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Experimental Phenylketonuria in Infant Monkeys

A high phenylalanine diet produces abnormalities simulating those of the hereditary disease.

Harry A. Waisman and Harry F. Harlow

The rapid progress in both biochemistry and genetics within the past generation has brought these disciplines closer together and enabled us to explain a number of hereditary diseases on the basis of an enzyme deficiency. It is now a well-accepted hypothesis that a gene, or a subunit of a gene, controls the action of a single enzyme, and when the gene is absent or defective, the lack of the enzyme gives rise to a distinct clinical condition with definite signs and symptoms. Some types of mental retardation are due to hereditary traits in which the deficiency of an enzyme causes abnormal metabolic products which are easily detected in the blood or urine. The awareness of the close relationship between biochemistry and genetics has resulted in the discovery of an increasing number of previously unrecognized hereditary diseases.

Phenylketonuria still remains the best model for the concept that in some cases of mental deficiency an inborn metabolic error is the basis for the brain damage. A knowledge of the biochemistry of this disease has led to the development of methods for early diagnosis and for prevention of the severe retardation usually found in untreated patients. However, no clearly acceptable explanation for the brain damage in these patients has been forthcoming, despite satisfactory treatment of children with phenylketonuria. The exact etiology of the damage is still not known, despite metabolic studies which deal with phenylalanine metabolites and despite experiments on the enzyme deficit.

It seemed worth while, therefore, to apply the biochemical knowledge at hand in an attempt to produce mental deficiency in animals. Use of such retarded animals would permit more extensive study of the brain, liver, and kidney than would be justifiable in human patients, and it would permit long-term metabolic experiments which could not be made in children without prejudicing the outcome of the experiments or the welfare of the patient. Because phenylketonuria is the foremost example of an inborn error of metabolism associated with mental retardation, efforts were first directed to the experimental production of phenylketonuria.

Phenylketonuria in humans is characterized not only by excretion of keto acids of phenylalanine but also by markedly elevated concentrations of phenylalanine in plasma (*1*). Therefore, the initial experiments were directed toward elevating the phenylalanine level in the blood. Waisman and his coworkers (*2, 3*) had shown previously that the disordered metabolism observed in experimental rat leukemia and in human leukemia was also characterized by elevated levels of phenylalanine in plasma. At that time it was thought likely that the phenylalanine hydroxylase enzyme, which is known to occur only in the liver and which normally converts phenylalanine to tyrosine, is somehow involved in the leukemic state, probably as a result of associated liver damage, but no evidence was at hand to prove this notion. In experiments with rats (*3*) injected with both tryptophan and tyrosine, the concentration of phenylalanine hydroxylase in liver was decreased to less than one-third the normal value. This reduction was attributed to a feedback mechanism operating through the action of tyrosine. Later experiments (*4*) showed that this reduction was probably due to the tryptophan, as injections of tyrosine alone had no effect on the phenylalanine hydroxylase activity, and injection of tryptophan alone caused a decrease in the activity. Since phenylalanine hydroxylase was wholly or almost wholly lacking in subjects with phenylketonuria (*5*), it seemed logical to try to produce phenylketonuria experimentally by suppressing the phenylalanine hydroxylase activity. On the basis of the misleading earlier work (*3*) with *injected* amino acids, it was supposed that addition of tyrosine *to the diet* would decrease the enzyme activity and therefore decrease the amount of phenylalanine converted to tyrosine. The excess phenylalanine would then, it was hoped, increase in concentration in the blood and be excreted in the urine or converted to pyruvate through transamination. Another approach was to supply so much substrate—in this case, phenylalanine—that the hydroxylase would be overwhelmed.

The initial experiments of Auerbach, Waisman, and Wyckoff (*6*) indicated that addition of 5 percent of

Dr. Waisman is professor of pediatrics and director of the Joseph P. Kennedy Jr. Laboratory in the Department of Pediatrics at the University of Wisconsin Medical Center, Madison. Dr. Harlow is professor of psychology, director of the Primate Laboratory, and director of the Wisconsin Regional Primate Center at the University of Wisconsin, Madison.

