulating subsequent abnormalities.

CHAPTER II

METHODS

Chemicals:

p-chlorophenylalanine (p-chlphe) and β-methyl-DL-phenylalanine hydrochloride (b-mephe) were purchased from Aldrich Chemical Company, Inc, Milwaukee, Wisconsin. α-methyl-DL-phenylalanine (a-mephe) and all other amino acids, amino acid derivatives, biogenic amines and metabolites were obtained from Sigma Chemical Co., St. Louis, Missouri. All radiochemicals used were of the highest specific activity available. L-[^{14}C(U)]-phenylalanine (500 mCi/mmol), L-[^{14}C(U)]-tryptophan (564 mCi/mmol), L-ring-3,5-^{3}H-tyrosine (50 Ci/mmol), ^{14}C carboxyl d,l,3-4 dihydroxyphenylalanine (49.4 mCi/mmol), ^{3}H water (1.0 mCi/g) and D-^{14}C(U)-glucose (329 mCi/mmol) were purchased from New England Nuclear Nuclear, Boston, Massachusetts. (1-^{14}C)n-Butanol (4 mCi/mmol) was purchased from ICN Radiochemicals, Irvine, California and inulin (^{14}C carboxylic acid) (2.2 mCi/g) from Amersham, Arlington Heights, Illinois.

Animals and Treatments:

Rats used in these studies were of the albino Fischer 344 (CDF) strain (Charles River Breeding Laboratories, Wilmington, Massachusetts) or the Long Evans Hooded strain (Blue Spruce Farms, Altamont, New York). The Fischer rats were used as a continuation of the originally developed model of experimental PKU (Greengard et al, 1976). The

use of the Long Evans strain for possible future experimental models of neurochemical deficiency was initiated following the demonstration of permanent cognitive deficits in adult rats of this strain which had been exposed to chronic hyperphenylalaninemia during the suckling period (Strupp et al, 1984). All animals were housed under conditions of 12 hours light/12 hours darkness with food (Purina Rodent Lab Chow) and water 'ad libitum'. Gestational age was determined following timed-matings, and the presence of a sperm-positive smear thus designated as day 1 of pregnancy. For pregnancies which came to term, the day of birth served as day zero. Suckling rat pups were weaned at 21-22 days of age.

For fetal studies of hyperphenylalaninemia, Purina Lab Chow was given to pregnant dams until the 12th day of gestation at which time the experimental rats were changed to a diet of the same chow containing 0.5% (w/w) a-mephe and concentrations ranging from 2.5 to 7.0% (w/w) phe depending upon the protocol.

In postnatal studies, intralitter variation was minimized by dividing litters equally on the basis of sex and body weight into control and experimental groups. All injection solutions were prepared in 0.9% NaCl, subjected to sonication and gentle heating if necessary and the pH adjusted to 7.2. According to the length of treatment protocol in the chronic hyperphenylalaninemic studies, daily subcutaneous injections were given in which

controls received 0.2 ml/10g body weight (B.Wt.) of 0.9% NaCl and the experimentals 0.2 ml/10g B.Wt.of a solution containing 12 mM a-mephe (2.4 umol/g B.Wt) plus 26 mM L-phe (5.2 umol/g B.Wt.) (DelValle et al, 1978). At weaning, the injection regimen was replaced by a dietary regimen of Purina rodent lab chow containing 0.5% a-mephe (w/w) plus 3.0% phe (w/w). B-mephe was also administered as 2.4 umol/g B. Wt. P-chlphe was injected as 0.9 umol/g B.Wt., but because of solubility difficulties, p-chlphe and phe (5.2 umol/g B.Wt.) were always injected as separate solutions. For studies of net accumulation of phe in the brain following induction of a sustained hyperphenylalaninemia, rats received a subcutaneous injection of a-mephe (2.4 umol/g B. Wt). L-phe was then administered 20 to 24 hours later in incremental amounts according to age and body weight. Other amino acids (except glycine) were also injected in amounts of 2.6 or 5.2 umol/g B.Wt. as indicated. Glycine was administered in a single dose of 7.7 umol/g B.Wt.(deGroot et al, 1979). Tyrosine supplementation was carried out using solutions of both L-p-tyrosine (5.2 umol/g B.Wt.) and the more soluble DL-p-tyrosine methyl ester (5.2 & 6.0 umol/g B.Wt.) (Oishi & Szabo, 1984). Phenylethylamine (PE) was injected at a dosage of 100 ug/g B.Wt.(Fuxe et al., 1967).

Behavioral Studies:

Individual rotometer measurements of spontaneous rotation were made during the 18 hour test period covering

6 hours light and 12 hours of darkness. The rotometer (Greenstein & Glick, 1975), permitted photoelectric differentiation between full (360^{o}) and quarter turns (90^{o}) to the left or to the right. For each rat, full rotations were measured as well as their sum (total full rotations) and their difference (net rotations). The direction in which more full rotations were made was designated as the direction of rotational preference and the percent preference calculated as net/total rotations x 100. 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 and well as the sum (total extra quarter turns) for each rat tested (Brass, 1983). In other studies, as indicated, parameters of spontaneous activity were quantified over the first 6 hours of the dark period using a Digiscan Animal Activity Monitor (Omnitech, Columbus, Ohio) with 16 x 16 inch plexiglass arena and a video camera. This enabled measurement of exploratory behavior, spontaneous rotations and hole poke activity (25 holes).

Brain Uptake Studies Using Intracarotid injection:

The uptake of radiolabelled tracer material was determined using modifications of the procedure of Oldendorf (1970). Animals were anesthetized with an intraperitoneal injection of Nembutal (25 ug/g B.Wt.) or Urethane (780 ug/g B.Wt.) They were positioned supine on

a rodent surgery board under a dissection microscope (Leitz) and the right common carotid artery was surgically exposed. A mixture containing ^{14}C amino acid or appropriate control substance (^{14}C Butanol or ^{14}C Inulin) together with ^{3}H Water as a diffusable reference standard, was prepared in Ringer's solution and buffered to pH 7.5.

The amount of tracer solution injected and syringe and needle sizes were standardized depending upon the age of the rats and were based on the principles used by Cornford et al.(1982). Rats of 24-70 days received 100 ul of the isotope mixture using a 1 cc tuberculin syringe fitted with a 28 gauge needle. The same syringe and needle sizes were used in 19-20 day old animals with 50 ul of solution delivered. In the 13-18 day range, 20 ul was injected using a 1/2 inch long, beveled 33 gauge needle attached to a 50 ul glass syringe (Hamilton Co., Reno, Nevada). For the smallest neonatal rats under the age of 13 days, borosilicate glass pipettes (1.5 mm O.D., 0.75 mm I.D., Frederick Haer, Brunswick, Maine) were attenuated on a micro-electrode puller, and then broken under a microscope to allow aspiration of 5 ul of the injectate. Lumen size of tips was standardized and only those with similar inside diameter were used. Delivery of the solution was accomplished by attaching the pipette to a 10 cc syringe via flexible tubing (Technicon) and lowering it into the artery with a micromanipulator.

Decapitation in all age groups was performed 10

seconds after injection. Approximately 0.2 g of ipsilateral frontal cortex of the brain was removed and placed in a glass scintillation vial. 1.5 ml of Protosol was added and the tissue homogenized by aspiration back and forth into a syringe attached to a 19 gauge needle. The vials were then covered with polyethylene-lined caps and placed in a water bath at 55°C for 2 hours, with occasional vortexing. After cooling, 10 mls of Econofluor were added and 1 hour later the $^{14}C/^{3}H$ ratio determined using a Packard Scintillation Counter. A standard of the tracer solution was prepared by adding 10 ul of the isotope mix to 0.2 g of unlabelled brain tissue and assaying in the aforementioned manner. For injection into the left ventricle of the heart, a small overlying rib section was removed and the tracer mixture given through a 26 gauge needle inserted about 1.5 mm into the left ventricle (Sershen & Lajtha, 1976). The procedures for decapitation and sample preparation were then followed as described above.

To calculate the Brain Uptake Index (BUI), the ratio of $^{14}C/^{3}H$ in the brain tissue was divided by the ratio of $^{14}C/^{3}H$ in the injectate; the BUI of inulin (at the corresponding age) was then subtracted in order to correct for the residual radioactivity remaining in the capillary space and the value converted to a % (Oldendorf, 1970; Pardridge, 1983).

Metabolic Radioactive Tracer Studies:

Rats which had received a standard daily dose of a-mephe plus phe or saline from the age of 2 to 10-12 days were given 5 uCi of U-^{14}C glucose (329 mCi/mmole) i.p. and 10 minutes later brain tissue was removed. The concentrations and radioactivities of amino acids were determined by the method of Shank and Aprison (1970a).

Biochemical Determinations:

All measurements were made at the specified ages and times after the last injection or treatment. Animals were sacrificed by decapitation and trunk blood collected in heparinized capillary tubes or microvettes (Sarstedt, West Germany). When indicated, tail blood was taken from pregnant rats under light ether anesthesia. The blood was centrifuged for 5 minutes in a microhematocrit or microcentrifuge, the plasma separated, deproteinized in trichloroacetic acid (TCA)(final concentration 0.3 M) and centrifuged for 20 minutes at 20,000xg. The supernatants were then separated and retained for analysis. Tissues were removed immediately, rinsed in saline and dissected. Skeletal muscle was taken from the gastrocnemius and gluteus maximus. The stomach and contents were separated and analysed separately. Small intestine was separated at the junction of the stomach and duodenum. The first 10 cm was removed and cleaned by saline irrigation. Homogenization was carried out in TCA (final concentration 0.3M) using a Polytron. Whole brains were also homogenized

in TCA, or for simultaneous enzyme and amino acid determination in 4 volumes of distilled water. An aliquot was removed for deproteinization in TCA to give a final concentration of 0.3 M. When required, brain was dissected into frontal cortex, mid-brain or striatum (Glowinski & Iversen, 1966). After homogenization of the tissue in TCA (final concentration 0.3M) and centrifugation at 20,000xg the supernatants were separated for analyses.

Amino Acid Analyses:

Amino acid concentrations were determined using either a Beckman 120C automatic amino acid analyser or more routinely using separate fluorometric and spectrophotometric quantifications as described. Non-specific tissue blank corrections were subtracted from the readings and recoveries of added standard ranged from 96 to 100 %. Addition of 'in vitro' amounts of a-mephe and/or phe equivalent to or greater than those present in tissues of hyperphenylalaninemic animals did not interfere with any of the following spectrophotometric or fluorometric procedures.

Phenylalanine:

The measurement of phenylalanine was made using the flourometric method of McCaman & Robins (1962) as described by Faulkner (1965).

Tyrosine:

Tyrosine was determined by the fluorometric method of Wong (1964) based on the spectrophotometric assay of Udenfriend & Cooper (1952).

Tryptophan:

Tryptophan determination was based on the original method of Denckla & Dewey (1967) but used a correction for light interference (Lehman, 1971) and a modification in the addition of $FeCl_3$ as revised and described by Bloxam & Warren (1974).

Glycine:

Brain tissue supernatants were prepared by addition of TCA to distilled water homogenates and centrifugation at 105,000 x g for 30 minutes. The glycine content was determined by a micromodification of the method of Goodwin & Stampwala, (1973). Final concentrations in 200 ul were 0.1 M H_2SO_4, 0.2% chloramine-T plus 0.15 M TCA to which deproteinized supernatant was added. After heating at 100^{o}C for 10 minutes, followed by rapidly cooling, 1.8 ml of chromotropic acid reagent in H_2SO_4 was added and the tubes heated at 100 ^{o}C for 30 minutes. Absorbance at 570 nm was then determined. To ensure against a possible circadian fluctuation in rodent cerebral glycine (Ross et al, 1980) samples for glycine measurements were taken at the same time (10-11 a.m.) each day.

Biogenic Amine and Metabolite Determination:

Using a Sorvall Omnimix, brain tissue was homogenized (final concentration 0.05 M perchloric acid, 0.1% cysteine and 0.5 uM dihydroxybenzylamine (DHBA) as internal standard) and centrifuged at 20,000 x g for 10 minutes. The supernatant was separated and if not analyzed immediately was maintained at -20^{o}C for no longer than two days. High Performance Liquid Chromotography (HPLC) with electrochemical detection (EC) was utilized for the determination of biogenic amines and metabolites. A Beckman Model 322 Liquid Chromatograph was equipped with a LC 4B Amperometric Detector and glassy carbon electrode (Bioanalytical Systems, Indiana). Using a 5um C18 Octyldecylsepharose column (ODS)(4.6 x 250 mm, Beckman) fitted with a Biophase C18 ODS guard column (Bioanalytical Systems, Indiana), reverse phase separation was carried out at a flow rate of 1.0 ml/min under isocratic conditions at an ambient temperature of 26^{o}C. The composition of the mobile phase was 0.15 M monochloroacetic acid, 1.0 mM sodium octyl sulfate, 0.75 mM disodium ethylenediamine tetraacetic acid, pH 2.8 with 10% methanol (v/v) (Mayer & Shoup, 1983; Warnhoff, 1984). By adjustment to these concentrations of ion pair reagent and organic modifier, the separation system was reproducible and the retention times obtained for each species reflected a compromise between prevention of a prolonged period of separation affecting the elution of

5HT and the avoidance of the co-elution of norepinephrine (NE) and 4-hydroxy, 3-methoxyphenylglycol (MHPG). Before use, the mobile phase was filtered using a 0.2 u Nylon Membrane (Schleicher & Schuell, New Hampshire) and degassed by stirring under vacuum. Prior to analysis, all samples were filtered through 0.2 u nylon Cameo filters (Scleicher & Schuell) and 20 ul of supernatant was routinely injected for HPLC analysis. The peak areas for all substances measured were individually adjusted to the peak area obtained for a known concentration of added dihydroxybenzylamine (DHBA). An oxidation potential of 700 mV vs a Ag/AgCl reference electrode was routinely used to avoid the detection of L-o-, m- and p-tyrosines. All three isomers were found to elute at the same retention time, close to that of DA, and were detected at higher oxidation potentials. There was also no detection of a-mephe, p-chlphe, b-mephe, PE or phe and the presence of these substances at equivalent 'in vivo' concentrations added to standards did not alter the qualitative or quantitive HPLC responses. Since many more substances were detected in the brain samples than were actually quantified, precautions were taken to ensure the identity of each peak and that there were no coeluting compounds at the same retention times as those of interest. In order to achieve maximum peak specificity, HPLC runs were repeated at selective oxidation potentials and with different concentrations of ion pair reagent and methanol

(±0.5%) to modify retention times.

Enzyme Assays:

Phenylalanine Hydroxylase (EC 1.14.16.1)

The activity of phenylalanine hydroxylase (PAH) was measured by the method of McGee et al,(1972). 10% post-mitochondrial supernatant fractions of liver in 0.15 M KCl were incubated in a total volume of 1.0 ml containing (final concentration), 100 mM potassium phosphate buffer pH 6.8, 10 mM phenylalanine, 0.75 mM 6,7,-dimethyl-5,6,7,8-tetrahydropteridine and 5.0 mM dithiothreitol. After incubation at 25 ^{o}C for 20 minutes the reaction was stopped by the addition of 1.0 ml of 12% TCA. The tubes were allowed to stand for 20 minutes, then remixed and centrifuged. Tissue blanks were prepared by adding the TCA at zero time. The concentration of tyrosine was then determined in the supernatant by the fluorometric method of Wong et al, (1964).

Tyrosine Hydroxylase (EC 1.14.16.2)

The measurement of tyrosine hydroxylase activity was carried out using a modified method of Nagatsu et al.(1964), as described by Brass (1983). Using purified ^{3}H-tyrosine, quantification of enzyme activity was based on the $^{3}H_2O$ formed. Striatal tissue was homogenized in 0.32 M sucrose containing 0.6% triton X-100. Homogenate, containing a maximum of 10 mg, was added to incubation

mixture (total volume 0.3 ml) which included: 200 mM Sodium Acetate buffer pH 6.0, 0.5 mM tyrosine, 280 mM NaCl, 200 units of catalase, 5 mM 6,7,-dimethyl-5,6,7,8 tetrahydropteridine HCl, 10 mM dithiothreitol and 10 mM $Fe(NH_4)_2SO_4.6H_2O$. Blanks consisted of all components except for the cofactor 6,7-dimethyl-5,6,7,8-tetrahydropteridine. Incubation was carried out for 8 minutes at 37^{o} C, and the reaction terminated by the addition of 0.2 ml of glacial acetic acid. Following centrifugation at 7,000 x g for 10 minutes, the supernatant was passed over a column (0.5 x 3 cm) of BioRad 50W-X8 cation exchange resin, washed twice with 0.5 ml of distilled water and the total effluent collected for determination of $^{3}H_2O$.

Dopa Decarboxylase (Aromatic L-amino acid decarboxylase) (EC4.1.1.26.)

The activity was determined in 0.32 M sucrose homogenates utilizing the $^{14}CO_2$ released from ^{14}C carboxyl DL 3,4-dihydroxyphenylalanine (DOPA) determined using a modified procedure of the method of Lamprecht & Coyle, (1973). 0.1 M sodium phosphate buffer was used without the addition of ascorbic acid and disodium EDTA. Because of the chemical interaction of substrate with the cofactor pyridoxal phosphate the reaction was started with the substrate and blanks consisted of zero time addition. Tubes capped with rubber stopper from which was suspended

a plastic well containing a 1.1 x 2.5cm piece of Whatman No 1 filter paper and 200 ul of Protosol (New England Nuclear, Boston, Massachusetts) to trap the liberated $^{14}CO_2$. Incubation was carried out for 20 minutes at 37°C. The reaction was terminated by the addition of 0.5 ml of 10% TCA. After an additional hour of shaking at 37° C, the wells were removed and for complete extraction of the product from the filter paper, 10% methanol was added to the Econofluor Scintillant.

Tryptophan Hydroxylase (EC 1.14.16.4.)

The assay of tryptophan hydroxylase activity was based on the method of Gal and Patterson (1973) as described (Greengard & Wolfe, 1987). Rat midbrain was homogenized in 20% Tris-acetate buffer (pH 7.6) containing 1 mM 2-mercaptoethanol. Samples were centrifuged at 30,000 x g for 30 minutes and the supernatant was used as the source of the enzyme, In the 1 ml of incubation mixture, final concentrations of substances were: 50 mM Tris-acetate (pH 7.6), 1 mM 2-mercaptoethanol. 0.2 mM pargyline, 0.176 mM L-tryptophan, 0.16 mM DL-6-methyl 5,6,7,8,tetrahydropteridine hydrochloride (Calbiochem, La Jolla, California), 10 ug catalase (Sigma, St. Louis, Missouri) and 0.05 to 0.15 ml of midbrain homogenate. Blanks consisted of all reagents except cofactor. The reaction was started by the addition of tissue supernatant and following incubation at 37 °C, terminated after 30 minutes by placing in boiling water

for 5 minutes. Following centrifugation at 3000 rpm for 10 minutes, 0.5 ml of the resultant supernatant was added to 0.1 ml of 1% cysteine, 1.15 ml 10 N HCl and 0.2 ml of 0.01% o-phthalaldehyde (Regis Chemical Co., Morton Grove, Illinois) in 10 N HCl. The reaction mixture was heated at 100 ^{o}C for 15 minutes, cooled and the fluorescence determined. Standard 5HT prepared in 0.059 M Tris-acetate buffer (pH 7.6) and carried throughout the assay (minus cofactor) gave a recovery of 100%. Addition of a-mephe and phe at greater levels estimated to be present in experimental brain did not interfere with the enzyme assay.

Dopamine B Hydroxylase (EC 1.14.17.1)

Dopamine B Hydroxylase activity was determined by modifications of the methods of Coyle & Axelrod, 1972; Sperk et al, 1980. Whole brains were homogenized to give a final concentration of 50 mM Acetate Buffer pH 6.0 with 0.2% Triton-X-100 and centrifuged at 20,000 x g for 10 minutes. Final concentrations in an incubation mixture of 400 ul total volume were: 50 mM sodium acetate buffer pH 5.8, 6 mM dopamine, 10 mM ascorbic acid pH 5.8, 40 mM fumarate pH 5.8, 0.6 mM pargyline, 1500 units catalase, 0.3 mM copper sulfate and up to 20 mg of brain tissue. Commercially available purified DBH from bovine adrenal gland (Sigma, St Louis, Missouri) was routinely used as an interassay control . Incubation for 30 minutes at 37^{o}C was carried out and the reaction was stopped by the addition

of 400 ul of 0.1 M perchloric acid containing 0.2% cysteine and 1.0 uM DHBA. Following centrifugation at 2500 rpm for 15 minutes, the supernatant was filtered through a 0.2 um Spartan filter (Schleicher & Schuell) and the NE was determined using HPLC with electrochemical detection as described for biogenic amine estimation. Both the known inhibitor of DBH, fusaric acid, and boiled enzyme served as negative controls. There was no effect of adding either a-mephe or phe in amounts equivalent to their cerebral concentrations in hyperphenylalaninemic rats.

Phosphoserine Phosphatase (EC 3.1.3.3.)

Tissue was homogenized in distilled water or 0.32 M sucrose and centrifuged at 105,000xg for 30 minutes. For quantification of total cerebral activity compared with that in synaptosomes, homogenates were prepared in 0.32 M sucrose with or without Triton X-100, 0.5% (v/v) final concentration. The undialyzed supernatant was then assayed using o-phospho-D-serine as substrate and measurement of the phosphate liberated (Knox et al, 1969).

Glycine Synthase (EC 2.1.2.10)

Activity of brain homogenized in 4 volumes of 0.32 M sucrose homogenates was determined by a microadaptation of the method of Bruin et al,(1973) using reagent concentrations as described (Isaacs & Greengard, 1980). As described above for the assay of dopa decarboxylase, stoppered 16 x 100 mm glass test-tubes holding a center

well containing Protosol (New England Nuclear) adsorbed to filter paper were found to provide a CO_2 trapping system of similar efficiency to that of 25 ml Erlenmeyer flasks, and enabled the total incubation volume of the assay to be reduced to 0.5 ml.

Protein:

Protein was determined by the use of the Folin phenol reagent after alkaline copper treatment as described by Lowry et al (1952).

Statistical Evaluation of Data:

As indicated in each legend for the tables and figures, the results are expressed as individual values, means ± S.D. or means ± SEM. The Student's t test was used for determination of the significance of differences between the means. Linear regression analysis was performed to determine the correlation between pairs of data.

