The Effects of Hyperphenylalaninemia on Fetal Development: a New Animal Model of Maternal Phenylketonuria

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Summary

A new model has been developed for the study of maternal phenylketonuria. Beginning on the 12th day of gestation the diet of pregnant rats was supplemented with 0.5% α -methylphenylalanine and 3% phenylalanine. This resulted in an 83% reduction of hepatic phenylalanine hydroxylase activity. The maternal plasma phenylalanine was elevated 10-20-fold for two-thirds of the day, but the degree and persistence of the fetal hyperphenylalaninemia may have been even greater. The brain phenylalanine concentrations in the fetus were raised up to 2900 nmole/g brain, whereas the highest level observed in the dam was 382 nmole/g. Experimentally-treated fetuses showed small reductions in both body and brain weight when compared to age-matched controls; however, no differences were seen in crown to rump length, litter size, DNA and protein concentrations per g, or in postnatal survival. Initiation of the diet at conception rather than on the 12th day caused a significantly greater inhibition of fetal growth, and 21% mortality.

The fetal cerebral concentrations of methionine and the branched chain amino acids (valine, leucine and isoleucine) were decreased by hyperphenylalaninemia. From the 16th day on, the concentration of the inhibitor neurotransmitter glycine was elevated. Cerebral serotonin showed a 20-30% deficit and its primary metabolite 5-hydroxyindoleacetic acid a 71-77% deficit.

Of twelve enzymes quantified in the brains of hyperphenylalaninemic fetuses only phosphoserine phosphatase showed any change. From the 20th to the 22nd day of gestation its activity was 46-67% higher in experimental than in normal fetuses. Measurement on the 22nd day of gestation showed that the increases in phosphoserine phosphatase activity and glycine content were present in brain stem, cerebellum, and forebrain.

Speculation

This new animal model of maternal phenylketonuria is suitable for elucidating the mechanisms of abnormal development of heterozygous children of phenylketonuric mothers. Hyperphenylalaninemia in the dam is responsible for gross elevations in fetal brain phenylalanine content and the biochemical consequences of this elevation, such as perturbations in the levels of glycine, serotonin and branched chain amino acids, which may be responsible for the lasting damage to cerebral function.

Several studies have indicated that mental retardation is associated not only with phenylketonuria but also with the majority of heterozygous infants of phenylketonuric mothers (8, 16, 17, 30, 38). This indicates that permanent damage of cerebral functions can occur if severe hyperphenylalaninemia is restricted to intra-uterine life. The common end result, mental retardation, does not necessarily imply a common pathogenic pathway: the immediate impact of the same metabolic abnormality may well be different

at different stages of ontogeny. Studies of phenylketonurics and rats subjected to experimental hyperphenylalaninemia have provided some insights into the postnatal biochemical changes; however, little is known about those occurring *in utero*. The unavailability of suitable animal models has been one difficulty. Among previously explored methods, only the injection to pregnant rats of phenylalanine together with *p*-chlorophenylalanine was reported to cause significant fetal hyperphenylalaninemia. Studies on this experimental system, however, were severely limited by the toxic side effects and lethality associated with this treatment (1, 7). An alternate suppressor of phenylalanine hydroxylase is α -methylphenylalanine (21), which at least in young postnatal rats, was found to be less toxic than *p*-chlorophenylalanine (11, 29). Animals rendered hyperphenylalaninemic with phenylalanine plus α -methylphenylalanine during infancy, therefore, are now extensively used for study of neurochemical and behavioral abnormalities (15, 18, 24, 25, 26, 29, 36, 43). Whether this treatment is suitable for inducing severe hyperphenylalaninemia in pregnant rats and their fetuses has not yet been tested. This was one purpose of the present study. The other was to compare some of the prenatal effects of excess phenylalanine with known postnatal effects of hyperphenylalaninemia. The results describe a regimen of α -methylphenylalanine plus phenylalanine that induces severe and prolonged hyperphenylalaninemia but does not interfere with the pre- or postnatal survival of the rats. Observations on chemical abnormalities in the developing brain are also presented.

