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City University of New York PH.D. 1986

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REGULATION OF CEREBRAL SEROTONIN METABOLISM IN EXPERIMENTAL PHENYLKETONURIA

by

Jessie M. Wolfe

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

1986

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

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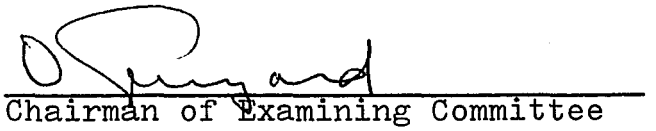
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Abstract

REGULATION OF CEREBRAL SEROTONIN METABOLISM IN EXPERIMENTAL PHENYLKETONURIA

by

Jessie M. Wolfe

Advisor: Dr. O. Greengard

The cerebral 5-HT deficiency characteristic of phenylketonuric children was reproduced in 2-16 day old rats by the administration of a-methylphenylalanine (a-me) plus phenylalanine (phe). The similar decrease in 5-HT found in the fetuses of pregnant rats rendered hyperphenylalaninemic indicates that the deficiency of this neurotransmitter is also present during gestation.

The mechanism of action of a-me and of p-chlorophenylalanine (p-cpa), the alternative agent used in the production of animal models of phenylketonuria, was studied under various conditions. One type of common action is the competitive inhibition of cerebral tryptophan hydroxylase and tyrosine hydroxylase in vitro. Suppression of hepatic phenylalanine hydroxylase exemplifies the other type of action of these agents which, inoperative in vitro, is characterized by a slow (24 hours) onset and by the fact that restitution of normal activity awaits resynthesis of the enzyme. Cerebral tryptophan hydroxylase but not tyrosine hydroxylase was also found to be subject to such suppression and this second type of action, rather than

competitive inhibition of the enzyme, was shown to account for the gradual diminution and slow recovery of the brain's 5-HT content that follows injection of a-me or p-cpa.

In contrast to a-me, p-cpa suppressed tryptophan hydroxylase activity even in the presence of severe hyperphenylalaninemia. In addition, it counteracted the cerebral tryptophan depleting effect of these high plasma phenylalanine levels. Therefore, the 5-HT deficiency in this model is due entirely to suppression of tryptophan hydroxylase. In contrast, the 5-HT deficiency in the a-me model for phenylketonuria, as in the human disease, is attributable to hyperphenylalaninemia per se since the tryptophan hydroxylase suppression became inoperative in the presence of hyperphenylalaninemia and a-me did not counteract the diminution of cerebral tryptophan caused by excess phenylalanine monopolizing the transport system for large neutral amino acids. Moreover, tryptophan injections specifically prevented the cerebral 5-HT deficiency in this model and did so despite the persistence of hyperphenylalaninemia. Analogous observations were made with tyrosine injections which prevented the catecholamine but not the 5-HT deficit.

Acknowledgements

It is with extreme gratitude that I acknowledge the following people, who made this endeavor possible and helped me through many difficult moments.

My husband Steven, whose love, caring and assistance were a tremendous source of inspiration.

My advisor Dr. Greengard, whose superior scientific guidance, editing skills and time involvement were generously provided.

My mother for her love and encouragement.

Jon Head and Ruth McChesney for their technical assistance and continued interest in the project.

Wendy for her excellent proof-reading, professional advice and support.

Edgar, Eva, Diana, Hanne, Gertrude, Elsa, Alice and Susan for their belief in me and valuable help.

My brothers Richard, Sasha and Charlie for their friendship and devotion.

TABLE OF CONTENTS

Title page	i
Copyright page	ii
Approval page	iii
Abstract	iv
Acknowledgements	vi
Table of Contents	vii
List of Tables	x
List of Figures	xiii
List of Abbreviations	xv
CHAPTER I INTRODUCTION	1
1. Human Phenylketonuria	3
A. History	3
B. Phenylalanine Metabolism	5
C. Subclassification of the Condition and Therapy	10
D. Infants of PKU Mothers	16
E. Structural and Biochemical Alteration in the PKU Brain	17
2. Experimental Models of Phenylketonuria	19
A. Criteria for a Model and Evaluation of Previous Models	19
B. Biochemical Basis of the Mental Disorder	24
3. Serotonin in the Central Nervous System	27
A. History	27
B. The Metabolic Pathway	27
C. The Role of 5-HT in the Central Nervous System	29
CHAPTER II METHODS	32
1. Assays	32
A. Serotonin and 5-hydroxyindole-acetic acid	32
B. Plasma and Brain Tryptophan and Phenylalanine	33
C. L-Aromatic Amino Acid Decarboxylase	34
D. Monoamine Oxidase	35
E. Tyrosine Hydroxylase	36
F. Tryptophan Hydroxylase	37
G. Catecholamines	38
2. Treatment of Animals	39
A. Injections to Postnatal Animals	39
B. Maternal Hyperphenylalaninemia	40
C. Behavioral Studies	40
D. Tissue Preparation	41

CHAPTER III DECREASE IN 5-HYDROXYINDOLES BY HYPERPHENYLALANINEMIA 43

1. Postnatal Hyperphenylalaninemia 44
2. Prenatal Hyperphenylalaninemia 50
3. Discussion 55

CHAPTER IV IN VIVO AND IN VITRO INHIBITION OF THE PTERIDINE-DEPENDENT HYDROXYLASES BY PHENYLALANINE AND SYNTHETIC ANALOGUES 58

1. The Development of a Quantitative Assay for Tryptophan Hydroxylase in Hyperaminoacidemic Animals 60
2. Interference of P-chlorophenylalanine with Aromatic Amino Acid Hydroxylases 67
3. Interference of A-methylphenylalanine with Aromatic Amino Acid Hydroxylases 69
4. Depletion of 5-HT and 5-HIAA Content after Acute Injection of A-methylphenylalanine or P-chlorophenylalanine 75
5. Discussion 75

CHAPTER V THE EFFECT OF EXPERIMENTAL PKU ON BRAIN AND PLASMA TRYPTOPHAN CONTENT 84

1. Brain and Plasma Tryptophan Content in PKU Models 85
   A. Changes in Brain Tryptophan 85
   B. Decrease in Plasma Tryptophan 88
   C. Reversibility with Tryptophan of the Cerebral 5-HT Deficit 90
2. Discussion 93

CHAPTER VI MECHANISMS OF MONOAMINE DECREASE IN EXPERIMENTAL PHENYLKETONURIA 97

1. Mechanism of the Decrease in Monoamine Levels in Experimental PKU 99
   A. Decrease in 5-hydroxyindoles 99
   B. Decrease in Catecholamines 102
   C. Effect of Chronic Treatment on Tryptophan Hydroxylase and Tyrosine Hydroxylase 103
   D. Decrease in Tyrosine and Tryptophan 103
   E. Competitive Inhibition of Tyrosine Hydroxylase and Tryptophan Hydroxylase 107
   F. Timing of Measurement 108

CHAPTER VII BIOCHEMICAL AND BEHAVIORAL STUDIES IN ADULT RATS HAVING UNDERGONE NEONATAL HYPERPHENYLALANINEMIA 119

1. Results on Rats Exposed to Hyperphenylalaninemia During Infancy 121
2. Sex Difference in 5-HT in Postnatal Rats 126
3. Discussion 128

GENERAL DISCUSSION 132

BIBLIOGRAPHY 144

LIST OF TABLES

I-1	Classification of hyperphenylalaninemia	11
I-2	IQ scores and ages of PKU patients versus sibling controls	13
I-3	Pathological and clinical observations concerning untreated phenylketonuric patients	20
III-1	Effect of a-methylphenylalanine and phenylalanine on the 5-HT assay	45
III-2	Effect of a-methylphenylalanine and phenylalanine on the 5-HIAA assay	47
III-3	Effect of chronic hyperphenylalaninemia on 5-HT and 5-HIAA concentrations in brain regions	48
III-4	Effect of chronic hyperphenylalaninemia on whole brain 5-HT and 5-HIAA content with increasing age in postnatal rats	49
III-5	Decrease in whole brain 5-HT and 5-HIAA content by phenylalanine treatment	51
III-6	The phenylalanine content of maternal and fetal plasma and brain and the plasma/brain phenylalanine ratio	52
III-7	Cerebral large neutral amino acids and neurotransmitter content of hyperphenylalaninemic fetuses	54
IV-1	Tryptophan hydroxylase assay blanks	61
IV-2	Cofactor saturation for the tryptophan hydroxylase assay	63
IV-3	Substrate saturation for the tryptophan hydroxylase assay	65
IV-4	Increase in tryptophan hydroxylase activity with time and enzyme concentration	66
IV-5	Effect of increased a-methylphenylalanine dose on midbrain 5-HT and 5-HIAA levels	77

IV-6	Phenylalanine analogues and kinetic parameters (Ki) of the pteridine dependent hydroxylases	81
V-1	Decrease in midbrain 5-HT and 5-HIAA and in whole brain tryptophan by phenylalanine and leucine	87
V-2	Effect of chronic hyperphenylalaninemia on plasma and brain tryptophan levels	89
V-3	Fluorescent measurement of tryptophan in the 5-HT assay	91
V-4	Reversal of 5-HT and 5-HIAA decrease with tryptophan injections after a-methylphenylalanine plus phenylalanine treatement	92
VI-1	Effect of acute injection of a-methylphenylalanine on brain 5-HT, 5-HIAA and tyrosine hydroxylase	101
VI-2	Comparison of the dopamine depleting effects of a-methylphenylalanine with that of phenylalanine, and of a-methylphenylalanine plus phenylalanine	106
VI-3	Reversal of 5-HT decrease with time. Measurement of 5-HT, tryptophan and tryptophan hydroxylase 4 to 6 and 24 hours after the last injection of a-methylphenylalanine and a-methylphenylalanine plus phenylalanine	110
VI-4	Catecholamine and serotonin depletion in response to various cerebral levels of phenylalanine and tyrosine	113
VII-1	Behavioral results from adult rats having undergone neonatal hyperphenylalaninemia	122
VII-2	Effect of chronic hyperphenylalaninemia on cerebral phenylalanine, 5-HT, tryptophan hydroxylase, dopamine, norepinephrine and plasma phenylalanine and tryptophan in male and female rats	124

VII-3 Biochemical results in adult rats having undergone neonatal hyperphenylalaninemia 125

VII-4 Sex difference in midbrain 5-HT and 5-HIAA content in control animals and in females that received an injection of testosterone at an early age 127

LIST OF FIGURES

I-1	Metabolism of phenylalanine by normal minor pathways	6
I-2	Phenylalanine hydroxylation and ancillary reactions	8
I-3	Diagram of 5-HT metabolism	28
IV-1	Time course of suppression of hepatic phenylalanine hydroxylase activity by p-chlorophenylalanine	68
IV-2	Time course of suppression of hepatic phenylalanine hydroxylase activity by a-methylphenylalanine	70
IV-3	Double reciprocal plot of tryptophan concentration against the rate of tryptophan hydroxylation with various levels of a-methylphenylalanine	71
IV-4	Double reciprocal plot of tyrosine concentration against the rate of tyrosine hydroxylation with various levels of a-methylphenylalanine	72
IV-5	Effect of acute treatment with a-methylphenylalanine on brain 5-HT and 5-HIAA and tryptophan hydroxylase	73
IV-6	Effect of acute treatment with p-chlorophenylalanine on brain 5-HT, 5-HIAA and tryptophan hydroxylase	76
IV-7	Double reciprocal plot of tyrosine concentration against the rate of tyrosine hydroxylation with various levels of phenylalanine	79
V-1	Comparison of the depleting effects of a-methylphenylalanine, a-methylphenylalanine plus phenylalanine, p-chlorophenylalanine and p-chlorophenylalanine plus phenylalanine on brain tryptophan	86
VI-1	Diagram of 5-HT and catecholamine metabolism	98

VI-2 Comparison of the depleting effects of a-methylphenylalanine, a-methylphenylalanine plus phenylalanine, p-chlorophenylalanine and p-chlorophenylalanine plus phenylalanine on brain serotonin 100

VI-3 Comparison of the inhibiting effects of a-methylphenylalanine, a-methylphenylalanine plus phenylalanine, p-chlorophenylalanine and p-chlorophenylalanine plus phenylalanine on brain tryptophan hydroxylase 104

VI-4 Time course of elevation of the cerebral phenylalanine concentration during chronic hyperphenylalaninemia 109

VI-5 Time course of dopamine depletion in response to phenylalanine plus a-methylphenylalanine or a-methylphenylalanine treatments 111

LIST OF ABBREVIATIONS

a-me	alpha-methylphenylalanine
CSF	cerebrospinal fluid
$DMPH_4$	6,7-dimethyltetrahydropteridine
g	gram
h	hour
5-HIAA	5-hydroxyindole-3-acetic acid
5-HT	5-hydroxytryptamine or serotonin
Ki	inhibition rate constant
Km	reaction rate constant
M	molar
min	minute
ml	milliliter
mM	millimolar
$6MPH_4$	6-methyltetrahydropteridine
ngm	nanogram
nM	nanomolar
p-cpa	para-chlorophenylalanine
phe	phenylalanine
PKU	phenylketonuria
try	tryptophan
try-ase	tryptophan hydroxylase
tyr	tyrosine
w	weight

CHAPTER 1

INTRODUCTION

Phenylketonuria (PKU) was first described by Folling fifty years ago (Folling, 1934). Extensive research has led to its classification as an inborn error of metabolism and as a model to study mental retardation resulting from this error. In classical PKU which is inherited as an autosomal recessive trait, the gene product, liver phenylalanine hydroxylase, is defective, leading to high blood and tissue phenylalanine levels. Although hyperphenylalaninemia is present in all body tissues, the most prominent and serious consequence of the disease is abnormal brain development. Other hyperphenylalaninemias, also leading to mental defects, can arise from genetic impairment of necessary components of phenylalanine oxidation.

PKU illustrates a historical principle formulated by Galton (Galton, 1932) which states that the phenotype of a genetic disease will vary with an individual's environmental exposure. The clinical manifestation of this disease results from the ingestion of phenylalanine. Since the phenylalanine hydroxylase deficiency precludes normal homeostatic control, the amount of dietary phenylalanine is the primary determinant of the degree of hyperphenylalaninemia and thus of the severity of nervous system abnormalities. This realization led to the

introduction of a diet containing no more phenylalanine than essential for protein synthesis in the growing organism. This is the only treatment that has succeeded in preventing the severe mental retardation of PKU children.

Classical PKU and other hyperphenylaninemias have been the subject of numerous review articles (Knox, 1972; Gaull et al.,1975; Scriver and Clow, 1980a and 1980b; Tourian and Sidbury, 1983).

The work presented in this thesis deals with neurochemical and behavioral changes in an experimental model of PKU. The chosen model, induced by daily injection of alpha-methylphenylalanine plus phenylalanine in rats, is thought to successfully mimic classical PKU and to be devoid of the toxicity associated with other models. The neurochemical studies are focused on abnormalities of serotonin metabolism which have been well documented in human cases. The mechanism of serotonin decrease in the model is examined and compared with other models and with the decrease in catecholamines. In addition, a correlation between behavioral performance and permanent neurochemical changes in monoamine synthesis is explored. It is hoped that these studies will contribute to the understanding of mental retardation associated with the aminoacidopathies and suggest alternative methods of treatment.

1. Human Phenylketonuria

A. History

In 1934, a Norwegian physician published his findings on a disease in children that led to mental retardation and that was associated with high urine levels of a phenylalanine metabolite (Folling, 1934). Asbjorn Folling's discovery was instigated by the examination of two mentally retarded siblings whose urine had an unpleasant musty odor. By reacting their urine with ferric chloride, he obtained a green color which was due to the presence of phenylpyruvic acid. He extended these studies to other children in mental institutions and proposed that the disease "imbecillitas phenylpyruvica" was due to abnormal phenylalanine metabolism. Since a number of children were siblings, it seemed likely that the disease was hereditary.

A few years later, high levels of phenylalanine as well as several other of its metabolites were detected in the urine of patients and high levels of phenylalanine in their blood (Folling, 1938). A more complete genetic study by Jervis led to the conclusion that the disease which is referred to as "PKU" since then, is inherited as an autosomal recessive trait that is due to a single gene defect (Jervis, 1939). After these initial descriptions, Jervis and his coworkers focused on elucidating the biochemical basis of the abnormality and demonstrated that

the liver of phenylketonurics is unable to oxidize phenylalanine to tyrosine (Jervis, 1947; Jervis, 1953). Bickel et al. (1953) and later Armstrong and Tyler (1955) showed that reduction of phenylalanine intake improved the mental condition of phenylketonurics. It became necessary therefore to develop a simple assay for phenylalanine detection in newborns. Until the 1960's, the ferric chloride test of the urine was the only available method for diagnosing PKU. A microbiological assay for blood phenylalanine developed by Guthrie was found to be more reliable and permitted mass screening of newborns for hyperphenylalaninemia (Guthrie, 1961; Guthrie and Susi, 1963).

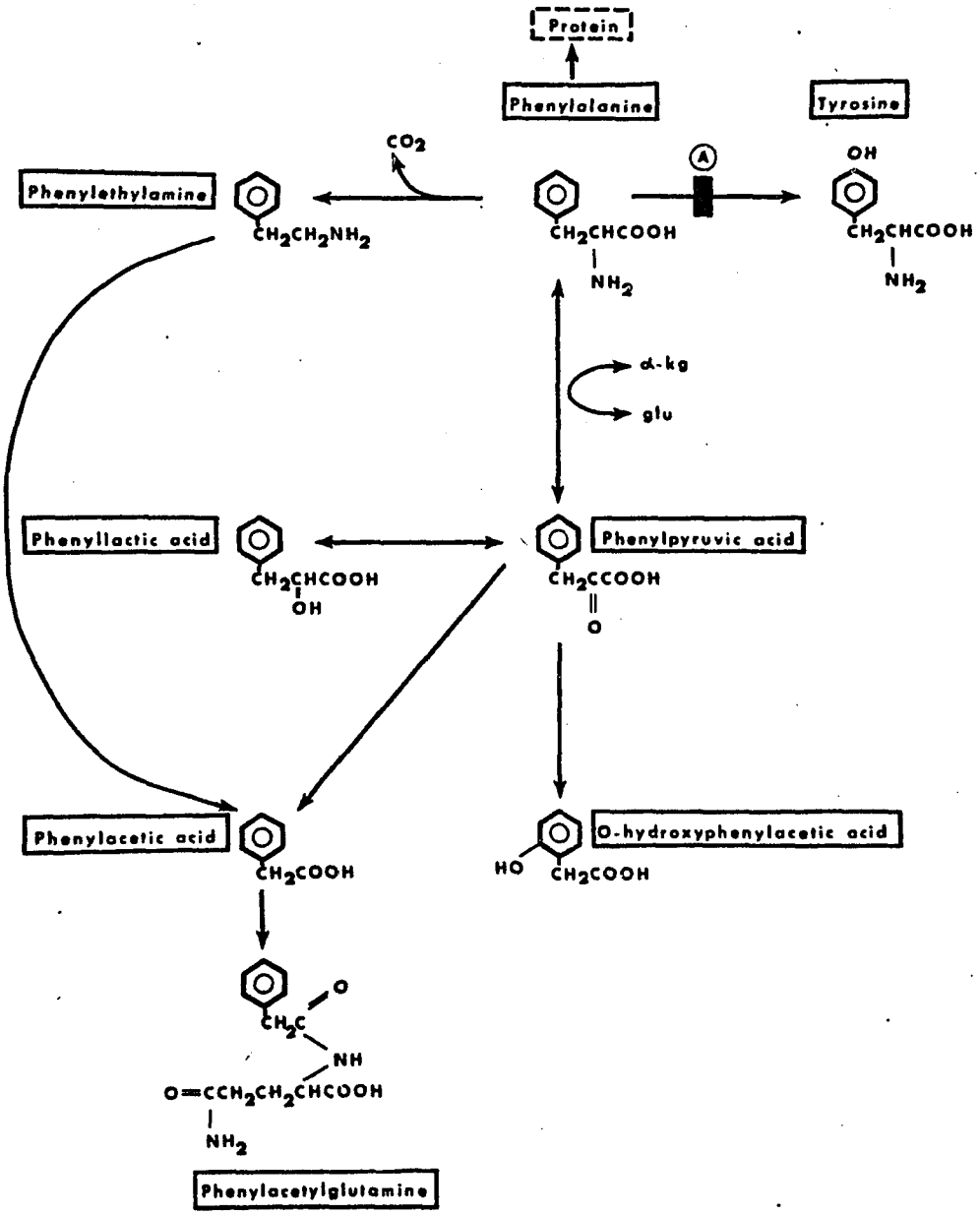
The screening program and the application of the dietary treatment resulted in a large decrease in the number of phenylketonuric patients who had to be placed in mental institutions (MacCready, 1974). The large scale screening and cases in which the treatment were unsuccessful also led to the realization of the complex etiology of the disease and to the identification of inborn hyperphenylalaninemias where the basic lesion is different from that in classical PKU.

B. Phenylalanine Metabolism

Phenylalanine is an essential amino acid and its steady state level is a function of dietary intake, protein turnover and metabolic degradation. In the reaction sequence responsible for 80% of phenylalanine metabolism, hydroxylation to tyrosine is the first step (Kaufman, 1977). A defect in the hydroxylation step (Jervis, 1953) thus explains the hyperphenylalaninemia of phenylketonurics. The alternative metabolic route, (see Figure I-1) which begins with the transfer of the amine group from phenylalanine and where the phenyl ring remains intact through several subsequent steps is operative in PKU. However, the large amount of blood phenylalanine entering the liver overloads the capacity of the "transamination" pathway and metabolites which are normally at negligible concentrations accumulate. This explains the production and excretion of appreciable amounts of phenylpyruvic acid, phenylethylamine, phenyllactic acid, phenylacetic acid, mandelic acid, o-hydroxyphenylacetic acid and phenylacetylglutamine (Knox, 1972). The characteristic musty odor of phenylketonuric urine is due to the presence of phenyllactic acid while phenylpyruvic acid is the substance primarily responsible for the positive ferric chloride reaction whereby the disease was initially diagnosed.

FIGURE I-1

METABOLISM OF PHENYLALANINE BY NORMAL MINOR PATHWAYS



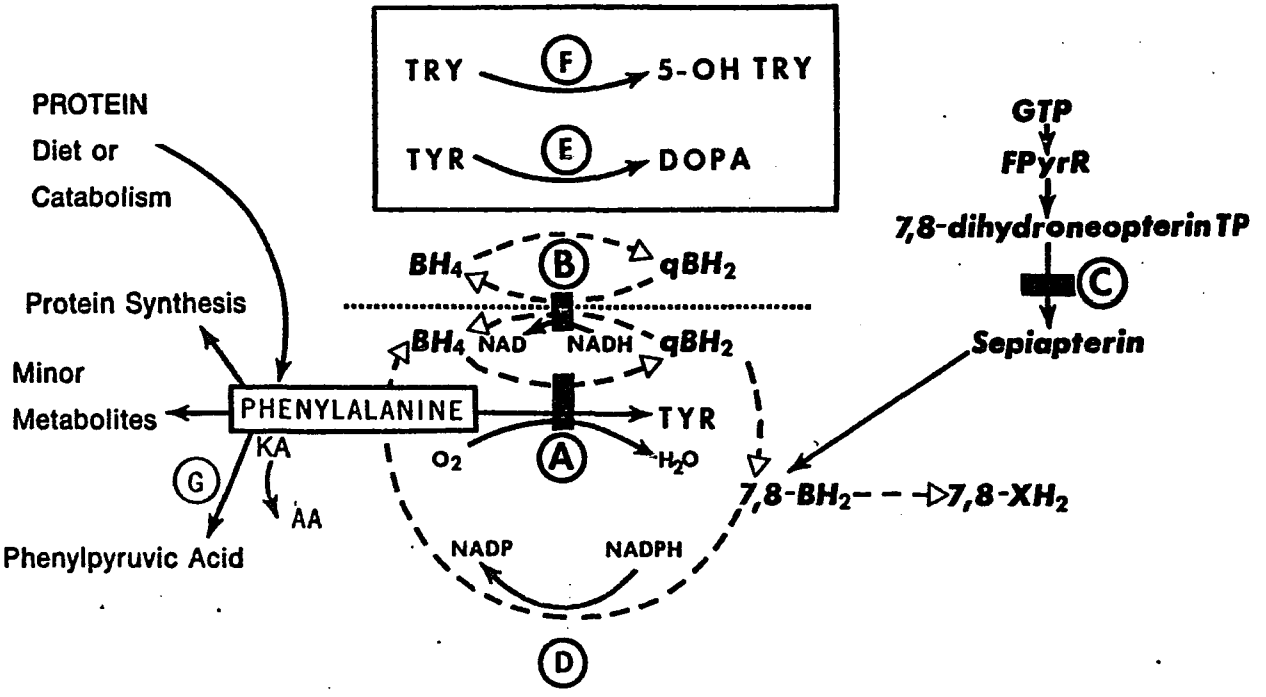
Site of block in phenylketonuria is shown at A.

