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The *PAH* Gene, Phenylketonuria, and a Paradigm ShiftCharles R. Scriver<sup>1–4\*</sup><sup>1</sup>Department of Human Genetics, Faculty of Medicine, McGill University, Montreal, Quebec, Canada; <sup>2</sup>Department of Biochemistry, Faculty of Medicine, McGill University, Montreal, Quebec, Canada; <sup>3</sup>Department of Pediatrics, Faculty of Medicine, McGill University, Montreal, Quebec, Canada; <sup>4</sup>Department of Biology, Faculty of Science, McGill University, Montreal, Quebec, Canada

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“Inborn errors of metabolism,” first recognized 100 years ago by Garrod, were seen as transforming evidence for chemical and biological individuality. Phenylketonuria (PKU), a Mendelian autosomal recessive phenotype, was identified in 1934 by Asbjörn Fölling. It is a disease with impaired postnatal cognitive development resulting from a neurotoxic effect of hyperphenylalaninemia (HPA). Its metabolic phenotype is accountable to multifactorial origins both in nurture, where the normal nutritional experience introduces L-phenylalanine, and in nature, where mutations (> 500 alleles) occur in the phenylalanine hydroxylase gene (*PAH*) on chromosome 12q23.2 encoding the L-phenylalanine hydroxylase enzyme (EC 1.14.16.1). The *PAH* enzyme converts phenylalanine to tyrosine in the presence of molecular oxygen and catalytic amounts of tetrahydrobiopterin (BH<sub>4</sub>), its nonprotein cofactor. PKU is among the first of the human genetic diseases to enter, through newborn screening, the domain of public health, and to show a treatment effect. This effect caused a paradigm shift in attitudes about genetic disease. The PKU story contains many messages, including: a framework on which to appreciate the complexity of PKU in which phenotype reflects both locus-specific and genomic components; what the human *PAH* gene tells us about human population genetics and evolution of modern humans; and how our interest in PKU is served by a locus-specific mutation database (<http://www.pahdb.mcgill.ca>; last accessed 20 March 2007). The individual Mendelian PKU phenotype has no “simple” or single explanation; every patient has her/his own complex PKU phenotype and will be treated accordingly. Knowledge about PKU reveals genomic components of both disease and health. *Hum Mutat* 28(9), 831–845, 2007. Published 2007 Wiley-Liss, Inc.†

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“Nature is no where accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path;... For it has been found, in almost all things, that what they [the rarer forms of disease] contain of useful or applicable is hardly perceived unless we are deprived of them, or they become deranged in some way.”

—Attributed to William Harvey and adapted by Archibald Garrod in the Harveian Oration at the Royal College of Physicians [Garrod, 1924].

## FOREWORD

This article in *Human Mutation*, a Wiley-Liss journal, joins the bicentennial celebration at John Wiley & Sons in the year 2007 when it celebrates 200 years of publishing in science and other fields. One hundred years ago, halfway into the Wiley bicentennial, Sir Archibald Garrod introduced his evidence for human chemical individuality, gleaned from his study of persons with rare “inborn errors of metabolism” [Garrod, 1908]. One of the disorders that interested Garrod was cystinuria, in which extravagant chemical individuality in the composition of urine is the cause of harmful calculi in the urinary tract of patients. The chemical basis of calculi had been identified much earlier, in 1810 actually, by Wollaston [1810], just 3 years after John Wiley & Sons had begun their odyssey in publishing.

## INTRODUCTION

My topic is an inborn error of metabolism called phenylketonuria (PKU; MIM# 261600) [Donlon et al., 2004]. The disease and our response to it has been called an epitome of human biochemical genetics [Scriver and Clow, 1980a, 1980b]. It 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 nurture and nature

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leads to hyperphenylalaninemia (HPA), which can have a toxic effect on brain.

Phenylketonuria, the name of the disease phenotype, is inherited as an autosomal recessive. It is a mutant homozygous phenotype with a metabolic component (HPA) and an important clinical component (impaired cognitive development and function). The untreated disease behaves like a quasigenetic lethal, yet the average birth incidence is considerable,  $10^{-4}$  in European and Oriental Asian populations and the aggregate (mutant) allele frequency in the population, assuming Hardy-Weinberg equilibrium, is polymorphic ( $\sim 0.01$ ). Of particular relevance, PKU was among the first, if not the first, “genetic” disease for which selection in the mutant homozygote was relaxed by medical treatment, thus showing how *Homo sapiens* could participate intentionally in its own evolution.

Six facets of the “PKU story” are selected from many for this essay: the historical milestones; the human *PAH* gene; the *PAH* gene mutation relational database; *PAH* alleles in the context of population genetics; evidence that the Mendelian phenotype is not “simple” in its manifestations; and various treatment options. The choice reflects the particular interests of the writer and comes with a warning: “this is not the whole PKU story!” Moreover, the danger in being selective in focus is that reductionist terms, phrases, and perspectives take on a prominence that does not reflect the actual complexity of the story [Weiss and Buchanan, 2003].

### The Beginning of the Story

PKU was discovered and described by Fölling in 1934 [Fölling, 1934]. He called the condition “phenylpyruvic oligophrenia,” soon thereafter to be renamed “phenylketonuria” in recognition of its unusual metabolic by-product [Penrose and Quastel, 1937]. The discovery of PKU proved to be an important event for Garrod, who had received correspondence from both Penrose and Fölling about the newly recognized disease (see p. 145 in Bearn [1993]); important because PKU offered further evidence of *human chemical individuality*, and *the existence of metabolic pathways*, the concepts that Garrod [1902] had formulated earlier and developed further in his Croonian lectures of 1908 [Garrod, 1908], but which had made little headway thereafter in general medical thinking (see page 128–133 in Olby [1974]).

Lionel Penrose also contributed importantly to the early understanding of PKU [Penrose, 1998]. He had an abiding interest in causes of mental retardation and he surmised that so-called “feeble-mindedness,” in the untreated PKU state, had an endogenous chemical cause. Because L-phenylalanine is an essential amino acid in human nutrition and metabolism, Penrose was among the first to consider the possibility that modification of “nurture” might neutralize the harmful effect of the mutant “nature” in PKU. Evidence did soon emerge [Bickel et al., 1954] that a low phenylalanine diet could prevent the HPA of PKU, with benefit to cognitive function. *PKU is now celebrated as one of the first human genetic diseases to have an effective rational therapy*. Such recognition constituted a “paradigm shift” in medical thinking about genetic disease in general; the idea here of a superior paradigm shift echoes Kuhn’s use of the term to describe scientific revolutions [Kuhn, 1970].

### Major Milestones Recognized in the History of PKU

In the 1930s, PKU reveals itself to be a disease associated with mental retardation and a deviant metabolic phenotype, explained best as the homozygous mutant phenotype of a condition inherited

in Mendelian fashion as an autosomal recessive [Fölling, 1934; Penrose, 1935]. The word “phenylketonuria” [Penrose and Quastel, 1937] is coined to link disease and metabolic phenotypes.

In the 1940s, Penrose gives his inaugural address as the newly appointed Galton Professor of Eugenics at University College, London [Penrose, 1998]. The thoughts and hypotheses appearing in his address generate momentum in research on PKU that continues today.

In the 1950s, hepatic phenylalanine hydroxylase activity (EC 1.14.16.1) is shown to be deficient in the PKU patient [Jervis, 1953]. It is also shown that the metabolic phenotype in PKU can be treated by dietary restriction of phenylalanine, with the potential to prevent mental retardation [Bickel et al., 1954; Woolf et al., 1955; Armstrong and Tyler, 1955]. Accordingly, PKU offers a new paradigm to medical thinking about hereditary disease.

In the 1960s, a simple laboratory test is developed, compatible with population screening in the newborn [Guthrie and Susi, 1963]. Newborn screening programs subsequently appear worldwide for purposes of early diagnosis and treatment to prevent mental retardation in the PKU patient. PKU becomes a prototype for genetic screening in human populations [National Academy of Sciences, Committee for the Study of Inborn Errors of Metabolism, 1975], and one cannot overestimate the importance of newborn screening and public health programs in generating information about PKU and the related population genetics [Scriver and Clow, 1980a, 1980b].

