Detection of Heterozygotes for Phenylketonuria by Constant Intravenous Infusion of L-Phenylalanine

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We measured the rate of elimination of phenylalanine by constant intravenous infusion of L-phenylalanine in 14 parents of children with phenylketonuria and in 21 subjects with a negative family history for this disease. When reciprocals of the observed elimination rates were plotted against the reciprocals of the increase in the plasma phenylalanine concentrations, approximately straight lines resulted. The theoretical maximum elimination rate, the mean value for which was 32 mmol/h in the reference subjects, was reduced by 41% in the phenylketonuric heterozygotes. The elimination rate at an increase in plasma phenylalanine concentration of 0.5 mmol/liter discriminated the phenylketonuric heterozygotes from normal homozygotes, with no overlap between the groups. A lower plasma tyrosine concentration in the phenylketonuric heterozygotes than in the reference subjects at the same rate of elimination of phenylalanine indicated an increased rate of elimination of tyrosine at a fixed concentration of this amino acid in these subjects.

Additional Keyphrases: tyrosine clearance • inherited disorders • diagnostic aids • phenylalanine/tyrosine relationships • sex- and age-related differences • elimination kinetics

For assessment of the metabolism of single amino acids, different kinds of loading tests can be used. We studied the kinetics of phenylalanine after a single intravenous loading and found that its elimination from plasma could be adequately described by assuming a two-compartment model and first-order kinetics. This implies that the elimination rate constant from the central compartment is assumed to be the same within a rather wide range of concentration. By this method we studied the kinetics of L-phenylalanine in patients with cirrhosis of the liver (1) and in heterozygotes for phenylketonuria (2).

In the present investigation we have studied the possibility of calculating the rate of elimination of phenylalanine by constant intravenous infusions of L-phenylalanine. By varying the infusion rate, different concentrations in plasma can be obtained. At equilibrium the elimination rate is equal to the infusion rate. The elimination rate determined in this way is compared with the results obtained from single intravenous injection.

The possibility of discriminating phenylketonuric heterozygotes from normal homozygotes by this loading test has also been examined.

Material

Reference group. This group consisted of 21 volunteers: 11 men 22 to 28 years old (mean age, 24 years) and 10 women 22 and 26 years old (mean, 24 years). The weight of the men ranged between 63 and 77 kg (mean, 71 kg) and that of the women between 42 and 62 kg (mean, 52 kg). None of the subjects had a family history of phenylketonuria.

One of the men was excluded from the statistical calculations because he most likely was a heterozygote for phenylketonuria (see below).

Heterozygotes for phenylketonuria. This group consisted of 14 previously described (2) parents (eight and six women) of phenylketonuric children.

Methods

Loading procedures. All studies were performed between 0800 and 1200 hours, after an overnight fast. The subjects were allowed to rest in a semirecumbent position in a comfortable chair, and no food, drink, or smoking were permitted during the experiment. The different loadings of one person were generally done within a three-month period, at intervals of one to four weeks. At the first study L-phenylalanine (300 μmol/kg body weight) was given intravenously as a single injection, as previously described (2), and blood was drawn before the infusion and thereafter at intervals of 5 min during the first hour and of 10 min during the next 2 h.

In the succeeding studies, L-phenylalanine was given by constant infusion. The solution contained 151 mmol

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of L-phenylalanine per liter, i.e., the same as used in the experiments with single intravenous injection. Before the start of the infusion a priming dose was given intravenously during 3 to 5 min. The dose \( (P) \) was calculated according to the formula \( P = s/\beta \), where \( s \) is the infusion rate and \( \beta \) the rate constant determined from the plasma elimination curve after the single injection of L-phenylalanine (2). The infusion rate was varied between 24 and 90 ml/h, corresponding to 3.64 and 13.64 mmol of L-phenylalanine per hour. The total duration of infusion was 240 min. A Braun infusion pump, Model 1830, was used. Two or three 50-ml syringes were coupled in parallel. At the infusion rate of 24 ml/h refilling of the syringes was not necessary. At higher infusion rates, refilling was necessary but was not done during the last 100 min of the infusion. During the refilling the pumps were stopped for 2 min and the infusion rate was therefore doubled for 2 min thereafter to compensate for this interruption. Blood samples were drawn before the loading and every 10 min for the last 100 min of the infusion.

