EXPERIMENTAL PHENYLKETONURIA IN RATS: AN INVESTIGATION OF THE CRITICAL INTERVAL HYPOTHESIS

by

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The purpose of this experiment was to simulate PKU in rats and to determine if there were a critical period during which an increase of PA in the system has its most detrimental effect.

Fifty-one Sprague-Dawley rats were paired at birth with a litter mate of the same sex and randomly assigned to one of three feeding conditions for administering the PA and control diets: beginning at birth (E-1, C-1), beginning at 18 days of age (E-2, C-2) and beginning at 36 days of age (E-3, C-3). Until weaning, E-1 and C-1 were fed by intubation. After weaning experimental animals were fed a 7% PA diet.

Six days after terminating the PA and control diets, experimental Ss were compared with their pair-fed controls in a six-unit water maze for six days. On the first day through the reverse path of the maze, E-1 made significantly more errors than C-1. On both reverse days, E-1 and E-2 made significantly more errors than E-3. The former results implied that E-1 had greater difficulty than C-1 in extinguishing a no-longer-correct response, a characteristic of brain injured and low mentality Ss as in PKU. The latter results also indicated that both E-1 and E-2 had difficulty extinguishing an incorrect response and in part supported a critical-period hypothesis. The lack of a systematic impairment of behavior in E-1 throughout the maze testing was attributed to a "ceiling effect" inherent in the maze.

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INTRODUCTION

In 1934 A. Folling, a medical researcher, examined two mentally retarded, Norwegian children (Folling 1934). Their mother had reported a peculiar odor arising from their urine. When Folling mixed their urine with 10% ferric chloride, it turned green. He subsequently deduced that the musty odor was produced by the excretion of phenylpyruvic acid (PPA), a metabolite of phenylalanine (PA), not found in the urine of normal people. This disease is now called phenylketonuria (PKU).

Phenylketonuria is an "inborn error of metabolism" transmitted by an autosomal recessive gene. It is one pleiotropic effect of a single genetic lesion that greatly reduces the production of phenylalanine hydroxylase (PAH), a liver enzyme which converts PA to tyrosine (TY). Consequently, there is an excess of PA in the system. Biochemically, this excess results in an increased PA plasma level (from 2 mg.% to 15-60 mg.%), an increase of PPA and other phenylketones in the urine, the inhibition of dihydroxyphenylalanine (DOPA) by derivatives of PA and a sequential reduction of norepinephrine and epinephrine, and a decrease of 5-hydroxy-tryptophane and its indole metabolites in the cerebrospinal fluid (Bickis, Kennedy, and Quastel, 1957; Jervis, 1953; Nadler and Hsia, 1961; Pare, Sandler, and Stacey, 1957; Pare, 1957).

The salient characteristic of PKU is the mental retardation which accompanies the disease. Bjornson (1964) stated that 98% of all phenyl-ketonurics have an IQ below 50; of these, 84% have an IQ below 30. However, Knox (1960) has shown that phenylketonurics improve mentally and the biochemical aberations disappear when they are placed on a low PA diet. An

interaction between nature and nurture is apparent; hence, producing a model of PKU is important. It may explain why an excess of PA in the system of phenylketonurics causes idiocy, and it would be an ideal means by which to study the relationships between genetics, biochemistry, and behavior.

Experimental Model: The first model for the experimental induction of PKU was proposed by Auerbach, Waisman, and Wyckoff (1958). Since the production of PAH is greatly reduced in the phenylketonuric organism, an increase of PA and a decrease of TY occurs. They attempted to simulate these biochemical conditions by administering larger amounts of PA than the system normally could metabolize coupled with an excess of TY, which should reduce the end product feedback. Accordingly, they administered a diet mixed with 2.5% L-TY and DL-PA to eight rats with another eight serving as controls. Under conditions of water deprivation, a marked retardation in temporal discrimination characterized the experimental rats. However, the experimenters failed to use pair-fed controls and the results may be simply attributable to malnutritional deficiencies. Also, the biochemical criteria for PKU were not adequately simulated. That is, the plasma PA level was raised only from 1.6 mg.% to 2.8 mg.%.