In the 1970s, individuals are recognized with a strange condition called “malignant HPA” [Danks et al., 1978]. It is already known that tetrahydrobiopterin is a necessary catalytic cofactor for the conversion of phenylalanine to tyrosine on the *PAH* enzyme [Kaufman, 1963]. Discovery of this new form of PKU reveals disorders in both the synthesis and recycling of tetrahydrobiopterin. PKU is seen as an “epitome of human biochemical genetics.”

In the 1980s, the cDNA for the human phenylalanine hydroxylase gene (*PAH*) is cloned, mapped to chromosome 12 [Woo et al., 1983] and catalogued in GenBank: GenBank NM\_000277 (mRNA); U49897.1 (cDNA). David Konecki and colleagues will later obtain the full-length genomic *PAH* sequence; GenBank : AF404777 (gDNA) (for details, see Scriver et al. [2003]).

In the 1990s, a *PAH* Mutation Analysis Consortium is formed, mutation analysis in PKU probands is carried out in diverse human populations, and extensive nonrandom allelic heterogeneity is discovered (> 500 alleles). The alleles acquire a standardized taxonomy [Antonarakis and the Nomenclature Working Group, 1998], and PAHdb, an online relational database, is created. The *PAH* enzyme is also crystallized and its structure is described at 2-Å resolution [Erlandsen and Stevens, 1999], making molecular modeling of mutation effects in silico feasible.

By the first decade of the 21st century, PKU is a prototype to show that a “simple” Mendelian phenotype can also be a complex disorder [Scriver and Waters, 1999]. It is also discovered that *PAH* alleles, which cause misfolding of proteins, may respond to pharmacological doses of tetrahydrobiopterin (BH4) with a chaperone-like therapeutic effect.

### WHAT’S IN A PHENOTYPE NAME?

If one assumes that any degree of HPA could cause cognitive impairment, then all of its forms might have only one name—“phenylketonuria” [Smith, 1994]. While one may concur, there

remains precedent, practice, and reality. First, different phenotypic features exist in the “PKU” and “non-PKU” forms of HPA, the latter being forms with less drastic clinical consequences, which are associated with different *PAH* alleles [Weiss and Buchanan, 2003]. Second, there are forms of HPA that do not map to the *PAH* locus. They involve mutations in genes encoding enzymes that control the synthesis and recycling of tetrahydrobiopterin (BH<sub>4</sub>), the catalytic cofactor for normal *PAH* enzyme function; these forms of HPA require specific BH<sub>4</sub> replacement for treatment to be effective. Third, in the absence of treatment, variant phenotypes associated with allelic variation at the *PAH* locus yield greater or lesser risk of impaired cognitive development according to the degree of HPA, a feature that has practical relevance because there is some evidence that blood phenylalanine levels below 600 μM (normal, <120 μM) in untreated patients may not be harmful to cognitive development [Weglage et al., 2001]. Accordingly, while we may use the term “phenylketonuria” to encompass a spectrum of phenotypes because it is convenient to do so, yet as phenotype becomes better explained by allelic (*PAH*) variation and genomic (modifier loci) variation, the better will we understand the process of genotype–phenotype relationships, and the more will we aspire to patient-specific therapy.

### A Framework to Understand PKU

***PKU is a mendelian phenotype.*** Fölling [1934] and Penrose [1935] both observed an increased frequency of consanguinity among parents of PKU patients, implying autosomal recessive inheritance. The condition will later be catalogued in Mendelian Inheritance in Man as MIM entry number 261600. *PAH* alleles are associated with a recessive metabolic phenotype in obligate heterozygotes, implying that the heterozygous genome contains loci that can buffer the effects of *PAH* mutations [Kacser and Burns, 1981]. Prevalence of the homozygous metabolic phenotype (both PKU and non-PKU HPA) in aggregate is  $\sim 10^{-4}$  live births in the populations of Europe and among Orientals in mainland Eurasia; on the other hand, the incidence is at least an order of magnitude less among sub-Saharan Africans and their descendant populations such as African-Americans and African-Britons [Scriver et al., 2006].

***There is extensive allelic heterogeneity.*** Silent polymorphic alleles at the *PAH* locus provide a large array of haplotypes and minihaplotypes that harbor over 500 disease-causing (phenotype-modifying) *PAH* mutations. In any particular population, fewer than 10 different disease-causing *PAH* alleles usually account for more than half the aggregate population frequency [Scriver et al., 1996].

***There is locus heterogeneity.*** Mutations in the genes encoding enzymes for the synthesis or recycling of BH<sub>4</sub> account for only 2% of patients with HPA; the aggregate population incidence, for BH<sub>4</sub>-deficient forms of PKU is 1 out of 1 million births [Thöny and Blau, 2006]. Although the homozygous mutant clinical phenotypes associated with primary disorders of BH<sub>4</sub> metabolism are rare, they must be identified in the follow-up investigation of a positive newborn screening test if counseling is to be accurate and the treatment appropriate.

Allelic heterogeneity also exists among BH<sub>4</sub>-deficient phenotypes that are a cause of deficient activities in other enzymes (phenylalanine-4-hydroxylase, tryptophan-5-hydroxylase, tyrosine-3-hydroxylase, nitric oxide synthase, and glycerol ether monooxygenase) because each of the latter enzymes is dependent on the BH<sub>4</sub> cofactor. Extensive information about these genes, alleles, and enzymes [Thöny and Blau, 2006; Blau, 2003] is accessible on a dedicated website ([www.bh4.org](http://www.bh4.org)).

***HPA is a multifactorial phenotype.*** The condition is multifactorial with proximate and ultimate events: the proximate one being dietary intake of L-phenylalanine; the ultimate event being paired mutant alleles in the *PAH* gene. Thus, mutation and nutrition are both necessary and sufficient conditions to create the variant metabolic phenotype.

***PKU is a complex trait.*** Genotype–phenotype relationships in PKU often show no robust correlation. While there is indeed a major locus (*PAH*) where mutations occur, and the mutations are a principal explanation for the Mendelian phenotype called PKU, nonetheless genotype is not a consistent predictor of phenotype. Genetic modifiers and other events can explain some of the observed discordances.

### THE HUMAN *PAH* GENE

*Comment:* Participants in the PKU story were beneficiaries of the Human Genome project and allied projects, from which came the sequences and maps of human genes.

A single gene, whose full-length human cDNA was first obtained and sequenced [Kwok et al., 1985], then expressed in an in vitro system [Ledley et al., 1985], is sufficient to encode tetrahydrobiopterin-dependent conversion of phenylalanine to tyrosine on the phenylalanine hydroxylase enzyme (EC 1.14.16.1). The human gene (symbol *PAH*) is expressed in liver and kidney [Wang et al., 1992; Lichter-Konecki et al., 1999; Tessari et al., 1999] as a monomer of 452 amino acids with regulatory, catalytic, and tetramerization domains; the monomer assembles to form the functional dimeric and tetrameric forms of the enzyme (see Erlandsen and Stevens [1999]).

The *PAH* gene maps to chromosome 12, region 12q23.2. The finished total sequence for chromosome 12 [Scherer et al., 2006] harbors 1,435 loci in 130,683,379 basepairs of nonoverlapping sequence accommodating  $\sim 4.5\%$  of the human genome. The rate of base substitutions on chromosome 12 in recent evolution is declining in hominids compared with primates and rodents. Chromosome 12 is particularly rich in disease-associated loci, with 487 loci accounting for 5.2% of known “disease genes.” The *PAH* gene itself ranked high (13th) in PubMed citations in 2006 ( $n = 5,485$ ) among the disease-associated genes on chromosome 12.

The locus for the gene encoding the enzyme phenylalanine hydroxylase, covers 1.5 Mbp and harbors five other genes. There are finished sequences for both cDNA [Kwok et al., 1985; Konecki et al., 1992] and gDNA [Konecki in Scriver et al. [2003]]; see PAHdb ([www.pahdb.mcgill.ca](http://www.pahdb.mcgill.ca)). The *PAH* genomic sequence and its flanking regions span 171,266 basepairs: the 5′ UTR upstream covers  $\sim 27$  kbp; the 3′ sequence downstream from the poly(A) site in the last exon (exon 13) covers  $\sim 64.5$  kbp. The 5′ UTR of the human *PAH* gene contains several *cis* control elements [Konecki et al., 1992]. SNP, multiallelic tandem repeat sequences, and RFLPs, embedded in the *PAH* genomic sequence, provide signatures of multiple haplotypes with particular associations for a wide range of disease-causing mutations.

