Unimpaired Learning Ability of Rats Made Artificially Phenylketonuric During Fetal or Neonatal Life.* (30159)

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The mechanism by which a failure of hydroxylation of phenylalanine to tyrosine in the liver in turn produces mental defect in human phenylketonuria is not known. Woolley and van der Hoeven(1,2) have suggested recently that mental defect in experimental phenylketonuria in mice is related to low brain serotonin concentrations in early life, and they report that mental defect can be prevented if these animals are given 5-hydroxytryptophan or melatonin. These authors suggest that the mental defect characteristic of human phenylketonuria may be the result of serotonin deficiency in the brain during infancy(1,3).

It has been demonstrated repeatedly that artificial phenylketonuria in experimental animals, produced by feeding excessive amounts of phenylalanine, results in a lowering of brain serotonin concentrations. This has been shown for rats(4-9), mice(10), and guinea pigs(11). Whether or not brain serotonin concentrations are low in naturally occurring phenylketonuria in man has not been determined. Pare *et al*(12) noted a defect in the 5-hydroxyindole pathway of tryptophan metabolism in phenylketonurics, and were the first to suggest that a failure in serotonin production might play some part in the pathogenesis of the mental deficiency. Subsequently it was shown that human phenylketonurics excrete much less serotonin in urine than do normal subjects(13), and that this is due to a defect in the 5-hydroxylation of tryptophan, which can be reversed by a reduction of tissue phenylalanine concentrations to normal levels with the use of a low-phenylalanine diet(14).

Mental defect or behavioral deficit has been reported to occur in artificially phenylketonuric monkeys(15) and rats(4,16). In each of these studies, however, animals were tested while still on high-phenylalanine diets, and the possibility was not excluded that their poor performance on various psychological tests was due merely to the immediate non-specific toxic effects of high tissue phenylalanine concentrations. Woolley and van der Hoeven(1,2) subjected artificially phenylketonuric mice to testing a short time after the conclusion of dietary phenylalanine loading; but even in their experiments it is not clear that the animals' learning ability was permanently impaired(17).

We undertook the investigation described below in an effort first to determine whether a true persistent learning defect can be produced in rats made artificially phenylketonuric during the period of rapid brain growth, and second to explore the possible causal relationship of low brain serotonin to any learning deficit which might be produced.

Methods. Artificial phenylketonuria was produced in newborn white rats of the Sprague-Dawley strain by means of repeated subcutaneous injections of a saturated aqueous solution of *L*-phenylalanine. Oral tube feeding of phenylalanine to newborn rats with sufficient frequency to cause a continuous elevation of tissue phenylalanine concentration did not seem technically feasible to us; and preliminary experiments showed that frequently repeated intraperitoneal injections into newborn rats were associated with excessive mortality.

In one experiment, newborn litter-mate pups were assigned at random immediately after birth to one of 3 groups, and were left undisturbed with foster mothers until approximately 24 hours old. One group then received 4 subcutaneous injections of phenylalanine per day at 6-hour intervals, to give a daily phenylalanine dose of 4 g/kg body weight.

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A second group received phenylalanine injections on the same schedule, but *DL*-5-hydroxytryptophan (5-HTP) was added to the phenylalanine solution in an amount sufficient to give each animal a daily dose of 5-HTP equal to 4 mg/kg. The third group of pups was injected every 6 hours with distilled water. Injections were continued for 72 hours, or until the rats were 4 days old. It was found impossible to inject rats every 6 hours for longer than 3 days without incurring excessive mortality.

In a second experiment, newborn littermate rats were similarly assigned at random to 3 groups, and were injected subcutaneously every 8 hours for 7 days, or from the ages of 1 day to 8 days. In this experiment, one group of pups received phenylalanine alone in a daily dosage of 2.4 g/kg, a second group received the same dosage of phenylalanine plus 5-HTP in a daily dosage of 4.2 mg/kg, and the third control group was injected with water only. Injections could not be continued for longer than 7 full days, because of marked growth failure in the phenylalanine-injected rats, and because abscesses developed at injection sites in some of the animals.

In a third experiment, artificial phenylketonuria was produced in pregnant female rats, in order to assess the effects of high tissue phenylalanine concentrations on the brains of fetal rats. Female rats were mated and were fed ground rat chow containing 7% added *L*-phenylalanine. When such a diet was fed pregnant rats during the last 10 days of pregnancy, all pups died within a few hours of birth, even if placed immediately with foster mothers having an adequate milk supply. When a high-phenylalanine diet was fed only during the last 8 days of pregnancy, however, about half of the newborn rats survived if placed promptly with foster mothers. In this manner viable litters were obtained from rats fed 7% added phenylalanine for the last 8 or 8½ days of pregnancy, as well as from phenylalanine-fed rats that had in addition been given 8-hourly injections of 5-HTP during the same period in a daily dosage of 9 mg/kg. Newborn rats from untreated pregnant females born at the same time served as controls.

