Increased Reliability for the Determination of the Carrier State in Phenylketonuria

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THE DETECTION of heterozygotes in phenylketonuria by means of phenylalanine tolerance tests was first described by Hsia, Driscoll, Troll, and Knox (1956) and Hsia, Price, and Driscoll (1957). Later, Knox and Messinger (1958) reported that with more precise analysis, the fasting plasma phenylalanine alone was as sensitive a test for distinguishing between normal and heterozygous individuals as the phenylalanine tolerance test. Unfortunately, neither test will identify with certainty the genotype of all suspected individuals, and the problem of correctly informing anxious prospective parents that they are or are not carriers of the trait still remains.

The present study was designed to improve the information available from the tolerance test by making additional measurements after administration of phenylalanine. Since some measurements may be more important than others, combination of selected measurements would be best for differentiating between the carriers and normals. Therefore, the data presented here are treated by the method of discriminant functions (Rao, 1952) and a procedure is established whereby an individual can be assigned to one or other of the groups with less error than for any single measurement.

METHODS

The 17 heterozygotes used in this study were parents of known phenylketonuric patients and the 19 control subjects were university students with no family history of a known genetic disease. The usual dosage of 0.1 gm. L-phenylalanine per kg. of body weight was given to both heterozygotes and control subjects after an overnight fast. The amino acid was mixed with orange juice and consumed immediately after the fasting blood sample was drawn. Additional blood samples were obtained at 1, 1½, 2, 3, and 4 hours after the administration of the L-phenyalanine. When the tolerance test was performed with the DL mixture, it was fed at a level of 0.2 gm. per kg. of body weight, and the test performed in a manner similar to that for the L isomer. The first 16 normals and 14 carriers were assigned to the DL mixture. While this allotment was not strictly random, there was no known difference between the individuals assigned to the two tests. Both tolerance tests were performed on 6 heterozygotes and 4 normals, as many as could easily be convinced to submit to the venepunctures on two separate oc-

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casions. Since there was no appreciable correlation between these replicates, they have been treated as independent observations. The plasma was separated after centrifuging and stored at −10° C. until analysis. The bacterial enzyme decarboxylation method of Udenfriend and Cooper (1953) was used for the L-phenylalanine determination and L-tyrosine was measured by the nitrosonaphthol reaction of Udenfriend and Cooper (1952).

To combine the determinations into a discriminant which maximizes the intraclass correlation for the carrier and normal classes, the most convenient procedure is to assign a binary variable which takes the value 1 for carriers and 0 for normals. The usual least squares regression of this variable on the phenylalanine and tyrosine determinations as independent variates gives the coefficients of the discriminant, and the regression analysis constitutes an analysis of discrimination. This gives an optimum discriminant when the independent variables are normally distributed with the same covariance matrix in the two groups, and a generally good but not optimum discriminant when these conditions are not satisfied.

Table 1 presents the average plasma L-phenylalanine and L-tyrosine levels in normal and heterozygous subjects prior and subsequent to the ingestion of L-or DL-phenylalanine. Fasting levels of plasma phenylalanine and tyrosine fell well within the ranges reported by other workers using the same methods. Normal subjects exhibited a peak in plasma level of phenylalanine at 1 or 1½ hours after the administration of L-phenylalanine, and at 2 hours after the administration of the DL mixture, after which the levels gradually approached the normal

TABLE 1. PLASMA PHENYLALANINE AND TYROSINE LEVELS IN HETEROZYGOUS AND IN NORMAL SUBJECTS AFTER ADMINISTRATION OF PHENYLALANINE

