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Comparison of α-Methylphenylalanine and *p*-Chlorophenylalanine as Inducers of Chronic Hyperphenylalaninaemia in Developing Rats

By JUAN A. DELVALLE, GERALD DIENEL 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.*

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α-Methylphenylalanine is a very weak competitive inhibitor of rat liver phenylalanine hydroxylase *in vitro* but a potent suppressor *in vivo*. The loss of the hepatic activity (the renal one is unaffected) becomes maximal (70–75% decrease; cf. control) 18h after the administration (per 10g body wt.) of 24μmol of α-methylphenylalanine with or without 52μmol of phenylalanine. Chronic suppression of hepatic phenylalanine hydroxylase was obtained by injections of α-methylphenylalanine plus phenylalanine to suckling rats, and by their addition to the diet after weaning. A series of comparisons of the effects of this treatment, and one with *p*-chlorophenylalanine, was then carried out. In both cases there was a rise (1.3–2-fold) in phenylalanine–pyruvate aminotransferase activity (but no change in four other enzyme activities) in the liver; in brain there was a rise in phosphoserine phosphatase activity, but the total activity and subcellular distribution of nine enzymes revealed no other abnormalities in cerebral development. Striking increases in the concentration of plasma phenylalanine during 26 of the 31 experimental days (with a transient fall at 18–22 days) were maintained by treatment with both analogues plus phenylalanine. However, *p*-chlorophenylalanine-treated animals had a 30–60% mortality rate and 27–52% decrease in body weight. Developing rats treated with α-methylphenylalanine, showing no growth deficit or signs of toxicity (e.g. cataracts), appear to be a more suitable model for the human disease of phenylketonuria. Their plasma phenylalanine concentrations exhibited at least 20–40-fold increases during 50% of each of the first 18 days of life, and 30-fold after weaning.

Among inborn errors in amino acid metabolism, phenylketonuria is the one in which the association between the primary lesion in the liver, the chemical alterations in the blood and the defective function in the brain is best established. A low-phenylalanine diet prevents mental deterioration, but only if started shortly after birth (Knox, 1972), indicating convincingly that it is not the irreversible hepatic lesion (the phenylalanine hydroxylase deficiency) but the consequent accumulation of phenylalanine that causes the brain defect and that it does so during early postnatal development. Hence hyperphenylalaninaemia and its presence during infancy, rather than the hepatic enzyme defect, are the essential features required in rat models for this disease. However, it is difficult to achieve a sustained increase in phenylalanine concentration in the plasma merely by increasing its intake. From birth onwards, the liver (Brenneman & Kaufman, 1965) and kidney (McGee *et al.*, 1972) of the rat contain considerable amounts of phenylalanine hydroxylase, which assures the rapid elimination of large excesses of phenylalanine. After the discovery of *p*-chlorophenylalanine, an inhibitor of phenylalanine hydroxylase (Koe & Weissman, 1966), infant rats treated with this analogue plus the natural substrate were proposed as suitable models for the human disease of phenylketonuria (Lipton *et al.*, 1967; Andersen & Guroff, 1972). We previously described a regimen for maintaining 20–30-fold increases in plasma phenylalanine in 3–15-day-old rats (DelValle & Greengard, 1976). However, the dosages of *p*-chlorophenylalanine required for this appeared to be toxic, which, in view of previously reported side effects of *p*-chlorophenylalanine (Koe & Weissman, 1966; Watt & Martin, 1969), was not surprising. In searching for an alternative suppressor of hepatic phenylalanine hydroxylase, we identified α-methylphenylalanine as a potent one, and suggested that this analogue may be an effective inducer of sustained hyperphenylalaninaemia in infant rats (Greengard *et al.*, 1976). The present study confirms this and provides some information about the specificity and mode of action of α-methylphenylalanine.

α-Methylphenylalanine proved to be much less toxic to the developing animal than was *p*-chlorophenylalanine. However, we consider that it is advantageous to continue to study both animal models for phenylketonuria. Like all foreign substances, α-methylphenylalanine may also eventually be found to have multiple targets, but the side effects are likely

to differ from those of *p*-chlorophenylalanine. Comparative studies can identify effects that are unique to one of the inhibitors and thus not attributable to hyperphenylalaninaemia itself. Biochemical changes in the central nervous system, which may be important in the pathogenesis of mental retardation, are among those exhibited by both models. Therefore this first description of the effectiveness of α-methylphenylalanine in imitating the conditions of phenylketonuria is reported together with further information on the improved utilization of *p*-chlorophenylalanine. We also report the effects of treatment with both compounds (plus phenylalanine) on the cerebral and hepatic activities of several enzymes and on DNA and protein concentrations.

Experimental

Rats used in this study were of the Fisher (CDF) strain. They were weaned at the age of 22 days and then maintained on Purina Chow diet *ad libitum*. Preparations of phenylalanine and *p*-chlorophenylalanine for use in injections were made as previously described (DelValle & Greengard, 1976); α-methylphenylalanine (Nutritional Biochemicals Division, ICN Life Science Group, Cleveland, OH, U.S.A.) was dissolved in 0.9% NaCl by heating. All solutions were adjusted to pH 7.2 with NaOH.

Chronic treatments

Unless otherwise specified, rats were injected subcutaneously from age 3 days to 21 days with α-methylphenylalanine (24 μmol/10g body wt.) plus phenylalanine (52 μmol/10g) daily, or with *p*-chlorophenylalanine (9 μmol/10g every other day) plus phenylalanine (52 μmol/10g daily). The control group received injections of 0.9% NaCl. From age 22 to 34 days, the two experimental groups were fed on powdered Purina Chow containing 0.5% α-methylphenylalanine plus 3% phenylalanine and 0.5% *p*-chlorophenylalanine plus 3% phenylalanine respectively. Since the weaned rats consume approx. 2g of food/day per 10g body wt., their daily intake of phenylalanine (363 μmol/10g) was higher than that given by injections to the suckling rats. The daily intake of the inhibitors was about 50 μmol/10g.