Serum phenylalanine concentrations were determined on infant rats and pregnant female rats by the method of McCaman and Robins(18), urinary phenylpyruvic acid was determined qualitatively by the ferric chloride test, and the urinary amino acids were determined semi-quantitatively by 2-dimensional paper chromatography. Brain serotonin concentrations were measured with a spectrophotofluorometric technique(19). Individual brain serotonin concentrations were determined on 8-day-old rats given phenylalanine injections during the neonatal period, but the small size of the brain necessitated pooling the brains of 4 or more pups from each litter born to rats given a high phenylalanine diet during the last 8 days of pregnancy. Such animals were sacrificed for brain serotonin determination within 3 hours of birth.

Young rats surviving the experimental procedures were weaned at the age of 21 days, and when 6 to 8 weeks old were tested for their ability to acquire visual discrimination. Testing was carried out by one of us (G.M.L.) who remained unaware of the treatment each group of animals had received until the evaluation was completed. The apparatus used for training and testing was a Lon Davis Skinner Box. All rats were first trained to press a lever for food on a variable ratio schedule (3:1) of reward until a steady rate of response was obtained. The period of training lasted 8 to 10 days. The rats then were tested for their ability to discriminate between the presence and absence of light. During the period when the light was on, lever-pressing was rewarded by food, while during the period when the light was off, bar-pressing was not reinforced. Tests of discrimination were performed on each animal for 25 minutes per day. The duration of each test period was 5 minutes, and the "light on" and "light off" periods were alternated. On alternate days, 3 of each of the 5-minute periods were reinforced and 2 were not, while on the other days the reverse order was followed. Marked reduction in the number of bar-presses during a 5-minute period when

TABLE I. Serum Phenylalanine and Brain Serotonin Concentrations of Control and Phenylketonuric Infant Rats.

Injections received	Serum phenylalanine, mg/100 ml	Brain serotonin, μg/g
Water (7)	2.5 ± .26	.44 ± .018
Phenylalanine (6)	45.8 ± 2.33	.32 ± .006
Phenylalanine and 5-HTP (8)	40.2 ± 2.59	.34 ± .024

Numbers in parentheses indicate No. of animals in each group.

Values expressed as mean and standard error for each group.

Injections given every 8 hr, with phenylalanine daily dosage 2.4 g/kg, and 5-HTP daily dosage 4.2 mg/kg.

the light was off was interpreted as a measure of the rats' ability to acquire visual discrimination, or to "learn."

Results. Biochemical findings. Urine voided by infant rats receiving 6-hourly injections of phenylalanine (daily dosage 4 g/kg) was found to contain both phenylpyruvic acid and large amounts of phenylalanine and tyrosine. Infant rats receiving 8-hourly injections of phenylalanine (daily dosage 2.4 g/kg) also excreted large amounts of phenylalanine and tyrosine, but phenylpyruvic acid was not regularly found in their urine. Some animals in the groups receiving the lower daily phenylalanine dosage (2.4 g/kg) and the higher 5-HTP dosage (4.2 mg/kg) were sacrificed at the age of 8 days, to obtain blood for serum phenylalanine determinations as well as to measure brain serotonin concentrations. The results of these determinations are shown in Table I. It can be seen that injections of phenylalanine on the lower of the 2 dosage schedules used did render the infant rats chemically phenylketonuric, and the elevated serum phenylalanine concentrations achieved were as high as those found in untreated human phenylketonuria. Brain serotonin concentration was reduced by approximately 25% in the phenylalanine-injected newborn rats killed on the last day of injections, as compared with their water-injected controls, and simultaneous injection of 5-HTP failed to elevate brain serotonin.

Pregnant rats fed a diet to which 7% phenylalanine had been added also had elevated serum phenylalanine concentrations, although these varied more widely than those of infant rats injected with phenylalanine, presumably due to variations in food consumption during the hours immediately before blood specimens were obtained. Eleven pregnant rats on the high phenylalanine diet had a mean serum phenylalanine concentration of 30 mg per 100 ml (range 6 to 61), while 17 rats fed a normal diet had a mean serum phenylalanine concentration of 1.8 mg per 100 ml (range 1.4 to 2.4). Pregnant rats fed the high-phenylalanine diet also excreted phenylpyruvic acid and large quantities of phenylalanine and tyrosine in urine, and they clearly had been made chemically phenylketonuric.

