The Regulation of Phenylalanine Hydroxylase in Rat Tissues *in vivo*

THE MAINTENANCE OF HIGH PLASMA PHENYLALANINE CONCENTRATIONS IN SUCKLING RATS: A MODEL FOR PHENYLKETONURIA

By JUAN A. DELVALLE and OLGA GREENGARD

*Department of Biological Chemistry, Harvard Medical School, and
the Cancer Research Institute, New England Deaconess Hospital, Boston, MA 02215, U.S.A.*

(Received 15 September 1975)

Maximum inhibition of phenylalanine hydroxylase activity in the liver (85%) and in the kidney (50%) of suckling rats required the administration of over 9 μ mol of *p*-chlorophenylalanine/10g body weight. Despite the decrease in the total activity from 184 to 34 units per 10g body weight, the injection of as much as 26 μ mol of phenylalanine was required for its concentration in plasma to be still considerably elevated 12h later. In rats injected with *p*-chlorophenylalanine every 48h and with phenylalanine every 24h from 3 to 18 days of age, the hepatic and renal phenylalanine hydroxylase remained inhibited, whereas the activities of three other hepatic enzymes were unchanged. There was about 20% inhibition of brain and body growth, but no interference with the developmental formation of several cerebral enzymes (four dehydrogenases, hexokinase and glutaminase) was detected. In the course of this prolonged treatment, the phenylalanine concentrations in plasma increased gradually; on day 2 and day 8 (measured 12h after the last injection) they were 800 and 1395 nmol/ml respectively; on day 15, 12 and 18h after the usual injection, the values were 2030 and 1030 respectively as opposed to the 96 nmol in untreated rats. This degree of hyperphenylalaninaemia, persisting for 18h per day throughout a critical period of development, fulfils the primary criterion of a suitable animal model for phenylketonuria.

Efforts to gain some understanding about the pathogenesis of mental retardation have led many investigators to focus on phenylketonuria, the inborn disease that manifests the closest association between a cognitive and a metabolic defect. Early attempts to reproduce in animals the condition of hyperphenylalaninaemia, characteristic of this human disease, consisted of maintaining rats on a high phenylalanine diet (e.g. Auerbach *et al.*, 1958). However, the efficiency of the normal organism to metabolize phenylalanine prevents it from accumulating in blood to the extent that it does in phenylketonuric subjects, owing to their inborn deficiency of hepatic phenylalanine hydroxylase (Knox, 1966). There is also the practical difficulty of maintaining rats on experimental diets while they are still suckling. Another approach was made possible by the discovery of an agent, namely *p*-chlorophenylalanine, which inhibited the hepatic phenylalanine hydroxylase activity of adult rats (Koe & Weissman, 1966; Guroff, 1969) and thereby decreased the rate of elimination of ingested phenylalanine (Lipton *et al.*, 1967). In view of the increasing realization that the irreversible cerebral damage of phenylketonurics is caused by the high concentrations of circulating phenylalanine during early infancy (Knox, 1966), studies in which suckling rats were treated with *p*-chlorophenylalanine

(and phenylalanine) are of particular interest. Several laboratories have reported promising results such as a learning deficit [tested by performance in a swimming maze (Watt & Martin, 1969)], retarded accumulation of oleic acid in cerebral lipids (Foote & Tao, 1968), and even behavioural symptoms akin to those in phenylketonuric subjects (Andersen & Guroff, 1972; Andersen *et al.*, 1974). However, these papers do not contain measurements of the concentration of phenylalanine in plasma, and it is therefore not clear whether the treatments induced sustained hyperphenylalaninaemia. Even the daily administration of phenylalanine plus *p*-chlorophenylalanine in the study of Andersen *et al.* (1974) may have been insufficient, since a report by Lipton *et al.* (1967) showed that, at least in adult *p*-chlorophenylalanine-treated rats, the concentrations of phenylalanine in plasma return to normal as early as 3h after the administration of 12 μ mol of phenylalanine/10g body wt. The extent of loss of phenylalanine hydroxylase activity was also uncertain, since the maximum effective dose of *p*-chlorophenylalanine in suckling rats has not been determined, nor was it known whether this agent inhibited the appreciable activity present in kidney (McGee *et al.*, 1972a).