The model proposed by Auerbach et al. (1958) raised many questions. Particularly, is the administration of L-TY and DL-PA an effective means of simulating PKU? What are the optimal amounts of such drugs to be administered to best simulate the disease? At what age should these drugs first be administered? What kind of controls should be used? And what behavioral tests best distinguish between experimental and control animals?

Some possible answers to these questions have been explored by various investigators during the last eight years.

Woolley and Hoeven (1964a) administered DL-PA and L-TY to newborn mice. They used DL-PA because of the longer persistence of the D-isomer and L-TY because it suppresses the biosynthesis of PAH. On a conditioned avoidance task the experimental animals did more poorly than the controls. Bender and Krebs (1960) investigated whether or not the administration of D-PA increased the PA plasma level. They demonstrated that D-PA could be deaminated by D-amino acid oxidase in the kidney to form PPA . This fact implies that the excretion of PPA in the urine may not be due to an excess of PA but rather to a characteristic of the D-isomer. Later experimenters have used the L-isomer with promising results (Yuwiler and Louttit, 1961; Wang and Waisman, 1961). Moreover, Woods and McCormick (1964) have shown that an increase of L-PA significantly depresses the PAH activity. The administration of L-TY as an effective inhibitor of PAH has also been questioned. Waisman (1960) concluded that L-TY fails to raise PA levels and, in fact, increases the PAH activity. It seems that the administration of L-PA by itself may be an adequate means of inducing PKU.

The effect of feeding varying amounts of L-PA has been explored by Wang and Waisman (1961). Weanling rats 18 days of age were fed 3.5, 5.0, or 7.0% L-PA in a ground commercial chow. In order to maintain uniform PA plasma levels, an attempt was made to regulate the feeding habits of some of the animals by alternating the lighting conditions from light to dark every three hours. This light-dark cycling had little effect on rats fed 5.0 or 7.0% L-PA. Nevertheless, 7.0% L-PA produced continuously high PA plasma levels, adverse growth and phenylketones as high as 4 mg. per

100 ml. PPA. Five per cent L-PA also affected growth but produced high PA plasma levels that subsided one-half hour after eating. Three and one-half per cent L-PA produced no change, either physically or chemically. However, the PAH activity was reduced 40-60% regardless of the dosage. Boggs and Waisman (1964) fed 21-day-old rats 5 or 7% L-PA until they were 50-60 days of age. They reported high plasma PA levels (26 mg.%) and high urinary phenylketone levels comparable to those found in PKU children. Although the high TY level was not similar to the low TY level of PKU children, the PAH activity was decreased by 50%. It appears that 7% L-PA is an optimal dosage to simulate PKU biochemically.

Investigators have initially administered PA to experimental organisms at varying ages. The ages of initial administration of PA fall into three groups: prenatally, at birth and after weaning.

Polidora, Boggs, and Waisman (1963) investigated the behavioral deficit associated with PKU in rats. They began feeding PA to 21-day-old weanlings. On a water-filled multiple-T maze, the experimental rats displayed a clearcut behavioral deficit. However, this deficit was questioned due to the absence of pair-fed controls. Yuwiler and Louttit (1961) used weanlings 17 days of age to discover the effects of a PA diet on brain serotonin levels. Experimentals received 5 gm./kg. body weight of L-PA and conflicting results were obtained. Animals fed PA made more errors on the Hebb-Williams maze yet needed fewer trials to meet a criterion on a successive discrimination problem than their controls.

Other researchers have administered PA to newborn animals. Perez and Schmidt (1963) injected PA intraperitoneally in rats from birth. The

injections were given daily for 60 days. Experimental males were found to be retarded both in growth and in their performance in a Lashley III maze. Females performed more poorly but not significantly more poorly than their controls. Woolley and Hoeven (1964a) fed neonatal mice PA plus TY by a stomach tube. Animals receiving these amino acids displayed subnormal learning ability in a T maze.

Loo, Diller, and Owen (1962) have attempted to insult the intermediary metabolism of PA in foetal rats by feeding PA to their mothers Beginning at four weeks of age the young experimental Ss were fed the same diet for 16 weeks. Control animals developed a conditioned avoidance response readily; PA fed rats failed to learn the same response.