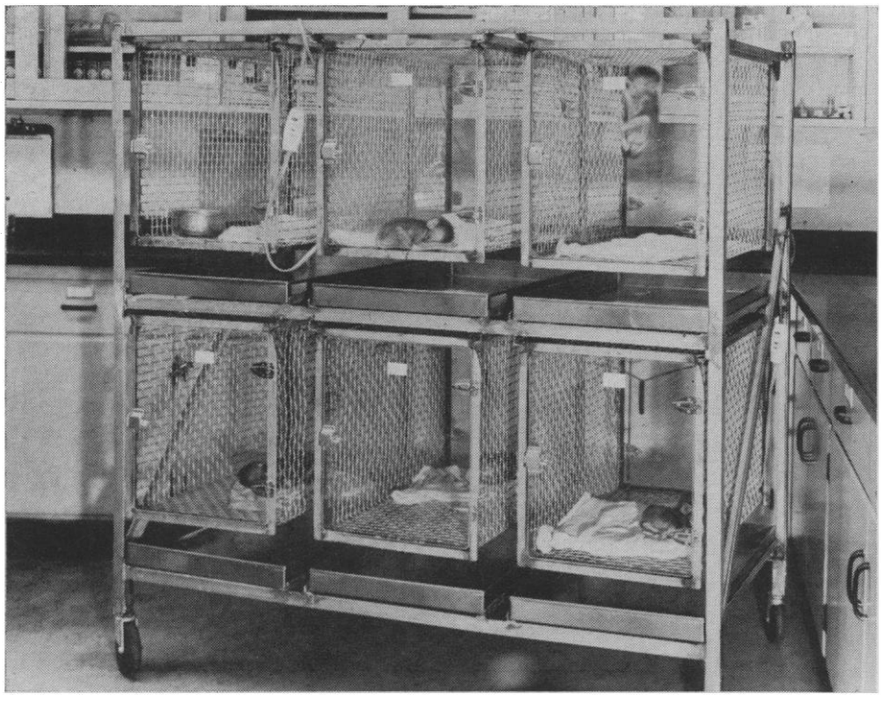


Fig. 1. The cage in which the experimental monkeys were placed shortly after birth.

