Liver Phenylalanine Hydroxylase Activity in Relation to Blood Concentrations of Tyrosine and Phenylalanine in the Rat

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The plasma concentration of phenylalanine and tyrosine decreases in normal rats during the first few postnatal days; subsequently, the concentration of phenylalanine remains more or less constant, whereas that of tyrosine exhibits a high peak on day 13. The basal concentrations of the two amino acids were not altered by injections of thyroxine or cortisol, except in 13-day-old rats, when an injection of cortisol decreased the concentration of tyrosine. In young rats (13–15 days old), treatment with cortisol increased the activity of phenylalanine hydroxylase in the liver (measured *in vitro*) and accelerated the metabolism of administered phenylalanine: the rate constant of the disappearance of phenylalanine from plasma and the initial increase in tyrosine in plasma correlated quantitatively with the activity of phenylalanine hydroxylase in the liver. In adult rats, the inhibition of this enzyme (attested by assay *in vitro*) by p-chlorophenylalanine resulted in a proportionate decrease in tyrosine formation from an injection of phenylalanine. However, the quantitative relationship between liver phenylalanine hydroxylase activity and phenylalanine metabolism within the group of young rats was different from that observed among adult rats.

The conversion of phenylalanine into tyrosine, catalysed by phenylalanine hydroxylase, is probably the major reaction responsible for the catabolism of phenylalanine in mammals: the high blood concentration of this amino acid associated with phenylketonuria in man is due to the inborn absence of phenylalanine hydroxylase, and carriers of one abnormal gene are identifiable by a partial incapacity to clear administered phenylalanine (Knox, 1966a). We decided to explore the extent to which the rate of phenylalanine catabolism in rats correlates with the activity of phenylalanine hydroxylase in liver. The examination of this relationship required animals with various and reliably measured enzyme levels. The preceding paper (McGee *et al.*, 1972) describes the sensitive assay method used for the determination *in vitro* of the total phenylalanine hydroxylase activity and the manner in which this activity changes in liver with age and cortisol treatment. Although we found that kidney also contains significant phenylalanine hydroxylase activity (one-sixth as much per g as liver), this organ will not be considered because its contribution to the total activity in the body is small and is not augmented by an injection of cortisol. The phenylalanine hydroxylase activities in liver at various ages and after treatments designed to alter the activity were correlated with the basal con-

centrations of tyrosine and phenylalanine and with changes in these concentrations after administration of test doses of phenylalanine.

Methods

Blood from rats (inbred NEDH strain) was collected in heparinized capillary tubes from partially amputated tails of adults and from the severed neck vessels of young animals. Plasma phenylalanine and p-chlorophenylalanine (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) were measured by the fluorimetric method of McCaman & Robins (1962) as modified by Faulkner (1965). The fluorescence of the complex with the latter compound was 17% greater per mol than that of the complex with phenylalanine. Plasma tyrosine was determined by the technique of Udenfriend & Cooper (1952). Amino acid concentrations are given in nmol/ml of plasma. Phenylalanine hydroxylase activity was determined by the method of McGee *et al.* (1972) and was expressed as units (nmol of tyrosine produced/min at 25°C) per g liver wet weight.

The amount of L-phenylalanine injected (intraperitoneally) was 130μmol/100g body wt. After such injections the blood concentration of phenylalanine rose to a maximum in 30min and declined during the next 2h. The basal phenylalanine concentration (i.e. that before injection) was subtracted from each value; these net values (phe) were then plotted against

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time (min) on semilogarithmic graph paper. The slope of the resulting straight line, calculated by least squares, gives the rate constant $(\log \mathrm{phe}_{t_2} - \log \mathrm{phe}_{t_1})/$ $(t_2 - t_1)$ for the disappearance of phenylalanine. It is treated as a positive rate.

