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The Quantitative Determination of Phenylalanine Hydroxylase in Rat Tissues

ITS DEVELOPMENTAL FORMATION IN LIVER

By MARGARET M. McGEE,* OLGA GREENGARD and W. EUGENE KNOX
Department of Biological Chemistry, Harvard Medical School, and the Cancer Research Institute, New England Deaconess Hospital, Boston, Mass. 02215, *U.S.A.*

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A sensitive method was developed for determining the phenylalanine hydroxylase activity of crude tissue preparations in the presence of optimum concentrations of the 6,7-dimethyl-5,6,7,8-tetrahydropterin cofactor (with ascorbate or dithiothreitol to maintain its reduced state) and substrate. Tissue distribution studies showed that, in addition to the liver, the kidney also contains significant phenylalanine hydroxylase activity, one-sixth (in rats) or half (in mice) as much per g as does the liver. The liver and the kidney enzyme have similar kinetic properties; both were located in the soluble phase and were inhibited by the nucleo-mitochondrial fraction. Phenylalanine hydroxylase, like most rat liver enzymes concerned with amino acid catabolism, develops late. On the 20th day of gestation, the liver (and the kidney) is devoid of phenylalanine hydroxylase and at birth contains 20% of the adult activity. During the second postnatal week of development, when the phenylalanine hydroxylase activity was about 40% of the adult value, an injection of cortisol doubled this value. Cortisol had no significant effect on phenylalanine hydroxylase in adult liver or on phenylalanine hydroxylase in kidney at any age.

The inborn deficiency of liver phenylalanine hydroxylase in the human disease of phenylketonuria, which is associated with mental deficiency, has been the major stimulus for studying the properties of this enzyme. In normal humans and rats (Reem & Kretchmer, 1957; Kenney *et al.*, 1958; Kenney & Kretchmer, 1959), phenylalanine hydroxylase was considered not to be present during foetal life, so that only after birth could the absence of this enzyme be diagnostic for phenylketonuria. Subsequently, Freedland *et al.* (1962) detected significant hydroxylase activity in foetal rat liver 1 day before birth, but confirmed the observation that adult livers contained much more activity than did those of newborn rats. However, after the elucidation of the nature and role of the pteridine cofactor in the hydroxylase for phenylalanine (Kaufman & Levenberg, 1959; Kaufman, 1958), Brenneman & Kaufman (1965) reported that immature livers of foetal or newborn mammals were deficient in this cofactor, but that they contained almost adult activities of the phenylalanine hydroxylase itself. Our main purpose was to re-study this question to establish the precise age at which phenylalanine hydroxylase first appears in developing rat liver. For this purpose we devised an assay system supplemented with an optimum amount of substrate and cofactors, in which the reaction rate was limited only by the concentration of phenylalanine hydroxylase itself. The results describe the rate of accumulation of the enzyme in liver and kidney throughout development. The subcellular distribution of phenylalanine hydroxylase and its response to hormones *in vivo* was also investigated.

* Present address: Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee, U.K.

Methods and Materials

Animals

Tissues were obtained from inbred NEDH albino rats and from mice of the albino CE/J strain. Estimation of foetal ages was based on timed matings and on the correlation between body weights and age according to Gonzalez (1932).

Chemicals

6,7-Dimethyl-5,6,7,8-tetrahydropterin was obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A., dithiothreitol from Sigma Chemical Co., St. Louis, Mo., U.S.A., and purified ox liver catalase from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.

Enzyme assay

For the standard assay of phenylalanine hydroxylase, freshly excised tissues were homogenized in 9vol. of 0.15M-KCl and were centrifuged at 16000*g*