Although different initial ages of administration were used in the above studies, there were no reliable behavioral results peculiar to a particular age. A lack of reliability may be attributed to other variables. Some of the studies attempted to produce PKU beginning with rats 17-21 days of age, which is equivalent to a human of age 1.7 - 1.9 years of age. However, the biochemical effects of PKU are most detrimental during the early period of the child's life. That is, up to four or five years of age, impairment of intelligence is commensurate with the age at which the phenylketonuric infant is introduced to a low PA diet (Knox, 1960). Since the impairment of IQ in PKU is developmental, one should begin to administer PA from birth to produce the greatest impairment of behavior in rats.

Three methods for administering PA in preweanlings have been used. Perry, Ling, Hansen, and MacDougal (1965), Perez (1964) and Perez and Schmidt (1963) injected PA into animals. Although the amount of the drug

injected was accurately regulated, mortality was high. As many as 70% died. Also, there was no consistent behavioral effect. Other investigators, for example, Boggs, Polidora, and Waisman (1962) and Loo et al. (1962) fed PA to neonates via their lactating mother whose diet was rich in the amino acid. Again, mortality was high in the young, and there was poor regulation of the amount of PA consumed by neonates. Woolley and Hoeven (1964a) fed PA to newborn mice by intubation. This method facilitated regulation of the consumption of this amino acid while minimizing infant mortality. In a pilot study by the author, Woolley and Hoeven's method of intubation was successfully modified. Neonates were permitted to suckle a polyethylene tube which was adapted to a syringe.

In order to account for the effect of an independent variable, adequate controls must be established. Wang and Waisman (1961) demonstrated the adverse effect of PA on growth in rats. Apparently, the amino acid imbalance produced by feeding rats an excess of PA results in a voluntary decrease in food intake. This self-imposed starvation may produce symptoms similar to malnutrition disorders like kwashiorkor. Cowley and Griesel (1964) have simulated the kwashiorkor syndrome by feeding rats low protein diets. These rats were retarded in growth and made more errors on the Hebb-Williams test of intelligence than their controls. In other words, the behavioral effect produced by feeding rats PA may simply be related to malnutrition.

Polidora et al. (1963) and Boggs and Waisman (1964) have attempted to control for the effects of malnutrition from feeding PA by using pair-fed , control animals. Pair-fed controls are fed the same amount of food

their experimental counterparts consume ad lib. Hence, paired control and experimental animals consume equal amounts of food. Although pair feeding may not take into account the amino acid imbalance, it does control for any malnutritional effects to the extent that the latter are not dependent on the amino acid imbalance.

Coleman (1960) reported that certain strains of mice had diminished PAH activity ranging from 14-86% of normal. They were characterized by diluted pigmentation, extreme nervousness and susceptibility to audiogenic seizures. Yuwiler and Louttit (1961) and Louttit (1962) accounted for possible genetic differences by employing a split litter technique wherein animals were randomly assigned to control and experimental groups. The split litter technique minimizes differences in PAH activity and other genetic abnormalities.

Many types of behavioral tests have been used by investigators to distinguish control from experimental animals fed PA. The Hebb-Williams test and a black and white successive discrimination problem were employed by Yuwiler and Louttit (1961). The results were conflicting. That is, animals fed PA made more errors on the Hebb-Williams maze and needed fewer trials to meet the criterion on the successive discrimination problem. Perez and Schmidt (1963) failed to obtain significant differences using a Lashley III maze. Auerbach et al. (1958) reported marked retardation in temporal discrimination by experimental animals fed PA. All of the above studies used either water or food as a reward.

Boggs and Polidora (1963) recognized that the self-imposed decrease in food intake by phenylketonuric rats may cause hypermotivation. In

other words, water and food are confounding types of reinforcement. Moreover, hypermotivation is not necessarily controlled for by pair feeding, since the pair-fed Ss essentially are on a deprivation schedule. According to Yuwiler and Louttit (1961) pair feeding eliminates group differences related to body weight but may introduce those related to differential hunger.

