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Phenylketonuria Heterozygote Detection in Families with Affected Children

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INTRODUCTION

Phenylketonuria (PKU) is a severely handicapping disorder if not diagnosed and treated early in life [1]. The "classical" untreated PKU patient presents with mental retardation, pigment dilution, hyperkinesis, microcephaly, and seizure activity, but with the establishment of mass neonatal screening programs [2] and the institution of early dietary treatment [3], the "classical" clinical presentation is becoming a medical rarity. Since mass screening occasionally misses a child, follow-up tests of newborns are important. Heterozygote detection followed by genetic counseling of individuals in families with PKU children should guarantee that future children in the families receive follow-up tests. Phenylalanine hydroxylase, the deficient enzyme in PKU, is almost exclusively a liver enzyme [4] which is not readily accessible for assay. Hence, indirect methods must be developed to detect abnormalities in the phenylalanine hydroxylase system. Previous investigators have recognized the need for heterozygosity testing [5–11]. Hsia et al. [5], using oral phenylalanine loading tests, demonstrated that obligate heterozygotes for phenylketonuria have lower capacities for metabolizing phenylalanine than do normal people. However, because of the wide range of normal variation, discrimination between heterozygotes and normals was not complete.

Knox and Messinger [6] showed that assay of fasting blood phenylalanine concentrations also permitted some distinction between PKU carriers and normals. Later, Perry

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et al. [7, 8], using ion-exchange chromatography, were able to demonstrate that fasting phenylalanine/tyrosine ratios provided better and easier discrimination than any test done after either oral or intravenous loading. Rosenblatt and Scriver [9] improved discrimination with a midday, semifasting blood sample, and plotted phenylalanine vs. the phenylalanine/tyrosine ratio. However, all of these procedures resulted in significant overlapping values between obligate heterozygotes and controls.

Another approach adopted by Blau et al. [10] and Olek et al. [11] measured phenylalanine metabolites in the urine to quantify the differences between normals and carriers. The technique is currently being refined, and no large studies have been reported demonstrating its efficiency.

More recent attempts at accurate carrier detection used Bayesian probability [12]. Gold et al. [13] used Bayesian probability and pedigree analysis to generate joint density functions for the maximum likelihood estimate of an individual's chance of being homozygous or heterozygous at a given locus. Westwood and Raine [14] similarly used heterozygous likelihood ratios to derive the probabilities of being a heterozygous carrier or homozygous normal. These approaches, however, involve the use of computer and statistical techniques not readily available to all investigators and do not solve the problem of overlap between carriers and normals.

Griffin et al. [15] and Griffin and Elsas [16] were successful in separating obligate heterozygotes from controls with multivariate linear discriminant analysis using semifasting, midday plasma phenylalanine and tyrosine concentrations.

Heterozygote detection should be efficient and convenient for genetic applicability. The methods discussed above, however, are not completely reliable. Factors that modify tyrosine or phenylalanine metabolism and complicate heterozygote detection include oral contraceptives, pregnancy, sex, age, obesity, renal disease, and protein deficiency [15, 17–21].

Our investigation quantified semifasting, midday serum phenylalanine, tyrosine, and tryptophan concentrations by ion-exchange chromatography and compared these values in controls and obligate heterozygotes who were pregnant or on birth control medication and in controls and obligate heterozygotes who were not. This is the first study to use the serum tryptophan concentrations to improve the discrimination between normals and PKU heterozygotes. Multivariate linear discriminant analysis was used to distinguish controls from heterozygotes. The techniques of parent-child exclusion, frequency of heterozygosity in unrelated spouses, and segregation analysis were used to check for genetic compatibility of the results. The results of these analyses should allow further genetic counseling for pregnancies at risk for PKU.