The present study was based on the hypothesis that a major, sustained elevation of the concentration

of phenylalanine in plasma is the primary criterion for the adequacy of an animal model system for phenylketonuria. We first determined the amount of *p*-chlorophenylalanine required to cause maximum inhibition of the hepatic and renal phenylalanine hydroxylase activity in immature rats and then the amount of phenylalanine to be administered to cause a prolonged (at least 12 h) and considerable increase in its concentration in plasma. The results indicate that such sustained elevations were unlikely to have occurred with the regimen used in the above studies reporting behavioural abnormalities.

Some of the observations made in the course of developing this animal model for phenylketonuria led to separate investigations of the mechanism of regulation of phenylalanine hydroxylase by *p*-chlorophenylalanine, phenylalanine and cortisol. These results are described in the following paper (Greengard & DelValle, 1976).

Experimental

Materials

Rats were of the albino CDF strain, maintained on Purina Chow diet fed *ad libitum*. The adult rats were 75- to 90-day-old males. *p*-Chlorophenylalanine (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) was suspended at room temperature (24°C) in 0.9% NaCl and sonicated to small particle size. This suspension was stirred, and 0.1–2 ml sucked into a syringe and injected immediately. L-Phenylalanine was dissolved in 0.9% NaCl by heating.

Methods

Determinations of phenylalanine, tyrosine and *p*-chlorophenylalanine concentrations. Blood was collected in heparinized microhematocrit capillary tubes, centrifuged for 5 min and the supernatant diluted with 4 vol. of trichloroacetic acid (final concn. 0.3 M). The precipitated proteins were left for 20 min at room temperature, then sedimented in a clinical centrifuge for 20 min at maximum speed. Portions of the supernatant to be assayed with a Beckman 120C automatic amino acid analyser were diluted with a 0.2 M-sodium citrate buffer, pH 2.2, and applied to a column (69 cm × 0.9 cm) of Beckman type PA-28 spherical resin at 55°C. The flow rate was 50 ml/h for the elution buffer (0.2 M-sodium citrate, pH 4.25) and 25 ml/h for the ninhydrin solution, peaks corresponding to phenylalanine, tyrosine and *p*-chlorophenylalanine appeared at 128, 215 and 280 min respectively. The concentrations (nmol) were then calculated from the areas under the peaks. The standard amino acid solution was purchased from Pierce Chemical Co., Rockford, IL, U.S.A. The phenylalanine concentration in plasma from animals that had not received

injections of *p*-chlorophenylalanine was also determined fluorimetrically (results not shown) by the method of McCaman & Robins (1962), as modified by Faulkner (1965), and were consistently found to be 15–20% higher than those obtained in the amino acid analyser.

Enzyme assays. The assay method for hepatic and renal phenylalanine hydroxylase activity was as described by Greengard & DelValle (1976). Previously established methods were used for the assay of tyrosine aminotransferase (EC 2.6.1.5) (Lin *et al.*, 1958), soluble and mitochondrial aspartate aminotransferase (EC 2.6.1.1) (Herzfeld & Greengard, 1971) and the malate dehydrogenase (EC 1.1.1.37) (Ochoa, 1955) activities of liver extracts. The assay for glutaminase (EC 3.5.1.2) and succinate dehydrogenase (EC 1.3.99.1) activities in the particulate fraction of cerebral hemispheres (hereafter referred to as 'brain') were those of Huang (1974) and Laatsch *et al.* (1962). The malate-NADP⁺ dehydrogenase (EC 1.1.1.40) (Greengard & Jamdar, 1971), glutamate dehydrogenase (EC 1.4.1.2) (MacDonnell & Greengard, 1974), hexokinase (EC 2.7.1.1) (MacDonnell & Greengard, 1974) and lactate dehydrogenase (EC 1.1.1.27) (MacDonnell & Greengard, 1974) activities were determined in cerebral homogenates after standing for 30 min at 0°C with Triton X-100 (final concn. 0.5%). Enzyme activities are reported in units/g fresh wt. of tissue; a unit is the amount of product formed/min at 25°C (nmol for phenylalanine hydroxylase and μ mol for all other enzymes).