	I L-phenylalanine (0.1 gm./kg.)		II DL-phenylalanine (0.2 gm./kg.)	
	Normal (7)	Carrier (9)	Normal (16)	Carrier (14)
	plasma L-phenylalanine (mg./100 ml.)		plasma L-phenylalanine (mg./100 ml.)	
Fasting	1.0 ± 0.2*	1.5 ± 0.2	1.0 ± 0.1	1.4 ± 0.1
1 hour	8.6 ± 1.1	12.2 ± 2.2	4.6 ± 0.4	5.7 ± 0.4
1½ hours	8.3 ± 0.9	14.5 ± 1.5	6.3 ± 0.4	7.7 ± 0.5
2 hours	7.4 ± 0.8	13.2 ± 0.6	7.3 ± 0.4	8.9 ± 0.6
3 hours	5.1 ± 0.5	10.8 ± 1.2	6.5 ± 0.4	10.2 ± 0.7
4 hours	3.4 ± 0.5	8.6 ± 1.1	5.7 ± 0.3	9.1 ± 0.7
	plasma L tyrosine (mg./100 ml.)		plasma L tyrosine (mg./100 ml.)	
Fasting	1.5 ± 0.2	1.6 ± 0.2	1.7 ± 0.2	1.5 ± 0.1
1 hour	3.0 ± 0.2	1.8 ± 0.2	3.1 ± 0.2	2.1 ± 0.2
1½ hours	3.3 ± 0.2	1.8 ± 0.2	3.5 ± 0.7	2.0 ± 0.2
2 hours	3.7 ± 0.3	2.1 ± 0.3	4.1 ± 0.2	2.5 ± 0.2
3 hours	3.8 ± 0.4	2.2 ± 0.2	4.3 ± 0.3	2.8 ± 0.2
4 hours	3.8 ± 0.4	2.4 ± 0.3	4.2 ± 0.1	2.7 ± 0.2

* ± = standard error

Figures in parentheses represent number of individuals.

TABLE 2. TEST OF HETEROGENEITY OF DISCRIMINANTS BASED ON DL- AND L-PHENYLALANINE TESTS, CONVERTED TO COMMON LOGARITHMS

Source	Sum of Squares due to discrimination (S.S.)	Degrees of freedom (d.f.)	Multiple correlation coefficient squared (R^2)
DL discriminant	6.337	12	.849
L discriminant	3.881	12	.986
Discriminants (pooled)	9.500	12	.833
Homogeneity	0.718	12	—

Error variance = 0.059, d.f. = 20
$F_{12, 20} = 1.01$, n.s.

value in the manner of the usual tolerance curve. The plasma tyrosine levels were also increased but to a lesser extent and they remained high for a longer period. The peak of plasma phenylalanine values was higher and attained later in heterozygous subjects than in normal. In contrast, the plasma tyrosine levels were found to be consistently lower than those of the normal individuals.

The plasma amino acid measurements were converted to common logarithms to stabilize the standard deviations which otherwise tend to be proportional to the means. The error variances for the two tests were found to be homogeneous and so were pooled. Table 2 shows that in this sample the tolerance test using the L-phenylalanine is better than that of the DL mixture for distinguishing between the normal individuals and the heterozygotes. However, there was no statistically significant difference between the two discriminants and they were therefore pooled for further calculations.

Table 3 gives the analysis of the pooled discriminant. The fasting phenylalanine level is the best single discriminator, followed by the 3 hour and 4 hour levels and the 1½ hour tyrosine level, in that order. When all measurements are combined into a single discriminant, they account for 83.3 percent of the total variation, which is a significant improvement over the 65.6 percent due to the fasting phenylalanine level alone. However, the tyrosine determinations do not contribute significantly to the discriminant, which still accounts for 79.4 percent of the variation when the tyrosine test is omitted. Even if all the tyrosine values are combined, the discriminant is less satisfactory than the fasting phenylalanine level alone, accounting for only 56.2 percent of the variation.

The best discriminant therefore is based on the phenylalanine measurements. The one hour and four hour values are least critical, and may be omitted without reducing the discrimination significantly. To this simplified discriminant, which accounts for 77.7 percent of the variation, the most important contributions are made by the fasting, 3 hour, 2 hour, and 1½ hour phenylalanine levels, in that order. The common logarithms of these values are given weights of 1, .6068, −.7627, and .3578, respectively. It is interesting that the 2 hour value is given a significantly negative weight, showing that the area under the tolerance curve is not the best discriminant.