MATERIALS AND METHODS

Sample Sources

Blood was collected from 498 volunteers including: (1) relatives of PKU patients seen in the Metabolic Clinic at Indiana University Medical Center (Indianapolis); (2) families from the Amish populations of Michigan and northern Indiana in which there was a previously ascertained proband with PKU; (3) relatives of PKU patients seen in the Genetic Clinic at Emory University (Atlanta, Georgia); (4) relatives of PKU patients followed in Pediatrics and Genetics Clinics at the Medical College of Virginia (Richmond); (5) relatives of PKU patients seen at the Henry Ford Hospital (Detroit, Michigan); and (6) staff, students, and their spouses at Indiana University

Medical Center, the Medical College of Virginia, and Henry Ford Hospital. Informed consent was obtained from all the participants in this study.

Sample Collection

Venous blood specimens were collected between 11:30 a.m. and 12:30 p.m. after an overnight fast and a light breakfast before 7:30 a.m. The blood was allowed to clot in the cold for approximately 1 – 1½ hr; the clotted cells were sedimented by centrifugation, and the serum was withdrawn, quickly frozen (dry ice and methanol), and stored at less than −70°C until analyzed. Some of the samples (20%) were stored from 1 – 4 days at −20°C before being transferred to −70°C. Since Perry and Hansen [22] demonstrated that storage at −20°C for 3 months resulted in decreased tryptophan levels, we compared the samples stored at −70°C with the samples stored at −20°C for 1 – 4 days and found no differences.

Amino Acid Analysis

The serum samples were deproteinized with sulfosalicylic acid [23]; the flocculent precipitate was compacted by centrifugation at 180,000 *g*, and the supernatant fluid withdrawn for analysis of tyrosine, phenylalanine, and tryptophan. Separation and elution of the amino acids were accomplished on a JEOL JLC-5AH amino acid analyzer with a lithium buffer (0.5 N) at pH 3.97 and at 55°C on a short column (0.8 cm × 15 cm). The amino acids were quantified as micromoles per liter, following reaction with ninhydrin at 95°C for 10 min, using a recording spectrophotometer at a wavelength of 570 nm and an Infotronics integrator (model CRS 110 A) wired in parallel with the recorder.

Discriminant Analysis

Discriminant analysis classified individuals into two or more groups (in this case, genotypes) in terms of several variables. In order to find a discriminant function, that is, a linear combination of the variables that would maximally differentiate between obligate heterozygotes and controls, BMD7M [24], a step-wise, multivariate discriminant procedure, was used to select the variables which maximized this separation. In this study, selection continued until all variables with F values greater than 1.0 had been included. In the final discriminant function, only those variables with F values significant at the 5% level were selected.

Misclassification

The method of Penrose [25] was used to calculate the theoretical proportion of each group which would be misclassified. When both genotypes (in this case normal homozygotes and PKU heterozygotes) have a normal distribution, the generalized distance between the means gives a good measure of the discriminatory power of the test. Hence, this procedure was also used to test which method provided the best discrimination.

Genetic Tests of the Discriminant Function

Samples were collected from extended families (i.e., grandparents, aunts, uncles, and cousins, in addition to the proband sibship), and the amino acid analysis and classification were performed without prior knowledge of family relationships. In addition to the method of Penrose, three other methods were available to test the accuracy and efficiency of the discriminant functions used in this study. (1) We classified the unknown individuals in the study and then checked for genetic incompatibility. (2) We calculated the frequency with which an unrelated spouse of a collateral relative was classified as a heterozygote. (3) Finally, we used segregation analysis [26]. Since only families with at least one affected PKU child could be ascertained, truncate selection was used to test for abnormal segregation frequencies. This was done for each sex separately and both sexes combined.

RESULTS

In the first discriminant function analysis, only those individuals who were neither pregnant nor on birth control medication were used to develop the following equation:

$$Z_1 = \ln Phe - 0.69 \ln Tyr - 0.20 \ln Trp, \quad (1)$$

where *Phe, Tyr,* and *Trp* were expressed in micromoles per liter. The estimated overlap area was 3.75%.

A similar analysis using individuals who were pregnant or on birth control medication resulted in a second equation:

$$Z_2 = \ln Phe - 0.72 \ln Tyr, \quad (2)$$

where *Phe* and *Tyr* were expressed in micromoles per liter. The estimated overlap area for this equation was 8.23%.