The 13 exons in the *PAH* gene comprise  $\sim 2.88\%$  of the genomic sequence lying between the start codon and the 3′ poly(A) tract. The shortest exon is 57 bp (exon 9); the longest is 892 bp (exon 13). The shortest intron is 556 bp (intron 10); the longest is 17,874 bp (intron 2).

The *PAH* genomic sequence has 40.7% GC nucleotide content, slightly above the modal value (37–38%) for a typical human gene. The density of interspersed repeats accounts for 42.2% in the *PAH* gene; the value is typical for a mammalian gene. Repetitive DNA

sequences are quite abundant, and are now seen as causes of previously unidentified large *PAH* gene deletions and duplications [Kozak et al., 2006]. Putative alu repeats also occur in the *PAH* genomic sequence; and CpG dinucleotides ( $n = 1198$ ) are potential sites for recurrent mutation in the *PAH* gene.

*PAH* gene mutation types and relative frequencies (%) comprise (broadly): missense, 63%; small deletions, 13%; splice, 11%; putative silent, 7%; stop/nonsense, 5%; small insertions, 1%. Large deletions, once thought to be rare, probably account for 3% of PKU-causing mutations [Kozak et al., 2006].

### PAHDB: A LOCUS-SPECIFIC MUTATION DATABASE (LSDB)

*Comment:* Participants in the PKU story were beneficiaries of the in silico revolution that gave rise to computers, databases, and the Internet.

Databases are legacies of science, yet they are often neglected by the communities they can serve [Maurer et al., 2000]. As discussed previously [Scriver et al., 2003], when a particular domain of science evolves, in genetics for example, first there is a stage of explanation with classification and taxonomy of its entities (e.g., mutations); then follows inquiry into mechanisms underlying the entities; the final stage synthesizes and recreates the entities. In genetics, mutation is both entity and mechanism; mutations in the integrity of DNA can be created, for example by site-directed mutagenesis and then expressed experimentally to discover mutation in phenotype. Accordingly, databases with information about genotypes, phenotypes, and attributes have become important, even essential, resources in genetics. Databases have become repositories of information about individual genes and over 600 locus-specific databases (LSDBs) now populate the Internet. PAHdb ([www.pahdb.mcgill.ca](http://www.pahdb.mcgill.ca)) is one such legacy and resource.

PAHdb is an LSDB of relational design. It began in a word processor, as a file of mutations being discovered at the *PAH* locus [John et al., 1990]; it then expanded to include annotations about the associations between mutations, haplotypes and populations of origin [Scriver et al., 1993]. As the number of entities (mutations and descriptors of attributes) grew, through the work of the *PAH* Mutation Analysis Consortium (81 members in 26 countries), PAHdb converted to a relational structure [Hoang et al., 1996], and the curators of PAHdb adopted recommendations for a mutation nomenclature system based on numbered genomic or cDNA sequences [Antonarakis and the Nomenclature Working Group, 1998; den Dunnen and Antonarakis, 2000]. Guidelines and recommendations for content and deployment of LSDBs also appeared [Scriver et al., 2000a].

The *PAH* Mutation Analysis Consortium became 88 members in 28 countries and its next report [Nowacki et al., 1998] contained information about 328 mutations and their attributes. Unique identifiers (UIDs) were given to each *PAH* mutation different by state. By 1998, PAHdb had acquired over 4,000 records, held in over 60 data tables, distributed among several conceptual modules containing information about, for example, the *PAH* gene reference sequence, the mutations, the associated polymorphic haplotypes, genotype–phenotype correlations, expression analyses of *PAH* mutations in vitro, sources of the data, and clinical information; in addition there is a module holding information about mutant mouse orthologs of human HPA. Many of the modules have a dedicated curator [Scriver et al., 2003]. PAHdb also acquired status as a repository of intellectual property, and its home page reminds the user that the information in

PAHdb is copyrighted, a factor of some importance because the pace of mutation discovery exceeds the capacity to publish mutation data by conventional means.

From analysis of the data in PAHdb, it is now apparent for example that: 1) as few as six alleles, each different by state, account for 60% of the mutant alleles causing PKU/HPA worldwide; 2) *PAH* mutations stratify by population and geographic region; and 3) Caucasian and Oriental *PAH* mutation sets are quite different [Nowacki et al., 1998].

PAHdb was reviewed in a large study of LSDBs and deemed to be a useful prototype [Claustres et al., 2002]. With its extensive information content, PAHdb is now seen as a “knowledgebase” [Scriver et al., 2000b]. If genomic databases are “inch deep but a mile wide” in character, *PAHdb* (and other LSDBs like it) is “an inch wide but a mile deep” in its character. Different needs are served, accordingly.

PAHdb, as a relational LSDB, has records and annotations of alleles (entities) both “pathogenic” and polymorphic (see a mutation map via the link [www.pahdb.mcgill.ca/Information/MutationMap/mutationmap.pdf](http://www.pahdb.mcgill.ca/Information/MutationMap/mutationmap.pdf)). The alleles are named by nucleotide number (systematic names); they also have “trivial” names, and they receive unique identifier numbers. The gDNA sequence for the *PAH* gene is annotated in PAHdb; as mentioned earlier, it is typical for a mammalian gene and the sequence is numbered so that cDNA and gDNA counterparts are interconvertible. A site map for PAHdb leads one to a large array of secondary data (attributes), for example: source of the allele (submitter, publication, population), polymorphic haplotype background; effect of the allele (as predicted by molecular modeling) on the phenylalanine hydroxylase enzyme (EC 1.14.16.1) or by in vitro expression analysis. The majority (63%) of the putative pathogenic *PAH* alleles are point mutations causing missense in translation. Fewer appear to have a primary effect on the enzyme kinetics; most apparently have a secondary effect on function through misfolding and instability, and intracellular degradation of the protein. Some point mutations create new splice sites. A subset of primary *PAH* mutations are tetrahydrobiopterin-responsive; these are discussed on the Curators’ Page of PAHdb. A “clinical module” linked to GeneReviews [Mitchell and Scriver, 2005] describes the corresponding human clinical disorders (MIM# 261600: HPA and PKU), and their treatment is also described. PAHdb also contains data on the mouse gene (*Pah*); it describes four orthologous mutant mouse models and discusses their use in the search for a new experimental treatment of PKU with the enzyme phenylalanine ammonia lyase (EC 4.3.1.5).

PAHdb is linked to other databases on the Internet. They include: 1) *central and core databases*, such as Online Mendelian Inheritance in Man ([www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM)) and the Human Gene Mutation Database ([www.hgmd.org](http://www.hgmd.org)); and 2) *distributed LSDBs* ([www.centralmutations.org](http://www.centralmutations.org)).

PAHdb has been used in the development of two new types of database: FINDbase and PhenCode. FINDbase (Frequency of Inherited Disorders; [www.FINDbase.org](http://www.FINDbase.org)) is a relational database containing information about relative frequencies of pathogenic mutations causing inherited diseases in particular populations and with ethnic affiliations [van Baal et al., 2007]. FINDbase creates links with LSDBs including PAHdb, to collect and retrieve mutation data relevant to diseases with elevated frequencies in particular populations, ethnic groups or geographic regions.

PhenCode (Phenotypes for ENCODE; [www.bx.psu.edu/phencode](http://www.bx.psu.edu/phencode)) is a collaborative project to explore variant phenotypes associated with human mutations in the context of sequence and functional data obtained from genome projects [Giardine et al.,

2007]. The PhenCode project uses selected LSDBs, including PAHdb, to connect human phenotype data with genomic sequences, evolutionary history and function from the ENCODE project. Mutations can be found in the University of California at Santa Cruz (UCSC) Genome Browser in a new human mutation track and follows links back to the relevant LSDB. On the other hand, one can start at the LSDB and via the Browser find complementary information including data describing chromatin modifications and protein binding, or data on evolutionary constraint and regulatory potential. The PhenCode project shows how these connections serve to adumbrate genotype–phenotype relationships.