Results

Plasma concentrations of phenylalanine and tyrosine during the development of the rat are shown in Fig. 1. The high concentrations of phenylalanine found in foetal and newborn animals declines sharply after birth. Thereafter, this concentration is maintained at a fairly constant value, decreasing slightly in adulthood. The neonatal decrease in blood phenylalanine coincides in age with the emergence of phenylalanine hydroxylase activity (McGee et al., 1972). This decrease in phenylalanine is not reflected in any accumulation of tyrosine: blood tyrosine also decreases after birth (Fig. 1), simultaneously with the emergence of tyrosine aminotransferase activity in liver (Greengard & Dewey, 1967; Franz & Knox, 1967). The prominent peak of blood tyrosine concentration between the 9th and 15th postnatal days does not coincide with striking changes in either phenylalanine hydroxylase or tyrosine aminotransferase activities. However, an injection of cortisol, which increases tyrosine aminotransferase activity (Franz & Knox, 1967), did decrease the tyrosine concentration in 13-day-old rats, but not in 8-day-old ones (Fig. 1). This treatment did not affect the basal plasma phenylalanine concentration at either age. The known increase in thyroid activity at the end of the second postnatal week (Samel, 1968) is unlikely to be responsible for the change in tyrosine concentration, since injections of thyroxine did not cause a premature increase in blood tyrosine (Fig. 1).

Typical experiments with normal adult rats are depicted in Figs. 2 and 3, in which the concentrations of phenylalanine and tyrosine were measured in blood at several time-points after an injection of L-phenylalanine. The plasma concentration of phenylalanine rose sharply within 30 min after the administration of phenylalanine. We injected 1.3μmol/g body wt., and found only about 0.6μmol/ml of plasma at this 30 min peak. The same proportions were found after injections of three times the usual dose (see Fig. 4). Most of the injected phenylalanine disappeared within 2 h. The unnatural analogue, p-chlorophenylalanine, rose to similar concentrations, but persisted in the animal much longer (Fig. 2). The maximum concentrations therefore reflect distribution in the body and the rapid decrease in phenylalanine must be largely due to its enzyme-catalysed metabolism. Values on the downward slope of the phenylalanine curve were used to obtain the rate constant (see inset in Fig. 2) for the disappearance
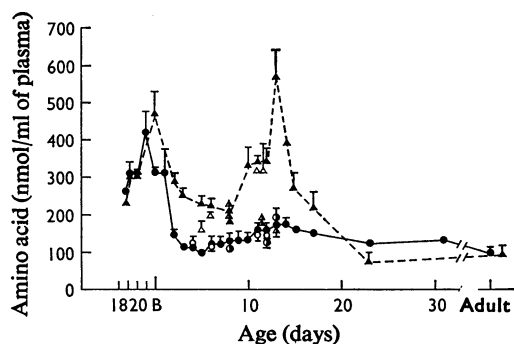

Fig. 1. *Plasma concentrations of phenylalanine and tyrosine as a function of age*

Phenylalanine (●, ○, ◑) and tyrosine (▲, △, ◮) were determined in untreated animals (●, ▲) and in rats injected with 25μg of thyroxine on each of 2 days before assay (○, △). The concentrations of phenylalanine and tyrosine in 13-day-old rats 24 h after the injection of cortisol are shown by half-filled symbols (◑, ◮). The points refer to individual animals or to means of results with 4–20 rats (each bracket represents 1 s.d.). B denotes birth.

of the amino acid; in six normal adult rats this averaged 0.009 ± 0.002 min^{-1} in the presence of liver phenylalanine hydroxylase activity of 980 ± 79 units/ g.

The concentration of tyrosine in plasma rose to a maximum about 30 min after phenylalanine administration (Fig. 3). This initial increment was used as an independent measure of phenylalanine catabolism. The subsequent rate of metabolism of tyrosine was faster than its rate of formation from the gradually declining plasma phenylalanine (cf. Figs. 2 and 3). Three times the usual dose of phenylalanine resulted in about three times higher peak concentration of plasma phenylalanine and indicated no limitation of absorption and distribution in the 30 min period. But the larger dose did not give higher concentrations of tyrosine (Fig. 4).