Figure 1 shows the separation achieved between heterozygotes and controls by the above equations. It can be seen that the observed overlap areas (fig. 1) are less than the estimated overlap areas which were calculated by the method of Penrose [25].

Genetic Tests of the Discriminant Function

Using the first method (i.e., classifying the unknown individuals in the study and then checking for genetic incompatibility), only two pedigrees (fig. 2) showed a parent-child exclusion. In both cases, all the children in the two sibships were

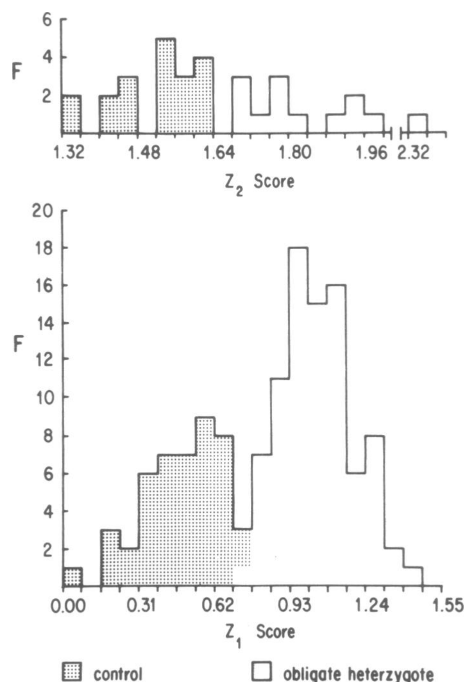

FIG. 1.—Distribution of Z_1 and Z_2 for controls and obligate heterozygotes in micromoles per liter.

compatible on blood markers with the parents, and indeed, the only discrepancy was at the PKU locus. However, both mothers with incompatible children (II-5 in pedigrees 1 and 2) were taking the antihypertensive agent aldomet (α-methyldopa), and there is evidence to suggest that this drug can induce alterations in tyrosine and tryptophan metabolism.

Using the second method (i.e., determining the frequency with which unrelated collateral spouses were classified as heterozygotes), two of the 47 spouses were classified as heterozygotes. One of these was later determined to have been pregnant at the time the blood sample was obtained, and when the appropriate equation [equation (2)] was used, she was reclassified as normal. The observed incidence of heterozygous carriers in genetically unrelated individuals in this study was 1/47.

The third method, (i.e., segregation analysis in which 55 normal by heterozygote matings and 15 heterozygote by heterozygote matings were analyzed [table 1] showed no significant χ^2 tests.

DISCUSSION

The investigation reported here is the first to consider the serum concentration of tryptophan and to demonstrate that inclusion of the serum concentration of tryptophan improved the separation between heterozygotes for PKU and homozygous normals. However, numerous reports have appeared in the literature showing a relationship between tryptophan metabolism and phenylalanine and tyrosine metabolism. Yarbo et al. [27] demonstrated that the elevated levels of phenylalanine in PKU children inhibit gut transport of tryptophan with concomitantly decreased levels of serum tryptophan.

TABLE 1

SEGREGATION ANALYSIS OF THE PKU GENE IN NORMAL BY HETEROZYGOTE (N × H) AND HETEROZYGOTE BY HETEROZYGOTE (H × H) MATINGS AFTER HETEROZYGOTE DETECTION

			Classification				
			Normals		Heterozygotes		
Group and Mating Type	No. Families	Sex	Observed	Expected	Observed	Expected	χ^2 (df = 1)*
N × H:							
Amish	17	M	22	21.5	21	21.5	0.02
		F	23	21.0	19	21.0	0.38
Non-Amish	38	M	16	20.5	25	20.5	1.98
		F	22	23.0	24	23.0	0.08
Total	55	M	38	42.0	46	42.0	0.76
		F	45	44.0	43	44.0	0.04
H × H:							
Amish	5	M	1	3.0	8	6.0	2.00
		F	4	3.0	5	6.0	0.50
Non-Amish	10	M	3	2.3	4	4.7	0.29
		F	4	2.3	3	4.7	1.80
Total	15	M	4	5.3	12	10.7	0.50
		F	8	5.3	8	10.7	2.00

* χ^2: $\alpha = 0.05_1 = 3.84$.