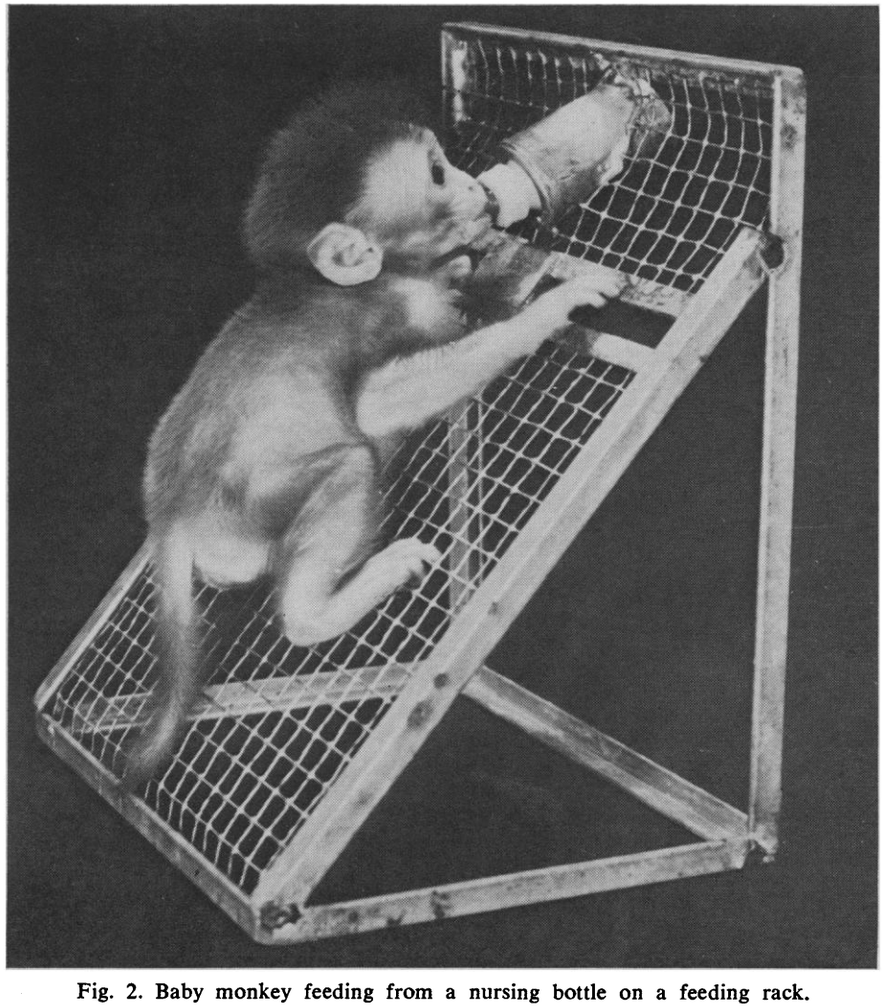


Fig. 2. Baby monkey feeding from a nursing bottle on a feeding rack.

either DL-phenylalanine or L-tyrosine to the diet would cause almost complete loss of phenylalanine hydroxylase activity. But Freedland, Krakowski, and Waisman (*4*), using an improved method for determining this enzyme, showed that feeding of tyrosine always caused an increase in the activity of the enzyme instead of a decrease. Therefore, tyrosine feedings would not be expected to raise the phenylalanine level in the blood. These studies confirmed the earlier finding that feeding phenylalanine in large amounts decreased the phenylalanine hydroxylase activity, but the decrease was only of the order of 50 percent. Since phenylalanine decreased the activity of phenylalanine hydroxylase, it seemed that the best method for increasing plasma levels of this amino acid was the direct method of feeding the subject phenylalanine. Additional data obtained by altering the amounts of both L-phenylalanine and L-tyrosine in the diet made it quite clear that the level of phenylalanine in plasma of the rat depends also on the influence of other enzymes in the liver (*7*) concerned with the disposition of the amino acids and their by-products. The kidney is also undoubtedly important in regulating levels of amino acids in plasma.

In the earlier experiments with rats fed DL-phenylalanine (at concentrations in the diet of 2.5 or 5 percent) (*6*), there was an increase in urinary phenylpyruvic acid, a result not obtained later when the rats were fed L-phenylalanine at a concentration of 3.5 percent (*8*). However, phenylketones were always found in the urine of rats fed a diet containing L-phenylalanine at a concentration of 7 percent.

Although the rat experiments provided valuable information, it seemed wise to use another animal for some studies of phenylketonuria. The rhesus monkey was selected because (i) the monkey's metabolism closely resembles man's, (ii) its behavior and intellectual performance can be tested with precision (*9*), and (iii) the dietary requirements were well documented by our previous research with this animal (*10*). The goal was to produce phenylketonuria experimentally by applying the clinical and biochemical information already available. This article describes the successful production, in rhesus monkeys, of experimental phenylketonuria, which is almost identical in all respects to the clinical disease.