Results

To determine the extent to which *p*-chlorophenylalanine can inhibit the phenylalanine hydroxylase activity of immature liver *in vivo*, various amounts were injected into 6-day-old rats 24 h before assay. Fig. 1 shows that at least 9 μ mol/10 g body wt. was required to cause 85% inhibition and that one-half of this dose resulted in only 35% inhibition. In adult livers with a higher basal phenylalanine hydroxylase activity (McGee *et al.*, 1972a), the percentage inhibition caused by a maximally effective dose of *p*-chlorophenylalanine was slightly higher than in immature rats (Table 1). (As shown by the values in line 3, a simultaneous administration of phenylalanine did not influence the degree of inhibition.) At both ages the activity remained minimal for at least 30 h; the values at 48 h suggest that the recovery of hepatic phenylalanine hydroxylase activity may begin somewhat earlier in the immature than in the adult rats.

McGee *et al.* (1972a) reported that kidney also contains significant phenylalanine hydroxylase activity, which increases during early postnatal life. Table 1 shows that *p*-chlorophenylalanine lowered the phenylalanine activity of immature kidney by 50% and that of adult kidney by only 30%. At both

ages the percentage at 24 or 48 h was significantly less than in liver and could not be further increased by a second dose of *p*-chlorophenylalanine administered 24 h after the first one.

The purpose of the experiments of Table 2 was to determine the amounts of *p*-chlorophenylalanine and phenylalanine that need to be injected into immature rats to cause substantial, prolonged rises in the concentration of plasma phenylalanine. In 6-day-old rats, as in adult rats (Lipton *et al.*, 1967), some increase was seen after an injection of *p*-chlorophenylalanine alone. The administration of *p*-chlorophenyl-

alanine, together with 13 and 26 μ mol of phenylalanine/10 g 12 h before assay, resulted in 480 and 841 nmol of phenylalanine/ml of plasma respectively. A second injection of either the lower or the higher dose of phenylalanine given 24 h after the *p*-chlorophenylalanine was as effective as the first. As expected, *p*-chlorophenylalanine decreased the concentration of tyrosine in plasma; only with the higher dose of phenylalanine did it reach the plasma concentration of normal untreated rats (356 nmol/ml).

The last line of Table 2 refers to rats injected with the same amounts of substrate and analogue that were used previously (Andersen & Guroff, 1972; Andersen *et al.*, 1974) in the prolonged treatment of infant rats. After 12 h the plasma concentration of phenylalanine was only 30% above normal, whereas that of tyrosine was very high. This can be explained by the small inhibition of the hepatic phenylalanine hydroxylase activity with 3 μ mol of *p*-chlorophenylalanine/10 g body wt. (see Fig. 1).

Since the phenylalanine hydroxylase activities (Table 1) and the metabolism of injected phenylalanine (Table 2) remained inhibited for 48 h after the administration of *p*-chlorophenylalanine, this agent was injected only every other day in the course of prolonged treatment. The first part of Table 3 illustrates the results obtained with intraperitoneal injections given from day 3 to day 11 and day 15 of life. The concentration of phenylalanine in plasma was still ten times above normal 18 h after the last injection. At 12 h, it was higher (2020 nmol/ml) in the 15-day-old than in the 11-day-old group (1390 nmol/ml), but even in the latter it was higher than seen in the acute experiments (841 nmol/ml, see Table 2) where the rats received the same doses of *p*-chlorophenylalanine plus phenylalanine, but only once. This apparently gradual accumulation of phenylalanine in the course of chronic treatment is not due to a further diminution of phenylalanine hydroxylase activity (cf. Tables 1 and 3). Repeated treatment with *p*-chlorophenylalanine alone also resulted in higher concen-

Fig. 1. Inhibition of hepatic phenylalanine hydroxylase activity by different amounts of injected *p*-chlorophenylalanine

The indicated amounts of *p*-chlorophenylalanine were injected into 6-day-old rats, 24 h before assay. The phenylalanine hydroxylase activities (units per g) are given as a percentage of that found in livers of untreated rats, i.e. 620 units/g. Each point is the mean of results with three animals, the S.D. being indicated by the vertical bar.

Table 1. Effect of *p*-chlorophenylalanine in vivo on the hepatic and renal phenylalanine hydroxylase activity of adult and immature rats

Phenylalanine hydroxylase activities were determined at the indicated time after the administration of a single dose of 18 μ mol of *p*-chlorophenylalanine/10 g body weight. Values are means $\pm$ S.D. with the numbers of animals in parentheses.