CHAPTER III

THE UPTAKE AND ACCUMULATION OF PHENYLALANINE IN DEVELOPING RAT BRAIN

Interference with cerebral growth and differentiation during the early postnatal period is the generally accepted explanation for the more profound damage that the brain can incur from a given metabolic insult during this period than in later life (Dobbing, 1974). However, a greater accessibility of the immature brain to the damaging substance itself may also play a role in conditions like PKU or other hyperaminoacidemias. A preliminary finding has indeed indicated that exposure to the same degree of hyperphenylalaninemia at sequential ages in rats resulted in a much higher cerebral phe concentration (i.e. net accumulation) in the early suckling period than in later life (Greengard & Brass, 1984). The following studies investigate this phenomenon and possible mechanisms underlying the decrease in the capacity to accumulate phe, notably, whether the influx of phe at physiological blood concentration changes during brain development. Studies carried out with a frequently employed approach to such a question, i.e. the rapid intracarotid injection technique (Oldendorf, 1970), have shown that the brain uptake index (BUI) for tryptophan in rabbits decreases during the first 28 postnatal days (Cornford et al, 1982). In rats, the BUI for tryptophan was reported to be higher in the late suckling period than

in adulthood (Sarna et al, 1982). Whether there is also an earlier change is not known, since the 19th postnatal day has been the youngest age at which the BUI of an amino acid (that of valine and tryptophan) has been determined in rats (Cremer et al, 1976; Sarna et al, 1982). Adaptation of the rapid intracarotid method (Oldendorf, 1970) to younger rats was, therefore, one of the objectives. The developmental profile of the BUI was determined and compared with the accumulation of phe in the brain at blood levels closely approximating those found in PKU.

RESULTS:

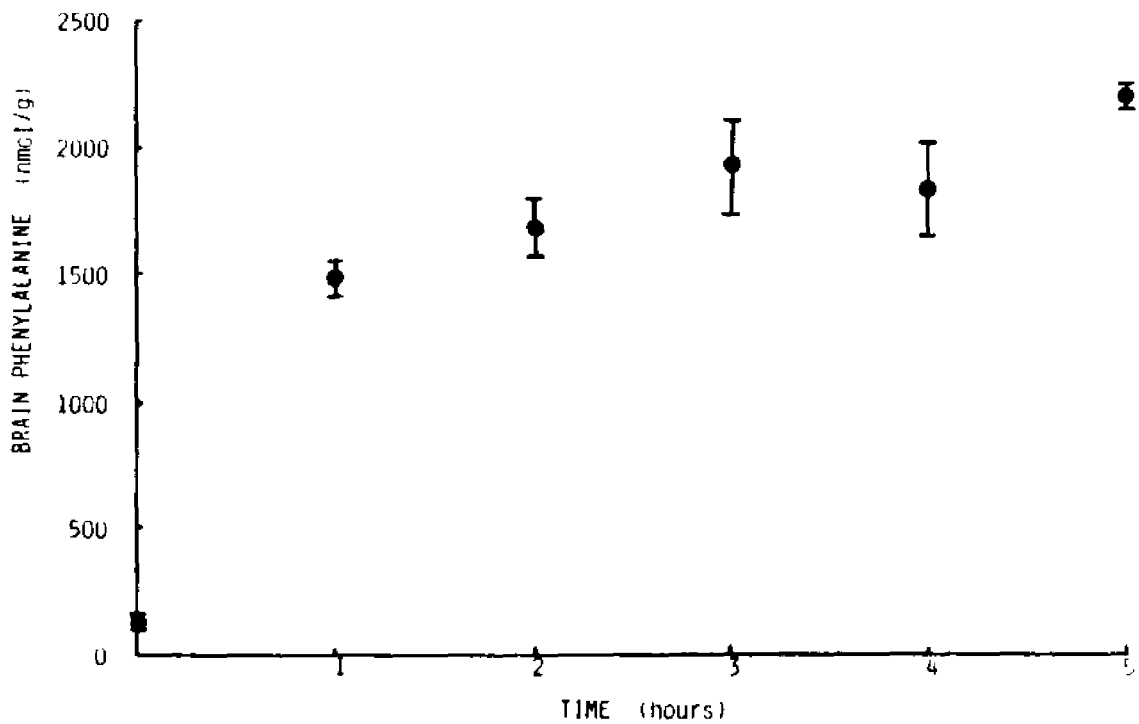
The standard rat model of experimental hyperphenylalaninemia involves daily injection of a-mephe plus phe (DelValle et al, 1987). In order to study the accumulation of phe by the postnatal developing rat brain, it was necessary to overcome the experimental fluctuation of plasma phe which may follow a single subcutaneous injection and the variation in blood phe levels with age (Greengard & Brass, 1984). A-mephe was injected on the day prior to the experiment, in order to suppress PAH activity. On the day of the experiment, the animals received phe injections in doses required to sustain a degree of hyperphenylalaninemia at a plasma concentration of 4000 nmol/ml (i.e. forty-fold above normal) comparable to phe levels in classical PKU, and the brain was assayed every hour. The time course of change in such a study of 16 day old rats is shown in Figure III-1. In neonatal and adult rats also, the concentration of phe in the brain has reached a steady state 5 hours after the induction of hyperphenylalaninemia. This time was therefore chosen for the studies of net cerebral accumulation.

The above method, using age-adjusted doses of phe, was then employed to achieve the same elevations in blood phe concentration on different days of age and to assure that they remained constant for at least 4 hours preceding assay of the brain. As illustrated in Figure III-2 for plasma phe elevations of 4000 and 7000 nmol/ml , the

FIGURE III-1

TIME COURSE OF BRAIN PHENYLALANINE ELEVATION FOLLOWING INDUCTION OF A SUSTAINED HYPERPHENYLALANINEMIA

Forty-fold elevations in blood phe were induced in 16 day old suckling rats whose PAH activity had been suppressed by prior injection of a-mephe (2.4 umol/g B.Wt.). At the indicated times after the first injection of phe, brain phe was determined. Results depicted represent means ± S.D. of values for 3 animals.



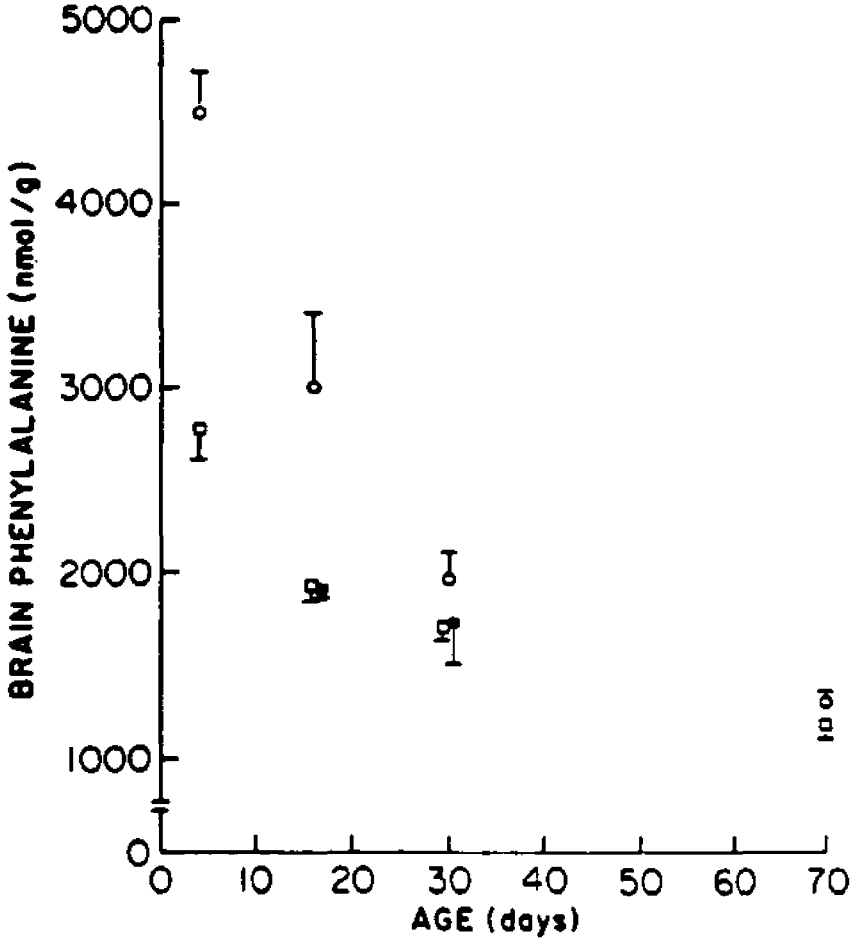
cerebral concentration of phe (resulting from the same elevation in blood level) decreased to a greater extent between the 4th and 16th or 30th day of age than between the 30th day and adulthood. The results also reveal that on the 4th day of age, the brain accumulates much more phe from blood levels standardized at 7000 than at 4000 nmol/ml, ($p<0.005$) whereas in 16, 30 or 70 day old rats the differences are insignificant. This indicates that after early infancy variation in the degree of hyperphenylalaninemia of PKUs has little effect on brain phe content, but that during the particularly vulnerable neonatal period the cerebral accumulation of phe can differ considerably depending on whether its blood concentration is 10,20 or 40 times above normal.

The effect of prior exposure to chronic hyperphenylalaninemia on this developmental pattern was subsequently determined. Since the brain uptake of phe has been shown to be increased following prolonged infusion of ammonium salts (Jonung et al, 1985), it was of interest to know whether uptake would be likewise altered as a consequence of chronic hyperaminoacidemia, a condition in which blood ammonia levels are elevated (Knox, 1972; Table IV-1). In one group of rats, the standard experimental PKU treatment was begun so as to impose chronic hyperphenylalaninemia from the third day of life until 48 hours prior to administering the above described protocol to maintain constant elevation in blood phe for 5 hours

FIGURE III-2

DEVELOPMENTAL CHANGES IN CEREBRAL PHENYLALANINE ACCUMULATION

On the indicated day of age, the blood phe levels of rats were raised to either 4000 (□) or 7000 (○) nmol/ml for a period of 5 hours. The numbers of rats (in the order of increasing age) in the former group were 3,5,3 and 4, and in the latter group they were 5,4,3 and 7. Additional rats (■) with blood phe levels raised to 4000 nmol/ml, on the 16th(n=6) or 30th (n=3) days had also received a daily injection of saline or a-mephe plus phe from the third day on (replaced by supplemented diet at weaning), so as to impose chronic hyperphenylalaninemia. Values are means ± SEM of results of 3 to 7 rats. At both blood levels, the differences between the 4 day mean and the means at 16 ($p<0.02$), 30 ($p<0.01$), and 70 ($p<0.001$) days were statistically significant (Student's t test). At 4 days the difference between the mean at 4000 nmol/ml and 7000 nmol/ml was also significant ($p<0.005$) but there were no differences at other ages.



before assay. The results are shown in Table III-1 and Figure III-2. Table III-1 also presents measurements of whole brain and cerebellum weights, which show decreases in the rats exposed to chronic hyperphenylalaninemia indicating the efficacy of the treatment. However, although brain and particularly cerebellar weights were significantly lower in rats exposed to chronic hyperphenylalaninemia, the brain/plasma phe ratios indicate that this condition did not alter the developmental decrease in cerebral phe content at blood levels elevated to 4000 nmol/ml (see also Figure III-2).

In view of the developmental change in phe accumulation, and in particular the significant decrease between the 4th and 16th days, determination of whether there is a change during these early days in the BUI for phe (i.e. influx at physiological blood levels) was of particular interest. This required that the rapid intracarotid injection technique for BUI determination be adapted to rats of an earlier age than hitherto studied. In addition, the frequent lethality in neonatal rats caused by the anesthetics (nembutal or pentobarbital) employed in those studies, was overcome by replacing them with the less hazardous urethane. This did not seem to introduce any artifact since the BUI for phe did not differ from that measured in survivors of nembutal anesthesia. Prior to these measurements, it was found that the 28 gauge metal needle, suitable for delivery of the

TABLE III-1

CHRONIC HYPERPHENYLALANINEMIA AND PHENYLALANINE ACCUMULATION BY THE DEVELOPING BRAIN

Suckling rats received daily injections of either saline (Acute) or a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) from day 2 of age which was replaced by diet supplementation (0.5% a-mephe plus 3.0% phe) at weaning (Chronic). Two days prior to receiving an acute load of phe, the chronic treatment was stopped and all rats were given a-mephe (2.4 umol/g). This was followed 20-24 hours later by phe administration to raise and sustain the blood levels at a forty-fold elevation for at least 4 hours. Samples for plasma and brain determination of phe were taken 5 hours after the first injection and the ratios of brain/plasma calculated. Results are expressed as means ± S.D. for the number of animals indicated in parentheses. Statistical analyses were made using Student's t test and the significance of differences between the means given by * $p<0.05$.

16 DAYS	Acute	Chronic
Body weight(g)	19.1 ± 3.4 (5)	17.3 ± 2.1 (6)
Brain weight(g)	1.14 ± 0.08(5)	1.03 ± 0.06(6)*
Cerebellum wt.(mg)	121 ± 9 (4)	91 ± 11 (3)*
Brain/Plasma Phe	0.50 ± 0.11 (5)	0.53 ± 0.14 (6)

30 DAYS	Acute	Chronic
Body weight(g)	52.0 ± 5.4 (3)	49.3 ± 6.8(4)
Brain weight(g)	1.33 ± 0.07 (3)	1.23 ± 0.06(4)
Brain/Plasma Phe	0.41 ± 0.09 (3)	0.44 ± 0.18(4)

test substances at the 18th postnatal day or later, had to be replaced by a beveled 33 gauge needle for use in 13 to 18 day old rats. The narrow carotid arteries of younger rats (especially those of the smaller Fischer strain) however, necessitated the preparation of borosilicate pipettes.

Since conformation to the criteria of a bolus requires that the injection is rapid, lasting no more than 0.25 seconds (Oldendorf, 1971), pipettes with lumen sizes resulting in slower delivery were discarded and only those permitting delivery within the correct time were used. Further tests for possible errors arising from application of the borosilicate pipettes to the youngest age group were carried out in Long Evans rats in which intracarotid insertion of the commercially obtained 33 gauge needles was feasible by the 7th postnatal day. The results (see Table III-2) showed that the BUIs thus determined did not differ from those obtained by delivering the test solution through the standardized borosilicate pipettes. In one experiment, unlabelled phe was added to the injectate raising its final concentration from the standard 0.012 mM to 0.08 mM. The lower cerebral radioactivity thus obtained was in harmony with previous studies in which the amount of cold phe injected into adult animals was varied (Pardridge & Oldendorf, 1975), but the difference in uptake between the 7th (BUI = 40 $\pm$ 8 %, n = 3) and 24th day of age (BUI = 32,33%) was also apparent at the 0.08 mM

phe concentration.

For comparative purposes, the cerebral phe uptake was also determined by injecting the test solutions into the left ventricle of the heart. The results were in qualitative agreement with a previous study utilizing this technique (Sershen & Lajtha, 1976) in which the BUI of phe thus obtained in neonatal rats was well above the BUI determined in mature animals with the standard intracarotid approach. However, such a comparison gives an exaggerated estimation of the change with age, since it was also found that the absolute values obtained with the former method were higher. They were also more variable; for these reasons, and because of the various errors arising from peripheral tracer administration (Oldendorf, 1971), use of the heart intraventricular method at each age would also not give accurate estimates of the developmental change in BUI.

The BUIs of phe determined with the intracarotid injection technique (as detailed under Methods) in Fischer rats from the first week of life up to 70 days of age are shown in the left side of Table III-2. It may be seen that there was an appreciable decrease between the 4th and 7th days, and that the value seen at 24 days was as low as in adulthood. The somewhat higher value at 30 compared with 24 and 70 days must represent a real discontinuity rather than an experimental variation since it was confirmed in Long Evans rats (see right side of Table III-

TABLE III-2

COMPARISON OF THE BRAIN UPTAKE INDEX OF PHENYLALANINE IN FISCHER AND LONG EVANS RATS DURING DEVELOPMENT

The BUI for phe was determined as described in Chapter II, except that in one 7 day old group (+), injection was given with a syringe and needle instead of the glass pipettes normally used in 7 day old and younger rats. Values are means ± SD of results with the number of animals in parentheses, or refer to individual animals.

	FISCHER STRAIN		LONG EVANS STRAIN	
AGE (days)	BODY WEIGHT (g)	BUI (%)	BODY WEIGHT (g)	BUI (%)
4	5.1± 0.2	74 ± 14 (4)	8.3 ± 1.0	76 ± 11 (5)
7	8.1± 0.8	63 ± 10 (7)	13.5 ± 0.5	65 ± 9 (4)[+] 60, 62
24	21 ± 2	49 ± 6 (9)	26 ± 6	43 ± 3 (4)
30	35 ± 4	55, 58	71 ± 5	52 ± 5 (3)
70	110 ± 11	48 ± 4 (4)	211 ± 24	48 ± 6 (3)

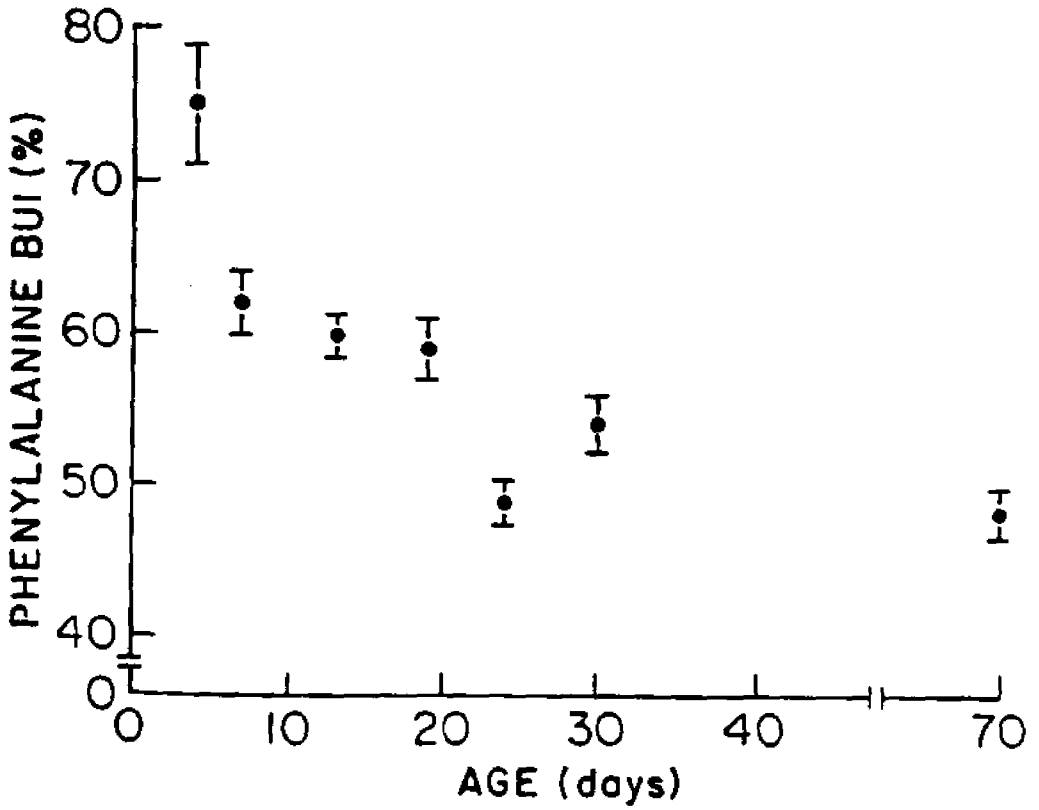
2). More importantly, comparison of the two strains suggests that conceptual age rather than body weight is the important factor in the rate of developmental change in BUI. For, even though Long Evans rats showed higher birth weight and more rapid body growth, the BUIs at any given age were indistinguishable from those in Fischer rats. The individual values for each age, therefore, could be averaged over the two strains. It was also found that the extraction of water as measured by the uptake relative to that of labelled butanol did not vary greatly with age [7 days, 93 ± 5 %(5) versus 30 days, 107 ± 6% (3)] nor was there interspecies difference. From these results it would appear that the age-related changes in BUI for phe cannot be accounted for by alterations in blood flow, a finding also reported by Cornford et al (1982) for tryptophan in rabbits.

Using the pooled data from both strains, the means ± SEM thus obtained (as well as the 13 day values which refer to Fischer rats only) are plotted in Figure III-3. The statistically significant decrease between 19 and 70 days might have been expected since the BUI of another large neutral amino acid (tryptophan) in rats was reported to be higher in adulthood than at the late suckling stage (Sarna et al, 1982). However, the novel finding is that already before this stage there is a decrease in the BUI of phe, and that even between the 4th and 7th days this decrease is significant (see legend). These results are

FIGURE III-3

BRAIN UPTAKE INDEX FOR PHENYLALANINE IN DEVELOPING RATS

The brain uptake index (BUI) was determined by intracarotid injection in two strains of rats. The results for Fischer and Long Evans rats of the same age are averaged together (see Table III-2). Each point represents the mean ± SEM of results for groups of rats, the numbers of which, in the order of increasing age, were 9,13,3,9,13,5 and 7, respectively. The differences between the 4 day mean and the means at 7 ($p<0.01$), 24 ($p<0.001$), 30 ($p<0.005$) and 70 ($p<0.005$) days were statistically significant (Student's t test).



not unlike the curves in Figure III-2: it appears that the early postnatal period of change in BUI overlaps with the developmental period (i.e. between the 4th to the 16th day) during which there is a rapid decrease in the capacity of the rat brain to accumulate phe at persistent and pathological elevations of blood level.