MATERIALS AND METHODS

Animals. Adult female Fischer (CDF) rats were time-mated with males of the same strain. Vaginal smears were taken daily and upon finding a sperm positive smear, the female was designated as one day pregnant. Dams were allowed food and water *ad libitum*. Except where noted, food consisted of Purina Lab Chow until the 12th day of gestation, at which time the experimental rats were switched to pellets of the same chow supplemented with 0.5% D,L- α -methylphenylalanine (Sigma, St. Louis, MO) and 3% phenylalanine. Rats were maintained on a 12-h light (0700-1900 h)/dark cycle.

Dams were decapitated between 10 and 11 A.M. on the appropriate gestational day and trunk blood was collected in a heparinized tube. The fetuses were then removed, blotted dry on a paper towel, weighed and measured for crown-to-rump length. Following decapitation, fetal blood was collected in heparinized capillary tubes. Fetal brains were removed, weighed, and when appropriate, dissected into cerebellum, brain stem and the remaining forebrain.

Assays. The blood was centrifuged for 5 min and the plasma deproteinized with 2 or 4 volumes of trichloroacetic acid (TCA, final concentration 0.3 M). Brain tissue for phenylalanine analysis was homogenized with 2 or 4 volumes of TCA (final concentration 0.3 M) and centrifuged for 20 min at 105,000 X g. Phenylalanine was measured in deproteinized plasma and brain supernatants

fluorometrically by the method of McCaman and Robins (37) as modified by Faulkner (14).

Brain tissue for glycine analysis was prepared by addition of TCA to water homogenates (final concentration 0.3 M). Glycine content was estimated by a micromodification of the method of Goodwin and Stampwala (19). A 200 μ l incubation mixture containing 0.1 M H_2SO_4 , 0.2% chloramine-T plus 0.15 M TCA (final concentrations) and 100 μ l of deproteinized supernatant was heated at 100°C for 10 min. It was rapidly cooled and then heated at 100°C for 30 min after the addition of 1.8 ml of chromotropic acid reagent in H_2SO_4 . Absorbance at 570 nm was determined using a Zeiss spectrophotometer PM6. Recovery of glycine was 95–100% and was not altered by the addition of α -methylphenylalanine or phenylalanine to a final concentration of 2.5 mM or 5 mM. This concentration of phenylalanine is greater than the highest one found in the brain of experimental rats. The concentrations of other amino acids were measured in a Beckman 120 C automatic analyzer. For this purpose brain samples were homogenized in 5 volumes of 0.6 M TCA and centrifuged. Plasma samples were deproteinized with 5 volumes of 3% sulfosalicylic acid. Serotonin and 5-hydroxyindoleacetic acid (5-HIAA) were measured by the method of Curzon and Green (9). Enzyme activities, assayed under optimal conditions, are reported in units per g. One unit represents the metabolism of (1) nmole of substrate per min. For the assay of phosphoserine phosphatase (E.C. 3.1.3.3.) fetal brain was homogenized in ice-cold water and (after removing a sample for glycine analysis) centrifuged at 105,000 X g for 30 min. The undialyzed supernatant was assayed by the method of Knox, *et al* (28). For the assay of the other enzymes a pool of fetal brains was homogenized in 9 volumes of 0.15 M KCl and centrifuged for 30 min at 105,000 X g. The resulting supernatant fluid was used for the determination of hexokinase (39) (E.C. 2.7.1.1.), glutamate dehydrogenase (39) (E.C. 1.4.1.2.), aspartate aminotransferase (39) (E.C. 2.6.1.1.) and malate dehydrogenase (47) (E.C. 1.1.1.37). The assay of hepatic phenylalanine hydroxylase (E.C. 1.14.16.1) was as described by DelValle and Greengard (12). Protein was measured by the method of Lowry, *et al* (34) and DNA by the procedure of Kissane and Robins (27) as modified by Hinegardner (23).