There was a much smaller increase in plasma tyrosine in rats given p-chlorophenylalanine 17 h before the usual injection of phenylalanine (Fig. 3). In these rats the phenylalanine hydroxylase activity was about 30% of the normal (see also Fig. 6). As shown previously (Lipton et al., 1967; Guroff, 1969), p-chlorophenylalanine added in vitro does not inhibit phenylalanine hydroxylase of normal liver preparations, but livers from rats that have been injected with this analogue show greatly decreased phenylalanine hydroxylase activities when assayed in vitro. In rats treated with p-chlorophenylalanine it was not possible to determine blood phenylalanine con-

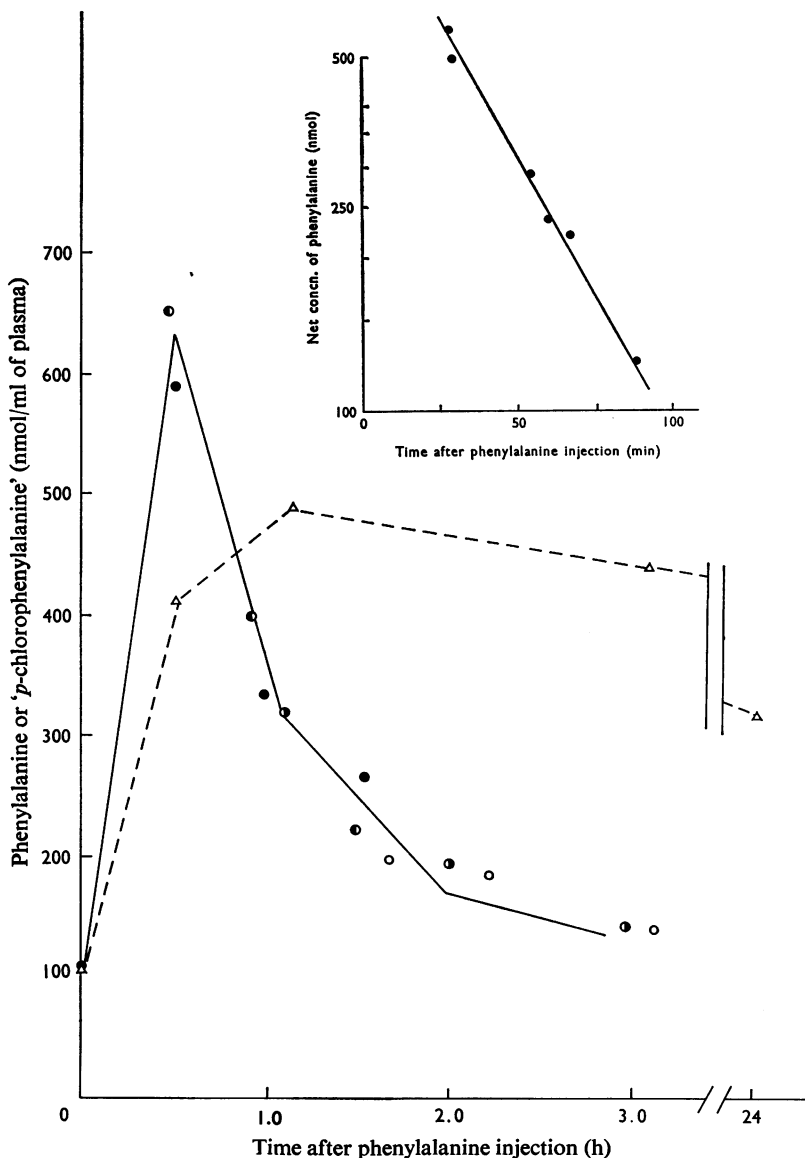Fig. 2. *Plasma concentrations of phenylalanine and p-chlorophenylalanine after their injections*

Phenylalanine (——) or *p*-chlorophenylalanine (----) (130μmol/100g body wt.) were injected at zero time. Different symbols (●, ○, ◐, ◑ for phenylalanine, and △, ▲ for *p*-chlorophenylalanine) represent different animals from which sequential blood samples were taken. The quotation mark around *p*-chlorophenylalanine is a reminder that the values obtained reflect, in part, the basal phenylalanine present, hence the value of 100 before the injection of *p*-chlorophenylalanine. The inset depicts the semi-logarithmic plot from which the rate constant of $0.009 \pm 0.002\,\mathrm{min}^{-1}$ for the disappearance of phenylalanine was calculated (see the Methods section).

centrations, because the chemical assay does not distinguish phenylalanine from *p*-chlorophenylalanine. The latter persists in the blood for at least 24h (see Fig. 2).

