Scriver's OMMBID The Online Metabolic & Molecular Bases of Inherited Disease
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PART 8: AMINO ACIDS

Chapter 77: Hyperphenylalaninemia: Phenylalanine Hydroxylase Deficiency

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Abstract

1. We describe an inborn error of metabolism called *phenylketonuria* (PKU; MIM No. 261600). The disease has been called an epitome of human biochemical genetics (Scriver and Clow, 1980a, 1980b). The disorder reflects a disadaptive interaction between nature and nurture. The component in nurture is an essential amino acid, L-phenylalanine; the one in nature is mutation in the *phenylalanine hydroxylase gene (PAH)* encoding the enzyme L-phenylalanine hydroxylase (EC 1.14.16.1). The discordance between nature and nurture leads to *hyperphenylalaninemia (HPA)*, which can have a toxic effect on brain development and function. The "proximal" phenotype (phenylalanine hydroxylase dysfunction) is under the control of one locus encoding the phenylalanine hydroxylase enzyme and additional loci encoding several other enzymes necessary for synthesis and recycling of the tetrahydrobiopterin cofactor essential for the catalytic reaction; *locus heterogeneity* thus enters the interpretation of HPA. The intermediate (metabolic) and distal (cognitive) phenotypes of PKU disease both behave as *complex traits* that elude consistent interindividual genotype-phenotype correlations. The phenylalanine hydroxylase gene harbors great allelic diversity; several hundred mutations, both disease-causing and polymorphic, are recorded in *PAHdb*, a public-locus-specific mutation database (www.pahdb.mcgill.ca).

2. The HPAs are disorders of phenylalanine hydroxylation. The minimum requirements for the normal reaction, which occurs in both liver and kidney in human subjects, are *phenylalanine hydroxylase enzyme* (a monooxygenase, EC 1.14.16.1), oxygen, L-phenylalanine substrate, and the 6*R* isomer of the tetrahydrobiopterin (BH_4) cofactor. For the pterin cofactor to function as a catalyst, BH_4 must be regenerated from the carbinolamine byproduct (4a-hydroxytetrahydropterin) of the hydroxylation reaction. This is achieved by a recycling pathway in which *4α-carbinolamine dehydratase* (formerly known as *phenylalanine hydroxylase–stimulating protein*) converts the carbinolamine to the quinonoid dihydropterin, which, as the substrate for *dihydropteridine reductase* in the presence of reduced pyridine nucleotide, is converted back to BH_4. A pathway exists for biosynthesis of this obligatory cofactor involved both here and in the function of other aromatic monooxygenases and of nitric oxide synthase; the enzymes in the pathway are *guanosine triphosphate cyclohydrolase*, *6- pyruvoyltetrahydropterin synthase*, and *sepiapterin reductase*. Diseases of BH_4 synthesis and recycling are discussed in Chap. 78.

3. *Hyperphenylalaninemia* is defined as a plasma phenylalanine value greater than 120 µmol/liter (>2 mg/dl). Whether forms of HPA owing to altered integrity of the enzyme should be subdivided into different forms—notably *phenylketonuria* (plasma phenylalanine >1000 µmol/liter, diet phenylalanine tolerance < 500 mg/day) and *non-PKU* forms (plasma phenylalanine < 1000 µmol/liter, diet tolerance > 500 mg/day)—is a moot point. Evidence suggests that mild degrees of persistent untreated HPA (<600 µmol/liter) may not be harmful to cognitive development (as yet an unproven hypothesis). For purposes of diagnosis, counseling and correct treatment of the non-PAH enzyme deficiencies affecting BH_4 homeostasis must be ruled out.

4. The human *PAH* gene covers approximately 100 kb of genomic DNA on chromosome 12, band region q23.2, and is embedded in a region of 1.5 Mbp harboring five other genes. The nucleotide sequences, both genomic (GenBank accession number AF404777) and cDNA (U49897.1), are now known (see www.pahdb.mcgill.ca); *PAH* has 13 exons and a complex 5' untranslated region containing *cis*-acting,

trans-activated regulatory elements. The gene is rich in intragenic polymorphic markers, including biallelic restriction-fragment-length polymorphism (RFLP) and single-nucleotide polymorphism (SNP) alleles, a tetranucleotide short tandem repeat (STR) acting as a fast molecular clock in intron 3, and a variable number of tandem repeats (VNTRs) (30-bp-length cassettes) in the 3' untranslated region (UTR). The polymorphic sites are in linkage disequilibrium and describe a large series of extended and miniature haplotypes. The *PAH* gene also harbors several hundred disease-causing alleles associated with HPA, of which more than 60 percent are missense alleles. Only a half-dozen different alleles account for the majority of mutant chromosomes in Europeans or Orientals; the remainder are rare, even private alleles.

5. The human *PAH* gene has both developmental- and tissue-specific transcription/translation. Its translation product is a 452-amino-acid polypeptide homologous in several domains with the subunits of tyrosine and tryptophan hydroxylases. The catalytic domain of the human PAH polypeptide has been resolved at 2 Å for residues 117–427. The enzyme is homo-oligomeric and functions in alternating activated and deactivated states in dimeric and tetrameric conformations. The effect of mutant alleles is being studied by molecular modeling *in silico* by using the protein design algorithm FoldX to predict the energetic impact on native-state stability of the PAH enzyme of missense *PAH* alleles and by expression analysis *in vitro*. Missense alleles can cause misfolding of the PAH enzyme subunit, leading to aggregation and disposal by the proteasome.

6. The effects of disease-causing *PAH* mutations on the patient can be measured at three levels: proximal (enzymic), intermediate (metabolic), and distal (cognitive function). Enzyme dysfunction can be measured in vitro either directly by hepatic biopsy or indirectly by expression analysis when the mutation is expressed in a transgenic construct in mammalian or bacterial cell systems or in a cell free transcription/translation system. The latter enables hepatic PAH activity in vivo to be broadly predicted. Flux rates in vivo for phenylalanine hydroxylation/oxidation also can be measured by two different isotopic methods. All studies of genotype-phenotype correlations reveal reasonable correlations at the proximal (enzyme) level; however, at intermediate (metabolic) and distal (cognitive) levels, the phenotypes have emergent properties and behave as complex traits in which the effects of PAH, the major locus, is modulated by "modifiers."

7. Pathogenic *PAH* alleles produce their effects on PAH enzyme by various mechanisms and behave in broad terms as *null* (no activity), *V*max *altering* (reduced activity), *kinetic* (altered affinity for substrate or cofactor), *unstable* (as a result of misfolding and increased turnover and loss of PAH protein), and *BH_4-responsive*. Findings occasionally have been taken to signify negative allelic complementation as an additional mechanism of mutant genotype expression. An important subset of missense alleles that do not map to the BH_4-binding region confers a 6*R*-BH_4-responsive phenotype *in vivo* by mechanisms that include stabilizing a misfolding subunit by chaperone-like therapy and by overcoming unfavorable BH_4-binding kinetics by saturation.

8. Newborn screening for PKU occurs in many societies and is a potent resource for ascertainment and sampling of mutant *PAH* genes. Prevalence data for HPA (5–350 cases per 1 million live births) and mutation analysis together reveal nonuniform distribution of patients and alleles in human populations. Human genetic diversity at the *PAH* locus complements data from analysis of mitochondrial DNA, the Y chromosome, and classic autosomal polymorphisms. The distribution and types of *PAH* alleles indicate how migration, genetic drift, natural selection (perhaps), recurrent mutation, and intragenic recombination over the past 100,000 years might account for the present-day incidence of PKU, the observed mutation-haplotype associations, and the nonuniform distribution of cases and major alleles in modern human populations. The prevalence rates for PKU in persons of African descent appear to be an order of magnitude lower than those for persons in European, Chinese, and Korean populations, where prevalence is similar (10^{-4}) in these populations.

9. Pathogenesis of the most important clinical (disease) phenotype (cognitive and neurophysiologic impairments) is undoubtedly complex, but there is an emerging consensus that phenylalanine itself, at elevated concentrations, is the harmful molecule that starts the process of allostasis. Several strains of mice mutagenized by *N*-ethyl-*N*-nitrosourea, with documented *PAH* gene mutations and deficient enzyme

activity, are orthologous resources to study pathogenesis, as well as treatment to control the phenotypic effects of the mutant genotype.

10. Newborn screening with measurement of blood phenylalanine is the most reliable method for early detection of HPA. The classification of phenotype includes severe and less severe forms of PAH deficiency, of BH_4-responsive and BH_4-nonresponsive primary deficiency, and of primary BH_4 deficiency. Classification requires measurements of phenylalanine, pterins, and neurotransmitter derivatives in urine, plasma, and cerebrospinal fluid (CSF), along with specific protocols and various assays of enzyme activity (see Chap. 78). If it is requested, prenatal diagnosis for PKU is feasible by DNA analysis of mutations and haplotypes.

11. Treatment of HPA requires restoration of blood phenylalanine to values as near normal as possible as early as possible in postnatal life and for as long as possible—perhaps for a lifetime. At present, it seems that any deviation from this policy may incur a cost in structure and function of brain in the PKU patient. Among the modalities of treatment, the low-phenylalanine diet is still paramount, but it needs to be improved in organoleptic properties and in nutrient composition—notably of the essential fatty acids and the relative ratios of amino acids. The BH_4-responsive *PAH* alleles may require only pharmacologic therapy (e.g., 10 mg BH_4/kg per day). Alternative modalities include the possibility of enzyme substitution with engineered recombinant phenylalanine ammonia lyase (promising) and gene therapy (in a holding pattern for human subjects but showing efficacy in the mouse model).

12. Maternal HPA, a toxic embryopathy/fetopathy, causes congenital malformations, microcephaly, and permanently impaired cognitive development. It is a consequence of intrauterine phenylalanine excess in the fetal compartment derived from a positive transplacental gradient. All females of reproductive age with HPA should receive reproductive counseling, social support, and continued or renewed treatment to restore euphenylalaninemia before conception and throughout pregnancy. Meticulous treatment of maternal HPA is compatible with a normal outcome for the fetus.

13. Virtually all the major themes and issues now considered to be important in PKU were recognized by the human geneticist Lionel Penrose half a century ago. The fundamental questions about PKU are the same then and now; only the tools and opportunities to address them have changed.

INTRODUCTORY COMMENT

This chapter is not a total overview of hyperphenylalaninemia (HPA) and its principal disease type,* even though it touches most aspects of the topic. Rather, it is a close analysis of the enzyme involved [phenylalanine hydroxylase (PAH)], the corresponding gene (*PAH*) with its alleles, their disease-causing effects, and how those diseases occur and can be prevented (Scriver, 2007). The primary disorders of BH_4 homeostasis affecting phenylalanine hydroxylation and other reactions are covered in Chap. 78.

In 1994, a symposium† was held to celebrate the sixtieth anniversary of Asbjørn Følling's German-language report (1934) on a new inborn error of metabolism; another symposium† in 1995 celebrated the fortieth anniversary of effective therapy of this genetic disease. The disease, renamed *phenylketonuria* in 1935 by Penrose, had been called *imbecilitas phenylpyrouvica* by Følling to recognize its effect on cognitive development.‡ Some 11 years later, for his inaugural address as Galton Professor at University College London (Penrose, 1946), Penrose chose phenylketonuria as his topic. Among the many themes running through his remarkable analysis of this now-classic genetic disease was Penrose's observation that phenylketonuria (PKU) was the first to exhibit a chemical explanation for mental retardation.

With hindsight, major milestones on this journey of discovery about PKU can be recognized: (1) In the 1930s, it was shown that both the disease (mental retardation) and the major metabolic abnormality (hyperphenylalaninemia) are accounted for by autosomal recessive inheritance of a deleterious gene

(Følling, 1934; Penrose, 1935) (MIM 261600). (2) In the 1950s, PKU patients were shown to have deficient activity of hepatic PAH (EC 1.14.16.1), the key enzyme controlling phenylalanine catabolism (Jervis, 1953). (3) In the same decade, a diet restricting intake of phenylalanine, an essential amino acid for human beings, was shown to ameliorate the HPA of PKU, thus offering the potential to prevent mental retardation (Bickel et al., 1953). (4) In the 1960s, a simple test for population screening was developed (Guthrie and Susi, 1963), providing the opportunity for early diagnosis, treatment, and prevention of PKU disease. (5) In the 1960s and 1970s, treatment of this particular genetic disease was seen as a prototype for the treatment of other genetic diseases (Scriver, 1967), and it was shown to be truly effective in preventing mental retardation in PKU (MacCready, 1974). (6) Mapping and cloning of the phenylalanine hydroxylase gene in the 1980s came first in the rodent (Robson et al., 1982) and then in *Homo sapiens sapiens* (Woo et al., 1983) (human gene symbol *PAH*; GenBank cDNA sequence U49897.1). This is followed in the 1990s by worldwide mutation analysis and recognition that extensive mutant allelic heterogeneity accounts for PKU and related forms of HPA (Nowacki et al., 1998). (7) In the 1970s, there was recognition (Danks et al., 1978; Kaufman, 1971) that locus heterogeneity must exist to account for all components of phenylalanine hydroxylation *in vivo* and to account for a mysterious subset of patients with "malignant" HPA (see Chap. 78). (8) In the 1990s, it became evident that PKU and the HPAs are more than Mendelian traits; they also have the features of multifactorial., multilocus, and complex traits (Scriver and Waters, 1999). Therefore, they serve as prototypes for thinking more broadly about the nature of so-called Mendelian disease. (9) In the 1990s, the catalytic core of the human PAH protein was crystallized and its structure visualized at 2-Å resolution (Erlandsen et al., 1997a, 1997b); thus (virtual) molecular modeling *in silico* of mutation effects could begin. (10) In the same decade, the vast allelic diversity, both disease-causing and neutral polymorphic in type, at the *PAH* locus began to be recorded in an online relational database (www.pahdb.mcgill.ca) (Nowacki et al., 1998).

Whatever one might like to call "progress," it is chastening to realize, by rereading Penrose's article (1946), that Penrose anticipated much of our present knowledge; the difference between then and now is simply the evidence, obtained with tools not available in Penrose's own lifetime; the evidence shows that he was a very foresighted geneticist—as were so many of his colleagues.

Footnotes

Standard abbreviations are listed preceding the index of this book. Nonstandard abbreviations used in this chapter are as follows:

BH_4 = tetrahydropterin[(*6R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin]; DHPR = dihydropteridine reductase; $DMPH_4$ = 6,7-dimethyltetrahydropterin; GTP-CH = guanosine triphosphate cyclohydrolase; HPA = hyperphenylalaninemia; 6-MPH_4 = 6-methyltetrahydropterin; PAH = phenylalanine hydroxylase enzyme (phenylalanine 4-monooxygenase); *PAH* = phenylalanine hydroxylase gene (human); *Pah* = phenylalanine hydroxylase gene (mouse); PAL = phenylalanine ammonia lyase; PKU = phenylketonuria; 6-PTS = 6-pyruvoyltetrahydropterin synthase; qBH_2 = quinonoid form of dihydrobiopterin; RFLP = restriction fragment length polymorphism; SNP = single-nucleotide polymorphism; STR = short tandem repeat; VNTR = variable number of tandem repeats.

*This version of Chap. 77 is an overview of both recent developments and well-established themes in the Mendelian HPAs. Many new reports have appeared since the online edition of MMBID-8 extended the print version of January 2001. The present version is much revised and contains significant large attachments (external updates) not found in the print version. The style for reference citation is also different.

[†] The symposium was published; it opens with an elegant essay about Asbjørn Følling and his discovery of PKU, written by his son Ivar (Følling, 1994). Another symposium, opened by Horst Bickel, "the father of PKU therapy," covering this and other topics was published thereafter (*European Journal of Pediatrics,* 1996).

[‡] Asbjørn Følling later renamed "his" disease, preferring to call it *oligophrenia phenylpyrouvica* (Følling et al., 1945), a name more compatible with the wide range of mental deficiency he had discovered in patients with PKU. The same article extends the evidence in his Norwegian cases for autosomal recessive inheritance of the disease, adding support to similar claims made early by himself (1934), Penrose (1935), and Jervis (1939).

INTRODUCTION

The generic term for a phenotype distinguished by phenylalanine concentrations persistently elevated above the distribution of its plasma values in healthy controls is *hyperphenylalaninemia* (HPA). This disturbance in metabolic homeostasis can have clinical consequences depending on its pathogenesis and its degree. The major associated clinical manifestation is impaired cognitive development and function resulting from neurochemical imbalance—postnatally in affected cases and prenatally in the fetus carried by an affected pregnant woman. The genetic causes of HPA are *primary*—mutations (alleles) in the gene (symbol *PAH*) encoding L-phenylalanine hydroxylase enzyme (PAH)—and *secondary*—at loci for at least two enzymes in the pathway for synthesis of tetrahydrobiopterin, also known as BH_4 [(6*R*)-L-*erythro*-5,6,7,8-tetrahydrobiopterin], the cofactor for the hydroxylation reaction, and at the loci for 4α-carbinolamine dehydratase and dihydropteridine reductase (DHPR), enzymes that regenerate BH_4 from the oxidized biopterin by-product of the hydroxylation reaction. So-called null mutations at the *PAH* locus cause PKU; others conferring some residual enzyme activity cause a lesser degree of HPA (so-called non-PKU HPA), where the associated risk of mental retardation is less than it is in classic PKU.

BH_4 homeostasis serves two additional hydroxylation reactions involving L-tryptophan and L-tyrosine, notably in brain. The hydroxylated derivatives of these substrates, 5-hydroxytryptophan and L-dopa, respectively, are precursors of serotonin and catecholamines, which, as neurotransmitters, influence brain development and function. Accordingly, diagnosis of BH_4-deficient variants of HPA is relevant for prognosis and treatment and has become an integral part of PKU screening programs even though fewer than 2 percent of newborn infants with persistent HPA (in European populations) have a disorder of BH_4 homeostasis (see Chap. 78 and the relevant website: www.bh4.org).

Nomenclature

This chapter deals with the HPAs associated with primary deficiency of PAH enzyme function caused by variant disease-causing alleles in the *PAH* gene. If all degrees of HPA are a risk factor for impaired cognitive development, then it may not matter whether we classify the trait into its more consistently severe (PKU) or usually milder (non-PKU HPA) forms; we will worry about prognosis in either form. One thus could argue for the use of a single name—*phenylketonuria*; indeed, Smith (1994a) recommended acceptance of the term *phenylketonuria* "as a scientifically sound, collective noun for the family and disorders due to PAH deficiency." We concur, but there is the issue of common practice. The literature is now well populated with papers naming the severe and mild forms of HPA as *PKU* and *non-PKU HPA,* respectively; we use both these terms here knowing that each implicates some degree of harm to the central nervous system (CNS). On the other hand, there is some evidence that untreated non-PKU HPA (at levels < 600 µmol/liter) is not harmful to cognitive development (Weglage et al., 2001); hence there is reason to retain the distinctive nomenclature.

The clinical distinction between PKU and non-PKU HPA currently rests on recognition of higher plasma phenylalanine values in PKU (>1000 µmol/liter; >16.5 mg/dl) in the untreated state and on lower tolerance for dietary phenylalanine in PKU (>500 mg/day) (Scriver et al., 1995). Others have suggested similar but quantitatively different criteria for classification of PAH enzyme–deficient HPA (Guttler, 1980), but the intention is similar: Higher ambient blood phenylalanine levels and lower dietary tolerance are associated with a more PKU-like phenotype and thus greater hazard of impaired cognitive development.

Hyperphenylalaninemia: A Framework for Understanding Why This "Simple" Phenotype Has "Complex" Explanations (See also Supplement: "Homeostasis, Complexity, and Monogenic Phenotypes: The View from Phenylketonuria")

Whereas PKU is a classic Mendelian disease, its metabolic phenotype (HPA) and the associated disease (mental retardation) are the result of more than the effect of a single mutant allele. To be aware of this is to have a framework for understanding HPA and its attendant diseases, as well as so-called Mendelian disease in general (Scriver, 2007).

HPA is a Mendelian inborn error of metabolism

PKU is listed under entry 261600 in the McKusick *Catalogs of Mendelian Inheritance in Man* (OMIM). When Følling found an increased frequency of consanguinity among the parents of his patients, he recognized evidence for autosomal recessive inheritance; he also found an aberrant metabolic state in the propositi he investigated (Følling, 1934, 1994). Lionel Penrose almost immediately recognized the disorder as a new "inborn error of metabolism" (Penrose, 1935) and as the first form of mental retardation to have an overt chemical feature (Penrose, 1946); he communicated accordingly with Garrod (Bearn, 1993). Since then, all human mutations causing primary impairment of PAH enzyme integrity have been shown to map to the *PAH* locus on chromosome 12q (Lidsky et al., 1985a), in keeping with the involvement of a major gene in PKU and related forms of hyperphenylalaninemia. *Incidence* of the autosomal recessive metabolic phenotype and its disease, when due to primary deficiency of phenylalanine hydroxylase function, is on average 1 in 10,000 live births in Europeans; *prevalence* of persons affected by and coping with the implications of persistent HPA will be the same. However, in a source population of 100 million people, the subset following contemporary recommendations for treatment of HPA would generate 500,000 patient treatment years in half a century.

Hyperphenylalaninemia in PKU is multifactorial

Dietary intake of L-phenylalanine, an essential nutrient for *Homo sapiens sapiens*, is required to produce HPA in the mutant phenotype. Dietary experience and mutant genotype thus are both necessary components of cause. Accordingly, the metabolic phenotype is multifactorial., and therein lay the original opportunity for treatment through restriction of dietary phenylalanine.

HPA is genetically heterogeneous

PKU indeed manifests HPA, but not all HPA is necessarily PKU. The phenylalanine hydroxylating reaction requires tetrahydrobiopterin (BH_4) cofactor. Mutation in a gene controlling any one of the several stages of synthesis or recycling of BH_4 can cause HPA. Recognition of this fact is a necessary part of the diagnosis, workup, counseling, and treatment of every patient with persistent HPA (see Chap. 78).

PKU disease and the attendant metabolic phenotype are complex traits (See also Supplement: "Homeostasis, Complexity, and Monogenic Phenotypes: The View from Phenylketonuria")

Analysis of genotype-phenotype correlations in untreated PKU patients shows no tight and consistent relationship, either inter- or intrafamilial., between predicted severity of the *PAH* mutation effect and cognitive development (IQ or DQ scores) (Ramus et al., 1993). Long ago, Penrose had noticed discrepancies in IQ values within PKU sibships (Penrose, 1946); he further recognized that whereas phenylalanine values in normal and PKU populations behaved as a discontinuous metric trait, IQ values resembled a *quasi-discontinuous* trait. Since intelligence itself is a complex trait, the failure to see a tight correlation between IQ and *PAH* genotype is not surprising. However, even blood phenylalanine values themselves, more proximate than cognitive function to the primary mutant gene effect on enzyme function, do not correlate consistently with predicted effect of the mutant *PAH* genotype. For example, the same mutant genotype can be associated with both severe (PKU) and mild (non-PKU HPA) forms of HPA (Guldberg et al., 1998; Kayaalp et al., 1997), and whereas *in vivo* measures of L-phenylalanine oxidation rates show the gene dosage expected for a Mendelian trait, the corresponding plasma phenylalanine values may not (Scriver, 1998a; Treacy et al., 1997). This evidence implies that whereas the oxidative step has a high sensitivity coefficient for phenylalanine homeostasis (Kacser and Burns, 1981), PAH enzyme function is not the only determinant of phenylalanine homeostasis. Phenylalanine homeostasis is apparently under the control of a set of *quantitative trait loci* (Scriver and Waters, 1999). It is a manifestation of a human *phenome* (Freimer and Sabatti, 2003).

Multiple *PAH* alleles

Even half a century after Følling's description of PKU, it was customary to refer to *the* PKU mutation and to record it by the single symbol *a* (for a recessive allele). In the molecular era of PKU studies, however, it is apparent that the mutant human *PAH* locus harbors hundreds of disease-causing alleles ($a_1 + a_2 + a_3 + \uparrow + a_n$). A few are prevalent; most are rare, even "private" (Nowacki et al., 1998; Scriver et al., 2000, 2003). PKU fits an emerging view of Mendelian disease in general. The majority of disease-causing alleles at the relevant locus will be rare; only a few will be prevalent (Weiss, 1996).

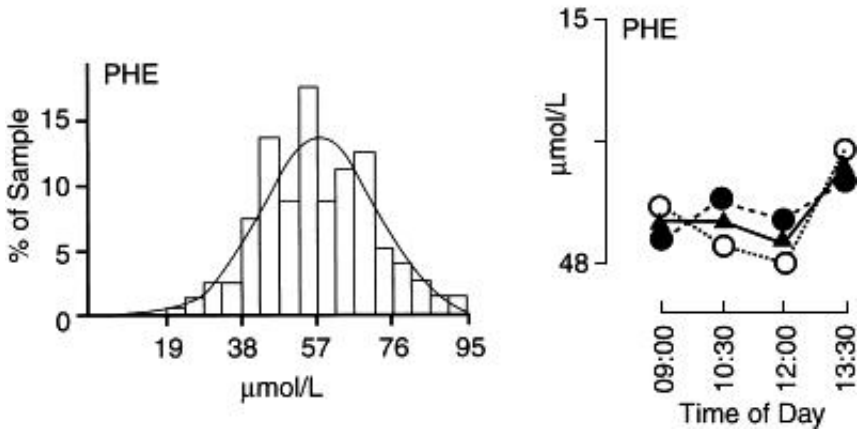
PHENYLALANINE HOMEOSTASIS

Claude Bernard (1878) recognized that constancy in the internal milieu was a necessary condition of life; Walter B. Cannon called it *homeostasis* (Cannon, 1929). The phenylalanine content of blood, or of any other body fluid, is a metric trait that observes a central tendency (homeostasis). HPA is recognized when the value for non-peptide-bound (free) phenylalanine is greater than the normal frequency distribution; the normal range of values represents one steady state, and the range of deviant values in HPA reflects a different steady state, which may or may not have consequences for health.

The metabolic steady state is a dynamic one in which the concentrations of metabolites in the system remain fixed in the face of fluxes through it (Cohn et al., 1980). Any persistent change in a flux eventually will change the steady-state value. Regulatory mechanisms control homeostatic systems so that steady-state values experience only minor, transitory changes within certain limits under usual circumstances (Murphy and Pyeritz, 1986). The dispersion of plasma phenylalanine values around the central tendency (the mean or *homing* value) fits this model (Fig. 77-1) and shows that the plasma phenylalanine value is a quantitative (complex) trait. It reflects both intraindividual or interindividual biologic variation (V_G) and the range of experience encountered by the individual (V_E) (Scriver et al., 1985). The normal value for plasma phenylalanine *within* an individual shows infradiem oscillation (Scriver et al., 1985) that is not greater than about half the nadir value (see Fig. 77-1). The broad similarity in values *between* individuals implies that their homeostatic mechanisms are similar and have been acquired through shared human evolution; the outlier values are of interest because they reflect unusual biologic

diversity or experience. Evidence for heritability (h_2), which in the broad sense is $V_G/(V_G + V_E)$, is found in twin studies (Scriver and Rosenberg, 1973); plasma phenylalanine values are more similar within monozygotic twin pairs than they are between nontwin subjects. Accordingly, an individual has a "private" plasma amino acid phenotype, with values distributed over a narrower range than the "public" distribution of values for a population of unrelated individuals (Scriver et al., 1985). Thus the blood phenylalanine value indeed should be viewed as a quantitative trait in which the controlling biologic factors are the *quantitative trait loci* (Langenbeck et al., 1988; Scriver et al., 1985). What those loci encode as proteins and mechanisms of homeostasis are scarcely known, but they eventually will yield to enquiries in the context of "systems biology."

Figure 1:



A: The frequency distribution of plasma phenylalanine values (n = 80) in adult human subjects (n = 10) studied twice (fasting and fed) at four different times of the day. B: Mean values (▲) for infradiem variation in plasma phenylalanine in fasted (•) and fed (○) subjects. (Both figures adapted from Scriver et al., 1985. Used by permission.)

Normal Plasma Values

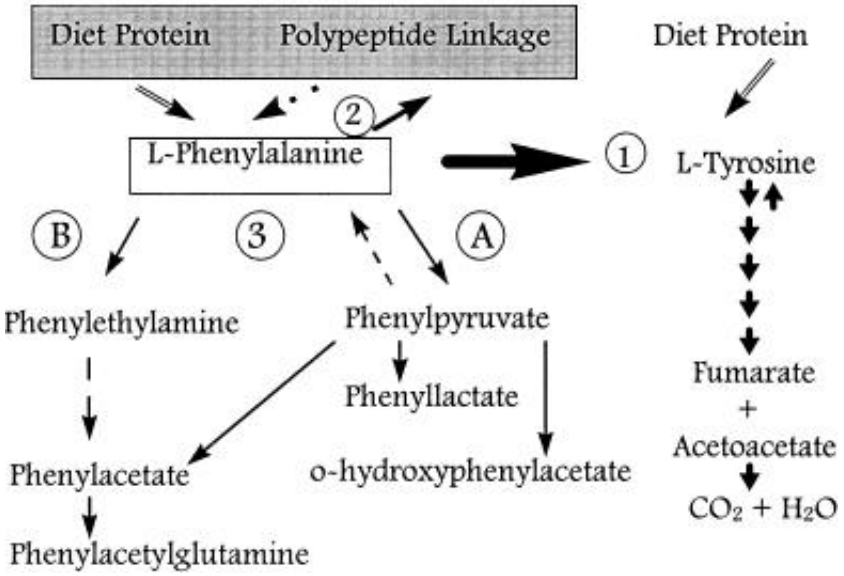
The normal plasma free phenylalanine value is not significantly different in young and adult-age subjects (Gregory et al., 1986; Scriver et al., 1985). The normal adult value under physiologic conditions is 58 ± 15 µmol/liter (mean ± SD); the corresponding values in children (mean age 8 years) and adolescents (mean age 16 years) are 62 ± 18 and 60 ± 13 µmol/liter, respectively. Gender affects the value only in adolescents (male values are higher) (Gregory et al., 1986). Values in newborn and older infants are similar to those in older subjects (Scriver and Rosenberg, 1973).

Determinants of the Steady State

Fluid compartments in the body (e.g., plasma and cerebrospinal fluid) may have quite different concentrations of free phenylalanine, but at the steady state the compartments are in a state of equilibrium, albeit at far from chemical equilibrium (Cohn et al., 1980). [This fundamental feature will lie behind the proposal., for example, to use enzyme substitution therapy with oral phenylalanine ammonia lyase to treat PKU (Sarkissian et al., 1999; see section on treatment).] The flux of free phenylalanine through pools in the human body comprises inputs and runouts (Fig. 77-2), and there are ways to measure net flux rates *in vivo* (Krempf et al., 1990). Input of phenylalanine has two major sources:

exogenous from dietary phenylalanine and *endogenous* from bound (polypeptide) and free amino acid stores, the latter located largely in muscle (Scriver and Rosenberg, 1973). Runout involves incorporation into bound pools, oxidation to tyrosine, and conversion to minor metabolites; net runout by oxidation can be measured *in vivo* by measurement of labeled CO_2 in expired air derived from a tracer dose of labeled L-phenylalanine (Lehmann et al., 1986; Treacy et al., 1997).

Figure 2:



Major inputs (υ) and runouts (τ) 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 (Table 77-1)] 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) transamination (A) and decarboxylation (B). The approximate proportional importance of the three runouts is 3:1:trace at normal steady state (see the discussions in Scriver et al., 1989 and Kaufman and, 1999).

Input

L-Phenylalanine is an essential amino acid in humans, and a (dietary) source is necessary both to maintain phenylalanine homeostasis and to meet requirements for endogenous protein synthesis (American Academy of Pediatrics, 1976; Young and Pellett, 1987) The nutritional requirement for L-phenylalanine is difficult to estimate in the normal subject because catabolic runout perturbs the estimate of the anabolic requirement. Accordingly, the blocked catabolic state (as in a person with totally deficient PAH enzyme activity) is useful to estimate the actual human requirement. Empirical estimates in treated PKU patients indicate that the minimum requirement to support protein synthesis is on the order of 200 to 500 mg/day in the infant and young child (depending in part on the mutant *PAH* genotype) and probably not more than 1.5 times greater in older children. The requirement for phenylalanine is greater in the absence of tyrosine in subjects with intact phenylalanine hydroxylation (American Academy of Pediatrics, 1976; Basile-Filho et al., 1997; Young and Pellett, 1987). Thus the range of phenylalanine requirement in PKU and normal subjects probably reflects interindividual differences in the biologic determinants of homeostasis, as well as allelic differences at the *PAH* locus in the PKU subjects. Estimates of the

requirement in adults (Table 77-1) are probably lower than the true value (Young and Pellett, 1987), and values in adults obtained by kinetic analysis with isotope-labeled amino acid resemble those for young children when expressed per unit of total protein requirement (see Table 77-1). This revisionist view may influence treatment of HPA in affected pregnant woman.

Table 77-1: Estimates of Phenylalanine Requirement in Humans* (American Academy of Pediatrics, 1976; Young, Pellett, 1987)

	mg/day	mg/kg/day	mg/g protein
Infant		25–90	
Preschool child	200–500†	69	63
Older child	200–500	22	22
Young adult		19–14	
Young male adult		39‡	

Estimates made in normal subjects with normal phenylalanine hydroxylase enzyme (PAH) activity and receiving tyrosine in the diet

†Estimates made in patients with severe deficiency of PAH activity (classic phenylketonuria) (American Academy of Pediatrics, 1976).

‡Estimated by oral tracer studies in subjects receiving various phenylalanine intakes without added tyrosine (Basile-Filho et al., 1997).

Turnover of endogenous peptide-bound pools contributes input to the plasma phenylalanine pool value (Basile-Filho et al., 1997; Berke et al., 1992; Sanchez et al., 1996). When nutrition is inadequate, protein catabolism occurs, and free L-phenylalanine is released; as a consequence, plasma phenylalanine values will rise initially in the PKU patient in early negative nitrogen balance. Failure to recognize this phenomenon can confound treatment of PKU patients during intercurrent illness.

Runout

Input of phenylalanine will expand the plasma phenylalanine pool unless there is compensating runout. Incorporation into protein, oxidation, and conversion of phenylalanine to other metabolites (see Fig. 77-2) provide runout on a background of interorgan amino acid flow and cellular uptake. The relative contributions of these components to phenylalanine homeostasis have theoretical interest and practical relevance.

In a series of important theoretical studies (Kacser and Burns, 1973, 1981; Kacser and Porteous, 1987), it was reasoned that each component in a network of steady-state determinants has a value, called the *sensitivity coefficient*, proportional to its importance in setting the summation property (value = 1) of the steady state maintained by the network. Relative quantitative values for the components of phenylalanine runout have been measured (Kaufman 1976; Salter et al., 1986). At physiologic concentrations, incorporation into protein and hydroxylation to tyrosine account for about one-quarter and three-quarters of total free phenylalanine runout, respectively; conversion to phenylpyruvic acid is of minor significance and occurs only at elevated plasma concentrations of phenylalanine (Kaufman, 1976). Conversion to phenylethylamine is a trivial part of the whole. Whereas stress, feeding, and fasting modulate phenylalanine oxidation (Basile-Filho et al., 1997; Berke et al., 1992), on average, about half the control of runout is accounted for by a first step, which is uptake of phenylalanine by cells, notably hepatocytes (Salter et al., 1986); the remainder occurs by events after this step (i.e., hydroxylation, protein incorporation, and metabolite formation). Direct measures in human subjects confirm that hepatic uptake and oxidation of phenylalanine are almost equally important determinants of the steady-state value for plasma phenylalanine in mammals (Sanchez et al., 1996); from the PKU phenotype, we see that the *PAH* gene behaves as the "major" locus in the genetic makeup of this complex trait. On the other hand, since the sensitivity coefficient of the hydroxylation component in the system is considerably less than 1.0 (because the system maintaining phenylalanine homeostasis has many components), it follows that the genetic basis of HPA does not confer a dominant phenotype and must be recessive—and furthermore that the heterozygote, under usual conditions, will not show significant HPA, in keeping with theory (Kacser and Burns, 1981).

Alternative phenylalanine conversion pathways

Conversion to tyrosine is the major metabolic pathway for phenylalanine runout (Kaufman, 1976) (see Fig. 77-2). Conversion of phenylalanine to nontyrosine derivatives constitutes only a minor alternative under normal conditions; more details of the latter reactions are given in previous editions of this text (Knox, 1960, 1966, 1972) and elsewhere (Scriver and Rosenberg, 1973).

The initial reaction in the most significant alternative pathway is transamination of L-phenylalanine to form phenylpyruvate; this and the subsequent metabolic transformations in the transamination pathway (see Fig. 77-2) are restricted to the alanine side chain of the molecule. Only when the major catabolic pathway is blocked and phenylalanine concentration is much increased does the transamination pathway become functionally significant. The reaction is induced by substrate and is not fully operative in the immature newborn or in the early phase of HPA (Scriver and Rosenberg, 1973). Rates of phenylalanine disposal by the alternative pathways do influence the mutant metabolic phenotype in PKU, and siblings with identical mutant *PAH* genotypes can have different rates for disposal of minor metabolites (Treacy et al., 1996).

Decarboxylation of phenylalanine to phenylethylamine (see Fig. 77-2) is not an important route for disposal of excess phenylalanine in humans at any time. Monoamine oxidase inhibitors, which block further metabolism of phenylethylamine, do little to alter its level in the human subject (Rampini et al., 1974).

Interorgan phenylalanine flow and uptake

PAH enzyme is active mainly in hepatocytes in humans, but significant PAH enzyme activity (Lichter-Konecki et al., 1999; Tessari et al., 1999) and metabolic conversion of phenylalanine to tyrosine (Moller et al., 2000) now have been identified in human kidney. As a result, the kidney is a major donor of tyrosine to the systemic circulation in the presence of normal PAH enzyme activity. Phenylalanine is incorporated into protein in all tissues, and metabolic conversion of phenylalanine to various metabolites

also occurs in tissues other than liver. Accordingly, interorgan fluxes are an integral part of runout for this amino acid (Christensen, 1982), and there must be phenylalanine transport across plasma membranes before it can enter its intracellular pathways. L-Phenylalanine uptake by mammalian cells is mediated by carriers that are coupled to the inward-oriented Na^+ gradient in apical membranes of renal (Kragh-Hansen et al., 1984; Samarzija and Fromter, 1982) and intestinal epithelia (Berteloot et al., 1982) and by Na^+-independent carriers in other plasma membranes (cited in Christensen, 1982, 1986, 1987). The nephron, which contains both high- and low-affinity carriers for phenylalanine (Kragh-Hansen et al., 1984), achieves near-total reabsorption of the amino acid from filtrate under physiologic conditions. The systems do not saturate even at high phenylalanine concentrations in filtrate (Owens, 1977; Scriver and Rosenberg, 1973), which means that the transporters continue to function in the homeostatic network and contribute to the HPA of PKU.

Phenylalanine enters parenchymal cells from plasma and extracellular fluid on an Na^+-independent, weakly concentrative carrier that accepts both branched-chain and aromatic amino acids; it exits from cells on a system shared by neutral-charge amino acids (Christensen, 1986, 1987). Hepatic uptake of phenylalanine is a significant component in the runout flux (Salter et al., 1986). Interactions between amino acids on the carriers can perturb these fluxes (Pardridge, 1987; Shershen et al., 1987; Smith, 1987) and may play a role in the pathogenesis of the brain phenotype in PKU.

By way of a synthesis for these themes about phenylalanine homeostasis, Kaufman (1999) proposed a quantitative model for metabolism of phenylalanine at steady state in fasted human subjects that takes into account disposal by the hydroxylation and transamination reaction (decarboxylation being a negligible reaction in this context) and input from net protein degradation. From available empirical data, Kaufman assigned kinetic values: K_m (mM) for hydroxylase and transaminase, 0.54 and 1.37, respectively; the corresponding V_{max} (µmol/ml per hour), 0.9 and 0.063, respectively; and phenylalanine release from protein degradation, 0.012 µmol/ml per hour. Assuming that blood levels reflect tissue levels, Kaufman then tested the validity of his model against data for normal subjects, heterozygotes, and mutant homozygotes with PKU. The model was satisfactory and sufficiently robust to show that HPA could not be explained by putative transaminase deficiency and that alternative pathways for significant phenylalanine disposal (e.g., on tyrosine hydroxylase) do not exist.

THE *PAH* GENE

Comment

More than 98 percent of mutations associated with human HPA occur at the *PAH* locus; the remainder are at the loci dedicated to synthesis and regeneration of tetrahydrobiopterin (see Chap. 78). The *PAH* gene has regulatory elements and an architecture typical of many housekeeping genes. The locus harbors several hundred known alleles, some of which are polymorphic and neutral in their effect on PAH enzyme activity; most are a cause of HPA. The online locus-specific mutation database (www.pahdb.mcgill.ca) is a prototype in the human mutation database initiative taking place at the interface between human genomics and genetics.

The human *PAH* gene

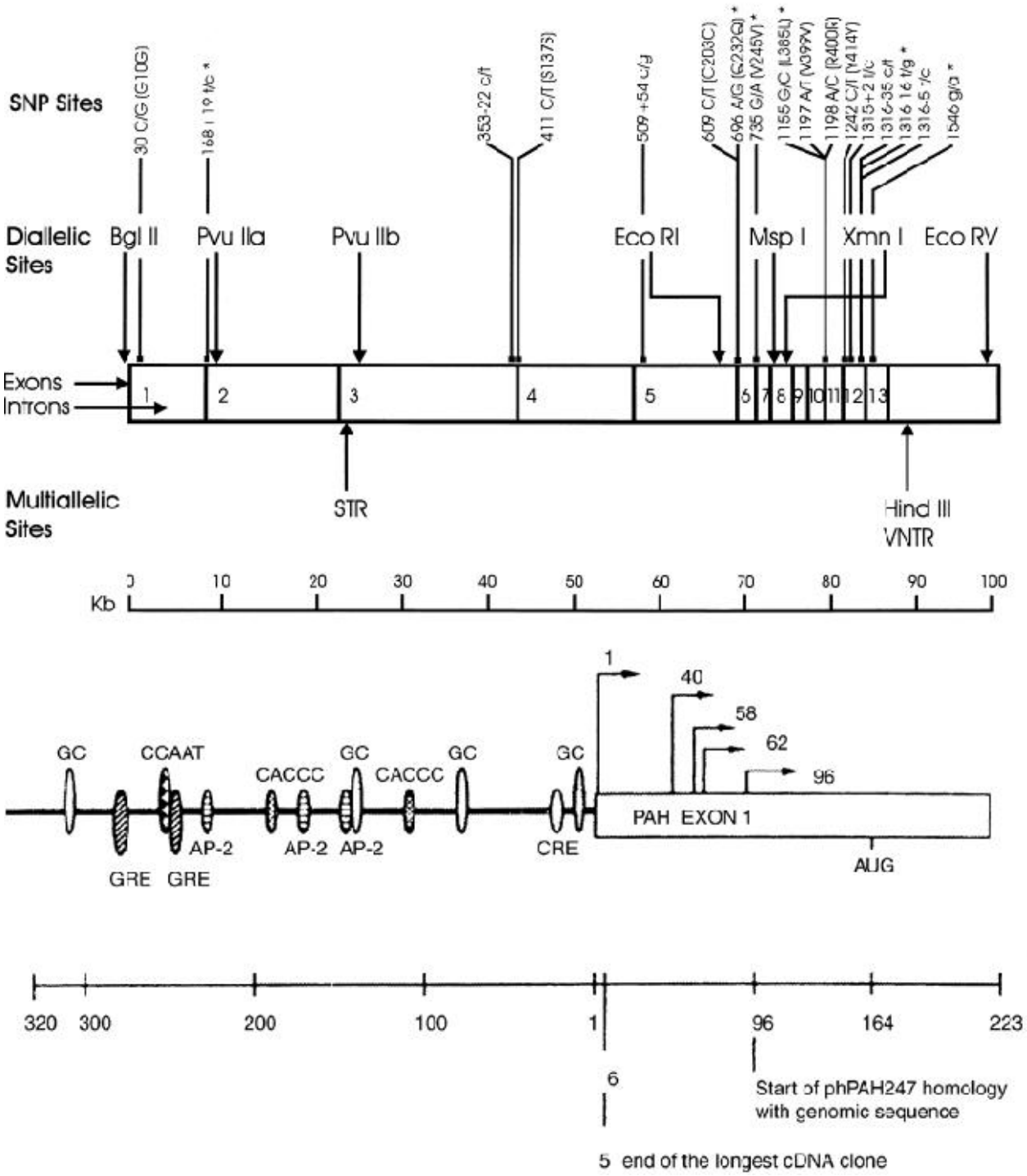
Isolation of a human *PAH* cDNA followed synthesis and authentication of a rat cDNA from liver mRNA purified by polysome immunoprecipitation (Robson et al., 1982, 1984). A human liver cDNA library probed with the rat clone produced a 2.4-kb cDNA clone (*hPAH* 247) encoding a polypeptide of 452 amino acids, of 51,862 relative molecular mass (Kwok et al., 1985). Others obtained cDNA clones from rat (Dahl and Mercer, 1986), mouse (Ledley et al., 1990), and human (Speer et al., 1986) genomes. Rodent and human

sequences, both DNA and protein, are similar, with 92 percent overall polypeptide homology and 96 percent similarity at the C-terminal end (Dahl and Mercer 1986; Kwok et al., 1985; Robson et al., 1984). *In vitro* expression of the human or rat *PAH* cDNA is sufficient to assemble a homopolymeric protein with phenylalanine hydroxylating activity in the presence of pterin cofactor (Choo et al., 1986; Ledley et al., 1985). The human *PAH* locus, mapped by *in situ* hybridization, is on chromosome 12 (Lidsky et al., 1985a), band region 12q23.21 (International Human Genome Sequencing Consortium, 2001), embedded in a 1.5-Mbp region containing five other genes of known or unknown function (Venter et al., 2001).

The finished total sequence for the whole of chromosome 12 (Scherer et al., 2006) harbors 1435 loci in a sequence of 130,683,379 bp of nonoverlapping sequence accommodating approximately 4.5 percent of the human genome. The rate of base substitutions on chromosome 12 in recent evolution is slowing in hominids compared with primates and rodents. Chromosome 12 is rich in disease-associated loci, with 487 loci accounting for 5.2 percent of currently known disease-causing genes.

The cDNA sequence (DiLella et al., 1986a; Konecki et al., 1992; Kwok et al., 1985) (GenBank U49897.1) contains 13 exons that constitute approximately 2.9 percent of the genomic *PAH* sequence. The intronic splice-site nucleotide sequences are all conventional. Exon border types vary; most are type 3 (beginning after the third nucleotide of a codon); codons 118 and 236 introduce type 1 borders spanning introns 3 and 6, respectively; codons 170, 281, and 400 introduce type 2 borders spanning introns 5, 7, and 11, respectively. The structure of the gene is represented to scale in Fig. 77-3.

Figure 3:



Basic structure of the human phenylalanine hydroxylase (PAH) gene. The locus covers approximately 100 kb of genomic DNA on chromosome 12p. The cDNA sequence is deposited at GenBank (NCBI) under U49897-1; the genomic sequence is under AF404777. The top shows to scale the relative sizes and

positions of exons and introns in PAH genomic sequence, and positions of polymorphic sites (multiallelic sites are shown below the gene). Biallelic restriction fragment length polymorphism sites and single-nucleotide polymorphic (SNP) sites are placed above it. SNPs are identified by their systematic (nucleotide) names, as recommended (Antonarakis et al., 2001). The other figure depicts the 5′ end of the PAH gene. (Reproduced from Konecki et al., 1992 with permission of the American Chemical Society.)

A genomic sequence of the *PAH* gene (Konecki, personal communication documented in www.pahdb.mcgill.ca, November 2001) and its flanking regions spanning 171,266 bp has approximately 027 kbp of 5' untranslated region (5'UTR) upstream from the transcription initiation site and approximately 64.5 kbp of 3' sequence downstream from the poly(A) site in the last exon (exon 13). The gDNA nucleotide numbers are in register with the cDNA sequence (which has long served *PAH* mutation nomenclature) because the *PAH* gDNA has been numbered in *PAHdb* so that the +1 nucleotide is the adenine of the transcription initiation site (ATG) in exon 1. Thus gDNA exons, introns, and the 3'UTR have positive numbers; the 5'UTR has negative numbers (Scriver et al., 2003). Single-nucleotide polymorphic sites (SNPs) and restriction fragment length polymorphism sites (RFLPs), currently used to create *PAH* polymorphic haplotypes, are annotated on the genomic sequence. Other sites of interest at the *PAH* locus can be identified by using tools such as the NEBcutter (www.neb.com).

Exonic sequences in the human *PAH* gene take up less than 3 percent of the genomic sequence between the 5' +1 position down to the 3' poly(A) tract. Amplicon primer sequences for all 13 exons are available (www.pahdb.mcgill.ca). The shortest and longest exons are 57 bp (exon 9) and 892 bp (exon 13), respectively; the mean exon size is 170 bp. Three polyadenylation signals [AATAAA] in exon 13 are annotated on the gDNA sequence; the third site is used most frequently. The shortest and longest introns are 556 bp (intron 10) and 17,874 bp (intron 2), respectively, whereas intron 3 is 17,187 bp in length, and the mean intron size is 6390 bp. These are typical mammalian gene dimensions.

The *PAH* genomic sequence consists of 40.7 percent GC, slightly above the modal value (37 to 38 percent) for human genes. RepeatMasker analysis shows the density of interspersed repeats to be 42.2 percent in the *PAH* gene, a typical value for a mammalian gene. *Alu* repeat elements are annotated on the gDNA sequence. Intron 2 contains an *Alu*-like repeat element, between bp 17,273 and bp 17,546, that might account for a 5' deletion causing PKU (Sullivan et al., 1985). Putative *Alu* repeats and CpG dinucleotide sites (n = 1198) are annotated on the *PAH* gene sequence (Scriver et al., 2003).

The 5' untranslated region of the gene (see Fig. 77-3) has five potential cap sites upstream from the actual methionine translation initiation codon in exon 1 (Konecki et al., 1992); multiple cap sites are a feature of many housekeeping genes within a 0.5-kb region upstream from the first codon. The *PAH* gene lacks a proximal TATA box but has several elements (see Fig. 77-3), including four GC-rich domains as putative Sp1-binding sites (another housekeeping feature), a CCAAT sequence (a target for factors regulating efficiency of transcription), GRE and CACCC sites (elements involved in regulation mediated by glucocorticoids), several activator protein 2 (Ap2)–binding sites, and one partial cAMP response element (CRE). The 5' region of human *PAH*, 3.5 kb upstream and beyond the 0.5-kb region described earlier, contains a sequence very similar to the mouse liver–specific hormone-inducible *PAH* gene enhancer; this region binds the hepatic nuclear factor 1 (HNF1) (Lei and Kaufman, 1998a); dose-dependent HNF1-mediated transactivation of gene expression is further potentiated by the dimerization cofactor of HNF1 (DcoH). Thus DcoH, which is identical to the phenylalanine hydroxylase simulator (PHS protein), is also a 4α-carbinolamine dehydratase that participates both in regeneration of tetrahydrobiopterin cofactor and in the regulation of *PAH* gene expression. A 5' *PAH* construct fused to a *CAT* reporter gene and expressed in transgenic mice showed that expression of the human *PAH* gene is specific both for tissue and for stage of development (Wang et al., 1992a). The 5' flanking regions of rat (McDowell and Fisher,

1995; Rees et al., 2001) and mouse (Faust et al., 1996) genes encoding phenylalanine hydroxylase have features and elements very similar to those of the human gene.

PAH has a rich repertoire of RFLPs and polymorphic markers (see Fig. 77-3) for analysis of the gene (Kwok et al. 1985), from which a large number of informative haplotypes are derived (DiLella et al., 1986a).

Evolutionary aspects

Aromatic amino acid hydroxylase genes exist in early complex organisms. A gene encoding phenylalanine hydroxylase is expressed in hypodermal cells of the nematode *C. elegans*, where it may play a role in cuticle formation (Loer et al., 1999). The *Drosophila melanogaster* genome harbors a phenylalanine hydroxylase locus (*PAH*) for which structure has been ascertained from the cDNA (Ruiz-Vazquez et al., 1996); the intron positions and the C-terminal domain are conserved with the mammalian gene counterpart. The fly gene may encode both tryptophan and phenylalanine hydroxylase activities, and it has an alternative promoter and transcript.

PAH is in a mammalian gene family (Eisensmith and Woo, 1995; Hufton et al., 1995) of aromatic L-amino hydroxylases that includes L-tyrosine 3-monooxygenase (gene symbol *TYH*; EC 1.14.16.2) (Fauquet et al., 1988) and L-tryptophan 5-monooxygenase (gene symbol *TPH*; EC 1.14.16.4) (Grenett et al., 1987); the latter are tetrahydrobiopterin-requiring apoenzymes catalyzing rate-limiting steps in pathways leading to the synthesis of neurotransmitters (see Chap. 78). Primary structures of the polypeptide products have remained similar throughout biological evolution (Dahl and Mercer, 1986; Erlandsen et al., 1997b; Grenett et al., 1987) with conserved intron/exon boundaries (Stoll and Goldman 1991); *PAH*, *TPH*, and *TYH* are likely to have evolved over the last 75 million years from a common ancestral entity by duplication and divergence (Grenett et al., 1987; Neckameyer and White, 1992), with *PAH* and *TPH* being more closely related to each other than to *TYH*.

The three hydroxylases share several features of their domain structures (Dahl and Mercer, 1986; Erlandsen et al., 1997b; Grenett et al., 1987). C-terminal regions are more conserved, and N-terminal regions are more divergent. The former region contains the determinants for hydroxylating activity (Iwaki et al., 1986) and BH_4 binding (Jennings and Cotton 1990; Jennings et al., 1991), whereas the latter contains the determinants for substrate specificity (Iwaki et al., 1986) and phosphorylation-mediated activation (Campbell et al., 1986; Pigeon et al., 1987). The genes have significant differences: Genomic *TYH* (smaller than 10 kb) (Kobayashi et al., 1988) is small relative to *PAH; TPH* is probably of intermediate size if one extrapolates from the corresponding mouse data (Stoll and Goldman, 1991). Human *TYH* transcribes several species of mRNA (Nagatsu, 1991), but this is not so with *PAH* and *TPH*.

The *PAH* Mutation "Knowledgebase" (*PAHdb*: www.pahdb.mcgill.ca)

Databases are a legacy in and of science, yet they have often been neglected by the communities they serve (Maurer et al., 2000; Scriver et al., 2003). Science is an explanatory process, and as a particular domain of it develops, there is first a stage of explanation in classification and nomenclature (taxonomy) of its entities; next, one of enquiry into mechanisms underlying the entities; finally, a stage where the entities are revealed (Keller, 2002). The science of genetics recognizes mutation as both entity and mechanism; mutation also can be created. Thus mutation databases, in their various ways, recapitulate the science and recreate it *in silico*. Accordingly, mutation databases have become resources in genetics as repositories of the vast wealth of data about individual genes and the genomes they inhabit. *PAHdb* is one such legacy and resource.

PAHdb (www.pahdb.mcgill.ca) is an online relational locus-specific "knowledgebase" (Scriver et al., 2000, 2003; Scriver 2007) originating in the *PAH* Mutation Analysis Consortium, serving it and other communities. *PAHdb* has emerged as a comprehensive and useful prototype of the locus-specific type of genetic database (Claustres et al., 2002).

The origins, development, and design of *PAHdb* have been described in detail (Scriver et al., 2000, 2003; Scriver 2007). The database is built on four core elements: (1) a *unique identifier* for each allele, (2) the *source* of the information, (3) the *context* of the allele (e.g., the species and name of the gene), and (4) the *name* of the allele. *PAHdb* contains *entities* (mutations) and annotates them with *attributes*. The Tables of Mutations arise from these core elements. Tables of *in vitro* expression data describe the functional effects of mutations on PAH enzyme integrity and function. The database provides a link to a site at the Scripps Institute (http://stevens.scripps.edu/) to visualize *in silico* how mutations map onto the 3D protein structure. A Curators' Page highlights unscheduled topics (e.g., discovery of BH_4-responsive *PAH* alleles) and novel data not readily handled by the existing tables. Among other options, *PAHdb* introduces visitors to the mouse *Pah* gene and a mouse model of PKU. The clinical significance of human *PAH* mutations is the subject of another module. A counter logs visits and records the last date of curation. A partial site map of *PAHdb* is depicted elsewhere (Scriver et al., 2003). The intellectual property of *PAHdb* is copyrighted.

PAHdb has served in the development of two new types of mutation databases: FINDbase (van Baal et al., 2007) and PhenCode (Giardine et al., 2007). FINDbase (www.FINDbase.org) is a relational database giving information about relative frequencies of pathogenic mutations in various genes in particular populations with ethnic affiliations in geographic regions. FINDbase has links with locus-specific databases (LSDBs), including *PAHdb*. PhenCode (phenotypes for ENCODE; www.bx.psu.edu/phencode) is a collaborative project exploring variant phenotypes associated with human mutations and relating sequence and functional data obtained from genome projects. The PhenCode project uses LSDBs, including *PAHdb*, to connect human phenotype data with genomic sequences, evolutionary history and function from the ENCODE project. The latter provided unexpected relevance to the contents of this chapter, as described in an accompanying article (Rhesus Macacque Genome Sequencing and Analysis Consortium, 2007). It showed that an allele in the human genome associated with HPA is not associated with HPA in the macacque—a fascinating observation.

Because *PAH* alleles are named according to conventions now widely accepted and used (Antonarakis et al., 2001), the *PAHdb* can be searched easily or, by using appropriate tools, used to retrieve and transfer its alleles to an experimental "WayStation" that will be linked to a comprehensive data warehouse in the making of a repository of all human genomic allelic variation (Teebi et al., 2001). The *PAHdb* is a participant in the Human Variome Project (Anon, 2007; Cotton et al.. 2007). The *PAHdb* is contributing to the development of "a database of LSDBs" serving as a hub in a network of LSDBs around the world (Horaitis et al., 2007).

The *PAH* Gene: Allelic Variation (Mutations)

In this chapter, the word *mutation* means allelic variation in the nucleotide sequence of the *PAH* gene (Cooper and Krawczak, 1993). *PAH* mutations are ascertained most frequently, but not exclusively, in persons with persistent postnatal HPA attributable to phenylalanine hydroxylase dysfunction; this constitutes a bias of ascertainment. The mutations are of two types: (1) polymorphic and neutral in their effect on phenotype or (2) pathogenic through impairment of PAH enzyme function. Alleles are called *polymorphic* when their frequency is 0.01 or greater in the population; whether every "polymorphic" allele is truly neutral and without a phenotypic effect cannot be stated without other considerations (see

Aulehla-Scholz and Heilbronner, 2003). Alleles are considered likely to be disease-causing (*pathogenic*) (Cotton and Scriver, 1998) when they segregate in affected persons, are inherited codominantly, are unambiguously null (e.g., frameshift, protein truncation, splice defective, or large deletion), or are missense and affect a conserved residue—which is the case for over half of missense *PAH* alleles (Eisensmith and Woo, 1995)—or the mutation is known to impair PAH enzyme function in an *in vitro* expression system (Waters, 2003; Waters et al., 1998).

Polymorphic Alleles

The *PAH* cDNA sequence contains recognized "polymorphisms" (Fig. 77-3), with the certainty that more will be recognized in the *PAH* gene now that the full genome sequence is known. Three forms of *PAH* polymorphism exist:

1. *Biallelic restriction fragment length polymorphisms* (RFLPs) (DiLella et al., 1986a; Lidsky et al., 1985b) named from the corresponding restriction enzyme (*Bgl*II, *Pvu*IIa, *Pvu*IIb, *Eco*RI, *Msp*I, *Xmn*I, and *Eco*RV). With the exception of the *Eco*R sites, which still require analysis by Southern blotting, the RFLPs can be analyzed by methods based on polymerase chain reaction (PCR) amplification [*Bgl*II (Dworniczak et al., 1991b), *Pvu*IIa (Dworniczak et al., 1991a), *Pvu*IIb (RC Eisensmith, unpublished), *Msp*I (Wedemeyer et al., 1991), and *Xmn*I (Goltsov et al., 1992a)].
2. *Multiallelic polymorphisms*, which include a hypervariable sequence [variable number of tandem repeats (VNTRs)] of 30-bp cassettes harboring at least 10 alleles (differing by number of repeats) in a *Hind*III fragment 3 kb downstream from the last exon in *PAH* (Goltsov et al., 1992b; Latorra et al., 1994), and a series of short tandem [tetranucleotide $(TCTA)_n$] repeats (STRs) harboring at least 9 alleles in the third intron of *PAH* (Giannattasio et al., 1997; Goltsov et al., 1993; Zschocke et al., 1994a).
3. *Single-nucleotide polymorphisms* (SNPs), which are silent (non-RFLP) alleles; for example, c.1546g/a, which occurs at about 0.20 frequency in the 3' UTR of *PAH* on both mutant and normal chromosomes (Ramus and Cotton, 1995), and a silent c.696A/G polymorphism (q = 0.08–0.63) in codon 232 (Q232Q) (Lichter-Konecki et al., 1994).

