Enzyme substitution therapy for hyperphenylalaninemia with phenylalanine ammonia lyase. An alternative to low phenylalanine dietary treatment; effective in mouse models.

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To Mum and Dad

who gave up all their comforts

and moved three continents, to provide

us with every opportunity

to be all that we wanted to be.

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ABSTRACT

Phenylketonuria (PKU) and related forms of non-PKU hyperphenylalaninemias (HPA) result from deficiencies in phenylalanine hydroxylase (PAH), the hepatic enzyme that catalyses the conversion of phenylalanine (phe) to tyrosine (tyr). Patients are characterised by a metabolic phenotype comprising elevated levels of phe and some of its metabolites, notably phenyllactate (PLA), phenylacetate (PAA) and phenylpyruvate (PPA), in both tissue and body fluids. Treatment from birth with low-phe diet largely prevents the severe mental retardation that is its major consequence.

Mechanisms underlying the pathophysiology of PKU are still not fully understood; to this end, the availability of an orthologous animal model is relevant. A number of *N*-ethyl-*N*-nitrosourea (ENU) mutagenized mouse strains have become available. I report a new heteorallelic strain, developed by crossing female ENU1 (with mild non-PKU HPA) with a male ENU2/+ carrier of a 'severe' PKU-causing allele. I describe the new hybrid ENU1/2 strain and compare it with control (BTBR/Pas), ENU1, ENU2 and the heterozygous counterparts. The ENU1, ENU1/2 and ENU2 strains display mild, moderate and severe phenotypes, respectively, relative to the control and heterozygous counterparts.

I describe a novel method using negative ion chemical ionization gas chromatography/mass spectrometry (NICI-GC/MS) to measure the concentration of PLA, PAA and PPA in the brain of normal and mutant mice. Although elevated moderately in HPA and more so in PKU mice, concentrations of these metabolites are not sufficient to explain impaired brain function; however phe is present in brain at levels associated with harm.

Finally, I describe a new modality for treatment of HPA, compatible with better human compliance: it involves enzyme substitution with non-absorbable and protected phenylalanine ammonia lyase (PAL) in the intestinal lumen, to convert L-phenylalanine to the harmless metabolites (*trans*-cinnamic acid and trace

ammonia). PAL, taken orally, substitutes for the deficient PAH enzyme and depletes body pools of excess phe. I describe an efficient recombinant approach to produce PAL enzyme. I also provide proofs of both pharmacologic and physiologic principles by testing PAL in the orthologous mutant mouse strains with HPA. The findings encourage further development of PAL for oral use as an ancillary treatment of human PKU.

RÉSUMÉ

La phénylcétonurie (PCU) et autres formes d'hyperphénylalaninémies (HPA) résultent du malfonctionnement de la phénylalanine hydroxylase (PAH), l'enzyme hépatique catalysant la conversion de la phénylalanine (phe) en tyrosine (tyr). Les patients atteints de PCU présentent dans leurs tissues et leurs liquides corporels des niveaux élevés de phe et certains métabolites, notamment la phényllactate (PLA), la phénylacétate (PAA) et la phénylpyruvate (PPA). Une diète (à la naissance) à faible teneur en phe prévient grandement le retard mental sévère, caractéristique de la PCU.

Les mécanismes impliqués dans la physiopathologie ne sont pas entièrement compris; c'est pourquoi un modèle animal orthologue est nécessaire. Il existe les souches de souris ENU1 (avec légère HPA) et ENU2 (avec PCU) obtenues par mutation induite à la *N*-ethyl-*N*-nitrourée (ENU). Je décris maintenant une nouvelle souche hétéroallélique, ENU1/2 (ENU1♀ x ENU2/+♂), et compare cette souche avec les contrôles (BTBR/Pas), ENU1, ENU2 et leurs hétérozygotes respectifs. Les phénotypes sont: ENU1 (léger), ENU1/2 (modéré), ENU2 (sévère), lorsque comparé aux contrôles et hétérozygotes.

Je décris une nouvelle méthode utilisant la chromatographie gazeuse d'ionisation chimique d'ions négatifs couplée à la spectrométrie de masse (NICI-GC/MS) pour mesurer la concentration de PLA, PAA et PPA dans le cerveau de souris. Même si les niveaux sont élevés dans la souris HPA (légèrement) et PCU (beaucoup), la concentration de ces métabolites est insuffisante pour expliquer le malfonctionnement du cerveau. Cependant, la quantité de phe est suffisante pour causer certains dommages.

Finalement, je décris un nouveau mode de traitement de HPA qui est compatible avec un usage chez l'humain. Ceci implique la substitution d'enzyme avec la lyase phenylalanine ammoniaque (LPA) protégée (non-absorbable) dans le lumen intestinale. Ceci permet de convertir la L-phenylalanine en métabolites

inoffensifs (l'acide trans-cinnamique et des traces d'ammoniaque). La LPA, prise oralement, peut se substituer à l'enzyme déficiente PAH et éliminer l'excès de phe corporel. Je décris une approche recombinante efficace pour produire l'enzyme PAL. Aussi, je fournis des preuves aux niveaux pharmacologique et physiologique en testant PAL chez les souches de souris mutantes orthologues avec HPA. Ces découvertes encouragent le développement de PAL pris oralement comme traitement auxiliaire de la PCU chez l'humain.

ABBREVIATIONS

ACRS	amplification created recognition site
ANOVA	analysis of variance
BBB	blood-brain barrier
BH_4	tetrahydrobiopterin
bp	base pairs
BSA	bovine serum albumin
BTBR/Pas	genetic background of ENU treated mouse models
cDNA	complementary deoxyribonucleic acid
CGDN	Canadian Genetic Diseases Network
CLEC	cross-linked enzyme crystals
CNS	central nervous system
CSF	cerebrospinal fluid
Da	dalton
DHPR	dihydropteridine reductase
DNA	deoxyribonucleic acid
ENU	*N*-ethyl-*N*-nitrosourea, a chemical mutagen
ENU1	$Pah^{enu1/1}$ mouse; mutant orthologue of human non-PKU HPA
ENU1/+	heterozygous counterpart to $Pah^{enu1/1}$ mouse
ENU1/2	$Pah^{enu1/2}$ heteroallelic mouse; orthologue of human non-PKU HPA
ENU2	$Pah^{enu2/2}$ mouse; mutant orthologue of human PKU
ENU2/+	heterozygous counterpart to $Pah^{enu2/2}$ mouse
ENU3	$Pah^{enu3/3}$ mouse; another mutant orthologue of human PKU
GC/MS	gas chromatography/mass spectrometry
GFAP	glial fibrillary acid protein
GI	gastro-intestinal
GTP-CH	guanosine triphosphate cyclohydrolase
HAL	histidine ammonia lyase
HPA	hyperphenylalaninemia
HPLC	high performance liquid chromatography
IFNG	interferon, immune gene
i.p.	intraperitoneal
IQ	intelligence quotient
IR	infrared
6-MPH_4	6-methyltetrahydropterin
MBTFA	*N*-methyl-bis-trifluoroacetamide
MRC	Medical Research Council (Canada)
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MRS	magnetic resonance spectroscopy

m/z	mass/charge
NICI	negative ion chemical ionization
non-PKU HPA	non-phenylketonuria hyperphenylalaninemia
OD	optical density
PAA	phenylacetic acid
$PAAd_5$	deuterium labeled PAA internal standard ($[^2H_5]$-phenylacetic acid)
Pah	mouse phenylalanine hydroxylase enzyme
Pah	mouse phenylalanine hydroxylase gene
PAH	human phenylalanine hydroxylase enzyme
PAH	human phenylalanine hydroxylase gene
PAL	phenylalanine ammonia lyase enzyme
PAL	phenylalanine ammonia lyase gene
PCR	polymerase chain reaction
PEA	phenylethylamine
PEPB	peptidase B gene
PFB	pentafluorobenzyl bromide
phe	phenylalanine
PKU	phenylketonuria
PLA	phenyllactic acid
$PLAd_1$	PLA labeled with a single deuterium atoms (reduced PPA)
$PLAd_3$	deuterium labeled PLA internal standard (3-phenyl-2,3,3-$^2H_3$-lactic acid)
PPA	phenylpyruvic acid
R^2	squared correlation coefficient
RNA	ribonucleic acid
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error
SEM	standard error of the mean
SIM	selected ion monitoring
s.c.	subcutaneous
TFA	trifluoroacetate
TMS	trimethylsilyl
tyr	tyrosine

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PREFACE

Format of the Thesis

This thesis is composed of three chapters (2 - 4) of scientific experimentation and analyses, all of which are manuscripts that have been published. The format is in accordance with the regulations stated in the "Guidelines for Submitting a Doctoral Thesis", which reads as follows:

"Guidelines for Thesis Preparation: (C) MANUSCRIPT-BASED THESIS

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: (a) a table of contents; (b) an abstract in English and French; (c) an introduction which clearly states the rational and objectives of the research; (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); (e) a final conclusion and summary.

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled" Contributions of Authors" as a preface to the thesis. The

supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

When previously published copyright material is presented in a thesis, the candidate must obtain, if necessary, signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition."

Thus, this thesis is submitted in the form of manuscripts, with slight modifications needed to meet the requirements for a uniform presentation.

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CONTRIBUTIONS OF AUTHORS

Throughout my course of study, I have collaborated with several people. As chapters 2 to 4 of this Thesis are published articles by multiple authors, I am listing the contributions of each author below.

All three manuscripts presented in this thesis have been co-authored by my thesis supervisor, Dr. Charles R. Scriver and myself. For each body of work, Dr. Scriver discussed concepts with me, provided both technical guidance and supervised the project; he also helped with manuscript preparations.

The non-supervisory co-authors of the manuscript "A heteroallelic mutant mouse model: A new orthologue for human hyperphenylalaninemia" (Chapter 2) are Danielle M. Boulais and Dr. J. David McDonald. Danielle Boulais is the Animal Health Technician working with Dr. C.R. Scriver; Dr. J. David McDonald is an Associate Professor and Chair of the Department of Biological Sciences at Wichita State University. Danielle Boulais was responsible for the general care of the animals, aided with blood and tissue collection, assisted in metabolic manipulation experiments and recorded birth rate-survival to weaning and behavioral assessment. Dr. David McDonald provided advice on weaning, genotyping and metabolic manipulation.

Dr. Orval A. Mamer is the non-supervisory co-author of the manuscript entitled "Measurement of phenyllactate, phenylacetate and phenylpyruvate by negative ion chemical ionization-gas chromatography/mass spectrometry (NICI-GC/MS) in brain of mouse genetic models of phenylketonuria and non-phenylketonuria hyperphenylalaninemia" (Chapter 3). Dr. Mamer is the Director of the Mass Spectrometry Unit at McGill University and was responsible for proposing and directing the development of the NICI-GC/MS method. He provided both technical guidance and supervision.

The non-supervisory co-authors of the manuscript entitled "A different approach to treatment of phenylketonuria: Phenylalanine degradation with

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CHAPTER 1

Introduction

Phenylketonuria (PKU) and related hyperphenylalaninemias (HPA) are Mendelian disorders resulting from deficient conversion of phenylalanine (phe) to tyrosine (tyr). They are explained by primary deficiencies of phenylalanine hydroxylase enzyme (PAH) (EC 1.14.16.1) activity. If untreated, the patient will most probably experience irreversible impairment of cognitive development. Although highly investigated, the mechanisms underlying the pathophysiology of the disease are still not fully understood (1).

Discovery of PKU

The story of PKU began in 1934 when Dr. Asbjørn Følling, a physician and biochemist at the University of Oslo School of Medicine, was contacted by the mother of two mentally challenged children. She was determined to find the cause of their retardation and of the mousy odour that seemed to be related to their condition. Dr. Følling asked the mother to bring him urine samples. She returned with over 5 gallons, from which he identified an excessive amount of phenylpyruvic acid (PPA) - a phenylketone. Følling, suspecting a link between the excretion of PPA and the mental retardation, surveyed several of the surrounding mental institutions and identified other such cases. He noted evidence of autosomal recessive inheritance. He further demonstrated that in addition to the high concentration of PPA, these patients also had elevated levels of circulating phe. He therefore hypothesised that metabolism of dietary phe was defective and suggested an inherited metabolic disorder as the cause for mental retardation. He named the disease "oligophrenia phenylpyrouvica" (2-4). Penrose and Quastel renamed the disease "phenylketonuria", literally meaning phenylketones in the urine (5). Penrose postulated that the mental retardation had an identifiable chemical cause (6).

Defining Hyperphenylalaninemia

PKU and related HPAs are among the most widely studied and well-documented of the hereditary metabolic disorders. Various classification schemes exist for the

different forms of HPA (the categories defining the level of severity). The one used here identifies 'hyperphenylalaninemia' as an event in individuals having plasma [phe] > 120 μM; 'phenylketonuria' as an event in those with plasma [phe] > 1000 μM and a diet phe tolerance < 500 mg/day; the term 'non-PKU HPA' is used for those with 120 μM < plasma [phe] < 1000 μM and a diet phe tolerance > 500 mg/day (1;7). 'Variant PKU' is the term used for the patients who do not strictly fit the description for either PKU or non-PKU HPA (8). In reality these terms simply describe places on the spectrum of a metrical trait called HPA.

The prevalence of the PAH-deficient forms of HPA is in its broadest terms 1 in 10,000 live births in Caucasian and East Asian populations, with a heterozygote carrier frequency of 1 in 50 (1;8). However, ethnic variation in the frequency of so-called PKU has long been evident (9), with population rates as high as 1 in 2000 (Turkish) and 1 in 4500 (Irish) and as low as 1 in 43,000 (Japanese) and 1 in 200,000 (Finn and the Ashkenazi Jewish); the frequency of the non-PKU HPA shows less stratification and is more uniform (1;8).

The Phenylalanine Hydroxylase Gene

The PAH-deficient forms of HPA are autosomal recessive traits at clinical and metabolic levels of phenotype and codominant at the enzymatic level (OMIM 261600). They result from mutations in the phenylalanine hydroxylase gene (*PAH*) which encodes the PAH enzyme (1). The human *PAH* gene is localized on the long arm of chromosome 12, band region q22-24.1 (10). It extends over ~100kb of genomic DNA (11). It encodes 13 exons, which cover less than 3% of the genomic length (11;12). The complete genomic sequence is soon to be reported (D. Konecki, personal communication); meantime the full-length complementary DNA (cDNA) has been cloned and the nucleotide sequence determined (GenBank U49897.1)(12). The corresponding mRNA species is approximately 2500 nucleotides in length and contains the complete genetic code for the functional PAH enzyme (11;13). The 5' untranslated region encodes *cis*-acting, *trans*-

activated regulatory elements and 5' potential transcript initiation (CAP) sites; it lacks a TATA box and has sequences similar to GC boxes, all features of house-keeping genes (11).

Mutations Associated with PAH Deficiency

To recognise the rapid evolution of mutation research, one only needs to go back 32 years. In 1968, Woolf et al. reported the possibility of a third allele at the *PAH* locus, which resulted in distinct phenotypic characteristics, different from both the 'normal' and the 'typical' PKU allele. They were not sure whether an observed phenotype was caused by a variant form of PAH enzyme or if it was an effect brought about by other enzymes involved in phe metabolism (14). In 1971, Ara Tourian suggested the possible presence of yet another site for phe-PAH interaction, distinct from its catalytic site, resulting in a genetic variant that caused 'atypical' PKU with PAH enzyme activity of around 5% normals in the affected patients (15). Today we know of several hundred (>380) disease-causing alleles harbored by the *PAH* gene on more than 80 different haplotypes (16). The phenotypic outcome of these mutations covers a complete spectrum, ranging from benign polymorphisms to non-PKU HPA and variant PKU to the severely affected PKU phenotype. In the majority of cases, the identification of mutant genotype can broadly predict severity of the disease (17;18) and could indicate the level of phe tolerance of the individual patient (17).

Disease-causing *PAH* mutations fall into 5 classes: missense, small deletions or insertions (usually with frame shift), modifiers of mRNA splicing, termination (nonsense) alleles; large deletions are rare in this gene (1). The missense alleles cover ~ 65% *PAH* mutations (19) in comparison to the ~50% of disease-causing human alleles (20). Approximately a half dozen *PAH* mutant alleles account for over 50% of HPA cases in the Caucasian population; the remaining mutations are either rare or private alleles (1;16). Different sets of alleles are found in Orientals; again a few prevalent, most rare (16;21). These

studies have provided interesting insight on the saying that the history of the population can be history of the alleles. They have shed light on the variety of genetic backgrounds on which mutations are found and the spatial distribution and relative frequencies of a number of these mutations (22). Therefore, human genetic diversity at the *PAH* locus can teach us a great deal about the occurrence of migration, genetic drift, natural selection (perhaps), recurrent mutation and intragenic recombination over the past 100,000 years (1;9).

Disease-causing mutation in *PAH* have their effect at three phenotypic levels: (*i*) 'proximate', affecting enzyme structure and function; (*ii*) 'intermediate', affecting metabolic homeostasis; and iii) 'distal', affecting brain structure/function, with associated cognitive developmental deficiencies (1).

PAH Expression

Transcription of the *PAH* gene with its TATA-less promoter is regulated by multiple *cis*-acting elements (11). The *PAH* gene has developmental- and tissue-specific transcription/translation, proposed to be regulated at the transcriptional level (1;23). Since the proximal promoter region of the human *PAH* gene has no tissue-specific transcription factor binding sites, it has been suggested that the tissue-specific expression of the gene results from the combined effect of the interaction between multiple protein factors and multiple *cis*-elements (23;24).

Phenylalanine hydroxylase is present in a number of organisms from bacteria to humans (25). PAH appears in the cytoplasm (26). In humans, PAH enzyme is active in hepatocytes, with new evidence that kidney also harbors significant phenylalanine hydroxylating activity (1;27-30). In addition, there is evidence of some illegitimate transcription as demonstrated by the presence of mRNA in lymphocytes (31). In rodents, phenylalanine hydroxylating (symbol Pah) (EC 1.14.16.1) activity can be found in liver, kidney and pancreas (32;33). It has been suggested that detection of Pah activity in the pancreas is due to the common developmental origin of the pancreas and liver (34).

The PAH Polypeptide and Enzyme

The *PAH* gene translates into a 452-amino acid polypeptide with a molecular weight of 52kD (one subunit 50Å x 45Å x 45Å) (35). The wild-type enzyme forms a homooligomer which is in equilibrium between its tetrameric and dimeric forms (36). The dimer is the minimum functional unit of purified PAH (37). The equilibrium can be shifted to the tetrameric form and activated by preincubation with phe and by lowering the pH (26;35;36;38;39). The minimum molecular requirements for the normal reaction are: PAH, oxygen, L-phenylalanine and tetrahydrobiopterin (BH_4) cofactor (1).

With the use of X-ray crystallography, the structure of residues 117-427, containing the catalytic domain of human PAH, was determined at a resolution of 2Å (40). These crystallization experiments showed dimeric forms of PAH missing the N-terminal regulation and C-terminal tetramerization domains. Therefore the same team went further to crystallize and determine the structure of a truncated form of human tetrameric PAH (residues 118-452) (38). Finally the conformational properties of the N-terminal domain were studied by the use of infrared (IR) spectroscopic methods (39).

The enzyme is composed of three domains: regulatory (residues 1-142), catalytic (residues 143-410), and tetramerization (residues 411-452) (13;35;36;41-43). The secondary structural conformations and their internal and intra-subunit (monomer) interactions have been studied indicating a pronounced asymmetry in the packing of the four subunits (38).

The subunit encoded off the *PAH* gene, then folds into tertiary and quaternary structures, determining the catalytic efficiency, substrate specificity and binding ability of the enzyme, each of which can be altered in the presence of a mutation (43). Patients with typical PKU display < 1% normal PAH activity while those with non-PKU HPA had more enzymatic activity (> 5% normal) (1). In some cases, interindividual variation in hepatic enzyme activity has been attributed to allelic heterogeneity. In addition, allelic interaction may play a role

where it is much more likely to reduce the catalytic activity than to enhance it (44).

Mutations affecting the PAH enzyme can be divided into four groups by their apparent effect: (*i*) 'null' alleles (no activity); (*ii*) 'Vmax' alleles (reduced activity); (*iii*) 'kinetic' alleles (altered affinity for substrate or cofactor); (*iv*) 'unstable' alleles (increased turnover and loss of PAH protein) (1;45).

The combined expression of two mutant alleles determines the metabolic phenotype. Yet the independent activity of the mutant alleles is important for broad prediction of the probable phenotype in newborn HPA cases. If a patient carries two 'severe' mutant alleles each associated with classical PKU, the clinical outcome is classical PKU. However, if there is a 'mild' mutation on one chromosome and a 'null' on the other, the result is likely to be a mild phenotype owing to the codominance of the 'mild' allele (46).

To study the effect of a mutation, a number of prokaryotic and eukaryotic *in-vitro* expression systems have been adopted, with the mammalian cell expression systems probably providing a more direct reflection of the human situation *in vivo* (47). Most expressed missense mutations in *PAH* provide evidence of misfolding with an associated increased aggregation (48;49); there are, however, a few stable mutant proteins that show normal levels of PAH immunoreactive protein with decreased enzymatic activity (47). Mutant proteins that have an increased tendency to aggregate tend to have enhanced degradation. Therefore, Waters *et al.* proposed that degradation of misfolded protein could be a general mechanism by which missense alleles cause PKU and related forms of HPA (48;50). Variability in the cellular handling of the protein (background genetics) may also have a significant impact explaining the range of PKU/HPA phenotypes (48;51).

Phenylalanine Homeostasis

Claude Bernard referred to the constancy of the internal milieu as a necessary condition for life (52). The phenylalanine concentration is a metrical trait with an observed steady-state and a central tendency (homeostasis). HPA results when the non-peptide bound (free) phenylalanine is greater than the normal frequency distribution, reflecting a different (higher) steady-state value (1).

The metabolic steady-state is dynamic. Concentrations of metabolites in the system are fixed through fluxes imposed during standard daily dietary intake. However, persistent change in a flux will eventually change the steady-state value (53;54). Enzymes do not act in isolation; these steady-state values are determined by kinetically-linked enzymes that interact via their substrates and products. The steady-state of phe is determined by processes that lead to the net disposal and the replenishing of the plasma pool (55). Therefore, the fluxes through the system are under the control of the loci covering the overall mechanism. The level of contribution by a particular locus (enzyme) is described by its sensitivity coefficient, Z, which is defined by the fractional change in flux over the fractional change in enzyme activity; the sum of all the coefficients combined is always unity *in vivo* (56).

The free phe pool in the normal subject is derived from two sources: intake of exogenous dietary protein and turnover of endogenous polypeptides. At normal physiological concentrations (<120 μM), hydroxylation of phe to tyr by PAH accounts for approximately 75% of the phe runout in the system with the remaining quarter incorporated into protein (see Fig. 1) (57;58). In addition, different urinary excretion and distribution of amino acids between extracellular and intracellular water and tissue compartments may also have small but significant roles in phe runout (46;59). Other metabolite pathways for the runout are normally of minor consequence (see Fig. 1). However, in the absence of, or at reduced levels of PAH activity (with elevated plasma concentration of phe ~

0.3 - 0.5 mM), the transamination pathway becomes significant. The correlation between the excretion of each of the individual phe metabolites becomes statistically significant at these levels (60). The reaction is not fully operative in the immature newborn (61).

Phenylalanine transaminase initiates this alternative pathway by modifying the alanine side-chain of phe (55). This pathway is also active in tissues other than liver (1). This initial step involves converting phe to phenylpyruvic acid. Phenylpyruvate is subsequently converted to phenyllactic acid (PLA), phenylacetic acid (PAA), and phenylacetylglutamine. Decarboxylation to phenylethylamine (PEA), a tertiary pathway, is a trivial part of the whole (see Fig. 1) (1;55;62).

Treatment Outcome

PKU is now a treatable genetic disease and occurrence of the associated mental retardation is unusual in the treated patient. The treatment entails dietary restriction of phe intake. A large ongoing collaborative study, evaluating the outcome of early-treated PKU cases, showed that IQ scores at 6 years of age were directly related to maternal intelligence, treatment age of onset, and average life-time plasma phe values during treatment. With early treatment, patients with good dietary maintenance attain an IQ score in the normal range. However, PKU probands, as a group, have a small IQ deficit relative to their normal sibs. Patients who terminated treatment early scored lower than those who continued it longer (63;64).

Short-term periods without treatment, however, tend not to have a devastating affect on the older patients. One study measuring neuropsychological outcomes showed that treated older children sustaining 3 months of recurrent HPA suffered minimal if any harm to psychological function (65). This supports the idea that by late childhood, the vulnerability of the nervous system to the neurotoxic effect of phe may be significantly reduced (65).

Patients, treated by diet, with satisfactory IQ outcome, may however display other (non-cognitive) problems. These include significantly increased tooth wear (66), osteopenia with prominent bone loss (67) and other physical phenotypes such as decreased skin and hair pigmentation, when compared with controls (8;68). Patients tend to meet their genetic potential and have normal pubertal development for height, although they tend to be mostly overweight (69-72). They show impaired abstract reasoning and executive functions, behavior may be more extroverted and they may have problems with task orientation. They also have twice the normal frequency of neurotic and emotional disorder and hyperkinetic behavior (73-77).

In the event of treatment termination in adulthood, as is often the case due to the difficulties associated with the current therapy, patients can suffer from agoraphobia, depression and anxiety, with impaired vigilance and reaction times. They are deficient in social quotients with substantial behavioral problems (75;78-81).

Brain Pathogenesis

There are 'threshold values' for plasma phe that result in apparent neurotoxicity in PKU patients. For an acute effect, a value of 1300 μM (82) is assumed; however chronic values >120 but < 600 μM (83) can still result (in treated patients) in changes visible by magnetic resonance imagine (MRI), and IQ score distributions below the normal range. It is therefore suggested that any degree of persistent HPA could be harmful to the brain, particularly during early life. If recurrence of HPA occurs in later life, a reversible acute neurotoxicity will most probably appear at first, and if the HPA persists, irreversible chronic neurotoxicity could result (1).

Pathogenesis of cognitive development in PKU has been studied for many years; however 'cause' is poorly understood. Phenylalanine can interfere with the development and function of the central nervous system (CNS) by different

mechanisms; however, no single process by itself seems sufficient to explain the brain phenotype in PKU (84). One of the suggested mechanisms involves phe transport across the blood-brain barrier (BBB). At least two components contribute to this effect. One is trans-membrane fluxes of phe into parenchymal cells (achieving net intracellular accumulation of phe). It requires a system independent of the one used by several other large neutral amino acids to enter the cell. However, these amino acids exit parenchymal cells on the same system as phe. This system is blocked in the presence of high phe concentration (85-91). The other is that large neutral amino acids are transported across the BBB by a common saturable carrier; phe has the highest affinity for the system, hence in the presence of excess phe, the cellular influx of these amino acids is impeded (the system is specific for large neutral amino acids). Therefore, the net concentration of these large neutral amino acids in brain will be reduced as they will be both trapped in the parenchymal tissues and will have to compete at the BBB (86;87;91).

The transport of large neutral amino acids is an important control point for the overall regulation of cerebral metabolism, including neurotransmitter production and protein synthesis. The inhibited uptake (by phenylalanine) of amino acids, tyrosine and tryptophan, into the brain results in reduction of serotonin and catecholamine (82;92;93). Serotonin, and the catecholamines (dopamine and norepinepherine) are neurotransmitters that act in both central and peripheral systems, are involved with the modulation of psychomotor function, aid in vascular tone maintenance and blood flow, help control thermoregulation, and modulate pain mechanisms. Hence, any changes in this system can have a profound impact on central and peripheral homeostasis and may lead to neurological complications (94).

Interindividual variation in phe transport is apparent with different concentrations of free phe in the brain tissue of affected individuals; this also influences the brain phenotype in PKU (1). Brain phe concentrations can be

measured by magnetic resonance spectroscopy (MRS) (95;96). In comparing the PKU patients, clinical outcome with various blood vs. brain phe levels, it was found that those individuals with high blood and brain phe levels displayed 'typical' PKU phenotype while those patients (some untreated) with high blood and low brain phe concentrations were of normal intelligence (95;97;98), suggesting that PKU adults with low brain phe concentration (below 0.25mM) are likely to do well clinically despite high blood concentrations (97). No statistically significant differences were found in the regional concentrations of phe (96).

Other studies indicate that cerebral protein synthesis is inversely related to plasma phe, and this effect is apparent at phe concentrations as low as 200-500 μM (99). Increased phe concentrations in the brain results in decrease proliferation and increased loss of neurons (100-102). DNA content is apparently reduced in the affected brain cells and its synthesis is impaired (102;103). Elevated phe levels result in dysmyelination and can cause decreased neurotransmitter receptor density and cell connectivity (104), decreased dendritic arborization and a number of synaptic spines (105), all associated with brain dysfunction in HPA (106;107). Toxic levels of phe are also shown to affect neuronal development during axonal maturation, resulting in hypomyelination in the outer cortical layers. Myelination appears to be severely inhibited during the critical developmental period (108).

Finally, histological and biochemical analyses of brain samples from PKU mouse orthologues and on *in vitro* cultured oligodendrocytes of wildtype mouse brain exposed to high concentrations of phe show evidence of myelinating oligodendrocytes, adopting a non-myelinating phenotype and over-expressing a glial fibrillary acid protein (GFAP). In addition, in the PKU mouse, the increased turnover of myelin is associated with loss of neurotransmitter (muscarinic acetylcholine) receptor density (104;109;110).

The Challenge of Maternal HPA

Following the control and normal development of the affected patient, there is one additional concern that plagues female patients. Maternal hyperphenylalaninemia is a form of teratogenesis, imposing its cost on the next generation. The phenotypic effect observed here is dependent on the two mutant alleles carried by the mother where the obligatory heterozygous *PAH* genotype of the fetus is of little functional significance (111). Because the placenta pumps phe to the fetus, untreated pregnancies generally result in severe embryopathy and fetopathy, with other complications. There is a greater than 80% chance of microencephaly, mental retardation, malformations of the heart (~7.5-12% probability) and other organs, esophageal atresia, dysmorphic facial features, and intrauterine growth retardation in the unborn child (112). Congenital cardiac defects occur at the rate of ~ 7.5-12% in children born to mothers with PKU, but only at 0.8% in the control population (113). Prevention is therefore essential and treatment necessary for the well being of the fetus.

The need for preconceptional and intrapartum treatment to prevent HPA teratogenesis was highlighted by Scriver in 1967 (114) and its relevance documented by Lenke and Levy in 1980 in their study of the outcome of over 500 untreated HPA mothers (115). It is very effective in preventing teratogenesis. The level of HPA is important when assessing the danger to the fetus, as no cases of congenital heart disease were observed in babies born to mothers with mild HPA (116). However, the ultimate reproductive outcome in maternal PKU is dependent on both prenatal metabolic control and postnatal environmental circumstances. These factors greatly depend on the educational resources provided for the PKU mother (117).

Unfortunately ~60% of female PKU patients become pregnant unintentionally and therefore are not in metabolic control. Such pregnancies are usually late-treated and expose fetuses to elevated maternal phe levels (118). In addition, there are women with undiagnosed PKU (who may be well in the range

of normal mental function) whose fetuses are at risk (119). Consequently, a very careful management of maternal HPA is necessary, albeit difficult. Without treatment the beneficial effects of newborn screening for PKU would be lost in one generation, as untreated PKU women would give birth to defective children (120;121). Prevention requires identification and education (with reproductive counseling) of women with HPA in the reproductive age group (112).

The Maternal Phenylketonuria Collaborative Study was established to assess the efficacy of phe-restricted dietary regime and estimate the quality of outcome in reducing morbidity among the offspring of women with PKU (116;118;122). The report states that the offspring derives the most benefit when treatment is instituted before conception or by 8 to 10 weeks' gestation (to levels $< 600\ \mu M$), significantly reducing the chances of teratogenesis. Preliminary data suggests that optimum intellectual status of the offspring follows preconceptional and intrapartum treatment (116).

Relevance of Animal Models

PKU-related pathologies have been very difficult to study and analyze. Some of the reasons for this are the inherent limitations in using human beings in controlled studies, including low participant numbers (due to the rarity of PKU), population heterogeneity with respect to genetic constitution, and participant compliance with the parameters of the experiment (123). To this end, the availability and use of animal models have been invaluable. Surrogate organisms with uniform genetic background allow the accumulation of statistically strong data (as a large sample size is possible), and the manipulation of environmental conditions to evaluate their contribution to the disease (for example, the control of phe intake by animals to reduce variation in blood phe caused by dietary non-compliance) (123).

For many years, hyperphenylalaninemic animal models were created by the use of exogenous phe loads and the introduction of chemical agents such as *p*-

chlorophenylalanine and α-methylphenylalanine to block the Pah reaction (it was necessary to inhibit Pah activity to obtain hyperphenylalaninemia without hypertyrosinemia). However, these chemical agents had additional effects, which resulted in secondary consequences (neurochemical, among others) (124). The obvious shortcoming of such methods is that clear conclusions often can not be drawn from studies using chemically induced models.

To eliminate these drawbacks, Dove and his group at the University of Wisconsin set out to create natural mammalian counterparts (orthologues) of human PKU. The mouse was chosen as the most appropriate surrogate organism.

In the past century, the status of the mouse has been elevated from pest to model, and it now serves as one of the best studied 'honorary humans' (125). Mice are 2000-3000 times lighter than humans; they have gestation and generation periods of 3 and 10 weeks; the average litter size is between 5-10 pups; they have a postpartum estrus; they are easy to handle with a robust health status; their pregnancies can be timed by the detection of a vaginal plug after mating; and they are the most cost-efficient mammal to maintain (123;126). Moreover, these animals allow for extensive genetic manipulation and have a high degree of physiological similarity to humans. There is a growing body of evidence detailing the similarity between the mouse and human genomes as well as the phenylalanine hydroxylase gene and protein sequences (123;127;128). There is currently an international effort to define the mouse structural and functional genome and to create a mouse mutant resource with database and bioinformatics facilities (125). The genetic map of the mouse is the most highly characterized mammalian map, rivaled only by the human map (127). In addition, the map of homology segments between mouse and man is the one nearest saturation for any pair of species in the genome initiative (128). In fact, localization of the mouse *PAH* gene (symbol *Pah*) was achieved by using the rapidly consolidating map of the human genome to identify a putative syntenic group including *Pah*, *PEPB* and *IFNG*. Since the latter two members of this group had been previously found on

mouse chromosome 10, the *Pah* gene was easily localized to chromosome 10 C2 → D1 (33). In 1988, the mouse *Pah* gene was mapped and the cDNA cloned and sequenced (GenBank X51942) (33;129).

Mouse and human genes have ~87% conservation of sequence, with ~ 10% of the nucleotide changes silent. No similarities are observed in the untranslated regions (129). The mouse Pah enzyme has 453 residues (molecular weight, 51,786 Da). Some divergence (~ 8%) exists between human and mouse amino acid sequences with the majority of differences in the N-terminal region; there is a 96% similarity at the C-terminal. (12;129).

To create animal models of a particular disease, two different approaches can be taken: (*i*) The genotype-driven approach is labor-intensive and generally entails large-scale genome-wide mutagenesis through gene trapping in embryonic stem cells. Here, identification of the mutated gene is relatively trivial; the focus is on characterization of genome mutational change. The very obvious drawback to this system is that the genotype-driven route gives no indication of the likely phenotypic outcome. Quite often the model does not closely resemble the corresponding human condition, compounded by the fact that homozygous animals carrying a null allele could die prior to any comprehensible analyses. (ii) Phenotype-driven approaches use mutagenic procedures that focus on the recovery of novel or sought-after phenotypes, without focusing on the underlying gene or pathway that has been disrupted. Here, the problem lies with the identification of the underlying gene (130-132).

One phenotype-driven approach is by chemical mutagenesis using *N*-ethyl-*N*-nitrosourea (ENU, $C_2H_5N(NO)CONH_2$). ENU is a DNA-alkylating agent, which is currently the most powerful mutagen known for mouse germline (131-133). It is a potent mouse spermatogonial stem cell mutagen (134) mainly creating point mutations (i.e. A-T base pair substitutions as well as small intragenic lesions rather than large deletions). ENU injected into male mice mutagenizes premeiotic spermatogonial stem cells. Therefore, one treated male can produce a

large number of F1 animals carrying different mutations (126;135). ENU induces mutations at a frequency of ~ 10^{-3} per locus. Therefore ~1000 gametes need to be examined from an ENU-treated male mouse for any specific mutant locus recovery (135;136).