The discriminant score of the normal group ranges from 1.221 to 1.407 with a mean of 1.315 and a standard deviation of .0536. The carriers range from

TABLE 3. CONTRIBUTIONS OF THE MEASUREMENTS OF PHENYLALANINE AND TYROSINE AT DIFFERENT TIMES TO THE DISCRIMINATION OF CARRIERS

	d.f.	S.S. due to discrimination	R^2	Significance
All measurements	12	9.500	0.833	fasting phenylalanine†
				2 hour phenylalanine*
X_1 to X_7	6	9.059	0.794	fasting phenylalanine†
			3.9% due to X_8 to X_{13}	1½ hr. phenylalanine*
				2 hr. phenylalanine†
X_1, X_2, X_4, X_5, X_6	4	8.866	0.777	fasting phenylalanine†
			1.7% due to X_3, X_7	1½ hr. phenylalanine*
				2 hr. phenylalanine†
				3 hr. phenylalanine†
X_1, X_2, X_5	2	7.498	0.657	fasting phenylalanine†
			12.0% due to X_4, X_6	
X_1, X_8 to X_{13}	6	6.412	0.562	1½ hr. tyrosine*
				2 hr. tyrosine*
X_1, X_2	1	7.477	.656	fasting phenylalanine†

X_1 = Normal (0), Carrier (1)
X_2 = Fasting level phenylalanine
X_3 = 1 hour level phenylalanine
X_4 = 1½ hr. " "
X_5 = 2 hour " "
X_6 = 3 hour " "
X_7 = 4 hour " "
X_8 = Fasting level tyrosine
X_9 = 1 hour " "
X_{10} = 1½ hr. " "
X_{11} = 2 hr. " "
X_{12} = 3 hr. " "
X_{13} = 4 hr. " "

* $P < .05$
† $P < .01$

TABLE 4. ESTIMATES, $D/\bar{s}$, OF THE POWERS OF VARIOUS METHODS OF DISCRIMINATING BETWEEN HETEROZYGOTES AND HOMOZYGOUS NORMALS

Measurement	This report	Renwick *et al.* (1960)	Hsia *et al.* (1956, 1957)	Knox and Messinger (1958)
Fasting phenylalanine	2.7	1.8	1.2	1.9
1 hour "	0.9	1.2	3.2	—
1½ hour "	1.2	—	—	—
2 hour "	1.3	2.4	2.7	—
3 hour "	2.2	—	—	—
4 hour "	2.1	2.7	2.5	—
Fasting, 1½, 2, 3 hours phenylalanine	3.6	—	—	—
Fasting tyrosine	0.3	—	—	—
1 hour "	1.6	—	—	—
1½ hour "	2.1	—	—	—
2 hour "	1.8	—	—	—
3 hour "	1.7	—	—	—
4 hour "	1.5	—	—	—

TABLE 5. SCORES FOR INDIVIDUALS TESTED TWICE

		Test	
	Subject	L-phenylalanine	DL-phenylalanine
Heterozygotes	W.M.	1.6028	1.5419
	S.M.	1.5653	1.4422
	C.H.	1.6530	1.6130
	J.H.	1.4990	1.6696
	R.L.	1.4824	1.5320
	M.L.	1.4445	1.5310
Normals	S.S.	1.3010	1.3002
	M.E.	1.2256	1.3594
	R.P.	1.3550	1.3384
	N.C.	1.2400	1.2590