Population Genetics

RFLP, STR, and VNTR alleles can be combined to generate core haplotypes at the extended *PAH* locus. An informative *minihaplotype* consisting of only the STR, *Xmn*I, and VNTR alleles and accessible to PCR-based analysis has been developed (Eisensmith et al., 1994), as have other approaches (Zschocke et al., 1995). The extended (*full*) *PAH* haplotypes are named with Arabic numbers (Eisensmith and Woo, 1992), and at least 87 are known (see *PAHdb*). A matrix (Fig. 77-4) summarizes *PAH* haplotype configurations derived from seven biallelic and two multiallelic sites in a population of European descent; the variety of configurations would be vastly increased if SNPs were included. Another system of haplotype analysis with yet more potential to classify haplotype disease mutation associations has been developed (Zschocke and Hoffmann, 1999).

Figure 4:

Haplotype	BglII	PvuIIa			S	T	R				PvuIIb	EcoRI	MspI	XmnI			V	N	T	R			EcoRV	N
			228	232	236	240	244	248	252	256					3	7		8	9	11	12	13		
10	–	+				o					–	+	+	–				o					–	2
1, 24, 25	–	+		o	o	o	o	o	o		–	–	+	–	o	o	o	o		o	o		–	63
2, 12, 27	–	+		o	o	o	o	o			–	–	+	–	o	o	o	o		o	o	o	+	28
8, 41	–	+				o	o	o	o		–	+	+	–	o	o			o				+	5
3, 31	–	+		o		o					–	+	–	+	o	o	o						–	8
4, 19	–	+		o	o	o	o				–	+	–	+	o		o						+	30
28	–	+		o	o	o	o				–	–	–	+	o								+	6
16	–	+			o	o	o				–	+	–	–	o								+	4
36	+	–				o	o				+	–	+	–		o			o				–	2
novel	+	–						o	o		+	–	+	–	o						o		+	3
5, 21	+	–				o	o	o	o	o	+	+	+	–					o	o			+	17
18	+	–			o						+	+	–	+	o								+	1
7	+	–	o	o	o	o					–	+	–	+				o					–	13
40	+	–	o	o							–	–	–	+				o					–	4
30	+	+	o								–	–	–	+				o					–	1
9	+	+					o				–	+	+	–				o					+	1
11, 69	+	–				o	o			o	–	+	+	–	o			o	o				+	4
34	+	–				o					–	+	+	–				o					–	1
32	+	–	o					o			–	–	+	–				o					–	3
26	+	+									–	–	+	–				o					–	1

Extended polymorphic haplotypes in the human phenylalanine hydroxylase (PAH) gene are derived from seven biallelic restriction fragment length polymorphisms and two multiallelic sites (STR, short tandem repeat; and VNTR, variable number of tandem repeats) (see Fig. 77-3 for their relative positions in the gene). Most, but not all, known haplotypes are summarized in this matrix. Fragment sizes (in base pairs) of the STR alleles are corrected for size (Zschocke et al., 1994a); they are 2 bp shorter than as described in the original report (Goltsov et al., 1992). The numbers in the right-hand column illustrate a typical frequency distribution of haplotypes in a human population [in this case, French Canadians from eastern Quebec (Byck et al., 1996)]. (Figure designed by Mary Fujiwara.)

PAH haplotypes could be generated from combinations of RFLP, STR, and VNTR alleles (Eisensmith and Woo, 1995), but far fewer have actually been observed on human chromosomes; an illustration is shown in Fig. 77-4. As stated for the general case (Eisensmith and Woo, 1995), only a few haplotypes are prevalent, and most are uncommon, and this is typical of all human populations analyzed up to the present. The apparent shortage of PAH haplotypes is explained by linkage disequilibrium across the 100-kb region of the extended haplotype (Chakraborty et al., 1987; Degioanni and Darlu, 1994; Kidd et al., 2000). PAH haplotype heterogeneity is greater on mutant and normal chromosomes in Europeans (Daiger et al., 1989a) than it is on chromosomes in Asians (Daiger et al., 1989b). PAH haplotype diversity is greater in African populations than it is in Europeans, assuming that the latter are descendant of a small founding group emerging out of Africa some 100,000 years ago (Kidd et al., 2000).

Particular PAH haplotypes tend to harbor the most prevalent disease-causing mutations in European populations (Eisensmith and Woo, 1995; Scriver et al., 1993, 1996a); for example, haplotype 7 is usually associated with the prevalent PKU-causing mutation G272X in Norway, haplotype 2 with R408W in eastern Europe, haplotype 1 with R408W on the northwestern fringes of Europe, haplotype 3 with IVS12nt1 in northern Europe, haplotype 9 with I65T in western Europe and the Iberian Peninsula, and haplotype 6 with IVS10nt-11 in Anatolia, southeastern Europe, and the Mediterranean. At the level of family rather than population, codominant segregation of polymorphic *PAH* haplotypes, in association with the known mutant genotype, is compatible with carrier detection and prenatal diagnosis (Eisensmith et al., 1994; Romano et al., 1994; Woo et al., 1983).

Polymorphic haplotypes at the PAH locus serve the study of human evolution and the histories of human populations (Kidd et al., 2000) (see Supplement to this chapter by Kidd and Kidd discussing the population genetics of PAH). Divergence between African, European, and Asiatic populations, with support for the "out of Africa" hypothesis, has been documented by PAH polymorphic haplotype analysis (Degioanni and Darlu, 1994; Kidd et al., 2000). The ancestral haplotypes on which some modern configurations arose can be postulated (Lichter-Konecki et al., 1994); the origins, by geographic region and population, of a particular allele can be surmised (Cali et al., 1997; Ramus and Cotton, 1995). The particular genetic structure (at the PAH locus) of a population can be recognized in its configuration of haplotypes and used to unravel localized demographic histories (Byck et al., 1996; Scriver et al., 1996b); haplotypes also can serve as migration traces over large geographic regions and time frames (Bender et al., 1994) (see the section "Population Genetics" below).

Pathogenic Alleles

When the allele is distinct by state from all others, *PAHdb* assigns unique identifiers to each new *PAH* mutation (Scriver et al., 2000). Over 500 putative pathogenic *PAH* alleles are documented in the database (see map at www.pahdb.mcgill.ca; click on Site Map; then on Mutation Map, which permits a detailed search for mutations). The vast majority of alleles are known (or presumed to be) causes of PKU or non-PKU HPA, having been ascertained through patients with a HPA phenotype. The disease-causing mutations fall into five classes: missense, 63 percent of all alleles; small deletions (<22 bp), 13 percent; modifiers of mRNA splicing, 12.8 percent; termination nonsense alleles, 5.4 percent; and small insertions, 1 percent; large deletions may account for approximately 3 percent of the pathogenic *PAH* mutations (Kozak et al., 2006).

PAH mutations follow two patterns (Scriver et al., 1996b), as noticed for many human genes (Weiss, 1996): (1) Only a few alleles, usually only five in the case of *PAH*, account for the majority of all the disease-causing mutations found in the population, whereas the remainder are rare. (2) The distribution of alleles, by geographic region or population, is usually nonrandom, so the history of a particular allele often corresponds with the history of the population in which it is found (Scriver, 1993; Scriver et al., 1996b).

POPULATION GENETICS OF PHENYLKETONURIA AND PATHOGENIC *PAH* ALLELES

Comment

Our motivation for describing, at considerable length, the evidence for population genetic variation at the human *PAH* locus is as follows. *First*, PKU is a harmful Mendelian trait in the homozygous state, yet incidence data show that allele frequencies can reach or exceed 0.01 in some extisting populations. If so, what is the mechanism to explain a polymorphic frequency for this harmful allele? *Second*, because newborn screening for PKU is so widely practiced, there is a unique opportunity to detect all persons with a rare variant phenotype and to identify all DNA allele(s) associated with the phenotype. Thus the history

of particular alleles will be found in the histories of the populations that contain them. *Third*, these inquiries and their findings help us to apply the relevant knowledge to individuals, families, and communities and to prevent harmful consequences when PKU-causing alleles segregate in populations and are inherited by individuals.

Relevant Characteristics of the *PAH* Locus

The locus is rich in allelic variation (see the section "The *PAH* Gene" above) and polymorphic both in its biallelic and multiallelic forms (see Fig. 77-3 and Fig. 77-4) and in a large number of so-called rare disease-causing alleles (see map at www.pahdb.mcgill.ca; click on Site Map; then on Mutation Map, which permits a detailed search for mutations). This autosomal locus behaves as a single 100-kb block of DNA in *Homo sapiens*, and having been sampled many thousand times in different persons on planet Earth, in its own small way it complements the information gained from the analysis of haploid, mitochondrial., and Y-chromosomal DNA and from protein polymorphisms (Cavalli-Sforza, 1998). From the classic studies, a mosaic human genetic geography has emerged to reflect demic expansion and human evolution during the past 100,000 years (Cavalli-Sforza et al., 1994; Cavalli-Sforza and Piazza, 1993; Mountain and Cavalli-Sforza, 1997). In their turn, *PAH* alleles can be viewed as a unique set of biologic memories connecting individuals, families, and communities who share the contingent histories that are echoes of the past. The historic and social accidents of migration, genetic drift, gene flow, assortative mating (endogamy, inbreeding), and recurrent mutation, alone or together, with or without selection by heterozygote advantage, have contributed to the particular frequencies and distributions of *PAH* alleles seen in contemporary human populations (Scriver et al., 1996b).

Polymorphic *PAH* haplotypes serve to analyze modern human evolution during the past 100 to 200 millennia (Degioanni and Darlu, 1994; Kidd et al., 2000). They also reveal particular associations between the haplotype and a pathogenic allele (Chakraborty et al., 1987; Eisensmith and Woo, 1995; Lichter-Konecki et al., 1994). The following text focuses on the rare pathogenic alleles as causes of HPA and what they offer to the ongoing study of human population genetics (see Chap. 11).

Hyperphenylalaninemia: A Special Opportunity in Human Population Genetics

The HPAs offer both advantages and limitations for the study of mechanisms by which pathogenic alleles achieve their particular frequencies and distributions in human populations.

Advantages

1. Frequencies and classifications of HPA can be systematically documented through newborn screening programs (Table 77-2).
2. The molecular basis of *PAH* allelic diversity can be determined by DNA analysis and codified (Antonarakis et al., 2001).
3. There are associations between the prevalent disease-causing mutations and their polymorphic background haplotypes.
4. Alleles and their associations with haplotypes, populations, and phenotypes are documented systematically in a dedicated relational online database (www.pahdb.mcgill.ca) (Scriver et al., 2003).

Table 77-2: Incidence of Hyperphenylalaninemia Phenotypes by Political or Population Associations: Examples

Phenotype	Association	Incidence(Cases/Million Births)	References*
Phenylketonuria (PKU)	In Africans living outside Africa	10	Hardelid et al., 2007
	In "Oriental" populations	Incomplete data	Okano and Isshiki, 1995
	China	60	Chen et al., 1989; Liu and Zuo, 1986; Wang et al., 1991a
	China	100	Gu and Wang, 2004; Song et al., 2005
	Korea	100	Lee et al., 2004
	Japan	8	Aoki, 1991
	In "European" populations	50–200	Thalhammer, 1975; Zschocke and, 2003
	Turkey	385	Ozalp et al., 1986
	Yemenite Jews (in Israel)	190	Avigad et al., 1990
	Scotland	190	
	Czechoslovakia	150	
	Hungary	90	
	Denmark	85	
	France	75	
	Norway	70	
	United Kingdom	70	
	Italy	60	
	Finland	<5†	
	Canada	45‡	Laberge et al., 1987
	In "Arabic" populations	Up to 165	Teebi and Farag, 1997§

Non-PKU Hyperphenylalaninemia	In "Oriental" Populations		
	Japan	4	Aoki, 1991; Okano and Isshiki,1995
	In "Europeans" (except Finland)	15–75	Thalhammer, 1975; Zschocke and, 2003
	In "Arabic" populations	"Low"	Teebi and, Farag, 1997§

Newborn screening is the direct source for incidence data. Incidence also has been estimated indirectly from consanguinity rates in Norway (Saugstad, 1975) and Italy (Romeo et al., 1983)

†Data also were taken from Woolf LI, Lentner C: Geigy Scientific Tables, 8th ed. Basle, Geigy, 1986.

‡Data are the average of eight provincial screening programs in Canada with an annual cohort of ~400,000 births.

§Arabic populations (including Bedouins) in Egypt, Jordan, Iraq, Kuwait, Lebanon, the Maghred (North Africa), and Palestine/Israel and Sudan. The data also cover Jewish populations in Arab nations, including Morocco, Tunisia, and Yemen (Teebi and Farag, 1997).

Limitations

1. Alleles are identified mainly through affected propositi, rarely otherwise, thus introducing a bias of sampling.
2. Populations are sampled mainly through screening programs that are not operative in all human societies; again, there is a bias of sampling.
3. Up to the present, mutation analysis has been feasible across only 3 percent of the whole *PAH* gene.
4. Analysis of population-specific mutant chromosomes is rarely 100 percent efficient (although it often exceeds 95 percent); allele frequencies are relative, rarely absolute, for the selected sample, and they are not true estimates of population frequencies.
5. Parental alleles are not uniformly analyzed (and reported), and *de novo* mutations are likely to be underestimated.

Implications in Nonuniform Incidence Rates

The following thought appears in the Preface to the Supplement on Population Genetics of *PAH* (attached to this chapter), coauthored by Kidd and Kidd (2005): "When *clinical geneticists* think about phenylalanine hydroxylase (PAH), they think about mutations in the gene causing phenylketonuria (PKU) and the other metabolic diseases of phenylalanine hydroxylase deficiency, their diagnosis, and their treatment. When *biochemical geneticists* think about PAH, they think about metabolic pathways, enzyme activity levels, and metabolic substrates and products. When *population geneticists* think about PAH, they think about allele frequencies of disease-causing mutations and normal polymorphisms in different populations, the patterns of these variations in the populations, and what these frequencies and patterns mean in terms of disease, gene, and—more broadly—human evolution" (italics added for emphasis).

Lionel Penrose had already reflected on the rather elevated frequency of this harmful autosomal recessive disease among Europeans (Penrose, 1998). He offered several explanations, including consanguinity, hypermutability, and heterozygous selective advantage. Nor did the nonuniform distribution of PKU cases in European populations escape his notice. It was never a dry season in Penrose's fertile mind.

Newborn screening provides data to corroborate Penrose's view of prevalence rates and allele frequencies. Prevalence rates reveal an aggregate frequency for pathogenic alleles (both PKU and non-PKU HPA) in the *polymorphic* range in certain geographic regions and populations in Europe (Zschocke, 2003). New data derived from newborn screening programs (Gu and Wang, 2004; Song et al., 2005; Lee et al., 2004) reveal prevalence rates in Chinese and Korean populations to be equivalent to those in European populations. Internal studies in the European populations, for example, show stratification by population or geographic region for PKU (see Table 77-2); the evidence for stratification is less prominent for non-PKU HPA.

There is a possibility that inbreeding will explain the high incidence of PKU in Turkey (Ozalp et al., 1986; Ozgüç et al., 1993; Woolf, 1994); the hypothesis has been confirmed by formal analysis in the Pakistani community of the West Midlands in the United Kingdom (Hutchesson et al., 1998) and in some Arabic countries (Teebi and Farag, 1997). Consanguinity does not explain the incidence of PKU in either Norway (Saugstad, 1975) or Italy (Romeo et al., 1983). The Italian study showed further, even before the human *PAH* gene had been cloned, that PKU is the result of mutation at only one locus and that locus heterogeneity need not be taken into account to explain the incidence of PKU—an interpretation that has proved to be realistic because only approximately 2 percent of cases of HPA are not explained by mutations at the *PAH* locus (see Chap. 78). The origins of deviant prevalence rates of PKU in human populations are still open to inquiry.

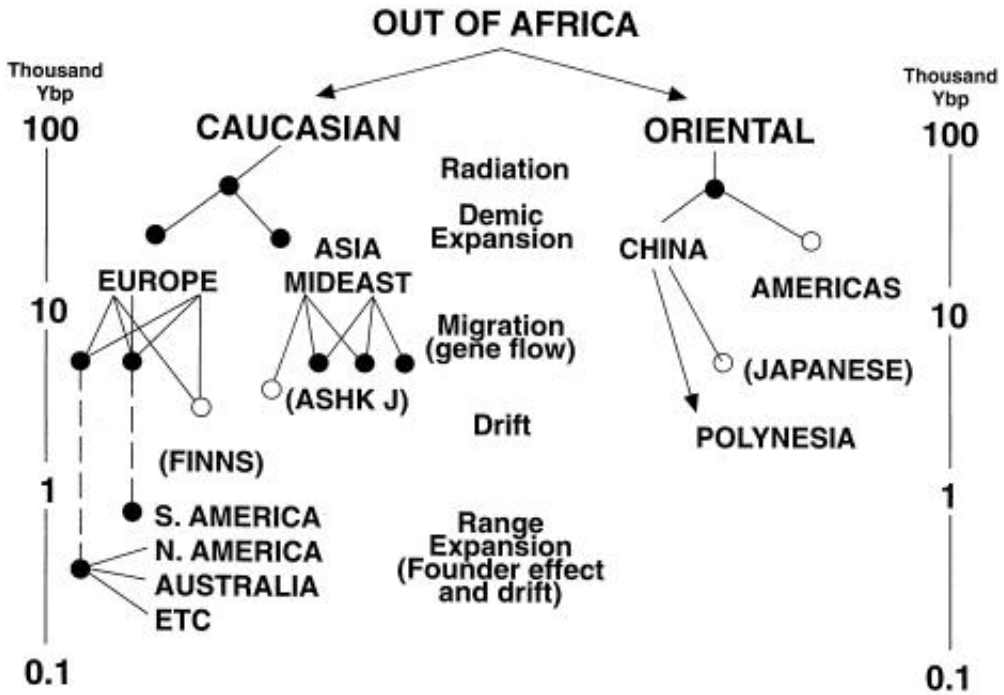
Implications in Nonuniform Allelic Distributions

The *PAH* locus of *Homo sapiens* has accumulated an impressive array of alleles, both disease-causing and neutral polymorphic, each different by state and identity (see Fig. 77-3 and www.pahdb.mcgill.ca), during demic expansion over the past 100,000 years (Fig. 77-5) [see also Chapter 77 Supplement "The Population Genetics of *PAH*" (Kidd, Kidd, 2005)]. Among the disease-causing *PAH* mutations, only a few (between four and six in most populations) make up the majority of the total at the locus (Table 77-3), and this pattern is emerging as the norm for most pathogenic alleles in different human genes (Weiss, 1996; Weiss and Buchanan, 2003).

Table 77-3: Different Sets of the Most Prevalent PKU-Causing *PAH* Alleles Occur in European and Oriental Populations

Rank Order	European (*n* = 3630)*		Oriental (*n* = 210)*	
	Allele	*%*	*Allele*	*%*
1	R408W†	31	R243Q	13
2	IVS12nt1	11	R413P	13
3	IVS10nt-11	10	c.611AG	13
4	I65T	5	IVS4-1	7
5	Y414C	5	R111X	7
	Cumulative	62		53

Figure 5:



Human history hypothesized from the viewpoint of allelic diversity at the human phenylalanine hydroxylase (PAH) gene locus. Following an "out of Africa" migration and divergence, different sets of phenylketonuria-causing alleles arose in Europeans (Caucasians) and Asian Orientals and were acted on by genetic drift. Founder effect is the likely explanation for relative rarity of PKU alleles in American aboriginals, Japanese, Ashkenazi, and Finnish populations (also in Polynesians). Demic expansion, migrations, and gene flow disrupt "trees of descent" in pre- and post-Neolithic eras (10,000 ybp). Range expansion and creation of neo-European populations overseas (from 1000 ybp) explain the "overseas"

distributions from European sources of certain PAH alleles.

Denominator (independent chromosomes) for calculation of relative frequencies (percent) of alleles

†R408W on haplotypes 1.8 and 2.3 combined

From Eisensmith and Woo, 1995;www.pahdb.mcgill.ca.

Distribution of disease-causing *PAH* mutations in populations with origins in different geographic regions is not uniform. Such alleles are almost invisible in sub-Saharan Africans (see Beighton, p. 90, in Scriver et al., 1996b), a finding supported by an epidemiologic study (Hardelid et al., 2007) and by a small survey of mentally retarded subjects living in Africa (Familusi and Bolodeoku, 1976). Although sampling of PKU alleles in Africans living in Africa is extremely limited in the absence of newborn screening in populations on the continent, there is supporting evidence that the prevalence rate of PKU in African descendants living outside Africa is indeed lower by an order of magnitude than their corresponding cohorts of European descent (Eisensmith et al., 1996; Epps, 1968; Gjetting et al., 2001; Graw and Koch, 1967; Guldberg et al., 1996a; Hardelid et al., 2007; Knox, 1972; Hofman et al., 1991; Tyfield et al., 1997). The apparent discrepancy between prevalence rates in populations rooted in Africa and in temperate zones suggests that the majority of disease-causing *PAH* mutations in Europeans and Orientals may not have originated in Africa but appeared later in association with selective advantage in the heterozygote following the out-of-africa diaspora (Hardelid et al., 2007).

Current evidence based on sampling in European and Oriental populations (see www.pahdb.mcgill.ca/ and Gu et al., 1995; Gu and Wang, 2004; Lee et al., 2004; Okano et al., 1992; Okano and Isshiki, 1995; Li et al., 1994; Song et al., 2005; Sun et al., 1997; Wang et al., 1989, 1992b) shows (1) that incidence of PKU is similar among Orientals (in mainland Asia) and Europeans (see Table 77-2) but much lower in the Japanese (Okano et al., 1992; Okano and Isshiki, 1995), (2) that the alleles among Orientals as a group are quite different from those in Europeans (see Table 77-3), and (3) that the alleles are different again between Japanese and other Orientals (Okano et al., 1992, 1994; Okano and Isshiki, 1995; Goebel-Schreiner and Schreiner, 1993). The Japanese data are compatible with genetic drift in the founding of this island population.

PKU is no longer seen as a rare trait in Arabic populations in the Middle East (Teebi and Farag, 1997); the mutations are often particular to Arabic chromosomes (Hashem et al., 1996; Kleiman et al., 1992a; Kleiman et al., 1992b, 1993). In Kuwait, prevalence rates are 1:6500 for PKU and 1:20,000 for non-PKU HPA (Teebi et al., 1988).

The supposedly low incidence of PKU in Pakistan and India, 29 cases per 1 million births (Hardelid et al., 2007), may reflect bias of ascertainment rather than absence of alleles. Unusual alleles, both deletion (Guldberg et al., 1997b) and missense (Guldberg et al., 1993b), are found in PKU propositi born in the Asian subcontinent. Moreover, screening of a Pakistani population living in Britain yielded a PKU incidence of 1:14,500 (equivalent to the corresponding European cohort in the United Kingdom) (Hutchesson et al., 1998); however, because of consanguinity in this Pakistani community, allele

frequency is lower (1:713) relative to the European cohort (1:112).

It is now evident that the human *PAH* locus harbors extensive allelic diversity. Therefore, wherever PKU is found, and where nonconsanguineous mating is the norm, the mutant phenotype is likely to reflect a heteroallelic mutant genotype. The high homozygosity rate for a pathogenic PAH allele found in Yemenite Jews reflects a major founder effect (Avigad et al., 1990), and wherever consanguineous mating is a convention, homoallelic PKU genotypes will be more prevalent. Otherwise, the genetic homozygosity value *j* at the *PAH* locus will be low (Table 77-4).

Table 77-4: Expected Homozygosity at the *PAH* Locus in Patients with Hyperphenylalaninemia (Largely Phenylketonuria)—a Measure of Allelic Heterogeneity in Human Populations in Different Geographic Regions

Population/Region	*J*[*]	*N*[†]	Mutation Detection Rate (%)	Reference
Yemenite Jews	1.0	44	100	Avigad et al., 1990
Southern Poland	0.44	80	91.3	Zygulska et al., 1994
Iceland	0.26	34	100	Guldberg et al., 1997a
Tataria	0.19	27	100	Kuzman et al., 1995
Denmark	0.17	378	98.4	Guldberget al., 1993; Guldberg et al., 1994[¶]
Northern Ireland	0.14	242	99.6	Zschocke et al., 1995
Australia (Victoria)	0.11	83	97.6	Ramus et al., 1995
Norway	0.10	236	99.6	Eiken et al., 1996a, 1996b
Netherlands	0.08	68	92.6	Van Der Sijs-Bos et al., 1996
Germany	0.06	90	95.6	Guldberg et al., 1996b[¶]
Quebec	0.06[‡]	142	96.5	Carter et al., 1998[¶]
USA	0.06[§]	294	94.9	Guldberg et al., 1996a
Sicily	0.06	106	98.1	Guldberg et al., 1993a

j* = Σx_i^2 *where* x_i *is frequency of each allele different by state and identity; when analysis of state was not 100 percent, the uncharacterized alleles are given the aggregate frequency 1/N.

†N number of chromosomes available

‡Variation in j by population and geographic subregion in Quebec was 0.05–0.08 (Carter et al., 1998).

§Variation in j by region in United States was 0.05–0.10 (Guldberg et al., 1996a).

¶Includes unpublished data from source.

Centers of Diffusion and Gene Flow

Demic expansion and migration across Europe and Asia (Cavalli-Sforza et al., 1993) (as implied in Fig. 77-5), were likely ways to spread *PAH* alleles. A large meta-analysis of almost 9000 European chromosomes harboring 29 different alleles, each at relative frequency greater than 3 percent (Zschocke, 2003), reveals distinctive geographic distributions for the most prevalent alleles (Table 77-5); the new study confirms and extends an earlier analysis of European alleles (Eisensmith et al., 1992). There is corresponding evidence for distribution of *PAH* alleles in Oriental populations (Wang et al., 1991a, 1991b). Clines of allele frequency can be derived, and corresponding maps have been created to suggest centers of diffusion for several *PAH* alleles in human populations (Eisensmith and Woo, 1995; Zschocke, 2003); these further imply genetic drift in comparative isolation at the time the centers of diffusion developed, a hypothesis compatible with other measures of human genetic diversity (Mountain and Cavalli-Sforza, 1997).

Table 77-5: Major Geographic Distributions of the Most Prevalent PAH Alleles in Europe and the Orient

In Europe		In the Orient	
Region	**Allele**	**Region**	**Allele**
Norway	G272X	China	R243Q
Denmark	IVS12+1	China	R413Q
Iceland	c.1129delT	China	IVS4-1
Ireland	R408W[H1.8]	Korea (South)	Y204C/IVS4-1
Central Germany	R408W[H2.2]	Japan	R413P
France	IVS12+1		
Switzerland	R261Q		
Spain and Portugal	IVS10-11		
Sicily	IVS10-11		
Turkey, others	IVS10-11		
Poland	R408W[H2.3]		
Russia	R408W[H2.3]		
Estonia	R408W[H2.3]		

From Europe, Zschocke and, 2003; Iceland, Guldberg et al., 1997a; Orient, Eisensmith and Woo, 1995 and Okano and Isshiki, 1995; southern Europe, Cali et al., 1997; Turkey, Ozgüç et al., 1993 and Cali et al., 1997.

Genetic drift

Pathogenic *PAH* alleles are useful records of the "dance to the music of time" that molds human societies. The extent of their variation ranges between extremes. At one end of the spectrum is the penetrating effect of drift on *PAH* alleles, as illustrated by (1) a unique (deletion) allele in Yemenite Jews living in Israel (Avigad et al., 1990) and (2) the example of two solitary alleles in European Gypsies (IVS10nt-11 on haplotype 34 and R252W, haplotype unspecified) (Desviat et al., 1997; Kalanin et al., 1994; Tyfield et al., 1989). At the other end of the spectrum is the virtual absence of *PAH* alleles (negative founder effect) in Finns, Ashkenazi Jews, and Japanese, among whom PKU is a very rare disorder (see Table 77-2). In between these extremes, in Europe, for example, there is the allelic intermingling that reflects population growth (Cavalli-Sforza and Piazza, 1993).

Migration

The allelic composition of the *PAH* gene among contemporary PKU patients in the non-Slavic population of Tataria (Kuzman et al., 1995), a region in the former USSR, reveals mutations that could have been introduced by conquest or movement along trade routes: from Eastern Europe (the R408W mutation on haplotype 2), from Anatolia (the IVS10nt-11 mutation on haplotype 6), and from Scandinavia (IVS12nt1 and a rare frameshift allele, the latter otherwise found only in Scandinavians). No Oriental mutations are found in Tatarian PKU patients. Together, the findings imply that this population was formed mainly by people from Caucasian rather than Oriental background, a hypothesis being tested by analysis of other nuclear genes and mitochondrial DNA.

The different patterns of allelic diversity among HPA patients in Denmark (Guldberg et al., 1993c, 1994) and in Sicily (Guldberg et al., 1993a), for example, reflect the different political and linguistic histories of the regions. Within Italy itself, there is a significant difference in the composition of *PAH* alleles in PKU patients from the northern and southern regions of the country (Dianzani et al., 1994, 1995), their distribution reflecting the different demographic and cultural histories of northern and southern regions.

Examples compatible with the effects of migration and demic diffusion are seen in outlier regions of Europe (Zschocke, 2003), such as Iceland (Guldberg et al., 1997a), the British Isles (Tyfield et al., 1997; Zschocke et al., 1997), Ireland (O'Donnell et al., 2003), and the Iberian Peninsula (Pérez et al., 1997; Rivera et al., 1998). Alleles identified in these regions appear to have arrived there in people who came from both elsewhere in Europe and over long stretches of time.

Complex "untreelike" allelic diversity, among both the more prevalent and the rarer *PAH* alleles, is seen toward the center of Europe—for example, in Denmark (Guldberg et al., 1993c), France (Abadie et al., 1993a), the Netherlands (Van Der Sijs-Bos et al., 1996), and Germany (Guldberg et al., 1996b; Zschocke and Hoffmann, 1999)—reflecting the fact that the populations of continental Europe have not evolved according to simple "trees" of descent; the reality is more like "networks" of lineages reflecting intermingling historical movement (Cavalli-Sforza and Piazza, 1993).

Range Expansion

PAH mutations make useful evocative records of range expansion by neo-Europeans (Crosby, 1986) from countries of origin out to the Atlantic islands, the Americas, and Australasia, for example (see Fig. 77-5). Evidence lies in studies, for example, from Iceland (Guldberg et al., 1997a), Australia (Ramus et al., 1995), the United States (Guldberg et al., 1996a), Canada (Carter et al., 1998), Mexico (Nicolini et al., 1995), Costa Rica (Santos et al., 1996), and South America (Perez et al., 1993, 1996). Emigration from Europe (range expansion) has been recurrent over the past half-millennium, usually initiated by small numbers of individuals. When colonization of the new territory was successful, demic expansion by natural increase would follow, with or without new immigration.

These are the conditions under which recent founder effects could still be manifest, and the *PAH* locus again offers examples: (1) The M1V allele, prevalent in Quebec (Carter et al., 1998) and rare in France (Abadie et al., 1993a), has narrow time and space clusters for its origin out of France and entry into New France (Lyonnet et al., 1992); its distribution in Quebec today clearly reflects the history of its population (Carter et al., 1998). (2) The c.1129delT mutation in Iceland accounts for 40 percent of mutant *PAH* alleles there (Guldberg et al., 1997a). The mutation is associated with biallelic haplotype 4, VNTR 3, STR 234. Genealogic reconstruction for five generations in families harboring the mutation identifies ancestors from an isolated part of southern Iceland. The mutation has not been seen on any European chromosomes

outside Iceland. Since the tetranucleotide STR locus is a "fast" molecular clock (Weber and Wong, 1993), the presence of only one species of STR allele in the *PAH* haplotype bearing c.1129delT is compatible with a "recent" origin for the pathogenic mutation [other considerations eventually may refute this hypothesis (Rannala and Slatkin, 1998)]. Several *PAH* mutations found in Iceland also appear in Scandinavia but not in Ireland or Scotland. The genetic evidence suggests that the Icelandic population is predominantly of Scandinavian origin, with little contribution from the British Isles (as was once proposed). (3) Mariners from the Iberian Peninsula discovered the Central and Southern Americas, and colonization by their followers apparently introduced two *PAH* alleles (IVS10nt-11 and V388M), both prevalent in the Iberian Peninsula (Pérez et al., 1997; Rivera et al., 1997, 1998) and now accounting for 5 to 30 percent of *PAH* mutations in the screened South American populations (Desviat et al., 1995; Perez et al., 1993, 1996).

Range expansion, with its potential founder effect in the newly created population, does not always produce greater homozygosity for the rare pathogenic alleles in the descendants (see Table 77-5); Quebec is an illuminating example in this respect (Carter et al., 1998). *PAH* homozygosity for pathogenic alleles in Quebec overall is low ($j = 0.06$); it is not significantly higher in the linguistic and cultural subsets of the population in eastern and western Quebec and in Montreal. Groups of settlers from France before 1759, from the British Isles and Ireland after 1759, and from eastern Europe and Mediterranean nations after 1945 have each introduced different and identifiable *PAH* alleles into the population, of which the geographic and demographic distributions in the province reflect the histories of the different communities that make up Quebec today.

Molecular Mechanisms Introducing Novel *PAH* Alleles and Haplotype Associations

Penrose (1998) suggested *recurrent mutation* as a possible explanation for the frequency of PKU in European populations. The hypothesis might be rephrased to ask: Is the *PAH* gene *hypermutable*?

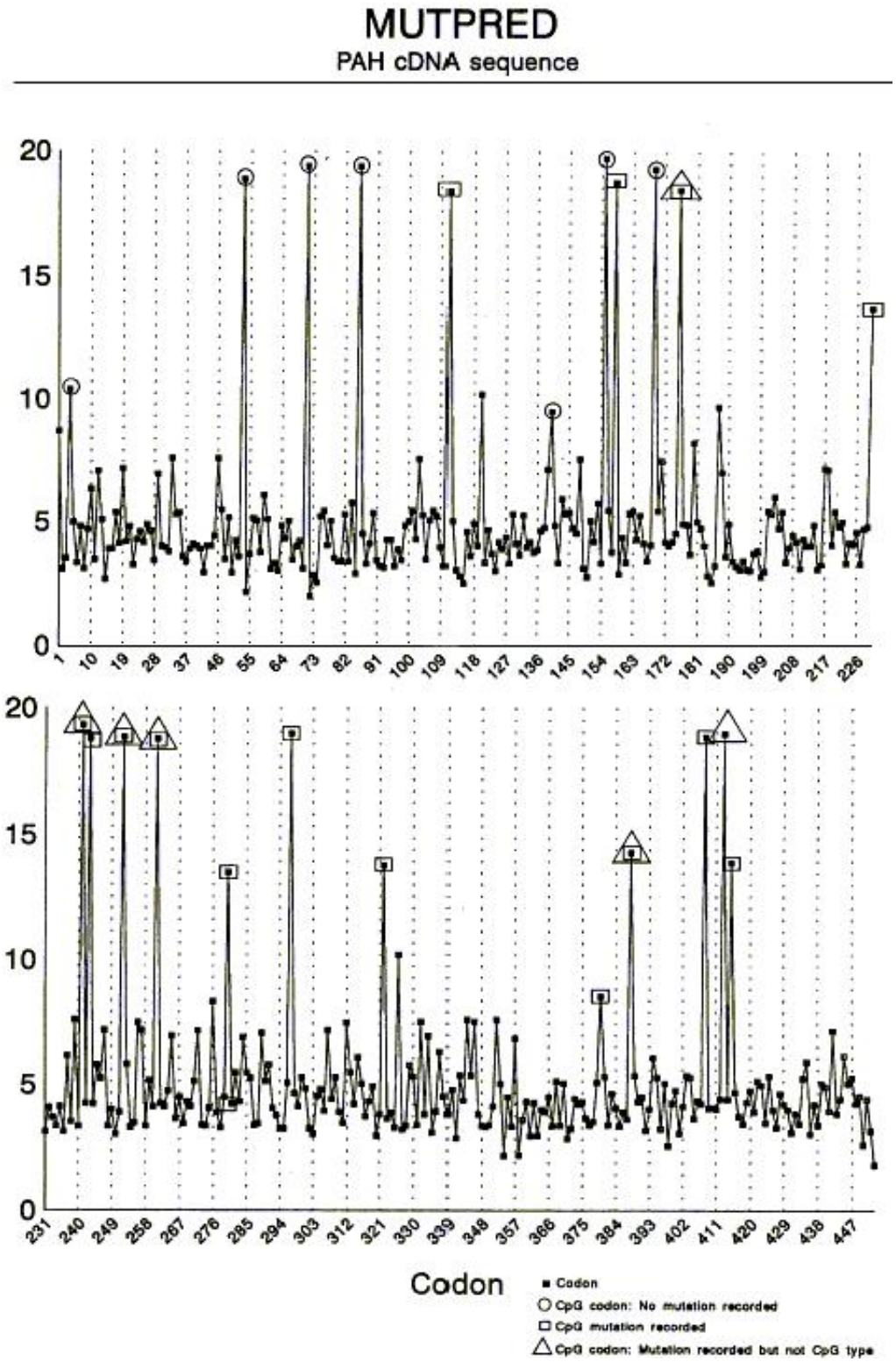
De novo mutations

Mutation analysis is done and reported more frequently in PKU propositi than in their biological parents; thus the actual rate of *de novoPAH* mutation is not known. Even so, $\mu = Sq^2$ is likely to be a small value at the *PAH* locus because *de novo* alleles are indeed rare, with only one report each so far of four different alleles on thousands of independent chromosomes (Aulehla-Scholz and Heilbronner, 2003; Chen et al., 2002; Eiken et al., 1996a).

Recurrent mutation

A predicted mutability profile exists for the cDNA sequence of *PAH* (Fig. 77-6). The majority of the *predicted hypermutable* regions coincide with the 24 CpG dinucleotide sites in the gene (Byck et al., 1997). CpG sites are 40 times more mutable than any other dinucleotide sequences (Cooper and Krawczak, 1993; Cooper and Youssoufian, 1988), and they can experience C→T or G→A transition mutations during deamination of the 5' methylcytosine if the cytosine in the dinucleotide was methylated initially. Among the pathogenic *PAH* mutations, at least 23 are known to be CpG-type alleles (Byck et al., 1997); another 8 occur at CpG sites but are not C→T or G→A transitions, and seven regions in the *PAH* gene containing CpG sites have no reported mutations (see Fig. 77-6).

Figure 6:

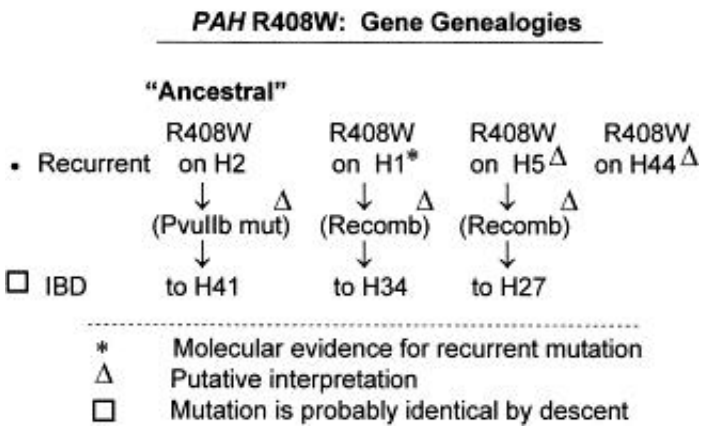


The mutability profile for the human phenylalanine hydroxylase (PAH) gene and cDNA sequence predicted by the MUTPRED software (Cooper, Krawczak and, 1993). Three classes of hypermutable CpG sites occur in the PAH gene: ○, sites with no reported alleles; □, sites with CpG-type (C→T or G→A) mutations; ▵, sites at which the mutation is not CpG type. (From Byck et al., 1997. Used by permission of Wiley-Liss Inc.)

There is evidence for recurrent mutation at the R408W codon in the *PAH* gene. Ramus and colleagues (1992) identified two different mutations in this codon: R408W (c.1222C→T) and R408Q (c.1223G→A); they proposed a mutational "hotspot." An earlier study (John et al., 1990) found the R408W mutation (c.1222C→T) on two different RFLP haplotypes in the Quebec population and proposed recurrent mutation, gene conversion, or intragenic recombination as a mechanism. Genealogic reconstructions in the Quebec families segregating on haplotype 1 found Celtic ancestors from Scotland and Ireland (Treacy et al., 1993). The finding suggested a center of diffusion in northwestern Europe for R408W[H1] different from R408W on haplotype 2. This hypothesis was tested by haplotype analysis of more than 1200 European chromosomes harboring the R408W mutation (Eisensmith et al., 1995). Those carrying haplotype 1 clustered on the northwestern fringes of Europe, whereas those carrying haplotype 2 clustered in eastern Europe. Further analysis of the flanking 5' STR alleles and 3' VNTR markers of normal and mutant *PAH* chromosomes (Byck et al., 1994; Tighe et al., 2003) produced more evidence for recurrent mutation. Thus R408W has identity only by state and not by descent in northwestern and eastern European populations; R408W on haplotype 1.8 might be the older of the two alleles (Tighe et al., 2003).

The R408W mutation now has been found on seven different haplotype backgrounds from which putative "gene genealogies" can be created (Fig. 77-7). It seems likely that recurrent mutation is the source of R408W on haplotypes 1.8, 2.3, and 5 in Europeans (Kalaydjieva et al., 1990; Kozak et al., 1995; L Kozak, personal communication, documented in www.pahdb.mcgill.ca; Zschocke et al., 1994b; Zygulska et al., 1993) and on haplotype 44 in the Orient (Guttler et al., 1999). A mechanism for the recurrent R408W allele has been proposed (Murphy et al., 2006). It involves spontaneous methylation-mediated deamination of the 5mC (cytosine) at position c.1222. This cytosine normally is methylated in human and nonhuman primates. On deamination, it converts to thymine, creating the mutation c.1222C→T and thus changing the codon from CGG to TGG and creating the missense allel p.R408W. On the other hand, the R408W mutation on haplotype 41 in both European (Kadasi et al., 1995) and Oriental populations (Lin et al., 1992), on haplotype 34 in Portuguese patients (Caillaud et al., 1992), and on haplotype 27 in a Belgian patient (L Michiels, personal communication, documented in www.pahdb.mcgill.ca) can be explained, in each case either by mutation (or gene conversion) at a single RFLP site or by an intragenic recombination. In each case, the R408W mutation would be identical by descent (see Fig. 77-7). The E280K (c.838G→A) allele occurs on haplotypes 1 and 2, a finding compatible with recurrence (Okano et al., 1990) and supported by molecular evidence (Byck et al., 1997).

Figure 7:



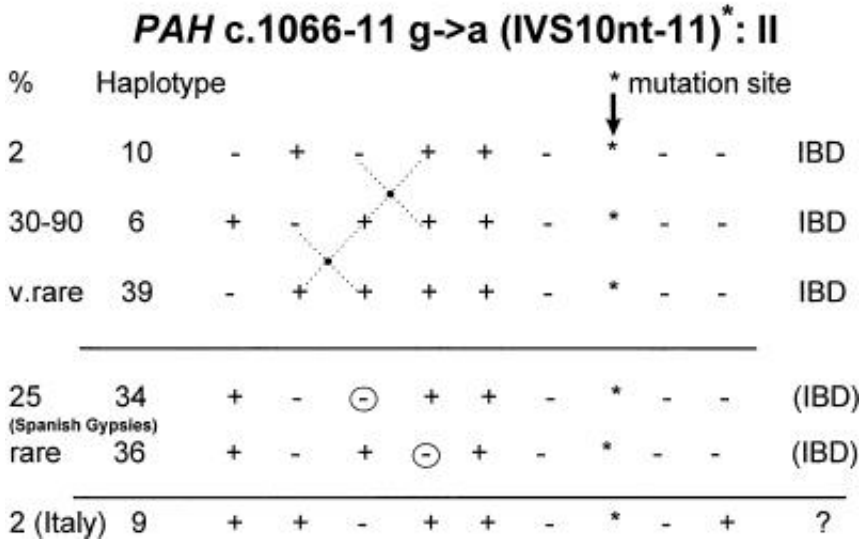
A "gene genealogy" for the phenylketonuria-causing R408W allele. The mutation, always identical by state (c.1222 C→T) is found on seven different extended haplotypes (H). Sequence analysis of normal and mutant (H1) and (H2) chromosomes reveals the likelihood that one of these versions of R408W in Europeans is a recurrent mutation at a CpG site. Inspection of the other haplotype associations with R408W point to a total of four recurrent alleles and three that are identical by descent on intragenic recombinant copies of the human phenylalanine hydroxylase (PAH) gene.

Intragenic recombination

PAH alleles are useful markers in evidence of a classic mechanism by which evolution has generated genomic diversity—genetic recombination. R408W mutations are not the only ones that appear to have changed haplotype by intragenic recombination. S76P alleles on haplotypes 1 and 4, G218W on haplotypes 2 and 1, and V245A on haplotypes 7 and 3 all have been identified on chromosomes in Europeans and in their descendants abroad; in each case, a single recombination within the *PAH* gene can explain the association of one mutation with two haplotypes (Carter et al., 1998). An IVS12nt1 allele, found almost exclusively on haplotype 3, has been found once on a foreign haplotype; this event is explained by a double intragenic recombination (Carter et al., 1998).

Multiple associations between a pathogenic *PAH* allele at a non-CpG site and a polymorphic haplotype have been identified in the case of the prevalent (and ancient) IVS10nt-11 splice allele. Recombination could explain some of these associations (Fig. 77-8).

Figure 8:



The human phenylalanine hydroxylase (PAH) gene mutation c.1066nt-11g→a (IVS10nt-11) is prevalent in populations of Southeastern Europe and the Mediterranean. The allele occurs on six different haplotypes at the relative frequencies (percent) shown (Cali et al., 1997). Its occurrence on different haplotypes is compatible with identity by descent (IBD) assuming (1) intragenic recombination between haplotypes 6, 10, and 39; or (2) mutation/gene conversion at a restriction fragment length polymorphism (RFLP) site. The origin of the IVS10 mutation on haplotype 9 is unclear. The RFLP sites not named here are shown in Fig. 77-5. The relative position (*) of the IVS10nt-11 mutation in the gene is shown.

Selective Advantage: Is It a Mechanism?

Are any of the foregoing mechanisms, by themselves, sufficient to explain the incidence of PKU in temperate-zone populations? Consider the evidence once more:

1. Involvement of *multiple loci* that could accumulate larger numbers of mutational hits to cause HPA can be discounted because more than 98 percent of cases reflect phenotypic homozygosity for mutations at the *PAH* locus alone.
2. Although some pathogenic *PAH* alleles are the result of *recurrent mutation*, an overall higher than average rate of disease-causing mutational events at the *PAH* locus can be dismissed.
3. *Inbreeding* is a likely contributor in some populations, but overall, it is not the explanation (Woolf, 1994).
4. *Founder effect* is only an occasional mechanism to account for the high relative frequency of a specific allele, and it always relates to a particular population.
5. The presence of many different prevalent alleles in different populations is compatible with *genetic drift* (see W Bodmer in Scriver et al., 1996b). Moreover, genetic drift could be responsible for the incidence of PKU in temperate-zone populations, and it would act independently on a wide variety of alleles because the subset of deleterious *PAH* alleles is still small relative to the whole set and thus susceptible to random genetic drift (see D Hartl, p. 93, in Scriver et al., 1996b).

The weight of evidence would seem to favor random genetic drift to account for the prevalence of PKU in most contemporary human populations. Meanwhile, the debate continues (see "Discussion" in Scriver et al., 1996b, p. 95), and fascination for *selective advantage* (heterozygous advantage or over dominant selection) persists (Krawczak and Zschocke, 2003; Hardelid et al., 2007).

When only a few in a large set of disease-causing recessive alleles account for the major fraction of the aggregate frequency distribution, selective advantage is a mechanism to consider (Flint et al., 1993). Selection of *PAH* alleles (the objects) will occur if the process acts on a phenotype that confers an advantage and is encoded by the corresponding gene. Selection can act indirectly or directly—in the former case through "hitchhiking" at a closely linked locus and in the latter on the primary locus. For example, the γ-interferon locus in region 12q24.1 is physically linked to *PAH*, but there is no evidence that it is involved in the selection of *PAH* alleles. Selection is more likely to have occurred if it could act on a phenotype encoded directly by the *PAH* locus in heterozygotes carrying disease-causing *PAH* alleles. Conventional wisdom predicts a disadaptive or, at best, neutral effect of the mutant *PAH* allele in the heterozygote, but if there is a disadaptive effect, it must be small (Vogel, 1985). Even so, a disadaptive effect will not increase allele frequency.

Increase can occur only through a process that confers an advantage in reproductive fitness. In the case of PKU, a simple calculation shows that heterozygote advantage, in terms of surviving offspring, can be only 1 percent (or less) greater than for normal homozygotes, so the advantage in fitness is marginal. Selective advantage was proposed (Kidd, 1987) when PKU-causing alleles were first characterized (DiLella et al., 1986b, 1987) but was rejected subsequently by its initial advocate (see Kidd in Scriver et al., 1996b, p. 94).

The selection process must act first on a phenotype (Sober, 1984); thereafter, it can influence frequency of the allele (the object). Might plasma phenylalanine levels in heterozygotes be the designated phenotype on which the process of selection acts? The effect of heterozygosity on this recessive metric phenotype is actually very small (Gold et al., 1974; Rosenblatt and Scriver, 1968), and it is difficult to imagine how the selection process would discern it. If selection acts at a more proximal level (e.g., on PAH enzyme itself), it will have been acting not only on *null* protein phenotypes caused by prevalent alleles such as IVS12nt1, IVS10nt-11, and R408W but also on a variant kinetic phenotype such as that caused by the prevalent I65T allele. How this would occur is unknown.

Selective advantage is expressed ultimately in either gametic selection, better survival to reproductive age, or higher rates of reproduction among heterozygotes. There is evidence both for (Saugstad, 1977; Woolf, 1994; Woolf et al., 1975) and against (Paul et al., 1979; Saugstad, 1973) these alternatives in the case of PKU. Such conflicting evidence may reflect bias in the original studies because the families sampled were ascertained through PKU probands. Corrections have since been made for such bias (ten Kate, 1978; Woolf, 1986), but the ambiguities remain.

It may never be possible to identify the agent of selection. For example, ochratoxin A, a mycotoxin in grains and lentils, has been proposed as a candidate (Woolf, 1986), but without experimental supporting evidence, it may never be possible to quantify the effects of the putative selective process. There are at least two reasons: (1) Such effects may have existed only in the past and are no longer acting, and (2) the effects of heterozygote selection may be too small to detect reliably because PKU is much less frequent than other genetic disorders where such a mechanism has been invoked (Flint et al., 1993). Thus, if the magnitude of the selective effect on *PAH* alleles is indeed small, yet still greater than the effect of genetic drift, then it would have acted on a large number of individuals or a particular demographic strata to

produce the observed incidence of mutant *PAH* alleles and of PKU disease, and it may have left a molecular signature in the region of the locus under selection (see "Discussion" in Hardelid et al., 2007). Furthermore, the selective advantage must have existed in several temperate regions of the world where populations were exposed to different climates, cultures, diets, and infective agents. On theoretical grounds (Thompson and Neel, 1997), there is no need to invoke heterozygous advantage to explain the very variable frequencies of these pathogenic recessive *PAH* alleles. Yet it remains an active hypothesis, and refutations of the arguments for genetic drift are combined with appeals to recognize that some "pathogenic" alleles have reached true polymorphic frequencies in some populations (Krawczak and Zschocke, 2003). Genetic drift will do as an explanation. So why not accept selective advantage? It is appealing to use this point of view when explaining the prevalence of PKU to the family of a newly diagnosed newborn: "The mutation was a ‘good’ allele in human history."

The IVS10nt-11 *PAH* Allele: A Paradigm

This *PAH* mutation, a splice allele with systematic name c.1066-11g→a (also known by the trivial name IVS10nt-11 or IVS10nt546), illustrates many of the themes in population genetics.

1. IVS10nt-11 in the homoallelic genotype unambiguously confers a PKU phenotype *in vivo* (Guldberg et al., 1998; Kayaalp et al., 1997). Its geographic distribution is nonuniform and is most prevalent in southern and southeastern Europe, particularly in the Anatolian region of modern-day Turkey, where it appears to have originated (Cali et al., 1997).
2. From observed frequencies in various Mediterranean populations and the corresponding incidence of PKU, the highest relative frequency of the allele (0.32) is found in Turkey (Ozgü et al., 1993), where the estimated absolute (population) frequency is approximately 0.003 (Cali et al., 1997; Woolf, 1994). Endogamy and consanguinity contribute to the high prevalence of this mutant genotype in this particular region (Woolf, 1994).
3. IVS10nt-11 is embedded in what has been called an "ancestral" haplotype [RFLP haplotype 6, STR 252 (Goltsov length), VNTR 7], but this particular association is not exclusive. IVS10nt11 is also found on several RFLP haplotypes, and in most cases, this finding is compatible with intragenic recombination or point mutation at an RFLP site (see Fig. 77-8). Since the allele does not involve a CpG site, it is unlikely to be recurrent, and the majority of its mutation-haplotype associations are compatible with identity by descent. Haplotype data indicate a time horizon (origin) for the mutation at least 5000 to 10,000 years ago (Cali et al., 1997).
4. Associations between IVS10nt-11 and haplotypes on chromosomes from patients in Turkey, Israel, and Italy (Cali et al., 1997), Greece (Traeger-Synodinos et al., 1994), and the Iberian Peninsula (Cali et al., 1997; Pérez et al., 1997; Rivera et al., 1997, 1998) indicate a post-Neolithic *demic expansion and diffusion* (gene flow) from the eastern regions westward across the Mediterranean basin. These data, derived from DNA analysis at the *PAH* locus, are concordant with the gradients of classic polymorphic protein markers in the same region (Cavalli-Sforza et al., 1994; Cavalli-Sforza and Piazza, 1993).
5. The IVS10nt-11 allele on haplotype 6 is a tracer or *migration* (gene flow) overland to Tataria (Kuzman et al., 1995) and of range expansion overseas to North America (Carter et al., 1998; Guldberg et al., 1996a), South America (Perez et al., 1996), and Australia (Ramus et al., 1995).
6. The IVS10nt-11 mutation is found on a unique haplotype [RFLP 34, STR 230 (Zschocke length), VNTR 7] in Spanish Gypsies (Desviat et al., 1997), where it is a marker for *founder effect* with drift in relative genetic isolation.

THE PHENYLALANINE HYDROXYLATING SYSTEM

The most important single determinant of phenylalanine homeostasis in humans is the hydroxylation reaction. To understand the mutant phenotypes associated with HPA, the normal components of the hydroxylation should be known.

General Characteristics

The phenylalanine hydroxylation reaction is an obligatory and rate-limiting step in the catabolic pathway that leads to the complete oxidation of phenylalanine to CO_2 and water (Milstien and Kaufman, 1975). The ketogenic (e.g., acetoacetate) and gluconeogenic (e.g., fumarate) products of phenylalanine catabolism (see Fig. 77-2) contribute to the organism's pool of two-carbon metabolites and glucose. In view of the brain's partial dependence on a peripheral supply of glucose, the ability of phenylalanine to provide gluconeogenic substrates, in this context, plays a role in normal brain development and function. Hydroxylation of phenylalanine plays another role in mammalian metabolism: It provides the organism with an endogenous supply of the nonessential amino acid tyrosine. When hydroxylation is deficient, tyrosine becomes an *essential* amino acid. The formal name for PAH enzyme is L-phenylalanine-4-monooxygenase (EC 1.14.16.1), a mixed-function oxidoreductase.

Tissue Distribution of Hydroxylating Activity

It was thought, at one time, that PAH is not present in nonhepatic mammalian tissues (Udenfriend and Cooper, 1952). However, subsequent studies demonstrated appreciable activity in mouse kidney and pancreas (Tourian et al., 1969) and in rat and guinea pig kidney (Berry et al., 1972). The putative presence of hydroxylase activity in pancreas has not been confirmed (Rao and Kaufman, 1986). These authors observed that rat kidney PAH is in an unusual state of activation relative to the rat liver enzyme, and at the resting blood phenylalanine concentration, kidney enzyme might account for as much as 20 to 30 percent of the total phenylalanine hydroxylase activity in rats. Until recently, the status of the kidney hydroxylase in humans was unclear. Ayling and colleagues (Ayling et al., 1974, 1975) reported hydroxylase activity in kidney, whereas others found none in either human or nonhuman primate kidney (Murthy and Berry, 1975). In support of the latter evidence, rat phenylalanine hydroxylase cDNA hybridizes with rat kidney mRNA but not with baboon kidney mRNA (House et al., 1997). Evidence based on analysis of mRNA, immunohistochemistry, and assay of enzymic activity indicates that human kidney expresses significant phenylalanine hydroxylase protein and enzyme activity (Lichter-Konecki et al., 1999; Tessari et al., 1999). The kidney is now considered to be an important site of conversion of phenylalanine to tyrosine in adult humans (Moller et al., 2000).

PAH is not present in brain (Abita et al., 1974), contrary to an earlier claim (Wapnir et al., 1971); the finding has bearing on pathogenesis of the brain phenotype in PKU. Brain does contain another enzyme, tyrosine hydroxylase, that catalyzes the conversion of phenylalanine to tyrosine at a rate comparable to its ability to hydroxylate tyrosine (Katz et al., 1976). Perhaps tyrosine hydroxylase, acting on phenylalanine, provides the developing brain with a significant fraction of the tyrosine it needs for protein synthesis (Kaufman, 1987); whether it might in PKU is not known. Catalytic properties of the human fetal liver PAH appear to be the same as those of the adult enzyme (Friedman and Kaufman, 1971; Raiha, 1973).

Evidence has been reported that PAH and 4α-carbinolamine dehydratase are present together in human melanocytes and keratinocytes, where they are postulated to play a role in melanin formation (Schallreuter et al., 1994). In the proposed scenario, hydroxylation of phenylalanine provides the tyrosine essential for melanin synthesis; furthermore, the depigmentation disorder vitiligo constitutes a lack of the dehydratase with accumulation of 7-biopterin, which inhibits phenylalanine hydroxylase (Davis et al.,

1992), leading to disruption of the supply of tyrosine and consequent impairment of melanin synthesis.

Although the presence of 4α-carbinolamine dehydratase in normal human epidermal keratinocytes has been demonstrated unequivocally, the conclusion that phenylalanine hydroxylase is also present (Schallreuter et al., 1994) is not strongly supported. The enzyme that hydroxylates phenylalanine in both melanocytes and keratinocytes could be tyrosine hydroxylase. Indeed, if as postulated phenylalanine hydroxylase were essential for melanin synthesis, PKU patients would be expected to show irreversible signs of this defect. PKU patients do tend to have fair skin, but this defect in pigmentation is reversed when the plasma phenylalanine level is reduced, a finding consistent with the evidence that excess phenylalanine inhibits tyrosinase-mediated melanin synthesis (Miyamoto and Fitzpatrick, 1957).

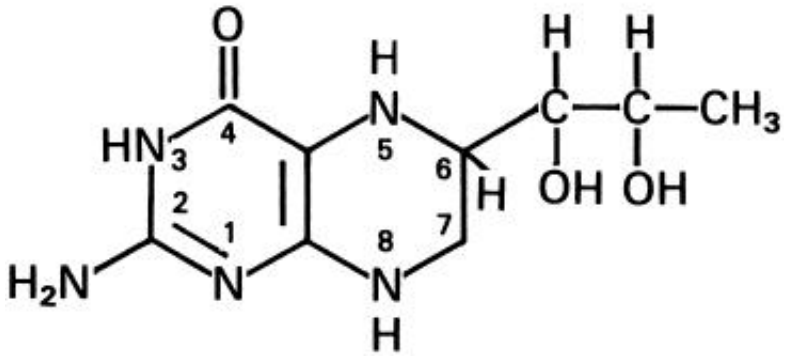
The phenomenon of *illegitimate transcription* is relevant to a discussion of the tissue distribution of phenylalanine hydroxylase. The term was coined to describe the low level of transcription of tissue-specific genes in nonspecific cells (Schallreuter et al., 1994). Gene transcripts of phenylalanine hydroxylase (as well as tyrosine hydroxylase) have been detected in white blood cells, erythroleukemic cells, and chorionic villus cells (Sarkar and Sommer, 1989). To date, the levels of transcripts detected are too low to know whether their occurrence is of any functional significance. The phenomenon has, however, proved to be of value in the identification of PKU-associated mutations in phenylalanine hydroxylase in circulating lymphocytes (Ramus et al., 1992b; Abadie et al., 1993b).