Identification of new recessive mutations requires mating of the ENU-treated males to wild-type females. The F1 progeny (G1) are bred to wild-type mice, establishing families of siblings (G2) sharing common mutations. G2 progeny can be back crossed to G1, producing G3 progeny that can be assessed for recessive mutant phenotypes. The initial strains of hyperphenylalaninemic mice were selected using this breeding strategy, including mutations in the gene for *Pah* (135;137).

To produce these mouse orthologues, inbred ENU-treated BTBR/Pas (genetic background) males were mated to normal BTBR/Pas females. The BTBR/Pas strain was chosen because of its excellent breeding ability and because it was inbred for many generations (carrying a uniform genetic background) (138). Selection required the three generation breeding scheme described above followed by the phenotype test (retarded ability of the animals to clear a phe load). Four different mutant autosomal recessive lines were initially selected using this strategy. In the first one (*hph-1*), HPA was mutated at the *guanosine triphosphate cyclohydrolase* locus (GTP-CH catalyzes the first step in biosynthesis of BH_4 - the essential cofactor for hydroxylase activity) (137). The Pah^{hph5} mutant (now renamed $Pah^{enu1/1}$, ENU1) was identified next. This model is mutated at the *Pah* locus (139). Once one *Pah* mutant strain was established (ENU1), further allelic variants were selected by mating the newly selected F1 animals to the homozygous established mutant; extended HPA following a phe challenge was again the selection criteria (127;136;139-141). ENU allowed the generation of multiple alleles increasing the depth of the mouse mutant resource at this locus (135).

To date, three mutant Pah enzyme-deficient homoallelic and one heteroallelic mouse orthologues of human PKU and non-PKU HPA have been described: ENU1, *Pah*$^{enu2/2}$ (ENU2), *Pah*$^{enu3/3}$ (ENU3) and *Pah*$^{enu1/2}$ (ENU1/2). The homozygous ENU1 mouse was reported in 1990 (139). ENU1 mice have a c.364 T → C transition (V106A) in exon 3. Val 106 is conserved among the mammalian species but is divergent in the others (142). This mutation falls in the N-terminal regulatory domain of the protein, distant from the catalytic residues of PAH/Pah, as evidenced by crystallographic analysis of the human enzyme (38;40). This factor, in conjunction with the conservative nature of the predicted Val to Ala substitution, may explain the mild phenotype of the ENU1 animals (142). On regular diet (Teklad, #8626), these animals display normal plasma phe levels and normal coloration with no abnormal behavior. No apparent effect of maternal phenotype on the fetus is observed; progeny shows normal growth and survival of progeny rates. Under conditions of elevated phe in diet, reduced growth is observed (139;141).

ENU2 and ENU3 strains were reported in 1993 (141). Both strains are classified as PKU counterparts. The ENU2 mutation is a c.835 T → C transition (F263S) in exon 7 (142). Phenylalanine 263 is in a highly conserved catalytic domain of the protein. Also present in the active site, which is proposed to be important for pterin binding (35;43). The presence of mutation in this critical region in conjunction with the radical phe to serine substitution may explain the severe phenotype observed in ENU2 animals.

The ENU3 mouse mutation is still not reported; however, it has been localized to a 301 bp portion of the coding sequence, spanning part of exon 11 and all of exons 12 and 13 (142). Neither ENU2 or ENU3 have measurable hepatic enzyme activity; on normal diets, they both have 10-20 fold elevated serum phe levels and elevated phenylketones in urine. These animals display retarded pre- and postnatal growth, microcephaly, pronounced hypopigmentation and behavioral abnormalities which start 2 weeks into adulthood. These include

toppling while grooming, lack of alertness, too uncoordinated to swim and impaired discrimination, reversal and latent learning (141;143). In addition, ENU2 mouse brain analyses have shown reduced amine contents, metabolism and release (144). Both strains display severe maternal effect on the fetus with complete loss of litters within several hours of birth (141). These reproductive problems solely depend on the maternal genotype and dietary phe intake; paternal or fetal genotype have no effect (123). Such pregnancies have also been terminated pre-term to analyze the heart tissue; a variety of cardiovascular defects beginning at 75% through gestation was observed. The defects ranged from mild to serious and were mainly vascular in the mouse (145).

A hybrid ENU1/2 mouse orthologue for a non-PKU HPA phenotype has been developed. It is the topic of Chapter 2 in this thesis and the corresponding publication in Appendix A.

These orthologous animal models have provided a surrogate organism for the study of potential therapies and made possible the study of mechanisms underlying the pathophysiology in PKU and related non-PKU HPA phenotypes.

Phenylalanine Transamination Metabolites

Treated PKU patients whose blood phe levels range between 300-600 μM excrete 6-16 times the normal amount of the phe transamination metabolites (60). In an *in vivo* study with stable isotopes measuring the disposal of phe in PKU, Treacy *et al.* proposed that the variation in formation of phe transamination metabolites, and differences in renal clearance of these metabolites, play a role in dietary phe tolerance in PKU (146). However, generally these pathways do not significantly reduce the excess phe in the system and therefore do not nullify its possible toxic effect. It is also extremely unlikely that pronounced HPA can be caused by a lack of transaminase (55). Therefore, the question remains whether phe itself or one of its metabolites is the cause of the neurotoxicity in HPA.

Urine, plasma and cerebrospinal fluid (CSF) sampling and analysis by various methods have shed some light on this issue. Elevated urinary levels of PEA do not result in clinical deterioration providing no support for the idea that elevations in peripheral levels of PEA will interfere with normal brain function (147). Loo *et al.* showed that elevated organic acid derivatives of phe cause microcephaly and myelin defects in PKU rat models exposed to phe and *para*-chlorophenylalanine (148). However, the actual metabolite concentrations required to bring about this effect were approximately of two orders of magnitude higher than the levels reached in HPA/PKU (147). In addition, Perry *et al.* who compared various biochemical characteristics in two adult brothers with untreated PKU (one with a severe mental defect and the other with superior intelligence), found no difference in urinary phe metabolite levels or in their degree of HPA (149), suggesting that toxicity was not caused by any one of the acid metabolites at the concentrations presented in HPA/PKU. Wadman later reported on two sisters with normal mental development that had permanently increased amounts of phe transamination products measured in their urine, while their levels of phe were normal (150). These findings provided more support for the non-toxic status of phe metabolites and further implicated phe as the chief villain associated with HPA pathology. No further substantial proof of neurotoxicity was gathered since these earlier studies (147) and the data highly suggest that "there are no abnormal metabolites in PKU, only normal metabolites in abnormal amount"(151). However an effect of phe metabolites in the brain could not be fully ruled out as no method directly measuring brain metabolites was available (55;84;147;151;152). Therefore, such a technique and a good comparable natural model (human surrogate) for brain sampling were two very useful resources to elucidate this issue. This is the focus of Chapter 3 in this thesis and the corresponding publication in Appendix B.

Treatment

Phenylalanine is an essential nutrient and in addition to its requirement for protein synthesis, it serves as a precursor for tyr and tyr derivatives. In its absence, tyr becomes an essential amino acid. Therefore, the treatment of HPA requires the balanced reduction of systemic phe concentration without excessive depletion plus the provision of satisfactory amounts of tyr, a by-product of phe hydroxylation (1).

Hyperphenylalaninemias are multifactorial disorders, as the dietary experience and mutant genotype are both necessary components of the cause. Accordingly, the metabolic phenotype is also multifactorial, providing the opportunity for dietary treatment (1). PKU treatment is a classic example of euphenic therapy (Lederberg's term) where the aim is to restore the normal phenotype without genetic modification. It is necessary to manipulate the experience to maintain health (1;153).

The experience here is control of dietary phe. An artificial diet to reduce phe intake became available in the mid-1950's (154-156). The story began when Louis Woolf, a biochemist at the Hospital for Sick Children, London, convinced his colleagues to put a PKU patient on a diet low in phenylalanine. Sheila Jones, 17 months of age at the time, was the child chosen for this project. Woolf prepared a diet low in phe, by passing a protein hydrolysate through active charcoal (157) and with the help of John Gerrard, Evelyne Hickmans and Horst Bickel, administered it to Sheila (154;155;158). In a short time, Sheila's systemic phenylalanine levels were reduced and although her mental status did not change appreciably, it was evident to the research team and her mother that she was benefiting from the treatment. She was able to make eye contact for the first time and smile; she had stopped drooling and was starting to walk. Because of the skepticism among their colleagues, Bickel and his team decided to add phe back into the diet without telling Sheila's mother. One week later, Sheila was no longer making eye contact or smiling, was again drooling and had stopped

walking (158;159). It was a long road from these initial experiments to the institution of dietary therapy, but the proof of principle was there. Efficacy of dietary treatment in the prevention of severe IQ loss, became uniformly accepted by the late 1960's (1), and a population screening test was soon developed (160) to identify new-born patients early and to initiate therapy to prevent postnatal cognitive impairment.

The current treatment regimen involves the careful regulation of dietary L-phenylalanine to < 500 mg/day, sufficient to support protein synthesis while preventing excess accumulation of phe in the free pool. The exact tolerance for phe (200-500 mg/day) varies between patients even when the same mutant *PAH* genotype is present (1). A semi-synthetic diet, low in phe, presumed to be adequate in other nutrients, is used to treat PKU and non-PKU HPA. Better plasma phe homeostasis is observed if the amino acid intake is distributed throughout the day (161;162).

Up to the early 1970's, it was suggested that diet, stopped completely at the age of 6-9, would result in normal intellectual performance and behavior. At that time, the main concern was reintroduction of diet during the reproductive years of the affected female population, as a possible teratogenic role of PKU on the unborn child was suspected (163). However, by the 1980's, reports on problems such as learning disabilities among patients with early treatment termination, urged reconsideration. It was at this time that recommendations for 'diet-for-life' treatment was introduced (163). In the 1990's, clinicians realized the difficulties inherent in lifetime dietary treatment. The need to consider the quality of life for adolescents and adult PKU patients played a major role in this debate (163), since adults with PKU can tolerate a much higher level of phe than children, without the same devastating outcome. Today, quality of life, dietary cost, and varying genetic and socioeconomic backgrounds have all become major components in dietary recommendations for adults (97). Treatment is currently recommended until at least the ages of 10-12 when the brain is suspected to be

fully developed (8;164;165); however, life-long compliance is advised (166;167). In addition, reintroduction of diet can be used as a definite option when a patient who has abandoned the diet develops associated shortcomings. A significant improvement in attentiveness, a reduction in hyperreflexia and shortening of the latencies of the evoked potentials has been observed, even if the patient has been treatment-free for a number of years (168). The optimal treatment recommendations today generally entail early onset (within one month of birth) continuous treatment throughout childhood, adolescence, conception and pregnancy, with more and more evidence for benefits with lifetime control (169). If controlled levels are kept below ~ 360 μM in early childhood, a positive outcome is expected. The individual should be able to attain normal executive function (defined as higher goal-directed mental activity, organizing function and dependent on good control of attention) (170). Delayed treatment generally results in an IQ deficit of ~ 4 points for each month's delay until treatment onset, for each 300 μM rise in average blood phe levels and for each 5 month period during infancy when blood phe concentration stays below 120μM (171). However, late diagnosis (~ 3 months of age) and treatment still results in significant intellectual improvement. Therefore it is suspected that society could benefit substantially by providing a phe-restricted diet for late-diagnosed mentally retarded persons with PKU (172).

Treatment Difficulties and Other Associated Drawbacks

Although dietary treatment is simple in theory, it is demanding for the patient and the caregiver in practice. In fact, controlled phe intake, while meeting the nutritional needs of the patient at various growth periods, is a science by itself. It involves disruption of culture (food), lifestyle (dietary choice), family behaviour (divided culinary activities) and so on. As the patients gets older, his/ her energy and protein requirements decline, the dietary needs evolve and must be adjusted accordingly (173). The importance of complete or almost complete intake of the

recommended amount of phe-free amino acid mixture cannot be overstated, as it plays a major role in the control of blood phe level of patients (174).

Maternal PKU also requires treatment and is now recommended when maternal phe levels exceed 400 μM (169). Nutrition takes top priority in any pregnancy, but in women with PKU, the goal is 2-fold: (*i*) providing adequate nutrients for fetal growth and (*ii*) controlling phe levels to protect the fetus. The women must consume the low-phe diet that includes multivitamins, vitamin B12, and folic acid before and during pregnancy. Furthermore, the gestational age at which the mother attains metabolic control is an important factor associated with outcome (118). The blood phe levels must be adjusted between 120 to 360 μM prior to conception and throughout the pregnancy (113). Duration of treatment prior to conception should take into consideration the fact that severe or moderate PKU patients, who are planning a pregnancy, may have more difficulty in getting their blood phe levels within the recommended range (117).

The principles of treatment for PKU, non-PKU HPA may be clear enough; however in practice, restoring normal homeostasis in tolerable fashion, is often something else (153). Although there are general guidelines for treatment of patients at specific developmental periods in life, other considerations also come in to play, not least of which is the genetic background of the patients. Each patient has his or her own form of HPA; accordingly, he/she requires his or her own stringency of therapy (153), further complicating the treatment process. The first consideration is the individuality of the HPA state. Diagnosis that characterizes severity of the disease provides objective and effective criteria for therapy of each particular case (175). Steady-state concentration of blood phe, blood phe clearance rate following an oral phe challenge, and dietary phe tolerance are all values that help in the decision about the degree of phenylalanine intake restriction necessary to achieve a satisfactory clinical outcome (55). The recent findings on brain phe concentration may also have a role in determining dietary stringency and adult need for life-long control, raising the fundamental

question of whether treatment should be based on the concentration of phe in the brain or levels in the blood, or both. It may mean that patients with high transport levels would require more stringent dietary restriction than recommended by the general guidelines; the reverse being true for those with low transport levels (97).

The overall advantages of dietary HPA treatment are obvious. Biochemical abnormalities are reversed, preventing neurological deterioration, which in turn improve neuropsychological performance and prevention of teratogenesis in maternal HPA. However, the diet is difficult to enjoy (poor in organoleptic qualities and boring in composition), and not perfect as full compliance is very difficult and requires a great deal of social support. There is also a continual worry about nutrient imbalance and deficiency (176-178). Cause for concern are imperfections in the composition of low phe diets, detected deficiencies in trace minerals: selenium, iron, copper and chromium; reduced levels of Vitamin D, cholesterol, carnitine, lack of the essential nutrient, the fatty acid decosahexanoic acid, and other nutrients which are necessary for normal development (152;153;179-181). In addition, protein catabolism occurs when nutrition is not adequate, releasing phe and increasing the free pool (1). Fever and infection can further complicate the problem as elevated blood phe is observed during such episodes (182). Finally, phe controlled diet initiated prior to conception, or early in the first trimester, may still result in abnormalities (183).

Compliance with dietary therapy remains a major challenge. Teaching and support of an experienced healthcare team of physicians, nutritionists, genetic counselors, social workers, nurses, and psychologists are very necessary components in improving the chances of successful treatment (8). Efforts to improve the organoleptic properties of the diet continue. Some of the simplest solutions have been the production of less offensive amino acid mixtures by varying the formulation (184), or by masking them through the use of gelatine encapsulation (initially introduced to accommodate the delicate tolerance of pregnant PKU

females) (185). Other research, focusing on expression of genes that translate into modified phe-free protein products in corn are in progress (186) and transgenic cow production of modified human protein (alpha-lactalbumin) lacking phe, are also currently in progress (187).

Non-Diet Treatment Options

Alternative forms of therapy deserve consideration.

Treatment by Liver Transplants

An orthotopic liver transplantation was conducted on a 10-year-old PKU patient from Italy who also had concomitant, unrelated end-stage chronic liver disease. The transplantation was successful and a complete correction of the metabolic abnormalities was observed. Liver function was promptly restored with normal phe tolerance; blood [phe] fell from 9.5 mg/dl (pre-transplant with phe restricted diet) to 1.0 to 1.2 mg/dl (post transplant on normal diet or with oral protein load). The success of liver transplantation observed confirmed that the liver is the chief site of the enzymatic defect in classic PKU (188). Although this method was effective in correcting HPA in the single patient, because of the shortage of donated organs, complications with anti-rejection therapies and the good results achieved with early dietary restriction, it is not an appropriate option for otherwise uncomplicated PKU in the population of patients (188).

Gene Therapy

Germline gene therapy could result in a potential 'cure' for the successive generations of PKU individuals, but is the least likely option because of technical and ethical reasons. On the other hand, research into somatic gene therapy has shown great potential. Human *PAH* cDNA was expressed in a series of cultured mammalian cells. Transient expression was observed here, as integration of the cDNA was not stable. This problem was overcome by the use of recombinant

retroviruses where the gene was transmitted to subsequent generations (1). Primary hepatocyte cultures from the Pah-deficient mouse have been transfected with wildtype mouse *Pah* cDNA. Transduced cells have shown high expression levels of mouse Pah-specific mRNA enzyme activity, and immunoreactive protein (189;190). ENU2 mice, treated with a recombinant adenoviral vector containing a human PAH cDNA, showed normal plasma phe levels for one week; however the effect did not persist (191). This method involves further complications such as surgical procedure (portal vein infusion) and the use of adenoviral vectors (reported to be cytopathic to host cells) (192). Other attempts at gene therapy with the ENU2 mouse have introduced the gene construct with a mouse muscle creatine kinase promoter element. Here they observed Pah expression in cardiac and skeletal muscle but not in liver and kidney. This experiment also required the repeated dosing of the cofactor BH_4; however it successfully demonstrated the locus-specific PAH enzyme activity (it expressed in heterologous tissue}. Euphenylalaninemia was restored when the animals were supplied with abundant cofactor (193). Finally, one other group reported the possibility of gene therapy using T cells from PKU patients. These cells have a small amount of biopterin, but significant dihydropteridine reductase (DHPR) activity. Intracellular biopterin content could again be increased by exogenous BH_4 supplementation. The cells were able to uptake the retrovirus containing the human *PAH* cDNA. The T lymphocytes were able to express all that is required for phenylalanine hydroxylation reaction. T lymphocyte-directed gene therapy is currently the safest established means of gene therapy (192). The above methods are at the experimental stage and will only be used in the absence or failure of all other forms of treatment.

Tetrahydrobiopterin-Responsive HPA Therapy

A subgroup of HPA patients is BH_4 responsive (194). Efficacy in these particular persons is attributed to a residual PAH activity stabilized when saturated

with BH_4 cofactor (so called BH_4-dependent or BH_4-responsive HPA). The particular combination of the mutant PAH subunits is likely to be important for BH_4 responsiveness.

Enzyme Therapy

Enzyme therapy could be done either by replacement of PAH (via transplantation) or by PAH enzyme substitution, with another protein involved in phe degradation.

PAH *Enzyme Replacement*: This is an unlikely option, as a series of problems exist associated with its large-scale isolation and purification of the protein. In addition, the enzyme requires the (very costly) cofactor to function. Replacement therapy with PAH will require the intact multi-enzyme complex for catalytic hydroxylating activity (1).

Enzyme Substitution Therapy with Phenylalanine Ammonia Lyase (PAL) *(EC 4.3.1.5):* This is a potential alternative, probably best seen as an adjunct to modest dietary control. PAL is a robust autocatalytic protein without need for a cofactor (195), an enzyme that stoichiometrically converts L-phenylalanine to *trans*-cinnamic acid and ammonia by nonoxidative deamination (196-198). PAL carries all the information required for catalytic activity (199). Some species that have PAL activity are capable of utilizing phe as a sole source of carbon and nitrogen (200). In fungal cells, this enzyme has a strictly catabolic role (201). In plants, PAL is a key biosynthetic enzyme catalyzing the first specialized reaction in the synthesis of a variety of polyphenyl compounds (i.e. flavonoids, phenylpropanoids and lignin in plants). Induced activity is observed in response to various stimuli, such as injury, light and hormones (202-204). Approximately 30 - 40% of plant organic matter is derived from L-phe through the cinnamate pathway (205).

The complete *PAL* sequence for *Rhodosporidium toruloides* yeast has been reported (GenBank X51513) (201); it contains a 2148-bp open reading frame (206). The genomic sequence contains six introns; relatively small in comparison with those found in higher eukaryotes, with sizes ranging from 50-88bp (201;202;207). It has three transcription initiation sites with non-translated leaders of 24-35 nucleotides (206). The 25 bases upstream of the start codon are A/C rich (76%) as observed in several other lower eukaryotic genes and the 45 bases preceding the transcription initiation sites are pyrimidine-rich (90%), a feature of genes from filamentous fungi and particularly striking in genes lacking the TATA box and in highly expressed genes (206). The size of *PAL* mRNA was shown to be ~2.5kb with at least 50 of these bases consisting of the 3' polyadenylated tail. The mRNA is monocistronic (one gene copy) (207).

The *PAL* sequence translates into a 716 amino acid polypeptide monomer with a molecular weight of ~77 kDa (206). The enzyme is tetrameric, consisting of four identical monomers, pairs of which form a single active site (199;208;209). The molecular weight of the tetramer is estimated to be from 275 to 300 kDa (197).

Little is known about functional groups of PAL; however, there is good evidence that a dehydroalanine is an essential part of the active site. Site-directed mutagenesis of conserved serine residue 143 in the sister enzyme histidine ammonia-lyase (HAL, EC 4.3.1.3) from *Pseudomonas putida*, resulted in complete loss of activity. HAL deaminates L-histidine to *trans*-urocanic acid (210). HAL and PAL are very similar enzymes with high sequence homology; they catalyze the same reaction type and possess the same prosthetic group (211). Serine 212 is the equivalent residue in *R. toruloides* PAL (210;212). Serine is the precursor of the active-site dehydroalanine; the conversion is autocatalytic (195;199). Dehydroalanine is made post-translationally by forming a prosthetic group covalently attached to the enzyme (199). This modification is suspected to be the rate-limiting step in the conversion of the PAL to a catalytically competent

form (212). PAL is also a sulfhydryl enzyme (200). The optimum pH range for PAL activity is 8.0 to 10.5 and the enzyme remains stable up to 50°C, but is destroyed above 60°C (200).

The product, *trans*-cinnamic acid, is a harmless metabolite. It is present in all vegetable matter, is used as a food additive and as a constituent of some cosmetics (196;213) and has no embryotoxic effects in laboratory animals (214). The liver metabolizes it to benzoic acid and it is then excreted principally as hippurate via urine (215). A small amount of cinnamate, unchanged, can also appear in the urine, together with some benzoic acid (196). The ~ 3g of cinnamic acid that would be generated daily with complete dietary phe conversion by PAL is predicted to be harmless to both PKU and normal individuals. The ammonia formed would be metabolically insignificant (196;213).

Route of PAL Administration

The proposed route of (protected) PAL administration is oral and it capitalizes on three prior observations: (*i*) amino acids are in equilibrium between the various compartments of body fluids and ultimately in equilibrium with the intestinal lumen, therefore this mode of treatment affects all body pools (53;85;216); (*ii*) it will modify phe content of body fluids in the whole organism (217;218); (*iii*) the proposed theory of extensive enterorecirculation of amino acids states that the most likely major source of intestinal amino acids is probably from gastric, pancreatic, intestinal and other intestinal secretions. Tryptic digestion converts the secreted proteins, enzymes, polypeptides and peptides into amino acids, which are then reabsorbed back into the body as they pass down the intestine. This forms the large enterorecirculation of amino acids between the body and intestine (219). Oral PAL therapy will deplete the phe pool in the intestine whether the phe source is dietary or from the endogenous run out of free phe from bound pools (219).

Oral administration of PAL avoids any possible immunological problems that may occur with injected enzymes. Such immunological reactions have been reported when animals have been injected with PAL (213;220).

Oral delivery of a protein 'drug', though a worthy objective, can be extremely difficult (221). PAL may have no toxic properties, however the unprotected enzyme, exposed to the gastro-intestinal (GI) environment, would not survive to complete the job; the strong acidic condition of the stomach and the abundance of proteases and peptidases in the GI tract are the reasons (221). Duodenal juices rapidly inactivate PAL, with chymotrypsin working approximately 30 times faster than trypsin, suggesting the presence of a hydrophobic region on the surface of the enzyme (209). PAL activity is stable in duodenal juice with inactivated chymotrypsin and trypsin.

In light of the problems associated with oral therapy, it is necessary to create a protective yet porous barrier that will surround the PAL enzyme before administration. The barrier must provide the following: first, it must prevent the relatively large molecular weight digestive proteases from gaining access to the enzyme; and second, it must provide full phe access to the PAL enzyme for conversion (see Fig. 2). The problem with the low gastric pH is overcome by enteric coating of the free or formulated enzyme. Enteric coating works with neutralization of the expelled stomach contents in the duodenum followed by dissolution of the capsules and intimate mixing of enzyme and duodenal contents. Preliminary studies in human PKU patients showed attenuated HPA following the administration of PAL in enteric coated gelatin capsules (213).

A number of different formulations and mechanisms have been tested to prevent protease inactivation. Artificial cells (semipermeable microcapsules) of cellular dimension with an ultrathin (200Å) membrane have been used to immobilize PAL (222). Administration to chemically induced HPA rats (223) as well as in naturally-occurring HPA in a mouse model has shown significant plasma phe reduction (218). Here, encapsulated PAL acts by the diffusion of phe

into the artificial cells where it is converted into *trans*-cinnamic acid. *Trans*-cinnamic acid is then released from the artificial cell. Entrapment of enzyme in silk fibroin was another proposed method of PAL protection. The investigators here showed effective activity and protection of PAL following direct injection into rat duodenum. Efficacy was measured by the appearance of *trans*-cinnamate (224). Recently, adenocarcinoma cell line Caco-2 (225) as a package delivery system with a protective cell wall has been successfully attempted. Finally, one other route, using a non-oral-extracorporeal multitubular enzyme reactor, with immobilized PAL, was developed to deplete circulating phe. Here a shunt was introduced and the phe-rich blood was pumped through the reactor, the phe-depleted blood pumped back into the system. This was successfully tried on a patient with PKU as the plasma phe levels dropped significantly without the enzyme entering the circulation (226). These studies were not continued because PAL was not available in sufficient amounts at reasonable cost.

Economical production of PAL is now possible, thanks to cloning technology. Protected free PAL 'enclosed' within the *E. coli* cells and studies with the protease inhibitor aprotinin (in the mouse model), provide positive proof of principle that PAL will reduce systemic phe levels in the PKU and HPA mouse models. Thus there is the potential for oral enzyme substitution therapy as adjunct alternative therapy for PKU independent of artificial diets. This project is the focus of Chapter 4 and the corresponding publication in Appendix C.

Proposal

As described above, the focus of a number of projects in the past two decades has been to improve the treatment of PKU. Yet not one of these attempts was effective enough to take the place of dietary therapy. However, with current advancements in animal modelling and related biotechnologies, such as cloning, some of the former difficulties associated with new drug development and measurement of efficacy have been overcome. This thesis focuses on one of these technologies, after an overview of other options. I approached my project on three levels:

I. An Animal Model:

PKU and HPA animal models serve as living systems to measure orthologous human phenotypic outcomes. Their use eliminates problems in population genetic heterogeneity, in standardizing experimental parameters, and with measurements that would otherwise be unacceptable in human subjects. The first objective of this project was to develop a compound heteroallelic ENU1/2 mouse model and to describe and compare their organismal phenotypes with those of the control (BTBR/Pas), mutant strains (ENU1 and ENU2) and their carrier counterparts. To achieve this, the corresponding metabolic parameters, quantitation of Pah protein levels, hepatic Pah enzyme activity, blood and brain phe levels, behavioral parameters and maternal HPA effect on the fetus were all measured.

II. A New Analytic Approach:

A method was devised for specific measurement of phe metabolites in the brain to further describe these animal models and to answer the question of whether phe itself, or one of its metabolites, is the cause of neurotoxicity in PKU. Such measurements must be sensitive enough to analyse samples of trivial volume. Hence the second objective of this project was to develop a very sensitive detection

method that incorporated negative ion chemical ionization gas chromatography/mass spectrometry (NICI-GC/MS) to measure these metabolites.

III. An Alternative to Diet Therapy for PKU

Improvement in the treatment of PKU was my ultimate goal. I used enzyme substitution therapy [with PAL] to replace the current dietary treatment. The relevance of this treatment mode was demonstrated by: (*i*) developing an economical industrial supply of PAL at IBEX; (*ii*) protecting the enzyme sufficiently to allow short-term passage through the GI tract; and (*iii*) providing proofs of pharmacological and physiological efficacy in the Pah-deficient genetic mouse models.

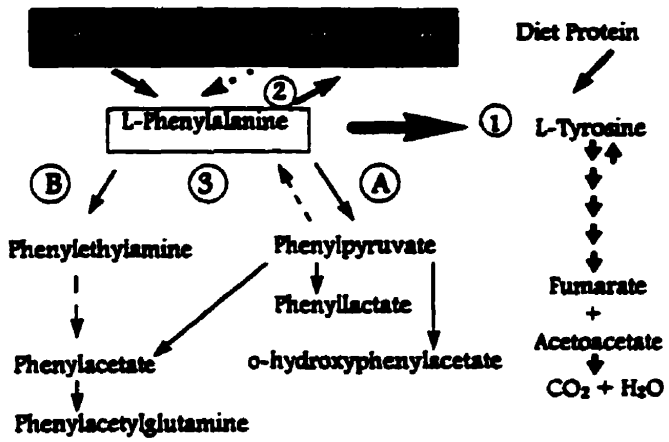

FIG. 1. Major inputs (⇒) and runouts (→) of free L-phenylalanine in human metabolism. Inputs of this essential amino acid to the pool of freely diffusible solute are from dietary protein [hence the minimal dietary requirement] and turnover of endogenous (bound, polypeptide) pools. Runout is by (1) hydroxylation to tyrosine (reaction 1 catalyzed by phenylalanine hydroxylase, followed by oxidation); (2) incorporation into bound (polypeptide) pools (reaction 2); and (3) by transamination (A) and decarboxylation (B). The approximate proportional importance of the three runouts is 3:1:trace at normal steady state.

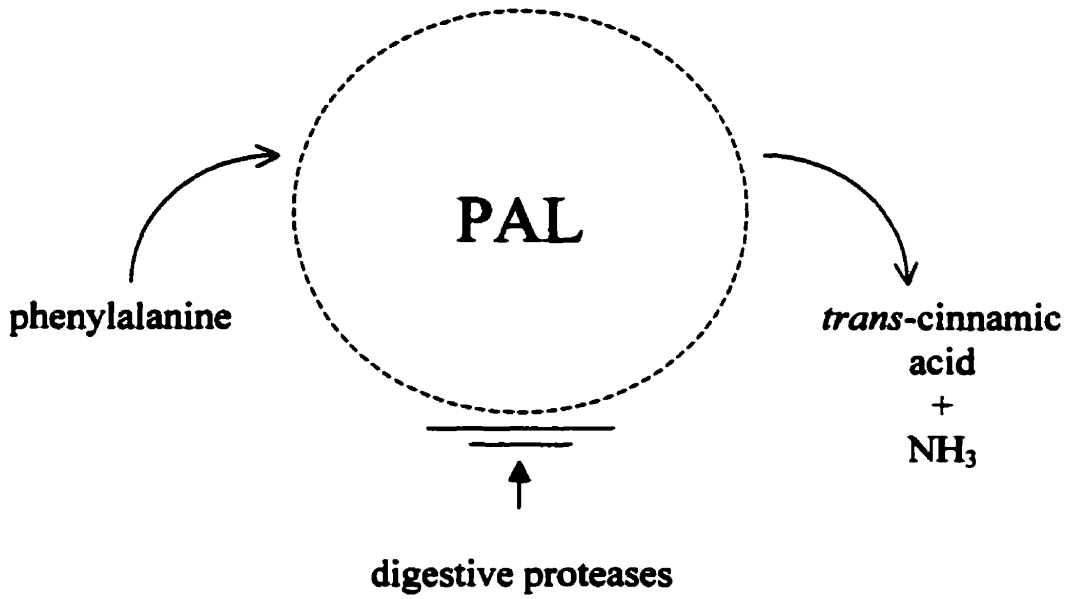

FIG. 2. Schematic representation of PAL enzyme protected from degradation for passage through the GI tract: A protective yet porous barrier (e.g. by encapsulation or cross-linked enzyme crystals) can shield PAL within a microenvironment, free of digestive proteases that are unable to cross the boundary. Free phenylalanine can be transported through the slightly porous barrier and be converted by PAL to *trans*-cinnamic acid.

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CONNECTING TEXT

In this Chapter, I describe the breeding of ENU1/2 compound hybrid strain. These animals were developed for two reasons, first, because the majority of patients are heteroallelic and second because we needed a model that was more pliant to metabolic manipulation; as the already available ENU1 animals were very difficult to control metabolically while the ENU2 animals were very fragile during long-term manipulation. To address these objectives, animals (ENU1, ENU2, ENU1/2, ENU1/+, ENU2/+, and BTBR/Pas-wildtype counterparts) were characterised and compared. The animals were genotyped to verify strain. Measuring concentration of phe in plasma and brain, presence of immunoreactive Pah protein and the level of Pah enzyme activity assessed their metabolic phenotype. They were also manipulated with a phe challenge and characterised according to their rate of plasma phe clearance. Finally, the clinical phenotypes were evaluated according to the effect of parental phenotype on fetal survival and by measuring behavioural outcome in each strain.

CHAPTER 2

A heteroallelic mutant mouse model: A new orthologue for human hyperphenylalaninemia

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ABSTRACT

Hyperphenylalaninemias (HPA) are Mendelian disorders resulting from deficiencies in the conversion of phenylalanine to tyrosine. The vast majority are explained by a primary deficiency of phenylalanine hydroxylase (PAH) activity. The majority of untreated patients experience irreversible impairment of cognitive development. Although it is one of the best known hereditary metabolic disorders, mechanisms underlying the pathophysiology of the disease are still not fully understood; to this end, the availability of an orthologous animal model is relevant. Various mutant hyperphenylalaninemic mouse models with an HPA phenotype, generated by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis at the *Pah* locus, have become available. Here we report a new hybrid strain, ENU1/2, with primary enzyme deficiency, produced by cross breeding. The ENU1, ENU1/2, and ENU2 strains display mild, moderate and severe phenotypes, respectively, relative to the control strain (BTBR/Pas). The Pah enzyme activities of the various models correlate inversely with the corresponding phenylalanine levels in plasma and brain and the delay in plasma clearance response following a phenylalanine challenge. The maternal HPA effect on the fetus correlates directly with the degree of hyperphenylalaninemia; but only the ENU2 strain has impaired learning.

INTRODUCTION

Phenylketonuria (PKU) and related forms of non-PKU hyperphenylalaninemia (HPA) (OMIM 261600) are human autosomal recessive traits, characterized by elevated levels of phenylalanine (phe) in body fluids. These Mendelian disorders result from deficiencies in phenylalanine hydroxylase enzyme (PAH) (EC 1.14.16.1), which catalyzes the conversion of phenylalanine to tyrosine. Untreated patients have elevated levels of phenylalanine in body fluids. Those with persistent HPA from birth are likely to have irreversible impairment of cognitive development. Furthermore, transplacental transport of excess phe from the maternal pool to the fetus, during intrauterine development, puts offspring at risk for microcephaly and birth defects. These disease-causing effects of PKU and maternal HPA are preventable by treatment that restores euphenylalaninemia (1).

Although PKU is one of the best known hereditary metabolic disorders, the mechanism underlying the pathophysiology is still not fully understood (2). Furthermore, the current dietary treatment of the human disease is not optimal for either compliance or fully normal cognitive outcome. To this end, the availability of an orthologous animal model is relevant (3;4).