### PAH ALLELES, POPULATIONS, AND HISTORIES

*Comment:* Participants in the PKU story have benefited from learning that “besides its obvious intrinsic value, knowledge of population history, and of the demographic and evolutionary changes that accompany it, has proven fundamental to address applied research in genetics” [Barbujani and Goldstein, 2004].

The *PAH* locus has been a privileged player in the pursuit of human population genetics. The locus is rich in allelic variation, both polymorphic (SNP, biallelic, and multiallelic) and in forms that are disease-causing and relatively rare; see current mutation map, tables, and descriptors at PAHdb ([www.pahdb.mcgill.ca/Information/MutationMap/mutationmap.pdf](http://www.pahdb.mcgill.ca/Information/MutationMap/mutationmap.pdf)). The mutation map is updated according to new data in PAHdb and shows the location of mutations, the exons and the introns in the *PAH* gene. Splice mutations are depicted below the gene; missense and other point mutations above the gene. Large deletions are indicated by horizontal double pointed arrows. Restriction enzyme sites used to create haplotypes are shown in open boxes below the gene. Short tandem repeats (STR) and variable number of tandem repeats (VNTR) sites are shown in solid boxes below the gene. The mutation names are in the trivial style; the systematic (formal) names appear in the database tables.

The locus behaves as a single block (~100 kb) of DNA in *Homo sapiens*, and having been sampled many thousand times recently during mutation analysis in probands, families, and populations, largely discovered through newborn screening programs, it complements the information gained from other projects analyzing human genomic variation in haploid, mitochondrial, and Y-chromosome DNA [Cavalli-Sforza, 1998]. From the latter studies, a mosaic human genetic geography has emerged reflecting demic expansion and migrations during the 100,000 years or more of Out-of-Africa human evolution (Fig. 1) [Cavalli-Sforza et al., 1994; Cavalli-Sforza and Piazza, 1993; Mountain and Cavalli-Sforza, 1997].

In their turn, *PAH* alleles, both polymorphic and disease-causing, are being seen as a particular set of biological memories connecting individuals, families, and populations who share contingent histories that are echoes of the past. The historical and social accidents of migration, genetic drift, gene flow, assortative mating, and recurrent mutation, alone or combined, with or without balanced selection, have generated the observed frequencies and distributions of *PAH* alleles in contemporary human populations [Scriver et al., 1996].

### Polymorphic PAH Haplotypes

“When population geneticists think about the *PAH* (gene), 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” [Kidd and Kidd, 2005].

Penrose [1998] had speculated, early on in the history of PKU, about “the original habitat” of the PKU gene and how often the genotype might have arisen by new mutation. Penrose also considered the significance of different PKU prevalence rates in different populations and regions of the world; even noting the relative absence of cases among Afro-Americans!

It became possible to address Penrose’s observations, from the perspectives of population genetics, when a *PAH* cDNA appeared that allowed one to probe and detect polymorphic alleles from which could be constructed haplotypes at the *PAH* locus [Lidsky et al., 1985]. When the first PKU-causing mutations were characterized [DiLella et al., 1986, 1987], it was noted that certain of the polymorphic haplotypes were disproportionately in linkage disequilibrium with the mutations. This led to the hypothesis that *PAH* haplotypes could be used to understand both the mutational genetics of PKU and the underlying population genetics revealed at the locus [Kidd, 1987]. Meanwhile, studies of *PAH* haplotypes on normal non-PKU chromosomes in European [Daiger et al., 1989a], Asian [Daiger et al., 1989b], Polynesian [Hertzberg et al., 1989], and African-American [Hofman et al., 1991] populations revealed greater diversity in African-Americans, and lesser diversity in Asians and Polynesians, relative to Europeans. These findings were seen to reflect evolutionary distance: lesser distance (younger history) was signaled by stronger linkage disequilibrium between pairs of polymorphic markers; greater distance (older history) was inferred when linkage disequilibrium was weaker between the markers [Chakraborty et al., 1987; Daiger et al., 1989a].

Individuals in populations from different regions of the world have been sampled and analyzed to reveal evidence for or against linkage disequilibrium among normal polymorphisms that differ significantly between populations [Kidd and Kidd, 2005]. Normal *PAH* polymorphic haplotypes were sufficiently informative to reveal divergence between African, European, and Asiatic populations [Degioanni and Darlu, 1994], but not sufficient to see the diversity among European populations. On the other hand, differences in haplotype frequencies on PKU and normal chromosomes were significant in the European populations.

The polymorphic alleles at the physical ends of the *PAH* gene have now been used, both pairwise and in other combinations, to measure linkage disequilibrium among individuals in up to 38 different populations from Africa, Europe, Asia, Oceania, and the Americas [Kidd and Kidd, 2005; Kidd et al., 2000]. The objective was to investigate origin and evolution of the normal *PAH* gene and to define further the background haplotypes upon which disease-causing *PAH* mutations arose. As in the corresponding studies at the *CD4*, *DM*, and *DRD2* loci [Tishkoff et al., 1996, 1998; Kidd et al., 1998], the findings at the *PAH* locus [Kidd et al., 2000] provide strong evidence for an Out-of-Africa model of human demic expansion [Barbujani and Goldstein, 2004], and for an ancestral haplotype that is more frequent in Africa and uncommon elsewhere. Moreover, African populations have a greater number of common haplotypes at similar relative frequencies, along with a greater number of rare haplotypes, than in other world populations.

### Pathogenic PAH Alleles

Over 500 different *PAH* alleles are recorded in PAHdb, of which a small number are quite prevalent while most are rare in relative frequency. As a result, three-quarters of phenotypic homozygotes

in admixed populations are compound genetic heterozygotes [Kayaalp et al., 1997] with implications for more intricate phenotypic heterogeneity (see Table 2 in Weiss and Buchanan [2003]). The alleles have been identified by various methods of mutation analysis, but most often in probands ascertained by newborn screening. Advantages in this mode of ascertainment include opportunities to measure prevalence rates of the phenotype, to classify it and to describe associations between allele, haplotype, phenotype, and population; limitations include biases in sampling and ascertainment and the reality of incomplete mutation detection. Nonetheless, it is apparent that population-specific PKU prevalence rates are not uniform and vary 70-fold among geographic regions (see Table 77-2 in Donlon et al. [2004]), that the aggregate of specific PAH allele frequencies can reach the polymorphic range in some populations and regions, that mutation profiles and their frequencies vary among populations, with many alleles being specific to regions and origins of the population, and that as few as five different alleles will often account for half the relative frequency in many populations [Weiss and Buchanan, 2003]. These general characteristics show that pathogenic PAH alleles, like other DNA markers, can reflect their geographic origins, their population history, and genetic drift among the alleles responsible for the PKU/HPA phenotypes [Weiss and Buchanan, 2003]. Accordingly, pathogenic PAH alleles are also being used to interrogate human genomic diversity and population structures, and to speculate about the forces, such as mutation, migration, genetic drift and selection, that shape our genomes.

### What PAH Alleles Can Tell Us

Diversity of pathogenic PAH alleles is visible, for example, within regional populations, such as Quebec and Iceland; between regional populations, as in Europe; and between so-called races such as Caucasian and Asian Oriental.

In Quebec, PAH alleles stratify by geographic region and reflect four centuries of population history [Carter et al., 1998; Scriver, 2001]. PAH alleles are markers of European range expansion to New France in North America during three different phases of immigration and subsequent demographic expansion by natural increase. The mutant Quebec chromosomes that were analyzed harbored 45 different alleles, some among the pathogenic set causing non-PKU HPA, but many more associated with PKU, 10 found first in Quebec, and five still unique there, only five occurring at relative frequencies above 5% but accounting for half the relative frequency in the population. Among the Quebec alleles is one (c.1222C>T, p.R408W) giving evidence for recurrent mutation as a molecular mechanism affecting its identity and frequency [John et al., 1990; Byck et al., 1994, 1997; Murphy et al., 2006], as well as its wide distribution in various populations [Eisensmith et al., 1995]. Penrose had already surmised that recurrent mutation could be a factor behind the ubiquity and prevalence of PKU. Did Penrose ever stop thinking . . . ? Analyses of the polymorphic haplotypes, gave additional evidence of intragenic recombinations and for other recurrent mutations at the PAH locus.