The Hydroxylating System

The hepatic phenylalanine hydroxylating system consists of three essential components: PAH, DHPR, and the unconjugated pterin tetrahydrobiopterin (BH_4), i.e., 2-amino-4-hydroxy-6-[L-*erythro*-1',2'-dihydroxypropyl]-tetrahydropteridine (Kaufman, 1963, 1971, 1997) in addition to a stimulating protein (Kaufman, 1970) isolated in pure form from rat liver (Huang et al., 1973), which, although not essential., can markedly stimulate the hydroxylation reaction *in vivo*. Originally called *phenylalanine hydroxylase stimulator* (PHS), this protein was later shown to be the aforementioned dehydratase (Lazarus et al., 1983).

The structure of BH_4 is shown in Fig. 77-9. As with all other naturally occurring compounds of this type, BH_4 is a 2-amino-4-hydroxypteridine (trivial name *pterin*); it is classified as an unconjugated pterin to distinguish it from its relatives, the folates. The latter compounds are called *conjugated pterins* because their pterin rings are conjugated with a *para*-aminobenzoyl-glutamate(s) substituent at position 6 of the pteridine ring. Unlike the folates, BH_4 is not a vitamin for mammals because they can synthesize it. Several synthetic tetrahydropterins with simple alkyl substituents at position 6, such as 6-methyltetrahydropterin ($6MPH_4$) and 6,7-dimethyltetrahydropterin ($DMPH_4$), are even more active than BH_4 in the phenylalanine hydroxylating system (Kaufman and Levenberg, 1992).

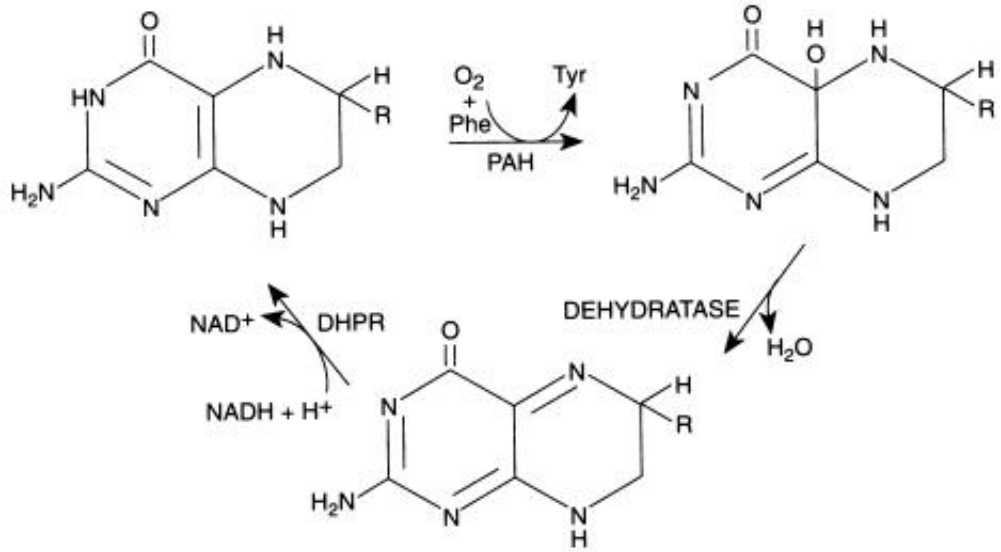
Figure 9:



Structure of tetrahydrobiopterin
[2-amino-4-hydroxy-6(L-erythro-1′,2′-dihydroxypropyl)-tetrahydropteridine], of BH_4, the natural coenzyme (cofactor) for phenylalanine hydroxylase.

The reactions catalyzed by PAH, DHPR, and PHS (the dehydratase) in the presence of the pterin appear in Fig. 77-10. PAH catalyzes a coupled reaction in which phenylalanine is oxidized to tyrosine and the tetrahydropterin is oxidized to the corresponding 4-hydroxytetrahydropterin (also called 4α-carbinolamine). The conversion of carbinolamine to quinonoid dihydropterin and water is catalyzed by 4α-carbinolamine dehydratase (Lazarus et al., 1983), formerly known as PAH-stimulating protein. The oxygen in the *para* position of the benzene ring of the tyrosine product is derived from molecular oxygen rather than from water (Kaufman et al., 1962); accordingly, the hydroxylase is an oxygenase. During the hydroxylation reaction, the second atom of oxygen in the oxygen molecule is normally reduced to the level of water.

Figure 10:



The conversion of phenylalanine to tyrosine is catalyzed by the phenylalanine hydroxylation system. The overall reaction is the sum of three reactions, each catalyzed by a separate enzyme. In the presence of an active tetrahydropterin cofactor like the naturally occurring substance BH_4 (R = —CHOH—CHOH—CH3), phenylalanine hydroxylase (PAH) catalyzes a coupled reaction in which phenylalanine is converted to tyrosine and BH_4 to a carbinolamine, the corresponding 4α-hydroxytetrahydropterin. The carbinolamine is then converted to the quinonoid dihydropterin by a dehydratase (4α-carbinolamine dehydratase). The cycle is completed by the action of dihydropteridine reductase (DHPR), which catalyzes the NADH-mediated reduction of the quinonoid dihydropterin back to the tetrahydropterin. (The pterin substrates for the three reactions are indicated in italics.)

The minimum requirements for phenylalanine hydroxylation are the hydroxylase, oxygen, L-phenylalanine, and BH_4, but under these conditions, BH_4 can function only stoichiometrically (i.e., the amount of tyrosine formed cannot exceed the amount of BH_4 present). For the pterin coenzyme to function catalytically, there must be another component of the system: DHPR (along with a reduced pyridine nucleotide). Although the reductase is active with both NADH and NADPH, NADH is the better substrate *in vitro* (Craine et al., 1972; Neilson, 1969; Scrimgeour and Cheema, 1971). It is not known whether NADH or NADPH or both function with the reductase *in vivo*. In addition to the NADH-dependent DHPR-catalyzed regeneration of BH_4 from quinonoid dihydrobiopterin (qBH_2), reduction of the latter compound to BH_4 also can proceed nonenzymatically in the presence of millimolar concentrations of reducing agents such as mercaptans and ascorbate (Kaufman, 1959). Attempts to demonstrate ascorbate-mediated regeneration of BH_4 in humans have not been successful.

The complete absence of phenylalanine hydroxylase, DHPR, or BH_4 leads to persistent HPA. By contrast, because the reaction catalyzed by the dehydratase can occur quite rapidly by nonenzymatic routes, it was predicted that the complete absence of this enzyme would cause only mild or transient HPA (Kaufman, 1983). This prediction was validated in a patient with the mild phenotype who excretes 7-BH_4 and harbors two mutations in the gene for the dehydratase, one inherited from the mother (C82R) and the other (E87X) from the father (Citron et al., 1993). The results of studies of the activity of these two engineered alleles *in vitro* also were coherent with this prediction. The C82R mutant had about 40 percent of the activity of the wild-type dehydratase enzyme (Johnen et al., 1995; Koster et al., 1995), whereas the E87X allele was devoid of activity, in part because of its instability (Johnen et al., 1995). The predicted effects of these two mutations, combined *in vivo*, would much reduce the dehydratase activity. (Dehydratase deficiency is further described in Chap. 78.)

In addition to its catalytic function in the regeneration of BH_4 (see Fig. 77-10), the dehydratase also plays a completely unrelated role in regulating gene transcription, a striking example of a phenomenon dubbed *molecular opportunism* (Doolittle, 1988). On cloning and sequencing the dehydratase, it was found that this protein is identical to DCoH, the dimerization cofactor of hepatocyte nuclear factor 1 (HNF1) (Citron et al., 1992a; Hauer et al., 1993). The dimeric form of the latter protein regulates transcription of a large number of genes in the liver, intestine, and kidney, including those coding for albumin, α_1-antitrypsin, and fibrinogen. DCoH enhances this transcriptional activity by combining with HNF1 dimers and stabilizing them (for a review, see Mendel and Crabtree, 1991). Mice lacking DCoH are viable and fertile but display HPA (Bayle et al., 2002). Involvement of the dehydratase in a gene transcription system raised questions about whether mutations that affect dehydratase activity also affect its transcription-enhancing activity. A detailed analysis of the effect of some mutations on both these activities showed that those decreasing dehydratase activity to less than 1 percent of wild type decrease transcriptional activity only modestly (by approximately 10 percent) and have no effect on binding DCoH to HNF1, a clear indication that dehydratase activity is not essential for the binding (Johnen and Kaufman, 1997). The same conclusion

was reached from a study that focused on the binding of allelic dehydratase (C82R) to HNF1 (Sourdive et al., 1997). These findings may explain why dehydratase-deficient patients do not appear to suffer from the global metabolic consequences that would be expected from disruption of the activity of the DCoH/HNF1 transcription system.

Although the DNA and amino acid sequences of mouse, rat, and human phenylalanine hydroxylases are strikingly similar (Ledley et al., 1990), there are noteworthy physiologic differences between the human enzyme and the rodent enzyme (Kaufman, 1997). Structural and functional studies of the 5'-flanking region of the phenylalanine hydroxylase gene have defined some of the features that are essential for the regulation of the phenylalanine hydroxylase gene and have identified some of the characteristics that may account for these differences (Konecki et al., 1992) (see the section "The *PAH* Gene" above). As shown in transgenic mice, a 9-kb DNA fragment upstream of the human gene contains all the *cis*-acting elements needed to direct tissue-specific and developmental-stage-specific expression of phenylalanine hydroxylase *in vivo* (Wang et al., 1992a). As with other typical housekeeping genes, human *PAH* gene uses multiple transcriptional initiation sites (Wang et al., 1994). The promoter regions of the mouse and human phenylalanine hydroxylase genes are similar in their lack of a TATA-like sequence (Faust et al., 1996). Expression of the mouse phenylalanine hydroxylase gene depends on a hormone-responsive and tissue-specific enhancer located 3.5 kb upstream, as well as on the presence of cAMP and steroid hormones such as dexamethasone. Furthermore, activity of this enhancer was found to require the hepatocyte-enriched transcription factors HNF1 and C/EBP.

Further studies of the promoter region in the mouse *PAH* gene (Pontoglio et al., 1996) reveal multiple DNase-hypersensitive sites in the normal animal, reflecting an open chromatin structure and a low degree of methylation, both characteristic of transcriptionally active genes. By contrast, the corresponding region of the gene in the HNF1 knockout mouse has a relatively closed chromatin structure and the hypermethylation pattern of a silent gene. These results suggest that HNF1 is essential for chromatin remodeling and DNA methylation that accompany transcriptional activation. Experiments carried out on HNF1 knockout mice complement the studies *in vitro* and show that phenylalanine hydroxylase belongs to the group of proteins whose genes are indeed regulated by HNF1 (Pontoglio et al., 1996); the gene coding for phenylalanine hydroxylase is completely silent in the knockout. These animals, however, are not true models for PKU because they also have a severe Fanconi syndrome caused by proximal renal tubule dysfunction; they die around the time of weaning.

Until recently, any attempt to relate the results obtained with the HNF1 knockout mice to PKU in humans had been hampered by the lack of evidence that HNF1 regulates the expression of human PAH. That gap was closed initially with the demonstration that the 9-kb human phenylalanine hydroxylase 5'-flanking fragment (Wang et al., 1994) contains two HNF1-binding sites located 0.5 kb upstream (Lei and Kaufman, 1998a). Cotransfection experiments showed that HNF1 markedly transactivated the 9-kb DNA fragment (linked to a reporter gene) in Chinese hamster ovary cells. Moreover, although DCoH by itself lacked this ability, it could enhance the HNF1-mediated transactivation of the 9-kb fragment. These effects of HNF1 and DCoH were observed in the absence of added hormones, in contrast to the hormonal dependence of the effect of HNF1 on the expression of the mouse *PAH* gene (Faust et al., 1996), an indication that regulation of expression of human PAH by hormones does not follow the same pattern as that for the mouse enzyme.

A study of a patient with HPA revealed a 3.7-kb deletion in the 5'-flanking region of the *PAH* gene. Characterization of the deleted sequence identified a novel liver-specific DNase I hypersensitive site located 3.3 kb upstream of the RNA initiation site of the *PAH* gene. Transient transfection assays, again using a reporter gene, demonstrated that a plasmid construct containing the deletion mutation was

severely impaired with respect to *PAH* transcriptional activity (Chen et al., 2002). The expression of PAH was suppressed in hepatoma HepG2 cells when there was overexpression of a truncated HNF1α mutant (Tanizawa et al., 1999).

Dihydrofolate reductase (DHFR; EC 1.6.99.7) also may play a role in hydroxylation of phenylalanine *in vivo*. This enzyme has a well-established role in one-carbon metabolism, catalyzing the reduction of 7,8-dihydrofolate:

[IMAGE]

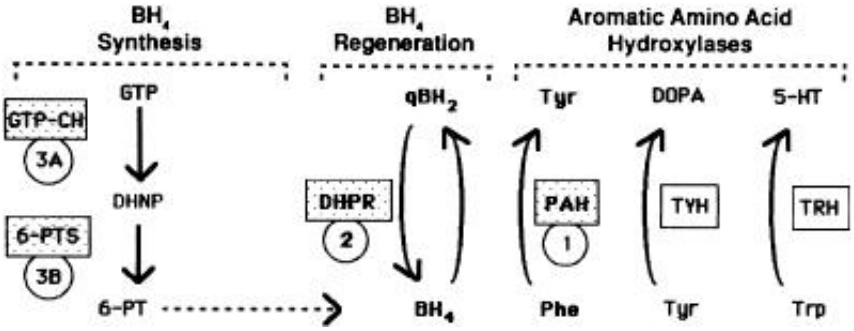
The enzyme also can catalyze the analogous reaction with 7,8-dihydrobiotperin (Kaufman, 1967):

[IMAGE]

The second reaction assumes importance for phenylalanine hydroxylation when the rate of the DHPR-catalyzed reduction of qBH_2 lags behind the rate of the PAH-catalyzed formation of the quinonoid derivative (see Fig. 77-10). Under such conditions, the extremely unstable qBH will undergo a rearrangement to the corresponding 7,8-dihydrobiopterin (Kaufman, 1967); the latter is not a substrate for DHPR. Therefore, when DHPR limits the rate of phenylalanine hydroxylation, DHFR could salvage some of the biopterin diverted to the 7,8-dihydro derivative and thereby potentially support some phenylalanine hydroxylation. However, since the near-total absence of DHPR leads to HPA (see Chap. 78), it is evident that neither DHFR nor any other enzyme is as effective as DHPR at sustaining normal rates of phenylalanine hydroxylation.

The reactions depicted in Fig. 77-10 were the first to establish a metabolic role for an unconjugated pterin and to reveal the coenzyme role of BH_4. It was shown subsequently that BH_4 and DHPR play precisely the same roles in the hydroxylating systems for phenylalanine, tyrosine, and tryptophan (Brenneman and Kaufman, 1964; Shiman et al., 1971; Friedman et al., 1972a) (Fig. 77-11). Accordingly, BH_4 and DHPR are essential for the biosynthesis of the neurotransmitters dopamine, norepinephrine, and serotonin. A fuller realization of the *in situ* role of BH_4 and DHPR in these other hydroxylating systems became clear with the discovery of variant forms of PKU caused by defects in BH_4 regeneration and synthesis (see Chap. 78).

Figure 11:



Scheme to show the hydroxylation reactions for phenylalanine, tyrosine, and tryptophan, each requiring BH_4 cofactor and therefore dependent on synthesis and maintenance of BH_4. The pathway for BH_4 synthesis is only sketched (see Chap. 78 for details). Maintenance of BH_4 requires regeneration from dihydropteridine reductase (DHPR) in the presence of dehydratase (not shown here; see Fig. 77-10) and DHPR. Enzymes involved in the Mendelian HPAs (shaded) are disorders of (1) primary hydroxylase activity (this chapter), (2) maintenance of BH_4 (Chap. 78), and (3) BH_4 synthesis (Chap. 78). GTP-CH, GTP cyclohydrolase; 6PTS, 6-pyruvoyl tetrahydropterin synthase; PAH, TYH, and TRH, phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase, respectively; DHNP, dihydroneopterin. (Reproduced from Scriver et al., 1988. Used by permission.)

Phenylalanine Hydroxylase

Physical properties

Many of the properties of PAH were first determined with enzyme purified from rat liver extracts (Kaufman and Fisher, 1970). The properties of the human liver enzyme are similar to those of the rat enzyme, with the exceptions mentioned below.

Rat enzyme

Essentially pure rat liver PAH appeared initially to be a mixture of two different polymeric forms. Based on a determination of their Stokes radii and sedimentation constants, molecular weights of 210,000 and 100,000 were calculated for the major and minor species, respectively. Since the molecular weight of the subunit(s) is about 49,000 to 51,000 (Kaufman and Fisher, 1970), the two forms are putative tetramers and dimers, respectively. When assayed with the synthetic pterin cofactor 6-MPH_4, both species had identical specific activities; when assayed with BH_4, the specific activity of the tetramer was five times that of the dimer. Preincubation of the hydroxylase with phenylalanine increases conversion of dimers to tetramers (Doskeland et al., 1982).

Human enzyme

When expressed in *E. coli* cells, the oligomeric composition of human phenylalanine hydroxylase at pH 6.8 is a mixture of tetramers and dimers (80:20 parts) of subunit size of approximately 52 kDa and with no evidence of higher aggregates (Kowlessur et al., 1996; Kaufman 1996). As with the rat liver enzyme, the proportion of dimeric human enzyme is augmented by increasing the pH to mildly alkaline values (Kappock et al., 1995). Preincubation of human phenylalanine hydroxylase with L-phenylalanine shifts the equilibrium in the direction of the tetrameric form (Martinez et al., 1995). The ability to determine distinctive catalytic properties of the two species and their ready separation during gel-permeation chromatography indicate that the dimer and tetramer are not in rapid equilibrium even under assay conditions.

Human phenylalanine hydroxylase resembles rat liver phenylalanine hydroxylase in being phosphorylated by cAMP-dependent protein kinase, phosphorylation resulting in activation (Kaufman, 1983). Recombinant human phenylalanine hydroxylase incorporates between 0.6 mol (Kowlessur et al., 1996) and 0.97 mol (Martinez et al., 1995) of phosphate per phenylalanine hydroxylase subunit, resulting in 1.5- and 1.2-fold activation, respectively, of the BH_4-dependent activity. This modest degree of human enzyme activation is less than the 2- to 4-fold activation observed with either rat liver (Abita et al., 1976; Donlon and Kaufman, 1977) or recombinant rat enzyme (Citron et al., 1992b).

An even more striking difference in regulatory properties between recombinant human phenylalanine hydroxylase and its rat counterpart has been observed with other known activators of the latter enzyme. Whereas preincubation with phenylalanine or lysolecithin activates the BH_4-dependent activity of the rat enzyme 8- and 25-fold, respectively, the human hydroxylase is activated only 2.2-fold by either of these treatments (Kowlessur et al., 1996). Similar results were reported for the uncleaved fusion products of human phenylalanine hydroxylase with the maltose-binding protein (Knappskog et al., 1996a).

The K_m for phenylalanine (measured with BH_4 as the cofactor) of recombinant human phenylalanine hydroxylase is 50 µM (Kowlessur et al., 1996), a value close to the normal level of phenylalanine in human plasma and notably lower than the K_m value (280 µM) for the recombinant rat liver enzyme (Citron et al., 1992b). The corresponding values for the human fusion protein and its cleavage products are closer to those of the recombinant rat hydroxylase (Martinez et al., 1995). Most of the other catalytic properties of human phenylalanine hydroxylase are similar to those of the rat enzyme (Knappskog et al., 1996a; Kowlessur et al., 1996; Martinez et al., 1995).

Just as was found with rat liver phenylalanine hydroxylase (Curtius et al., 1990; Davis et al., 1992), 7-tetrahydrobiopterin (7-BH_4) has cofactor activity with human recombinant phenylalanine hydroxylase (Kowlessur et al., 1996). Significantly, this pterin is also a potent inhibitor of the human enzyme when tested against the likely hepatic concentration of BH_4. Moreover, the inhibition shows the same peculiar pattern as the one that was originally reported for rat liver phenylalanine hydroxylase, i.e., greater inhibition at higher phenylalanine concentrations (Davis et al., 1992) with 50 percent inhibition by 5 µM 7-BH_4 occurring at 100 µM phenylalanine. Based on the inhibition of rat phenylalanine hydroxylase by 7-BH_4, it was proposed that this inhibition could account for the mild HPA observed in patients with suspected deficiency of 4α-carbinolamine dehydratase that excrete 7-BH_4 (Davis et al., 1992). The demonstration that HPA patients who excrete 7-BH_4 are indeed deficient in the dehydratase (Citron et al., 1993), together with the evidence that this pterin is a potent inhibitor of human phenylalanine hydroxylase (Kowlessur et al., 1996), strongly supports the proposed mechanism for the HPA seen in dehydratase-deficient patients.

Human phenylalanine hydroxylase is only moderately stimulated by the several agents (e.g., phenylalanine and lysolecithin) that markedly activate rat hydroxylase; this finding suggests that the human enzyme is in a relatively activated state (Kowlessur et al., 1996). If these unusual *in vitro* properties of the human enzyme *in vitro* accurately reflect the enzyme's state of activation *in vivo*, it would mean that some earlier notions about phenylalanine homeostasis in humans that were derived from properties of rat liver phenylalanine hydroxylase would have to be modified. At resting blood and hepatic levels of phenylalanine, the rat enzyme has very low activity, but it is poised to be massively activated in response to a physiologic demand, such as that resulting from eating a protein-rich meal (Donlon and Beirne, 1982) or from glucagon-stimulated phosphorylation activation (Beirne et al., 1985; Donlon and Kaufman, 1977). By contrast, the properties of recombinant human phenylalanine hydroxylase indicate that its basal activity is relatively high even without phenylalanine- or glucagon-mediated activation. This higher basal activity of human enzyme may explain why the mean normal plasma phenylalanine concentration in adult human subjects is 58 µM (Scriver et al., 1985) and in the rat is 96 µM (Delvalle and Greengard, 1976). Based on site-directed mutagenesis, the structural basis for the relatively activated state of human phenylalanine hydroxylase can be explained on the basis of a cysteine residue being present at residue 29 in human PAH, whereas a serine occurs at that position in rat PAH (Wang et al., 2001).

The human phenylalanine hydroxylase subunit consists of 452 amino acids (Kwok et al., 1985). The initial breakthrough on the structural biology of phenylalanine hydroxylase came when the structure of the catalytic domain of human enzyme was determined by x-ray crystallography (Erlandsen et al., 1997b); it was quickly followed by further information including the regulatory and tetramerization domains (reviewed in Erlandsen and Stevens, 1999; Flatmark and Stevens, 1999). (See Supplement: "Structural Studies of Phenylalanine Hydroxylase Enzyme"; also see http://stevens.scripps.edu/pkuweb/tsld001.htm.) Despite the enforced use of truncated species of protein to generate crystals, these findings have provided unique insights into the way the enzyme functions and how various mutations affect integrity and function. However, it should be borne in mind that the properties of the truncated species of the enzyme differ from those of the intact protein in several important respects. First, the crystallized truncated species lacks the N-terminal regulatory domain, and the N-terminus as a whole is important for maintaining rat PAH in the optimal catalytic conformation (Wang et al., 2001); the formation of an Arg-13 to Ser-16 phosphate salt bridge and the resulting conformational change of the N-terminal tail also can explain the higher stability toward limited tryptic hydrolysis of the phosphorylated human enzyme (Miranda et al., 2002). Second, as originally shown for the rat liver enzyme (Fisher and Kaufman, 1973; Iwaki et al., 1986), proteolytic removal of 11 kDa (approximately 100 amino acid residues) from the N-terminus stimulates by approximately 30-fold the BH_4-dependent activity of the protease-resistant catalytic domain that is located toward the central portion of the molecule. As a result, the isolated remaining species can no longer be activated by preincubation with L-phenylalanine. Further recent results for recombinant rat enzyme (Horne et al., 2002) support the model whereby on phenylalanine binding, the mobile N-terminal residues associate with the folded core of PAH, and phosphorylation may facilitate this interaction. Third, results of limited proteolysis of rat hydroxylase (Iwaki et al., 1986) show that removal of a 5-kDa portion (approximately 45 amino acid residues) from the C-terminus does not lead to activation, destroys the enzyme's ability to form tetramers, eliminates the cooperative binding of phenylalanine, and implicates the C-terminus in the formation of tetramers from two dimers. The properties of the engineered truncated species of human phenylalanine hydroxylase used in the crystallization studies are coherent with these earlier results of studies of the rat liver enzyme. The truncated human enzyme occurs almost exclusively as dimers and does not show positive cooperativity of binding of phenylalanine (Knappskog et al., 1996a); the stretch of amino acids that determines these two properties had been narrowed to 25 residues at the C-terminus. Dimerization of phenylalanine hydroxylase monomers is mediated by the interaction of two symmetry-related loops (residues 414–420) located close to the C-terminus of the crystallized truncated species (Erlandsen et al., 1997b).

With further regard to structural and stability effects of phosphorylation, nature does not use aspartate (D) or glutamate (E) at position 16 but an autoregulatable, phosphorylatable serine. The negative charge on the side chain at that residue owing to phosphorylation is the key, analogous to other phosphoproteins. As discussed earlier, phosphorylation results in a salt bridge between Arg13 and Ser16, and futher support for limited conformational change localized to the region of the phosphoserine has been provided (Miranda et al., 2004). This study also provides an explanation for the resistance to tryptic proteolysis (at Arg13) in the modified protein owing to interaction with phosphoserine. All in all, it does seem plausible that the local conformational change induced by phosphorylation has consequences for the turnover of the enzyme in vivo, in addition to short-term reversible activation and activation by phenylalanine.

Rat liver phenylalanine hydroxylase contains one atom of iron per subunit. The iron is essential for catalytic activity (Parniak and Kaufman, 1981; Fisher et al., 1972). The Fe^{3+} atom sits in a crevice 10 Å below the surface of the protein on the floor of the active center. It is coordinated to residues H285 and H290 and one oxygen atom in residue E330 (Erlandsen and Stevens, 1999). Through the use of site-directed mutagenesis, the two equivalent histidine residues in rat phenylalanine hydroxylase had been shown previously to be necessary for iron binding (Gibbs et al., 1993). A close-up of the active site surrounding the iron also showed three water molecules liganded to the iron (Erlandsen and Stevens,

1999). The advent of the structural information described earlier (Erlandsen and Stevens, 1999), coupled with x-ray absorption spectroscopy (Wasinger et al., 2002) and hybrid density functional theory (Bassan et al., 2003), has yielded insights into the role of iron in the mechanism of action of PAH. When both substrate and cofactor are bound, water is lost from the ferrous active site of the enzyme. This opens a coordination center in the iron(II) center for the reaction with O_2 to generate an active intermediate for the direct, coupled hydroxylation of the cosubstrates.

Some PKU mutations are associated with residues in the region of the iron at the active site, i.e., T278, E280, P281, and F331. Single-point mutations in the *PAH* gene, not including silent and missense mutations, have been mapped onto the protein (Erlandsen and Stevens, 1999). The effects of these mutations, which can range from mild to severe HPA phenotypes, now can be interpreted in the context of their effects on the structure/conformation of the resulting protein. Four cofactor-binding regions have been identified in the vicinity of the active-site iron (Erlandsen and Stevens, 2001). Some residues adjacent to cofactor-binding motifs are also associated with mutations resulting in recently discovered mild or variant forms of HPA that are responsive to BH_4 (Kure et al., 1999).

More recently, it has been observed (Miranda et al., 2005) that Tyr325 in human PAH appears to have an important role in ensuring stoichiometric binding of iron, correct geometry of the complexes with substrate and cofactor, and a favorable coupling efficiency of the PAH reaction. That amino acid residue also appears to be important for the correct cooperative regulation by L-phenylalanine.

Two mutations together account for almost 50 percent of PKU patients in the northern European population; they are both in the C-terminal end of the catalytic domain. The splice defect allele (IV12nt1g→a) deletes exon 12 (Marvit et al., 1987) and interferes with tetramer formation; when expressed in COS cells, the allele is a null, mainly owing to instability of the protein (DiLella et al., 1987; Waters et al., 2000). The other common mutation, R408W, is located at the start of the tetramerization helix; replacement of the arginine by the larger tryptophan residue could interfere with proper folding of the tetramer. When expressed in COS cells, it has less than 1 percent wild-type activity (DiLella et al., 1987; Waters et al., 1998), the level of immunoreactive phenylalanine hydroxylase is comparably low, and the low activity results from protein instability, at least when expressed in COS cells (Okano et al., 1991; Waters et al., 1998).

Regulation of Phenylalanine Hydroxylase

Human liver PAH and rat liver PAH have some significant differences in their regulated properties, and the following should be read with this in mind. Since PAH catalyzes the rate-limiting step in the major pathway by which phenylalanine is catabolized to CO_2 and water and thus has the highest sensitivity coefficient (Kacser and Burns, 1981) for metabolic runout of phenylalanine (see Fig. 77-2), it is a likely site for regulation of phenylalanine homeostasis (Milstien and Kaufman, 1975). The enzyme can play this role because its catalytic activity is exquisitely sensitive to changes in concentrations of its substrate, phenylalanine. This sensitivity ensures that exposure of tissues to high levels of phenylalanine will be kept to a minimum, and it also ensures that the hydroxylase-catalyzed conversion of phenylalanine to tyrosine will not lead to depletion of phenylalanine to the point where normal protein synthesis is compromised.

This delicate balance is accomplished by a synergistic interaction between two types of regulating mechanisms: activation by phenylalanine and activation/deactivation by phosphorylation/dephosphorylation. Together they accommodate short-term regulation of PAH activity. These mechanisms enable a more responsive coupling between hydroxylase activity and tissue levels of

phenylalanine than could be achieved by an enzyme having simple Michaelis-Menten kinetics. The Michaelis-Menten relationship describes a rectangular hyperbolic response in the initial velocity to variation in substrate concentration; it shows that activity of the enzyme is geared to availability of substrate. While it may constitute an adequate regulatory mechanism at substrate concentrations at or below K_m values, it is a relatively insensitive coupling device at higher substrate concentrations.

Regulation by substrate and cofactor

The first evidence for short-term regulation of rat liver PAH was the 20- to 30-fold increase in the BH_4-dependent activity on brief exposure to a phospholipid such as lysolecithin (Fisher and Kaufman, 1972, 1973); by contrast, activity of the enzyme in the presence of $DMPH_4$, a synthetic pterin cofactor, was only slightly increased by lysolecithin treatment (Fisher and Kaufman, 1972). The sigmoid relationship between initial velocity and phenylalanine concentration in the presence of BH_4 changed to hyperbolic with lysolecithin (Fisher and Kaufman, 1972, 1973). Diverse treatments of PAH such as limited proteolysis (Fisher and Kaufman, 1973) and alkylation of a single sulfhydryl group (Parniak and Kaufman, 1981) also markedly increased the BH_4-dependent hydroxylase activity. In the presence of BH_4, PAH is predominantly in a low-activity form, expressing only 3 to 5 percent of its potential activity.

Although there is no evidence to indicate that any of the aforementioned modes of activation are of physiologic significance, they delineate some of the characteristics of the activated hydroxylase. Activation by substrate is probably involved in acute physiologic regulation of PAH, a process discovered first when 6-MPH_4 was used to assay the enzyme (Nielsen, 1969) and then when BH_4, the natural cofactor, was used (Kaufman, 1970). This activation process was further investigated later (Phillips and Kaufman, 1984; Shiman and Gray, 1980). In the presence of BH_4, the results of these studies can be accommodated by a single model that depicts PAH in equilibrium between a low-activity conformation E and an active conformation E′ (Phillips and Kaufman, 1984):

[IMAGE]

[IMAGE]

According to this formulation, the E′ conformation of PAH can be stabilized by the binding of phenylalanine to a regulatory site, whereas the E conformation can be stabilized by the binding of BH_4 in the absence of or prior to phenylalanine binding. In contrast to the natural coenzyme BH_4, synthetic analogues such as $DMPH_4$ are not effective in pushing the equilibrium in the direction of E; hence more of the enzyme would exist as E′.

The preceding model assumes that there is a second site distinct from the catalytic site that binds phenylalanine, which, when occupied, leads to activation. Phenylalanine increases the binding of PAH to a hydrophobic matrix (Shiman et al., 1979), and it changes the fluorescence of the enzyme (Phillips et al., 1984). These observations support the model because they imply that phenylalanine changes the conformation of the enzyme. The amount of phenylalanine bound by rat liver hydroxylase is 1.5 mol phenylalanine per 1 mol PAH subunit (Parniak and Kaufman, 1981); this finding provides direct experimental support for the existence of a second phenylalanine-binding site.

Studies of the inactivation of rat liver phenylalanine hydroxylase by bombarding it with high-energy electrons have provided additional structural details about how the pterin cofactor and phenylalanine interact to regulate the activity of the enzyme. From the loss of activity as a function of radiation dose, it was shown that the target size or minimum mass necessary for the 6-MPH_4-dependent activity is the dimer, with radiation causing physical destruction of one monomer at a time. By contrast, low doses of irradiation actually increase the BH_4-dependent activity; this phase is followed by a decrease in activity at higher doses, with the target size again corresponding to a dimer. The results with BH_4 support the important conclusion that this pterin inhibits or inactivates tetramers but not dimers and that a radiation hit in any part of the tetramer relieves the inhibition, resulting in activation at low doses of radiation (Davis et al., 1996).

Parallel studies of the radiation-induced loss of the phenylalanine-activated hydroxylase indicate that phenylalanine increases the interactions between the subunits in a dimer and weakens the interactions between dimers in a tetramer. Furthermore, pretreatment of the enzyme with phenylalanine prevents the increase in BH_4-dependent activity seen at low doses of radiation, which appears to require a tetrameric structure. Phenylalanine activation, therefore, appears to be due in part to its ability to remove the BH_4-mediated inhibition of the activity of tetramers (Davis et al., 1997).

In relation to substrate activation and negative regulation by the cofactor, binding parameters and the conformational states of the enzyme have been studied using isothermal titration calorimetry (Pey et al., 2004; Pey and Martinez, 2005) and molecdular dynamic simulations (Teigen et al., 2003, 2004). These studies have thrown further light on the conformation of the N-terminal autoregulatory region in the presence and absence of phenylalanine and on the inhibition caused by specific interactions of the dihydroxypropyl side chain of the cofactor (see Fig. 77-9).

Regulation by phosphorylation/dephosphorylation of subunits

Activity of the hydroxylase is increased severalfold by phosphorylation, a reaction catalyzed by cAMP-dependent kinase (Abita et al., 1976). Activation by phosphorylation is fully expressed when the enzyme is assayed in the presence of BH_4 but not in the presence of $DMPH_4$ or 6-MPH_4. Activation is accompanied by the incorporation of about 0.70 mol inorganic phosphate per subunit M_r = 50,000. Because less than stoichiometric amounts of phosphate are incorporated into the pure hydroxylase *in vitro*, it seems likely that PAH isolated from rat liver is already partially phosphorylated, and indeed, five different preparations of the native enzyme had an average of 0.31 mol (range 0.23–0.42 mol) phosphate per 1 mol of subunit M_r = 50,000. The phosphorylated form of human hepatic PAH is recognized by a monoclonal antibody, and this reagent was used to show that binding of antibody correlates closely with the phosphorylation state of PAH in crude cell extracts from rat liver (Green et al., 1990). It also was shown that dibutyryl cAMP stimulates phosphorylation of PAH in isolated rat kidney tubules.

The amino acid sequence at the serine [^{32}P]phosphorylation site of rat liver phenylalanine hydroxylase is SRK[^{32}P]SNFGQQ (Wretborn et al., 1980). The amount of this peptide is at least twice that calculated from the radioactivity of the sample, implying that it contains a substantial amount of endogenous phosphate. The finding provides independent evidence that hepatic PAH, in untreated rats, is a mixture of phosphorylated and nonphosphorylated forms. It also provides an explanation for an earlier claim (Barranger et al., 1972) that rat PAH exists as three isozymes (designated *pi*, *kappa*, and *upsilon*). Chemical analysis showed that the preparation contained different amounts of protein-bound phosphate, with the predominant form corresponding to the monophosphorylated tetramer (M_r = 200,000) and the second most prevalent form corresponding to the diphosphorylated tetramer (Donlon and Kaufman,

1980). The catalytic properties of the two forms of PAH were fully consistent with their states of phosphorylation: Relative PAH activity (in the presence of BH_4) was higher for the diphosphorylated tetrameric form than for the monophosphorylated species. These results indicate that the major, if not the sole, structural determinant for elution time of different forms of PAH on a calcium phosphate column (the method used for isolation of these multiple forms) is the amount of protein-bound phosphate in PAH. Phosphoprotein phosphatase 2A catalyzes the dephosphorylation of rat hepatic PAH (Ingebritsen and Cohen, 1983; Jedlicki et al., 1977).

Activation of rat liver PAH by phosphorylation has physiologic significance. Glucagon causes fourfold activation of rat hepatic PAH *in vivo* (Donlon and Kaufman, 1978) and *in vitro* (Abita et al., 1980). The effect is rapid and transient, it involves phosphorylation (Carr and Pogson, 1981; Donlon and Kaufman, 1978; Garrison and Wagner, 1982), and it can be elicited by repeat injections, implying that decay of the activated state is not due to proteolytic degradation of the hydroxylase. The effect of glucagon is detectable when PAH is assayed in the presence of BH_4 but not in the presence of synthetic cofactor analogues such as $DMPH_4$.

Glucagon and insulin have broadly opposing metabolic effects *in vivo*. Insulin depletion increases *in situ* phenylalanine hydroxylation activity in rats (Santana et al., 1985). Addition of glucagon to hepatocytes from diabetic rats further stimulates this elevated activity (Carr and Pogson, 1981). These results were confirmed in studies of PAH activity in liver extracts from rats 3 days after the onset of diabetes (Donlon and Beirne, 1982); PAH appeared to be more highly phosphorylated in diabetic livers. The diabetic state increases not only the BH_4-dependent PAH activity but also the $DMPH_4$-dependent (and 6-MPH_4-dependent) activity. The increased PAH activity seen in diabetic rats is due not only to an increased degree of phosphorylation but also to an increased amount of hydroxylase protein (Guerin et al., 1968).

Studies in cultured cells elucidate further the multiple forms of PAH. Normal adult rat liver contains different forms of PAH in different states of phosphorylation (Donlon and Kaufman, 1977, 1978), with different isoelectric points (Tourian et al., 1975). The form corresponding to half-phosphorylated tetramersx, designated *form III* (Donlon and Kaufman 1977, 1980), has an isoelectric point of 5.60; form II is the most and form I the least prevalent. H_4 hepatoma cells contain a single form of the hydroxylase (Miller and Shiman, 1976; Tourian, 1976) that is similar in its behavior to the half-phosphorylated tetramers. By immunochemical criteria, the single species of PAH in hepatoma cells is distinct from the three forms present in normal adult rat liver and the single form in rat kidney (Tourian, 1976; Tourian et al., 1975). Treatment of hepatoma cells with hydrocortisone selectively "induces" the expression of the two forms that are present in adult liver but missing in hepatoma cells (Miller and Shiman, 1976); i.e., the pattern after hydrocortisone treatment of cultured cells is similar to that in rat liver. The notion that hydrocortisone induced the expression of different hydroxylase isozymes is not relevant because the so-called isozymes (Barranger et al., 1972) differ only in their states of phosphorylation (Donlon and Kaufman, 1980). The hydrocortisone effect is a complex one involving some kind of posttranslational modification of the enzyme, in addition to its effect on the amount of the enzyme in the cells.

Effect of ligands on PAH

Phenylalanine and BH_4 have opposite effects on the rate of phosphorylation and activation of purified rat liver PAH. Significant concentrations of the naturally occurring 6-*R* diastereoisomer of BH_4 (6–8 μM) inhibit phosphorylation and activation by 80 percent, whereas 200 μM L-phenylalanine stimulates both processes to a modest extent; phenylalanine can completely overcome the inhibition caused by BH_4

(Phillips and Kaufman, 1984). Inhibition of phosphorylation is quite specific for the 6-*R* diastereoisomer of BH_4; relatively large concentrations of 6-MPH_4 or $DMPH_4$ do not inhibit it (Doskeland et al., 1984; Phillips and Kaufman, 1984). The phosphorylated PAH requires less phenylalanine to be activated than does the nonphosphorylated form (Doskeland et al., 1984; Shiman et al., 1982): 29 and 51 μM phenylalanine were required to obtain half-maximal activation of the phosphorylated and nonphosphorylated forms, respectively. These findings are again consistent with the notion that the enzyme exists as an equilibrium mixture of high-E′-activity and low-E-activity conformations. Inhibition of phosphorylation in the presence of BH_4 and stimulation in the presence of phenylalanine imply that the active form of PAH (E′) is a better substrate for phosphorylation than the low-activity form (E).

It seems likely that the opposing effects of BH_4 and phenylalanine on direct activation of the enzyme and on kinase-mediated activation are a dominant feature of the physiologic regulation of hepatic PAH. In the case of the kinase reaction, the effect of BH_4 could be to limit the extent of phosphorylation, and thus activation, when the levels of hepatic phenylalanine are very low and high hydroxylase activity is not required. This would be true under basal conditions. Higher concentrations of phenylalanine then would be able to overcome the inhibitory effect of BH_4, allowing an increase in the extent of phosphorylation and activation of the hydroxylase when the organism needs higher hydroxylase activity to catabolize excess phenylalanine. This inhibitory effect of BH_4 then would serve to protect against depletion of the organism's pool of phenylalanine below essential levels.

Activation of PAH by phosphorylation and phenylalanine are probably synergistic modes of regulation. Phosphorylation (and activation) by cAMP-dependent protein kinase is stimulated by phenylalanine (Doskeland et al., 1984; Phillips and Kaufman, 1984), whereas phosphorylation sensitizes the enzyme to activation by phenylalanine (Carr and Pogson, 1981; Doskeland et al., 1984; Shiman, 1980). A useful adaptive consequence of these interlocking control mechanisms is enhanced responsiveness of PAH activity to altered levels of phenylalanine.

Pancreatic glucagon secretion is stimulated by protein feeding (Muller et al., 1970). Blood glucagon increases in association with the postprandial rise in blood amino acids, and amino acids are potent stimulators of pancreatic glucagon release (Guttler et al., 1978; Rocha et al., 1972). Since glucagon activates hepatic adenylate cyclase with an increase in hepatic cAMP levels, it follows that activation of cAMP-dependent protein kinase and phosphorylation-mediated activation of PAH will attend protein feeding. A consequence of this regulatory response to a postprandial rise of blood phenylalanine is accelerated catabolism of the amino acid to maintain homeostasis. Since phenylalanine is also a glycogenic amino acid (see Fig. 77-2), activation of phenylalanine hydroxylase also may be geared to increased gluconeogenesis. This limb of the regulatory process would be coupled to the blood glucose level. A fall in blood glucose would increase glucagon release and suppress insulin release, resulting in phosphorylation-mediated activation of PAH with a resulting gluconeogenic effect.

Dihydropteridine Reductase

(This enzyme and its role in BH_4 metabolism are discussed in more detail in Chap. 78.) DHPR, an essential dimeric enzyme in the hydroxylating systems for phenylalanine, tyrosine, and tryptophan (Fig. 77-11), is widely distributed in animal tissues (Craine et al., 1972). Whereas its occurrence in brain and adrenal medulla is not surprising in view of its role in the tyrosine hydroxylation system in these tissues and in the tryptophan hydroxylation system in brain, why DHPR should be found in tissues such as heart and lung, which have little or no aromatic amino acid hydroxylating activity, is obscure. Its wide tissue distribution, together with BH_4, hints at undiscovered roles for both BH_4 and DHPR. For example, BH_4 is

the cofactor for nitric oxide synthase (Tayeh and Marletta, 1989), an important enzyme active in lung, and recycling of BH_4 may be required there (see Chap. 78). In addition to its role in regenerating BH_4, it has been proposed that DHPR plays an ancillary role (together with dihydrofolate reductase) in brain to keep folate in the tetrahydro form (Pollock and Kaufman, 1978). DHPR deficiency is a cause of human HPA (Kaufman et al., 1975a), so it should be considered in the diagnostic investigation and treatment adapted accordingly (see Chap. 78).

Biosynthesis of BH_4

(This topic and its relevance to HPA are described in further detail in Chap. 78.) The cofactor function of BH_4 in the hydroxylation reaction with aromatic amino acids is related to its ability to reduce molecular oxygen; BH_4 provides electrons and, in turn, is oxidized to qBH_2 (Kaufman, 1971, 1997). The consumption of BH_4 is stoichiometric during hydroxylation of substrate. Whereas the DHPR-catalyzed reaction regenerates (recycles) BH_4 from qBH_2 moment by moment, so the cofactor functions catalytically, the steady state of BH_4 ultimately depends on biosynthesis from precursors. Its fundamental role in cellular biochemistry and pathogenesis of disease is a large topic (Kaufman, 1997; Thony et al., 2000; Werner-Felmayer et al., 2002); its specific relevance to the human HPAs is the subject of Chap. 78.

As outlined earlier, a dominant theme in the acute regulation of phenylalanine hydroxylase is the balance between the activating effect of phenylalanine and the deactivating effect of BH_4. A variation of this theme also operates to regulate the mammalian biosynthesis of BH_4 and explains how phenylalanine itself controls the synthesis of BH_4 through a regulatory protein that interacts with guanosine triphosphate cyclohydrolase (GTP-CH). All three genes involved in the *de novo* biosynthesis pathway yielding BH_4 have been cloned, sequenced, and mapped to chromosomes. There is evidence that elevated amounts of the end products of hydroxylase-dependent tryptophan and tyrosine pathways downregulate BH_4 synthesis.

PHENOTYPIC EFFECTS OF *PAH* MUTATIONS

Comment

A disease-causing *PAH* mutation has a measured effect at three levels of the phenotypic components that compose the "phenome": (1) at the enzyme level—on enzyme integrity and function (a proximal level)—and (2) at the nonenzymic levels—(a) on phenylalanine homeostasis and thus on its concentration in body fluids (the intermediate level) and (b) on the brain, its function, and cognitive development (a distal level). The likelihood that categorical genotype-phenotype correlations can be found at all phenotype levels of this autosomal recessive trait is confounded by the extent of allelic heterogeneity, the complexity in the buffering that results in phenylalanine homeostasis, and the fact that the distal phenotype (IQ) is itself a complex trait (Scriver and Waters, 1999). We discuss the relationships first at the most proximal level (PAH enzyme), then at the most distal level (brain), and finally at the intermediate level, where phenylalanine appears to be the mediator between a mutant *PAH* genotype and its association with hazard to cognitive development and function. A better understanding of the related phenome (Freimer and Sabatti, 2003) and how it can be perturbed is necessary to understand fully the *disease* called PKU.

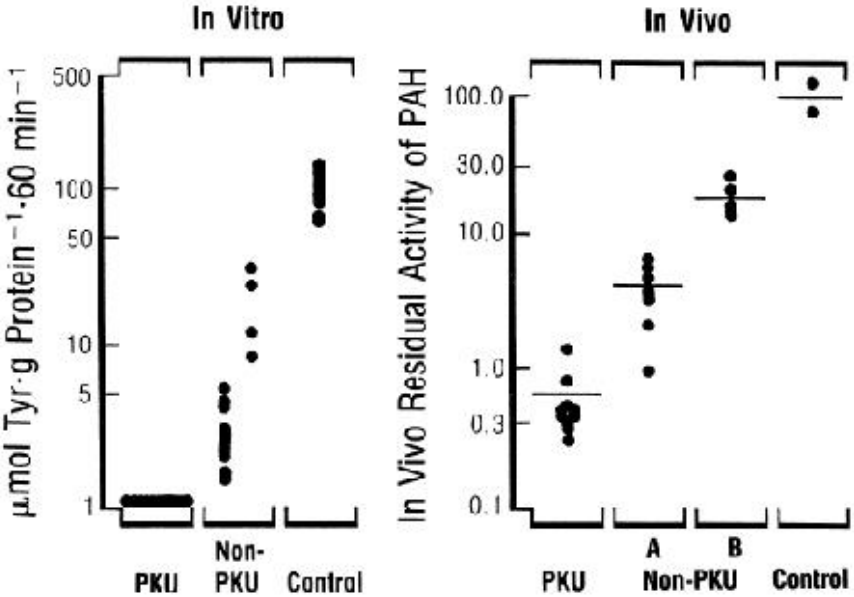
Effect of *PAH* mutation on enzymic (hydroxylating) function

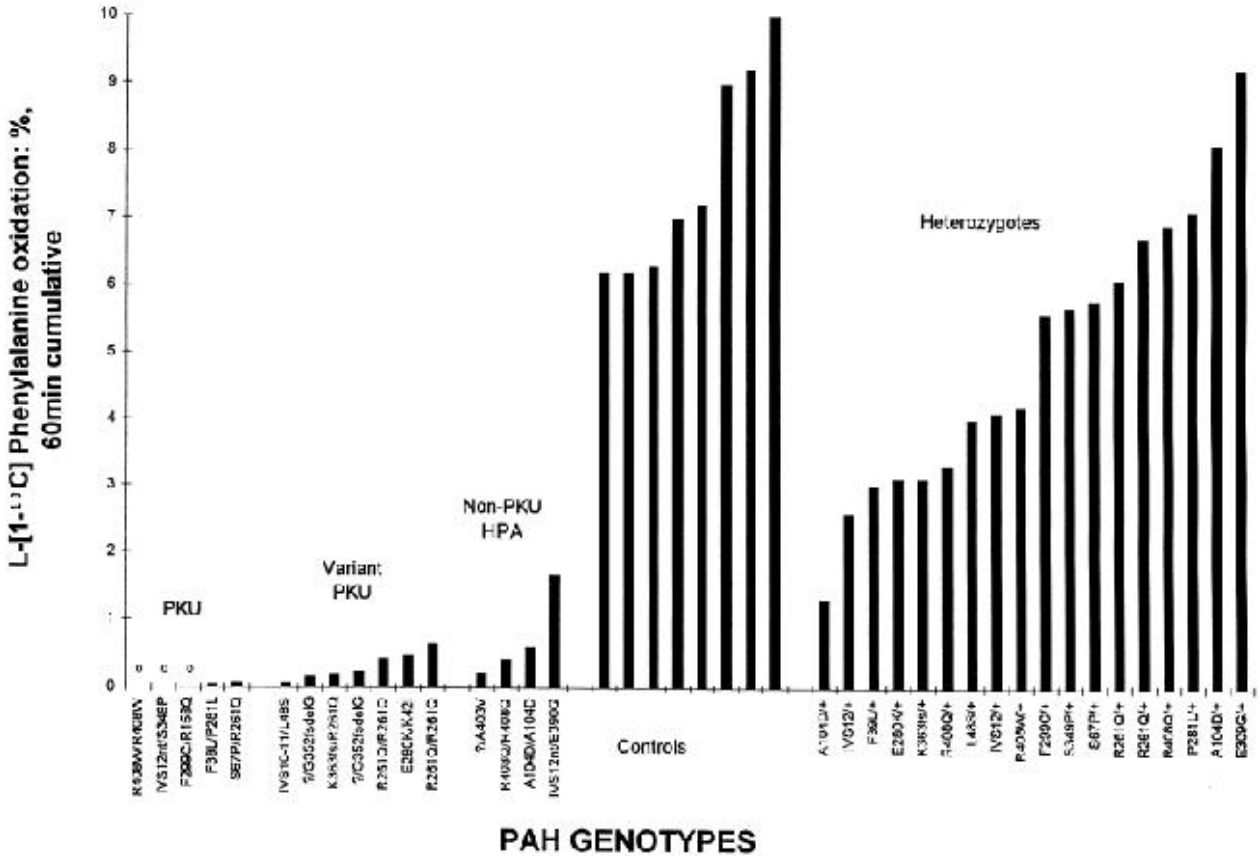
The effect of *PAH* mutations on PAH protein function can be assessed either directly or indirectly by three quite different approaches: (1) by enzyme assay on a tissue biopsy sample *in vitro*, (2) by an isotopic method *in vivo*, and (3) by gene expression analysis *in vitro*. The first two methods measure the effect of mutant genotype (as homoallelic or heteroallelic forms of homozygosity or in the heterozygote) on enzymatic function in patients, whereas the third measures the effect of a particular allele placed in a recombinant gene construct on homopolymeric PAH (enzyme) activity.

Hepatic Enzyme Activity in Biopsy Samples

Several early studies using the relatively crude assay of the day identified deficient enzymatic activity by direct assay on liver samples from PKU patients (Mitoma et al., 1957; Udenfriend and Bessman, 1953; Wallace et al., 1957). Corroboration with more refined assays followed (Choo et al., 1979; Cotton, 1977; Kaufman, 1958). These and other studies identified cross-reacting material (CRM+ and CRM–) hepatic enzyme phenotypes in PKU (Bartholomé and Dresel, 1982; Choo et al., 1979, 1980; Yamashita et al., 1985). Very low enzyme activity with apparently normal affinity for substrate also was observed (Friedman et al., 1973, 1972b). The largest single study of hepatic PAH enzyme activity (Bartholomé et al., 1975) used a reliable assay on liver biopsy material obtained from a group of very well-monitored patients with HPA; patients with typical PKU had less than 1 percent normal activity, whereas those with non-PKU HPA had more enzymatic activity, usually more than 5 percent of normal (Fig. 77-12). This particular study also showed that impaired hydroxylating activity and primary deficiency of PAH enzyme integrity are not synonymous; some patients who had HPA *in vivo* and yet had "intact" phenylalanine hydroxylating activity *in vitro* actually had a primary disorder of BH_4 cofactor metabolism (Bartholomé et al., 1975). Another variant of deficient PAH enzyme activity owing to mutation at the *PAH* locus, noteworthy because it is BH_4-responsive, has been discovered. In this form, a primary *PAH* gene allele alters PAH protein in some way that is correctible with pharmacologic doses of BH_4 *in vivo* (www.pahdb.mcgill.ca; see the section on BH_4-responsive HPA below).

Figure 12:





Measured activity of phenylalanine hydroxylase enzyme. Top: In vitro on hepatic needle-biopsy material (left), in vivo by isotope infusion (right). Bottom: Measured in vivo by ingestion of isotope and analysis of expired 13CO2. The in vivo data (top) were obtained by infusion of heptadeuterated phenylalanine followed by measurement of the rate of tyrosine labeling at steady state. The in vivo assay in the bottom figure measures cumulative expired 13CO2 for 80 min after ingestion of a tracer dose of L-[1-13C]phenylalanine; propositi in this study were persons with phenylketonuria, variant PKU, and non-PKU HPA and the corresponding heterozygotes of known genotype (indicated on abscissa). The figure is a composite of data from three sources (Bartholomé et al., 1975; Trefz et al., 1981; Treacy et al., 1997).

Interindividual variation in hepatic enzyme activity in PKU patients (Bartholomé et al., 1975) has been attributed largely to allelic heterogeneity (Bartholomé et al., 1984). The issue of *PAH* allelism and possible allelic interaction, usually of negative type, at the polypeptide level came to prominence in several studies. For example, propositi with non-PKU HPA had *in vitro* hepatic enzyme activity approximately 5 percent of normal, and their parents had activity approximately 3 percent of normal (mean 6 subjects), which in the latter is a level much lower than the expected half-normal value for heterozygotes (Kaufman et al., 1975b). Some obligate heterozygotes for the PKU phenotype again had enzyme activity well below expectation (14–44 percent of normal) (Bartholomé, 1979; Bartholomé and Dresel, 1982; Grimm et al., 1977). The deviant gene-dose effects *in vitro* seen in these subjects imply negative cooperativity at the subunit level of PAH enzyme, perhaps a dominant negative mutation effect. Whether this unusual dose effect also occurs *in vivo* is not yet known. Meanwhile, one must question why heterozygotes with low activity *in vitro* would not have HPA and why patients with considerable activity *in vitro* nonetheless have HPA. One could say that the HPA phenotype, a so-called Mendelian trait, has the attributes of a complex trait *in vivo* (Scriver and Waters, 1999); there is much yet to be learned about the buffering processes and the boundary conditions for this or any other metabolic phenotype (Scriver, 2002; Strohman, 2002).

Isotopic Studies in Vivo

Phenylalanine hydroxylating activity can be estimated by an indirect assay *in vivo* following intravenous infusion of isotopically labeled substrate: The rate at which labeled phenylalanine is converted to tyrosine is measured in plasma (see Fig. 77-12) (Clarke and Bier, 1982; Curtius et al., 1972; Lehmann et al., 1983; Lehmann and Heinrich, 1985; Milstien and Kaufman, 1975; Trefz et al., 1978, 1981; Van Spronsen et al., 1998). Phenylalanine hydroxylating activity measured in this way is clearly deficient in the patient with a PKU phenotype (Scriver, 1998a; Van Spronsen et al., 1998). There was earlier conflicting evidence (Thompson and Halliday, 1990) that now can be explained. The discrepancy between earlier and recent findings is the subject of an editorial (Scriver, 1998a) accompanying a paper by van Spronsen and colleagues (1998). A reader (Scott C Denne, personal communication, September 11, 1998) of the editorial and the article explained how a technical artifact accounts for the difference between earlier findings and the new data. In the earlier paper (Thompson and Halliday, 1990), d_2-tyrosine was used to measure the tyrosine Ra, whereas van Spronsen used [^{13}C]-labeled tyrosine. The different methods have consequences for the background shift effect in the measurements by mass spectrometry. A larger magnitude of background shift effect would account for essentially all the apparent phenylalanine hydroxylation measured in the earlier study. Accordingly, the apparent flux through the hydroxylation reaction reported in the earlier study was an artifact of method and measurement. In other studies, the findings *in vivo* have been corroborated by direct measurements *in vitro* of hepatic enzyme activity in biopsy material (Trefz et al., 1981). The *in vivo* method itself is further validated by evidence for regulated adaptation of PAH enzyme activity under physiologic conditions (Clarke and Bier, 1982).

Phenylalanine hydroxylating activity (more precisely, the *oxidation rate*) also can be assayed *in vivo* as a flux through the whole pathway by a noninvasive approach where [1-^{14}C] or [1-^{13}C]-labeled phenylalanine (Lehmann et al., 1986; Treacy et al., 1997; Muntau et al., 2002) is ingested, and labeled CO_2 is measured in expired air. This approach has the virtue of simplicity. It shows the predicted gene-dose effect (see Fig. 77-12), it confirms by an independent approach that *hydroxylating* activity is deficient in PKU patients, and it is sensitive enough to distinguish between the two phenotype classes (PKU and non-PKU HPA phenotype) (Treacy et al., 1997), although it is not sufficiently sensitive to correlate a particular mutant genotype with phenotype within its major class.

Nonisotopic Studies *in Vivo*

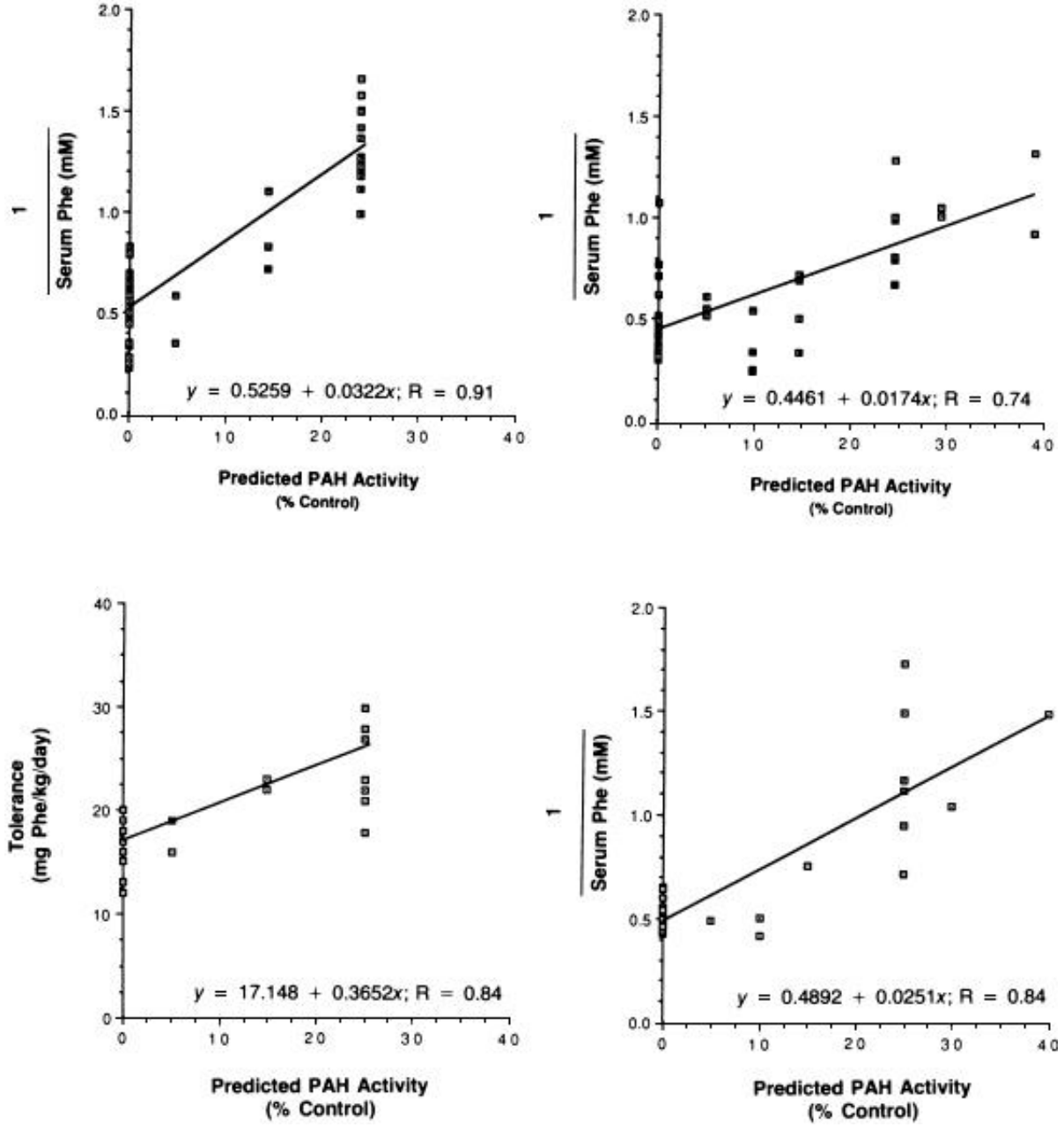
A nonisotopic method that involves an oral load of phenylalanine (0.6 mmol/kg of body weight) and several blood samples taken over 72 hours has been devised to assess PAH activity *in vivo* (Guldberg et al., 1995). Patients with genotypes predicted to harbor severe, intermediate, or mild *PAH* alleles manifest corresponding PKU, variant PKU, and non-PKU HPA phenotypes in the rates at which they clear the load from plasma. The observed rates were concordant with the clinical classification based on pretreatment plasma phenylalanine values and daily diet phenylalanine tolerance (Güttler, 1980). The method, of course, measures all parameters of phenylalanine runout and not PAH enzyme activity alone; that is, it measures both complex and Mendelian phenotypes.