Table II shows brain serotonin concentrations of newborn rats from litters produced by pregnant females given high-phenylalanine diet alone, high-phenylalanine diet plus injections of 5-HTP, as well as no treatment. In addition, figures are included for the brain serotonin concentrations of litters of normal fetal rats removed from the uterus during the last 5 days of pregnancy. Although the number of litters examined was small, exposure to high maternal serum phenylalanine concentrations *in utero* did not appear to have low-

TABLE II. Brain Serotonin Concentrations of Offspring of Normal and Phenylketonuric Pregnant Rats.

Age of litter	Treatment received by pregnant rat	Brain serotonin of litter, μg/g
17-day fetuses (12)	None	<.15
18 " " (12)	"	.20
19 " " (11)	"	.24
20 " " (11)	"	.26
21 " " (11)	"	.29
Newborn rats (5)	None	.31
" " (5)	"	.37
" " (4)	"	.38
" " (4)	"	.40
Newborn rats (6)	Phenylalanine	.29
" " (4)	"	.31
" " (4)	"	.35
Newborn rats (6)	Phenylalanine and 5-HTP	.27
" " (4)	*Idem*	.36

Numbers in parentheses indicate No. of brains pooled.

7% *L*-phenylalanine added to diet last 8 days of pregnancy. 5-HTP given in 8-hourly injections for daily dosage of 9 mg/kg for last 8 days of pregnancy.

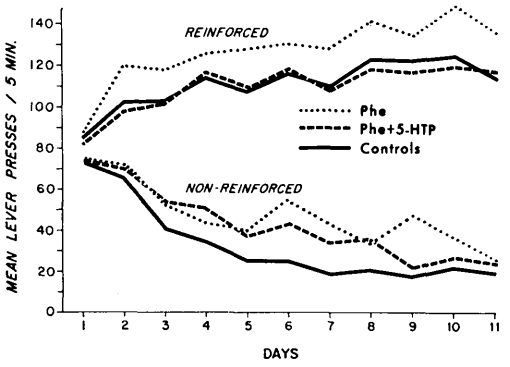

FIG. 1. Mean lever presses during periods of reinforcement and non-reinforcement of rats exposed to excessive phenylalanine during fetal life.

ered the brain serotonin concentration of newborn rats appreciably. It was also apparent that brain serotonin concentration is very low in normal fetal rats, and begins to rise only at or shortly before the time of birth.

Learning ability. Of the newborn rats injected every 6 hours for 3 days, 7 control rats, 4 phenylalanine-injected rats, and 3 rats injected with both phenylalanine and 5-HTP survived to be tested for their ability to acquire visual discrimination. Of the newborn rats injected every 8 hours for 7 days, 14 control rats, 21 phenylalanine-injected rats, and 19 rats given both phenylalanine and 5-HTP survived to the completion of testing. No significant difference in ability to acquire visual discrimination could be demonstrated between these groups of rats, and the tester was unable to ascertain what treatment each group had been given.

Six offspring of pregnant rats fed a high-phenylalanine diet, and 13 offspring of rats given injections of 5-HTP in addition to the high-phenylalanine diet survived to be tested. They were compared with a group of 11 animals born at the same time to control pregnant rats. Again no significant difference in ability to acquire visual discrimination could be demonstrated between the 3 groups. The data for these groups of rats are illustrated graphically in Fig. 1.

Inspection of Fig. 1 might suggest that the phenylalanine-treated rats acquired visual discrimination more slowly than the control animals. To test this possibility, an analysis of variance was made of scores taken from points on the graph in which the largest difference appeared between groups (day 6). The scores used in the analysis were the differences between the number of bar presses for the "light on" and "light off" periods for each rat. The F value obtained for the 3 groups yielded a figure of 1.13, which for 2 and 27 degrees of freedom is 2.51 at the 10% level.

Discussion. The experiments described above demonstrate that induction of artificial phenylketonuria in the rat in the perinatal period failed to produce a detectable permanent learning defect. Elevated tissue phenylalanine concentrations achieved in our newborn animals were comparable to those which occur in human phenylketonuria. Although serum phenylalanine was not measured in the rats exposed to the amino acid *in utero,* it is probable that tissue concentrations in the fetuses were very high. Boggs and Waisman (20) found that fetal plasma phenylalanine concentrations averaged twice as high as the maternal concentrations when pregnant rats were fed a high-phenylalanine diet. Administration of phenylalanine in greater amounts to newborn rats, or exposure of fetal rats to excessive phenylalanine for longer than the last 8 or 9 days of pregnancy, is not compatible with survival of the animals.

Exposure of the human fetus to high maternal serum phenylalanine concentrations is known to produce mental defect, since non-phenylketonuric children born to phenylketonuric mothers are mentally retarded(21). In addition, a delay of several months in instituting the low-phenylalanine diet in phenylketonuric infants usually results in readily detectable permanent mental deficiency. The periods of exposure to excessive phenylalanine of the fetal and infant rats in our experiments were probably comparable to those which are known to produce mental defect in man. It is entirely possible that learning defect cannot be produced in the rat at any age by artificial phenylketonuria. Our experience agrees with that of Perez(22), who failed to demonstrate mental defect in rats injected for as long as 60 days from birth with a somewhat lower dosage of phenylalanine than we used. It is not un-

reasonable to suppose that abnormally elevated tissue phenylalanine concentrations during the period of rapid brain growth might damage the highly developed central nervous system of man, and yet leave the more primitive brain of rodents unharmed.