Chemically induced HPA in the rat had long served as the animal "model" for "PKU", however, it had many imperfections (5). These were avoided when a mutant HPA mouse model generated by *N*-ethyl-*N*-nitrosourea mutagenesis (6;7) manifesting all forms of the harmful HPA phenotype, became available (1;8). The mice thus generated had autosomal recessive forms of HPA representing both locus and allelic heterogeneity; for example, the strain first identified (9), called *hph*-1, was mutated at the GTP-CH locus (10); GTP-CH catalyzes the first step in biosynthesis of tetrahydrobiopterin, a cofactor essential for hydroxylase activity. Subsequently, other strains all mutated at the *Pah* locus, but with varying degrees of phenotypic severity[1] (orthologues of human PKU and non-PKU HPA), became

[1] $Pah^{enu1/1}$ mice with an HPA phenotype were initially named the Pah^{hph-5} strain; $Pah^{enu2/2}$ mice are the PKU counterpart.

available (3;8). Here we report a hybrid strain (*Pah*$^{enu1/2}$), produced by crossing a homozygous female *Pah*$^{enu1/1}$ non-PKU HPA mouse with a heterozygous male *Pah*$^{enu2/+}$ PKU carrier. We describe the organismal phenotypes of the control (BTBR/Pas) and mutant strains (*Pah*$^{enu1/1}$ and *Pah*$^{enu2/2}$, the heteroallelic *Pah*$^{enu1/2}$ strain, and their carrier counterparts), their corresponding metabolic parameters, hepatic Pah enzyme activity, blood and brain phe levels, behavioral parameters and corroborate prior evidence of maternal HPA effect on the fetus. We also discuss how this mouse model is being used to measure the response to an alternative therapy for PKU by enzyme substitution with phenylalanine ammonia lyase (EC 4.3.1.5) to degrade excess dietary and endogenous phenylalanine (11).

MATERIALS AND METHODS

ENU Mice

Wildtype mice (BTBR/Pas background) were treated with alkylating agent (*N*-ethyl-*N*-nitrosourea) and mutations mapping to the mouse phenylalanine hydroxylase locus (*Pah*) were identified by hyperphenylalaninemia, a metrical metabolic trait (3;7). The original strains *Pah*$^{enu1/1}$ (phenotype name, ENU1) and *Pah*$^{enu2/2}$ (ENU2), kindly given to us by W. Dove and A. Shedlovsky, were bred in Madison, Wisconsin (3;7) and later genotyped (12). The hybrid heteroallelic strain (*Pah*$^{enu1/2}$, ENU1/2) was bred in Montreal, Canada as described here.

The homozygous mutant ENU1 mouse is a counterpart of human non-PKU HPA (7;8); it expresses a missense mutation (c.364T → C, V106A) in exon 3 of the *Pah* gene (12). The homozygous mutant ENU2 mouse is a counterpart of human PKU (3;8); it expresses a missense mutation (c.835T → C, F263S) in exon 7 of the *Pah* gene (12). The ENU1/2 strain was developed by selective mating of ENU1♀ x *Pah*$^{enu2/+}$(ENU2/+)♂. Genotypes at the *Pah* locus of all breeders and offspring were verified by DNA analysis. All procedures described below were reviewed and approved by the Animal Care Committee at McGill University.

Metabolic Manipulation of Animal Models

Plasma and brain phe levels were measured in all mice when fed on breeder mouse chow (Teklad 8626) (Madison, WI). ENU2 mice were then made euphenylalaninemic by placing them, for 3 consecutive days, on solid diet devoid of phe (Teklad 97152) supplemented with water containing 30 mg/L L-phe; then injected with L-phe (1.1 mg/g body wt, subcutaneously) to produce a controlled predictable HPA state. ENU1 and ENU1/2 mice do not show the HPA state on normal diet; a predictable degree of HPA was produced in these strains by subcutaneous injection of L-phe (1.1 mg/g).

Metabolic characterization

Time-dependent plasma clearance of phe was measured after subcutaneous injection of the standard dose of L-phe in the ENU1, ENU1/2 and euphenylalaninemic ENU2 animals. Plasma phe was measured at baseline (zero hr) and 1, 2 and 3 hr post-challenge. We use this parameter as a measure of phe "runout".

Pah Enzyme Assay

Liver tissues were prepared and stored at -80°C until analysis. Protein concentration was measured using the Bio-Rad protein assay kit (Hercules, CA). The assay (13-15), measuring the conversion of [U-^{14}C]-L-phe to labeled tyrosine, was modified as follows. Homogenate (100 μg) and 0.4 mM 6-methyltetrahydropterin (6-MPH$_4$) (Schircks Labs, Jona, Switzerland) were added to the reaction mixture (0.1 M potassium phosphate buffer (pH 6.8), 1000 units catalase (Sigma-Aldrich, Oakville, Ontario, Canada), 0.3 mM L-phe, 4 x 10^5 cpm [U-^{14}C]phe (DuPont NEN, Boston, MA) and 10 mM dithiothreitol (ICN, Costa Mesa, CA)) (final reaction volume = 250 μl). The samples were incubated (with shaking) at 25° C for 1 hr, then boiled for 5 min, placed on ice, and analyzed

according to Ledley *et al.* (15). Preliminary experiments established linearity of activity versus protein concentration and incubation time.

Western Blots

Liver was homogenized in buffer containing 50 mM Trizma (pH 8.0), 150 mM NaCl, 1% (v/v) NP-40, 0.3 μM aprotinin (Roche Molecular Biochemicals, Laval Quebec, Canada), and 1 mM Pefabloc (Roche Molecular Biochemicals). Samples were electrophoresed on 10% SDS-polyacrylamide gel, transferred to Hybond-C extra nitrocellulose (Amersham, Little Chalfont, Buckinghamshire, England) and immunoblotted, to detect mouse Pah protein, with 1 μg/ml anti-mouse PH8 primary antibody (PharMingen, Missisauga, Ontario, Canada) (16), detected with peroxidase-labeled anti-mouse antibody (1:1000 dilution) (Amersham) and developed with RPN 2109 ECL (Amersham). Human PAH was used as a positive control.

Effect of Parental Phenotype on Fetal Survival

Females were introduced to their breeding partners immediately following sexual maturity. The phenotypes for the following female homozygous and compound genotypes were studied: wildtype BTBR/Pas (Control), ENU1, ENU1/2, ENU2, and heterozygous ENU1/+, ENU2/+. Various male genotypes (and phenotypes) were also studied for possible paternal affect. The effect of maternal HPA on progeny was measured by counting the number of litters and number of progeny at birth and weaning. Blood phe was measured in the dam prior to breeding and at delivery and weaning.

Tests of Behavior

The T-maze alternation test was conducted as described by Nasir *et al.* (17). A Win-Stay eight-arm radial maze task was conducted 1 week following the completion of the T-maze tests. The latter task, modified as described by

Seamans and Phillips (18) and Nasir *et al.* (17), consists of a central octagonal platform with eight arms radiating from the middle (35 x 9 x 12 cm); eight 6-W light bulbs mounted directly above food cups (~4cm from the base) were placed at the end of each arm. On the first 2 days the mice were acclimated to the maze for 10 minutes; no food was introduced. For the following three days, the animals were tested (two trials/day). On each trial day, four randomly selected arms were lit and baited with Kellogg's Froot Loops (Etobicoke, Ontario, Canada) cereal. Following food consumption, the light was turned off. After all four pellets had been retrieved (max. time allowed, 10 min) the animal was removed for a 5 min period and then placed back into the maze where the same four arms were lit and baited once again. The arm choices, the time latencies (s/min) to reach the food in the first arm chosen, and the total time required to complete the daily trial was recorded.

DNA Analysis

Animals were genotyped for wildtype and mutant alleles as described (11;12).

Brain and Blood Samples

The brain was removed within 5 s after death and homogenized (on ice) in 0.1 %(w/v) sodium dodecyl sulfate (19). Allo-isoleucine was added as an internal standard; homogenate was then incubated for 15 min at room temperature followed by the addition of 1.5 % (v/v) 5-sulfosalicylic acid dihydrate solution then vortexed and centrifuged at 14,000*g* for 15 min. The supernatant was extracted and frozen at -80°C until analysis. Blood was collected from tail into heparinized tubes and plasma was obtained by centrifugation.

Amino Acid Analysis

Amino acids were measured by HPLC on a Beckman 6300 automatic amino acid analyzer (Palo Alto, CA).

Statistical Analysis

We used single-factor analysis of variance (ANOVA) between the different genotypes (conducted for each factor measured). The Poisson heterogeneity test confirmed homogeneity within genotypes. All values reported on figures and text are expressed as mean ± SEM.

RESULTS

Metabolic Manipulation and Catabolic Rates for Phe

ENU2 mice exhibit 10- to 20-fold elevated plasma phe levels and 10-fold increase in brain phe levels ($P = 1.13 \times 10^{-11}$ and 4.7×10^{-15}, respectively) when fed with breeder mouse chow (Fig. 1); the corresponding measurements for the ENU1/2, ENU1, ENU1/+, ENU2/+ and BTBR/Pas wildtype strains show near normal or normal values (ANOVA conducted between these genotypes showed $P = 0.17$ and 0.7 respectively). After the standardized injection to induce HPA in the ENU2 (initially made euphenylalaninemic, see materials and methods), ENU1, and ENU1/2 animals, the time-dependent plasma clearance rates (catabolic rates) (Fig. 2) show that ENU2 animals have the most severe metabolic phenotype, ENU1 animals the least severe, and ENU1/2 mice an intermediate phenotype.

Pah Enzyme Activity

We examined the relationship between hepatic Pah enzyme activity and plasma phe levels (Fig. 3). The ENU2 mice, with undetectable enzyme activity, have the most elevated plasma phe levels. ENU1 and ENU1/2 mice, with ~24 and 5% normal enzyme activity, respectively, have near normal plasma phe levels under standard diet conditions. ENU2/+, ENU1/+ heterozygotes with ~49 and

61% normal enzyme activity, have normal plasma phe levels as expected for a recessive trait.

Enzyme Protein Levels

Hepatic Pah protein levels, as visualized by immunoblotting of liver extracts, are different in the 3 ENU phenotypes (Fig. 4). These findings imply that enzyme stability may vary with genotype: The ENU1 mutation apparently affects stability (and therefore activity) of the protein, evident by the clearly reduced level of immunoreactive Pah. The ENU2 mutation, which impairs enzyme activity, may also affect stability; it produces somewhat diminished but easily detectable protein, evident by the substantial amounts of immunoreactivity. The ENU1/2 compound is intermediate in both protein stability and enzyme activity. These findings were consistent in 6 replicates.

Maternal Effect

The ENU2 dams with severe HPA conferred a severe maternal effect on offspring; the other strains did not, the effect being dependent on the degree of HPA (Table 1). ANOVA analyses showed a significant reduction in the number of litters and of progeny at birth and of those surviving to weaning only from the ENU2 mothers ($P < 0.00014$ and 0.0008, respectively).

Behavior

The T-maze alternation test assesses simple discrimination learning and short-term memory; we observed impaired behavior only in the ENU2 animals (Table 2). The Win-Stay eight-arm radial maze task measures reference memory, habit learning, and memory for a visual stimulus. Only the ENU2 model showed impaired behavior (Table 2).

DISCUSSION

Animal models assist the study of disease phenotypes and potential therapies (20). Mouse models offer the added advantage in that their genomes are highly homologous with the human genome (21). In addition the mouse and human phenylalanine hydroxylase genes have ~87% conservation of the nucleotide sequence, with ~10% of the changes silent; the corresponding proteins have 95% conserved amino acid sequences (15). Here we have described and compared phenotypes of control (BTBR/Pas) and mutant strains (*Pah*$^{enu1/1}$, *Pah*$^{enu2/2}$, *Pah*$^{enu1/2}$, and their heterozygous counterparts), the mutants being orthologues of human PKU and non-PKU HPA (Fig. 5).

The ENU1 mouse, which displays a mild phenotype (non-PKU HPA), carries a mutation in exon 3 (12) affecting the N-terminal region of the enzyme distant from the catalytic residues of PAH/Pah, recently identified by crystallographic analysis of the human enzyme (22;23). The ENU2 counterpart has a mutation in exon 7, which includes the catalytic region; the site is predicted to have π-stacking interactions with the substrate (24). The resulting phenotype is PKU-like (12). The ENU1/2 heteroallelic counterpart displays an intermediate phenotype.

Pah enzyme activities of the various models correlate inversely with the corresponding phe levels in plasma and brain and with plasma phe clearance time. ENU2 mice with undetectable enzyme activity have ~20-fold elevated plasma and brain phe levels, and the slowest clearance rate, while ENU1 and ENU1/2 mice with ~24 and 5% normal enzyme activity, respectively, have near normal plasma and brain phe levels and more normal clearance rates. The carrier counterparts, having approximately half the normal enzyme activity, display normal plasma and brain phe levels. These metrical traits are those expected for an autosomal recessive phenotype (25).

Pah protein shows different states of stability in the different ENU phenotypes. The ENU1 mutation results in protein instability, whereas the ENU2

mutation has only a minor effect on this parameter. Stability in the ENU1/2 compound is intermediate in comparison.

The behavioral assessment of ENU2 mice shows impaired simple discrimination in short-term memory, reference memory, habit learning, and memory for a visual stimulus. The other genotypes were correspondingly unaffected in clinical measures. These observations concur with earlier findings (26).

The maternal effect on offspring correlates directly with the degree of HPA and is apparent only with the ENU2 mothers; the effect is completely independent of paternal genotype. These findings resemble human data where maternal plasma phe levels below the 400 μM range are usually associated with normal fetal outcome (1).

The ENU1/2 mouse model has been particularly useful for measuring the response to an alternative therapy for PKU, namely oral administration of phenylalanine ammonia lyase in an effort to degrade excess phe from the accumulated pools (11). Our present studies demonstrate proof of principle, both pharmacological and physiological. The present report describes in detail the parameters available to measure the short- and long-term responses to this therapy and document the advantages of the ENU1/2 strain for this work.

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TABLE 1
Maternal effect on number and size of litters and survival to weaning (Mean ± SE, $n = 5$)

Maternal Genotype	Average No. of litters/mated female	Average No. of litters surviving to weaning/mated female	Average No. of progeny at birth/litter	Average No. of progeny surviving to weaning/litter
Control	1.9 ± 0.2	1.6 ± 0.1	8.0 ± 0.7	7.1 ± 0.8
ENU1/+	2.0 ± 0.3	1.6 ± 0.5	8.1 ± 1.2	7.6 ± 1.4
ENU2/+	3.1 ± 0.4	1.9 ± 0.3	6.6 ± 0.6	4.9 ± 0.7
ENU1	2.7 ± 0.2	1.9 ± 0.2	6.2 ± 0.5	5.0 ± 0.5
ENU1/2	3.1 ± 1.3	2.1 ± 0.4	6.0 ± 0.8	5.3 ± 0.8
ENU2	1.3 ± 0.2	0 ± 0[a]	1.3 ± 0.9	0 ± 0[b]

[a]ANOVA, $P < 0.00014$.
[b]ANOVA, $P < 0.0008$.

TABLE 2
Behavioral assessment (Mean ± SE, $n = 6$)

Test	ENU2	Remaining genotypes combined	Significance (P)
T-maze alternation test			
Increase in average time required to reach food (s)	20.7 ± 6.7	5.2-10.3 ± 0.4-2.1	< 0.005
The Win-Stay eight-arm radial maze task			
Reduction in the No. of entries into initially lit arms	5.5 ± 0.3	6.2-7.5 ± 0.4-0.5	< 0.03
Increase in average time required to reach food (s)	58.3 ± 15.5	5.6-13.1 ± 1.3-1.9	$< 4 \times 10^{-9}$
Increase in average time to complete trials (s)	290.0 ± 36.0	71.8-138.6 ± 6.4-13.4	$< 8 \times 10^{-12}$

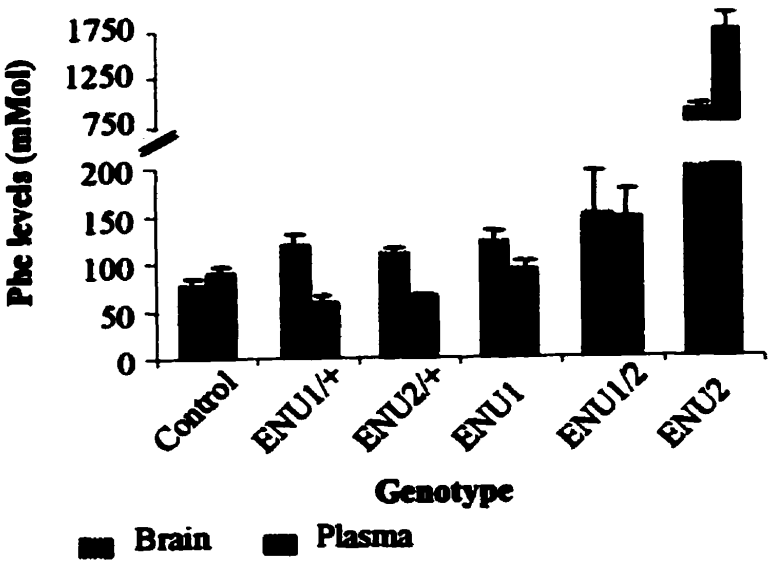

FIG. 1. Plasma phe values (μM, mean $\pm$ SE, $n = 4$): Control (BTBR/Pas) mice, 91.2 $\pm$ 6 μM; ENU1/+, 58.5 $\pm$ 7; ENU2/+, 65.7 $\pm$ 1; ENU1, 93.4 $\pm$ 8; ENU1/2, 147.0 $\pm$ 29; ENU2, 1697.4 $\pm$ 175. The corresponding brain phe values (μM, mean $\pm$ SE) are Control, 76.9 $\pm$ 6; ENU1/+, 120.3 $\pm$ 10; ENU2/+, 112.5 $\pm$ 5; ENU1, 123.6 $\pm$ 1; ENU1/2, 150.7 $\pm$ 42; ENU2, 876.4 $\pm$ 30.

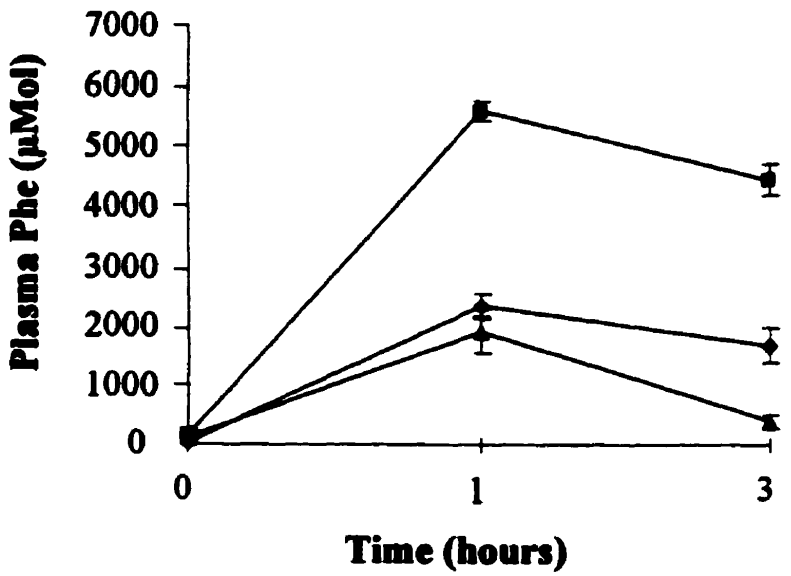

FIG. 2. Plasma phe clearancè rates for ▲, ENU1; ◆, ENU1/2; and ■, ENU2 animals loaded with a standard dose of phe (mean ± SE; $n = 5$).

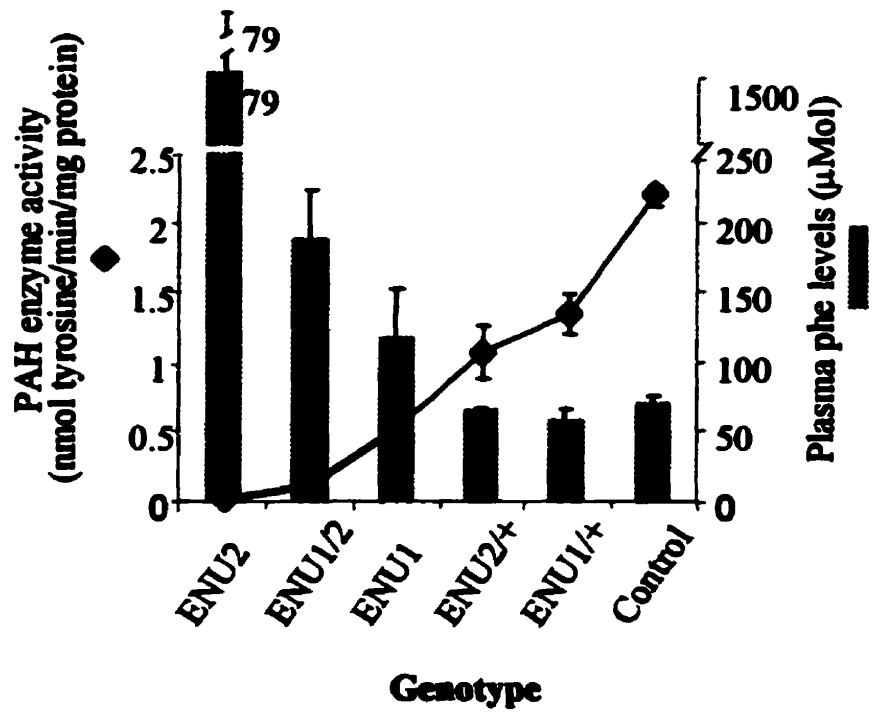

FIG. 3. Pah enzyme activity vs plasma phe values in control and HPA phenotypes (mean ± SEM; $n = 4$). Enzyme activity and plasma phe levels of the ENU1/2 animals are intermediate compared to those of ENU1 and ENU2 animals ($P < 0.05$).

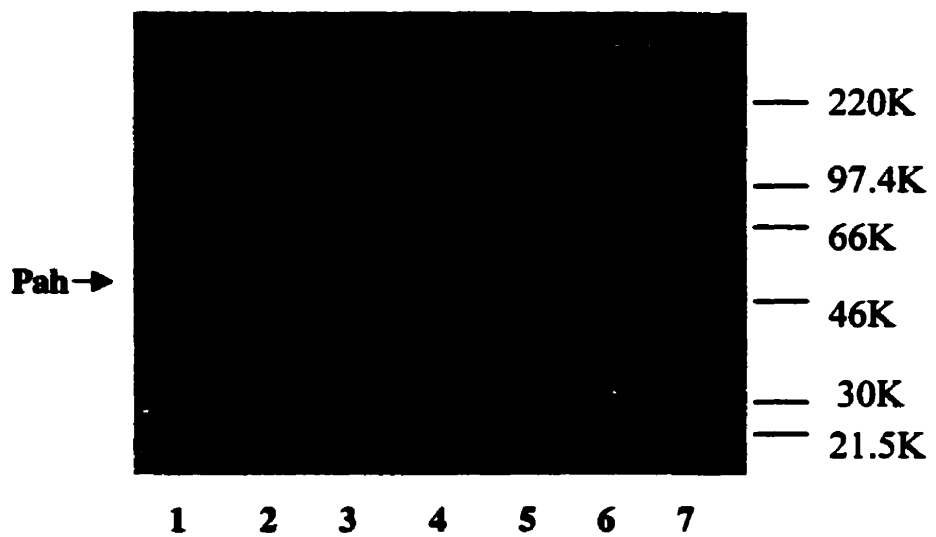

FIG. 4. Western blot for Pah protein in liver extracts (representative of 6 replicates). The ~52-kDa band (arrow) is the Pah monomer; other faint bands represent non-specific binding, confirmed by their absence with purified human PAH (lane 1) and their presence in the wildtype mouse strain (lane 7). Lane 1: purified human PAH (hPAH); Lane 2: ENU2, Lane 3: ENU1/2; Lane 4: ENU1; Lane 5: ENU1/+; Lane 6: ENU2/+; Lane 7: Wildtype BTBR/Pas. Rainbow Marker used for reference.

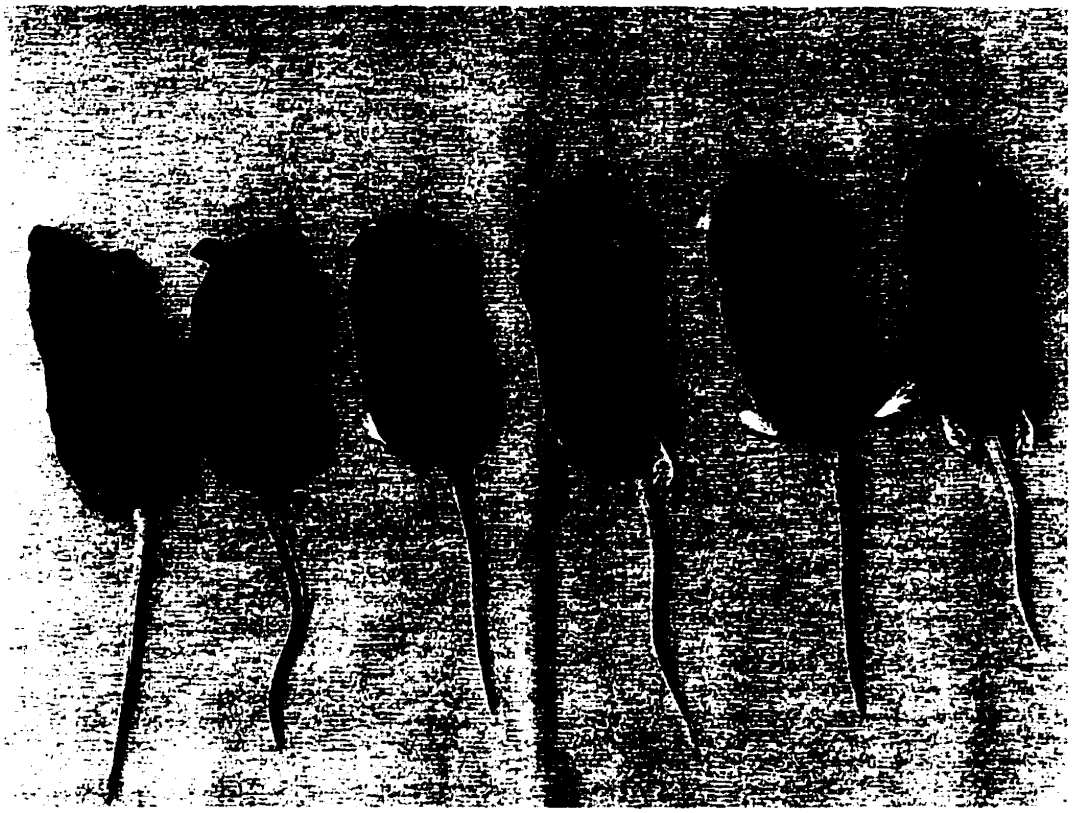

FIG. 5. Classic mouse models of PKU and non-PKU HPA, induced by *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis: A, control BTBR/Pas; B, *Pah*$^{enu1/+}$ heterozygous carrier/wildtype; C, *Pah*$^{enu1/1}$ non-PKU HPA orthologue; D, *Pah*$^{enu2/+}$ heterozygous carrier/wildtype; E, *Pah*$^{enu2/2}$ PKU orthologue; F, *Pah*$^{enu1/2}$ heteroallelic orthologue.

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CONNECTING TEXT

In Chapter 3 I use the animal models to measure brain concentrations of phenylalanine derived metabolites, PPA, PAA and PLA. The pathogenic effect of these metabolites in PKU and non-PKU HPA have long been debated and the objective of this study was to understand better their possible contribution towards the phenotypic outcome. The availability of human surrogates allowing access to brain tissue sampling and a method sensitive enough to measure these metabolites were two prior problems that prevented the completion of this study. The first problem was overcome with the availability of non-PKU HPA, PKU and control mouse orthologues, as described in Chapter 2. The solution to the second obstacle is described here. A method has been developed based upon stable isotope dilution techniques, coupled with negative ion chemical ionization gas chromatography/mass spectrometry to measure PPA, PAA, PLA. I have used this method to measure these phe metabolites in the mouse brain tissue.

CHAPTER 3

Measurement of phenyllactate, phenylacetate and phenylpyruvate by negative ion chemical ionization – gas chromatography/mass spectrometry in brain of mouse genetic models of phenylketonuria and non-phenylketonuria hyperphenylalaninemia

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ABSTRACT

Phenylketonuria (PKU) (OMIM 261600) is the first Mendelian disease to have an identified chemical cause of impaired cognitive development. The disease is accompanied by hyperphenylalaninemia (HPA) and elevated levels of phenylalanine metabolites (phenylacetate (PAA), phenyllactate (PLA), and phenylpyruvate (PPA)) in body fluids. Here we describe a method to determine the concentrations of PAA, PPA, and PLA in the brain of normal and mutant orthologous mice, the latter being models of human PKU and non-PKU HPA. Stable isotope dilution techniques are employed with the use of [$^2H_5$]-phenylacetic acid and [2,3,3-$^2H_3$]-3-phenyllactic acid as internal standards. Negative ion chemical ionization NICI-GC/MS analyses are performed on the pentafluorobenzyl ester derivatives formed *in situ* in brain homogenates. Unstable PPA in the homogenate is reduced by NaB^2H_4 to stable PLA, which is labelled with a single deuterium and discriminated from endogenous PLA in the mass spectrometer on that basis. The method demonstrates that these metabolites are easily measured in normal mouse brain and are elevated moderately in HPA mice and greatly in PKU mice. However, their concentrations are not sufficient in PKU to be "toxic"; phenylalanine itself remains the chemical candidate causing impaired cognitive development.

INTRODUCTION

Phenylketonuria (PKU) and related forms of non-PKU hyperphenylalaninemia (HPA) (1) are autosomal recessive disorders of amino acid metabolism, which result from primary dysfunction of phenylalanine hydroxylase (PAH), the hepatic enzyme responsible for catalysing the conversion of phenylalanine to tyrosine. PKU and HPA patients have elevated levels in body fluids of phenylalanine (phe) and of metabolites derived from phenylalanine (phenylpyruvate (PPA), phenylacetate (PAA), and phenyllactate (PLA)) (2;3). Untreated PKU probands usually have severe irreversible mental retardation; the risk of mental retardation is less in the conditions with a lower degree of HPA (non-PKU HPA).

The free phe pool in the normal subject is derived from two sources: intake of exogenous dietary protein and turnover of endogenous polypeptides. Approximately 25% of the free pool is normally incorporated into protein; most of the remaining 75% is hydroxylated to tyrosine and only a trivial fraction is transaminated to PPA under normal conditions (4). PAH enzyme catalyzes the hydroxylation reaction; when its activity is absent or reduced (as in PKU and to a lesser degree in non-PKU HPA), the free phe pool expands, if dietary phe input is not reduced. At this stage, the degradative transamination pathway, involving conversion of phe to PPA (the initial reaction in this pathway) becomes significant at a modal phe value of ~0.5 mM (2-5). PPA is subsequently converted to PLA and PAA and phenylacetylglutamine (1). Whether these metabolites actually contribute to pathogenesis of cognitive impairment has long been debated (3;5).

Orthologous mouse models of PKU and non-PKU HPA exist (6-8) which allow us to measure phe and its metabolites in brain in various degrees of HPA. Here we describe a GC/MS method to measure phenylalanine metabolites based upon stable isotope dilution techniques, coupled with negative ion chemical ionization (NICI).

MATERIALS AND METHODS

The method measures PAA as the pentafluorobenzyl (PFB) ester with [$^2H_5$]-phenylacetic acid (PAAd$_5$) as internal standard, and PLA as the PFB and trifluoroacetate (TFA) diester with [2,3,3-$^2H_3$]-phenyllactic acid (PLAd$_3$) as internal standard. PPA is reduced to [2-^{2}H]-phenyllactic acid (PLAd$_1$) by addition of sodium borodeuteride (NaB^2H_4) to the supernatant of the tissue homogenate, and is measured in the manner similar to that for PLA.

Mouse Models

The homozygous mutant strain *Pah$^{enu2/2}$* (phenotype name, ENU2) and *Pah$^{enu1/1}$* (ENU1) are orthologues of human PKU and non-PKU HPA, respectively (http://www.mcgill.ca/pahdb/mouse). They were developed by treating wildtype mice (BTBR/Pas background (used as controls)) with the alkylating agent *N*-ethyl-*N*-nitrosourea (6;7;9). Produced in Wisconsin, they were kindly given to us by W. Dove and A. Shedlovsky. They display a range of phenotypic characteristics comparable to those of affected human individuals (10).

Preparation of Internal Standard Solution

PAAd$_5$: A 0.071 mM PAAd$_5$ stock solution was prepared by dissolution of 1.0 mg (CDN isotopes) in 100 ml deionized H_2O.

PLAd$_3$: PPA (17 mg, 0.1 mmol, Sigma Chemical Co.) was dissolved in deuterium oxide (25 ml, CDN Isotopes) and made basic (pH > 12) with 3 drops of 40% NaO^2H in ^2H_2O. The resulting solution was held at 60° C for 1 hr and then rotary evaporated to near-dryness. The residue was taken up in a 10 ml aliquot of ^2H_2O and held at 60°C for 1 hr. This solution was cooled to room temperature and NaB^2H_4 (approximately 5 mg, CDN Isotopes) was added. The resulting solution was warmed to 50°C for 10 min, cooled in an ice bath, and slowly acidified to pH < 2 with 2 N HCl (caution: vigorous evolution of hydrogen). The solution was saturated with NaCl and extracted with three 10 ml volumes of diethyl ether. The ether extracts were combined, made anhydrous by addition of

solid anhydrous Na_2SO_4 (2 successive 1-g lots), and then evaporated to dryness in a dry nitrogen stream. The product, $PLAd_3$, was obtained (14 mg, 83% crude yield) in oil form, free of residual PPA as determined by GC/MS analysis of the trimethylsilyl derivatives. Extensive experience with reductions of ketoacids with NaB^2H_4 in this laboratory shows that these reductions are quantitative. The isotopic purity was determined to be 97% as the triply deuterium- labeled isotopomer. Approximately 14 mg of the crude product was dissolved in deionized water (100 ml) to be used as the internal standard for PLA and $PLAd_1$ determinations. The concentration of this internal standard was determined by a reverse stable isotope dilution assay, measuring relative ion intensities in a solution with unlabeled PLA of known concentration. The final concentration of the $PLAd_3$ internal standard solution was 0.787 mM.

Brain Sample Preparation

Brains were removed within 5 s following decapitation of ENU2, ENU1 and control animals fed with standard rodent diet (Teklad |No. 8604) (n = 6/genotype) and immediately homogenized in minimal 20°C deionized water (1:1, w/v) to which we added the labeled internal standards (100 μl each of the $PLAd_3$ and $PAAd_5$ solutions). The total volume was made up to 1 ml, adjusted to pH 10-12 with dilute KOH, NaB^2H_4 (2 mg) added immediately, and the tubes placed in 50°C water for 10 min. PFB derivatives were prepared in the manner previously reported (11) with the following stock solutions: A, methylene chloride (20 ml) and pentafluorobenzyl bromide (0.4 ml, Aldrich Chemical Co.); B, potassium phosphate buffer (pH 7.4, 100 ml) and tetrabutylammonium hydrogen sulfate (3.4 g, Aldrich Chemical Co.) adjusted to pH 7.4 with 2 N KOH. Solutions A (0.25 ml) and B (0.25 ml) were combined in a separate tube, 0.25 ml of tissue homogenate supernatant was added, the mixture was vortexed for 2 min and then placed in an ultrasonic bath for 20 min at room temperature. Hexane (2 ml) was added, the mixture vortexed for 1 min, the hexane layer then removed and dried by addition of anhydrous sodium sulfate (10 mg) with vortexing for 1 min. The

hexane layer was next decanted into a separate tube, *N*-methyl-bis-trifluoroacetamide (MBTFA, 50 μl, Pierce Chemical Co.) added then vortexed and placed into a 50°C water bath for 10 min. A 1 N sodium bicarbonate solution (2 ml) was added to the tube and the mixture vortexed for 1 min. The hexane layer was finally removed into a separate tube, anhydrous sodium sulfate was added and the mixture vortexed for 1 min. An aliquot of this final hexane solution was transferred to an autoinjector vial for GC/MS analysis.

Blank Sample

Blank samples were prepared with deionized water equal in volume to the brain tissue homogenate supernatants. These samples were processed and analyzed as described for the tissue samples.