In Vitro Expression Analysis

Yet another way to demonstrate the effect of *PAH* alleles on the corresponding hydroxylating activity is by expression analysis *in vitro*. This method was used early in the study of PKU alleles to analyze the effect of the human R408W allele (DiLella et al., 1987). An expression vector [p91023(B)] containing the mutant *PAH* cDNA, created by site-directed mutagenesis, and a plasmid construct containing normal cDNA sequence were separately transfected into cultured mammalian (COS) cells; PAH enzyme activity was then assayed in the presence of synthetic cofactor (6-MPH_4). When related to wild-type activity, the expression levels for R408W-mutant enzyme activity and for PAH immunoreactive protein were less than 1 percent, whereas they were 100 percent for mRNA. When the R408W allele was reanalyzed in a different expression system in the presence of natural cofactor (BH_4), the original findings were corroborated (Svensson et al., 1992).

In a landmark study (Fig. 77-13), the molecular basis of phenotype heterogeneity in PAH-deficient HPA was analyzed for several different alleles by expression analysis *in vitro*, the results being correlated with the metabolic phenotype *in vivo* (Okano et al., 1991). *PAH* mutations, such as R408W, with a "severe" effect on enzyme function *in vitro* were associated with a severe PKU phenotype *in vivo*, whereas mutations with a "mild" effect *in vitro* conferred a corresponding phenotype *in vivo*.

Figure 13:



Relationships between predicted phenylalanine hydroxylase activity (by in vitro expression analysis) and biochemical phenotypes in HPA patients. Least-squares analysis was used to calculate regressions, for which equations and correlation coefficients are shown. (Top left) Relationship between predicted phenylalanine hydroxylase activity and 1/pretreatment serum phenylalanine levels in Danish phenylketonuria (PKU) patients (n = 51). (Top right) Relationship between predicted phenylalanine hydroxylase activity and 1/pretreatment serum phenylalanine levels in German PKU patients (n = 44). (Bottom left) Relationship between predicted phenylalanine hydroxylase activity and phenylalanine tolerance at age 5 years in Danish PKU patients (n = 48). (Bottom right) Relationship between predicted

phenylalanine hydroxylase activity and 1/serum phenylalanine 72 hoursafter an oral protein load at age 6 months in German PKU patients (n = 23). (From Okano et al., 1991. Used by permission.)

At the present time, many different human *PAH* mutations have been analyzed by expression analysis *in vitro* (see table under the heading "IVE–Human" in www.pahdb.mcgill.ca). The database also contains information on 12 artificially created mutations in the human *PAH* nucleotide sequence (see table under "IVE–Artificial") and on 41 artificially created mutations in the rat *Pah* gene (see table under "IVE–Rat"). (There are search options on the home page of *PAHdb*: "IVE–Human" is listed under *user-defined queries;* the other two tables are under *prequeried data*. There is a link to "IVE–Commentary" where the use and interpretation of the "IVE–Human" table is described.) The advantages and disadvantages of *in vitro* expression analysis of the *PAH* gene in one or another of the available systems have been discussed in detail (Waters, 2003; Waters et al., 1998).

Data from expression analysis *in vitro* provide the evidence that *PAH* mutations actually alter protein function; they document the severity of the mutation effect; and they help to describe the mechanism of the effect. Mutations affecting protein integrity and function are grouped under four broad headings (Flatmark et al., 1997): (1) knockout or null alleles with undetectable activity, (2) alleles that specifically affect the catalytic center and impair V_{max}, (3) alleles with a kinetic effect altering affinity (K_m) for substrate or cofactor, and (4) alleles mapping to regulatory, catalytic, or tetramerization domains that affect folding of the protein, thus altering its stability and thereby increasing turnover and loss of the protein. A fifth group has emerged—missense alleles conferring BH_4 responsiveness that do not map to BH_4-binding residues (see below).

About two thirds of phenotype-altering *PAH* mutations are predicted from the DNA sequence to be missense alleles, where the substitution of one amino acid for another could affect folding and stability of the protein. Numerous *PAH* mutations have been identified to have this latter effect (Gamez et al., 2000; Pey et al., 2003; Waters et al., 1999; Waters et al., 2000). A recent study (Pey et al., 2007) confirms that decreased protein stability associated with missense mutations is the major molecular pathogenic mechanism in PKU. Pey and colleagues studied the effect of *PAH* mutations by using the protein design algorithm FoldX to predict the energetic impact on the PAH protein and its native-state stability. Eighty of the missense mutations studied in their project had data for in vitro expression analysis in eukaryotic systems, allowing comparison with the corresponding metabolic data. Pey and colleagues examined 238 other *PAH* mutations with the algorithm, leading to the conclusion that the residues encoded in exons 7, 8, and 9 and in the interdomain regions of the protein play important structural roles and constitute hotspots for destabilizing the enzyme. Pey and colleagues further considered the mechanism underying BH_4 responsiveness, leading to the suggestion that in BH_4- induced stabilization of PAH protein mutants with mild stability defects, response to BH_4 occurs when the latter molecule acts as a chemical/pharmacologic chaperone, thus interpreting earlier observations on the therapeutic effect of BH_4 on the phenotype (Muntau et al., 2002; Erlandsen et al., 2004). Additional studies using a yeast two-hybrid system to analyze mutations mapping to the regulatory domain detected subtle aberrations in PAH enzyme oligomerization suggestive of a dominant negative-like effect on interactions between mutant and wild-type subunits in the tetrameric enzyme (Waters et al., 2001).

Very few disease-causing *PAH* alleles directly affect kinetic behavior and specific activity of the enzyme; examples include D143G in one study (Knappskog et al., 1996b) and Y277D and E280K in another (Pey et al., 2003). Some mutations have a combined effect on protein folding and on binding of substrate or cofactor (Gjetting et al., 2001a; Leandro et al., 2000).

Artificial alleles in the homologous rat *PAH* gene have been analyzed by expression analysis *in vitro*. Replacement of serine 16, a phosphorylation site, by anion residues (E16 or D16) activates the enzyme constitutively (Kowlessur et al., 1995). The P281L allele increases the K_m value for phenylalanine (Quinsey et al., 1996), whereas various substitutions of the E286 residue alter pterin binding and function (Dickson et al., 1994). Removal of the C-terminal domain of the rat enzyme subunit reduces enzymic activity (Hufton et al., 1998). An artificial rat mutation (L448A) with the potential to disrupt a leucine zipper/coiled-coil domain affects assembly of dimers into tetramers (Hufton et al., 1998). Accordingly, it is of interest that the disease-causing human mutation A447D affects a residue adjacent to L448 (Guldberg et al., 1996a). Further studies of the rat gene indicate that residues 16 to 26 in the regulatory domain of the PAH enzyme subunit are critical for a regulatory effect involving interaction with the natural BH_4 cofactor (Wang et al., 2001). The rat S29C allele increases enzyme activity fourfold. The human enzyme has cysteine at this position, and this difference between rat and human enzymes might explain the different regulatory properties of rat and human enzyme (Wang et al., 2001). In the catalytic domain, the S249 residue appears to facilitate critical interaction between the amino group of phenylalanine and the enzyme (Jennings et al., 2000).

"Virtual" Molecular Modeling (*in Silico*)

The crystal structures of the catalytic domain and the regulatory domain (N-terminal region) of human PAH enzyme (residues 118–424) have been resolved at 2.0 Å and provide the first structural view of how mutations occurring in exons of the gene could affect protein integrity (Erlandsen et al., 1997b, 2000; Erlandsen and Stevens, 1999; Flatmark and Stevens, 1999; Fusetti et al., 1998). The truncated enzyme crystallizes as a homotetramer, with each monomer containing a catalytic domain and a tetramerization domain (Fusetti et al., 1998). The latter functions as a domain-swapping arm that interacts with the other monomers to form an antiparallel coiled-coil. R408W, the most prevalent of all PKU-causing alleles, affects the highly conserved arginine position on the hinge loop connecting the tetramerization arm to the core of the monomer; R408 also forms a hydrogen bond with the main chain carbamyl of L308, A309, and L311 (Fusetti et al., 1998). Mutations affecting the three latter residues all cause PKU. Replacement of R408 by tryptophan severely disrupts alignment of the arms; replacement by glutamine would be less disruptive, and the R408Q allele is indeed associated with milder forms of HPA in patients (Kayaalp et al., 1997) and with considerable enzyme activity by expression analysis *in vitro* (Waters et al., 1998).

A supplement describes in great detail the putative mechanisms by which missense alleles affecting amino acid residues could affect PAH protein integrity and function (see Supplement: "Structural Studies of Phenylalanine Hydroxylase Enzyme," by Erlandsen and Stevens – Prisc, please check spelling)).

Effects of *PAH* mutation on nonenzymic phenotypes

Comment

Despite limitations in translating data from analysis of *PAH* alleles *in vitro* into the clinical equivalent, broad correlations do exist between predicted PAH enzyme activity *in vivo* and either the metabolic phenotype (plasma phenylalanine level, clearance after loading, and dietary tolerance) (Eiken et al., 1996c; Eisensmith and Woo, 1995; Guldberg et al., 1995; Okano et al., 1991; Svensson et al., 1993) or the IQ score attained in a patient (Di Silvestre et al., 1991; Güttler et al., 1993; Ramus et al., 1993; Trefz et al., 1993). Yet problematic and interesting deviations from that predicted have been noted (Langenbeck et al., 1988) in the observed phenotype in untreated PKU patients.

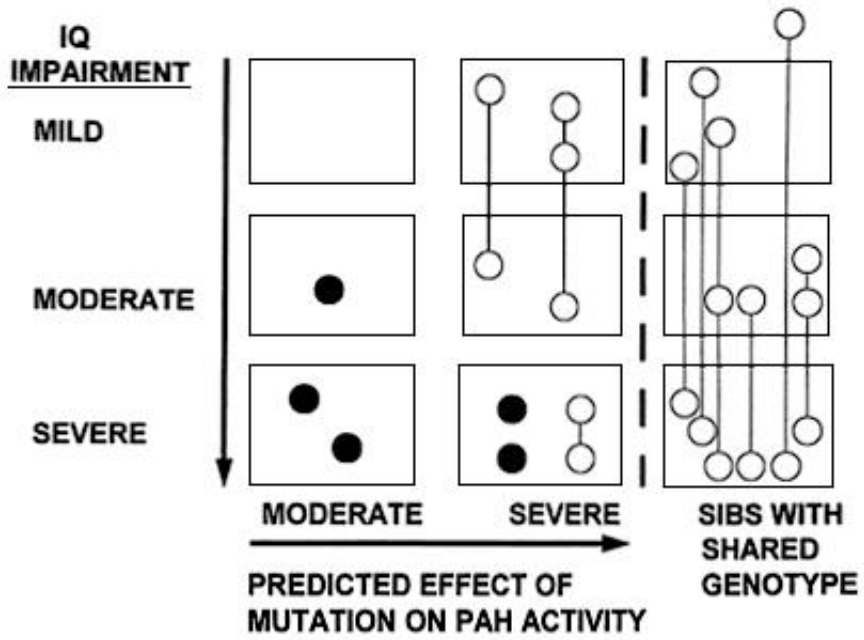
Discordant correlations between genotype and (clinical) phenotype should not be a surprise because they are readily apparent in patients with "monogenic" diseases (Romeo and McKusick, 1994; Summers, 1996), diseases in which phenotype is accountable to more than penetrant alleles at a major locus (Scriver, 2002; Scriver and Waters, 1999).

One can ask either of two questions and expect the answers to be still largely hidden: Can one predict phenotype from genotype? Can one predict genotype from phenotype? Interpretations of discrepancies tend to focus on possible modifiers of phenotype in single-gene disorders; the modifiers include boundary conditions, stochastic and environmental variation, and genetic factors. Among the latter may be allelic variation (in the same gene; so-called malleable genotypes) and postzygotic mutation. Modifier genes may produce their effects by direct interaction with the major gene, by modulating interactions between proteins, by effects on alternative pathways or parallel systems, or by more distant effects, all of which tend to shift our view from the simple highly penetrant Mendelian trait to the biological reality that most phenotypes are in fact *complex traits* (Nakai et al., 2003; Scriver and Waters, 1999; Summers, 1996). In brief, no genetic disease is uninfluenced by other genes (Lander and Schork, 1994). A careful study of phenotypes in PKU and non-PKU HPA will show that the same mutant genotype is not necessarily associated with an identical phenotype in different individuals. For clinical purposes, *treat the actual phenotype, not that predicted from genotype* (Scriver, 2002).

Effect on IQ and brain function

Penrose (1946/1998) knew that untreated siblings with PKU could attain quite different levels of cognitive development. Discrepancies between a particular *PAH* genotype and the predicted effect on phenylalanine metabolism—and thus on the IQ score in an individual untreated PKU patients—were "rediscovered" many years later and observed both within and between families (Di Silvestre et al., 1991; Güttler et al., 1993; Koch et al., 1997; Langenbeck et al., 1988; Ramus et al., 1993; Trefz et al., 1993) (Fig. 77-14). Since IQ must be one of the most complex of complex human traits, it is perhaps surprising that any correlation, even a weak one, between IQ score and genotype at the solitary *PAH* locus should be found. It does imply that the mutant genotype is a principal determinant of the metabolic phenotype (Benit et al., 1999) that, in turn, affects cognitive phenotype.

Figure 14:



IQ impairment classified as severe, moderate, or mild from IQ scores in untreated phenylketonuria (PKU) adults is related to mutant human phenylalanine hydroxylase (PAH) activity as predicted from genotype. Filled circles are data for singletons. Open circles with joining lines are siblings. Mutation data for "sibs with shared genotype" are incomplete, and enzymic activity could not be predicted. Nonetheless, in this series, the untreated PKU sibs, with the same (unknown) genotype, have different cognitive phenotypes. (Adapted from data by Ramus et al., 1993)

There *is* broad concordance: *PAH* alleles with a "severe effect *in vitro*" are likely to cause PKU with low IQ scores in the untreated state, whereas mild *PAH* alleles are likely to cause non-PKU HPA with higher IQ scores. A correlation further exists between predicted severity of an allele, the metabolic phenotype, and findings by noninvasive magnetic resonance imaging (MRI) in brain (Walter et al., 1993). All this we know, and it implies that one mediator of the genotypic effect on cognitive development and brain function is likely to be the phenylalanine level itself in body fluids (Ramus et al., 1993): The level, on average, is higher in PKU than in non-PKU HPA patients. But something other than the *PAH* genotype and its effect on the metabolic (phenylalanine) phenotype now can be taken into account.

Blood-Brain Barrier: Modifier of Phenotype

Noninvasive in vivo ^{1}H-magnetic resonance spectroscopy (MRS) has been used (Weglage et al., 1998a) to measure free phenylalanine concentrations in brain in four untreated adult patients with classic PKU (genotypes unknown), all with similar plasma phenylalanine values in a similar range (1200–1500 µM). Of the four patients, two were retarded and had severe abnormalities of brain myelin, as revealed by MRI, whereas two had normal IQ scores and minimal myelin abnormalities. The former (low-IQ) pair had high brain phenylalanine levels (650 and 670 µM), whereas the latter (normal-IQ) pair had brain phenylalanine levels below detectability. In a second study (Weglage et al., 1998b) using similar analytic techniques, the same group evaluated two siblings with the homoallelic R408W genotype, poor treatment compliance, similar high blood phenylalanine levels [yet different WAIS-R IQ scores (90 and 77)], and different degrees

of brain white matter change (detected by MRI). After a standard-load test, the sibling with the higher IQ and less severe MRI signs accumulated less phenylalanine in brain and cleared it more quickly over 40 hours than did the sibling with the more severe phenotype. A third study (Moller et al., 1998) in 11 typical PKU patients and 3 untreated adult PKU patients with normal intelligence corroborated the findings under both static and kinetic conditions. These important observations are further corroborated by independent observations (Moats et al., 1999).

Whether impaired flux of phenylalanine from plasma into brain (on an L-type carrier for large neutral amino acids) is a significant explanation for discordance between a "brain phenotype" and a "plasma phenylalanine phenotype" in phenylketonuria has become a topic for debate. Whereas it could be seen as a variant adaptive biologic mechanism to explain a favorable discordance (normal IQ outcome in untreated classic PKU), it seems unlikely that modest and putative physiologic variations in the blood-brain barrier (BBB) flux of phenylalanine (Weglage et al., 2002) play a significant role in the interindividual variations in outcome of IQ values in treated PKU patients (Pietz et al., 2002; Rupp et al., 2001). There is also a cautionary tale to tell about the rigor of the quantitative measurements by MRS of brain free phenylalanine concentrations and the associated blood-brain amino acid ratio (Kreis, 2000). On the other hand, there can be little doubt that phenylalanine has access to the brain compartment on a mediated saturable membrane transport process and that its flux on this L-type amino acid carrier can be modified in the presence of other substrates such as the branched-chain amino acids that share the carrier (Pietz et al., 1999; Koch et al., 2003).

The findings imply that rare and extreme outlier interindividual variation in brain phenylalanine transport is another factor to explain interindividual differences in IQ scores in PKU (Weglage et al., 1998a). Phenylalanine transport from blood to cellular organ is a mediated and complex process (see the section "Pathogenesis: Metabolic Phenotypes and Neurotoxicity" below). A transport component from blood across the hepatic cell plasma membrane has been shown elsewhere to be a significant component in whole-body phenylalanine homeostasis (Salter et al., 1986). Transporter genes, like enzyme genes, are subject to allelic variation. Perhaps (polymorphic) allelic variation in a (high-K_m) brain phenylalanine transporter is a modifier of PKU-causing *PAH* genotypes. The NMR observations *in vivo* are preliminary, but their importance is obvious, and they must be further confirmed.

Effect on Phenylalanine Homeostasis

One of the early analyses of correlations between mental and biochemical phenotypes in untreated PKU (Langenbeck et al., 1988) suggested that *modifiers* must be involved to explain a discordant genotype-phenotype relationship. Treacy and colleagues (1996) have shown, by isotope tracer analysis in two siblings with PKU of known mutant *PAH* genotype (R408W/I65T), that significant differences in metabolic runout of phenylalanine, to form the minor metabolites (pathway 3B in Fig. 77-2), have a modifier effect that accounts for consistent and significant differences in dietary phenylalanine tolerances and in the metabolic phenotypes of the siblings.

In another study (Treacy et al., 1997), phenylalanine hydroxylation rates *in vivo* were analyzed by measuring the flux of ingested [^{13}C]-labeled phenylalanine appearing as expired $^{13}CO_2$. The study was done in patients with different clinical forms of HPA. There were broad correlations between mutant genotypes (of predicted severe, intermediate, or mild effect) and the corresponding metabolic phenotype; however, there also was discordance in the correlations among individuals. This led to the conclusion that whereas phenylalanine oxidation *in vivo* is largely under the control of the *PAH* locus—this being evident in the gene-dose effect on phenylalanine oxidation in controls, obligate heterozygotes, and propositi (Treacy et al., 1997)—the individualized "homeostasis value" cannot be explained solely by hepatic

hydroxylating activity. Accordingly, as expected of an important biochemical and physiologic process, whole-body phenylalanine homeostasis has features of a complex quantitative trait (Treacy et al., 1997); it is an *emergent property* (Mayr, 1982) in which the whole is more than the sum of its parts, probably reflecting the scale-free networks that characterize the intricate hubs and links of the "metabolome" (Oltvai and Barabasi, 2002).

PAH Gene Molecular Complexities That Influence Metabolic Phenotype

Discordance between predicted PAH enzyme activity and phenylalanine oxidation rates *in vivo* may reflect interactions between different species of mutant PAH subunits, and a yeast two-hybrid system *in vitro* for expression of *PAH* alleles (Waters et al., 2001) begins to reveal how mutant heteroallelic *PAH* genotypes could affect the enzyme and thus the metabolism of phenylalanine. Most PKU patients in outbred populations are not homoallelic in mutant genotype; about three-quarters of PKU propositi are heteroallelic in diverse populations, and the corresponding values (*j*) for homozygosity are accordingly low (Carter et al., 1998; Guldberg et al., 1996a).

Interindividual phenotypic variation *within* a family is sometimes explained by an unusual degree of allelic variation within the family. Families are known in which three different *PAH* alleles, instead of two, segregate to cause different intrafamilial forms of HPA (Avigad et al., 1991; Guldberg et al., 1996c; Ledley et al., 1986a; Tyfield et al., 1990). Molecular evidence of two mutant alleles occurring in *cis* on a *PAH* haplotype have been reported (Aulehla-Scholz and Heilbronner, 2003; Gjetting et al., 2001b) (see summary list of *cis* mutations at www.pahdb.mcgill.ca for further data).

Meta-analyses of genotype-phenotype correlations have now been done on data from more than 1000 patients expressing more than 100 different pathogenic *PAH* alleles (Desviat et al., 1999; Guldberg et al., 1998; Kayaalp et al., 1997). Analysis was restricted to individuals in whom the genotype was either homoallelic or *functionally hemizygous*, where a missense allele is paired with a null (Guldberg et al., 1995). Several alleles, whose effects were not explained by genotypic or phenotypic misclassification, were associated with more than one metabolic and clinical phenotype. This flexible discordance between genotype and phenotype awaits a full explanation. All the alleles in this category are missense (e.g., L48S, I65T, R158Q, R261Q, and Y414C).

These studies yield the following conclusions: (1) *PAH* genotype classification is broadly predictive of metabolic (and clinical) phenotype and therefore useful for counseling and treatment, and (2) a small subset of ambiguities highlights the fact that HPA owing to PAH enzyme deficiency involves more than simple predictable *PAH* allele expression (i.e., a *single-gene* effect).

The theme of variation in the homozygous phenotype for a so-called recessive disease has been examined by Weiss and Buchanan (2003). Whereas historically the Mendelian homozygous state was assumed to be homoallelic, it is now apparent that one must go beyond the conventional two-allele classification (mutant, wild type) to accommodate the heteroallelic state of the mutant homozygous phenotype. As revealed in the meta-analyses mentioned earlier (Guldberg et al., 1998; Kayaalp et al., 1997), there is a quasi-continuous genotype-phenotype distribution reflecting what is "phenogenetic equivalence" (Weiss and Buchanan, 2003) among the vast number of diploid genotypes that could result from coinheritance of any pairing among the hundreds of mutant *PAH* alleles. Table 77-6 indicates how that phenotypic variation could reflect the inheritance of a genotype with an effect predicted from expression analysis of the individual alleles *in vitro*. Once again, consideration of the phenogenetic relationship brings one back to the maxim "treat the phenotype rather than the genotype" (Scriver, 2002).

Table 77-6: *PAH* Genotype-Phenotype Relationships: Observed versus. Expected Phenotypes in

184 Individuals with PAH Deficiency

		Observed Phenotype PKU					
Genotype Score*	**Expected Phenotype†**	**Classic**	**Moderate**	**Mild**	**MHP**	**Total**	**Obs = Exp, %**
2	Classic PKU	42‡	11	4	—	57	73.7
3	Moderate PKU	10	13‡	6	—	29	44.8
4	Moderate/mild PKU	1	6‡	6‡	—	13	92.3
5 and 6	Mild PKU	1	3	23‡	2	29	79.3
8	Mild PKU/MH	—	—	10c	10‡	20	100.0
9-16	MHP	—	—	1	35‡	36	97.2
Total		54	33	50	47	184	

Sum of estimated effects of individual alleles, where 1 = classic PKU, 2 = moderate PKU, 4 = mild PKU, and 8 = mild HPA (MHP) symptoms.

†Determined, for each patient, on the basis of the sum of the observed alleles of the two PAH mutations.

‡Groups in which observed matched the expected phenotype (Guldberg et al., 1996a).

From Weiss and Buchanan, 2003.

PAH Alleles Conferring a BH_4-Responsive Phenotype

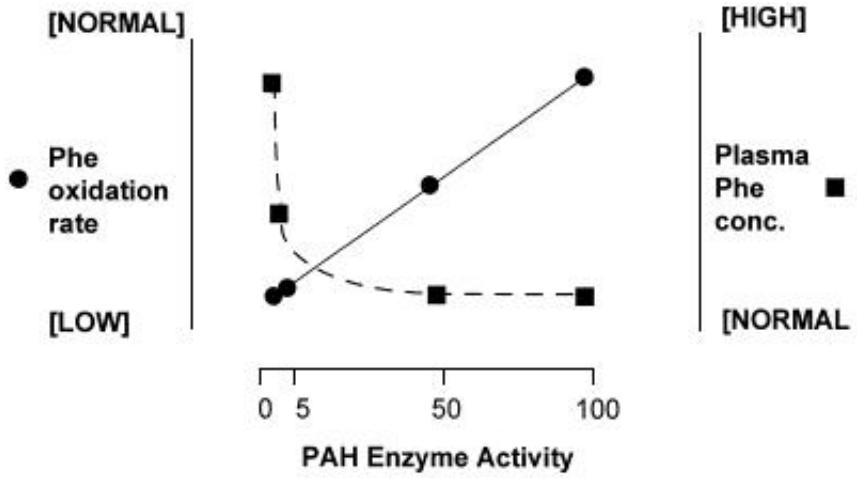
BH_4 is a catalytic cofactor for PAH hydroxylating activity. Mutations at human loci encoding enzymes for BH_4 synthesis and recycling *in vivo* (see Chap. 78) both impair PAH enzyme function and confer a dependency for therapeutic replacement of BH_4. However, mutations also exist at the *PAH* locus itself; these confer a variant enzymic and metabolic phenotype responsive to BH_4 (in pharmacologic doses up to 20 mg/kg of body weight per day) in the absence of any impairment of BH_4 homeostasis. Since its initial description (Kure et al., 1999), the condition has become widely recognized (see list of cases in Spaapen and Rubio-Gozalbo, 2003). Its significance has produced molecular considerations about the interaction between the 6-*R*-BH_4 isomer and the surface of the PAH protein (Erlandsen and Stevens, 2001). Detailed investigations of phenylalanine clearance rates from plasma and the simultaneous rates of phenylalanine oxidation to CO_2 *in vivo* have been investigated in this class of BH_4-responsive HPA

(Muntau et al., 2002). BH_4 increases phenylalanine oxidation in the responsive patient. The majority of responsive patients express a missense *PAH* allele that can be in any region of the PAH protein. It has been proposed that perhaps 60 percent of patients with HPA and phenylalanine values less than 800 µmol/liter are BH_4-responsive (Blau, 2003). The tetrahydropterin home page (www.BH_4.org) lists relevant clinical reports; the corresponding *PAH* alleles are listed there and in www.pahdb.mcgill.ca. The mechanism of the 6-*R*-BH_4 effect is likely to be specific to the *PAH* allele and its effect on the protein; a beneficial chaperone-like effect on the folding, assembly, and/or thermodynamic stability of the mutant PAH protein to slow its intracellular degradation is a hypothesis awaiting the test (Waters, 2003). Nonetheless, the BH_4-responsive phenotype shows the clinical relevance of *PAH* mutation analysis and of performing a BH_4 loading test in all patients with persistent postnatal HPA.

Mutation Effects in Heterozygotes: Significance for Classification

Identification of a heterozygote carrying a disease-causing *PAH* allele has long been a challenge (see McDonald and Charlton, 1997) because the phenotypic effect of a single mutant *PAH* allele on phenylalanine homeostasis is highly buffered and thus "recessive," as both predicted (Kacser and Burns, 1973, 1981) and observed (Fig. 77-15). Nonetheless, discerning whether predicted severity of a *PAH* allele is reflected in a corresponding metabolic phenotype continues to attract attention. For example, a subject (Scriver, 2002) identified by newborn screening had persistent non-PKU HPA (<325 µM); studies later in life revealed euphenylalaninemia and molecular "heterozygosity" by DNA analysis (c.970-6G→T/+); another allele was not recognized, and the unusual circumstance of heterozygosity with neonatal HPA was declared. There could be another explanation: heteroallelic mutant genotype—one null allele and one very "mild" allele unidentified.

Figure 15:



A stylized summary of evidence for (1) the recessive nature of the plasma amino acid phenotype in the phenylalanine hydroxylase (PAH)-deficient HPAs (plasma phenylalanine values, right ordinate), and (2) gene-dosage effect on the rate of hydroxylase-dependent oxidation of phenylalanine in vivo from 13CO2 in expired air (left ordinate). Original data from Rosenblatt and Scriver and, 1968 and Treacy et al., 1997. (Adapted from Scriver (1998a); used by permission of the American Society for Clinical Investigation.)

Several studies have examined the metabolic phenotype in obligate heterozygotes, attempting to find a genotype-phenotype correlation. Overnight fasting followed by an oral phenylalanine load (100 mg/kg) segregated heterozygotes as a group with varying degrees of efficiency (Spada et al., 1998) according to the fasting plasma phenylalanine value, the postload plasma clearance of phenylalanine, and the postload rise in tyrosine; there was no correlation in any of the metric parameters with the predicted severity of the *PAH* allele.

In an earlier study, a discriminate function of plasma amino acid values for phenylalanine and tyrosine (the dependent variable), related to *in vitro* expression activity of the *PAH* allele (the independent variable), did yield a significant correlation ($r = 0.40$; $n = 140$; $p < .001$) (Svensson et al., 1994). In another study (Treacy et al., 1997), a measure *in vivo* of hydroxylating activity using $^{13}CO_2$ excretion after ingestion of labeled phenylalanine revealed a gene-dose effect in heterozygotes as a group (see Fig. 77-12 and Fig. 77-15); however, considerable interindividual variation was found among different heterozygotes expressing the same mutant allele (see Fig. 77-12). The latter finding makes it no easier than it was to classify a heterozygote by this new measure of phenotype. Heterozygosity is still classified just as well by a simple plasma amino acid algorithm (Rosenblatt and Scriver, 1968; Treacy et al., 1997) and best by DNA analysis.

PATHOGENESIS: METABOLIC PHENOTYPES AND NEUROTOXICITY

Whereas the enzyme deficiency is a hepatic phenotype, the major clinical effect of HPA in the PKU phenotype is on brain development and function. Thus the variant metabolic phenotype must be at least a necessary, if not perhaps a sufficient, explanation for the neurotoxicity. Herein we discuss metabolic phenotypes associated with mutation at the *PAH* locus affecting integrity of the PAH enzyme and how, through this effect on the metabolic phenotype, mutation ultimately may affect the brain.

Phenylalanine and Neurotoxicity

Phenylalanine is increased severalfold in brain in mentally retarded individuals with PKU (McKean and Peterson, 1970) and is probably the chief villain in neurotoxicity (Knox, 1960). Derivatives of phenylalanine (see Fig. 77-2) are not present at sufficient concentrations to be toxic in PKU (Kaufman, 1989); their concentrations, in cerebrospinal fluid (CSF), for example (Antoshechkin et al., 1991), bear no relation to those used to show toxic effects *in vitro* or in animal experiments (Scriver et al., 1989), and a recent study in the mutant mouse orthologue of PKU (Sarkissian et al., 2000a) denies the relevance of metabolites other than phenylalanine itself. The report of normal intelligence in two euphenylalaninemic sisters who excreted large amounts of phenylalanine metabolites provides further evidence that the abnormal amounts of these metabolites are not the cause of neurotoxicity in PKU (Wadman et al., 1975).

Induction of temporary HPA in treated patients with PKU provokes acute measurable impairment of higher integrative brain function (Huijbregts et al., 2002; Krause et al., 1985) and abnormal electroencephalographic tracings (Epstein et al., 1989; Krause et al., 1986). Under these conditions, urine dopamine excretion (Krause et al., 1985) and plasma L-dopa levels (Krause et al., 1986) correlate inversely with plasma phenylalanine and directly in the measures of brain dysfunction. These effects on brain dysfunction appear when plasma phenylalanine exceeds 1300 µM (Krause et al., 1985), a significant value that correlates with concentrations that alter transport and distribution of phenylalanine in the brain (see below). Whereas such measurements say little about the profile of brain metabolites, direct (Sarkissian et al., 2000a) and indirect (Avison et al., 1990) evidence from NMR studies in animal models and in patients with PKU (see below), from analysis of CSF metabolites (Antoshechkin et al., 1991; Bach et al., 1991; Lukkelund et al., 1988) and from miscellaneous studies (Lou et al., 1987; Smith, 1985), indicates that high plasma phenylalanine levels indeed do have an effect on brain chemistry and thus on

brain function.

The threshold value for plasma phenylalanine for its acute neurotoxic effect (1300 µM) does not necessarily correspond with the value associated with chronic neurotoxicity in PKU. Evidence for a lower value in the latter case is apparent—first in brain white matter, where the changes visible by MRI can be found in *treated* patients with chronic HPA whose levels are less than 600 µM (Bick et al., 1991) and second in *treated* patients whose IQ scores are distributed below the normal range despite long-term maintenance of plasma phenylalanine values of less than 300 µM (Michel et al., 1990). On the other hand, in *untreated* non-PKU HPA, where plasma phenylalanine levels are less than 600 µM, there may be no significant abnormalities (Weglage et al., 2001).

These findings have implications for treatment because they suggest that the putative threshold level for plasma phenylalanine is different for acute and chronic effects on the brain. They also hint that something about the phenylalanine-restricted diet may yet be less than totally adequate in preventing the neurotoxicity. Finally, they indicate that if severe HPA recurs in later life for whatever reason, a reversible acute neurotoxicity will appear first; if this degree of HPA persists, irreversible chronic neurotoxicity could be a consequence.

How Might Deviant Phenylalanine Metabolism Be Neurotoxic?

Pathogenesis can be considered from three viewpoints: (1) a putative deficiency of tyrosine in the brain, (2) the effect of increased phenylalanine on transport and distribution of metabolites in the brain, and (3) an effect on neurochemical processes. No single effect by itself seems sufficient to explain the PKU brain phenotype, but however complex the process, the ultimate effect will be a disturbance of normal chemical homeostasis in the brain.

Tyrosine deficiency

Complete deficiency of PAH enzyme activity promotes tyrosine to the status of an essential dietary amino acid from which a line of reasoning called the *justification hypothesis* (Bessman et al., 1978) proposes jeopardy for the fetus with PKU and then in postnatal life for two reasons: (1) The affected fetus/newborn cannot obtain tyrosine from its own supply of phenylalanine, and (2) the maternal supply of tyrosine to the fetus is compromised by maternal heterozygosity. However, at least five lines of evidence refute this hypothesis: (1) Postnatal tyrosine supplementation alone without reduction of phenylalanine intake does not prevent mental retardation in PKU (Batshaw et al., 1981), (2) postnatal phenylalanine restriction by itself should not be beneficial, yet it is, and it appears largely to prevent neurotoxicity (see the section "Treatment of PKU" below), (3) there is no consistent or pathologic reduction in plasma tyrosine content in untreated patients with PKU (Koepp and Held, 1977), (4) there is no evidence of significant tyrosine deficiency in cord blood samples obtained from newborns with PKU (Scriver et al., 1980), and (5) tyrosine supplements during treatment of PKU sufficient to increase plasma tyrosine levels do not improve neurophysiologic parameters or neuropsychological functions (Pietz et al., 1995a; Smith et al., 1998).

Although the findings pertain only to extracellular tyrosine, the corresponding intracellular values may be higher than the extracellular values in PKU because the mechanisms by which tyrosine might be reduced in phenylketonuric plasma lead to intracellular accumulation (Christensen, 1986, 1987) and because not only is lymphocyte tyrosine increased in persons with PKU as well as in heterozygotes (Thalhammer et al., 1980, 1982), but brain tyrosine is also increased in PKU (McKean and Peterson, 1970).

Effect on Transport Processes and Metabolic Distribution in the Brain

Transport of phenylalanine across the BBB has been measured *in vitro* in a human capillary preparation (Choi and Pardridge, 1986; Hargreaves and Partridge, 1988). A high-affinity system ($K_m \approx 20$ μM) operates on the blood side of the capillary endothelium, whereas a very high-affinity system ($K_m \approx$ 0.25–0.30 μM) operates on the brain side. This arrangement keeps the phenylalanine concentration in the interstitial fluid low and stable during the diurnal fluctuations in its plasma level (Maher et al., 1984; Svensson et al., 1991).

Amino acid uptake across the BBB has been measured noninvasively *in vivo* by positron-emission tomography (PET) using ^{11}C-labeled substrate (aminocyclohexane carboxylate) (Koeppe et al., 1990). These and other studies *in vivo* (Momma et al., 1987) show that affinity for phenylalanine uptake is the same both *in vivo* and *in vitro* (Choi and Pardridge, 1986). The studies *in vivo* (Momma et al., 1987) also show competition on the BBB transport system between phenylalanine and other large neutral amino acids (LNAAs), such as branched-chain amino acids, methionine, tyrosine, and tryptophan. Phenylalanine has the highest affinity for the system. Consequently, elevated concentrations of phenylalanine could impair uptake of other LNAAs into the brain, and the availability for the brain of these amino acids from blood is predicted to be impaired by even modest supraphysiologic concentrations of phenylalanine in the range of 200 to 500 μM (Pardridge, 1998). Inhibition had been demonstrated *in vivo* in patients with PKU and fits the prediction closely (Koeppe et al., 1990).

The transporter responsible for LNAA brain uptake has been partially characterized. It is a sodium-independent system originally designated *LAT* by Christensen and colleagues (1994) and now designated *LAT1* (Boado, 2002), and it appears to be a heterodimer of the 4F2hc heavy chain and LAT1 light chain (Kanai et al., 1998). Both cDNAs have been isolated from BBB cDNA libraries (Boado et al., 1999; Boado, 2002). The LAT1 transcript enhanced tryptophan transport into frog oocytes coinjected with the 4F2hc transcript, a substrate transport inhibited by other LNAAs, and was highly expressed in brain capillaries and not detected in liver, heart, lung, or kidney, indicating selective expression at the BBB (Boado et al., 1999; Boado, 2002).

Brain uptake and content of phenylalanine *in vivo* in patients with PKU have been measured by other methods. The intravenous double-indicator technique estimates *in vivo* transport of phenylalanine and other amino acids across the BBB (Knudsen 1994; Knudsen et al., 1994) and has shown saturation in the hyperphenylalaninemic state with inhibition of leucine transport (Knudsen et al., 1995). MRS measures free phenylalanine content and distribution in brain (Moller et al., 1995, 1997; Novotny et al., 1995; Pietz et al., 1995b). Subjects with PKU have elevated phenylalanine levels and show saturation of the separate transport processes from plasma to interstitial fluid and from extracellular space into brain cells (Moller et al., 1997).

The important studies using MRS to measure free phenylalanine in the brain (Moller et al., 1998; Pardridge 1998; Weglage et al., 1998a; see the section "Effect [of *PAH* alleles] on IQ and Brain Function" above) imply that interindividual variation exists in the transport of phenylalanine into brain cells and that concentrations of free phenylalanine in brain tissue are one of the ultimate determinants of brain phenotype in PKU. Interindividual variation in MRS among patients with PKU has been substantial (Moats et al., 2000; Weglage et al., 2002), even between siblings who have similar blood phenylalanine levels and an identical *PAH* genotype (Weglage et al., 2002). This variation is probably not artifactual and may explain the variation in cognitive ability observed in untreated PKU in patients and their siblings who have similar mutant PAH phenotypes (Ramus et al., 1993; Weglage et al., 2002).

Competition for uptake between amino acids occurs on carriers in other membranes in the brain. Phenylalanine inhibits transport of tyrosine (Aragon et al., 1982) and tryptophan (Herrero et al., 1983) in synaptosomal plasma membrane vesicles. Hence the combined effects of tyrosine sequestration in somatic cells and inhibition of uptake in the brain seem to reduce tyrosine availability for synthesis of catecholamine neurotransmitters (McKean, 1972). These mechanisms also may affect tryptophan availability for serotonin synthesis (McKean, 1972). Both brain catecholamines and serotonin are deficient in the *Pah* enu2 mouse model for PKU (Puglisi-Allegra et al., 2000).

There is MRS evidence (Pietz et al., 1999) in a crossover study that LNAAs compete with phenylalanine at the BBB, and whereas the adverse effect of excess phenylalanine on other LNAAs may be pathogenic in PKU, dietary supplements of LNAAs, if harmless themselves, could block the potentially harmful influx of phenylalanine into the brain. Kaufman earlier (1976) suggested that the interaction between the LNAAs during transport into brain may be used to advantage in the treatment of PKU, and this has been examined (Berry et al., 1982; Huether et al., 1985; Jordan et al., 1985). Dietary supplements of branched-chain amino acids sufficient to double their plasma values in patients with PKU will reduce phenylalanine levels in plasma and CSF (Berry et al., 1982) and improve their psychological test performance (Jordan et al., 1985). Renal handling of phenylalanine and derivatives has intrinsic interest but has only marginal influence on neurotoxicity and is not discussed here (see Scriver et al., 1989, for details).

Effects on Neurochemistry and Metabolism

The cause of defective brain myelination in PKU has long been a focus of interest. There are many explanations for this abnormality (Baba et al., 1987; Dwivedy and Shah, 1982; Hommes, 1991; Huether et al., 1982; Johnson and Shah, 1980; Matsuo and Hommes, 1987; Shah, 1979), but none is comprehensive. Additional evidence from MRI (Shaw et al., 1991; Thompson et al., 1991a) indicates that the process of dysmyelination (Ullrich et al., 1994) is more prevalent than had been suspected even in well-treated patients (Thompson et al., 1991a) and in non-PKU HPA.

The myelination problem has been studied in the *enu*2 mouse, a counterpart of human PKU (see the section "Animal Models" below). MRI and histologic studies do not reveal a dysmyelination (Kornguth et al., 1994) or abnormalities in cytoarchitecture or histomorphometry. However, more recent histologic and biochemical studies of the *enu*2 mouse brain and on *in vitro* cultured oligodendrocytes from brain of wild-type control mice (*BRBR* strain) exposed to abnormal concentrations of L-phenylalanine in the medium (up to 3500 µM), suggest that myelinating oligodendrocytes adopt a nonmyelinating phenotype and overexpress a glial fibrillary acid protein (GFAP) (Dyer et al., 1996). This effect has been attributed to a reduction of cholesterol synthesis. In the *enu*2 mouse model, Shefer and colleagues (2000) found that forebrain, but not hindbrain, was hypomyelinated and that there was a corresponding reduction of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), the rate-limiting enzyme in cholesterol synthesis, in microsomes isolated from the forebrain but not in microsomes isolated from the hindbrain. The reduced activity of HMGR seemed to result from downregulation by increased phenylalanine because the number of oligodendrocytes remained normal and because HMGR activity and cholesterol synthesis also were decreased in microsomes of the oligodendrocyte-like 4C8 glioma cells on exposure to 2424 µM phenylalanine for 3 weeks (Shefer et al., 2000).

Increased turnover of myelin as a component of brain dysfunction (Changeux and Danchin, 1976; Hommes and Moss, 1992) is associated with loss of neurotransmitter (muscarinic acetylcholine) receptor density in the *enu*2 mouse model (Friedman and Kaufman, 1971; Hommes, 1993). Related studies in a rat model (made hyperphenylalaninemic by phenylalanine loading with PAH inhibition by

DL-α-methylphenylalanine) that used measurements of neural cell adhesion molecule (NCAM), GFAP, and hyaluronate-binding activity found that these parameters were grossly altered in brain exposed to HPA (Hommes, 1994). A similar model demonstrated deficit in myelin basic protein neurofilament staining and maturation of myelin and axons; early-onset HPA produced permanent deficits in axon myelination in outer cortical layers (Reynolds et al., 1993).

Brain protein synthesis is perturbed by excessive phenylalanine, as shown by PET in PKU patients (Paans et al., 1996). This effect has been attributed to polysome disaggregation (Binek et al., 1981) and inhibition of translation initiation (Okano et al., 1986). The effect can be offset by augmenting the pool of LNAAs (Binek-Singer and Johnston, 1982). These findings may be relevant to treatment (see below).

Brain histology and cellular development are altered in human PKU (Bauman and Kemper, 1982), the corresponding "artificial" animal models (Bauman and Kemper, 1982; Cordero et al., 1983; Huether et al., 1983; Huether and Neuhoff, 1981; Johnson and Shah, 1984; Reynolds et al., 1993; Swaiman and Wu, 1984), and the *enu*2 mouse (Dyer et al., 1996). The number and spread of dendritic basilar processes of large pyramidal cells are reduced by HPA in rat pups (Cordero et al., 1983); high levels of phenylalanine and its metabolites, both in culture (Swaiman and Wu, 1984) and *in vivo* (Huether et al., 1983; Huether and Neuhoff, 1981), decrease proliferation and increase loss of neurons. DNA content is decreased in affected brain cells (Huether et al., 1983), and its synthesis is impaired (Johnson and Shah, 1984). The net effect is impaired brain growth (Reynolds et al., 1993). Long exposure to the deviant metabolic phenotype impairs development of brain architecture in untreated patients with PKU, with abnormalities in myelination, width of the cortical plate, cell density and organization, dendritic arborization, and number of synaptic spines (Bauman and Kemper, 1982).

To summarize, phenylalanine itself is probably the neurotoxic agent in PKU. Metabolites of phenylalanine are not found in the human (or mouse) disease at sufficiently high concentrations to disturb metabolic and chemical relationships in brain. Whatever the mechanism, the neurotoxic consequences of PKU are either acute and reversible or chronic and irreversible; they both affect neuropsychological function.

ANIMAL MODELS

Animal models have long served to study pathogenesis of the disease phenotype in PKU; certain of them also enable studies of potential new ways to treat HPA.

Artificial Models

Hyperphenylalaninemic animal models are not homologues of human PKU when they are achieved by the use of exogenous phenylalanine loads and chemical agents to block the phenylalanine hydroxylation reaction (Kaufman, 1976). Such studies, done mainly in rats, were a major source of the early data about putative effects of HPA on brain metabolites and chemistry (Lane and Neuhoff, 1980; Vorhees et al., 1981). However, the phenylalanine load used to produce HPA in the animals produced an additional burden of tyrosine when PAH enzyme activity was left intact. Accordingly, it was necessary to inhibit the enzyme to obtain the requisite HPA without hypertyrosinemia. Unfortunately, some of the agents used for this purpose (*p*-chlorophenylalanine and α-methylphenylalanine, for example) had additional effects, notably inhibition of tetrahydrobiopterin-requiring hydroxylating reactions in brain (Hoshiga et al., 1993) with secondary consequences for neurochemistry.

A Natural Model

Better opportunities to obtain a mammalian counterpart of human PKU now exist in mice (McDonald, 1994; McDonald et al., 2002; Shedlovsky et al., 1993). The mouse gene (*Pah*) has been cloned and characterized (GeneBank Accession Number X51942, cDNA); it controls expression of hepatic enzyme activity, and there are strong homologies between mouse and human phenylalanine hydroxylase genes and enzymes (Ledley et al., 1990). The mouse *Pah* locus is in a linkage group on chromosome 10 (Ledley et al., 1988a), homologous to the region on human chromosome 12 where human *PAH* is located. Mutations at the mouse *Pah* locus in the *BTBR* line have been produced by chemical mutagenesis with *N*-ethyl-*N*-nitrosourea (McDonald et al., 1990a). The first strain with evidence of mutant phenylalanine hydroxylation to be produced by this method had a defect in GTP-CH 1 (Bode et al., 1988; Hyland et al., 1996). Other strains, subsequently identified, had mutation at the *Pah* locus affecting phenylalanine hydroxylase function (McDonald et al., 1990b; Shedlovsky et al., 1993). These strains are orthologues of human PKU and non-PKU HPA. The mutations and the associated phenotype have been characterized (McDonald and Charlton, 1997). The enu1 mouse ($Pah^{enu1/enu1}$) is a counterpart of non-PKU HPA and homozygous for the mutation c.364T→C in exon 3 (V106A); the *enu*2 mouse, a counterpart for human PKU, is homozygous for a mutation in exon 7 [c.835T→C (F263S)].

A third variant, *enu*3, is now historic; its splice mutation has been described (Haefele et al., 2001). The *enu*1 mouse has normal plasma phenylalanine and normal behavior on the mouse breeder diet (Teklad Number 8626), whereas the *enu*2 mouse has a 10- to 20-fold elevation of plasma phenylalanine, excretes phenylketones in the urine when fed the breeder diet, and has changes in behavior and in coat color. A hybrid strain ($Pah^{enu1/enu2}$), heteroallelic for the exon 3 and exon 7 mutations, offers advantages for the manipulation of blood phenylalanine levels (Sarkissian et al., 2000b); negative complementation may exist in the heteropolymeric tetrameric Pah enzyme of this strain. These animals are described in more detail on the *PAHdb* Web site and elsewhere (Scriver et al., 2003).

It has been noted that breeding of the *enu* mice is difficult. To offset this difficulty, the mutant *Pahenu*2 allele has been transferred from the *BTBR* background to the *Bl/6* genomic background, with much improved breeding efficiency for the mutant strain (B Thony, personal communication, 2005).

The orthologous *enu* mouse strains are being used to study pathogenesis of brain disease (Hommes, 1994; Kornguth et al., 1994), the effect of maternal HPA on fetal cardiac organogenesis (McDonald et al., 1997), and the efficacy of enzyme-substitution therapy with phenylalanine ammonia lyase (Safos and Chang, 1995; Sarkissian et al., 1999; Sarkissian et al., 2000b).

Measurement of phenylalanine metabolites (i.e., phenylpyruvate, phenyllactate, and phenylacetate) in brains of normal., *enu*1, and *enu*2 mice shows that metabolite levels are minimally elevated in the *enu*1 (non-PKU HPA) mouse and increased in the *enu*2 (PKU) mouse (Sarkissian et al., 2000a). However, concentrations of these metabolites are not sufficient to be toxic; phenylalanine itself remains the best candidate for a chemical cause of impaired cognitive development.

The search for the mechanism behind the aberrant brain phenotype in PKU continues with new studies in the *enu*2 mouse (Smith and Kang, 2000). Saturation by phenylalanine of the L-amino acid carrier at the BBB and distortion of the ratio of free phenylalanine to large neutral amino acids in the intracerebral total acid-soluble metabolite pool reduced local rates of protein synthesis by one-fifth in the adult homozygous mutant mouse. On the other hand, there was no decrease of tRNA-bound neutral amino acids in the pool. The latter finding is rather surprising and suggests that distorted function at the BBB may not directly underlie the mechanism of impaired brain protein synthesis in the PKU phenotype. An excellent summary

of research in the *enu* mouse models has appeared as a meeting report from the principle investigators (McDonald et al., 2002).

SCREENING AND DIAGNOSIS

Comment

Guthrie and Susi published their landmark description of a simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants in 1963 (Guthrie, 1996; Guthrie and Susi, 1963), and many years later it is apparent that applications of the method and its derivatives have "gone around the world, changed the natural history of a disease (phenylketonuria), and through genetic screening, have introduced new concepts and approaches to the practice of medicine and health care" (Scriver, 1998b). This section describes the principles and practices that made screening for PKU, a population-based procedure, a prototype for (population) genetic screening.

Principles

The rationale for population screening of newborns is *early* medical intervention (National Academy of Sciences, 1975). The goal in diagnosis of HPA is *correct* medical intervention. These objectives each require processes and resources.

A *medical* screening test identifies individuals who probably have a disease from those who probably do not (Wilson and Jungner, 1968). A *genetic* screening test finds persons who are apparently at risk of incipient or established disease in themselves or in their relatives because of genotype (National Academy of Sciences, 1975). On the order of 10 million newborn infants are screened annually worldwide for HPA, and relatives of affected probands are now interested in knowing their genotype, and they seek testing. Accordingly, PKU screening and directed testing have become some of the more widely applied "genetic" tests in health care (American Academy of Pediatrics Committee on Genetics, 1989). The procedure is now seen as the gold standard against which screening for other inborn errors of metabolism can be judged (Seymour et al., 1997), and it is now enhanced by the use of tandem mass spectrometry (Chace et al., 1993, 1998; Pollitt et al., 1997).

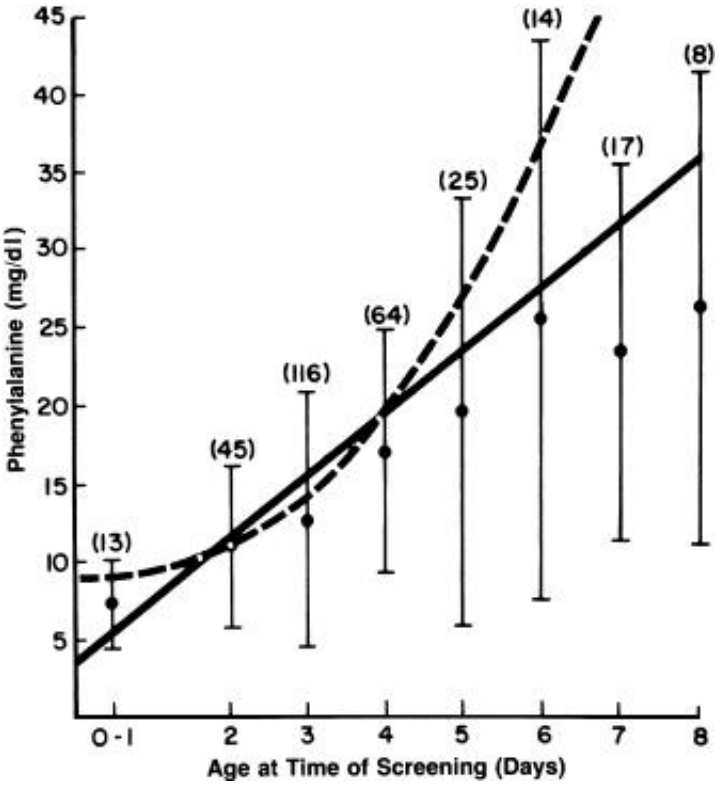
Screening

Screening tests for HPA are performed in the newborn and identify the level of phenylalanine in blood. The original method, urine screening for phenylpyruvic acid, is unreliable to identify PKU (Medical Research Council Steering Committee for the MRC/DHSS Phenylketonuria Register, 1981). Screening at the level of the *PAH* genotype, by DNA (mutation) analysis (Cotton, 1997), will not replace a reliable phenotype test for HPA because of vast allelic heterozygosity in the *PAH* gene and locus heterogeneity in HPA.

The most reliable newborn screening tests employ microbiologic, enzymatic, chromatographic, fluorometric, or most recently, mass spectrometric methods to measure the phenylalanine content of dried capillary (not cord) blood samples collected on filter paper. Phenylalanine in blood spots on properly stored filter paper is stable for years, and accurate retrospective measurements are feasible. How the blood sample is taken from the newborn, its effect on the baby, and the efficiency of the procedure have been analyzed. A Microlance needle for venipuncture on the dorsum of the hand (compared with two sizes of lancets for sampling by heel prick) has proved to be less painful, more successful on the first attempt, and faster, although collecting capillary blood by puncturing the heel remains the most frequently employed procedure. The microbiologic (Guthrie and Susi, 1963) and chromatographic methods are both

semiquantitative, with limitations on accuracy at low phenylalanine concentrations. Enzymic and fluorometric (McCaman and Robins, 1992) methods are fully quantitative down to the lowest plasma phenylalanine levels and have the added advantage of a low coefficient of variation. Tandem mass spectrometry (MS/MS) is also fully quantitative and has the added advantages of simultaneously measuring tyrosine as well as combining screening for PKU with that for a number of other inborn errors in a single analysis (Chace et al., 1993). The technical issues are relevant. A crucial attribute of a screening test is its sensitivity, which is its ability to minimize the frequency of false-negative results. An enzymic test based on an NADH-detecting biosensor, as well as MS/MS, has special promise for this purpose (Chace et al., 1998; Huang et al., 1998). Because PKU screening is best done soon after birth, and because blood phenylalanine begins to rise in the affected infant only after separation from the placenta, capillary blood phenylalanine values in affected cases will be lower the closer the day of testing is to the day of birth (Fig. 77-16). This has been believed to impair the sensitivity of the test when performed in specimens collected before day 2 of life (American Academy of Pediatrics Committee on Genetics, 1982; McCabe et al., 1983), but prospective studies of early newborn screening indicate that HPA is readily detectable in specimens collected by the end of day 1 (Doherty et al., 1991; Hanley et al., 1997; Meryash et al., 1981). Regardless, it is desirable to use a fully quantitative test to separate abnormal from normal values as accurately as possible; the issue is particularly relevant when an infant has non-PKU HPA (Hanley et al., 1997). A review of neonatal screening for HPA in Britain (Smith et al., 1991a) found that all false-negative tests in that program were performed by the semiquantitative microbiologic inhibition assay (*Guthrie test*) and that none was associated with the fully quantitative fluorometric assay.

Figure 16:



Retrospective analysis of blood phenylalanine values (mean ± 2 SD), related to age, in neonates with

confirmed persistent HPA and a diagnosis of phenylketonuria. Regressions are for male infants (solid line) and female infants (broken line). Original data are from Holtzman et al., 1974a and Holtzman et al., 1974b. (From Scriver and, 1982. Used by permission.)

Despite the apparent reliability of newborn screening for HPA on days 1–2 of life, even earlier discharge from hospital nurseries is quite frequent. For example, one-quarter of term newborns in the United States are discharged at or before 24 hours (Sinai et al., 1995). Because the obstetric practice is unlikely to change in the culture of cost saving, the search for a better screening practice has to focus elsewhere (American Academy of Pediatrics Committee on Genetics, 1982; Kirkman et al., 1982; Levy et al., 1984; McCabe et al., 1983; Schoen et al., 1983; Scriver, 1983; Smith et al., 1991a). Routine follow-up (repeat) testing of infants to capture those with false-negative first tests is deemed to be inefficient and expensive (Schoen et al., 1983; Sepe et al., 1979; U.S. Congress, 1988) but nonetheless is being recommended in parts of the United States (American Academy of Pediatrics Committee on Genetics, 1992; Sinai et al., 1995). Most screening programs that do not include routine repeat testing request a repeat specimen whenever the initial screening specimen was collected before 24 hours (Walraven et al., 1995). To convert the test from semiquantitative to fully quantitative status has technical merits (Kirkman et al., 1982; Scriver, 1983; Smith et al., 1991) and is now underway in many screening programs as a result of the introduction of MS/MS (Levy and Albers, 2000). Any (quantitative) method applied to filter-paper blood samples should compensate for the effect of climate and season on the phenylalanine value (Hill, 1969; Lambert, 1994). The threshold (cutoff) value signifying HPA can be lowered to improve sensitivity but at some loss in specificity and predictive value (Clemens et al., 1990; Doherty et al., 1991). A phenylalanine value of 150 μM (2.5 mg/dl) should detect all neonatal cases of PKU unless there is a biologic reason (Hanley et al., 1997) for a normal value in an affected infant at the time of the test. However, MS/MS technology seems to have the capacity to improve both sensitivity and predictive value by allowing for a phenylalanine:tyrosine ratio with 2.5 as the discriminator (cutoff) above which persistent HPA is highly likely (Chace et al., 1998).