DISCUSSION:

Studies of the kinetics of nutrient influx into the brain have shown that the specific carrier systems of the BBB play an important role in regulating cerebral metabolism (Pratt, 1979; Pardridge, 1983). The subnormal concentrations of several amino acids (e.g. leucine, valine, tryptophan and methionine) in the autopsied brains of PKU subjects (McKean, 1972) has been found to be consistent with the fact that the high circulatory level of phe, competing for the common L-transport system, limits the influx of other large neutral amino acids across the BBB (Oldendorf et al, 1971, 1973). Since this competitive mechanism is fully operative by the time of birth (Braun et al, 1980; Banos et al, 1978), and probably even prenatally (Brass et al, 1982), the resulting diminished supply to the growing brain of essential precursors of protein and neuroactive amine synthesis has been proposed to underlie the major or minor degrees of cerebral maldevelopment seen in several conditions associated with elevations in the blood level of a large neutral amino acid (Pratt, 1979). In the case of hyperphenylalaninemia, however, additional, more specific factors have been implicated in the pathogenic process. The diversion of excess phe to normally minor metabolic pathways results in the accumulation of several metabolites which, by inhibiting cerebral protein and neurotransmitter synthesis , would interfere with normal

brain growth and differentiation (Blau, 1979; Scriver & Clow, 1980). Moreover, excess phe itself directly inhibits protein synthesis (Hughes & Johnson, 1978) as well as the hydroxylation of tyrosine (Nagatsu et al, 1964) and tryptophan (Lovenberg et al, 1968), and experimental hyperphenylalaninemia (comparable to that in classical PKU) has been found to cause cerebral catecholamine deficiency even if the cerebral concentration of the precursor, tyrosine, is as high or somewhat higher than in normal animals (Brass & Greengard, 1982). For these reasons, and because some of the potentially harmful phe metabolites are synthesized in the brain itself (Mosnaim & Wolf, 1978), the extent of interference with the above mentioned synthetic processes is clearly dependent on the magnitude of rise in the cerebral concentration of phe. Thus, if this concentration is especially high in neonatal brain, then the damage sustained during early infancy would be particularly severe not only because of the heightened vulnerability of the growing brain but also because of the greater degree of chemical abnormality it was exposed to during this period. Indeed, by subjecting rats on different days of age to the same degree of hyperphenylalaninemia, it was shown not only that the cerebral phe concentration was highest in neonatal life, but that most of the developmental decrease in the accessibility of the brain to phe occurs prior to the 16th postnatal day. This was true at blood phe standardized at

either 7000 or 4000 nmol per ml. The present study also indicates that the magnitude of decrease with age in cerebral capacity to accumulate phe is the same whether or not the animals are exposed to chronic hyperphenylalaninemia throughout the developmental period studied. This occurs despite the decreased weight of the cerebrum and cerebellum caused by chronic treatment. The weight deficit is probably related to the fact that during the first 3 postnatal weeks there is active cell division in the cerebellum and, although 97% of the final number of cells are acquired (Balazs & Richter, 1973), only 70% of adult synapses are present by the end of this time (Cragg, 1974). In rats exposed to chronic hyperphenylalaninemia during early postnatal development, cellular abnormalities have also been observed in the cerebellum (Huether et al, 1982).

At normal blood levels as well, evidence has been obtained for an age-dependent decrease in the cerebral uptake of several amino acids. The higher value for immature animals is usually referred to as an "increase", and the biological utility of the phenomenon is invariably stated to lie in assuring that the rapidly growing brain receives an ample supply of essential amino acids (Cornford et al, 1982; Banos et al, 1978; Pratt, 1979). However, it seems likely that the decrease in uptake capacity with age has been the change in evolution that conveyed a selective advantage to the brain of higher

animals for, as intermittent feeding begins, this would protect the brain against sudden increases in the blood levels of these amino acids. It is thus of interest, that according to the only detailed age curve in the literature for the decreasing cerebral uptake of an amino acid in any species, namely of tryptophan in rabbits, the BUI attained minimal values of day 28, i.e. shortly before the usual time of weaning in rabbits (Cornford et al, 1982). This age curve is not unlike the one now obtained in rats for the BUI of phe, in that most of the developmental decline was accomplished by the usual time of weaning (21-22 days) (Figure III-3). It may also be seen from Table III-2 that the results on Fischer and Long Evans rats were essentially identical despite considerable differences in body weight.

Most studies on cerebral nutrient supply under physiological conditions have employed Oldendorf's rapid intracarotid injection method. BUIs thus determined reflect unidirectional transport since no appreciable efflux occurs during the 10-15 seconds required for a single passage through the brain (Pardridge, 1983). While the Oldendorf method has numerous advantages over other approaches (for example, it avoids the peripheral metabolism of the injected substances), its recently suggested potential sources of inaccuracy include two which may be pertinent to developmental studies. A possible mixing of the intracarotid bolus with the blood

(Takasato et al, 1984) would result in the addition of amino acids competing for transport and thus in the underestimation of the BUI. However, the salient conclusion from Figure III-3, i.e. that the BUI declines during the early suckling period, would not be weakened by such an error. On the contrary, it would mean that the decrease with age could be even steeper than appears since, owing to the normal early postnatal decline in the blood concentration of competing amino acids (Pratt, 1979; Sarna et al, 1982), any mixing of the bolus with blood would have underestimated the BUI to a greater extent in neonatal than in 2 or 3 week old rats. The other suggested fault of the Oldendorf method is that it fails to adequately correct for changes in cerebral blood flow rate (Hardebo & Nilsson, 1979; Clark et al, 1981). If so, then the known increase of flow rate in rats during the first 20 days of life (Moore et al, 1976) rather than a developmental change in the properties of the BBB itself may have been responsible for the concomitant decline of the BUI of phe shown in Figure III-3. This possibility cannot be absolutely disproven. However, changes in blood flow are unlikely to have had any significant effect on the results since, in accord with a previous study (Cornford et al, 1982), no marked age differences were found in the cerebral uptake of labelled butanol relative to water. In addition, control of cerebral blood flow presents no problem in the alternative, intravenous

infusion technique and yet with this technique, too, the cerebral influx rate of leucine at least was found to be much higher in neonatal than in 2.5 week old rats (Banos et al, 1978).

The observations at pathological blood phe levels were qualitatively similar to the BUI results in normal rats, in that the curves in both Figures III-2 and III-3 show a steeper decrease in the first 2 postnatal weeks than during subsequent life. It is during the same early period, therefore, that the brain appears to develop the ability to protect itself from physiological and pathological rises in blood phe. A developmental change in the BBB limiting capillary transport probably accounts for the evolving control of amino acid uptake during physiological fluctuations in blood level. However, at prolonged, pathological elevations in blood phe its cerebral concentration depends on several processes such as efflux and cellular as well as capillary transport into the brain (Lajtha & Toth, 1962). The relationship (in terms of structure or specificity) between the carriers involved in these different processes is not well understood. Whether the above mentioned resemblence between the age curves in Figures III-2 and III-3 may also be true for other amino acids is also not known. The results indicate, however, that simultaneous developmental changes in the properties of several systems involved in transport underlie the evolving efficacy of the control of

cerebral phe homeostasis.

Little is known about the ontogeny of brain transport mechanisms. Capillaries isolated from the brains of 1-45 day old rats showed distinct developmental patterns for brain capillary functions, including the appearance of sodium-dependent neutral amino acid transport after 21 days of age which is postulated to be responsible for active efflux from brain to blood (Betz & Goldstein, 1981). In another study using isolated brain capillaries, phe transport was found to be characterized by two saturable transport systems and a non-saturable component. The km values were virtually identical at the rat and human BBB (Choi & Pardridge, 1986).

One explanation for the observation that some untreated PKUs demonstrate relatively normal mental development (McKean & Peterson, 1970; Gaull et al, 1973) may be that in the brains of these individuals there has been a precocious maturation of mechanisms involved in the transport of phe. The cerebral phe concentration may not have been as high during childhood as in others with similarly severe hyperphenylalaninemia, and thus the likelihood of interference by phe with crucial biosynthetic processes in the brain was reduced.

CHAPTER IV

PLASMA AND TISSUE AMINO ACIDS IN EXPERIMENTAL HYPERPHENYLALANINEMIA

In the previous chapter the age-related effect of circulating blood phe on brain phe level was described. This section investigates other consequences of hyperphenylalaninemia on plasma and tissue amino acids. In untreated classical PKU abnormal levels of amino acids have been demonstrated in CSF (Van Sande et al, 1970; Quentin et al, 1974 and in autopsy brain samples (McKean, 1972). Accompanying the elevation of phe in the blood, plasma profiles have shown depletions in a number of other amino acids (Linnewah & Ehrlich, 1962; Efron et al, 1969), the reason for which remains undetermined.

Experimental animal studies have also shown an association between hyperphenylalaninemia and diminished levels of large neutral amino acids in the brain including tryptophan (McKean et al, 1968; Isaacs & Greengard, 1980; Lane et al, 1980; Huether et al, 1984; Greengard & Wolfe, 1987), which is also decreased in the blood (Huether et al, 1984; Greengard & Wolfe, 1987). One report in young suckling hyperphenylalaninemic rats of decreased blood and brain tryptophan levels but increased peripheral tissue tryptophan concentrations, has speculated that it is this accumulation of tryptophan in extra-cerebral tissues which is responsible for the loss from the blood and hence from the brain (Huether et al, 1984). These findings have been

discussed in presentation of a unifying hypothesis of cellular transport involving the sequestering of amino acids in tissues (Christensen, 1986 & 1987). However, no consideration was given to the clinical findings of abnormal tryptophan metabolites in untreated PKU (Anderson et al, 1967) and to the postulate that hyperphenylalaninemia may interfere with amino acid transport out of the gut (Efron et al, 1969; Blau, 1979).

To further investigate these phenomena, the following experiments were undertaken. Utilizing the protocol for sustaining the phe concentration in the blood at a constant steady state level, the effect of hyperphenylalaninemia on plasma, tissue and gastric phe and tryptophan content was determined. The results are discussed in relation to how competitive mechanisms operating in hyperphenylalaninemia may affect amino acid balance in various tissues.

RESULTS:

The daily injection of a-mephe plus phe in infant rats results in elevations in blood and brain phe (DelValle et al, 1978) equivalent to the hyperphenylalaninemia associated with clinical PKU (Knox, 1972). As illustrated by Table IV-1, the blood amino acid profile determined at 4 hours after the last injection of a-mephe plus phe, shows a decrease in the concentration of large neutral amino acids, including tryptophan, leucine, isoleucine, valine, methionine and histidine. Tryptophan, in view of its role as precursor of cerebral 5HT synthesis, was at the focus of the studies to be described. According to several investigators (Fernstrom, 1983), the cerebral uptake rate of tryptophan is governed by its total plasma concentration irrespective of the free and albumin-bound portion. Thus, even though it has been suggested that the concentration of the free relative to bound tryptophan influences its availability for cerebral 5HT synthesis (Bourgoin et al, 1974), only the total (free plus bound) tryptophan was measured in the present study.

Although the treatment regimen of a-mephe plus phe assures the maintenance of hyperphenylalaninemia, the greater constancy of blood phe levels required for the present study necessitated that the rise and fall of phe following the daily injection be avoided. This, together with minimization of any possible side effect of the synthetic analog a-mephe, was achieved by prior

TABLE IV-1

PLASMA AMINO ACID COMPOSITION FOLLOWING EXPERIMENTAL HYPERPHENYLALANINEMIA

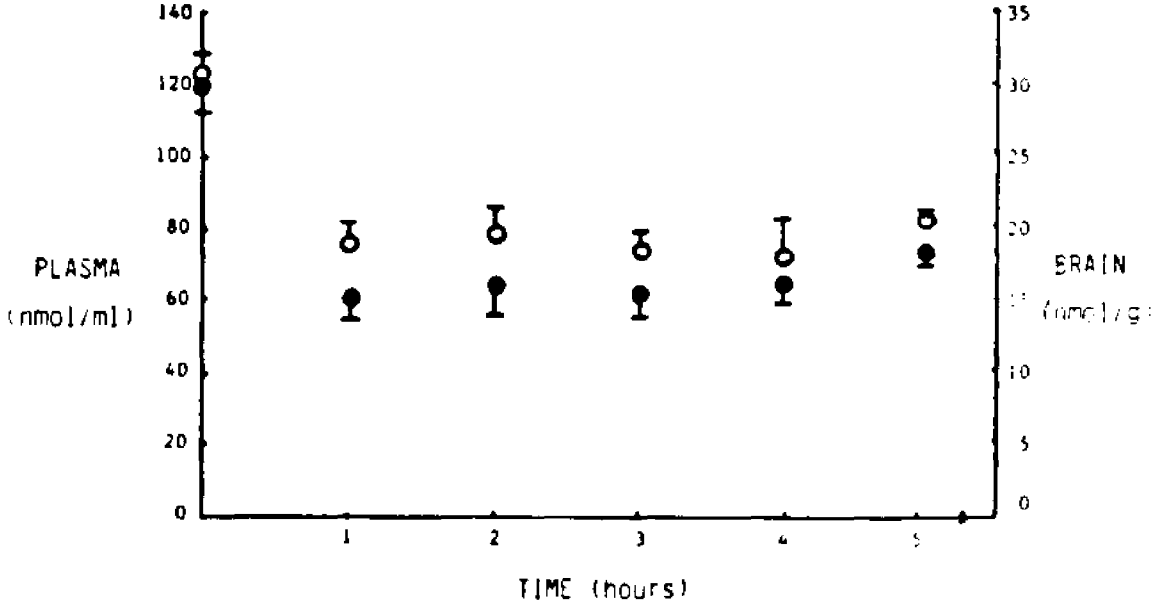
Suckling rats received daily injections of either saline(C) or a-mephe (2.4 umol/g) plus phe (5.2 umol/g) (E) from day 3 of age. At 4 hours after the last injection, plasma samples were taken from 8 day old rats for analysis by Amino Acid Analyzer or, in the case of tryptophan (+) by fluorometric assay. Values refer to pooled samples from 2 to 3 control (C) or experimental (E) rats and are in nmol/ml.

	C	E
Phenylalanine	69	3808
Tyrosine	318	517
Tryptophan^{+}	127	61
Leucine	204	171
Isoleucine	83	84
Valine	245	228
Methionine	63	47
Histidine	165	117
Ornithine	146	137
Lysine	269	312
Alanine	500	339
Glycine	463	338
Serine	601	499
Threonine	451	411
Citrulline	129	132
Glutamine	1003	650
Glutamate	226	188
Aspartate	46	46
Taurine	172	299
Urea	2429	4261

FIGURE IV-1

TIME COURSE OF PLASMA AND BRAIN TRYPTOPHAN LEVELS FOLLOWING INDUCTION OF HYPERPHENYLALANINEMIA

Liver PAH activity was suppressed in 16 day old rats by injection of a-mephe (2.4 umol/g B.Wt.). On the following day (20 hours later) forty-fold elevations in blood phe were induced and the levels of tryptophan in plasma (O) (nmol/ml and brain (●) (nmol/g) determined at the indicated times after the first injection of phe. Results are means ± S.D. for 3 animals.



suppression of PAH with a-mephe and injections of phe 20-24 hours later (see also Chapter III). Both prior to the injections and following maintenance of a forty-fold elevation in plasma phe, the tryptophan concentration in the plasma and brain was monitored hourly for up to 5 hours, the results being shown for 16 day old rats in Figure IV-1. At the beginning of the time course the tryptophan levels in the rats to receive phe were not different from control values but as the phe increased and was maintained at a steady forty-fold level, the concentration of tryptophan declined and remained reduced in the blood as well as in the brain.

Since Huether et al (1984) have reported elevations in extra-cerebral tissue tryptophan concentration and have implicated this sequestering as a major cause for the plasma depletion, it was determined whether peripheral tissues exhibit altered tryptophan levels. The same elevations in plasma phe were maintained for 5 hours, and the levels of tryptophan and phe measured in the plasma, brain, lung, liver, skeletal muscle and small intestine of rats aged 16 and 30 days. As shown in Table IV-2, apart from significant decreases in plasma and brain, the tryptophan levels in other tissues, were unchanged by hyperphenylalaninemia. Further increasing the amount of phe administered enhanced the reductions in cerebral and blood tryptophan levels but still had no effect on other tissue levels. The phe concentrations determined for

TABLE IV-2

THE EFFECT OF HYPERPHENYLALANINEMIA ON THE TRYPTOPHAN CONCENTRATIONS OF PLASMA AND PERIPHERAL TISSUES

Rats received an acute load of phe 20 hours after receiving a-mephe (2.4 umol/g B.Wt.) and were divided into 2 groups according to the level of hyperphenylalaninemia: E1 indicates forty-fold above normal whereas the blood levels in E2 were raised to exceed this. Controls received saline (C). Samples were taken after a 5 hour period of sustained hyperphenylalaninemia and the phe concentrations determined for plasma (nmol/ml) and tissues (nmol/g). Results are expressed as means ± S.D. with the number of rats given in parentheses. Satistical analyses of the significance of the difference from the control value was determined by Student's t test and is indicated by *p<0.05, **p<0.01,*** p<0.001.

TRYPTOPHAN
(nmol/ml or nmol/g)

16 DAYS

	C	E1	E2
Plasma	116 ± 19 (10)	88 ± 17 (4)**	81 ±8 (7)**
Brain	30.0±3.4(10)	17.9±2.5 (4)***	16.2±2.6(7)***
Lung	45.3±5.5 (7)	45.0±3.2 (4)	44.9 ±4(7)
Intestine	124 ± 42 (6)	120 ±50 (3)	119 ±44 (3)
Muscle	44.1± 7.2 (8)	42.6±8.9 (4)	42.2 ±9.2(7)
Liver	44.9± 7.7 (3)	49.8±4.3(3)	

30 DAYS

	C	E1
Plasma	102 ± 22(3)	80± 12(3)*
Brain	18.3 ± 3.1(3)	12.9±3.7(3)**
Lung	41.1 ± 7.4(3)	38.7±5.2(3)
Intestine	102 ± 48(3)	82 ± 25(3)
Muscle	29.0±6.8(3)	28.3±6.9(3)

TABLE IV-3

THE EFFECT OF HYPERPHENYLALANINEMIA ON THE PHENYLALANINE CONCENTRATIONS OF PLASMA AND PERIPHERAL TISSUES

Rats received an acute load of phe, 20 hours after receiving a-mephe (2.4 umol/g B.Wt.) and were divided into 2 groups according to the levels of hyperphenylalaninemia: E1 denotes forty-fold above normal whereas the blood levels in E2 were raised to exceed this. Controls received saline (C). Samples were taken after a 5 hour period of sustained hyperphenylalaninemia and the phe concentrations determined for plasma (nmol/ml) and tissues (nmol/g). Values given are means ± S.D. with the number of rats given in parentheses.

PHENYLALANINE
(nmol/ml or nmol/g)

16 DAYS

	C	E1	E2
Plasma	94 ± 12 (10)	4142 ± 1038 (4)	6680 ± 993 (7)
Brain	105 ± 9 (10)	1870 ± 416 (4)	2842 ± 829 (7)
Lung	118 ± 22 (7)	2987 ± 520 (4)	5040 ±1328 (7)
Intestine	649 ±143 (6)	3378 ± 540 (3)	6594 ± 1902(3)
Muscle	294 ± 34 (8)	4231 ± 1103 (4)	6741 ± 1437(7)
Liver	168 ± 10 (3)	3512 ± 420 (3)	

30 DAYS

	C	E1
Plasma	83 ± 4 (3)	4284 ± 529 (3)
Brain	97 ± 8 (3)	1615 ± 34 (3)
Lung	151 ± 11 (3)	3948 ± 493 (3)
Intestine	648 ± 130 (3)	4654 ± 529 (3)
Muscle	197 ± 30 (3)	4496 ± 530 (3)
Liver	159 ± 10 (3)	3074 ± 350 (3)

these tissues are shown in Table IV-3. The levels of phe in the muscle and small intestine were found to reflect those found in the plasma, while the phe content of liver and, in the case of 16 day old rats, the lung, did not achieve the same phe concentration as the plasma, although in neither case, were these values as low as in the brain. The degree of hyperphenylalaninemia did not alter the relative tissue distribution of phe.

Since no changes in tryptophan levels in tissues other than the brain could be found, other possible reasons for the plasma tryptophan decrease were considered. In hyperphenylalaninemia, interference with gastrointestinal tryptophan absorption by elevated phe has been postulated to occur (Anderson et al, 1967; Efron et al, 1969). The levels of phe and tryptophan in gastric contents of 16 day old rats with a sustained forty-fold elevation in phe are given in Table IV-4. A significant increase in the stomach tryptophan as well as phe content was found to follow this treatment. Both increases over the corresponding values for control animals were evident when expressing the amino acid concentrations per total, as well as per g stomach content. These results are also presented graphically in Figure IV-2.

According to a recently put forward hypothesis (Huether et al, 1984), the cerebral tryptophan of young suckling rats exposed to hyperphenylalaninemia is due

TABLE IV-4

THE EFFECT OF HYPERPHENYLALANINEMIA ON THE PHENYLALANINE AND TRYPTOPHAN LEVELS IN GASTRIC CONTENTS

Rats aged 16 days, received a-mephe (2.4 umol/g B.Wt.) followed 20 hours later by a standardized load of phe to raise and sustain blood levels forty-fold (E) or received saline (C). Plasma, stomach and gastric contents were taken 5 hours after induction of hyperphenylalaninemia and the phe and tryptophan concentrations determined. Analyses of statistical significance of difference from controls by Student's t test is indicated by *p<0.05, ***p<0.001.