The confounding effects of food and water as reinforcement may be prevented by using avoidance conditioning. Loo et al. (1962) trained rats to turn a wheel to avoid shock when a sound was presented. Control animals developed the conditioned avoidance readily, while the PA fed rats failed. Woolley and Hoeven (1963) have shown promising results using a T maze with escape from bright lights and the view of the experimenter as the reward. Phototropism in rats was described by Crozier and Pincus (1926). Neonatal rats are heliotropic but after their eyes open they tend to move toward darkened places. In a pilot study by the author, Woolley and Hoeven's assay was slightly modified. However, the results failed to differentiate performances better than chance. Consequently, this behavioral assay was discarded. Moreover, escape from shock may be confounded by skin resistant changes produced by the PA diet. Also, escape from light may be increased by heightened skin sensitivity from an excess of PA which, in fact, decreases pigmentation.

A six-choice, water maze similar to the one described by Biel (1940) has been successfully used by Polidora et al. (1963). The use of water avoidance has been explored with promising results in a pilot study by the author. Biel's maze was slightly modified and test animals easily learned the conditioned avoidance response. The Wisconsin group has

recently published an account of their refinement of the water maze assay (Polidora et al., 1966). This procedure will be used in the proposed study for it appears to be ideally suited to the study of PKU.

Bruhl, Arnesen and Bruhl (1964) distinguished between the toxic syndrome and the irreversible component in experimental PKU. The toxic syndrome is characterized by a reversal in behavioral impairment when excess PA is reduced. The irreversible component refers to the permanent brain damage caused by an excess of PA. Perry et al. (1965) inferred that in many cases the poor performance by experimental animals fed PA was possibly due to the immediate, nonspecific, toxic effects of high PA levels. He cites in particular, studies by Yuwiler and Louttit (1961) and Polidora et al. (1963) in which Ss were still on the PA diet when tested.

McMillan (1964) shed further light on the distinction between the toxic syndrome and the irreversible component in PKU when he criticized Woolley's failure to distinguish between learning and performance (Woolley and Hoeven 1963, 1964a). Woolley had purported to show "changes in learning ability" as a function of the amount of serotonin in the brain produced by an excess of PA in the diet. McMillan claimed that the phrase "changes in learning ability" was not justified by the experimental design, inasmuch as the drug effect was not restricted to the learning period. Woolley, himself, found no difference in performance when mice were retested several days after the cessation of treatments. This last finding seems to indicate that the treated mice showed neither better nor poorer "learning ability" than the untreated mice. In other words, it was performance rather than learning that was affected by the experimental condition.

To completely simulate PKU in rats, one must produce an irreversible impairment of behavior. The irreversible component should be distinguished from the toxic syndrome. This distinction can be made by permitting the high PA levels produced by a PA diet to drop before testing.

Serotonin Hypothesis: The question of why an excess of PA in the system causes idiocy has not yet been adequately answered. It is known that an excess of PA produces a decrease of 5-hydroxytryptophane (5-HTP) in the brain. This decrease indicates that the serotonin (5-HT) pathway via 5-HTP is suppressed by PA and its immediate metabolic products (Culley 1962). In fact, Renson, Weissbach, and Undenfriend (1962) demonstrated that the enzyme PAH which hydroxylates PA also hydroxylates tryptophane to make 5-HTP, the precursor of 5-HT. If PAH is absent or inhibited, as in PKU, then tryptophane is not hydroxylated to 5-HT and a consequent reduction of 5-HT follows. Some researchers have suggested that it might be this 5-HT deficiency, which is present from birth in phenylketonuric humans, that gives rise to idiocy. In other words, the primary genetic lesion may be only remotely related to the metabolic phenomena which produce the clinical syndrome.

If a deficiency of 5-HT produced by an excess of PA causes idiocy, one might prevent this deficiency by administering 5-HT congeners. Woolley and Hoeven (1964b) induced PKU in mice by administering PA and TY from birth until maturity. These mice displayed subnormal maze performance. However, this performance deficit was not displayed by mice which received 5-HT congeners, such as melatonin or 5-HTP, simultaneously with the experimental diet. This study indicates that it is not the direct effect of PA but rather a decrease in 5-HT which produced the deficit in maze performance.