Whereas a screening program will not detect every case of persistent HPA, the error rate is low [two studies found that only about 1 in 70 cases of PKU is missed (Holtzman et al., 1986a; Medical Research Council Steering Committee for MRC/DHSS Phenylketonuria Register, 1981)] and can be lower still (Hanley et al., 1997). The principal causes for missed cases are noncompliance with the process and errors of procedure (Holtzman et al., 1986a; McCabe and McCabe, 1983; Medical Research Council Steering Committee for MRC/DHSS Phenylketonuria Register, 1981), but biologic causes may occur occasionally (Hanley et al., 1997). Reanalysis of the original filter-paper blood sample obtained from several "missed" HPA cases has revealed normal blood phenylalanine values at the time of the original screening test (American Academy of Pediatrics Committee on Genetics, 1982; Hanley et al., 1997). The phenomenon is especially rare in the typical PKU phenotype but occurs more frequently in non-PKU HPA and seems more likely to affect female infants (Hanley et al., 1997; Holtzman et al., 1974; Laberge et al., 1987; Scriver, 1982) (see Fig. 77-16). Accordingly, it is possible that some females with HPA may escape detection and will be ignorant of the risk to offspring in their maternal HPA later in life.

False results, either positive or negative, also arise from artifacts; they include ampicillin contamination of the sample (Mabry et al., 1988; Kremensky and Kalalydjieva, 1989; Wilcken et al., 1989), total parental nutrition with some proprietary amino acid solutions (Mitton et al., 1988), and even lot-to-lot variability in the filter paper affecting absorbency and metabolite recovery (Slazyk et al., 1988).

As experience with neonatal screening for PKU and allied disorders continues to grow, the need to improve education and follow-up increases (Meaney, 1988; Smith et al., 1991). Meanwhile, society will accept a modest rate of false-positive tests (Sorensen et al., 1984) as a "cost" for maximizing the sensitivity of screening. In overall economic terms, screening for PKU is cost-effective (Bush et al., 1973; Dagenais et al., 1985; U.S. Congress, 1988).

Tests for Diagnosis and Differential Diagnosis

A positive screening test identifies a newborn with HPA. The diagnostic test identifies the *cause* of the phenotype in the particular infant.

Some infants with positive screening tests have only transient HPA of no further clinical significance. Rarely, transient HPA will be caused by 4α-carbinolamine dehydratase deficiency (see Chap. 78), where the HPA may persist for days or weeks; it also could be an effect of maternal HPA but only for the first day or two of life (Levy and Lobbregt, 1995). Among the infants with persistent HPA, the major cause (>98 percent of cases) is mutation at the *PAH* locus. Some *PAH* alleles cause the PKU phenotype, in which the plasma phenylalanine concentration exceeds 600 µM (10.5 mg/dl) on a normal diet. Other alleles cause non-PKU HPA in which the phenylalanine value is consistently below 600 M on a normal diet. The distinction is relevant because harm to cognitive development is more likely to occur at the higher levels of phenylalanine in PAH deficiency.

Some infants (<2 percent) with persistent HPA have impaired synthesis or recycling of tetrahydrobiopterin (BH_4) (see Chap. 78), for which there is an international register (Blau et al., 1996) (see www.BH_4.org), and these patients require specific treatment to offset the deficiency of BH_4. Since the plasma phenylalanine value alone does not distinguish between BH_4-impaired and BH_4-sufficient forms of HPA, every case of persistent HPA must be investigated further to rule out the disorders of BH_4 metabolism.

Recently, it has become evident that some patients with PAH deficiency also can be treated with BH_4. These patients do not have a deficiency of BH_4 but respond with a reduction of phenylalanine when given BH_4 (Kure et al., 1999; Muntau et al., 2002; Shintaku et al., 2003). This response is a result of enhanced PAH activity, as shown by increased phenylalanine oxidation (Muntau et al., 2002). In HPA, BH_4-responsive PAH deficiency always has been considered to be mild PKU (plasma phenylalanine 600–1200 µM) or non-PKU HPA (plasma phenylalanine < 600 µM) and has been associated with several *PAH* mutations (Muntau et al., 2002; Shintaku et al., 2003).

Differential diagnosis

A detailed discussion of diagnostic tests and procedures is given in the sixth edition of this text; a précis, including Table 77-7, is given here.

Table 77-7: Diagnostic Tests for Follow-up of Neonatal Hyperphenylalaninemia

Test Procedure	Approximate Normal Values or Response in Affected Case
1. Plasma (or blood) phenylalanine and tyrosine (µM) with normal protein intake	Phenylalanine 40-130 Tyrosine 50-140
2. Urine total biopterin and neopterin concentrations (mmol/mol creatinine); neonates have the higher neopterin values	Biopterin 0.4-2.5 Neopterin 0.1-5.0% Biopterin 20-80
3. BH_4 load (7.5 mg/kg orally), plasma phenylalanine and tyrosine at 0, 1, 2, 4, 6 and 24 hours (0-h phenylalanine should be > 200 µM)	Fall in phenylalanine to normal or near normal indicates BH_4 synthesis is deficient
4. Plasma (or dried blood spot) total biopterin (ng/mL; *Crithidia fasciculata* assay)	Plasma biopterin 1.4-3 BH_4 synthesis is deficient
5. CSF concentrations of HVA and 5-HIAA (nmol/ml; neonates have the higher values)	HVA 400-1000 5-HIAA 200-400
6. CSF total biopterin and neopterin (nmol/ml; neonates have the higher values)	Biopterin 12–40 Neopterin 10–3
7. percentage of total biopterin as BH_4 in urine and CSF	Urine 60–80 CSF 90–98
8. BH_4 load (20 mg/kg orally as the 6*R* epimer of BH_4), plasma phenylalanine at 0, 4, 8, 24, 48 hours(0-h phenylalanine should be >360 µM)	Fall of ≥30-50% in phenylalanine indicates BH_4-responsive PAH deficiency

CSF, cerebrospinal fluid; HVA, 5-HIAA-, 5-hydroxyindoleacetic acid; HVA-, homovanillic acid.

Adapted from Smith and Lee, 2000.

Tests at the metabolite level

For Phenylalanine (Whole Blood or Plasma)

The normal value is less than 150 µM in neonates and less than 120 µM in older subjects (Gregory et al., 1986; Scriver et al., 1985; Scriver and Rosenberg, 1973).

For Phenylalanine Metabolites

There are no reliable alternatives to measurement of phenylalanine itself for diagnosis (or screening). Measurement of phenylpyruvic acid and/or related metabolites (see Fig. 77-2) is not recommended because formation of phenylpyruvate, the "classic" urine metabolite, depends on transaminase activity (Brenneman and Kaufman, 1964), and this may be attenuated in the neonate (Armstrong and Binkley, 1956). Moreover, there is almost fourfold interindividual variation in the substrate (phenylalanine) concentration in plasma at which the keto acid is produced in PKU patients (Knox, 1970).

Phenylalanine Loading Test

A loading test is not recommended. Formerly, it was used to ascertain the presence of PKU by the degree of phenylalanine intolerance (Woo, 1984). However, with greater understanding of the relationship between the ambient plasma phenylalanine level and the classification of HPA and the ability to determine mutations at the *PAH* locus along with genotype-phenotype correlation (Guldberg et al., 1998; Kayaalp et al., 1997), phenylalanine loading is no longer necessary. It is especially contraindicated in the newborn infant, in whom it might raise the phenylalanine to a level that could produce irreversible neurotoxicity and would delay dietary treatment.

The Plasma Phenylalanine Response to BH_4

The BH_4 dose (see Table 77-7) is given orally. The test is done with the 6*R* epimer of BH_4. The phenylalanine level must be elevated when the test is done; a fall in the level after BH_4 loading usually to normal or near normal (and usually with an increase in tyrosine) and within the first 6 hours (rarely, not until 24 hours) indicates BH_4 deficiency. The test could be misinterpreted when BH_4 cannot function as a catalytic component of the hydroxylating reaction, as in DHPR deficiency (Kaufman, 1986). After BH_4 deficiency has been excluded and PAH deficiency established, the larger BH_4 load (20 mg/kg) may be given to determine BH_4 responsiveness with a 30 percent or greater fall in the phenylalanine level indicating responsiveness. (*Note:* BH_4 is available for clinical use from B Schircks, Jona, Switzerland CH8645.)

For Pterin Metabolites

Measurements are performed reliably only in laboratories with expertise. Pterin metabolite patterns are abnormal in plasma, CSF, and urine in generalized disorders of BH_4 homeostasis. The so-called peripheral form of 6-pyruvoyltetrahydropterin synthase (6-PTS) deficiency may be present when initial pterin levels in CSF are normal (Allanson et al., 1991). Biopterin can be measured in dried blood spots on filter paper. BH_4 levels are high in untreated cases with ambient HPA and intact BH_4 synthesis (Leeming et al., 1976) and low in disorders of BH_4 homeostasis.

Pterin metabolites (total biopterin and neopterin) can be measured in urine by several methods; relative amounts and normal values are age-dependent. PAH enzyme deficiency confers elevated pterin levels with normal neopterin:biopterin ratios; DHPR deficiency produces elevated total pterin levels and low BH_4 levels; 6-PTS deficiency elevates neopterin levels and neopterin:biopterin ratios; and GTP-CH deficiency produces low pterin values and normal ratios.

For Neurotransmitter Metabolites

CSF levels of homovanillic acid and 5-hydroxyindoleacetic acid, derivatives of tyrosine and tryptophan, respectively, are usually depressed in disorders of BH_4 synthesis and recycling but not equivalently in PKU. CSF neurotransmitter metabolite levels are normal in the peripheral form of 6-PTS deficiency (Allanson et al., 1991).

Tests at the enzyme level

For PAH Enzyme

Direct measurement of PAH enzyme activity requires liver biopsy. Indirect *in vivo* assays are feasible by stable isotope infusions (Trefz et al., 1978) and by ingestion of ^{14}C-labeled (Lehmann et al., 1986) or ^{13}C-labeled phenylalanine (Treacy et al., 1997); they show high intraindividual correlation, they correlate with *in vitro* assays of PAH activity, and they will demonstrate deficient phenylalanine hydroxylating activity *in vivo*. (For the four tests listed next, see Chap. 78 for a full discussion.)

For DHPR

Activity is measured in many tissues, including liver biopsy material; cultured skin fibroblasts and amniocytes, erythrocytes, leukocytes, and platelets; and in dried blood spots on filter paper. An automated assay of DHPR activity in eluates from dried blood spots on filter paper has been developed (Surplice et al., 1990).

For 4α-Carbinolamine Dehydratase

Activity can be measured in blood lymphocytes and scalp hair root cells (Lei and Kaufman, 1998b).

For GTP-CH

Activity is measured in liver biopsy material and phytohemagglutinin-stimulated mononuclear leukocytes. GTP-CH activity is normally low in unstimulated white blood cells and should not be mistaken for an inherited deficiency.

For 6-PTS

Activity is measured in liver biopsy material and erythrocytes.

Diagnosis by DNA Analysis

The *PAH* locus can be analyzed both for the disease-causing mutation and for associated polymorphic haplotype. Venous blood, dried blood spots, buccal cells (obtained by saline mouthwash), cultured skin fibroblasts, and other cells are convenient sources of DNA or mRNA transcripts. Analytic methods include Southern blotting (for large deletions), heteroduplex analysis, single-strand conformational polymorphism, denaturing gradient gel electrophoresis, chemical cleavage of mismatch, restriction enzyme digestion,

allele-specific oligonucleotide hybridization (Cotton, 1993, 1997; Grompe, 1993), and analysis of "illegitimate" (mRNA) transcripts generated by reverse-transcript PCR (Chelly et al., 1989; Sarkar and Sommer, 1989). Detection of a mutation requires *proof of causation* (Cotton and Scriver, 1998) to rule out an artifact or the possibility that it is a neutral polymorphic variant. A negative finding by any one method, however, does not exclude a mutation of clinical significance because no single method can detect all *PAH* alleles. Diagnosis of the mutant *PAH* genotype has some clinical relevance because it is often possible to predict a severe or a mild effect on enzyme function (Guldberg et al., 1994, 1995, 1998; Okano et al., 1991) and to counsel accordingly.

Prenatal Diagnosis

Indications for prenatal diagnosis exist in the HPAs (Ledley et al., 1988b; Scriver and Clow, 1988). Treatment under some conditions may be difficult to obtain or administer, and prognosis for a fully normal outcome still can be uncertain (Barwell and Pollitt, 1987; Riess et al., 1987). Accordingly, there may be an interest in avoidance of recurrent disease. Where the option is permitted, families at risk should know that prenatal diagnosis is feasible (Cleary and Wraith, 1991).

Fetal diagnosis of PAH deficiency is feasible by DNA analysis (Woo, 1984). Unless the mutant alleles in the proband are known, polymorphic markers (RFLPs), in gametic association with the PKU alleles, are the mainstay of fetal diagnosis. The approach requires analysis of parental haplotypes and of the association between haplotype and *PAH* mutation. Three-quarters of affected cases are likely to be genetic compounds for PKU alleles, and this has implications for prenatal diagnosis by either haplotype or mutation analysis. Amniocytes and chorionic villus samples can be sources of DNA for analysis; maternal contamination of the chorionic villus sample is a hazard.

Diagnosis of Heterozygotes (*PAH* Locus)

Identification of *PAH* heterozygosity is sometimes required. Two relatively simple approaches are available. One involves the use of isotopes (Lehmann et al., 1984, 1986; Treacy et al., 1997). The tests are done with small substrate loads, and measurements are made under quasi-steady-state conditions—circumstances that maximize the sensitivity coefficient of PAH enzyme activity *in vivo*.

The second approach without the use of substrate loads or isotopic tracers can be achieved by taking a single semifasting noontime blood sample in which phenylalanine and tyrosine are measured quantitatively (Guneral et al., 1991; Hilton et al., 1986; Rosenblatt and Scriver, 1968); the effect of circadian variation should be taken into account if another time is chosen (Scriver et al., 1985). A quadratic discriminant function that incorporates the prior probability of heterozygosity (Gold et al., 1974; Saraiva et al., 1993; Westwood and Raine, 1975) and linear discriminant functions that do not (Freehauf et al., 1984; Paul et al., 1978; Sartorio et al., 1988; Wenger et al., 1986), are about equally efficient for purposes of carrier classification. Measurement of labeled CO_2 following administration of isotope-labeled phenylalanine offers no particular advantage over the discriminant-function approach applied to ambient plasma phenylalanine levels (Lehmann et al., 1986; Treacy et al., 1997).

The advantages and disadvantages of genetic testing by DNA analysis are those already described. On the other hand, it is a very reliable method if the alleles have been identified in an affected family member.

TREATMENT OF PHENYLKETONURIA

Comment

In 1965, the Third International Congress of Human Genetics addressed the topic of treatment in medical genetics. In the discussion, reference was made to "treatment undertaken to modify the environment in which the [person] lives. The otherwise... potentially deleterious mutation is thus offset, and the individual prospers. The principles of therapy in this realm are generally clear. In most instances, they are immediately applicable to [*Homo sapiens*], and many examples of practical success can be documented" (Scriver, 1967). Treatment of PKU is one of the classic examples of "euphenic therapy" (Lederberg's term), where a normal (or near-normal) phenotype is restored without modification of the mutant genotype. Because recognized approaches to treatment have been in use for sufficient time to assess the degree of success (Holton, 1995), and its big success is prevention of mental retardation (MacCready, 1974), PKU is one of those diseases in which process and outcome variables deserve ongoing analysis. In the meantime, more stringent treatment of HPA is being recommended [Cockburn et al., 1993; Medical Research Council (UK), 1993; National Institutes of Health Consensus Development Panel, 2001; Smith, 1994b]. Some 40 years of PKU treatment have been reviewed at international symposia (J Inherit Metab Dis **25:**605, 2002; Eur J Pediatr **155**(Suppl 1), 1996), where the major good news was reiterated along with evidence that refinement of treatment protocols is indicated. The PKU story, in both good and less good parts, reveals how successful but how difficult it is for *Homo modificans* (a descendant of *H. sapienssapiens*) to act as a substitute for the normal *PAH* genotype. The search for new and improved therapy for PKU continues (Blau and Scriver, 2003).

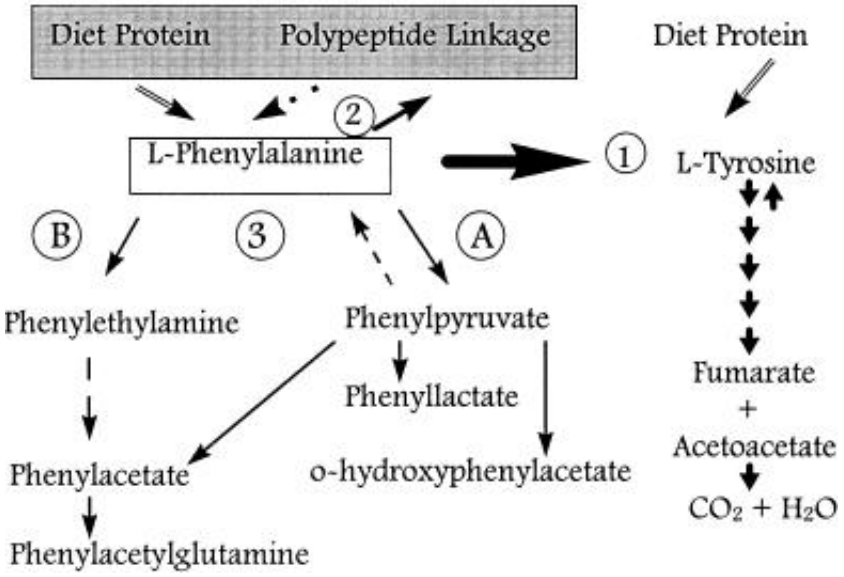
Treatment by Low-Phenylalanine Diet

The mainstay of treatment for PKU is the low-phenylalanine diet (Bickel et al., 1953; Woolf and Vulliamy, 1951), and treatment by diet has been feasible since the mid-1950s (Bickel et al., 1954; Woolf et al., 1955; Armstrong and Tyler, 1955). When started in the neonatal period, it modifies the metabolic phenotype and prevents the neuropsychological consequences of HPA. Optimal treatment of PKU emphasized in the National Institutes of Health Consensus Statement on Screening and Management of Phenylketonuria (National Institutes of Health Consensus Development Panel, 2001) requires (1) early onset of treatment (within 1 month of birth), (2) continuous treatment for life, including through conception and pregnancy in the affected female (to benefit the fetus), and (3) severe restriction of phenylalanine intake to the small amounts sufficient to hold plasma phenylalanine values as close as possible to the normal range yet sufficient to support protein synthesis (excessive restriction will impair growth and development). The precise tolerance for phenylalanine (200–500 mg/day) varies among patients with PKU, even among those with the same mutant *PAH* genotype—which implies the familiar maxim: Treat the patient, not the genotype! Help for the patient to comply with treatment remains important; long-term compliance is difficult (Walter et al., 2002).

Response to Early Treatment

A prospective, controlled trial of treatment by selective restriction of phenylalanine intake, beginning early in postnatal life, has never been attempted. Nevertheless, alternatives to a restricting phenylalanine have proved to be unpromising: (1) A single patient treated purposefully from early infancy only by means of a tyrosine supplement and with no restriction of phenylalanine intake nonetheless expressed the PKU phenotype (Batshaw et al., 1981). (2) L-Dopa therapy alone in adult PKU patients off treatment since 10 years of age does not improve frontal lobe and other brain functions (Ullrich et al., 1996). Conversely, while never-treated contemporary PKU patients continue to manifest the classic PKU phenotype (Langenbeck et al., 1988; Pitt and Danks, 1991), patients treated continuously and carefully with phenylalanine following neonatal diagnosis avoid severe impairment of cognitive development and function (see Fig. 15-11 in Scriver et al., 1989).

Figure 2:



Major inputs (υ) and runouts (τ) 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 (Table 77-1)] 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) transamination (A) and decarboxylation (B). The approximate proportional importance of the three runouts is 3:1:trace at normal steady state (see the discussions in Scriver et al., 1989 and Kaufman and, 1999).

An early study of 28 PKU sibling pairs (Smith and Wolff, 1974) matching the index case who presented with a late diagnosis and mental retardation with the sibling who had been diagnosed and treated for PKU from the neonatal period showed that the difference in intellectual development between the early-treated sibling (IQ values all > 80) and late-diagnosed sibling (mean IQ = 45; range 30–81) was significant. In another historic study (Dobson et al., 1976), when 36 early-treated PKU patients and their unaffected siblings were compared, the mean IQ values were 94 and 99, respectively.

In a large collaborative study in the United States that evaluated the outcome of early-treated PKU patients (Williamson et al., 1977), IQ scores measured in 111 PKU children at 4 years of age, whose treatment began between 3 and 92 days after birth (Dobson et al., 1977), yielded a mean score of 93 (Stanford Binet Intelligence Scale) for the whole group, and patients treated from the first month had a higher mean score (IQ = 95) than those first treated between 31 and 65 days of age (IQ = 85). Evaluation of 132 of these children at 6 years of age (Williamson et al., 1981) found a mean IQ score of 98; regression analysis showed that scores at 6 years were related to maternal intelligence, age at onset of treatment, and average lifetime plasma phenylalanine values during treatment. Evaluation of outcome in 55 of these children at 8 years of age (Koch et al., 1984) found early treatment compatible with attainment of a "normal" IQ score (WISC Full Scale IQ score = 100). However, the PKU probands, as a group, had a small IQ deficit relative to their normal siblins, whose mean IQ score was 107 (p = 0.001), and patients who terminated treatment early scored lower than those who continued it longer (Koch et al., 1984). Among the 95 evaluated at 12 years of age (Azen et al., 1991), 23 had maintained blood phenylalanine

levels of less than 900 µM for the full 12 years, whereas the remainder had not been able to maintain this degree of control. IQ scores correlated positively with age reached at loss of dietary control and with the midparental IQ score and negatively with age at which treatment began and with mean blood phenylalanine value during treatment. The best test scores were obtained by the patients treated longest and with blood phenylalanine values kept consistently less than 900 µM. Recently, 70 of the 125 patients who had been followed to 10 years of age were studied as adults (Koch et al., 2002). The 9 who had continued diet reported fewer problems and tended to have higher cognitive and achievement scores than those who had discontinued treatment.

Independent corroborating evidence exists for both the efficacy of treatment and outcome in early-treated children with PKU (Smith and Beasley, 1988; Smith et al., 1988, 1990). For example, 263 PKU patients born between 1964 and 1971 in the United Kingdom for whom outcomes were known and followed prospectively had a mean IQ of 96 compared with the estimated population norm of 109.8, and 545 born between 1972 and 1980 had a mean IQ of 104 compared with the estimated population norm of 112.4; in this study, IQ scores were adjusted to account for the rise in normal scores (and 0.3 IQ points per year) since standardization of the test in 1932 (Smith et al., 1990; Flynn, 1984). PKU patients showed a 4-point deficit (1) for each month's delay after birth until onset of treatment, (2) for each rise of 300 µM in average blood phenylalanine level with insufficient treatment, and (3) for each 5-month period during infancy when blood phenylalanine values remained less than 120 µM owing to excessive treatment (Smith et al., 1990).

More recent studies from the United Kingdom (Griffiths et al., 1998) and the United States (Arnold et al., 1998) confirm that dietary treatment achieving mean blood phenylalanine levels of less than 360 µM in early childhood is compatible with normal executive function (higher goal-directed mental activity, organized in nature and dependent on good control of attention)(Griffiths et al., 1998): The longer and better the control of phenylalanine levels, the better was the cognitive and motor function, behavioral temperament, and executive function (Arnold et al., 1998; Smith et al., 2000; Weglage et al., 2000). These studies uphold the recommendations for more stringent treatment (Cockburn et al., 1993; National Institutes of Health Consensus Development Panel, 2001).

Many early-treated PKU patients have been found to have subtle performance deficits in conceptual, visual spatial, and language-related tasks (Henderson et al., 2000; Holtzman et al., 1986b; Melnick et al., 1981; Pennington et al., 1985; Rapoport et al., 1983; Smith, 1985); in reading and arithmetic skills, as well as overall school achievement (Azen et al., 1991; Koch et al., 1984; Stemerdink et al., 2000); and in motor coordination, attention span, response time, problem-solving ability, working memory, and executive functioning (Berry et al., 1979; Diamond et al., 1997; Faust et al., 1986; Feldmann et al., 2002; Fishler et al., 1987; Griffiths et al., 1997a; Holtzman et al., 1986b; Koch et al., 1984; Melnick et al., 1981; Netley et al., 1984; Pennington et al., 1985; Smith et al., 2000; Welsh et al., 1990; White et al., 2002). Moreover, behavior may be more extroverted along with negative task orientation (Kalverboer et al., 1994), and the frequency of neurotic and emotional disorders (Ris et al., 1997; Weglage et al., 2000), as well as hyperkinetic behavior, can be twice normal (Burgard et al., 1994). Disordered function in the prefrontal cortex is thought to be at the source of these problems (Diamond, 1994; Eisensmith et al., 1994; Smith et al., 2000). Despite these selective deficits, well-treated PKU patients have satisfactory lives (Koch et al., 1985): They meet their genetic potential for height (Holm et al., 1979; Lyonnet et al., 1989)—although they tend to be overweight (McBurnie et al., 1991; White et al., 1982)—and they have normal pubertal development (Scaglioni et al., 1986) and are gainfully employed (Koch et al., 1997).

Whereas social class can have an effect on outcome (Smith et al., 1990), and parental skills influence compliance with diet (Fehrenbach and Petersen, 1989), treatment of PKU need not disrupt the family (Kazak et al., 1988)—a fact of some relevance when counseling a family about recurrence risks and the burden of PKU (see Scriver and Clow, 1988, Riess et al., 1987, and Barwell and Pollitt, 1987, for views of the "burden" by PKU families).

To summarize, (1) the benefits of early treatment in ameliorating the clinical impact of PKU are well established, (2) early-treated children have mean IQ scores approximately half a standard deviation lower than scores for their unaffected siblings and the corresponding population norm, and (3) early-treated subjects may exhibit some degree of intellectual impairment attributable at least in part to events in early childhood. Nevertheless, while subtle neuropsychological impairments are cause for concern, most early-treated PKU children function within the broad normal range of ability and can attend ordinary schools.

Does Non-PKU Mild Hyperphenylalaninemia Require Treatment?

There has been an abiding question whether mild HPA (<600 µM) should be treated. The question was tentatively answered over 30 years ago (Levy et al., 1971) when a small group of untreated individuals with mild HPA were observed to have normal cognitive development, and the conclusion was drawn that dietary therapy is not needed for mild HPA. A recent paper (Weglage et al., 2001) supports the earlier proposal. In this analysis, 31 never-treated adolescents or adult individuals with persistent HPA were evaluated with comprehensive psychological testing, cranial MRI, MRS, and *PAH* gene mutation analysis. Their mutant genotypes were compatible with the non-PKU HPA phenotype. There were no deficits in the various measurements, leading to the conclusion that "dietary treatment is unlikely to be of value to patients with mild HPA (<600 µM)." The findings also suggest that dietary treatment of such individuals could paradoxically incur a cost of cognitive development. The authors were prudent and advised that every patient identified in the newborn period and potentially in this class be followed carefully in the first year of life to detect any change of status. Occasionally children who, as infants, had phenylalanine values considered to be in the mild HPA range and continued on a normal diet later have been found to have levels in the range of PKU requiring dietary therapy (Berlin et al., 1995; Weglage et al., 1998). They also reminded the readers that the situation of maternal HPA always must be kept in mind.

Response to termination of treatment

There has long been a concern that premature termination of treatment in midchildhood might impair later intellectual and neuropsychological performance (Cabalska et al., 1977; Smith et al., 1977, 1978). As a result, quite early in the history of PKU treatment there was a small controlled trial of continued versus terminated treatment (Holtzman, 1977; Holtzman et al., 1975). Only a small deficit in IQ (4 points) was found in 6-year-olds 2 years after termination of treatment. Thus it seemed that to terminate treatment at this early age would not be cause for concern; nonetheless, the authors of the report recommended a longer period of observation in a larger number of subjects (Holtzman et al., 1975). Conditions for that larger study were fulfilled (Cabalska et al., 1977; Koch et al., 1982; Smith et al., 1977, 1978; Williamson et al., 1979); this provided unambiguous evidence that IQ scores in PKU patients usually are compromised by premature termination of their treatment (Table 77-8).

Table 77-8: Effect of Treatment Termination on IQ scores of Patients with Phenylketonuria (AUTHOR: Please supply name, date references for the "Reference" column.)

Design	*n*	IQ Points (Change)	Significance	Tested at Age (Year)	Months off Diet (Mean)	Reference
Controlled Trial	14	–3.8	NS	6	24	731
Longitudinal	17	–6.3	Yes*	8	41	733
Longitudinal	6	–7.8	Yes*	3.6	12	734
Longitudinal	6	–14.2	Yes*	6.6	48	734
Longitudinal	16	–8.3	<.001†	11	34	735
Longitudinal	7	–9.1	<.05†	13	44	735
Longitudinal	30	–0.08	NS	6	24	736
Controlled Trial	55	–5	<.01	6	24	737
Controlled Trial	115	–4.5‡	NS	8	24	738
Longitudinal	14	–14	<.005	11	6	740

***Stated to be significant.**

***† Significance applies to treatment-terminated patients only; those who continued treatment on a relaxed regimen had less change in IQ scores. (Smith et al, 1978).**

***‡ The group that terminated diet before age 8 years also showed deterioration in reading and spelling achievement (WRAT scores) relative to the treatment continuation group.**

The phenomenon has been called *late-onset phenylalanine* intoxication (Woolf, 1979), the initial features of which are like those that follow any induced increase in blood phenylalanine levels of the PKU subject (Epstein et al., 1989; Krause et al., 1985, 1986). A significant fall in the IQ score (by 5–30 points) is observed when treatment is terminated in midchildhood (Cabalska et al., 1977; Smith et al., 1978); the decrease is smaller when treatment is relaxed rather than terminated (Smith et al., 1978). It is now generally agreed that premature termination of treatment is followed by deficits in performance in most patients (Behbehari, 1985; Koch et al., 1997; Lou et al., 1985; Matthews et al., 1986; Seashore et al., 1985; Smith, 1985). One controlled, prospective study of early- and well-treated PKU patients, however, found no deterioration in cognitive ability following termination of low-phenylalanine diets over the next 10 years (Rey et al., 1996).

Termination of treatment affects more than the IQ score. There are abnormal neurologic features in later life (McDonnell et al., 1998), deviant electroencephalographic findings (Behbehari 1985; Seashore et al., 1985), and decreased levels of neurotransmitter metabolites in body fluids (Lou et al., 1985; Neilsen et al., 1988). The last are analogous to the findings produced by purposeful phenylalanine intoxication (Epstein et al., 1989; Krause et al., 1986); impaired vigilance and reaction times (Lou et al., 1985), deficits in social quotients measured on the Vineland scale (Matthews et al., 1986), and neuropsychological and behavioral problems (Koch et al., 2002; Ris et al., 1997; Smith, 1985) are also seen.

Termination of treatment is also associated with abnormal brain white matter visualized by MRI (Bick et al., 1991; McDonnell et al., 1998; Shaw et al., 1991; Thompson et al., 1990, 1991a; Villasana et al., 1989). The clinical relevance of this finding is unclear because it occurs even in well-treated patients still on treatment (Thompson et al., 1991a). Nonetheless, there is some evidence, not all of it consistent (Bick et al., 1991; Pearsen et al., 1990; Shaw et al., 1991), that the MRI changes are more severe in patients with a history of higher levels of phenylalanine (Shaw et al., 1991; Thompson et al., 1991a). For example, two adolescent patients with non-PKU HPA, both untreated, had normal MRI findings; their phenylalanine levels had apparently never exceeded 600 µM (Bick et al., 1991). The MRI findings, if of a shorter duration in treated PKU patients, disappear when treatment reduces blood phenylalanine levels (Battistini et al., 1991; Thompson et al., 1990; Walter et al., 1997), implying that the abnormality in this circumstance is one of dysmyelination rather than demyelination (Walter et al., 1997). Although the changes in white matter are more common than overt neurologic changes [Medical Research Council (UK), 1993], and perhaps more prevalent than measurable neuropsychological deficits, their association with long-term HPA of some degree seems undeniable.

Stringency of treatment: The threshold argument

Is there a tolerable degree of HPA in the treated patient with PKU and in the untreated patient with non-PKU HPA? There is, so far, no clear answer to this question. Until all causes of cognitive dysfunction, MRI abnormalities, and the like—including the diet itself (Cockburn et al., 1996; Eiken et al., 1996b)—have been ferreted out, we are left with the concern that any increase in phenylalanine could be a troublemaker at every stage of life in patients with HPA. Nevertheless, persons with untreated non-PKU HPA achieve normal cognitive function in the presence of persistent but modest HPA, as shown by absence of significant abnormalities in untreated adolescents and adults with well-documented non-PKU HPA (<600 µM) (Weglage et al., 2001). The latter findings require us to question whether the phenylalanine-restricted diet modality has some undesirable consequences that were avoided in the untreated HPA patients (see the section "Pathogenesis: Metabolic Phenotypes and Neurotoxicity" above).

In reality, for *treated* patients with PKU, there may be no threshold value for blood phenylalanine at which the brain will escape an effect of persistent HPA [Medical Research Council (UK), 1993]. The results in one study (Michel et al., 1990) imply that an even very modest HPA level (<300 µM in the treated patient) may incur cognitive dysfunction, but again, this finding could implicate something harmful in the process or modality of treatment itself. Evidence for the argument against safety in an arbitrary threshold value has come mainly from treated patients with classic PKU (Flynn, 1984; Michel et al., 1990; Smith et al., 1988, 1990) but also has been advanced from other observations (Diamond, 1994; Smith et al., 1991): In all treated patients, outcome correlates with the quality of treatment and the ambient blood phenylalanine value. Meanwhile, the prudent position remains the one proposed in recent guidelines: Treat PKU as early, as well, and for as long as possible [Cockburn et al., 1993; Medical Research Council (UK) 1993; National Institutes of Health Consensus Development Panel, 2001].

Stringency of treatment: Prolongation

Although some patients were advised long ago that treatment would be for life (Naughten, 1989), most received different advice at the time of diagnosis. In the United States, for example, there was no standard policy for continuation or termination of treatment until the late 1970s (Schuett et al., 1980). When the evidence began to emerge that early termination of treatment might have undesirable consequences, the majority of American clinics changed policy and began to recommend treatment for the duration (Schuett and Brown, 1984). Unfortunately for patients who had already discontinued treatment, it was difficult to reinstate it successfully (Finkelson et al., 2001; Hogan et al., 1986; Schuett et al., 1985). With hindsight, it seems illogical to terminate treatment in patients with classic PKU, but patients, families, and advisors all had their reasons to do so—among them, the adverse social conditions of the treatment (restrictions in lifestyle), the inadequate organoleptic properties of the phenylalanine-restricted diet products (poor flavor and offensive smell), and the awkward nutritional considerations (how to keep the intake of phenylalanine far below normal when the intake of other nutrients must be increasing while the patient is growing).

Risk of brain dysfunction owing to HPA, however, persists throughout life, albeit less in adulthood than in childhood during the rapid development of brain functions. Accordingly, guidelines recommend that treatment should continue beyond childhood, through adolescence and into adulthood, certainly during conception and pregnancy, and perhaps for the lifetime of all patients [Cockburn et al., 1993; Medical Research Council (UK) 1993; National Institutes of Health Consensus Development Panel, 2001]. Compliance can be beneficial (Koch et al., 1999) but is clearly difficult to achieve (Walter et al., 1993) and will require better support programs (Levy and Waisbren, 1994) and better resources than are presently in place for treatment during late adolescence and into later life (Fisch et al., 1997; McDonnell et al., 1998). As long as the dietary mode of treatment has the imperfections familiar to anyone who has experienced it, compliance will be a problem beyond childhood. Adolescent patients, well treated in the first decade, can tolerate 3 months of HPA (at levels 1000–1300 μM) without evidence of neurotoxicity (Griffiths et al., 1997b, 1998), but whether their executive functions are immune to harm or whether a longer period of HPA can be tolerated has yet to be analyzed. Abnormal neurologic features can occur in adult patients after termination of treatment with a rise of blood phenylalanine to the aforementioned levels (McDonnell et al., 1998).

Treatment for the previously untreated: Benefit

It was in an untreated child with PKU who was mentally retarded and had behavior abnormalities that Bickel and colleagues first demonstrated the benefit of the phenylalanine-restricted diet (Bickel et al., 1954). On diet, her blood phenylalanine levels decreased, her behavior improved with increased awareness and responsiveness, and she made developmental gains. This initial result of dietary treatment and subsequent further evidence of benefit in mentally retarded, never-treated children (Armstrong et al., 1957; Hsia et al., 1958; Woolf et al., 1955)—presumably the result of relieving the intoxication of extreme HPA (Woolf et al., 1955)—has led to an examination of dietary benefit in retarded adults with PKU, usually those in group homes or institutions (Baumeister and Baumeister, 1998; Harper and Reid, 1987; Hoskin et al., 1992; Koch et al., 1999; Marholin et al., 1978; Williams, 1998; Yannicelli and Ryan, 1995). The objective is not to increase the presumably unchangeable cognitive function but to have a positive effect on behavior by reducing hyperactivity and developing some degree of appropriate social interaction, thereby lessening the threat of physical injury to self and others and reducing need for intensive nursing care and psychotropic medication (Levy, 2000). Studies of benefit from the institution of a phenylalanine-restrictive diet in these adults have had mixed results. Individual case reports generally have reported marked reductions in disturbed behavior (Harper and Reid, 1987; Hoskin et al., 1992; Williams, 1998), but controlled studies have shown inconsistent results, with some patients benefiting but others unchanged (Marholin et al., 1978). A survey of the experience among residential facilities for the mentally retarded found that 46 percent of the patients with PKU had positive behavioral changes when

on a phenylalanine-restricted diet (Yannicelli and Ryan, 1995). From an economic standpoint, the greatest benefit seems to be in untreated patients who require a large amount of nursing care (Brown and Guest, 1999). It would seem that diet should be attempted in these patients and continued if there is a significant reduction in the need for care. However, this may not be evident until the degree of HPA has been substantially reduced for several months. Consequently, if there is no improvement over a shorter period of diet, it should not be concluded that diet is ineffective.

The modalities for dietary treatment: Implications

Protein intake by itself cannot be reduced sufficiently to prevent HPA in PKU without causing deficiencies of other essential amino acids. PKU patients are "consumers with special needs" (Scriver, 1971), and they require elective restriction of phenylalanine intake. Tolerance for dietary phenylalanine [200–500 mg/day (Acosta et al., 1983)] to maintain plasma phenylalanine levels at "nontoxic" levels in young PKU patients is far below the normal intake. Tolerance increases significantly only during the latter half of a pregnancy (Thompson et al., 1991b).

A synthetic diet free of phenylalanine and presumed to be adequate in other nutrients is used to treat PKU. Several commercial products provide the amino acids in adequate amounts (American Academy of Pediatrics, 1976). Although these products have nutrient compositions and proportions vastly different from human nutrient compositions and proportions vastly different from human milk, some have been formulated to resemble human milk in most aspects (Link and Wachtel, 1984), and efforts are constantly being made to improve their composition and organoleptic properties (Acosta et al., 1977; Kindt et al., 1983; Kindt and Halvorsen, 1980). However, they are still unlike human milk in their content of essential fatty acids (Agostoni et al., 1998; Cockburn, 1994; Farquharson et al., 1993; Gu et al., 1995; Riva et al., 1996). Whereas PKU and normal subjects have similar nitrogen requirements (Acosta et al., 1977; Kindt et al., 1983; Kindt and Halvorsen, 1980), classic PKU patients provide evidence that the recommendations for protein requirements in humans can be refined (Kindt et al., 1984, 1988). The artificial diets used to treat PKU alter body composition. They incur lower levels of trace minerals (e.g., zinc, selenium, iron, copper, and chromium) and cholesterol (Gropper et al., 1988; Scriver et al., 1987). Absence of preformed arachidonic acid and the C22:6(*n*-3) fatty acid [docosahexaenoic acid (DHA)] in treatment diets (Agostoni et al., 1998; Giovannini et al., 1966) distorts the fatty-acid composition of plasma and erythrocytes in patients and perhaps also in the brain (Acosta et al., 2001a; Cockburn et al., 1996; Galli et al., 1991; Giovannini et al., 1966; Moseley et al., 2002).

Treatment of PKU can be made more physiologic and plasma phenylalanine homeostasis can be improved (MacDonald et al., 1998) by distributing protein intake throughout the day (Acosta and Yannicelli, 1994) and by increasing the nonphenylalanine protein intake to at least 3 g/100 kcal (Acosta and Yannicelli, 1994). These measures also may avoid the poor growth seen in some treated patients (van der Schot et al., 1994; Van Spronsen et al., 1997) and, when combined with more appealing treatment products, may prevent feeding problems (MacDonald et al., 1994).

The dietary mode of treatment has many pitfalls. First, there can be long-term HPA from excessive treatment (Hanley et al., 1970; Smith et al., 1990) or persistent HPA with inadequate treatment, and both will affect outcome adversely. Second, the poor organoleptic properties (taste and smell) of most low-phenylalanine products affect compliance adversely. Third, in the absence of full understanding of pathogenesis, and without assurance that phenylalanine levels in the brain can be normalized by dietary means alone in PKU, it is still unclear whether supplements of certain free L-amino acids (i.e., leucine, isoleucine, valine, tyrosine, tryptophan, and lysine) are helpful, necessary, or advisable (Kaufman, 1976; Hommes, 1989; Hommes and Lee, 1990; Berry et al., 1982; Jordan et al., 1985; Huether et al., 1985) (see

section "Pathogenesis: Metabolic Phenotypes and Neurotoxicity" above, as well as Pietz et al., 1999). A supplement of L-tyrosine (100 mg/kg of body weight daily) increased plasma tyrosine but produced no change in neuropsychological performances according to double-blind crossover studies with measurements of three neurophysiologic parameters (Pietz et al., 1995a; Smith et al., 1998). Others claim some benefit from tyrosine supplements at a higher dose (180 mg/kg per day) (Lou, 1994), although tyrosine supplementation has not consistently been found to improve neuropsychological function in treated PKU (Kalsner et al., 2001; Poustie and Rutherford, 2000). Supplements of breast milk are considered beneficial in young PKU infants on diet treatment (McCabe et al., 1989), and the supply of decosahexaenoic acid (DHA) in breast milk may contribute to higher IQ scores in PKU patients treated early with breast-milk supplements (Riva et al., 1996).

An alternative to full dietary treatment in PKU has been proposed by the Danish group for use in never-treated adults, previously treated but off-diet adolescents, and adults supposedly on diet but who cannot comply with the rigid requirements of the diet. This alternative consists of reduced natural protein intake and a supplement of large neutral amino acids (LNAAs) excluding phenylalanine and enriched in tyrosine and tryptophan. The concept is that by competition with other LNAAs at the BBB, brain influx of phenylalanine is reduced, thereby lowering the level of phenylalanine in the brain (Anderson and Avins, 1976) (see the section "Pathogenesis: Metabolic Phenotypes and Neurotoxicity" above). The supplement has been used extensively by the Danish group with reported improvement in behavior domains such as motor skills, socialization, communication, emotionality, attention span, and frustration tolerance in a majority of the patients (Kalkanoglu et al., 2002). Reports from Poland and the United States indicate reduction in brain phenylalanine (Koch et al., 2003) and suggest improvement in psychological and emotional health (Giewska et al., 2001; Koch et al., 2003). A recently published double-blinded crossover study of LNAAs or placebo from Australia indicated that LNAAs had some effect in lowering the blood phenylalanine level and in improving executive function, especially in those who were not in dietary compliance (Schindeler et al., 2007). Preliminary data from a very recent study of 4 patients on LNAAs for 1 year who were on a normal diet indicated a decline of approximately 50% in the blood phenylalanine level (Michals-Matalon et al., 2008). An accurate assessment of its benefits awaits objective studies that may soon be underway.

Is the diet modality for PKU therapy without risk?

Outcome with dietary treatment is not optimal for many patients with PKU (see the section "Treatment of PKU" above). The cause generally has been assumed to be imperfect compliance with the treatment protocol leading to attendant HPA. But could suboptimal outcome be a function of the treatment modality itself—the phenylalanine-restricted diet—rather than persistence of HPA? Perhaps toxicity or important deficiency in the diet modality is the problem.

Cockburn (1994) noted that "much of the increase in gray matter weight [of human brain] is due to the development of the complex arborisations and synaptosome formation, which subserve neuronal function and the learning processes. Myelination proceeds rapidly after birth and in this process neuroglial cells envelop the axons of cortical neurons…. 60 percent of the total energy intake of the infant during the first year is utilized by the brain and much of the energy used to construct neuronal membranes and deposit myelin comes from fat in human milk and infant formulas. Fat, however, is not simply a source of hydrocarbon for energy production but is comprised of a series of complex hydrocarbon structures [fatty acids] necessary for the creation of membranes."

Cockburn, his colleagues, and others (Agostoni et al., 1998; Giovannini et al., 1966) have built a case for the importance of long-chain polyunsaturated fatty acids, notably DHA, in the diet of human infants. This fatty acid is present in human milk but at much lower concentrations in the infant formulas currently in use. Moreover, the liver is unable to synthesize DHA from α-linolenic acid [C18:3*n*-3] in the first months of life (Farquharson et al., 1995a). The DHA content of cerebral cortex is significantly higher in breast-fed infants than in formula-fed infants (Farquharson et al., 1995b). The "requirement" for DHA could be met by a dietary supplement of 30 mg/day (approximately 0.2 g/100 g fatty acid) in the diet of formula-fed infants (Farquharson et al., 1993). Cockburn (1994) cites evidence "that preterm infants fed human milk have a higher developmental status at 18 months and a higher intelligence quotient in late childhood than those fed infant formula." This outcome is thought not to be explained by the social environment (Porter, 1996). Are these findings relevant to PKU?

Long-chain polyunsaturated fatty acids such as DHA and arachidonic acid are present as such only in animal foods (Agostoni et al., 2000). Thus patients with PKU can have a very low dietary intake of DHA [and arachidonic acid (Agostoni et al., 1998)] when phenylalanine-restricted diet products replace cow or human milk feedings. The majority of PKU diets in infancy also provide only a low intake of α-linolenic acid, which might replace DHA when hepatic synthesis of DHA matures later in infancy (Cockburn et al., 1996). Patients with PKU have significantly reduced concentrations of DHA (and other long-chain polyunsaturated fatty acids) in plasma and erythrocytes (Moseley et al., 2002). Diet-treated children with PKU have erythrocyte membranes that are poorly populated in the DHA molecules (Cockburn et al., 1996; Giovannini et al., 1966), a deficit likely to be reflected in membranes of the nervous system. Breast-feeding, in the 20- to 40-day postnatal interval before phenylalanine-restricted dietary treatment usually began, is linked to higher IQ scores among patients with PKU (Riva et al., 1996). Accordingly, disappointments in the outcome of treatment might be related as much to deficiency of DHA in early infancy as to chronic HPA. The hypothesis deserves further investigation, careful review of dietary compositions, and possible supplementation with DHA, particularly during infancy, in the treated patient with PKU. The findings also heighten the relevance in seeking possible alternatives to the dietary mode of treatment (see the section "Treatment of PKU" above).

The special problem of aspartame

L-Aspartyl-L-phenylalanine methyl ester (trivial name Aspartame) is an artificial sweetener; on hydrolysis, it releases free L-phenylalanine, L-aspartic acid, and methanol. Aspartame is marketed widely. For example, it has replaced more than 12 percent of the total sweeteners consumed annually in America. Its wide availability makes it a potential hazard in the dietary management of PKU (e.g., 1 quart of Kool-Aid contains 280 mg phenylalanine, more than half the daily allowance of the amino acid for a PKU patient). Accordingly, those with HPA who are on diet and their providers must know about Aspartame, read product labels, and adjust diet accordingly. Despite claims to the contrary, Aspartame seems to present no hazard to PKU heterozygotes, including pregnant heterozygotes (Caballero et al., 1986). Studies in normal subjects and heterozygotes, done at ninetieth and ninety-ninth centiles of projected Aspartame intakes with single-bolus ingestion (the worst-case scenario), found no significant disturbance of blood phenylalanine, tyrosine, and large neutral amino acid levels in response to the load (Filer and Stegink, 1989); these findings have been corroborated (Curtius et al., 1994).

PET following ingestion of Aspartame showed a nonsignificant decrease in brain amino acid transport, as measured with an inert marker (Koeppe et al., 1991). Aspartame loading does not change behavior in rats (Mullenix et al., 1991) and has no effect on photically induced myoclonus in baboons. Notwithstanding such esoteric studies, PKU heterozygotes will find reassurance that a hamburger and a milk shake can safely replace a craving for Aspartame-flavored sustenance.

In summary, the overall advantages of treating HPA with the phenylalanine-restricted diet are clear: (1) reversal of key biochemical abnormalities, (2) improved neuropsychological performance, and (3) prevention of neurologic deterioration. The disadvantages are also clear: (1) difficulty in obtaining full compliance with the treatment process, (2) the need for complex social support, (3) risks of nutrient imbalance and deficiency, and (4) persisting uncertainty that dietary treatment in its present formulations and by itself can achieve all that is desired (Cockburn et al., 1996; Riva et al., 1996; Smith, 1994a).

Cofactor Therapy: Tetrahydrobiopterin-Responsive *PAH* Alleles

Over 30 years ago, Milstien and Kaufman suggested the possibility that a tetrahydropterin might be used to treat those with PKU who still possess some residual phenylalanine hydroxylase activity (Milstien and Kaufman, 1975a). This has now been shown to be true. Beginning in 1999 (Kure et al., 1999) with the demonstration that a mild form of PKU responded to pharmacologic doses of tetrahydrobiopterin by a substantial reduction in the serum phenylalanine concentration, studies have shown that there is a subset of patients with PKU in whom tetrahydrobiopterin therapy lowers phenylalanine (Fiege and Blau, 2007; Bernegger and Blau, 2002; Fiori et al., 2005; Matalon et al., 2004; Mitchell et al., 2004; Leuzzi et al., 2006; Levy at al., 2007; Muntau et al., 2002, Lindner et al., Spaapen and Rubio-Gozalbo, 2003; Burton et al., 2007; Hennermann et al., 2005). None of these BH_4-responsive patients has an abnormality in the synthesis or recycling of BH_4 cofactor.

The overall frequency of responsiveness in studies of largely unselected patients has varied from 20 to 40 percent (Burton et al., 2007; Fiege and Blau, 2007; Mitchell et all, 2005; Hennermann et al., 2005; Boveda et al., 2007). Proving correct the prediction of Milstien and Kaufman that residual phenylalanine hydroxylase activity would be required for therapeutic benefit from a tetrahydropterin (Milstien and Kaufman, 1975a), most responders have mild PKU or mild hyperphenylalaninemia (Muntau et al., 2002; Fiege and Blau, 2007; Fiore et al., 2005; Leuzzi et al., 2006). Specifically, the frequencies have been 80 to 95 percent among patients with mild hyperphenylalaninemia, 50 to 75 percent among patients with mild or moderate PKU, but only 5 to 15 percent among those with classic PKU (Fiege and Blau, 2007; Mitchell et al., 2005; Leuzzi et al., 2006; Fiore et al., 2005; Hennermann et al., 2005; Desviat et al., 2004). The most frequent regimen for determining BH_4 responsiveness in these studies was to administer 20 mg/kg tetrahydrobiopterin (6-*R*-BH_4) as a single or divided dose and to compare the blood phenylalanine concentrations obtained before and within 24 to 36 hours after dosing. A notable exception was a large multicenter study of 485 patients administered a daily dose of 10 mg/kg for 8 days with responsiveness determined by the blood phenylalanine concentration on the eighth day (Burton et al., 2007). A 30 percent or greater reduction in the blood phenylalanine level has been the standard for responsiveness.

The critical question about the efficacy of BH_4 in the treatment of PKU is whether long-term therapy is effective in reducing the stringency of the phenylalanine-restricted diet or eliminating the need for diet. Several studies have examined this question by treating patients with BH_4 from 12 months to over 5 years (Hennermann et al., 2005; Lambrushchini et al., 2005; Shintaku et al., 2004; Cerone et al., 2004; Steinfeld et al., 2004; Trefz et al., 2005; Belanger-Quintana et al., 2005). These reports show that BH_4 responsiveness continues and that some patients can maintain blood phenylalanine control without diet, whereas others can maintain control with a substantially liberalized diet.

The primary biochemical effect of BH_4 in this new phenotype increases the catalytic flux of phenylalanine by increasing PAH enzyme function; secondarily, it ameliorates the metabolic phenotype (Mutau et al., 2002; Okano et al., 2004). This important PAH enzyme phenotype also has been analyzed from the viewpoint of structural biology (Erlandsen and Stevens, 2001; Erlandsen et al., 2004). Mapping 15

different *PAH* mutations associated with BH_4 responsiveness onto their structural model of a PAH monomer, Erlandsen and colleagues (2004) determined multiple mechanisms for BH_4 responsiveness. For some alleles, BH_4 therapy overcomes kinetic variants affecting binding of BH_4 and cooperativity of substrate binding. Additional BH_4-binding mutants have been reported subsequently (Aguado et al., 2007; Perez et al., 2005). For other alleles, BH_4 therapy acts as a chemical chaperone to prevent misfolding of mutant PAH and its consequent proteolytic degradation, thus maintaining PAH in an active configuration (although only rarely restored to wild-type levels). This seems to be the more frequent mechanism of pharmacologic BH_4 action.

The BH_4-responsive alleles are a particular subset of those causing PKU and related forms of hyperphenylalaninemia (see www.pahdb.mcgill.ca and www.BH_4.org/for catalogues and commentary). The majority of BH_4-responsive *PAH* alleles map to the catalytic domain of the enzyme monomer in one of two regions: in the cofactor-binding regions themselves or in regions that interact with the secondary elements involved in binding (Zurfluh et al., 2008). The remaining alleles map to the regulatory or tetramerization domain. Wherever the allele has caused misfolding of the protein with loss of integrity and function, the response to BH_4 may be revealing a chaperone-like effect, in which case the BH_4-responsive HPA phenotype may be prototypes for "pharmacologic chaperone" therapy of genetic disease (see Scriver and Waters, 1999; Blau and Erlandsen, 2004). Consistent with the phenotype of mild PKU or mild hyperphenylalaninemia, the BH_4-responsive mutations express a PAH with residual activity (Erlandsen et al., 2004).

A clinical trial to identify BH_4 responsiveness should be considered in every patient with PKU or a variant non-PKU form of HPA. Recommendations for performing this trial and its interpretation have been published recently (Levy et al., 2007). *PAH* mutation analysis may be very helpful in predicting responsiveness as well as interpreting unclear results.

A proprietary form of BH_4 (sapropterin dihydrochloride, 6*R*-BH_4; KUVAN; BioMarin Pharmaceutical, Inc., Novato, CA 90907) has become available recently. A phase III clinical trial of this preparation indicated that a daily dose of 10 mg/kg for 6 weeks was safe and efficacious in reducing the blood phenylalanine level by 30% or more in responsive patients (Levy et al., 2007).

BH_4 therapy is likely to be very helpful in controlling the blood phenylalanine level as well as reducing the stringency of diet in BH_4-responsive patients with a maternal PKU pregnancy. However, the safety of BH_4 during pregnancy has not yet been assessed in the dose required to treat PKU.

Enzyme Therapy

If enzymatic activity could be restored in PKU patients, it would deal with the primary problem, PAH enzyme deficiency, and there would be no need for a phenylalanine-restricted therapeutic diet. Two approaches to *enzyme therapy* have been considered.

Enzyme replacement

Orthotopic liver transplantation (in a patient with chronic end-stage liver disease) completely corrected the biologic phenotype of PKU (Vajro et al., 1993). Accordingly, heterologous partial transplantation of normal liver tissue or implantation of normal hepatocytes could replace lost enzyme activity in PKU patients. It would constitute somatic *multigene* therapy. No dedicated initiatives in this direction have been reported. In theory, only a small replacement of enzyme activity would be needed to convert this mutant autosomal recessive phenotype from homozygous mutant to heterozygous (Kacser and Burns, 1981).

A novel approach to enzyme therapy using phenylalanine hydroxylase–based fusion proteins has been described recently (Eavri and Lorberboum-Galski, 2007). The authors used small peptides called *protein transduction domains* that facilitate the penetration of fused proteins through the membranes of eukaryotic cells. The HIV transactivator of transcription (TAT) peptide has been most widely studied in this role, and TAT fusion proteins are delivered efficiently into cultured cells intact tissue and live tissues when the agent is injected into mice. The pharmacokinetics of the TAT fusion protein reveals that the liver is its main target. Accordingly, the authors first contructed a TAT-PAH fused protein. They also constructed a different set of PAH-based fusion proteins using human hepatocyte growth factor. Again, liver was the main target. The PAH-based fusion proteins were produced in bacterial cells, and when used in the mouse, they lowered plasma phenylalanine levels within minutes of intravenous administration of the fusion protein. The authors suggest this method as an alternative concept for the treatment of PKU among other metabolic diseases.

Enzyme substitution

Phenylalanine ammonia lyase (PAL) (EC 4.3.1.5) is a robust autocatalytic protein with no requirement for a cofactor (Hodgins, 1971). The enzyme will convert L-phenylalanine stoichiometrically to a nontoxic derivative (*trans*-cinnamic acid) and trace amounts of ammonia (Hoskins et al., 1984). PAL is similar in nature to histidine ammonia lyase (see Chap. 80); the catalysis-competent form of PAL requires posttranslational autocatalytic conversion of a serine residue to dehydroalanine to create the catalytic center of the enzyme (Hodgins, 1971). Efficacy of the enzyme to treat hyperphenylalaninemia *in vivo* has been examined and shown to be promising for clinical use (Gamez and Sarkissian, 2005).

PAL has been administered to human PKU patients using a reactor placed in the extracorporeal circulation (Ambrus et al., 1987; Larue et al., 1986); the effect of enteric-coated capsules given orally to such patients also has been examined (Hoskins et al., 1980, 1984). In both circumstances, the trials were brief and limited in scope but sufficient to observe a modest fall in blood phenylalanine values. The high initial cost of PAL enzyme was partially overcome, for the animal studies described here, by using a recombinant gene from *Rhodosporidium toruloides* (Gilbert et al., 1985) expressed in nonpathogenic *E. coli* and purified by column chromatography (Sarkissian et al., 1999). PAL also has been expressed in *Lactococcus lactis* to "treat" hyperphenylalaninemic rats (Liu et al., 2002).

In a rat model of chemically induced HPA, microencapsulated PAL placed in the intestinal lumen lowered phenylalanine concentration in intestinal and somatic tissues and in plasma (Bourget and Chang, 1985, 1986, 1989).

The mutant hydroxylase-deficient mouse model (McDonald et al., 2002; Sarkissian et al., 2000b) has been used to demonstrate that recombinant protected PAL administered orally will ameliorate HPA in the natural mutant state, providing proofs of principle both pharmacologic and physiologic for this form of enzyme substitution therapy (Safos and Chang, 1995; Sarkissian et al., 1999). Parenteral administration

of PAL reduces brain phenylalanine levels (Sarkissian et al. 2003) and PEGylated conjugates of PAL reduce the systemic phenylalanine pool size in the mutant animal model over the long term (Sarkissian and Gamez, 2005). Structure-based chemical modification, with polyethylene glycol (the pegylation process), of *R. toruloides* PAL, for which there is a crystal structure at 1.6 Å (Wang et al., 2005), enhances *in vivo* activity of PAL and its metabolic efficacy; it also reduces the immunogenicity of its injectable form (Gamez et al., 2005). Human T-cell epitope mapping has been performed on PAL for MHC class I and class II associated T-cell epitomes. PEGylation of surface lysine residues covered the immunogenic regions of PAL. Lysine residues have been removed or added in an unsuccessful attempt to improve the therapeutic enzyme (Gamez et al., 2007).

PAL-PEG conjugates other than those with *R. toruloides* include *Anabaena variabilis*, *Nostoc punctiforme*, and *Petroselinum crispum*. They each have defined characteristics, and some of their derivatives (Gamez et al. 2007), e.g., site-directed mutants, show short- and long-term dose-related correction of phenylalanine concentrations in the mutant mouse model. These conjugates are not toxic, they have a gender effect (male mice respond better), and they restore pigmentation in the treated animals (CN Sarkissian, personal communication, 2007). When biochemical and structural features are taken into account, a double mutant (C503S, C565S) of *A. variabilis* has enhanced thermal stability and resistence to proteolic cleavage (Wang et al. 2008) and may have the most promise as a substitute therapeutic enzyme for treatment of PKU.