Time of assay after <i>p</i> -chlorophenylalanine administration (h)	Phenylalanine hydroxylase activity (units/g)			
	Liver		Kidney	
	6-day-old rat	Adult	6-day-old rat	Adult
—	680 $\pm$ 52 (7)	946 $\pm$ 98 (5)	145 $\pm$ 18 (6)	156 $\pm$ 16 (3)
24	103 $\pm$ 21 (10)	96 $\pm$ 17 (3)	68 $\pm$ 12 (10)	98 $\pm$ 6 (3)
24	100 $\pm$ 24 (3)*	—	—	—
30	89 $\pm$ 8 (3)	—	—	—
48	172 $\pm$ 19 (7)	100 $\pm$ 20 (3)	78 $\pm$ 6 (4)	107 $\pm$ 12 (3)

* Rats received 13 μ mol of phenylalanine/10 g simultaneously with the *p*-chlorophenylalanine.

Table 2. Concentrations of phenylalanine, *p*-chlorophenylalanine and tyrosine in plasma of 6-day-old rats

Injections of *p*-chlorophenylalanine (18 μ mol/10g) and phenylalanine, given at the indicated times before assay, were intraperitoneal except for the experiments shown in the last line in which the injections were subcutaneous and the amount of *p*-chlorophenylalanine was only 3 μ mol/10g. Phenylalanine concentrations (μ mol/10g) injected alone (line 2), with *p*-chlorophenylalanine (lines 5, 7, 9) or twice (with, and 24 h after, *p*-chlorophenylalanine, lines 6, 8), are given in parentheses. Values refer to individual animals or means $\pm$ s.d. of results with three or four rats.

Time of assays (h)				
After <i>p</i> -chlorophenylalanine injection	After phenylalanine injection	Phenylalanine (nmol/ml of plasma)	<i>p</i> -Chlorophenylalanine (nmol/ml of plasma)	Tyrosine (nmol/ml of plasma)
—	—	97 $\pm$ 13	—	356 $\pm$ 23
—	12 (26)	100, 96	—	—
12	—	192 $\pm$ 21	1082 $\pm$ 151	178 $\pm$ 33
36	—	252 $\pm$ 74	725 $\pm$ 95	287 $\pm$ 63
12	12 (13)	480 $\pm$ 53	1410 $\pm$ 387	201 $\pm$ 81
36	36, 12 (13)	510 $\pm$ 46	783 $\pm$ 121	234 $\pm$ 17
12	12 (26)	841 $\pm$ 242	1155 $\pm$ 157	420 $\pm$ 35
36	36, 12 (26)	797 $\pm$ 290	663 $\pm$ 53	383 $\pm$ 57
12 (3)	12 (20)	126 $\pm$ 5	160 $\pm$ 9	569 $\pm$ 91

Table 3. Effects of chronic treatment with *p*-chlorophenylalanine and phenylalanine

Starting on postnatal day 3, rats were injected with *p*-chlorophenylalanine every other day and with phenylalanine (26 μ mol/10g) every day. Control rats received 0.9% NaCl of the same volume and frequency. Lines 1–5 refer to rats injected intraperitoneally; the doses of *p*-chlorophenylalanine were 18 μ mol/10g. Values refer to means that agreed within 5%, for duplicate measurements of livers or plasma pooled from three rats.

Injections	Age when assayed (days)	Time after last in- jection (h)	Concentration (nmol/ml of plasma)			Phenylalanine hydroxylase activity (units/g)	
			Phenyl- alanine	<i>p</i> -Chloro- phenylala- nine	Tyrosine	Liver	Kidney
0.9% NaCl	11–12	12	96	—	350	958–988	160
<i>p</i> -Chlorophenylalanine	11	12	770	1730	160	—	—
<i>p</i> -Chlorophenylalanine+phenylalanine	11	12	1395	1500	430	211	—
<i>p</i> -Chlorophenylalanine+phenylalanine	15	12	2020	1970	540	284	95
<i>p</i> -Chlorophenylalanine+phenylalanine	15	18	1030	1650	170	—	—
<i>p</i> -Chlorophenylalanine+phenylalanine*	12	12	1230	1258	510	339	92

* Refers to rats injected subcutaneously; the doses of *p*-chlorophenylalanine were 9 μ mol/10g.

trations of plasma phenylalanine (and of *p*-chlorophenylalanine) than those expected from observations on rats given a single injection of *p*-chlorophenylalanine (cf. Tables 2 and 3).