Adapted from Scriver and Rosenberg (1973)

Although the site of the metabolic block was known in the 1950's, understanding the nature of this block awaited elucidation of the properties of the phenylalanine hydroxylating system and of its critical components. The system was first described by Udenfriend and Cooper in 1952, who found high hydroxylating activity in a soluble extact from rat liver. The activity utilizing molecular oxygen appeared to require enzymes and a reducing agent which was a derivative of biopterin. (Mitoma, 1956; Kaufman, 1957; Kaufman, 1962). The details of the reaction which hydroxlates L-phenylalanine in the 4 position of the phenyl ring to form L-tyrosine are now well understood and is classified as a mixed function oxidase. As shown in Figure I-2, the system is composed of phenylalanine hydroxylase (E.C. 1.14.16.1) and 7,8-dihydrobiopterin which is reduced by an NADPH dependent dihydrofolate reductase (E.C. 1.5.1.3.) (Kaufman, 1967). It is thought that the essential cofactor, tetrahydrobiopterin, is oxidized to a quinonoid form and that its regeneration by an NADH dependent dihydropteridine reductase (E.C. 1.6.99.7) is closely coupled to the phenylalanine hydroxylase reaction.

Short term regulation of the enzyme by the concentration of substrate and other modulating agents has been studied in vitro. Phenylalanine is thought to cause a shift in the enzyme to a more active form (Kaufman and Fisher, 1970). Lysolecithin phospholipid decreases the Km for phenylalanine and increases the Vmax (Fisher and

FIGURE I-2
PHENYLALANINE HYDROXYLATION AND ANCILLARY REACTIONS



A denotes phenylalanine hydroxylase (PH), B dihydropteridin reductase (DHPR), C L-*erythro*-7,8-dihydrobiopterin synthetase, D dihydrofolate reductase (DHFR), E tyrosine hydroxylase, F tryptophan hydroxylase, and G phenylalanine aminotransferase. Reactions E and F in nonhepatic tissue are dependent on a mechanism of BH_4 homeostasis similar to that maintaining reaction A in the liver. Other abbreviations include: BH_4, tetrahydrobiopterin; BH_2, dihydrobiopterin in the quinonoid (q) and tautomeric (7,8) forms; XH_2, dihydroxanthopterin; TRY, tryptophan; 5-OHTRY, 5-hydroxytryptophan; TYR, tyrosine; DOPA, 3,4-dihydroxyphenylalanine; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADP, NAD phosphate; NADPH reduced NAD phosphate; KA, ketoacid; AA, amino acid; GTP, guanosine triphosphate; FPyrR, formamido pyrimidine ribotide; and TP, triphosphate. Enzyme deficiencies affecting A, B, or C are associated with PKU and with various forms of hyperphenylalaninemia. Relations between phenylalanine and metabolic pathways other than the hydroxylating step are also shown.

FROM SCRIVER AND CLOW (1980a)

Kaufman, 1972). Long term regulation of phenylalanine hydroxylase is thought to be mediated by hydrocortisone, insulin, and diet (Tourian and Sidbury, 1983).

The physical properties of phenylalanine hydroxylase have been studied in human and rat after purification from liver (Kaufman and Fisher, 1970; Woolf, 1976). The rat enzyme has a native molecular weight of 100,000 to 110,000 daltons and is thought to be a single species. (However, some of the isolation procedures may cause aggregation of enzyme molecules resulting in an apparent molecular weight of 250,000 daltons (Woo et al., 1974)). The enzyme molecule contains two atoms of iron which are necessary for catalytic activity (Fisher et al., 1972). Many similarities exist between the rat and human liver enzyme, and the same form of the enzyme is thought to be present from the fetal to the adult stage in humans (Woo et al., 1974). In addition to liver, phenylalanine hydroxylase activity is present in mammalian kidney and pancreatic tissue (Tourian et al., 1969).

Experiments with enzyme and with antisera generated by its administration to rabbits indicated that the phenylalanine hydroxylase molecule itself is the affected component in classical PKU (Friedman et al., 1972a). With a sensitive assay, the hepatic phenylalanine hydroxylase concentration in PKU was found to be equal to about 0.27% of that present in control liver (Friedman et al., 1973). The deficiency is attributable to a mutation in the

structural gene. Quite recently, it has been possible to isolate the defective gene (Ledley et al., 1985).

C. Subclassification of the Condition and Therapy

As a consequence of neonatal screening, infants with hyperphenylalaninemia are now classified into different clinical categories as shown in Table I-1 (Tourian and Sidbury, 1983). Patients with hyperphenylalaninemia type I or classic PKU are characterized by undetectable or barely detectable phenylalanine hydroxylase activity. The enzyme is also not inhibited effectively by its substrate and antiserum nor is it stimulated as well by lysolecithin (Friedman et al., 1973; Bartholome et al., 1975). In the caucasian population, the frequency of type I PKU phenotype is about 1 in 11,000 persons and is most common among Celtic and Central European populations (Scriver and Clow, 1980b). In untreated classic PKU, the symptoms of the disease become obvious after the first year of life. These include skin pigment loss and eczema, urine with a musty odor, severe delays in psychomotor development, poor language ability, decreased muscle tone, irritability and agressive behaviour, seizures, microcephaly, and slow growth rate (Knox, 1972). The primary and most consistent effect is the abnormal development of the central nervous system. Approximately 98% of untreated phenylketonuric children will have an I.Q. of less than 50 in later childhood (Koch et al., 1974).

TABLE I-1

CLASSIFICATION OF HYPERPHENYLALANIMIA

Type	*Condition*	*Clinical aspects*	*Defect*	*Blood Phe*	*Blood Tyr*	*Urine*	*Treatment*
I	Phenylketonuria	Mental retardation and associated symptoms if untreated	Phe hydroxylase absent	>20 mg/100 dl on regular diet	Normal–low	Elevation of Phe metabolites	Low Phe diet
II	Persistent hyperphenylal-aninemia	Normal; may show retardation without treatment in more severe cases	Decreased Phe hydroxylase	May be same as PKU early; later 4–20 mg/100 dl on regular diet	Normal–low	Normal or transiently increased Phe metabolites	None—or temporary dietary therapy
III	Transient mild hyperphenylal-aninemia	Normal	Maturational delay of hydroxylase	May be same as PKU early; progressively declines toward normal	Normal–low	Same as type II	Same as type II
IV	Dihydropteri-dine reductase deficiency	Initially normal; seizures, abnormal development evident within first year of life	Deficient or absent dihydropteridine reductase	Variable—may be as in type I	Normal	Variable, dependent on age and Phe concentration in blood	Dopa, 5-OH-tryptophan, carbidopa
V	Abnormal dihydrobiop-terin function	Myoclonus, uncontrolled movements, tetraplegia, greasy skin, recurrent hyperthermia	Dihydrobiopterin synthesis defect	May be >20 mg/100 dl	Normal	Abnormal biopterin metabolites	Dopa, 5-OH-tryptophan, carbidopa

From Tourian and Sidbury (1983)

In the treatment of classical PKU, the main objective is to control blood phenylalanine and, thus, to prevent abnormal cerebral development. If the patient's blood phenylalanine is above 10 mg/100ml, the patient is usually placed on a low phenylalanine diet. Maintaining blood phenylalanine within a desired range is achieved by limiting the phenylalanine intake from 27 to 90 mg/kg per day (Snyderman, 1974). Children with a blood phenylalanine level maintained below 5.5 mg/dl seem to have the most promising outcome (Dobson et al., 1977). The prescribed diet often has to be adjusted for the individual patient and probably reflects differences in the amount of residual enzyme activity. The standard diet is a low phenylalanine milk preparation such as Lofenalac and limited amounts of fruits and vegetables (American Academy of Pediatrics, 1976).

Treatment has been evaluated by I.Q. testing in the adult. Comparison of treated and normal sibling pairs shows that the treated siblings have a slightly lowered I.Q. score (Table I-2) and a low risk of mental retardation (Wrona, 1979). Furthermore, treatment in older children and in adults can improve some of the clinical symptoms (Knox, 1972; Marholin et al., 1978). An undesirable outcome after treatment may be due to poor compliance, or to a blood phenylalanine level too low to permit normal body growth. Complications from the latter also include osteoporosis, hypoglycemia, repeated infections, and

TABLE I-2

IQ SCORES AND AGES OF PKU PATIENTS VERSUS SIBLING CONTROLS

Matched Pairs Code	Age When Tested (mo)		Stanford Binet IQ 1972 Norms		Difference Between IQs
	PKU Index Case	Sibling	PKU Index Case	Sibling	
1	72	73	89	77	PKU + 12
2	72	73	98	110	PKU – 12
3	49	48	116	94	PKU + 22
4	37	34	67	91	PKU – 24
5	50	49	128	122	PKU + 6
6	36	34	81	94	PKU – 13
7	48	47	96	121	PKU – 25
8	48	48	116	114	PKU + 2
9	36	35	110	88	PKU + 22
10	48	53	90	91	PKU – 1
11	48	54	76	99	PKU – 23
12	36	35	71	93	PKU – 22
13	48	51	100	104	PKU – 4
14	48	51	108	102	PKU + 6
15	48	49	74	82	PKU – 8
16	48	44	94	115	PKU – 21
17	49	49	82	90	PKU – 8
18	37	36	91	105	PKU – 14
19	47	49	102	110	PKU – 8
20	72	69	74	107	PKU – 33
21	75	75	86	78	PKU + 8
22	72	79	98	93	PKU + 5
23	72	72	99	102	PKU – 3
24	48	44	82	94	PKU – 12
25	37	36	103	105	PKU – 2
26	35	37	96	103	PKU – 7
27	51	51	63	77	PKU – 14
28	38	38	72	86	PKU – 14
29	50	48	106	120	PKU – 14
30	46	47	127	129	PKU – 2
31	38	41	88	87	PKU + 1
32	36	41	73	78	PKU – 5
33	37	41	79	94	PKU – 15
34	48	54	88	68	PKU + 20
35	71	74	126	123	PKU + 3
36	70	72	119	111	PKU + 8
Mean	50.0	50.9	94	99	PKU – 5.25
SE	13.0	13.6	17.6	15.0	2.23

From Dobson et al. (1976)

neurologic symptoms (Dodge et al., 1975). Alternatives to the diet such as intake of phenylalanine ammonia lyase (Hoskins et al., 1980) or other forms of enzyme replacement therapy (Scriver, 1977) are not presently effective.

It has clearly been shown that children will benefit most from the diet if it is started at an early age (Smith and Wolf, 1974; Dobson et al., 1977). The developing brain is therefore highly susceptible to the metabolic insult and is subject to irreversible effects by late childhood. The question of when to terminate the diet remains controversial. Some studies suggest that termination of the diet at five years of age is sufficient to maximize future I.Q. scores (Holtzman et al., 1975; Koff et al., 1979), whereas others argue that the diet should be continued longer (Smith et al., 1978; Williamson et al., 1979). Variations in diet management may hinder definite conclusions.

In patients with non-PKU hyperphenylalaninemia types II and III, phenylalanine hydroxylase activity is between 1 and 34.5% of normal (Bartholome et al., 1975). The frequency of these phenotypes is about 1 in 43,000 in the caucasian population. Their blood phenylalanine level is generally not greater than 10 mg/100 ml. If their tolerance for dietary phenylalanine is low, they are treated with a low phenylalanine diet. In many cases, their tolerance for phenylalanine improves with age and the diet is discontinued.

Hyperphenylalaninemia types IV and V are more serious variants in which patients have a deficiency in either dihydropteridine reductase activity (Milstien et al., 1976) or in dihydrobiopterin biosynthesis (Curtius et al., 1979). The percentage of patients with this form of the disease is low at 1 to 3% of all hyperphenylalaninemias (Tourian and Sidbury, 1983). Blood phenylalanine is usually elevated. The important consequence in these variants is an interference in neurotransmitter synthesis. Phenylalanine hydroxylase utilizes the same cofactor as tryptophan and tyrosine hydroxylase; a deficiency in the cofactor utilization will result in a deficiency in the biogenic amine products of these enzymes. Defective catecholamine and indoleamine biosynthesis has been confirmed in a number of cases (Butler et al., 1978). In spite of better tolerance of dietary phenylalanine, these patients develop numerous central nervous system abnormalities. There has been some success by treatment with administration of dopa, 5-OH-tryptophan, and carbidopa to restore normal biogenic amine levels (Butler et al., 1981).

D. Infants of PKU Mothers

Early detection and treatment of PKU has resulted in an increased reproductive rate of PKU women. It has become apparent that their offspring, even if heterozygous, have a high incidence of postnatal complications such as mental retardation, microcephaly, and to a lesser degree, low birth weight and congenital heart disease (Lenke and Levy, 1980). These complications are of great concern in that phenylketonuric women who were treated during infancy have become a new source of mental retardation in children. Lenke and Levy's study showed that the frequency of mental retardation and the extent of this retardation (based on I.Q. measurement) correlated with the mother's blood phenylalanine content. For example, at concentrations of maternal phenylalanine greater than 20 mg per deciliter, the frequency of mental retardation was 92% whereas, at 3 to 10 mg per deciliter, it was 21%. Similar correlations were found between maternal blood phenylalanine and the degree of microcephaly (Levy and Waisbren, 1983).

Treatment of women with classic and severe atypical forms of PKU seems to be effective if the low phenylalanine diet began at conception and continued during the entire pregnancy (Levy and Waisbren, 1983). Mild hyperphenylalaninemia, on the other hand, does not seem to damage the fetus and is usually not treated (Berry et al., 1975).

E. Structural and Biochemical Alteration in the PKU Brain

The metabolic insult in PKU causes pathological changes in the brain. Nevertheless, these changes are small, in marked contrast to the degree of mental retardation in these patients. One of the more consistent neuropathological findings is a reduction in brain weight (Jervis, 1963). An alteration in white matter myelin and a decrease in lipids appear to be common findings. Pathological studies of myelin have revealed a reduction in myelin staining (Alvord et al., 1950; Crome et al., 1962), structural changes in myelin, and spongy lesions (Malamud, 1966). Decreases in proteolipids (Menkes, 1968) and in galactolipids (Agrawal and Davison, 1973) have been observed, as well as alteration in the fatty acid composition of myelin lipids (Foote et al., 1965). In addition to pale myelin staining in certain fiber tracts, Bauman and Kemper (1982) noted a lack of maturity in selective neurons in adult phenylketonurics. This lack of maturity is described as a reduction in the size and in the number of dendritic processes of the neuron and disturbances in the development of Nissl granules. Both the fiber tracts and the neurons that are affected undergo a long developmental process suggesting that neuronal development may be primarily delayed with secondary effects on myelin staining. Therefore, it seems unlikely that the

neuropathological changes are due to a destructive process of mature cerebral components.

The biochemical changes that have been studied in the PKU brain are limited to the concentration of amino acids and neurotransmitters. Phenylalanine and other large neutral amino acids utilize a common transport system for entry into the brain (Christensen, 1969). The lowering of these amino acids in brain by high circulating phenylalanine levels in PKU patients has been demonstrated (Oldendorf et al., 1971). In autopsied phenylketonuric subjects, brain levels of dopamine, norepinephrine, and serotonin as well as their precursors, tyrosine, and tryptophan (Mckean, 1972) are decreased.

2. Experimental Models of Phenylketonuria

A. Criteria for a Model and Evaluation of Previous Models

The most common biochemical and behavioral findings in human PKU are listed in Table I-3 (Lane and Neuhoff, 1980). It is fairly straightforward to determine whether or not the appropriate increases in phenylalanine and its metabolites have been reproduced in the experimental model. The relationship between mental retardation and changes in animal behavior is less clear; the best approximation appears to be an impaired performance in a multitude of learning tasks.

In order to mimic the developmental nature of PKU, treatment should begin at an early age when brain development is most vulnerable to trauma. As the brain matures, successive events take place, all of which are potential targets of a metabolic insult. Human brain growth is pronounced up to six months of age (Winick, 1969) and a rapid phase of myelination occurs before birth (Davison and Dobbing, 1966). Although it is not known which developmental events are particularly sensitive to hyperphenylalaninemia, any changes measured during the first year of life in humans are of interest in trying to understand the basis for future mental retardation in infant PKU. Behavioral testing in experimental PKU should begin after cessation of treatment to assure that the developmental changes that occurred are irreversible.

TABLE I-3

PATHOLOGICAL AND CLINICAL OBSERVATIONS CONCERNING UNTREATED PHENYLKETONURIC PATIENTS

Parameter	Observation
Plasma phenylalanine	10-30-fold increase (greater than 25 mg/dl or 1.5mM)
Serum phenylpyruvate	elevated (normally not detected)
Urinary phenylalanine	elevated
Urinary phenylketones (phenylpyruvate, phenylacetate, phenylacetylglutamine, o-hydroxyphenylacetate)	gram quantities excreted
Urinary indoleorganic acids	approx. 35% decrease
Liver biopsy findings	decreased or absent phenylalanine hydroxylase activity. normal dihydropteridine reductase activity.
Brain autopsy findings	
volume	decreased
mass	decreased
phenylalanine	6-10 fold increase
serotonin	decreased
dopamine	decreased
norepinephrine	decreased
tryptophan	decreased
tyrosine	decreased
white matter	general myelin deficit
Intelligence quotient	reduced, often below 50

Adapted from Lane and Neuhoff (1980)

Biochemical changes that occur in utero are relevant in defining abnormalities that lead to mental retardation in maternal PKU. Although the clinical features are similar in both types of PKU, they may originate by different processes. Brain growth curves vary among different species, suggesting that the ages at which to treat animals should be chosen to fall at stages of maximal growth. In the rat, a rapid phase of growth occurs after birth, suggesting that the neonatal stage is appropriate to begin induction of infant PKU. However, it has not been demonstrated that brain damage in PKU occurs during a rapid growth stage.

Early attempts to induce PKU were largely based on feeding phenylalanine to raise plasma phenylalanine levels (Boggs and Waisman, 1964) or breeding genetic strains of animals with low phenylalanine hydroxylase levels (Seller, 1972). These models were problematic in that phenylanine hydroxylase activity converts phenylalanine to tyrosine and results in a phenylalanine to tyrosine ratio of approximately 1. In spite of high tyrosine levels, a few studies fulfilled some other essential criteria and showed that treatment with phenylalanine at an early age can lead to poor performance of behavioral tasks in adult rats (Schalock and Klopfer, 1967) and monkeys (Chamove et al., 1973).

More successful models involve the use of inhibitors of phenylalanine hydroxylation. Koe and Weissman reported

in 1966 that p-chlorophenylalanine is a potent inhibitor of phenylalanine hydroxylase. Esculin (Valdiviesco et al., 1975) and clotrimazole (Dhondt et al., 1977) are inhibitors of dihydropteridine reductase and when administered with p-chlorophenylalanine cause greater inhibition of phenylalanine hydroxylase then does p-chlorophenylalanine alone. However, since these inhibitors of dihydropteridine reductase have toxic side effects, and block the hydroxylation of not only phenylalanine but also of the other aromatic amino acids, they are not considered to be very useful in producing appropriate models of PKU. Neonatal p-chlorophenylalanine leads to permanent behavioral deficits in adult rats (Pryor and Mitoma, 1970) and monkeys (Chamove and Molinaro, 1978). However, since p-chlorophenylalanine causes a 70-90% rather than a complete inhibition of the hepatic phenylalanine hydroxylase, its injection to animals on a normal diet does not result in sufficiently increased blood phenylalanine levels. Therefore, administration of phenylalanine as well as of p-chlorophenylalanine is required to maintain severe hyperphenylalaninemia. Studies with rats injected with p-chlorophenylalanine plus phenylalanine (Lipton et al., 1967) suggest that this model reproduces the essential biochemical changes in PKU. Plasma phenylalanine levels are maintained above 1 mM for most of the day. The plasma phenylalanine to tyrosine ratio is about 10. Other similarities with PKU include increased urinary

phenylalanine and phenylketones, decreases in brain myelin, in monoamine neurotransmitters, and in brain weight (Lane and Neuhoff, 1980).

Several behavioral abnormalities were demonstrated in animals after termination of treatment with p-chlorophenylalanine plus phenylalanine. Butcher and his associates noted reduced activity and a deficiency in water maze learning in rodents (Vorhees et al., 1972; Butcher et al., 1970). The p-chlorophenylalanine plus phenylalanine treatment initially appeared promising, in that many of the characteristics of the disease were reproduced. However, negative aspects of the treatment include a large decrease in body weight and a high mortality compared to saline treated controls (Lane et al., 1980). Other non specific effects are hypophagia, cataracts, loss of body hair, and skin lesions. The toxicity of the treatment is such that interpretation of the results is often ambiguous. Toxicity of p-chlorophenylalanine has also been demonstrated in vitro in cell cultures (Kelly and Johnson, 1978).

Another inhibitor of phenylalanine hydroxylase, a-methylphenylalanine, was introduced by Greengard et al., in 1976. A-methylphenylalanine plus phenylalanine treatment causes no mortality or obvious toxicity, and a slight reduction in body weight is no longer present after termination of treatment. In addition the main biochemical characteristics of PKU were reproduced (Lane and Neuhoff, 1980). Behavioral changes in adult rats seen 3 months

after termination of treatment with a-methylphenylalanine plus phenylalanine include deficiencies in passive and active avoidance tests (Luttges and Gerren, 1979), diminished maze learning, increased running wheel and open field activity (Greengard et al., 1979) and exaggerated cerebral lateralization (Glick and Greengard, 1980). These animals also show impaired "advantageous learning" (Strupp et al., 1984) which is thought to test permanent deficits in cognitive ability by eliminating immediate biological needs as a factor in the tasks performed.

B. Biochemical Basis of the Mental Disorder

Many studies have focused on defects in myelin in experimental models of PKU because they are one of the few permanent pathological features in adult phenylketonurics. Huether et al., (1982), describe a number of transient changes in myelin characteristics and a permanent reduction in myelin content in the "a-methylphenylalanine model" of PKU. Other investigators have found a deficit in the basic proteins of the central nervous system myelin in the same model (Figlewicz and Druse, 1980). It is possible that neuronal differentiation is dependent on structural characteristics of myelin. However, defects in myelination may also be a consequence of a more generalized delay in the maturation of neurons (Bauman and Kemper, 1982).

Impairment in energy metabolism is thought to be another factor responsible for abnormal brain development.

Inhibition of glycolytic enzymes in experimental PKU has been demonstrated in vitro with phenylalanine and p-chlorophenylalanine by some investigators (Weber, 1969; Schwark et al., 1970) but not confirmed by others (Akeson et al., 1979).

Phenylalanine metabolites also have the potential to disturb brain metabolism in vitro. However, the doses of phenylalanine metabolites used in these experiments are usually above the amount present in phenylketonuric brain (Loo et al., 1977; Loo et al., 1980).

A decrease in cerebral large neutral amino acids by high circulating phenylalanine was demonstrated in rats (Oldendorf, 1973; Mckean et al., 1968). The importance of the lowering of brain amino acids such as tryptophan and tyrosine can be interpreted in various ways since they constitute the building blocks of protein synthesis and are precursors for neurotransmitters. Evidence for a decrease in protein synthesis relies mainly on problematic models of phenylketonuria: decreases in brain polyribosomes (Taub and Johnson, 1975), in acylation of transfer ribonucleic acid (Hughes and Johnson, 1975), and of amino acid incorporation into protein (Agrawal et al., 1969) were found in rodents given phenylalanine alone.

A number of neurotransmitters have been implicated in a pathogenic role. Elevation of glycine occurs in a experimental model (Isaacs and Greengard, 1980) but has not been confirmed in phenylketonuric subjects. Depletion of

brain serotonin (Isaacs and Greengard, 1980) and of dopamine and norepinephrine (Brass and Greengard, 1982) has been demonstrated. As described in a later section, certain behavioral tasks that are modified in experimental animals are closely related to monoamine levels in the central nervous system and this relationship constitutes one of the few links between biochemical and behavioral changes in experimental PKU. In particular, Ponzio and Jonsson (1978) describe how neonatal treatment with 5-7-dihydroxytryptamine in rats leads to a redistribution of 5-HT nerve terminals in selected brain areas. It has also been shown that 5-HT inhibits neurite outgrowth and prevents formation of electrical synapses between specific neurons in the Helisoma snail (Haydon et al., 1984). The mechanism of monoamine decrease in experimental PKU is therefore of particular importance and the studies described in this thesis are focused on this phenomenon. Maternal phenylketonuria is a recent problem and biochemical alterations in the fetal brain which may be the basis for the mental retardation in offspring of these mothers have not been explored. An animal model, developed by Brass et al., (1982), provides some answers to this question by looking at changes in neurotransmitter and amino acid levels in fetal brain. Although they present numerous practical difficulties, comparable studies in humans would be essential.

3. Serotonin in the Central Nervous System

A. History

Serotonin, referred to as enteramine initially, was first detected in the enterochromaffin cells of rabbit gastrointestinal tract. It had the property of contracting smooth muscle (Erspamer, 1966). In the 1940's, a plasma component with vasoconstrictor properties, named vasotonin, was isolated in a crystalline form. Its structure, identified as 5-hydroxytryptamine, (Rapport, 1949) was the same as that of enteramine or serotonin (5-HT) as shown by Erspamer and Asero in 1952. The discovery of 5-HT in the brain occurred in 1953 (Twarog and Page, 1953). Approximately 90% of the 5-HT present in the body is stored in enterochrommaffin cells in the gastro-intestinal tract. The remaining 10% is in platelets and the central nervous system.