GC/MS Analysis

Aliquots (1 μl) of the derivatized mixtures were analyzed in NICI mode with a Hewlett-Packard 5988A GC/MS fitted with a 30 m x 0.25 mm i.d. capillary column (J & W Scientific) coated with a 0.25 μm DB-1 film. The helium flow rate was 2 ml/min; the injector and interface temperatures were 250° C. The column was temperature programmed from 100° C after a 1 min hold to 120°C at 40°C/min and then at 10°C/min to 280°C. The column was baked out at 280°C for 5 min at the completion of each sample analysis. Methane was used as the moderator gas at an indicated source pressure of 0.6 mbar and the ion source temperature was 120°C. Selected ion mode was used to measure the intensities of negative ion fragments *m/z* 135, 140, 261, 262, and 264 with dwell times of 50 ms each. These fragments arise by the loss of the pentafluorobenzyl radical from the molecular anions of the PFB derivatives of PAA and $PAAd_5$, and the PFB/TFA derivatives of PLA, $PLAd_1$, and $PLAd_3$, respectively.

Amino Acid Quantitation

Whole blood was collected from mouse tails into heparinized tubes, plasma was separated by centrifugation, and after deproteinization the amino acid content was analyzed by HPLC on a Beckman 6300 automatic amino acid analyzer.

Whole brain amino acids were analyzed according to the method described by Diomede *et al.* (12). Brain tissue was removed within 5 s after decapitation and homogenized (on ice) in 0.5% sodium dodecyl sulfate solution (1:4, w/v). Alloisoleucine was added as an internal standard and the homogenate was then incubated for 15 min at room temperature. A 4% solution of 5-sulfosalicylic acid dihydrate solution (1:0.6, v/v) was added, and the mixture was centrifuged at 14,000*g* for 15 min. The supernatant was decanted and frozen at -80°C until analyzed by HPLC as above.

RESULTS AND DISCUSSION

NICI mass spectra obtained for the PFB esters of authentic PAA and $PAAd_5$ standards are shown in Fig. 1. The most intense ions correspond to the carboxylate anions (*m/z* 135 and 140, respectively) produced by the loss of the pentafluorobenzyl radical (181 Da) from the molecular anions (*m/z* 316 and 321, respectively, not detected). Loss of HF (20 Da) from the molecular anions, which is commonly observed in derivatives of this nature, is detectable, although not apparent in Fig. 1. The 140-Da fragment in the spectrum of $PAAd_5$ confirms that all labelling is intact in the ion measured. SIM analysis of $PAAd_5$ shows that the unlabeled content is 1.39% relative to the labelled. This is taken into account in the calculations of the endogenous concentrations of PAA.

NICI spectra for the PFB esters of the trifluoroacetyl esters of unlabelled and labelled PLA are shown in Fig. 2. The most intense ions (*m/z* 261, 262 and 264) represent the carboxyl anions produced by the loss of the pentafluorobenzyl radical from the molecular anions *m/z* 442, 443 and 445 (not detected) of unlabelled authentic PLA, $PLAd_1$ synthesized from PPA by reduction with NaB^2H_4, and synthesized $PLAd_3$ (internal standard), respectively. The $PLAd_1$ isotopomer

corresponds to PPA in the original homogenate. It is essential that the 2-hydroxyl group of the PLA isotopomers be derivatized because decomposition by dehydration at GC temperatures would lead to deuterium label loss. The $PLAd_3$ synthesized and used as internal standard was found by SIM analysis to be 97.17% $PLAd_3$, 2.66% $PLAd_2$, 0.001% $PLAd_1$, and 0.16% $PLAd_0$. Reduction of authentic PPA by NaB^2H_4 yielded $PLAd_1$ that was 0.29% unlabelled, a measure of the isotopic purity of the lot of NaB^2H_4 which was used for the entire study.

SIM chromatograms obtained for one of the calibrating mixtures containing PAA, $PAAd_5$, and unlabelled PLA are shown in Fig. 3. The PAA isotopomers are represented by the carboxylate anions (*m/z* 135 and 140) formed by the loss of the pentafluorobenzyl moiety from the molecular anions (not detectable). The PLA chromatograms (*m/z* 261, 262, and 264) would normally represent the carboxylate anions of unlabelled PLA, $PLAd_1$ (formed by the reduction of PPA by NaB^2D_4), and the internal standard $PLAd_3$, respectively formed by the loss of the pentafluorobenzyl moiety from the molecular anions of the PFB–TFA derivatives. In this instance, the measured relative intensities are for unlabelled PLA substituted with only natural abundance heavy isotopes. The intensities of *m/z* 262 and 264 measured in the ion cluster relative to *m/z* 261 are 12.47 and 0.13%, respectively, and compare well with the calculated values of 12.51 and 0.12%, respectively.

The calibration curves for all three metabolites, over the expected physiological ranges, are shown in Fig. 4. A linear response is demonstrated for each metabolite ($R^2 > 0.98$ for each metabolite). The response for PPA (measured as $PLAd_1$) is approximately 72% that of $PLAd_0$. This is possibly due to lack of purity or homogeneity in the PPA originally weighed out. Analysis of the PPA as the TMS derivative shows the presence of small but significant quantities of PLA and PAA. The sensitivity of our method (for all three metabolites) is estimated to be 0.1 nmol/g brain tissue.

Typical chromatograms for normal (Fig. 5), ENU1 (Fig. 6) and ENU2 (Fig. 7) mouse brain are shown. In normal mouse brain PAA is detectable (*m/z* 135) at low levels, while PLA and PPA (*m/z* 261 and 262) are essentially absent (Fig. 5).

The apparently inverted peak (retention time, 9.02 min) in the responses for *m/z* 135 and 140 results from the elution of a large unidentified peak which temporarily depletes the thermal electron atmosphere in the ion source.

The ENU1 (non-PKU HPA) mouse brain (Fig. 6) contains elevated levels of PLA and PPA (*m/z* 261 and 262). The minor variation in the PAA levels between the ENU1 and normal mice demonstrates that at normal or near normal (physiological) phe levels the transamination pathway contributes insignificantly to the brain metabolic phenotype in the variant HPA state.

The ENU2 (PKU-like) mouse (Fig. 7) shows greatly increased concentrations of all three metabolites. The well-known tendency for deuterium-labeled analogs to elute slightly earlier than the corresponding unlabelled compounds is apparent.

The correlation between plasma phe and brain metabolites is shown in Table 1. BTBR/Pas-wildtype and ENU1 animals, displaying normal (<100 μM) or low (150-400 μM) plasma phe levels, respectively, have low phe metabolite levels in brain. This finding implies that the transamination pathway comes into play only at phe levels above 0.4 mM, as implied by the findings in the ENU2 animals, moreover correlating well with previous observations (see Fig. 15-1 in ref. (5)). When present, metabolite levels have a rank order PLA > PAA > PPA. The relationship between plasma and brain phenylalanine levels in the mouse models is given in Table 2.

Our data are among the first reporting direct measurements of brain phe metabolites in PKU and non-PKU HPA. A preliminary report by Evans (13) compared brain and body fluid metabolites in control and ENU2 mice. Here we present a formal analysis of PLA, PAA, and PPA in brains of orthologous mice with PKU and non-PKU HPA and compare them with control values. Our interest was to study phe metabolite concentrations in brain independent of their levels in blood or urine; the latter were the focus of most earlier studies. The data show that brain metabolite concentrations correlate positively with plasma phe levels. More important, the levels of the metabolite measured here do not reach levels of toxicity predicted for human subjects by Kaufman (3) and documented in earlier studies (14).

Taking into account assumptions about distributions of metabolites in the intracellular and extracellular space of brain, the levels of metabolites measured here are 10-fold lower than those associated with toxicity in brain (14). Nonetheless, our PKU (ENU2) mice exhibit the behavioural and cognitive impairment expected in PKU (10), and which we attribute primarily to the effect of phenylalanine itself. On the other hand, in certain untreated PKU patients with normal cognitive function, brain phe values are not elevated in the presence of high blood values, as measured by MRI (see ref. (15)); an independent impediment of blood/brain phe transport has been offered as an explanation. Our use of both the analytical method and the orthologous mouse model of the human is offered here as a contribution toward resolving a long standing controversy about pathogenesis of the cognitive phenotype in PKU.

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TABLE 1

Relationship between brain phe metabolites and plasma phe levels in three phenotypes (normal, ENU1, and ENU2)

(n = 6/genotype, Mean ± SE)

Mouse Models	Plasma Phenylalanine Concentration (μM)	PPA (nmol/g brain)	PAA (nmol/g brain)	PLA (nmol/g brain)
Control	<100	1.2 ± 0.1	2.7 ± 0.3	0.2 ± 0.1
ENU1	150-400	1.2 ± 0.1	2.2 ± 0.5	0.9 ± 0.3
ENU2	1400-3000	2.2 ± 0.3	7.4 ± 1.6	59.3 ± 21.8

TABLE 2

Relationships between plasma and brain phe values in three mouse models (μM, n=6, Mean ± SE)

Mouse Models	Plasma phenylalanine concentration (μM)	Brain phenylalanine concentration (μM)
Control	74.4 ± 8.8	70.1 ± 6.5
ENU1	181.5 ± 23.3	120.6 ± 8.1
ENU2	1882.7 ± 156.5	886.97 ± 26.6

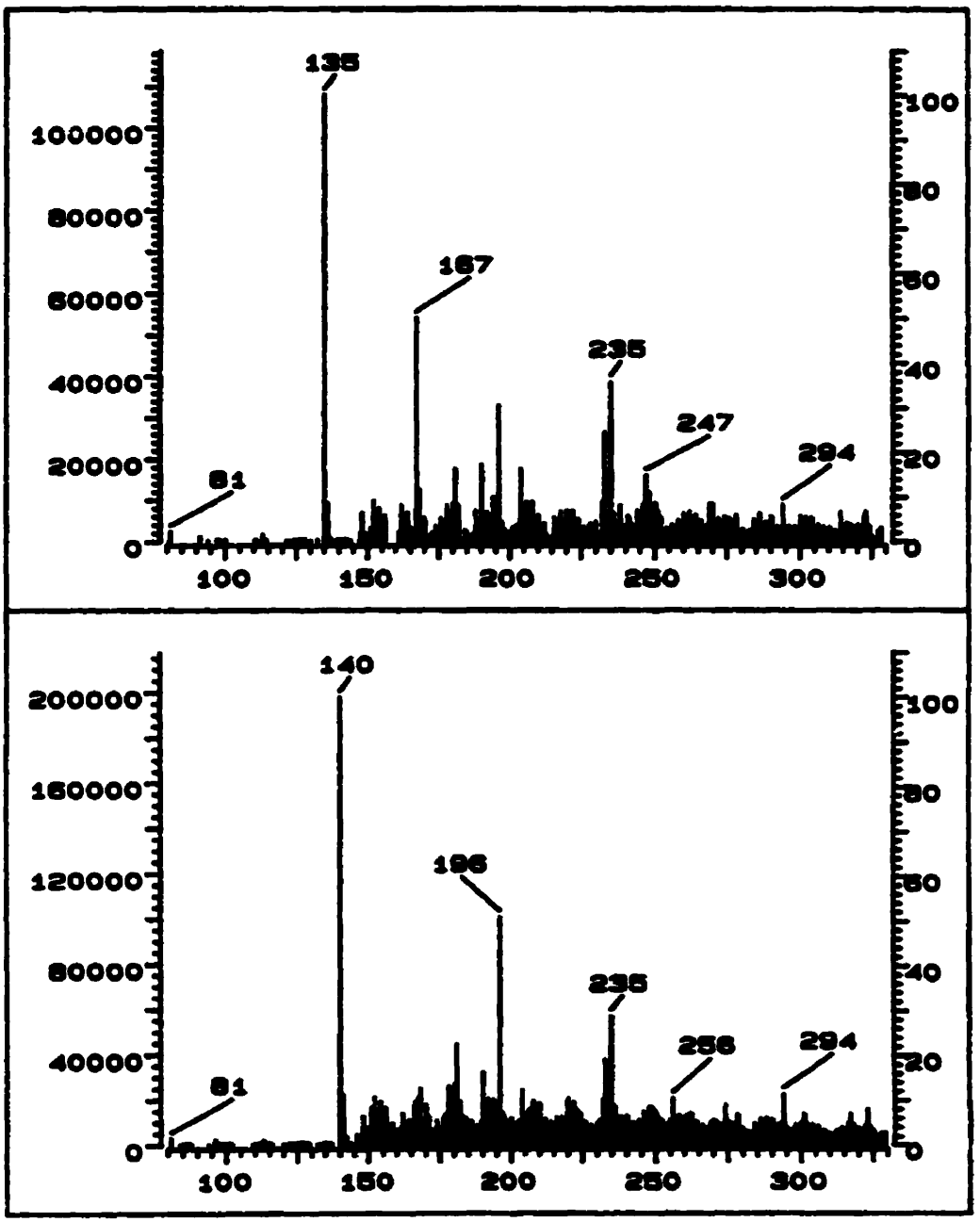

FIG. 1. NICI mass spectra obtained for the PFB derivatives of unlabelled PAA and PAAds. Intensities of ions with masses greater than 145 Da have been multiplied by 20, and appear not to be related to the sample. The intense ions at *m/z* 135 and 140 carry nearly the entirety of the ion current produced and correspond to the carboxylate anions formed by the loss of the pentafluorobenzyl radical from the molecular anions. While the molecular anions expected at *m/z* 316 and 321, respectively, are not detected, very weak ion currents attributable to loss of HF from the molecular anions (not shown) confirm the nature of the derivatives.

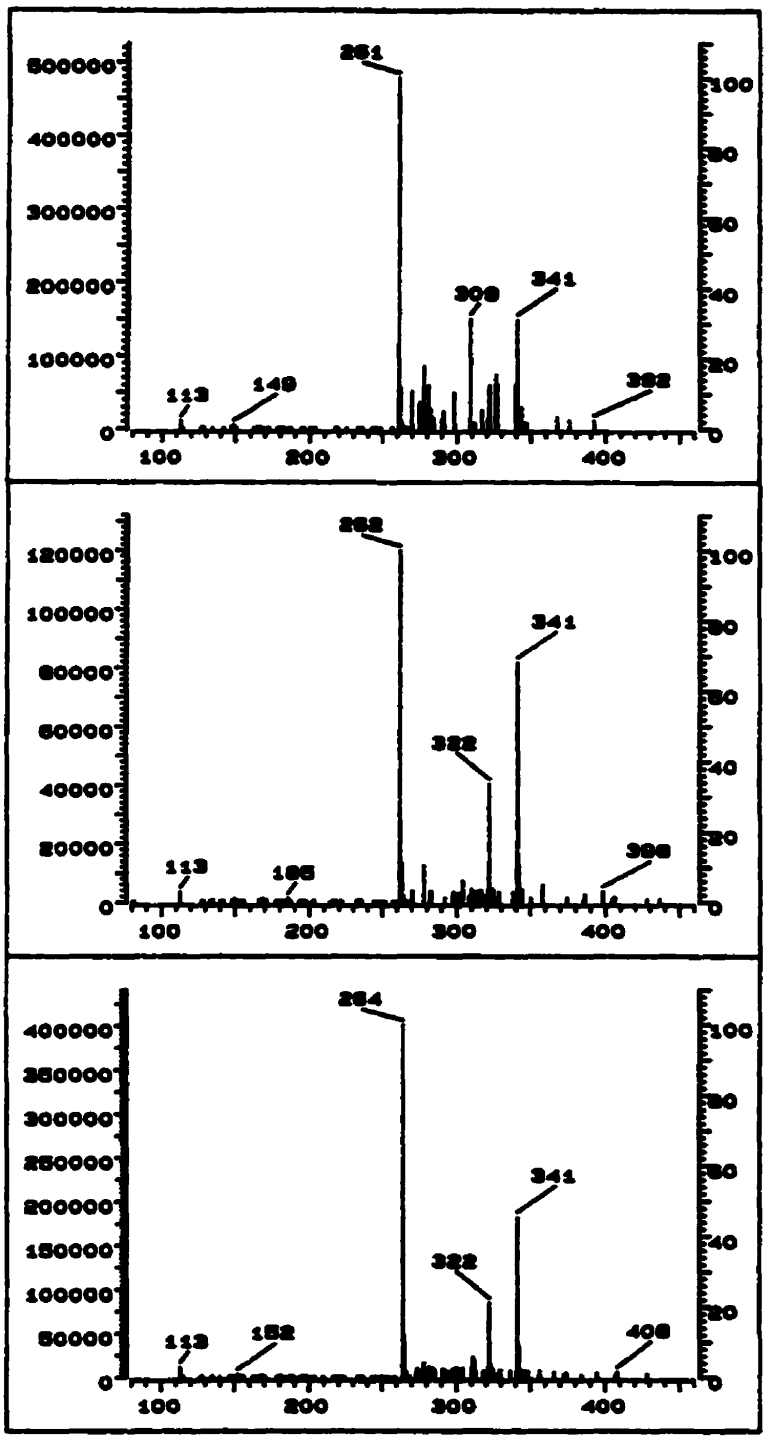

FIG. 2. NICI mass spectra obtained for the PFB derivatives of the TFA esters of unlabelled PLA, $PLAd_1$, and $PLAd_3$. Intensities of ions with masses greater than 270 have been multiplied by 50 to show that they appear not to be related to the sample, as the masses show no incrementing dependent upon labelling. The carboxylate anions at *m/z* 261, 262, and 264 formed by the loss of the pentafluorobenzyl radical from the molecular anions (*m/z* 442, 443, and 445, not detected) carry nearly all the ion current.

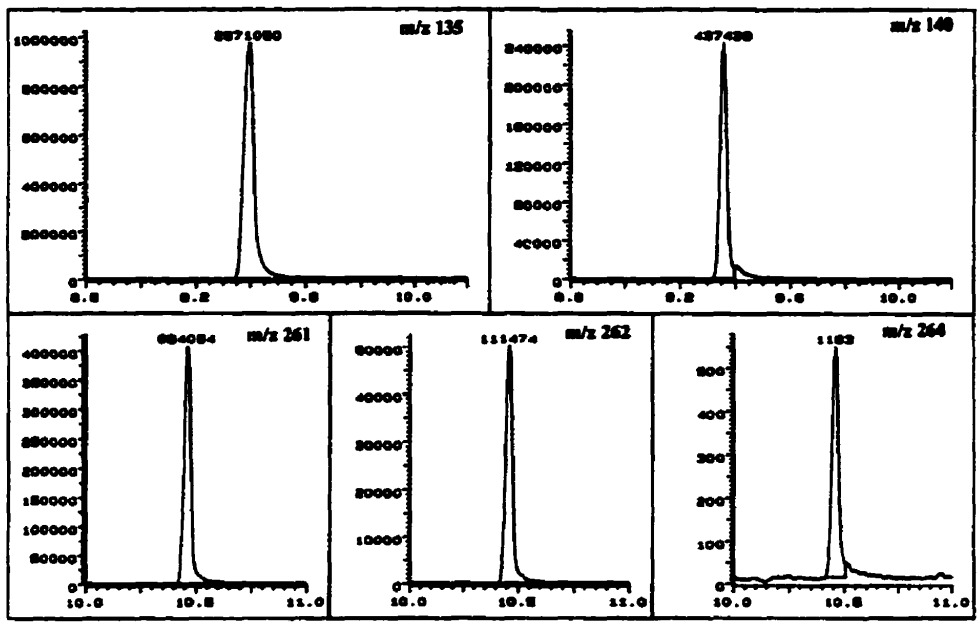

FIG. 3. SIM chromatograms for a calibrating mixture containing unlabelled PAA (*m/z* 135), internal standard PAAds (*m/z* 140), and unlabelled PLA (*m/z* 261). Ion currents at *m/z* 262 (12.47% relative to *m/z* 261) and 264 (0.13%) are due to natural abundance heavy isotope inclusion in the carboxylate anion formed from the unlabelled PLA derivative.

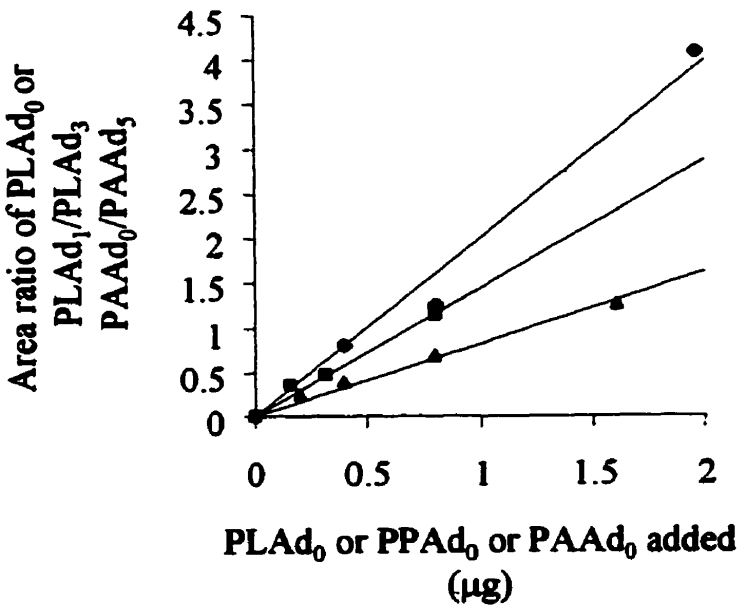

FIG. 4. Calibration curves, showing linear responses for ◆, PLA; ■, PPA; and ▲, PAA; over physiological ranges. The abscissa represents weights of unlabeled metabolites added to calibrating aqueous solutions of 1 ml volume containing 1 μg of $PLAd_3$ and 1 μg of $PAAd_5$. The ordinate represents the ratio of areas of unlabelled to labeled analogues obtained for these solutions.

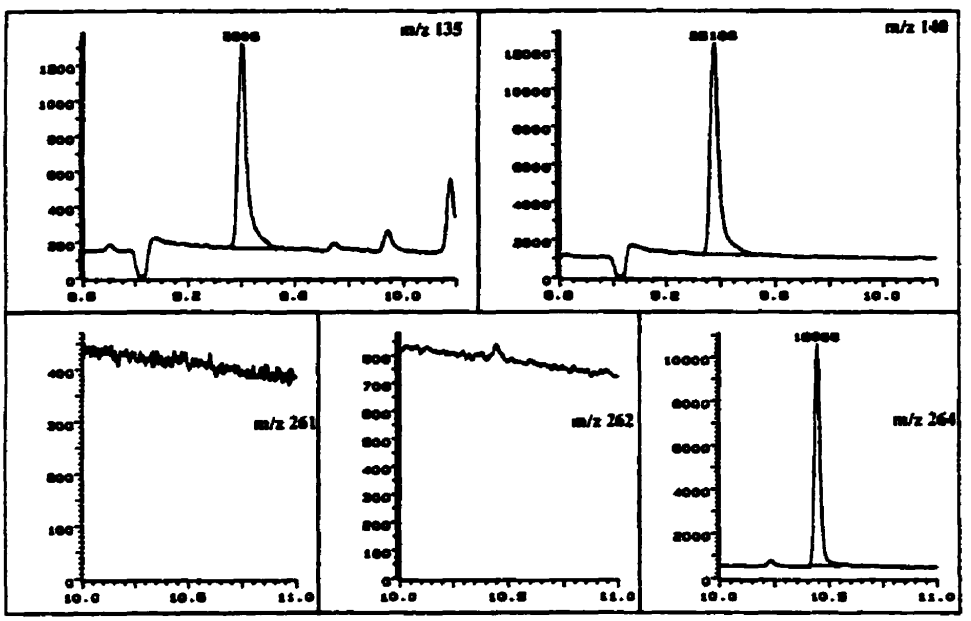

FIG. 5. SIM chromatograms obtained for phe metabolites in the brain of a normal mouse. PAA (*m/z* 135) is clearly present, while PLA and PPA (*m/z* 261 and 262) are not detected in the present example. The ion current at *m/z* 264 is due to the $PLAd_3$ internal standard.

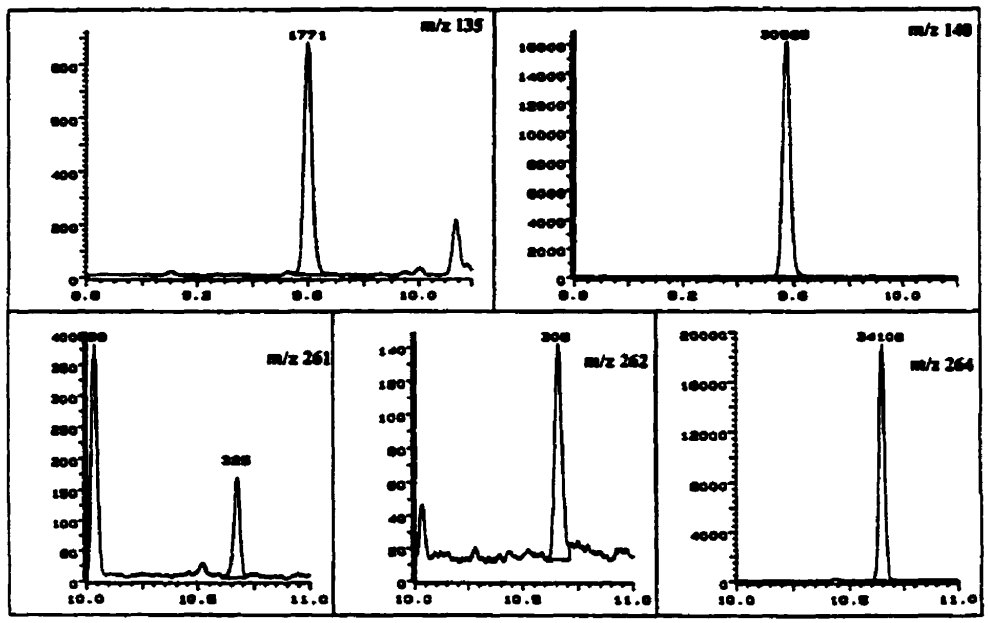

FIG. 6. SIM chromatograms obtained for phe metabolites in the brain of an ENU1 mouse. PAA is present at levels somewhat lower than in the normal mouse, and PLA and PPA are elevated slightly relative to the normal mouse. The retention times seen here are slightly different from Fig. 5 because in the interim the analytical column was replaced.

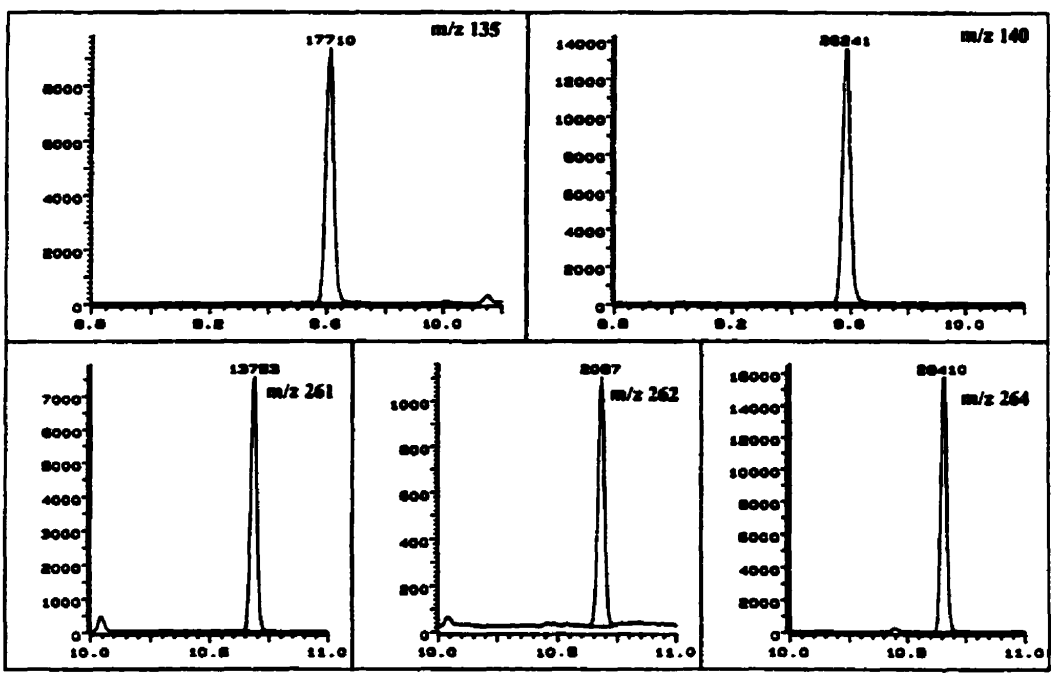

FIG. 7. SIM chromatograms obtained for phe metabolites in the brain of an ENU2 mouse. PAA, PLA, and PPA are all clearly elevated. The labelled internal standards (*m/z* 140 and 264) elute slightly earlier than their unlabelled analogues. The retention times seen here are again slightly different from Fig. 5 because in the interim the analytical column was replaced.

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CONNECTING TEXT

In Chapter 4, I describe a different approach for the treatment of Phenylketonuria. The current dietary therapy is difficult to follow and although efforts to improve the organoleptic properties are ongoing, imperfections in the composition of the diet and compliance with it are a continuous concern. Therefore an alternative form of treatment using PAL enzyme substitution is suggested here. Access to human surrogates for pre-clinical drug administration testing and the availability of a commercial supply of PAL were two prior obstacles that needed to be resolved to make this work possible. Once again the first problem was overcome when mouse orthologues for PKU and non-PKU HPA became available. These animals were characterised and the protocol required for metabolic manipulation established in Chapters 2 and 3. A solution to the second obstacle is detailed here in Chapter 4, where an efficient recombinant approach to produce PAL enzyme is described and the product tested in orthologous ENU mutant mouse strains with HPA. Proofs of pharmacological and physiological principles for the efficacy of PAL therapy are established.

CHAPTER 4

A different approach to treatment of phenylketonuria: Phenylalanine degradation with recombinant phenylalanine ammonia lyase

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ABSTRACT

Phenylketonuria (PKU), with its associated hyperphenylalaninemia (HPA) and mental retardation, is a classic genetic disease and the first to have an identified chemical cause of impaired cognitive development. Treatment from birth with low phenylalanine diet largely prevents the deviant cognitive phenotype by ameliorating HPA and is recognized as one of the first effective treatments of a genetic disease. However, compliance with dietary treatment is difficult and when it is for life, as now recommended by an internationally used set of guidelines, is probably unrealistic. Herein we describe experiments on a mouse model using another modality for treatment of PKU compatible with better compliance using ancillary phenylalanine ammonia lyase (PAL, EC 4.3.1.5) to degrade phenylalanine, the harmful nutrient in PKU; in this treatment, PAL acts as a substitute for the enzyme phenylalanine monooxygenase (PAH, EC 1.14.16.1) which is deficient in PKU. PAL, a robust enzyme without need for a cofactor, converts phenylalanine to *trans*-cinnamic acid, a harmless metabolite. We describe (*i*) an efficient recombinant approach to produce PAL enzyme, (*ii*) testing of PAL in orthologous *N*-ethyl-*N*-nitrosourea (ENU) mutant mouse strains with HPA, and (*iii*) proofs of principle (PAL reduces HPA) — both pharmacologic (with a clear dose-response effect vs. HPA after PAL injection) and physiologic (protected enteral PAL is significantly effective vs. HPA). These findings open a new way to facilitate treatment of this classic genetic disease.

INTRODUCTION

Phenylketonuria (PKU) (1) is the prototypical human Mendelian disease (OMIM 261600) demonstrating benefits from treatment (2). PKU and a related form of less harmful hyperphenylalaninemia (HPA, termed non-PKU HPA) result from impaired activity of phenylalanine hydroxylase (PAH; EC 1.14.16.1), the enzyme catalyzing conversion of the essential amino acid nutrient phenylalanine to tyrosine. The enzyme is responsible for disposal (by oxidative catabolism) of the majority of nutrient phenylalanine intake. The untreated PKU patient with persistent postnatal HPA is likely to experience irreversible impairment of cognitive development. Antenatal HPA, caused by transplacental transport of phenylalanine from the maternal pool to the fetus during a pregnancy in which there is maternal HPA, will harm the embryo and fetus. These disease-causing effects of PKU and maternal HPA are preventable by treatment to restore euphenylalaninemia (1).

Present treatment relies on the observation that a (semi-synthetic) diet low in phenylalanine (3-5) will prevent HPA and thus the disease. Because it involves a major alteration of lifestyle, dietary treatment is difficult. Moreover, dietary therapy can be associated with deficiencies of several nutrients (1), some of which may be detrimental to brain development (6;7). Moreover, most low phenylalanine treatment products have organoleptic properties sufficiently unsatisfactory that compliance with the treatment is compromised (8;9). Such concerns have greater relevance now that better and longer compliance with therapy of PKU and non-PKU HPA in all persons at risk has been recommended (10;11).

A combination of oral enzyme therapy with phenylalanine ammonia lyase (PAL; EC 4.3.1.5) and controlled low protein diet might replace dependence on the semisynthetic diet, for treatment of PKU after infancy (10;12). PAL is a robust, autocatalytic enzyme that, unlike PAH, does not require a cofactor (13). PAL converts phenylalanine to metabolically insignificant amounts of ammonia and *trans*-cinnamic acid, a harmless metabolite; the latter is converted to benzoic acid and rapidly excreted in urine as hippurate (14). A preliminary report indicates that HPA

is attenuated by oral administration of microencapsulated PAL in the rat with chemically induced HPA (15) and also, in preliminary studies, in naturally occurring HPA in a mouse model (16-18). Preliminary studies in human PKU patients showed analogous responses after the administration of PAL in enteric-coated gelatin capsules (19) or during use of an extracorporeal enzyme reactor (20). The human and even the animal studies were not continued because PAL was not available in sufficient amounts at reasonable cost.

(*i*) We used a construct of the *PAL* gene from *Rhodosporidium toruloides* (21) under the control of a high-expression promoter and expressed it in a strain of *Escherichia coli* to obtain large amounts of PAL (22). (*ii*) We used existing (23) and new strains (C.N.S., J. D. McDonald, and C.R.S. at http://data.mch.mcgill.ca/pahdb/mouse/sarkiss.html) of the mutant *N*-ethyl-*N*-nitrosourea (ENU)-treated mouse as orthologous models of human PKU and HPA to study enzyme substitution therapy with PAL. (*iii*) We showed that i.p. PAL injection lowers plasma phenylalanine in the mouse model (proof of pharmacological principle), and oral gavage of these mice with PAL enzyme, protected from inactivation by digestive enzymes, lowers plasma phenylalanine (proof of physiological principle). These developments point to an alternative approach to treatment of PKU, compatible with current guidelines (10;11).

MATERIALS AND METHODS

Synthesis of Recombinant PAL

Amplification of the PAL Gene: R. toruloides [ATCC no. 10788] was purchased from the American Type Culture Collection . Cells were grown in minimal medium containing phenylalanine as the sole carbon source (24), total RNA was extracted with hot acidic phenol (25) from a mid-logarithmic-phase culture, and mRNA was isolated with the PolyATtract mRNA Isolation System (Promega). A cDNA pool was then synthesized using the RiboClone cDNA Synthesis System (Promega).

Oligonucleotides (RTJP, 5'-AAGAATTCATGGCACCCTCGCTCGACT CGATCTCG-3', and RT2, 5'-CCGAATTCTAAGCGATCTTGAGGAGGACGT-3'), synthesized on an Ecosyn D300 DNA synthesizer (Eppendorf) were designed to feature an *Eco*RI site at their 5' end and to be homologous to 5' and 3' ends of the published sequence of the *R. toruloides PAL* gene (refs. (26) and (27); GenBank accession no. X51513). PCR amplification (28) was performed in 100 μl containing 100 pmol of each primer, 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton^X-100, nuclease-free BSA at 10 mg/ml, all four dNPTs (each at 0.2 mM), 30 ng of cDNA as the template, and 5 units of *Pfu* DNA polymerase (Stratagene). Samples were incubated in a DNA thermal cycler (Barnstead/Thermolyne) at 95°C for 30 s, at 50°C for 1 min, and 72°C for 3 min; repeated for 35 cycles. The PCR product was analyzed on a 1% agarose gel containing 0.6 mg of ethidium bromide per ml and subsequently cloned in pBluescript KS+ (Stratagene). Identity of the *PAL* gene was verified by sequence analysis using an AutoRead sequencing kit and an automated A.L.F. DNA sequencer (Pharmacia).