The ratio phenylalanine/tyrosine in the plasma of rats injected with *p*-chlorophenylalanine plus phenylalanine appears to be higher in the chronic (3–4, see Table 3) than in the acute experiments (about 2, see Table 2). More important, when the absolute concentration of phenylalanine in the plasma was 14–20 times above normal, that of tyrosine was elevated only 1.2 and 1.5 times (Table 3).

The last line of Table 3 shows that repeated subcutaneous injections of *p*-chlorophenylalanine

(at one-half the dose) and phenylalanine (usual dose) also gave the desired increase in concentrations of phenylalanine in plasma. The death rate in this group (10% or less by the age of 18 days) was lower than those in the upper part of Table 3 (about 50%).

Table 4 shows that prolonged treatment with *p*-chlorophenylalanine and phenylalanine resulted in 24, 21 and 17% decreases of body, brain and liver weights respectively. The treatment did not affect the hepatic concentration of three soluble enzymes and of the mitochondrial isoenzyme of aspartate aminotransferase. Of the cerebral enzymes studied, some are exclusively particulate (glutamate dehydrogenase, glutaminase and succinate dehydrogenase); the

Table 4. *Effect on development of chronic treatment with p-chlorophenylalanine and phenylalanine*

Two litters of rats were treated with phenylalanine plus *p*-chlorophenylalanine and two litters with 0.9% NaCl (Control) as described for Table 3. At the age of 15 days (i.e. 12 days after the beginning of treatment) three or four rats of each litter were killed. The enzyme activities in pools of three livers or brains were measured in duplicate, as described under 'Methods'. Weights are given as means $\pm$ s.d. with the numbers of rats in parentheses. In a similar experiment, but terminated on day 11, the body weights were 12.15 g (3) for the 'Treated' and 13.33 g (3) for the 'Control' rats.

	Weight (g)	
	Control	Treated
Body	20.11 $\pm$ 2.30 (6)	15.36 $\pm$ 3.07 (7)
Brain	0.66 $\pm$ 0.04 (6)	0.52 $\pm$ 0.03 (7)
Liver	0.57 $\pm$ 0.08 (6)	0.47 $\pm$ 0.12 (7)
	Activity (units/g of liver)	
	Control	Treated
Aspartate amino-transferase		
Mitochondrial	127	142
Soluble	47.9	48.4
Malate dehydrogenase	459	501
Tyrosine amino-transferase	0.74	0.71
	Activity (units/g of brain)	
	Control	Treated
Hexokinase	4.7	4.5
Glutamate dehydrogenase	4.9	4.3
Lactate dehydrogenase	19.4	18.3
Malate-NADP ⁺ dehydrogenase	0.36	0.39
Glutaminase	23.4	24.3
Succinate dehydrogenase	0.97	0.95

activities given for the others refer to the sum of their soluble and particulate portions. These enzymes were chosen on the basis of their known developmental behaviour in normal rat brain: major increases in their activities (per g) occur during the first 3 weeks of postnatal life (E. Ryder, G. Dienel & O. Greengard, unpublished work; MacDonnell & Greengard, 1974; Banik & Davison, 1969). Table 4 shows that the prolonged treatment did not appear to interfere with this process. In studies of rats treated with high doses of *p*-chlorophenylalanine and phenylalanine (Prohaska & Wells, 1974), the cerebral hexokinase and cytochrome *c* oxidase (EC 1.9.3.1) activities remained normal.