1.424 to 1.670 with a mean of 1.560 and a standard deviation of .0833. Each of the means is based on 23 observations and each of the standard deviations on 20 degrees of freedom. The discriminating power is measured by $D/\bar{S} = 3.6$ (Penrose 1951) where D is the difference between the means and $\bar{S}$ is the average of the two standard deviations. This compares with 2.7 for the fasting level alone and 3.3 or 3.2 for the best discriminants proposed by Renwick, Lawler and Cowie (1960) and by Hsia, Driscoll, Troll and Knox (1956), and Hsia, Price and Driscoll (1957). Using the antimode as the critical value to discriminate between carriers and normals, the classification error is less than four per cent for our discriminant, but at least nine percent for the fasting level alone. The practice of choosing among several variates the one with the greatest value of $D/\bar{S}$ leads to a spuriously high estimate of the discriminating power, and therefore the classification error associated with the best discriminant of Renwick, Lawler and Cowie (1960) cannot be evaluated. The conclusion that a more complete discriminant would be better than any single measurement was anticipated by Renwick, Lawler and Cowie (1960) who stated that "There are indications that, in spite of the marked correlations among the 0, 1, 2, and 4 hour phenylalanine levels, a more time-consuming criterion based on all four of the phenylalanine levels would have been slightly more powerful". The question arises whether our discriminant would be best in the hands of other investigators. Renwick, Lawler and Cowie (1960) found the four hour test to be superior, Hsia, Driscoll, Troll and Knox (1956) and Hsia, Price and Driscoll (1957) obtained most satisfactory results with a one hour test, while we find the fasting level to be the best single discriminator, and all of these single determinations are significantly inferior to the combined discriminator in our material. Hsia and Steinberg (1960) used the sum of the one and two hour phenylalanine levels, which Steinberg (personal communication) had earlier concluded to be as satisfactory as a discriminant using all of the data. Some of the discordances among studies may be nonsignificant, but others may be be due to the conduct of the test, including duration of fast and details of the phenylalanine assay. Until these differences are resolved, the best procedure is for each investigator to determine the discriminant which minimizes misclassification in his material.

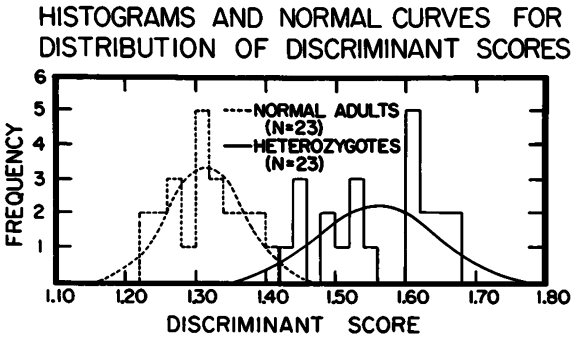

FIG. 1

Several methods may be used to reduce the classification error, including increased precision of assay, multiple determinations, taking account of the prior odds of the two genotypes, and putting borderline values of the discriminant into a doubtful group for retesting or exclusion from the analysis. For any case where the correct specification of the genotype is critical, but there is overlap between carriers and normals for any single measurement, the increased precision which the discriminant gives will amply justify the slight arithmetical labor involved. Fig. 1 gives the histogram of the scores between carriers and normals, which in our sample do not overlap. The distribution of carriers is not only significantly more variable than the normals, but shows two sharp peaks which, if supported by more observations, would suggest either multiple alleles at the phenylketonuria locus or a major modifier. However, other studies of carriers did not indicate heterogeneity, and the standard deviation of replicate scores on the six individuals tested twice is .0812, or almost as great as for different individuals. Thus heterogeneity of phenylketonuria carriers is unlikely to be detected by present methods.

SUMMARY

The best and most economical discriminant for phenylketonuric carriers is found to be

$$D = \phi_0 + .3578\ \phi_{1.5} - .7626\ \phi_2 + .6068\ \phi_3,$$

where ϕ_0 is the common logarithm of the fasting plasma phenylalanine value in mgm/1000 ml. plasma and $\phi_{1.5}$, ϕ_2, and ϕ_3 are the logarithmic levels at 1½, 2, and 3 hours respectively, after the administration of phenylalanine. Tyrosine determinations and phenylalanine levels at one hour and four hours do not contribute significantly to this discriminant. The D value for normal subjects lies between 1.221 to 1.407, whereas for the carriers it lies between 1.424 to 1.670. If values less than 1.415 are classified as normal homozygotes, and larger values are classified as heterozygous carriers, the classification error is calculated to be less than four per cent, compared with at least nine per cent for the best single measurement.

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