E. coli Strains and Plasmids: *E. coli* XL-1Blue was the host used for general cloning and vector construction; *E. coli* Y1091 was the host for fermentation to produce PAL. *E. coli* IBX-4, used in the animal study, was isolated from a Sprague-Dawley rat and identified by using api20E® bacterial identification kit (BioMerieux, Charbonnier les Bains, France). Plasmid pBluescript was used for cloning PCR-amplified *PAL* fragments.

Construction of High-Expression PAL Plasmid pIBX-7: Plasmid pIBX-1 (29) was modified by site-directed mutagenesis, changing the unique *Bam*HI site to an *Eco*RI site to allow cloning of the 2.2-kb *PAL* PCR fragment; the product was then further modified, which resulted in the deletion of the *Eco*RI sites. To increase expression, an additional *tac* promoter (*Ptac*) was synthesized by PCR and cloned downstream

of the existing *Ptac*. The 5' Shine-Delgarno sequence (AGGAG) is separated from the ATG codon by a 9-nt sequence (ACAGAATTT). Kanamycin resistance for selection of *PAL*-containing cells was conferred by substituting the Kanamycin resistance gene for the ampicillin resistance gene of pIBX-1. The plasmid was transformed into *E. coli* (hosts Y1091 and IBX-4) and induced with isopropyl-β-D-thiogalactoside (1 mM) for expression. The expression levels are similar in both hosts when cultured in shaken flasks (data not shown).

Purification of PAL *Enzyme from Cell Extract:* Frozen *E. coli* cells (Y1091; 500 g) expressing plasmid pIBX-7 were suspended in 2 L of Buffer A (30 mM Tris-HCL, pH 8.0/10 mM phenylalanine/2 mM cysteine) to which DNase I (5 mg/L) and 5 mM $CaCl_2$ were added. The cell suspension was homogenized three times in a Rannie MiniLab 8.30H high-pressure homogenizer (APV Canada, Montreal) at 700 bar (1 bar = 100 kPa); between passages, the suspension was cooled to 12°C. The homogenate was centrifuged (14,100 x g) for 1 hr at 4°C, the supernatant containing PAL protein diluted 2-3 fold in water and loaded on a column containing 1.25 L Q-Sepharose Big Beads (Pharmacia). Washes were performed at a linear flow rate of 153 ml/hr with three column volumes of Buffer A followed by 3 column volumes of Buffer B (30 mM Tris-HCl, pH 8.0/10 mM phenylalanine/2 mM cysteine/1 M NaCl). PAL protein is eluted with Buffer B in a linear gradient (3% - 100%) in six column volumes; the fractions with PAL activity were pooled. Ammonium sulphate was added slowly to the eluates (final concentration, 50%), stirred (30 min) at room temperature and then centrifuged (14,700 x g for 30 min at 4°C). The final PAL protein pellet was dissolved in a minimal amount of Buffer C (50 mM sodium phosphate, ph7.5/5 mM phenylalanine/1 mM glutathione).

ENU Mice

The ENU mouse models, deficient in hepatic Pah enzyme activity, were created by treating wildtype mice (BTBR/Pas background) with the alkylating agent

ENU. The original strains *Pah*$^{enu1/1}$(ENU1) and *Pah*$^{enu2/2}$(ENU2), from W. Dove and A. Shedlovsky, (University of Wisconsin, Madison), were produced in Wisconsin (23;30) and genotyped by J. D. McDonald (31). We produced the hybrid heteroallelic strain *Pah*$^{enu1/2}$(ENU1/2) in our facilities (see http://www.mcgill.ca/pahdb/ Pah Mouse / Mouse Pah Homologues). (All procedures described below have been reviewed and approved by the Animal Care Committee, McGill University).

The homozygous mutant ENU1 mouse is a counterpart of human non-PKU HPA. It has a missense mutation in the *Pah* gene [c. 364T→C in exon 3 (V106A)] (30;31), and on breeder diet (product 8626, Teklad, Madison, WI), it has both a normal plasma phenylalanine level and normal behavior but can be made hyperphenylalaninemic under controlled conditions with a phenylalanine load [by s.c. injection or gavage of L-phenylalanine at 1.1 mg/g (body weight)]. The homozygous mutant ENU2 mouse is a counterpart of human PKU. It has a missense mutation [c. 835T→C in exon 7 (F263S)] (23;31). On breeder diet, it has 10- to 20-fold elevated plasma phenylalanine and phenylketones in urine; euphenylalaninemia in this strain was achieved for these studies by placing mice on a diet free of phenylalanine (product 2826, Teklad) with ad libitum water containing L-phenylalanine (30 mg/L), for 3 consecutive days. After establishing euphenylalaninemia, ENU2 animals received standardized s.c. injections of L-phenylalanine [0.1mg/g (body weight)] to achieve reproducible HPA.

We developed the new ENU1/2 strain by crossing female ENU1 and male ENU2/+; all parents and offspring were typed by DNA analysis. The mutant heteroallelic animals have a normal plasma phenylalanine level on breeder diet but easily achieve a modest elevation, to levels between that of untreated ENU1 and ENU2, by s.c. injection or gavage of L-phenylalanine [1.1mg/g (body weight)]. The ENU strains are further described on our web site (see above).

Protocols to Study the Effect of PAL Enzyme on Phenotype

i.p. Administration of Recombinant PAL *(for Proof of Pharmacological Principle):* Studies were done on mice, younger than 1 year of age (all three strains). Each animal served as its own control; a sham (saline) injection was given in the first week, followed by the PAL injection 1 week later (same day of the week and time of day). Efficacy of i.p. PAL was measured by change in the plasma phenylalanine between the first and second week; animals (n = 5 per trial) received 2, 20, and 100 units of PAL. ENU1 and ENU1/2 animals were fasted overnight before to the experiment. Food was reintroduced after PAL administration. During the first week, 0.2ml of saline i.p. was followed directly by an L-phenylalanine challenge [1.1mg/g (body weight) by gavage] for the ENU1 and ENU1/2 mice; tail blood samples taken at zero min and 1, 2, 3, and 24 hr after the phenylalanine challenge. For the second-week protocol, PAL (replacing saline) was diluted to the dosage required with 0.1M Tris·HCl (pH 8.5).

Gavage of Recombinant PAL *(for Proof of Physiological Principle):* Studies were done in mice younger than 1 year of age. Each animal served as its own control and efficacy of PAL treatment was measured by change in plasma phenylalanine. We used two different PAL preparations: (*i*) recombinant PAL inside *E. coli* cells (IBX-4) and (*ii*) unprotected PAL (purified from Y1091 *E. coli* cells) in solution with aprotinin (protease inhibitor). Saline gavage was used as the control treatment. Plasma phenylalanine levels were adjusted in ENU2 animals (n = 4) to achieve euphenylalaninemia. In the first week, the animals received a phenylalanine load [0.1mg/g (body weight) by s.c. injection] on day 4, followed at 1hr and 2hr by gavage of saline bicarbonate (6 mg, to neutralize gastric acidity). Tail blood samples were taken before treatment (at time zero minus 5 min), and four times at hourly intervals after the phenylalanine challenge. In the second week, the gavage contained (*i*) 25 units of PAL (as induced recombinant *E. coli* cells, OD_{500}) or (*ii*)

200 units of PAL, in combination with 10 mg of aprotinin in the sodium bicarbonate buffer.

In Vitro Assays of PAL *Effect:* We measured efficacy of the PAL preparation *in vitro* by analyzing its effect on a solution containing 4mM phenylalanine (initial concentration) at 37°C and pH 8.5. We compared (*i*) the individual effects of non-recombinant *E. coli* cells (OD_{600} = 50), chymotrypsin (100 mg/ml), and mouse intestinal fluid (diluted 1:10 with Tris buffer at pH 8.5) with (*ii*) the effect of naked recombinant PAL either alone or in the presence of chymotrypsin (100 mg/ml) or of intestinal fluid (1:10 dilution with Tris buffer at pH 8.5) and (*iii*) the effect of *E. coli* cells (OD_{600} = 50) expressing PAL (5 units) alone or in the presence of chymotrypsin (100 mg/ml) or mouse intestinal fluid (1:10 dilution with Tris buffer at pH 8.5).

Analytical

Plasma Phenylalanine Concentration: We collected blood from tail into heparinized tubes, extracted plasma, and measured phenylalanine by HPLC (Beckman System Gold™, DABS amino-acid analysis Kit).

DNA Analysis: Animals were genotyped, as described (31), for the Pah^{enu2} mutation. We developed a method to detect the Pah^{enu1} mutation that eliminates a amplification created recognition site (ACRS) for the restriction enzyme *Taq*I . We used PCR, with primers 5'-GAGAATGAGATCAACCTGACA-3' and 5'-TTGTCTCGGGAAAGCTCATCG-3', to amplify a 169-bp segment of exon 3 (mouse *Pah*) from blood spots collected on Guthrie cards. The product subjected to *Taq*I digestion yields a distribution of fragments with a distinct banding pattern for each of three possible genotypes: $Pah^{+/+}$ generates two fragments (148bp and 21bp) , $Pah^{enu1/+}$ generates three (169bp, 148bp and 21bp) and $Pah^{enu1/1}$ generates one fragment (169bp).

RESULTS

Synthesis and Purification of PAL

We obtained PAL by expressing the pIBX-7 construct (Fig. 1) in *E. coli*, followed by purification on a Q-Sepharose column. The product is a yeast (*R. toruloides*) PAL enzyme, at 80% purity (Fig. 2). The yield in our present system is 100-150 units/g *E. coli* cells, with a K_m of 250 μM, at specific activity of 2.2-3.0 units/mg of PAL protein; 1 unit of PAL deaminates 1.0 μmol L-phenylalanine to *trans*-cinnamate (and NH_3) per minute at pH 8.5 and 30°C.

Effect of PAL in ENU Mice

We used the ENU mouse orthologues of human PKU and non-PKU HPA to obtain proof of pharmacologic and physiologic principles by demonstrating efficacy of PAL enzyme against the hyperphenylalaninemic phenotype.

Pilot Study

PKU mice (ENU 2; $n = 15$) on regular diet were treated with PAL (2, 20 or 100 units) by i.p. injection without any additional manipulations. Within 3 hr, the postinjection value for blood phenylalanine had fallen (range, 0-984 μM) from the pretreatment value (range, 389-2,012 μM). These preliminary observations demonstrated both an apparent treatment response and troublesome inter- and intra-individual variation. Accordingly, we controlled for the latter by adopting the protocols described above.

Proof of Pharmacological Principle: A single i.p. injection of PAL enzyme significantly lowered plasma phenylalanine in PKU mice (ENU2; $P < 0.05$) and showed a dose-response (Fig. 3); at each point, data are normalized to the control (sham-treated) values for each animal to accommodate inter- and intra-individual variation. The non-PKU HPA mice (ENU1) and the heteroallelic HPA stain

(ENU1/2) also responded to PAL treatment (data not shown). The effect of a single i.p. PAL injection in ENU1 mouse model persisted for 24 hr ($P < 0.02$; Fig. 4); ENU1/2 and ENU2 mice both had similar 24 hr responses (data not shown).

Proof of Physiological Principle: To demonstrate the effect of orally administered PAL on plasma phenylalanine levels required protease-resistant PAL formulations. Recombinant PAL enzyme activity was protected against inactivation by gastric acidity and intestinal digestive enzymes by retaining and shielding it in the *E. coli* cells where it was synthesized. We used these cells in the absence of a different form of PAL protection.

Recombinant PAL enzyme protected by the cell wall and membrane of *E. coli* resists inactivation by proteolytic intestinal enzymes *in vitro*; otherwise activity of naked enzyme is abolished (data not shown). PAL enclosed in *E. coli* cells was shown to reduce phenylalanine content of the *in vitro* solution (Table 1). When given by oral gavage, recombinant PAL (25 units) expressed in an *E. coli* strain isolated from Sprague-Dawley rat lowered plasma phenylalanine in ENU2 mice by 31% in 1 hr ($P < 0.04$) and 44% in 2 hr ($P < 0.0004$) (Fig. 5).

Other experiments *in vivo*, conducted with aprotinin (protease inhibitor) and recombinant PAL enzyme purified from *E. coli* (Y1091), gave further proof of physiological principle. This formulation resists inactivation by chymotrypsin and mouse intestinal fluid *in vitro* (data not shown). Oral gavage of naked PAL (200 units) combined with aprotinin (protease inhibitor) lowered plasma phenylalanine in ENU2 mice: by 50% in 1 hr ($P < 0.017$) and 54% in 2 hr ($P < 0.023$) (Fig. 6).

DISCUSSION

We describe a method to produce recombinant PAL (Fig. 1) that may enable the use of this enzyme to degrade excess phenylalanine in PKU where the normal pathway for its disposal is impaired. Why would this alternative be useful if, as is generally assumed, early treatment of PKU by a semisynthetic low phenylalanine diet is one

of the success stories of medical genetics (1;2)? The answers lie in the incidence of PKU, our expectations for its treatment, and the anticipated "prevalence" of patient treatment years.

The combined incidence of PKU and non-PKU HPA is on the order of 10^{-4} live births in populations of European descent. Current evidence reveals similar rates in Asian-Oriental and Arabic populations (1). New guidelines (10;11) for the treatment of HPA have appeared so that residual imperfections in outcome (1) can be overcome and expectations met; the guidelines advise treatment to restore blood phenylalanine levels as near normal as possible, as early as possible, for as long as possible, perhaps for a lifetime. In this context, the "prevalence" for patient treatment years takes on new meaning when it involves the difficult existing modes of treatment. The predicted "prevalence" in patient treatment years for a population of 10^8 persons, assuming 50 years of treatment per patient, would be 500,000 patient treatment years in half a century.

PKU is a multifactorial disease; mutation in the *PAH* gene and dietary exposure to the essential nutrient amino acid L-phenylalanine are equally necessary causes of the mutant phenotype (1). Treatment of HPA is feasible because the dietary experience can be purposefully modified, and the low phenylalanine diet has been its mainstay.

Untreated PKU is a disease at three phenotypic levels. At the proximal (enzyme) level, many different mutations in the *PAH* gene impair PAH integrity and function (32). Because PAH enzyme is the principle determinant of phenylalanine homeostasis *in vivo* (1), its impairment leads to HPA, which is the intermediate (metabolic) level of the variant phenotype. Impaired cognitive development and neurophysiological function is the distal (clinical phenotype); phenylalanine is the neurotoxic molecule (1), and hence the rationale to restore euphenylalaninemia.

Three modalities for treatment exist in theory or in practice: gene therapy, enzyme therapy, and diet therapy. The first has its appeal but is only at the experimental stage (33) (also C. Harding, personal communication) and unlikely to

be put into practice unless the alternatives fail. The third method (low phenylalanine diet therapy) was inaugurated in the 1950s (3-5) and has achieved its primary goal: it has prevented mental retardation in the adequately treated patient (1;34). However, dietary treatment of PKU and HPA is difficult; it involves rigorous compliance, a major alteration in lifestyle, and use of treatment products that have unusual organoleptic qualities. Moreover, measurements of cognitive and neurophysiologic outcomes show subtle deficits (IQ scores are 0.5 SD below normal), and there are lacunae in neuropsychological and neuro-physiological performance. Accordingly, there is growing interest in the second form of treatment (enzyme therapy).

Enzyme therapy could be done by replacement (of PAH enzyme) or by substitution (with another enzyme to degrade excess phenylalanine). Replacement of PAH requires the intact multienzyme complex for catalytic hydroxylating activity (1); it could be done best perhaps only by hepatic transplantation and this approach has not been pursued. But if the PAL enzyme could be administered by mouth to the PKU patient, it would have a certain appeal; therapy with enzymes protected from inactivation is feasible for the treatment of metabolic conditions (35;36). We propose that PAL will "substitute" for deficient hepatic PAH activity and degrade phenylalanine in the PAH-deficient organism. To test this hypothesis, we used the induced mutant (ENU) mouse orthologues of human PKU and non-PKU HPA (23). We first demonstrated proof of pharmacologic principle: given by injection, PAL acts *in vivo* to lower ambient blood phenylalanine levels (Fig. 3). We then demonstrated proof of physiologic principle; PAL placed in the intestinal lumen acts *in vivo* to suppress HPA (Figs. 5 and 6). The latter is a significant finding that capitalizes on three prior concepts and observations: (*i*) amino acids are in equilibrium between various compartments of body fluids (37;38) and ultimately in equilibrium with the intestinal lumen (39), so that treatment of intestinal lumen phenylalanine will affect all body pools. (*ii*) PAL placed in the intestinal lumen will modify phenylalanine content of body fluids in the whole animal (15;18). (*iii*)

PAL placed in the intestinal lumen will act on both the dietary phenylalanine and the endogenous runout of free phenylalanine from its bound pools (40). Accordingly, the appropriate dosage and schedule (to avoid under- or overtreatment) of oral PAL, perhaps in combination with a controlled and modestly low protein diet, should control the phenylalanine pool size without need of the drastic restriction of dietary phenylalanine as now practiced and requiring artificial diets. Ancillary treatment of PKU with PAL and prudent protein intake would become analogous to treatment of diabetes mellitus with insulin, with an additional feature — the enteral route would avoid problems with immune recognition of PAL.

Until now, the cost of PAL has prohibited any consideration of therapy; even animal studies were curtailed. The recombinant enzyme we describe here may avoid this constraint and has enabled our investigations. The relatively low specific activity of the recombinant PAL product and relative inefficiency at pH 7.0 (IBEX, unpublished data), may be offset by the long contact time between enzyme and substrate during passage through small and large intestine. The formulation currently under development is focused on completely protecting the PAL enzyme against a protease environment.

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TABLE 1
Change in phenylalanine content of *in vitro* solutions under various treatments

Experimental Conditions	Treatment[1]	Decrease in Phenylalanine (% control)[◷]
Control	Non-recombinant *E. coli* cells	0
	Chymotrypsin	0
	intestinal fluid	0
PAL	In induced recombinant *E. coli* cells	76
	In induced recombinant *E. coli* cells + chymotrypsin in medium	72
	In induced recombinant *E. coli* cells + intestinal fluid in medium	66

[1] Treatments were for 1hr followed by measurement of L-phenylalanine content.
[◷] Initial concentration, 4 mM L-phenylalanine.

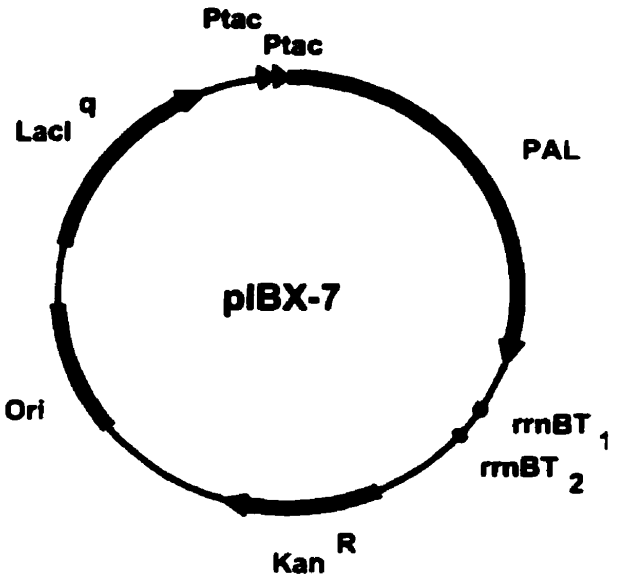

FIG. 1. *PAL* gene from yeast *R. toruloides* was cloned in the expression vector pIBX-7 where transcription is controlled by the strong inducible *tac* promoter and terminated by the rRNA transcription terminator sequences rrnBT1 and rrnBT2. *LacI*^{q} represses the *tac* promoter, hence isopropyl β-D-thiogalactoside is required to release it from the promoter. The kanamycin resistance gene (Kan^{R}) is included in the construct to allow selection of cells containing the plasmid.

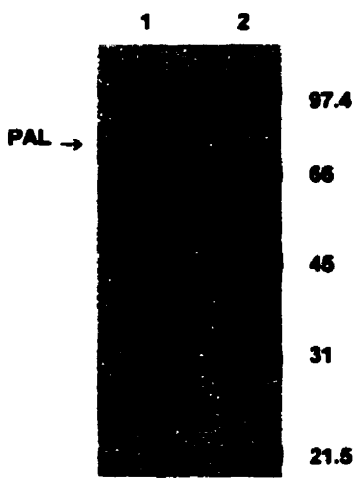

FIG. 2. Purified PAL enzyme (5 μg) separated on 4-15% gradient SDS/PAGE. Molecular mass markers in kDa are to the right. Lanes: 1, sample of PAL with ~20% impurities indicated by additional bands; 2, low-range molecular mass standards (Bio-Rad).

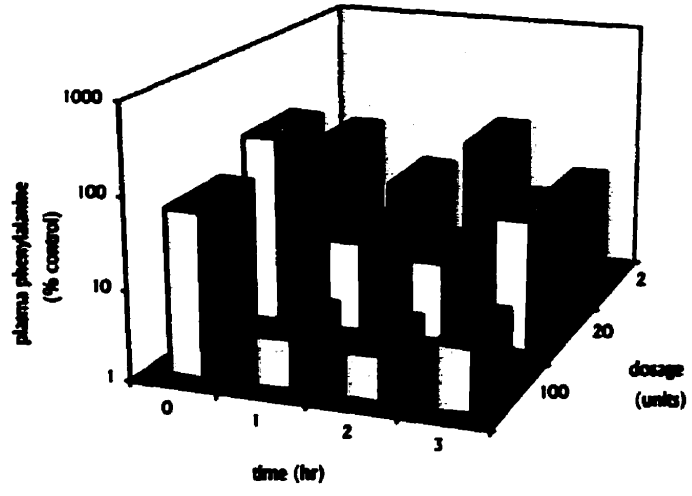

FIG. 3. Injection i.p. of recombinant PAL enzyme reduces plasma phenylalanine in the ENU2 mouse (*y* axis is logarithmic scale) over time (*x* axis) ($P < 0.05$). Reduction of plasma phenylalanine by PAL shows a dose-response relationship (*z* axis). Data are normalized to the control (sham-treated) values for each animal at each point. Data depicted are the average of 5 paired series. The range of control (100%) values was 390-2,013 μM for animals receiving 2 units of PAL, 572-1,488 μM for animals receiving 20 units, and 504-1,474 μM for animals receiving 100 units.

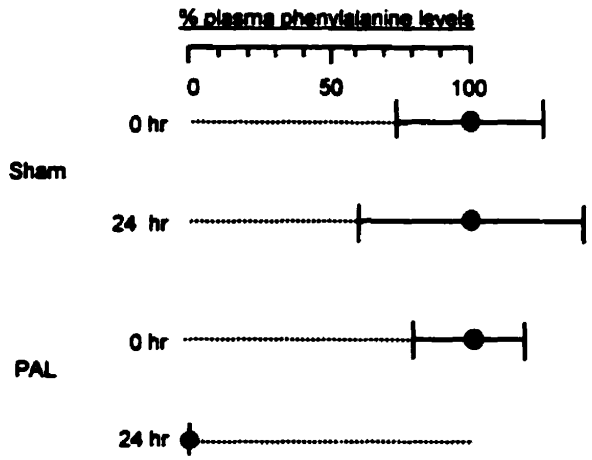

FIG. 4. A single i.p. injection of recombinant PAL enzyme (100 units at zero time), reduces plasma phenylalanine level in ENU1 mice by 95% at 24 hr ($P < 0.02$) relative to sham-treated controls. Data for five paired series (means $\pm$ 1 SD) are normalized to paired control values to accommodate inter- and intra-individual variation, The range of control values (100% at zero time) was 41-528 μM; every animal showed a response to PAL.

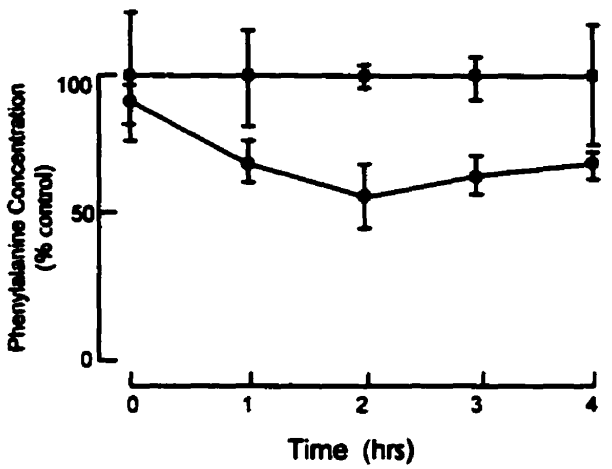

FIG. 5. Plasma phenylalanine levels in ENU2 mice after oral administration (25 units per mouse) of induced recombinant *E. coli* cells expressing PAL: 31% reduction within 1 hr ($P < 0.04$) and 44% reduction in 2 hr ($P < 0.004$). Data are normalized to control values (mean ± 1 SD). ■, sham; •, PAL enclosed in *E. coli*. The range of control values (100%) was 425-800 μM; every animal showed a response to PAL.

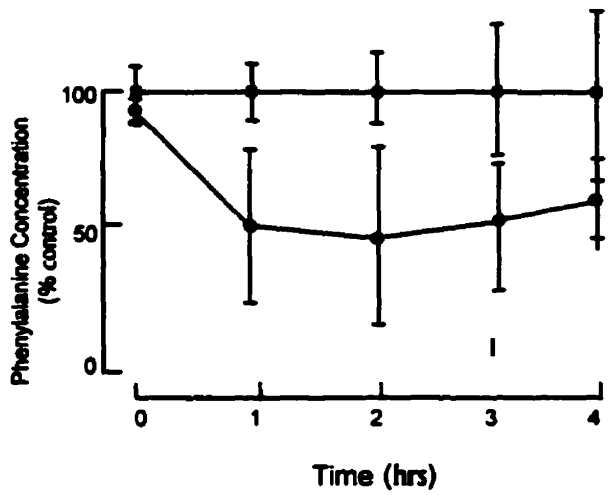

FIG. 6. Plasma phenylalanine levels in ENU2 mice after combined oral administration of naked PAL enzyme (200 units per mouse) and protease inhibitor (aprotinin): 50% reduction within 1 hr ($P < 0.017$) and 54% in 2 hr ($P < 0.023$). Data are normalized to control values (means $\pm$ 1 SD). ■, sham; ●, unprotected PAL plus aprotinin. The range of control values (100%) was 458-1,051μM; every animal showed a response to PAL.

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CHAPTER 5

Discussion

In the history of life sciences, mutants have been important resources for gaining insight into the biological function of genes (1). Orthologous animal models have served science by providing surrogates for human subjects in the study of disease phenotypes and their potential therapies (2). The particular advantages in mice as the orthologue are (*i*) highly homologous genome segments between mouse and man (3), (*ii*) the short mouse generation time, (*iii*) large litter size, (*iv*) their relatively small cost in care and maintenance and (*v*) the possibility of producing a model (transgenic) if a 'natural' one is not available (1;4). Access to mouse models has been a great help towards the understanding of underlying disease mechanisms (1).

The initial development of mouse models of human disease entails either a genotype-driven approach which required lengthy process of gene mutagenesis followed by isolation of the mutant strain and further inbreeding, or a phenotype-driven approach (i.e. by ENU treatment) which focuses on the recovery of novel or sought after phenotypes without focusing on the underlying gene. Identification of the associated gene is secondary. Either method results in models that only have the potential to measure the metrical traits of a specific mutant allele, and since conclusions about the role of a gene product drawn from a single mutation may be misleading (especially with the genotype-driven approach where the mutation may produce an atypical phenotype), it is important, if at all possible, to try and look at more than one mutant allele at a particular locus (5;6).

The Heteroallelic Mouse

In the presence of formerly established mutant lines, the potential development of heteroallelic animal models becomes an added advantage. Generation of heteroallelic mouse models provide the opportunity to produce additional variants of disease phenotypes, in some instances, also allowing the expression of mutant genes that would otherwise not be viable in the homoallelic form. In addition, when various alleles are available, series of heteroallelic models can be developed

providing a spectrum of phenotypic characteristics associated with the disease in question. For HPA, heteroallelism at the (human) *PAH* locus is representative of ~75% of probands in the population (7), therefore the corresponding mouse models can provide a better understanding of phenotypes associated with this disorder. To this end, the heteroallelic mouse model developed here contributes to a more complete understanding of the human condition.

In Chapter 2 and in the mouse genetic *Pah* homologues web page presented in Appendix D, we report on a new heteroallelic ENU1/2 mouse model and describe and compare phenotypes of control (BTBR/Pas) and mutant orthologues of human PKU and non-PKU HPA strains (ENU2, ENU1 and ENU1/2) and their heterozygous counterparts. The mutations associated with each of the homoallelic models were described in Chapters 1 and 2. The ENU1 mouse displays a mild phenotype (non-PKU HPA) and carries a mutation that affects amino acid residue 106 (V106A). Val 106 is conserved among mammalian species but is divergent in others; it is in a region that carries the majority of divergence (8). To date, no human *PAH* mutation has been identified affecting this amino acid residue. The nearest affected human amino acid changes occur at alanine 104 (A104D) - associated with variant PKU (7;9) or at serine 110 (S110L) (9); both mutants appear to affect secondary structure and protein stability (10). They are both located in N-terminal regulatory domain of the PAH enzyme (10). The ENU2 mouse has a severe PKU-like phenotype and carries a mutation affecting amino acid residue 263 (F263S). One human *PAH* mutation has been reported at this residue (F263L) (9). Position 263 is located 4.9 Å away from the Fe atom in the active site; substitution with leucine in the human protein is suspected to interfere with the protein's π-stacking interactions with the substrate (10). The substitution occurs at a position directly adjacent to amino acids involved in the pterin binding (10-15). In the ENU2 allele (F263S), a similar relationship may exist. The majority of amino acid changes in this region are associated with variant to severe

PKU phenotypes (7;16). As shown here, the heteroallelic (V106A/F263S) ENU1/2 mouse counterpart displays an intermediate HPA phenotype.

Metabolic Manipulation

Like human subjects, the mice are pliant to metabolic manipulation. (See Chapter 2, Fig. 2). An extended HPA can be induced in the ENU1 and ENU1/2 mice by injection or oral gavage with phe-containing solutions. For the ENU2 animals, euphenylalaninemia can be established after a three-day dietary control of phe intake. An oral dose or injection of phe can quickly reestablish PKU phenotype in the ENU2 animal. Although HPA can be easily induced in the ENU1 strain, the plasma phe clearance rate is fast and therefore levels do not remain high for an extended period of time; in contrast, the plasma phe clearance rate is slowest in the ENU2 animal making controlled HPA difficult to establish and maintain. The ENU1/2 model, with its attenuated clearance rate, displays HPA, suitable for experiments requiring highly monitored plasma phe regulation.

Pah Enzyme Activity

Hepatic Pah enzyme activities of the various models correlate inversely with the corresponding plasma and brain phe levels (See Chapter 2, Fig. 1 and 3). The ENU2 mice on normal diet with highly elevated plasma and brain phe levels have no detectable Pah activity, while ENU1 and ENU1/2 mice with ~24 % and 5% normal enzyme activity respectively have near normal plasma and brain phe levels. Enzyme activity in the kidney (as measured in humans by Lichter-Konecki et al. 1988 (17)), may also have an affect on the overall phenotype of the mice. This was not measured in our studies, however one would expect these mutations to have similarly deleterious effects. The ENU1/2 animals could be considered functionally hemizygous for their ENU1 alleles since they carry the ENU2 allele that apparently abolishes PAH activity (18). However, the fact that the activity of the enzyme in the heteroallelic mouse is lower than the average of the ENU1 and

ENU2 hints at a possible negative complementation between the alleles. The carrier counterparts with approximately 50% normal enzyme activity, display normal plasma and brain phe levels, an expected result for autosomal recessive phenotypes. These observations agree with the suggestion by Kacser and Burns that "enzymes do not act in isolation" (19). All systems function in response to a series of enzymes that are kinetically linked to other enzymes via their substrates. Fluxes through such systems are therefore a product of the systemic properties, and so variation at one locus must be measured as part of the whole system (sensitivity coefficient). There are as many coefficients for a given flux as there are enzymes in the system; the sum of these coefficients is equal to 1, with each coefficient being small. Therefore any large change in a specific enzyme's activity (a single coefficient) results in a negligible change in the flux. Fifty percent activity loss, as is the case with our heterozygote, results in a minimal change in phenotype (19).

Pah Enzyme Stability

Variation in Pah protein stability is observed in the different ENU treated HPA mouse models. Premature degradation of mutant protein can be triggered by structural abnormalities that are generally associated with altered oligomerization and increased aggregation of the protein (20). This may explain the results observed from the western blots for the ENU1 mutant protein, which displayed apparent protein instability. The ENU1 mutation does not lie in close vicinity to the PAH epitope (amino acids 139-155) recognised by the PH8 monoclonal antibody (13). Therefore loss of the epitope does not explain the lack of detectable PAH on the western blots. On the other hand, some missense mutations have minimal or no effect on the stability of the enzyme but result in loss of activity, as the affected residue is critical for proper enzyme function. This is the suspected cause of the phenotype observed in the ENU2 mouse where the protein is stable but does not have any detectable activity. In the ENU1/2 model the oligomer assembly, with interaction between the two different mutant subunits, results in a compound

with an intermediate stability in comparison to the ENU1 and ENU2 mutant proteins.

Measuring Behavior

Behavior measurement is difficult in the animal model. However maze-tests have been developed that measure 'executive function' in animals (21-23). The T-maze alternation test and the Win-Stay eight-arm radial maze task, which were used here, measure simple discrimination learning, short-term memory on spatial-reversal and reference memory, habit learning and memory for visual stimulus, respectively. In studying and comparing the various mouse models, the ENU2 mice (on normal diet) were the only ones showing impaired behaviour. No significant difference was observed between wildtype, ENU1, ENU1/2 and their heterozygous counterparts (none of these mutant animals was dietarily manipulated).

Reproductive Problems

Reproductive problems associated with maternal HPA in humans are also observed with the PKU mouse. These are again independent of either paternal or fetal genotype, depending solely on the maternal genotype and dietary phe intake (24). The maternal effect on offspring correlates directly with the degree of HPA, with loss of progeny only observed with ENU2 mothers. The level of HPA has also been effectively manipulated in the ENU2 mouse model allowing a measure of the dose-response relationship between maternal HPA and pregnancy outcome (25). A number of investigators have looked at the possible cause of these problems. It is apparent that mice, like humans, have a transplacental gradient for phe, favoring the fetus (25). This elevated HPA is the cause of cardiovascular developmental defects, different from those observed with humans. They were vascular (26), in comparison to those most reported in humans, which were cardiac (2). Once again, no significant differences were observed between wildtype, ENU1, ENU1/2 and their heterozygous counterparts (again, none of the

animals were manipulated dietarily). The natural HPA experienced by the ENU1 and ENU1/2 animals was not high enough to significantly affect reproductive status. These findings do resemble human data since maternal plasma phe levels below 400 μM range are usually associated with normal fetal outcome (2;27).

Phenylalanine Metabolites

The steady state level of plasma phenylalanine is determined by those processes that lead to the net disposal of phe and those that replenish the plasma pool (28). For the normal population, the disposal pathway mainly involves hydroxylation of phenylalanine to tyrosine with a partial fraction of phe used for net increase in body protein in growing children. However in the absence or reduced function of PAH, a secondary route, the transamination pathway, becomes significant. This pathway converts phe to PPA, which is subsequently converted to PLA and PAA and phenylacetylglutamine. Whether these products are the ones responsible for the brain abnormalities associated with PKU has long been debated. In the past, only analyses of urine, plasma and CSF samples were possible. These studies strongly suggested that the levels of transamination products achieved *in* vivo would not be toxic to brain (29). However, their potential toxicities in the brain could not be fully ruled out as no method was available that directly measured brain metabolite levels.

In Chapter 3 we described a novel method for measuring the concentrations of PPA, PLA and PAA in mouse brain tissue. To achieve this, we used NICI-GC/MS. This method requires the reduction of the PPA metabolite to a singly deuterium-labeled PLA ($PLAd_1$) as PPA is very unstable and decomposes in basic environments necessary for sample extraction. In addition, as $PLAd_1$, it can be measured in a standard stable isotope dilution assay, using $PLAd_3$ as internal standard, which is also used to measure the natural PLA metabolite. PLA, $PLAd_1$ and $PLAd_3$ are then derivatized with PFB and TFA, and PAA and $PAAd_5$ (internal standard) with PFB. The extra derivatization of PLA isotopomers with TFA is

necessary, as the 2-hydroxyl group of the phenyllactic acid would otherwise decompose by dehydration when passing through the gas chromatograph. Derivatization transforms the acids into thermally stable, chemically inert compounds that are volatile at temperatures below 300^{0}C. The gas chromatography column separates the compounds based on volatility.