RESULTS

Although subcutaneous or intraperitoneal injections of phenylalanine plus α -methylphenylalanine have been our usual method of inducing severe hyperphenylalaninemia (12, 21, 25), some observations on 22–33-day-old rats indicated that similar results could be obtained by the incorporation of these substances into solid food (11). To avoid daily injections, this diet (see "Materials and Methods") was given to pregnant rats. It reduced their hepatic phenylalanine hydroxylase activity by 83%, *i.e.*, from 601 to 104 units/g. The inhibition was thus similar to that seen in immature rats (21). Because rats are nocturnal eaters, the degree of hyperphenylalaninemia induced by the experimental diet might be expected to be higher during the night than during the daytime. Observations on blood sampled at different times throughout the day indicated that this indeed was the case. The plasma phenylalanine levels of the pregnant rats on the experimental diet were highest during the dark period (*i.e.*, at midnight, 2 A.M. or 6 A.M.). They were 30–50 and 60% lower at 10 P.M. and 6 P.M., respectively. In all subsequent experiments the animals were killed between 10 and 11 A.M.; therefore, the 10–20-fold elevations in maternal plasma phenylalanine (see Table 1) represent low estimates, that is, lower than those prevailing during two-thirds of each day. This should also be true for elevations in the phenylalanine content of fetal plasma since these correlate ($r = 0.92$ $P < 0.0001$) with the levels seen in the corresponding dam (see Fig. 1); however, the absolute levels were nearly three times higher in the fetuses. This is apparent from the values averaged for the different dams (1086 ± 359 nmole/ml as opposed to 2922 ± 801 in the fetuses) as well as from individual comparisons (see Table 1).

Table 1. The phenylalanine content of maternal and fetal plasma and brain¹

	Gestational day	Dam		Fetus	
		nmole/ml plasma	nmole/g brain	nmole/ml plasma	nmole/g brain
Control	19–22	96 $\pm 31(6)$	80 $\pm 17(5)$	243 $\pm 62(6)$	292 $\pm 52(6)$
Experimental	17	965			2935
	18	878	376		2173
	19	1166	372		1521
	20	895			2400
		810	177	2850	1176
	21	1440		3447	1894
		1822	191	3660	1606
	22	885	382	1647	1414
		1254	291	3843 F; 3414 M	2056 F; 1981 M
		690	282	2454 F; 2064 M	1751
NB		472		822	726
		120	82	936	588

¹Rats were started on the experimental diet on the 12th day of gestation. They were killed, as were the dams on the control diet, between 10–11 A.M. on the gestational day indicated. The control values are means $\pm$ S.D. of results on the indicated number of dams, or of results on one fetal brain and pooled fetal blood from six different litters. Under "Experimental," data on each horizontal line refer to the same dam, and to one brain and pooled plasma of fetuses born to that dam. Values on brain or pooled plasma followed by M and F refer to male and female fetuses, respectively. NB refers to pups of experimental dams assayed 4–6 h after birth.

The results of experiments in which we also determined the phenylalanine concentration of the fetal brains are illustrated in Table 1. There was a 4–10-fold (mean $\pm$ S.D. = 6.5 ± 1.7) elevation in the brain phenylalanine concentration of experimental fetuses compared with controls; however, only a 2–5-fold elevation (3.7 ± 1.1) was seen in the maternal brain. This is clear not only from the absolute values (third and last columns of Table 1) but also from the brain/plasma phenylalanine ratios, which were 0.10–0.43 (0.29 ± 0.13) in the dams but no less than 0.41 (0.67 ± 0.18) in their fetuses analyzed at the same time.

The elevations in the phenylalanine content of fetal plasma or brain showed no consistent variation with gestational age. Nor, as illustrated for the last prenatal day, did they differ between male and female fetuses of the same dam. The degree of hyperphenylalaninemia decreased rapidly during neonatal life. Three to five h after birth, fetal brain and plasma phenylalanine levels were only about four times that of controls (last two lines of Table 1).

The experimental interference caused small decreases in body and brain weight, but not in the crown-to-rump length of the fetuses (Table 2). The weight deficits were apparent by the 20th day, and were of the same magnitude (10–11%) on the 22nd day of gestation. The litter sizes of the experimental (9.5 ± 1.8) and control (8.9 ± 2.1) dams were not significantly different. Also, all of the dams appeared to be healthy and to sustain normal weight gains during pregnancy. Table 2 also shows that, in the experimental fetuses, the amount of DNA and protein per g brain was normal.