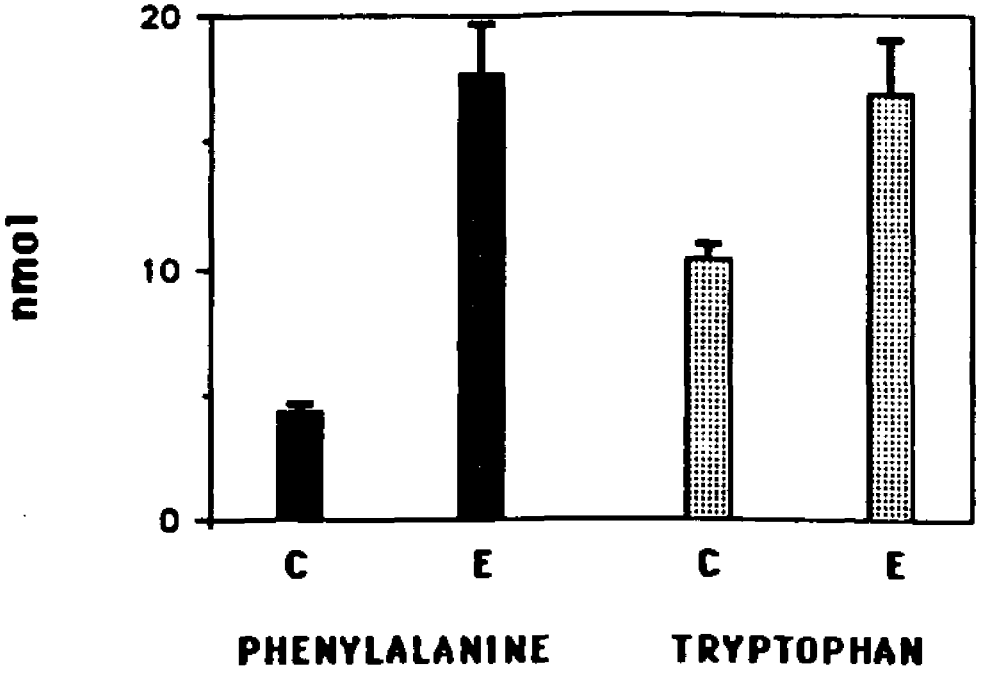
	C	E
PLASMA		
Phenylalanine (nmol/ml)	90 ± 5 (3)	4457 ± 558 (3)
Tryptophan (nmol/ml)	120 ± 7 (5)	89 ± 15 (5)**
STOMACH		
Phenylalanine (nmol/g)	259 ± 41 (3)	4524 ± 532 (3)
Tryptophan (nmol/g)	86.7 ± 15.2(3)	72.5 ±11.5 (3)
GASTRIC CONTENTS		
Total Weight (g)	0.41 ± 0.17 (3)	0.47 ± 0.15 (3)
Phenylalanine (nmol/g)	11.4 ± 2.7 (5)	37.8 ± 3.2 (5)***
(nmol)	4.3 ± 0.5 (5)	17.6 ± 4.6 (5)***
Tryptophan (nmol/g)	23.5 ± 10.7(5)	32.6 ± 8.8 (5)
(nmol)	10.3 ± 1.5 (5)	16.9 ± 4.9 (5)*

FIGURE IV-2

HYPERPHENYLALANINEMIA AND AMINO ACID LEVELS

IN GASTRIC CONTENTS

Rats aged 16 days received a standardized load of phe to raise and sustain blood levels forty-fold (E) or received saline (C). Stomach contents were removed 5 hours after induction of hyperphenylalaninemia and the phe and tryptophan concentrations determined. Mean values are shown by the height of the columns with bars representing SEM.



simply to their diminished plasma tryptophan level. This is unlikely, for there is extensive evidence that large neutral amino acids are transported across the BBB by a common carrier, and that increased amounts of one of these amino acids can reduce the uptake of the others. Findings of decreased large neutral amino acids in hyperphenylalaninemic fetal rats (Brass et al, 1982) also indicate that this mechanism develops early, however, no direct evidence has been obtained for the competition between phe and tryptophan for cerebral uptake in young rats. To seek such evidence, the rapid intracarotid injection (Oldendorf, 1970) was used since this technique avoids problems arising from peripheral amino acid metabolism or efflux and the BUI thus measured reflects unidirectional transport via the BBB. The results obtained by administration of tracer amounts of ^{14}C tryptophan for weanling rats are shown in Table IV-5. As expected from the minimal mixing of the injected bolus with the blood (see Discussion), the BUI for tryptophan was only slightly lower in hyperphenylalaninemic than in control animals. The significantly lower values obtained in normal rats by addition of unlabelled phe to the bolus indicates, however, that phe elevated to levels comparable to the blood in PKU inhibits the uptake of tryptophan. These results demonstrate that cerebral tryptophan depletion in hyperphenylalaninemic weanling rats can occur in the absence of diminished blood tryptophan levels. A

TABLE IV-5

CEREBRAL UPTAKE OF LABELLED TRYPTOPHAN

Rats aged 28 to 30 days received the standard protocol to achieve a forty-fold sustained hyperphenylalaninemia (E) or saline (C). They were then given an intracarotid injection containing a tracer dose of labelled tryptophan, plus the indicated amount of phe. Statistical analyses by Student's t test of the significance of the differences from the saline group (C) in line 1 are indicated by * $p<0.05$, *** $p<0.001$.

RATS (PRIOR TREATMENT)	PHENYLALANINE IN INTRACAROTID INJECTION (mM)	BUI TRYPTOPHAN (%)
Control(C)	0.0	48 ± 8 (4)
Hyperphenylalaninemic(E)	0.0	36 ± 3 (4)*
Control(C)	4.0	4 ± 2 (3)***

similar conclusion may be drawn from some observations on euphenylalaninemic animals at various hours after the last food intake. Their blood tryptophan level showed considerable variation, and even in cases with blood tryptophan levels as low as in hyperphenylalaninemic animals, the brain tryptophan concentration remained in the normal range.

By monopolizing the L transport system, excess phe reduces the concentration of not only tryptophan but also that of other large neutral amino acids. It has been postulated however, that the hyperphenylalaninemia-associated depletion of plasma tryptophan is a specific phenomenon (Huether et al, 1984). To address this question, the effect of other experimental hyperaminoacidemias on several large neutral amino acid concentrations was studied. An acute load of a series of amino acids was given and tryptophan, tyrosine and phe determined in plasma and brain. Table IV-6 shows that with leucine, methionine and histidine, which share the L transport system with phe, there were decreases in tryptophan, tyrosine and phe concentration in the plasma as well as in the brain. However after administration of large amounts of glycine and lysine, which are not transported by the L system, this effect was not seen.

TABLE IV-6

AMINO ACID ADMINISTRATION AND PHENYLALANINE, TYROSINE AND TRYPTOPHAN LEVELS IN PLASMA AND BRAIN

Acute loads of amino acids (10.4 umol/g B.Wt.) or saline were administered to 10 day old rats , in 2 doses 1 hour apart. Plasma and brain samples were taken 3 hours after the first injection for the analyses of tryptophan, tyrosine and phe. Results are expressed as single values or means ± S.D. of the number of animals given in parentheses. Statistical analyses by Student's t test of the significance of difference of the means from the saline-injected group is indicated by *p<0.05, **p<0.01, ***p<0.001.

TREATMENT	TRYPTOPHAN	TYROSINE	PHENYLALANINE
		PLASMA (nmol/ml)	
Saline	114 ± 8 (6)	271 ± 42 (6)	101 ± 4(6)
Phe	66 ± 7 (3)***	555 ± 180(3)	7555 ± 42(3)
Leucine	80 ± 6 (4)***	132 ± 8 (4)***	61 ± 11(4)***
Methionine	93 ± 3 (4)**	137 ± 32 (4)***	73 ± 10(4)***
Histidine	88 ± 3 (3)**	146 ± 28 (3)**	67 ± 10(3)***
Lysine	123	251	107
Glycine	94	173	60
		BRAIN (nmol/g)	
Saline	32.3 ± 1.9 (6)	229 ± 37 (6)	104 ± 9 (6)
Phe	16.5 ± 1.7 (3)***	291 ± 84(3)	2965± 384(3)
Leucine	19.3 ± 2.3 (4)***	115 ± 19(4)***	59 ± 25 (4)***
Methionine	23.9 ± 2.2 (4)**	102 ± 21(4)***	66 ± 26 (4)**
Histidine	24.9 ± 3.7 (3)*	138 ± 5 (3)**	90 ± 16 (3)*
Lysine	31.7	226	104
Glycine	35.9	218	91

DISCUSSION:

This study has attempted to more fully explore the effect of hyperphenylalaninemia on plasma and cerebral L-amino acid imbalance by using a model of sustained experimental hyperphenylalaninemia. Determination of tryptophan levels under such conditions circumvents potential problems associated with fluctuating phe levels or analog effects as a result of co-injection of a-mephe with phe, neither of which were controlled for in the experiments of Huether et al, 1984. Thus the elevations of tryptophan concentration in peripheral tissues of hyperphenylalaninemic rats could not be confirmed, and the theory that this elevation is responsible for the diminished tryptophan concentration in the plasma remains unsupported by experimental evidence.

Although studies of untreated phenylketonurics showing decreased plasma concentrations in a number of amino acids (Linnewah & Erlich, 1962; Efron et al, 1969) did not include measurement of tryptophan, observations on several models of experimental hyperphenylalaninemia indicate that tryptophan must also be at diminished concentrations in the plasma of PKU subjects. Moreover, since abnormally high concentrations of various indole metabolites believed to be formed from the action of intestinal microflora have been found in the urine of untreated phenylketonurics, it has been suggested that reduction of tryptophan absorption from the

gastrointestinal tract is responsible for the increased formation of these indole derivatives (Anderson et al, 1967). The present study, showing elevated tryptophan as well as phe in the stomach contents of PKU rats, provides experimental support for this hypothesis, and indicates that it is the movement of excess phe into the gut that may inhibit the absorption of tryptophan and contribute to the eventual depletion of its plasma concentration. To further characterize the effect of phe on the absorption of tryptophan from the gut, and of the specificity of this effect, it would be necessary to quantify amino acid concentrations in the intestinal lumen and to perform additional transport studies.

Unlike the suggestion by Huether, 1984, the hyperphenylalaninemia-associated reduction of plasma tryptophan is not a specific phenomenon in that administration of leucine, histidine and methionine also reduced the plasma concentration of other neutral amino acids. These results were not surprising since diminutions in the plasma level of these amino acids have also been noted in human subjects with Maple Syrup Urine Disease and Hypermethioninemia (Rosenberg & Scriver, 1980). However, the lack of effect of lysine and glycine (Table IV-5) indicates that the plasma levels of tryptophan are depleted only by amino acids that are transported by the L-system.

It has been hypothesized early on that transport of

amino acids into cells is responsible for their decreased concentration in the serum (Nyhan et al, 1961). A recent explanation for the sequestering of amino acids in tissues other than brain has been put forward (Christensen, 1986, 1987). An amino acid present in excess can inhibit either the tissue uptake or release of other amino acids, so that, depending on the kms regulating these competitive transport processes, it can either deplete or elevate the tissue concentration of those amino acids. The possibility that an elevation in peripheral tissues contributes to the diminished plasma tryptophan in PKU rats is not entirely disproven by the normal tryptophan concentrations now found in these tissues. For, unchanged steady state concentration may conceivably be due to the fact that the hyperphenylalaninemia also stimulated the utilization of tryptophan. Examination of this question thus requires study of the turnover of radiolabelled tryptophan.

Although high affinity membrane transport systems for amino acids have been shown to be present in brain neurons themselves (Hamon & Glowinski, 1974), kinetic evidence indicates that the BBB, residing in the capillary endothelial cell, is the key regulator of amino acid supply to the brain (Pardridge, 1977). Plasma amino acid concentrations approximate within one order of magnitude the km values for transport across the BBB, and recent studies of isolated brain capillaries have shown very similar phe transport km values for the rat and human BBB

(Choi & Pardridge, 1986). Competition between amino acids for transport sites can be expected to occur 'in vivo' when the km of a particular tissue and plasma concentration are similar. Accordingly increased plasma phe has been shown to lead to decreased uptake of other amino acids sharing the same transport system into the brain (Guroff & Udenfriend, 1962; Oldendorf, 1971; Fernstrom & Wurtman, 1972) but not into other tissues (Guroff & Udenfriend, 1962; Young & Friedman, 1971).

Measurement of BUI may be rendered inaccurate by a mixing of the injected bolus with the blood, although it has been reported that the resulting inaccuracy is only 5.5% for circulating plasma and 3.3% for efflux from the brain (Pardridge et al, 1985). The present results support the latter view, since the BUI for tryptophan was only slightly lower in young hyperphenylalaninemic than control animals. If, however, a corresponding excess of phe was added to the bolus itself, then the BUI of tryptophan was greatly diminished. Similar results have also been obtained previously in adult animals with several different amino acids and with alternative techniques. The relevance of this competitive mechanism to the cerebral amino acid profile in PKU was suggested however, from earlier studies by McKean (McKean et al, 1968) who reported that within 1/2 hour after phe loading of adult or young suckling rats, there were decreases in cerebral amino acids which share the same transport system

as phe but that the serum concentrations of some of these amino acids (including tryptophan) in the young rats was not diminished but actually slightly increased. The present studies under more standardized conditions, also demonstrate that at certain degrees of hyperphenylalaninemia, the blood level of tryptophan shows no decrease.

The recent hypothesis that such a decrease accounts for the depletion of brain tryptophan (Huether et al, 1984) is thus untenable. The alternative, more generally held view is that competition with excess phe for cerebral uptake accounts for diminished cerebral concentration of large neutral amino acids. This view may also require some qualification, however. For, even though competition of excess phe for transport across the BBB plays a pivotal role at very high degrees of hyperphenylalaninemia the depleted blood level may also be a contributory factor.

CHAPTER V

CEREBRAL GLYCINE CONTENT AND PHOSPHOSERINE PHOSPHATASE ACTIVITY IN HYPERPHENYLALANINEMIA AND OTHER HYPERAMINOACIDEMIAS

In addition to abnormalities in the cerebral concentrations of large neutral amino acids sharing the same L transport system as phe, elevated cerebral levels of glycine and of phosphoserine phosphatase (PSP) activity have been found in rats rendered chronically hyperphenylalaninemic (DelValle et al, 1978; Isaacs & Greengard, 1980; Lane et al, 1980; Dienel, 1981). Both these increases were found to be present in several brain areas (Isaacs & Greengard, 1980) including the brain stem, a region in which glycine is believed to be a neurotransmitter (Pycock & Kerwin, 1981). One reason for the interest in this phenomenon is that, although PSP is thought to catalyse the rate limiting step in cerebral glycine synthesis (Bridgers, 1965; Shank & Aprison, 1970b), conditions associated with concomitant quantitative changes in the two parameters have not been previously found. Also, metabolic or dietary deviations evoking such a change may represent hazards to the developing brain, for increases in brain glycine concentration have been linked to the neurological abnormalities in children with the inborn disease of non-ketotic hyperglycinemia (Perry et al, 1977) and in the mutant mouse strains "staggerer", "weaver" and "nervous"

(McBride et al, 1976). The main objectives of the present investigation was to elucidate mechanisms regulating the cerebral glycine content during gestational and postnatal hyperphenylalaninemia, and to determine whether the glycine response was specific or could be evoked by elevations in amino acids other than phe, and to quantitatively compare this response with alterations in cerebral PSP activity.

RESULTS:

Since increased cerebral glycine content and PSP activity have been demonstrated to accompany hyperphenylalaninemia (Isaacs & Greengard, 1980), the question was addressed of whether this phenomenon could also be observed in other experimentally induced hyperaminoacidemias. It had also been noted previously that elevations of glycine content and PSP activity in the brain of suckling rats given a daily injection of a-mephe plus phe is seen only after three or more days of this treatment (Isaacs & Greengard, 1980). The measurements therefore, were carried out after 3 to 5 days of treatment with the indicated substances. Included in the study were aromatic, branched chain and neutral amino acids. In Table V-1 it may be seen that phe caused almost as much increase in cerebral glycine concentration as did phe plus a-mephe, while the response to a-mephe alone was smaller. Tyrosine (even when injected twice daily) and isoleucine, proline, threonine and alanine were without effect. The observations on cerebral PSP were similar, except that methionine as well as phe or phe plus a-mephe caused increased activity. The results of tissue specificity studies in Table V-2, demonstrated that these effects of phe or methionine on PSP were specific for brain, since the renal or hepatic enzyme activities were unchanged by hyperphenylalaninemia and were in fact decreased by methionine treatment. When methionine and a-mephe plus phe

TABLE V-1

THE EFFECT OF AMINO ACID ADMINISTRATION ON CEREBRAL GLYCINE CONTENT AND PHOSPHOSERINE PHOSPHATASE ACTIVITY

All amino acids were injected daily (or twice daily, as # indicates on last line) in amounts of 5.2 umol/g B.Wt. (except a-mephe 2.4 umol/g B.Wt.) from days 2 to 7 of age, and brain tissue was analyzed 24 hours after the last injection. Values given are means ± S.D. with the number of animals in parentheses. As determined using Student's t test, *p<0.05, **p<0.005, ***p<0.001 indicate statistically significant differences from the mean for saline-injected or (+p<0.001)from a-mephe-alone injected rats.

TREATMENT	GLYCINE (umol/g)	PSP (nmol/min/g)
Saline	1.31 ± 0.05(6)	192 ± 7 (6)
A-mephe + phe	1.76 ± 0.11(7)***+	246 ± 17 (7)***+
Phe	1.60 ± 0.15(3)**	227 ± 13 (3)**
A-mephe	1.43 ± 0.08(6)	194 ± 14 (6)
Methionine	1.33 ± 0.09(3)	252 ± 37 (3)**
Isoleucine	1.32, 1.35	182, 180
Proline	1.32 ± 0.01(4)	189 ± 1 (4)
Threonine	1.31 ± 0.02(3)	184 ± 11 (3)
Alanine	1.35 ± 0.05(3)	204 ± 14 (3)
Tyrosine	1.34 ± 0.06(4)	194 ± 7 (4)
Tyrosine#	1.34 ± 0.06(5)	201 ± 14 (5)

TABLE V-2

HYPERAMINOACIDEMIA AND TISSUE PHOSPHOSERINE PHOSPHATASE ACTIVITY

Following daily injections of saline, a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) or methionine (5.2 umol/g B.Wt.) from the third day of age, brain, kidney and liver were assayed for PSP activity 24 hours after the last injection at the age of 8 days. Results are means ± S.D. for the number of rats given in parentheses. Statistical significance of difference from saline-injected controls as determined by Student's t test is indicated by *p<0.05, **p<0.01, ***p<0.001.

	PSP (nmol/min/g)		
	BRAIN	KIDNEY	LIVER
Saline	192 ± 7 (6)	928 ± 47(3)	947 ± 86(3)
A-mephe + phe	246 ± 17(7)***	960 ± 85(3)	839 ± 57(3)
Methionine	252 ± 37(3)**	702 ± 74(3)*	661 ± 66(3)*

TABLE V-3

THE EFFECT OF HYPERPHENYLALANINEMIA AND HYPERMETHIONINEMIA ON CEREBRAL GLYCINE CONTENT AND PHOSPHOSERINE PHOSPHATASE ACTIVITY

Rats were injected from day 2 until day 11 with saline or a-mephe plus phe. In each group, 3 rats were given in addition, from days 9 to 11, saline or methionine (5.2 umol/g) 3 hours before the injection of a-mephe plus phe. Samples were analyzed 24 hours after the last injection. Values are means ± S.D.with the number of animals given in parentheses. Results of analyses by Student's t test are indicated by ***p<0.001 versus saline-injected controls, ++p<0.01 versus saline + methionine group and #p<0.002 versus a-mephe + phe group.

	GLYCINE (umol/g)	PSP (nmol/min/g)
Saline	1.11 ± 0.03 (4)	247 ± 4 (4)
Saline + Methionine	1.14 ± 0.05 (3)	271 ± 18 (3)
A-mephe + phe	1.46 ± 0.05 (4)***	289 ± 6 (4)***
A-mephe + phe + Methionine	1.62 ± 0.09 (3)++	349 ± 15 (3)++#

were administered to the same rats (Table V-3), the increase in cerebral PSP activity was greater than that for animals receiving either regimen alone.

During late fetal and early postnatal development there is a decrease in the glycine content and increase in PSP activity of the normal rat brain. Both parameters show, however, a positive response to chronic hyperphenylalaninemia (Table V-4). In fetal rats these responses become significant in the last two gestational days, i.e. on the 9th day of dietary treatment. The effect of postnatal treatment, on the other hand, is evident by the third daily injection, and more prolonged treatment (c.f. 8 day old rats in Tables V-1 and V-4) causes no further rise in glycine or PSP.

Since the total cerebral content of glycine is decreasing during neonatal life (Bayer & McMurray, 1967) there is the question of whether the hyperphenylalaninemia-associated increase in glycine is a true increase or reflects an interruption or prevention of what would be the normal physiological decline. In order to study this, an age was selected when the rate of fall in glycine was less pronounced. If hyperphenylalaninemia is introduced on day 9, by day 12, there is a measurable elevation in glycine albeit not as great as that seen when treatment was begun on day 2 of age (see 12 day values for E and E1 in Table V-4). After the age of two weeks the standard phe plus a-mephe treatment does not maintain

TABLE V-4

THE EFFECT OF HYPERPHENYLALANINEMIA ON CEREBRAL GLYCINE METABOLISM

Pregnant dams were maintained on a diet supplemented with a-mephe (0.5%) plus phe (3.0%) from the 12th day of gestation. In postnatal rats, treatment was begun on day 2, or in the case of E1, on day 9 only; experimental animals (E) were given a daily injection of the standard amount of a-mephe (2.4 umol/g B.Wt.) plus the standard amounts of phe (5.2 umol/g B.Wt.)or (see Ea) plus twice the standard amount of phe from days 13-15. Animals in group Eb received the standard a-mephe plus phe treatment until day 21 when they were weaned on to a diet containing 0.5% a-mephe plus 3% phe. Values are means ± S.D. of results on the number of animals in parentheses. Statistically significant differences from saline-injected littermate controls of the same age (C) are indicated by *p<0.05, **p<0.01, ***p<0.001 (Student's t test).

AGE (days)	TREATMENT	GLYCINE (umol/g)	PSP (nmol/min/g)	GLYCINE SYNTHASE (nmol/min/g)
Fetal				
17	C	1.73±0.07(3)	125±13 (3)	
	E	1.84±0.02(3)	132±18 (3)	
20-22	C	1.33±0.09(4)	170± 8 (5)	
	E	1.99±0.26(5)**	247±32 (6)**	
Postnatal				
4	C	1.79,1.82	187,197	
	E	2.00,2.11	219,225	
8	C	1.28±0.08(6)	204±11 (6)	4.1±0.45(6)
	E	1.83±0.05(6)***	251±15 (6)***	5.0±0.53(6)
12	C	1.13±0.05(4)	222±12 (4)	
	E1	1.51±0.16(3)*	252±12 (3)*	
	E	1.76±0.11(4)***	297±13 (3)***	
16	C	0.85±0.02(3)	287±10 (3)	4.20±0.06(3)
	E	1.04±0.03(3)**	320±22 (3)	4.59±0.26(3)
	Ea	1.39±0.09(4)***	378±21 (4)**	4.66±0.18(4)*
32	C	1.45±0.07(4)	242± 9 (4)	
	Eb	1.55±0.06(4)	280±15 (4)	

quite as severe a hyperphenylalaninemia as it does earlier so that, as illustrated in Table V-4, twice the dose was needed (on the last three days preceeding assay) to maintain the same significant rises in glycine and PSP in the brain of 16 day old rats. The distribution of PSP between the perikaryal and the synaptosomal cytoplasm does not appear to be altered by hyperphenylalaninemia, since this represented 38 ± 3% (n=3) of the total activity in control brain and essentially the same (43 ± 5% (n=3) for experimental brain, and was also not altered (43 ± 3%,n=3) when the amount of phe given was further increased.

The principal pathway of glycine metabolism involves the glycine cleavage system (Yoshida & Kikuchi, 1972), information about the activity of which can be obtained by determination of the rate of decarboxylation of glycine by the enzyme glycine synthase. Measurements at both 8 and 16 days showed a small but significant increase (see Table V-4) in cerebral glycine synthase activity in hyperphenylalaninemic as compared to control rats. In fetal rats, control or experimental, the glycine synthase activity was found to be too low to be reproducibly measured by the same method.