Indwelling catheters ("Venflon") in the cubital veins were used for sampling blood from one arm and infusion into the other. The blood was drawn without stasis into heparinized tubes. After centrifugation the plasma was stored at \(-18^\circ\)C until analysis.

**Determination of renal amino acid clearance.** The renal clearance of phenylalanine in three subjects. The urine produced during the last 100 min of the infusion was collected in one portion.

**Glomerular filtration rate** was determined by the \(^{51}\text{Cr}-\text{EDTA} \) method (3).

**Calculations.** The change in the plasma phenylalanine concentration during the last 100 min of the constant infusion was determined from the regression line calculated by the method of least squares, assuming linear regression and using all 11 observations. The rate of elimination of phenylalanine \( (E) \) was calculated from the equation: \( E = I - k \cdot V_{d,\beta} \cdot BW \) (equation 1), where \( I \) is the infusion rate, \( k \) is the regression coefficient of the question describing the change in the plasma phenylalanine concentration during the last 100 min of the infusion, \( V_{d,\beta} \) is the apparent distribution coefficient of phenylalanine calculated from the plasma elimination curve after the single intravenous injection of L-phenylalanine as previously described (2), and \( BW \) is the body weight.

The rate of elimination of phenylalanine was also determined from the experiment with single intravenous injection from the equation: \( E = k_{el} \cdot V_{c} \cdot C \), where \( k_{el} \) is the elimination rate constant from the central compartment \( (V_{c}) \) and \( C \) is the increase in the plasma phenylalanine concentration at which the elimination rate is calculated.

**Chemical analyses and statistical analysis** were done as previously described (2).

**Results**

**Studies of the Experimental Procedure**

**Reproducibility of the method.** The reproducibility of the method was tested in three subjects. In two of them (A and B) identical infusions were performed twice at an interval of six months. Almost identical plasma concentrations were obtained on both occasions. In a third subject the effect of repeated phenylalanine loadings was studied. L-Phenylalanine, 45 mmol/day, was given orally for nine days, with breakfast. The same plasma concentrations were obtained when identical infusions were performed before phenylalanine was given and the day after the last phenylalanine intake.

**Change in plasma concentration during constant infusion.** The 21 subjects in the reference group were loaded three to five times, the 14 heterozygotes twice. The infusion rate was varied between 3.64 and 13.64 mmol/h. In 26 of the 98 experiments we saw a small but significant \( (P < 0.05) \) change in plasma phenylalanine concentration during the last 100 min. As judged from the regression equation for all experiments, the change was less than \( \pm 1\% \) per hour in 36, between \( \pm 1 \) and \( \pm 3\% \) in 48, and between \( \pm 3 \) and \( \pm 4.2\% \) in the remaining 14 experiments. When calculating the rate of elimination of phenylalanine a correction was made for this change in the plasma concentration (equation 1). The use of this equation resulted in a correction of the elimination rate of less than \( \pm 5\% \) in 60, between \( \pm 5 \) and \( \pm 10\% \) in 26 experiments, and between \( \pm 10 \) and \( \pm 14.2\% \) in the remaining 12 experiments. One subject was loaded on three occasions with an interval of three days between each loading. The priming dose given was varied, but the infusion rate was constant (3.64 mmol/h). On the first occasion the priming dose was calculated as described above (see loading procedure). On the other two occasions the priming dose was 18% smaller and 18% larger than on the first occasion.

When the correct priming dose was given, there was an increase of 2% per hour in the time interval 140–240 min after the start of the infusion, to a final concentration of 238 \( \mu \)mol/liter at 240 min. When the low priming dose was given, the plasma concentration increased with 5% per hour to a final concentration of 226 \( \mu \)mol/liter; it decreased with 3% to a final concentration of 244 \( \mu \)mol/liter when the large priming dose was given.