In a similar experiment, Woolley and Hoeven (1964a) fed mice reserpine and chlorpromazine from birth until maturity. Reserpine and chlorpromazine are antimetabolites of 5-HT and consequently reduce the 5-HT content. Each of these drugs produced subnormal learning abilities similar to those produced by administering PA and TY. These two experiments seem to corroborate the serotonin hypothesis about PKU.

Proposal: The proposed investigation was designed to determine if there is a critical period during which the administration of PA has its greatest effect. To determine if there is a critical period, the drug is initially administered to rats at varying ages. Polidora, Cunningham, and Waisman (1966a) rejected the critical interval hypothesis and suggested that the magnitude of the induced behavioral effects was directly related to the total quantity of PA consumed. Unlike Polidora's experiment which dealt with postweanling rats, the proposed experiment, in addition to dealing with postweanlings, also deals with neonatal rats during their first 18 days of life. These first 18 days are hypothesized to be the critical period for inducing experimental PKU.

METHOD

Subjects: Fifty-one Sprague-Dawley rats were used. At birth Ss were randomly paired with a litter mate of the same sex. Sexing eliminated possible hypermotivation due to the differential amounts of food consumed by opposite sexes. One member of each pair was randomly designated the pair-fed control for the other S, who was assigned to the PA diet. All unpaired Ss were assigned to the PA diet.

Diet: Each pair was randomly assigned to one of three feeding conditions for administering the experimental and control diets: beginning at birth (E-1, C-1), beginning at 18 days of age (E-2, C-2), and beginning at 36 days of age (E-3, C-3). In order to control for Polidora's hypothesis (Polidora et al., 1966a, 1966b) that the magnitude of the induced behavioral effects was directly related to the total consumption of PA, E-1, E-2, and E-3 needed to consume equivalent amounts of PA. Since E-1 consumed less than one gm. of PA during their first 18 days, there was a negligible difference in total PA consumption between E-1 and E-2; hence, E-1 and E-2 were both fed the PA diet until 54 days of age. The PA diet was fed to E-3 until they had consumed as much as their litter mates in E-1.

Two types of L-PA diet were used in the experiment. An L-PA suspension was administered to E-1 from birth until 18 days of age. Since L-PA is relatively insoluble in water, an optimal concentration was established: 35 mg. of L-PA per 1 ml. of a 7% sucrose solution heated to 40 degrees C. Both the sugar and the temperature made the suspension appear to be more palatable to the neonate. A 7% L-PA mixture was fed to postweanlings.

This mixture was prepared so that x gm. of L-PA divided by x gm. of L-PA plus y gm. of ground Purina Rat Chow equalled 7%; this was equivalent to mixing 75 gm. of L-PA with 1000 gm. of chow. The control for all feeding intervals was ground Purina Rat Chow.

To insure that the amount of PA ingested by experimental rats was consistent throughout the experiment, the amount of PA administered to preweanlings was proportional to their PAH activity and the amount consumed ad lib. by postweanlings. Freedland, Krakowski, and Waisman (1961) demonstrated that the PAH activity varies with age, increasing asymptotically as the rat matures. That is, from age seven to eighteen days the PAH activity is constant at 23.0 units per gm. of liver. This is a 230% increase from age one to six days, when it is also constant at 10 units per gm. of liver. About 28 days of age the PAH activity levels off with four times the activity on day one. In other words, if 50-day old rats fed a 7% PA diet consumed .525 gm. per 100 gm. body weight per day (demonstrated in a pilot study by the author), then one to six day old neonates should consume .13 gm. of PA per 100 gm. of body weight per day because the PAH activity of the latter is 25% of the former. Likewise, rats seven to eighteen days of age, whose PAH activity is 58% of the mature rat, should consume .3 gm. of PA per 100 gm. of body weight per day.

The method of administering PA to neonates was patterned after the technique of intubation described by Woolley and Hoeven (1964a). Their technique of forced feeding was modified permitting neonates to suckle a polyethylene tube that was adapted to a one ml. syringe. Within 36 hours after birth the PA suspension was administered to E-1 with C-1 receiving

an equal amount of the same solution without the PA. Both E-1 and C-1 were fed five times a day at two-hour intervals. Depending on their body weight and PAH activity, E-1 was administered .05 ml. to .6 ml. of the PA suspension per feeding. All animals were permitted to feed ad lib. from their mothers. Dams were fed Purina Rat Chow throughout pregnancy and during nursing.