McKean et al. [28] demonstrated that hyperphenylalaninemia in rats produces a rapid and extensive decrease in the intracellular concentration of tryptophan in plasma and brain cells. Kaufman and Fisher [29] showed that, like phenylalanine hydroxylase, tryptophan hydroxylase requires the pterin cofactor system. Drugs such as birth control medication [30–32] and aldomet [33–35] interfere with both tryptophan and tryosine metabolism. Although it is not known which factors are affecting tryptophan metabolism in heterozygotes, there is ample evidence for metabolic interaction between phenylalanine, tryosine, and tryptophan. Hence, alterations in the metabolism of one or more of these amino acids probably leads to secondary changes in the metabolism of the other amino acids.

Since attempts to discriminate between obligate heterozygotes and controls without a separate analysis for individuals on birth control medication were not successful [36], a second equation derived from data on individuals who were pregnant or on birth control medication was necessary. Our results substantiated previous investigations [15, 18, 20] which demonstrated that birth control medication and pregnancy interfere with heterozygote detection for PKU.

Figure 2 shows the pedigrees from the two families where genetic incompatibility was found. As noted above, both mothers (individuals II-5 in pedigrees 1 and 2) were taking the antihypertensive agent aldomet (α-methyldopa). Maxwell [33] and Briggs and Holland [34] have shown that α-methyldopa enters catecholamine biosynthesis by replacing dopa (3,4-dihydroxyphenylalanine) as a substrate for L-dopa decarboxylase, and the resulting α-methylnorepinephrine replaces norepinephrine as a neurotransmitter. They also demonstrated that α-methyldopa inhibits the decarboxylation of 5-hydroxytryptophan. Recently, Markovitz and Fernstrom [35] reported that aldomet competes with natural large neutral amino acids for brain uptake. Thus, by replacing the tyrosine derivative dopa in catecholamine biosynthesis, by inhibiting an enzyme in one of the metabolic pathways of tryptophan metabolism, and by competing in the brain uptake of these amino acids, it is postulated that aldomet results in slightly

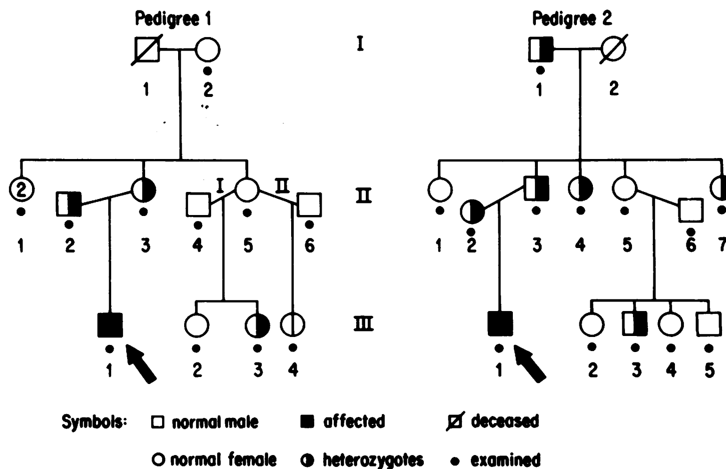

FIG. 2.—Pedigrees with parental-child exclusion for the PKU gene.

elevated concentrations of tyrosine and tryptophan in the serum. Of the 500 individuals in the present study, only one other individual (also classified as normal) was taking aldomet. This individual's tyrosine and tryptophan serum concentrations were both approximately 2 standard deviations above the respective means. To test with certainty whether this association between false negative classification and aldomet ingestion is an artifact or a real phenomenon will require examination of the serum of obligate heterozygotes on aldomet treatment.

Two other methods were used to test the accuracy of the equations developed in the present investigation: examination of the frequency with which unrelated collateral spouses were classified as heterozygotes and performance of segregation analysis on the families. The observed 1/47 incidence of heterozygotes among genetically unrelated individuals in the present study is similar to previous population estimates of about 1/40 to 1/70 based on an assumed gene frequency of 1/80 to 1/140. Since the results in table 1 showed no significant χ^2 tests, there was no evidence of disturbed segregation of the PKU gene in the different populations, mating types, or sexes.