In order to further explore the association between the cerebral glycine and PSP contents, these parameters were determined in animals exposed to different degrees of gestational and postnatal hyperphenylalaninemia. As depicted in Figure V-1, for each brain or pool of two

fetal cerebra, the glycine concentration varied in proportion to the respective PSP activity. The points for fetal and postnatal animals appear to fit the same linear correlation which was highly significant ($r=0.948$, $p<0.001$).

Once present after 3 days of chronic treatment, the increases in glycine content and PSP activity are known to persist throughout the day, despite the rise and fall of phe (Isaacs & Greengard, 1980). The present study of the effect of withdrawal of hyperphenylalaninemia (Table V-5 and Figure V-2) shows that one day after the termination of treatment with a-mephe plus phe, the cerebral glycine concentration and PSP activity are still elevated, and that about 72 hours are required to revert to control values. Rats treated from days 2 to 21 showed normal PSP and glycine levels in adulthood.

For further characterization of these increases in glycine and PSP, both the effect of a glycine load and the formation of glycine from administered radiolabelled precursor were studied. In a-mephe plus phe treated animals with elevated cerebral glycine content, the plasma concentration of glycine is unchanged (Chapter IV and Table V-6). Injection of large amounts of glycine does raise its cerebral concentration in hyperphenylalaninemic animals, however, the maximal increment (see 3 hour values in Table V-6) was similar to that in untreated rats. By 24 hours, the concentration had reverted to the respective

FIGURE V-1

RELATIONSHIP BETWEEN CEREBRAL GLYCINE CONTENT AND PHOSPHOSERINE PHOSPHATASE ACTIVITY IN DEVELOPING RATS WITH VARYING DEGREES OF HYPERPHENYLALANINEMIA

Open symbols represent glycine and PSP estimations on the same pool of brains from two fetuses on the 22nd day of gestation; they were from dams which, from the 12th day of pregnancy, were on a normal diet (0) or on one containing 0.5% a-mephe plus 3% or 7% phe.(△) Suckling rats (closed symbols) were given saline (●), or standard doses of a-mephe (2.4 umol/g B.Wt.) supplemented with 1.3, 5.2 or 7.8 umol/g B.Wt. phe (▲) daily from days 2 to 7; they were assayed 24 hrs after the last injection. The linear regression coefficient was 0.9484 (n=20), p<0.001.

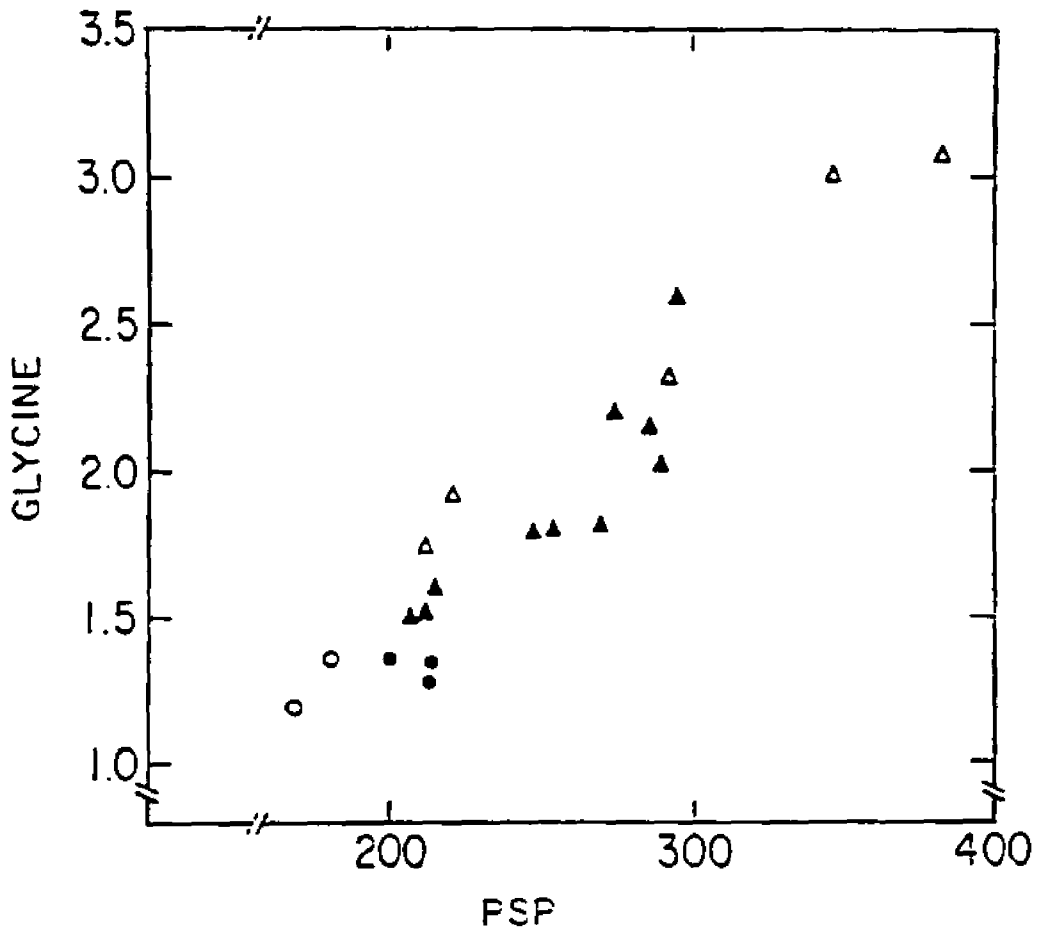


TABLE V-5

WITHDRAWAL OF HYPERPHENYLALANINEMIA AND GLYCINE METABOLISM

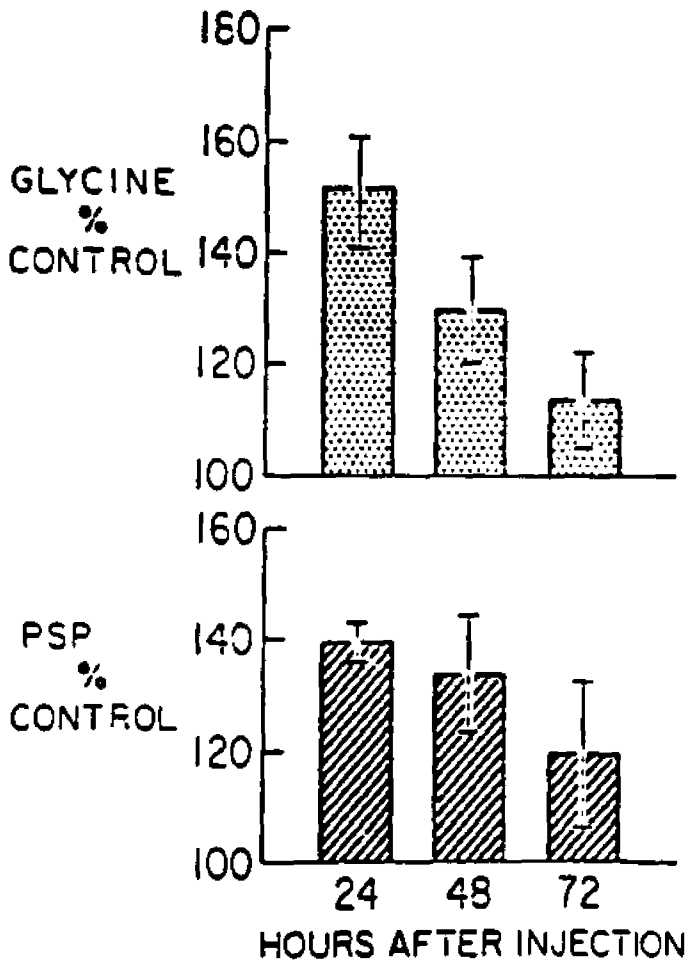
Beginning at day 2, rats received daily injections of saline (C) or a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) (E). Measurements of cerebral glycine concentration and PSP activity were made at the indicated times after the last injection. Results are means ± S.D. with the numbers of animals in parentheses or refer to single animals. As determined by Student's t test, statistical significance of differences from control (C) values at the same age, is indicated by *p<0.05, ** p<0.01, *** p<0.001.

AGE & TIME AFTER LAST INJECTION	GLYCINE (umol/g)		PSP (nmol/min/g)	
	C	E	C	E
12 DAYS				
24 hours	1.24±0.03(5)	1.80±0.10(5)***	242±4(5)	337±8(5)***
Range		1.69-1.96		332-347
48 hours	1.15±0.08(4)	1.50±0.09(4)*	263±8(4)	354±38(4)*
Range		1.38-1.59		309-392
72 hours	1.15±0.03(3)	1.32±0.12(3)	260±3(3)	309±35(3)
Range		1.20-1.44		270-339
ADULT				
200 days	1.46,1.53	1.47,1.49	225,214	229,231

FIGURE V-2

REVERSIBILITY OF ELEVATIONS IN CEREBRAL GLYCINE CONTENT AND PHOSPHOSERINE PHOSPHATASE ACTIVITY FOLLOWING WITHDRAWAL OF HYPERPHENYLALANINEMIA

Rats received saline or a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) daily from days 2 to 11 of age. At the indicated time after the last injection, glycine content (umol/g) and PSP activity (nmol/min/g) were determined in individual brains. Mean values for 3 to 8 rats, as % of control, are shown by the height of the columns, with bars representing 1 standard deviation.



levels present before the glycine injection. To determine the effect of hyperphenylalaninemia on glycine synthesis from precursor, the specific activity of cerebral glycine and serine isolated from animals injected with ^{14}C-glucose was measured and is shown in Table V-7. The values for glycine, but not for serine, were higher in hyperphenylalaninemic animals than in their littermate controls. Increased 'de novo' synthesis or turnover may thus be associated with the rise in cerebral glycine evoked by hyperphenylalaninemia.

TABLE V-6

THE EFFECT OF AN ACUTE LOAD OF GLYCINE ON PLASMA AND CEREBRAL GLYCINE LEVELS

Suckling rats treated from days 2 to 7 with saline (C) or a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) (E) were given on day 8, a single injection of glycine (7.7 umol/g B.Wt.). Plasma and cerebral glycine were measured at the indicated hours thereafter. The values are means ± S.D. of the number of animals given in parentheses, or refer to individual animals. Results of statistical analyses by Student's t test are indicated by ***p<0.001.

	PLASMA (umol/ml)	
	C	E
0 hour	0.48 ± 0.02 (6)	0.47 ± 0.03 (6)
1 hour	3.23 ± 0.69 (3)	3.62 ± 0.56 (3)
3 hours	1.82 ± 0.03 (3)	1.70 ± 0.08 (4)
5 hours	1.10, 1.40	1.51 ± 0.21 (4)
24 hours	0.42	0.52

	BRAIN (umol/g)	
	C	E
0 hour	1.32 ± 0.06 (5)	1.81 ± 0.15 (5)***
1 hour	2.01	2.84
3 hours	2.69, 2.40	3.46 ± 1.86 (3)
5 hours	2.11	2.56
24 hours	1.27 ± 0.05 (3)	1.85 ± 0.09 (4)***

TABLE V-7

CEREBRAL GLYCINE SYNTHESIS FROM LABELLED GLUCOSE

The radioactivities of glycine and serine were determined 10 minutes after intraperitoneal injection of radiolabelled glucose to 10 to 12 day old suckling rats which had received daily injections of saline (C) or a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) (E) from the age of 2 days. Values are means ± S.D. of results on 3 pools of two cerebra each from control or experimental rats of the same litter.

	CEREBRAL RADIOACTIVITY (cpm/g)	
	C	E
Glycine	54 ± 44	572 ± 314
Serine	500 ± 305	475 ± 80
Glycine/Serine	0.098 ± 0.027	1.292 ± 0.805
Alanine-Proline	6315 ± 4694	5929 ± 701

DISCUSSION:

The increase of cerebral glycine concentration in hyperphenylalaninemic rats of the Fischer strain (Isaacs & Greengard, 1980) has been confirmed in Wistar rats (Lane et al, 1980) but whether excess phe itself was specifically responsible for this increase remained to be explored. In this animal model for PKU there is a 2-fold increase in plasma tyrosine concentration due to incomplete (75-90%) suppression of the hepatic PAH by a-mephe allowing some of the injected phe to be converted to tyrosine (DelValle et al, 1978). However, this is unlikely to be the cause of the cerebral glycine effect since administration of large amounts of tyrosine, like that of five other amino acids, did not raise the cerebral glycine content. The small rise caused by injection of a-mephe is not a direct effect but is attributable to the small degree of hyperphenylalaninemia caused by treatment with this analogue alone. Threonine, although reported to induce an elevation of glycine in the rat spinal cord (Maher & Wurtman, 1980), did not induce an increase in the brain and this latter observation was confirmed in the present study. The possibility that subnormal cerebral concentrations of other large neutral amino acids whose uptake is inhibited by excess phe, has also been excluded. Excess isoleucine or tyrosine, which would monopolize the same transport system as does phe and reduce the levels of the same amino acids (e.g. valine, leucine, and

tryptophan) did not cause any increase in glycine concentration. It seems, therefore, that the increase in phe itself is the cause of the cerebral glycine change.

There is greater permeability of the brain to essential rather than non-essential amino acids and in accord with this trend, the BUI of glycine is very low (Pardridge, 1983). Previous studies have indicated that hyperphenylalaninemia does not influence the cerebral transport of glycine (Antonas & Coulson, 1975). Moreover, such alterations in transport rate might be expected to result in immediately apparent and rapidly reversible concentration changes, whereas the rise in glycine concentration in the brain (which is unaccompanied by that in plasma), is detectable only after 2 to 3 days of hyperphenylalaninemia and it takes at least as long to return to normal levels after the treatment has been stopped. Also, when glycine is administered in large doses, the rise and fall of glycine in the brain of hyperphenylalaninemic animals was not different from that in controls, whereas the specific radioactivity of glycine upon ^{14}C-glucose injection was higher than in control animals. These series of observations together with the elevated PSP levels are in accord with the postulate that hyperphenylalaninemia does not affect the transport but rather the intracerebral metabolism of glycine. This is also consistent with the belief that unlike many amino acids, the glycine concentration in the brain is regulated

by cerebral metabolism rather than by its level in the plasma (Aprison et al, 1975).

The role of glycine in the brain encompasses both metabolic and neurotransmitter functions. It serves as a precursor in porphyrin, purine and creatine biosyntheses. Although a non-essential amino acid, it has been shown that it is possible to outstrip the synthetic capacity of glycine by administration of drugs such as benzoate which require glycine to form the excretory conjugate hippuric acid (Bender, 1975). Although the presence of glycine acyl transferase in the liver has been demonstrated, this enzyme activity is low in the brain (MacDermott et al, 1982). The degradation of excess phe via the normally minor transamination pathway resulting in the accumulation of phenylacetic acid may increase glycine utilization because in rats (unlike in man) phenacetylglycine is the major excretory product (Meister, 1965; James et al.1972). However, there is little known about the occurence of these degradative and conjugation processes in the brain or about their possible influence on cerebral glycine metabolism. Other direct connections between cerebral phe and glycine metabolism are also unknown. It is conceivable that the rise in glycine denotes an overcompensation of its increased demand for processes such as purine synthesis. There is some evidence that this occurs in neuroblastoma cells deficient in hypoxanthine phosphoribosyl transferase (necessary for the alternative

route of purine formation) (Skaper & Seegmiller, 1976). However, there are no data that would support the assumption that this phenomenon is pertinent to hyperphenylalaninemia.

The remarkably close quantitative correlation between cerebral glycine and PSP in both pre- and postnatal animals with various degrees of elevation in plasma phe content (Figure V-1) leaves little doubt that hyperphenylalaninemia affects the intracerebral synthesis of glycine via the "phosphorylating" pathway and that it may do so by increasing the level of PSP, the rate limiting enzyme on this pathway. The greater than normal amount of serine thus produced (as indicated by its essentially unchanged steady state concentration (Isaacs & Greengard, 1980) is converted to glycine. The requirement for serine in the metabolism of excess methionine via the transsulfuration pathway (which is operative in the brain, but not as significant in other tissues such as the kidney (Mudd et al, 1965; Mudd & Levy, 1983) is the most probable explanation of the finding that methionine was the only amino acid other than phe which increased the cerebral PSP level and that this did not result in glycine accumulation. Thus, concomitant rises in PSP activity and glycine concentration seem to be specific for the condition of hyperphenylalaninemia.

The optimal substrate concentrations used in the assay of PSP, and the additivity of the normal and

hyperphenylalaninemic brains' activity in admixtures of the two, excluded the possibility that the effect of hyperphenylalaninemia was mediated by changes in the concentration of dissociating inhibitors or activators of PSP. The slow onset further suggests that hyperphenylalaninemia acted on the synthetic system resulting in a larger number or in qualitatively different (more active) PSP molecules.

The significance of the glycine elevation to the cerebral maldevelopment in PKU is unclear since there is no evidence for (or against) this elevation in the brain of human subjects of the disease. Nonetheless, the fact that chronic elevations in the brain level of this inhibitory neurotransmitter (which injection of glycine, owing to its sluggish brain uptake, cannot achieve easily (de Groot et al, 1979)) can be maintained by experimental hyperphenylalaninemia is pertinent to pathological states in man. For, a similar degree of cerebral glycine elevations (brought about by an entirely different mechanism) underlies the severe neurological symptoms and mental maldevelopment of children with inborn, non-ketotic hyperglycinemia (Perry et al, 1977).

CHAPTER VI

EXPERIMENTAL HYPERPHENYLALANINEMIA AND BIOGENIC AMINE METABOLISM IN DEVELOPING RAT BRAIN

In untreated PKU, diminished levels of neuroactive amines and their metabolites have been observed in the blood (Weil-Malherbe, 1955; Pare et al, 1957), the cerebrospinal fluid (CSF) and in autopsied brain samples (McKean, 1972; Curtius et al, 1972). More recently in treated PKU children who were no longer receiving the low-phe dietary regimen, a correlation between elevated blood phe levels, neuropsychological impairment and a reduction in urinary and CSF amines and metabolites has also been noted (Krause et al, 1985; Lou et al, 1985). In the experimental model of PKU in suckling rats achieved by suppression of PAH with a-mephe (Greengard et al, 1976), cerebral 5HT, 5HIAA (Isaacs & Greengard, 1980; Lane et al, 1980;) DA and NE levels are also diminished (Brass & Greengard, 1982). Post-weanling rats receiving a diet supplemented with a-mephe plus phe have also demonstrated reduced levels of cerebral biogenic amines and metabolites (Taylor et al, 1983). In contrast to studies demonstrating the interference by phe with tyrosine hydroxylation (Nagatsu & Udenfriend 1964; Brass & Greengard, 1982), there is a lack of information available about the underlying cause of the NE decrease in hyperphenylalaninemia. In this chapter, the effect of hyperphenylalaninemia on biogenic amines and their

metabolites was determined. In addition, measurement was made of the 'in vitro' activity of the enzyme Dopamine-B-Hydroxylase (DBH), which is responsible for NE biosynthesis. The effects of two other phe analogs were also determined: p-chlphe which is also used in studies of hyperphenylalaninemia, and B-methylphenylalanine (b-mephe) which has not been studied previously.

RESULTS:

For determination of the effects of hyperphenylalaninemia on cerebral biogenic amines, suckling rats were administered a-mephe and p-chlphe either with or without phe and all substances were injected for at least three days. For studies of biogenic amine metabolism, equal intralitter numbers of males and females were allocated per treatment group, since lower basal levels of catecholamines in infant female rat brain has been previously noted (Brass, 1983). The analyses of all monoamines and metabolites were carried out using HPLC with electrochemical detection. The effects of three daily injections of a-mephe with or without phe on cerebral biogenic amines are shown in Table VI-1 with a typical HPLC chromatogram depicted in Figure VI-I. The 5HT and 5HIAA levels were significantly decreased in the experimentally hyperphenylalaninemic group while a small loss was noted with a-mephe alone. A dramatic effect on the catecholamines was observed with both treatments; without phe, DA levels were 54% and in the presence of phe, 67% of controls. NE was similarly decreased to 44% and 62% respectively.

Information on the net metabolism and turnover of the catecholamines can be obtained by quantification of metabolites formed through the sequential action of monoamine oxidase and catechol-O-methyl-transferase. Among DA metabolites are homovanillic acid (HVA),

TABLE VI-1

THE EFFECT OF A-METHYLPHENYLALANINE ALONE OR WITH PHENYLALANINE ON CEREBRAL BIOGENIC AMINES AND METABOLITES

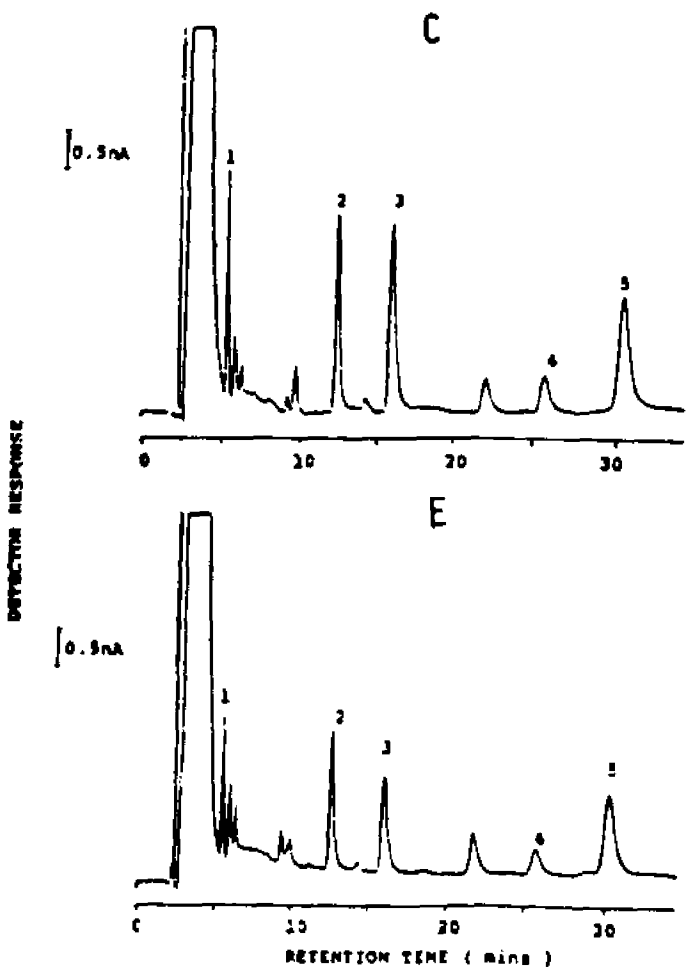
Suckling rats received 3 daily injections of saline, a-mephe alone (2.4 umol/g B.Wt.) or a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.). Brain tissue was taken for analysis of biogenic amines and metabolites at the age of 10 days, 5 hours after the last injection. Results are expressed as means ± S.D. of the number of animals given in parentheses. Statistical analyses by Student's t test of the significance of difference of the mean from saline-injected controls is indicated by * $p<0.05$, *** $p<0.001$.