Determination of phenylalanine and tyrosine concentrations

Blood was collected and processed as previously described (DelValle & Greengard, 1976). In control and α-methylphenylalanine-treated animals, plasma phenylalanine was determined by the fluorimetric method of McCaman & Robins (1962), as modified by Faulkner (1965). Plasma tyrosine was measured by the fluorimetric method of Udenfriend (1962), as modified by Wong *et al.* (1964). The colorimetric assay for tyrosine of Udenfriend & Cooper (1952), as modified by McGee *et al.* (1972), was used to test for the formation of α-methyltyrosine from α-methylphenylalanine *in vitro*.

In *p*-chlorophenylalanine-treated animals, plasma phenylalanine, tyrosine and *p*-chlorophenylalanine concentrations were determined with a Beckman amino acid analyser as previously described (DelValle & Greengard, 1976); the elution times were 128, 118 and 280min respectively.

Enzyme assays

Animals were killed by a blow to the neck. Phenylalanine hydroxylase (EC 1.14.16.1) in liver and kidney was assayed in freshly excised tissues as previously described (Greengard & DelValle, 1976). Liver and cerebral hemispheres (hereafter referred to as brain) were routinely homogenized in 4vol. of 0.15M-KCl and 0.32M-sucrose respectively, except that brain to be used for the assay of phosphoserine phosphatase was homogenized in water. Supernatant and particulate fractions were prepared by centrifugation at 45000*g* for 60min at 4°C in a Beckman model L ultracentrifuge. Previously described methods (MacDonnell & Greengard, 1974) were used to measure hexokinase (EC 2.7.1.1), lactate dehydrogenase (EC 1.1.1.27), glutamate dehydrogenase (EC 1.4.1.2), aspartate aminotransferase (EC 2.6.1.1) and pyruvate kinase (EC 2.7.1.40). The assay methods for malate–$NADP^+$ dehydrogenase (EC 1.1.1.40) (Ochoa, 1955), phosphoserine phosphatase (EC 3.1.3.3) (Knox *et al.*, 1969) and phenylalanine–pyruvate aminotransferase (Brand & Harper, 1974*a*) have also been previously established. The measurement of glutamate decarboxylase (EC 4.1.1.15) activity was that based on $^{14}CO_2$ production (MacDonnell & Greengard, 1975).

Particulate fractions were usually resuspended in the original homogenizing medium containing Triton X-100 (final concentration 0.5%), and left at 0°C for 30min. However, the assay of the following two enzymes did not involve pretreatment with Triton. Succinate dehydrogenase (EC 1.3.99.1) activity was measured as described by Laatsch *et al.* (1962), except that the acceptor INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride; Sigma Chemical Co., St. Louis, MO, U.S.A.] was used at a final concentration of 1.0mg/ml. β-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) was measured after samples were subjected to sonication at 9kHz for 5min in 0.32M-sucrose containing 10mM-succinate, 1mM-EDTA, 1mM-ATP and 1mM-NAD^+, pH 7.4 (Lehninger *et al.*, 1960). The assay was modified by replacing NaCN with antimycin A (Calbiochem, San Diego, CA, U.S.A.) at a final concentration of 10 μM.

All enzyme activities were determined at 25°C except glutamate decarboxylase, which was measured at 37°C. The results are expressed in units (nmol for phenylalanine hydroxylase, and μmol for all other enzymes/min) per g fresh wt. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard, and DNA was measured by the method of Burton (1956), with calf thymus DNA as the standard.

Results

As previously reported (Greengard *et al.*, 1976), the administration of α-methylphenylalanine to rats suppresses the activity of hepatic phenylalanine hydroxylase, but addition of this analogue (0.8–8 mM) to the standard assay system *in vitro* is without effect (Table 1). It was decided to test further whether α-methylphenylalanine can serve as a substrate and/or inhibitor of the enzyme reaction. The expected product, α-methyltyrosine, forms a nitrosonaphthol derivative. Its spectrum is similar to that of tyrosine and its absorbance (at 450 nm) is only about 32% lower (Fig. 1). The sensitivity of the method would permit the accurate measurement of the formation, under standard phenylalanine hydroxylase assay conditions, of 10 nmol of α-methyltyrosine/min per g of liver. However, very little if any appeared after 20 min of incubation with the liver extract (Table 1, line 4). Thus phenylalanine hydroxylase, under conditions optimal for the conversion of phenylalanine, does not catalyse to any significant extent the hydroxylation of the α-methyl analogue of the natural substrate.

Fig. 2 shows that at non-saturating concentrations of phenylalanine (0.5–5 mM), α-methylphenylalanine appears to be a competitive inhibitor of the phenylalanine hydroxylase activity. The inhibition by 8 mM-α-methylphenylalanine, even at the lowest concentration of phenylalanine tested (0.5 mM), was only 32%. The K_I, calculated from Lineweaver–Burk plots, was 10 mM. However, this inhibition does not

Table 1. *Effect of α-methylphenylalanine on the assay of phenylalanine hydroxylase*

Livers of 6-day-old rats were assayed for phenylalanine hydroxylase but in the presence of various amounts of α-methylphenylalanine and phenylalanine.