**Renal phenylalanine clearance.** In three subjects with normal renal function (glomerular filtration rate >6 liters/h) the renal phenylalanine excretion was determined at different plasma phenylalanine concentrations. The clearance was not correlated to the plasma concentration within the range 50–450 \( \mu \)mol/liter, and was in all subjects between 0.017 and 0.034 liter h \(^{-1} \) m \(^{-2} \). The loss into the urine was always less than 0.5% of the amount infused.

**Relationship between the elimination rate and plasma level of phenylalanine.** The rates of elimination of phenylalanine in the individual reference subjects calculated according to equation were plotted against the plasma concentrations in the middle of the observation period, i.e., 190 min after the start of the infusion. The relationship was not linear. However, when the reciprocals of the elimination rates were plotted vs. the
reciprocals of the increase in the plasma phenylalanine concentrations, approximately straight lines were obtained. In the subject who was studied on three occasions with the same infusion rate (3.64 mmol/h) but with various priming doses, the calculated rate of elimination of phenylalanine was 3.18, 3.48 and 3.84 mmol/h, respectively, at attained plasma phenylalanine concentrations of 216, 234, and 249 μmol/liter at 190 min; i.e., the relation between the observed plasma phenylalanine concentration and the calculated elimination rate was similar to that observed when the infusion rate was varied.

Elimination rates determined from constant infusion and from single intravenous injection. In the calculation of the elimination rate from data on experiments with single injection it is assumed that the elimination rate constant is the same within the range of concentration obtained in the experiment; i.e., there should be a linear relationship between plasma phenylalanine concentration and elimination rate. A comparison between the elimination rates determined from constant infusion and single intravenous injections shows that the elimination rate is somewhat underestimated at low phenylalanine concentrations when determined from single intravenous injections (Figure 1). In the concentration range 0.3–0.6 mmol/liter, the difference between the methods was less than ±10%. At higher plasma phenylalanine concentrations the elimination rate was overestimated by the single-injection technique.

Comparison between Control Subjects and Phenylketonuric Heterozygotes

From the regression lines expressing the relation between the reciprocals of the elimination rates and the reciprocals of the increase in the plasma phenylalanine concentrations, the elimination rate at any phenylalanine concentration can be calculated. The elimination rate calculated at an increase in the plasma phenylalanine concentration of 0.5 mmol/liter in the reference subjects was higher in the men than in the women (Figure 2), but when the elimination rate was corrected for differences in body size, no sex-related difference was obtained. The mean value for the elimination rate was on average 5.25 ± 0.48 (SD) mmol-h⁻¹-m⁻² in all reference subjects. The theoretical elimination rate at an infinite plasma phenylalanine concentration was 18.1 ± 6.2 mmol-h⁻¹-m⁻².

The phenylketonuric heterozygotes reached higher phenylalanine concentrations than did the control subjects at the corresponding infusion rates. The rate of elimination of phenylalanine at an increase of 0.5 mmol/liter was significantly lower in the phenylketonuric heterozygotes than in the controls and there was no overlapping between the groups (Figure 2). The mean elimination rate at an increase in phenylalanine concentration of 0.5 mmol/liter was 3.44 ± 0.37 mmol-h⁻¹-m⁻² in the heterozygotes, i.e., a 34% decrease as compared to the controls. The mean value for the calculated maximum elimination rate was 10.6 ± 3.8 mmol-h⁻¹-m⁻², i.e., a 41% decrease in the heterozygotes, but overlapping between the groups was considerable.

The intercept with the abscissa of the line expressing the reciprocal of the increase in the plasma concentration and the reciprocal of the elimination rate corresponded to a plasma concentration of 1.21 ± 0.51 mmol/liter (mean ± SD) in the reference subjects and 1.07 ± 0.59 mmol/liter in the phenylketonuric heterozygotes.