	TREATMENT GROUP		
(nmol/g)	SALINE (n=4)	A-MEPHE (n=3)	A-MEPHE + PHE (n=4)
5HT	1.66 ± 0.06	1.31 ± 0.12*	0.73 ± 0.07 ***
5HIAA	1.58 ± 0.12	1.09 ± 0.18*	0.47 ± 0.05 ***
DA	2.59 ± 0.19	1.39 ± 0.09***	1.74 ± 0.15 ***
HVA	1.25 ± 0.08	0.52 ± 0.06***	0.68 ± 0.15 ***
NE	1.33 ± 0.04	0.59 ± 0.05***	0.82 ± 0.07 ***

FIGURE VI-1

HPLC CHROMATOGRAMS OF CEREBRAL MONOAMINES AND METABOLITES FOLLOWING ADMINISTRATION OF A-METHYLPHENYLALANINE PLUS PHENYLALANINE

Chronic treatment with saline (C) or with the standard regimen of a-mephe plus phe was administered to suckling rats. At 10 days of age, 5 hours after the last injection brains were prepared for HPLC/EC analyses of catecholamines, indoleamines and metabolites. Peaks are indicated by : 1 = norepinephrine, 2 = dopamine, 3 = 5-hydroxyindoleacetic acid, 4 = homovanillic acid, 5 = serotonin.



dihydroxyphenylacetic acid (DOPAC) and 3-methoxytyramine (3MT) (Westerink, 1985). HVA is present in 10 day old whole rat brain in measurable amounts. Although 3MT is found in adult rat brain, at 10 days of age it was not detectable using the present HPLC system. The analysis of DOPAC presented another difficulty in that, even though it was present in postnatal rat brain, with the HPLC conditions optimized for monoamine determination it eluted in a region of the chromatogram where clear separation from other compounds such as epinephrine did not always occur. Thus, the only catecholamine metabolite routinely quantified in these studies was HVA. As seen in Table VI-1, this compound is reduced to 42% of controls with a-mephe administration and to 54% with a-mephe plus phe. Using the same HPLC conditions, the major NE metabolite in the CNS, 3-hydroxy,4-hydroxyphenylglycol (MHPG) (Schanberg et al, 1968; Mass & Landis, 1968), could be separated from NE. Most of this substance is present in the brain as a sulfated conjugate. However although it has been reported that digestion with an aryl sulfatase from Helix Pomatia (Warnhoff, 1984) will release the sulfate and permit determination of total metabolite present, this procedure was not found to yield a post-digestion supernatant which could be chromatographed and the peaks readily identified.

One aspect of catecholamine metabolism that has not been previously investigated in experimental hyperphenylalaninemia is the reason for the NE deficit.

Assay of the enzyme responsible for NE biosynthesis from DA, Dopamine-B-Hydroxylase (DBH) (Coyle & Axelrod,1972), has been beset by technical difficulties, mostly because of the presence of endogenous interferring substances in brain homogenates (Laduron, 1975). Improved analysis based on the use of the natural substrate DA and the direct measurement of the product NE by HPLC/EC has now been achieved (Sperk et al, 1980). These investigators have also reported the inclusion of Concanavalin A to bind the enzyme and assay its activity free from endogenous tissue inhibitors. In the present study this procedure was not found to completely or consistently bind DBH and consequently whole homogenates were used instead (Coyle & Axelrod, 1972). The already developed HPLC/EC system for biogenic amine analysis however, permitted the use of this approach for assay of DBH activity with some minor modifications as described in Chapter II. The effect of chronic hyperphenylalaninemia on rat brain DBH activity was determined at 10 and 14 days of age following at least 3 daily injections of either a-mephe alone or together with phe. However, as shown in Table VI-2, these treatments had no effect on DBH activity. The results are shown together with measurements of enzyme activity in cerebral monoamine synthesis.

In addition to DA, other B phenylethylamine compounds can act as substrates for DBH. Normally a trace substance, phenylethylamine (PE) has been found to be elevated in

TABLE VI-2

HYPERPHENYLALANINEMIA AND THE ACTIVITIES OF MONOAMINE BIOSYNTHETIC ENZYMES

Chronic treatment with either saline, a-mephe alone (2.4 umol/g B.Wt.) or a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) was given to suckling rats. The activities of the cerebral enzymes dopamine B Hydroxylase (DBH) in whole brain, tyrosine hydroxylase (TYR H) and dopa decarboxylase (DDC) in the striatum and tryptophan hydroxylase (TRP H) in midbrain were measured at the ages indicated, 4 to 6 hours after the last injection. Results are expressed as means ± S.D. for the number of animals given in parentheses or are single values, + indicating adaptation of values from Brass,(1983) and # from Greengard & Wolfe,(1987).

	TREATMENT GROUP		
	SALINE	A-MEPHE	A-MEPHE + PHE
DBH (pmol/min/g)			
10 days	184 ± 21 (4)	206 ± 15 (4)	190 ± 17 (4)
14 days	208 ± 13 (3)		206 ± 10 (3)
TYR H (umol/min/g)+			
15 days	24.7, 21.8		22.1 ± 3.3(4)
DDC (umol/min/g)+			
15 days	2.28 ± 0.51(12)		2.33 ± 0.60(12)
TRP H (nmol/hr/g)#			
9-12 days	96 ± 17(14)	53 ± 12 (9)	86 ± 22 (12)

untreated PKU (Oates et al,1963; Michals & Matalon,1985). In adult rats administration of PE can decrease brain catecholamines, particularly NE (Fuxe et al. 1967; Jackson & Smythe, 1973). However in neonatal rats, the role of PE in relation to the catecholamine deficit has not been studied previously. The injection protocol utilized previously for adult rats was adapted (Fuxe et al, 1967), and biogenic amine profiles were examined. As shown in Table VI-3, the loss of both DA, NE as well as 5HT was dramatic. Concomitant with these deficits, was the emergence of increased levels of metabolites, including 3MT, (0.30, 0.31 nmol/g) which as mentioned above, was normally undetectable in postnatal rat brain during the first 10 days of life.

In the model of experimental hyperphenylalaninemia induced by a-mephe plus phe, the question of how this biogenic amine deficit relates to the loss of cerebral capacity to accumulate phe (described in Chapter III) was also of interest. The strategy already developed to determine age-related phe and trp levels was thus also used to investigate the effect on cerebral biogenic amines. Blood phe levels were raised and sustained for 5 hours in rats from the suckling period up to 70 days of age. The results outlined in Table VI-4 indicate that the significant 5HT deficit at 16 days, can still occur even at 70 days. However, the decreases in DA and NE, appreciable at 16 days, become progressively smaller with

TABLE VI-3

CEREBRAL BIOGENIC AMINES AND METABOLITES FOLLOWING THE ADMINISTRATION OF PHENYLETHYLAMINE

Suckling rats were given saline, 3 daily injections of a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) or a single injection of phenylethylamine (PE)(100 ug/g B.Wt.). Brain samples were taken at the age of 10 days, either 5 hours (a-mephe plus phe) or 1 hour after injection (PE). Results are given as % of saline injected controls. Statistical analyses by Student's t test are indicated by *** $p<0.001$.

	TREATMENT GROUP		
	SALINE	A-MEPHE + PHE	PE
5HT	100 ± 5(4)	51, 48	65 ± 8 (3)***
5HIAA	100 ± 7(4)	40, 42	119 ± 13 (3)
DA	100 ± 7(4)	64, 65	48 ± 16 (3)***
HVA	100 ± 5(4)	51, 56	161 ± 6 (3)***
NE	100 ± 6(4)	62, 60	34 ± 24 (3)***

TABLE VI-4

AGE-RELATED EFFECT OF HYPERPHENYLALANINEMIA

ON CEREBRAL BIOGENIC AMINES AND METABOLITES

At the indicated ages, rats received a-mephe (2.4 umol/g) 20 hours before an acute load of phe, age-adjusted so as to elevate and sustain plasma levels. Brains were taken for analysis 5 hours after induction of hyperphenylalaninemia. Results are expressed as means ± S.D. as % of saline-injected control values for the appropriate age and the number of animals is given in parentheses. Statistical analyses by Student's t test of the significances of the differences from corresponding saline-injected age-matched controls is indicated by * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

	AGE (days)		
	16	30	70
% SALINE -INJECTED CONTROLS			
5HT	43 ± 7 (4)***	62 ± 22 (4)***	68 ± 6 (4)***
5HIAA	37 ± 9 (4)***	43 ± 18 (4)***	54 ± 11(4)***
DA	78 ± 9 (4)*	87 ± 4 (4)	96 ± 3 (4)
NE	77 ± 8 (4)*	83 ± 3 (4)	93 ± 9 (4)

age and eventually non-significant. This age-dependent change coincides with the declining ability of the mature brain to achieve the cerebral phe concentrations that are seen during the early suckling period (Chapter III).

An alternative means of producing experimental hyperphenylalaninemia in developing rats is by the regimen of p-chlphe with phe. Although in adult rats p-chlphe alone has been reported to alter NE (Miller et al, 1970), the effect of this combination of treatment with phe on catecholamines has also not been studied previously in suckling rats. Samples were therefore taken in both an acute and chronic study: for the latter, three daily injections were given and the results are shown in Table VI-5 with typical chromatograms in Figure VI-2. By 24 hours after the first injection of p-chlphe, the cerebral 5HT and 5HIAA are reduced, but have not reached minimum levels, as shown by the further marked diminution in both groups treated chronically for three days with either p-chlphe or p-chlphe plus phe. By comparison, the concentrations of catecholamines were not markedly altered after three days of treatment, although the DA and HVA levels in both groups treated chronically did show a barely significant decrease from controls ($p<0.05$). In rats treated with p-chlphe alone, tyrosine levels were reduced both in the plasma (saline 274 $\pm$ 20, n=4, versus p-chlphe 191 $\pm$ 38, n=4 nmol/ml) and in the brain (saline 197 $\pm$ 12, n=4 versus p-chlphe 151 $\pm$ 12, n=4 nmol/g),

TABLE VI-5

THE EFFECT OF P-CHLOROPHENYLALANINE ALONE OR WITH PHENYLALANINE ON CEREBRAL BIOGENIC AMINES AND METABOLITES

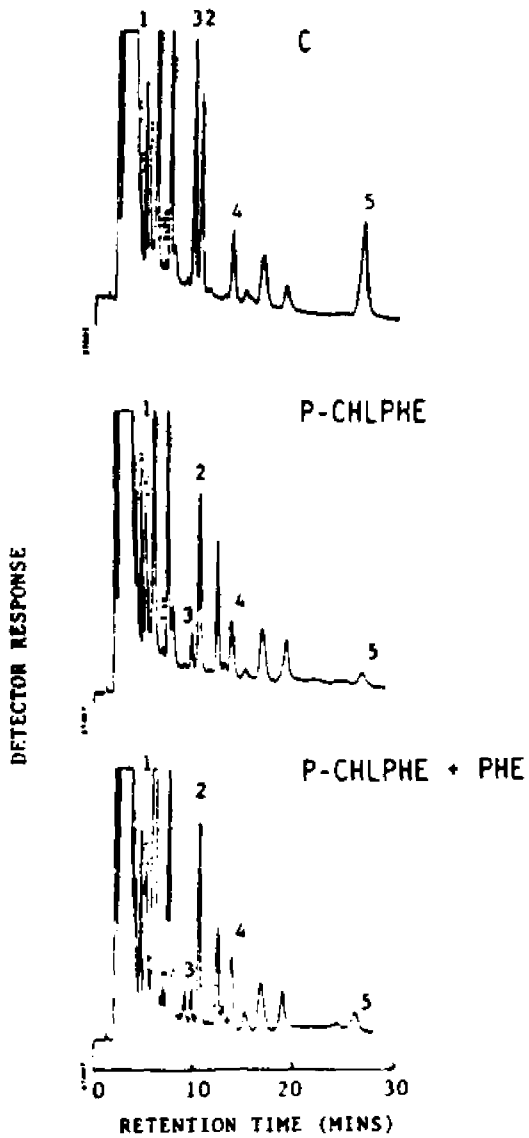
Suckling rats received either saline, one single injection of p-chlphe alone (0.9 umol/g B.Wt.) or 3 daily injections of p-chlphe alone (0.9 umol/g B.Wt.), or p-chlphe (0.9 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.). At the age of 10 days, (24 hours after the first injection or 5 hours after the third treatment), whole brains were analyzed for biogenic amines and metabolites by HPLC/EC. Results are expressed as means ± SD in nmol/g for the number of animals given in parentheses, Analysis of significances of differences between means by Student's t test is indicated by * $p<0.05$, *** $p<0.001$.

	TREATMENT GROUP			
	SALINE (n=10)	ACUTE P-CHLPHE (n=3)	CHRONIC P-CHLPHE (n=6)	CHRONIC P-CHLPHE + PHE (n=6)
(nmol/g)				
5HT	1.62±0.11	0.81±0.13***	0.40±0.12***	0.34±0.02***
5HIAA	1.41±0.16	0.53±0.05***	0.29±0.08***	0.19±0.04***
DA	2.52±0.22	2.10±0.20	2.13±0.08*	2.09±0.12
HVA	1.29±0.18	0.93±0.07	1.05±0.09*	1.10±0.14
NE	1.36±0.09	1.28±0.08	1.28±0.10	1.28±0.12

FIGURE VI-2

HPLC CHROMATOGRAMS OF CEREBRAL MONOAMINES AND METABOLITES FOLLOWING ADMINISTRATION OF P-CHLOROPHENYLALANINE ALONE OR WITH PHENYLALANINE

Rats received three daily injections of saline, p-chlphe only, or p-chlphe plus phe. The final injection was given at the age of 10 days and 5 hours later whole brains were taken for HPLC/EC analyses. Representative chromatograms are shown for each treatment group. Peak identitiy is indicated by: 1 = norepinephrine, 2 = dopamine, 3 = h-hydroxyindoleacetic acid, 4 = homovanillic acid, 5 = serotonin.



suggesting an effect of diminished precursor availability. However, this effect was not operative in rats treated with p-chlphe plus phe, since the tyrosine levels were in fact slightly elevated (plasma, 430 ± 81 nmol/ml, n=4 and brain 274 ± 69 nmol/g, n=4). The DA and HVA deficits are thus consistent with the competitive action of phe at the tyrosine hydroxylase or dopa decarboxylase steps and the elevated tyrosine is apparently not sufficient to overcome this inhibition. Closer observation of the chromatograms obtained using the p-chlphe or p-chlphe plus phe treatments (Figure V-2) revealed the presence of an additional peak which eluted between DA and HVA with a retention time of approximately 13 minutes. Under the HPLC conditions used in the present study, neither p-chlphe nor p-chlorophenylethylamine were detected. Both these substances as well as the above unidentified compound(s) did not interfere with the determination of biogenic amines and metabolites of interest.

Although two phe analogs, a-mephe and p-chlphe, are available to inhibit PAH, an alternative analog was sought. The effect of another methyl derivative of phe, B-methylphenylalanine (b-mephe) was thus examined. Suckling rats were injected with b-mephe in amounts equimolar with a-mephe and the PAH activity in the liver determined. As shown in Table VI-6, b-mephe does reduce PAH activity but not quite to the same degree as a-mephe. When rats were injected with daily doses of b-mephe their growth was not

TABLE VI-6

THE EFFECT OF B-METHYLPHENYLALANINE ON LIVER PHENYLALANINE HYDROXYLASE ACTIVITY AND CEREBRAL BIOGENIC AMINES AND METABOLITES

At the indicated time after injection of either saline, a-mephe (2.4 umol/g B.Wt.) or b-mephe (2.4 umol/g B.Wt), livers from 7 day old rats were prepared for the analysis of PAH activity. Other rats were injected for 3 days with saline, a-mephe (2.4 umol/g B.Wt.) b-mephe (2.4 umol/g B.Wt.) and brains were analyzed for biogenic amines and metabolites. Results are expressed as means ± S.D. for the number of animals given in parentheses or are individual values. Statistical analyses of the significance of differences by Student's t test from saline-injected controls is given by * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

	TREATMENT GROUP		
	SALINE (n=4)	A-MEPHE (n=3)	B-MEPHE (n=3)
LIVER PAH (umol/min/g) 18-24 hours	651 ± 21	170 ± 4 ***	276 ± 17***
BRAIN (nmol/g)			
5HT	1.66 ± 0.06	1.31 ± 0.12*	1.58 ± 0.08
5HIAA	1.58 ± 0.12	1.09 ± 0.18*	1.37 ± 0.10
DA	2.59 ± 0.19	1.39 ± 0.09 ***	2.79 ± 0.15
HVA	1.25 ± 0.08	0.52 ± 0.06 ***	1.21 ± 0.06
NE	1.33 ± 0.04	0.59 ± 0.05 ***	1.34 ± 0.05

impaired and no toxic effects were observed. As also shown in Table VI-6, no effect was seen on monoamines and their metabolites in the brain. The blood phe levels remained close to normal, and injection of b-mephe together with phe was required to induce significant hyperphenylalaninemia (see Chapter VII).

DISCUSSION:

Analyses using HPLC/EC in the present study have permitted not only simultaneous determination of indoleamines and catecholamines but also a more detailed quantitative examination of cerebral biogenic amines and metabolites than was possible using previous fluorometric and radioenzymatic assays (Isaacs & Greengard, 1980; Lane et al, 1980; Brass & Greengard, 1982; Greengard & Wolfe, 1987). Following a regimen of a-mephe plus phe, the decreased 5HT and 5HIAA levels in the brain measured by HPLC in the present experiments confirm previous observations (Isaacs & Greengard,1980; Lane et et al, 1980; Greengard & Wolfe, 1987). In this model, there is no cumulative effect of the treatment on indole parameters and the reduced indoles are most likely accounted for by the concomitant reduction in tryptophan level rather than by an effect on tryptophan hydroxylase activity (Greengard & Wolfe, 1987).

Both DA and NE concentrations are decreased by a regimen of a-mephe plus phe. It was now found that HVA was decreased as well, suggesting that the turnover and/or release of DA is also reduced (Westerink, 1985). This finding is in accord with the low HVA found in the CSF in PKU (McKean, 1972; Curtius et al, 1972). The diminished catecholamine levels are consistent with the known inhibition of tyrosine hydroxylase by phe (Nagatsu et al, 1964; Brass & Greengard, 1982). There is evidence

that m- and o-tyrosines which are normally present at trace levels in the brain, may actually be the products of the action of tyrosine hydroxylase on phe (Ishimitsu et al,1986).

A-mephe is known to inhibit the activity of tyrosine hydroxylase 'in vitro' and to do so at lower concentrations than does phe (Brass & Greengard, 1982). However, probably because phe reduces the cerebral uptake of a-mephe, the effect of the two substances on catecholamine synthesis 'in vivo' is not additive. In fact, treatment with a-mephe caused a greater cerebral deficit than did a-mephe with phe, and deficits in NE and HVA were also somewhat greater (Table VI-1). It should be noted in this connection that, while a-mephe is an irreversible inhibitor of the hepatic PAH 'in vivo' causing prolonged suppression, its inhibition of the cerebral tyrosine hydroxylase and of catecholamine synthesis 'in vivo' is a rapidly reversible effect (Brass & Greengard, 1982). Thus, in the PKU model, a-mephe plays only an indirect role (i.e. prevention of phe metabolism) and the cerebral catecholamine deficit is attributable to hyperphenylalaninemia itself. This is demonstrated by observations that one day after an injection of a-mephe (i.e. when PAH is still suppressed but a-mephe inhibition of tyrosine hydroxylase is no longer present) administration of phe alone results in significantly diminished cerebral DA, HVA and NE concentrations (see

Table VI-4).

In view of the significant reduction of NE, there may be an effect of hyperphenylalaninemia on DBH although the assay of DBH activity at saturating substrate concentrations 'in vitro' does not indicate an inhibitory effect of chronic hyperphenylalaninemia, phe or a-mephe on this enzyme. However, the Km of dopamine for DBH in stellate ganglion is 0.6 mM (Molinoff et al, 1971) and it is possible that owing to the fall in cerebral DA, the enzyme was far from being saturated thus leading to a decline in NE production. The effect on NE, therefore, may be attributable not only to an effect on tyrosine hydroxylase but also to the effect of diminished substrate availability for the DBH catalysed conversion of DA to NE.

The present study shows that cerebral DA, NE and 5HT of young suckling rats can be significantly depleted upon the administration of PE. These results are of interest because, in addition to an the earlier report of elevated PE levels in PKU (Oates et al, 1963), a more recent examination of metabolite levels in PKU children after termination of the dietary restriction has also showed increased PE (Michals & Matalon, 1985). PE is actively taken up from the blood by the brain (Oldendorf, 1971), and can be formed in the brain itself through decarboxylation by dopa decarboxylase (L-aromatic amino acid decarboxylase). In addition, it has been suggested that interference with monoamine synthesis may also occur

through the formation of the PE metabolite, phenylethanolamine, by DBH (Blau, 1979). The reduced NE formation in hyperphenylalaninemia may thus be aggravated by this interference with the DBH reaction. Continuing investigation of the role of PE and phenylethanolamine in the catecholamine loss in hyperphenylalaninemia is hampered by difficulties in studying and quantifying PE levels. The rapid metabolism of PE 'in vivo' can be overcome by administering the specific monoamine oxidase B inhibitor deprenyl, but present methodology available for quantification of PE is believed to be questionable (Boulton & Juorio, 1983).

In infant rats, p-chlphe treatment alone or with phe caused a dramatic reduction in the indoles 5HT and 5HIAA. With neither of these regimens was there as marked an effect on the catecholamines. Repeated injections of p-chlphe had no cumulative effect on the catecholamine levels, despite the continuing presence of a marked decrease in 5HT. Thus the results do not lend support to the hypothesis that the two neurotransmitter systems are synergistically regulated and that indole status will control DA and NE levels (Fuenmayor & Bermudez, 1985). However the possibility cannot be ruled out that this regulation may operate in some adult brain areas and that it was obscured in the present study of the whole brain of immature rats.