Phenylalanine (mM)	α-Methylphenylalanine (mM)	Tyrosine or α-methyltyrosine formed (μmol/min per g of liver)
10	—	554
10	0.8	579
10	8	533
—	10	<10

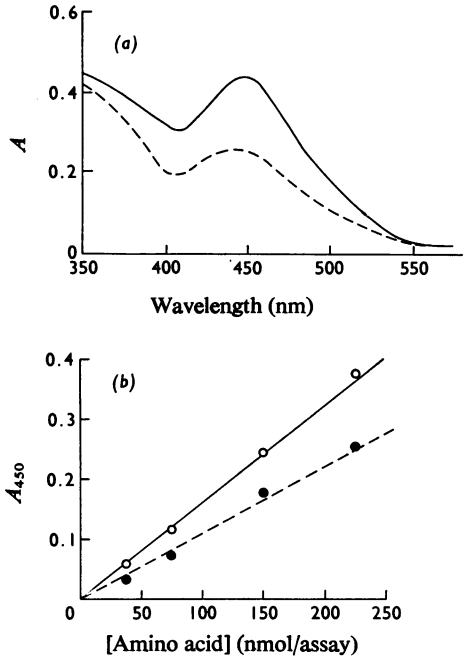

Fig. 1. *Absorbance of nitrosonaphthol derivatives of tyrosine and α-methyltyrosine*

——, Tyrosine; – – – –, α-methyltyrosine. (*a*) Spectrum obtained with 225 nmol of each. (*b*) A_{450} versus concentration. The final volume of the assay mixture was 3.5 ml.

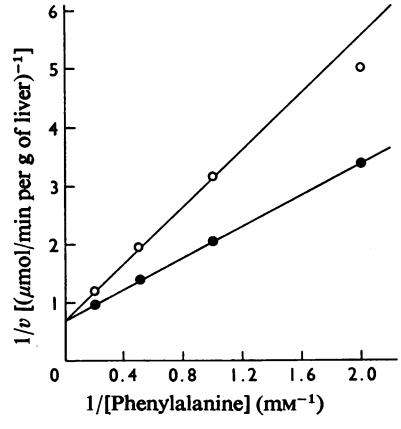

Fig. 2. *Effect of α-methylphenylalanine on rat liver phenylalanine hydroxylase activity at non-saturating concentrations of phenylalanine*

Liver extracts (20 mg of protein per assay) from 7-day-old rats were assayed in the standard manner, but at various concentrations of phenylalanine in the absence (●) or presence (○) of 8 mM-α-methylphenylalanine.

explain the effect of α-methylphenylalanine on phenylalanine hydroxylase *in vivo*. The activity of livers excised 24h after the injection of α-methylphenylalanine (24μmol/10g) was low (20–30% of normal), even though assayed at saturating concentrations of substrate (Greengard *et al.*, 1976; see also Table 2). In any case, the amount of the analogue in this liver extract, and thus in the assay mixture, can be calculated (assuming uniform tissue distribution) to be too low (less than 0.1mM) to be inhibitory at even non-saturating concentrations of phenylalanine.

The low rate at which the phenylalanine hydroxylase activity decreases in rats injected with α-methylphenylalanine further indicates that the effect of α-methylphenylalanine *in vivo* is quite unlike that observed *in vitro*. Maximal loss was seen only at 18h (Fig. 3). The enzyme activity remained low for 24h and reverted to almost normal on day 4.

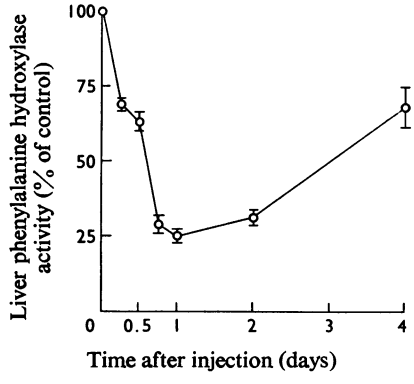

Fig. 3. *Time course of inhibition of hepatic phenylalanine hydroxylase activity by α-methylphenylalanine in vivo* Assays were performed at the indicated times after the injection of α-methylphenylalanine (24μmol/10g body wt.) to 6-day-old rats. Points represent means of results with three or four animals (bar = ±1S.D.).

To design a treatment regimen that would maintain minimal phenylalanine hydroxylase activity throughout postnatal development, we compared the effects of different dosages of α-methylphenylalanine at various ages. Table 2 shows that in both 6- and 22-day-old rats, the loss of hepatic phenylalanine hydroxylase was greater when 24 rather than 12μmol of α-methylphenylalanine/10g body wt. was injected. Higher doses (48μmol/10g) caused no further suppression. The basal activity changed with age, showing a peak at 22 days. The activity remaining after treatment with maximally effective doses was also highest at 22 days. At this age, the inhibition was the least marked (43 versus 64–75%), but the absolute decrement was as great as at 6, 15 or 90 days of age. Enzyme activity in kidney was not affected, whereas administration of the other phenylalanine analogue, *p*-chlorophenylalanine, caused a 50% inhibition (DelValle & Greengard, 1976).

p-Chlorophenylalanine has long been known to suppress the activity of phenylalanine hydroxylase (Koe & Weissman, 1966). Our previous studies showed that in 6-day-old rats 9μmol/10g was the most effective dose (DelValle & Greengard, 1976). Phenylalanine hydroxylase activity was minimal between 24 and 48h after the administration of *p*-chlorophenylalanine. However, repeated injections of phenylalanine on this second day caused a doubling of phenylalanine hydroxylase activity (Greengard & DelValle, 1976). The purpose of the experiments of Table 3 was to test whether the two analogues are synergistic and whether phenylalanine injections can also reverse the effect of α-methylphenylalanine on phenylalanine hydroxylase. The two compounds were not synergistic: the inhibition was no higher than that with either compound alone. The phenomenon illustrated in the second part of Table 3 was qualitatively similar in α-methylphenylalanine- and *p*-chlorophenylalanine-treated rats; however, in the former, the three injections of phenylalanine induced only a 1.4-fold rise in phenylalanine hydroxylase activity.