As the molecules elute from the column and pass through the ionization chamber, they acquire an electron and become negatively charged. This ionization destabilises the molecular structure, which results in the loss of the pentafluorobenzyl radical for each of the PAA and PLA molecules and their isotopomers. The ions are then passed through a quadrupole mass analyzer, which sorts the ions according to their mass/charge ratio.

The ions produced by PAA and PLA were initially identified by a complete scan of their NICI mass spectra to identify the most intense ions. SIM was then applied, which measures the intensities of only the intense-significant ions identified in the full scans of the PFB esters of authentic PAA and PAAd$_5$ internal standard and the PFB and trifluoroacetyl esters of unlabelled and labelled PLA. SIM enhances the measured intensities by a few orders of magnitude.

To calculate the final endogenous concentrations of the compounds, the isotopic impurities of the internal standards and the natural abundances of heavy isotopes in the constituent atoms were measured and taken into account in the subsequent calculations. Calibration curves for all three metabolites, over the expected physiological ranges, demonstrated a linear response. The sensitivity of this method is estimated to be ~0.1 nmol/g brain tissue.

The use of deuterium-labelled analogues as internal standards contributes greatly to the accuracy of this method, as endogenous PPA (as reduced to PLAd$_1$), PLA and PAA are compared with virtually the same molecules, affected in the same manner during each step of sample preparation and analysis.

This NICI-GC/MS method was used to measure the concentration of PPA, PLA, and PAA in the brain tissue of wildtype, ENU1 and ENU2 mice. Metabolite

levels correlated directly with the corresponding plasma and brain phe levels indicating that at normal or near normal phe levels, the transamination pathway contributes insignificantly to the brain metabolic profile. The ENU2 mouse levels of metabolites measured in brain tissue did not reach levels of toxicity necessary for pathogenesis, as documented in earlier studies (29;30); levels were in fact 10-fold lower than those associated with toxicity, yet the ENU2 mice exhibit behavioural and cognitive impairment. We attribute this effect to phe itself until evidence indicates otherwise, and therefore agree that "there are no abnormal metabolites in PKU, only normal metabolites in abnormal amounts" (31). In this way the combined use of both the analytical method and the orthologous mouse models has made a contribution toward resolving a long-standing controversy about PKU associated pathologies.

Logic and Role of Low Phenylalanine Diet

With phenylalanine once again verified as 'the villain' associated with the natural course of the mutant phenotype, it is reassuring that the mode of therapy applied for the past four and a half decades has been functioning to eliminate the problem directly at the source. Low-phe dietary therapy was inaugurated in the 1950's (32-34) and has since been the mainstay of treatment. It is effective because the dietary experience can be purposefully modified affecting the internal milieu (2;35). It lowers and normalizes the steady-state value of phe concentration, reestablishing euphenylalaninemia. This treatment modality achieved its primary goal by preventing mental retardation and providing a positive pregnancy outcome (27;36;37). Yet the process is anything but simple, requiring rigorous compliance and a major alteration in lifestyle. Difficulties stem in part from the restrictions associated with constant dietary constraints and in part from the poor organoleptic qualities of the treatment itself (2;38). In addition, outcomes (whether a product of imperfect overall compliance or deficits in diet composition) remain less than

optimal, displaying subtle deficits in IQ scores and neuropsychological and physiological performance (2).

The problems associated with the diet-based treatment are even more cogent with recent recommendations for 'life long treatment' (37). There is now a growing interest in other forms of treatment that may be less rigorous and far less socially imposing.

Various alternative treatments of PKU and their apparent feasabilities were described in Chapter 1. Enzyme substitution therapy with phenylalanine ammonia lyase (Chapter 4) in this context is relevant.

Prior attempts (39-43) at PAL treatment did not progress beyond the occasional experiment, as the limited supply and costly acquisition of the purified form of the PAL enzyme prohibited any consideration of human therapy. Treatment with PAL, purified from natural sources, has a predicted daily cost of 10 to 10,000 times the yearly-approximated $5000 (CDN) cost/patient of the diet therapy (IBEX Technologies, personal communication). For the PAL modality of treatment to be economically feasible, the cost had to be lower or equal to that of dietary treatment.

PAL Protection and Use as an Alternative to Diet Therapy

Cloning technology offers the opportunity for an economical supply of PAL to enable enzyme substitution as an alternative treatment for PKU. The *PAL* gene from yeast *R. toruloides* has been inserted into a plasmid under the control of a high expression modified double *Ptac* promoter and expressed in non-invasive strains of *E. coli*. Active enzyme is easily isolated and purified from the bacteria. The recombinant enzyme described here provides a virtually limitless supply of PAL at sustainable cost.

Stabilized (protected) PAL, taken orally, could 'substitute' for the native mutant PAH protein; the hypothesis is as follows: PAL would function by depleting both dietary phe and the endogenous runout of free phe from its bound

pools (44) as all systemic phe pools are in flux equilibrium with the intestinal lumen (41;45-47). Recent profiles of the amino acids in the duodenum, jejunum and ileum have indicated extremely high (10-100 fold) concentration of intestinal free amino acids as compared to the plasma (44;48). Chang et al. proposed that the major sources of intestinal amino acids are from gastric, pancreatic and intestinal and other secretions and not from the dietary source, thus this phe pool would be endogenous in origin (44;48). The secretions are highly concentrated with peptides, polypeptides, proteins and enzymes, which are broken down into amino acids by tryptic digestion in the intestine. These amino acids are reabsorbed as they pass down the intestine (indicated by the steady decrease in amino acid concentrations down the intestine), constituting the large recirculation of amino acids between the body and the intestine (44;48). Oral ingestion of stabilized PAL would metabolize phe from ingested foods as well as the gastric, pancreatic and intestinal secretions, preventing the undesirable amino acid from reabsorption back into the body (44). The enzyme is protected (stabilized) to avoid degradation and digestion in the gastro-intestinal tract. In addition, the enzyme will pass through the intestine, metabolize phe and then be excreted in the stool, bypassing problems with immune response associated with accumulation of synthetic injectable drugs (39;41;49-51).

PAL, not requiring a cofactor, converts phenylalanine to the metabolites *trans*-cinnamic acid and trivial amounts of ammonia (52). The *trans*-cinnamate metabolized by the liver to benzoic acid, is then excreted in the urine as hippurate (53); it has no toxic properties (52;54;55).

To test the efficacy of recombinant PAL, we used mutant ENU mouse orthologues of non-PKU HPA and PKU and demonstrated both proof of pharmacological and physiological principles. The first objective was necessary to verify the efficacy of the recombinant PAL in depleting systemic phe levels. Intra-peritoneal injection of PAL acted *in vivo* to lower ambient blood phe levels. The effect persisted over 24 hours following PAL treatment, with plasma phe

levels markedly lower than those measured at zero time prior to PAL administration.

The second objective was to show that PAL can be administered orally and protected from degradation by intestinal digestion while actively depleting phenylalanine. This was effectively demonstrated using two different systems. First, recombinant PAL was expressed in a non-pathogenic *E. coli* organism that provided an environment for PAL, protecting it from the action of digestive enzymes, which were unable to penetrate the cells. The free phe, in the intestinal lumen, was transported through the bacterial membrane and converted by PAL to *trans*-cinnamic acid.

Preliminary experiments were carried out in two stages to measure the efficacy of the *E. coli* system. *In vitro* phe conversion was measured. Control cells (empty of PAL), chymotrypsin solution and intestinal fluid were compared to cells filled with active PAL, in protease free environment and exposed to chymotrypsin solution and intestinal fluid. The first three had no effect on phe levels in solution, whereas the cells filled with active PAL rapidly reduced phe levels by an average of ~71%, with a minimal impairment of the PAL effect on phe by chymotrypsin or intestinal enzymes in the media. In addition, naked PAL, unprotected by cellular membrane, lost all activity within minutes upon exposure to chymotrypsin and intestinal fluid. Our second approach was to observe the *in vivo* effect of PAL protected from degradation inside the *E. coli* vehicle. Gavage of ENU mice with this system acted to offset hyperphenylalaninemia in the mouse model.

We then studied a second system. PAL was delivered in solution with aprotinin protease inhibitor. This mixture, placed in the intestinal lumen, again acted *in vivo* effectively depleting the systemic phenylalanine levels.

The long contact time between enzyme and substrate during passage through small and large intestine can offset, in part, the relatively low specific activity of recombinant PAL (2.2-3.0IU/mg). In addition, systems adopted here

were interim experimental approaches used purely for proof-of-physiological principle. The application of this form of therapy in humans would ideally require a PAL enzyme of higher specific activity and require a formulation that will (*a*) effectively protect the protein against protease attack, and (*b*) not disturb the intestinal environment, allowing for normal digestion. With such a formulation, a more appropriate prediction of the dosage and treatment regiment for the various forms of HPA will be possible. The assumption stands that as with dietary therapy, PAL treatment will be adjusted on an individual basis. However, unlike dietary treatment, PAL should control the phenylalanine pool size without drastic life style restriction, making it far more user friendly. What would be considered an awesome feat with the current treatment could be more easily achieved with PAL; far better compliance on a day to day basis and potentially for a lifetime. It will also eliminate the worries associated with diet related nutrient imbalance and deficiency (56-58) as no major dietary adjustments (from the norm) will be required with the PAL treatment modality.

In *The Metabolic and Molecular Bases of Inherited Disease*, Scriver et al. recognized the major milestones in the PKU journey since its discovery in 1934. Among them were the discoveries in the 1950's of deficient PAH enzyme as the major cause of PKU and the restriction of dietary phe intake, a functional treatment for this disease; in 1963, the Guthrie test was introduced which allowed for population screening and in the 1980's, the *PAH* gene was mapped and cloned (2). Here we recognized the 1990's development of mouse models which opened the door to investigations that would have otherwise been impossible with human subjects. For the future decade, we hope to go a step further by introducing a new oral drug therapy for HPA. The work we have done here could transform the treatment of PKU to a more compatible approach (adding a positive effect with a mechanism vs. removing a negative influence by drastically changing life style and culture) and more importantly, improve the lives of the affected patients.

Future Aims

The findings in this thesis may have implications on a number of levels. The heteroallelic ENU1/2 mouse model, displaying HPA through metabolic manipulation, is suitable for experiments requiring a model with plasma phe levels that don't fluctuate constantly. Different severities of HPA, easily induced and maintained in this animal model, would be helpful in measuring clinical parameters such as maternal effect (viz McDonald, 2000) (25) and behavior.

We see three ongoing initiatives with PAL: 1. The goal here is to produce a PAL formulation that will have long-term efficacy. Various oral protein drug strategies have been attempted at IBEX with the aim of improving PAL protection, bioavailability and producing a formulation that is fit for repeated administration. Cross-Linked Enzyme Crystal (CLEC) formulation, involving batch crystallization of PAL and chemical cross-linking, has been suggested as a better option for PAL delivery; both steps are equally critical in producing a stable protein with good mechanical properties (IBEX unpublished data). CLEC technology has been used with other proteins to produce catalysts that have increased stability against denaturation by heat, organic solvents and exogenous proteases (59). This stability may be explained by the exclusion of proteases, owing to the size of the solvent channels (60). With PAL, the small substrate (phe) will easily penetrate the body of the crystal to react with the active sites.

2. Animals continue to be a tool for ongoing studies with PAL therapy as we have now developed a protocol for long-term PAL administration, using a route that provides direct access to the duodenum (See Appendix E). This route will serve at the interim experimental stage while enteric coating, which protects against the acid environment of the stomach (a possible contributor to enzyme inactivation), is not applied.

3. Long-term efficacy of oral formulated PAL will be demonstrated by extended (week-long) stable plasma and brain euphenylalaninemia and normal phe metabolite (PAA, PPA and PLA) levels (measured by NICI-GC/MS) in the mouse.

First-in-man studies will follow proof of long-term efficacy established in the mouse model. Like in animals, the end point here will be to establish long-term euphenylalaninemia in patients, following routine oral dosing of PAL. This form of treatment will ultimately replace the current dietary treatment of PKU.

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CLAIMS TO ORIGINALITY

1. Creation of a hybrid heteroallelic ENU1/2 (non-PKU HPA like) mouse strain, with descriptions and comparisons of control (BTBR/Pas), mutant orthologues of human PKU and non-PKU HPA strains (ENU2, ENU1 and ENU1/2) and the heterozygous counterparts.

2. Development of an amplification-created recognition site (ACRS) method for fast detection of the ENU1 mutation.

3. Metabolic manipulation and measures of the rate of plasma phe clearance for the HPA mouse strains. Demonstration of an intermediate phenotype for the ENU1/2 animals, lying between severe ENU2 and mild ENU1 phenotypes. The ENU1/2 mouse, more pliant to metabolic manipulation, displays an HPA phenotype that is suitable for experiments requiring highly monitored plasma phe regulation.

4. Comparison of measured hepatic Pah enzyme activities of the various mouse strains, showing inverse correlation between activity and the corresponding phe levels in plasma and brain and plasma phe clearance time. ENU1/2 animals display levels intermediate to those of the ENU2 and ENU1 mice.

5. Variation in Pah protein stability is shown for the different HPA mouse models. Stability of the ENU1/2 mutant protein is intermediate to the ENU1 (unstable mutant) and the ENU2 proteins.

6. Measures of the maternal HPA effect on offspring correlate directly with the degree of maternal HPA; abnormal phenotype observed only with the ENU2

mouse model. Measures of behaviour demonstrate an abnormal phenotype, again observed only with the ENU2 mouse (resembling human data where low levels of HPA are associated with normal CNS function).

7. A NICI GC/MS method was developed for analyses of PFB ester derivatives of PAA, PPA and PLA, when formed *in situ* in brain homogenates. A stable isotope dilution technique was used with $PAAd_5$ and $PLAd_3$ as internal standards.

8. Unstable PPA in the homogenate was reduced by NaB^2H_4 to stable $PLAd_1$, labeled with a single deuterium and discriminated from endogenous PLA in the mass spectrometer on this basis.

9. NICI mass spectra were obtained for the PFB esters of authentic PAA and $PAAd_5$ and for the PFB esters of the trifluoroacetyl esters of unlabelled and labelled PLA and their associated ions were identified.

10. Concentrations of PLA, PAA and PPA in the brain tissue of wildtype, ENU1 and ENU2 mice correlated directly with the corresponding plasma and brain levels of phe. The highest levels of the metabolites measured in the ENU2 brain tissue were 10-fold lower than those linked with toxicity (as described in literature reports). PKU-related CNS dysfunction is thus associated with elevated levels of phe itself.

11. Use of the ENU mouse orthologues to obtain proof of <u>pharmacological principle</u>: Intraperitoneal injection of recombinant PAL acted *in vivo* to lower ambient blood phe levels. The effect persisted for over 24 hours.

12. Use of the ENU mouse orthologues to obtain proof of physiological principle: Two recombinant PAL formulations (protected against degradation by intestinal digestion) were delivered into the intestinal lumen. Depletion of the endogenous pool (plasma phe) was demonstrated.

13. Development of a protocol for long-term PAL administration, using a route that provides direct access to the duodenum.

APPENDIX A

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A Heteroallelic Mutant Mouse Model: A New Orthologue for Human Hyperphenylalaninemia

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Hyperphenylalaninemias (HPA) are Mendelian disorders resulting from deficiencies in the conversion of phenylalanine to tyrosine. The vast majority are explained by a primary deficiency of phenylalanine hydroxylase (PAH) activity. The majority of untreated patients experience irreversible impairment of cognitive development. Although it is one of the best known hereditary metabolic disorders, mechanisms underlying the pathophysiology of the disease are still not fully understood; to this end, the availability of an orthologous animal model is relevant. Various mutant hyperphenylalaninemic mouse models with an HPA phenotype, generated by *N*-ethyl-*N'*-nitrosourea (ENU) mutagenesis at the *Pah* locus, have become available. Here we report a new hybrid strain, ENU1/2, with primary enzyme deficiency, produced by cross breeding. The ENU1, ENU1/2, and ENU2 strains display mild, moderate, and severe phenotypes, respectively, relative to the control strain (BTBR/Pas). The Pah enzyme activities of the various models correlate inversely with the corresponding phenylalanine levels in plasma and brain and the delay in plasma clearance response following a phenylalanine challenge. The maternal HPA effect on the fetus correlates directly with the degree of hyperphenylalaninemia, but only the ENU2 strain has impaired learning. © 2000 Academic Press

Key Words: **phenylketonuria; hyperphenylalaninemia; new orthologous mouse model; *Pah* gene; heteroallelic.**

Phenylketonuria (PKU) and related forms of non-PKU hyperphenylalaninemia (HPA) (OMIM 261600) are human autosomal recessive traits, characterized by elevated levels of phenylalanine (phe) in body fluids. These Mendelian disorders result from deficiencies in phenylalanine hydroxylase enzyme (PAH) (EC 1.14.16.1), which catalyzes the conversion of phenylalanine to tyrosine. Untreated patients have elevated levels of phenylalanine in body fluids. Those with persistent HPA from birth are likely to have irreversible impairment of cognitive development. Furthermore, transplacental transport of excess phe from the maternal pool to the fetus, during intrauterine development, puts offspring at risk for microcephaly and birth defects. These disease-causing effects of PKU and maternal HPA are preventable by treatment that restores euphenylalaninemia (1).

Although PKU is one of the best known hereditary metabolic disorders, the mechanism underlying its pathophysiology is still not fully understood (2). Furthermore, the current dietary treatment of the human disease is not optimal for either compliance or fully normal cognitive outcome. To this end, the availability of an orthologous animal model is relevant (3,4).

Chemically induced HPA in the rat had long served as the animal "model" for "PKU"; however, it had many imperfections (5). These were avoided when a mutant HPA mouse model generated by *N*-ethyl-*N'*-nitrosourea mutagenesis (6,7), manifesting all forms of the harmful HPA phenotype, became available (1,8). The mice thus generated had autosomal recessive forms of HPA representing both

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locus and allelic heterogeneity; for example, the strain first identified (9), called *hph*-1, was mutated at the GTP-CH locus (10); GTP-CH catalyzes the first step in biosynthesis of tetrahydrobiopterin, a cofactor essential for hydroxylase activity. Subsequently, other strains all mutated at the *Pah* locus, but with varying degrees of phenotypic severity[2] (orthologues of human PKU and non-PKU HPA), became available (3,8). Here we report a hybrid strain ($Pah^{enu1/2}$), produced by crossing a homozygous female $Pah^{enu1/1}$ non-PKU HPA mouse with a heterozygous male $Pah^{enu2/+}$ PKU carrier. We describe the organismal phenotypes of the control (BTBR/Pas) and mutant strains ($Pah^{enu1/1}$ and $Pah^{enu2/2}$, the heteroallelic $Pah^{enu1/2}$ strain, and their carrier counterparts), their corresponding metabolic parameters, hepatic Pah enzyme activity, blood and brain phe levels, behavioral parameters and corroborate prior evidence of maternal HPA effect on the fetus. We also discuss how this mouse model is being used to measure the response to an alternative therapy for PKU by enzyme substitution with phenylalanine ammonia lyase (EC 4.3.1.5) to degrade excess dietary and endogenous phenylalanine (18).

MATERIALS AND METHODS

ENU mice. Wild-type mice (BTBR/Pas background) were treated with alkylating agent (*N*-ethyl-*N'*-nitrosourea) and mutations mapping to the mouse phenylalanine hydroxylase locus (*Pah*) were identified by hyperphenylalaninemia, a metrical metabolic trait (3,7). The original strains $Pah^{enu1/1}$ (phenotype name, ENU1) and $Pah^{enu2/2}$ (ENU2), kindly given to us by W. Dove and A. Shedlovsky, were bred in Madison, Wisconsin (3,7) and later genotyped (11). The hybrid heteroallelic strain ($Pah^{enu1/2}$, ENU1/2) was bred in Montreal, Canada, as described here.

The homozygous mutant ENU1 mouse is a counterpart of human non-PKU HPA (7,8); it expresses a missense mutation (c.364T → C, V106A) in exon 3 of the *Pah* gene (11). The homozygous mutant ENU2 mouse is a counterpart of human PKU (3,8); it expresses a missense mutation (c.835T → C, F263S) in exon 7 of the *Pah* gene (11). The ENU1/2 strain was developed by selective mating of ENU1♀ × $Pah^{enu2/+}$ (ENU2/+)♂. Genotypes at the *Pah* locus of all breeders and offspring were verified by DNA analysis. All procedures described below were reviewed and approved by the Animal Care Committee at McGill University.

Metabolic manipulation of animal models. Plasma and brain phe levels were measured in all mice when fed on breeder mouse chow (Teklad 8626) (Madison, WI). ENU2 mice were then made euphenylalaninemic by placing them, for 3 consecutive days, on solid diet devoid of phe (Teklad 97152) supplemented with water containing 30 mg/L L-phe, and then injected with L-phe (1.1 mg/g body wt, subcutaneously) to produce a controlled predictable HPA state. ENU1 and ENU1/2 mice do not show the HPA state on normal diet; a predictable degree of HPA was produced in these strains by subcutaneous injection of L-phe (1.1 mg/g).

Metabolic characterization. Time-dependent plasma clearance of phe was measured after subcutaneous injection of the standard dose of L-phe in the ENU1, ENU1/2, and euphenylalaninemic ENU2 animals. Plasma phe was measured at baseline (zero h) and 1, 2 and 3 h postchallenge. We use this parameter as a measure of phe "runout."

Pah enzyme assay. Liver tissues were prepared and stored at −80°C until analysis. Protein concentration was measured using the Bio-Rad protein assay kit (Hercules, CA). The assay (12–14), measuring the conversion of [U-^{14}C]-L-phe to labeled tyrosine, was modified as follows. Homogenate (100 μg) and 0.4 mM 6-methyltetrahydropterine (6-MPH_4) (Schircks Labs, Jona, Switzerland) were added to the reaction mixture (0.1 M potassium phosphate buffer (pH 6.8), 1000 units catalase (Sigma–Aldrich, Oakville, Ontario, Canada), 0.3 mM L-phe, 4×10^5 cpm [U-^{14}C]phe (DuPont NEN, Boston, MA), and 10 mM dithiothreitol (ICN, Costa Mesa, CA)) (final reaction volume = 250 μl). The samples were incubated (with shaking) at 25°C for 1 h, then boiled for 5 min, placed on ice, and analyzed according to Ledley *et al.* (14). Preliminary experiments established linearity of activity versus protein concentration and incubation time.

Western blots. Liver was homogenized in buffer containing 50 mM Trizma (pH 8.0), 150 mM NaCl, 1% (v/v) NP-40, 0.3 μM aprotinin (Roche Molecular Biochemicals, Laval Quebec, Canada), and 1 mM Pefabloc (Roche Molecular Biochemicals). Samples were electrophoresed on 10% SDS–polyacrylamide gel, transferred to Hybond-C extra nitrocellulose (Amersham, Little Chalfont, Buckinghamshire, En-

[2] $Pah^{enu1/1}$ mice with an HPA phenotype were initially named the Pah^{hph-1} strain; $Pah^{enu2/2}$ mice are the PKU counterpart.

gland), and immunoblotted to detect mouse Pah protein, with 1 μg/ml anti-mouse PH8 primary antibody (PharMingen, Missisauga, Ontario, Canada) (15), detected with peroxidase-labeled anti-mouse antibody (1:1000 dilution) (Amersham), and developed with RPN 2109 ECL (Amersham). Human PAH was used as a positive control.

Effect of parental phenotype on fetal survival. Females were introduced to their breeding partners immediately following sexual maturity. The following female homozygous and compound phenotypes were studied: wild-type BTBR/Pas (Control), ENU1, ENU1/2, ENU2, and heterozygous ENU1/+, ENU2/+. Various male genotypes (and phenotypes) were also studied for possible paternal affect. The effect of maternal HPA on progeny was measured by counting the number of litters and number of progeny at birth and weaning. Blood phe was measured in the dam prior to breeding and at delivery and weaning.

Tests of behavior. The T-maze alternation test was conducted as described by Nasir *et al.* (16). A Win–Stay eight-arm radial maze task was conducted 1 week following the completion of the T-maze tests. The latter task, modified as described by Seamans and Phillips (17) and Nasir *et al.* (16), consists of a central octagonal platform with eight arms radiating from the middle (35 × 9 × 12 cm); eight 6-W light bulbs mounted directly above food cups (−4 cm from the base) were placed at the end of each arm. On the first 2 days the mice were acclimated to the maze for 10 min; no food was introduced. For the following 3 days, the animals were tested (two trials/day). On each trial day, four randomly selected arms were lit and baited with Kellogg's Froot Loops (Etobicoke, Ontario, Canada) cereal. Following food consumption, the light was turned off. After all four pellets had been retrieved (max. time allowed, 10 min) the animal was removed for a 5-min period and then placed back into the maze where the same four arms were lit and baited once again. The arm choices, the time latencies (s/min) to reach the food in the first arm chosen, and the total time required to complete the daily trial were recorded.

DNA analysis. Animals were genotyped for wild-type and mutant alleles as described (11,18).

Brain and blood samples. The brain was removed within 5 s after death and homogenized (on ice) in 0.1% (w/v) sodium dodecyl sulfate (19). Allo-

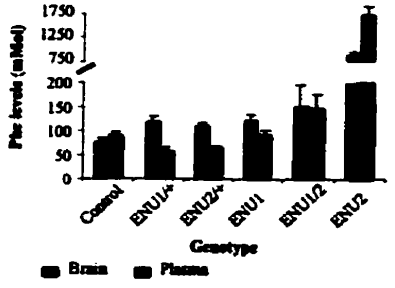

FIG. 1. Plasma phe values (μmol, mean ± SE, n = 4): control (BTBR/Pas) mice, 91.2 ± 6 μmol; ENU1/+, 58.5 ± 7; ENU2/+, 65.7 ± 1; ENU1, 93.4 ± 8; ENU1/2, 147.0 ± 29; ENU2, 1697.4 ± 175. The corresponding brain phe values (μmol, mean ± SE) are control, 76.9 ± 6; ENU1/+, 120.3 ± 10; ENU2/+, 112.5 ± 5; ENU1, 123.6 ± 1; ENU1/2, 150.7 ± 42; ENU2, 876.4 ± 30.

isoleucine was added as an internal standard; homogenate was then incubated for 15 min at room temperature followed by the addition of 1.5% (v/v) 5-sulfosalicylic acid dihydrate solution and then vortexed and centrifuged at 14,000g for 15 min. The supernatant was extracted and frozen at −80°C until analysis. Blood was collected from the tail into heparinized tubes and plasma was obtained by centrifugation.

Amino acid analysis. Amino acids were measured by HPLC on a Beckman 6300 automatic amino acid analyzer (Palo Alto, CA).

Statistical analysis. We used single-factor analysis of variance (ANOVA) between the different genotypes (conducted for each factor measured). The Poisson heterogeneity test confirmed homogeneity within genotypes. All values reported on figures and text are expressed as means ± SEM.

RESULTS

Metabolic manipulation and catabolic rates for Phe. ENU2 mice exhibit 10- to 20-fold elevated plasma phe levels and 10-fold increase in brain phe levels ($P = 1.13 \times 10^{-11}$ and 4.7×10^{-15}, respectively) when fed with breeder mouse chow (Fig. 1); the corresponding measurements for the ENU1/2, ENU1, ENU1/+, ENU2/+, and BTBR/Pas wild-type strains show near normal or normal values (ANOVA conducted between these genotypes showed P = 0.17 and 0.7, respectively). After the standardized injection to induce HPA in the ENU2 (initially made euphenylalaninemic, see Materials and Methods), ENU1, and ENU1/2 animals, the time-dependent

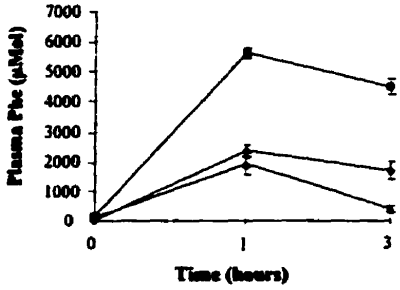

FIG. 2. Plasma phe clearance rates for ▲, ENU1; ◆, ENU1/2; and ■, ENU2 animals loaded with a standard dose of phe (mean ± SE; $n = 5$).

plasma clearance rates (catabolic rates) (Fig. 2) show that ENU2 animals have the most severe metabolic phenotype, ENU1 animals the least severe, and ENU1/2 mice an intermediate phenotype.

Pah enzyme activity. We examined the relationship between hepatic Pah enzyme activity and plasma phe levels (Fig. 3). The ENU2 mice, with undetectable enzyme activity, have the most elevated plasma phe levels. ENU1 and ENU1/2 mice, with ~24 and 5% normal enzyme activity, respectively, have near normal plasma phe levels under standard diet conditions. ENU2/+, ENU1/+ heterozygotes with ~49 and 61% normal enzyme activity, have normal plasma phe levels as expected for a recessive trait.

Enzyme protein levels. Hepatic Pah protein levels, as visualized by immunoblotting of liver extracts, are different in the three ENU phenotypes (Fig. 4). These findings imply that enzyme stability may vary with genotype: The ENU1 mutation apparently affects stability (and therefore activity) of the protein, evident by the clearly reduced level of immunoreactive Pah. The ENU2 mutation, which impairs enzyme activity, may also affect stability; it produces somewhat diminished but easily detectable protein, evident by the substantial amounts of immunoreactivity. The ENU1/2 compound is intermediate in both protein stability and enzyme activity. These findings were consistent in six replicates.

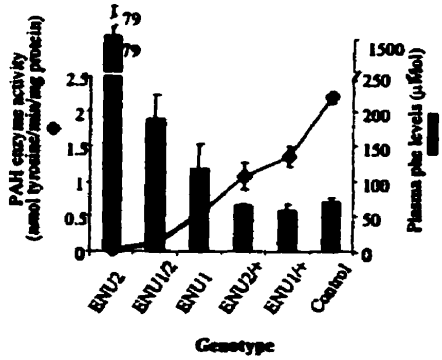

FIG. 3. Pah enzyme activity vs plasma phe values in control and ENU phenotypes (mean ± SEM; $n = 4$). Enzyme activity and plasma phe levels of the ENU1/2 animals are intermediate compared to those of ENU1 and ENU2 animals ($P < 0.05$).

FIG. 4. Western blot for Pah protein in liver extracts (representative of six replicates). The ~52-kDa band (arrow) is the Pah monomer; other faint bands represent nonspecific binding, confirmed by their absence with purified human PAH (lane 1) and their presence in the wild-type mouse strain (lane 7). Lane 1: purified human PAH (hPAH); lane 2: ENU2, lane 3: ENU1/2; lane 4: ENU1; lane 5: ENU1/+; lane 6: ENU2/+; lane 7: wild-type BTBR/Pas. Rainbow marker used for reference.

Maternal effect. The ENU2 dams with severe HPA conferred a severe maternal effect on offspring; the other strains did not, the effect being dependent on the degree of HPA (Table 1). ANOVA analyses showed a significant reduction in the number of litters and progeny at birth and of those surviving to weaning only from the ENU2 mothers ($P < 0.00014$ and 0.0008, respectively).

Behavior. The T-maze alternation test assesses simple discrimination learning and short-term memory; we observed impaired behavior only in the ENU2 animals (Table 2). The Win–Stay eight-arm radial maze task measures reference memory, habit learning, and memory for a visual stimulus. Only the ENU2 model showed impaired behavior (Table 2).

DISCUSSION

Animal models assist the study of disease phenotypes and potential therapies (20). Mouse models

TABLE 1
Maternal Effect on Number and Size of Litters and Survival to Weaning (Mean ± SE, $n = 5$)

Maternal genotype	Average No. of litters/mated female	Average No. of litters surviving to weaning/ mated female	Average No. of progeny at birth/litter	Average No. of progeny surviving to weaning/litter
Control	1.9 ± 0.2	1.6 ± 0.1	8.0 ± 0.7	7.1 ± 0.8
ENU1/+	2.0 ± 0.3	1.6 ± 0.5	8.1 ± 1.2	7.6 ± 1.4
ENU2/+	3.1 ± 0.4	1.9 ± 0.3	6.6 ± 0.6	4.9 ± 0.7
ENU1	2.7 ± 0.2	1.9 ± 0.2	6.2 ± 0.5	5.0 ± 0.5
ENU1/2	3.1 ± 1.3	2.1 ± 0.4	6.0 ± 0.8	5.3 ± 0.8
ENU2	1.3 ± 0.2	0 ± 0[a]	1.3 ± 0.9	0 ± 0[b]

[a] ANOVA, $P < 0.00014$.
[b] ANOVA, $P < 0.0008$.

offer the added advantage in that their genomes are highly homologous with the human genome (21). In addition the mouse and human phenylalanine hydroxylase genes have ~87% conservation of the nucleotide sequence, with ~10% of the changes silent; the corresponding proteins have 95% conserved amino acid sequences (14). Here we have described and compared phenotypes of control (BTBR/Pas) and mutant strains ($Pah^{enu1/1}$, $Pah^{enu2/2}$, $Pah^{enu1/2}$, and their heterozygous counterparts), the mutants being orthologues of human PKU and non PKU-HPA (Fig. 5).

The ENU1 mouse, which displays a mild phenotype (non-PKU HPA), carries a mutation in exon 3 (11) affecting the N-terminal region of the enzyme distant from the catalytic residues of PAH/Pah, recently identified by crystallographic analysis of the human enzyme (22,23). The ENU2 counterpart has a mutation in exon 7 which includes the catalytic region; the site is predicted to have π-stacking interactions with the substrate (24). The resulting phenotype is PKU-like (11). The ENU1/2 heteroallelic counterpart displays an intermediate phenotype.

Pah enzyme activities of the various models correlate inversely with the corresponding phe levels in plasma and brain and with plasma phe clearance time. ENU2 mice with undetectable enzyme activity have ~20-fold elevated plasma and brain phe levels and the slowest clearance rate, while ENU1 and ENU1/2 mice with ~24 and 5% normal enzyme activity, respectively, have near normal plasma and brain phe levels and more normal clearance rates. The carrier counterparts, having approximately half the normal enzyme activity, display normal plasma and brain phe levels. These metrical traits are those expected for an autosomal recessive phenotype (25).

Pah protein shows different states of stability in the different ENU phenotypes. The ENU1 mutation results in protein instability, whereas the ENU2 mutation has only a minor effect on this parameter. Stability in the ENU1/2 compound is intermediate in comparison.

The behavioral assessment of ENU2 mice shows impaired simple discrimination in short-term memory, reference memory, habit learning, and memory for a visual stimulus. The other genotypes were cor-

TABLE 2
Behavioral Assessment (Mean ± SE, $n = 6$)

Test	ENU2	Remaining genotypes combined	Significance (P)
T-maze alternation test			
Increase in average time required to reach food (s)	20.7 ± 6.7	5.2–10.3 ± 0.4–2.1	<0.005
The Win-Stay eight-arm radial maze task			
Reduction in the No. of entries into initially lit arms	5.5 ± 0.3	6.2–7.5 ± 0.4–0.5	<0.03
Increase in average time required to reach food (s)	58.3 ± 15.5	5.6–13.1 ± 1.3–1.9	$<4 \times 10^{-9}$
Increase in average time to complete trials (s)	290.0 ± 36.0	71.8–138.6 ± 6.4–13.4	$<8 \times 10^{-12}$

FIG. 5. Classic mouse models of PKU and non-PKU HPA, induced by *N*-ethyl-*N'*-nitrosourea (ENU) mutagenesis: A, control BTBR/Pas; B, $Pah^{enu1/+}$ heterozygous carrier/wild-type; C, $Pah^{enu1/1}$ non-PKU HPA orthologue; D, $Pah^{enu2/+}$ heterozygous carrier/wild-type; E, $Pah^{enu2/2}$ PKU orthologue; F, $Pah^{enu1/2}$ heteroallelic orthologue.

respondingly unaffected in clinical measures. These observations concur with earlier findings (26).