The decreased tyrosine values in the rats treated

with p-chlphe alone lends support to a proposal that there is a catecholamine precursor shortage resulting from such a treatment (Gibson & Wurtman, 1978). However these authors have suggested that the loss of tyrosine in the brain occurs due to competition with p-chlphe at the BBB. An alternative explanation may be the diminished plasma concentration of tyrosine. This did not occur when p-chlphe was administered together with phe; the plasma and brain tyrosine levels became elevated which is probably the reason for the fact that, despite severe hyperphenylalaninemia, the DA deficiency is small and the NE concentration remains unchanged.

In the search for an alternative suppressor of PAH, b-mephe was tested and was found to cause a 58% inhibition. Chronic treatment with b-mephe did not reduce cerebral biogenic amine concentrations (Table VI-6) and thus does not appear to exert inhibitory effects on tyrosine, tryptophan or dopamine-B hydroxylases. Such a substance should thus be useful for studies involving inhibition of PAH only, without effect on other hydroxylases.

The role of biogenic amines in the production of the cerebral abnormalities in PKU has been recently reexamined in the light of the finding of reduced 5HT and DA excreted by children after terminating the low phe diet (Krause et al, 1985). In the present study determination of age-related effects of hyperphenylalaninemia on

monoamine levels in the brain through the use of PAH suppression by a-mephe followed 20-24 hours later by age-adjusted injections of phe (as also applied in the experiments of Chapters III and IV) allowed the effect of hyperphenylalaninemia 'per se' to be determined. It is seen that the 5HT depletion, which in this model is principally a consequence of reduced concentration of precursor tryptophan, can still be produced at 70 days of age. However production of the catecholamine deficit by this age is very difficult, because the cerebral phe concentration can be raised only to a limited extent. This limitation is consistent with the declining capacity of the developing brain to accumulate the same phe levels as during the early postnatal period. Information on these age-related differences was thus extended to studies described in the following chapter designed to selectively deplete and restore biogenic amine levels and produce variants of the experimental animal model for future behavioral assessments.

CHAPTER VII

GESTATIONAL AND POSTNATAL EXPERIMENTAL HYPERPHENYLALANINEMIA: BIOCHEMICAL AND BEHAVIORAL MODULATIONS

Establishment of an animal model of PKU requires that the treatment utilized to experimentally induce hyperphenylalaninemia will also produce lasting behavioral anomalies analogous to the mental retardation found in the human disease (Karrer & Cahilly,1965; Vorhees et al, 1980). However inherent in this requirement is the selection of the appropriate window through which recognition of the cerebral dysfunction may be made. Using standard tests of maze learning and spontaneous locomoter activity, adult rats which were rendered hyperphenylalaninemic during the first three weeks of life using a regimen of a-mephe plus phe, have demonstrated behavioral and learning deficits (Glick & Greengard, 1980; Lane et al, 1980; Brass, 1983). Traditionally such tests examine 'essential learning' i.e the acquisition of some information necessary to obtain food or avoid an aversive event. However, tests of 'advantageous learning', in which the information to be learned is not critical for meeting immediate biological needs, are believed to offer a more appropriate and sensitive means for revealing behavioral deficits which may be analogous to mental retardation in humans. It has been shown that as judged by these tests also, early neonatal exposure to hyperphenylalaninemia

results in permanent cognitive deficits (Strupp et al, 1984).

Characterization of biochemical and behavioral abnormalities using a-mephe and phe were originally carried out using the albino Fischer 344 strain of rats (Greengard et al, 1976; DelValle et al, 1978; Glick & Greengard, 1980). A number of these same defects were reproduced in the Wistar, another albino strain (Lane et al, 1980). Long Evans hooded rats which had been exposed to the same daily treatment regimen during the first three weeks of life were found to exhibit cognitive deficits in adult life (Strupp et al, 1984). However, since no biochemical measurements were carried out in this strain, the phe elevations in the blood and the brain that could be achieved by the standard experimental treatment during the prenatal and postnatal periods were unknown.

This chapter describes pre- and postnatal studies using the Long Evans strain. A model of gestational hyperphenylalaninemia was established to enable cognitive testing in adulthood. The studies also provide additional information about the effect of postnatal hyperphenylalaninemia on cerebral biogenic amine metabolism, and describe the basis for creating variant models by selective depletion and restoration of monoamines whereby it is possible to probe the separate role these substances may play in the development of cognitive abnormalities.

RESULTS:

Gestational Hyperphenylalaninemia:

Gestational hyperphenylalaninemia has been recently described in Fischer rats (Brass et al.,1982, Brass, 1983). Using this already established procedure, a-mephe and phe were added to purina chow at concentrations of 0.5% and 5.0% respectively. This level of phe was intermediate between the 3.0% and 7.0% amounts utilized previously, both concentrations of which from day 11 of gestation had assured the birth of live Fischer rat pups. When pregnant Long Evans rats were maintained on this diet from day 11 of gestation, the number and the size of litters that came to term was normal and birth occurred within the expected time. However the newborns showed a 28% decrease in body weight (4.4 $\pm$ 0.6g, n=20 versus 6.1 $\pm$ 0.4 g, n=22) and the entire litter did not survive more than 72 hours after birth. The phe concentration in the diet was thus reduced in decremental amounts but it was not until the phe was lowered to 2.5% that the litters showed 100% survival. The levels of phe in the maternal, fetal and newborn plasma and brain from these gestational studies comparing Fischer and Long Evans rats are shown in Table VII-1. The brain/plasma phe ratios were greater in the fetus than in the dam indicating a concentration gradient of phe across the placenta. Since plasma and brain samples were taken from the Long Evans rat pups immediately after birth, the newborn phe concentrations

are still elevated. The reduced plasma and brain tryptophan levels were also consistent with the degree of hyperphenylalaninemia present.

The selected composition of the diet was thus 0.5% a-mephe and 2.5% phe. The offspring of pregnant Long Evans rats, exposed to hyperphenylalaninemia from day 11 of gestation until birth only, were studied in adulthood to determine parameters of rotational behavior and lateralization. Table VII-2 outlines results of the tests performed. No significant differences were found between the control and experimental groups for either males or females. Comparison of this data with that obtained in Fischer rats showed that the behavioral indices of locomoter activity in both strains of rats were similar although the net rotations were fewer in the Long Evans rats.

Postnatal Hyperphenylalaninemia:

Suckling Long Evans rats received the standard daily regimen of a-mephe plus phe from day 3 of age and biochemical parameters were determined. The results are presented in Table VII-3. The liver PAH activity was suppressed. The blood phe showed forty-fold elevations in 10 day old rats assayed 4 hours after the last injection, but by 16 days the ability of the treatment to elevate plasma and cerebral phe has diminished somewhat. This pattern is quantitatively similar to that observed for Fischer rats. The effect of chronic hyperphenylalaninemia

TABLE VII-1

BODY WEIGHT, BRAIN WEIGHT AND AROMATIC AMINO ACID LEVELS AFTER EXPOSURE TO GESTATIONAL HYPERPHENYLALANINEMIA

Pregnant dams were placed on a high phe diet from the twelfth day of gestation, as Purina Lab Chow supplemented with a-mephe (0.5% w/w) plus phe (3.0% w/w for Fischer rats and 2.5% w/w for Long Evans rats)(E) or continued to receive the unsupplemented Purina Lab Chow (C). Plasma and brain amino acids were measured in the dams, fetuses and newborns taken within 8 hours of birth. The concentration of plasma phe in the dam was determined on day 21-22 of pregnancy by estimation in tail blood. Values given are means ± S.D. of the number of rats indicated in parentheses. Results of statistical analyses by Student's t test are indicated by *** $p<0.001$.

	FISCHER		LONG EVANS	
	C	E	C	E
DAM (21-22 days)				
Plasma phe	96 ± 31(6)	1218 ± 449(5)***	60	2018
trp			71	62
Brain phe	80 ± 15(5)	287 ± 78(4)	-	-
FETUS (19-22 days)				
Plasma phe	243 ± 62(6)	2922 ± 801(7)***		
Brain phe	292 ± 52(6)	1755 ± 372(9)***		
NEWBORN				
Body Weight(g)			6.1±0.4(22)	5.2±0.3(15)***
Brain Weight(mg)			266	224
Plasma phe		822,936	64,77	1898,1867
trp			83,95	89,68
Brain phe		726,588	138,150	1759,1802
trp			87,88	70,70

TABLE VII-2

COMPARISON OF BEHAVIORAL RESULTS FROM FISCHER AND LONG EVANS RATS EXPOSED TO GESTATIONAL HYPERPHENYLALANINEMIA

From Day 11 of pregnancy until the day of parturition, experimental dams received Purina Chow with 0.5% a-mephe plus 3.0% phe (Fischer 344 rats) or 2.5% (Long Evans rats). Control animals were from separate litters raised at the same time, but whose dams received a diet of Purina Lab Chow only. In adulthood, the progeny of these animals underwent behavioral testing. The data for Fischer rats are adapted from Brass (1983). Values given are means ± SEM with the number of animals indicated in parentheses.

	CONTROL		EXPERIMENTAL	
Strain	Fischer	Long Evans	Fischer	Long Evans
Tests				
Net Rotations				
Male	65±18 (5)	45±12(8)	89±33(11)	21±6(7)
Female	132±112(4)	21± 8(8)	53±11(15)	21±6(8)
Extra 1/4 Turns				
Male		455± 87(8)		355± 82(7)
Female		650±276(8)		621±152(8)
Total 1/4 Turns				
Male		886±141 (8)		685±163(7)
Female		906±350 (8)		986±235(8)
% Preference				
Male	69±11 (5)	70± 4(8)	73±5(11)	65±4(7)
Female	71±10 (4)	63± 4(8)	71±3(15)	61±3(8)

induced until 16 days of age using a-mephe plus phe, was compared with that utilizing b-mephe plus phe. The group injected with b-mephe plus phe showed a similar decrease in brain weight as that observed in rats treated with a-mephe plus phe. The levels of phe achieved in the plasma and in the brain were, however, less and are consistent with the lesser degree of inhibition of PAH by b-mephe than by a-mephe (see Chapter VI, Table VI-6).

Brain tissue from animals which had been chronically injected with a-mephe plus phe through the first 21 days was analyzed for phe, tryptophan and biogenic amine content and the results are outlined in Table VII-4. Significant decreases in cerebral tryptophan, 5HT, 5HIAA, DA, HVA and NE, were present in the experimental animals. Behavioral parameters and regional brain weights of adult female rats which had been exposed to postnatal hyperphenylalaninemia during the suckling period are shown in Table VII-5. A significant decrease compared with saline-treated controls was seen in the vertical activity, a parameter which is believed to correlate with exploratory behavior and which is also depicted in Figure VII-1 as the cumulative results for each hour after the onset of the testing period. Other behavioral indices now tested showed no abnormalities. However, the effect of early postnatal hyperphenylalaninemia on brain development was evident in the decreased brain weight,particularly that of the cerebellum.

TABLE VII-3

HYPERPHENYLALANINIA AND PARAMETERS OF GROWTH AND PLASMA AND CEREBRAL PHENYLALANINE LEVELS IN INFANT LONG EVANS RATS

From the third day of life, suckling rats received daily injections of saline (C), or phe (5.2 umol/g B.Wt.) together with a-mephe (2.4 umol/g B.Wt.)(Ea), or b-mephe (2.4 umol/g B.Wt.)(Eb). At 10 and 16 days of age, 4 hours after the last injection, plasma and brain samples were taken for analysis of phe concentration. Liver PAH activity was also determined. Results are expressed as means ± S.D. with the number of rats in parentheses. Analyses of significance of differences from means for saline-injected controls by Student's t test are represented by **p<0.01, ***p<0.001.

	10 DAYS	
	C	Ea
Body Weight (g)	18.8 ± 1.7 (4)	17.9 ± 2.1 (5)
Brain Weight (g)	0.92 ± 0.02(4)	0.82 ±0.03 (5)**
Plasma phe (nmol/ml)	95 ± 10(4)	4169 ± 211(3)***
Brain phe (nmol/g)	97 ± 5 (4)	2463 ± 114(3)***
Liver PAH (umol/min/g)	854 ± 25 (4)	260 ± 28 (3)***

	16 DAYS		
	C	Ea	Eb
Body Weight (g)	30.2 ± 1.1 (3)	27.2 ± 1.3 (3)	26.5 ± 2.8(5)
Brain Weight (g)	1.31 ± 0.02(3)	1.15 ±0.03 (3)**	1.12 ±0.05(5)**
Plasma phe (nmol/ml)	92 ± 5 (3)	1179 ± 292 (3)	798 ± 520(5)
Brain phe (nmol/g)	90 ± 4 (3)	985 ± 80 (3)	614 ± 377(5)

TABLE VII-4

THE EFFECT OF HYPERPHENYLALANINEMIA ON CEREBRAL AMINO ACIDS, MONOAMINES AND METABOLITES IN RATS AGED 21 DAYS

From days 3 to 21, suckling Long Evans rats received a daily injection of either saline (C) or a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) (E). Samples for the analyses of cerebral amino acids, biogenic amines and metabolites were taken 5 hours after the last injection. Results are means ± S.D. for the number of animals given in parentheses. Analysis of the significances of the differences by Student's t test are indicated by *p<0.005,**p<0.01, ***p<0.001.

	C	E
AMINO ACID (nmol/g)		
PHENYLALANINE	100 ± 11 (3)	1358 ± 348 (4)
TRYPTOPHAN	29.2 ± 1.1 (3)	23.9 ± 1.1 (4)*
BIOGENIC AMINES & METABOLITES (nmol/g)		
5HT	2.64 ± 0.24 (3)	1.16 ± 0.12 (4)***
5HIAA	1.61 ± 0.13 (3)	0.46 ± 0.06 (4)***
DA	4.97 ± 0.60 (3)	3.70 ± 0.25 (4)*
HVA	1.16 ± 0.24 (3)	0.76 ± 0.15 (4)
NE	2.26 ± 0.26 (3)	1.56 ± 0.08 (4)**

TABLE VII-5

THE EFFECT OF POSTNATAL HYPERPHENYLALANINEMIA ON ADULT BEHAVIOR AND REGIONAL BRAIN WEIGHTS

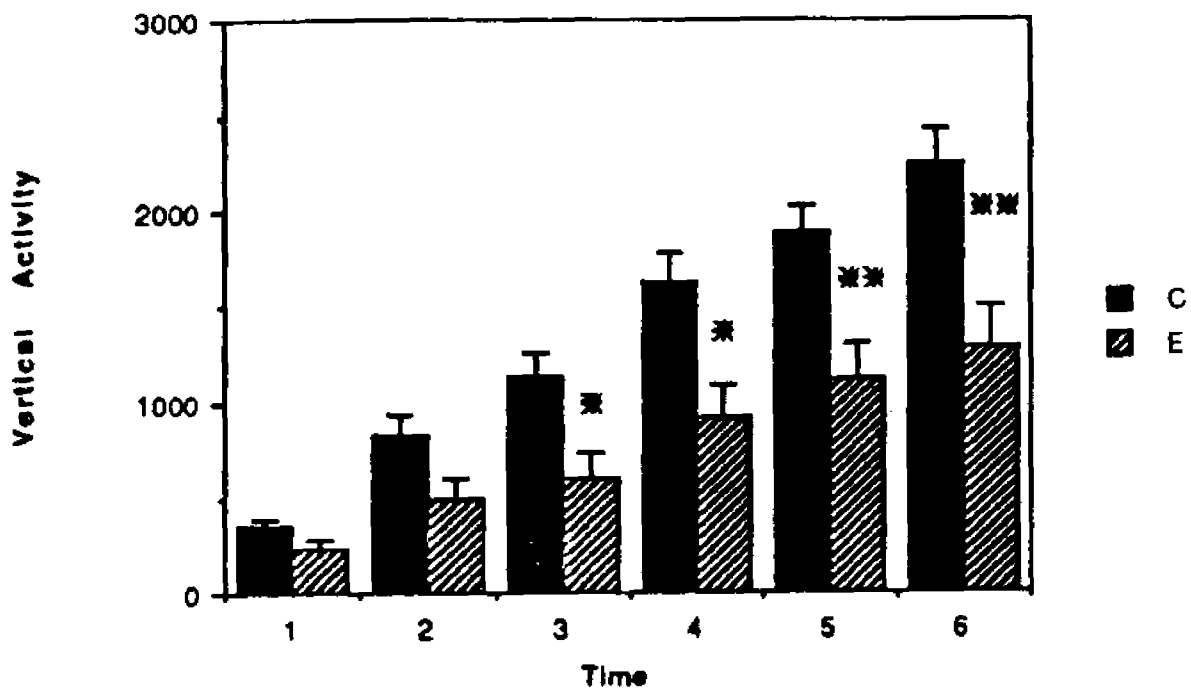
Adult female Long Evans rats which had been injected with either saline (C) or a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) from days 3 to 21 (E), were studied and behavioral indices measured over 6 hours of testing except for hole poke which was 1/4 hour. At the time of sacrifice, body weights and regional brain weights were determined. Results are means ± S.D. of the number of animals given in parentheses. Statistical analyses by Student's t test of the significance of the difference from saline-injected littermate controls (C) are indicated by * $p<0.05$, ** $p<0.01$.

	C	E
Left 1/4 Turns	195 ± 40 (6)	156 ± 74 (6)
Right 1/4 Turns	232 ± 75 (6)	134 ± 49 (6)
Left Full Turns	19 ± 6 (6)	16 ± 9 (6)
Right Full Turns	23 ± 11 (6)	14 ± 7 (6)
Total Full Turns	42 ± 15 (6)	30 ± 13 (6)
Net Full Turns	7 ± 9 (6)	6 ± 8 (6)
Holeboard Poke	13 ± 14 (7)	29 ± 28 (7)
Vertical Activity		
6 p.m.	348 ± 70 (6)	238 ± 95 (6)
7 p.m.	490 ± 207(6)	249 ± 152(6)
8 p.m.	303 ± 116(6)	117 ± 102(6)*
9 p.m.	487 ± 79 (6)	323 ± 115(6)*
10 p.m.	259 ± 126(6)	192 ± 138(6)
11 p.m.	357 ± 187(6)	172 ± 131(6)
12 midnight	264 ± 316(6)	252 ± 149(6)
Body Weight (g)	379 ± 37 (6)	363 ± 63 (6)
Brain Weight (g)	2.06 ± 0.06 (6)	1.83 ± 0.07 (6)**
Cerebellum Weight (mg)	281 ± 8 (6)	232 ± 10 (6)**

FIGURE VII-1

VERTICAL ACTIVITY IN ADULT RATS EXPOSED TO POSTNATAL HYPERPHENYLALANINEMIA

Female Long Evans rats which had received daily injections of a-mephe plus phe from day 3 to 21 were raised to adulthood. The vertical activity was measured by Omnitech Digiscan apparatus from 6 p.m.until 12 midnight and is presented as the cumulative activity for each hour. Results are given as means with the height of the bars representing ± SEM. The number of rats for each group was 5. Statistical analysis by Student's t test is indicated by *p<0.05, **p<0.02.



Depletion and Restoration of Cerebral Monoamines:

One approach for the determination of the causative factor(s) in the cerebral maldevelopment is to selectively induce or preclude one or another of the chemical abnormalities (e.g. those in phe, DA, NE and 5HT levels) suspected to be pathogenic factors. Based on the information obtained about the biogenic amine depletions in hyperphenylalaninemic Long Evans rats, the development of models of selective depletion and restoration was undertaken. As described in Chapter VI, compounds known to deplete cerebral monoamines without changing the cerebral phe concentration, are a-mephe (catecholamines) and p-chlphe (indoleamines). Thus catecholamine deficits were evoked using three different doses of a-mephe, and indoleamine deficiencies were induced through the use of p-chlphe. Suckling rats were injected with these compounds and the results of HPLC analysis are shown in Table VII-6. Significant decreases in both DA and NE were produced with a-mephe, even at a concentration of 1.2 umol/g, which is one half the usual amount administrered in the chronic treatment regimen. Lowering the amount of p-chlphe from 0.9 umol/g (i.e. the amount injected in the studies described in Chapter VI) to 0.5 umol/g was still an effective means of depleting 5-HT and even at a dose of 0.1 umol/g, reductions in cerebral indoles could be seen. Since the inhibition by PAH by a-mephe or p-chlphe is not complete, the phe levels were close to normal.

TABLE VII-6

DEPLETION OF CEREBRAL MONOAMINES WITH

PHENYLALANINE ANALOGS

Suckling Long Evans rats received three daily injections of p-chlphe, as 0.9, 0.5 or 0.1 umol/g or a single injection of a-mephe given as three different doses; 2.4, 1.8 and 1.2 umol/g. At the age of 10 days, brains were analyzed at the indicated times after the last injection. Results are expressed as means ± S.D. for the number of animals indicated in parentheses. Analyses by Student's t test of significances of differences of means from saline-injected controls are indicated by ***p<0.001.

	5HT	5HIAA	DA	NE
	(% SALINE-INJECTED CONTROLS)			
P-CHLPHE				
5 HOURS				
DOSE				
(umol/g)				
0.1(n=3)	53 ± 3(3)***	54 ± 4(3)***	97 ± 7(3)	87 ± 6(3)
0.5(n=3)	18 ± 2(3)***	16 ± 2(3)***	94 ± 5(3)	87 ± 8(3)
0.9(n=3)	17 ±10(3)***	17 ± 8(3)***	89 ± 8(3)	86 ± 8(3)
A-MEPHE				
6 HOURS				
(umol/g)				
1.2	101 ± 3(4)	96 ± 4(4)	58 ± 7(4)***	64 ± 4(4)***
1.8	99 ± 2(4)	96 ± 5(4)	55 ± 6(4)***	61 ± 4(4)***
2.4	97 ± 4(4)	97 ± 6(4)	54 ± 8(4)***	58 ± 6(4)***
12 HOURS				
1.2	100 ± 5(4)	102 ± 3(4)	80 ± 8(4)**	79 ± 5(4)***
1.8	101 ± 7(4)	104 ± 7(4)	74 ± 5(4)**	72 ± 6(4)***
2.4	99 ± 6(4)	102 ± 8(4)	71 ± 6(4)**	61 ± 4(4)***

An alternative method of producing a model for study of the pathogenic role of monoamine depletion involves selective restoration of the amine through amino acid precursor administration to hyperphenylalaninemic animals. In order to determine the dosage and effectiveness of the amino acid precursor, rats received the chronic daily treatment with a-mephe plus phe and on the day of biochemical measurement, they were also given injections of either tyrosine or tryptophan. Tables VII-7 and VII-8 describe the results of HPLC analyses of biogenic amines and metabolites while amino acid levels in the plasma and the brain are presented in Table VII-9. At an equimolar concentration to phe, tyrosine was found to only partially restore catecholamines and was thus replaced by the more soluble tyrosine methyl ester, previously demonstrated to elevate dopamine levels in adult rat brain (Oishi & Szabo,1984). Administration of 6.0 umol/g of this compound restored DA to control levels (Table VII-7) but by contrast, there was a failure of NE to be completely restored.