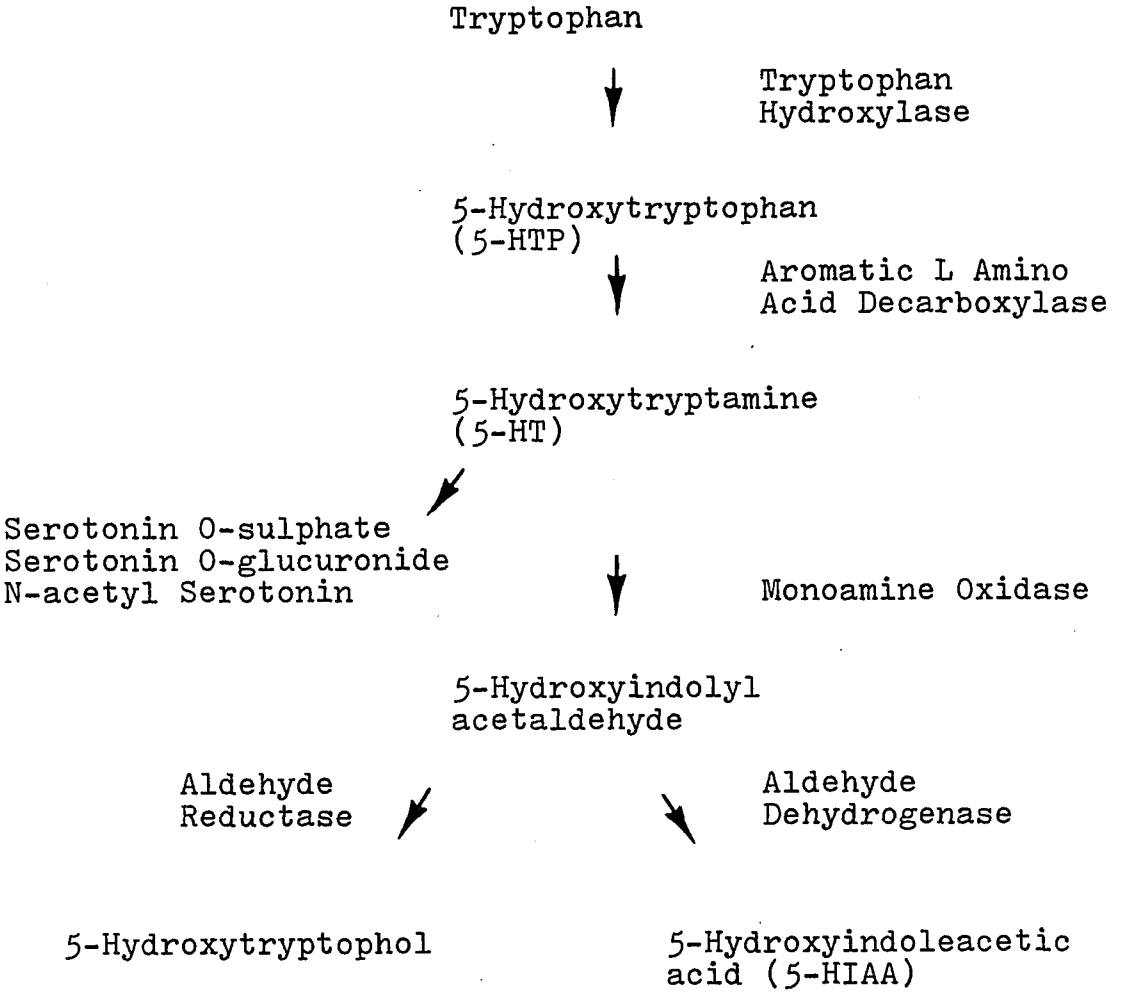
B. The Metabolic Pathway

The 5-HT in neurons, as in most other cells, is synthesized in situ from L-tryptophan. The first reaction on the pathway (see Figure I-3) is catalyzed by tryptophan hydroxylase (E.C.1.14.16.4) and proceeds according to the following reaction:

L-tryptophan + tetrahydrobiopterin + oxygen $\rightarrow$
5-hydroxy-L-tryptophan + dihydrobiopterin + water.

FIGURE I-3

DIAGRAM OF 5-HT METABOLISM



As in the case of the phenylalanine hydroxylase system, the reaction is coupled to dihydrobiopterin reductase which reconstitutes the cofactor to its reduced form. The next step, decarboxylation of 5-hydroxytryptophan to 5-HT is catalyzed by aromatic L amino acid decarboxylase or dopa decarboxylase (E.C.4.1.1.26) which is also involved in the synthesis of catecholamines.

On the main degradative pathway of 5-HT in the brain, deamination by monoamine oxidase or MAO (E.C.1.4.3.4) yields 5-hydroxyindoleacetaldehyde which is rapidly converted to 5-hydroxyindoleacetic acid (5-HIAA) by aldehyde dehydrogenase. MAO type A is thought to preferentially deaminate 5-HT and norepinephrine whereas beta-phenylethylamine is one of the preferred substrates for MAO type B.

C. The Role of 5-HT in the Central Nervous System

The function of 5-HT in the brain has been widely studied and it has been proposed to be involved in such diverse functions as sleep, neuroendocrine control, sexual behavior, and temperature regulation. Its role as a neurotransmitter is well documented. It has a heterogeneous distribution in the CNS with higher levels in the midbrain raphe nuclei and its projections, engaged in serotonergic functions (Maickel et al., 1968). 5-HT is stored in intraneuronal granules and is released by reserpine (Brodie et al., 1957). After depolarization, its

activity is terminated by a high affinity reuptake mechanism (Snyder et al., 1973) and by metabolism by MAO in the neuronal cytoplasm. 5-HT changes the firing rate of neurons when applied microiontophoretically and is released from synaptosomal preparations after depolarization by K+ (Mulder et al., 1975).

Several studies have implicated the serotonergic system in mental disorders. Decreased 5-HIAA levels were found in the cerebrospinal fluid of depressive patients (Mendels et al., 1972) and a decreased 5-HT turnover was noted after probenicid treatment (Van Praag et al., 1973). Autopsy studies of depressive suicides have revealed that they have decreased brain 5-HT and 5-HIAA content (Pare et al., 1969). Attempts to treat patients by increasing their brain 5-HT content have yielded mixed results. These included administration of tryptophan, of MAO inhibitors, and of tricyclic anti-depressants. In bipolar depression and mania, treatment of patients with tryptophan is more effective in the manic state (Murphy et al., 1974) than in the depressive state (Carroll et al., 1970). The effects of these treatments are not restricted to the serotonergic pathway and it usually takes a week before they become effective. It is therefore difficult to assign a specific role to 5-HT in the etiology of affective disoders.

Woolley and Shaw (1954) proposed that 5-HT may have to do with hallucinogenic symptoms as suggested by its synergistic effect with LSD (Costa, 1956). However, an

antagonistic effect of LSD on 5-HT action occurs at high doses (Gaddum, 1953). The administration of tryptophan to schizophrenics causes an exacerbation of their symptoms (Alexander et al., 1963). It has been postulated that psychotomimetic compounds are formed in these patients by abnormal methylation of 5-HT. Among the postulated hallucinogens are N,N'dimethyltryptamine, its 5-hydroxy derivative bufotenine (Wyatt et al., 1974) and 5-methoxytryptamine (Koslow, 1976). Although elevations of these metabolites have been found in patients' urine, a few control subjects also had elevations, suggesting that the methylated derivative hypothesis is questionable. Another metabolic pathway, the cyclization of indoleamines to beta-carbolines (Barchas et al., 1974) has no known physiological significance.

CHAPTER II

METHODS

1. Assays

A. Serotonin and 5-Hydroxyindole Acetic Acid

Serotonin (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) were measured by the fluorimetric method of Curzon and Green (1970). This method is sensitive, reproducible, and fairly rapid. Approximately 0.01 ug 5-HT and 5-HIAA can be detected. The fluorimetric method is based on the formation of fluorescent complexes when 3,5 substituted indoles are reacted with o-phthalaldehydes. Whole brain or separated regions were homogenized in cold acidified butanol and centrifuged at 3000 rpm for 5 minutes. 2.5 ml of the supernatant was added to 5 ml n-heptane and 0.4 ml of 0.1% cysteine. The aqueous phase was separated by centrifugation at 3000 rpm for 5 minutes and 5-HT was determined fluorometrically after reaction with 0.01% o-phthalaldehyde in 10N HCL. For the determinaton of 5-HIAA, 5 ml of the organic phase was mixed with 0.6ml of 0.5 M sodium phosphate buffer (pH 7.0), centrifuged at 3000 rpm for 3 minutes and fluorescence was determined after reaction of the aqueous phase with 0.1% o-phthalaldehyde in 10 N HCL. All preparations were assayed immediately or stored under nitrogen gas at -20°C for less than one week. Fluorescence, at Emax 470 nm with activation at 360 nm, was

found to increase linearly by 5-HT addition in the range of 30 ng to 600 ng and with 5-HIAA in the range of 60 ng to 600 ng. All determinations in rat brain were restricted to this range.

The o-phthalaldehyde reagent was purchased from Regis Chemical Co., Morton Grove, Ill. Standard solutions of 5-HT (Sigma, St. Louis, Mo) were made up in 0.1% cysteine. Standard solutions of 5-HIAA (Sigma, St. Louis, Mo) were made up in 0.5 M sodium phosphate buffer (pH 7.0).

B. Plasma and Brain Tryptophan and Phenylalanine

Brain tissue was homogenized in 12 volumes of 10% trichloroacetic acid and centrifuged at 20,000 g for 10 minutes. Heparinized trunk blood was centrifuged for five minutes, and the plasma was deproteinized by mixing with 10% trichloroacetic acid after a 100 fold dilution and centrifuged at 20,000 g for 10 minutes. The measurement of tryptophan is based on the fluorimetric method of Denckla and Dewey (1967) as revised by Bloxam and Warren (1974) and Lehmann (1971). The volume of brain supernatant used in the tryptophan assay was 0.1 to 0.2 ml, and that of plasma supernatant was 0.5 to 1.0 ml. The samples were kept at 0-4° C until assay. 0.2 ml of 2% formaldehyde was added to the tubes, followed by 0.1 ml of 6 mM $FeCl_3$. (For the maximal production of fluorescence, $FeCl_3$ has to be added immediately before the heating step, otherwise, some of the tryptophan is degraded.) The samples were then heated at

100°C for 1 hour and were read at Emax 450 nm with activation at 370 nm. Phenylalanine (0.52 mM), a-methylphenylalanine (0.44 mM) and p-chlorophenylalanine (0.16 mM) did not interfere with the assay.

Phenylalanine in the same plasma or brain samples were measured by the fluorimetric method of McCaman and Robins (1962) as modified by Faulkner (1965). A-methylphenylalanine did not interfere with the assay, but in animals treated with p-chlorophenylalanine (which did interfere), phenylalanine was measured in a Beckman automatic amino acid analyzer.

C. L-Aromatic Amino Acid Decarboxylase

L-aromatic amino acid decarboxylase (E.C. 4.1.1.26) activity was determined by a modification of the method of Lamprecht and Coyle (1972). Brain tissue was homogenized in 0.32 M sucrose plus 0.6% triton. 50 ul or 100 ul homogenate was added to the reaction mixture consisting of 0.1 M sodium phosphate buffer, pH 7, 25 ul of 0.1 mM pyridoxal phosphate (Sigma, St. Louis, Mo), 30 ul of 0.017 M L-dopa (Sigma, St. Louis, Mo), 2 ul of DL-[^{14}C] dopa (49.4 mCi/mmole, New England Nuclear, Boston, Mass.) and 68 ul H_2O. Samples were incubated at 37°C for 20 minutes. 0.5 ml of 10% trichloroacetic acid was added to stop the reaction and during an additional incubation at 37°C for 60 minutes, released $^{14}CO_2$ was collected on filters with 200ul NCS. The samples were counted for $^{14}CO_2$

after addition of 10 ml of 10% methanol in econofluor (New England Nuclear, Boston, Mass.) to the filters. A blank was made up by adding 0.5 ml of trichloroacetic acid to the assay before incubation. Activity is expressed in umoles/hour/g brain tissue.

D. Monoamine Oxidase

Monoamine oxidase (E.C. 1.4.3.4.) activity was measured by the method of Wurtman and Axelrod (1963). Brain tissue was homogenized in 9 volumes of 0.32 M sucrose plus 0.2% triton and diluted in 0.15 M KCl to a 1% homogenate. The assay mixture contained 50 or 100 ul of brain homogenate, 100 ul of 0.5 M sodium phosphate buffer, pH 7.4, 50 ul of 1 mM tryptamine (Sigma, St. Louis, Mo), 1 ul of 2-^{14}C tryptamine bisuccinate (59 mCi/mmole; New England Nuclear, Boston, Mass.), 49 ul of 0.01 N HCl and 0.15 M KCl in a final volume of 300 ul. After incubation at 37°C for 20 minutes, the reaction was stopped with 200 ul 2 N HCl and 6 ml toluene was added to extract the deaminated product. Samples were centrifuged at 1500 rpm for 5 minutes and 3 ml of the supernatant was added to 10 ml of insta-gel scintillation fluid (Packard Instrument Company, INC., Downers Grove, Ill.). Complete reaction mixtures to which 200 ul 2 N HCl was added before incubation constituted the blanks. Activity is expressed in nmoles/min/g brain tissue.

E. Tyrosine Hydroxylase

Tyrosine hydroxylase (E.C. 1.14.16.2) was measured by a modification of the method of Nagatsu et al. (1964), as described by Brass (1983), and is based on the quantification of tritiated H_2O release from L-[ring-3,5-^{3}H] tyrosine (50 Ci/mM; New England Nuclear Corp., Boston, MA). 0.1 ml of this substrate (plus 0.05 ml glacial acetic acid and 1.0 ml H_2O) was purified by putting it on a cation exchange resin (BioRad Ag 50W-x8, H+ form, 0.5x3 cm, 200-400 mesh; BioRad Laboratories, Richmond, CA) and washing with approximately 1 liter of distilled H_2O. Tyrosine was eluted with 15 ml of 2N HCl, lyophilized, and kept at -20°C for less than a month. Striatal tissue was homogenized in 9 volumes of cold 0.32 M sucrose containing 0.6% Triton X-100. 50 or 100 ul of the homogenate was added to the incubation mixture which included: 200 mM NaAcetate buffer (pH 6), 280 mM NaCl, 200 units catalase (Sigma, St. Louis, MO), 5 mM 6,7-dimethyl-5,6,7,8 tetrahydropteridine HCL or $DMPH_4$ (Sigma, St. Louis, MO), 10 mM DTT, 10 mM $Fe(NH_4)_2SO_4 6H_2O$, and 0.5 mM tyrosine which included the above labelled tyrosine (0.04 to 0.08 uCi). The reaction was run at 37 C for 8 minutes and terminated by addition of 200 ul of glacial acetic acid. After centrifugation at 7,000g for 10 minutes, the supernatant was passed on a column, identical to the one used to purify the labelled substrate, and washed twice with 0.5 ml

distilled H_2O. This effluent was counted for tritiated H_2O in 10 ml of Instagel (Packard Instrument Co., Downers Grove, IL). A blank was run under the same assay conditions minus the cofactor $DMPH_4$. The reaction was linear up to 10 minutes and with 1 to 10 mg tissue homogenate. Results are expressed in umol/min/g striatal tissue.

F. Tryptophan Hydroxylase

The assay of tryptophan hydroxylase (E.C.1.14.16.4) involving fluorimetric measurement of the 5-hydroxyindoles (after reaction with o-phthalaldehyde) produced during incubation of brain extracts with tryptophan, was based on the method of Gal and Patterson (1973).

Rat midbrain was homogenized in 20% 50mM Tris-acetate buffer (pH 7.6) containing 10^{-3}M 2-mercaptoethanol. Samples were centrifuged at 30,000 g for 30 minutes and the supernatant was used as the source of enzyme. In the 1 ml of incubation mixture, final concentrations of substances were as follows: 50 mM Tris-acetate (pH 7.6), 1 mM 2-mercaptoethanol (Sigma, St. Louis, Mo), 0.2 mM pargyline, 0.176 mM L-tryptophan, 0.16 mM DL-6-methyl 5, 6, 7, 8, tetrahydropterine hydrochloride or 6-MPH_4 (Calbiochem, La Jolla, Ca), 10 ug catalase (Sigma, St. Louis, Mo), and 0.050 to 0.150 ml midbrain homogenate. Blanks were prepared by omitting $6MPH_4$. All reactions were started by addition of the tissue supernatant. 30 minutes after

incubation at 37°C in a shaking water bath the tubes were placed in boiling water for 5 minutes and centrifuged at 3000 rpm for 10 minutes. 0.5 ml of the supernatant was added to 0.1 ml 1% cysteine, 1.15 ml 10 N HCl and 0.2 ml of 0.01% o-phthalaldehyde (Regis Chemical Co., Morton Grove, Ill.) in 10 N HCl. After heating the reaction mixture at 100°C for 15 minutes, the samples were cooled and fluorescence was read at activation 360 nm and Emax 470 nm. Standard 5-HT (Sigma, St. Louis, Mo) was made up in 0.059 M Tris-acetate buffer (pH 7.6) and carried throughout the entire assay minus the cofactor $6MPH_4$.

With 10-40 mg tissue, the reaction rate was linear up to 30 minutes of incubation. Recovery of added 5-HT was 100%. Addition of a-methylphenylalanine and phenylalanine, at 20 times greater levels than estimated to be present in experimental brains did not interfere with the enzyme assay. Enzyme activity is expressed in nmoles/hour/g midbrain tissue.

G. Catecholamines

Dopamine and norepinephrine were measured by the method of Coyle and Henry (1973) as modified by Saller and Zigmond (1978).

2. Treatment of Animals

A. Injections to Postnatal Animals

Albino Fischer (CDF) rats were used in these studies and were given food and water ad libitum. DL-a-methylphenylalanine (Sigma, St. Louis, Mo), L-phenylalanine (Sigma, St. Louis, Mo), DL-a-methylphenylalanine plus phenylalanine, and L-tryptophan (Eastman Kodak Co., Rochester, NY) solutions were dissolved in 0.9% NaCl with heating. P-chlorophenylalanine (Aldrich, Milwaukee, Wi) and p-chlorophenylalanine plus phenylalanine were added to 0.9% NaCl sonicated and heated, and were injected as a fine suspension. Testosterone propionate (Sigma, St. Louis, Mo) was dissolved in sesame oil. All injections were subcutaneous. Control animals (littermates) received vehicle only. A-methylphenylalanine (24 umol/10 g body wt.) and p-chlorophenylalanine (9 umol/ 10 g body wt.) with or without phenylalanine (52 umol/ 10 g body wt.) were injected once ("acute experiments") or once a day for at least 3 days ("chronic experiments").

The long term treatment in experiments of chapter VII involved a daily injection with standard doses of a-methylphenylalanine (24 umol/ 10 g body wt.) plus phenylalanine (52 umol/ 10 g body wt.) from the 3rd to the 21st day of age. In one experiment, the treatment was

modified to include a second daily injection of the same dose of phenylalanine on days 7 to 10 for one litter, and on days 13, 14, 18, and 21 for the other. The animals were weaned at 21 to 23 days and were placed on a 7% phenylalanine diet up to 30 days of age. Biochemical and behavioral studies on these rats were carried out 3 to 4 months later.

B. Maternal Hyperphenylalaninemia

Female Fischer rats were mated with males of the same strain and the first day of pregnancy was determined by the presence of sperm in a vaginal smear. Experimental dams received a diet containing 0.5% D,L-a-methylphenylalanine plus 3% or 7% phenylalanine. All pregnant animals were maintained on a 12:12:h light:dark cycle and had access to food and water ad libitum.

C. Behavioral Studies

Rotometer studies were carried out in an environmentally controlled room (22°C, 60% humidity, and 12:12:h light:dark cycle). Adult rats (above 100 days old) were placed individually into a rotometer for a 24 h period with food and water provided "ad libitum". The animal was harnessed by a wire to a shaft leading to a photoelectric position sensing device. This device recorded rotations automatically and distinguished between full (360°) and quarter (90°) turns to the left and to the right

(Greenstein and Glick, 1975). For expression of the results, the net magnitude of rotation (the difference between full turns in the dominant and non-dominant directions), the percent preference (dominant full turns multiplied by 100 and divided by total full turns) and the amount of random activity or extra quarter turns (subtracting 4 times the number of full turns from the total number of quarter turns) were quantitated. The sum of full rotations to the left and to the right (total full rotations) and their difference (net rotations) were also calculated from the recorded data.

D. Tissue Preparation

Studies were done on fresh tissue or blood obtained after decapitation. Animals were sacrficed between 11 A.M. and 1 P.M. on the appropriate day. Brain tissue was kept at 0-4°C during dissection. Blood was collected in heparinized tubes. Whole brain or midbrain was used for 5-HT and 5-HIAA measurement. Tryptophan was measured in whole brain or in whole brain minus the midbrain. Dopa decarboxylase and monoamine oxidase were measured in striatum and tryptophan hydroxylase in midbrain. The midbrain dissection was defined dorsally by the rostral border of the superior colliculi and caudal border of the inferior colliculi. It was limited ventrally by the rostral border of the pons and the caudal border of the

mammillary glands. The striata were removed by the method of Glowinski and Iversen (1966).

CHAPTER III

DECREASE IN 5-HYDROXYINDOLES BY HYPERPHENYLALANINEMIA

A study by Pare et al. (1957) showed that phenylketonuric children had low levels of serum 5-HT and urinary 5-HIAA compared to normal children and to non phenylketonuric children with mental defects. This subnormal 5-hydroxyindole concentration was confirmed and extended to the cerebrospinal fluid (CSF) by Tu and Partington (1972). In a more recent study of Butler et al. (1981), they showed that in phenylketonuric children from birth to at least the 4th year, the 5-HIAA concentration of the CSF was 50% below normal. Reversal of this deficit by reduction of phenylalanine intake indicated that alteration of 5-HT metabolism in PKU is secondary to the severe hyperphenylalaninemia characteristic of untreated subjects of this disease. Information about intracerebral 5-hydroxyindole metabolism in PKU comes from autopsy studies by McKean (1972), demonstrating that the 5-HT content in several brain areas was 50-70% below that in corresponding areas of age-matched control subjects. This deficiency must also be a consequence of hyperphenylalaninemia since, experimentally at least, it can be evoked by phenylalanine administration to normal rats. However, repeated phenylalanine administration is required to maintain a chronic 5-HT deficiency (without suppression of the hepatic phenylalanine hydroxylase).

This treatment is also toxic to developing rats, which is one reason why such animals do not constitute appropriate models for PKU. This chapter describes the decrease, at various developmental stages, of the cerebral 5-HT and 5-HIAA concentration of rats treated with a suppressor of phenylalanine hydroxylase (a-methylphenylalanine) plus phenylalanine.

1. Postnatal Hyperphenylalaninemia

The decreases in brain 5-HT and 5-HIAA caused by a-methylphenylalanine plus phenylalanine, or phenylalanine alone, could be due to the interference of these substances with the assay system. The results in Table III-1 show the effects of a-methylphenylalanine, phenylalanine, and the two compounds simultaneously on the 5-HT assay when added in vitro. An assay has not been developed for the measurement of a-methylphenylalanine. In order to estimate the quantity of a-methylphenylalanine present in the assay after injection, it is assumed that a-methylphenylalanine is equally distributed in all tissues. The standard injection of 2.4 umol/g body wt. would thus result in an approximate tissue concentration of 2.4 mM. By taking into account the dilutions for the 5-HT assay, the final concentration in the assay is 0.42 mM. (This calculation in fact overestimates the amount of a-methylphenylalanine present since not all of the injected material is absorbed.) The brain phenylalanine concentration after

TABLE III-1

EFFECT OF A-METHYLPHENYLALANINE AND PHENYLALANINE ON THE 5-HT ASSAY

A-Me added (mM)	0	0.042	0.42	4.2	42
F.U.	35±1.7(3)	36	34	33	32
Phe added (mM)	0	0.050	0.50	5.0	50
F.U.	35±1.7(3)	36	36	36	38
A-Me + Phe (mM)	0	0.42+0.52	2.1+2.5	4.2+5.0	
F.U.	31	28	28	31	

A standard amount of 5-HT (60ng) was measured by the fluorimetric method of Curzon and Green (1970) in the presence of the indicated amounts of phenylalanine or a-methylphenylalanine or both. F.U. denotes fluorescent units. Values are means ±1 S.D. or single values.

injection of the standard amount of a-methylphenylalanine plus phenylalanine was 2.94 mM and in the assay system, in vitro, it becomes diluted to 0.50 mM. These compounds do not interfere with the assay. Up to 42 mM a-methylphenylalanine, 50 mM phenylalanine, and 4.2 mM a-methylphenylalanine plus 5.0 mM phenylalanine did not change the fluorescence of a standard amount of 5-HT (Table III-1). The estimate for a-methylphenylalanine inadvertently present in the 5-HIAA assay system is 0.14 mM and for phenylalanine, it is 0.17 mM. Twenty times these amounts did not interfere with the assay (Table III-2).

As described by Isaacs and Greengard (1980), experimental PKU induced by a-methyphenylalanine plus phenylalanine results in a decrease in cerebral 5-HT of approximately 30% in young rats. Table III-3 shows that this decrease is present in all the brain regions measured in 11 day old rats. The decrease in 5-HIAA is more pronounced, at 60 to 70%, and is also present in all brain regions (Table III-3).

In addition to brain areas, the decrease in brain 5-HT and 5-HIAA levels in our model of phenylketonuria was examined with increasing duration of treatment. As shown in Table III-4, there is a 30 to 40% decrease in 5-HT that persists up to 16 days in age. This decrease is of the same magnitude in 8 day old animals treated for 6 days or 3 days, suggesting the lack of a cumulative effect.