At 18 days of age all Ss were weaned and placed in individual cages. The 7% L-PA diet was administered to E-1 and E-2 immediately after weaning and to E-3 18 days after weaning. From weaning until 36 days of age, E-3 consumed the control diet. All experimentals were fed ad lib. throughout the experiment and the amount was recorded. Each control S was fed as much of the control diet as its pair-fed litter mate consumed of the L-PA diet. C-3 was fed ad lib. until 36 days of age. Six days prior to maze testing all Ss were fed the control diet ad lib. Ad lib. water was available to all Ss.

Apparatus: A six-unit, water-filled T-maze was fashioned after the one described by Biel (1940). The maze was 60 x 60 in. across by 18 in. high with a six in. wide channel. The traverse length was 150 in. which terminated in a 60 in. straight channel. It was constructed out of 1/2 in. marine plywood and placed in a plastic tank 96 in. in diameter and 20 in. high. The tank was filled with 12 in. of water and maintained at a temperature of 23.5 degrees C. plus or minus one degree.

Procedure: E-1 and C-1 were weighed every day from birth until 18 days of age to determine the amount of L-PA suspension to be administered. After weaning, all Ss were weighed at nine-day intervals until the cessation of the experimental diet and thereafter at six day intervals until the end of the maze testing to determine relative growth rates.

Two biochemical criteria were chosen as standards by which to measure the degree of PKU in rats. One was the excretion of PPA in the urine. The other was the concentration of PA in the blood plasma. Either one or both of these criteria have been used by investigators as standards for measuring the degree of experimental PKU (Boggs and Waisman, 1964; Perez, Loo et al., 1962; Perry et al., 1965).

Beginning at 18 days of age and thereafter at 9 day intervals urine was collected from experimental Ss ingesting L-PA and from their respective controls. The urine was collected in a metabolic cage and then tested with Phenistix. Phenistix has been demonstrated to be a reliable means of detecting PPA in the urine (Baird, 1958). The test is performed by wetting the impregnated end of the paper strip with urine and comparing it to a color scale.

Beginning at 9 days of age and thereafter at 9 day intervals blood was collected from experimental Ss ingesting L-PA and from their respective controls. The method of collection was similar to the one described by Burhoe (1940) for securing small quantities of blood. The distal end of the tail was anesthetized by spraying it with Frigiderm, a topical anesthesia. One of three caudal veins was cut with a scapel and blood was absorbed with a preweighed 1/4 in. disc of filter paper. The wound was treated with collodion. The blood-discs were stored at -10 degrees C. until weighed again prior to being autoclaved. The two weighings determined the amount of blood on each disc and hence, the relative amount of PA per unit volume of blood. The PA concentration was assayed by the test designed by Gutherie (1961). The test is a modified inhibition technique

using Bacillus subtilus which forms clones around the blood-discs, their size varying with the amount of PA in the blood.

Maze testing was begun six days after terminating the experimental and paired control diets and lasted six days. On day one, each S received five pretraining trials, each trial consisting of placing the S in one end of the 60 in. terminal channel. Elapsed time between entering the water and touching the exit ramp was recorded.

On days two, three, and four, Ss received five "forward" trials per day through the maze, during which the S was placed at the end of the terminal channel. Four measures were recorded during each trial: elapsed time between start and entrance into the terminal channel (maze transit time); elapsed time between entry of the nose into the terminal channel and finish (terminal transit time); total number of entries of the whole body into a cul (errors); and the sequence of errors from start to finish.

On days five and six, total transit time and errors were recorded on five "reverse" trials per day. Reverse trials differed from forward trials in that Ss were placed in the maze at the end of the terminal channel and required to swim to the starting point -- the reverse of the route learned on days two, three, and four, though not the reverse sequence of turns.

The Ss were allowed to retrace in the maze, but if on any trial the S had not gained exit in 150 seconds, it was removed from the water and run again when next scheduled. If this occurred, the scores recorded were 150 seconds for maze transit time, 20 seconds for terminal transit time (on days two through four), and the actual number of errors made to that point. At least ten minutes intervened between trials for a given S. Each S was dried with a towel after each trial.