To compare further the phenylalanine hydroxylase activity with the capacity to metabolize phenylalanine *in vivo*, we turned to young rats. As shown in the preceding paper (McGee *et al.*, 1972), cortisol
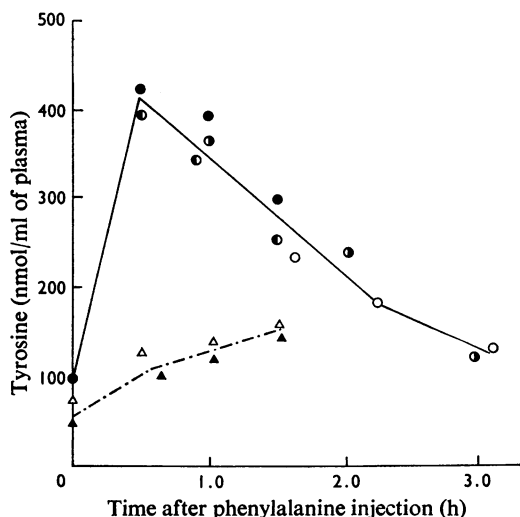
Fig. 3. *Plasma tyrosine concentration after administration of phenylalanine and p-chlorophenylalanine*

The solid line refers to rats (●, ○, ◑, ◐, same as those used in Fig. 2) each given phenylalanine. The broken line refers to two rats (△, ▲) given 125 μmol of p-chlorophenylalanine/100g 17h before the injection of phenylalanine.

Fig. 4. *Plasma concentrations of phenylalanine and tyrosine after the injection of different concentrations of phenylalanine*

The solid lines refer to rats given 605 μmol of phenylalanine/100g and the broken lines to rats given 130 μmol of phenylalanine/100g as in Fig. 2. The circles (●, ○) denote phenylalanine concentrations and the triangles (▲, △) those of tyrosine.

elevates the activity of liver phenylalanine hydroxylase in young but not in adult animals. The rate constant for the clearance of blood phenylalanine was determined by the same method used in experiments with adult rats; however, more animals were required for each experiment, since only one blood sample was taken from each rat. These animals were either untreated or given an injection of cortisol 24h before the administration of the test dose of phenylalanine. In Fig. 5 the phenylalanine hydroxylase activity determined in their livers is plotted against the rate constants observed. There is good correlation between the two parameters (correlation coefficient, $r = 0.92$): a doubling in the activity of phenylalanine hydroxylase is linked with a two- to three-fold increase in the capacity of the rats to eliminate phenylalanine. However, as shown by comparison with Fig. 2, adult rats have more hydroxylase activity but a lower rate constant and so do not fit the correlation between phenylalanine hydroxylase activities and the rate of phenylalanine elimination *in vivo* established for young rats.