Method for Producing Phenylketonuria

Each of six newly born rhesus monkeys (*Macaca mulatta*), three males (A08, A18, A32) and three females (A23, A24, A39), was separated from its mother shortly after birth and placed on a warming pad in a cage equipped with two doors for easy access (Fig. 1). The infant was gently wrapped in a cloth diaper and held while fed from a toy nursing bottle and nipple every 2 hours, in accordance with the procedure developed in the Primate Laboratory (*11*). A warmed mixture of colostrum and regular cow's milk formula (in the ratio 1:2) was fed for the first 5 days. Thereafter, the monkey was fed the milk formula to which had been added small quantities of L-phenylalanine. The amount of phenylalanine was increased gradually until the optimum level of 3 grams of phenylalanine per kilogram of body weight per day was fed (*12*). Not all the monkeys maintained this level of phenylalanine intake, but the attempt was made to feed as much phenylalanine-containing milk as possible. The infant monkeys accepted the milk and phenylalanine mixture during the hand-feeding period if the phenylalanine concentration was kept at one-eighth to one-fourth the final concentration for the first 3 or 4 days. The desired concentration of phenylalanine in the milk was reached by the 19th day. Originally, whole cow's milk was diluted (in the ratio 1:1, by volume) with a 2.5-percent solution of L-phenylalanine. In later experiments the milk was prepared from a commercial modified liquid milk concentrate or from whole-milk powder, so that increasing amounts of phenylalanine solution could be used when necessary. The reconstituted milks were nutritionally complete in all factors such as carbohydrate, protein, fat, vitamins, and minerals, including iron, and they were accepted by the infants even though they had a somewhat repulsive taste. The first feeding of the day was additionally supplemented by a polyvitamin preparation to insure an adequate intake of vitamin C, despite the fact that the milk concentrate contained this and other added vitamins. Hand-feeding was continued until the animals were 15 days old, or until the animal would accept a sufficient amount of the formula. After that, the nursing bottle and nipple were placed in a holder on a "false mother" (mother surrogate), as illustrated in Fig. 2. The monkeys crawled onto the "mother," completed the feeding, and slept or played until the next feeding. As the concentration of phenylalanine was increased, it was often necessary to revert to hand-feeding because of the infants' resistance to the dietary change. Numerous monkeys fed normal diets have been separated from their mothers and fed in this manner (*11*) in the Primate Laboratory, and some of them served as controls in this experiment.

The infant monkeys were fed every 2 hours for the first several days. When they could take larger quantities of milk at each feeding they were fed 4 or 5 times a day, and they still maintained adequate intake. Five feedings per day was the preferred number for this experiment, but the monkeys would sometimes refuse one or more of the feedings. Later, between days 12 and 30, the animals were weaned and fed from a small cup; on or after day 31 they were fed from a large cup.

Milk intake was carefully recorded for each feeding, so that the amount of amino acid consumed per day per kilogram of body weight could be calculated, allowance being made for spillage. When the animals were 3 months old, a quarter of an apple and a quarter of an orange were placed in the cage once a day, but the monkeys' coordination was poorer than that of normal 3-month-old monkeys, so they had some difficulty in holding the fruit during the first few months after it was offered. Two of the six animals remained on the diet for 2 years, and four have continued on the diet for 3 years.

Blood specimens were drawn from the femoral vein into a heparinized syringe after the monkeys had fasted overnight for at least 8 hours. The phenylalanine and tyrosine concentrations in the plasma were determined by the methods of Udenfriend and Cooper (*13*) and of La Du and Michael (*14*). Specimens were obtained at weekly, at biweekly, and then at monthly intervals after it was apparent that the blood concentrations of phenylalanine were stabilized at high levels, above the desired minimum of 10 milligrams per 100 milliliters. Tests of the urine for phenylketones were made at daily intervals throughout the initial months of the experiments, with Phenistix (*15*), ferric chloride, or 5 percent 2,4-dinitrophenylhydrazine, but this routine testing was discontinued when the urine was consistently found to test four plus.

Twenty-four-hour collections of urine, properly preserved with thymol and kept in ice, were obtained by means of a metabolic chair designed at the Primate Laboratory (*16*).