All of the genetic tests on the data support the hypothesis that the equations developed in the present investigation were accurate discriminators between PKU heterozygotes and normal homozygotes. Other investigators, however, should apply these values cautiously by establishing their own cut-off points, and maybe even their own discriminant function coefficients.

SUMMARY

Improved approaches to the problem of heterozygote detection for phenylketonuria (PKU) were developed in this study. The discrimination was based on 85 obligate heterozygotes and 45 controls who were neither pregnant nor on birth control medication. The best separation between heterozygotes and normals was achieved with a linear discriminant function involving the logarithms of the serum concentrations of phenylalanine, tyrosine, and tryptophan. The theoretical overlap area between the distributions of heterozygotes and controls, based on the above function, was 3.75%. In the 19 obligate heterozygotes and 13 controls who were either pregnant or on birth control medication, the best separation was achieved with a linear discriminant function involving the logarithms of the serum concentrations of phenylalanine and tyrosine. The theoretical overlap area was 8.23%. The genetic accuracy of the discriminant function was confirmed by testing the results with parental-child exclusions, segregation analysis, and the frequency of heterozygosity in nonrelated collateral spouses. Finally, there was evidence suggesting that the antihypertensive agent, aldomet, alters serum tyrosine and tryptophan levels.

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REFERENCES

1. FØLLING A: Über Ausscheidung von Phenylbrenztraubensäure in den Harn als Stoffwechsel Anomalie in Verbindung mit Imbezillitat. *Z Physiol Chem* 227:169–176, 1934

2. GUTHRIE R, SUSI A: A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* 32:338–343, 1963
3. HUNT MM, SUTHERLAND BS, BERRY HK: Nutritional management in phenylketonuria. *Am J Dis Child* 122:1–5, 1971
4. KAUFMAN S: The phenylalanine hydroxylating system from mammalian liver. *Adv Enzymol* 35:245–319, 1971
5. HSIA DYY, DRISCOLL KW, TROLL W, KNOX WE: Detection by phenylanine tolerance tests of heterozygous carriers of phenylketonuria. *Nature* 178:1239–1240, 1956
6. KNOX WE, MESSINGER EC: The detection in the heterozygote of the metabolic effect of the recessive gene for phenylketonuria. *Am J Hum Genet* 10:53–60, 1958
7. PERRY TL, TISCHLER B, HANSEN S, MACDOUGALL L: A simple test for heterozygosity for phenylketonuria. *Clin Chim Acta* 15: 47–55, 1967
8. PERRY TL, HANSEN S, TISCHLER B, BUNTING T: Determination of heterozygosity for phenylketonuria on the amino acid analyzer. *Clin Chim Acta* 18:51–56, 1967
9. ROSENBLATT D, SCRIVER CR: Heterogeneity in genetic control on phenylalanine metabolism in man. *Nature* 218:677–678, 1968
10. BLAU K, SUMMER GK, NEWSOM HC, EDWARDS CH, MAMER OA: Phenylalanine loading and aromatic acid excretion in normal subjects and heterozygotes for phenylketonuria. *Clin Chim Acta* 45:197–205, 1971
11. OLEK K, OYANAGI K, WARDENBACH P: Quantitative analysis of phenylalanine metabolites in urine to detect heterozygotes of phenylketonuria. *Humangenetik* 22:85–88, 1974
12. MURPHY EA, MUTALIK GS: The application of Bayesian methods in genetic counselling. *Hum Hered* 19:126–151, 1969
13. GOLD RJM, MAAG U, NEAL J, SCRIVER CR: The use of biochemical data in screening for mutant alleles and in genetic counseling. *Ann Hum Genet* 37:315–326, 1974
14. WESTWOOD A, RAINE DN: Heterozygote detection in phenylketonuria: measurement of discriminatory ability and interpretation of the phenylalanine loading test by determination of the heterozygote likelihood ratio. *J Med Genet* 12:327–333, 1975
15. GRIFFIN RF, HUMIENNY ME, HALL EC, ELSAS LJ: Classic phenylketonuria: heterozygote detection during pregnancy. *Am J Hum Genet* 25:646–654, 1973
16. GRIFFIN RF, ELSAS LJ: Classic phenylketonuria: diagnosis through heterozygote detection. *J Pediatr* 86:512–517, 1975
17. HSIA DYY: Phenylketonuria: the phenylalanine-tyrosine ratio in the detection of the heterozygous carrier. *J Ment Defic Res* 2:8–16, 1958
18. YAKYMYSHYN LY, REID DWJ, CAMPBELL DJ: Problems in the biochemical detection of heterozygotes for phenylketonuria. *Clin Biochem* 5:73–81, 1972
19. JACKSON SH, HANLEY WB, GERO T, GROSSE GD: Detection of phenylketonuric heterozygotes. *Clin Chem* 17:538–543, 1971
20. BROWN EA, WAISMAN HA, SWANSON MA, COLWELL RE, BANKS ME, GERRITSEN T: Effects of oral contraceptives and obesity on carrier tests for phenylketonuria. *Clin Chim Acta* 44:183–192, 1973
21. YOUNG GA, PARSONS FM: Impairment of phenylalanine hydroxylation in chronic renal insufficiency. *Clin Sci Mol Med* 45:89–97, 1973
22. PERRY TL, HANSEN S: Technical pitfalls leading to errors in the quantitation of plasma amino acids. *Clin Chim Acta* 25:53–58, 1969
23. HAMILTON PB: Ion-exchange chromatography of amino acids-microdetermination of free amino acids in serum. *Ann NY Acad Sci* 102:55–75, 1962
24. DIXON WJ: *University of California Publications in Automatic Computation*, no. 2, *BMD Biomedical Computer Programs*. Berkeley, Univ. California Press, 1967
25. PENROSE LS: Measurement of pleiotropic effects in phenylketonuria. *Ann Eugen (Lond)* 16:134–141, 1951
26. CAVALLI-SFORZA LL, BODMER WF: *The Genetics of Human Populations*. San Francisco, W. H. Freeman, 1971
27. YARBO MT, ANDERSON JA: L-tryptophan metabolism in phenylketonuria. *J Pediatr* 68:895–904, 1966