for 15 min in a Spinco model L ultracentrifuge. Portions (0.1 or 0.2 ml) of the supernatant fraction were incubated in a total volume of 1.0 ml with 100 mM-potassium phosphate buffer (pH 6.8), 10 mM-phenylalanine, 0.75 mM-6,7-dimethyl-5,6,7,8-tetrahydropterin and 5.0 mM-dithiothreitol or -ascorbate (the latter two compounds were dissolved immediately before use in 1.00 mM-potassium phosphate buffer). The pterin and enzyme were the last two additions. After 20 min of incubation at 25°C the reaction was stopped by the addition of 1.0 ml of 12% (v/v) trichloroacetic acid. The contents were left for 20 min and re-mixed before centrifugation for 20 min in a clinical centrifuge at maximum speed. Tyrosine was determined in 1.5 ml portions of the protein-free supernatant fluid by the method of Udenfriend & Cooper (1952) with one modification: as recommended by Nielsen (1969), the concentration of $NaNO_3$ in the nitric acid reagent was increased to 0.12%. Tyrosine standards consisted of known amounts of tyrosine added to complete assay mixtures treated with trichloroacetic acid as described above. After subtraction of the tyrosine content of the complete assay mixture before incubation, the phenylalanine hydroxylase activities were expressed in units (nmol of tyrosine formed at 25°C/min) per g wet weight of tissue. The standard tissue, adult male rat liver, contained 1130±122 units of phenylalanine hydroxylase/g (mean±S.D. for eight determinations). This value was obtained with dithiothreitol as the reducing agent; with ascorbate the mean value was 980±79 units/g.

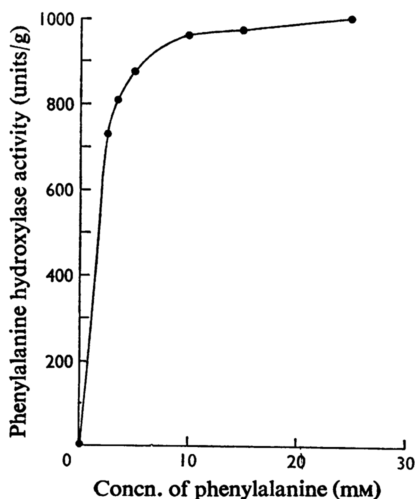

Fig. 1. *Effect of phenylalanine concentration on phenylalanine hydroxylase activity*

For experimental details see the text.

Results

Rat liver phenylalanine hydroxylase activities at various concentrations of phenylalanine are shown in Fig. 1. The calculated K_m value was 1.25×10^{-3}M. Incubation of assay mixtures without phenylalanine did not result in detectable tyrosine production. The amount of substrate used in the standard incubation was the optimum 10 mM. Higher concentrations were not inhibitory. No significant changes in activity were observed between pH 6.7 and 7.3 in potassium phosphate buffer. At the optimum pH, 6.9, the activity with tris buffer was the same as with phosphate buffer.

Fig. 2 demonstrates the proportionality between the amount of liver extract added (in the range of 5–10 mg of tissue for adults and 10–30 mg for 1-day-old rats) and the amount of tyrosine formed. Tyrosine production was linear for at least 25 min.

Under the present assay conditions, the endogenous cofactor in liver extract was insufficient to promote measurable phenylalanine hydroxylase activity. In the absence of added 6,7-dimethyl-5,6,7,8-tetrahydropterin, with the standard amounts of re-

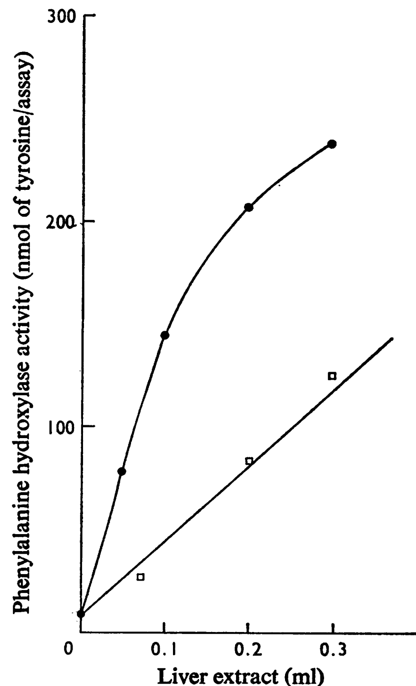

Fig. 2. *Phenylalanine hydroxylase activity as a function of amount of enzyme*

In 0.1 ml of extract there is 1.0 mg of liver. The indicated amounts of adult female (●) and 1-day-old liver extracts (□) were incubated for 20 min.

ducing agents, virtually no tyrosine was produced (Fig. 3); in routine assays 0.75mM-6,7-dimethyl-5,6,7,8-tetrahydropterin, which gave maximum activities, was used. Previously, in an assay system designed for partially purified phenylalanine hydroxylase, dithiothreitol but not ascorbate was found to be stimulatory (Bublitz, 1969). In the present assay, only slightly higher activities were obtained with optimum amounts of dithiothreitol than with optimum amounts of ascorbate; half the standard concentrations of these reducing agents gave somewhat lower activities (Fig. 3). With optimum amounts of 6,7-dimethyl-5,6,7,8-tetrahydropterin the absence of any added reducing agent decreased enzyme activity by about 50%, presumably because under these conditions the cofactor is autoxidized and not reduced again (Nielsen, 1969). Catalase influenced the activity of partially purified phenylalanine hydroxylase in some previous assay systems (Nielsen, 1969; Bublitz, 1969); added purified catalase was without effect in our assay on the reaction in adult or developing liver and kidney.