Gene Therapy

The limitations of conventional dietary therapy and the tardiness of clinical initiatives in enzyme therapy give gene therapy for PKU a profile it might not otherwise have. The promise lies in putting a normal human *PAH* gene in place of or in addition to the mutant gene in somatic cells of the patient. Germ-line therapy would constitute a potential "cure" for the next and successive generations of PKU individuals, but it will not be used soon for ethical and technical reasons. Meanwhile the possibilities of somatic cell gene therapy for PKU are at hand: A cloned *PAH* cDNA is available, ways exist to deliver the incoming gene to target cells, targeted integration of the gene into the somatic nuclear genome is feasible, and there is reasonable assurance that the incoming gene will be expressed and transmitted to daughter cells. Of particular relevance, an orthologous (PKU) mouse model (McDonald et al., 2002) is available on which to practice somatic cell gene therapy.

A human *PAH* cDNA has already been expressed in a variety of cultured mammalian cells (Ledley et al., 1986b; Peng et al., 1988). Expression is transient when integration of the cDNA is not stable, but when a recombinant retrovirus is used as a vector to infect the cells, integration is stable, and the *PAH* gene is transmitted to subsequent generations. A functional *PAH* gene thus had been introduced successfully into NIH 3T3 mouse fibroblasts, Hepa1-a mouse hepatoma cells, and normal mouse hepatocytes in primary culture (Pey et al., 2007). However progress with *PAH* gene transfer ultimately must be measured in the living PKU organism.

Cultures of primary hepatocytes from the phenylalanine hydroxylase–deficient mouse have been transfected with a wild-type mouse *Pah* cDNA (Liu et al., 1992) using a variant of the LNCX retrovirus (Miller and Rosman, 1989) and with an asialo-orosomucoid-poly-(L-lysine) DNA complex (Cristiano et al., 1993). These transduced cells express high levels of mouse *Pah*-specific mRNA enzyme activity, as well as immunoreactive protein. Phenylalanine hydroxylase–deficient mice also have been treated *in vivoet situ* with a recombinant adenoviral vector containing a human *PAH* cDNA (Fang et al., 1994). Although the HPA phenotype was normalized in 1 week, the effect did not persist.

A recombinant adenoviral vector carrying the human *PAH* cDNA and a control element, when infused via the portal vein into liver of the PKU mouse, produced significant PAH enzyme activity, but the effect did not persist, and repeat administration of the vector met neutralizing antibodies (Eisensmith and Woo, 1996).

There have been no reports of success, either *ex vivo* or *in vivo*, with retrovirus-mediated gene transfer in the PKU mouse model. Moreover, the report of a leukemia-like disorder following a retroviral-based gene therapy trial is a somber reminder that correctly targeted gene insertion is a critical element of retroviral gene therapy in the human subject (Thomas et al., 2003).

Recombinant adeno-associated viral (rAAV) vectors have become popular, being capable of long-term transgene expression and of transducing nondividing cells while evoking only trivial immune response (Thomas et al., 2003). Successful reconstitution of the normal phenotype (i.e., pigmentation, blood phenylalanine level, and behaviour) in the PKU *enu*2 mouse and restoration of sufficient hepatic phenylalanine hydroxylase activity followed infusion, through the tail or portal vein, of an rAAV vector (ideally of serotype 8) containing mouse *Pah* cDNA (Ding et al.,0 2006; Harding et al., 2006; Mochizuki et al., 2004) or the human PAH-cDNA (Oh et al., 2004, 2005). The therapeutic effect could persist up to 40 weeks (Mochizuki et al., 2004), and the neuropathologic changes in brain have shown signs of reversal in the treated PKU enu2 mouse (Embury et al. 2007). Site-specific genome integration of a Pah-cDNA, achieved in the PKU enu2 mouse with the phiBT1 phage integrase corrected the variant phenotype (Chen and Woo, 2005), tolerated repeated dosage of the transgene (Chen and Woo, 2007) and overrode a gender effect (Laipis et al. 2003) in female mice where estrogen limited the supply of BH_4 cofactor in hepatocytes (Chen et al., 2007). However, contrary to predictions that their integration efficiency would be low, actively transcribing genes in nondividing cells have have actually experienced deletions following rAAV vector integration (Nakai et al., 2003). Heterologous, non-liver gene therapy for PKU has been examined. It is understood in all such work that catalytic amounts of cofactor (BH_4) must be present wherever the site of expression of the incoming gene may be, if there is to be sustained PAH enzyme function. *In vitro* experiments with retroviral transduced human T cells from PKU patients obtained high levels of PAH enzyme activity (Lin et al., 1997). *Pah* gene expression in erythrogenic bone marrow in PKU mice was successful at the molecular level but had no effect on the metabolic phenotype (Harding et al., 2003). Human skin fibroblasts and keratinocytes were transduced in culture with independent retroviral vectors expressing *PAH* and *GTPCH* genes, the latter providing the BH_4 cofactor; phenylalanine clearance was enhanced in this preparation (Christensen et al., 2000). Would transformed skin grafts act as a metabolic sink in the human patient?

A human *PAH* gene placed in a construct with promoter elements from mouse muscle creatine kinase has been studied in the PKU mouse model (Harding et al., 1998). This transgene expressed human PAH enzyme activity in mouse cardiac and skeletal muscle cells but not in cells of liver or kidney. The heterologous PAH enzyme expression *in vivo* required large repeated doses of BH_4 to produce the metabolic effect. If hepatocyte transplantation becomes feasible in human subjects [it has been done in rabbits and dogs (Kay et al., 1992)], the patient's own hepatocytes could be obtained, transduced *ex vivo* by one of the methods mentioned here, and then reimplanted. In the meantime, one surmises that any real progress in human somatic cell gene therapy is likely to reflect a perceived failure of all other forms of treatment, and it will depend on better methods of transfer and better gene expression *in vivo* (Ding et al., 2003).

MATERNAL HYPERPHENYLALANINEMIA

Comment

Inborn errors of metabolism interact with the process of human reproduction (Vargas and Levy, 1998) in several ways: (1) They may cause infertility, (2) the pregnancy may affect maternal metabolic control, (3) a fetal metabolic defect may affect the pregnancy, and (4) a maternal metabolic phenotype may affect fetal development. Maternal HPA is in the last category.

Maternal HPA is harmful to the embryo and fetus because it is a form of metabolic teratogenesis (American Academy of Pediatrics, 2001; Levy and Ghavami, 1996) and, as such, resembles the fetal alcohol syndrome (Costa et al., 2002; Levy and Ghavami, 1996) and the effects of maternal diabetes mellitus. The problem has long been recognized (Dent, 1957; Levy, 2003; Mabry et al., 1963), as has the potential for intrapartum treatment to prevent harm to the fetus (Allan and Brown, 1968; Levy, 2003). However, achievements still fall short of expectations because we have yet to learn how to identify, counsel, and treat every mother with HPA and the fetus at risk (Hanley et al., 1999; Scriver, 1967). Should we fail, the incidence of HPA-associated mental retardation could rebound to levels experienced before newborn screening came into practice (Kirkman, 1992; Scriver, 1967).

A Metabolic Embryopathy

Maternal HPA is unquestionably a cause of embryopathy and fetopathy (Lenke and Levy 1980; Levy and Ghavami, 1996; Lipson et al., 1984; Scriver, 1967). Its consequences include microcephaly, impaired cognitive development, congenital heart disease, dysmorphic facial features, and intrauterine growth retardation (Lenke and Levy, 1980). Fetal ultrasonography in the second trimester will detect congenital heart disease but not microcephaly; in the first trimester it will date gestational age and determine whether the pregnancy is viable—facts relevant to counseling and treatment (Levy et al., 1996a). Maternal HPA can cause hypoplasia of the corpus callosum in the fetus, but the changes in cerebral white matter seen in postnatal PKU are absent (Levy et al., 1996b).

Paternal HPA does no harm to the product of conception (Fisch et al., 1991; Levy et al., 1991). The occurrence and pattern of congenital heart disease in maternal HPA have been studied (Levy et al., 2001). It seems to appear only when the maternal phenylalanine level is 900 µM or more and is especially likely to occur when the level is greater than 1800 µM. However, even at the highest levels of maternal phenylalanine, only a third of the offspring have congenital heart disease, in contrast to the almost invariable occurrences of microcephaly and mental retardation at those levels (Lenke and Levy, 1980). This seems to imply that HPA is necessary but not sufficient for congenital heart disease and that a second factor, possibly a polymorphism in a cardiogenic gene, is also required. In addition, heart defects with coarctation of the aorta and hypoplastic left heart syndrome are significantly overrepresented, and tetralogy of Fallot and patent ductus arteriosus are more frequent relative to children with congenital heart disease in the general population.

Whereas there is no doubt that unmodified maternal HPA is an overall hazard to the fetus, there are thresholds for particular effects: a threshold at 900 µM for congenital heart disease and a possible threshold at the 400-µM level for reduced cognitive development (Levy et al., 1994). Conversely, there seems to be a linear dose-dependent effect on head circumference beginning at the lowest elevations of maternal phenylalanine (Drogari et al., 1987). The prudent interpretation would view any degree of maternal HPA as hazardous to the fetus, but with lower risk when the maternal phenotype is the milder non-PKU form of HPA (Levy et al., 1994, 2003).

Pathogenesis

The normal transplacental gradient for phenylalanine favors the fetus. The fetal:maternal ratio is 1.5 on average when there is maternal HPA (Brenton, 1988; Hanley et al., 1987), but there is interindividual variation, with higher values in early pregnancy. Values overall range from 1.1 to 2.9 (Hanley et al., 1987; Schoonheyt et al., 1994). It follows that one cannot predict the phenylalanine pool size in a particular fetus from the maternal phenylalanine level other than to say that it will be the same as or greater than the maternal value. From this, it follows that the aim of treatment is to keep the maternal phenylalanine value as near to normal as possible as early as possible in the pregnancy.

An excess of phenylalanine is harmful to the embryo (Denno and Sadler, 1990; Roux et al., 1992), probably through the same mechanisms that make it harmful to the CNS of the postnatal human infant (Brenton and Haseler, 1990; Levy amd Ghavami, 1996). The phenylalanine effect in the fetus is totally dependent on maternal HPA and its transport across the placenta; the fetus seems to have no ability to alter the phenylalanine level (Levy et al., 1984) despite evidence *in vitro* of almost complete PAH activity as early as the thirteenth gestational week (Raiha, 1973). This accounts for the lack of a difference in teratogenic effect between the fetus with PKU and the non-PKU heterozygous fetus (Levy et al., 1992). The effect does not require accumulation of metabolites derived from phenylalanine itself (Levy et al., 1988) because they are apparently harmless to the fetus (Dorland et al., 1993). Among the likely mechanisms for the effect are competition between phenylalanine and other amino acids for uptake by fetal tissues and perhaps by the placenta (Brenton, 1988; Brenton and Haseler, 1990; Gardiner, 1990; Kudo and Boyd, 1990; Vorhees and Berry, 1989).

Several experiments have been used to study the pathogenesis of maternal HPA (Brass et al., 1982; Kirby, Mihagawa, 1990; Loo et al., 1983; Sadova and Sutcliffe, 1988; Sato et al., 1988), but many of these have used pharmacologic manipulations that have included inhibitors of phenylalanine hydroxylation combined with phenylalanine load to produce HPA in the maternal compartment. Should the inhibitors have reached the fetus, their own effect cannot be dissociated from the effect of phenylalanine alone. Accordingly, the preferred model is a PAH-deficient mouse strain (Shedlovsky et al., 1993) in which the effect of the maternal phenylalanine level could be manipulated purposefully and is the result of mutation affecting phenylalanine hydroxylation activity in the maternal compartment. Cho and McDonald (2001) used this model to show teratogenic effects similar to those in human maternal PKU.

A recent study deserves special mention. Oberdoerster and colleagues (2000) found that exposure of human astrocytes, including fetal astrocytes, to phenylalanine decreased cell proliferation and DNA synthesis. Since the microcephaly observed in maternal PKU is really fetal microencephaly, these findings suggest that inhibition of DNA synthesis could be the mechanism for the fetal neuropathology of maternal PKU.

One further idea about pathogenesis should be mentioned. The *justification hypothesis* indicts tyrosine deprivation rather than phenylalanine excess as the important pathogenic event in PKU (Bessman et al., 1978). A formal test of the hypothesis, made by measuring phenylalanine and tyrosine in cord-blood samples from infants with PKU or non-PKU HPA and from matched controls, found no deficiency of tyrosine in term offspring with HPA born to mothers heterozygous for a mutant *PAH* allele (Scriver et al., 1980). Nonetheless, the hypothesis persists as an echo (Bessman, 1998) in a newer study (Rohr et al., 1998) that found midpregnancy tyrosine values lower in women being treated for maternal HPA than in controls (untreated pregnant non-HPA women). Because the plasma tyrosine levels of the HPA women could be increased by a dietary supplement of tyrosine, these findings might be seen to validate the justification hypothesis (Bessman, 1998). In response to this suggestion (Levy, 1998) and with a careful

reading of the primary paper (Rohr et al., 1998), however, one finds no support for the idea that the harmful effects of maternal HPA are anything but a result of phenylalanine excess. Whether one supplements maternal tyrosine intake or not, the primary goal in the management of the HPA pregnancy remains reduction of the maternal phenylalanine pool size to safe levels.

Preventing the Fetal Effects of Maternal Hyperphenylalaninemia

Prevention requires broad awareness of the problem, recognition and identification of women with HPA in the reproductive age group, and a planned pregnancy so that with a phenylalanine-restricted diet, if necessary, the maternal plasma phenylalanine level will be normal or at least less than 400 µM from conception to delivery (American Academy of Pediatrics, 2001; Cockburn et al., 1993; Levy and Ghavami, 1996).

Better awareness of the hazard in maternal HPA requires better education of all those involved in maternal health, obstetrics, and prenatal care; even well-established programs do not track all women at risk (Mowat et al., 1999). Better detection of the women with HPA in the reproductive age group will come with a combination of initiatives (Mowat et al., 1999). The first initiative is to track women already known to regional treatment programs (Waisbren et al., 1988), perhaps through patient registers (Cartier et al., 1982; Smith and Wolff, 1978), while recognizing that tracking and follow-up of cases will be better for persons whose HPA was treated in childhood and adolescence and less so for those not treated (Waisbren et al., 1988). The second method is to realize that women 35 years of age and older and those from areas of the world without routine newborn screening might not have been screened in the newborn period—and thus among them will be women with unsuspected HPA (Hanley et al., 1999; Mowat et al., 1999). They can be detected by measuring their blood phenylalanine, and a case for doing so systematically as a health care policy has been proposed (Hanley, 1994; Hanley et al., 1999; Mowat et al., 1999). The third method, by all accounts the least desirable, is through the birth of a microcephalic infant signaling unsuspected maternal HPA (Gungor et al., 1996; Koch et al., 1990; Mowat et al., 1999; Naughten and Saul, 1990; Superti-Furga et al., 1991).

A woman with HPA should be counseled about the merits of treatment and the need to normalize her phenylalanine levels before conception or as early as possible in the first month of pregnancy. When the maternal phenylalanine level clearly exceeds 400 µM (Levy et al., 1994), treatment should be prescribed and should begin before conception. With increasing recognition that the phenylalanine-restricted diet should continue after childhood (National Institutes of Health Consensus Development Panel, 2001), more women with HPA entering reproductive age should be on diet. Nevertheless, because of lowered compliance with diet therapy in older subjects, their phenylalanine level will likely be too high for optimal metabolic control of a maternal PKU pregnancy (Schmidt et al., 1996). Accordingly, even these women will require intensive dietary intervention when planning pregnancy or when pregnant.

Data from the prospective international Maternal Phenylketonuria Collaborative Study (MPKUCS) indicate that with optimal metabolic control, including maintenance of the phenylalanine level at 120 to 360 µM from before conception to delivery, risk to the fetus should be no greater than for the general population (Koch et al., 2000; Platt et al., 2000; Roch, 2003; Waisbren and Azen, 2003). Metabolic control is entirely achievable through strict adherence to the phenylalanine-restricted diet and is independent of the maternal or fetal *PAH* genotype (Guttler et al., 2003). The degree of risk with delay of treatment, once conception has occurred, depends on the gestational age at which metabolic control is achieved. Regardless of the blood phenylalanine level, metabolic control can be achieved within 2 to 3 days of strict dietary compliance (Duran et al., 1999). If control is established within the first 8 gestational weeks, congenital heart disease is prevented (Levy et al., 2001), but IQ at 7 years of age is reduced to a mean of

100 as compared with the mean IQ of 105 when metabolic control is achieved before conception (Waisbren and Azen, 2003). Notably, offspring cognitive outcome is negatively correlated with the number of elapsed gestation weeks until metabolic control is achieved (Waisbren and Azen, 2003). When metabolic control is not achieved until 10 gestational weeks or later, the likelihood increases for congenital heart disease, microcephaly or reduced head circumference, low IQ or frank mental retardation, and poor somatic growth (Levy et al., 2003; Roch, 2003; Waisbren and Azen, 2003). Accordingly, counseling to the family should be cautious, and they should know that any delay in onset treatment can have severe adverse effects on the fetus. They also should know that such delay is seen by some as reason to consider termination of pregnancy.

Now that the efficacy of rigorous preconception-intrapartum treatment of maternal HPA is no longer in doubt, many studies (Brown et al., 2002; Clark and Cockburn, 1991; Drogari et al., 1987; Güttler et al., 1990; Naughten and Saul, 1990; Thompson et al., 1991b; Waisbren et al., 1988) are showing how difficult it can be to achieve the desired effect. It is clear that social support, awareness, and education are important for compliance with treatment (Levy and Waisbren, 1994; Shiloh et al., 1993; Waisbren et al., 1988, 1991, 1995, 1997). For women without social support, a "resource mother" (usually the mother of a child with PKU who is trained to be a social resource to a woman with HPA who is planning a pregnancy or is pregnant) may provide this support (St. James et al., 1999). Intensive psychotherapy also may be required (Antshel et al., 2002). The diet must be strictly maintained, blood phenylalanine measured weekly, and phenylalanine depletion avoided by recognizing the striking increase in phenylalanine tolerance that begins toward the end of the second trimester and accelerates severalfold during the third trimester (Clark and Cockburn, 1991; Hyanek et al., 1988; Michaels-Matalon, 2003). This represents both enhanced protein synthesis in the fetal-maternal unit and perhaps a capacity for phenylalanine metabolism gained during the latter part of pregnancy by the heterozygous fetus.

The diet consists of a phenylalanine-free amino acid mixture (which may include vitamins, minerals, fat, and a source of carbohydrates) and low-protein natural and special foods (Acosta, 1995). The qualitative (organoleptic) features of the amino acid medical product often have a strong influence on dietary compliance. Some products are better tolerated than others for some women (Owada et al., 1988; Rohr et al., 2001; Thompson et al., 1991b; Wardley and Taitz, 1988), and their use can be associated with satisfactory fetal outcomes (Drogari et al., 1987; Thompson et al., 1991b). Gelatin encapsulation of the mixture to mask the disagreeable odor and taste may be important for some women (Kecskemethy et al., 1993), provided they can swallow the required large number of capsules (Rohr et al., 2001). Supplementation of branched-chain amino acids (Vorhees and Berry, 1989) seems not to be required for optimal metabolic control and offspring outcome (Acosta et al., 2001b). Tyrosine supplementation will improve maternal tyrosine levels, if indicated (Rohr et al., 1998).

The newborn offspring born to an untreated (or poorly treated) mother with HPA may be thought to have PKU if the newborn screening specimen is collected within the first 12 hours postpartum; the phenomenon reflects maternally transmitted phenylalanine. If the baby has not itself inherited HPA, the phenylalanine increase clears by 24 hours postpartum, and the infant should be given a normal diet (Levy and Lobbregt, 1995).

The mother with HPA may safely breast-feed her offspring. The relatively small increase in breast milk total phenylalanine (Fox-Bacon et al., 1997) can be easily metabolized by the heterozygous offspring or incorporated in a phenylalanine-restricted dietary regimen should the offspring have HPA; the breast-fed heterozygous offspring of maternal HPA maintains normal phenylalanine levels (Fox-Bacon et al., 1997).

Despite progress in reducing its potential impact on the frequency of mental retardation (Kirkman and Frazier, 1996), maternal HPA continues to be an important challenge even after more than 40 years of awareness that it would become such in the global management of PKU (Dent, 1957; Kirkman, 1992; Mabry et al., 1963; Scriver, 1967). It is a problem that must be resolved; otherwise, achievements in the prevention of mental retardation associated with PKU and related forms of HPA will have been gained at the cost of a terrible Faustian bargain.

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REFERENCES

1. Abadie V et al: Molecular basis of phenylketonuria in France. *Dev Brain Dysfunct.* **6**:120, 1993a.

2. Abadie V et al: Illegitimate transcription of the phenylalanine hydroxylase gene in lymphocytes for identification of mutations in phenylketonuria. *Hum Mol Genet.* **2**:31, 1993b. PMID 8098245

3. Abita J et al: Further studies on the nature of phenylalanine hydroxylation in brain. *Pediatr Res.* **8**:714, 1974. PMID 4152145

4. Abita JP et al: In vitro activation of rat liver phenylalanine hydroxylase by phosphorylation. *J Biol Chem.* **251**:5310, 1976. PMID 182695

5. Abita XX et al: Hormonal control of phenylalanine hydroxylase activity in isolated rat hepatocytes. *Biochem Biophys Res Commun.* **92**:912, 1980. PMID 6244820

6. Acosta PB: Nutrition support of maternal phenylketonuria. *Semin Perinatol.* **19**:182, 1995. PMID 7570070

7. Acosta PB et al: Nutrient intake of untreated infants with phenylketonuria. *Am J Clin Nutr.* **30**:198, 1977. PMID 835506

8. Acosta PB et al: Phenylalanine intakes of 1- to 6-year old children with phenylketonuria underoing therapy. *Am J Clin Nutr.* **38**:694, 1983. PMID 6637861

9. Acosta PB et al: Intake of blood levels of fatty acids in treated patients with phenylketonuria. *J Pediatr Gastroenterol Nutr.* **33**:253, 2001a. PMID 11593118

10. Acosta PB et al: *Intake of major nutrients by women in the maternal phenylketonuria (MPKU) study and effects on plasma phenylalanine conentrations Am J Clin Nutr.* **73**:792, 2001b. PMID 11273855

11. Acosta PB, Yannicelli S: Protein intake affects phenylalanine requirements and growth of infants with phenylketonuria. *Acta Paediatr Suppl.* **407**:66, 1994. PMID 7766962

12. Agostini C et al: Plasma arachidonic acid and serum thromboxane B_2 concentrations in phenylketonuric children are correlated with dietary compliance. *Z Emahrungswiss.* **37**:122, 1998. PMID 9558743

13. Agostoni C et al: Effects of long-chain polyunsaturated fatty acid supplementation on fatty acid status and visual function in treated children with hyperphenylalaninemia. *J Pediatr.* **137**:504, 2000. PMID 11035829

14. Aguado C et al: BH_4 responsiveness associated to a PKU mutation with decreased binding affinity for the cofactor. *Clin Chim Acta.* **380**:8, 2007. PMID 17408607

15. Allan JD, Brown JK: Maternal phenylketonuria and foetal brain damage: *An attempt at prevention by dietary control, in KS Holt, VP Coffey (eds).* : Some Recent Advances in Inborn Errors of Metabolism. Edinburgh, Livingston, 1968. PMID 17408607

16. Allanson J et al: Combined transient and peripheral defects in tetrahydrobiopterin synthesis. *J Pediatr.* **118**:261, 1991. PMID 1993959

17. Ambrus CM et al: Extracorporeal enzyme reactors for depletion of phenylalanine in phenylketonuria. *Ann Intern Med.* **106**:531, 1987. PMID 3826953

18. New issues in newborn screening for phenylketonuria and congenital hypothyroidism. *Pediatrics.* **69**:104, 1982. PMID 7054742

19. Newborn screening fact sheets. *Pediatrics.* **83**:449, 1989. PMID 2919149

20. *Issues in newborn screening Pediatrics.* **89**:345, 1992. PMID 1530685

21. *Maternal phenylketonuria Pediatrics.* **107**:427, 2001. PMID 11158484

22. Special diets for infants with inborn errors of amino acid metabolism. *Pediatrics.* **57**:783, 1976. PMID 940719

23. Anderson AE, Avins L: Lowering brain phenylalanine levels by giving other large neutral amino acids. *Arch Neurol.* **33**:684, 1976. PMID 987768

24. What is the Human Variome Project. *Nat Genet.* **39**:423, 2007.

25. Antonarakis SE et al: The nature and mechanisms of human gene mutation. *In CR Scriver et al: (eds).* : The Metabolic and Molecular Bases of Inherited Disease, 8th ed. New York, McGraw-Hill, 2001.

26. Antoshechkin AG et al: Content of phenylalanine, tyrosine and their metabolties in CSF in phenylketonuria. *J Inherit Metab Dis.* **14**:749, 1991. PMID 1779620

27. Antshel KM et al: A case study suggesting the use of prenatal psychotherapy to help control phenylalanine levels. *Am J Orthopsychiatr.* **72**:577, 2002.

28. Aoki K: Follow-up study of the patients detected by newborn screening in Japan. *Jpn J Pediatr Med.* **23**:1887, 1991.

29. Aragon MC et al: Implications in the pathogenesis of phenylketonuria. *J Neurochem.* **39**:1185, 1982. PMID 6889630

30. Armstrong MD, Binkley EL Jr: Studies in phenylketonuria: V. Observations on a newborn infant with phenyketonuria. *Proc Soc Exp Biol Med.* **93**:418, 1956. PMID 13389478

31. Armstrong MD et al: IX. Further observations on the effect of phenylalanine-restricted diet on patients with phenylketonuria. *Am J Clin Nutr.* **5**:543, 1957. PMID 13469756

32. Tyler FH, Armstrong MD: Studies on phenylketonuria: I. Restriction phenylalanine intake in phenylketonuria. *J Clin Invest.* **34**:565, 1955. PMID 14367510

33. Arnold GL et al: Factors affecting cognitive, motor, behavioural and executive functioning in children with phenylketonuria. *Acta Paediatr.* **87**:565, 1998. PMID 9641741

34. Aulehla-Scholz C, Heilbronner H: Mutational spectrum in German patients with phenylalanine hydroxylase deficiency. *Hum Mutat.* **21**:399, 2003. PMID 12655553

35. Avigad S et al: A single origin of phenylketonuria in Yemenite Jews. *Nature.* **344**:168, 1990. PMID 1968617

36. Avigad S et al: The contribution of mutations for classical phenylketonuria. *Am J Hum Genet.* **49**:393, 1991. PMID 1867197

37. Avison MJ et al: Proton NMR observation of phenylalanine and an aromatic metabolite in the rabbit brain in vivo. *Pediatr Res.* **27**:566, 1990. PMID 2162514

38. Ayling JE et al: Comparison with the liver enzyme. *Biochemistry.* **13**:78, 1974. PMID 4808705

39. Ayling JE et al: Comparison with the liver enzyme. *Enzyme.* **20**:6, 1975. PMID 1126332

40. Azen CG et al: Intellectual development in 12-year old children treated for phenylketonuria. *Am J Dis Child.* **145**:35, 1991. PMID 1985428

41. Baba H et al: Study on experimental hyperphenylalaninemia. *Neurochem Res.* **12**:459, 1987. PMID 2438567

42. Bach FW et al: Correlation between cerebrospinal fluid phenylalanine and β-endorphin in patients with phenylketonuria. *Neurosci Lett.* **129**:131, 1991. PMID 1922962

43. Barranger JA et al: Isozymes of phenylalanine hydroxylase. *Science.* **175**:903, 1972. PMID 4257648

44. BartholomÃ© K: Genetics and biochemistry of the phenylketonuria: Present state. *Hum Genet.* **51**:241, 1979. PMID 511154

45. BartholomÃ© K, Dresel A: Studies on the molecular defect in phenylketonuria and hyperphenylalaninemia using antibodies against phenylalanine hydroxylase. *J Inherit Metab Dis.* **5**:7, 1982. PMID 6820417

46. BartholomÃ© K et al: Determination of phenylalanine hydroxylase activity in patients with phenylketonuria and hyperphenylalaninemia. *Pediatr Res.* **9**:899, 1975. PMID 1196708

47. BartholomÃ© K et al: Compound heterozygotes in hyperphenylalaninemia. *Hum Genet.* **65**:405, 1984. PMID 6693130

48. Barwell BE, Pollitt RJ: Attitude des parents vis-à-vis du diagnostic prénatal de la phenylcétonurie. *Arch Fr Pediatr.* **44**:665, 1987. PMID 3449010

49. Basile-Filho A et al: Continuous 24-h L-[1-^{13}C]phenylalanine and L-[3,3-$^{2}H_2$]tyrosine oral-tracer studies at an intermediate phenylalanine intake to estimate requirements in adults. *Am J Clin Nutr.* **65**:473, 1997. PMID 9022533

50. Bassan A et al: Mechanism of diozygen cleavage in tetrahydrobiopterin-dependent amino acid hydroxylases. *Chemistry.* **9**:106, 2003. PMID 12506369

51. Batshaw ML et al: Unsuccessful treatment of phenylketonuria with tyrosine. *J Pediatr.* **99**:159, 1981. PMID 7252655

52. Battistini S et al: Unexpected white matter changes in an early treated PKU case and improvement after dietary treatment. *Funct Neurol.* **6**:177, 1991. PMID 1916459

53. Bauman ML, Kemper TL: Morphologic and histoanatomic observations of the brain in untreated human phenylketonuria. *Acta Neuropathol.* **58**:55, 1982. PMID 7136517

54. Baumeister AA: Dietary treatment of destructive behaviour associated with hyperphenylalaninemia. *Clin Neuropharmacol.* **21**:18, 1998. PMID 9579281

55. Bayle JH et al: Hyperphenylalaninemia and impaired glucose tolerance in mice lacking the bifunctional DCoH gene. *J Biol Chem.* **277**:28884, 2002. PMID 12011081

56. Bearn AG: *Archibald Garrod and the Individuality of Man*. Oxford, Clarendon Press, 1993

57. Behbehani AW: Termination of strict diet therapy in phenylketonuria: A study on EEG sleep patterns and computer spectral analysis. *Neuropediatrics.* **16**:92, 1985. PMID 4010896

58. Beirne E et al: Effect of glucagon on hepatic phenylalanine hydroxylase in vivo. *Biosci Rep.* **5**:463, 1985. PMID 4041562

59. Belanger-Quintana A et al: Evolution of seven patients on long-term treatment with tetrahydrobiopterin. *Mol Genet Metab.* **86**:S61, 2005. PMID 16165389

60. Bender C et al: Haplotype analysis and a new Msp1-polymorphism at the phenylalanine hydroxylase gene in the Arabian population. *Eur J Pediatr.* **153**:392, 1994. PMID 8033936

61. Benit P et al: The mutant genotype is the main determinant of the metabolic phenotype in phenylalanine hydroxylase deficiency. *Mol Genet Metab.* **68**:43, 1999. PMID 10479481

62. Berke XX et al: Resting metabolic rate and the influence of the pretesting environment. *Am J Clin Nutr.* **55**:626, 1992. PMID 1550034

63. Berlin CM et al: Delayed increase in blood phenylalanine concentration in phenylketonuric children initially classified as mild hyperphenylalaninemia. *Screening.* **4**:35, 1995.

64. Bernard C: *Les Phenomenes de la Vie, 1st ed*. Paris, Bailliere, 1878

65. Blau N, Bernegger C: High frequency of tetrahydrobiopterin-responsiveness among hyperphenylalaninemias: A study of 1919 patients observed from 1988 to 2002. *Mol Genet Metab.* **77**:304, 2002. PMID 12468276

66. Berry HD et al: Intellectual development and academic achievement of children treated early for phenylketonuria. *Dev Med Child Neurol.* **21**:311, 1979. PMID 467815

67. Berry HK et al: Reduction of cerebrospinal fluid concentrations of phenylalanine, after oral administration of valine, isoleucine, and leucine. *Pediatr Res.* **16**:751, 1982. PMID 7133808

68. Berry HK et al: Development of phenylalanine hydroxylase activity in guinea pig liver. *Biochim Biophys Acta.* **261**:315, 1972. PMID 5004151

69. Berteloot A et al: K^+ and Na^+ -gradient-dependent transport of L-phenyelalanine by mouse intestinal brush border membrane vesicles. *Biochim Biophys Acta.* **691**:321, 1982. PMID 6291610

70. Bessman SP: Historical perspective: Tyrosine and maternal phenylketonuria, welcome news. *Am J Clin Nutr.* **67**:357, 1998. PMID 9497176

71. Bessman SP et al: Evidence in support of the justification hypothesis. *Proc Natl Acad Sci USA.* **75**:1562, 1978. PMID 274741

72. Bick U et al: Evaluation with magnetic resonance imaging. *Eur J Pediatr.* **150**:185, 1991. PMID 2044589

73. Bickel H et al: Influence of phenylalanine intake on phenylketonuria. *Lancet.* **265**:812, 1953. PMID 13098090

74. Bickel XX et al: Influence of phenylalanine intake on the chemistry and behaviour of a phenylketonuric child. *Acta Paediatr.* **43**:64, 1954. PMID 13138177

75. Binek PA et al: Effect of α-methylphenylalanine and phenylalanine on brain polyribosomes and protein synthesis. *J Neurochem.* **36**:1476, 1981. PMID 7264644

76. Binek-Singer P, Johnston TC: The effects of chronic hyperphenylalaninemia on mouse brain protein synthesis can be prevented by other amino acids. *Biochem J.* **206**:407, 1982. PMID 7150251

77. Blau N: Tetrahydrobiopterin control in phenylketonuria. *Genet Med.* **5**:57, 2003. PMID 12544478

78. Blau N, Erlandsen H: The metabolic and molecular phenylalanine hydroxylase deficiency. *Hum Mutat.* **29**:167, 2008. PMID 17935162

79. Blau N, Trefz FK: Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency: Possible regulation of gene expression in a patient with the homozygous L48S mutation. *Mol Genet Metab.* **75**:186, 2002. PMID 11855940

80. Blau N et al: International database of tetrahydrobiopterin deficiencies. *J Inherit Metab Dis.* **19**:8, 1996. PMID 8830181

81. Scriver CR, Blau N: *New approaches to treat PKU.* : How far are we? Mol Genet Metab (in press). PMID 8830181

82. Boado RJ: Blood-brain barrier large neutral amino acid transporter. *J Inherit Metab Dis.* **25**:609, 2002.

83. Boado RJ et al: Selective expression for the large neutral amino acid transport at the blood-brain barrier. *Proc Natl Acad Sci USA.* **96**:12079, 1999. PMID 10518579

84. Bode V et al: A mouse mutant with hereditary hyperphenylalaninemia induced by ethylnitrosourea mutagenesis. *Genetics.* **118**:299, 1988. PMID 3360305

85. Chang TM, Bourget L: Phenylalanine ammonia-lyase immobilized in semipermeable microcapcules for enzyme replacement in phenylketonuria. *FEBS Lett.* **180**:5, 1985. PMID 4038490

86. Chang TMS, Bourget L: Phenylalanine ammonia-lyase immobilized in microcapsules for the depletion of phenylalanine in plasma in phenylketonuric rat model. *Biochim Biophys Acta.* **883**:432, 1986. PMID 3756210

87. Chang TMS, Bourget L: Effects of oral administration of artificial cells immobolized phenylalanine ammonia-lyase on intestinal amino acids in phenylketonuric rats. *Biomater Artif Cells Artif Org.* **17**:161, 1989. PMID 2775877

88. BÃ³veda MD et al: Evaluation of response and subsequent treatment. *J Inherit Metab Dis.* **30**:812, 2007. PMID 17603758

89. Brass CA et al: A new animal model of maternal phenylketonuria. *Pediatr Res.* **16**:388, 1982. PMID 7201630

90. Kaufman S, Brenneman AR: The role of tetrahydropteridine in the enzymatic conversion of tyrosine to 3,4-dihydroxyphenylalanine. *Biochem Biophys Res Commun.* **17**:177, 1964.

91. Brenton DP: Maternal phenylketonuria. *Eur J Clin Nutr.* **43**:13, 1988. PMID 2786806

92. Brenton DP, Haseler ME: Maternal phenylketonuria, in J Fernandes et al: (eds): *Inborn Metabolic Diseases.* : Diagnosis and Treatment. Berlin, Springer Verlag, 1990. PMID 2786806

93. Brown AS et al: Barrier to successful dietary control among pregnant women with phenylketonuria. *Genet Med.* **4**:84, 2002. PMID 11882785

94. Brown MC, Guest JF: Economic impact of feeding a phenylalanine-restricted diet to adults with previously untreated phenylketonuria. *J Intellect Disabil Res.* **43**:30, 1999. PMID 10088966

95. Burgard P et al: Results of the German collaborative study of phenylketonuria. *Acta Paediatr Suppl.* **407**:108, 1994. PMID 7766943

96. Burton B et al: A phase II, multicentre, open-label, screening study. *J Inherit Metab Dis.* **30**:700, 2007. PMID 17846916

97. Bush JW et al: *Analysis of PKU program, in Berg RL (ed).* : Health Status Indexes Hospital Research and Educational Trust. London, Health Status Indexes Hospital Research and Educational Trust, 1973. PMID 17846916

98. Byck S et al: Evidence for origin, by recurrent mutation, of the phenylalanine hydroxylase R408W mutation on two haplotypes in European and Quebec populations. *Hum Mol Genet.* **3**:1675, 1994. PMID 7833927

99. Byck S et al: The Quebec example. *Am J Hum Genet.* **59**:A33, 1996.

100. Byck S et al: Codon 280 contains recurrent mutations in Quebec and other populations. *Hum Mutat.* **9**:316, 1997. PMID 9101291

101. Caballero B et al: Plasma amino acid levels after single-dose aspartame consumption in phenylketonuria, mild-hyperphenylalaninemia, and heterozygous state for phenylketonuria. *J Pediatr.* **109**:668, 1986. PMID 3761084

102. Cabalska B et al: Termination of dietary treatment in phenylketonuria. *Eur J Pediatr.* **126**:253, 1977. PMID 590278

103. Caillaud C et al: Linkage disequilibrium between phenylketonuria and RFLP haplotype-1 at the phenylalanine hydroxylase locus in Portugal. *Hum Genet.* **89**:69, 1992. PMID 1349566

104. Cali F et al: The STR252-IVS10nt546-VNTR7 phenylalanine hydroxylase mini haplotype in five Mediterranean samples. *Hum Genet.* **100**:350, 1997. PMID 9272154

105. Campbell DG et al: Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase. *J Biol Chem.* **261**:10489, 1986. PMID 2874140

106. Cannon WB: Organization for physiological homeostasis. *Physiol Rev.* **9**:399, 1929.

107. Carr FPA, Pogson CI: Phenylalanine metabolism in isolated liver cells: Effects of glucagon and diabetes. *Biochem J.* **198**:655, 1981. PMID 7326031

108. Carter KC et al: The Quebec experience. *Eur J Hum Genet.* **6**:61, 1998. PMID 9781015

109. Cartier L et al: Prevention of mental retardation in offspring of hyperphenylalaninemic mothers. *Am J Pub Health.* **72**:1386, 1982. PMID 7137436

110. Cavalli-Sforza LL: The DNA revolution in population genetics. *Trends Genet.* **14**:60, 1998. PMID 9520599

111. Cavalli-Sforza LL et al: Demic expansions and human evolution. *Science.* **259**:639, 1993. PMID 8430313

112. Cavalli-Sforza LL: *et al:.* : The History and Geography of Human Genes. Princeton, NJ, Princeton University Press, 1994. PMID 8430313

113. Cavalli-Sforza LL, Piazza A: Human genomic diversity in Europe: A summary of recent research and prospects for the future. *Eur J Hum Genet.* **1**:3, 1993. PMID 7520820

114. Cerone R et al: Long-term follow-up of a patient with mild tetrahydrobiopterin-responsive phenylketonuria. *Mol Genet Metab.* **81**:137, 2004. PMID 14741196

115. Chace DH et al: Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry. *Clin Chem.* **39**:66, 1993. PMID 8419060

116. Chace DH et al: Use of phenylalanine-to-tyrosine ratio determined by tandem mass spectrometry to improve newborn screening for phenylketonuria of early discharge specimens collected in the first 24 hours. *Clin Chem.* **44**:2405, 1998. PMID 9836704

117. Chakraborty R et al: Polymorphic DNA haplotypes at the human phenylalanine hydroxylase locus and their relationship with phenylketonuria. *Hum Genet.* **76**:40, 1987. PMID 2883110

118. Changeux JP, Danchin A: Selective stabilization of developing synapses as a mechanism for the specification of neuronal networks. *Nature.* **264**:705, 1976. PMID 189195

119. Chelly J et al: Transcription of any gene in any cell type. *Proc Natl Acad Sci USA.* **86**:2617, 1989. PMID 2495532

120. Chen K-J et al: Identification and characterization of a novel liver-specific enhancer of the human phenylalanine hydroxylase gene. *Hum Genet.* **110**:235, 2002. PMID 11935335

121. Chen L: Complete and persistent phenotype correction of phenylketonuria in mice by site-specific genomic integration of murine phenylalanine hydroxylase cDNA. *Proc Natl Acad Sci USA.* **102**:15581, 2005. PMID 16230623

122. Chen L: Correction of female PKU mice by repeated administration of mPAH cDNA using phiBT1 integration system. *Mol Ther.* **15**:1789, 2007. PMID 17637719

123. Chen L et al: Metabolic basis of sexual dymorphism in PKU mice after genome-targeted PAH gene therapy. *Mol Ther.* **15**:1079, 2007. PMID 17406346

124. Chen RG et al: Twenty-one cases of phenylketonuria out of 348,767 newborns in Shanghai, China. *J Inherit Metab Dis.* **12**:485, 1989. PMID 2516180

125. McDonald JD, Cho S: Effect of maternal blood phenylalanine level on mouse maternal phenylketonuria offspring. *Mol Genet Metab.* **74**:420, 2001. PMID 11749047

126. Choi TB, Pardridge WM: Phenylalanine transport at the human blood-brain barrier. *J Biol Chem.* **261**:6536, 1986. PMID 3700406

127. Choo KH et al: Studies of human liver phenylalanine hydroxylase subunit structure and of mutations in phenylketonuria. *Biochem J.* **181**:285, 1979. PMID 496890

128. Choo KH et al: Observations indicating the nature of the mutation in phenylketonuria. *J Inherit Metab Dis.* **2**:79, 1980. PMID 6796764

129. Choo KH et al: Expression of rat phenylalanine hydroxylase. *DNA.* **5**:529, 1986. PMID 3028740

130. Christensen HN: Interorgan amino acid nutrition. *Physiol Rev.* **62**:1193, 1982. PMID 6752985

131. Christensen HN: *Where do the depleted plasma amino acids go in phenylketonuria? Biochem J.* **236**:929, 1986. PMID 3790098

132. Christensen HN: Hypothesis: Where the depleted plasma amino acids go in phenylketonuria, and why. *Perspect Biol Med.* **30**:186, 1987. PMID 3309879

133. Christensen HN et al: Gene-product designations for amino acid transporters. *J Exp Biol.* **196**:51, 1994. PMID 7823044

134. Christensen R et al: Development of a skin-based metabolic sink for phenylalanine by overexpression of phenylalanine hydroxylase and GTP cyclohydrolase in primary human keratinocytes. *Gene Ther.* **7**:1971, 2000. PMID 11175307

135. Citron BA et al: Purification and biochemical characterization of recombinant rat liver phenylalanine hydroxylase produced in Escherichia coli. *Protein Exp Purif.* **3**:93, 1992. PMID 1422220

136. Citron BA et al: Identity of 4α-carbinolamine dehydratase, a component of the phenylalanine hydroxylation system, and DCoH, a transregulator of homeodomain proteins. *Proc Natl Acad Sci USA.* **89**:11891, 1992. PMID 1465414

137. Citron BA et al: Mutation in the 4α-carbinolamine dehydratase gene leads to mild hyperphenylalaninemia with defective cofactor metabolism. *Am J Hum Genet.* **53**:768, 1993. PMID 8352282

138. Cockburn F, Clark BJ: Management of inborn errors of metabolism during pregnancy. *Acta Paediatr Scand Suppl.* **373**:43, 1991. PMID 1927527

139. Clarke JTR, Bier DM: The conversion of phenylalanine to tyrosine in man: Direct measurement by continuous intravenous tracer infusions of L-(ring-$^{2}H_{5}$) phenylalanine and L-(1-^{13}C) tyrosine in the postabsorptive state. *Metabolism.* **31**:999, 1982. PMID 7132735

140. Claustres M et al: A review of locus-specific mutation databases. *Genome Res.* **12**:680, 2002. PMID 11997335

141. Cleary MA, Wraith JE: Antenatal diagnosis of inborn errors of metabolism. *Arch Dis Child.* **66**:816, 1991. PMID 1863132

142. Clemens PC et al: *Is 240 µmol/liter the most appropriate cutoff level? Prev Med.* **19**:54, 1990. PMID 2181433

143. Cockburn F: Neonatal brain and dietary lipids. *Arch Dis Child.* **70**:F1, 1994. PMID 8117119

144. Cockburn F et al: Recommendations on the dietary management of phenylketonuria. *Arch Dis Child.* **68**:426, 1993. PMID 8466250

145. Cockburn F et al: Relevance to PKU. *Internat Pediatr.* **11**:56, 1996.

146. Cohn RM et al: *Non equilibrium thermodynamics, non covalent forces, and water, in RH Herman et al: (eds).* : Principles of Metabolic Control in Mammalian Species. New York, Plenum Press, 1980.

147. Krawczak M, Cooper DN: *Human Gene Mutation*. Oxford, Bios Scientific Publishers, 1993

148. Cooper DN, Youssoufian H: The CpG dinucleotide and human genetic disease. *Hum Genet.* **78**:151, 1988. PMID 3338800

149. Cordero ME et al: Histological maturation of the neocortex in phenylketonuric rats. *Early Hum Dev.* **8**:157, 1983. PMID 6684021

150. Costa LG et al: Do similar phenotypes indicate a common mode of action? A comparison of fetal alcohol syndorme, tolvene embryopathy and maternal phenylketonuria. *Toxicol Lett.* **127**:197, 2002. PMID 12052659

151. Cotton RGH: The primary molecular defects in phenylketonuria and its variants. *Int J Biochem.* **8**:333, 1977.

152. Cotton RGH: Current methods of mutation detection. *Mutat Res.* **285**:125, 1993. PMID 7678126

153. Cotton RGH: *Mutation Detection*. New York, Oxford University Press, 1997

154. Cotton RGH: Recommendations of the 2006 Human Variome Project meeting. *Nat Genet.* **39**:433, 2007.

155. Scriver CR, Cotton RGH: Proof of “disease-causing” mutation. *Hum Mutat.* **12**:1, 1998. PMID 9633813

156. Craine JE et al: The isolation and characterization of dihydropteridine reductase from sheep liver. *J Biol Chem.* **247**:6082, 1972. PMID 4405600

157. Cristiano RJ et al: adenovirus enhancement of receptor-mediated gene delivery and expression in primary hepatocytes. *Proc Natl Acad Sci USA.* **90**:2122, 1993. PMID 8384712

158. Crosby AW: *Ecological Imperialism.* : The Biological Expansion of Europe 900–1900. Oxford, Cambridge University Press, 1986. PMID 8384712

159. Curtius HC et al: The use of deuterated phenylalanine for the elucidation of the phenylalanine-tyrosine metabolism. *Clin Chim Acta.* **37**:277, 1972. PMID 5022091

160. Curtius HC et al: 7-Substituted pterins. *J Biol Chem.* **265**:3923, 1990. PMID 2303485

161. Curtius HC et al: Effect of high-protein meal plus aspartame ingestion on plasma phenylalanine concentrations in obligate heterozygotes for phenylketonuria. *Metabolism.* **43**:413, 1994. PMID 8159095

162. Dagenais DL et al: A cost-benefit analysis of the Quebec Network of Genetic Medicine. *Soc Sci Med.* **20**:601, 1985. PMID 3923626

163. Dahl H-H, Mercer JFB: Isolation and sequence of a cDNA clone which contains the complete coding region of rat phenylalanine hydroxylase: Structural homology with tyrosine hydroxylase, glucocorticoid regulation, and use of alternate polyadenylation sites. *J Biol Chem.* **261**:4148, 1986. PMID 2869038

164. Daiger SP et al: Polymorphic DNA haplotypes at the phenylalanine hydroxylase (PAH) locus in European families with phenylketonuria (PKU). *Am J Hum Genet.* **45**:310, 1989a. PMID 2569271

165. Daiger SP et al: Polymorphic DNA haplotypes at the phenylalanine hydroxylase (PAH) locus in Asian families with phenylketonuria (PKU). *Am J Hum Genet.* **45**:319, 1989b. PMID 2569272

166. Danks DM et al: Current status. *J Inherit Metab Dis.* **1**:49, 1978. PMID 117241

167. Davis MD et al: 7-Tetrahydrobiopterin, a naturally occurring analogue of tetrahydrobiopterin, is a cofactor for and a potential inhibitor of the aromatic amino acid hydroxylases. *Proc Natl Acad Sci USA.* **89**:10109, 1992. PMID 1359535

168. Davis MD et al: Structure-function relationships of phenylalanine hydroxylase revealed by radiation target analysis. *Arch Biochem Biophys.* **325**:235, 1996. PMID 8561502

169. Davis MD et al: The role of phenylalanine in structure-function relationships of phenylalanine hydroxylase revealed by radiation target analysis. *Proc Natl Acad Sci USA.* **94**:491, 1997. PMID 9012811

170. Degioanni A, Darlu P: Analysis of the molecular variance at the phenylalanine hydroxylase (PAH) locus. *Eur J Hum Genet.* **2**:166, 1994. PMID 7834276

171. Delvalle JA, Greengard O: The regulation of phenylalanine hydroxylase in rat tissues in vivo. The maintenance of high plasma phenylalanine concentrations in suckling rats: A model for phenylketonuria. *Biochem J.* **154**:613, 1976. PMID 133670

172. Denno KM, Sadler TW: Phenylalanine and its metabolites induce embryopathies in mouse embryos in culture. *Teratology.* **42**:565, 1990. PMID 2278031

173. Dent CE: Relation of biochemical abnormality to development of mental defect in phenylketonuria. Discussion to paper by Armstrong MD in Ross Labs (ed): *Report of 23rd Ross Pediatric Research Conference.* : Etiological Factors in Mental Retardation. Columbus, Ross Labs, 1957. PMID 2278031

174. Desviat LR et al: Evidence in Latin America of recurrence of V388M, a phenylketonuria mutation with high in vitro residual activity. *Am J Hum Genet.* **57**:337, 1995. PMID 7668259

175. Desviat LR et al: Prevalence of the IVS10nt546 mutation on haplotype 34. *Hum Mutat.* **9**:66, 1997. PMID 8990013

176. Desviat LR et al: Molecular survey by regions. *Eur J Hum Genet.* **7**:386, 1999. PMID 10234516

177. Desviat LR et al: Results of the BH_4 loading test in 31 Spanish PKU patients and correlation with their genotype. *Mol Genet Metab.* **83**:157, 2004. PMID 15464430

178. Di Silvestre et al: Different clinical manifestations of hyperphenylalaninemia in three siblings with identical phenylalanine hydroxylase genes. *Am J Hum Genet.* **48**:1014, 1991. PMID 2018035

179. Diamond A: Phenylalanine levels of 6–10 mg/dL may not be as benign as once thought. *Acta Paediatr Suppl.* **407**:89, 1994. PMID 7766969

180. Diamond A et al: Prefrontal cortex cognitive deficits in children treated early and continuously for PKU. *Monogr Soc Res Child Dev.* **62**:1, 1997. PMID 9421921

181. Dianzani I et al: Genetic history of phenylketonuria mutations in Italy. *Am J Hum Genet.* **55**:851, 1994. PMID 7942862

182. Dianzani I et al: Characterization of phenylketonuria alleles in the Italian population. *Eur J Hum Genet.* **3**:294, 1995. PMID 8556304

183. Dickson PW et al: Delineation of the catalytic core of phenylalanine hydroxylase and identification of glutamate 286 as a critical residue for pterin function. *J Biol Chem.* **269**:20369, 1994. PMID 7914195

184. DiLella AG et al: Molecular structure and polymorphic map of human phenylalanine hydroxylase gene. *Biochemistry.* **25**:743, 1986a. PMID 3008810

185. DiLella AG et al: Tight linkage between a splicing mutation and a specific DNA haplotype in phenylketonuria. *Nature.* **322**:799, 1986b. PMID 3018584

186. DiLella AG et al: An amino-acid substitution involved in phenylketonuria is in linkage disequilibrium with DNA haplotype 2. *Nature.* **327**:333, 1987. PMID 2884570

187. Ding Z et al: State-of-the-art 2003 on PKU gene therapy. *Mol Genet Metab.* **81**:3, 2004. PMID 14728985

188. Ding Z et al: Administration-route and gender-independent long-term therapeutic correction of phenylketonuria (PKU) in a mouse model by recombinant adeno-associated virus 8 pseudotyped vector-mediated gene transfer. *Gene Ther.* **13**:587, 2006. PMID 16319947

189. Dobson JC et al: Intellectual performance of 36 phenylketonuria patients and their nonaffected siblings. *Pediatrics.* **58**:53, 1976. PMID 934784

190. Dobson JC et al: Intellectual assessment of 111 four-year-old children with phenylketonuria. *Pediatrics.* **60**:822, 1977. PMID 600594

191. Doherty LB et al: Detection of phenylketonuria in the very early newborn blood specimen. *Pediatrics.* **87**:240, 1991. PMID 1987536

192. Donlon J, Beirne D: Modulations of rat hepatic phenylalanine hydroxylase due to induced diabetes or high protein diet. *Biochem Biophys Res Commun.* **108**:746, 1982. PMID 6756405

193. Kaufman S, Donlon J: Modification of the multiple forms of rat hepatic phenylalanine hydroxylase by in vitro phosphorylation. *Biochem Biophys Res Commun.* **78**:1011, 1977. PMID 911322

194. Kaufman S, Donlon J: Glucagon stimulation of rat hepatic phenylalanine hydroxylase through phosphorylation in vivo. *J Biol Chem.* **253**:6657, 1978. PMID 690116

195. Kaufman S, Donlon J: Relationship between the multiple forms of rat hepatic phenylalanine hydroxylase and degree of phosphorylation. *J Biol Chem.* **255**:2146, 1980. PMID 7354083

196. Doolittle RF: Lens proteins: More molecular opportunism. *Nature.* **336**:18, 1988. PMID 3185715

197. Dorland L et al: Phenylpyruvate, fetal damage, and maternal phenylketonuria syndrome. *Lancet.* **341**:1351, 1993. PMID 8098485

198. Doskeland AL et al: Some physical and chemical properties. *Neurochem Res.* **7**:407, 1982. PMID 7110509

199. Doskeland P et al: The effect of ligands of phenylalanine 4-monooxygenase on the cAMP-dependent phosphorylation of the enzyme. *J Biol Chem.* **259**:11242, 1984. PMID 6470001

200. Drogari E et al: An International collaborative study by the MCR/DHSS Phenylketonuria Register. *Lancet.* **2**:927, 1987. PMID 2889860

201. Duran GP et al: Necessity of complete intake of phenylalanine-free amino acid mixture for metabolic control of phenylketonuria. *J Am Dietet Assoc.* **99**:1559, 1999. PMID 10608951

202. Shah SN, Dwivedy AK: Effect of hyperphenylalaninemia on polyphosphoinositides content of rat brain. *Experientia.* **38**:1458, 1982. PMID 6295805

203. Dworniczak B et al: PCR detection of the PvuII (Ea) RFLP at the human phenylalanine hydroxylase (PAH) locus. *Nucleic Acids Res.* **19**:1958, 1991a. PMID 1674373

204. Dworniczak B et al: PCR detection of the Bg1II RFLP at the human phenylalanine hydroxylase (PAH) locus. *Nucleic Acids Res.* **19**:1958, 1991b. PMID 1674372

205. Dyer CA et al: Evidence for central nervous system glial cell plasticity in phenylketonuria. *J Neuropathol Exp Neurol.* **55**:795, 1996. PMID 8965095

206. Eavri R, Lorberboum-Galski H: A novel approach for enzyme replacement therapy: The use of phenylalanine hydroxylase–based fusion proteins for the treatment of phenylketonuria. *J Biol Chem.* **282**:23402, 2007.

207. Eiken HG et al: Relative frequency, heterogeneity and geographic clustering of PKU mutations in Norway. *Eur J Hum Genet.* **4**:205, 1996a. PMID 8875186

208. Eiken HG et al: Dection of eight (one de novo, seven inherited) of nine remaining Norwegian PKU mutation. *Hum Mutat.* **8**:19, 1996b. PMID 8807331

209. Eiken HG et al: Phenylketonuria genotypes correlated to metabolic phenotype groups in Norway. *Eur J Pediatr.* **155**:544, 1996c. PMID 8831077

210. Eisensmith RC et al: Multiple origins for phenylketonuria in Europe. *Am J Hum Genet.* **51**:1355, 1992. PMID 1361100

211. Eisensmith RC et al: A simple, rapid, and highly informative PCR-based procedure for prenatal diagnosis and carrier screening of phenylketonuria. *Prenat Diagn.* **14**:1113, 1994. PMID 7899279

212. Eisensmith RC et al: Recurrence of the R408W mutation in the phenylalanine hydroxylase locus in Europeans. *Am J Hum Genet.* **56**:278, 1995. PMID 7825588

213. Eisensmith RC et al: Molecular basis of phenylketonuria and a correlation between genotype and phenotype in a heterogeneous southeastern US population. *Pediatrics.* **97**:512, 1996. PMID 8632937

214. Eisensmith RC, Woo SLC: Letters to the Editor: Updated listing of haplotypes at the human phenylalanine hydroxylase (PAH) locus. *Am J Hum Genet.* **51**:1445, 1992. PMID 1361103

215. Eisensmith RC, Woo SLC: Molecular genetics of phenylketonuria: *From molecular anthropology to gene therapy, in JC Hall, JC Dunlap (eds).* : Advances in Genetics, vol 32. 1995. PMID 1361103

216. Eisensmith RC, Woo SL: Somatic gene therapy for phenylketonuria and other hepatic deficiencies. *J Inherit Metab Dis.* **19**:412, 1996. PMID 8884565

217. Embury JE et al: PKU is a reversible neurogenerative process within the nigrostriatum that begins as early as 4 weeks of age in Pah (enu2) mice. *Brain Res.* **1127**:136, 2007. PMID 17112485

218. ENCODE Project: Identification and analysis of functional elements in 1 percent of the human genome by the ENCODE pilot project. *Nature.* **447**:799, 2007.