The maternal effect on offspring correlates directly with the degree of HPA and is apparent only with the ENU2 mothers; the effect is completely independent of paternal genotype. These findings resemble human data where maternal plasma phe levels below the 400-μmol range are usually associated with normal fetal outcome (1).

The ENU1/2 mouse model has been particularly useful for measuring the response to an alternative therapy for PKU, namely oral administration of phenylalanine ammonia lyase in an effort to degrade excess phe from the accumulated pools (18). Our present studies demonstrate proof of principle, both pharmacological and physiological. The present report describes in detail the parameters available to measure the short- and long-term responses to this therapy and document the advantages of the ENU1/2 strain for this work.

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APPENDIX B

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Measurement of Phenyllactate, Phenylacetate, and Phenylpyruvate by Negative Ion Chemical Ionization–Gas Chromatography/Mass Spectrometry in Brain of Mouse Genetic Models of Phenylketonuria and Non-Phenylketonuria Hyperphenylalaninemia

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Phenylketonuria (PKU) (OMIM 261600) is the first Mendelian disease to have an identified chemical cause of impaired cognitive development. The disease is accompanied by hyperphenylalaninemia (HPA) and elevated levels of phenylalanine metabolites (phenylacetate (PAA), phenyllactate (PLA), and phenylpyruvate (PPA)) in body fluids. Here we describe a method to determine the concentrations of PAA, PPA, and PLA in the brain of normal and mutant orthologous mice, the latter being models of human PKU and non-PKU HPA. Stable isotope dilution techniques are employed with the use of [$^2H_5$]-phenylacetic acid and [2,3,3-$^2H_3$]-3-phenyllactic acid as internal standards. Negative ion chemical ionization (NICI)–GC/MS analyses are performed on the pentafluorobenzyl ester derivatives formed *in situ* in brain homogenates. Unstable PPA in the homogenate is reduced by NaB^2H_4 to stable PLA, which is labeled with a single deuterium and discriminated from endogenous PLA in the mass spectrometer on that basis. The method demonstrates that these metabolites are easily measured in normal mouse brain and are elevated moderately in HPA mice and greatly in PKU mice. However, their concentrations are not sufficient in PKU to be "toxic"; phenylalanine itself remains the chemical candidate causing impaired cognitive development. © 2000 Academic Press

Key Words: **PKU; non-PKU hyperphenylalaninemia; phenyllactate; phenylacetate; phenylpyruvate; phenylalanine metabolites; GC/MS; deuterium; selected ion monitoring; stable isotopes; mouse models.**

Phenylketonuria (PKU)[2] and related forms of non-PKU hyperphenylalaninemia (HPA) (1) are autosomal recessive disorders of amino acid metabolism, which result from primary dysfunction of phenylalanine hydroxylase (PAH), the hepatic enzyme responsible for catalyzing the conversion of phenylalanine to tyrosine. PKU and HPA patients have elevated levels in body fluids of phenylalanine (phe) and of metabolites derived from phenylalanine (phenylpyruvate (PPA), phenylacetate (PAA), and phenyllactate (PLA)) (2, 3). Untreated PKU probands usually have severe irreversible mental retardation; the risk of mental retardation is less in the conditions with a lower degree of HPA (non-PKU HPA).

The free phe pool in the normal subject is derived from two sources: intake of exogenous dietary protein and turnover of endogenous polypeptides. Approximately 25% of the free pool is normally incorporated into protein; most of the remaining 75% is hydroxy-

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[2] Abbreviations used: d, suffix denotes substitution by x atoms of deuterium in the molecule; ENU, *N*-ethyl-*N*′-nitrosourea; (ENU1/1) orthologous mouse model for human non-PKU hyperphenylalaninemia; (ENU2/2), orthologous mouse model for human PKU; HPA, hyperphenylalaninemia; MBTFA, *N*-methyl-bis-trifluoroacetamide; NICI, negative ion chemical ionization; PAA, phenylacetic acid; PFB, pentafluorobenzyl; phe, phenylalanine; PKU, phenylketonuria; PLA, phenyllactic acid; PPA, phenylpyruvic acid; SIM, selected ion monitoring; TFA, trifluoroacetate.

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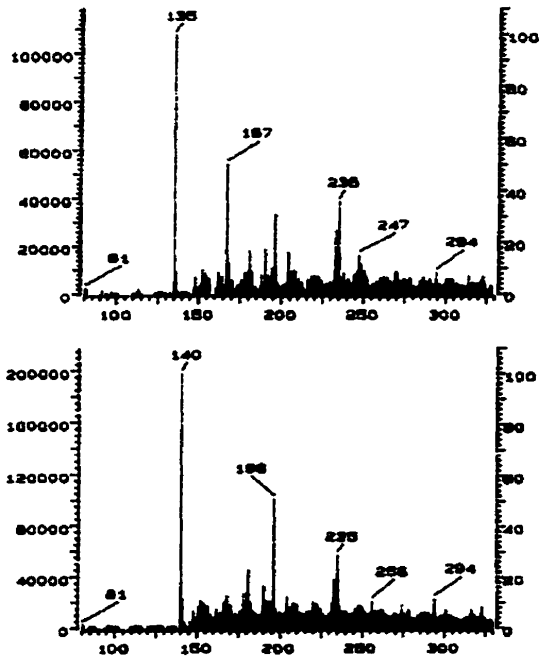

FIG. 1. NICI mass spectra obtained for the PFB derivatives of unlabeled PAA and $PAAd_5$. Intensities of ions with masses greater than 145 Da have been multiplied by 20, and appear not to be related to the sample. The intense ions at *m/z* 135 and 140 carry nearly the entirety of the ion current produced and correspond to the carboxylate anions formed by the loss of the pentafluorobenzyl radical from the molecular anions. While the molecular anions expected at *m/z* 316 and 321, respectively, are not detected, very weak ion currents attributable to loss of HF from the molecular anions (not shown) confirm the nature of the derivatives.

lated to tyrosine and only a trivial fraction is transaminated to PPA under normal conditions (4). PAH enzyme catalyzes the hydroxylation reaction; when its activity is absent or reduced (as in PKU and to a lesser degree in non-PKU HPA), the free phe pool expands, if dietary phe input is not reduced. At this stage, the degradative transamination pathway, involving conversion of phe to PPA (the initial reaction in this pathway), becomes significant at a modal phe value of ~0.5 mM (2–5). PPA is subsequently converted to PLA and PAA and phenylacetylglutamine (1). Whether these metabolites actually contribute to pathogenesis of cognitive impairment has long been debated (3, 5).

Orthologous mouse models of PKU and non-PKU HPA exist (6–8) which allow us to measure phe and its metabolites in brain in various degrees of HPA. Here we describe a GC/MS method to measure phenylalanine metabolites based upon stable isotope dilution techniques, coupled with negative ion chemical ionization (NICI).

MATERIALS AND METHODS

The method measures PAA as the pentafluorobenzyl (PFB) ester with [$^2H_5$]-phenylacetic acid ($PAAd_5$) as internal standard, and PLA as the PFB and trifluoroacetate (TFA) diester with [2,3,3-$^2H_3$]-phenyllactic acid ($PLAd_3$) as internal standard. PPA is reduced to [2-^2H]-phenyllactic acid ($PLAd_1$) by addition of sodium boro-

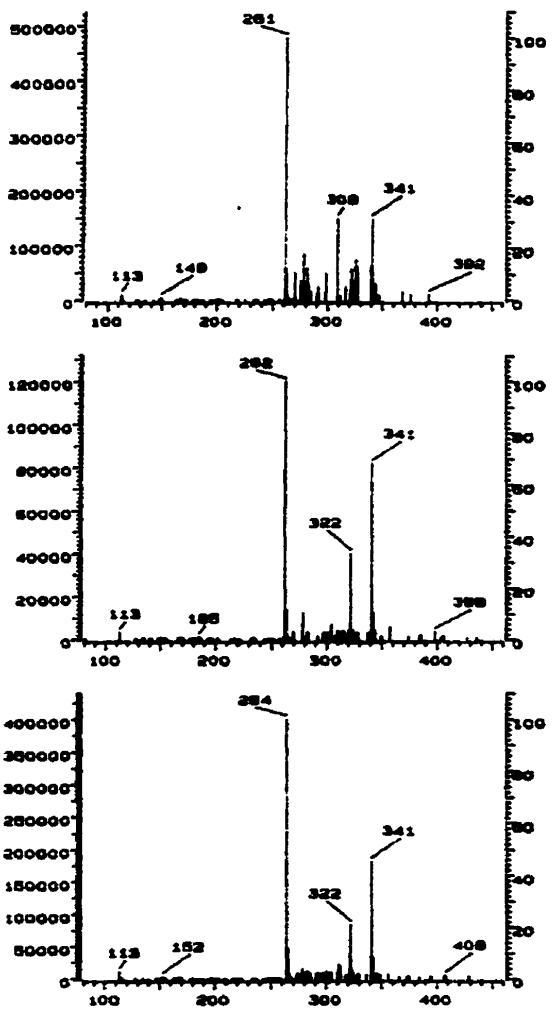

FIG. 2. NICI mass spectra obtained for the PFB derivatives of the TFA esters of unlabeled PLA, $PLAd_1$, and $PLAd_3$. Intensities of ions with masses greater than 270 have been multiplied by 50 to show that they appear not to be related to the sample, as the masses show no incrementing dependent upon labeling. The carboxylate anions at *m/z* 261, 262, and 264 formed by the loss of the pentafluorobenzyl radical from the molecular anions (*m/z* 442, 443, and 445, not detected) carry nearly all the ion current.

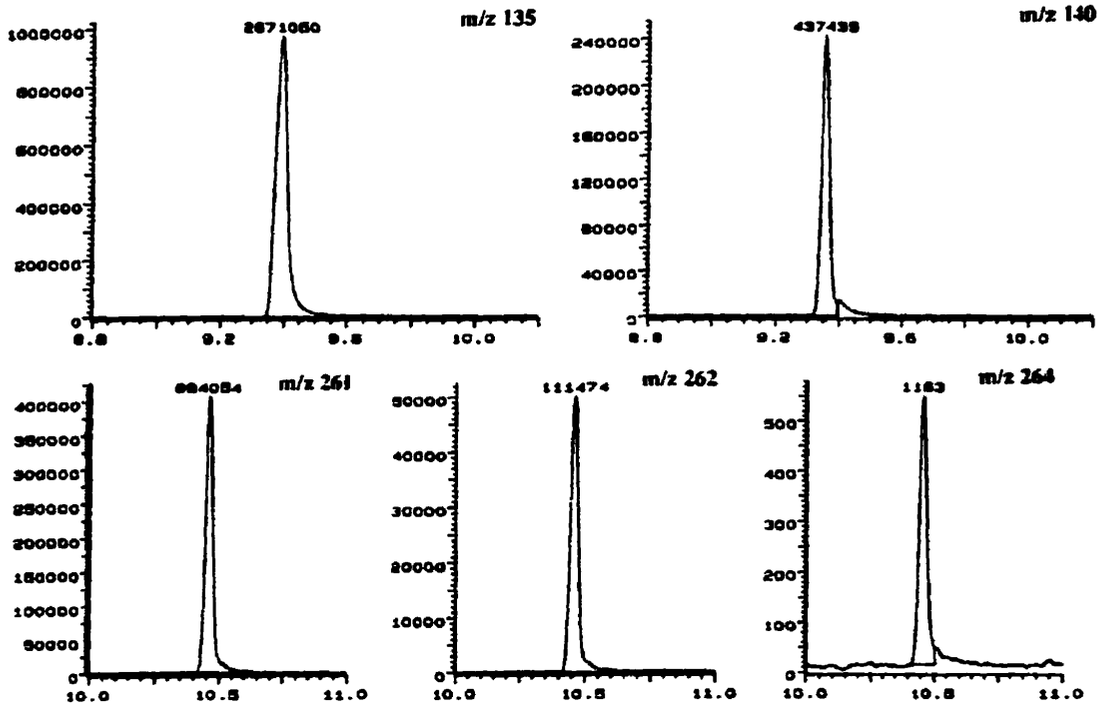

FIG. 3. SIM chromatograms for a calibrating mixture containing unlabeled PAA (*m/z* 135), internal standard $PAAd_5$ (*m/z* 140), and unlabeled PLA (*m/z* 261). Ion currents at *m/z* 262 (12.47% relative to *m/z* 261) and 264 (0.13%) are due to natural abundance heavy isotope inclusion in the carboxylate anion formed from the unlabeled PLA derivative.

deuteride (NaB^2H_4) to the supernatant of the tissue homogenate, and is measured in the manner similar to that for PLA.

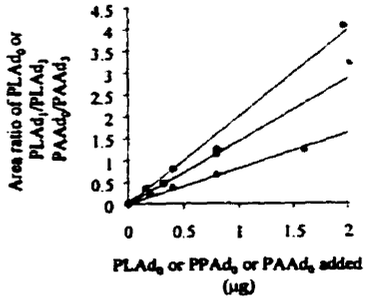

FIG. 4. Calibration curves, showing linear responses for PLA, PPA, and PAA, over physiological ranges. The abscissa represents weights of unlabeled metabolites added to calibrating aqueous solutions of 1 ml volume containing 13.31 μg of $PLAd_2$ and 1 μg of $PAAd_5$. The ordinate represents the ratio of areas of unlabeled to labeled analogues obtained for these solutions.

Mouse Models

The homozygous mutant strain *Pah*$^{enu2/enu2}$ (phenotype name, ENU2/2) and *Pah*$^{enu1/enu1}$ (ENU1/1) are orthologues of human PKU and non-PKU-HPA, respectively (http://www.mcgill.ca/pahdb/mouse). They were developed by treating wild-type mice (BTBR background (used as controls)) with the alkylating agent *N*-ethyl-*N'*-nitrosourea (6, 7, 9). Produced in Wisconsin, they were kindly given to us by W. Dove and A. Shedlovsky. They display a range of phenotypic characteristics comparable to those of affected human individuals (10).

Preparation of Internal Standard Solutions

$PAAd_5$. A 0.071 mM $PAAd_5$ stock solution was prepared by dissolution of 1.0 mg (CDN isotopes) in 100 ml deionized H_2O.

$PLAd_2$. PPA (17 mg, 0.1 mmol, Sigma Chemical Co.) was dissolved in deuterium oxide (25 ml, CDN Isotopes) and made basic (pH >12) with 3 drops of 40% NaO^2H in ^2H_2O. The resulting solution was held at 60°C for 1 h and then rotary evaporated to near-dry-

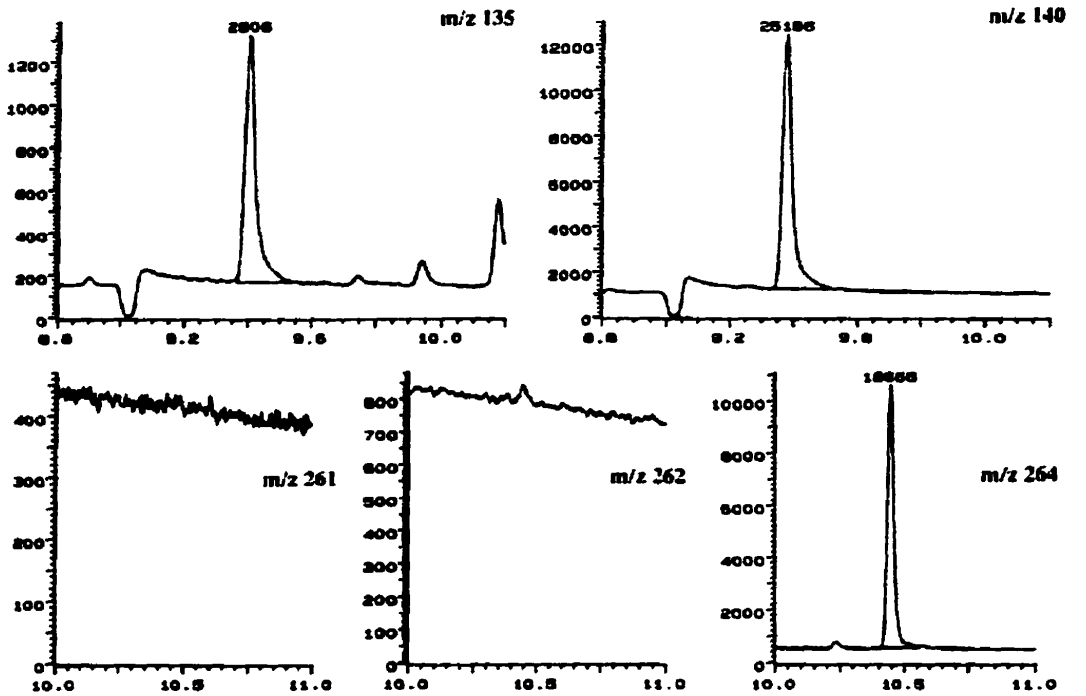

FIG. 5. SIM chromatograms obtained for phe metabolites in the brain of a normal mouse. PAA (*m/z* 135) is clearly present, while PLA and PPA (*m/z* 261 and 262) are not detected in the present example. The ion current at *m/z* 264 is due to the $PLAd_5$ internal standard.

ness. The residue was taken up in a 10-ml aliquot of ^2H_2O and held at 60°C for 1 h. This solution was cooled to room temperature and NaB^2H_4 (approximately 5 mg, CDN Isotopes) was added. The resulting solution was warmed to 50°C for 10 min, cooled in an ice bath, and slowly acidified to pH < 2 with 2 N HCl (caution: vigorous evolution of hydrogen). The solution was saturated with NaCl and extracted with three 10-ml volumes of diethyl ether. The ether extracts were combined, made anhydrous by addition of solid anhydrous Na_2SO_4 (two successive 1-g lots), and then evaporated to dryness in a dry nitrogen stream. The product, $PLAd_3$ was obtained (14 mg, 83% crude yield) in oil form, free of residual PPA as determined by GC/MS analysis of the trimethylsilyl derivatives. Extensive experience with reductions of keto acids with NaB^2H_4 in this laboratory shows that these reductions are quantitative. The isotopic purity was determined to be 97% as the triply deuterium-labeled isotopomer. Approximately 14 mg of the crude product was dissolved in deionized water (100 ml) to be used as the internal standard for PLA and $PLAd_t$ determinations. The concentration of this internal standard was determined by a reverse stable isotope dilution assay, measuring relative ion intensities in a solution with unlabeled PLA of known concentration. The final concentration of the $PLAd_3$ internal standard solution was 0.787 mM.

Brain Sample Preparation

Brains were removed within 5 s following decapitation of ENU2/2, ENU1/1, and control animals fed with standard rodent diet (Teklad No. 8604) (n = 6/genotype) and immediately homogenized in minimal 20°C deionized water (1:1, w/v) to which we added the labeled internal standards (100 μl each of the $PLAd_3$ and $PAAd_5$ solutions). The total volume was made up to 1 ml, adjusted to pH 10–12 with dilute KOH, NaB^2H_4 (2 mg) was added immediately, and the tubes were placed in 50°C water for 10 min. PFB derivatives were prepared in the manner previously reported (11) with the following stock solutions: A, methylene chloride (20 ml) and pentafluorobenzyl bromide (0.4 ml, Aldrich Chemical Co.); B, potassium phosphate buffer (pH 7.4, 100 ml) and tetrabutylammonium hydrogen sulfate (3.4 g, Aldrich Chemical Co.) adjusted to pH 7.4 with 2 N KOH. Solutions A (0.25 ml) and B (0.25 ml) were combined in a separate tube, 0.25 ml of tissue homogenate supernatant was added, the mixture was vortexed for 2 min and then placed in an ultrasonic bath for 20 min at

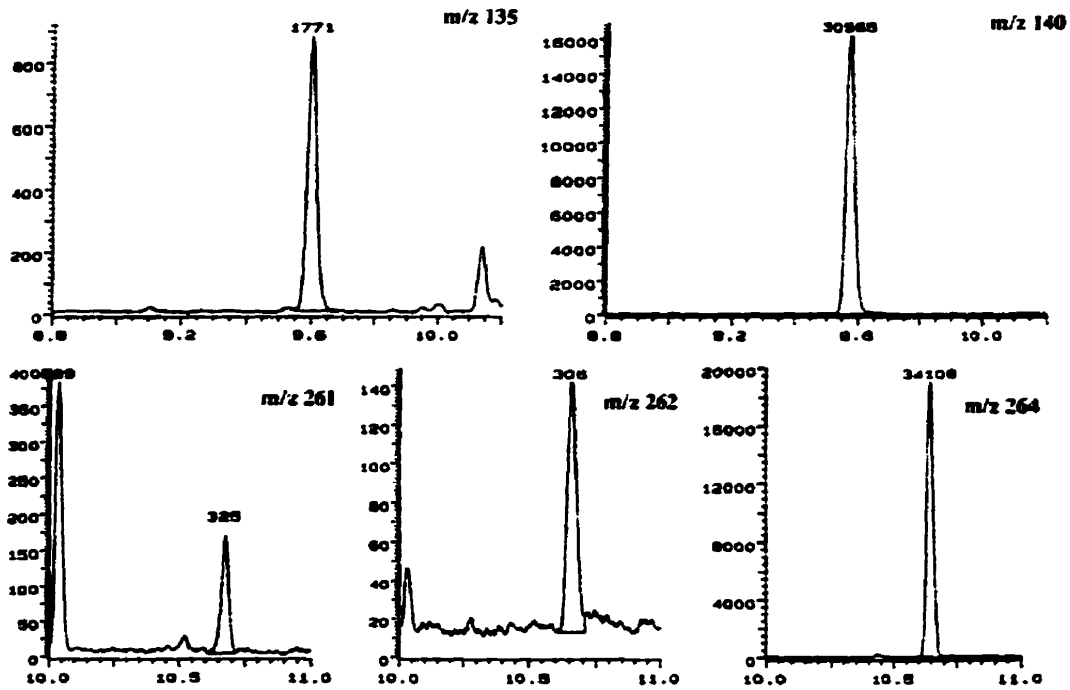

FIG. 6. SIM chromatograms obtained for phe metabolites in the brain of an ENU-1 mouse. PAA is present at levels somewhat lower than those in the normal mouse, and PLA and PPA are elevated slightly relative to the normal mouse. The retention times seen here are slightly different from Fig. 5 because in the interim the analytical column was replaced.

room temperature. Hexane (2 ml) was added, the mixture was vortexed for 1 min, the hexane layer was then removed and dried by addition of anhydrous sodium sulfate (10 mg) with vortexing for 1 min. The hexane layer was next decanted into a separate tube, *N*-methyl-bis-trifluoroacetamide (MBTFA, 50 μl, Pierce Chemical Co.) added was then vortexed and placed into a 50°C water bath for 10 min. A 1 N sodium bicarbonate solution (2 ml) was added to the tube and the mixture was vortexed for 1 min. The hexane layer was finally removed into a separate tube, anhydrous sodium sulfate was added, and the mixture was vortexed for 1 min. An aliquot of this final hexane solution was transferred to an autoinjector vial for GC/MS analysis.

Blank Sample

Blank samples were prepared with deionized water equal in volume to the brain tissue homogenate supernatants. These samples were processed and analyzed as described for the tissue samples.

GC/MS Analysis

Aliquots (1 μl) of the derivatized mixtures were analyzed in NICI mode with a Hewlett–Packard 5988A GC/MS fitted with a 30-m × 0.25-mm i.d. capillary column (J & W Scientific) coated with a 0.25-μm DB-1 film. The helium flow rate was 2 ml/min; the injector and interface temperatures were 250°C. The column was temperature programmed from 100°C after a 1-min hold to 120°C at 40°C/min and then at 10°C/min to 280°C. The column was baked out at 280°C for 5 min at the completion of each sample analysis. Methane was used as the moderator gas at an indicated source pressure of 0.6 mbar and the ion source temperature was 120°C. Selected ion mode was used to measure the intensities of negative ion fragments *m/z* 135, 140, 261, 262, and 264 with dwell times of 50 ms each. These fragments arise by the loss of the pentafluorobenzyl radical from the molecular anions of the PFB derivatives of PAA and $PAAd_5$, and the PFB/TFA derivatives of PLA, $PLAd_1$, and $PLAd_3$, respectively.

Amino Acid Quantitation

Whole blood was collected from mouse tails into heparinized tubes, plasma was separated by centrifugation, and after deproteinization the amino acid content was analyzed by HPLC on a Beckman 6300 automatic amino acid analyzer.

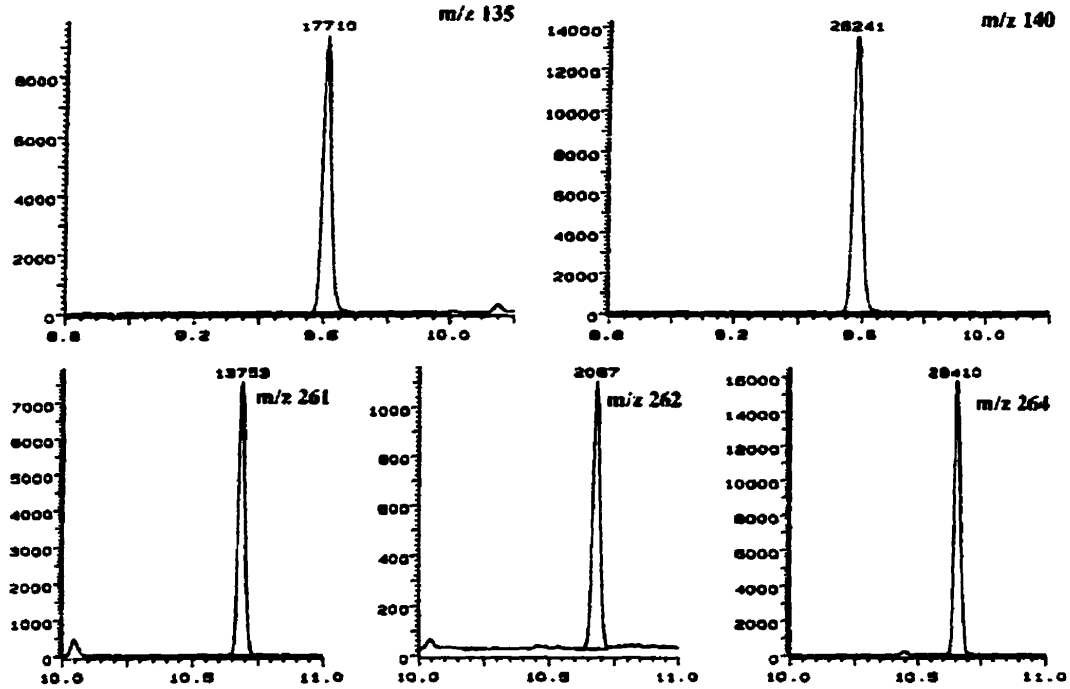

FIG. 7. SIM chromatograms obtained for phe metabolites in the brain of an ENU-2 mouse. PAA, PLA, and PPA are all clearly elevated. The labeled internal standards (*m/z* 140 and 264) elute slightly earlier than their unlabeled analogues. The retention times seen here are again slightly different from Fig. 5 because in the interim the analytical column was replaced.

Whole brain amino acids were analyzed according to the method described by Diomede *et al.* (12). Brain tissue was removed within 5 s after decapitation and homogenized (on ice) in 0.5% sodium dodecyl sulfate solution (1:4, w/v). Alloisoleucine was added as an internal standard and the homogenate was then incubated for 15 min at room temperature. A 4% solution of 5-sulfosalicylic acid dihydrate solution (1:0.6, v/v) was added, and the mixture was centrifuged at 14,000*g* for 15 min. The supernatant was decanted and frozen at −80°C until analyzed by HPLC as above.

RESULTS AND DISCUSSION

NICI mass spectra obtained for the PFB esters of authentic PAA and $PAAd_5$ standards are shown in Fig. 1. The most intense ions correspond to the carboxylate anions (*m/z* 135 and 140, respectively) produced by the loss of the pentafluorobenzyl radical (181 Da) from the molecular anions (*m/z* 316 and 321, respectively, not detected). Loss of HF (20 Da) from the molecular anions, which is commonly observed in derivatives of this nature, is detectable, although not apparent in the Fig. 1. The 140-Da fragment in the spectrum of $PAAd_5$ confirms that all labeling is intact in the ion measured. SIM analysis of $PAAd_5$ shows that the unlabeled content is 1.39% relative to the labeled. This is taken into account in the calculations of the endogenous concentrations of PAA.

NICI spectra for the PFB esters of the trifluoroacetyl esters of unlabeled and labeled PLA are shown in Fig. 2. The most intense ions (*m/z* 261, 262, and 264) represent the carboxyl anions produced by the loss of the pentafluorobenzyl radical from the molecular anions *m/z* 442, 443, and 445 (not detected) of unlabeled authentic PLA, $PLAd_1$ synthesized from PPA by reduction with NaB^2H_4, and synthesized $PLAd_3$ (internal standard), respectively. The $PLAd_1$ isotopomer corresponds to PPA in the original homogenate. It is essential that the 2-hydroxyl group of the PLA isotopomers be derivatized because decomposition by dehydration at GC temperatures would lead to deuterium label loss. The $PLAd_3$ synthesized and used as internal standard was found by SIM analysis to be 97.17% $PLAd_3$, 2.66% $PLAd_2$, 0.001% $PLAd_1$, and 0.16% $PLAd_0$. Reduction of authentic PPA by NaB^2H_4 yielded $PLAd_1$ that was

TABLE 1

Relationship between Brain Phe Metabolites with Plasma Phe Levels in Three Phenotypes (normal, ENU1, and ENU2) (n = 6/genotype, $\bar{X} \pm$ SE)

Mouse models	Plasma phenylalanine concentration (μM)	PPA (nmol/g brain)	PAA (nmol/g brain)	PLA (nmol/g brain)
Control	<100	1.2 ± 0.1	2.7 ± 0.3	0.2 ± 0.1
ENU-1	150–400	1.2 ± 0.1	2.2 ± 0.5	0.9 ± 0.3
ENU-2	1400–3000	2.2 ± 0.3	7.4 ± 1.6	59.3 ± 21.8

0.29% unlabeled, a measure of the isotopic purity of the lot of NaB^2H_4 which was used for the entire study.

SIM chromatograms obtained for one of the calibrating mixtures containing PAA, $PAAd_5$, and unlabeled PLA are shown in Fig. 3. The PAA isotopomers are represented by the carboxylate anions (m/z 135 and 140) formed by the loss of the pentafluorobenzyl moiety from the molecular anions (not detectable). The PLA chromatograms (m/z 261, 262, and 264) would normally represent the carboxylate anions of unlabeled PLA, $PLAd_1$ (formed by the reduction of PPA by $NaBD_4$), and the internal standard $PLAd_3$, respectively, formed by the loss of the pentafluorobenzyl moiety from the molecular anions of the PFB–TFA derivatives. In this instance, the measured relative intensities are for unlabeled PLA substituted with only natural abundance heavy isotopes. The intensities of m/z 262 and 264 measured in the ion cluster relative to m/z 261 are 12.47 and 0.13%, respectively, and compare well with the calculated values of 12.51 and 0.12%, respectively.

The calibration curves for all three metabolites, over the expected physiological ranges, are shown in Fig. 4 A linear response is demonstrated for each metabolite ($R^2 > 0.98$ for each metabolite). The response for PPA (measured as $PLAd_1$) is approximately 72% that of $PLAd_0$. This is possibly due to lack of purity or homogeneity in the PPA originally weighed out. Analysis of the PPA as the TMS derivative shows the presence of small but significant quantities of PLA and PAA. The sensitivity of our method (for all three metabolites) is estimated to be 0.1 nmol/g brain tissue.

Typical chromatograms for normal (Fig. 5), ENU-1 (Fig. 6), and ENU-2 (Fig. 7) mouse brain are shown. In normal mouse brain PAA is detectable (m/z 135) at low levels, while PLA and PPA (m/z 261 and 262) are essentially absent (Fig. 5). The apparently inverted peak (retention time, 9.02 min) in the responses for m/z 135 and 140 results from the elution of a large unidentified peak which temporarily depletes the thermal electron atmosphere in the ion source.

The ENU-1 (non-PKU HPA) mouse brain (Fig. 6) contains elevated levels of PLA and PPA (m/z 261 and 262). The minor variation in the PAA levels between the ENU-1 and normal mice demonstrates that at normal or near normal (physiological) phe levels the transamination pathway contributes insignificantly to the brain metabolic phenotype in the variant HPA state.

The ENU-2 (PKU-like) mouse (Fig. 7) shows greatly increased concentrations of all three metabolites. The well-known tendency for deuterium-labeled analogs to elute slightly earlier than the corresponding unlabeled compounds is apparent.

The correlation between plasma phe and brain metabolites is shown in Table 1. BTBR-wild-type and ENU1 animals, displaying normal (<100 μM) or low (150–400 μM) plasma phe levels, respectively, have low phe metabolite levels in brain. This finding implies that the transamination pathway comes into play only at phe levels above 0.4 mM, as implied by the findings in the ENU2 animals, moreover correlating well with previous observations (see Fig. 15-1 in Ref. 5). When present, metabolite levels have a rank order PLA > PAA > PPA. The relationship between plasma and brain phenylalanine levels in the mouse models is given in Table 2.

Our data are among the first reporting direct measurements of brain phe metabolites in PKU and non-PKU HPA. A preliminary report by Evans (13) compared brain and body fluid metabolites in control and ENU-2 mice. Here we present a formal analysis of PLA, PAA, and PPA in brains of orthologous mice with PKU and non-PKU HPA and compare them with control values. Our interest was to study phe metabolite concentrations in brain independent of their levels in blood or urine; the latter were the focus of most earlier studies. The data show that brain metabolite concen-

TABLE 2

Relationships between Plasma and Brain Phe Values in Three Mouse Models (μM, n = 6, $\bar{X} \pm$ SE)

Mouse models	Plasma phenylalanine concentration (μM)	Brain phenylalanine concentration (μM)
Control	74.4 ± 8.8	70.1 ± 6.5
ENU-1	181.5 ± 23.3	120.6 ± 8.1
ENU-2	1882.7 ± 156.5	886.97 ± 26.6

trations correlate positively with plasma phe levels. More important, the levels of the metabolite measured here do not reach levels of toxicity predicted for human subjects by Kaufman (3) and documented in earlier studies (14). Taking into account assumptions about distributions of metabolites in the intracellular and extracellular space of brain, the levels of metabolites measured here are 10-fold lower than those associated with toxicity in brain (14). Nonetheless, our PKU (ENU-2) mice exhibit the behavioral and cognitive impairment expected in PKU (10), which we attribute primarily to the effect of phenylalanine itself. On the other hand, in certain untreated PKU patients with normal cognitive function, brain phe values are not elevated in the presence of high blood values, as measured by MRI (see Ref. 15); an independent impediment of blood/brain phe transport has been offered as an explanation. Our use of both the analytical method and the orthologous mouse models of the human is offered here as a contribution toward resolving a long-standing controversy about pathogenesis of the cognitive phenotype in PKU.

ACKNOWLEDGMENTS

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APPENDIX C

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Medical Sciences

A different approach to treatment of phenylketonuria: Phenylalanine degradation with recombinant phenylalanine ammonia lyase

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ABSTRACT **Phenylketonuria (PKU), with its associated hyperphenylalaninemia (HPA) and mental retardation, is a classic genetic disease and the first to have an identified chemical cause of impaired cognitive development. Treatment from birth with a low phenylalanine diet largely prevents the deviant cognitive phenotype by ameliorating HPA and is recognized as one of the first effective treatments of a genetic disease. However, compliance with dietary treatment is difficult and when it is for life, as now recommended by an internationally used set of guidelines, is probably unrealistic. Herein we describe experiments on a mouse model using another modality for treatment of PKU compatible with better compliance using ancillary phenylalanine ammonia lyase (PAL, EC 4.3.1.5) to degrade phenylalanine, the harmful nutrient in PKU; in this treatment, PAL acts as a substitute for the enzyme phenylalanine monooxygenase (EC 1.14.16.1), which is deficient in PKU. PAL, a robust enzyme without need for a cofactor, converts phenylalanine to trans-cinnamic acid, a harmless metabolite. We describe (*i*) an efficient recombinant approach to produce PAL enzyme, (*ii*) testing of PAL in orthologous *N*-ethyl-*N*′-nitrosourea (ENU) mutant mouse strains with HPA, and (*iii*) proofs of principle (PAL reduces HPA)—both pharmacologic (with a clear dose–response effect vs. HPA after PAL injection) and physiologic (protected enteral PAL is significantly effective vs. HPA). These findings open another way to facilitate treatment of this classic genetic disease.**

Phenylketonuria (PKU) (1) is the prototypical human Mendelian disease (OMIM 261600) demonstrating benefits from treatment (2). PKU and a related form of less harmful hyperphenylalaninemia (HPA, termed non-PKU-HPA) result from impaired activity of phenylalanine hydroxylase (PAH; EC 1.14.16.1), the enzyme catalyzing conversion of the essential amino acid nutrient phenylalanine to tyrosine. The enzyme is responsible for disposal (by oxidative catabolism) of the majority of nutrient phenylalanine intake. The untreated PKU patient with persistent postnatal HPA is likely to experience irreversible impairment of cognitive development. Antenatal HPA, caused by transplacental transport of phenylalanine from the maternal pool to the fetus during a pregnancy in which there is maternal HPA, will harm the embryo and fetus. These disease-causing effects of PKU and maternal HPA are preventable by treatment to restore euphenylalaninemia (1).