Table 2. *Inhibition of liver phenylalanine hydroxylase activity by α-methylphenylalanine at different ages*
Animals at denoted ages were given a single subcutaneous injection of the indicated amounts of α-methylphenylalanine 24h before assay. The values refer to individual animals or to means±S.D. for the numbers of animals in parentheses.

Age (days)	α-Methylphenylalanine injected ...	Phenylalanine hydroxylase activity (units/g of tissue)		
		None	12μmol/10g	24μmol/10g
Liver				
6		680± 28 (5)	238± 20 (3)	173± 3 (3)
14		988±158 (3)	—	356± 82 (3)
22		1791±126 (5)	1380±102 (3)	1035±110 (3)
90		948± 98 (5)	—	243± 25 (3)
Kidney				
6		109± 13 (3)	—	114± 16 (3)
60		226± 15 (3)	—	231, 199

Since the experiments to be described next involved the study of plasma concentrations of phenylalanine and tyrosine, it was necessary to test whether the analogues might interfere with these measurements. α-Methylphenylalanine does not interfere with the fluorimetric measurement of phenylalanine, whereas *p*-chlorophenylalanine does (Table 4), so that the phenylalanine concentration in the plasma of rats treated with *p*-chlorophenylalanine had to be determined in the amino acid analyser; neither analogue interferes with the determination of tyrosine. (It should be noted, however, that in the autoanalyser, α-methylphenylalanine, but not *p*-chlorophenylalanine, is eluted together with tyrosine.)

In suckling rats, a deficiency in hepatic phenylalanine hydroxylase activity and an increase in the plasma concentration of phenylalanine were achieved by injections of α-methylphenylalanine plus phenylalanine (Greengard *et al.*, 1976). In weaned rats, this route of administration can be avoided. They consumed normal amounts of solid food, containing α-methylphenylalanine plus phenylalanine, and exhibited increased plasma concentrations of phenylalanine as well as decreased hepatic phenylalanine hydroxylase activity. Both changes were already detectable at 24h. By day 4 of treatment, the loss of phenylalanine hydroxylase was 75%, and the blood concentrations of phenylalanine exhibited considerable, though highly variable, increases (Table 5). [The diet-induced hyperphenylalaninaemia was more reproducible in rats that had previously been given injections of α-methylphenylalanine plus phenylalanine (Fig. 4).] Since neither α-methylphenylalanine

Table 3. *Effect of combined injection of α-methylphenylalanine, p-chlorophenylalanine and phenylalanine on rat liver phenylalanine hydroxylase in vivo*

In experiments in the first four lines, 6-day-old rats were injected intraperitoneally with α-methylphenylalanine (24μmol/10g) or *p*-chlorophenylalanine (18μmol/10g), or both, 24h before assay. The last four lines refer to experiments of 33h duration. α-Methylphenylalanine (24μmol/10g) was injected at 0 and 12h, and *p*-chlorophenylalanine (9μmol/10g) at 0h; injections of phenylalanine (13μmol/10g) or 0.9% NaCl were given at 24, 27 and 30h. Values are means±S.D. with the numbers of animals in parentheses.

Injections	Phenylalanine hydroxylase (units/g of liver)
0.9% NaCl	660±80 (3)
α-Methylphenylalanine	173± 3 (3)
p-Chlorophenylalanine	100±14 (3)
α-Methylphenylalanine +*p*-chlorophenylalanine	155±29 (3)
α-Methylphenylalanine, then 3×0.9% NaCl	215±38 (7)
α-Methylphenylalanine, then 3×phenylalanine	306±27 (7)
p-Chlorophenylalanine, then 3×0.9% NaCl	104±27 (9)
p-Chlorophenylalanine, then 3×phenylalanine	235±19 (9)

Table 4. *Fluorimetric assays for phenylalanine and tyrosine in the presence of α-methylphenylalanine or p-chlorophenylalanine*

The relative fluorescence of α-methylphenylalanine and *p*-chlorophenylalanine (dissolved in 0.3M-trichloroacetic acid) was measured under conditions for the assay of phenylalanine (upper half of Table) and tyrosine (lower half of Table).

Amino acids added	Relative fluorescence (%)
'Phenylalanine' assay	
Phenylalanine (100nmol)	100
α-Methylphenylalanine (25–500nmol)	<0.5
Phenylalanine+α-methylphenylalanine (100nmol each)	105
p-Chlorophenylalanine (100nmol)	133
Phenylalanine+*p*-chlorophenylalanine (50nmol each)	114
'Tyrosine' assay	
Tyrosine (5.5nmol)	100
α-Methylphenylalanine (10–550nmol)	<0.8
Tyrosine+α-methylphenylalanine (5.5nmol each)	97
p-Chlorophenylalanine (10–80nmol)	<4
Tyrosine (5.5nmol)+*p*-chlorophenylalanine (10–80nmol)	103

Table 5. *Dietary induction of hyperphenylalaninaemia*

Previously untreated rats were weaned at the age of 21 days and were fed, *ad lib.*, on a solid diet containing α-methylphenylalanine (0.5%) plus phenylalanine (3%). They were killed at 10:00h, 24 or 96h after beginning treatment. Values refer to individual animals.