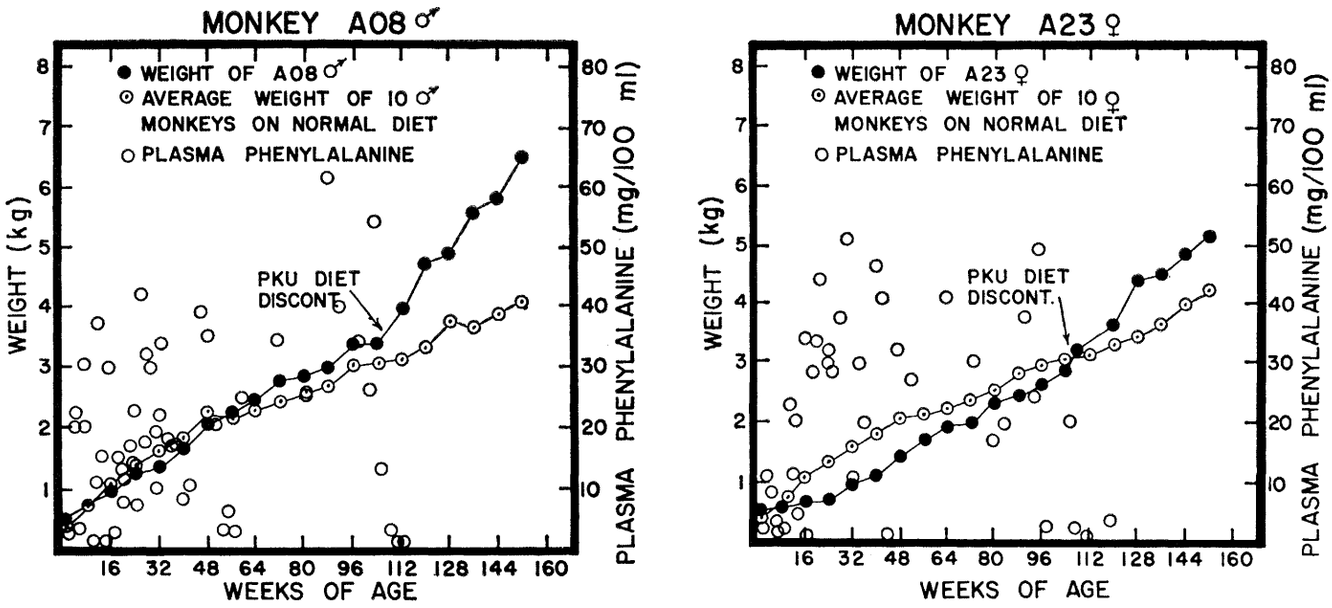
Methods for Studying Behavior

Earlier work (*12*) had indicated that the performance of adolescent monkeys fed phenylalanine might be inferior to that of control monkeys on learning tests. Consequently, it was expected that monkeys given a high-phenylalanine diet from early infancy, when brain growth and intellectual development are proceeding at a rapid rate, might show marked intellectual deficits, as do untreated human infants with phenylketonuria. To test such possible

Table 1. Test schedule for phenylketonuric subjects; values are ages (in days) during testing. WGTA, Wisconsin General Test Apparatus.

Subject	Test						
	Mazes	WGTA adaptation	Discrimination learning	Delayed response	Learning set	Discrimination reversal	Parallel strings
A08 ♂	15–63	118–163	164–191	164–288	295–416	462–643	178–204
A18 ♂	15–75	76–118	122–147	122–249	250–371	417–541	Could not be adapted
A32 ♂	Not tested	45–129	130–166	130–221*	Not tested	241–377	130–164
A23 ♀	15–78	79–132	133–193	132–258	260–379	402–602†	Could not be adapted
A24 ♀	Not tested	46–116	121–150	121–255	260–283†	317–517†	Could not be adapted
A39 ♀	Not tested	45–117	120–151	120–201*	Not tested	217–357	120–146

* Testing discontinued after 60 test days. † Testing discontinued.



Figs. 3 and 4. Plots of weights and of plasma levels of phenylalanine for monkey A08 ♂ (left) and monkey A23 ♀ (right).

deficits in the infant monkeys, a battery of learning tests was used to measure abilities known to mature in normal infant monkeys in the first year of life (*17*).

The learning-test schedule for the phenylalanine-fed animals is shown in Table 1. The age of each subject, in days, at the start and at the completion of each test is indicated. The variation in the ages at the time of testing resulted from difficulties in adapting these subjects to the test situation and in maintaining sufficient motivation to obtain responses. Unlike most normal rhesus monkeys, they balked frequently and tended to work slowly. Test sessions often had to be terminated because of refusal to respond or because of extreme disturbance and hyperactivity; in some instances the refusal to respond in a given situation extended over periods of weeks, necessitating discontinuance of the test.