In Iceland, a founding mutation (c.1129delT, p.Y377>Tfs) accounts for over 40% of the mutant PKU chromosomes; haplotype data imply it has a common ancestral origin [Guldberg et al., 1997]. Acting as a specific genetic marker in an isolated population, combined with corresponding genealogical reconstructions, and with comparisons to PAH allele profiles in Scandinavia, Ireland, and the United Kingdom, an old controversy was revisited

to propose a predominantly Norse heritage, rather than Irish, of the Icelandic people; incidentally, an hypothesis already under attack from new mitochondrial DNA haplotype data [Helgason et al., 2000].

Extensive allelic heterogeneity underlies the PKU and non-PKU HPA phenotypes in Europe [Zschocke, 2003]. Analysis of 9,000 European chromosomes revealed 29 different mutations, each occurring at relative frequencies above 3%. Different sets of these alleles account for the PKU and non-PKU HPA phenotypes, the alleles are not randomly distributed and particular ones show regional associations, for example: c.1315+1G>A, IVS12+1G>A in Northern Europe; c.1241A>G, p.Y414C in Scandinavia; c.1222C>T, p.R408W-H2 in Eastern Europe; c.1066–11G>A, IVS10–11G>A in Asia Minor, Southeast and Southern Europe and Iberia; c.1222C>T, p.R408W-H1 in Northwest Europe; and c.194T>C, p.I65T in Iberia and Western Europe and Ireland. These findings indicate multiple origins of PKU in Europe (another of Penrose's speculations!), with numerous independent mutation events. No single mutation is prevalent in all European populations, yet some mutations reach polymorphic absolute population-based frequencies in particular populations and regions, for example p.R408W on haplotype 1 in Western Ireland, and p.R408W on haplotype 2 in Estonia. In aggregate, the PAH allelic data, along with the evidence from protein polymorphisms [Cavalli-Sforza and Piazza, 1993], are significant in that they reveal network-like rather than tree-like genome structures in European populations.

We now know that PKU is not rare outside of Europe. Caucasians (Europeans) and Asian Orientals (Chinese and Koreans) have similar PKU/HPA birth prevalence rates [Zschocke, 2003; Gu and Wang, 2004; Song et al., 2005; Lee et al., 2004], while the prevalence rate in the Japanese population, which is about 1 out of 20 that in the mainland populations, is thought to reflect the effects of genetic drift in an Island population (Table 77-2 in Donlon et al. [2004]). Of further significance, PAH mutation profiles are different in European and Oriental populations; the prevalent alleles in Europeans are those mentioned above; in Northern Chinese PKU patients [Song et al., 2005] the most prevalent alleles are c.728G>A, p.R243Q (relative frequency 22%); c.611A>G, p.EX6-96A>G (11%); c.331C>T, p.R111X (9%); c.1238G>G, p.R413P (6.5%); and c.1068C>A, p.Y356X (6.5%). The Korean patients [Lee et al., 2004] harbor an overlapping set of prevalent mutations accounting for one-third of the total set: R243Q; c.442–1G>A, IVS4–1G>A, and p.EX6–96A>G. In Japanese patients, the most prevalent allele, accounting for one-third of their PKU mutations, is c.1238G>C, p.R413P. These findings corroborate earlier data [Wang et al., 1991a, 1991b; Okano et al., 1992] that suggest the effects of multiple origins of PAH mutations, diffusion and genetic drift in these Oriental populations. (The Oriental populations are also among the first to reveal that certain PAH alleles are BH<sub>4</sub>-responsive [Lee et al., 2004]).

PKU frequencies and mutations have not yet been ascertained from newborn screening in African sub-Saharan populations. A robust measurement of PKU frequency among persons of Black African descent living in the United Kingdom [Scriver et al., 2006] indicates that, in this sample of the African population, the prevalence is an order of magnitude less than in Caucasian and Asian Orientals. This finding may indicate that PKU appeared only in particular human populations (Caucasian and Oriental), and only during or after the Out-of-Africa range expansion of modern humans. But, in the absence of primary PKU prevalence data for populations living in Africa today, and with the awareness

that the ancestral African population was stratified [Falush et al., 2003; Garrigan et al., 2005] and further evidence that not all components participated in the range expansion [Satta and Takahata, 2004], the data may only reflect selective sampling of the African population [Hardelid et al., in press].

Stratification of *PAH* allele frequencies and phenotypes in human populations invites speculation about underlying origins and mechanisms. Historical demography seems to mingle with classical evolutionary forces (Fig. 1). From the clines of allele frequencies, centers of diffusion and gene flow could be surmised (for examples, see Eisensmith et al. [1995]), along with genetic drift in comparative isolation at the time the centers of diffusion developed [Mountain and Cavalli-Sforza, 1997]. Recognizable patterns of migration and demic diffusion also exist; while the effects of range expansion, by Europeans, during the past half millennium [Crosby, 1986] reveal themselves in the distribution of *PAH* alleles, for example in the Americas and Australia [Scriver et al., 1996]. Social and cultural practices, such as inbreeding, can occasionally account for a high prevalence of PKU in some regions and communities [Scriver et al., 1996].

In addition to the accidental mechanisms of population behavior accounting for allele stratification, there is evidence that molecular mutational events play some role in generating *PAH* allelic diversity. An example is found in the prevalence of the p.R408W allele whose codon contains a methylated cytosine [Murphy et al., 2006], which can experience methylation-mediated deamination of the 5mC nucleotide to create thymine (c.1222C>T). This process of recurrent mutation provides one explanation for the high relative frequency (~10%) of the R408W allele in European PKU genomes, and why it occurs today on at least four different haplotypes (see Fig. 77-7 in Donlon et al. [2004]), and why the p.R408W-H1 and p.R408W-H2 alleles have

different ages, the former apparently being the older one [Tighe et al., 2003]. Methylation of cytosine, or lack of it, in so-called hypermutable CpG codons in the *PAH* gene may explain why some codons harbor PKU-causing mutations as predicted and are associated with multiple haplotypes, and other so-called hypermutable codons have no mutations, perhaps because the 5mC signature is absent [Byck et al., 1997]. A formal search for distribution and prevalence of methylated cytosines in the coding regions of the *PAH* gene is indicated.

Does the prevalence and distribution of PKU in human populations reflect positive selection of genetic drift? Why is PKU apparently more prevalent in populations of northerly latitudes relative to the more equatorial populations from which the former evolved? The apparent scarcity of PKU/HPA in Black Africans, the much higher prevalence of this autosomal recessive phenotype among Caucasians and Asian Orientals, and the different allelic diversity in the two populations, has renewed one's interest in the possibility of overdominant (positive) selection acting during the history of PKU in modern human populations [Krawczak and Zschocke, 2003].

PKU is an autosomal recessive phenotype and it has behaved during modern human evolution as a genetic lethal in the mutant homozygote. One then asks why is the aggregate allele frequency ( $q$ ) ~0.01 in Caucasian and Asian Oriental populations? Some fitness advantage in the heterozygote to balance the disadvantage to the homozygote, has been proposed at various times as an explanation for the polymorphic genotype/phenotype [Woolf et al., 1975; Glazier et al., 2002; Krawczak and Zschocke, 2003; Penrose, 1998]. The magnitude of the advantage in reproductive success has been estimated to be small (1.5–5%) [Woolf et al., 1975; Krawczak and Zschocke, 2003]. Whether the distribution of *PAH* alleles in human populations reflects selective advantage or whether it is attributable to change (genetic drift) remains a controversial issue (see discussion in Scriver et al. [1996]). Moreover there is no way to reconstruct the selective forces under which positive selection might have occurred. On the other hand, the HapMap project might provide persistent molecular echoes of positive selection on the *PAH* locus. The selection process has left molecular signatures at other loci, for example at loci for intestinal lactase, the Duffy blood group, the G6PD enzyme, and in association with certain beta globin alleles [McVean et al., 2005]. The signature is revealed in linkage disequilibrium between polymorphic markers at the locus, in lower than expected polymorphic diversity, in skewed allele frequency spectra and, in the ideal case, in predominance of a single haplotype. In addition, there are statistical signatures for selection [Tajima, 1989; Fay and Wu, 2000], in which case a search for the molecular and statistical echoes of selection in the DNA molecular at the *PAH* locus could help to resolve the debate between positive selection and genetic drift and help to explain the unusual prevalences and distributions of PKU in modern human populations.