Several dams were allowed to give birth and their offspring were followed for growth and survival. None of the dams on the experimental diet delivered before term. Their litter sizes were normal and no obvious anatomical malformations could be detected. Of the offspring followed until 170 days of age, one whole control litter (out of five) and two of six experimental litters died for unknown reasons before the 60th day of life. In the remaining

Fig. 1. Relationship of fetal to maternal plasma phenylalanine concentrations. Blood was sampled between 10–11 A.M. on the 20th, 21st or 22nd day of gestation in normal and hyperphenylalaninemic dams. Each point refers to the plasma of one dam (ordinate) and to a pool of plasma collected from its fetuses (abscissa).

litters 36 of the 37 control offsprings reached weaning age (22nd postnatal day) and 34 adulthood. Forty-one of 44 experimental animals reached weaning age and 34 of 34 (one whole litter host) attained adulthood (lines 6 and 7 of Table 3).

The experimental offspring showed a small but significant body weight deficit at birth, but this was made up by the time of weaning (Table 3). The decrease in brain growth seen in the offspring is also recovered by females in adulthood; however, male rats continue to show a very small but significant brain weight deficit, even though body weight had recovered from the early insult (lines 4 and 5 of Table 3).

The offspring of rats on the experimental diet from conception exhibited even greater deficits in body weight at birth, but had normal weights at the time of weaning. Survival at the time of weaning in this group was only 79%.

The results on the measurements of amino acids in brain and plasma are shown in Table 4. The cerebral concentrations of methionine and the branched chain amino acids valine, leucine, and isoleucine were all decreased in both the dam and fetus exposed to hyperphenylalaninemia. There was no consistent re-

lationship between the cerebral level of these amino acids in the fetus and the fetal plasma values. This contrasts with increases in the cerebral concentrations of phenylalanine and tyrosine, which reflect changes in the plasma. Serine, lysine, threonine and glutamate plus glutamine are examples of amino acids whose concentrations were not changed in the fetal brain in response to hyperphenylalaninemia.

Changes in the fetal cerebral concentration of another amino acid and putative inhibitory neurotransmitter, glycine, are described in detail in Table 5. In normal fetal brain it decreased between the 16th and last day of gestation. This is in general agreement with the observations of Davis and Himwich (22). Hyperphenylalaninemia prevented this decrease. Every experimental fetus had higher brain glycine concentrations than age-matched controls on gestational days 16–22. This increase was seen in forebrain, cerebellum and brainstem on day 22; however, the level of another putative neurotransmitter, serotonin, showed a 20–30% deficit in the brains of hyperphenylalaninemic 20-, 21-, and 22-day-old, fetuses (Table 4). The concentration of the major serotonin metabolite, 5-HIAA, decreased by 71–77%; control values on fetal days 20, 21 and 22 were 0.81 , 0.46 and 1.33 ± 0.14 (3) $\mu\text{moles per g}$ respectively, whereas fetuses (of the same age) exposed to high phenylalanine had only 0.19 , 0.11 and 0.39 ± 0.05 (3) $\mu\text{mole/g}$ respectively. During the first postnatal day, cerebral levels of serotonin (Table 4) and 5-HIAA (control = 1.04 , 1.14 experimental = 1.08) returned to normal.

Gestational hyperphenylalaninemia had no effect on the activity per g brain of hexokinase, malate dehydrogenase or aspartate amino transferase (Table 5). Of twelve enzymes quantitated in the brain only phosphoserine phosphatase, which is involved in glycine synthesis, responded to hyperphenylalaninemia (Table 5). In normal brain its activity rose during the 16–18th days of gestation. In experimental fetal brains, its activity was clearly above normal from the 19th to the 22nd day of gestation. As shown for the 22nd gestational day, the increase was seen in the brain stem and cerebellum as well as in the forebrain.

DISCUSSION

This study shows that by incorporating α -methylphenylalanine and excess phenylalanine into the solid diet of pregnant rats it is possible to sustain high levels of phenylalanine in the fetal blood and brain. In light of clinical reports implicating even modest hyperphenylalaninemia in the subsequent damage to offspring (38), the 7–15-fold elevation in rat fetal plasma phenylalanine seems to constitute an appropriate model for human gestational phenylketonuria. The maternal plasma phenylalanine is elevated 10–20-fold for two-thirds of the day, but the degree and persistence of the fetal hyperphenylalaninemia may be even greater (see Table 1 and Text). This follows from our demonstration of a 2.5–3-fold concentration ratio of fetal to maternal plasma phenylalanine, and from studies which indicate that the placenta acts as a reservoir and lengthens the time during which elevations of phenylalanine in the fetal plasma (in response to those in the maternal plasma) persist (31, 50).