219. Epps RP: Phenylketonuria in an American Negro infant. *Clin Pediatr (Phila).* **7**:607, 1968. PMID 5682378

220. Epstein CM et al: EEG mean frequencies are sensitive indices of phenylalanine effects on normal brain. *Neurophysiology.* **72**:133, 1989. PMID 2464484

221. Erlandsen H et al: Correction of kinetic and stability defects by the tetrahydrobiopterin in phenylketonuria patients with certain phenylalanine hydroxylase mutations. *Proc Natl Acad Sci USA.* **101**:16903, 2004. PMID 15557004

222. Erlandsen H et al: Crystallization and preliminary diffraction analysis of a truncated homodimer of human phenylalanine hydroxylase. *FEBS Lett.* **406**:171, 1997a. PMID 9109411

223. Erlandsen H et al: Crystal structure of the catalytic domain of human phenylalanine hydroxylase reveals the structural basis for phenylketonuria. *Nat Struct Biol.* **4**:995, 1997b. PMID 9406548

224. Erlandsen H et al: Crystal structure and site-specific mutagenesis of pterin-bound human phenylalanine hydroxylase. *Biochemistry.* **39**:2208, 2000. PMID 10694386

225. Stevens RC, Erlandsen H: The structural basis of phenylketonuria. *Mol Genet Metab.* **68**:103, 1999. PMID 10527663

226. Stevens RC, Erlandsen H: A structural hypothesis for BH_4 responsiveness in patients with mild forms of hyperphenylalaninemia and phenylketonuria. *J Inherit Metab Dis.* **24**:213, 2001. PMID 11405341

227. Erlandsen H et al: Correction of kinetic and stability defects by tetrahydrobiopterin in phenylketonuria patients with certain phenylalanine hydroxylase mutations. *Proc Natl Acad Ssi USA.* **101**:16903, 2004. PMID 15557004

228. European Journal: Phenylketonuria, an international survey of management over 40 years. Proceedings of a workshop. Fulda, November 16–19, 1994. *Eur J Pediatr.* **155**:S1, 1996. PMID 8991792

229. Familusi JB, Bolodeoku JO: Blood phenylalanine levels in mentally retarded African children: A study of 138 patients from Ibidan, Nigeria. *Trop Geogr Med.* **28**:96, 1976. PMID 973249

230. Fang B et al: Phenotypic correction in a genetically deficiency mouse model by adenovirus-mediated hepatic gene transfer. *Gene Ther.* **1**:247, 1994. PMID 7584088

231. Farquharson J et al: Effect of diet on infant subcutaneous tissue triglyceride fatty acids. *Arch Dis Child.* **69**:589, 1993. PMID 8257182

232. Farquharson J et al: Age- and dietary-related distributions of hepatic arachidonic and docasahexaenoic acid in early infancy. *Pediatr Res.* **38**:361, 1995a. PMID 7494660

233. Farquharson J et al: Effect of diet on the fatty acid composition of the major phospholipids of infant cerebral cortex. *Arch Dis Child.* **72**:198, 1995b. PMID 7741563

234. Fauquet M et al: Amino acid homology with other hydroxylases discloses functional domains. *J Neurochem.* **50**:142, 1988. PMID 2447231

235. Faust D et al: Neuropsychological functioning in treated phenylketonuria. *Int J Psychiatr Med.* **16**:169, 1986. PMID 3744685

236. Faust DM et al: The activity of the highly inducible mouse phenylalanine hydroxylase gene promoter is dependent upon a tissue-specific, hormone-inducible enhancer. *Mol Cell Biol.* **16**:3125, 1996. PMID 8649424

237. Fehrenbach AMB, Petersen L: Parental problem-solving skills, stress, and dietary compliance in phenylketonuria. *J Consult Clin Psychol.* **57**:237, 1989. PMID 2708611

238. Feldmann R et al: No specific frontal lobe-dependent neurospsychological deficits of early-treated patients in comparison with diabetics. *Pediatr Res.* **51**:761, 2002. PMID 12032274

239. Blau N, Fiege B: Assessment of tetrahydrobiopterin (BH_4) responsiveness in phenylketonuria. *J Pediatr.* **150**:627, 2007. PMID 17517248

240. Stegink LD, Filer LJ: Aspartame metabolism in normal adults, phenylketonuric heterozygotes, and diabetic subjects. *Diabetes Care.* **12**:67, 1989. PMID 2653751

241. Finkelson L et al: Predicting diet continuation and maintenance. *J Inherit Metab Dis.* **24**:515, 2001. PMID 11596658

242. Fiori L et al: Incidence of BH_4-responsiveness in phenylalanine-hydroxylase-deficient Italian patients. *Mol Genet Metab.* **86**:S67, 2005. PMID 16198137

243. Fisch RO et al: current dietary treatment practices in the United States and Canada. *J Am College Nutr.* **16**:147, 1997. PMID 9100215

244. Kaufman S, Fisher DB: The stimulation of rat liver phenylalanine hydroxylase by phospholipids. *J Biol Chem.* **247**:2250, 1972. PMID 5062822

245. Fisch RO et al: An international survey. *J Pediatr.* **118**:739, 1991. PMID 2019929

246. Fisher DB et al: Rat liver phenylalanine hydroxylase, an iron enzyme. *J Biol Chem.* **247**:5161, 1972. PMID 4341541

247. Kaufman S, Fisher DB: The stimulation of rat liver phenylalanine hydroxylase by lysolecithin and alpha-chymotrypsin. *J Biol Chem.* **248**:4345, 1973. PMID 4145799

248. Fishler K et al: Psychoeducational findings among children treated for phenylketonuria. *Am J Ment Defic.* **92**:65, 1987. PMID 3618658

249. Flatmark T et al: *Molecular characterization of disease related mutant forms of human phenylalanine hyroxylase and tyrosine hydroxylase, in Pfleiderer W, Rokos hours(eds).* : Chemistry and Biology of Pteridines and Folates. Germany, 1997. PMID 3618658

250. Flatmark T, Stevens RC: Structural insight into the aromatic amino acid hydroxylases and their disease-related mutant forms. *Chem Rev.* **99**:2137, 1999. PMID 11849022

251. Flint XJX et al: Why are some genetic disease common? Distinguishing selection from other processes by molecular analysis of globin gene variants. *Hum Genet.* **91**:91, 1993. PMID 8462981

252. Flynn JR: The mean IQ of Americans: Massive gains 1932–1978. *Physiol Bull.* **95**:29, 1984.

253. FÃ¸lling A: Uber Ausscheidung von Phenylbrenztraubensaure in den Harn als Stoffwechselanomalie in Verbindung mit Imbezillitat. *Hoppe-Seylers Z Physiol Chem.* **277**:169, 1934.

254. FÃ¸lling A et al: A recessive syndrome in man. *Skrifter Det Norske Vitenskapsakademi i Oslo I Mat Naturv Klasse.* **1**:14, 1945.

255. FÃ¸lling I: The discovery of phenylketonuria. *Acta Pediatr Suppl.* **407**:4, 1994. PMID 7766954

256. Fox-Bacon C et al: Case report of identical twin mothers. *Clin Pediatr (Phila).* **36**:539, 1997. PMID 9307089

257. Freehauf CL et al: Comparison of two discriminant analysis procedures. *Am J Hum Genet.* **36**:1180, 1984. PMID 6517048

258. Freimer N, Sabatti C: The Human Phenome Project. *Nat Genet.* **34**:15, 2003. PMID 12721547

259. Friedman PA et al: Nature of the molecular defect in phenylketonuria and hyperphenylalaninemia. *Nature.* **240**:157, 1972. PMID 4118080

260. Friedman PA et al: Partial purification and characterization of tryptophan hydroxylase from rabbit hindbrain. *J Biol Chem.* **247**:4165, 1972. PMID 4402511

261. Friedman PA et al: Detection of hepatic phenylalanine 4-hydroxylase in classical phenylketonuria. *Proc Natl Acad Sci USA.* **70**:552, 1973. PMID 4405625

262. Kaufman S, Friedman PA: A study of the development of phenylalanine hydroxylase in fetuses of several mammalian species. *Arch Biochem Biophys.* **146**:321, 1971. PMID 5144032

263. Fusetti F et al: Structure of tetrameric human phenylalanine hydroxylase and its implications for phenylketonuria. *J Biol Chem.* **273**:16962, 1998. PMID 9642259

264. Galli C et al: Reduced plasma C-20 and C-22 polyunsaturated fatty acids in children with phenylketonuria during dietary intervention. *J Pediatr.* **119**:562, 1991. PMID 1919887

265. Gamez A et al: Development of pegylated forms of recombinated Rhodosporidium toruloides phenylalanine ammonia-lyase for the treatment of classical phenylketonuria. *Mol Ther.* **11**:986, 2005. PMID 15922970

266. Gamez A et al: Expression analysis of phenylketonuria mutations. *J Biol Chem.* **275**:29737, 2000. PMID 10875932

267. Gamez A et al: Development of pegylated forms of recombinant Rhodosporidium toruloides phenylalanine ammonia-lyase for the treatment of classical phenylketonuria. *Mol Ther.* **11**:986, 2005.

268. Gamez A et al: Structure-based epitope and PEGylation sites mapping of phenylalanine ammonia-lyase for enzyme substitution treatment of phenylketonuria. *Mol Genet Metab.* **91**:325, 2007.

269. Gardiner RM: Transport of amino acids across the blood-brain barrier: Implications for treatment of maternal phenylketonuria. *J Inherit Metab Dis.* **13**:627, 1990. PMID 2122125

270. Garrison JC, Wagner JD: Glucagon and the Ca^{2+}-linked hormones angiotensin II, norepinephrine, and vasopressin stimulate the phosphorylation of distinct substrates in intact hepatocytes. *J Biol Chem.* **257**:13135, 1982. PMID 6290494

271. Giardine B et al: Connecting ENCODE data with mutations and phenotype. *Hum Mutat.* **28**:554, 2007.

272. Giannattasio S et al: The analysis of an STR system in the human phenylalanine hydroxylase gene. *Mol Cell Probes.* **11**:81, 1997. PMID 9076721

273. Gibbs BS et al: Expression of rat liver phenylalanine hydroxylase in insect cells and site-directed mutagenesis of putative non-heme iron-binding sites. *J Biol Chem.* **268**:8046, 1993. PMID 8385134

274. Giewska L et al: Preliminary data. *J Inherit Metab Dis.* **24**: 2001.

275. Gilbert HJ et al: Molecular cloning of the phenylalanine ammonia lyase gene from Rhodosporidium toruloides in Escherichia coli K-12. *J Bacteriol.* **161**:314, 1985. PMID 2981805

276. Giovannini M et al: Fatty acid metabolism in phenylketonuria. *Eur J Pediatr.* **155**:S132, 1966. PMID 8828629

277. Gjetting T et al: Missense mutations in the N-terminal domain of human phenylalanine hydroxylase interfere with binding of regulatory phenylalanine. *Am J Hum Genet.* **68**:1353, 2001a. PMID 11326337

278. Gjetting T et al: A phenylalanine hydroxylase amino acid polymorphism with implications for molecular diagnostics. *Mol Genet Metab.* **73**:280, 2001b. PMID 11461196

279. Schreiner R, Goebel-Schreiner B: Identification of a new missence mutation in Japanese phenylketonuric patients. *Inherit Metab Dis.* **16**:950, 1993. PMID 8068076

280. Gold RJM et al: The use of biochemical data in screening for mutant alleles and in genetic counselling. *Ann Hum Genet.* **37**:315, 1974. PMID 4812952

281. Goltsov A et al: Detection of the XmnI RFLP at the human phenylalanine hydroxylase locus by PCR. *Nucleic Acids Res.* **20**:927, 1992a. PMID 1347420

282. Goltsov AA et al: Associations between mutations and a VNTR in the human phenylalanine hydroxylase gene. *Am J Hum Genet.* **51**:627, 1992b. PMID 1353941

283. Goltsov AA et al: A single polymorphic STR system in the human phenylalanine hydroxylase gene permits rapid prenatal diagnosis and carrier screening for phenylketonuria. *Hum Mol Genet.* **2**:577, 1993. PMID 8100164

284. Koch R, Graw RG: Phenylketonuria in two American Negroes. *Am J Dis Child.* **114**:412, 1967. PMID 6047241

285. Green AK et al: Experimental determination of the phosphorylation state of phenylalanine hydroxylase. *Biochem J.* **265**:563, 1990. PMID 2302187

286. Gregory DM et al: Plasma free amino acid values in normal children and adolescents. *Metabolism.* **35**:967, 1986. PMID 3762400

287. Grenett HE et al: Functional domains and evolution of aromatic amino acid hydroxylases. *Proc Natl Acad Sci USA.* **84**:5530, 1987. PMID 3475690

288. Griffiths P et al: Correlation with historical and concurrent phenylalanine levels. *J Intellect Disabil Res.* **41**:317, 1997. PMID 9297609

289. Griffiths P et al: Transitory hyperphenylalaninemia in children with continuously treated phenylketonuria. *Am J Ment Retard.* **102**:27, 1997. PMID 9241405

290. Griffiths P et al: Executive function in treated phenylketonuria as measured by the one-back and two-back versions of the continuous performance test. *J Inherit Metab Dis.* **21**:125, 1998. PMID 9584263

291. Griffiths P et al: Neuropsychological outcome of experimental manipulation of phenylalanine intake in treated phenylketonuria. *J Inherit Metab Dis.* **21**:29, 1998. PMID 9501267

292. Grimm U et al: Phenylalaninhydroxylase-Aktivität beiketerozygoten Analgeträgen für das Phenylketerurie-Gen. *Acta Biol Med Germ.* **36**:1179, 1977. PMID 612095

293. Grompe M: The rapid detection of unknown mutations in nucleic acids. *Nat Genet.* **5**:111, 1993. PMID 8252035

294. Gropper SS et al: Trace element status of children with PKU and normal children. *J Am Diet Assoc.* **88**:459, 1988. PMID 3351165

295. Gu XF et al: Phenylketonuria mutations in southern Chinese detected by denaturing gradient gel electrophoresis in exon 7 of PAH gene. *J Inherit Metab Dis.* **18**:753, 1995. PMID 8750617

296. Wang Z, Gu X: Screening for phenylketonuria and congenital hypothyroidism in 5.8 million neonates in China. *Chin J Prevent Med.* **38**:99, 2004. PMID 15061917

297. Guerin T et al: Correlation of rat hepatic phenylalanine hydroxylase, with tetrahydrobiopterin and GTP concentrations. *Int J Biochem Cell Biol.* **30**:1047, 1968. PMID 9785468

298. Guldberg P et al: 99 percent of the mutations detected by denaturing gradient gel electrophoresis. *Genomics.* **17**:141, 1993. PMID 8406445

299. Guldberg P et al: Implications for diagnosis of hyperphenylalaninemia in southern Europe. *Hum Mol Genet.* **2**:1703, 1993a. PMID 8268925

300. Guldberg P et al: A novel missense mutation in the phenylalanine hydroxylase gene of a homozygous Pakistani patient with non-PKU hyperphenylalaninemia. *Hum Mol Genet.* **2**:1061, 1993b. PMID 8364546

301. Guldberg P et al: Molecular heterogeneity of nonphenylketonuria hyperphenylalaninemia in 25 Danish patients. *Genomics.* **21**:453, 1994. PMID 8088845

302. Guldberg P et al: Characterization of seven common mutations. *Eur J Pediatr.* **154**:551, 1995. PMID 7556322

303. Guldberg P et al: Report from the Maternal PKU Collaborative Study. *Am J Hum Genet.* **59**:84, 1996a. PMID 8659548

304. Guldberg P et al: Mutational profile and nine novel mutations. *Hum Mutat.* **8**:276, 1996b. PMID 8889590

305. Guldberg P et al: Implications for diagnosis and genetic counselling. *J Med Genet.* **33**:161, 1996c. PMID 8929956

306. Guldberg P et al: Identification of a founding mutation and evidence of predominant Norse settlement. *Eur J Hum Genet.* **5**:376, 1997a. PMID 9450182

307. Guldberg P et al: Large deletions in the phenylalanine hydroxylase gene as a cause of phenylketonuria in India. *J Inherit Metab Dis.* **20**:845, 1997b. PMID 9427161

308. Guldberg P et al: Classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype. *Am J Hum Genet.* **63**:71, 1998. PMID 9634518

309. Guneral F et al: Detection by a spectrofluorimetric method. *J Inherit Metab Dis.* **14**:741, 1991. PMID 1843186

310. Gungor N et al: Microcephaly in a hyperphenylalaninemic infant leading to the diagnosis of maternal hyperphenylalaninemia. *Eur J Pediatr.* **155**:257, 1996. PMID 8929743

311. Guthrie R: The introduction of newborn screening for phenylketonuria. *Eur J Pediatr.* **155**:S4, 1996. PMID 8828599

312. Guthrie R, Susi A: A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics.* **32**:338, 1963. PMID 14063511

313. GÃ¼ttler F: Hyperphenylalaninemia: Diagnosis and classification of the various types of phenylalanine hydroxylase deficiency in childhood. *Acta Paediatr Scand.* **280**:S1, 1980. PMID 7006308

314. GÃ¼ttler F et al: Effects of oral phenylalanine load on plasma glucagon, insulin, amino acid and glucose concentrations in man. *Scand J Clin Lab Invest.* **38**:255, 1978. PMID 663549

315. GÃ¼ttler F et al: Report from the Maternal Phenylketonuria Collaborative Study. *Pediatrics.* **104**:258, 1999. PMID 10429004

316. GÃ¼ttler F et al: Impact of the phenylalanine hydroxylase gene on maternal PKU children. *Pediatrics.* **112**:1530, 2003. PMID 14654659

317. GÃ¼ttler F: Hyperphenylalaninemia: Diagnosis and classification of the various types of phenylalanine hydroxylase deficiency in childhood. *Acta Paediatr Scand.* **280**:1, 1980. PMID 7006308

318. GÃ¼ttler F et al: Cognitive development in offspring of untreated and preconceptionally treated maternal phenylketonuria. *J Inherit Metab Dis.* **13**:665, 1990. PMID 2122130

319. GÃ¼ttler F et al: Molecular basis for the phenotypical diversity of phenylketonuria and related hyperphenylalaninaemias. *J Inherit Metab Dis.* **16**:602, 1993. PMID 7609462

320. Haefele M et al: Characterization of the mouse phenylalanine hydroxylase mutation Pah^{enu3}. *Mol Genet Metab.* **72**:27, 2001. PMID 11161825

321. Hanley WB: Prenatal testing for maternal phenylketonuria (MPKU). *Int Pediatr.* **9**:S33, 1994.

322. Hanley WB et al: Malnutrition with early treatment of phenylketonuria. *Pediatr Res.* **4**:318, 1970. PMID 5447890

323. Hanley WB et al: A review. *Clin Biochem.* **20**:149, 1987. PMID 3308176

324. Hanley WB et al: Guthrie (BIA) screening and early hospital discharge. *Early Hum Dev.* **47**:87, 1997. PMID 9118832

325. Hanley WB et al: The need for prenatal selective screening or case finding. *Am J Obstet Gynecol.* **180**:986, 1999. PMID 10203668

326. Hardelid P et al: The birth prevlance of PKU in populations of European, South Asian and sub-Saharan African ancestry living in South East England. *Ann Hum Genet.* **71**:1, 2007.

327. Harding CO et al: Development of mice with phenylalanine hydroxylase expression in muscle. *Gene Ther.* **5**:683, 1998. PMID 9797873

328. Harding CO et al: Expression of phenylalanine hydroxylase (PAH) in erythrogenic bone marrow does not correct hyperphenylalaninemia in Pah^{enu2} mice. *J Gene Med.* **5**:984, 2003. PMID 14601136

329. Harding CO et al: Complete correction of hyperphenylalaninemia following liver-directed recombinant AAV2/8 vector-mediated gene therapy in murine phenylketonuria. *Gene Ther.* **13**:457, 2006. PMID 16319949

330. Hargreaves KM, Partridge WM: Neutral amino acid transport at the human blood-brain barrier. *J Biol Chem.* **263**:19392, 1988. PMID 2848825

331. Harper M, Reid AH: Use of a restricted protein diet in the treatment of behaviour disorder in a severely mentally retarded adult female phenylketonuric patient. *J Ment Defic Res.* **31**:209, 1987. PMID 3625767

332. Hashem N et al: Preliminary studies on the molecular basis of hyperphenylalaninemia in Egypt. *Hum Genet.* **98**:3, 1996. PMID 8682503

333. Hauer CR et al: Phenylalanine hydroxylase-stimulating protein/pterin-4α-carbinolamine dehydratase from rat and human liver. *J Biol Chem.* **268**:4828, 1993. PMID 8444860

334. Henderson RM et al: Visual event-related potentials in children with phenylketonuria. *Acta Paediatr.* **89**:52, 2000. PMID 10677058

335. Hennermann JB et al: Long-term treatment with tetrahydrobiopterin increases phenylalanine tolerance in children with severe phenotype of phenylketonuria. *Mol Genet Metab.* **86**:S86, 2005. PMID 16051511

336. Herrero E et al: Implications in the pathogenesis of phenylketonuria. *J Inherit Metab Dis.* **6**:32, 1983. PMID 6408306

337. Hill JB: A climatological factor influencing the determination of phenylalanine in blood of newborn infants in North Carolina. *Biochem Med.* **2**:261, 1969.

338. Hilton MA et al: A simple method for detection of heterozygous carriers of the gene for classic phenylketonuria. *J Pediatr.* **109**:601, 1986. PMID 3761073

339. Hodgins D: Yeast phenylalanine ammonia lyase: Purification, properties of the identification of catalytically essential dehydroalanine. *J Biol Chem.* **246**:2977, 1971. PMID 5102931

340. Hofman KJ et al: Molecular analysis of the phenylalanine hydroxylase gene. *Am J Hum Genet.* **48**:791, 1991. PMID 2014802

341. Hogan SE et al: Experience with adolescents with phenylketonuria returned to phenylalanine-restricted diets. *J Am Diet Assoc.* **86**:1203, 1986. PMID 3745744

342. Holm VA et al: II. Growth of treated children in the PKU collaborative study from birth to 4 years of age. *Pediatrics.* **63**:700, 1979. PMID 312490

343. Holton JB: Long-term results of treatment of some inherited metabolic diseases. *Pediatr Rev Commun.* **8**:139, 1995.

344. Holtzman C et al: Descriptive epidemiology of missed cases of phenylketonuria and congenital hypothyroidism. *Pediatrics.* **78**:553, 1986a. PMID 3763262

345. Holtzman NA: Anatomy of a trial. *Pediatrics.* **60**:932, 1997. PMID 600611

346. Holtzman NA et al: II. Age dependence of initial phenylalanine in infants with PKU. *Pediatrics.* **53**:353, 1974. PMID 4815254

347. Holtzman NA et al: III. Altered sex ratio; extent and possible causes. *J Pediatr.* **85**:175, 1974. PMID 4842793

348. Holtzman NA et al: A randomized, controlled study. *N Engl J Med.* **293**:1121, 1975. PMID 1102976

349. Holtzman NA et al: Effect of age at loss of dietary control on intellectual performance and behaviour of children with phenylketonuria. *N Engl J Med.* **314**:593, 1986b. PMID 3945244

350. Hommes FA: The role of the blood-brain barrier in the aetiology of permanent brain dysfunction in hyperphenylalaninemia. *J Inherit Metab Dis.* **12**:41, 1989. PMID 2501582

351. Hommes FA: On the mechanism of permanent brain dysfunction in hyperphenylalaninemia. *Biochem Med Metabol Biol.* **46**:277, 1991. PMID 1793607

352. Hommes FA: The effect of hyperphenylalaninaemia on the muscarinic acetylcholine receptor in the HPH-5 mouse brain. *J Inherit Metab Dis.* **16**:962, 1993. PMID 8127072

353. Hommes FA: Loss of neurotransmitter receptors by hyperphenylalaninemia in the HPH-5 mouse brain. *Acta Paediatr Suppl.* **407**:120, 1994. PMID 7766946

354. Hommes FA, Lee JS: The effect of plasma valine, isoleucine and leucine on the control of the flux through tyrosine- and tryptophan-hydroxylase in the brain. *J Inherit Metab Dis.* **13**:151, 1990. PMID 1974311

355. Hommes FA, Moss L: Myelin turnover in hyperphenylalaninaemia: A reevaluation with the HPH-5 mouse. *J Inherit Metab Dis.* **15**:243, 1992. PMID 1527991

356. Horaitis O et al: A database of locus-specific databases. *Nat Genet.* **39**:425, 2007.

357. Horne J et al: Structural characterization of the N-terminal autoregulatory sequence of phenylalanine hydroxylase. *Protein Sci.* **11**:2041, 2002. PMID 12142458

358. Hoshiga M et al: Autoradiographic distribution of [14C]tetrahydrobiopterin and its developmental change in mice. *J Pharmacol Exp Ther.* **267**:971, 1993. PMID 8246172

359. Hoskin R et al: The use of a low phenylalanine diet with amino acid supplement in the treatment of behavioural problems in a severely mentally retarded adult female with phenylketonuria. *J Intellect Disabil Res.* **36**:183, 1992. PMID 1591502

360. Hoskins JA et al: Enzymatic control of phenylalanine intake in phenylketonuria. *Lancet.* **1**:392, 1980. PMID 6101846

361. Hoskins JA et al: A kinetic study. *Biomed Mass Spectrom.* **11**:296, 1984. PMID 6743769

362. House JD et al: Phenylalanine requirements determined by using L-[^{14}C]phenylalanine in neonatal piglets receiving total parenteral nutrition supplemented with tyrosine. *Am J Clin Nutr.* **65**:984, 1997. PMID 9094883

363. Hsia DYY et al: A one-year controlled study of the effects of low-phenylalanine diet on phenylketonuria. *Pediatrics.* **21**:178, 1958. PMID 13505012

364. Huang CY et al: Purification and characterization of phenylalanine hydroxylase-stimulating protein from rat liver. *J Biol Chem.* **248**:4235, 1973. PMID 4711606

365. Huang T et al: Determination of L-phenylalanine based on an NADH-detecting biosensor. *Anal Chem.* **70**:991, 1998. PMID 9511473

366. Huether G et al: Myelination. *Neuropediatrics.* **13**:177, 1982. PMID 6891443

367. Huether G et al: Disturbed proliferation and reduced cell numbers in the cerebellum. *Neuropediatrics.* **14**:12, 1983. PMID 6682186

368. Huether G et al: A contribution to the understanding of the metabolic defects in phenylketonuria. *Biochem Med.* **33**:334, 1985. PMID 3925942

369. Huether G, Neuhoff V: Use of α-methylphenylalanine for studies of brain development in experimental phenylketonuria. *J Inherit Metab Dis.* **4**:67, 1981. PMID 6790851

370. Hufton SE et al: Structure and function on the aromatic amino acid hydroxylases. *Biochem J.* **311**:353, 1995. PMID 7487868

371. Hufton SE et al: Structure/function analysis of the domains required for the multimerisation of phenylalanine hydroxylase. *Biochim Biophys Acta.* **1382**:295, 1998. PMID 9540801

372. Huijbregts SC et al: Effects on neuropsychological outcome of a well-controlled populations. *J Inherit Metab Dis.* **25**:419, 2002. PMID 12555935

373. Hutchesson AC et al: A comparison of disease and gene frequencies of inborn errors of metabolism among different ethnic groups in the West Midlands, U.K. *J Med Genet.* **35**:366, 1998. PMID 9610798

374. Hyanek J et al: Changes in phenylalanine tolerance while monitoring the dietetic treatment of pregnant women suffering from hyperphenylalaninemia. *J Inherit Metab Dis.* **11**:427, 1988. PMID 3149703

375. Hyland K et al: Tetrahydrobiopterin and biogenic amine metabolism in the hph-1 mouse. *J Neurochem.* **67**:752, 1996. PMID 8764604

376. Cohen P, Ingebritsen TA: Protein phosphatases: Properties and role in cellular regulation. *Science.* **221**:331, 1983. PMID 6306765

377. International Human: Initial sequencing and analysis of the human genome. *Nature.* **409**:860, 2001. PMID 11237011

378. Iwaki M et al: Proteolytic modification of the amino-terminal and carboxyl-terminal regions of rat hepatic phenylalanine hydroxylase. *J Biol Chem.* **261**:2051, 1986. PMID 3944127

379. Jedlicki E et al: Partial purification and characterization of rat liver phenylalanine hydroxylase phosphatase. *J Biol Chem.* **252**:7711, 1977. PMID 199597

380. Cotton R, Jennings I: Structural similarities among enzyme pterin binding sites as demonstrated by a monoclonal anti-idiotypic antibody. *J Biol Chem.* **265**:1885, 1990. PMID 1967605

381. Jennings IG et al: Phenlylanine hydroxylase. *Proc Natl Acad Sci USA.* **88**:5734, 1991. PMID 2062852

382. Jennings IG et al: Funtional analysis, using in vitro mutagenesis, of amino acids located in the phenylalanine hydroxylase active site. *Arch Biochem Biophys.* **384**:238, 2000. PMID 11368310

383. Jervis GA: The genetics of phenylpyruvic oligophrenia. *J Mental Sci.* **1**:1939.

384. Jervis GA: Phenylpyruvic oligophrenia: Deficiency of phenylalanine oxidizing system. *Proc Soc Exp Biol Med.* **82**:514, 1953. PMID 13047448

385. John SWM et al: Evidence in French-Canadians and a catalog of mutations. *Am J Hum Genet.* **46**:970, 1990. PMID 1971147

386. Kaufman S, Johnen G: Studies on the enzymatic and transcriptional activity of the dimerization cofactor for hepatocyte nuclear factor 1. *Proc Natl Acad Sci USA.* **94**:13469, 1997. PMID 9391049

387. Johnen G et al: Characterization of the wild-type of 4α-carbinolamine dehydratase and two naturally occurring mutants associated with hyperphenylalaninemia. *Proc Natl Acad Sci USA.* **92**:12384, 1995. PMID 8618906

388. Johnson RC, Shah SM: Effects of α-methylphenylalanine plus phenylalanine treatment during development of myelin in rat brain. *Neurochem Res.* **5**:709, 1980. PMID 7422057

389. Johnson RC, Shah SN: Effect of hyperphenylalaninemia induced during suckling on brain DNA metabolism in rat pups. *Neurochem Res.* **9**:517, 1984. PMID 6235457

390. Jordan MK et al: Preliminary support for the oral administration of valine, iosleucine and leucine for phenylketonuria. *Dev Med Child Neurol.* **27**:33, 1985. PMID 3979670

391. Kacser H, Burns JA: The control of flux. *Symp Soc Exp Biol.* **27**:65, 1973. PMID 4148886

392. Kacser H, Burns JA: The molecular basis of dominance. *Genetics.* **97**:639, 1981. PMID 7297851

393. Kacser H, Porteous JW: Control of metabolism: *What do we have to measure? Trends Biochem Sci.* **12**:5, 1987. PMID 7297851

394. Kadasi L et al: Mutation screening and haplotype analysis. *Hum Genet.* **95**:112, 1995. PMID 7814013

395. Kalanin J et al: A point mutation of the phenylalanine hydroxylase gene in Gypsy families from Slovakia. *Am J Med Genet.* **49**:235, 1994. PMID 8116675

396. Kalaydjieva L et al: RFLP haplotypes and frequency of the major mutations. *J Med Genet.* **27**:742, 1990. PMID 1981599

397. Kalkanoglu HS et al: Experiences from Denmark. *J Inherit Metab Dis.* **25**:624, 2002.

398. Kalsner LR et al: Diurnal blood tyrosine levels and presumptive brain influx of tyrosine and other large neutral amino acids. *J Pediatr.* **139**:421, 2001. PMID 11562623

399. Kalverboer AF et al: Social behaviour and task orientation in early-treated PKU. *Acta Paediatr Suppl.* **407**:104, 1994. PMID 7766941

400. Kanai Y et al: Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). *J Biol Chem.* **273**:23629, 1998. PMID 9726963

401. Kappock JT et al: Comparison of resting and activated states. *J Biol Chem.* **270**:30532, 1995. PMID 8530485

402. Katz I et al: Studies on the phenylalanine and tyrosine hydroxylation by rat brain tyrosine hydroxylase. *Biochim Biophys Acta.* **445**:567, 1976. PMID 9989

403. Kaufman S: Phenylalanine hydroxylation cofactor in phenylketonuria. *Science.* **128**:1506, 1958. PMID 13615302

404. Kaufman S: Studies on the mechanism of the enzymatic conversion of phenylalanine to tyrosine. *J Biol Chem.* **234**:2677, 1959. PMID 14404870

405. Kaufman S: The structure of phenylalanine hydroxylation cofactor. *Proc Natl Acad Sci USA.* **50**:1085, 1963. PMID 14096182

406. Kaufman S: Metabolism of phenylalanine hydroxylation cofactor. *J Biol Chem.* **242**:3934, 1967. PMID 4382563

407. Kaufman S: A protein that stimulates rat liver phenylalanine hydroxylase. *J Biol Chem.* **245**:4751, 1970. PMID 5456148

408. Kaufman S: The phenylalanine hydroxylating system from mammalian liver. *Adv Enzymol Relat Areas Mol Biol.* **35**:245, 1971. PMID 4150152

409. Kaufman S: Phenylketonuria: Biochemical mechanisms. *Adv Neurochem.* **2**:1, 1971.

410. Kaufman S: Phenylketonuria and its variants. *Adv Hum Genet.* **13**:217, 1983. PMID 6362361

411. Kaufman S: Unsolved problems in diagnosis and therapy of hyperphenylalaninemia caused by defects in tetrahydropterin metabolism. *J Pediatr.* **109**:572, 1986. PMID 3489828

412. Kaufman S: *Aromatic amino acid hydroxylases, in E Krebs (ed), The Enzymes*. *Phorphorylation Control, 3d ed*. New York, Academic Press, 1987

413. Kaufman S: An evaluation of the possible neurotoxicity of metabolites of phenylalanine. *J Pediatr.* **114**:895, 1989. PMID 2654351

414. Kaufman S: *Tetrahydropterin, Basic Biochemistry and Role in Human Disease*. Baltmore, Johns Hopkins University Press, 1997

415. Kaufman S: A model of human phenylalanine metabolism in normal subjects and in phenylketonuria patients. *Proc Natl Acad Sci USA.* **96**:3160, 1999. PMID 10077654

416. Kaufman S et al: The source of oxygen in the phenylalanine hydroxylase and the dopamine-β-hydroxylase catalyzed reaction. *Biochem Biophys Res Acta.* **9**:497, 1962. PMID 14031389

417. Kaufman S et al: Phenylketonuria due to a deficiency of dihydropteridine reductase. *N Engl J Med.* **293**:785, 1975. PMID 1160969

418. Kaufman S et al: Deviation from proportionality with gene dosage. *Pediatr Res.* **9**:632, 1975. PMID 1153238

419. Kaufman S, Fisher DB: Purification and some physical properties of phenylalanine hydroxylase from rat liver. *J Biol Chem.* **245**:4745, 1970. PMID 5456147

420. Kaufman S, Levenberg B: Further studies on the phenylalanine hydroxylation cofactor. *J Biol Chem.* **234**:2683, 1992. PMID 14404871

421. Kay MA et al: Expression of human α^1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. *Proc Natl Acad Sci USA.* **89**:89, 1992. PMID 1729724

422. Kayaalp E et al: A metanalysis of genotype-phenotype correlations. *Am J Hum Genet.* **61**:1309, 1997. PMID 9399896

423. Kazak AE et al: A study of phenylketonuria. *Pediatrics.* **81**:224, 1998. PMID 3340473

424. Kecskemethy HH et al: The use of gelatin capsules for ingestion of formula in dietary treatment of maternal phenylketonuria. *J Inherit Metab Dis.* **16**:111, 1993. PMID 8487491

425. Keller M: *Making Sense of Life.* : Explaining Biological Development with Models, Metaphors and Machines, 1st ed. Cambridge, MA, Harvard University Press, 2002. PMID 8487491

426. Kidd JR et al: Haplotypes and linkage disequilibrium at the phenylalanine hydroxylase locus, PAH, in a global representation of populations. *Am J Hum Genet.* **66**:1882, 2000. PMID 10788337

427. Kidd KK, Kidd JR: The population genetics of PAH: *Online supplement to "The Hyperphenylalaninemias," in CR Scriver et al: (eds).* : The Metabolic and Molecular Bases of Inherited Disease. New York, McGraw-Hill, 2005 PMID 10788337

428. Kidd KK: Phenylketonuria: Population genetics of a disease. *Nature.* **327**:282, 1987. PMID 2884567

429. Kindt E et al: A longitudinal study of children treated for phenylketonuria. *Am J Clin Nutr.* **37**:778, 1983. PMID 6687778

430. Kindt E et al: *Is phenylalanine requirement in infants and children related to protein intake? Br J Nutr.* **51**:435, 1984. PMID 6722086

431. Kindt E et al: Fasting plasma amino acid concentrations in PKU children on two different levels of protein intake. *Acta Paediatr Scand.* **77**:60, 1988. PMID 3369307

432. Kindt E, Halvorsen Halvorsen: The need of essential amino acids in children. An evaluation on the intake of phenylalanine, tyrosine, leucine, isoleucine, and valine in children with phenylketonuria, tyrosine amino transferase defect and maple syrup urine disease. *Am J Clin Nutr.* **33**:279, 1980. PMID 6101930

433. Kirby ML, Mihagawa ST: The effects of high phenylalanine concentration on chick embryonic development. *J Inherit Metab Dis.* **13**:634, 1990. PMID 2122126

434. Kirkman HN: Projections of a rebound in frequency of mental retardation from phenylketonuria. *Appl Res Ment Retard.* **3**:319, 1992. PMID 7149708

435. Kirkman HN et al: Fifteen-year experience with screening for phenylketonuria with an automated fluorometric method. *Am J Hum Genet.* **34**:743, 1982. PMID 7124729

436. Kirkman HN, Frazier DM: Thirteen years after epidemological projections. *Int Pediatr.* **11**:279, 1996.

437. Kleiman S et al: A 22-bp deletion in the phenylalanine hydroxylase gene causing phenylketonuria in an Arab family. *Hum Mutat.* **1**:344, 1992a. PMID 1301943

438. Kleiman S et al: A defective splice site at the phenylalanine hydroxylase gene in phenylketonuria and benign hyperphenylalaninemia among Palestinian Arabs. *Hum Mutat.* **1**:340, 1992b. PMID 1301942

439. Kleiman S et al: Inactivation of phenylalanine hydroxylase by a missense mutation, R270S, in a Palestinian kinship with phenylketonuria. *Hum Mol Genet.* **2**:605, 1993. PMID 8518802

440. Knappskog PM et al: Effect of terminal deletions on the oligimerization, activation and cooperativity of substrate binding to the enzyme. *Eur J Biochem.* **242**:813, 1996a. PMID 9022714

441. Knappskog PM et al: Expression of a kinetic variant form of phenylalanine hydroxylase in three different systems. *Hum Mutat.* **8**:236, 1996b. PMID 8889583

442. Knox WE: *Phenylketonuria, in JB Stanbury et al: (eds).* : The Metabolic Basis of Inherited Disease, 1st ed. New York, McGraw-Hill, 1960. PMID 8889583

443. Knox WE: *Phenylketonuria, in JB Stanbury et al: (eds).* : The Metabolic Basis of Inherited Disease, 2nd ed. New York, McGraw-Hill, 1966. PMID 8889583

444. Knox WE: *Retrospective study of phenylketonuria.* : Relation of phenyl- pyruvate excretion to plasma phenylalanine. PKU Newsletter 2, 1970. PMID 8889583

445. Knox WE: *Phenylketonuria, in JB Stanbury et al: (eds).* : The Metabolic Basis of Inherited Disease, 3rd ed. New York, McGraw-Hill, 1972. PMID 8889583

446. Knudsen GM: Application of the double-indicator technique for measurement of blood-brain barrier permeability in humans. *Cerebrovasc Brain Metab Rev.* **6**:1, 1994. PMID 8186068

447. Knudsen GM et al: Blood-brain barrier permeability measurements by double-indicator method intraveneous injection. *Am J Physiol.* **266**:H987, 1994. PMID 8160848

448. Knudsen GM et al: Blood-brain barrier transport of amino acids in healthy control and in patients with phenylketonuria. *J Inherit Metab Dis.* **18**:653, 1995. PMID 8750601

449. Kobayashi K et al: Alternative splicing from a single gene accounts for generation of four mRNA types. *J Biochem.* **103**:907, 1988. PMID 2902075

450. Koch R et al: Preliminary report on the effects of diet discontination in PKU. *J Pediatr.* **100**:870, 1982. PMID 7086586

451. Koch R et al: Paired comparisons between early treated PKU children and their matched sibling controls on intelligence and school achievement test results at eight years of age. *J Inherit Metab Dis.* **7**:86, 1984. PMID 6434835

452. Koch R et al: Successful adjustment to society by adults with phenylketonuria. *J Inherit Metab Dis.* **8**:209, 1985. PMID 3939545

453. Koch R et al: A preliminary report of the collaborative study of maternal phenylketonuria in the United States and Canada. *J Inherit Metab Dis.* **13**:641, 1990. PMID 2122127

454. Koch R et al: The relationship of genotype to phenotype in phenylalanine hydroxylase deficiency. *Biochem Mol Med.* **60**:92, 1997. PMID 9169088

455. Koch R et al: Long-term beneficial effects of the phenylalanine-restricted diet in late-diagnosed individuals with phenylketonuria. *Mol Genet Metab.* **67**:148, 1999. PMID 10356314

456. Koch R et al: An international study. *Mol Genet Metab.* **71**:233, 2000. PMID 11001815

457. Koch R et al: A collaborative study. *J Inherit Metab Dis.* **25**:333, 2002. PMID 12408183

458. Koch R et al: A promising approach to treatment. *Mol Genet Metab.* **79**:110, 2003. PMID 12809641

459. Held KR, Koepp P: Serum-tyrosine in patients with hyperphenylalaninemaemia. *Lancet.* **2**:92, 1977. PMID 69180

460. Koeppe RA et al: Use of [11C] aminocyclohexane carboxylate for the measurement of amino acid uptake and distribution volume in human brain. *J Cereb Blood Flow Metab.* **10**:727, 1990. PMID 2384544

461. Koeppe RA et al: A positron emission tomography study. *J Neurochem.* **56**:1526, 1991. PMID 2013754

462. Konecki DS et al: Structural characterization of the 5' region of the human phenylalanine hydroxylase gene. *Biochemistry.* **31**:8363, 1992. PMID 1326329

463. Kornguth S et al: Near-microscopic magnetic resonance imaging of the brains of phenylalanine hydroxylase-deficient mice, normal littermates, and of normal BALB/c mice at 9.4 Tesla. *Neuroimage.* **1**:220, 1994. PMID 9343573

464. KÃ¶ster S et al: Characterization and kinetic analysis of wild-type and mutant enzymes. *Eur J Biochem.* **231**:414, 1995. PMID 7635153

465. Kowlessur D et al: Further studies of the role of Ser-16 in the regulation of the activity of phenylalanine hydroxylase. *Proc Natl Acad Sci USA.* **92**:4743, 1995. PMID 7761394

466. Kowlessur D et al: Novel regulatory and structural properties. *Arch Biochem Biophys.* **333**:85, 1996. PMID 8806757

467. Kozak L et al: Phenylketonuria mutations and their relation to RFLP haplotypes at the PAH locus in Czech PKU families. *Hum Genet.* **96**:472, 1995. PMID 7557973

468. Kozak L et al: Evidence for both homologous and non homologous mechanisms of rearrangement. *Mol Genet Metab.* **89**:300. 2006.

469. Kragh-Hansen U et al: Tubular localization of Na^+ -dependent phenylalanine and glucose-transport systems. *Biochemistry.* **220**:15, 1984. PMID 6743259

470. Krause W et al: Biochemical and neuropsychological effects of elevated plasma phenylalanine in patients with treated phenylketonuria. *J Clin Invest.* **75**:40, 1985. PMID 3880775

471. Krause W et al: Phenylalanine alters the mean power frequency of electroencephalograms and plasma L-dopa in treated patients with phenylketonuria. *Pediatr Res.* **20**:1112, 1986. PMID 3797105

472. Krawczak M, Zschocke J: A role for overdominant selection in phenylketonuria? Evidence from molecular data. *Hum Mutat.* **21**:394, 2003. PMID 12655549

473. Kreis R: Comments on in vivo proton magnetic resonance spectroscopy in phenylketonuria. *Eur J Pediatr.* **159**:S126, 2000. PMID 11221741

474. Kremensky I, Kalalydjieva L: Avoiding sources of error in PKU screening. *Am J Clin Pathol.* **92**:396, 1989. PMID 2773857

475. Krempf M et al: Estimates with different tracers and route of administration. *Metabolism.* **39**:560, 1990. PMID 2352476

476. Kudo Y, Boyd CAR: Transport of amino acids by the human placenta: Predicted effects thereon of maternal hyperphenylketonuria. *J Inherit Metab Dis.* **13**:617, 1990. PMID 2122124

477. Kure S et al: Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *J Pediatr.* **135**:375, 1999. PMID 10484807

478. Kuzmin AI et al: Presence of Slavic, Turkic and Scandinavian mutations. *Eur J Hum Genet.* **3**:246, 1995. PMID 8528673

479. Kwok SCM et al: Nucleotide sequence of a full-length complementary DNA clone and amino acid sequence of human phenylalanine hydroxylase. *Biochemistry.* **24**:556, 1985. PMID 2986678

480. Laberge C et al: Expérience canadienne et québecoise. *Arch Fr Pediatr.* **44**:643, 1987. PMID 3329493

481. Laipis PJ et al: Supply article title. *]] Mol Ther.* **7**:S391, 2003.

482. Lambert DM: *The genetic epidemiology of hyperphenylalaninemia in Quebec*. Thesis submitted to the Faculty of Graduate Studies and Research, McGill University, Montreal, 1994

483. Lambruschini N et al: Clinical and nutritional evaluation of phenylketonuric patients on tetrahydrobiopterin monotherapy. *Mol Genet Metab.* **86**:S54, 2005. PMID 16040265

484. Lander ES, Schork NJ: Genetic dissection of complex traits. *Science.* **265**:2037, 1994. PMID 8091226

485. Neuhoff V, Lane JD: Phenylketonuria: Clinical and experimental considerations revealed by the use of animal models. *Naturwissenschaften.* **67**:227, 1980. PMID 6446685

486. Langenbeck U et al: Correlative study of mental and biochemical phenotypes in never treated patients with classic phenylketonuria. *Brain Dysfunct.* **1**:103, 1988.

487. Larue C et al: An extracorporeal hollow fibre reactor for phenylketonuria using immobilized phenylalanine ammonia lyase. *Dev Pharmacol Ther.* **9**:73, 1986. PMID 3956347

488. Latorra D et al: Characterization of human AFLP systems apolipoprotein B, phenylalanine hydroxylase, and D1S80. *PCR Methods Appl.* **3**:351, 1994. PMID 7920240

489. Lazarus RA et al: Phenylalanine hydroxylase stimulator protein is a 4α-carbinolamine dehydratase. *J Biol Chem.* **258**:10960, 1983.

490. Leandro P et al: The V388M mutation results in a kinetic variant form of phenylalanine hydroxylase. *Mol Genet Metab.* **69**:204, 2000. PMID 10767175

491. Leandro P et al: Glycerol increases the yield and activity of human phenylalanine hydroxylase mutant enzymes produced in a prokaryotic expression system. *Mol Genet Metab.* **73**:173, 2001. PMID 11386853

492. Ledley FD et al: Molecular analysis of the inheritance of phenylketonuria and mild hyperphenylalaninemia in families with both disorders. *N Engl J Med.* **314**:1276, 1986. PMID 3702929

493. Ledley FD et al: Gene transfer and gene expression of human phenylalanine hydroxylase. *Science.* **228**:77, 1985. PMID 3856322

494. Ledley FD et al: Retroviral-mediated gene transfer of human phenylalanine hydroxylase into NIH 3T3 and hepatoma cells. *Proc Natl Acad Sci USA.* **83**:409, 1986. PMID 3455778

495. Ledley FD et al: Localization of mouse phenylalanine hydroxylase locus on chromosome 10. *Cytogenet Cell Genet.* **47**:125, 1988a. PMID 3378451

496. Ledley FD et al: Phenylalanine hydroxylase expression in liver of a fetus with phenylketonuria. *Pediatrics.* **113**:463, 1988b. PMID 2900886

497. Ledley FD et al: Homology and divergence from human phenylalanine hydroxylase. *Biochem J.* **267**:399, 1990. PMID 2334400

498. Lee DH et al: The molecular basis of phenylketonuria in Koreans. *J Hum Genet.* **49**:617, 2004.

499. Leeming RJ et al: Biopterin derivatives in normal and phenylketonuric patients after oral loads of L-phenylalanine, L-tyrosine, and L-tryptophan. *Arch Dis Child.* **51**:771, 1976. PMID 1008581

500. Lehmann WD et al: Stereospecificity of phenylalanine plasma kinetics and hydroxylation in man following oral application of a stable isotope-labelled pseudo-racemic mixture of L- and D-phenylalanine. *Clin Chim Acta.* **128**:181, 1983. PMID 6851137

501. Lehmann WD et al: Detection of heterozygous carriers for phenylketonuria by a L-[$^{2}H_{5}$]phenylalanine stable isotope loading test. *Clin Chim Acta.* **138**:59, 1984. PMID 6713688

502. Lehmann WD et al: Metabolic conversion of L-(U-$^{14>}$C) phenylalanine to respiratory $14CO_2$ in healthy subjects, phenylketonuria heterozygotes and classic phenylketonurics. *Clin Chim Acta.* **157**:253, 1986. PMID 3089645

503. Lehmann WD, Heinrich HC: Oral versus intravenous L-phenylalanine loading compared by simultaneous application of L-($^{2}H_{5}N$) phenylalanine. *Clin Chim Acta.* **147**:261, 1985. PMID 3995775

504. Kaufman S, Lei X-D: Identification of hepatic nuclear factor 1 binding sites in the 5' flanking region of the human phenylalanine hydroxylase gene: Implication of a dual function of phenylalanine hydroxylase stimulator in the phenylalanine hydroxylation system. *Proc Natl Acad Sci USA.* **95**:1500, 1998a. PMID 9465044

505. Kaufman S, Lei X-D: Human white blood cells and hair follicles are good sources of mRNA for the pterin carbinolamine dehydratase/dimerization cofactor of HNF1 for mutation detection. *Biochem Biophys Res Commun.* **248**:432, 1998b. PMID 9675155

506. Levy HL, Lenke RR: Maternal phenylketonuria and hyperphenylalaninemia: An international survey of untreated and treated pregnancies. *N Engl J Med.* **303**:1202, 1980. PMID 7421947

507. Leuzzi V et al: The spectrum of phenylalanine variations under tetrahydrobiopterin load in subjects affected by phenylalanine hydroxylase deficiency. *J Inherit Metab Dis.* **29**:38, 2006. PMID 16601866

508. Levy H, Ghavami M: Maternal phenylketonuria: A metabolic teratogen. *Teratology.* **53**:176, 1996. PMID 8761885

509. Levy HL: Reply to SP Bessman. *Am J Clin Nutr.* **67**:488, 1998. PMID 9497195

510. Levy HL: Comments on final intelligence in late treated patients with phenylketonuria. *Eur J Biochem.* **2**:S149, 2000. PMID 11221743

511. Levy HL: Historical background for the maternal PKU syndrome. *Pediatrics.* **112**:1516, 2003. PMID 14654656

512. Levy HL, Albers S: Genetic screening of newborns. *Annu Rev Genomics Hum Genet.* **1**:139, 2000. PMID 11701628

513. Levy HL et al: Persistent mild hyperphenylalaninemia in the untreated state. *N Engl J Med.* **285**:424, 1971. PMID 5557279

514. Levy HL et al: Lack of fetal effect on blood phenylalanine concentration in maternal phenylketonuria. *J Pediatr.* **104**:245, 1984. PMID 6694019

515. Levy HL et al: Newborn screening. *Pediatrics.* **73**:417, 1984. PMID 6701077

516. Levy HL et al: Implications for the pathogenesis of fetal damage in MPKU. *Am J Hum Genet.* **49**:43, 1988.

517. Levy HL et al: Paternal phenylketonuria. *J Pediatr.* **118**:741, 1991. PMID 2019930

518. Levy HL et al: Comparison of phenylketonuric and nonphenylketonuric sibs from untreated pregnancies in a mother with phenylketonuria. *Am J Med Genet.* **44**:439, 1992. PMID 1442884

519. Levy HL et al: An international survey of offspring outcome. *Lancet.* **344**:1589, 1994. PMID 7983992

520. Levy HL et al: Fetal ultrasonography in maternal PKU. *Prenat Diagn.* **16**:599, 1996a. PMID 8843468

521. Levy HL et al: MRI of the brain in offspring. *J Pediatr.* **128**:770, 1996b. PMID 8648535

522. Levy HL et al: Report from the Maternal PKU Collaborative Study. *Pediatr Res.* **49**:636, 2001. PMID 11328945

523. Levy HL: *et al:.* : Pregnancy experiences in the mildly hyperphenylalaninemic woman. Pediatrics (in press). PMID 11328945

524. Levy HL, Lobbregt D: Postnatal clearance of maternally derived phenylalanine in offspring of maternal phenylketonuria: Implications for newborn screening. *Screening.* **4**:79, 1995.

525. Levy HL, Waisbren SE: PKU in adolescents: Rationale and psychosocial factors in diet continuation. *Acta Pediatr Suppl.* **407**:92, 1994. PMID 7766970

526. Levy HL et al: a phase III randomized placebo-controlled study. *Lancet.* **370**:504, 2007. PMID 17693179

527. Li J et al: Identification and characterization of three novel nucleotide substitutions in the human phenylalanine hydroxylase gene. *Hum Mutat.* **3**:312, 1994. PMID 8019568

528. Levy H et al: Recommendations for evaluation of responsiveness to tetrahydrobiopterin (BH_4) in phenylketonuria and its use in treatment. *Mol Genet Metab.* **92**:287, 2007. PMID 18036498

529. Levy HL et al: A phase III randomized, placebo-controlled study. *Lancet.* **370**:504, 2007. PMID 17693179

530. Lichter-Konecki U et al: DNA sequence polymorphisms in exonic and intronic regions of the human phenylalanine hydroxylase gene aid in the identification of alleles. *Hum Genet.* **94**:307, 1994. PMID 8076950

531. Lichter-Konecki U et al: Human phenylalanine hydroxylase gene expression in kidney and other nonhepatic tissues. *Mol Genet Metab.* **67**:308, 1999. PMID 10444341

532. Lidsky AS et al: Regional mapping of the phenylalanine hydroxylase gene and the phenylketonuria locus in the human genome. *Proc Natl Acad Sci USA.* **82**:6221, 1985a. PMID 3862128

533. Lidsky AS et al: Extensive restriction site polymorphism at the human phenylalanine hydroxylase locus and application in prenatal diagnosis of phenylketonuria. *Am J Hum Genet.* **37**:619, 1985b. PMID 9556654

534. Lin C-H et al: Identification of a missence phenylketonuria mutation at codon 408 in Chinese. *Hum Genet.* **89**:593, 1992. PMID 1355066

535. Lin CM et al: Expression of human phenylalanine hydroxylase activity in T lymphocytes of classical phenylketonuria children by retroviral-medicated gene transfer. *J Inherit Metab Dis.* **20**:742, 1997. PMID 9427141

536. Lindner M et al: Tetrahydrobiopterin sensitivity in German patients with mild phenylalanine hydroxylase deficiency. *Hum Mutat.* **21**:400, 2003. PMID 12655554

537. Link RM, Wachtel U: Clinical experiences with an amino acid preparation in children with phenylketonuria. *Rev Med Liege.* **39**:422, 1984. PMID 6463465

538. Lipson A et al: Maternal hyperphenylalaninemia fetal effects. *J Pediatr.* **104**:216, 1984. PMID 6694013

539. Liu J et al: Study on a novel strategy to treatment of phenylketonuria. *Artif Cells Blood Substit Immobil Biotechnol.* **30**:243, 2002. PMID 12227645

540. Liu SR, Zuo QH: Newborn screening for phenylketonuria in eleven districts. *Chin Med J (Engl).* **99**:113, 1986. PMID 3093157

541. Liu T-J et al: Reconstitution of enzymatic activity in hepatocytes of phenylalanine hydroxylase-deficient mice. *Somat Cell Mol Genet.* **18**:89, 1992. PMID 1312261

542. Loer CM et al: A phenylalanine hydroxylase gene from the nematode C. elegans is expressed in the hypodermis. *J Neurogenet.* **13**:157, 1999. PMID 10928216

543. Loo YH et al: An examination of two animal models. *Dev Neurosci.* **6**:227, 1983. PMID 6240395

544. Lou HC: Dopamine precursors and brain function in phenylalanine hydroxylase deficiency. *Acta Pediatr Suppl.* **407**:86, 1994. PMID 7766968

545. Lou HC et al: Decreased vigilance and neurotransmitter synthesis after discontinuation of dietary treatment for phenylketonuria in adolescents. *Eur J Pediatr.* **144**:17, 1985. PMID 2410277

546. Lou HC et al: Increased vigilance and dopamine synthesis by large doses of tyrosine or phenylalanine restriction in phenylketonuria. *Acta Paediatr Scand.* **76**:560, 1987. PMID 2442957

547. Lykkelund C et al: Increased neurotransmitter biosynthesis in phenylketonuria induced by phenylalanine restriction of by supplementation of unrestricted diet with large amounts of tyrosine. *Eur J Pediatr.* **148**:238, 1988. PMID 2463918

548. Lyonnet S et al: A mutation associated with partial phenylalanine hydroxylase deficiency. *Am J Hum Genet.* **44**:511, 1989. PMID 2564729

549. Lyonnet S et al: Time and space clusters of the French-Canadian M1V phenylketonuria mutation in France. *Am J Hum Genet.* **51**:191, 1992. PMID 1609797

550. Mabry CC et al: A cause of mental retardation in children without the metabolic defect. *N Engl J Med.* **269**:1404, 1963. PMID 14064329

551. Mabry CC et al: A source of error in phenylketonuria screening. *Am J Clin Pathol.* **90**:279, 1988. PMID 3137803

552. MacCready RA: Admissions of phenylketonuric patients to residential institutions before and after screening programs of the newborn infant. *J Pediatr.* **85**:383, 1974. PMID 4431499

553. Macdonald A: Feeding problems in young PKU children. *Acta Paediatr Suppl.* **407**:73, 1994. PMID 7766964

554. Macdonald A et al: *Does a single plasma phenylalanine predict quality of control in phenylketonuria? Arch Dis Child.* **78**:122, 1998. PMID 9579152

555. Maher TJ et al: Effects of dietary protein intake. *Am J Clin Nutr.* **39**:722, 1984. PMID 6538743

556. Marholin D et al: An experimental analysis. *Pediatr Res.* **12**:179, 1978. PMID 347364

557. Martinez A et al: Expression of recombinant human phenylalanine hydroxylase as fusion protein in Escherichia coli circumvents proteolytic degradation by host cell proteases. *Biochem J.* **306**:589, 1995. PMID 7887915

558. Marvit J et al: GT to AT transition at a splice donor site causes skipping of the preceding exon in phenylketonuria. *Nucleic Acids Res.* **15**:5613, 1987. PMID 3615198

559. Matalon R et al: Biopterin responsive phenylalanine hydroxylase deficiency. *Genet Med.* **6**:27, 2004. PMID 14726806

560. Matsuo K, Hommes F: Regional distribution of the phenylalanine-sensitive ATP-sulphurylase in brain. *J Inherit Metab Dis.* **10**:62, 1987. PMID 3033396

561. Matthews WS et al: Social quotients of children with phenylketonuria before and after discontinuation of dietary treatment. *Am J Ment Defic.* **91**:92, 1986. PMID 3740120

562. Maurer SM et al: Science's neglected legacy. *Nature.* **405**:117, 2000. PMID 10821250

563. Mayr E: *The Growth of Biological Thought*. Diversity, Evolution and Inheritance Cambridge, MA, Belknap Press of Harvard Univversity Press, 1982

564. McBurnie MA et al: Physical growth of children treated for phenylketonuria. *Ann Hum Biol.* **18**:357, 1991. PMID 1750755

565. McCabe ERB et al: Predictive validity as a function of age. *Pediatrics.* **72**:390, 1983. PMID 6889045

566. McCabe ERB, McCabe L: Screening for PKU in sick or premature neonates. *J Pediatr.* **103**:502, 1983. PMID 6886931

567. McCabe L et al: The management of breast feeding among infants with phenylketonuria. *J Inherit Metab Dis.* **12**:467, 1989. PMID 2516178

568. McCaman MW, Robins E: Fluorometric method for the determination of phenylalanine in serum. *J Lab Clin Med.* **59**:885, 1992.

569. McDonald JD: The PKU mouse project: Its history, potential and implications. *Acta Paediatr Suppl.* **407**:122, 1994. PMID 7766947

570. McDonald JD, Charlton CK: Characterization of mutations at the mouse phenylalanine hydroxylase locus. *Genomics.* **39**:402, 1997. PMID 9119379

571. McDonald JD et al: The use of *N*-ethyl-*N*-nitrosurea to produce mouse models for human phenylketonuria and hyperphenylalaninemia. *Prog Clin Biol Res 340C.* : 407, 1990a. PMID 2381939

572. McDonald JD et al: A mouse mutant deficient in phenylalanine hydroxylase. *Proc Natl Acad Sci USA.* **87**:1965, 1990b. PMID 2308957

573. McDonald JD et al: Cardiovascular defects among the progeny of mouse phenylketonuria females. *Pediatr Res.* **42**:103, 1997. PMID 9212044

574. McDonald JD et al: A meeting review. *Mol Genet Metab.* **76**:256, 2002. PMID 12208130

575. McDonnell GV et al: A neurological evaluation of adult phenylketonuria in Northern Ireland. *Eur Neurol.* **39**:38, 1998. PMID 9476722

576. McDowall IL, Fisher MJ: The immediate 5′-flanking region of the rat phenylalanine hydroxylase-encoding gene. *Gene.* **153**:289, 1995. PMID 7875607

577. McKean CM: The effects of high phenylalanine concentrations on serotonin and catecholamine metabolism in the human brain. *Brain Res.* **47**:469, 1972. PMID 4642573

578. McKean CM, Peterson NA: Glutamine in the phenylketonuric central nervous system. *N Engl J Med.* **283**:1364, 1970. PMID 5481755

579. Meaney FJ: Computerized tracking for newborn screening and follow-up: A review. *J Med Syst.* **12**:69, 1988. PMID 3045243

580. Medical Research: Phenylketonuria due to phenylalanine hydroxylase deficiency: An unfolding story. *Br Med J.* **306**:115, 1993. PMID 8435608

581. Medical Research: Routine neonatal screening for phenylketonuria in the United Kingdom 1964–1978. *Br Med J.* **282**:1680, 1981. PMID 6786433

582. Melnick CR et al: Linguistic development of children with phenylketonuria and normal intelligence. *J Pediatr.* **98**:269, 1981. PMID 7463228

583. Crabtree GR, Mendel DB: HNF-1, a member of a novel class of dimerizing homeodomain proteins. *J Biol Chem.* **266**:677, 1991. PMID 1985954

584. Meryash DL et al: Prospective study of early newborn screening for phenylketonuria. *N Engl J Med.* **304**:294, 1981. PMID 7442762

585. Michaels-Matalon K: *TBA*. Pediatrics (in press)

586. Michaels-Matalon K et al: Experience with long term use of LNAA in the treatment of phenylketonuria. *Mol Genet Metab.* **93**:256, 2008.