TABLE III-2

EFFECT OF A-METHYLPHENYLALANINE AND PHENYLALANINE ON THE 5-HIAA ASSAY

A-Me added (mMM)	0	0.14	1.40	2.80
F.U.	14	16	12	13
Phe added (mM)	0	0.17	1.70	3.40
F.U.	14	16	16	15
A-Me + Phe (mM)	0	0.14+0.17	1.40+1.70	2.80+3.40
F.U.	14	15	15	14

A standard amount of 5-HIAA (30ng) was measured by the fluorimetric method of Curzon and Green (1970) in the presence of the indicated amounts of phenylalanine or a-methylphenylalanine or both. F.U. denotes fluorescent units.

TABLE III-3

EFFECT OF CHRONIC HYPERPHENYLALANINEMIA ON 5-HT and 5-HIAA CONCENTRATIONS IN BRAIN REGIONS

control 5-HT (nmol/g)	experimental 5-HT (nmol/g)	control 5-HIAA (nmol/g)	experimental 5-HIAA (nmol/g)	tissue
1.62±0.11	1.13±0.23	1.11±0.03	0.47±0.20	whole brain
3.67±0.15	2.13±0.19	3.46±0.56	0.85±0.13	brain stem
1.61±0.18	1.37±0.20	1.19±0.10	0.41±0.15	cerebellum
1.23±0.22	0.80±0.12	0.61±0.09	0.22±0.07	cortex
1.81±0.25	1.27,1.03	1.55±0.61	0.62±0.05	diencephalon

Analyses of 11 day old rat brain tissue was done 4 hours after the last daily injection, started on day 2, of a-methylphenylalanine (2.4 umol/g body weight) plus phenylalanine (5.2 umol/g body weight) or NaCl. Values are expressed as means ±1 S.D. for 3 animals or refer to a single animal. Control values are significantly different from experimental ($P<0.01$) in whole brain, brain stem and cortex for 5-HT and in all tissues for 5-HIAA.

TABLE III-4

EFFECT OF CHRONIC HYPERPHENYLALANINEMIA ON WHOLE BRAIN 5-HT AND 5-HIAA CONTENT WITH INCREASING AGE IN POSTNATAL RATS

days of age	control 5-HT	experimental 5-HT	control 5-HIAA	experimental 5-HIAA
1	1.26,1.10		1.04	
6	1.13±0.05	0.80±0.01*	0.35±0.03	0.11±0.03**
8	1.60±0.09	1.13±0.14**	0.95±0.22	0.43±0.05**
8#	1.63±0.27	1.05±0.05**		
11	1.62±0.11	1.13±0.23**	1.11±0.03	0.47±0.20**
14	2.00±0.20	1.12±0.08**	0.81±0.17	0.27±0.05**
16	1.93±0.17	0.89±0.09**	1.62±0.43	0.24±0.08**

Whole brain 5-HT and 5-HIAA depletion was measured 4 to 6 hours after the last treatment with a-methylphenylalanine plus phenylalanine or saline as described in the Methods section. All animals were treated from 3 days of age except for, as indicated by the #, one group of 8 day old animals which were treated during the preceding 2 days only. Values are expressed as nmoles of 5-HT or 5-HIAA/g wet weight of brain and are means ±1 S.D. for 3 animals or refer to a single animal. A significant difference from control is indicated by * ($P<0.05$) and ** ($P<0.01$).

The effect of phenylalanine alone was also examined. In order to raise plasma phenylalanine levels sufficiently in the presence of uninhibited phenylalanine hydroxylase, multiple injections were required. In this experiment, 5.2 umol/g phenylalanine was given twice and animals were sacrificed 4 hours after the first injection. There was an appreciable decrease in whole brain 5-HT and 5-HIAA in both 6 and 10 day old animals (Table III-5). Thus, as in adulthood (Yuwiler and Louttit, 1961), so during early postnatal development, treatment with phenylalanine alone causes a decrease in 5-hydroxyindole levels.

2. Prenatal Hyperphenylalaninemia

In a model of maternal PKU, pregnant animals were placed on a phenylalanine plus a-methylphenylalanine diet 12 days after conception. The results in Table III-6 confirm that this diet is appropriate for inducing maternal PKU. Maternal plasma phenylalanine with treatment 1 (3% phenylalanine) or treatment 2 (7% phenylalanine) is elevated 10 to 20 fold by the experimental diet. A higher increase in plasma phenylalanine is observed in fetal plasma phenylalanine. Brain phenylalanine levels are increased in dams 2 to 5 fold. In the fetuses, however, the elevation in brain phenylalanine is more pronounced (4 to 10 fold). Thus, the average plasma/brain ratio is 3.9 in the dams but only 1.92 in the fetuses. Furthermore, the higher dose of dietary phenylalanine (cf. last line in

TABLE III-5

DECREASE IN WHOLE BRAIN 5-HT AND 5-HIAA BY PHENYLALANINE TREATMENT

	days of age	5-HT (nmoles/g)	5-HIAA (nmoles/g)
control	6	1.49±0.11(4)	0.69 ±0.01(4)
experimental	6	1.00±0.05(5)	0.38 ±0.02(5)
control	10	1.60±0.20(7)	0.98 ±0.27(7)
experimental	10	0.70±0.10(3)	0.33 ±0.07(3)

Rats were injected twice with phenylalanine (5.2 umoles/g body weight) 2 hours apart and sacrificed 4 hours after the first injection. All values are expressed in nmoles/g wet weight of whole brain and are means ±1 S.D.(n). Control values are significantly different from experimental ($P<0.01$) for 5-HT and 5-HIAA.

TABLE III-6
THE PHENYLALANINE CONTENT OF MATERNAL AND FETAL PLASMA AND BRAIN AND THE PLASMA/BRAIN PHENYLALANINE RATIO

DAM	plasma phe. (nmole/ml)	brain phe. (nmole/g brain)	plasma/brain ratio
control	96±31(6)	80±17(5)	
treated 1	1080±344(10)*	296±86(7)*	3.90±2.76(7)
treated 2	1743±1485(6)*	314±66(3)*	
FETUS			
control	243±62(6)	292±52(6)	
treated 1	2922±801(8)*	1893±520(10)*	1.92±0.57(4)
treated 2	3300±383(3)*	3315±1304(4)*,**	

Rats were placed on the experimental diet.
(treated 1=0.5% a-methylphenylalanine plus 3% phenylalanine: treated 2=0.5% a-methylphenylalanine plus 7% phenylalanine) as described in the Methods section. Dams and fetuses were sacrificed between 10-11 AM on gestational days 18-22. Control values are means ±1 S.D.(n) of results on the indicated number of dams, or of results on one fetal brain and pooled fetal blood from six different litters. Experimental values refer to individual dams and to one brain and pooled plasma of fetuses born to those dams.
* significant differences from control with $p<0.01$
** significant differences from treated 1, with $p<0.01$

Table III-6) results in a ratio in the fetuses which is close to 1 but is not further lowered in the dams, again indicating the limited phenylalanine uptake capacity of the mature brain.

Results for brain amino acids and neurotransmitter levels in the fetuses of hyperphenylalaninemic dams are shown in Table III-7. Levels of the large neutral amino acids valine, leucine, and isoleucine are decreased whereas tyrosine levels are increased. The amount of the putative neurotransmitter glycine is increased. This change in glycine levels was also observed in postnatal phenylketonuric rats (Isaacs and Greengard, 1980). Brain 5-HT content is decreased by 21% and 5-HIAA by 71% but a difference between controls and experimentals is no longer observed at birth. No statistically significant changes are observed in the dopamine or norepinephrine levels in these rats.

From these studies, it appears that both prenatal and postnatal forms of hyperphenylalaninemias can reduce 5-hydroxyindole levels in developing rat brain. This decrease caused by our model treatment is not dependent on the duration of treatment or on the age at which treatment is initiated. The most relevant factor appears to be the maintenance of high circulating phenylalanine.

TABLE III-7

CEREBRAL LARGE NEUTRAL AMINO ACIDS AND NEUROTRANSMITTER CONTENT OF HYPERPHENYLALANINEMIC FETUSES

	Control	Experimental
phenylalanine	80	1065
tyrosine	148	178
valine	193	130
leucine	148	123
isoleucine	80	60
methionine	28	20
glycine	1.37±0.06(3)	1.90±0.18(4)*
5-HT	1.28±0.10(3)	1.01±0.09(3)*
5-HIAA	1.33±0.14(3)	0.39±0.05(3)*
norepinephrine	0.61±0.26(5)	0.50±0.16(6)
dopamine	1.06±0.12(5)	1.17±0.20(6)

Experimental rats were placed on the a-methylphenylalanine (0.5%) plus phenylalanine (3%) diet 12 days after conception. Animals were sacrificed between days 20 and 22 of gestation. Brains of fetuses pooled from one dam were assayed in the automatic amino acid analyzer. For 5-HT and 5-HIAA, two fetal brains were pooled for one measurement. For glycine, dopamine and norepinephrine, measurements were made on one fetal brain. Values for 5-HT, 5-HIAA, glycine, dopamine and norepinephrine are means ±1 S.D.(number of litters). All values are expressed in nmoles/g brain tissue.
* indicates a significant difference ($P < 0.01$) from control.

3. Discussion

The decrease in brain 5-HT and 5-HIAA that occurs in human PKU was also found in our animal model of the disease. In order to be certain that an in vivo mechanism was responsible for these changes, it was necessary to test the effects of the injected agents on the fluorescent assay of 5-hydroxyindoles. The results showed that the addition of 20 times the amount of a-methylphenylalanine (or phenylalanine) that might have been present in brain tissues did not interfere with either the 5-HT or 5-HIAA assay (Tables III-1 and III-2). A decrease of these compounds was found in all brain regions tested (Table III-3) and was of the same magnitude up to 16 days of age (Table III-4). The change in 5-HIAA was about 30% greater than that of 5-HT, suggesting that a relatively small decrease in 5-HT appreciably lowers the rate of its oxidation by monoamine oxidase in vivo.

Prolonged a-methylphenylalanine plus phenylalanine treatment does not appear to have adverse effects on developing rats. They were devoid of obvious signs of toxicity and their weight gain was the same as in controls. The decrease in 5-HT was the same after 2 as after 6 days of treatment. These results demonstrate that the decrease in 5-hydroxyindoles in our experimental model of PKU is not due to progressive toxicity. Injection of phenylalanine alone also decreases 5-HT and 5-HIAA levels (Table III-5).

This would suggest that hyperphenylalaninemia is responsible for the decreases in 5-hydroxyindoles in animals receiving the dual treatment and that the role of a-methylphenylalanine was merely to permit (by suppression of phenylalanine hydroxylase) the maintenance of chronically elevated plasma phenylalanine levels. However, in view of additional experiments showing that a-methylphenylalanine can inhibit cerebral tryptophan hydroxylase (see next chapter), the question of whether the 5-HT depletion in the rat model occurs by the same mechanism as in human PKU required further investigation.

In addition to homozygous phenylketonurics, heterozygous children of phenylketonuric mothers are at high risk for developing mental retardation. There is no biochemical information on the brains of these children during or after gestation. The treatment used to produce an animal model of the condition (i.e. giving to pregnant rats a-methylphenylalanine and phenylalanine in their diet from the 12th day after conception on) was not toxic. It caused only a small temporary body weight deficit and postnatal survival was unchanged. The diet was successful in elevating fetal plasma phenylalanine about 10 fold. It has been shown previously that the competition of large neutral amino acids for the brain's L-transport system is operative in neonatal as well as in adult animals but that uptake capacity of the former is higher. The results in Table III-7 extend both these observations to prenatal

brain. For, hyperphenylalaninemia 1) reduced the fetal brains' concentration of branched chain amino acids and methionine (but not glycine) and 2) the brain to plasma phenylalanine ratio was higher in fetal than in postnatal rats. The fact that tyrosine levels are also increased somewhat is probably a consequence of residual phenylalanine hydroxylase activity which converts some of the administered phenylalanine to tyrosine.

The fetuses, aged 20 to 22 days, also had a 20-30% deficit in 5-HT and a 71-77% deficit in 5-HIAA (Table III-7) which was similar to the deficits seen in young postnatal animals exposed to hyperphenylalaninemia. Study of the mechanism of 5-HT decrease in experimental hyperphenylalaninemia will be restricted to young postnatal rats because current assays are not sensitive enough for the accurate measurement of the low tryptophan hydroxylase activity of fetal rat brain.

CHAPTER IV

IN VIVO AND IN VITRO INHIBITION OF THE PTERIDINE-DEPENDENT HYDROXYLASES BY PHENYLALANINE AND SYNTHETIC ANALOGUES

As described in chapter III, a decrease in brain 5-HT metabolism is observed in classical PKU and is mimicked in experimental models. Models that involve the use of synthetic agents to inhibit phenylalanine hydroxylase are complicated by the potential effects that these agents may have on tryptophan hydroxylase and tyrosine hydroxylase. It is conceivable that a potent inhibitor of one enzyme will also act on the other hydroxylases because these enzymes share many properties and are considered members of the same family. They require molecular oxygen to hydroxylate their substrate according to the following reaction:

substrate + tetrahydrobiopterin + oxygen ⟶
product + quinonoid dihydrobiopterin + water

The reaction involves tight coupling of the oxidation of the pteridine and hydroxylation of the substrate. The exact mechanism of the reaction is not known but is thought to result in the formation of a quaternary complex with reduced enzyme, oxidized pteridine, substrate, and oxygen (Massey and Hemmerich, 1975). Although tetrahydrobiopterin is thought to be the natural cofactor of all three

hydroxylases, other tetrahydropteridines such as the 6-methyl and 6,7 dimethyl derivatives can also serve as electron donors (Gal, 1981). Dihydropteridine reductase regenerates these and the natural cofactor in a NADH dependent reaction. Purified rat liver phenylalanine hydroxylase (Fisher et al., 1972) and tyrosine hydroxylase (Petrack et al., 1972) were shown to be iron containing enzymes and it seeme likely that tryptophan hydroxylase is as well (Kuhn et al., 1980). While measurements of phenylalanine hydroxylase or tyrosine hydroxylase with the well established methods available are reproducible from laboratory to laboratory, there are several-fold discrepancies in the tryptophan hydroxylase values that different investigators (even if using the same method) report for normal rat brain. The following series of experiments were therefore necessary to explore the problems and to standardize the conditions of the tryptophan hydroxylase assay. Suitable assay conditions were also tested in immature brain with abnormal as well as normal endogenous amino acid concentrations.

1. The Development of a Quantitative Assay for Tryptophan Hydroxylase in Hyperaminoacidemic Animals.

The fluorimetric method devised by Gal and Patterson (1973) appears to be sufficiently sensitive to detect tryptophan hydroxylase activity in the midbrain of young animals. It involves incubation with unlabelled tryptophan and fluorimetric assay of the reaction product. In crude homogenates of adult brain the 5-hydroxytryptophan is probably instantly converted to 5-HT by the endogenous L aromatic amino acid decarboxylase. The fact that this conversion may or may not occur in extracts of immature abnormal brain presents no problem because the derivatised 5-hydroxytryptophan and 5-HT yields identical fluorescence per mole. However, the original study of Gal and Patterson and of others using the same assay provide no information about variables in the incubation conditions which influence the activity of tryptophan hydroxylase and also the "blank" reaction. Thus, a standard method permitting reproducible quantification of tryptophan hydroxylase in normal as well as abnormal immature brain remained to be established.

It may be seen from Table IV-1 that substrate-free reaction mixtures (which should control for endogenous 5-hydroxytryptophan and 5-HT and depend on the amount of tissue extract added, see line 1) do not constitute an

TABLE IV-1
TRYPTOPHAN HYDROXYLASE ASSAY BLANKS

Midbrain Homogenate:	F.U. 0	F.U. 50(ul)	F.U. 100(ul)
no tryptophan, incubation		3.0±0.8(7)	4.8,5.0
tryptophan, incubation (0.18mM)	5,2		
tryptophan, no incubation (0.18mM)		7.8±1.2(7)	11.0±2.6(3)
tryptophan, no incubation (0.36mM)		8.0	10.0
tryptophan, no incubation (0.72mM)		11.0	15.0
tryptophan, incubation (0.18mM), no cofactor		7.9	12,12
tryptophan, incubation		15.2	31.0

The assay was carried out at different tryptophan concentrations, without or after 30 minutes incubation, and with the indicated omissions of enzyme (column 1) or cofactor (6MPH4, line 6) from the reaction mixture. Enzyme volumes (ul) refer to a 20% rat midbrain homogenate. Values are expressed in fluorescent units (F.U) and are single values or means ± S.D. (n).

adequate blank. It is necessary to have a blank carried through the incubation, as well as to control for the contribution of tryptophan and endogenously present 5-HT. Incubates lacking cofactor only were included in each experiment. The fluorescent units obtained with the complete reaction mixture minus that obtained with the cofactor free blank (e.g. 31-12, see last column, Table IV-1) are considered to quantify the product of the tryptophan hydroxylase reaction specifically. Sensitivity, though limited by high blanks, was adequate for the present purposes. The results on the incubated cofactor free blanks, as well as on the complete reaction mixtures were well reproducible, indicating that differences in endogenous cofactor (or 5-HT) concentrations do not constitute a significant variable. If the fluorescent units of the homogenate alone are used to estimate the endogenous 5-HT content, a value of 5.17±1.41 (n=4) is obtained. This value is comparable to that measured in adult rat midbrain of 4.84±0.40 (n=7) (Curzon and Green, 1970). Values from which the blank has already been subtracted are presented (unless otherwise indicated) in all subsequent tables and figures. 5-HT standards added to the assay mixture were recovered 100% so that product was not lost during the incubation or various assay steps.

The cofactor 6-methyl tetrahydropteridine ($6MPH_4$) was saturating at a final assay concentration of 0.16 mM (Table IV-2) and this concentration was used in the

TABLE IV-2

COFACTOR SATURATION FOR THE TRYPTOPHAN HYDROXYLASE ASSAY

Cofactor	F.U. 0.16mM	F.U. 0.32mM
6MPH4	11.5+1.6(3)	13.6+1.7(4)
DMPH4	7.3	7.7

6MPH4= 6-methyl tetrahydropteridine
DMPH4= 6,7-dimethyl tetrahydropteridine

Standard assay conditions were used. All samples were incubated with 50 ul of a 20% rat midbrain homogenate. Results are expressed in fluorescent units (F.U.) minus blank per 30 minutes incubation and are means $\pm$ 1 S.D. (n) or single values.

standard assay. 6,7-dimethyl tetrahydropteridine ($DMPH_4$) was less active (Table IV-2) but was also saturating at this concentration. Table IV-3 shows the effect of increasing substrate concentration. At a concentration of 44 uM tryptophan, the enzyme is not saturated. Maximal activity was obtained with 180 uM tryptophan and, although higher concentrations were not inhibitory, the blank value increased. Therefore, 180 uM tryptophan was routinely used. Inhibition of tryptophan hydroxylase at high substrate concentrations is thought to occur in the presence of the natural cofactor tetrahydrobiopterin but not in the presence of $DMPH_4$ or $6MPH_4$ (Friedman et al., 1972).

The results in Table IV-4 show that the fluorescence units (F.U.) (i.e. the amount of product formed) increased linearly with the volume (ul) of supernatant added and with incubation time (up to 30 minutes). Centrifugation at 30,000xg yields a more active supernatant than centrifugation at 100,000xg. The 30,000xg supernatant was therefore routinely used.

In adult whole brain minus midbrain, enzyme activity was too low above blank to be accurately measured but it was easily determined in midbrain. This result confirms the high midbrain activity and heterogeneous distribution of the enzyme (Brownstein et al., 1975). In subsequent studies, enzyme activity was only measured in midbrain.

TABLE IV-3

SUBSTRATE SATURATION FOR THE TRYPTOPHAN HYDROXYLASE ASSAY

Substrate:	F.U. 0	F.U. 44uM	F.U. 88uM	F.U. 180uM	F.U. 352uM
Homogenate 50ul	3.0±0.8(7)	2.0,6.2	13.6±3.8(4)	16.2	15

Standard assay conditions were used except for dilutions of the standard substrate concentraton (176uM). Samples were incubated with 50 or 100 ul of a 20% rat midbrain homogenate. Results are expressed in fluorescent units minus blank per 30 minutes incubation and are means ± 1 S.D.(n) or single values.

TABLE IV-4
INCREASE IN TRYPTOPHAN HYDROXYLASE ACTIVITY WITH TIME AND ENZYME CONCENTRATION

enzyme homogenate(ul)	incubation time	F.U. 100,000g fraction	F.U. 30,000g fraction
50	30	9.1±3.3(5)	
75	30	15.3±4.0(3)	
100	30	23.0±3.9(4)	47.3±3.3(10)
50	10		9.9±2.3(4)
50	20		15.5±1.3(4)
50	30		28.6±1.8(10)

Assay conditions were standard except for the midbrain supernatant which was a 100,000g fraction or a 30,000g fraction. Enzyme activity is expressed in fluorescent units-blank (F.U.). Values are single points or means ±1 S.D.(n).

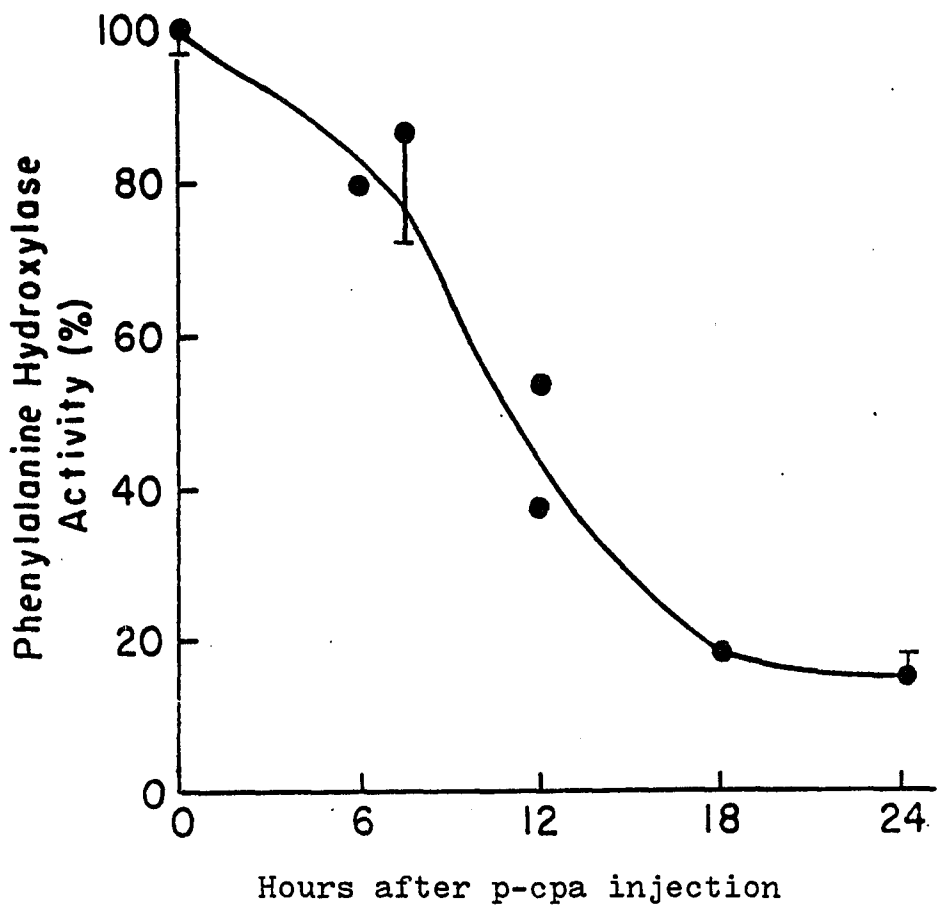
2. Interference of P-chlorophenylalanine with Aromatic Amino Acid Hydroxylases

P-chlorophenylalanine (p-cpa) was the first synthetic substance used to produce animal models of PKU. It is not an effective inhibitor of phenylalanine hydroxylase in vitro, (even at a concentration of 8mM, Lipton et al., 1967) but in animals given an injection of p-cpa the activity of this hepatic enzyme is greatly reduced (Koe and Weissman 1966, Lipton et al., 1967). This reduction is irreversible, in that new protein synthesis is required for recovery of phenylalanine hydroxylase activity in the course of 2-4 days. The loss of activity was then also found to be a slow process (Greengard and DelValle, 1976). As shown in Figure IV-1, a 20% loss is seen 7 hours after injection and a minimal level is attained as late as 24 hours. Such an effect on phenylalanine hydroxylase or on other enzymes will be referred to as "suppression" (see also General Discussion), while "inhibition" will denote effects which are immediately apparent and rapidly reversible, and which p-cpa (or other agents) can also exert in vitro.

The slow loss of tryptophan hydroxylase activity following an injection of p-cpa was first described in 1966 (Koe and Weissman). The approximate 90% decrease seen at 24 hours, and still apparent at 48 hours, is irreversible in the sense that removal of p-cpa by dialysis does not restore the tryptophan hydroxylase acitivity. However, the

FIGURE IV-1

TIME COURSE OF SUPPRESSION OF HEPATIC PHENYLALANINE HYDROXYLASE ACTIVITY BY P-CHLORORPHENYLALANINE



Rats (6 days old) were injected with 18 umoles of p-chlorophenylalanine/10 g body weight. The phenylalanine hydroxylae activities (units/g of liver), assayed at the indicated times thereafter, are given as a percentage of that found in the untreated controls (i.e. 562 units/g). The points refer to individual animals or to means of results with three or four animals, the vertical bar representing 1 S.D.