RESULTS

The L-PA diet markedly retarded the growth of experimental Ss. At weaning the average weight of all Ss was approximately 35 gm. with an average difference in weight of 4% between experimental Ss and their pair-fed controls. Thereafter, until the end of the experiment, E-1 grew in weight about 50% less than C-1, E-2 grew in weight about 40% less than C-2, while E-3 grew in weight only four per cent less than C-3. Of the total sample of 51 Ss, nine died in the course of the experiment, five on the L-PA diet and four on the control diet. Of those who died the average weight at weaning was 28 gm.

Due to the death of nine Ss, the original 24 matched pairs were reduced to 16 matched pairs, five in E-1, C-1, five in E-2, C-2, and six in E-3, C-3. In view of this small number of matched pairs, the use of nonparametric statistics seemed appropriate.

The Wilcoxon matched-pairs signed-ranks test was used to determine if there were significant differences between experimental animals and their pair-fed controls within each feeding condition (Siegel, 1956). A one-tailed test of significance was employed. All comparisons of swimming speed on test days one, two through four, five through six, and terminal speed as well as errors on test days two through four and five through six were nonsignificant. On test day five, the first day through the reverse path of the maze, E-1 made more errors than C-1. The difference was statistically significant at the .05 level. There was no significant difference between E-2 and C-2 or E-3 and C-3 on day five.

The Kruskal-Wallis one-way analysis of variance by ranks was used to determine if there were significant differences between E-1, E-2, and E-3 and between E-1 minus C-1, E-2 minus C-2, and E-3 minus C-3 (Siegel 1956). A one-tailed test of significance was used. All comparisons of swimming speed as well as errors on test days two through four were non-significant. On test days five and five through six, E-1 and E-2 made more errors than E-3, significant at the .05 level.

The administration of L-PA to experimental Ss increased their average plasma PA level from four to above twenty mg.% and increased their average PPA level from zero to forty mg.%. Six days after the termination of the L-PA diet these levels had returned to normal. The plasma PA level and the PPA level of the pair-fed controls increased slightly from four to ten mg.%, respectively. These increases were directly proportional to the duration the control Ss were fed the regulated control diet. These levels also returned to normal after six days of ad lib. feeding on the control diet.

DISCUSSION

The adverse effect of PA on growth in rats was evident in this experiment. Although each member of each matched pair received the same amount of food, E-1 and E-2 grew in weight 40-50% less than their pair-fed controls. Since the growth rates in E-1 and E-2 were similar to each other as were the growth rates in C-1 and C-2, one may assume that an excess of L-PA had a similar retarding effect on the growth in both E-1 and E-2. After the L-PA diet was terminated, the original growth rate of E-1 and E-2 increased 12 fold as compared to the three fold increase of C-1 and C-2. In other words, the growth in E-1, E-2, C-1, and C-2 increased at a similar rate. The difference in growth rates before and the similar rate of growth after terminating the L-PA diet may be attributable to a metabolic imbalance produced by the amino acid.

The mortality rate of Ss may be a selective factor whereby stronger Ss that are perhaps less susceptible to biochemically produced brain injury survive. In this experiment the 20% mortality rate was evenly split between experimental and control animals. Apparently, the type of diet was not the cause of death. However, there was a positive relationship between the size of the weanling and its viability. That is, the greater the weight of the weanling, the greater were its chances for survival. Weaning Ss at 21 days of age, when their average weight is greater than 40 gm., might have reduced this high mortality rate.

In the introduction it was hypothesized that the critical period, during which the administration of PA has its greatest effect, occurred

between birth and weaning. If there is such a critical period, E-1 should have made more errors than their pair-fed controls. Only on day five, the first day through the reverse path of the maze, did E-1 make significantly more errors than C-1.

Eighty per cent of C-1 initially reached the "reverse" goal box on the first trial of day five, while 80% of E-1 failed to reach the "reverse" goal box until the fourth trial of the same day. Moreover, E-1 made almost twice as many incorrect responses to the "forward" goal box as C-1. It appears that E-1 had greater difficulty than C-1 in extinguishing their response to the "forward" goal box.