Age when assayed (days)	Phenylalanine hydroxylase (units/g of liver)	Phenylalanine (nmol/ml of plasma)	Tyrosine (nmol/ml of plasma)
Control	2244–2619	82–120	91–178
22	1609, 1129, 1277	299, 963, 299	123, 240, 159
25	557, 832, 430	2876, 1274, 699	335, 305, 432

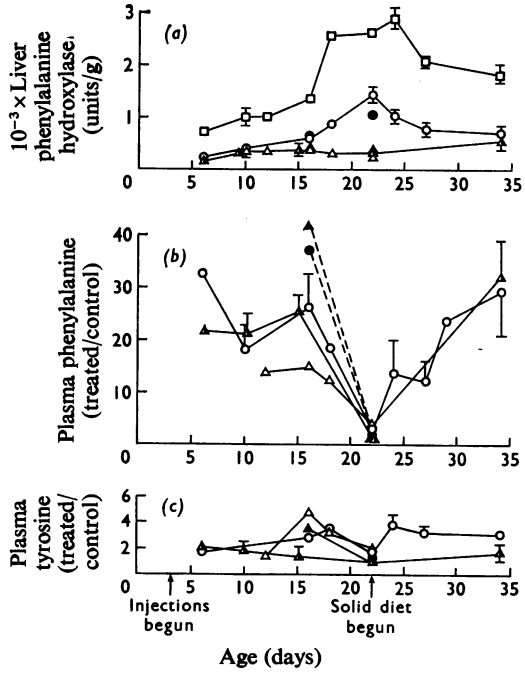

Fig. 4. *Plasma phenylalanine and tyrosine concentrations in rats with chronic phenylalanine hydroxylase deficiency*
Phenylalanine plus α-methylphenylalanine (●, ○) or plus *p*-chlorophenylalanine (▲, ▲, △) were given as daily injections (from day 3 to day 21) or in the diet (from day 22 to day 34). Results with standard dosages (see the Experimental section under 'Chronic treatments') are indicated by ○ and △. One group of *p*-chlorophenylalanine-treated animals was injected with half the standard amount of phenylalanine (26 μmol/10g) (△). In some cases, the standard treatment was supplemented from day 15 to day 21 with a second daily dose (9h after the first) of α-methylphenylalanine plus phenylalanine (●) or phenylalanine (52 μmol/10g) (▲). Animals were killed either 12h after the last injection (age 6–22 days) or at 08:00–12:00h (age 24–34 days). Their liver phenylalanine hydroxylase activities are compared with values for control animals (□) in (*a*). In (*b*) and (*c*) the results are expressed as ratios of values for treated animals to those for controls. The plasma phenylalanine in untreated rats remained at 70–100nmol/ml; tyrosine decreased from 350nmol/ml at 6–10 days to 98nmol/ml at 24–27 days. Points are either means (bar = ±1S.D.) or the averages of duplicate measurements on pools of three or four livers or plasma samples.

nor *p*-chlorophenylalanine (injected or incorporated into the solid diet) inhibited the enzyme completely, there was always some increase in plasma concentrations of tyrosine (Table 5 and Fig. 4*c*).

In the chronic experiments, rats were treated from days 3 to 34 of age and killed at frequent intervals for analysis of their livers and plasma. Fig. 4(*a*) indicates that injections (up to day 22) or the dietary intake (after day 22) of α-methylphenylalanine or *p*-chlorophenylalanine (plus phenylalanine) resulted in chronically diminished phenylalanine hydroxylase activity. Whereas in *p*-chlorophenylalanine-treated rats the activity remained low throughout the experiment, in those treated with α-methylphenylalanine the activity (as in the acute experiments of Table 2) was somewhat higher around the age of 22 days. However, as shown in Fig. 4(*b*), this difference in the effect of the two analogues on the hepatic phenylalanine hydroxylase was not reflected in the plasma phenylalanine concentrations.

During this chronic treatment with either analogue (plus phenylalanine), plasma phenylalanine concentration (Fig. 4*b*) remained high up to about day 18, and was again considerably increased from day 23 onward. On day 22, concentrations diminished to control values in both treatment groups (even after increasing the phenylalanine dosages; see the legend). Thus only for about 3–5 out of the 31 days of treatment was the degree of hyperphenylalaninaemia inadequate.

The results in Fig. 4(*b*), referring to the first 18 days of the chronic experiments, underestimate the degree of hyperphenylalaninaemia prevailing during the first half of each day. Much higher concentrations of phenylalanine existed before 12h after each injection, but this period was not systematically studied. At 12h, the concentrations were still 20–30-fold above normal in rats treated with standard dosages of α-methylphenylalanine plus phenylalanine. The standard dosages of *p*-chlorophenylalanine combined with 26 μmol (open triangles) and 52 μmol (half-open triangles) of phenylalanine gave 15- and 24-fold increases respectively in the plasma phenylalanine concentration. The few measurements (in 10–15-day-old rats) made as late as 18h after the last injection (600–950nmol/ml of plasma) indicate that only during the last 6h of each day did the plasma concentration fall below 6–9 times that of the untreated controls.

In both experimental groups, the plasma concentrations of tyrosine, like those of phenylalanine, were minimal in 22-day-old rats, as low as in the controls. The higher degree of hyperphenylalaninaemia in the younger and older rats was accompanied by some (1.4–4.8-fold) increase in plasma tyrosine concentration (Fig. 4*c*).

Fig. 5 shows the effect of the chronic hyperphenylalaninaemia on the growth of infant rats. *p*-Chlorophenylalanine treatment clearly inhibited the normal gain in body weight; the effect was already noticeable at 9 days and was more striking at later ages. The mortality rate was 30–60%. Neither growth deficit nor significant mortality was found in α-methyl-

phenylalanine-treated animals. As shown in Tables 6 and 7, the decrease in liver (2–6%) and brain weight (8–17%) was also less pronounced than in *p*-chlorophenylalanine-treated animals (27–52 and 22–24%).