The tyrosine concentration became nearly constant during the last 100 min of the phenylalanine infusion.
As a rule it increased with an increase in the infusion rate, but not as regularly as did the phenylalanine concentration. Especially in women, the results were variable.

Among the males the plasma tyrosine concentration at corresponding elimination rates of phenylalanine (Figure 3) was significantly \((P < 0.05)\) lower in the heterozygotes than in the control subjects. If we excluded those taking oral contraceptives, the same difference between heterozygotes and controls was observed among the females.

**Discussion**

On constant intravenous infusion of a compound, a constant concentration will be reached in the plasma sooner or later. The elimination rate of the compound is equal to the infusion rate when equilibrium is reached. To obtain a plasma concentration that is 90% of the concentration at equilibrium, the duration of the infusion must be fourfold the half-life of the infused substance \((4)\). A constant concentration can be reached considerably faster, theoretically within about 1 h in the case of phenylalanine, if a suitable priming dose is given. The priming dose can be calculated from the rate constant \(\beta\) \((5)\) determined from the slope of the plasma phenylalanine elimination curve after a single intravenous injection of L-phenylalanine \((2)\). When this priming dose was given, we found the concentrations in plasma to vary by less than \(\pm 5\%\) per hour in the time interval 140–240 min after the start of the infusion, assuming linear regression. Even when equilibrium is not reached, the elimination rate can be determined if the change in the total body pool of the compound is known. This change can be calculated from the distribution volume of the substance and the change in the concentration according to equation 1 \((6, 7)\). This resulted in a correction of the calculated elimination rate by less than \(\pm 10\%\) in 86 of the 98 experiments. The correction never exceeded 14.2%. It was thus possible to estimate the elimination rates with reasonable accuracy.

The elimination takes place through both metabolism and excretion. The renal excretion of phenylalanine was found to be negligible, less than 0.5% of the amount infused at all infusion rates. The loss through the gastrointestinal tract was not determined, but probably is also minute. Under normal conditions hydroxylation is the main metabolic route and the calculated elimination thus reflects the irreversible conversion of phenylalanine to tyrosine.

The relationship between the calculated rates of elimination of phenylalanine and the concentrations achieved in plasma was not linear, but rather slightly curved, similar to the relation seen for enzyme reactions that are following Michaelis–Menten kinetics. The basal phenylalanine elimination is not included in the calculated elimination rate. From the slopes of the curves, the intercepts with the ordinate can be obtained by extrapolation. This intercept was found to be about 1.3 mmol/h, suggesting a phenylalanine elimination of about 30 mmol per day, which approximately corresponds to the daily phenylalanine intake. This elimination however, is five- to 10-fold greater than the splanchnic uptake determined in the postabsorptive state \((8)\).

When the reciprocal of the increase in the plasma phenylalanine concentration was plotted vs. the reciprocal of the elimination rate, the relation was approximately linear. From these lines the elimination rates at different increases in the phenylalanine concentration can be calculated. We have chosen to give the elimination rate at an increase of 0.5 mmol/liter, which is within the experimental range.

The elimination rate at this concentration in plasma was significantly greater in men than in women when allowance was not made for differences in body size, but when the elimination was corrected to constant body surface area this difference vanished. The control subjects had an elimination rate exceeding 4.25 mmol-h\(^{-1}\)-m\(^{-2}\); it never exceeded 3.95 mmol-h\(^{-1}\)-m\(^{-2}\) in the heterozygotes for phenylketonuria. There was thus no overlapping between the two groups (Figure 2). By discriminant analysis the probability of erroneous classification was found to be 2% and the discriminatory level 4.34 mmol-h\(^{-1}\)-m\(^{-2}\). The method thus seems to offer a possibility of discriminating between heterozygotes for phenylketonuria and normal homozygotes with a high degree of probability.