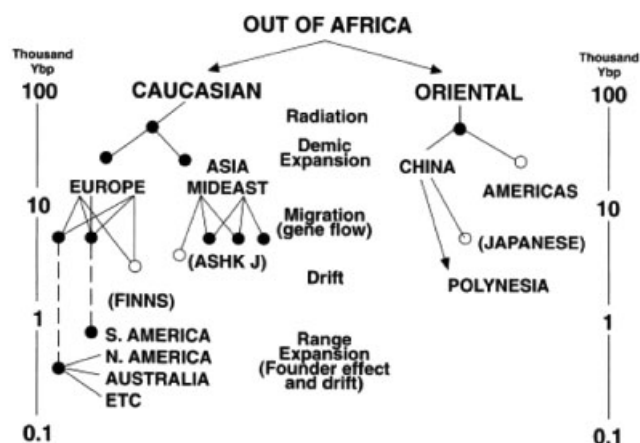


FIGURE 1. Human history hypothesized from the viewpoint of allelic diversity at the human phenylalanine hydroxylase (*PAH*) gene locus. Following an “out of Africa” range expansion and divergence, different sets of phenylketonuria-causing alleles arose in Europeans (Caucasians) and Asian Orientals. Their distribution reflects various forces of evolution including genetic drift (and perhaps natural selection). Drift with a founder effect (open circles) is the likely explanation for the relative rarity of pathogenic *PAH* alleles in American aboriginals, Ashkenazi, Finnish, and Japanese populations (also in Polynesians). Demic expansion, migration, and gene flow disrupted trees of descent in pre- and post-Neolithic eras (10,000 ybp). European range expansion, and the creation of neo-European populations overseas during the recent millennium, explain redistribution of certain *PAH* alleles from their European sources. (Taken from Donlon et al. [2004] with permission).

### PKU: SIMPLE MENDELIAN INHERITANCE; COMPLEX PHENOTYPE

*Comment:* Participants in the PKU story have been learning that a complex organism is comprised of molecular and cellular modules and networks [Oltvai and Barabasi, 2002]; learning also about the mechanisms that buffer genetic variation, and how mutant alleles can undermine the integrity of modules and networks.

Homeostasis of a soluble diffusible metabolite, such as phenylalanine, describes a state of relatively stable equilibrium

or, one might say, it is the tendency toward such a state, between different but interdependent compartments of the organism [Donlon et al., 2004; Scriver, 2006]. The metabolic steady state in the living organism is dynamic, yet the concentration of the metabolite, in the absence of large disruption, remains fixed even when flux through the space is altered. Thus the metrical trait value, for example the concentration of phenylalanine in the circulating blood volume of a normal person, experiences only minor transitory changes, under usual conditions, and is maintained within limits around a central tendency. The PKU phenotype reflects altered (disrupted) homeostasis of phenylalanine concentrations in body fluids.

### Individuality in the Normal Population

Darwin observed that no individual of the same species is cast in the same mould. A formal study [Scriver et al., 1985] of plasma amino acid values in healthy adult males and females, between ages 25 and 55 years, measured the effects of some standardized common physiological variables on the distributions of the phenylalanine and other amino acid values. The study showed that intraindividual private variation is significantly smaller than the interindividual public variation; the persons contributing to the public distribution of phenotype values themselves possess their own private phenotypes within the population. The findings imply that genotype is a significant determinant of the amino acid phenotype, a feature revealed in studies of amino acid values in twins (see Fig. 3-1 in Scriver and Rosenberg [1973]).

The biological events contributing to the measured phenotype value include: 1) absorption from the diet of the essential amino acid on a dedicated membrane transporter; 2) distribution into metabolic amino acid pools in the body, including controlled transport into liver [Salter et al., 1986], and into kidney, the other organ where phenylalanine is converted to tyrosine [Lichter-Konecki et al., 1999; Tessari et al., 1999] and where phenylalanine can be an important donor of tyrosine to the systemic circulation; 3) endogenous metabolism including degradation, synthesis, and transport; and 4) excretion from the organism. The genes encoding membrane transporters, enzyme-dependent reactions and controls of circadian variation will all influence the metrical trait value. In other words, the intraindividual and interindividual variations in measured phenotype values (plasma phenylalanine levels) point to a network of genetically determined processes acting to establish phenylalanine (and tyrosine) homeostasis, in what could be called “the metabolome” [Oltvai and Barabasi, 2002].

Homeostasis requires a network of catalysts and components that operate at far from chemical equilibrium in the living state, and in which buffering mechanisms exist to offset the effects of phenotype-modifying alleles. The set of buffering mechanisms endowed on the diploid organism by evolution [Hartman et al., 2001] is built up through redundancy in the genome, reduced specificity in function through flexibility and versatility in the proteins involved, and negative feedback regulation. The buffering can be intrinsic and focused on the pathways or components directly involved in the homeostatic process; or it can be extrinsic when independent circuits are involved. These features of the system confer an “emergent property” upon phenotype in which the whole (phenome) is greater than the sum of its parts [Kirschner et al., 2000]. The network confers complexity of response on the links between genotype and phenotype and, in the presence of potentially harmful allelic variation, it sustains robustness and health to offset risk of vulnerability and disease

[Dipple et al., 2001]. The effects of buffering are nicely manifest in the metabolic phenotype of PKU heterozygotes.

### Measured Phenotypes in the PKU Heterozygote

PKU is an autosomal recessive phenotype in which the heterozygote is demonstrably “silent” in the associated metabolic phenotype (HPA); yet the catalytic step encoded by the mutant *PAH* gene (hydroxylation of phenylalanine to form tyrosine) is presumed to show a gene-dosage effect with only half normal function (Fig. 2).

When actual plasma phenylalanine values are used to discriminate heterozygous from homozygous normal phenotypes, the PKU heterozygote is distinguishable only in a slight predominance of plasma tyrosine over phenylalanine and only by subtle statistical methods under particular conditions [Rosenblatt and Scriver, 1968; Gold et al., 1974]. The metrical trait values for plasma phenylalanine are essentially within the normal range; they behave as if buffered to prevent displacement. At this phenotypic level, the PKU heterozygote is indeed silent. It is hard to see how this heterozygous phenotype would be seen as a component upon which selection would act. However, when the actual metabolic flux of phenylalanine through the relevant space is measured *in vivo*, the flux value in PKU heterozygotes shows the predicted gene dose effect and it is half normal [Treacy et al., 1997].

Analysis of phenylalanine flux values in heterozygotes measures the response to mutation at one place in a systemic network, at a place where one gene encodes one enzyme and where gene dosage effect is anticipated. On the other hand, analysis of the plasma phenylalanine value measures the response of phenotype to heterozygosity over the whole system. Since there is no significant displacement of plasma phenylalanine values in the heterozygote, under normal physiological conditions, one takes this as evidence for the presence of a complex network of factors that buffer potential disturbances of metabolic homeostasis; and in this manner, they account for the recessive metabolic phenotype in PKU, and in other inborn errors of metabolism like it [Kacser and Burns, 1981].

### Individuality in the PKU Homozygote

Many monogenic diseases show an inconsistent relationship between mutant genotype and variant metabolic phenotype [Dipple and McCabe, 2000b, 2000a; Summers, 1996] and there are many invitations at phenomic levels to discover why genotype

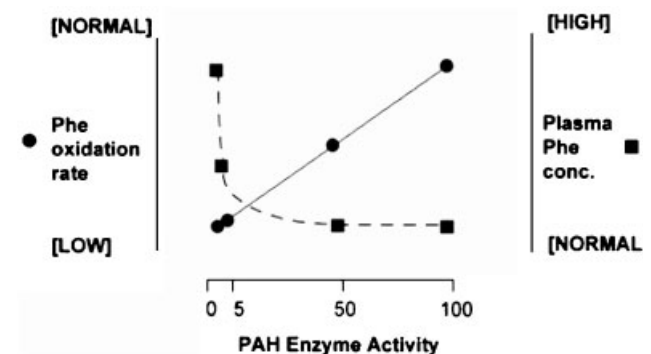


FIGURE 2. A diagram, based on measured phenotype values, to show that the mutant *PAH* gene has a salient effect on *PAH* enzyme activity and on phenylalanine oxidation; but the same gene effect is buffered in the “metabolome” of the person when measured by plasma amino acid values. (Taken from Donlon et al. [2004]) with permission).

is not a sufficient predictor of clinical and metabolic phenotypes [Scriver, 2002, 2004]. Metabolic homeostasis is a noisy business [McAdam and Arkin, 1999] and because buffering of allelic variation to modulate its effects must act through networks [Barkai and Leibler, 1997; Hartman et al., 2001; Hartwell et al., 1999; Jeong et al., 2000] it ought not to be surprising that a mutant genotype at the major locus of a Mendelian phenotype such as PKU will not always predict the finer features of the corresponding mutant phenotype. Whereas there indeed is a broad correlation between genotype and phenotype in PKU [Okano et al., 1991; Scriver, 1991] the exceptions attract notice because they may reveal things of interest [Scriver and Waters, 1999]—as Garrod anticipated.