587. Michel U et al: Results of psychological testing of patients aged 3–6 years. *Eur J Pediatr.* **149**:S34, 1990. PMID 2091929

588. Miller AD, Rosman GJ: Improved retroviral vectors for gene transfer and expression. *Biotechniques.* **7**:980, 1989. PMID 2631796

589. Shiman R, Miller MR: Hydrocortisone induction of phenylalanine hydroxylase isozymes in cultured hepatoma cells. *Biochem Biophys Res Commun.* **68**:740, 1976. PMID 177008

590. Kaufman S, Milstien S: Studies on the phenylalanine hydroxylase system in vivo: An in vivo assay based on the liberatin of deuterium or tritium into the body water from ring-labelled L-phenylalanine. *J Biol Chem.* **250**:4782, 1975a. PMID 124733

591. Kaufman S, Milstien S: Studies on the phenylalanine hydroxylase system in liver slices. *J Biol Chem.* **250**:4777, 1975b. PMID 124732

592. Miranda FF et al: Phosphorylation and mutations of Ser-16 in human phenylalanine hydroxylase. *J Biol Chem.* **277**:40937, 2002. PMID 12185072

593. Miranda FF et al: Localized structural changes in phenylalanine hydroxylase. *Protein Sci.* **13**:1219, 2004. PMID 15096628

594. Miranda FF et al: The active site residue tyrosine 325 influences iron binding and coupling efficiency in human phenylalanine hydroxylase. *J Inorg Biochem.* **99**:1320, 2005. PMID 15917086

595. Mitchell JJ et al: The New South Wales experience. *Mol Genet Metab.* **86**:S81, 2005 PMID 16091307

596. Mitoma C et al: On the nature of enzymatic defect in phenylpyruvic oligophrenia. *Proc Soc Exp Biol Med.* **94**:634, 1957. PMID 13431903

597. Mitton SG et al: Hyperphenylalaninemia in parenterally fed newborn infants. *Lancet.* **2**:1497, 1988. PMID 2904618

598. Fitzpatrick TB, Miyamoto M: Competitive inhibition of mammalian tyrosinase by phenylalanine and its relationship to hair pigmentation in phenylketonuria. *Nature.* **179**:199, 1957. PMID 13400136

599. Moats RA et al: MR Imaging and spectroscopy in PKU. *Ment Retard Dev Disab Res Rev.* **5**:132, 1999.

600. Moats RA et al: Brain phenylalanine concentration in the management of adults with phenylketonuria. *J Inherit Metab Dis.* **23**:7, 2000. PMID 10682303

601. Mochizuki S et al: Long-term correction of hyperphenylalaninemia by AAV-mediated gene transfer leads to behavioural recovery in phenylketonuria mice. *Gene Ther.* **11**:1081, 2004. PMID 15057263

602. Moller HE et al: Changes of cerebral phenylalanine levels under dietary treatment. *Neuropediatrics.* **26**:199, 1995. PMID 8544958

603. Moller HE et al: Kinetics of phenylalanine transport at the human blood-brain barrier investigated in vivo. *Brain Res.* **778**:329, 1997. PMID 9459550

604. Moller HE et al: Blood-brain barrier phenylalanine transport and individual vulerability in phenylketonuria. *J Cereb Blood Flow Metab.* **18**:1184, 1998. PMID 9809507

605. Moller N et al: A metabolic role of the kidney. *Proc Natl Acad Sci USA.* **97**:1242, 2000. PMID 10655515

606. Momma S et al: Phenylalanine transport across the blood-brain barrier as studied with the situ brain perfusion technique. *J Neurochem.* **48**:1291, 1987. PMID 3819731

607. Moseley K et al: Lipid status and long-chain polyunsaturdated fatty acid concentrations in adults and adolescents with phenylketonuria on phenylalanine-restricted diet. *J Inherit Metab Dis.* **25**:56, 2002. PMID 11999981

608. Cavalli-Sforza LL, Mountain JL: Multilocus genotypes, a tree of individuals, and human evolutionary history. *Am J Hum Genet.* **61**:705, 1997. PMID 9326336

609. Mowat DR et al: A continuing problem. *Med J Aust.* **170**:592, 1999. PMID 10416429

610. Mullenix PJ et al: No change in spontaneous behaviour of rats after acute oral doses of aspartame, phenylalanine, and tyrosine. *Fund Appl Toxicol.* **16**:495, 1991. PMID 1677339

611. Muller WA et al: Abnormal α-cell function in diabetes. *N Engl J Med.* **283**:109, 1970. PMID 4912452

612. Muntau AC et al: Tetrahydrobiopterin as an alternative treatment for mild phenylketonuria. *N Engl J Med.* **347**:2122, 2002. PMID 12501224

613. Murphy EA, Pyeritz RE: Homeostasis: VII. A conspectus. *Am J Med Genet.* **24**:735, 1986. PMID 3526891

614. Scriver CR, Murphy BC, Singh SM: CpG methylation accounts for a recurrent mutation (c.1222C→T) in the human PAH gene. *Hum Mutat.* **27**:975, 2006 PMID 16917891

615. Berry HK, Murthy LI: Phenylalanine hydroxylase activity in liver from humans and subhuman primates: Its probable absence in kidney. *Biochem Med.* **12**:392, 1975. PMID 1172439

616. Nagatsu T: Genes for human catecholamine-synthesizing enzymes. *Neurosci Res.* **12**:315, 1991. PMID 1684650

617. Nakai H et al: AAV serotype 2 vectors preferentially integrate into active genes in mice. *Nat Genet.* **34**:297, 2003. PMID 12778174

618. *Genetic Screening.* : Programs, Principles, and Research. Washington, DC, National Research Council, 1975. PMID 12778174

619. National Institutes of Health Consensus Development Conference Statement Screening and Management. October 16–18, 2000. *Pediatrics.* **108**:972, 2001. PMID 11581453

620. Naughten E, Saul IP: Maternal phenylketonuria: The Irish experience. *J Inherit Metab Dis.* **13**:658, 1990. PMID 2122129

621. Naughten ER: Continuation vs discontinuation of diet on phenylketonuria. *Eur J Clin Nutr.* **43**:7, 1989. PMID 2567666

622. Neckameyer WS, White K: A single locus encodes both phenylalanine hydroxylase and tryptophan hydroxylase activities in Drosophila. *J Biol Chem.* **267**:4199, 1992. PMID 1371286

623. Neilsen JB et al: Effects of diet discontinuation and dietary tryptophan supplementation on neurotransmitter metabolism in phenylketonuria. *Brain Dysfunction.* **1**:51, 1988.

624. Netley C et al: Observations on intellectual functioning. *Can Med Assoc J.* **131**:751, 1984. PMID 6478363

625. Nicolini H et al: Molecular analysis of the phenylalanine hydroxylase gene in Mexican phenylketonuric patients. *Arch Med Res.* **26**:53, 1995. PMID 7711448

626. Nielsen KH: Rat liver phenylalanine hydroxylase: A method for the measurement of activity, with particular reference to the distinctive features of the enzyme and the pteridine cofactor. *Eur J Biochem.* **7**:360, 1969. PMID 5791581

627. Novotny EJ et al: In vivo measurement of phenylalanine in human brain by proton nuclear magnetic resonance spectroscopy. *Pediatr Res.* **37**:244, 1995. PMID 7731764

628. Nowacki PM et al: 1997. Prototype for relational locus-specific mutation databases. *Nucleic Acids Res.* **26**:220, 1998. PMID 9399840

629. O'Donnell KA et al: The population history of the Irish revisited. *Eur J Hum Genet.* **10**:530, 2003. PMID 12173030

630. Oberdoerster J et al: Possible relevance in maternal phenylketonuria. *J Pharmacol Exp Ther.* **295**:295, 2000. PMID 10991993

631. Oh HJ et al: Long-term enzymatic and phenotypic correction in the phenylketonuria mouse model by adeno-associated virus vector-mediated gene transfer. *Pediatr Res.* **56**:278, 2004. PMID 15181195

632. Oh HJ et al: Reversal of gene expression provile in the phenylketonuria mouse model after adeno-associated virus vector-mediated gene therapy. *Mol Genet Metab.* **86**:S124, 2005. PMID 16150627

633. Okano Y et al: An experimental approach to maternal phenylketonuria. *J Inherit Metab Dis.* **9**:15, 1986. PMID 2941618

634. Okano Y et al: Recurrent mutation in the human phenylalanine hydroxylase gene. *Am J Hum Genet.* **46**:919, 1990. PMID 1971144

635. Okano Y et al: Molecular basis of phenotypic heterogeneity in phenylketonuria. *N Engl J Med.* **324**:1232, 1991. PMID 2014036

636. Okano Y et al: Frequency and distribution of phenylketonuric mutations in Orientals. *Hum Mutat.* **1**:216, 1992. PMID 1301927

637. Okano Y et al: Molecular characterization of phenylketonuric mutations in Japanese by analysis of phenylalanine hydroxylase mRNA from lymphoblasts. *Hum Mol Genet.* **3**:659, 1994. PMID 7915167

638. Okano Y, Isshiki G: Newborn mass screening and molecular genetics of phenylketonuria in East Asia. *Southeast Asian J Trop Med Public Health.* **26**:123, 1995. PMID 8629090

639. Okano Y et al: diagnosis of tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency. *Pediatr Res.* **56**:714, 2004. PMID 15319459

640. Oltvai ZN, Barabasi AL: Life's complexity pyramid. *Science.* **298**:763, 2002. PMID 12399572

641. Owada M et al: Successful treatment of maternal phenylketonuria with a formula consisting of low phenylketonuria peptide as a protein source. *J Inherit Metab Dis.* **11**:341, 1988. PMID 3148086

642. Owens CWI: Effects of phenylalanine analogues on renal tubular reabsorption of amino acids in the rat. *Clin Sci Mol Med.* **53**:355, 1977. PMID 913060

643. Ãzalp I et al: Incidence of phenylketonuria and hyperphenylalaninemia in a sample of the newborn population. *J Inherit Metab Dis.* **9**:237, 1986.

644. OzgÃ¼Ã§ M et al: Mutation analysis in Turkish phenylketonuria patients. *J Med Genet.* **30**:129, 1993. PMID 8445616

645. Paans AMJ et al: Neurotransmitter positron emission tomographic studies in adults with phenylketonuria, a pilot study. *Eur J Pediatr.* **155**:S78, 1996. PMID 8828616

646. Pardridge WM: *Phenylalanine transport at the human blood-brain barrier, in S Kaufman (ed).* : Amino Acids in Health and Disease. New York, Liss, 1987. PMID 8828616

647. Pardridge WM: Blood-brain barrier carrier-mediated transport and brain metabolism of amino acids. *Neurochem Res.* **23**:635, 1998. PMID 9566601

648. Parniak MA, Kaufman S: Rat liver phenylalanine hydroxylase: Activation by sulfhydryl modification. *J Biol Chem.* **256**:6876, 1981. PMID 7240248

649. Paul TD et al: Phenylketonuria heterozygote detection in families with affected children. *Am J Hum Genet.* **30**:293, 1978. PMID 677126

650. Paul TD et al: *Is there a heterozygote advantage in the birthweight and number of children born to PKU heterozygotes? Am J Hum Genet.* **31**:A104, 1979. PMID 677126

651. Pearsen KD et al: MR imaging of the brain with clinical correlation. *Radiology.* **177**:437, 1990. PMID 2217781

652. Peng H et al: Retroviral-mediated gene transfer and expression of human phenylalanine hydroxylase in primary mouse hepatocytes. *Proc Natl Acad Sci USA.* **85**:8146, 1988. PMID 3186716

653. Pennington BF et al: Effect of age at loss of dietary control on intellectual performance and behaviour of children with phenylketonuria. *Am J Ment Defic.* **89**:467, 1985.

654. Penrose LS: Inheritance of phenylpyruvic amentia (phenylketonuria). *Lancet.* **2**:192, 1935.

655. Penrose LS: Phenylketonuria: A problem in eugenics. *Lancet.* **1**:949, 1946.

656. Penrose LS: Phenylketonuria: A problem in eugenics. *Ann Hum Genet.* **62**:193, 1998. PMID 9803263

657. Perez B et al: Presence of the Mediterranean PKU mutation IVS10 in Latin America. *Hum Mol Genet.* **2**:1289, 1993. PMID 8401510

658. Perez B et al: Mutation analysis of phenylketonuria in South Brazil. *Hum Mutat.* **8**:262, 1996. PMID 8889586

659. Perez B et al: Mutation profile and association with intragenic polymorphic markers. *Am J Hum Genet.* **60**:95, 1997. PMID 8981952

660. Perez B et al: Kinetic and stability analysis of PKU mutations identified in BH_4-responsive patients. *Mol Genet Metab.* **86**:S11, 2005. PMID 16091306

661. Pey AL, Martinez A: The activity of wild-type and mutant phenylalanine hydroxylase and its regulation by phenylalanine and tetrahydrobiopterin at physiological and pathological concentrations: An isothermal titration calorimetry study. *Mol Genet Metab.* **86**:S43, 2005. PMID 15936235

662. Pey AL et al: Genotype-phenotype correlations based on expression analysis of structural and functional mutations in PAH. *Hum Mutat.* **21**:370, 2003. PMID 12655546

663. Pey AL et al: Thermodynamic characterization of the binding of tetrahydropterins to phenylalanine hydroxylase. *J Am Chem Soc.* **126**:13670, 2004. PMID 15493924

664. Pey AL et al: Predicted effects of missense mutations on native-state stability account for phenotypic outcome in phenylketonuria, a paradigm of misfolding diseases. *Am J Hum Genet.* **81**:1006, 2007.

665. Phillips RS et al: Evidence for a conformational change associated with activation. *Biochemistry.* **23**:3836, 1984. PMID 6487579

666. Kaufman S, Phillips RS: Ligand effects on the phosphorylation states of hepatic phenylalanine hydroxylase. *J Biol Chem.* **259**:2474, 1984. PMID 6698976

667. Pietz J et al: Effect of high-dose tyrosine supplementation on brain function in adults with phenylketonuria. *J Pediatr.* **127**:936, 1995a. PMID 8523192

668. Pietz J et al: The dynamics of brain concentrations of phenylalanine and its clinical significance in patients with phenylketonuria determined by in vivo 1H magnetic resonance spectroscopy. *Pediatr Res.* **38**:657, 1995b. PMID 8552430

669. Pietz J et al: Large neutral amino acids block phenylalanine transport into brain tissue in patients with phenylketonuria. *J Clin Invest.* **103**:1169, 1999. PMID 10207169

670. Pietz J et al: No evidence for individual blood-brain barrier phenylalanine transport influence clinical outcome in typical phenylketonuria patients. *Ann Neurol.* **52**:382, 2002. PMID 12205657

671. Pigeon D et al: Rat pheochromocytoma tyrosine hydroxylase is phosphorylated on serine 40 by an associated protein kinase. *J Biol Chem.* **262**:6155, 1987. PMID 2883182

672. Danks DM, Pitt DB: The natural history of untreated phenylketonuria over 20 years. *J Paediatr Child Health.* **27**:189, 1991. PMID 1909536

673. Platt LD et al: Report of a 12-year study. *Am J Obstet Gynecol.* **182**:326, 2000. PMID 10694332

674. Pollitt RJ et al: Cost, yield and outcome. *Heath Technol Assess.* **1**:i, 1997. PMID 9483160

675. Kaufman S, Pollock RJ: Dihydropteridine reductase may function in tetrahydrofolate metabolism. *J Neurochem.* **31**:115, 1978. PMID 209138

676. Pontoglio M et al: Hapatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. *Cell.* **84**:575, 1996. PMID 8598044

677. Porter CA: Breastfeeding, dummy use, and adult intelligence. *Lancet.* **347**:1766, 1996. PMID 8656924

678. Poustie VJ, Rutherford P: Tyrosine supplementation for phenylketonuria. *Cochrane Database Syst Rev.* **2**:CD001507, 2000. PMID 10796799

679. Puglisi-Allegra S et al: Dramatic brain aminergic deficit in a genetic mouse model of phenylketonuria. *Neurochemistry.* **11**:1361, 2000. PMID 10817622

680. Quinsey NS et al: Identification of Gln313 and Pro327 as residues critical for substrate inhibition in tyrosine hydroxylase. *J Neurochem.* **66**:908, 1996. PMID 8769848

681. Raiha NCR: Phenylalanine hydroxylase in human liver during development. *Pediatr Res.* **7**:1, 1973. PMID 4686999

682. Rampini S et al: Aromatic acids in urine of healthy infants, persistent hyperphenylalaninemia, and phenylketonuria, before and after phenylalanine load. *Pediatr Res.* **8**:704, 1974. PMID 4837567

683. Ramus S et al: CpG hotspot causes second mutation in codon 408 of the phenylalanine hydroxylase gene. *Hum Genet.* **90**:147, 1992. PMID 1358783

684. Cotton RGH, Ramus SJ: Polymorphism in the 3' untranslated region of the phenylalanine hydroxylase gene detected by enzyme mismatch cleavage: Evolution of haplotypes. *Hum Genet.* **96**:741, 1995. PMID 8522340

685. Ramus SJ et al: Illegitimate transcription of phenylalanine hydroxylase for detection of mutations in patients with phenylketonuria. *Hum Mutat.* **1**:154, 1992. PMID 1301202

686. Ramus SJ et al: Comparison of genotype and intellectual phenotype in untreated PKU patients. *J Med Genet.* **30**:401, 1993. PMID 8320703

687. Ramus SJ et al: Origin of alleles and haplotypes. *Am J Hum Genet.* **56**:1034, 1995. PMID 7726156

688. Slatkin M, Rannala B: Likelihood analysis of disequilibrium mapping, and related problems. *Am J Hum Genet.* **62**:459, 1998. PMID 9463316

689. Kaufman S, Rao DN: Purification and state of activation of rat liver phenylalanine hydroxylase. *J Biol Chem.* **261**:8866, 1986. PMID 3722178

690. Rapoport D et al: Psychological prospects and scholastic performance of 33 children with early diagnosis of hyperphenylalaninemia. *Arch Fr Pediatr.* **1**:273, 1983. PMID 6882147

691. Rees D et al: Sequence analysis of the rat phenylalanine hydroxylase gene promoter. *DNA Seq.* **12**:191, 2001. PMID 11762195

692. Rey F et al: Long-term follow up of patients with classical phenylketonuria after diet relaxation at 5 years of age. The Paris study. *Eur J Pediatr.* **155**:S39, 1996. PMID 8828607

693. Reynolds R et al: Retarded development of neurons and oligodendroglia in rat forebrain produced by hyperphenylalaninemia results in permanent deficits in myelin despite long recovery periods. *Exp Neurol.* **124**:357, 1993. PMID 7507064

694. Rhesus Macacque: Evolutionary and biomedical insights from the Rhesus macacque genome. *Science.* **316**:222, 2007.

695. Riess O et al: Introduction of genomic diagnosis of classical phenylketonuria to the health care system of the German Democratic Republic. *Clin Genet.* **32**:209, 1987. PMID 2890455

696. Ris MD et al: Adult psychosocial outcome in early-treated phenylketonuria. *J Inherit Metab Dis.* **20**:499, 1997. PMID 9266385

697. Riva E et al: Early breastfeeding is linked to higher intelligence quotient scores in dietary treated phenylketonuric children. *Acta Paediatr.* **85**:56, 1996. PMID 8834980

698. Rivera I et al: Relative frequency of IVS10nt546 mutation in a Portuguese phenylketonuric population. *Hum Mutat.* **9**:272, 1997. PMID 9090531

699. Rivera I et al: Population genetics of hyperphenylalaninemia resulting from phenylalanine hydroxylase deficiency in Portugal. *J Med Genet.* **35**:301, 1998. PMID 9598724

700. Robson KJH et al: Polysome immunoprecipitation of phenylalanine hydroxylase mRNA from rat liver and cloning of its cDNA. *Proc Natl Acad Sci USA.* **79**:4701, 1982. PMID 6750607

701. Robson KJH et al: Sequence comparison of rat liver phenylalanine hydroxylase and its cDNA clones. *Biochemistry.* **23**:567, 1984. PMID 6098294

702. Rocha DM et al: Glucagon-stimulating activity of 20 amino acids in dogs. *Clin J Invest.* **51**:2346, 1972. PMID 4639019

703. Rohr FJ et al: Tyrosine supplementation in the treatment of maternal phenylketonuria. *Am J Clin Nutr.* **67**:473, 1998. PMID 9497192

704. Rohr FJ et al: Acceptability of a new modular protein substitute for the dietary treatment of phenylketonuria. *J Inherit Metab Dis.* **24**:623, 2001. PMID 11768582

705. Romano V et al: Prenatal diagnosis by minisatellite analysis in Italian families with phenylketonuria. *Prenat Diagn.* **14**:959, 1994. PMID 7899270

706. McKusick VA, Romeo G: Phenotypic diversity, allelic series and modifier genes. *Nat Genet.* **7**:451, 1994. PMID 7951309

707. Romeo G et al: Incidence of classic PKU in Italy estimated from consanguineous marriages and from neonatal screening. *Clin Genet.* **24**:339, 1983. PMID 6652943

708. Scriver CR, Rosenblatt D: Hetergeneity in genetic control of phenylalanine metabolism in man. *Nature.* **218**:677, 1968. PMID 5655958

709. Roux C et al: An animal model for maternal phenylketonuria. *J Med Genet.* **28**:718, 1992. PMID 1941970

710. Ruiz-Vazquez P et al: Structure of the phenylalanine hydroxylase gene in Drosophila melangaster and evidence of alternative promoter usage. *Biochem Biophys Res Commun.* **225**:238, 1996. PMID 8769124

711. Rupp A et al: Variability of blood-brain ratios of phenylalanine in typical patients with phenylketonuria. *J Cereb Blood Flow Metab.* **21**:276, 2001. PMID 11295882

712. Sadava D, Sutcliffe D: The effects of maternal hyperphenylalaninemia on learning in mature rats. *Life Sci.* **43**:1119, 1988. PMID 3172977

713. Safos S, Chang TM: Enzyme replacement therapy in ENU2 phenylketonuric mice using oral microencapsulated phenylalanine ammonia-lyase: A preliminary report. *Artif Cells Blood Substit Immobil Biotechnol.* **23**:681, 1995. PMID 8556141

714. Salter M et al: Quantification of the importance of individual steps in the control of aromatic amino acid metabolism. *Biochem J.* **234**:635, 1986. PMID 2872885

715. Samarzija I, Fromter E: Electrophysiological analysis of rat renal sugar and amino acid transport: III. Neutral amino acids. *Pflugers Arch.* **393**:119, 1982. PMID 7099922

716. Sanchez M et al: Twenty-four hour intravenous and oral tracer studies with L-[1-13C] phenylalanine and L-[3,3-2H2] tyrosine at a tyrosine-free generous phenylalanine intake in adults. *Am J Clin Nutr.* **63**:532, 1996. PMID 8599317

717. Santana MA et al: The effect of experimental diabetes on phenylalanine metabolism in isolated liver cells. *Biochem J.* **227**:169, 1985. PMID 3888193

718. Santos M et al: Preliminary spectrum of PAH mutations and their associations with highly polymorphic haplotypes. *Hum Hered.* **46**:128, 1996. PMID 8860005

719. Saraiva JM et al: Plasma phenylalanine and tyrosine levels revisited in heterozygotes for hyperphenylalaninaemia. *J Inherit Metab Dis.* **16**:105, 1993. PMID 8487490

720. Sommer SS, Sarkar G: Access to a messenger RNA sequence on its protein product is not limited by tissue or species specificity. *Science.* **244**:331, 1989. PMID 2565599

721. Sarkissian C et al: Phenylalanine degradation with recombinant phenylalanine ammonia lyase. *Proc Natl Acad Sci USA.* **96**:2339, 1999. PMID 10051643

722. Sarkissian C et al: A new orthologue for human hyperphenylalaninemia. *Mol Genet Metab.* **69**:188, 2000. PMID 10767173

723. Sarkissian CN et al: Measurement of phenyllacetate, phenylacetate, and phenylpyruvate by negative ion chemical ionization-gas chromatography/mass spectrometry in brain of mouse genetic models of phenylketonuria and nonphenylketonuria hyperphenylalaninemia. *Anal Biochem.* **280**:242, 2000. PMID 10790306

724. Sarkissian CN et al: First evidence that extra-cerebral recombinant phenylalanine ammonia lyase treatment will reduce brain phenylalanine in phenylketonuria. *Am J Hum Genet.* **73**:459, 2003.

725. Sarkissian CN, Gamez A: *Phenylalanine ammonia lyase, enzyme substitution therapy for phenylketonuria.* : Where are we now? Mol Genet Metab (in press). PMID 16165390

726. Sartorio R et al: A simplified test to detect PKU heterozygotes by discriminant analysis in mentally retarded children and their mothers. *Clin Genet.* **33**:241, 1988. PMID 3359681

727. Sato T et al: Effects of maternal phenylalanine or tyrosine hydroxylase inhibition on postnatal maturation of catecholamine and amino acid metabolism in rats. *Acta Pediatr Jpn.* **30**:56, 1988. PMID 2906779

728. Saugstad LF: Increased “reproductive casualty” in heterozygotes for phenylketonuria. *Clin Genet.* **4**:105, 1973. PMID 4730939

729. Saugstad LF: Frequency of phenylketonuria in Norway. *Clin Genet.* **7**:40, 1975. PMID 1116309

730. Saugstad LF: Heterozygote advantage for the phenylketonuria allele. *J Med Genet.* **14**:20, 1977. PMID 839495

731. Scaglioni S et al: Pubertal maturation and classical phenylketonuria. *J Inherit Metab Dis.* **9**:285, 1986. PMID 3099074

732. Schallreuter KU et al: Defective tetrahydrobiopterin and catecholamine biosynthesis in the depigmentation disorder vitiligo. *Biochim Biophys Acta.* **1226**:181, 1994. PMID 8204666

733. Scherer SE et al: The finished DNA sequence of human chromosome 12. *Nature.* **440**:346, 2006.

734. Schindeler S et al: An MRS and neuropsychological study. *Mol Genet Metab.* **91**:48, 2007. PMID 17368065

735. Schmidt E et al: Effects of concurrent phenylalanine levels on sustained attention and calculation speed in patients treated early for phenylketonuria. *Eur J Pediatr.* **155**:S82, 1996. PMID 8828617

736. Schoen EJ et al: Recommendations of the Committee on Genetics. *Pediatrics.* **72**:139, 1983. PMID 6866585

737. Schoonheyt WE et al: Feto-maternal plasma phenylalanine concentration gradient from 19 weeks gestation to term. *Clin Chim Acta.* **225**:165, 1994. PMID 8088005

738. Schuett VE, Brown ES: Diet policies of PKU clinics in the United States. *Am J Public Health.* **74**:501, 1984. PMID 6711729

739. Schuett VE et al: Diet discontinuation policies and practices of PKU clinics in the United States. *Am J Public Health.* **70**:498, 1980. PMID 7377420

740. Schuett VE et al: Reinstitution of diet therapy in PKU patients from twenty-two US clinics. *Am J Public Health.* **75**:39, 1985. PMID 3966596

741. Scrimgeour KG, Cheema S: Quininoid dihydropterin reductase. *Ann NY Acad Sci.* **186**:115, 1971. PMID 4332032

742. Scriver CR: *Treatment in medical genetics, in JF Crow, JV Neel (eds).* : Proceedings of the Third International Congress of Human Genetics. Baltimore, Johns Hopkins Press, 1967. PMID 4332032

743. Scriver CR: Mutants: Consumers with special needs. *Nutr Rev.* **29**:155, 1971. PMID 4950571

744. Scriver CR: Screening for medical intervention: The PKU experience in human genetics. *Prog Clin Biol Res.* **103**:437, 1982. PMID 7163238

745. Scriver CR: More on newborn screening for phenylketonuria: In reply. *Pediatrics.* **72**:141, 1983.

746. Scriver CR: Every disease has two histories. *Trans R Soc Can.* **6**:25, 1993.

747. Scriver CR: An ongoing debate over phenylalanine hydroxylase deficiency in phenylketonuria. *J Clin Invest.* **101**:2613, 1998a. PMID 9637693

748. Scriver CR: Commentary 318–343. *Pediatrics.* **102**:236, 1998b. PMID 9651440

749. Scriver CR: Why mutation analysis does not always predict clinical consequences: Explanations in the era of genomics. *J Pediatr.* **140**:502, 2002. PMID 12032513

750. Scriver CR: The PAH gene, phenylketonuria, and a paradigm shift. *Hum Mutat.* **28**:831, 2007.

751. Scriver CR, Clow CL: Epitome of human biochemical genetics, part I. *N Engl J Med.* **303**:1336, 1980a.

752. Scriver CR, Clow CL: Epitome of human biochemical genetics, part II. *N Engl J Med.* **303**:1394, 1980b.

753. Scriver CR, Clow CL: Avoiding phenylketonuria: Why parents seek prenatal diagnosis. *J Pediatr.* **113**:495, 1988. PMID 3045280

754. Scriver CR, Waters PJ: Monogenic traits are not simple: Lessons from phenylketonuria. *TIGS.* **15**:267, 1999. PMID 10390625

755. Scriver CR et al: What a locus-specific knowledgebase can do. *Hum Mutat.* **21**:333, 2003. PMID 12655543

756. Scriver CR et al: Cord-blood tyrosine levels in the full-term phenylketonuric fetus and the "justification hypothesis. *" Proc Natl Acad Sci USA.* **77**:6175, 1980. PMID 6934544

757. Scriver CR et al: The influence of some common physiological variables. *Metabolism.* **34**:868, 1985. PMID 4033427

758. Scriver CR et al: Unusual gene dosage effect in heterozygotes. *Hum Genet.* **77**:168, 1987. PMID 3308682

759. Scriver CR et al: Mendelian hyperphenylalaninemia. *Ann Rev Genet.* **22**:301, 1988. PMID 3071251

760. Scriver CR et al: *The hyperphenylalaninemias, in CR Scriver et al: (eds).* : The Metabolic Basis of Inherited Disease, 6th ed. New York, McGraw-Hill, 1989. PMID 3071251

761. Scriver CR et al: An overview. *Dev Brain Dysfunct.* **6**:11, 1993.

762. Scriver CR et al: *The hyperphenylalaninemias, in CR Scriver et al: (eds).* : The Metabolic and Molecular Bases of Inherited Disease, 7th ed. New York, McGraw-Hill, 1995.

763. Scriver CR et al: *A marker for the history of phenylketonuria and human genetic diversity, in KM Weiss (ed).* : Variation in the Human Genome. Ciba Foundation Symposium No. 197. Chichester, England, Wiley, 1996a.

764. Scriver CR et al: *A marker for the history of phenylketonuria and human genetic diversity, in D Chadwick, G Cardew (eds).* : Variation in the Human Genome. Chichester, England, Wiley, 1996b.

765. Scriver CR et al: A locus-specific knowledgebase. *Hum Mutat.* **15**:99, 2000. PMID 10612829

766. Scriver CR, Rosenberg LE: *Amino Acid Metabolism and Its Disorders*. Philadelphia, Saunders, 1973

767. Waters PJ, Scriver C: Monogenic traits are not simple: Lessons from phenylketonuria. *Trends Genet.* **15**:267, 1999. PMID 10390625

768. Seashore MR et al: Loss of intellectual function in children with phenylketonuria after relaxation of dietary phenylalanine restriction. *Pediatrics.* **75**:226, 1985. PMID 3969322

769. Sepe SJ et al: An evaluation of routine follow-up blood screening of infants for phenylketonuria. *N Engl J Med.* **300**:606, 1979. PMID 33334

770. Seymour CA et al: A systematic review. *Heath Technol Assess.* **1**:i, 1997. PMID 9483156

771. Shah SN: Minireview Changes in phenylketonuria and Down's syndrome. *Int J Biochem.* **10**:477, 1979. PMID 156657

772. Shaw DWW et al: MR imaging of phenylketonuria. *Am J Nutr Res.* **12**:403, 1991. PMID 2058482

773. Shedlovsky A et al: Mouse models of human phenylketonuria. *Genetics.* **134**:1205, 1993. PMID 8375656

774. Shefer S et al: *Is there a relationship between 3-hydroxy-3-methylglutaryl coenzyme a reductase activity and forebrain pathology in the PKU mouse? J Neurosci Res.* **61**:549, 2000. PMID 10956425

775. Shershen H et al: *Amino Acids in Health and Disease.* : New Perspectives. New York, Liss, 1987. PMID 10956425

776. Shiloh S et al: Cross-cultural perspectives on coping with the risks of maternal phenylketonuria. *Psychol Health.* **8**:435, 1993.

777. Shiman R: Relationship between the substrate activation site and catalytic site of phenylalanine hydroxylase. *J Biol Chem.* **225**:10029, 1980. PMID 7430114

778. Shiman R et al: Solubilization and partial purification of tyrosine hydroxylase from bovine adrenal medulla. *J Biol Chem.* **246**:1330, 1971. PMID 5545077

779. Shiman R: *et al:.* : A simple purification of phenylalanine hydroxylase by substrate-induced hydrophobic chromatography. J Biol Chem 11300, 1979. PMID 500646

780. Shiman R et al: Regulation of phenylalanine hydroxylase activity by phenylalanine in vivo, in vitro, and in perfused rat liver. *J Biol Chem.* **257**:11213, 1982. PMID 6981644

781. Gray DW, Shiman R: Substrate activation of phenylalanine hydroxylase: A kinetic characterization. *J Biol Chem.* **255**:4793, 1980. PMID 7372612

782. Shintaku H et al: Long-term treatment and diagnosis of tetrahydrobiopterin-responsive hyperphenylalaninemia with a mutant phenylalanine hydroxylase gene. *Pediatr Res.* **55**:425, 2004. PMID 14681498

783. Shintaku H: *et al:.* : Mol Genet Metab (in press). PMID 14681498

784. Sinai LN et al: Effect of early newborn discharge. *Pediatrics.* **96**:605, 1995. PMID 7567318

785. Slazyk WE et al: Effect of lot-to-lot variability in filter paper on the quantification of thyroxin, thyrotopin, and phenylalanine in dried-blood specimens. *Clin Chem.* **34**:53, 1988. PMID 3338186

786. Smith CB, Kang J: Cerebral protein synthesis in a genetic mouse model of phenylketonuria. *Proc Natl Acad Sci USA.* **97**:11014, 2000. PMID 11005872

787. Smith I: *The hyperphenylalaninemias, in JK Lloyd, CR Scriver (eds).* : Genetic and Metabolic Disease in Pediatrics. London, Butterworths, 1985. PMID 11005872

788. Smith I: Treatment of phenylalanine hydroxylase deficiency. *Acta Pediatr Suppl.* **407**:60, 1994a. PMID 7766961

789. Smith I, Beasley M: Intelligence and behaviour in children with early treated phenylketonuria. *Eur J Clin Nutr.* **43**:1, 1989. PMID 2737171

790. Smith I et al: Effect of stopping the low phenylalanine diet on the intellectual progress of children with phenylketonuria. *Ann Clin Biochem.* **14**:134, 1977. PMID 869489

791. Smith I et al: Effect of stopping low-phenylalanine diet on intellectual progress of children with phenylketonuria. *Br Med J.* **2**:723, 1978. PMID 698696

792. Smith I et al: Behaviour disturbance in the 8-year-old children with early-treated phenylketonuria. *J Pediatr.* **112**:403, 1988. PMID 3346777

793. Smith I et al: Intelligence and quality of dietary treatment in phenylketonuria. *Arch Dis Child.* **65**:472, 1990. PMID 2357082

794. Smith I et al: Effect of intelligence of relaxing the low phenylalanine diet in phenylketonuria. *Arch Dis Child.* **66**:311, 1991. PMID 2025007

795. Smith I et al: Review of neonatal screening programme for phenylketonuria. *Br Med J.* **303**:333, 1991. PMID 1912773

796. Smith I, Wolff OH: Natural history of phenylketonuria and influence of early treatment. *Lancet.* **2**:540, 1974. PMID 4140266

797. Smith I, Wolff OH: MRC/DHSS Phenylketonuria Register. *Newsletter.* **5**:2, 1978.

798. Smith ML et al: Randomised controlled trial of tyrosine supplementation on neuropsychological performance in phenylketonuria. *Arch Dis Child.* **78**:116, 1998. PMID 9579151

799. Smith ML et al: Executive function in school-aged children with phenylketonuria. *J Dev Phys Disabil.* **12**:317, 2000.

800. Smith QR: Kinetic analysis of neutral amino acid transport across the blood-brain barrier, in S Kaufman (ed): *Amino Acids in Health and Disease.* : New Perspectives. New York, Liss, 1987.

801. Sober E: *The Nature of Selection*. Evolutionary Theory in Philosophical Focus Cambridge, MA, MIT Press, 1984

802. Song F et al: Phenylketonuria mutations in Northern China. *Mol Genet Metab.* **86**:S107, 2005.

803. Sorenson JR et al: Parental response to repeat testing of infants with “false-positive” results in a newborn screening program. *Pediatrics.* **73**:183, 1984. PMID 6694876

804. Sourdive DJD et al: The bifunctional DCoH protein binds to HNF1 independently of its 4α-carbinolamine dehydratase activity. *Nucleic Acids Res.* **25**:1476, 1997. PMID 9092652

805. Spaapen JM et al: Tetrahydrobiopterin response phenylalanine hydroxylase deficiency in Dutch neonates. *J Inherit Metab Dis.* **24**:352, 2001. PMID 11486900

806. Rubio-Gozalbo ME, Spaapen JM: Tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency, state-of-the-art. *Mol Genet Metab.* **78**:93, 2003. PMID 12618080

807. Spada M et al: influence of different phenylalanine hydroxylase mutations. *J Inherit Metab Dis.* **21**:236, 1998. PMID 9686365

808. Speer A et al: Typing of families with classical phenylketonuria using three alleles of the HindIII linked restriction fragment polymorphism, detectable with a phenylalanine hydroxylase cDNA probe. *Clin Genet.* **29**:491, 1986. PMID 3017615

809. St James et al: The Resource Mothers’ Program for maternal phenylketonuria. *Am J Public Health.* **89**:762, 1999. PMID 10224992

810. Steinfeld R et al: Efficacy of long-term tetrahydrobiopterin monotheraphy in phenylketonuria. *J Inherit Metab Dis.* **27**:449, 2004. PMID 1530300

811. Stemerdink BA et al: Behaviour and school achievement in patients with early and continuously treated phenylketonuria. *J Inherit Metab Dis.* **23**:548, 2000. PMID 11032330

812. Goldman D, Stoll J: Isolation and structural characterization of the murine tryptophan hydroxylase gene. *J Neurosci Res.* **28**:457, 1991. PMID 1714508

813. Strohman R: Maneuvering in the complex path from genotype to phenotype. *Science.* **296**:701, 2002. PMID 11976445

814. Sullivan SE et al: Phenylalanine hydroxylase deletion mutant from a patient with classical PKU. *Am J Hum Genet.* **37**:A177, 1985.

815. Summers KM: Relationship between genotype and phenotype in monogenic diseases: Relevance to polygenic diseases. *Hum Mutat.* **7**:283, 1996. PMID 8723677

816. Sun G et al: Novel mutations identified in exon 7 of phenylalanine hydroxylase gene in Chinese. *Yi Chuan Xue Bao.* **24**:492, 1997. PMID 9575658

817. Superti-Furga A et al: Maternal phenylketonuria syndrome in cousins caused by mild, unrecognized phenylketonuria in their mothers homozygous for the phenylalanine hydroxylase Arg-261-G1n mutation. *Eur J Pediatr.* **150**:493, 1991. PMID 1915502

818. Surplice IM et al: Automation of an assay for a national screening service. *J Inherit Metab Dis.* **13**:169, 1990. PMID 2116547

819. Svensson E et al: Polymorphic DNA haplotypes at the phenylalanine hydroxylase locus and their relation to phenotype in Swedish phenylketonuria families. *Hum Genet.* **87**:11, 1991. PMID 1674714

820. Svensson E et al: Two missense mutations causing hyperphenylalaninemia associated with DNA haplotype 12. *Hum Mutat.* **1**:129, 1992. PMID 1301200

821. Svensson E et al: Relation between genotype and phenotype in Swedish phenylketonuria and hyperphenylalaninemia patients. *Eur J Pediatr.* **152**:132, 1993. PMID 8444221

822. Svensson E et al: Severity of mutation in the phenylalanine hydroxylase gene influences phenylalanine metabolism in phenylketonuria and hyperphenylalaninemia heterozygotes. *J Inherit Metab Dis.* **17**:215, 1994. PMID 7967476

823. Swaiman KK, Wu SR: Phenylalanine and phenylacetate adversely affect developing mammalian brain neurons. *Neurology.* **34**:1246, 1984. PMID 6540412

824. Tanizawa Y et al: Overexpression of dominant negative mutant hepatocyte nuclear factor (HNF)-1α inhibits arginine-induced insulin secretion in MIN6 cells. *Diabetologica.* **42**:887, 1999. PMID 10440133

825. Tayeh MA, Marletta MA: Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate: Tetrahydrobiopterin is required as a cofactor. *J Biol Chem.* **264**:19654, 1989. PMID 2584186

826. Teebi AS et al: *A preliminary report, in Skeels MR et al: (eds).* : Proceedings of the 6th National Neonatal Screening Symposium. 1988, p 150. PMID 2584186

827. Teebi SA et al: A centralized structure for the collection of genetic variations. *Am J Hum Genet.* **69**:452, 2001.

828. Teebi AS, Farag TI: *Genetic Disorders Among Arab Populations*. New York, Oxford University Press, 1997

829. Martinez A, Teigen K: Probing cofactor specificity in phenylalanine hydroxylase by molecular dynamics simulations. *J Biomol Struct Dyn.* **20**:733, 2003. PMID 12744702

830. Teigen K et al: Ligand recognition and specificity. *J Med Chem.* **47**:5962, 2004. PMID 15537351

831. Ten Kate: On estimating the actual rate of foetal loss in families with an autosomal recessive disorder and Woolf's data on PKU. *Ann Hum Genet.* **41**:463, 1978. PMID 655635

832. Tessari P et al: Phenylalanine hydroxylation across the kidney in humans rapid communication. *Kidney Int.* **56**:2168, 1999. PMID 10594792

833. Thalhammer O et al: Frequency of inborn errors of metabolism especially PKU, in some representative newborn screening centres around the world. *Humangenetik.* **30**:273, 1975. PMID 1218857

834. Thalhammer O et al: Intracellular concentrations of phenylalanine, tyrosine and α-aminobutyric acid in 13 homozygotes and 19 heterozygotes for PKU compared with 26 normals. *Hum Genet.* **54**:213, 1980. PMID 7390492

835. Thalhammer O et al: Intracellular phenylalanine and tyrosine concentration in homozygotes and heterozygotes for phenylketonuria (PKU) and hyperphenylalaninemia compared with normals. *Hum Genet.* **60**:320, 1982. PMID 7106768

836. Thomas CE et al: Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet.* **4**:346, 2003. PMID 12728277

837. Thompson AJ et al: Neurological deterioration in young adults with phenylketonuria. *Lancet.* **336**:602, 1990. PMID 1975386

838. Thompson AJ et al: MRI changes in early treated patients with phenylketonuria. *Lancet.* **2**:1224, 1991a.

839. Neel JV, Thompson EA: Allelic distribution and allele frequency distribution as a function of social demographic history. *Am J Hum Genet.* **60**:197, 1997. PMID 8981963

840. Thompson GN et al: Dietary treatment aimed at normalizing maternal plasma phenylalanine concentration. *Arch Dis Child.* **66**:1346, 1991b. PMID 1755650

841. Thompson GN, Halliday D: Significant phenylalanine hydroxylation in vivo in patients with classical phenylketonuria. *J Clin Invest.* **86**:317, 1990. PMID 2365821

842. ThÃ¶ny B et al: Tetrahydrobiopterin biosynthesis, regeneration and functions. *Biochem J.* **347**:1, 2000. PMID 10727395

843. Tighe O et al: Genetic diversity within the R408W phenylketonuria mutation lineages in Europe. *Hum Mutat.* **21**:387, 2003. PMID 12655548

844. Tourian A: A unique identity of rat hepatoma phenylalanine hydroxylase. *Biochem Biophys Res Commun.* **68**:51, 1976. PMID 174569

845. Tourian A et al: Phenylalanine hydroxylase activity in mammalian cells. *J Cell Physiol.* **73**:159, 1969. PMID 5789918

846. Tourian A et al: Three immunologically distinct isozymes of phenylalanine hydroxylase. *Biochemistry.* **14**:4055, 1975.

847. Traeger-Synodinos J et al: Preliminary mutation analysis in the phenylalanine hydroxylase gene in Greek PKU and HPA patients. *Hum Genet.* **94**:573, 1994. PMID 7959699

848. Treacy E et al: “Celtic” phenylketonuria chromosomes found? Evidence in two regions of Quebec province. *Eur J Hum Genet.* **1**:220, 1993. PMID 7913864

849. Treacy E et al: A study of two siblings. *J Inherit Metab Dis.* **19**:595, 1996. PMID 8892014

850. Treacy EP et al: A pilot study. *Pediatr Res.* **42**:430, 1997. PMID 9380432

851. Trefz FK et al: In vivo determination of phenylalanine hydroxylase activity using heptadeutero-phenylalanine and comparison to the in vitro assay values. *Monogr Hum Genet.* **9**:108, 1978. PMID 732825

852. Trefz FK et al: In vivo residual activities of the phenylalanine hydroxylating system in phenylketonuria and variants. *J Inherit Metab Dis.* **4**:101, 1981. PMID 6790838

853. Trefz FK et al: Genotype-phenotype correlations in phenylketonuria. *Clin Chim Acta.* **217**:15, 1993. PMID 8222278

854. Trefz FK et al: Long-term treatment of patients with mild and classical phenylketonuria by tetrahydrobiopterin. *Mol Genet Metab.* **86**:S75, 2005. PMID 16242984

855. Tyfield LA et al: Identification of the haplotype pattern associated with the mutant PKU allele in the Gypsy population of Wales. *J Med Genet.* **26**:499, 1989. PMID 2570158

856. Tyfield LA et al: Genetic analysis of treated and untreated phenylketonuria in one family. *J Med Genet.* **27**:564, 1990. PMID 1977916

857. Tyfield LA et al: Sequence variation at the phenylalanine hydroxylase gene in the British Isles. *Am J Hum Genet.* **60**:388, 1997.

858. Udenfriend S, Bessman S: The hydroxylation of phenylalanine and antipyrene in phenylpyruvic oligophrenia. *J Biol Chem.* **203**:961, 1953. PMID 13084666

859. Udenfriend S, Cooper JR: The enzymatic conversion of phenylalanine to tyrosine. *J Biol Chem.* **194**:503, 1952.

860. Ullrich K et al: Results of magnetic resonance measurements. *Acta Pediatr Suppl.* **407**:78, 1994. PMID 7766966

861. Ullrich K et al: Effect of L-dopa on visual evoked potentials and neuropsychological tests in adult phenylketonuria patients. *Eur J Pediatr.* **155**:S74, 1996. PMID 8828615

862. U.S. Congress: *Healthy Children.* : Investing in the Future. Newborn Screening for Congenital Disorders. Washington, DC, US Government Printing Office, 1988. PMID 8828615

863. Vajro P et al: Correction of phenylketonuria after liver transplantation in a child with cirrhosis. *N Engl J Med.* **329**:363, 1993. PMID 8321274

864. van Baal et al: A relational databases recording frequencies of genetic defects leading to inherited disorders worldwide. *Nucl Acids Res.* **35**:D690, 2007.

865. van der et al: The phenylalanine response curve in relation to growth and mental development in the first years of life. *Acta Paediatr Suppl.* **407**:68, 1994. PMID 7539309

866. van der et al: 93 percent of the mutations are detected by single-strand conformation analysis. *Hum Hered.* **46**:185, 1996. PMID 8807319

867. Van Spronsen et al: *Does impaired growth of PKU patients correlate with the strictness of dietary treatment? Acta Paediatr.* **86**:816, 1997. PMID 9307159

868. Van Spronsen et al: The in vivo hydroxylation rate of phenylalanine into tyrosine is decreased. *J Clin Invest.* **101**:2875, 1998. PMID 9637722

869. Levy HL, Vargas JE: Maternal and fetal considerations in metabolic disorders. *J Jap Soc Mass Screen.* **8**:S29, 1998.

870. Venter JC et al: The sequence of the human genome. *Science.* **291**:1304, 2001. PMID 11181995

871. Villasana D et al: Neurological deterioration in adult phenylketonuria. *J Inherit Metab Dis.* **12**:451, 1989. PMID 2516176

872. Vogel F: Phenotypic deviations in heterozygotes of phenylketonuria (PKU). *Prog Clin Biol Res.* **177**:337, 1985. PMID 4011607

873. Berry HK, Vorhees CV: Branched chain amino acids improve complex maze learning in rat offspring prenatally exposed to hyperphenylalaninemia: Implications for maternal phenylketonuria. *Pediatr Res.* **25**:568, 1989. PMID 2740145

874. Vorhees CV et al: A critical review. *Neurosci Biobehav Rev.* **5**:177, 1981. PMID 6453302

875. Wadman SK et al: Permanent chemical phenylketonuria and a normal phenylalanine tolerance in two sisters with a normal mental development. *Clin Chim Acta.* **65**:197, 1975. PMID 1183053

876. Waisbren SE, Azen C: *Cognitive and behavioural development in maternal PKU offspring*. Pediatrics (in press)

877. Waisbren SE et al: Identification of at-risk women. *Am J Public Health.* **78**:789, 1988. PMID 3381953

878. Waisbren SE et al: Prevention of unplanned pregnancies. *Am J Public Health.* **81**:299, 1991. PMID 1994738

879. Waisbren SE et al: Women's adherence to medical recommendations. *Am J Public Health.* **85**:1636, 1995. PMID 7503337

880. Waisbren SE et al: Results of a maternal phenylketonuria summer camp. *J Inherit Metab Dis.* **20**:21, 1997. PMID 9061563

881. Wallace HW et al: Studies on conversion of phenylalanine to tyrosine in phenylpyruvic oligophrenia. *Proc Soc Exp Biol Med.* **94**:632, 1957. PMID 13431902

882. Walraven C et al: Survey of practices among newborn screening programs in the United States. *Screening.* **4**:1, 1995.

883. Walter JH et al: Biochemical control, genetic analysis and magnetic resonance imaging in patients with phenylketonuria. *Eur J Pediatr.* **152**:822, 1993. PMID 7901023

884. Walter JH et al: Complete reversal of moderate/severe brain MRI abnormalities in a patient with classical phenylketonuria. *J Inherit Metab Dis.* **20**:367, 1997. PMID 9266357

885. Walter JH et al: *How practical are recommendations for dietary control in phenylketonuria? Lancet.* **360**:55, 2002. PMID 12114043

886. Wang GA et al: Mutagenesis of the regulatory domain of phenylalanine hydroxylase. *Proc Natl Acad Sci USA.* **98**:1537, 2001. PMID 11171986

887. Wang L et al: Structure-based chemical modification strategy for enzyme replacement treatment of phenylketonuria. *Mol Genet Metab.* **86**:134, 2005. PMID 16006165

888. Wang L: *et al:.* : Structural and biochemical characterization of the CYS503 SER and CYS565 SER double mutant of phenylalanine ammonia-lyase from A. variabilis. Submitted 2007. PMID 16006165

889. Wang T et al: Linkage disequilibrium between a termination mutation and haplotype 4 of the phenylalanine hydroxylase gene. *Am J Hum Genet.* **45**:675, 1989. PMID 2816939

890. Wang T et al: Founder effect of a prevalent phenylketonuria mutation in the Oriental population. *Proc Natl Acad Sci USA.* **88**:2146, 1991a. PMID 2006152

891. Wang T et al: Further evidence for multiple origins of PKU in Asia. *Am J Hum Genet.* **48**:628, 1991b. PMID 1998345

892. Wang Y et al: Tissue- and development-specific expression of the human phenylalanine hydroxylase/chloramphenicol acetyltransferase fusion gene in transgenic mice. *J Biol Chem.* **267**:15105, 1992a. PMID 1321825

893. Wang Y et al: Evidence for recombination or recurrent mutation at the PAH locus. *Genomics.* **13**:230, 1992b. PMID 1349576

894. Wang Y et al: Functional characterization of a unique liver gene promoter. *J Biol Chem.* **269**:9137, 1994. PMID 8132651

895. Wapnir RA et al: Ontogenesis of phenylalanine and tryptophan hydroxylation in rat brain and liver. *Biol Neonate.* **18**:85, 1971. PMID 5109765

896. Wardley BL, Taitz LS: Clinical trial of a concentrated amino acid formula for older patients with phenylketonuria. *Eur J Clin Nutr.* **42**:81, 1988. PMID 3371301

897. Wasinger EC et al: X-ray absorption spectroscopic investigation of the resting ferrous and cosubstrate-bound active sites of phenylalanine hydroxylase. *Biochemistry 41X.* : 6211, 2002. PMID 12009881

898. Waters PJ: How PAH gene mutations cause hyperphenylalaninemia and why mechanism matters: Insights from in vitro expression. *Hum Mutat.* **21**:357, 2003. PMID 12655545

899. Waters PJ et al: Linking genotype to phenotype and structure to function. *Hum Mutat.* **11**:4, 1998. PMID 9450897

900. Waters PJ et al: A mechanism underlying phenylketonuria. *J Inherit Metab Dis.* **22**:208, 1999. PMID 10384369

901. Waters PJ et al: Characterization of phenylketonuria missense substitutions, distant from the phenylalanine hydroxylase active site, illustrates a paradigm for mechanism and potential modulation of phenotype. *Mol Genet Metab.* **69**:101, 2000. PMID 10720436

902. Waters PJ et al: Evaluation of two-hybrid approaches for functional analyses of mutations causing hyperphenylalaninemia. *Mol Genet Metab.* **73**:230, 2001. PMID 11461190

903. Weber JL, Wong C: Mutation of human short tandem repeats. *Hum Mol Genet.* **2**:1123, 1993. PMID 8401493

904. Wedemeyer N et al: PCR detection of the MspI (Aa) RFLP at the human phenylalanine hydroxylase (PAH) locus. *Nucleic Acids Res.* **19**:1959, 1991. PMID 1709499

905. Weglage J et al: Delayed onset of phenylketonuria. *Eur J Pediatr.* **157**:171, 1998. PMID 9504799

906. Weglage J et al: Clinical significance of interindividual differences in brain phenylalanine concentrations. *J Inherit Metab Dis.* **21**:81, 1998a. PMID 9501277

907. Weglage J et al: Pathogenesis of different clinical outcomes in spite of identical genotypes and comparable blood phenylalanine concentration in phenylketonuria. *J Inherit Metab Dis.* **21**:181, 1998b. PMID 9584274

908. Weglage J et al: Behavioural and emotional problems in early-treated adolescents with phenylketonuria in comparison with diabetic patients and healthy controls. *J Inherit Metab Dis.* **23**:487, 2000. PMID 10947203

909. Weglage J et al: Normal clinical outcome in untreated subjects with mild hyperphenylalaninemia. *Pediatr Res.* **49**:532, 2001. PMID 11264437

910. Weglage J et al: Individual blood-brain barrier phenylalanine transport in siblings with classical phenylketonuria. *J Inherit Metab Dis.* **25**:431, 2002. PMID 12555936

911. Weiss KM: Is there a paradigm shift in genetics? Lessons from the study of human diseases. *Mol Phylogenet Evol.* **5**:259, 1996. PMID 8673294

912. Weiss KM, Buchanan AV: Evolution by phenotype: A biomedical perspective. *Perspect Biol Med.* **46**:159, 2003. PMID 12721519

913. Welsh MC et al: Specific executive function deficits. *Child Dev.* **61**:1697, 1990. PMID 2083493

914. Wenger SL et al: Relative reliability of three different discriminant analysis methods for detecting PKU gene carriers. *Clin Genet.* **30**:38, 1986. PMID 3757295

915. Werner-Felmayer G et al: Tetrahydrobiopterin biosyntheseis, utilization and pharmacological effects. *Curr Drug Metab.* **3**:159, 2002. PMID 12003348

916. Westwood A, Raine DN: Heterozygote detection in phenylketonuria. *J Med Genet.* **12**:327, 1975. PMID 1219115

917. White DA et al: Age-related working memory impairments in children with prefrontal dysfunction associated with phenylketonuria. *J Int Neuropsychol Soc.* **8**:1, 2002. PMID 11843066

918. White JE et al: Excess weight among children with phenylketonuria. *J Am Coll Nutr.* **1**:293, 1982. PMID 7185860

919. Wilcken B et al: Eliminating some possible errors in phenylketonuria screening. *Am J Clin Pathol.* **92**:396, 1989. PMID 2773856

920. Williams K: Benefits of normalizing plasma phenylalanine: Impact on behaviour and health. *J Inherit Metab Dis.* **21**:785, 1998. PMID 9870203

921. Williamson M et al: Study design. *Pediatrics.* **60**:815, 1977. PMID 600593

922. Williamson M et al: Diet discontinuation in phenylketonuria. *Pediatrics.* **63**:823, 1979. PMID 440916

923. Williamson ML et al: Correlates of intelligence test results in treated phenylketonuric children. *Pediatrics.* **68**:161, 1981. PMID 7267221

924. Wilson JMG, Jungner G: *Principles and Practice of Screening for Disease*. Public Health Papers No 34 Geneva, World Health Organization, 1968

925. Woo SLC: Prenatal diagnosis and carrier detection of classic phenylketonuria by gene analysis. *Pediatrics.* **74**:412, 1984. PMID 6472974

926. Woo SLC et al: Cloned human phenylalanine hydroxylase gene allows prenatal diagnosis and carrier detection of classical phenylketonuria. *Nature.* **306**:151, 1983. PMID 6316140

927. Woolf LI: Late onset phenylalanine intoxication. *J Inherit Metab Dis.* **2**:19, 1979. PMID 6796756

928. Woolf LI: The heterozygote advantage of phenylketonuria. *Am J Hum Genet.* **38**:77, 1986. PMID 3717163

929. Woolf LI: Phenylketonuria in Turkey, Ireland and West Scotland. *J Inherit Metab Dis.* **17**:246, 1994. PMID 7967483

930. Woolf LI et al: Treatment of phenylketonuria with a diet low in phenylalanine. *Br Med J.* **1**:57, 1955. PMID 13219342

931. Woolf LI et al: The nature of the heterozygote advantage. *Ann Hum Genet.* **38**:461, 1975. PMID 1190737

932. Woolf LI, Vulliamy DG: Phenylketonuria with a study of the effect upon it of glutamic acid. *Arch Dis Child.* **26**:487, 1951.

933. Wretborn M et al: Amino acid sequence at the phosphorylation site of rat liver phenylalanine hydroxylase and phosphorylation of a corresponding synthetic peptide. *Biochem Biophys Res Commun.* **93**:403, 1980. PMID 7387651

934. Yamashita M et al: Purification of phenylalanine hydroxylase from human adult and foetal livers with a monoclonal antibody. *Biochem Biophys Res Commun.* **133**:202, 1985. PMID 4074362

935. Ryan A, Yannicelli S: Improvements in behaviour and physical manifestations in previously untreated adults with phenylketonuria using a phenylalanine-restricted diet: A national survey. *J Inherit Metab Dis.* **18**:131, 1995. PMID 7564227

936. Young VR, Pellett PL: Protein intake and requirements with reference to diet and health. *Am J Clin Nutr.* **45**:1323, 1987. PMID 3554971

937. Zschocke J et al: True fragment length obtained with fluorescent labelled PCR primers. *Acta Paediatr Suppl.* **407**:41, 1994a. PMID 7766955

938. Zschocke J et al: Automated sequencing detects all mutations in Northern Irish patients with phenylketonuria and mild hyperphenylalaninaemia. *Acta Pediatr Suppl.* **407**:37, 1994b. PMID 7766952

939. Zschocke J et al: A rapid stepwise approach. *Am J Hum Genet.* **57**:1311, 1995. PMID 8533759

940. Zschocke J et al: Phenylketonuria mutations in Europe. *Hum Mutat.* **21**:345, 2003. PMID 12655544

941. Zschocke J et al: Phenylketonuria and the peoples of Northern Ireland. *Hum Genet.* **100**:189, 1997. PMID 9254847

942. Hoffmann GF, Zschocke J: Phenylketonuria mutations in Germany. *Hum Genet.* **104**:390, 1999. PMID 10394930

943. Zygulska M et al: Molecular analysis of phenylketonuria in the population of southern Poland. *Dev Brain Dysfunct.* **6**:129, 1993.

944. Zygulska M et al: A new splice mutation in intron 9 at the PAH locus. *Hum Mutat.* **4**:297, 1994. PMID 7866411