The enduring consequences of hyperphenylalaninemia may be due to the accumulation of excess phenylalanine in the brain. In our model system, we see the preferential accumulation of phenylalanine in the fetal brain. There is a 4–10-fold elevation in fetal brain, in contrast to a 2–5-fold increase in the brain of the dam. Also, the brain to plasma phenylalanine ratios are much higher in the fetus (0.67) than in the dam (0.29). Thus, the brain phenylalanine concentrations in the fetus were raised up to 2900 nmole/g brain, whereas the highest level observed in the dam is 382 nmole/g. The fetal/maternal plasma ratio, and the brain/plasma phenylalanine ratios in the dam and fetus are all in agreement with previous work using Fischer rats (1). These differences, seen at two age extremes (fetal and adult) point to the importance of brain transport processes in the age-dependent vulnerability of the central nervous system to hyperphenylalaninemia. Similarly, genetic differences in transport processes between individuals with similar plasma phenylalanine elevations (2) may lead to variation

Table 2. Effect of hyperphenylalaninemia on fetal body and brain growth¹

Days of gestation	Body length (cm)		Body weight (g)		Brain weight (mg)		Brain DNA concentration (mg/g)		Brain protein concentration (mg/g)	
	C	E	C	E	C	E	C	E	C	E
16	1.08	1.06	0.23	0.24	44.4	34.3	5.7	6.49	67	64
			0.24		34.1				57	
17	1.28	1.25	0.41	0.38	52.4	46.8			67(3)	70
	±0.14	±0.13	±0.02	±0.02	±3.7	±4.5			±1	±1(3)
18	1.76	1.53	0.82	0.75	85	71				70
19	2.05	2.02	1.10	1.14	90.5	91	4.57	4.70	69	71
									61	58
20	2.67	2.73	1.98	1.78	122.6	110.7			59	70
	2.61	2.69	1.86	1.80	113.8	104.1			66	
21	3.29	2.98	3.06	2.81	153	129.7			59	
	3.10	3.11	3.21	2.83	150	139			±6(3)	
22	3.65	3.52	4.46	3.95	183.9	165.5	3.74	3.95		61
	±0.18	±0.18	±0.12	±0.26 ¹	±4.6	±9.3 ¹	±0.39	±0.36	±2	±8

¹Dams were placed on the experimental diet during the 12th day of gestation and sacrificed on the indicated day. Values without S.D. refer to the average of measurements (of length, body or brain weight) on individual fetuses within a litter, or to measurements of (DNA and protein) on pooled brains of fetuses of the same litter. Values with S.D. refer to the means of such results on 3–4 different litters. The superscript ¹ in the table indicates significant differences ($P < 0.01$) between the experimental (E) and control (C) results.

Table 3. The postnatal growth and survival of rats hyperphenylalaninemic during gestation¹

	Newborn		Adult	
			Male	Female
	Body weight (g)			
C	4.59 ± 0.25(37)		260(14)	153(19)
			±20	±12
E ₁	4.25 ± 0.32 ² (44)		240(16)	159(18)
			±36	±14
E ₂	3.90 ± 0.44 ^{2, 4} (37)			
	Brain weight (mg)			
	Male	Female		
C	219(11)	211(14)	1790(14)	1680(18)
	±13	±13	±30	±60
E ₁	197 (6) ²	200(10) ³	1720(16) ²	1660(18)
	±10	±11	±60	±60
	Survival			
	From birth to 21 days		From 22 days to 170 days	
C	97%		92%	
E ₁	91%		100%	
E ₂	79%			

¹ Offspring of control dams (C) and those on the experimental diet from the 12th to the last gestational days (E₁) were counted and weighed at birth on the 22nd day after birth (just before weaning) and at the age of 170 days. Rats exposed to hyperphenylalaninemia from the first day of gestation (E₂) were weighed at birth and at 22 days of age. The adult brain weights refer to the same rats whereas the newborn brain weights refer to a separate group that received the same treatment during gestation but were sacrificed on the first postnatal day. The values for brain and body weights are means ± S.D. of results on the number of individuals in parentheses. Survival is expressed in % born, with the numbers at birth (see in parentheses under newborn body weight) taken as 100.