### Misfolded Proteins and Allelic Variation

Analyses of diploid *PAH* genotypes and their correlations with metabolic and clinical phenotypes show that some mutations (usually null alleles) confer the classical PKU phenotype, whereas others (usually missense alleles) confer forms of HPA in a quasicontinuous genotype–phenotype distribution reflecting “phenogenetic equivalence” [Weiss and Buchanan, 2003]. But within the latter, phenotypes are not always consistent with predictions from genotype [Kayaalp et al., 1997; Guldberg et al., 1998; Desviat et al., 1999]. Moreover, patients with similar mutant genotypes can have dissimilar phenotypes.

Most *PAH* alleles are missense (see PAHdb). They can cause loss of *PAH* enzyme function indirectly through misfolding of the protein and its subsequent degradation [Eiken et al., 1996; Waters et al., 1998, 1999; Bjorgo et al., 1998]. Misfolding can be modulated by cellular chaperones. Thus “the challenge for PKU as for many other disorders is to identify what is happening to the mutant protein in vivo. Does it in fact interact with chaperones [Netzer and Hartl, 1998]. If so, with which ones? And with which components of the proteolytic pathways? If these interactions are important for stability in vivo, and thus for the disease phenotype, they could hold promise for combinatorial drug design in the treatment of disorders of misfolded proteins [Betts et al., 1997]” (quoted from Scriver and Waters [1999]). Expression analysis of various missense *PAH* alleles has shown that BH<sub>4</sub> can act as a natural chemical chaperone [Erlandsen et al., 2004] with the potential for use as a therapeutic agent against certain mutant *PAH* genotypes (see section on Treatment). The phenotypic effect of the allele may also be modulated by the degree of saturation of enzyme by cofactor (see below).

### Are There Modifier Loci?

Correlations exist between clinical phenotype, such as the cognitive phenotype in untreated PKU patients, and the corresponding metabolic phenotypes [Langenbeck et al., 1988] or the *PAH* genotypes [Ramus et al., 1993]. However, outlier findings point to the involvement of modifiers to explain discordant genotype–phenotype relationships (see below: modifier locus acting as a “protector”).

There is a candidate modifier locus in PKU [Treacy et al., 1996]. Isotope tracer analyses of excess phenylalanine disposal via the transaminase-dependent reaction (L-phenylalanine:pyruvate aminotransferase, E.C. 2.6.1.58) was performed in two siblings each with the same mutant *PAH* genotype (R408W/I65T), both siblings had HPA in the absence of treatment with a low phenylalanine diet, but one had a very different tolerance for dietary phenylalanine relative to the other sib. The difference in tolerance was explained precisely by the difference in runout of

phenylalanine via the transaminase reaction; greater runout conferred higher dietary phenylalanine tolerance.

Transaminase enzymes were of much interest during the era when protein polymorphisms were investigated. The difference between the siblings here could be explained if one of the them possessed a polymorphic variation in transaminase activity relative to the other. There is other evidence that polymorphic variation in transaminase activity exists in the population; it is the evidence for interindividual variation in the formation of phenylpyruvic acid relative to the degree of HPA [Knox, 1970]. For the moment, one can propose that the locus for L-phenylalanine-pyruvate amino transferase itself, or a factor acting on it, could be a candidate modifier locus in PKU.

### A Modifier Locus Acting as a “Protector”

Penrose knew that some untreated PKU patients could achieve near-normal cognitive development [Penrose, 1998]. The link between a variant *PAH* genotype and an IQ score is tortuous and it is not surprising that discordances between the metabolic and cognitive phenomes in PKU patients should be reported in the decades following Penrose’s initial observations [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]. IQ is a complex trait and if there is any correlation at all between IQ score, ambient blood phenylalanine levels, and *PAH* genotypes, it does suggest the strength, however much it is diluted by other factors, that exists in the links between genotype, metabolic phenotype, and the cognitive phenotype in the PKU population. Therefore, exceptions that distort the otherwise broad relationship are of interest. One of these exceptions resembles a “suppressor” or “protective” event on the cognitive phenotype in PKU.

Among four untreated PKU patients, all with similar and severe degrees of HPA, two had normal IQ scores and two had low scores. Magnetic resonance spectroscopy analysis showed that the high-scoring patients had undetectable elevations of phenylalanine in the brain compartment, whereas the low-scoring subjects had pathological elevated phenylalanine levels in their brains [Weglage et al., 1998a]. Further studies in additional patients measured brain phenylalanine content and flux rates of phenylalanine at the blood brain barrier between vascular and brain spaces [Weglage et al., 1998b; Moller et al., 1998; Moats et al., 1999]; the latter corroborated the initial observations. The findings suggest an effect of allelic variation (perhaps polymorphic) on a high-*K<sub>m</sub>* L-type transporter for large neutral amino acids shared by phenylalanine. The allelic variation acts as a suppressor of phenotype by impairing flux of phenylalanine into brain but only at high substrate concentrations in the PKU phenotype. A search for the putative variant allele, in the context of the L-carrier protein complex, has so far been unrewarding [Møller et al., 2005]. Nonetheless the putative protector is a site that could be a target for therapeutic intervention in the treatment of PKU [Matalon et al., 2006; Pietz et al., 1999].

These reflections on the effects of so-called modifier/suppressor loci on the PKU phenotype and on the relationship between genotype and phenotype are illustrations of a more general theme. Genetic modifiers encompass boundary conditions, stochastic and environmental variables, and genetic factors. Among the latter, are allelic variations that confer malleable genotypes and the possibility of postzygotic (somatic) mutation [Weiss, 2004]. Modifier genes in monogenic conditions may produce their effects by direct interaction with a major locus, by modulating interactions between protein products, by effects on alternative pathways

or parallel systems, or by distant effects—more surmised than revealed; all of which shifts our view away from Mendelian traits as simple phenotypes and toward the biological reality that they are complex traits harboring buffers, modifiers and suppressors [Kacser and Burns, 1981; Scriver and Waters, 1999]. Indeed there is no genetic disease that is not influenced by other genes [Lander and Schork, 1994]. An important practical lesson to take from this aspect of the PKU story and its counterparts is obvious: *treat the actual phenotype not the one predicted from genotype at the major locus.*

### TREATMENT OF GENETIC DISEASE— THE PARADIGM SHIFT IN PKU

*Comment:* During much of the 20th century, genetic disease held a low profile in the theory and practice of medicine. Genetic diseases, while legion, were individually odd and rare in prevalence, their causes lay hidden in the mysteries of heredity, and one could apparently do nothing to change or ameliorate their effects on the patient. Then came the evidence that a euphenic dietary form of treatment could prevent cognitive damage in the untreated Mendelian disorder called PKU. Evidence began to accumulate that other genetic diseases might also respond to treatment [Scriver, 1967]; and systematic measurements of progress in the treatment of inborn errors of metabolism followed [Costa et al., 1985; Hayes et al., 1985; Treacy et al., 2001, 1995; Campeau et al., 2006]. The “paradigm shift” in our outlook on genetic diseases and their treatment began with PKU.

*A cautionary note:* Until recently, treatment of PKU has always depended on a change in the nutrient environment of the patient and it takes place during critical stages of post-natal development. Epigenetics tells us that the boundaries between nature and nurture are far less distinct than imagined in the world of Mendelian genetics. Are we certain that the variant therapeutic nutritional experience in the early life of the PKU patient has had no effect on the epigenetic program of development in the infant? It is an important question awaiting an answer.