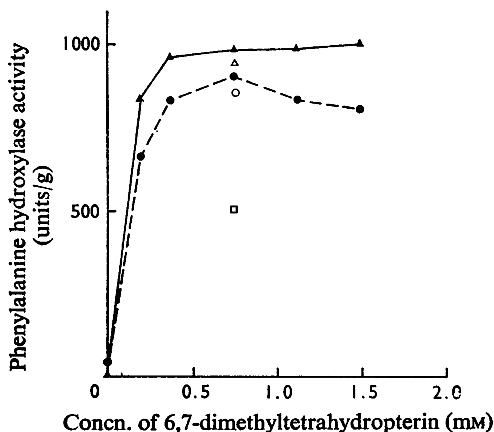

Fig. 3. *Effect of tetrahydropteridine and reducing agents on the phenylalanine hydroxylase activity of adult male liver*

Assays were carried out in the presence of the indicated amounts of 6,7-dimethyl-5,6,7,8-tetrahydropterin without reducing agents (□) and with ascorbate (○, ●) or dithiothreitol (△, ▲). Filled symbols refer to standard amounts of reducing agents (see the Methods and Materials section) and open symbols to half of these amounts.

In agreement with previous observations (Udenfriend & Cooper, 1952; Tourian *et al.*, 1969), most of the phenylalanine hydroxylase activity was found to be concentrated in liver (Table 1). We confirmed the presence of the enzyme in mouse kidney (Tourian *et al.*, 1969) and detected significant activity in rat kidney (17% of that in liver). Kidneys from male and female rats exhibited the same activity, whereas livers of adult male rats contained more activity than those of adult females. The five other adult tissues examined exhibited no phenylalanine hydroxylase activity (Table 1). An activity of 30 units/g could have been detected; such low activities have been reproducibly measured (see Figs. 4 and 5) in livers and kidneys of developing rats. At least in crude preparations, the properties of the kidney enzyme were similar to those of the liver hydroxylase. Their affinities for phenylalanine were almost identical; the K_m values were 1.3mM for liver and 2.0mM for kidney. The requirement for 6,7-dimethyl-5,6,7,8-tetrahydropterin was also the same in the two tissues. Both the kidney and the liver enzymes were located in the soluble fractions of their respective cells and were inhibited by particles sedimented at up to 16000*g*.

Subcellular fractionation of liver and kidney homogenates established that phenylalanine hydroxylase was a soluble enzyme in both tissues (Table 2). Sediments obtained after centrifugation at 16000 or 100000*g* contained very little activity. The supernatant fraction obtained after centrifugation at 16000*g* was more active than whole homogenate, corroborating the results of LaDu & Zannoni (1967).

Table 1. *Distribution of phenylalanine hydroxylase in rat and mouse tissues*

Activities were measured under standard conditions with ascorbate as the reductant; assay mixtures for pancreas, brain, spleen, diaphragm, heart and leg muscle contained 20 and 50mg of tissue. Values are means ±S.D. for the numbers of experiments given in parentheses.

	Activity (units/g)	
Adult tissues	Rat	Mouse
Liver, male	979 ± 77 (8)	655 ± 76 (3)
Liver, female	719 ± 79 (12)	—
Kidney	165 ± 31 (9)	359 ± 36 (3)
Pancreas	0	0
Brain, spleen, heart, muscle	0	0

Table 2. *Subcellular localization of phenylalanine hydroxylase in rat liver and kidney*

For experimental details see the text. Tissues were from an adult male rat.

Preparation	Phenylalanine hydroxylase activity (units/g of tissue)
Liver	
Whole homogenate	745
16000*g* sediment	39
16000*g* supernatant	1222
100000*g* sediment	43
100000*g* supernatant	1226
100000*g* supernatant + 16000*g* sediment (1:1)*	858
100000*g* supernatant + 16000*g* sediment (1:2)*	662
100000*g* supernatant + 16000*g* sediment (1:6)*	552
Kidney	
Whole homogenate	64
16000*g* supernatant	178
100000*g* sediment	14
100000*g* supernatant	149

* The ratio (v/v) of the particulate to the supernatant fraction was the same as in the whole homogenates (1:1) or 2 and 6 times higher.