In the experiments depicted in Fig. 5, the blood concentrations of tyrosine were also measured (Fig. 6). The initial increases in blood tyrosine concentration after phenylalanine administration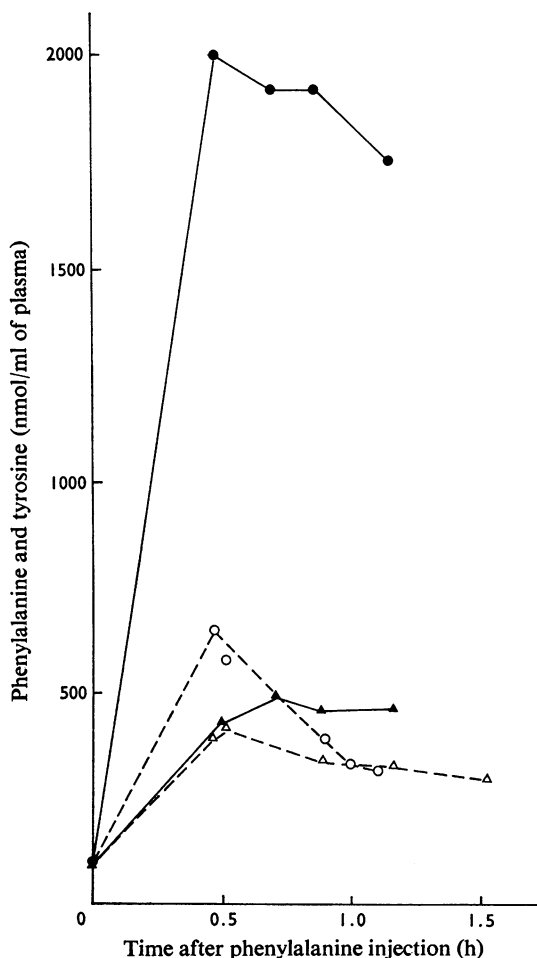 also correlated with the activities of liver phenylalanine hydroxylase in young rats with and without cortisol treatment. The different phenylalanine hydroxylase activities in normal adult male and female rats and in those given p-chlorophenylalanine also correlated with the initial increment in plasma tyrosine, but the line depicting this correlation does not coincide with that obtained for the young rats (Fig. 6). A given enzyme activity in adult rats was less effective in decreasing phenylalanine or forming tyrosine than in young rats.

Discussion

It is well recognized that variations in the amount of an enzyme, estimated by its total measurable activity *in vitro*, reflect changes in the functional capacity of the enzyme *in vivo* (Knox & Greengard, 1965). However, there are relatively few instances of quantitatively well established correlations between enzyme amounts and activities *in vivo* (Knox *et al.*, 1964; Altman & Greengard, 1966). The present studies, which demonstrate correlations between the activity of liver phenylalanine hydroxylase measured in a fortified system *in vitro*, and the capacity to convert phenylalanine into tyrosine *in vivo*, further emphasize that the amount of the enzyme is the primary determinant of its catalytic capacity. Superimposed fine regulations, which operate by changes of the activity of a given amount of enzyme (not detectable when assayed *in vivo*), may explain some of the observed discrepancies.

Young rats with liver phenylalanine hydroxylase activities increased by cortisol exhibit a proportionate increase in their capacity to clear injected phenylalanine. The initial rise in blood tyrosine concentration also correlates with the enzyme activity. In adult rats also, there is proportionality between the enzyme activity and the formation of tyrosine from administered phenylalanine; however, this correlation is quantitatively different from that established for young rats. Normal adult rats do not appear to eliminate phenylalanine faster than normal 2-week-old rats, although their phenylalanine hydroxylase activity is twice as high (see Fig. 5). The discrepancy may reflect, for example, differences in cofactor availability that would influence the capacity of a given amount of enzyme and that would not be detectable by assays *in vitro*. However, different age-dependent factors, such as the contribution of unsuspected interrelations, or those which influence the distribution of injected phenylalanine in the body, are equally likely to be responsible for the observation that young rats with less enzyme can eliminate the phenylalanine as fast as can adult rats with higher activities of phenylalanine hydroxylase.

It is known (Kerr *et al.*, 1968; Lines & Waisman, 1971) that rat and human foetuses have high phenylalanine concentrations. The present results show that in rats most of the decrease towards the adult value occurs during the neonatal period, and is probably due to the coincident emergence of phenylalanine hydroxylase described in the preceding paper (McGee *et al.*, 1972). However, in 2-week-old rats (not given a loading dose of phenylalanine) the rise in phenylalanine hydroxylase activity on injection of
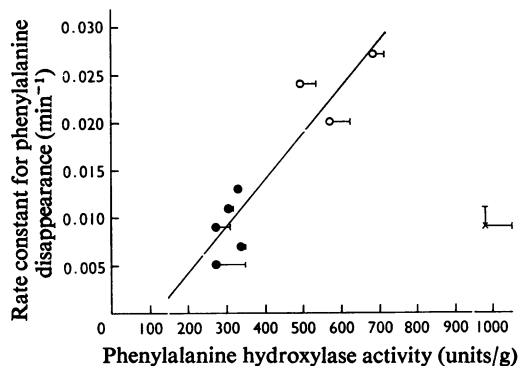