#### Treatment Modalities

There is no “cure” for PKU—yet. But there are modes of treatment that will prevent its effect on brain [Mitchell and Scriver, 2005]. The evidence is taken from treatment practices in human beings and in experimental protocols with animals. The latter have benefited from access to orthologous PKU mouse models [Shedlovsky et al., 1993; Sarkissian et al., 2000; McDonald et al., 2002].

**Low phenylalanine diet treatment.** PKU is a disease phenotype for which there is an explanation of pathogenesis (HPA) and for which, in turn, there is cause (mutation in the *PAH* gene and a relative excess of L-phenylalanine in the normal diet). Accordingly, the ability to manipulate the dietary experience and to ameliorate its metabolic consequence [Bickel et al., 1954; Woolf et al., 1955; Armstrong and Tyler, 1955] provided the evidence in the 1950s that this genetic disease could be treated; from this emerged the rationale for newborn screening and early diagnosis to introduce timely treatment.

In the subsequent half-century of experience with PKU treatment, the accumulated evidence (extensively cited in Donlon et al. [2004]) shows that early postnatal, aggressive and long-term use of a low phenylalanine diet permits normal or near-normal cognitive development. However, the available diet products, until very recently, have had significant deficiencies in both organoleptic properties and nutrient content [Scriver, 1971; Nayman et al., 1979] and this affected compliance with the dietary treatment

among the adolescent and adult patients; the treatment may also have affected epigenetic programs in postnatal development. (One way to appreciate the problem would be for the reader to adopt a PKU diet for a month). Furthermore, within the nuclear PKU family, there is likely to be a serious disruption of commensality. Fortunately, many of the deficiencies in the diet products are being overcome. However, when guidelines for more stringent dietary treatment of PKU emerged [Medical Research Council (UK), 1993; Cockburn and Clark, 1996; National Institutes of Health Consensus Development Panel, 2001], there was impetus to seek therapeutic alternatives compatible with the new guidelines recommending “earlier, more aggressive and longer term, even lifelong treatment”.

#### Treatment with large neutral amino acids (LNAAs).

L-phenylalanine is a large aromatic amino acid of neutral charge. It is transported into parenchymal cells on an L-type carrier—a  $\text{Na}^+$ -independent carrier shared by the aromatic and branched chain neutral amino acids. Phenylalanine exits from cells on another system shared with LNAAs. It also moves from the vascular space into brain across the blood brain barrier on this L-type carrier.

Treatment of PKU patients with a diet containing a defined amount of phenylalanine and supplements of LNAAs has reduced ambient HPA by half [Matalon et al., 2006]. Influx of excess phenylalanine into the brain in PKU is also blocked by LNAAs [Pietz et al., 1999]. These findings suggest that supplementary therapy with LNAAs might improve the treatment of PKU. A clinical trial will examine the safety and efficacy of LNAAs therapy in PKU [Matalon et al., 2006].

**Enzyme therapy.** The above-mentioned factors gave rise to an interest in enzyme therapy for PKU [Sarkissian, 2006]. Enzyme replacement therapy, with intact phenylalanine hydroxylase and the full set of enzymes to maintain  $\text{BH}_4$  is a daunting prospect not soon to be achieved. Orthotopic liver transplantation in one patient with end stage liver failure eliminated an incidental PKU phenotype [Vajro et al., 1993], but this is not a treatment option for all but the most unusual PKU patient. Therapeutic postnatal liver repopulation with an effective complement of normal PAH enzyme requires a selective growth advantage in donor hepatocytes over the recipient enzyme-deficient host liver. These are conditions that have not yet been achieved for the human PKU patient [Harding et al., 2006].

Enzyme substitution therapy with robust phenylalanine ammonia lyase (EC 4.3.1.5), which requires no cofactor, is appealing and probably feasible [Sarkissian, 2006]. PAL converts phenylalanine to trace amounts of ammonia and trans-cinnamic acid, a harmless metabolite. Both pharmacological and physiological principles have been demonstrated following the use of a recombinant yeast PAL given orally or by injection in a mouse model [Sarkissian et al., 1999]. Subsequent work [Sarkissian and Gamez, 2005; Sarkissian, 2006] with a PAL molecule, modified by pegylation for subcutaneous injection, has achieved a sustained fall in plasma phenylalanine levels, down to normal levels, with once-weekly injections over 12 weeks in the orthologous PKU mouse model (C.N. Sarkissian, personal communication, 2006).

**Treatment with  $\text{BH}_4$  (6-R-L-erythro-5,6,7-tetrahydrobiopterin).** HPA due to PAH enzyme deficiency, caused by missense alleles, can be responsive to pharmacological doses of 6- $\text{BH}_4$  in vivo [Kure et al., 1999]; there is no primary abnormality of cofactor metabolism here. Such patients may account for up to 70% of those with the milder non-PKU HPA phenotypes [Bernegger and Blau, 2002; Spaapen and Rubio-Gozalbo, 2003]. Their metabolic response to  $\text{BH}_4$  involves activation of phenylalanine oxidation via the PAH enzyme [Muntau et al., 2002].

The response is dependent on the particular PAH allele and its effect on PAH enzyme integrity. Recent work [Erlandsen et al., 2004] shows that the response sometimes involves a chaperone-like effect on enzyme stability, while at other times it is a kinetic response involving binding of BH<sub>4</sub> in the catalytic domain of the enzyme. The response may also involve a corrective restoration of optimal concentrations of BH<sub>4</sub> cofactor in hepatocytes [Kure et al., 2004].

**Gene therapy.** PKU will be a candidate for gene therapy only when it is safe, when there is an efficient delivery system for lasting correction of the PKU phenotype, and when all other forms of treatment are perceived to be less satisfactory by comparison [Donlon et al., 2004]. Meanwhile, gene therapy has been studied in the orthologous mouse models of PKU. A recent report [Ding et al., 2004] on nonviral and viral approaches to gene transfer found none to hold promise until the appropriate transfer vector is found. The latter may exist in a recombinant adenoassociated virus of serotype 8 [Harding et al., 2006]. Such a vector, containing the mouse Pah-cDNA, achieved long-term euphenylalaninemia in the mouse model after a single intravenous injection. The phiBT1 bacteriophage integrase achieves site-specific transgene integration into the mouse genome and when used to deliver the cDNA for Pah enzyme it corrects the hyperphenylalanemic phenotype of the Pahenu2 (PKU) mouse [Chen and Woo, 2005]. The formidable challenges to gene therapy, and to cell therapy and hepatocyte repopulation in PKU, continue to attract interest.

### Maternal HPA

Maternal HPA is a form of metabolic teratogenesis. It is a metabolic embryopathy and it permanently harms development of the fetus [Dent, 1957; Scriver, 1967; Lenke and Levy, 1980]. It is necessary to identify, counsel, and treat every mother with HPA during her pregnancy. Otherwise the prevalence of HPA-associated mental retardation will rebound to the levels existing in the population before newborn screening became a universal practice [Scriver, 1967; Kirkman, 1992; Hanley et al., 1999]. Maternal HPA also contributes to the incidence of congenital heart disease [Levy et al., 2001]. Maternal HPA is a poignant reason to continue the search for optimal treatment of the PKU/HPA phenotype.

### ENVOI

Phenylketonuria, a Mendelian disease, and the fifth inborn error of metabolism to be identified after this class of human individuality was announced in 1902, has its primary, salient and necessary genetic cause in pathogenic alleles at the PAH locus (~100 kb) on chromosome 12 band region q23.2. But pathogenesis of the disease phenotype requires a second and equally necessary condition—normal nutrition and thus exposure to the essential amino acid, L-phenylalanine. It was this second condition that provided an opening for an effective but difficult treatment—by means of a low phenylalanine diet. It has since become evident that the relationships between genotype and phenotype are subtle and if PKU is a very revealing disease entity, well off the beaten path of health, it is because it also reveals components that modulate that disease phenotype and defy some of the predicted relationships between genotype and phenotype. These components occur on the path of health, and they deserve equal notice, since they may point to other genes and products that buffer potentially harmful genetic variation and experience, and thus contribute to health. As components in the modules and networks

of living organisms, they are participants in the “healthy gene” paradigm, as others have called it [Nadeau and Topol, 2006].

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