The effects of chronic hyperphenylalaninaemia on the composition of the liver are shown in Table 6. The protein concentration, both soluble and particulate, was normal at all ages in both experimental groups. The amounts of four of the five enzymes assayed remained near normal. However, the phenylalanine–pyruvate aminotransferase activity was increased (see also Sanchez-Urrutia & Greengard, 1977). At 22–24 days, the change was significant ($P<0.01$ by Student's t test) in both groups, whereas at 16 days the change was more pronounced in the *p*-chlorophenylalanine-treated rats.

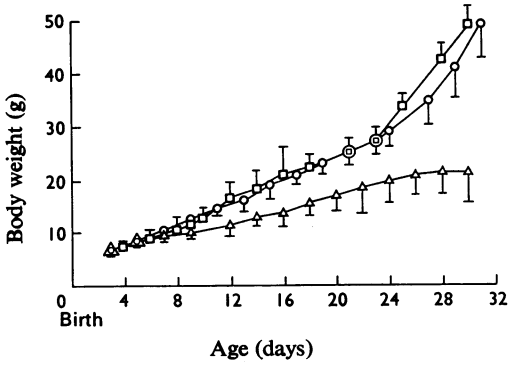

Fig. 5. *Growth of chronically hyperphenylalaninaemic rats*
Animals were injected (see the Experimental section, under 'Chronic treatments') with 0.9% NaCl (□), α-methylphenylalanine plus phenylalanine (○) or *p*-chlorophenylalanine plus phenylalanine (26 or 52 μmol/10g body wt.) (△). Each point represents the mean body weight of 7–24 animals (bar = ±1 S.D.).

Changes in the biochemical composition of brain during prolonged hyperphenylalaninaemia are shown in Table 7 and Fig. 6. Both soluble and particulate protein concentrations were normal at all ages examined. DNA concentration was increased (36%) in *p*-chlorophenylalanine-treated animals at 16 days, but not at 33 days; it was normal at both ages in α-methylphenylalanine-treated animals. The cerebral enzymes determined were selected so as to represent several subcellular compartments, different pathways, and to include some for which the activity is known to increase, decrease or remain unchanged during the first 2 or 3 postnatal weeks (MacDonnell & Greengard, 1974; Klee & Sokoloff, 1967; Banik & Davison, 1969). Table 7 shows that the treatments did not alter the activities of the three mitochondrial enzymes (glutamate dehydrogenase, β-hydroxybutyrate dehydrogenase or succinate dehydrogenase) nor influence the distribution of the last three enzymes between the particulate fraction [which includes the synaptosomes (Dienel *et al.*, 1977; Gray & Whittaker, 1962)] and the soluble fraction. Hyperphenylalaninaemia did not alter the normal developmental increase in five enzymes shown in Fig. 6. However, the concentration of cerebral phosphoserine phosphatase was increased in both models, more so in those treated with *p*-chlorophenylalanine. This increase, seen in several age groups, is a significant one and is specific

Table 6. *Effect of chronic hyperphenylalaninaemia on the liver*
Treatments with α-methylphenylalanine plus phenylalanine or *p*-chlorophenylalanine plus phenylalanine as described in the Experimental section began at age 3 days. Values followed by ±S.D. are means of results on three rats; the others are averages of closely agreeing duplicate measurements of pools of three to four livers. The amounts of soluble and particulate protein were measured separately, but since their ratio was constant, only the sums of the two are tabulated. All enzymes were measured in the soluble fraction, except β-hydroxybutyrate dehydrogenase. Activities are expressed in units/g of liver.

	Age (days)	Control	α-Methylphenylalanine +phenylalanine	*p*-Chlorophenylalanine +phenylalanine
Weight (g)	16	0.52±0.02	0.51±0.03	0.33±0.04
	33	3.10±0.04	2.93±0.24	1.50±0.14
Protein (mg/g)	16	170	175	192
	33	197	196	237
Phenylalanine–pyruvate aminotransferase	16	1.1, 1.2	1.2, 2.3	1.6, 3.4
	22–24	1.5 ± 0.2	2.6±0.1	2.8±0.4
	27	1.7	2.6	2.5
	33	1.4	1.5	1.9
Phosphoserine phosphatase	16	0.74	0.77	0.60
	33	0.85	1.04	0.83
Aspartate aminotransferase	16	47.9	—	48.4
	33	32.3, 23.0	36.4, 23.0	36.4, 41.7
β-Hydroxybutyrate dehydrogenase	33	17.2, 14.9	16.1, 14.8	12.1, 15.5
Lactate dehydrogenase	33	162	191	173

Table 7. *Effect of chronic hyperphenylalaninaemia on brain components*

See Table 6 for description of method and explanations. Enzyme activities are expressed in units/g of brain. Sucrose (0.25M) homogenates centrifuged for 60min at 45000*g* yielded the supernatant and particulate fractions; the latter includes the synaptosomes, which remained unlysed under these conditions. Values followed by ±S.D. are means of results on three rats; the others are averages of closely agreeing duplicate measurements of pools of three to four livers.