Artificial phenylketonuria in our newborn rats resulted in a reduction in brain serotonin concentrations, but these were not corrected by administration of 5-HTP in a dosage more than ten times that which might be expected to be synthesized by a normal animal. This could well be explained by the known interference of high tissue concentrations of phenylalanine with the active transport of 5-HTP into brain(8,23,24). Although the number of pooled brains of newborn rats examined for serotonin content after intrauterine exposure to excessive phenylalanine was small, there was no evidence that artificial phenylketonuria results in depression of brain serotonin in the fetal rat. The low serotonin concentrations found in brain in newborn rats and in rats during the last 4 days of pregnancy agree with those found by Karki *et al*(25).

The very low serotonin content of brain in late fetal and newborn normal rats, as well as the fact that low brain serotonin concentrations in infant rats with neonatal phenylketonuria were not followed by impaired learning ability, lend no support to the theory that the mental defect of phenylketonuria in man is mediated through serotonin deficiency at a crucial period during brain growth. Our findings in the rat do not exclude the possible validity of this theory in man. They do, however, suggest that further experiments to study any association between brain serotonin deficiency and permanent learning defect in phenylketonuria might be more profitably conducted in primates. In addition, administration of 5-HTP to human phenylketonuric infants to correct a hypothesized deficiency of brain serotonin, as previously suggested(14,26), would not appear to be warranted, in view of the failure of large amounts of 5-HTP to elevate depressed brain serotonin concentrations in phenylketonuric rats.

Summary. Rats made artificially phenylketonuric during fetal or early neonatal life by administration of large amounts of phenylalanine showed no defect in learning ability when tested later for capacity to acquire visual discrimination. Administration of large amounts of 5-HTP failed to correct the low brain serotonin concentrations characteristic of artificial phenylketonuria. The results of this investigation do not provide support for the theory that a serotonin deficiency in brain during infancy causes the mental defect characteristic of human phenylketonuria. In addition, it appears unlikely that the rat is a suitable experimental animal for exploring the exact mechanism of the mental defect of phenylketonuria in man.

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Differentiation *in vitro* of Embryonic Cartilage and Bone in a Chemically-Defined Medium.* (30160)

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Fell(1) first reported the cultivation of limb bud mesenchyme of the chick embryo and described osteogenesis *in vitro*; this study has been confirmed by other investigators(2-4). The purpose of the present communication is to report *in vitro* osteogenesis in posterior limb rudiments from 14-day-old embryonic mice. A completely defined chemical medium, containing neither plasma, embryo extract, nor any biologic supplement, was employed.

Materials and methods. Fourteen-day-old mouse (C57BL/6J) embryos were used. The mating system was as follows: (1) males were placed with females at about 4 PM; (2) between 8 and 9 AM the next day, the females were examined for vaginal plugs; (3) most mating occurred between midnight and 2 AM; therefore a "14-day embryo" is actually about 14 days and 8 hours post-mating.

The culture medium MAB87/3 (Table I) used in these experiments is one designed by one of us (C.W.) primarily for establishment of cultures from freshly explanted cells and tissues. The only notable way in which it differs from media designed for cell strains, *e.g.*, medium MD705/1(5), is in the inclusion of insulin. Other differences are mainly quantitative.

The entire embryo was separated from the uterus and fetal membranes and immersed promptly in Hanks' solution(6) at room temperature. After the embryos were removed from this solution, a posterior limb was dissected from each. This was then placed in a small drop of MAB87/3 culture medium on a tissue culture coverglass (3 × 0.7 cm). The coverglass was introduced into a 3.5 cm diameter Carrel flask (containing 1 ml of the liquid medium) which was closed by a sterile rubber stopper. The flasks were incubated at 37°C for periods of 3, 5, and 12 days. The culture medium was changed every 4 or 5 days. At the end of the appointed time of incubation, the limb was placed in Bouin's fixative, dehydrated and embedded in paraffin. Serial sections of the undecalcified limbs were cut at 10 μ. They were stained with hematoxylin and eosin, or with the periodic acid-Schiff stain.

Four embryos of the same age from the same litter served as controls. Without dissection, the entire embryos were placed in fixative and prepared histologically like the experimental series.

Results. Serial sections of the limbs from the controls showed the bone primordia to be composed of only cartilage. No collagen, osteoid or osteocytes were visible by trans-

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