² $P < 0.01$ from control.

³ $P < 0.025$ from control.

⁴ $P < 0.01$ from E₁.

in central nervous system phenylalanine content, possibly explaining the relatively normal brain development of some hyperphenylalaninemic individuals.

Several workers have used dietary phenylalanine or a daily

maternal injection of phenylalanine to mimic maternal phenylketonuria (4, 35), but in doing so monitored plasma phenylalanine levels at one time only. Such treatments are ineffective in raising phenylalanine concentrations for any length of time (32). Other workers have administered phenylalanine plus the phenylalanine hydroxylase inhibitor *p*-chlorophenylalanine to the dam to induce fetal hyperphenylalaninemia (1, 5, 7). This treatment was more successful in elevating plasma phenylalanine levels than previous models of maternal phenylketonuria. It was accompanied by pup mortality and has not been clearly shown to drastically elevate fetal plasma phenylalanine levels for long periods each day. Our present treatment maintained a 10–20-fold elevation in fetal plasma phenylalanine for over 16 h per day. Even the fluctuations in phenylalanine levels in our model mimic the human condition, where plasma phenylalanine levels also vary during the day in response to food intake.

The quantitative alterations in amino acid levels, of course, are not identical to the human disease state. In particular, the plasma tyrosine levels of adult phenylketonuric women (or men) are normal or low normal (42), whereas in our experimental dams, owing to the incomplete suppression by α -methylphenylalanine of the hepatic phenylalanine hydroxylase (21), the tyrosine levels are elevated. With respect to fetal tyrosine content, however, the discrepancy should be less pronounced. The human fetal liver, by about the tenth wk, attains at least 50% of its adult phenylalanine hydroxylase activity (13, 45). Although the phenylketonuric mother is devoid of the enzyme, its heterozygous fetus (with at least 25% of the normal adult phenylalanine hydroxylase activity) should be able to convert some of the excess circulating phenylalanine to raise its own tyrosine levels to even above normal. This may explain the 'high normal' tyrosine content of the amniotic fluid of phenylketonuric women (48). Furthermore, since the plasma tyrosine levels of these women do not correlate with the I.Q. of their children (30) (whereas phenylalanine levels do), it does not appear that low maternal plasma tyrosine is central to the pathogenesis of maternal phenylketonuria. Among the inevitable shortcomings of an animal model of gestational phenylketonuria, the deviation here with respect to maternal tyrosine levels is thus not a crucial one; however, the possibility that an undesirable interaction between the elevated tyrosine and phenylalanine takes place cannot be excluded.

Neither α -methylphenylalanine nor the hyperphenylalaninemia produced by its dietary introduction to the pregnant rat with 3% phenylalanine is lethal to the developing fetus. Rats maintained on this diet from the 12th day of gestation showed no changes in litter size or in the postnatal survival of offspring until adulthood (Table 3). Thus, the diet used in these studies is not accompanied

Table 4. *The effect of gestational hyperphenylalaninemia on branched chain amino acids in brain and plasma*¹

Amino acid	Maternal plasma		Maternal brain		Fetal plasma		Fetal brain	
	C	E	C	E	C	E	C	E
Phenylalanine	36	1095	32	188	136	1572	80	1065
Tyrosine	59	178	30	30	274	384	148	178
Valine	104	227	48	28	426	418	193	130
Leucine	76	155	40	28	279	310	148	123
Isoleucine	53	115	20	12	161	152	80	60
	25	45	8	5	92	65	28	20
Serotonin, gestational age	$\mu\text{mole/g}$							
20	0.94							
21	1.11							
22	1.29 $\pm$ 0.10							
NB	1.26, 1.10							

¹Experimental rats (E) were placed on the phenylalanine plus α -methylphenylalanine diet 12 days after conception. One such rat and a control (C) were sacrificed on the 22nd day of gestation. The maternal tissues, the pooled plasma, and brains of all fetuses from the same dam were assayed in the automatic amino acid analyzer (see first six lines of data). Values are expressed as nmol/ml of plasma or brain. Another set of dams given the same diet was killed on the indicated gestational day or allowed to give birth. The serotonin results on their progeny refer to a pool of two brains from a fetal or a new newborn litter or to means $\pm$ S.D. (see 22nd gestational day) of results on such pools from three different experimental and three different control litters. For concentrations of 5-hydroxyindoleacetic acid, see text.