(From Greengard and Delvalle, 1976)

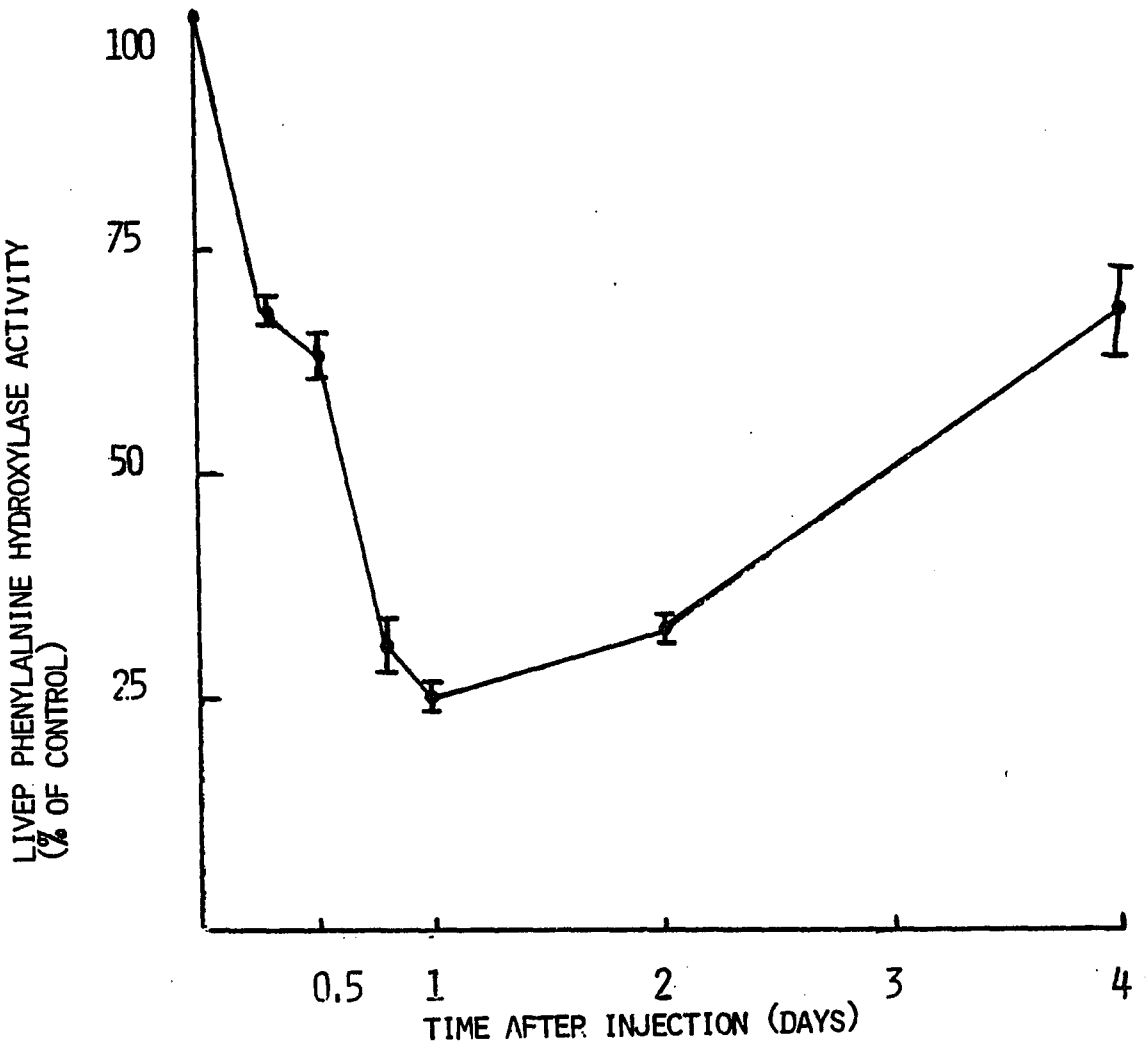
restore the tryptophan hydroxylase acitivity. However, the rapid loss of activity caused by addition of p-cpa in vitro (which is a competitive inhibition with an apparent Ki of 0.3mM) is reversible by dialysis (Jequier et al., 1967; Gal et al., 1970). Thus p-cpa, which is a suppressor of phenylalanine hydroxylase as well as of tryptophan hydroxylase, is also an inhibitor of the latter enzyme. Tyrosine hydroxylase, on the other hand, is not suppressed by p-cpa in vivo but is inhibited by this substance in vitro (Gal and Whitacre, 1982).

3. Interference of A-methylphenylalanine with Aromatic Amino Acid Hydroxylases

A-methylphenylalanine (a-me) is a weak competitive inhibitor of hepatic phenylalanine hydroxylase in vitro (apparent Ki 10mM), but is a very effective suppressor in vivo (DelValle et al, 1978). As shown in Fig IV-2, minimal activity is attained 20 hours after an injection and recovery takes at least 2 days. A-me is also a weak competitive inhibitor of brain tryptophan hydroxylase (Figure IV-3) and of tyrosine hydroxylase (Figure IV-4; see also Brass, 1983) with Ki's estimated at 1.2 mM and 1.46 mM respectively from a Lineweaver-Burk plot. The Ki for inhibition of tryptophan hydroxylase by a-me is also estimated at 1.1 mM from a Dixon plot. Similarly to p-cpa, a-me does not cause suppression of tyrosine hydroxylase. After a 3 day treatment with the standard dose, tyrosine

FIGURE IV-2

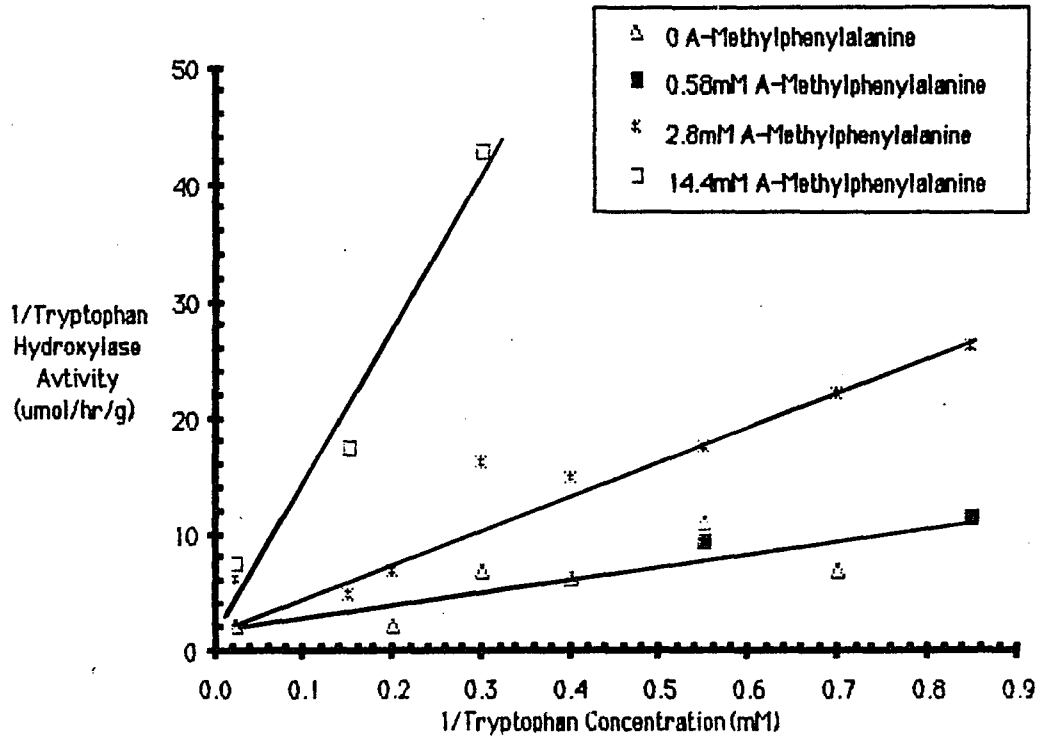
TIME COURSE OF SUPPRESSION OF HEPATIC PHENYLALANINE HYDROXYLASE ACTIVITY BY A-METHYLPHENYLALANINE



Assays were performed at the indicated times after the injection of a-methylphenylalanine (24 umoles/10 g body weight) to 6 day old rats. Points represent means of results with three or four animals (bar = ± S.D.).

FIGURE IV-3

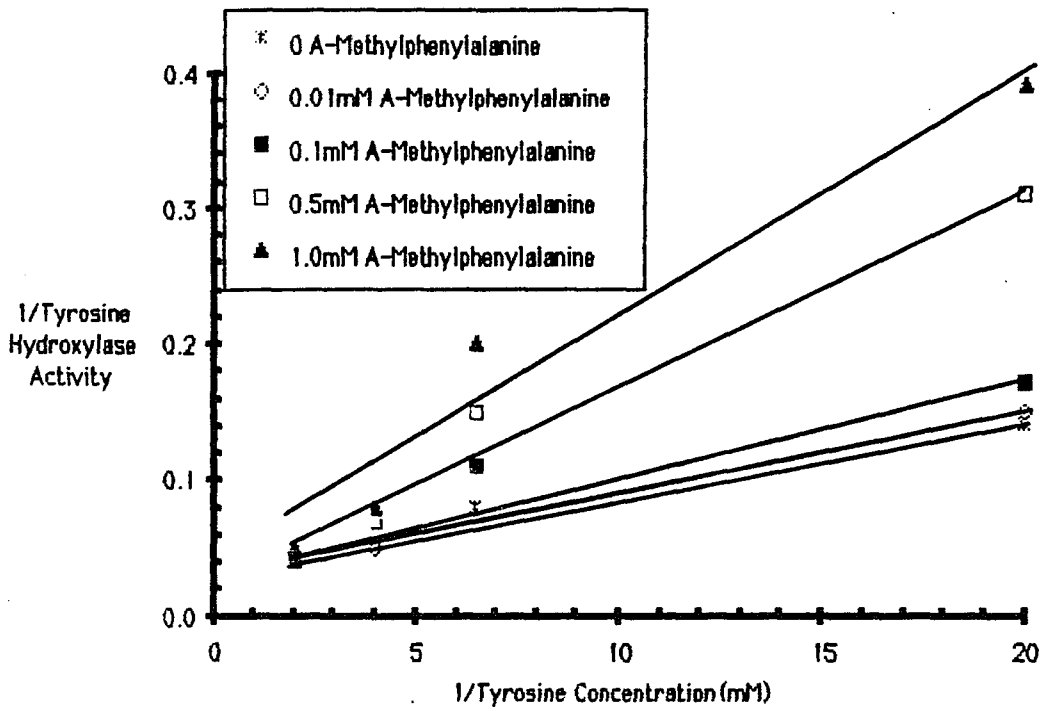
DOUBLE RECIPROCAL PLOT OF TRYPTOPHAN CONCENTRATION AGAINST THE RATE OF TRYPTOPHAN HYDROXYLATION WITH VARIOUS LEVELS OF A-METHYLPHENYLALANINE



The standard incubation mixture but various concentrations of tryptophan and a-methylphenylalanine was used. Enzyme was from a pool of the midbrain of 4 adult rats. The Ki, as calculated from the curves whose slope and intercept values were significant (with $p<0.01$) was 1.2mM.

FIGURE IV-4

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

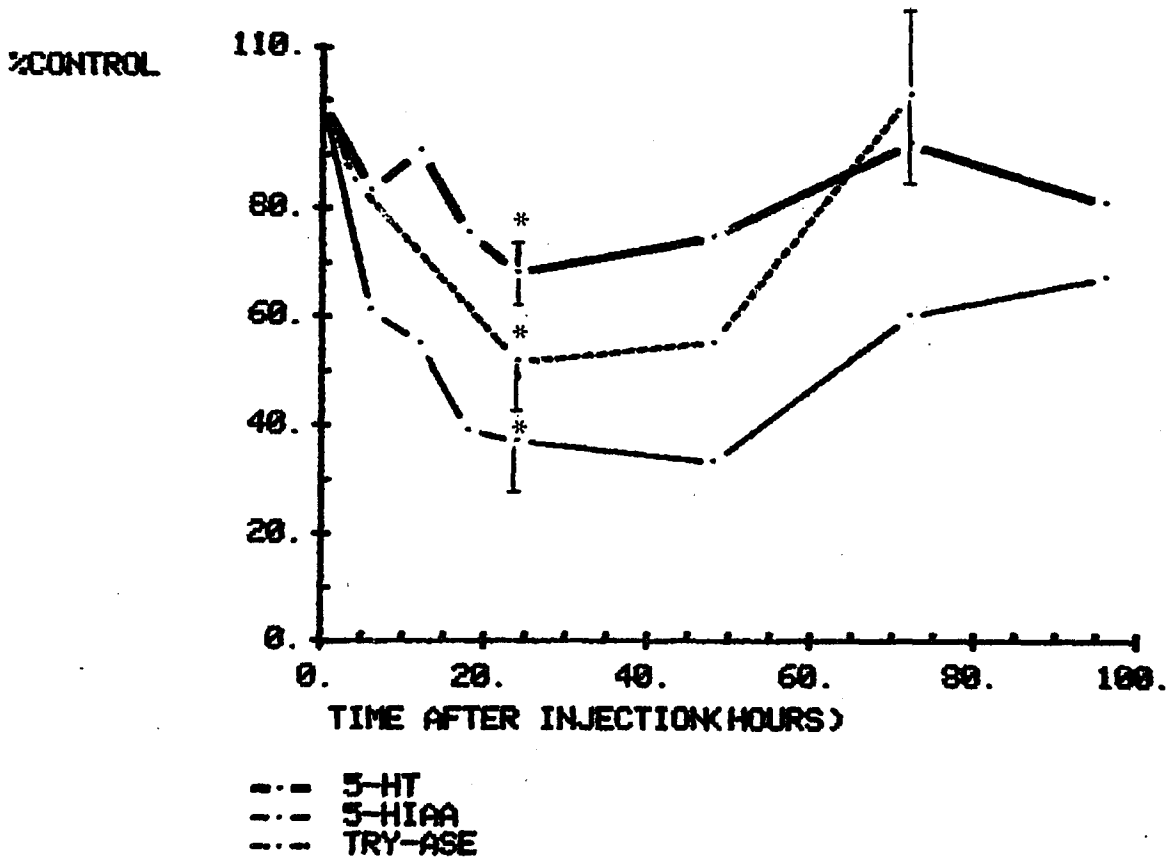


The standard incubation mixture plus various concentrations of a-methylphenylalanine was used. Enzyme was from a pool of striatum of 4 adult rats. Assays were performed in triplicate for each point and the average value used. The Ki, as calculated from the curves whose slope and intercept values were significant (with $p<0.05$), was 1.46×10^{-3}M.

From Brass (1983)

FIGURE IV-5

EFFECT OF ACUTE TREATMENT WITH A-METHYLPHENYLALANINE ON BRAIN 5-HT, 5-HIAA AND TRYPTOPHAN HYDROXYLASE



Assays were performed at the indicated time after injection of a-methylphenylalanine (2.4 umoles/g) to 7-10 day old rats. Results are means + 1 S.D. or single values.
* indicates significant difference ($p<0.01$) from control.

hydroxylase activity is identical to control (see Chapter VI, Table VI-1). As shown in Figure IV-5, the suppression of tryptophan hydroxylase by the standard amount of a-me (see Methods) is a long term process that is maximal at 50% after 24 hours ($P<0.01$). The enzyme activity recovers after 3 days.

The in vitro inhibition of tryptophan hydroxylase by a-me is of particular interest in that suppression of the enzyme might be an artifact due to the presence of a-me in the assay system of tissues from experimental animals. To rule out this possibility, this amount was estimated and tested for its effects on enzyme activity. After injection of 2.4 umol/g body weight, it was assumed that equal distribution in all tissues leads to a brain concentration of 2.4 mM. If the various dilutions are taken into account, the final concentration of a-me in the tryptophan hydroxylase assay is 0.024 mM. As shown in Figure IV-3, a much higher, 0.58 mM, a-me concentration does not cause in vitro inhibition of the enzyme even at lower tryptophan concentrations (e.g.0.06 mM) than used in the standard assay (0.176 mM). Therefore, a-me present in the assay system does not account for suppression of the enzyme.

4. Depletion of 5-HT and 5-HIAA Content after Acute Injection of A-methylphenylalanine or P-chlorophenylalanine

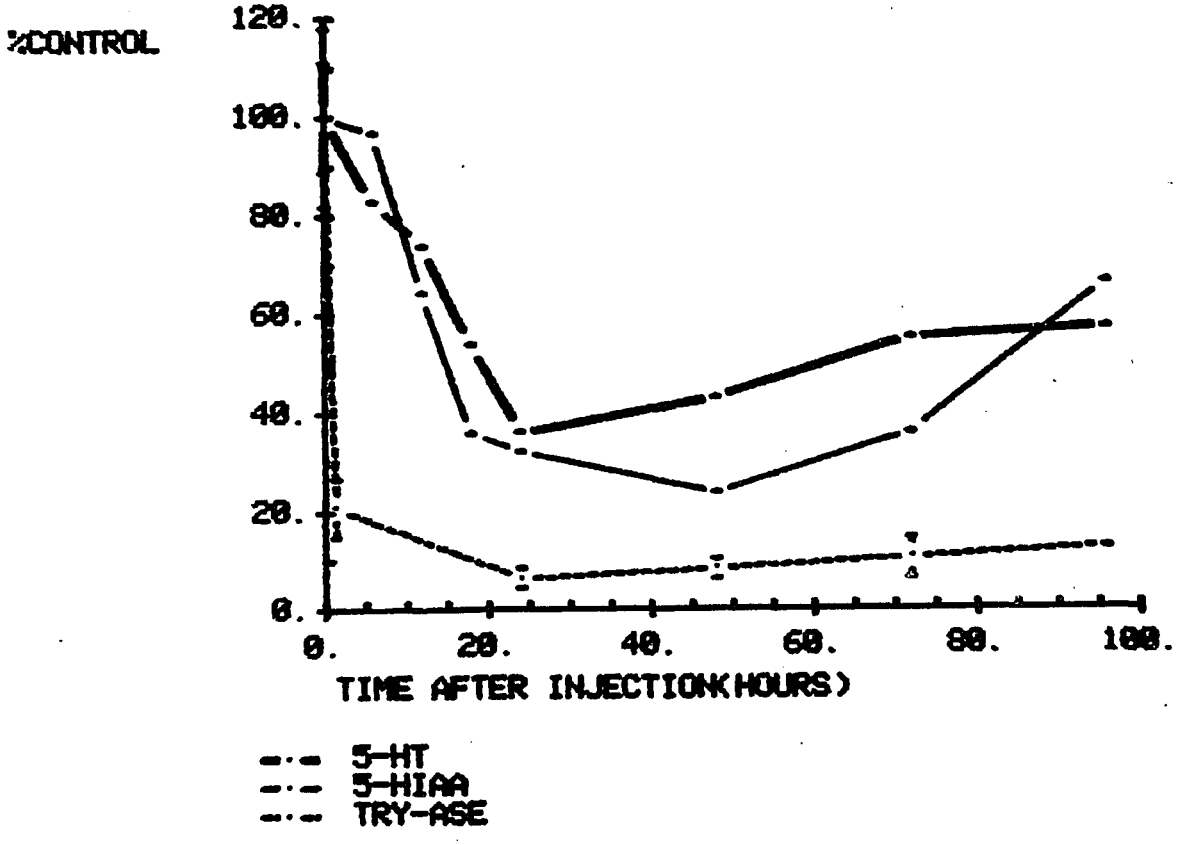
5-HT and 5-HIAA depletion after a-me treatment follows a similar time course with minimal levels of 5-HT and 5-HIAA after 24 hours (Figure IV-5). The recovery of 5-HIAA proceeds more slowly than 5-HT or tryptophan hydroxylase, with a 30 to 40% inhibition still present at three days. The acute effect of p-cpa on 5-HT and 5-HIAA is shown in Figure IV-6. The inhibition of 5-HT is pronounced after 24 hours at 64% and is still present at 45% after 4 days. A-me and p-cpa also suppress tryptophan hydroxylase with the same time course (Figures IV-5 and IV-6) although p-cpa is a more potent suppressor. Increasing the dose of a-me does not cause a greater decrease in 5-HT after 24 hours (Table IV-5), indicating that the inhibition is maximal at the dose used.

5. Discussion

The natural cofactor in tryptophan hydroxylation is thought to be tetrahydrobiopterin (BH_4) (Gal, 1981). The Km values for enzyme activity with BH_4 and $6MPH_4$ were reported to be lower than that for $DMPH_4$ in rabbit hindbrain (Friedman et al., 1972). Other investigators have found higher Km values with $6MPH_4$ than $DMPH_4$ in rat midbrain (Kuhn et al., 1978, 1979). The Km of tryptophan

FIGURE IV-6

EFFECT OF ACUTE TREATMENT WITH P-CPA ON BRAIN 5-HT, 5-HIAA AND TRYPTOPHAN HYDROXYLASE



Assays were performed at the indicated time after injection of p-chlorophenylalanine (0.9umoles/g) to 7-8 day old rats. The tryptophan hydroxylase results are obtained from Jequier et al. (1967). Results are means $\pm$ 1 S.D. or single values. The Spearman rank correlation coefficient r_s between 5-HT and 5-HIAA levels = 0.89($P<0.01$).

TABLE IV-5

EFFECT OF INCREASED A-ME DOSE ON MIDBRAIN 5-HT AND 5-HIAA LEVELS

	5-HT	5-HIAA
Control	4.50±0.27(7)	1.83±0.18(6)
A-ME 2.4 umol/g	3.84±0.36(7)	1.25±0.38(7)*
A-ME 4.8 umol/g	4.06±0.40(3)	0.98±0.18(3)*

Animals were injected once with 2.4 or 4.8 umoles/g body weight of a-methylphenylalanine at 7-8 days of age. Controls were injected with saline. 24 hours after the injection animals were sacrificed and whole brain 5-HT and 5-HIAA measured as described in the Methods section. All values were expressed in nmoles/g brain weight and are means ±1 S.D.(n). * indicates a significant difference ($P<0.01$) from control.

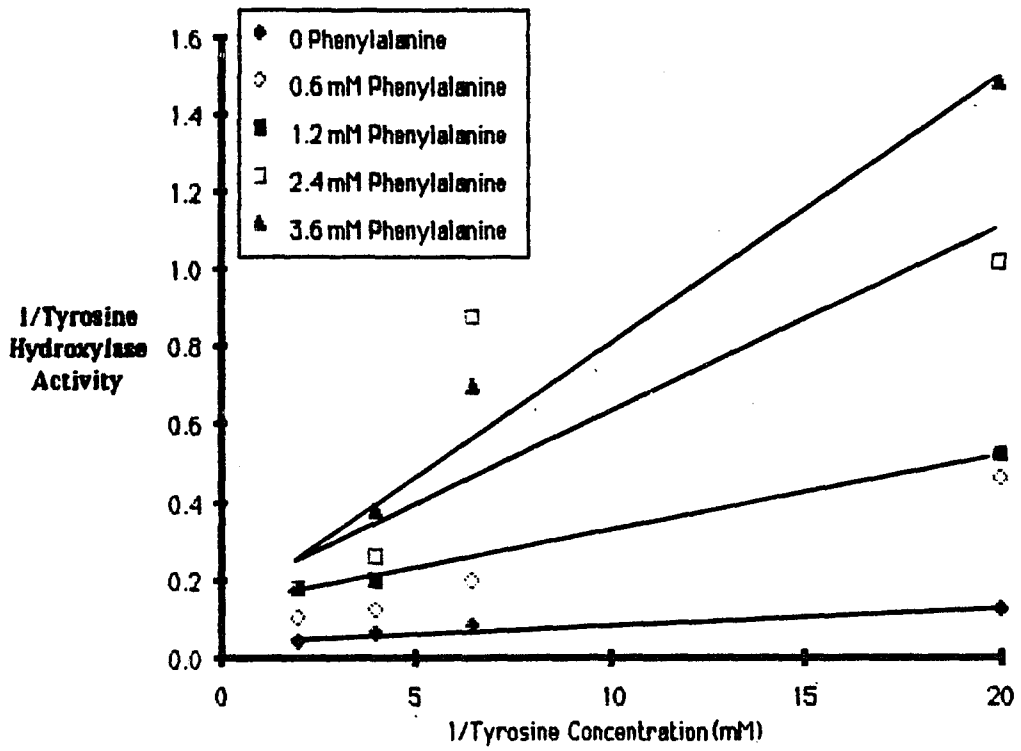
hydroxylase for its substrate also depends on the cofactor used. In rabbit hindbrain, the Km of tryptophan for tryptophan hydroxylase with BH_4 is similar to that with 6MPH_4 whereas the Km for DMPH_4 is lower. In fact, values within a 100 fold range have been reported depending on the species studied and cofactor used (Friedman et al., 1972; Youdim et al., 1975).

In the present study of the inhibition of tryptophan hydroxylase, the fluorimetric assay described by Gal and Patterson (1973) was used. A modification in substrate concentration was made to obtain a saturating concentration of tryptophan (0.180 mM). The substrate contributes to the product fluorescence so that a blank without cofactor was routinely included in all experiments. Numerous previous studies of the relationship between increased brain tryptophan levels and 5-HT content determined by a fluorimetric method have led to ambiguous results because they have not taken into account the fluorescent property of tryptophan. The synthetic cofactor, 6MPH_4, was found to be more active than DMPH_4 in the assay. The higher rate of tryptophan hydoxylation with 6MPH_4 compared to DMPH_4 in this assay might be due to a lower Km for substrate in the presence of 6MPH_4.