28. McKean CM, Boggs DE, Peterson NA: The influence of high phenylalanine and tyrosine on the concentrations of essential amino acids in brain. *J Neurochem* 15:235–241, 1968
29. Kaufman S, Fisher DB: Pterin-requiring aromatic amino acid hydroxylases, in *Molecular Mechanisms of Oxygen Activation,* edited by Hayaishi O, New York, Academic Press, 1974, pp 285–369
30. Rose DP, Braidman IP: Oral contraceptives, depression and amino acid metabolism. *Lancet* 1:1117–1118, 1970
31. Rose DP, Braidman IP: Excretion of tryptophan metabolites as affected by pregnancy, contraceptive steroids and steriod hormones. *Am J Clin Nutr* 24:673–683, 1971
32. Rose DP, Cramp DG: Reduction of plasma tyrosine by oral contraceptives and oestrogens: a possible consequence of tyrosine aminotransferase induction. *Clin Chim Acta* 29:49–53, 1970
33. Maxwell RA: Adrenergic blocking drugs, adenergic neuron blocking drugs, and drugs altering biochemical mechanisms in adenergic neurons, in *Drill's Pharmacology in Medicine*, 4th ed., edited by DiPalma JR, New York, McGraw-Hill, 1971
34. Briggs AH, Holland WC: Antihypertensive drugs, in *Drill's Pharmacology in Medicine,* 4th ed., edited by DiPalma JR, New York, McGraw-Hill, 1971
35. Markovitz DC, Fernstrom JD: Diet and uptake of aldomet by the brain: competition with natural large neutral amino acids. *Science* 197:1014–1015, 1977
36. Paul TD: Heterozygote detection and genetic analysis of serum amino acid metabolism in Amish and non-Amish families with a phenylketonuric proband. Ph.D. thesis, Indianapolis, Indiana Univ., 1977

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