Age (days) ...	16			33		
	Control	α-Methylphenylalanine +phenylalanine	*p*-Chlorophenylalanine +phenylalanine	Control	α-Methylphenylalanine +phenylalanine	*p*-Chlorophenylalanine +phenylalanine
Weight (g)	0.68±0.02	0.57±0.06	0.52±0.02	0.86±0.04	0.79±0.07	0.67±0.05
Total protein (mg/g)	100	99	90	122	94	103
DNA (mg/g)	1.30	1.42	1.77	0.87	0.78	0.84
Glutamate dehydrogenase						
Particulate	4.6	4.4	4.4	5.1	5.6	4.8
β-Hydroxybutyrate dehydrogenase						
Particulate	2.1	1.7	2.0	1.6, 1.6	1.7, 1.6	2.1
Succinate dehydrogenase						
Particulate	1.5	1.3	1.2	1.12, 1.54	1.15, 1.30	1.16, 1.44
Glutamate decarboxylase						
Soluble	0.05	0.06	0.04	0.07	0.06	0.08
Particulate	0.21	0.21	0.20	0.22	0.26	0.23
Hexokinase						
Soluble	1.68	2.40	2.40	0.98	0.98	0.98
Particulate	7.76	7.28	6.79	9.80	7.80	9.80
Lactate dehydrogenase						
Soluble	34.4	28.8	26.8	33.6	24.0	26.4
Particulate	47.2	34.4	34.4	45.6	38.4	38.4

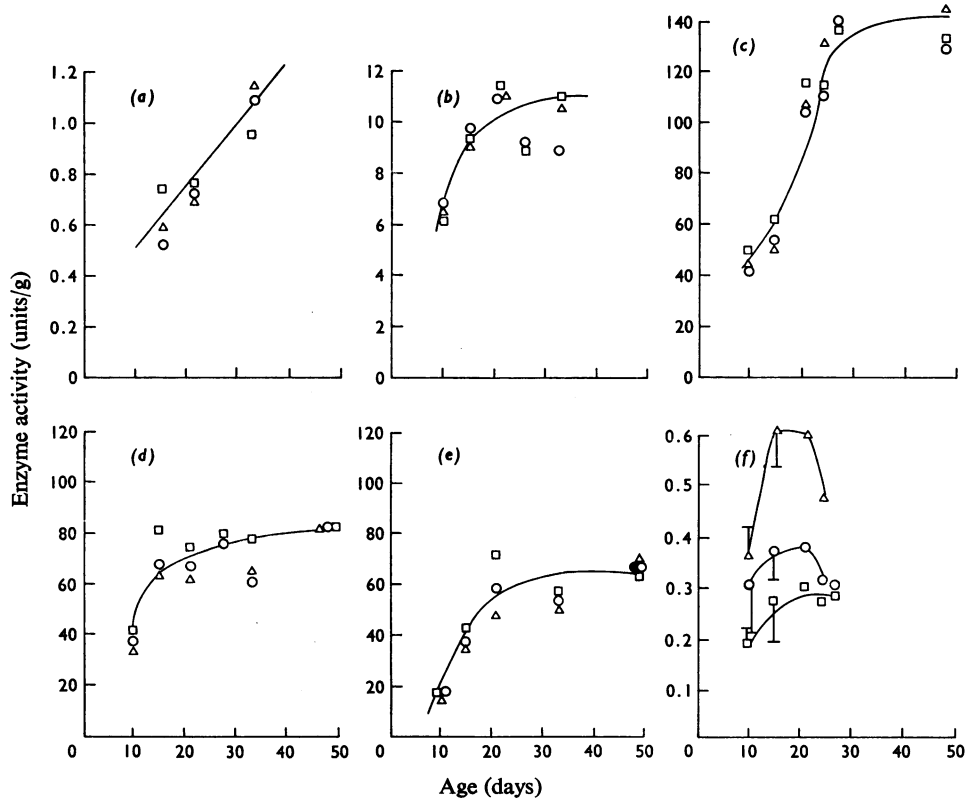

Fig. 6. *Changes in brain enzyme activities with age during chronic hyperphenylalaninaemia*

Animals were chronically treated with NaCl (□), α-methylphenylalanine (○) or *p*-chlorophenylalanine plus phenylalanine (△) as described in Table 6. They were killed 12h after the last injection or at 10:00h during the solid-feeding regimen. Five enzymes (malate–$NADP^+$ dehydrogenase, *a*; hexokinase, *b*; pyruvate kinase, *c*; lactate dehydrogenase, *d*; aspartate aminotransferase, *e*) were determined in the soluble and also in the particulate fraction of sucrose homogenates (as described in the Experimental section). However, since their distributions did not vary, the sum of the two activities was plotted. The other enzyme, phosphoserine phosphatase (*f*), was measured in the soluble fraction of water homogenates. Each point represents an average of duplicate determinations on a sample pooled from three or four rats. Bars indicate ±1 S.D. (at day 10, $n = 4$; at day 15, $n = 3$).

for brain in that the hepatic phosphoserine phosphatase activity of the same animals was unaltered (see also Table 6).

Discussion

The present results indicate that the recently discovered suppressor of phenylalanine hydroxylase, α-methylphenylalanine (Greengard *et al.*, 1976), is a suitable agent for inducing chronic hyperphenylalaninaemia in immature rats. Daily injections of α-methylphenylalanine, or its incorporation into the solid diet, maintains low hepatic phenylalanine hydroxylase activity. During at least 2 weeks of the suckling period, the plasma phenylalanine concentration was 20–40 times above normal throughout 50% of each day and exceeded 6–9 times normal in the ensuing 6h. This degree of hyperphenylalaninaemia, also attained in weaned rats (as determined each morning), is comparable with that reported for phenylketonuric patients (Partington & Lewis, 1963; Knox, 1972).

From the growth curves it is evident that the α-methylphenylalanine-treated animals are healthier than the *p*-chlorophenylalanine-treated animals, and may thus be better candidates for a rat model for phenylketonuria. There is little evidence for a decreased body weight in human subjects with this disease (Jervis, 1954, 1963). Other characteristics of the 'α-methylphenylalanine model', such as negligible mortality rates, absence of cataracts (only one case

out of 36 animals followed for 18 months), also contrast with those of *p*-chlorophenylalanine-treated animals (see also Gralla & Rubin, 1970; Andersen & Guroff, 1972). The slightly decreased brain weights, less pronounced in α-methylphenylalanine- than in *p*-chlorophenylalanine-treated animals, are in accordance with the somewhat smaller brain size and head circumference of phenylketonurics (Crome & Pare, 1960; Knox, 1972).