Brain injury as defined by Robinson and Robinson (1965) is any "injury to intact cells which occurs after conception and destroys or permanently disorganizes functioning cell systems in the brain." By this definition PKU belongs in the category of brain injury . In a card-sorting task McMurrary (1964) found that brain injured children took a longer time to abandon a no-longer-correct response in favor of a new one than did cultural-familial retarded Ss. The tendency of Ss with low mentality to take a longer time to extinguish a response was also demonstrated by Ellis (1962). The implication of the above statement is that E-1 suffered a pathogenic condition of the brain in the form of PKU.

If E-1 were phenylketonuric, one must ask why there was not a systematic deficit of behavior throughout the maze test. In learning the forward path of the maze, E-1 made as few errors as C-1. During the reverse test days, only 11% of the total errors made by E-1 occured after they had initially reached the "reverse" goal box. Once E-1 had extinguished their response to the "forward" goal box, they learned the reverse path of the maze as

easily as their pair-fed controls. The ease with which E-1 learned the maze on test days two through four and five through six may possibly be attributable to a "ceiling effect" inherent in the water maze. That is, the maze was not difficult enough to distinguish between brain injured and normal rats.

The critical-period hypothesis also predicts that E-1 should make more errors than E-2 or E-3. On test days five and five through six, both E-1 and E-2 had difficulty extinguishing their response to the "forward" goal. It is possible that the critical period in rats extends beyond weaning. According to Horner, Streamer, Alejandrine, Reed, and Ibbott (1962) phenylketonuric humans have been intellectually benefited by a PA deficient diet as late as three years of age, comparable to an age of one month in the rat. If the critical period does extend beyond weaning in the rat, then E-2 should have made significantly more errors than C-2 on test day five. Although there was no significant difference between them, the trend was in the predicted direction.

The levels of the biochemical criteria used to determine the degree of PKU in experimental rats correlated highly with those of phenylketonuric humans. This indicated that the L-PA diet produced the prerequisite biochemical effects. The six day interim prior to beginning the maze testing was sufficient time for the PA and PPA levels to return to normal. Hence, any behavior deficit could not be attributed to the direct toxic effect of excessive PA. It was also noted that the levels of these biochemical criteria increased slightly above normal in the Ss who were fed the regulated control diet. Apparently the reduced food intake and the concomitant reduction in PA consumption inhibited to a small degree the PAH activity.

The evidence supporting a critical period is tenuous. However, there appears to be some statistical basis for such an hypothesis. This experiment has shown that PKU can be biochemically simulated in rats and that a behavioral deficit on test day five, the first day of reversal, was evident in the experimental rats fed from birth. The lack of a systematic impairment of behavior in E-1 was attributed to a "ceiling effect" inherent in the maze. In view of the data and the small number of Ss employed, further investigation of the critical-period hypothesis seems merited.

SUMMARY

The purpose of this experiment was to simulate PKU in rats and to determine if there were a critical period during which an increase of PA in the system has its most detrimental effect.

Fifty-one Sprague-Dawley rats were paired at birth with a litter mate of the same sex and randomly assigned to one of three feeding conditions for administering the PA and control diets: beginning at birth (E-1, C-1), beginning at 18 days of age (E-2, C-2) and beginning at 36 days of age (E-3, C-3). Until weaning, E-1 and C-1 were fed by intubation. After weaning experimental animals were fed a 7% PA diet.

Six days after terminating the PA and control diets, experimental Ss were compared with their pair-fed controls in a six-unit water maze for six days. On the first day through the reverse path of the maze, E-1 made significantly more errors than C-1. On both reverse days, E-1 and E-2 made significantly more errors than E-3. The former results implied that E-1 had greater difficulty than C-1 in extinguishing a no-longer-correct response, a characteristic of brain injured and low mentality Ss as in PKU. The latter results also indicated that both E-1 and E-2 had difficulty extinguishing an incorrect response and in part supported a critical-period hypothesis. The lack of a systematic impairment of behavior in E-1 throughout the maze testing was attributed to a "ceiling effect" inherent in the maze.

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