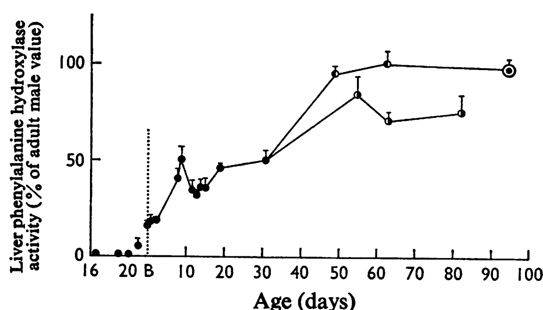

Fig. 4. *Developmental formation of rat liver phenylalanine hydroxylase*

The points are means (each bracket represents 1S.D.) of results with 3–18 rats. The foetal measurements showing zero activity were repeated on pools from 20 different litters. The activity of adult male liver [1134±122 units/g (9)] was taken as 100%. The circle around the last point indicates that the addition of an equal amount of a foetal liver preparation (20th day of gestation) did not alter the activity. ●, both sexes; ◐, male; ◑, female. B denotes birth.

Addition of the nucleo-mitochondrial preparation of liver inhibited the activity of the particle-free fraction, but a sixfold enrichment with the particulate fraction over the normal resulted in only about 50% inhibition. This inhibition could not be reversed by increasing the concentrations of 6,7-dimethyl-5,6,7,8-tetrahydropterin, dithiothreitol or ascorbate. The microsomal fraction was devoid of inhibitory action, since supernatants from centrifugation at 16000 or 100000*g* gave identical activities. Kidney enzyme was inhibited in the same way.

Before the 21st gestational day rat liver exhibited no phenylalanine hydroxylase activity (Fig. 4) either with the routine assay method or at increased or decreased concentrations of phenylalanine, 6,7-dimethyl-5,6,7,8-tetrahydropterin or reducing agent. Mixtures of preparations of foetal liver on the 20th day of gestation with those of adult liver resulted in the activity expected from the assay of adult liver alone (Fig. 4). Thus, the absence of activity in the foetal preparations is unlikely to be due to the presence of inhibitors or to the deficiency of unknown activators that might be present in the adult liver. On the 21st day, the low value probably represents real activity, since on the 22nd day, i.e. at term, and 1–2h after birth, liver activity was in the highly reproducible range of 19% of the adult value. On the 18th postnatal day 50% of the adult value was attained and there was no further rise during the ensuing 11 days. A sex difference became evident between 50 and 60 days of age, with the female having about 70% of the male value.

Phenylalanine hydroxylase was not detectable in foetal kidney before birth (Fig. 5). Newborn animals demonstrated about 10% of the activity found in adult male kidney, and the activity rose to 40% of this value by the 8th day of age, paralleling at a lower activity the values in liver, but without a sex difference.

Injection of foetuses with thyroxine, glucagon or

cortisol (Greengard, 1969; Greengard & Dewey, 1970) when *in utero* or shortly after birth was ineffective in hastening or increasing the formation of phenylalanine hydroxylase in liver. Table 3 shows that the injection of cortisol increased the phenylalanine hydroxylase activity in livers of 8–14-day-old rats but did not do so in livers of adult males. Adult females showed a small increase in enzyme activity after this treatment. Concurrent measurements of the kidney enzyme revealed no effect of this hormone even in 8–14-day-old animals.

Discussion

Previous quantitative analyses of phenylalanine hydroxylase in tissues have yielded discrepant results, which are most obvious in the relative activities reported during rat liver ontogeny. The present assay is the result of systematic adjustments of each condition (e.g. concentrations of substrate, cofactor and reducing agent; Knox, 1972) to give maximum activity, so it was more sensitive than most earlier assays. In addition, reaction time was limited to the period of constant (initial) rate, and enzyme concentrations in the range used were proportional to the activities found. We could be reasonably certain that differences in tissue activity did not reflect endogenous factors other than the concentrations of phenylalanine hydroxylase. With this sensitive assay we found the enzyme was not limited strictly to liver, that none was detected in foetal liver 2 days before birth, and that it was less than 20% of the adult value at term. These findings are not very different from those obtained with less adequate assays by Reem & Kretchmer (1957) and Freedland *et al.* (1962), but are different from the more recent reports in which the adult value was reported, at term (during labour) (Friedman & Kaufman, 1971) and within the first day of life (Brenneman & Kaufman, 1965). The latter paper reports a single value of 60% of adult value '2 days' before birth, but, using a different technique for estimating foetal age, the former paper reports a 77% adult value achieved during the 24h before the time estimated for birth.