Table 5. *The hyperphenylalaninemia-induced elevation of glycine and phosphoserine phosphatase activity in fetal rat brain*¹

Gestational day	Treat- ment	Glycine	Phosphoserine phos- phatase (PP)	Hexokinase	Aspartate ami- notransferase	Malate dehydro- genase	
16	C	1.83, 2.02	67, 68				
	E	2.38	74				
17	C	1.73 $\pm$ 0.07 (3)	125 $\pm$ 13 (3)				
	E	1.84 $\pm$ 0.02 (3)	132 $\pm$ 18 (3)				
18	C	1.35	161				
19	C	1.34, 1.46	174, 176	3.00	17.5		
	E	1.60	195				
20-21	C	1.37 $\pm$ 0.06 (3)	168, 159	2.70, 2.74	12.2, 13.6	36.2	
	E	1.90 $\pm$ 0.18* (4)	252 $\pm$ 20 (4)	2.50 $\pm$ 0.36	13.4	32.2, 37.0	
22	C	1.37, 1.19	166 $\pm$ 15 (3)	2.90, 2.91	13.1	49.1	
	E	2.01 $\pm$ 0.32 (3)	242 $\pm$ 44* (3)	2.90 $\pm$ 0.35 (3)	11.1, 12.8	48.8, 36.0	
NB	C	1.89 $\pm$ 0.14 (8)	172 $\pm$ 4 (3)				
	E	2.01 $\pm$ 0.21 (3)	253				
		Forebrain		Cerebellum		Brain stem	
		<u>GLY</u>	<u>PP</u>	<u>GLY</u>	<u>PP</u>	<u>GLY</u>	<u>PP</u>
C		1.34	176, 179	1.17	115, 99	2.56	143, 131
E		2.12	345	2.17	203	3.18	202

¹Dams placed on the experimental diet on day 12 of gestation (E) or controls (C) were killed on the indicated days. The attainment of elevated fetal brain phenylalanine levels (as represented in Table 1) was verified in each case. A single fetal brain was used for each glycine determination, whereas a pool of fetal brains from one litter was used for the measurement of enzyme activities (units/g fetal brain). The values refer to a single litter or to the mean $\pm$ S.D. of results on the number of litters in parentheses. Values for brain parts refer to the 22nd gestational day. NB, 10-24 h after birth.

*Significantly different ($P < 0.01$) from control.

by any gross toxic side effects and allows animals to be raised for behavioral studies. The high rate of mortality (1) or maternal muricide (7), resulting from treatment with *p*-chlorophenylalanine (plus phenylalanine), must be specific to this analogue since these effects are not seen when a more severe hyperphenylalaninemia over a longer period of gestation is induced using α -methylphenylalanine (plus phenylalanine). Similarly, high mortality is observed when suckling rats are made hyperphenylalaninemic with injections of *p*-chlorophenylalanine plus phenylalanine but not when α -methylphenylalanine plus phenylalanine is used (11, 29).

Hyperphenylalaninemia itself can be lethal to fetal development, but only at levels higher than those achieved with our usual treatment. Although all five dams placed on our standard experimental diet from day one of conception gave birth to normal size litters, 79% of which survived to 22 days, none of the ten dams

placed on a diet containing 7% phenylalanine (plus 0.5% α -methylphenylalanine) gave birth at all. Neither fetuses nor placentae were found in two dams on the latter diet when examined at 17 days of gestational age. This suggests that very high phenylalanine may block implantation of the embryo or cause very early fetal resorption.