Neither α-methylphenylalanine nor *p*-chlorophenylalanine suppressed phenylalanine hydroxylase activity completely. Thus (unlike in phenylketonurics) the plasma concentration of tyrosine was elevated throughout the period of chronic hyperphenylalaninaemia, though at most to 1.5–4.5 times above that in control rats of the same age. The renal phenylalanine hydroxylase activity, only 4–7% of the total in the organism, was unchanged in α-methylphenylalanine-treated rats and decreased by 50% in response to *p*-chlorophenylalanine (DelValle & Greengard, 1976).

It was not possible to obtain a more complete suppression of hepatic phenylalanine hydroxylase by the combination of α-methylphenylalanine and *p*-chlorophenylalanine. The simultaneous injection of optimal doses was no more effective than that of either substance alone. The two analogues may act on hepatic phenylalanine hydroxylase by a common mechanism. A further similarity is that loss of the enzyme activity proceeds slowly; minimal activities are attained 18–24h after an injection of either α-methylphenylalanine or *p*-chlorophenylalanine. The activities of four of the hepatic enzymes so far tested were unchanged in both groups of hyperphenylalaninaemic rats, although during *p*-chlorophenylalanine treatment there was an appreciable deficit in total liver weight.

The transient fall in the plasma phenylalanine concentration in α-methylphenylalanine-treated rats just before weaning cannot be attributed to a peak in hepatic phenylalanine hydroxylase, because the same fall, but with no peak in enzyme activity, was seen during treatment with *p*-chlorophenylalanine (cf. Figs. 4*a* and 4*b*). The increase in hepatic phenylalanine–pyruvate aminotransferase seen in both models (Table 6) might contribute to the more rapid clearance of phenylalanine, but this possibility is rendered unlikely by the fact that glucagon-induced increases in phenylalanine–pyruvate aminotransferase were not reflected by enhanced conversion of phenylalanine into phenylpyruvate (Fuller *et al.*, 1972; Brand & Harper, 1974*b*). It is also quite possible that excretory regulations become more efficient shortly before weaning. Whatever developmental changes are responsible for the increase in phenylalanine clearance, the high dietary intake of phenylalanine (initiated at the age of 22 days) compensated for them and restored the degree of hyperphenylalaninaemia seen in the 3–18-day-old experimental animals. Insofar as critical steps in rat brain differentiation may occur between days 18 and 22 after birth, both animal models may be disadvantaged by the trough in plasma phenylalanine concentration during this time. However, more frequent injections of phenylalanine (three or four per day) during this short period can overcome this difficulty.

Behavioural alterations have previously been observed in rats treated with *p*-chlorophenylalanine plus phenylalanine during infancy (Andersen & Guroff, 1972; Andersen *et al.*, 1974). Our own preliminary studies (P. A. de Villier, M. S. Yoss & O. Greengard, unpublished work) indicate an impaired learning performance in 5-month-old rats that had been treated with α-methylphenylalanine and phenylalanine (in standard dosages; see the Experimental section under 'Chronic treatments') from day 3 to day 33 of age. In a running-wheel test, they were slower than the controls ($P<0.03$) and exhibited significantly greater variability in speed of running ($P<0.01$). In a maze-learning test, the experimental animals performed less well than the controls with respect to the number of trials to reach the criterion for acquisition ($P<0.02$) and in the number of errors made in acquisition ($P<0.025$).

Deficiencies in learning ability would be expected to be associated with rather subtle biochemical changes in the brain. The concentration of ten different cerebral enzymes (as judged by their assay in optimally supplemented systems) was unchanged in the chronic hyperphenylalaninaemic animals now studied. The subcellular distribution of hexokinase, lactate dehydrogenase and glutamate decarboxylase suggests that synaptosomes developed normally and were not more fragile than in control brains. Prolonged hyperphenylalaninaemia did not alter the concentration of pyruvate kinase (Fig. 6), although its activity (Miller *et al.*, 1972; Weber *et al.*, 1970) was reported to be inhibited by high concentrations of phenylalanine. Abnormalities in cerebral lipid composition observed in phenylketonurics (see Menkes, 1967) and hyperphenylalaninaemic rats (Geison & Josephson, 1974) have not yet been studied in α-methylphenylalanine-treated rats.

An injection of *p*-chlorophenylalanine decreases the cerebral concentration of 5-hydroxytryptamine, whereas that of α-methylphenylalanine, at least in 6-day-old rats, does not (Greengard *et al.*, 1976). The two analogues, owing to their different structures, would be expected to have different side effects. Only those changes that, like the ones in behaviour, are common to both models, may be attributable to hyperphenylalaninaemia itself. The common chemical abnormality described here is reflected by phosphoserine phosphatase. Its activity under optimal conditions was greater than normal in the brain,

but not in the liver, of the experimental animals. This is the first indication of an increase in the concentration of a gene product in brain during chronic hyperphenylalaninaemia during infancy. Whether or not this alteration and a related modification of serine metabolism are necessarily associated with the behavioural deficit remains to be investigated. Either model is suitable for such studies: in choosing between them, the quantitatively greater increase in cerebral phosphoserine phosphatase, in the *p*-chlorophenylalanine model, has to be weighed against the advantages of using the alternative non-toxic agent α-methylphenylalanine, which permits normal growth.

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