Table VII-8 presents indoleamine parameters following the same treatment protocols. The supplementation of equimolar tryptophan more than adequately restored the 5HT to normal and 5HIAA levels were increased three-fold above controls. Even when the dose of tryptophan was halved, indoles still rose to above normal concentrations.

The results shown in Table VII-8 also demonstrate

that administration of tyrosine is without effect on the depressed cerebral concentrations of 5HT and 5HIAA in hyperphenylalaninemic animals. Similarly, supplemented tryptophan does not change the diminished levels of DA,HVA and NE. While more information was obtained for rats aged 10 days, the patterns of response to tyrosine methyl ester or tryptophan administration were the same at 16 days. Examination of the phe levels in Table VII-9 revealed that elevations of plasma and brain phe concentrations were not altered by supplementation with tyrosine or tryptophan. This was not an artifact of the presence of a-mephe, since administration of phe alone (5.2 umol/g) lead to similar plasma phe concentration (nmol/ml), 3235 $\pm$754 (n=3) as did administration of phe together with equimolar tyrosine, 3943 $\pm$ 405 (n=3).

TABLE VII-7

THE EFFECT OF HYPERPHENYLALANINEMIA AND AMINO ACID PRECURSOR ADMINISTRATION ON CEREBRAL CATECHOLAMINES AND METABOLITES

Rats were injected chronically with a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) (E). At the age of 10 or 16 days, they were given a final injection of the regular hyperphenylalaninemic treatment and simultaneously received a s.c. injection of tyrosine (tyr), tyrosine methyl ester (tyrME) or tryptophan (trp) in the amounts indicated in umol/g. Controls received saline only (C). Samples for cerebral biogenic amine analysis were taken 5 hours after the last injection. Results are single values or means ± S.D. of the number of animals given in parentheses and are expressed as % of saline injected sex-matched controls. Analysis by Student's t test is shown by **p<0.005, ***p<0.001.

	NE	DA	HVA
	(% SALINE-INJECTED CONTROLS)		
	10 DAYS		
C	100 ± 3(6)	100 ± 5(6)	100 ± 4(6)
E	58 ± 7(5)***	67 ± 7(5)***	57 ± 8(5)***
E + tyr (5.2)	61 ± 6(3)***	75 ± 8(3)**	66 ±10(3)***
E + tyrME(5.2)	75 ± 3(4)***	92 ± 12(4)	105 ± 14(4)
E + tyrME(6.0)	80 ± 7(4)**	104 ± 10(4)	118 ± 21(4)
E + trp (5.2)	57,54	76,66	34,41
E + trp (2.6)	59 ±10(3)***	66 ± 8(3)***	45±7(3)***
		16 DAYS	
E	64,68	62,83	53,84
E + tyrME(5.2)	75,78	90,100	109,115
E + trp (2.6)	66,60	68,60	50,46

TABLE VII-8

THE EFFECT OF HYPERPHENYLALANINEMIA AND AMINO ACID PRECURSOR ADMINISTRATION ON CEREBRAL INDOLEAMINES AND METABOLITES

Rats were injected chronically with a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.) (E). At the age of 10 or 16 days, they were given a final injection of the regular hyperphenylalaninemic treatment and simultaneously received a s.c. injection of tyrosine (tyr), tyrosine methyl ester (tyrME) or tryptophan (trp) in the amounts indicated in umol/g. Controls received saline only (C). Samples for cerebral biogenic amine analysis were taken 5 hours after the last injection. Results are single values or means ± S.D. for the number of animals indicated in parentheses and are expressed as % of saline injected sex-matched controls. Analysis by Student's t test is shown by **p<0.005, ***p<0.001.

	5-HT (% SALINE-INJECTED CONTROLS)	5-HIAA
	10 DAYS	
C	100 ± 5(6)	100 ± 6(6)
E	49 ± 6(6)***	34 ±7 (6)***
E + tyr (5.2)	47 ± 7(3)***	35 ±8 (3)***
E + tyrME(5.2)	48 ± 8(4)***	37 ±5 (4)***
E + tyrME(6.0)	43 ±11(4)***	34 ±3 (4)***
E + trp(5.2)	152, 126	452, 371
E + trp(2.6)	153 ± 50 (3)	307 ± 50 (3)
	16 DAYS	
E	34, 33	21, 22
E + tyrME(5.2)	44, 42	27, 26
E + trp (2.6)	134,127	395,402

TABLE VII-9

AMINO ACID LEVELS IN HYPERPHENYLALANINEMIC RATS FOLLOWING TYROSINE AND TRYPTOPHAN SUPPLEMENTATION

Rats were injected chronically with a-mephe (2.4 umol/g B.Wt.) plus phe (5.2 umol/g B.Wt.)(E). At the age of 10 days, they were given a final injection of the regular hyperphenylalaninemic treatment and simultaneously received a s.c. injection of tyrosine (tyr), tyrosine Methylester (tyrME) or tryptophan (trp) in the amounts indicated. Controls received saline only (C). Plasma and brain samples were taken 5 hours later for amino acid analyses. Results are means ± S.D. of the number of animals in parentheses, or are single values.

	PHENYLALANINE	TYROSINE	TRYPTOPHAN
		PLASMA (nmol/ml)	
C	105±11 (10)	270 ± 39 (6)	119± 8 (6)
E	3942±493 (8)	427 ± 66 (6)	96 ± 8 (6)
E + tyr (5.2)	4163±528 (4)	756 ± 41 (4)	88 ± 4 (4)
E + tyrME(5.2)	4228±528 (4)	1569,1830	98 ± 15 (4)
E + trp (5.2)	4342,3450	385, 398	3378, 3432
E + trp (2.6)	4138±568(3)	359	1720±62(3)
		BRAIN (nmol/g)	
C	99 ± 17 (3)	171 ± 15 (6)	33.3±0.7(4)
E	1626 ± 66 (3)	196 ± 30 (6)	27.8±1.6(4)
E + tyr(5.2)	1662 ± 179(3)	364 ± 86 (4)	26.8±1.1(4)
E + tyrME(5.2)	1680,1932	1270,1086	29.8,27.3
E + trp (5.2)	1639,1074	165,171	657
E + trp (2.6)	1533	190	

DISCUSSION:

Using methodology already developed to quantify biochemical and behavioral parameters in Fischer rats, these studies attested to the suitability of the Long Evans hooded strain of rat for establishment of models of experimental hyperphenylalaninemia. As shown in Chapter III, developmental changes in BUI were similar to those observed in Fischer rats despite consistently larger body weights at all postnatal ages. The present experiments have compared other chemical and behavioral parameters in the two strains with the view to further study of the permanent cognitive deficits in Long Evans rats resulting as a consequence of postnatal hyperphenylalaninemia (Strupp et al. (1984).

There is evidence from studies of mentally retarded humans that their impaired performance is more marked on tasks which reflect 'advantageous learning' whereas their performance is not different in tests of 'essential learning' (Zeaman & House, 1967; Estes, 1970). The deficiency in vision which is a characteristic of the albino rat, may be a disadvantage in behavioral studies. Therefore in applying tests of 'advantageous learning', Strupp et al (1984) used Long Evans rats. They found that while the ability for "observational learning" was present in both groups, the PKU rats demonstrated a diminished degree of cognition and possessed no capacity for "latent learning" at all. The a-mephe plus phe treatment given to

these rats during infancy was the same as the standard regimen used for Fischer rats (DelValle et al, 1978) and the present study shows that the degree of hyperphenylalaninemia was also similar. In addition, it was found that administration of this standard treatment to Long Evans female rats during the suckling period led in adulthood, to an abnormality in spontaneous vertical activity. This anomaly was seen during the first 6 hours of the test i.e. during the active evening period, and was less if calculated per hour during particular times of the total test period (Table VII-5) or plotted as the cumulative activity (Figure VII-1). Previous behavioral studies employing Long Evans rats have tested only males (Strupp et al, 1984). The present results indicate that Long Evans female rats (as do Fischer females) rendered hyperphenylalaninemic in neonatal life present with permanent neurobiological impairment in adulthood.

In view of the high incidence of mental retardation among offspring of PKU mothers, one might expect that experimental hyperphenylalaninemia during gestation only, should also lead to irreversible cognitive deficits. A recent study, applying the a-mephe plus phe treatment to Fisher rats through the second half of gestation indeed showed that in adulthood, these offspring performed less well in tests of 'advantageous learning' than did control animals (Sadava & Sutcliffe, 1988). It was therefore relevant to develop an experimental model of maternal PKU

in the Long Evans rats which are more suitable for behavioral studies. It was found that addition of 3% or more phe to the diet (containing 0.5% a-mephe) compromises postnatal survival. However, a phe concentration of 2.5% was sufficient to assure high maternal phe levels and deficits in newborn body and brain weights which are comparable to those in human maternal PKU. Thus a model for this condition is now available in a rat strain particularly suitable for studies of cognition. The only tests so far carried out on these animals were to examine the lateralization of circling activity, which is thought to be regulated by nigrostriatal dopaminergic pathways (Glick et al, 1977). Although anomalies with respect to this behavioral parameter have been demonstrated in adult Fischer rats subjected to hyperphenylalaninemia during the first three postnatal weeks (Glick & Greengard, 1980), the results for both the Long Evans and Fischer rat models of maternal PKU were negative. Therefore, while cognitive deficits may be the consequence of prenatal as well as early postnatal hyperphenylalaninemia, it appears that only the latter condition interferes with the normal development of cerebral lateralization.

Like Fischer rats, hyperphenylalaninemia in Long Evans rats induces abnormalities in monoamine metabolism. The selective depletion and restoration of biogenic amines described in this Chapter show that models can be produced for further definition of the role of these substances in

the production of cognitive and behavioral abnormalities. In the absence of hyperphenylalaninemia, the selective depletion of either 5-hydroxyindoles using p-chlphe, or of catecholamines using a-mephe, demonstrates an alternate yet parallel means of studying the role of one particular monoamine.

In the presence of hyperphenylalaninemia, the selective restoration of indoles was readily achieved by administration of tryptophan. The 50% greater than normal 5HT level thus obtained was consistent with the known overshoot effect of precursor administration on indoleamine synthesis. In contrast, catecholamines do not show this effect and their levels in normal animals cannot readily be elevated above normal by tyrosine administration (Milner & Wurtman, 1986). In hyperphenylalaninemic animals the diminished cerebral DA concentration could be raised towards normal level, and the present studies show that tyrosine methyl ester is particularly suitable for this purpose. However, although NE levels were raised somewhat by giving tyrosine methyl ester, at the highest amount tested (6.0 umol/g) the cerebral concentration still remained lower than controls. Other studies have also noted that NE synthesis is not necessarily commensurate with tyrosine precursor flux to DA (Milner & Wurtman, 1986). Another possible explanation of the present results follows from the observations that B-phenylethylamines 'in vivo' can interfere at the DBH

step. The PE produced in hyperphenylalaninemia may have prevented the restoration of NE. In addition, interference with catecholamine synthesis may arise from tyramine formation (Boulton & Juorio, 1983)

Amino acid supplementation is already a treatment regimen in the management of biopterin deficiencies (Kaufman, 1983), and has been suggested as an adjunct therapy for the treatment of PKU children, particularly when complete dietary phe restriction is no longer feasible. There is very little information however, about the extent to which amino acid administration restores the hyperphenylalaninemia-associated decreases in cerebral large neutral amino acid and monoamine levels. The disruption in steps of cerebral protein synthesis in mice rendered chronically hyperphenylalaninemic by treatment with a-mephe plus phe, has been shown to be prevented by giving a mixture of 7 large neutral amino acids (Binek-Singer & Johnson, 1982). Amino acid measurements indicated that the levels were partially restored but that the phe concentration was unchanged. The measurements of phe in the present experiments are in accord with this, in that the concentrations of phe in the plasma and brain in hyperphenylalaninemic rats were unaltered by tyrosine or tryptophan supplements. A previous study in 7 day old rats which had been given an injection of a-mephe on the day before, also showed that following administration of phe together with tyrosine, the phe levels were as high or

even more elevated than in littermates given phe alone (Brass, 1983).

Adult rats maintained for 10 days on a diet containing p-chlphe plus phe were found to exhibit deficits in water maze problem solving which could be ameliorated by concomitant administration of a mixture of valine, isoleucine and leucine (McSwigan et al, 1981). No information was given, however, as to the amino acid levels present in the brain following such treatment. Similarly, no data on amino acid concentrations, or comparison of 5HIAA and HVA levels in the CSF with those of age-matched controls was given in a study in which tyrosine and tryptophan was administered to PKU children undergoing low phe dietary termination and reported to normalize CNS relaxation time (Lou,1985). Such studies, including the above mentioned one on adult rats, are concerned with explaining and reversing the immediate toxic effects of hyperphenylalaninemia. The more profound mental defect in PKU untreated during early childhood, cannot be ameliorated by treatment introduced after this period, even though the cessation of hyperphenylalaninemia reverses all the known abnormalities in cerebral amino acid and biogenic amine concentrations. Thus, to study the pathogenic process, early infancy or gestation is the time when it is necessary to induce in experimental animals hyperphenylalaninemia with all the associated abnormalities in amino acid and monoamine profile. The

animal models for maternal and postnatal PKU conform to these criteria. However, the next step, identification of the pathogenic principles, requires variants of the model. The present study therefore, describes methods for inducing not the whole spectrum of cerebral chemical abnormalities present in PKU but only one or another of these abnormalities. The results show, for example, that the DA and 5HT deficits can be selectively restored by tyrosine and tryptophan administration respectively, and that this does not alter the extent of elevation of phe in the brain. Therefore, variant models are now available for determination of whether particular biogenic amine depletions or other hyperphenylalaninemia-associated chemical abnormalities are responsible for the CNS abnormalities characteristic of PKU.

CHAPTER VIII

CONCLUSIONS

Irreversible damage incurred as a result of exposure of the developing central nervous system to hyperphenylalaninemia is a frequent consequence of both gestational and neonatal PKU. The objective of this study was to characterize biochemical changes in cerebral amino acid and biogenic amine metabolism induced in animal models of PKU and, by study of transport, precursor availability and enzyme inhibition, relate these to the developmental profiles of phe uptake and accumulation in the maturing brain.

At constant raised blood phe levels, the accumulation of phe by the maturing rat brain was greater during the first week of life than at any subsequent age. This declining capacity of the developing rat brain to accumulate phe was unaltered by prior exposure to chronic hyperphenylalaninemia. By adaptation of the intracarotid injection technique to neonatal rats, phe uptake at physiological blood phe concentration could be studied for the first time through the suckling period. The BUI thus measured at 4 days of age was found to be higher than at all subsequent ages up to ten weeks, with the greatest magnitude of decline occurring during the first three weeks and no change after weaning. The qualitative similarity of these developmental curves is consistent with a decrease in phe influx with age contributing to the

fall in accumulative capacity. The results indicate that by the time intermittent feeding begins, the brain has developed a considerable ability both to protect itself against physiological fluctuations in circulating phe levels, and to restrict the cerebral accumulation of phe from pathologically elevated blood concentrations such as those in PKU. Thus, it is not only rapid growth and differentiation that renders the neonatal brain particularly vulnerable to hyperphenylalaninemia, but also the greater ease of cerebral phe uptake in this period than during subsequent development.

Decreases in the plasma concentration of amino acids, in particular tryptophan and the branched chain amino acids, are known to accompany hyperphenylalaninemia in both PKU and experimental animal models, yet the reason for the diminished blood levels and whether this is a major contributing factor to the decreased levels in the brain continue to be unexplained. One hypothesis for the plasma loss implicates an inhibitory effect of phe in the gut, preventing the absorption of alimentary amino acids (Anderson et al, 1967; Efron et al, 1969). Alternatively it has been suggested that hyperphenylalaninemia causes an increased accumulation of amino acids (in particular, tryptophan) in extra-cerebral tissues which leads to their depletion from the blood (Huether et al, 1984). The method now devised for sustaining the phe concentration in the blood at a constant steady state levels, permitted more

precise study of these questions. No support could be obtained for the latter of the above mentioned hypotheses. Hyperphenylalaninemia did not alter the tryptophan concentrations of the liver, lung, muscle, stomach and small intestine in either suckling or weaned rats. The results did provide, however, experimental support for the postulated role of the gut in the diminished plasma amino acid levels in PKU. The gastric content of the hyperphenylalaninemic rats was rich in phe and showed significantly elevated tryptophan concentration. This is consistent with an inhibition of tryptophan absorption by phe, although the less likely possibility of excessive secretion of several amino acids into the intestinal fluids cannot be entirely excluded. Thus, while the mechanism for the loss of plasma amino acids in hyperphenylalaninemia may be multifaceted, this study has highlighted the role of the gastrointestinal tract and nutritional status in the control of plasma amino acid balance.

It has recently been proposed (Huether et al, 1984) that tryptophan deficiency in the brain of phenylketonurics is not due to excess phe competing with other neutral amino acids for the common cerebral transport system but to their diminished plasma levels. Physiological variations in plasma levels can indeed evoke some response in the brain concentration (Fernstrom, 1983). However, by varying the degree of

hyperphenylalaninemia and following the time course of events, it could be shown that tryptophan depletion in the brain is not necessarily predicated on tryptophan depletion in the blood. The observation that the Brain Uptake Index of radiolabelled tryptophan was decreased by the addition of phe to the bolus injected via the carotid artery of rats also indicates that cerebral tryptophan deficiency can arise at normal plasma level. The conclusion thus is that competition for transport across the BBB is the mechanism primarily responsible for the cerebral tryptophan deficit in PKU; depressed plasma tryptophan may play a role during severe hyperphenylalaninemia but cannot account for the cerebral deficit at more moderate degrees of hyperphenylalaninemia.

The amino acid depletions in the plasma and brain were not unique to hyperphenylalaninemia. Administration of loads of leucine, methionine and histidine had a similar effect on other large neutral amino acids including tryptophan. On the other hand, the cerebral concentration of the essential amino acid and neurotransmitter, glycine, was specific for hyperphenylalaninemia: it was not produced by repeated administration of large doses of other amino acids. The plasma level of glycine remained unchanged, and its increase in the brain was associated with elevations in PSP catalyzing the rate limiting step in the cerebral glycine and serine synthesis. When varying the degrees of

gestational or early postnatal hyperphenylalaninemia, a significant linear correlation was found between the PSP and glycine concentrations. The slow onset and reversal of both these coordinated increases, and the similar clearance rate of administered glycine in controls and chronically hyperphenylalaninemic rats, are also consistent with an impact of phe on intracerebral metabolism of glycine rather than on its transport.

The alterations in the rat model of experimental PKU in the cerebral concentrations of the different neurotransmitters (glycine, 5HT and the catecholamines DA and NE) appear to be due to quite different mechanisms of action. The 5HT depletion is caused by precursor shortage resulting from the fact that high plasma concentrations of phe compete for the same transport system and inhibit the uptake of tryptophan into the brain. The reductions in cerebral DA or NE which arose despite the normal levels of the precursor, tyrosine, were due to the action of phe or its derivatives on steps in the catecholamine biosynthetic pathway. The 5HT and catecholamine depletions occur within hours after induction of plasma phe elevations and there is no evidence for changes in concentrations of enzymes involved in their metabolism. In contrast, the onset and reversal of the increases cerebral concentration of glycine occurred in parallel with that of its biosynthetic enzyme, PSP, and both changes required at least 3 days of hyperphenylalaninemia. However, as in the

case the catecholamine deficits, the brain concentration of phe is a primary regulator of the increased glycine concentration. Therefore, a particularly high cerebral accumulation of phe in gestational and early neonatal hyperphenylalaninemia enhances the risk of amino acid and biogenic amine abnormalities.

Although hyperphenylalaninemia during development has been shown to lead to irreversible behavioral changes in rats of the Fischer strain, the Long Evans strain appears to be more suitable for behavioral investigation and has been used to develop particularly sensitive tests of cognition. Further studies of the pathogenic mechanism should, therefore, be facilitated by the present demonstration that biochemical abnormalities akin to those in PKU can also be evoked in Long Evans rats by a-mephe plus phe treatment, and that such treatment can maintain gestational hyperphenylalaninemia in this as well as in the Fischer strain. The availability of experimental systems was further extended by the finding of a new suppressor of PAH, b-mephe, and by comparison of the chemical changes in the brain following hyperphenylalaninemia induced with the aid of the 3 suppressors, b-mephe, a-mephe and p-chlphe.

Some of the cerebral neurotransmitter deficits could be selectively restored by precursor administration. Tyrosine and tryptophan supplementation restored DA and 5HT levels, respectively, without diminishing the degree

of hyperphenylalaninemia. Thus, by behavioral comparison of these variants and of the original model, it will be possible to study the separate role of 5HT and DA in the pathogenic process and determine whether these monoamine deficits or other chemical consequences of hyperphenylalaninemia are primarily responsible for the cerebral maldevelopment. The answer to such questions is also pertinent to the clinical management of PKU. If, for example, the DA deficit is found to be the major factor in mental retardation, then supplementation with tyrosine and a less rigorous restriction of phe intake may offer an as beneficial and more easily instituted treatment.

Theories of the pathogenesis of mental retardation in PKU, have implicated a series of factors, such as interference of hyperphenylalaninemia with brain growth, myelinization or neuroactive amine metabolism. It has also been postulated that the interference with these processes is attributable to the accumulation of various phe metabolites, normally present in trace amounts only. However, it is known that excess phe itself inhibits, for example, protein synthesis as well as the activites of cerebral enzymes such as the tyrosine and tryptophan hydroxylases. For these reasons, and because some of the potentially harmful phe metabolites are produced in the brain itself, the magnitude of the pathogenic changes depends on the cerebral rather than the plasma concentration of phe. The complex relationship between

the two concentrations is governed by the BBB which, as indicated by influx studies at physiological blood level, becomes less permeable to phe during postnatal development. A similar change is seen in hyperphenylalaninemia: the blood to brain phe ratio is higher in fetal, than infant or adult hyperphenylalaninemic rats. More detailed studies at different pathological blood phe levels showed that the steady state concentration of phe in the brain, resulting from the same degree of sustained hyperphenylalaninemia at each age, is much higher in 4 than 16-20 day old rats and that no significant further decrease occurs during subsequent life. It appears, therefore, that there is a stage in infancy when the brain no longer accumulates damaging amounts of phe, and that knowledge of the developmental period at which this occurs in the human brain would help to define the age at which it would be safe to terminate or to render less rigorous the dietary treatment in PKU.

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