Fig. 5. *Correlation between rate of phenylalanine disappearance and phenylalanine hydroxylase activity in young rats*

Circles refer to 13–14-day-old animals given 130 μmol of phenylalanine/100 g body weight without (●) or 24 h after (○) injection of cortisol. Each of these points refers to one rate constant (min⁻¹) calculated from the analysis of blood samples from three or four rats and to a mean (each bracket represents 1 s.d.) of enzyme activities measured in each individual liver. The cross refers to six normal adult rats from experiments such as that illustrated in Fig. 2.
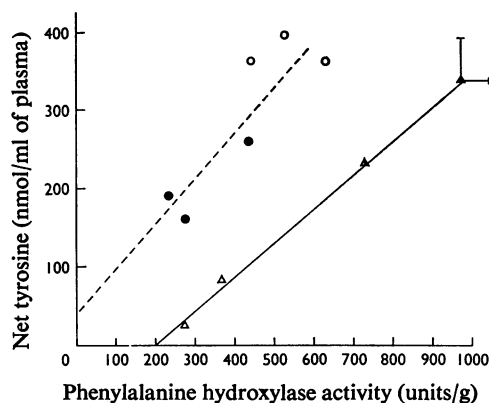

Fig. 6. *Increment of blood tyrosine concentration after phenylalanine administration*

The ordinate represents the difference between the basal concentration of plasma tyrosine and that determined in the same rat 30 min after phenylalanine administration. Each circle refers to 13–14-day-old rats untreated (●) and treated (○) with cortisol 24 h before phenylalanine administration. Adult rats were given phenylalanine without (▲, six males; ▲, one female) and 24 h after (△, two males) an injection of *p*-chlorophenylalanine.

cortisol is not reflected in a decrease of basal plasma phenylalanine concentration. Only by injecting an excess of phenylalanine is it possible to demonstrate its increased metabolism by more enzyme. The same phenomenon has been observed previously: the rise in the activity of tryptophan oxygenase, on injection of cortisol, is not followed by decreases in endogenous tryptophan (P. Feigelson, unpublished work), presumably because the low concentrations do not adequately saturate the enzyme and because any loss of this substance is counteracted by its increased provision from protein breakdown stimulated by the same hormone. Only the rate of elimination of high doses of tryptophan reveals the increased capacity of more tryptophan oxygenase to oxidize tryptophan (Knox, 1966b). In general, when testing correlations between amount of an enzyme and its activity *in vivo*, one cannot rely on changes in basal substrate concentrations, since this is conditioned by other factors in addition to catabolism.

Observations on the normal developmental changes in plasma tyrosine concentration (Fig. 1) provide new and potentially important information. The neonatal decrease is probably due to the coincident emergence of tyrosine aminotransferase (Greengard & Dewey, 1967), the first enzyme on the major pathway of tyrosine catabolism. In the third postnatal week there are major alterations in endocrine functions of the rat and in the enzymic composition of its liver (Greengard, 1970). We do not know which of these changes (apparently not that of the secretion of thyroxine; see Fig. 1) would account for the sudden rise in plasma tyrosine concentration at the age of 12 days, but the ensuing rapid decrease in plasma tyrosine may well be related to the normal increase in pituitary–adrenocortical function (Levine & Mullins, 1966) and to a further rise in tyrosine aminotransferase activity occurring around the 14th postnatal day (Franz & Knox, 1967). An injection of cortisol, which induces tyrosine aminotransferase at any postnatal age (Franz & Knox, 1967), decreases the blood concentration of tyrosine only at the age of 13 days, when the basal concentration is at its highest (Fig. 1).

The usual diagnosis of phenylketonuria by the concentrations of phenylalanine and tyrosine, if applied to newborn rats, would detect increases in both phenylalanine and tyrosine, but these could be evidence of immaturity owing to delayed appearance of the appropriate enzymes. Similar abnormalities are encountered in premature infants who do not have phenylketonuria (Bremer *et al.*, 1966; Cahalane, 1968).

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