Discussion

The amount of hepatic phenylalanine hydroxylase (judged by its activity in an optimally fortified assay system *in vitro*) appears to be the primary determinant of the capacity to metabolize phenylalanine *in vivo*: in adult rats, this was demonstrated with the aid of the inhibitor *p*-chlorophenylalanine (Guroff, 1969); in suckling rats, as we previously reported (McGee *et al.*, 1972b), cortisol-induced elevations of phenylalanine hydroxylase activity correlated quantitatively with increases in the rate constant of disappearance of a loading dose of phenylalanine from plasma. However, major changes in rate constant altered by only about 30 min the time-period during which the plasma phenylalanine concentration remained considerably above normal. Thus a near-complete inhibition of phenylalanine hydroxylase activity plus large doses of phenylalanine were expected to be necessary to obtain the degree of sustained hyperphenylalaninaemia aimed at in the present study. In our 6-day-old rats, 9–18 μ mol of *p*-chlorophenylalanine decreased the total potential phenylalanine hydroxylase activity (renal plus hepatic) of the organism from 188 to 34 units and the injection of as much as 26 μ mol of phenylalanine was then required for its concentration in plasma to be still considerably elevated 12 h later. The best-documented neurological and behavioural abnormalities were reported for rats that had been given repeated doses of only 3 μ mol of *p*-chlorophenylalanine and 20 μ mol of phenylalanine/10 g body wt. during infancy (Andersen & Guroff, 1972; Andersen *et al.*, 1974). We, however, found that an injection of these amounts did not inhibit the hepatic phenylalanine hydroxylase activity nor cause sustained hyperphenylalaninaemia. Thus the abnormalities observed might have been caused by factors other than the circulating phenylalanine unless, after prolonged treatment with even the low doses, there was a gradual accumulation of phenylalanine in plasma. It will of course be necessary to test whether these behavioural abnormalities in rats given low doses of *p*-chlorophenylalanine plus phenylalanine (Andersen & Guroff, 1972; Andersen *et al.*, 1974) are different or less extensive than those which may be found in hyperphenylalaninaemic rats treated with the regimen described in Table 3.

An almost threefold increase did occur in the course of prolonged treatment with the high doses of *p*-chlorophenylalanine plus phenylalanine (cf. Tables 2 and 3); by the end of the second week the concentrations of phenylalanine in plasma were over 20 times higher than those in control rats throughout 50% of each day and 10–20-fold above normal values during another 25% of the day. We did not determine the much higher concentrations of phenylalanine in plasma that must have prevailed during the first few hours after the daily injections.

Since the injection of *p*-chlorophenylalanine did not completely abolish the phenylalanine hydroxylase activity, the administration of phenylalanine was expected to raise the concentration of tyrosine in plasma above the low values found after treatment with *p*-chlorophenylalanine alone. However, compared with the plasma of untreated rats the increase (at 12 or 18 h after the last injection of phenylalanine) was small or insignificant. The phenylalanine/tyrosine ratio, which was 0.27 in untreated rats, rose to about 4. This 15-fold difference in ratio compares favourably with that between normal human subjects and untreated phenylketonurics, with ratios of 0.8 and about 20 respectively (Lipton *et al.*, 1967; Partington & Lewis, 1963).

The inhibition of body and organ growth in the treated animals accords with observations on developing rats (Clarke & Lowden, 1969) as well as with the lower brain size and head circumference of phenylketonurics (Knox, 1966). Chronic hyperphenylalaninaemia did not alter the hepatic concentration of the three enzymes nor did it appear to interfere with the developmental formation of the six cerebral enzymes so far measured. Since in rats, cerebral development [N.B. myelination (Davison, 1971)] is not accomplished by the fourth postnatal week (Benjamins & McKhann, 1972), it may be appropriate to test for cerebral abnormalities (biochemical, ultrastructural and behavioural) after an even more prolonged treatment with *p*-chlorophenylalanine and phenylalanine. The assumption that the regimen described in the present paper can produce a better animal model for phenylketonuria than those designed up to now, rests at present on the sustained hyperphenylalaninaemia achieved. Throughout a critical period of early postnatal development, at least 20-fold elevations of phenylalanine concentration in plasma persisted for the better part of each day. This approaches the 20–30-fold difference between untreated phenylketonuric and normal human subjects (Lipton *et al.*, 1967; Partington & Lewis, 1963).

This investigation was supported by the U.S. Public Health Service, the National Cancer Institute (grant no. CA 08676) and by the U.S. Energy and Research Development Administration Contract [E(11-1)-3085] with the New England Deaconess Hospital. We are grateful to Dr. W. Eugene Knox for valuable discussions about the present paper, to Ms. Barbara Green for assays with the automatic amino acid analyser and to Mr. Gerald A. Dienel for some of the cerebral-enzyme activity determinations. J. A. D. is the recipient of a fellowship from the Fundacion Juan March, Madrid, Spain.

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