A number of characteristics reported for the reaction of phenylalanine hydroxylase in crude tissue extracts were controlled or clarified by the assay developed here. Inhibition by an excess of phenylalanine did not occur, and catalase did not affect the activity. The enzyme in liver and kidney was soluble and, as reported previously (LaDu & Zannoni, 1967), was inhibited by the particles sedimenting at 16000*g*. The highly significant activity found in rat kidney (Table 2) was not previously noted, probably because of the insensitivity of earlier assays. However, the higher activity in mouse kidney (Table 1) has been observed (Tourian *et al.*, 1969). In different tissues the same enzyme frequently develops in a different

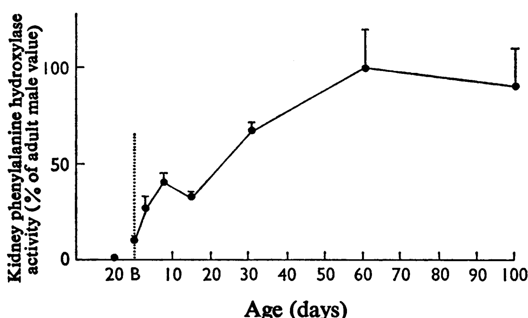

Fig. 5. *Phenylalanine hydroxylase activity in rat kidney as a function of age*

The points refer to means of results for 3–20 rats (each bracket represents 1 S.D.). The activity of adult male kidney [193±32 units/g (5)] was taken as 100%. The indicated zero activity was observed with pools of kidneys from four different litters. B denotes birth.

Table 3. *Effect of cortisol on liver phenylalanine hydroxylase in young rats*

Cortisol acetate (2.5mg/100g body weight) was injected intraperitoneally 24h before assay. For experimental details see the text. Results are means±S.D., for the numbers of experiments given in parentheses.

Rat tissues	Activity (units/g) Controls	After cortisol treatment
Liver, 8 days old	387±60 (6)	649±31 (3)
Liver, 13 days old	305±34 (19)	624±75 (15)
Liver, adult male	1013±79 (3)	978±76 (3)
Liver, adult female	685±49 (3)	809±25 (3)
Kidney, 8 days old	71± 1 (3)	65±14 (3)
Kidney, 15 days old	56± 5 (3)	55±10 (3)

time-pattern. Phenylalanine hydroxylase, however, starts from insignificant activity in late rat foetuses and accumulates in liver and kidney in a very similar manner.

Jakubovic (1971) has reported phenylalanine hydroxylase activity in livers of 12-week-old human foetuses, but since no determinations were made in adult livers by the same assay, it is impossible to evaluate how significant is this reported activity.

The developmental behaviour of phenylalanine hydroxylase in normal rat liver is not unique. Enzymes concerned with amino acid catabolism tend to appear late in the course of differentiation: only after birth does the rat liver gradually acquire tyrosine aminotransferase, serine dehydratase, asparaginase, ornithine aminotransferase, tryptophan oxygenase, histidinase and alanine aminotransferase activities (Greengard, 1970).

We have not been able to identify the stimulus that initiates the synthesis of phenylalanine hydroxylase at term. Hormones that trigger the synthesis of other enzymes during late foetal or neonatal life (cortisol, thyroxine and glucagon; Greengard, 1969; Greengard & Dewey, 1970) did not prematurely evoke that of phenylalanine hydroxylase. However, in the second postnatal week, an injection of cortisol can double the activity of liver phenylalanine hydroxylase. It is thus possible that pituitary–adrenocortical activity, which is known to increase around the 14th day (Levine & Mullins, 1966; Haltmeyer *et al.*, 1966), is the natural stimulus for the second phase of rise of phenylalanine hydroxylase activity, which occurs after the 14th postnatal day in the course of normal development. Two other enzymes, asparaginase (McGee *et al.*, 1971) and arginase (Greengard *et al.*, 1970), have been found to exhibit a second phase of rise around the third postnatal week; these enzymes also respond to an injection of cortisol in the 2-week-old rat but not in the adult.

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