An estimation of the maximum rate of elimination of phenylalanine is possible with the present method. It was found to be 32 mmol/h \((37 \, ^{\circ}\mathrm{C})\) in the control subjects, in agreement with in vitro experiments in which the maximum conversion of phenylalanine to tyrosine has been reported to be 7–17 \(\mu\)mol/h per gram of liver at 25 \(^{\circ}\mathrm{C}\) \((9)\), corresponding to about 15 mmol/h in a liver of normal weight. In the phenylketonuric heterozygotes the maximum elimination rate was 19 mmol/h, i.e., a 41% decrease. The total body clearance of phenylalanine, as determined from the curve for elimination from plasma after a single intravenous injection of phenylalanine \((2)\), and the elimination rates at different concentrations in plasma, as determined in
the present study, show a decrease in phenylalanine elimination by 32 to 41% in heterozygotes for phenylketonuria, a smaller decrease than reported by Woolf et al. (10), who found a decrease of 50%.

The method presented offers a possibility of determining the maximum elimination rate \(V_{\text{max}}\) but not the \(K_m\) value of the enzyme, as the intracellular concentration and the basal elimination rate are not known. A change in the \(K_m\) value of the enzyme, however, will probably result in a change of the intercept with the abscissa of the line expressing the relationship between the reciprocal of the increase in the plasma concentration and the reciprocal of the elimination rate. This intercept, similar in the control subjects and in the heterozygotes, corresponded to a concentration of 1.1 mmol/liter of plasma. The \(K_m\) value for phenylalanine 4-hydroxylase (EC 1.14.16.1) determined in vitro depends on the cofactors used, and other experimental conditions and values from 0.03 to 1.5 mmol/liter have been reported (11).

It has previously been observed that after phenylalanine loading there is a smaller increase in the plasma tyrosine concentration in phenylketonuric heterozygotes than in normal homozygotes (12). This has generally been assumed to reflect a decreased in tyrosine formation from phenylalanine. From the experiments with constant infusions of phenylalanine it has been shown that in heterozygotes the plasma tyrosine concentration is lower than in normal homozygotes, even at the same rate of elimination of phenylalanine. To get the same rate of elimination of phenylalanine, the concentration of this amino acid in plasma must be about 50% greater in heterozygotes than in normal homozygotes. The observed difference in the plasma tyrosine concentration might be explained by elimination of phenylalanine by metabolic routes other than by hydroxylation in the heterozygotes. Significant formation of phenylpyruvic acid and other aromatic acids is generally considered to take place only at plasma phenylalanine concentrations exceeding 0.5–1 mmol/liter (13, 14). The phenylalanine concentrations we attained in plasma were generally smaller than this, and the difference in the tyrosine concentration was observed also at a phenylalanine concentration of about 0.3 mmol/liter in the heterozygotes. It is therefore more likely that the difference observed was due to an increased rate of elimination of tyrosine at a fixed concentration in the phenylketonuric heterozygotes. This might explain why the discrimination between heterozygotes for phenylketonuria and normal homozygotes is generally better when changes not only in phenylalanine concentration but also in tyrosine concentration are considered (10, 12). The reason for this assumed change in the tyrosine metabolism in heterozygotes for phenylketonuria is not known.

We conclude that phenylalanine metabolism can be studied with high accuracy by the technique of constant infusion of it. The method can be used to discriminate heterozygotes for phenylketonuria from normal homozygotes, but is time- and resource-consuming. However, it can be simplified. As shown from the experiments in one of our subjects, it is not necessary to determine the rate constant \(\beta\) and the total distribution volume \(V_{d,\beta}\) in each individual for an exact calculation of the priming dose and correction of the change in the phenylalanine pool during the infusion. Instead, a fixed \(\beta\)-value of 0.40 h\(^{-1}\) (corresponding to a \(\beta\) value that is between that found in normal homozygotes and phenylketonuric heterozygotes) and a fixed value of \(V_{d,\beta}\) of 0.68 liter/kg, the mean value for all observations in a previous study (2), can be used. This makes the first loading, when phenylalanine was given intravenously as a single dose, unnecessary.

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References