Phenylalanine is a competitive inhibitor in vitro of brain tryptophan and tyrosine hydroxylase with Ki's estimated at 0.3 mM (Renson et al., 1962) and 0.22 mM (Figure IV-7; see also Brass, 1983) respectively. A-me and

FIGURE IV-7

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



The standard incubation mixture plus various concentrations of phenylalanine were used. Enzyme was from a pool of the striatum of several adult rats. Assays were performed for each point and the average value used. The Ki, as calculated from the curves whose slope and intercept were significant (with $P<0.05$), was 2.19×10^{-4} M.

From Brass (1983)

p-cpa are also weak competitive inhibitors with Ki values in the millimolar range (Table IV-6). However, their effect on 5-HT in vivo is attributable to suppression of the cerebral tryptophan hydroxylase. The decrease and eventual restoration of 5-HT and 5-HIAA levels followed the same time course as did changes in tryptophan hydroxylase activity measured at optimal susbstrate concentrations in the midbrain of animals sacrificed at various times after a-me or p-cpa injection. A further resemblance between these two analogues is that they are equally effective suppressors of the hepatic phenylalanine hydroxylase but neither a-me or p-cpa cause suppression of the cerebral tyrosine hydroxylase in vivo (Gal and Whitacre, 1982; Chapter VI, Table VI-1).

The mechanism of suppression is intriguing in that it occurs in the living animal only. It has been proposed that a metabolite of p-cpa is responsible for the inhibition (Jequier et al., 1967), however no such metabolite could be found. The action of a chloro-containing metabolite of p-cpa is certainly unlikely since the chlorine atom of p-cpa disappears rapidly. It has also been postulated that p-cpa may cause a decrease in the synthesis of phenylalanine hydroxylase by interfering with protein synthesis or be incorporated into the enzyme and thus change its properties (Gal et al., 1970). However, Chang et al., (1979), found that p-cpa had no effect on the incorporation of labeled leucine or arginine

TABLE IV-6

PHENYLALANINE ANALOGUES AND KINETIC PARAMETERS (Ki) OF THE PTERIDINE-DEPENDENT HYDROXYLASES

	A-ME	P-CPA	PHE	Reference
phenylalanine hydroxylase	10mM			Delvalle et al, 1978
tryptophan hydroxylase	1.2mM			Figure IV-3
tryptophan hydroxylase		0.3mM		Jequier et al., 1967; Gal et al., 1970
tryptophan hydroxylase			0.3mM	Renson et al., 1962
tyrosine hydroxylase	1.5mM			Figure IV-4
tyrosine hydroxylase			0.2mM	Figure IV-7

into phenylalanine hydroxylase nor was there a change in the half life of p-cpa treated enzyme. Furthermore, the immunologic properties of the treated enzyme were not altered. In a study by Gal and Whitacre (1982), similar observations with tryptophan hydroxylase were made. Gal and Whitacre proposed that p-cpa forms a complex with tRNA which is then hydroxylated to a tyrosine-tRNA complex. They argue that if tyrosine then replaces phenylalanine in protein synthesis, interference with the active site of tryptophan hydroxylase will occur. However, it has not been demonstrated that phenylalanine is a critical component of the active site. A recent, plausible suggestion is that p-cpa combines with a precursor of phenylalanine hydroxylase, thus slowing down the synthesis of the enzyme (Miller et al., 1976). A-me, especially since it is not yet available in radiolabelled form has not been used in the above studies. It is almost certain, however, that its action on phenylalanine hydroxylase and tryptophan hydroxylase is analogous to that of p-cpa. Further investigation of this mechanism of suppression might clarify critical components at the active site of the hydroxylases and provide increased insight into the regulation of the synthesis of these pteridine-dependent enzymes.

The importance of establishing competitive inhibition and suppression of the pteridine-dependent hydroxylases by p-cpa and a-me is directly related to the study of

experimental PKU. As synthetic agents, they may have actions other than the specific one desired, namely suppression of phenylalanine hydroxylase. However, the assessment of side effects requires the study of animals receiving these agents plus phenylalanine (see Chapter VI).

CHAPTER V

THE EFFECT OF EXPERIMENTAL PKU ON BRAIN AND PLASMA TRYPTOPHAN CONTENT

The concept that amino acids enter cells via transport systems selective for groups of amino acids led to the characterization of the L system that is specific for large neutral amino acids (Christensen, 1969). The fact that they include precursors of neuroactive amines, as well as essential amino acids mandatory for protein synthesis lends particular importance to the system. Extensive studies have therefore been carried out to compare the affinity of the different amino acids to their carrier. A technique, introduced by Oldendorf (1970), involves the rapid injection into the carotid artery of the ^{14}C labelled amino acid (along with [^{3}H] water as a control) and measurement of uptake 15 seconds later (i.e. after a single passage through the brain). Pardridge (1977), using this technique determined the Km for each amino acid of the L transport system. He also showed that the albumin bound as well as the free tryptophan competes for transport, so that the sum of the two concentrations determines the rate of entry. The inhibition of tryptophan uptake by plasma phenylalanine concentrations comparable to those in PKU led investigators to propose that the depletion of cerebral

5-HT in this disease might be due to the diminished tryptophan content of the brain (Daniel et al., 1976). However, as described in the next section (and also in the previous Chapter), other factors can contribute to the 5-HT depletion in hyperphenylalaninemic animals.

1. Brain and Plasma Tryptophan Content in PKU Models

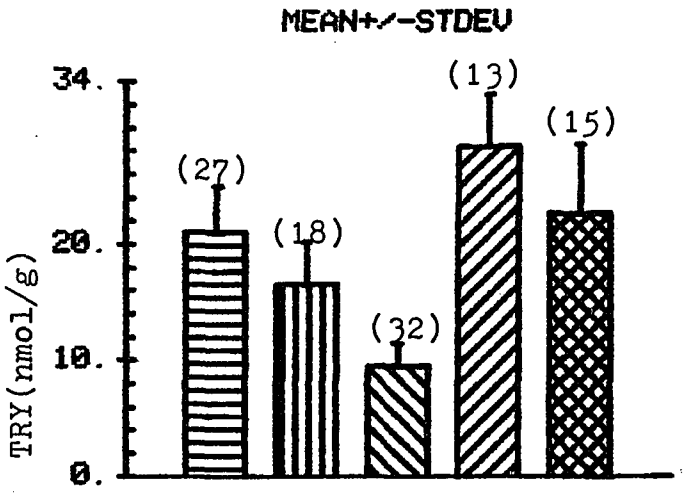
A. Changes in Brain Tryptophan

In the following experiments, the decrease in brain tryptophan in our experimental model was examined and compared with that in animals treated with a-me alone. Experimental PKU was also induced by p-cpa plus phenylalanine (phe) and the effects of this treatment as well as p-cpa alone on brain tryptophan were studied. The decrease in brain tryptophan levels by a-me alone (Figure V-1) is small and of borderline significance ($P<0.05$). If a-me plus phe is given, the decrease is much greater at 54 % ($P<0.01$). The effects of p-cpa and p-cpa plus phe on tryptophan levels are more complex. The results presented in Figure V-1 are for animals that were treated for 3 to 4 days and show that with p-cpa plus phe, tryptophan levels are the same as control whereas p-cpa alone causes a significant increase in tryptophan levels ($P<0.01$) compared to control.

The effects of leucine, an amino acid that utilizes the L-transport system, are shown in Table V-1, and

FIGURE V-1

COMPARISON OF THE DEPLETING EFFECTS OF A-METHYLPHENYLALANINE, A-METHYLPHENYLALANINE PLUS PHENYLALANINE, P-CHLOROPHENYLALANINE AND P-CHLOROPHENYLALANINE PLUS PHENYLALANINE ON BRAIN TRYPTOPHAN



SALINE	
A-ME	versus saline NS, versus a-me+phe $p<0.01$
A-ME+PHE	versus saline $p<0.01$
P-CPA	versus saline $p<0.01$, versus p-cpa+phe $p<0.01$
P-CPA+PHE	versus saline NS

As described in the Methods section, rats aged 9 to 12 days were injected daily for at least three days with a-me (2.4 umoles/g body w.), a-me (2.4 umoles/g) plus phe (5.2 umoles/g), p-cpa (0.9 umoles/g), or p-cpa (0.9 umoles/g) plus phe (5.2 umoles/g). Animals were sacrificed 4 to 6 hours after their last injection. Brain tryptophan is expressed in nmoles/g tissue and values are means ± 1 S.D.(n). NS indicates no statistically significant difference.

TABLE V-1

DECREASE IN MIDBRAIN 5-HT AND 5-HIAA AND IN WHOLE BRAIN TRYPTOPHAN BY PHENYLALANINE AND LEUCINE

	Midbrain 5-HT (nmol/g)	Midbrain 5-HIAA (nmol/g)	Whole Brain Try (nmol/g)
Control	4.7±0.6(7)	1.84±0.51(7)	22 ±7(25)
Phenylalanine	2.0±0.4(3)*	0.61±0.13(3)*	11 ±3(3)*
Leucine	2.7±0.4(3)*	1.18±0.08(3)*	14 ±4(3)

10 day old rats were injected twice with 0.2 ml/10g body weight phenylalanine (5.2 umol/g) or with leucine (5.2 umol/g) at 4 hours after the first injection. Controls were injected with the same volume of saline. All results are expressed in nmol/g wet weight of brain and are means ±1 S.D.(n). * indicates a significant difference ($P<0.01$) from control.

compared with phe. Both amino acids lower 5-HT, 5-HIAA, and brain tryptophan although phe is a somewhat more potent depletor than leucine. Phe lowers 5-HT by 58% and tryptophan by 52% whereas leucine lowers 5-HT by 43% and tryptophan by 36%. The action of phe is therefore not specific as other large neutral amino acids have depleting effects on brain 5-HT and tryptophan content.

B. Decrease in Plasma Tryptophan

The premise that brain tryptophan levels are lowered by competitive inhibition with phe for transport is mainly based on the numerous studies of the transport system. These studies have shown that the levels of circulating phe in experimental PKU are sufficiently raised to inhibit entry of tryptophan into the brain (Oldendorf, 1973; Daniel et al., 1976). However, it is assumed in these studies, that while one amino acid level in the plasma is abnormally high, the others remain at normal levels. In our experimental model, as shown in Table V-2, we find that plasma tryptophan levels are decreased 27% by a-me or p-cpa, and 35% by a-me plus phe or by p-cpa plus phe ($P<0.01$). These decreases in plasma tryptophan may contribute to the decrease in brain tryptophan in the a-me plus phe treated rats. However, in the p-cpa plus phe treated rats, brain tryptophan levels were identical to control.

TABLE V-2

EFFECT OF CHRONIC HYPERPHENYLALANINEMIA ON PLASMA AND BRAIN TRYPTOPHAN LEVELS

	PLASMA	BRAIN	
			24 hours
CONTROL	110±26(26)	23.0±6.4(27)	
A-ME	80±10(8)	17.5±4.7(18)	
A-ME+PHE	71±17(28)*	10.6±3.5(32)*	13.0±3.3(6)
P-CPA	81±19(13)	28.4±4.5(13)*	
P-CPA+PHE	73±25(17)*	21±7(15)	

Rats aged 9 to 12 days were injected daily for at least 3 days with a-methylphenylalanine (A-ME) (2.4 umoles/g body wt.), A-ME (2.4 umoles/g) plus phenylalanine (PHE) (5.2 umoles/g), p-chlorophenylalanine (P-CPA) (0.9 umoles/g), or P-CPA (0.9 umoles/g) plus PHE (5.2 umoles/g). Animals were sacrificed 4-6 hours or, in one case, 24 hours after the last injection. Brain tryptophan is expressed in nmol/g tissue and plasma tryptophan in nmol/ml plasma. Values are means ± 1 S.D.(n). * indicates a significant difference ($P<0.01$) from control.

C. Reversibility by Tryptophan of the Cerebral 5-HT Deficit

Since treatment with a-me plus phe did not cause long term inhibition of tryptophan hydroxylase, it seemed likely that the decrease in 5-HT caused by this treatment could be reversed by tryptophan injections. As revealed by studies of tryptophan hydroxylase (Chapter IV), tryptophan is fluorescent under the conditions used for the 5-HT assay. As shown in Table V-3, 0.1 mM tryptophan is sufficient to produce a high blank. The appropriate blank was therefore subtracted for 5-HT determinations. The approximate 0.07 mM tryptophan present in the assay system of brain from animals injected with tryptophan did not influence the measurement of 5-HIAA. A high blank in this assay is produced only by tryptophan concentrations above 0.4 mM.

Animals were given the standard treatment of a-me plus phe for 2 days. On the third day this was combined with 3 injections of tryptophan (300 mg/kg), which resulted in considerable increases in plasma and brain tryptophan levels (Table V-4). Brain 5-HT concentration was restored to control levels whereas brain 5-HIAA concentration was increased 4 fold, suggesting that there was an increased 5-HT turnover. In order to avoid the use of multiple tryptophan injections to reverse the 5-hydroxyindole decrease, a slow release of tryptophan by binding to the inert polymer carboxymethylcellulose (CMC)

TABLE V-3

FLUORESCENT MEASUREMENT OF TRYPTOPHAN IN THE 5-HT ASSAY

Tryptophan (mM)	5-HT assay (F.U.)
0	35 ± 1.7(3)
$3.4x10^{-4}$	37
$3.4x10^{-3}$	37
$3.4x10^{-2}$	38
0.10	60
0.20	63
0.34	48
0.40	69
4.00	58

A standard amount of 5-HT (60ng) was measured in the presence of the indicated amounts of tryptophan. F.U. denotes fluorescent units. The tryptophan concentration in the assay after in vivo injection is estimated at 0.2 mM. Values are means ±1 S.D. (n) or single values.

TABLE V-4
REVERSAL OF 5-HT AND 5-HIAA DECREASE WITH TRYPTOPHAN INJECTIONS AFTER A-METHYLPHENYLALANINE PLUS PHENYLALANINE TREATMENT

	5-HT (nmol/g)	5-HIAA (nmol/g)	Plasma Try (nmol/ml)	Brain Try (nmol/g)
Control	1.61±0.09(5)	0.90±0.07(4)	110±21(8)	19.8±2.8(8)
A-ME+PHE	0.97±0.13(3)	0.19±0.06(3)	72±11(8)	9.9±1.7(8)
A-ME+PHE+TRY	1.47±0.11(4)	3.45±0.17(4)	2604±463(4)	935±132(4)

Rats were treated for 3 days with a-me plus phe and were sacrificed 6 hours after the last injection at 11 days of age. Tryptophan (300mg/kg) was injected 1,3, and 5 hours after the a-me plus phe injection on day 11. Values are means ± 1 S.D.(n).

was investigated. This attempt proved to be unsuccessful since even at optimal conditions (50 mM tryptophan and pH 4.4 to 6) too little tryptophan was adsorbed to be useful for in vivo administration

2. Discussion

Characterization of the large neutral amino acid or L transport system resulted in the proposal that saturation of the blood brain barrier system by phe and the resulting amino acid deprivation could be responsible for abnormal brain development (Udenfriend, 1961). It was suggested that this deprivation might perturb cerebral protein (McKean et al., 1968) or neurotransmitter synthesis (Daniel et al., 1976). Competition by phe for the L transport system was thought to cause decreases in brain large neutral amino acids in human PKU and in experimental models (McKean, 1972; McKean et al., 1968).

The elevations in brain phe in our experimental model are approximately 2 to 3 times what is required to halve the rate of entry of tryptophan into the rat brain (Daniel et al., 1976). This estimate is based on maintaining a steady influx of phe into the rat bloodstream. This constancy does not quite apply to the a-me plus phe treatment. Since a-me only suppresses phenylalanine hydroxylase by 10-20%, each daily injection is followed by a rise and eventual decline in plasma phe levels. This may be the reason for a limited decrease (54%) in cerebral

tryptophan 4-6 hours after the last injection of a-me plus phe and for the fact that this decrease was less at 24 hours (Table V-1). With a-me alone, the decrease in brain tryptophan was not statistically significant, suggesting that this agent does not have high affinity for the transport system. The results with p-cpa treatment (Figure V-1) appear more complex since tryptophan levels are in fact raised compared to controls. Although p-cpa is a potent inhibitor of tryptophan hydroxylase, it is unlikely that tryptophan levels are raised due to the lack of conversion of tryptophan to 5-hydroxytryptophan. Only a small fraction of total brain tryptophan is metabolized via this pathway. In addition, Tagliamonte et al. (1971) found that adult rats treated with p-cpa have a lowered brain tryptophan content. P-cpa may have other effects in young rats such as the inhibition of protein synthesis which implies an underutilization of tryptophan and thus, conceivably an elevation in its tissue levels. In this case, levels of other amino acids might also be elevated but these were not measured.

Huether and his coworkers (1984) reported that treatment of immature rats with a-me plus phe (in doses somewhat higher than used in the present study) caused a greater depletion of tryptophan and other amino acids in the plasma than in the brain. They challenge, therefore, the generally accepted viewpoint that excess phe reduces tryptophan uptake into the brain by monopolizing the

L-transport system. Their theory is that stimulation of protein turnover in peripheral tissues (such as muscle and lung) is a specific effect of excess phe and results in an increased uptake of tryptophan and other amino acids into these tissues. Consequently, subnormal blood levels are the reason for the diminished tryptophan and 5-HT content of the PKU brain. The blood concentration of tryptophan which in approximate agreement with Huether et al., was now found to be 35% below normal after 3 days of a-me plus phe treatment (Table V-2), may well contribute to the brain deficit. However, the fact that these diminutions can also be evoked by leucine administration (Table V-1) argues against this theory. Interference by excess phe of transport into the brain remains the most likely, major mechanism underlying the cerebral tryptophan deficit in PKU and in its "a-me model". The absence of this deficit in the "p-cpa model" despite its severe hyperphenylalaninemia, does not invalidate this conclusion since the above mentioned positive effect of p-cpa on the brain content of tryptophan could counterbalance its inhibited transport.

Gal et al., (1978) reported that a tryptophan dose below 25 mg/kg resulted in an increase in cerebral 5-HT and 5-HIAA content and in their rate of synthesis. A higher dose of tryptophan caused a decrease in cerebral 5-hydroxyindole levels which they postulated was due to substrate inhibition of tryptophan hydroxylase. However, this decrease did not occur in the present study; on the

contrary, high tryptophan doses raised the 5-HT as well as the 5-HIAA levels and there was no reason to assume an inhibition of tryptophan hydroxylase by excess substrate. More importantly, evidence was obtained for the first time that tryptophan supplements can prevent or reverse the cerebral tryptophan deficit. The practical significance of such reversibility, i.e. the fact that one can produce hyperphenylalaninemic animals with and without a cerebral deficit in 5-HT (or in catecholamines) is outlined in the General Discussion.

CHAPTER VI

MECHANISMS OF MONOAMINE DECREASE IN EXPERIMENTAL PHENYLKETONURIA

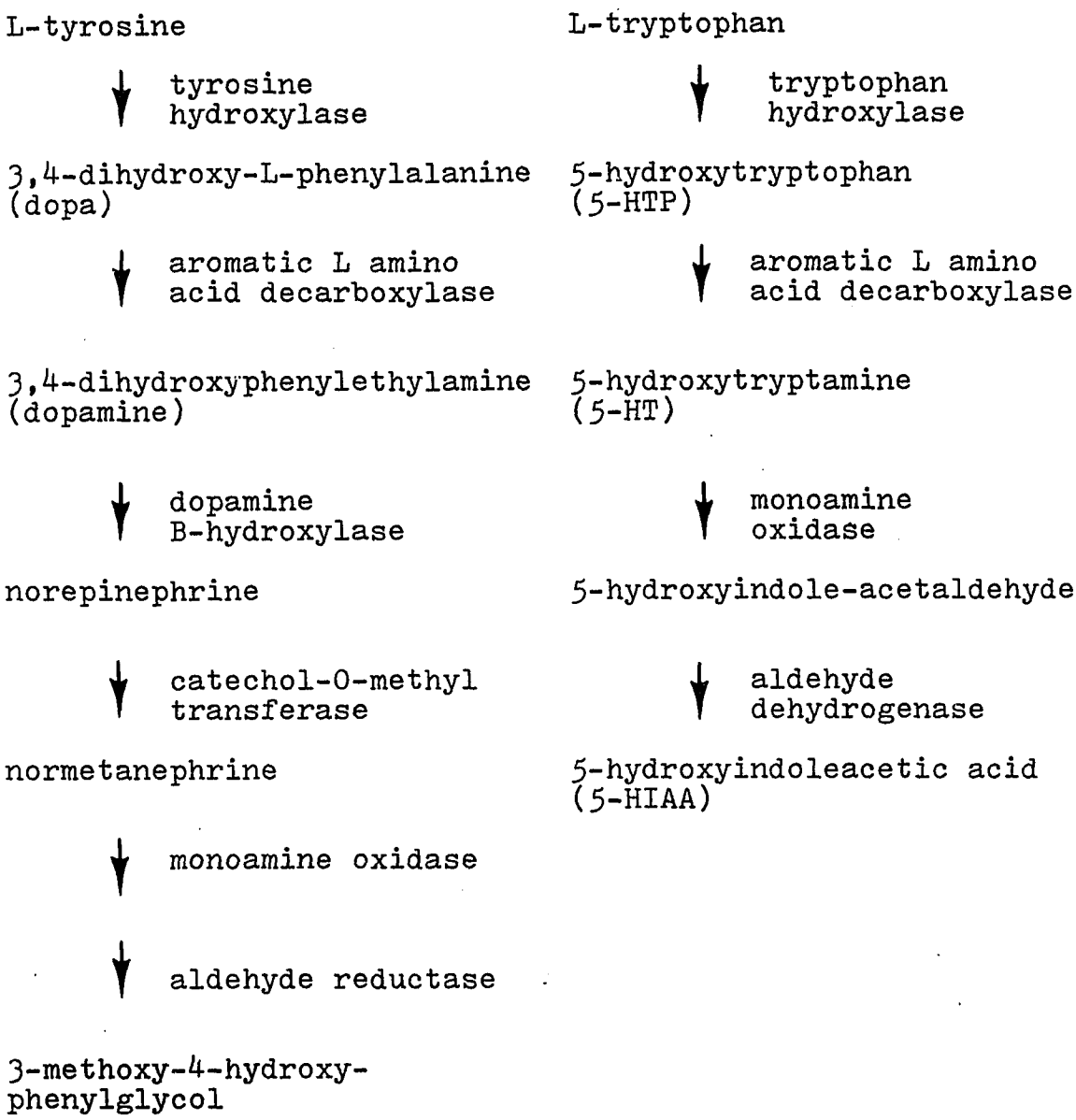
In a state of hyperphenylalaninemia, the decrease in a neurotransmitter such as 5-HT that is synthesized from its amino acid precursor, tryptophan, can be explained by three possible mechanisms.

1-The amino acid precursors are at diminished levels.
2-Phenylalanine inhibits enzymatic steps in the synthesis of the neurotransmitter.
3-Phenylalanine metabolites are responsible for the neurotransmitter deficits.

These theories can also be applied to the decrease in the catecholamine concentration of the brain in human (Mc Kean et al., 1972) and experimental PKU (Brass and Greengard, 1982). The metabolic pathways of the monoamines are briefly illustrated in Figure VI-1. On both pathways, the first reaction is dependent not only on substrate but also on two other constituents, tetrahydrobiopterin and the reductase which reconverts this cofactor to its active, reduced form. Deficiency in these constituents, and thus in both 5-HT and catechloamines, underlies the severe neurological abnormalities in some variants of PKU, but does not play a role in classical PKU or in experimental

FIGURE VI-1

DIAGRAM OF 5-HT AND CATECHOLAMINE METABOLISM



hyperphenylalaninemia (Lovenberg and Kuhn, 1982). The present study, therefore, does not extend to biopterin metabolism but focuses on some other analogies and differences between the abnormal regulation of catechloamines and 5-HT metabolism.

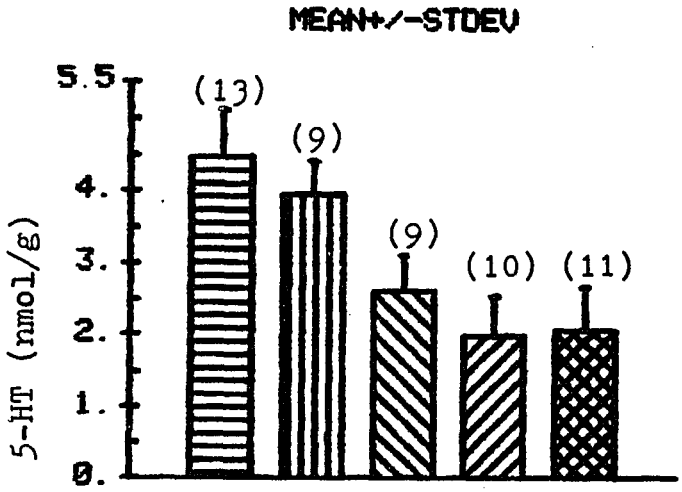
1. Mechanism of the Decrease in Monoamine Levels in Experimental PKU

A. Decrease in 5-Hydroxyindoles

Figure VI-2 shows the effect of chronic treatment for at least 3 days with a-me, a-me plus phe, p-cpa, and p-cpa plus phe on midbrain 5-HT content in 9 to 12 day old rats. All of the treatments, except for a-me alone, lower 5-HT content significantly ($P<0.01$). A-me alone lowers midbrain 5-HT content by only 12 %. The decrease in whole brain 5-HT after a single injection of a-me is also not significant at 15% (Table VI-1). For the acute study, measurements were made 24 hours after the last injection at which time this decrease is most pronounced (Chapter IV, Figure IV-5). Increasing the a-me dose by a factor of 2 does not cause a greater decrease in 5-HT (Chapter IV, Table IV-5), suggesting that the dose used produces the maximal decrease in 5-HT. The decrease in midbrain 5-HIAA at 46% is also not significantly greater with 4.8 umol/g a-me compared to 32% with 2.4 umol/g a-me (Table IV-5). Treatment with a-me plus phe differs from a-me

FIGURE VI-2

COMPARISON OF THE DEPLETING EFFECTS OF A-METHYLPHENYLALANINE, A-METHYLPHENYLALANINE PLUS PHENYLALANINE, P-CHLOROPHENYLALANINE AND P-CHLOROPHENYLALANINE PLUS PHENYLALANINE ON BRAIN SEROTONIN



	CONTROL	
	A-ME	versus control NS, versus a-me+phe p<0.01
	A-ME+PHE	versus control p<0.01
	P-CPA	versus control p<0.01, versus p-cpa+phe NS
	P-CPA+PHE	versus control p<0.01

As described in the Methods section, rats aged 9 to 12 days were injected daily for at least three days with a-me (2.4 umoles/g body w.), a-me (2.4 umoles/g) plus phe (5.2 umoles/g), p-cpa (0.9 umoles/g), or p-cpa (0.9 umoles/g) plus phe (5.2 umoles/g). Animals were sacrificed 4 to 6 hours after their last injection. Midbrain 5-HT is expressed in nmoles/g tissue and values are means +1 S.D.(n). NS indicates a non significant statistical difference.