Present treatment relies on the observation that a (semisynthetic) diet low in phenylalanine (3–5) will prevent HPA and thus the disease. Because it involves a major alteration of lifestyle, dietary treatment is difficult. Moreover, dietary therapy can be associated with deficiencies of several nutrients (1), some of which may be detrimental to brain development (6, 7). Moreover, most low phenylalanine treatment products have organoleptic properties sufficiently unsatisfactory that compliance with the treatment is compromised (8, 9). Such concerns have greater relevance now that better and longer compliance with therapy of PKU and non-PKU-HPA in all persons at risk has been recommended (10, 11).

A combination of oral enzyme therapy with phenylalanine ammonia lyase (PAL; EC 4.3.1.5) and controlled low protein diet might replace dependence on the semisynthetic diet, for treatment of PKU after infancy (10, 12). PAL is a robust autocatalytic enzyme that, unlike PAH, does not require a cofactor (13). PAL converts phenylalanine to metabolically insignificant amounts of ammonia and trans-cinnamic acid, a harmless metabolite; the latter is converted to benzoic acid and rapidly excreted in urine as hippurate (14). A preliminary report indicates that HPA is attenuated by oral administration of microencapsulated PAL in the rat with chemically induced HPA (15) and also, in preliminary studies, in naturally occurring HPA in a mouse model (16–18). Preliminary studies in human PKU patients showed analogous responses after the administration of PAL in enteric-coated gelatin capsules (19) or during use of an extracorporeal enzyme reactor (20). The human and even the animal studies were not continued because PAL was not available in sufficient amounts at reasonable cost.

(*i*) We used a construct of the *PAL* gene from *Rhodosporidium toruloides* (21) under the control of a high-expression promoter and expressed it in a strain of *Escherichia coli* to obtain large amounts of PAL (22). (*ii*) We used existing (23) and new strains (C.N.S., J. D. McDonald, and C.R.S. at http://www.mcgill.ca/pahdb/Pah Mouse/Mouse Pah Homologues) of the mutant *N*-ethyl-*N*′-nitrosourea (ENU)-treated mouse as orthologous models of human PKU and HPA to study enzyme substitution therapy with PAL. (*iii*) We showed that i.p. PAL injection lowers plasma phenylalanine in the mouse model (proof of pharmacological principle), and oral gavage of these mice with PAL enzyme, protected from inactivation by digestive enzymes, lowers plasma phenylalanine (proof of physiological principle). These developments point to an alternative approach to treatment of PKU, compatible with current guidelines (10, 11).

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Abbreviations: ENU, *N*-ethyl-*N*′-nitrosourea; PKU, phenylketonuria; HPA, hyperphenylalaninemia; PAL, L-phenylalanine ammonia lyase; PAH, human L-phenylalanine hydroxylase.

A Commentary on this article begins on page 1811.

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MATERIALS AND METHODS

Synthesis of Recombinant PAL. *Amplification of the PAL gene. R. toruloides* [ATCC no. 10788] was purchased from the American Type Culture Collection. Cells were grown in minimal medium containing phenylalanine as the sole carbon source (24), total RNA was extracted with hot acidic phenol (25) from a mid-logarithmic-phase culture, and mRNA was isolated with the PolyATtract mRNA isolation system (Promega). a cDNA pool was then synthesized with the RiboClone cDNA synthesis system (Promega).

Oligonucleotides (RTJP, 5'-AAGAATTCATGGCACCCTCGCTCGACTCGATCTCG-3', and RT2, 5'-CCGAATTCTAAGCGATCTTGAGGAGGACGT-3'), synthesized on an Ecosyn D300 DNA synthesizer (Eppendorf) were designed to feature an *Eco*RI site at their 5' end and to be homologous to 5' and 3' ends of the published sequence of the *R. toruloides PAL* gene (refs. 26 and 27; GenBank accession no. X51513). PCR amplification (28) was performed in 100 μl containing 100 pmol of each primer, 20 mM Tris·HCl (pH 8.0), 2 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton^X-100, nuclease-free BSA at 10 mg/ml, all four dNTPs (each at 0.2 mM), 30 ng of cDNA as the template, and 5 units of *Pfu* DNA polymerase (Stratagene). Samples were incubated in a DNA thermal cycler (Barnstead/Thermolyne) at 95°C for 30 sec, at 50°C for 1 min, and 72°C for 3 min; repeated for 35 cycles. The PCR product was analyzed on a 1% agarose gel containing 0.6 mg of ethidium bromide per ml and subsequently cloned in pBluescript KS+ (Stratagene). Identity of the *PAL* gene was verified by sequence analysis using an AutoRead sequencing kit and an automated A.L.F. DNA sequencer (Pharmacia).

E. coli strains and plasmids. E. coli XL-1Blue was the host used for general cloning and vector construction; *E. coli* Y1091 was the host for fermentation to produce PAL. *E. coli* IBX-4, used in the animal study, was isolated from a Sprague–Dawley rat and identified by using api20E bacterial identification kit (BioMerieux, Charbonnier les Bains, France). Plasmid pBluescript was used for cloning PCR-amplified *PAL* fragments.

Construction of high-expression PAL plasmid pIBX-7. Plasmid pIBX-1 (29) was modified by site-directed mutagenesis, changing the unique *Bam*HI site to an *Eco*RI site to allow cloning of the 2.2-kb *PAL* PCR fragment; the product was then further modified, which resulted in the deletion of the *Eco*RI sites. To increase expression, an additional *tac* promoter (*Ptac*) was synthesized by PCR and cloned downstream of the existing *Ptac*. The 5' Shine–Delgarno sequence (AGGAG) is separated from the ATG codon by a 9-nt sequence (ACAGAATTT). Kanamycin resistance for selection of *PAL*-containing cells was conferred by substituting the kanamycin resistance gene for the ampicillin resistance gene of pIBX-1. The plasmid was transformed into *E. coli* (hosts Y1091 and IBX-4) and induced with isopropyl β-D-thiogalactoside (1 mM) for expression. The expression levels are similar in both hosts when cultured in shaken flasks (data not shown).

Purification of PAL enzyme from cell extract. Frozen *E. coli* cells (Y1091; 500 g) expressing plasmid pIBX-7 were suspended in 2 liters of buffer A (30 mM Tris·HCl, pH 8.0/10 mM phenylalanine/2 mM cysteine) to which DNase I (5 mg/liter) and 5 mM $CaCl_2$ were added. The cell suspension was homogenized three times in a Rannie MiniLab 8.30H high-pressure homogenizer (APV Canada, Montreal) at 700 bar (1 bar = 100 kPa); between passages, the suspension was cooled to 12°C. The homogenate was centrifuged (14,100 × *g*) for 1 hr at 4°C, the supernatant containing PAL protein diluted 2- to 3-fold in water and loaded on a column containing 1.25 liters of Q-Sepharose Big Beads (Pharmacia). Washes were performed at a linear flow rate of 153 ml/hr with three column volumes of buffer A followed by three column volumes of buffer B (30 mM Tris·HCl, pH 8.0/10 mM phenylalanine/2 mM cysteine/1M NaCl). PAL protein is eluted with buffer B in a linear gradient (3%–100%) in six column volumes; the fractions with PAL activity were pooled. Ammonium sulfate was added slowly to the eluates (final concentration, 50%), stirred (30 min) at room temperature, and then centrifuged (14,700 × *g* for 30 min at 4°C). The final PAL protein pellet was dissolved in a minimal amount of buffer C (50 mM sodium phosphate, pH 7.5/5 mM phenylalanine/1 mM glutathione).

ENU Mice. The ENU mouse models, deficient in hepatic PAH enzyme activity, were created by treating wild-type mice (BTBR background) with the alkylating agent ENU. The original strains $Pah^{enu1/enu1}$(ENU1/1) and $Pah^{enu2/enu2}$(ENU2/2), from W. Dove and A. Shedlovsky (University of Wisconsin, Madison), were produced in Wisconsin (23, 30) and genotyped by J. D. McDonald (31). We produced the hybrid heteroallelic strain $Pah^{enu1/enu2}$(ENU1/2) in our facilities (see http://www.mcgill.ca/pahdb/Pah Mouse/Mouse Pah Homologues). (All procedures described below have been reviewed and approved by the Animal Care Committee, McGill University).

The homozygous mutant ENU1/1 mouse is a counterpart of human non-PKU-HPA. It has a missense mutation in the *Pah* gene [c. 364T → C in exon 3 (V106A)] (30, 31), and on breeder diet (product 8626, Teklad, Madison, WI), it has both a normal plasma phenylalanine level and normal behavior but can be made hyperphenylalaninemic under controlled conditions with a phenylalanine load [by s.c. injection or gavage of L-phenylalanine at 1.1 mg/g (body weight)]. The homozygous mutant ENU2/2 mouse is a counterpart of human PKU. It has a missense mutation [c. 835T → C in exon 7 (F263S)] (23, 31). On breeder diet, it has 10- to 20-fold elevated plasma phenylalanine and phenylketones in urine; euphenylalaninemia in this strain was achieved for these studies by placing mice on a diet free of phenylalanine (product 2826, Teklad) with ad libitum water containing L-phenylalanine (30 mg/liter), for 3 consecutive days. After establishing euphenylalaninemia, ENU2/2 animals received standardized s.c. injections of L-phenylalanine [0.1 mg/g (body weight)] to achieve reproducible HPA.

We developed the new ENU1/2 strain by crossing female ENU1/1 and male ENU2/+; all parents and offspring were typed by DNA analysis. The mutant heteroallelic animals have a normal plasma phenylalanine level on breeder diet but easily achieve a modest elevation, to levels between that of untreated ENU1/1 and ENU2/2, by s.c. injection or gavage of L-phenylalanine [1.1 mg/g (body weight)]. The ENU strains are further described on our web site (see above).

Protocols to Study the Effect of PAL Enzyme on Phenotype. *i.p. administration of recombinant PAL (for proof of pharmacological principle).* Studies were done on mice, younger than 1 year of age (all three strains). Each animal served as its own control; a sham (saline) injection was given in the first week, followed by the PAL injection 1 week later (same day of the week and time of day). Efficacy of i.p. PAL was measured by change in the plasma phenylalanine between the first and second week; animals (n = 5 per trial) received 2, 20, and 100 units of PAL. ENU1/1 and ENU1/2 animals were fasted overnight before the experiment. Food was reintroduced after PAL administration. During the first week, 0.2 ml of saline i.p. was followed directly by an L-phenylalanine challenge [1.1 mg/g (body weight) by gavage] for the ENU1/1 and ENU1/2 mice; tail blood samples taken at zero min and 1, 2, 3, and 24 hr after the phenylalanine challenge. For the second-week protocol, PAL (replacing saline) was diluted to the dosage required with 0.1M Tris·HCl (pH 8.5).

Gavage of recombinant PAL (for proof of physiological principle). Studies were done in mice younger than 1 year of age. Each animal served as its own control and efficacy of PAL treatment was measured by change in plasma phenylalanine. We used two different PAL preparations: (*i*) recombinant PAL inside *E. coli* cells (IBX-4) and (*ii*) unprotected PAL (purified from Y1091 *E. coli* cells) in solution with aprotinin

(protease inhibitor). Saline gavage was used as the control treatment. Plasma phenylalanine levels were adjusted in ENU2/2 animals (n = 4) to achieve euphenylalaninemia. In the first week, the animals received a phenylalanine load [0.1 mg/g (body weight) by s.c. injection] on day 4, followed at 1 hr and 2 hr by gavage of saline bicarbonate (6 mg, to neutralize gastric acidity). Tail blood samples were taken before treatment (at time zero minus 5 min), and five times at hourly intervals after the phenylalanine challenge. In the second week, the gavage contained (*i*) 25 units of PAL (as induced recombinant *E. coli* cells, OD_{500}) or (*ii*) 200 units of PAL, in combination with 10 mg of aprotinin in the sodium bicarbonate buffer.

***In Vitro* Assays of PAL Effect.** We measured efficacy of the PAL preparation *in vitro* by analyzing its effect on a solution containing 4 mM phenylalanine (initial concentration) at 37°C and pH 8.5. We compared (*i*) the individual effects of nonrecombinant *E. coli* cells (OD_{600} = 50), chymotrypsin (100 mg/ml), and mouse intestinal fluid (diluted 1:10 with Tris buffer at pH 8.5) with (*ii*) the effect of naked recombinant PAL either alone or in the presence of chymotrypsin (100 mg/ml) or of intestinal fluid (1:10 dilution with Tris buffer at pH 8.5) and (*iii*) the effect of *E. coli* cells (OD_{600} = 50) expressing PAL (5 units) alone or in the presence of chymotrypsin (100 mg/ml) or mouse intestinal fluid (1:10 dilution with Tris buffer at pH 8.5).

Analytical. *Plasma phenylalanine concentration.* We collected blood from tail into heparinized tubes, extracted plasma, and measured phenylalanine by HPLC (Beckman System Gold, DABS amino acid analysis kit).

DNA Analysis. Animals were genotyped, as described (31), for the Pah^{enu2} mutation. We developed a method to detect the Pah^{enu1} mutation that eliminates a recognition site for the restriction enzyme *TaqI*. We used PCR, with primers 5′-GAGAATGAGATCAACCTGACA-3′ and 5′-TGTCTCGG-GAAAGCTCATCG-3′, to amplify a 169-bp segment of exon 3 (mouse *Pah*) from blood spots collected on Guthrie cards. The product subjected to *TaqI* digestion yields a distribution of fragments with a distinct banding pattern for each of three possible genotypes: $Pah^{+/+}$ generates two fragments (148 bp and 21bp), $Pah^{enu1/+}$ generates three (169 bp, 148 bp, and 21bp), and $Pah^{enu1/enu1}$ generates one fragment (169 bp).

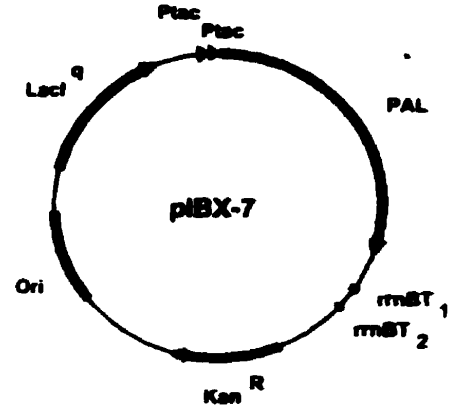

FIG. 1. *PAL* gene from yeast *R. toruloides* was cloned in the expression vector pIBX-7 where transcription is controlled by the strong inducible *tac* promoter and terminated by the rRNA transcription terminator sequences rrnBT1 and rrnBT2. $LacI^q$ represses the *tac* promoter, and hence isopropyl β-D-thiogalactoside is required to release it from the promoter. The kanamycin resistance gene (Kan^R) is included in the construct to allow selection of cells containing the plasmid.

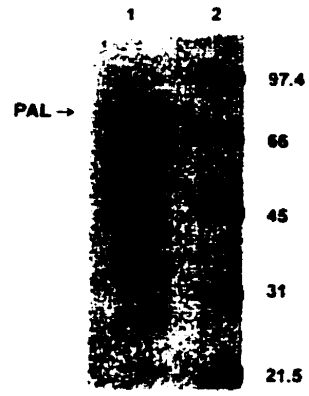

FIG. 2. Purified PAL enzyme (5 μg) separated on 4–15% gradient SDS/PAGE. Molecular mass markers in kDa are to the right. Lanes: 1, sample of PAL with ~20% impurities indicated by additional bands; 2, low-range molecular mass standards (Bio-Rad).

RESULTS

Synthesis and Purification of PAL. We obtained PAL by expressing the pIBX-7 construct (Fig. 1) in *E. coli*, followed by purification on a Q-Sepharose column. The product is a yeast (*R. toruloides*) PAL enzyme, at 80% purity (Fig. 2). The yield in our present system is 100–150 units/g of *E. coli* cells, with a K_m of 250 μmol/liter, at specific activity of 2.2–3.0 units/mg of PAL protein; 1 unit of PAL deaminates 1.0 mmol of L-phenylalanine to *trans*-cinnamate (and NH_3) per min at pH 8.5 and 30°C.

Effect of PAL in ENU Mice. We used the ENU mouse orthologues of human PKU and non-PKU-HPA to obtain proof of pharmacologic and physiologic principles by demonstrating efficacy of PAL enzyme against the hyperphenylalaninemic phenotype.

Pilot Study. PKU mice (ENU 2/2; n = 12) on regular diet were treated with PAL (2, 20, or 100 units) by i.p. injection without any additional manipulations. Within 3 hr, the postinjection value for blood phenylalanine had fallen (range, 0–984 μmol/liter) from the pretreatment value (range, 389–2,012 μmol/liter). These preliminary observations demonstrated both an apparent treatment response and troublesome inter-

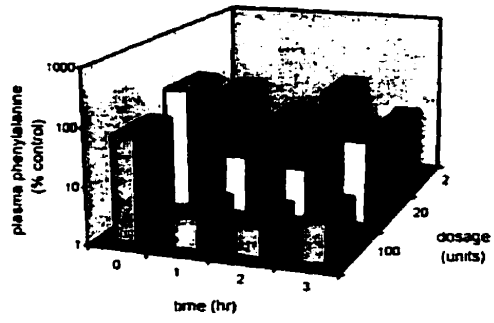

FIG. 3. Injection i.p. of recombinant PAL enzyme reduces plasma phenylalanine in the ENU2/2 mouse (*y* axis is logarithmic scale) over time (*x* axis) ($P < 0.05$). Reduction of plasma phenylalanine by PAL shows a dose–response relationship (*z* axis). Data are normalized to the control (sham-treated) values for each animal at each point. Data depicted are the average of five paired series. The range of control (100%) values was 390–2,013 μmol/liter for animals receiving 2 units of PAL, 572–1,488 μmol/liter for animals receiving 20 units, and 504–1,474 μmol/liter for animals receiving 100 units.

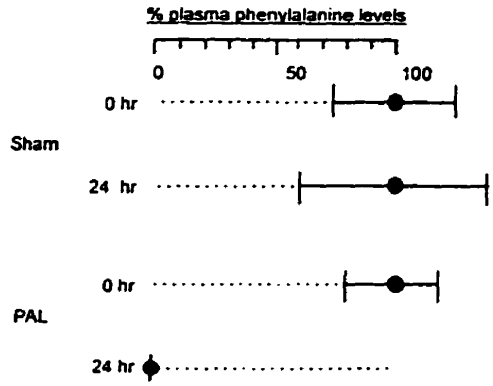

FIG. 4. A single i.p. injection of recombinant PAL enzyme (100 units at zero time), reduces plasma phenylalanine level in ENU1/1 mice by 95% at 24 hr ($P < 0.02$) relative to sham-treated controls. Data for five paired series (mean ± 1 SD) are normalized to paired control values to accommodate inter- and intraindividual variation. The range of control values (100% at zero time) was 41–528 μmol/liter; every animal showed a response to PAL.

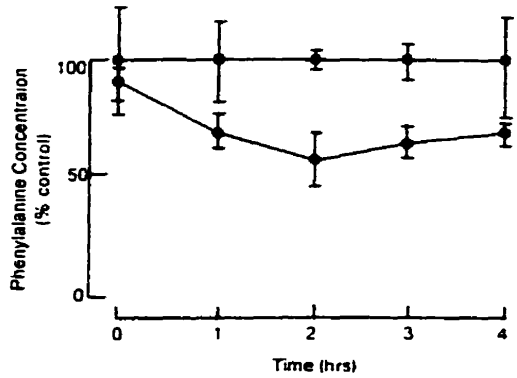

FIG. 5. Plasma phenylalanine levels in ENU2/2 mice after oral administration (25 units per mouse) of induced recombinant *E. coli* cells expressing PAL: 31% reduction within 1 hr ($P < 0.04$) and 44% reduction in 2 hr ($P < 0.004$). Data are normalized to control values (mean ± 1 SD). ■, Sham; ●, PAL enclosed in *E. coli*. The range of control values (100%) was 425–800 μmol/liter; every animal showed a response to PAL.

and intra-individual variation. Accordingly, we controlled for the latter by adopting the protocols described above.

Proof of pharmacological principle. A single i.p. injection of PAL enzyme significantly lowered plasma phenylalanine in PKU mice (ENU2/2; $P < 0.05$) and showed a dose-response effect (Fig. 3); at each point, data are normalized to the control (sham-treated) values for each animal to accommodate inter- and intra-individual variation. The non-PKU-HPA mice (ENU1/1) and the heteroallelic HPA stain (ENU1/2) also responded to PAL treatment (data not shown). The effect of a single i.p. PAL injection in ENU1/1 mouse model persisted for 24 hr ($P < 0.02$; Fig. 4); ENU1/2 and ENU2/2 mice both had similar 24-hr responses (data not shown).

Proof of physiological principle. To demonstrate the effect of orally administered PAL on plasma phenylalanine levels required protease-resistant PAL formulations. Recombinant PAL enzyme activity was protected against inactivation by gastric acidity and intestinal digestive enzymes by retaining and shielding it in the *E. coli* cells where it was synthesized. We used these cells in the absence of a different form of PAL protection.

Recombinant PAL enzyme protected by the cell wall and membrane of *E. coli* resists inactivation by proteolytic intestinal enzymes *in vitro*; otherwise activity of naked enzyme is abolished (data not shown). PAL enclosed in *E. coli* cells was shown to reduce phenylalanine content of the *in vitro* solution (Table 1). When given by oral gavage, recombinant PAL (25 units) expressed in an *E. coli* strain isolated from Sprague–Dawley rat lowered plasma phenylalanine in ENU2/2 mice by 31% in 1 hr ($P < 0.04$) and 44% in 2 hr ($P < 0.0004$) (Fig. 5).

Other experiments *in vivo*, conducted with aprotinin (protease inhibitor) and recombinant PAL enzyme purified from *E. coli* (Y1091), gave further proof of physiological principle. This formulation resists inactivation by chymotrypsin and mouse intestinal fluid *in vitro* (data not shown). Oral gavage of naked PAL (200 units) combined with aprotinin (protease inhibitor) lowered plasma phenylalanine in ENU2/2 mice: by 50% in 1 hr ($P < 0.017$) and 54% in 2 hr ($P < 0.023$) (Fig. 6).

DISCUSSION

We describe a method to produce recombinant PAL (Fig. 1) that may enable the use of this enzyme to degrade excess phenylalanine in PKU where the normal pathway for its disposal is impaired. Why would this alternative be useful if, as is generally assumed, early treatment of PKU by a semisynthetic low phenylalanine diet is one of the success stories of medical genetics (1, 2)? The answers lie in the incidence of PKU, our expectations for its treatment, and the anticipated "prevalence" of patient treatment years.

The combined incidence of PKU and non-PKU-HPA is on the order of 10^{-4} live births in populations of European descent. Current evidence reveals similar rates in Asian-Oriental and Arabic populations (1). New guidelines (10, 11) for the treatment of HPA have appeared so that residual imperfections in outcome (1) can be overcome and expectations met; the guidelines advise treatment to restore blood phenylalanine levels as near normal as possible, as early as possible, and for as long as possible, perhaps for a lifetime. In this context, the "prevalence" for patient treatment years takes on new meaning when it involves the difficult existing modes of treatment. The predicted "prevalence" in patient treatment years for a population of 10^8 persons, assuming 50 years of treatment per patient, would be 500,000 patient treatment years in half a century.

Table 1. Change in phenylalanine content *in vitro* solutions under various treatments

Experimental conditions	Treatment	Decrease in phenylalanine, % control†
Control	Nonrecombinant *E. coli* cells	0
	Chymotrypsin	0
	Intestinal fluid	0
PAL	In induced recombinant *E. coli* cells	76
	In induced recombinant *E. coli* cells + chymotrypsin in medium	72
	In induced recombinant *E. coli* cells + intestinal fluid in medium	66

*Treatments were for 1 h followed by measurement of L-phenylalanine content.
†Initial concentration, 4 mM of L-phenylalanine.

PKU is a multifactorial disease; mutation in the *PAH* gene and dietary exposure to the essential nutrient amino acid L-phenylalanine are equally necessary causes of the mutant phenotype (1). Treatment of HPA is feasible because the dietary experience can be purposefully modified, and the low phenylalanine diet has been its mainstay.

Untreated PKU is a disease at three phenotypic levels. At the proximal (enzyme) level, many different mutations in the *PAH* gene impair PAH integrity and function (32). Because PAH enzyme is the principal determinant of phenylalanine homeostasis *in vivo* (1), its impairment leads to HPA, which is the intermediate (metabolic) level of the variant phenotype. Impaired cognitive development and neurophysiological function is the distal (clinical phenotype); phenylalanine is the neurotoxic molecule (1), and hence the rationale to restore euphenylalaninemia.

Three modalities for treatment exist in theory or in practice: gene therapy, enzyme therapy, and diet therapy. The first has its appeal but is only at the experimental stage (33) (also C. Harding, personal communication) and unlikely to be put into practice unless the alternatives fail. The third method (low phenylalanine diet therapy) was inaugurated in the 1950s (3–5) and has achieved its primary goal: it has prevented mental retardation in the adequately treated patient (1, 34). However, dietary treatment of PKU and HPA is difficult; it involves rigorous compliance, a major alteration in lifestyle, and use of treatment products that have unusual organoleptic qualities. Moreover, measurements of cognitive and neurophysiologic outcomes show subtle deficits (IQ scores are 0.5 SD below normal), and there are lacunae in neuropsychological and neurophysiological performance. Accordingly, there is growing interest in the second form of treatment (enzyme therapy).

Enzyme therapy could be done by replacement (of PAH enzyme) or by substitution (with another enzyme to degrade excess phenylalanine). Replacement of PAH requires the intact multienzyme complex for catalytic hydroxylating activity (1); it could be done best perhaps only by hepatic transplantation and this approach has not been pursued. But if the PAL enzyme could be administered by mouth to the PKU patient, it would have a certain appeal; therapy with enzymes protected from inactivation is feasible for the treatment of metabolic conditions (35, 36). We propose that PAL will "substitute" for deficient hepatic PAH activity and degrade phenylalanine in the PAH-deficient organism. To test this hypothesis, we used the induced mutant (ENU) mouse orthologues of human PKU and non-PKU-HPA (23). We first demonstrated proof of pharmacologic principal: given by injection, PAL acts *in vivo* to lower ambient blood phenylalanine levels (Fig. 3). We then demonstrated proof of physiologic principal: PAL placed in the intestinal lumen acts *in vivo* to suppress HPA (Figs. 5 and 6). The latter is a significant finding that capitalizes on three prior concepts and observations: (*i*) Amino acids are in equilibrium between various compartments of body fluids (37, 38) and ultimately in equilibrium with the intestinal lumen (39), so that treatment of intestinal lumen phenylalanine will affect all body pools. (*ii*) PAL placed in the intestinal lumen will modify phenylalanine content of body fluids in the whole animal (15, 18). (*iii*) PAL placed in the intestinal lumen will act on both the dietary phenylalanine and the endogenous run out of free phenylalanine from its bound pools (40). Accordingly, the appropriate dosage and schedule (to avoid under- or overtreatment) of oral PAL, perhaps in combination with a controlled and modestly low protein diet, should control the phenylalanine pool size without need of the drastic restriction of dietary phenylalanine as now practiced and requiring artificial diets. Ancillary treatment of PKU with PAL and prudent protein intake would become analogous to treatment of diabetes mellitus with insulin, with an additional feature—the enteral route would avoid problems with immune recognition of PAL.

Until now, the cost of PAL has prohibited any consideration of therapy; even animal studies were curtailed. The recombinant enzyme we describe herein may avoid this constraint and has enabled our investigations. The relatively low specific activity of the recombinant PAL product and relative inefficiency at pH 7.0 (R.H., unpublished data) may be offset by the long contact time between enzyme and substrate during passage through small and large intestine. The formulation currently under development is focused on completely protecting the PAL enzyme against a protease environment.

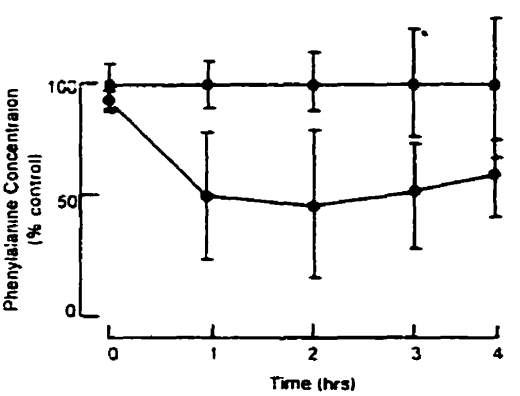

FIG. 6. Plasma phenylalanine levels in ENU2/2 mice after combined oral administration of naked PAL enzyme (200 units per mouse) and protease inhibitor (aprotinin): 50% reduction within 1 hr ($P < 0.017$) and 54% in 2 hr ($P < 0.023$). Data are normalized to control values (mean ± 1 SD). ■, Sham; ●, unprotected PAL plus aprotinin. The range of control values (100%) was 458–1,051 μmol/liter; every animal showed a response to PAL.

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APPENDIX D

PAHdb

The Mouse Genetic Pah Homologues Page.

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Mouse enzyme and gene for phenylalanine hydroxylase

The mouse phenylalanine hydroxylase gene (symbol *Pah*) was mapped to chromosome 10 in 1988 [7] (Reviewed in 8); the cDNA was cloned and sequenced in the same lab [6] (GenBank UID: X51942, (EC 1.14.16.1)).

The cDNA is 1978 bp and begins its coding sequence at position 48, followed by an open reading frame (1359 bp) and a 17-residue poly A signal (beginning at position c1965). Mouse and human genes have ~87% conservation of sequence, with ~10% of the changes silent (in the third codon position). No similarities are observed in their untranslated regions [6].

The murine Pah enzyme has 453 residues (molecular weight, 51,786 Da). There is ~8% divergence between human and mouse sequences; the majority of differences are in the N-terminal region (thought to be involved in regulatory or binding function rather than catalytic activity). In addition, there are reciprocal substitutions of cysteine residues in mouse and human enzymes, preserving the number of cysteines but altering their relative positions. Differential steady-state concentrations of the two enzymes in the cell, potential topological changes, which may alter structure and function, possible differences in the rates of transcription, translation, stability, activation or intrinsic catalytic activity are all possible contributors to the still unexplained difference in mouse and human PAH activities; mouse enzyme has an order of magnitude greater specific activity than human enzyme [6].

Genetic Mouse Models of Phenylketonuria and non-PKU hyperphenylalaninemia

Natural (genetic) mouse models for PKU and non-PKU Hyperphenylalaninemia (HPA) now exist. Inbred BTBR males were treated with *N*-ethyl-*N*-nitrosourea (†), mated to normal BTBR females, and the potentially heterozygous progeny from this cross were mated and their progeny screened for HPA. Three mutant phenotypes were identified; *Pah(enu1)*, *Pah(enu2)* and *Pah(enu3)*.

The homozygous *Pah(enu1)* mouse was reported in 1990 [9]. On the basis of its retarded clearance of an injected phenylalanine load, it was classified as a non-PKU HPA counterpart. *Pah(enu1)* mice have a c.364T>C transition (V106A) in exon 3(†)

[11]. Note: Structure/function studies (rat Pah) show that the region in which this mutation falls (the N-terminal regulatory domain) can be cleaved by chymotryptic digestion with the enzyme still retaining its catalytic activity [1]. Crystallographic analysis of the human enzyme reveals that this mutation is distant from the catalytic residues of PAH/Pah [2,5]. This factor in conjunction with the conservative nature of the predicted Val to Ala substitution may explain the mild phenotype of the *Pah(enu1)* animals [11]. These animals display normal plasma phenylalanine levels, normal colouration and no abnormal behaviour on regular diet (Teklad. #8626). There is no apparent effect of maternal phenotype on the fetus; and progeny show normal survival and growth rate. Pah enzyme activity is ~5-24% normal [10,12]. Under conditions of elevated Phe in diet, reduced growth is observed [9,13].

Pah(enu2) and *Pah(enu3)* strains were reported in 1993 [13]. Both strains are classified as PKU counterparts. The *Pah(enu2)* mutation is a c.835T>C transition (F263S) in exon 7 (‡) [11]. Note: This mutation affects a residue shown (by the crystal structure analysis of the human PAH catalytic and tetramerization domains) to be in the active site. This residue is implicated in pterin binding with predicted π -stacking interactions with the substrate [3,4]. The region is highly conserved. [1,2,5]. The presence of mutation in the critical region in conjunction with the non-conservative phe to ser substitution may explain the sever phenotype observed in *Pah(enu2)* animals. The *Pah(enu3)* mouse mutation remains to be identified, however, it has been localized to a 301 bp portion of the coding sequence, spanning part of exon 11 and all of exons 12 and 13 (‡) [11]. Neither *Pah(enu2)* or *Pah(enu3)* have measurable hepatic enzyme activity; on normal diets, they both display a 10-20 fold elevated serum phenylalanine level and elevated phenylketones in urine. These animals have retarded pre- and postnatal growth, smaller heads, behavioral abnormalities and, like their human PKU counterparts, show pronounced hypopigmentation. Both strains have a severe maternal effect on the fetus, resulting in the loss of litters within several hours of birth [12,13].

We have bred a variant of the *Pah(enu)* mouse using *Pah(enu1)* (female) x *Pah(enu2)* (male) crosses. Metabolic profiling, following L-phenylalanine challenge, reveals a HPA phenotype intermediate between *Pah(enu1)* and *Pah(enu2)*. On regular mouse chow, this *Pah(enu1/2)* heteroallelic strain has near normal plasma phe levels and normal coloration. Pah enzyme activity is ~5% normal. This strain has no abnormal behavior, a low maternal effect on the fetus and is pliant to metabolic manipulation [12].

Footnotes:

(†)N-ethyl-N-nitrosourea, an alkylating agent, induces mutations at about 10^{-3} frequency per locus, in spermatogonial stem cells.

(‡) Mouse Pah cDNA has been sequenced but not the complete gene, therefore, each mutation has been assigned to a specific exon(s) by regional homology between mouse and human genes.

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For further information about this site and its contents, please contact J.D.McDonald or C.N.Sarkissian.

APPENDIX E

Protocol for Long-Term PAL Administration Providing Direct Access to the Duodenum

Animals are injected with Tribrissen 24hrs prior to, the day of and the day following surgery. On the day of the surgery, they are injected (i.p.) with Pentobarbital, followed by an injection of Atropine sulfate (s.c.). The mouse is shaved around the abdominal area and the back (between the scapulae) and disinfected with isopropyl-alcohol, Hibitane and Iodovet. An incision is made on the back and abdominal area. A piece of silicone tubing is inserted through the back incision and directed (s.c.) towards the abdominal incision. The end, which appears at the posterior opening, is sealed with a sterile-removable plug (this can be accessed for administration of the enzyme). The peritoneal membrane is incised, and the duodenum located. Then a circular suturing is introduced and an incision made in the middle where the silicone tubing is inserted. Following insertion, the suture material is tightened around the tubing and Vetbond used to reinforce the seal. The abdominal cavity and skin is closed by suturing. The animals receive Buprenorphine (SC) prior to waking up and for the following 3 days.

One week following the surgery, the PAL formulation can be tested by injecting the solution, through the catheter, directly into the intestine. Efficacy of the enzyme can be measured by reduction in plasma phenylalanine levels over a period of a week.