The treatment caused a deficit in two specific growth parameters, fetal body and brain weight (Table 2), but did not delay all aspects of maturation. Other indices of gestational age, such as crown-to-rump length (Table 2) and several enzyme activities were unchanged. This reduction is in accord with clinical observations on the children of phenylketonuric mothers (16, 17, 30, 38). Although the concentration (mg/g) of cerebral protein and DNA (Table 2) remained normal, the decrease in total brain weight in the hyperphenylalaninemic fetuses must be due to a

reduction in the total number of cells and not a decrease in cell size.

Chemical interferences during ontogeny are usually seen to affect systems that develop particularly rapidly at the time of insult. It was not surprising that hyperphenylalaninemia during the 12th–22nd days of gestation, a period during which more than 95% of the fetal increment in body weight occurs, resulted in deficits in this parameter; however, it was unexpected that extending our experimental treatment back from day 12 to day 1 of gestation would cause a significantly increased deficit in the neonatal weight of gestationally hyperphenylalaninemic rats (line 3 in Table 3). This deficit was overcome by the 22nd postnatal day, but only 79% of them survived to this age. These results have important clinical implications. They indicate that very early hyperphenylalaninemia can interfere with fetal growth and postnatal survival. These effects emphasize the vulnerability of the young fetus to hyperphenylalaninemia and thus the necessity of immediate dietary treatment of identified pregnant phenylketonurics.

The only well understood mechanism underlying chemical alterations in the brains of phenylketonurics, and of animal models of the disease, is the inhibition by excess phenylalanine of the cerebral uptake of amino acids sharing a common transport system (3, 6, 33, 41). The present results show that this mechanism is operative in the 22-day-old fetus as well (see methionine, leucine, isoleucine and valine in Table 4). We also observed a decrease in the cerebral concentration of the putative neurotransmitter, serotonin, and its major metabolite, 5-HIAA. This is similar to decreases seen in phenylketonuric children (40) and in neonatal rats made hyperphenylalaninemic (25, 29). The fact that cerebral serotonin (and 5-HIAA) are not lowered in the experimental neonate, whose plasma phenylalanine levels rapidly return toward normal, is consistent with observations on suckling rats. The reduction in serotonin concentration appears to be a transient response directly related to several-fold elevations in phenylalanine within the past several h (25). This contrasts with the change in cerebral glycine in suckling rats which lasts several days after the cessation of hyperphenylalaninemia.

As previously demonstrated in suckling rats (25), the imposition of hyperphenylalaninemia in the fetus is also associated with increased cerebral glycine content as well as an elevation in the activity of phosphoserine phosphatase. This is the enzyme catalyzing the conversion of phosphoserine to serine, the principle precursor of cerebral glycine (10). These brain constituents were not measured in adult rats, which had been subjected to gestational hyperphenylalaninemia, but have been shown to return to normal levels in older rats that were hyperphenylalaninemic in early postnatal life. That altered glycine may play a central role in the neuropathogenesis of phenylketonuria is suggested by the fact that another inborn error of metabolism, nonketotic hyperglycinemia, is hallmarked by a 2–3-fold elevation in cerebral glycine as well as by severe neurologic abnormalities and mental retardation (44). Although the cerebral glycine content of phenylketonurics is unknown and their cerebrospinal fluid levels of glycine are normal, the elevated serine concentration in their cerebrospinal fluid (49) may possibly suggest some abnormalities in the intracerebral regulation of this pathway.

This new model should facilitate the study of the effects of hyperphenylalaninemia at different stages of neural development. Such studies are important for the information they may yield on normal neural development, on the selective effects of early hyperphenylalaninemia, and more generally, on the neural changes that may underlie 'mental retardation' states. Chemical interferences applied to intact systems have often proved useful in gaining insight into 'normal' processes. Hyperphenylalaninemia has been shown to have dramatic effects postnatally on the cytoarchitectural development of the rat cerebellum (24), a brain area maturing relatively late. Gestational hyperphenylalaninemia might have an impact on other earlier developing brain areas. Because information on the timing of cell differentiation and migration of various cell groups in the rodent brain now exists (46), application of our

experimental treatment during restricted periods of gestation may elucidate the effects of hyperphenylalaninemia on different brain areas or particular cell groups. Secondary effects of temporally selective interferences may also provide insight into the unfolding of normal ontogeny.

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