TABLE VI-1

EFFECT OF ACUTE INJECTION OF A-METHYLPHENYLALANINE ON BRAIN 5-HT, 5-HIAA AND TYROSINE HYDROXYLASE

	5-HT (nmol/g)	5-HIAA (nmol/g)	tyrosine hydroxylase (nmol/min/g)
Control	1.58±0.13	1.18±0.10	6.27±0.61
Experimental	1.35±0.09	0.56±0.12*	5.64±0.65

Animals, aged 16 days, were injected once with a-methylphenylalanine (2.4 umoles/g body wt.). Measurements were made 24 hours after the injection. Enzyme activity was determined in the striatum. 5-HT and 5-HIAA content were determined in whole brain minus the striatum. Values represent means of three animals ±1 S.D.. Significant differences from control are denoted by *($P<.01$).

alone in that the decrease in 5-HT content is more pronounced at 42% (Figure VI-2). The decrease with p-cpa or p-cpa plus phe is slightly greater at 55% ($P<0.01$). These results show that a-me plus phe treatment causes a greater decrease in 5-HT compared to a-me alone whereas p-cpa and p-cpa plus phe cause a decrease of the same magnitude.

B. Decrease in Catecholamines

As shown by Brass and Greengard (1982), catecholamine levels in neonatal rats are significantly decreased by the standard a-me plus phe treatment. Cerebral norepinephrine is decreased by 37% ($P<0.01$) in 7 day old rats, 5 to 7 hours after the last injection of a-me plus phe and cerebral dopamine is decreased by 27% ($P<0.01$). Comparison between a-me and a-me plus phe treatment reveals that a-me alone causes a greater decrease in dopamine content whether given chronically or acutely. The more potent effect of a-me compared to a-me plus phe with respect to dopamine levels contrasts with the effect of these agents on 5-HT levels as described in the previous section. A-me plus phe causes a 42% decrease in 5-HT whereas a-me alone only causes a 12% decrease.

C. Effect of Chronic Treatment on Tryptophan Hydroxylase and Tyrosine Hydroxylase

Chronic inhibition of tryptophan hydroxylase is shown in Figure VI-3. Suppression of tryptophan hydroxylase by a-me is pronounced at 44%. On the other hand, if a-me plus phe are given, the suppression of tryptophan hydroxylase is no longer significant at 11%. As expected from acute studies (Chapter IV), p-cpa is a potent suppressor of tryptophan hydroxylase when given chronically. P-cpa alone causes an 81% inhibition of the enzyme and p-cpa plus phe a 61% inhibition ($P<0.01$). Therefore, the presence of phe does not significantly lessen the degree of suppression by p-cpa alone.

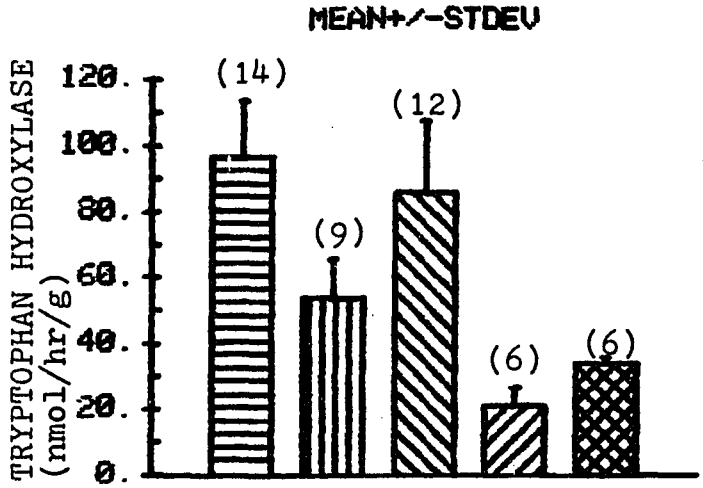
In contrast with tryptophan hydroxylase, neither a-me (Table VI-1) nor p-cpa (Gal and Whitacre, 1982) suppress tyrosine hydroxylase. With either agent, activity levels are identical to control 24 hours after injection.

D. Decrease in Tyrosine and Tryptophan

Since suppression of tyrosine hydroxylase by the standard dose of a-me after 24 hours does not occur (Table VI-1), the mechanism of the decrease in catecholamines was explored by measurement of brain tyrosine levels after a slight modification in the standard treatment. After receiving a standard dose of a-me, the animals were treated 24 hours later with phe (12.5 umol/g) or standard amounts

FIGURE VI-3

COMPARISON OF THE SUPPRESSING EFFECTS OF A-METHYLPHENYLALANINE, A-METHYLPHENYLALANINE PLUS PHENYLALANINE, P-CHLOROPHENYLALANINE AND P-CHLOROPHENYLALANINE PLUS PHENYLALANINE ON BRAIN TRYPTOPHAN HYDROXYLASE



CONTROL
A-ME versus control p<0.01, versus a-me+phe p<0.01
A-ME+PHE versus control NS
P-CPA versus control p<0.01, versus p-cpa+phe NS
P-CPA+PHE versus control p<0.01

As described in the Methods section, rats aged 9 to 12 days were injected daily for at least three days with a-me (2.4 umoles/g body w.), a-me (2.4 umoles/g) plus phe (5.2 umoles/g), p-cpa (0.9 umoles/g), or p-cpa (0.9 umoles/g) plus phe (5.2 umoles/g). Animals were sacrificed 4 to 6 hours after their last injection. Midbrain tryptophan hydroxylase is expressed in nmoles/hr/g tissue and values are means ± 1 S.D.(n). NS indicates a non significant statistical difference.

of a-me or a-me plus phe (Table VI-2). At the time of sacrifice, 5 to 7 hours after the last injection, phenylalanine hydroxylase activity is still maximally inhibited. This protocol offers the advantage of looking at the effects of phe alone in comparison to a-me and a-me plus phe without phenylalanine hydroxylase converting phe to tyrosine. From the tyrosine values obtained by these different treatments, it is clear that a decrease in tyrosine is not responsible for the decrease in dopamine (Table VI-2). Tyrosine levels are in fact increased by phe and a-me plus phe treatment and slightly above control after a-me alone. Dopamine levels, on the other hand, are significantly decreased by all of these treatments.

As described in Chapter V (Figure V-1), a-me plus phe but not a-me alone treatment significantly lowers the brain tryptophan content so that the first of the above mentioned 3 theories (the amino acid precursors are at diminished levels) would apply to the decrease in 5-HT but not in catecholamines in the "a-me model". In the "p-cpa model", tryptophan levels are raised by the treatment.

TABLE VI-2

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

Treatment	Phenylalanine (nmoles/g)	Tyrosine (nmoles/g)	Dopamine (% control)
Saline	80±17(5)	77±13(3)	100±14(6)
Phenylalanine	3194±323(4)	94±34(3)	76±14(3)
A-methylphenylalanine	269±32(3)	82±11(3)	50±12(6)
A-methylphenylalanine plus phenylalanine	2620±420(6)	120±27(4)	73±20(6)

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

Adapted from Brass (1983)

E. Competitive Inhibition of Tyrosine Hydroxylase and Tryptophan Hydroxylase

Since a-me and phe are competitive inhibitors of tryptophan hydroxylase, it is possible that this property accounts for the decrease in 5-hydroxyindole levels. The decrease in tryptophan hydroxylase activity could be calculated, knowing the brain tryptophan content after a-me plus phe treatment and Ki values of a-me and phe for tryptophan hydroxylase. However, these values are not revealing since Ki values vary with the pteridine cofactor used and are not accurate enough to predict the percent inhibition of the enzyme that occurs in vivo. Nevertheless, in addition to a decrease in tryptophan, competitive inhibition of the enzyme may contribute to the 5-HT deficit in the "a-me model".

Although a-me does not cause long term suppression of tyrosine hydroxylase, both a-me and phe are competitive in vitro inhibitors (Chapter IV). The Ki of phe is lower than that of a-me but the greater decrease in dopamine with a-me compared to a-me plus phe could be due to residual phenylalanine hydroxylase activity that converts phe to tyrosine when phe is injected with a-me. A rise in tyrosine can competitively overcome the inhibition caused by phe and a-me as shown by the Lineweaver Burke plots (Chapter IV, Figures IV-4 and IV-7; see also Brass and Greengard, 1982).

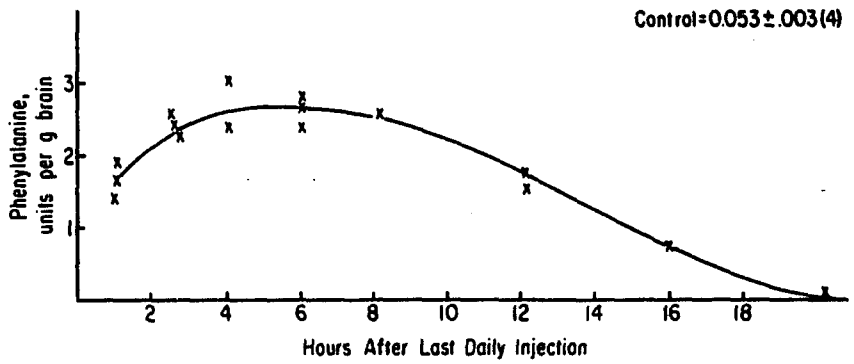
F. Timing of Measurement

As shown in Figure VI-4, brain phe is no longer raised 24 hours after the injection of the standard daily dose of a-me plus phe. Measurement at different time points, therefore, would reveal changes specifically related to the concentration in the blood which shows a sharper early peak and also declines by 24 hours. As expected from this change in blood level, the brain tryptophan content falls and rises. Consequently, and in accord with the first of the above mentioned theories which implicates precursor shortage, the decrease in 5-HT content is less pronounced 24 hours than 4-6 hours after the last injection of a-me plus phe (see lines 3 and 5, Table VI-3). For reasons discussed later, no such fluctuation is seen in animals treated with a-me alone.

On the other hand, both treatments result in a lesser cerebral dopamine deficit at 24 than at 6 hours (Figure VI-5) indicating that this deficit is directly related to the brain concentration of phe (or a-me) and that it is attributable to the fact that tyrosine hydroxylase is subject to reversible, competitive inhibition (but no suppression) by phe and a-me. For these reasons, and because the brain of these animals show no decrease but a slight increase in tyrosine content, the second rather than the first of the 3 theories (interference by phe with monoamine synthesis) is applicable to the catecholamine

FIGURE VI-4

TIME COURSE OF ELEVATION OF THE CEREBRAL PHENYLALANINE CONCENTRATION DURING CHRONIC HYPERPHENYLALANINEMIA



A-methylphenylalanine (2.5umol/g) plus phenylalanine (5.2 umol/g) was given as a daily injection (from day 3 to 8). Animals were sacrificed on day 8 and their brain phenylalanine content was measured at the indicated times after their last injection. The brain phenylalanine in untreated rats was 0.053±0.003 umoles per g brain.
1 unit = 1 umole.

TABLE VI-3

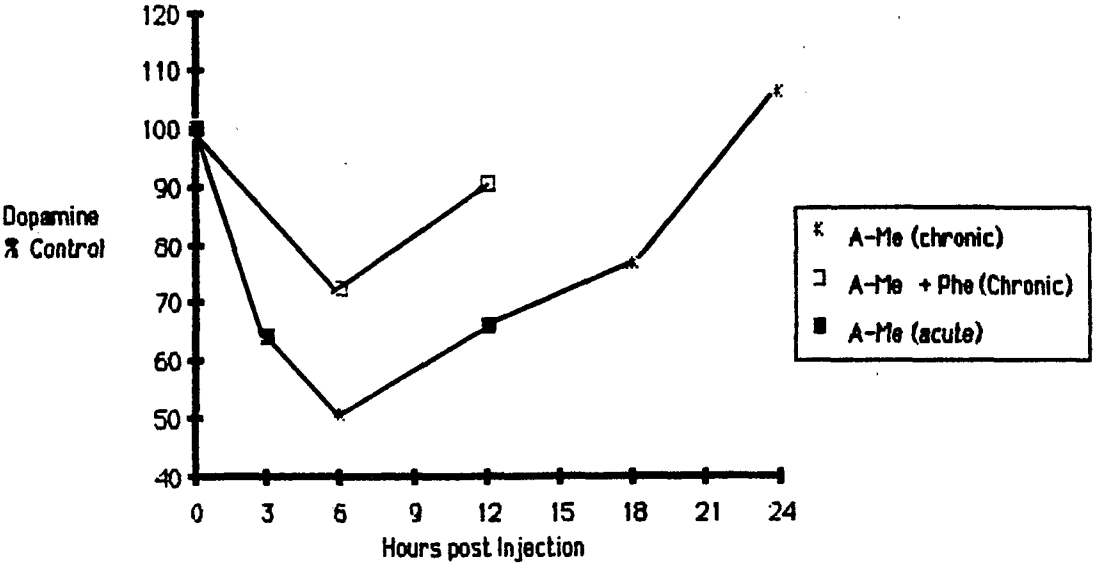
REVERSAL OF 5-HT DECREASE WITH TIME. MEASUREMENT OF 5-HT, TRYPTOPHAN, AND TRYPTOPHAN HYDROXYLASE 4-6 AND 24 HOURS AFTER THE LAST INJECTION OF A-ME AND A-ME+PHE.

	5-HT (nmol/g)	Brain Try (nmol/g)	Try Hydroxylase (nmol/hr/g)
CONTROL	4.46±0.65(13)	23±6(27)	96±17(14)
	4-6 HOURS		
A-ME	3.93±0.46(9)	17±4(18)	53±12(9)
A-ME+PHE	2.60±0.49(9)	10±3(32)	86±22(12)
	24 HOURS		
A-ME	3.40±0.45(4)	15,18	53±17(3)
A-ME+PHE	4.02±0.90(20)	13±3(3)	76±20(3)

Rats, aged 9 to 12 days, were injected daily for 3 days with a-me (2.4 umoles/g body w.) or a-me (2.4 umoles/g) plus phe (5.2 umoles/g) and were sacrificed 4-6 and 24 hours after the last injection. Values are means ±1 S.D.(n) or single values.

Figure VI-5

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



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

From Brass (1983)

deficit in the "a-me model" of PKU and probably in the human disease as well.

2. Prevention of the Monoamine Deficit

The purpose of this study was to investigate the depletion of catecholamines by phe alone and to reverse the catecholamine depletion by a coupled injection of phe plus tyrosine. All animals (Table VI-4) received an injection of a-me 24 hours prior to sacrifice thus causing inhibition of phenylalanine hydroxylase at the time of study. Measurements of midbrain 5-HT levels were also made to test whether this reversal is selective for catecholamines. Injection of 5 umol/g phe caused an increase in brain phe to 2900 umol/g after 6 hours and dopamine levels were decreased by 24%. 5-HT levels were decreased by 47% by the same treatment. Phe (5 umol/g) plus tyrosine (5 umol/g) injection elevated brain phe to 3835 umol/g and tyrosine to 472 umol/g. With the combined treatment, a loss in catecholamine levels was no longer observed. However, the decrease in 5-HT persisted at 42% with this treatment (Table VI-4).

5-HT levels are increased to control values if tryptophan is given in conjunction with the standard treatment (Chapter V, Table V-4). Therefore, both the catecholamine and the 5-HT deficit in our experimental model can be prevented by precursor administration.

TABLE VI-4

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

TREATMENT (umoles/g)		BRAIN AMINO ACIDS (nmoles/g)		BRAIN MONOAMINES (% control)		
Phenylalanine	Tyrosine	Phenylalanine	Tyrosine	Dopamine	Norepinephrine	Serotonin
-	-	85±12(4)	63±17(3)	110	105	72±9(3)
5	-	2900±303(6)	94±34(3)	76±14(3)	90±19(3)	53±4(4)
5	5	3835±930(4)	472±219(4)	104±11(3)	116±16(3)	58±10(3)

Rats in group two (second line) received 5.0 umoles/g body weight phenylalanine. The third group of animals (line 3) was injected with 5 umoles/g phenylalanine plus 5 umoles/g tyrosine. All of these animals had their liver phenylalanine hydroxylase activity reduced by prior treatment with a-methylphenylalanine. All rats were 7 days old and were sacrificed 6-7 hours after injection of phenylalanine. Monoamine levels are expressed as a per cent ± 1 S.D. (n) of the monoamine levels of control (saline injected littermates treated and assayed at the same time). Phenylalanine and tyrosine levels (nmoles/g brain ± 1 S.D. (n)) were determined in the brains of a separate group of animals treated similarly to those in which cerebral catecholamines were assayed.

3. Discussion

Since phenylketonurics are unable to convert phe to tyrosine, and since severe hyperphenylalaninemia can interfere with the cerebral entry of dietary tyrosine, the brains of subjects of this disease were expected to and were found to have a diminished tyrosine concentration. This diminution is not seen in experimental hyperphenylalaninemia, since a-me (or p-cpa) achieves a 80-90% rather than a complete suppression of the hepatic phenylalanine hydroxylase and the residual activity is sufficient to convert significant amounts of phe to tyrosine. The cerebral dopamine and norepinephrine deficiency in these animals is thus associated with a somewhat elevated rather than diminished tyrosine concentration. It is clear, therefore, that a subnormal precursor concentration is not a necessary condition for the catecholamine deficit. On the other hand, the possibility that precursor shortage may be a major factor limiting 5-HT synthesis cannot be excluded since the cerebral tryptophan concentration did decrease in this model. The tryptophan concentration in the "p-cpa model" is not pertinent here since catecholamine depletion has not been studied in this model and since the cerebral 5-HT deficit is due to tryptophan hydroxylase suppression which is a non-physiological mechanism unrelated to hyperphenylalaninemia per se (see Chapter IV).

If the second theory is examined, namely that the hydroxylation step in monoamine synthesis is inhibited, a distinction between reversible inhibition and lasting suppression must be made. Direct measurement of the enzymes as well as the time course of monoamine depletion can be used to assess whether or not suppression occurs. Treatment with a-me plus phe caused no change in tryptophan or tyrosine hydroxylase activity that could be detected by the in vitro assays of the experimental tissue at saturating substrate and cofactor concentrations, indicating that no lasting suppression occurs in the "a-me model" in vivo. The possibility that lasting suppression of the enzyme is operative here is also excluded by the fact that 5-HT and dopamine concentrations of the brain fluctuate with daily variations in the degree of hyperphenylalaninemia (Table VI-3 and Figure VI-5). It is clear that we are dealing with a rapidly reversible inhibition of the hydroxylases in vivo as well as in vitro. In agreement with the competitive nature of this inhibition, substrate injection restored normal monoamine levels despite persistent hyperphenylalaninemia. This particular finding also disproves the theory implicating substances produced by the overflow of excess phe to the normally minor transamination pathway. For, it is highly unlikely that the tryptophan or tyrosine supplements reduced the production of these metabolites, and yet they restored normal cerebral 5-HT and catecholamine levels.

One may conclude therefore, that in PKU and in the "a-me model", the mechanism postulated by the second theory, i.e. reversible inhibition of the first step on the synthetic pathway, is the major cause of the cerebral monoamine depletion. In fact, this is the only cause for the catecholamine depletion in the "a-me model" since there was no decrease and a slight increase in the cerebral tyrosine concentration of these animals.

The competitive inhibition of tryptophan hydroxylase by excess phenylalanine is a long known phenomenon. It is therefore surprising that 1) the effects of tryptophan administration on cerebral 5-HT have been studied in normal but not in hyperphenylalaninemic animals and that 2) tryptophan administration to phenylketonurics, although suggested to be desirable for assuring adequate protein synthesis, has not been contemplated as a means for restoring normal 5-HT levels. From the point of view of research on animal models, the feasibility of preventing the cerebral deficit in 5-HT without affecting catecholamines and vice versa is of considerable practical significance: it enables one to produce variants of the PKU model for the purposes of comparing the putative pathogenic action of the monoamine depletions. Therefore, one can determine whether the 5-HT or the catecholamine deficit plays a more important role in the hyperphenylalaninemia associated cerebral maldevelopment.

A few comments should be made about possible artifacts associated with the use of synthetic analogues of phenylalanine. The slowly developing and lasting reduction in vivo by a-me of cerebral tryptophan hydroxylase is not an artifact in the "a-me model", since in animals treated with phe as well, this suppression does not occur. Therefore, as with PKU, the cerebral 5-HT and 5-HIAA depletions are attributable to hyperphenylalaninemia per se. The competitive inhibition of tryptophan hydroxylase by a-me also does not play a role here since 5-HT showed the same response to phe and to a-me plus phe. Somewhat different considerations apply to the catecholamines. In normal animals injected with phe (or in previous models of PKU with insufficiently suppressed phenylalanine hydroxylase) the tyrosine level is high so that no catecholamine depletion occurs. It could be demonstrated, nevertheless, that their depletion in the "a-me model" can also be explained by hyperphenylalaninemia alone since 24 hours after a-me administration (at the time when phenylalanine hydroxylase activity is still suppressed) an injection of phenylalanine alone could reduce the catecholamine content rapidly, and to levels as low as in the chronic model (i.e. in animals given repeated, simultaneous injections of a-me plus phe). A complication not seen in the case of 5-HT was that even though a-me and phe are both inhibitors of tyrosine hydroxylase, treatment with a-me alone caused a greater rather than smaller

reduction of dopamine than treatment with a-me plus phe (Table VI-2). One possible explanation is that the small increase in tyrosine associated with the combined treatment is enough to preclude the inhibition of tyrosine hydroxylase by a-me, and thus mask the full depleting effect of a-me. Another possibility is that excess phe blocks the transport of a-me into the brain. In either case, a general, practical implication of these observations (and of the prevention by phe of the a-me suppression of tryptophan hydroxylase) is that, since elevated concentrations of an amino acid can minimize the side effects of synthetic analogues, study of the organism treated with such an analogue alone does not predict the occurrence of this side effect in animals treated with both substances.

CHAPTER VII

BIOCHEMICAL AND BEHAVIORAL STUDIES IN ADULT RATS HAVING UNDERGONE NEONATAL HYPERPHENYLALANINEMIA

One of the most consistent findings in phenylketonuric children is the development of mental retardation. Although experimental models reproduce the major biochemical abnormalities of the disease, the relationship between mental retardation and behavioral abnormalities in animal models is less straightforward. Rats that received the standard treatment of a-me plus phe during infancy, were found to perform poorly in T-mazes (Glick and Greengard, 1980) and in passive and active avoidance tests (Luttges and Gerren, 1979). These disabilities in tests of "essential learning" (i.e. pertinent to immediate biological needs and survival) might be considered as analagous to some aspects of the subnormal intelligence of PKU subjects. It is thought, however, that the impairment in human retardates are examples of "advantageous learning" defects. This type of learning ability, as shown recently (Strupp et al., 1984), is also impaired in rats that have been treated with a-me plus phe during infancy. In keeping with the hyperactvity of PKU children, these animals also exhibited increased locomotor activity (Glick and Greengard, 1980; Lane et al., 1980). Cerebral lateralization has not been studied in phenylketonurics. However, abnormal or precocious

lateralization is thought to underlie learning disabilities and reading difficulties in children with minor brain damage (Kershner, 1978). It was of interest, therefore, that "PKU rats" exhibit a behavioral change which pointed to abnormal cerebral lateralization. This was the exaggerated side preference that they exhibited in the spontaneous circling movement (Glick and Greengard, 1980). Deviations from the normal moderate degree of intrinsic nigrostriatal asymmetry (which explains this side preference) is known to interfere with the learning of tasks dependent on spatial orientation (Glick et al., 1977). It is also known that the circling pattern is regulated by nigrostriatal dopaminergic pathways influenced by 5-HT input (Glick et al., 1976). Abnormalities in the metabolism of cerebral monoamines may therefore be related to the defective rotational behavior and learning disabilities in experimental PKU.

All of these behavioral defects were seen 2-3 months after the cessation of hyperphenylalaninemia and some of them were more severe in female than in male rats. Therefore, studies in this chapter include examination of the brain for possible irreversible biochemical abnormalities as well as comparisons of 5-HT metabolism in male, female and hormone treated rats.

1. Results on Rats Exposed to Hyperphenylalaninemia During Infancy

Rats were treated with a-me plus phe in standard doses from the age of 3 to 21 days and were tested for rotatory and locomotor activity, and T-maze learning at approximately 100 days of age (Glick and Greengard, 1980). The degree of nigrostriatal lateralization in normal rats is manifested in the fact that, when allowed to circle spontaneously, they turn about 60% of the time in their preferred (clockwise or anti-clockwise) direction. Table VII-1 shows that the same was true for rats given daily saline injections during infancy but that maintenance of hyperphenylalaninemia with daily injections of a-me plus phe during this period led to a significant 81% side preference in the female group. These experimental females also performed an increased number of net rotations per day while the extra 1/4 turns, which measure random activity, were not sigificantly different. In T-mazes too, a significantly diminished performance was seen in the experimental female rats but not in male rats (see trials to criterion, Table VII-1).

These sex differences in behavior (seen also in locomotor tests in Table VII-1) may have been due to the fact that the metabolic abnormality in brain during infancy, as judged by the phe or tryptophan concentration, was greater in the female than in the male rats.

TABLE VII-1

BEHAVIORAL RESULTS FROM ADULT RATS HAVING UNDERGONE NEONATAL HYPERPHENYLALANINEMIA

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

Rats were given daily subcutaneous injections with phenylalanine (5.2 um/g body wt.) plus a-methylphenylalanine (2.4 um/g body wt.) (experimental) or saline diluent (control) on their 2nd to 21st days of life. At 90-120 days of age they were tested for rotational behavior, locomotor activity and T-maze learning ability. All values are means ± SD of results from 12 control males, 15 control females, 11 experimental males and 14 experimental females. Significant differences from control ($p<0.01$) are denoted by *.

From Brass, 1983

Table VII-2 shows, however, that elevations in phe and the decrease in brain or plasma tryptophan were the same in the two sexes; consequently, the 5-HT deficits were also similar. The absolute levels of dopamine and norepinephrine were somewhat different in control male and female brains, but the decrease caused by hyperphenylalaninemia was the same in the two sexes (Table VII-2).

Another possibility is that there are some permanent changes in the experimental brains which exhibit a sex difference. The 5-HT level, known to be normalized shortly after the cessation of hyperphenylalaninemia (Brass et al., 1982), was at control levels in adulthood and showed no significant sex difference (Table VII-3). Although a multitude of cerebral enzymes including tryptophan hydroxylase are at normal levels during exposure to hyperphenylalaninemia (Chapter VI; Delvalle et al., 1978), it is conceivable that the early exposure to hyperphenylalaninemia interfered with their subsequent development since these enzymes had not reached their adult levels by the end of that period. Table VII-3 shows however that this is not the case for enzymes involved in 5-HT metabolism: L aromatic amino acid decarboxylase, monoamine oxidase and tryptophan hydroxylase measured 2-3 months after the cessation of hyperphenylalaninemia revealed no significant sex difference from those in normal adult brain. The values

TABLE VII-2

EFFECT OF CHRONIC HYPERPHENYLALANINEMIA ON CEREBRAL PHENYLALANINE, 5-HT, TRYPTOPHAN HYDROXYLASE, DOPAMINE, NOREPINEPHRINE AND PLASMA PHENYLALANINE AND TRYPTOPHAN IN MALE AND FEMALE RATS

	CONTROL		EXPERIMENTAL	
	Male	Female	Male	Female
Plasma phenylalanine (nmol/ml)	70±20(6)	80±10(6)	2660±400(6)	2840±600(6)
Cerebral phenylalanine (nmol/g)	70±10(6)	70±20(6)	2040±20(6)	2050±510(6)
Plasma tryptophan (nmol/ml)	102±10(6)	111±25(7)	72±9(3)	65±13(8)
Cerebral tryptophan (nmol/g)	19±5(6)	21±4(7)	8±2(3)	9±2(8)
Cerebral 5-HT (nmol/g)	4.2±1.0(4)	4.5±0.5(3)	2.8±0.2(5)	2.9±0.2(3)
Cerebral tryptophan hydroxylase (nmol/hr/g)	94±10(4)	89±15(6)	72±9(3)	89±14(5)
Cerebral dopamine (nmol/g)	2.63±0.74(6)	2.15±0.31(6)	1.91±0.20(7)	1.58±0.42(6)
Cerebral norepinephrine (nmol/g)	2.96±0.34(3)	2.32±0.14(4)	1.81±0.71(4)	1.54±0.42(4)

9-12 day old animals were injected daily with a-methylphenylalanine (2.4 umoles/g body wt.) plus phenylalanine (5.2 umoles/g body wt.) or saline (controls) for 3 to 9 days. Animals were sacrificed 4-7 hours after their last injection. Values are means ± S.D.(n).

TABLE VII-3

BIOCHEMICAL RESULTS IN ADULT RATS HAVING UNDERGONE NEONATAL HYPERPHENYLALANINEMIA

	Control		Experimental	
	Male	Female	Male	Female
Whole brain weight (g)	1.68±0.10(3)	1.62±0.07(5)	1.58, 1.56(2)	1.41±0.09*
L aromatic amino acid decarboxylase (umol/hr/g)	5.82±0.50(3)	4.62±0.92(5)	6.5, 6.00(2)	5.7±1.2(5)
Monoamine oxidase (nmol/min/g)	140±14(3)	118±10(4)	126, 139(2)	126±22(5)
Tryptophan hydroxylase (nmol/hr/g)	194±7(3)	204±10(5)	183, 169(2)	198±32(5)
5-HT (nmol/g)	6.01±1.04(4)	5.00(1)	5.29, 4.73(2)	4.78(1)

Rats were given daily injections of a-methylphenylalanine (2.4 um/g body wt.) plus phenylalanine (5.2 um/g body wt.) or saline from day 3 to 21 (see Methods). They were then raised to 130-150 days of age and biochemical measurements were made. All values are means ± S.D. or single values. The number of animals in each group is listed in parenthesis. Significant differences from control (p 0.01) are denoted by *.

for experimental males and females were similar. The only permanent change (see first line Table VII-3) was that the brain weight deficit seen during the treatment was not completely recovered and that only in females was this slightly subnormal brain size statistically significant ($P<.01$).

2. Sex Difference in 5-HT in Postnatal Rats

There are a number of sex differences in the monoaminergic pathway that have been described in the literature but they are not generally confirmed. Vaccari et al. (1977), for example, describe a multitude of sex differences in neurotransmitter precursors, in tyrosine hydroxylase, dopa decarboxylase and tryptophan hydroxylase activities which do not quite agree with results by us (see later) and by others (Weil-Fuugazza et al., 1980; Brass, 1983). Similar considerations apply to reports on 5-HT and its response to androgens. In attempting to confirm results by Wilson and Agrawal (1979), a small sex difference in midbrain 5-HT content was apparent at 11 to 12 days of age but not at 8 to 9 or 15 days. (Table VII-4). The male 5-HT content was 10% and the 5-HIAA content was 23% above the female. However these differences, and the increase in 5-HT and 5-HIAA content above male levels if female rats are given an injection of testosterone at 2 days of age, were not statistically significant.

TABLE VII-4

SEX DIFFERENCE IN MIDBRAIN 5-HT AND 5-HIAA IN CONTROL ANIMALS AND IN FEMALES THAT RECEIVED AN INJECTION OF TESTOSTERONE AT AN EARLY AGE

Age (days)		Vehicle male	Vehicle female	Testosterone male	Testosterone female
8-9	5-HT	4.1±1.0(3)	3.8±0.3(3)	3.5,3.5	4.1±1.0(3)
	5-HIAA	0.75	0.75,0.60	0.92,0.65	0.72,0.65
11-12	5-HT	5.4±0.9(14)	4.9±0.7(9)		5.8±1.2(12)*
	5-HIAA	2.1±0.7(14)	1.7±1.0(9)		2.3±0.8(12)
15	5-HT	4.8±0.7(4)	4.5±1.2(5)	4.3±1.2(3)	4.7±1.7(4)
	5-HIAA	1.0±0.4(4)	1.0±0.3(5)	1.2±0.4(4)	1.2±0.5(5)

Testosterone propionate (1.25 mg/pup) was dissolved in sesame oil and administered on day 2. Control animals received the sesame oil vehicle only. Values are means ± 1 S.D. or single values.
* indicates a significant difference ($P<0.06$) from control of the same sex.

3. Discussion

Some sign of what one might consider a lack of intelligence in a rat is clearly a necessary criterion for a model of a disease characterized by severe mental retardation. Maze learning ability is an obvious parameter which is examined (and has been found to be diminished (Lane et al., 1980; Glick and Greengard, 1980) in PKU rats. Examination of how often they turn to the right or left when circling spontaneously may be of less obvious relevance to PKU. However, the side preference that rats exhibit while circling is the most clear-cut manifestation of their cerebral lateralization which is of interest for several reasons. One is that there have been several human studies exploring the intellectual and emotional implications of anomalies in cerebral lateralization. Such anomalies have been noted to be associated with psychoses (e.g. Gur, 1979), with the learning disability of infants with minimal brain damage (Gazzaniga, 1975), and precocious development of cerebral laterality has been suggested (Kershner, 1978) to underlie the reading difficulty of some

apparently normal children. Also, the exaggerated side preference of rats shown in Table VII-1 was associated with an 1) increased number of total turns and with 2) diminished T-maze performance, which are reminiscent of the well known hyperactivity and learning disability of phenylketonurics as well as with their more recently noted difficulty in position discrimination (Anderson and Siegal, 1976).

The behavioral abnormalities previously studied in PKU models are usually too vague or generalized to be traceable to a particular brain structure or a specific biochemical process. Thus, another reason for the interest in the anomalous circling activity of rats is that it is the first one which can be traced to a specific structure (the striatum) and to functions (dopaminergic and serotonergic) which further support the pathogenic role of altered 5-HT or catecholamine metabolism in hyperphenylalaninemia.

Studies in Table VII-2, prompted by the particular severity of this behavioral abnormality in female experimental rats, showed that the cerebral 5-HT and catecholamine deficit as well as the accumulation of phenylalanine and the loss of tryptophan were similar in males and females. Thus, an unequal sensitivity to the same biochemical changes must underlie the sex difference in the behavioral anomaly. This difference, abolished by a neonatal testosterone injection to female experimental rats (Brass, 1983), is probably related to the fact that

exposure to hyperphenylalaninemia coincided with the days when the sexual dimorphism of rat brain (attributable to the action of testosterone in the neonatal male) is irreversibly imprinted (Maclusley and Naftolin, 1981). For, an early and transient manifestation of this dimorphism during the suckling period, such as the higher thymidine kinase activity and faster DNA synthesis in females may imply increased sensitivity and provide a possible explanation for the more lasting brain growth deficit (Table VII-3) as well as functional damage in the female. Since in humans, the imprinting period is not postnatal but occurs during the 3rd to 4th month of gestation (Erhardt and Meyer-Bahlburg, 1981), this theory is not applicable to PKU children of heterozygous mothers (who are not hyperphenylalaninemic during gestation), and only gestation in mothers hyperphenylalaninemic during pregnancy would be expected to lead to particularly severe behavioral defects in the females. Comparison of these populations which would test the above theory have, unfortunately, not been carried out.

A more fundamental problem than the sex difference which is merely a quantitative one (in that male PKU rats also showed significant cognitive defects in alternative tests (Lane et al., 1980; Strupp et al., 1984) is to explain in chemical terms the enduring nature of the abnormalities. As is well known, the mental deficit associated with PKU is irreversible if the correction of

the metabolic state is delayed beyond the first few months or years of life (Knox, 1972). In animal models too, there is evidence for the lasting nature of the behavioral deficits. In contrast, none of the known abnormalities in chemical constituents of the brain (with the possible exception of myelin) persist for more than a few hours or days after the cessation of hyperphenylalaninemia. The present study shows that adult rats treated with a-me plus phe during infancy have normal concentrations of 5-HT as well as of tryptophan hydroxylase, monoamine oxidase, and L aromatic amino acid decarboxylase. In order to explain the functional and behavioral abnormalities in these animals, one will have to explore more subtle parameters, such as the turnover rate of the transmitter amines, the concentration and efficacy of the receptors, or fine structural characteristics of neural elements.

GENERAL DISCUSSION

The studies presented here examine several biochemical approaches to cerebral 5-HT depletion in the rat, the mechanisms involved in each, and their application to modeling the human disease PKU. They aid in our understanding of this disease process and in developing methods for inducing selective neurotransmitter deficits in immature, experimental animals.

Since animals with a mutation equivalent to that in PKU are not available, the synthetic substances p-cpa and a-me have been used to imitate the defective phenylalanine hydroxylase gene and consequent hyperphenylalaninemia characteristic of this disease. The latter agent is now usually employed in studies concerned with the lasting cerebral damage resulting from exposure to hyperphenylalaninemia during early postnatal or fetal development because a-me is devoid of the toxicity, lethality and prohibitive growth inhibitory effect of repeated p-cpa injections to developing rats. However, animals treated with p-cpa provide useful alternative models, and comparison of its mechanism of action with those of a-me facilitates insight into the various ways in which derangements of aromatic amino acid metabolism can arise.

There are several possible biochemical actions which may lead to a cerebral 5-HT depletion in the experimental systems studied. These include a decrease in the substrate (tryptophan), alteration in the activity of preformed enzyme or cofactors and irreversible inhibition of newly formed enzyme.

An example of this last mechanism is the loss of rat hepatic phenylalanine hydroxylase activity after p-cpa injection. It has been shown that this enzyme inhibition is a slow process in vivo, occurring only 24 hours after injection, with a recovery phase of 2-3 days requiring resynthesis of enzyme (Koe and Weissman, 1966; Lipton et al., 1967). This phenomenon, which does not occur in vitro (Lipton et al., 1967), was therefore termed "irreversible inhibition" and based on the above evidence is thought to be due to the action of p-cpa on the dynamic state of phenylalanine hydroxylase turnover in the living cell (e.g. alteration in covalent ligands, structure or amount of enzyme synthesized) and not to alteration of preformed enzyme. We refer to an alteration in enzyme activity brought about by this type of mechanism as "suppression" in contrast to "inhibition" which refers to a more rapid effect which can also be seen in vitro.

Although p-cpa and a-me are qualitatively similar in their actions on the three aromatic amino acid hydroxylases, we show in this study that quantitative differences of their alteration of tryptophan hydroxylase

actvity lead to different roles in causing cerebral 5-HT depletion in model systems of PKU. P-cpa and a-me have both been previously shown to be potent suppressors of phenylalanine hydroxylase (Koe and Weissman, 1966; Delvalle et al., 1978) and only competitive inhibitors of tyrosine hydroxylase (Gal and Whitacre, 1982; Brass, 1983). We now demonstrate that a-me suppresses cerebral tryptophan hydroxylase activity (Figure IV-5) as does p-cpa (Jequier et al., 1967). These agents also act as weak competitive inhibitors with Km values of 1.2 mM (Figure IV-3) and 0.3 mM (Jequier et al., 1967) respectively. Our data further indicate that the suppressive effect of p-cpa on tryptophan hydroxylase may be its exclusive mechanism of action in 5-HT depletion in the "p-cpa model" of PKU, whereas this action is much less important in the 5-HT depletion seen in the "a-me model" of this disease.

Although a-me alone causes a slight decrease in cerebral 5-HT, a much greater depletion is caused when this is combined with an injection of phe as done in our model system of PKU. This greater depletion is accompanied by a significant decrease in cerebral tryptophan due to decreased transport of plasma tryptophan across the blood-brain barrier as the elevated phe competes with tryptophan for sites on the shared L-neutral amino acid transport system. That substrate deficiency is a probable cause of 5-HT depletion in this model is further evidenced by the fact that injection of additional tryptophan (along

with a-me and phe) overcomes this 5-HT depletion (Table V-4). This is in marked contrast to the hyperphenylalaninemia caused by the addition of phe to p-cpa injections where the p-cpa induced depletion of cerebral 5-HT is unaltered. Furthermore, neither the p-cpa nor the p-cpa plus phe treatments caused a decrease in cerebral tryptophan levels. These findings have several implications in terms of animal models of PKU, investigation of selective neurotransmitter deficiencies and amino acid metabolism.

Modeling Phenylketonuria

With respect to the alterations in cerebral tryptophan noted in PKU, the "p-cpa model" of this disease appears to be a poor mimic. The decreased concentration of several neutral amino acids in the brains of phenylketonurics is thought by many to be a major contribution to the abnormal development of these children due to a decreased protein synthesis. However, p-cpa itself appears to affect the metabolism of cerebral tryptophan in a poorly understood way by raising it to levels not seen in PKU. This is seen by both the small but significant increase in cerebral tryptophan in rats injected with this substance alone (Figure V-1) as well as by the normal brain tryptophan levels in animals injected with p-cpa plus phe. In the latter case, the elevation in plasma phe (as high as in the "a-me model") would be expected to cause a decrease in the

cerebral tryptophan levels via competition of tryptophan transport into the brain by phe. The mechanism of this effect on tryptophan metabolism or on transport into the brain is unclear at present. The most simple explanation would be that the greater inhibition of tryptophan hydroxylase by p-cpa compared to a-me resulted in a tryptophan build-up in the brain due to a decreased conversion to 5-HT. This seems plausible in that the treatment causing greater tryptophan hydroxylase inhibition (p-cpa compared to p-cpa plus phe or a-me, see Figure VI-3) resulted in higher cerebral tryptophan levels. Therefore, the action of p-cpa in reversing the hyperphenylalaninemia induced decrease in brain tryptophan in the PKU models may only differ from that of a-me in a quantitative manner. However, the more likely alternative is that p-cpa has a separate action from a-me such as directly inhibiting protein synthesis and thus decreasing cerebral tryptophan utilization or affecting tryptophan efflux or influx from and into the brain.

Another observation in relation to modeling PKU revolves around the interference that increasing amounts of phe causes on the primary effects of its unnatural analogues, a-me and p-cpa. The principle that excess amounts of a normal amino acid may counteract some of the actions of its analogue by competing with enzymic targets or cerebral transport systems has been previously demonstrated in several instances. For example, large and

frequent injections of phenylalanine have been shown to partially counteract the suppression and enhance the recovery of hepatic phenylalanine hydroxylase caused by p-cpa and a-me injections (Delvalle et al., 1978). It has also been shown that although hyperphenylalaninemia induced by a-me plus phe injections causes catecholamine depletion in the brain of treated animals, a-me alone has a greater effect than if combined with phe (Brass and Greengard, 1982). Similarly, concomitant phe administration entirely prevents the body growth inhibition and high mortality caused by prolonged treatment of developing rats with a-me alone (Brass, 1983). These findings are quite relevant to the problem of separating the artifacts of synthetic substances (e.g. a-me and p-cpa) used to initiate disease states from the metabolic abnormalities attributable to the disease itself. For they indicate that this problem is not necessarily solved if, as is customary, animals treated with the analogue alone are used as "controls". Indeed, one may arrive at erroneous conclusions about artifacts present in the model. For example, the in vivo inhibition of tryptophan hydroxylase by a-me alone as shown in Figure VI-3 might suggest that this non-physiologic mechanism may cause the 5-HT depletion in our PKU model when in fact a-me does not suppress this enzyme in the presence of excess phe. Indeed, the data in Figure V-1 indicate that a physiologic mechanism, decreased cerebral tryptophan availability secondary to transport inhibition by elevated

plasma phe, is the mediator of 5-HT depletion in our model. This contrasts with the role of p-cpa itself causing 5-HT depletion in the alternative model of PKU.

Therefore, this study lends further evidence to the unsuitability of using p-cpa plus phe treatment to mimic PKU when compared to the use of a-me plus phe for this purpose. The former causes severe body weight deficits and mortality which poorly mimics the disease and makes behavioral studies of the mature animals difficult to interpret. Furthermore, this treatment clearly fails to mimic certain abnormalities of human PKU (cerebral tryptophan depletion) while causing others (5-HT depletion) through unclear mechanisms more attributable to artifacts of the model than imitation of the disease. In contrast, the a-me plus phe protocol results in animals with cerebral tryptophan and 5-HT depletions (Figure V-1 and Figure VI-2) as well as catecholamine deficits (Table VI-2) due to the effects of hyperphenylalaninemia and not artifacts of the a-me treatment. In addition, this treatment in the developing rat results in several behavioral abnormalities such as hyperactivity (Glick and Greengard, 1980) and cognitive deficits (Strupp et al., 1984) in mature animals which are similar to the adult behavioral disturbances seen in PKU.

Neurotransmitter Modulation and Behavior

PKU is of particular interest in the study of neurochemical development because of the clear link between an early insult to the maturing organism and later behavioral abnormalities. Rigorous restriction of phe intake is of no significant benefit to phenylketonurics unless initiated within weeks after birth, indicating that early infancy is the time when the brain incurs its permanent damage. Similarly, rats treated with a-me plus phe from days 2 to 21 of age exhibit several behavioral abnormalities when tested as adults despite reversal of the known neurochemical changes after cessation of the hyperphenylalaninemic state.

In view of this dichotomy, the most prevalent theories of pathogenesis in PKU implicate the altered concentration of certain amino acids which are reversible but, if present during early development, may result in permanent growth inhibition. The microcephaly of phenylketonurics may in fact be explained by an imbalanced precursor pool for protein synthesis. Since such an imbalance associated with other diseases or malnutrition does not lead to as severe a mental retardation as in PKU, the causal relationship of the growth deficit to the cognitive deficit may be questioned. Although the diminished concentrations of 5-HT and catecholamines in the PKU brain were also suggested to be involved in the pathogenic process, the reversibility of

these decreases do not easily concord with the permanent nature of the functional damage. However, it is now being suggested that the consequences of these neuroactive amine deficits may be as lasting, and more specific, than inhibition of overall protein synthesis and small brain size. In particular, evidence has been emerging in the last few years for a role of 5-HT and catecholamines in fetal brain development, namely that each promotes the differentiation in terms of morphology, function or contact formation of the specific neural structures that will depend on it as a transmitter later on (Lauder, 1983; Patel et al., 1979).

One behavioral anomaly noted in adult rats treated with a-me plus phe early in life is the exaggerated lateralization of spontaneous circling activity (Glick and Greengard, 1980) which is regulated by the dopaminergic nigrostriatal pathways and influenced by 5-HT input (Glick et al., 1976). This appears to be of particular interest because the anomaly in circling patterns is one which is known to interfere with orientation in space (Glick et al., 1977) and may underlie the learning deficits that these rats exhibit in maze paradigms (Glick and Greengard, 1980).

The role of catecholamine perturbations in this altered behavior has been investigated in adults. However, no consistent changes in adult levels of catecholamines, metabolic enzymes involved in their regulation, cerebral lateralization of catecholamine levels or alteration in

circling activity induced by drugs affecting catecholaminergic systems (amphetamine, apomorphine) have been found between control and experimental animals (Brass, 1983). Also, no differences in adult levels of tryptophan hydroxylase have been found. However, the role of possible long term serotonergic changes has not been fully investigated. It will therefore be of interest to compare the circling activity of experimental animals after injection of the 5-HT antagonist methysergide.

Another unclear behavioral finding in adult phenylketonuric rats is the female predominance of behavioral effects. The present data add little insight to this problem since no sex differences were found in 5-HT levels or related enzyme activities observed in control and experimental animals. However, sex-based behavioral differences found in experimental animals may be due not only to different biochemical responses to hyperphenylalaninemia but could also be the result of the same biochemical change in both sexes interacting with some other sexually dimorphic system. For example, normal adult rats have sexually dimorphic circling responses to amphetamine and apomorphine which may imply sexual differences in dopamine receptors (Robinson et al., 1980).

Future Applications

The demonstration that hyperphenylalaninemia-induced 5-HT depletion can be easily reversed with tryptophan supplementation (Table V-4) has important experimental and clinical implications. The combination of this approach with that of reversing the hyperphenylalaninemia-induced catecholamine depletion (Brass, 1983) gives rise to methods for studying selective neurotransmitter deficits without using overtly toxic drugs or physical lesions. We now know that experimentally induced hyperphenylalaninemia per se can cause cerebral 5-HT and catecholamine depletion. Moreover, the 5-HT depletion can be specifically reversed with tryptophan supplementation whereas the catecholamine depletion can be reversed with tyrosine supplementation. Evidence in fetal rats also indicates that the dopamine and norepinephrine deficits may be able to be separated out by careful adjustment of the phe to tyrosine ratio (Brass, 1983). Therefore, one could combine a-me plus phe treatment with tryptophan, tyrosine, or low-level tyrosine supplementation to selectively study cerebral dopamine, norepinephrine or 5-HT depletions. Refinement of this technique of producing selective neurotransmitter deficits via alteration of cerebral amino acid levels would provide a great advance in the study of the biochemical and behavioral correlates of such neuroactive amine depletions since cytotoxic interventions would be avoided.

In addition, the concept of the reversal of hyperphenylalaninemia-induced neurotransmitter deficits with amino acid supplementation is of potential clinical importance in the treatment of PKU. One great difficulty in the present therapeutic regimen for this disease is the poor tolerance for the phenylalanine restricted diet. This is true for both phenylketonuric children and for the increasing number of phenylketonuric women who may be resistant to diet changes during pregnancy. If catecholamine and 5-HT deficits do indeed play a major role in the developmental defects caused by the disease, then a more palatable diet of a less severe phe restriction combined with tyrosine and tryptophan supplementation, may improve patient compliance and thus be clinically useful.

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