The first test, started with three subjects on day 15 of life, was the Hebb-Williams maze test, administered with an adaptation of the procedure developed by Rabinovitch and Rosvold (*18*). Preliminary trials with six different mazes were given to adapt the animal to the test situation, and these were repeated until the animal performed promptly and without error. These trials were followed by trials with 12 test patterns, readaptation trials on three of the preliminary mazes, and then trials with the 12 mirror images of the test patterns. Five trials were given per day at intervals of 2 hours or more, and performance on a given test pattern was continued until the animal met the performance criterion. Testing was conducted 5 days per week. The reward at the finish box on each trial was the regular milk and phenylalanine mixture. The minimum number of test days required to complete the series was 33.

Adaptation to the Wisconsin General Test Apparatus (*17*) was begun after the completion of maze training or, if the subjects were not given such training, when they were 45 or 46 days old. This basic apparatus was used for all subsequent learning tests. Adaptation training consisted of placing the animal in the apparatus 5 days each week and giving it 15 trials per day in which it had only to displace a simple wooden block that covered a food well on the tray. Training was continued until the subject had completed its 15 trials smoothly and quickly.

Small wedges of grape were used as the incentive, but when the monkey balked, small pieces of apple, banana, or other foods were substituted. Normal monkeys of comparable ages, on a diet of milk, dry monkey food, lettuce, and fruit, were used as controls. Initially the balking of the phenylketonuric subjects in the adaptation training and subsequent testing with solid-food rewards stemmed, at least in part, from their unfamiliarity with solid foods, which had not been a part of their regular diet.

After preliminary adaptation, which required the abnormally long time of 6 to 12 weeks, training was begun simultaneously on discrimination learning, delayed response, and the parallel-strings test. Details of the test procedures have been reported (*9, 17*), and only general methods are described here.

Discrimination learning involved the presentation, in 25 trials per day for 20 days, of a single pair of three-dimensional objects. Response to one of the objects was consistently rewarded, and its position on the board (left or right) was varied randomly in a predetermined order. Delayed response tests consisted of presentation of two identical objects, one of which was baited in full view of the subject. The monkey was not permitted to respond until after a delay of 0 seconds or 5 seconds, the two delays being intermixed in the 20 daily trials. Four subjects were tested in this way for 90 days (see Table 1), but tests were discontinued for two other monkeys after 60 days when their performance was found to be no better than chance.

The parallel-strings test is a simple perceptual task in which two metal chains are laid parallel to each other and at right angles to the upright subject. A piece of fruit is attached to the distal end of one string, and the monkey is to pull in the baited chain. The position (left or right) of the baited string is varied in accordance with a balanced, predetermined order. This test was presented 20 times per day for 20 days. Before presentation of

the test the animal had been given adaptation tests with a single baited string for at least 15 days. Three subjects completed the adaptation and test trials; three failed completely to adapt to the single baited string and consequently could not be tested with the parallel strings.

The four subjects that completed both the discrimination learning and the delayed response tests were then started on a discrimination learning set (*17, 19*), in which they were presented with four six-trial discrimination problems each day for 85 to 100 days. Each problem introduced a new pair of objects, one of which was rewarded consistently. The position of the correct object was varied in a predetermined, random order. The two subjects that had failed to complete the delayed response tests were started, instead, on a discrimination reversal problem (*20*), while the remaining four monkeys were given this reversal problem after completion of the learning-set series.

Discrimination reversal consisted of presentation of a single set of two three-dimensional objects, designated A and B, for 20 trials per day, until the animal met the criterion of 10 correct responses in the first 11 trials with object A rewarded. On the following day object B was rewarded. The trials were continued, 20 per day, until the subject met the criterion of 10 correct choices of B in the first 11 trials. On the day after the subject had met this criterion, object A was rewarded. The procedure was continued until 20 reversals had been obtained, or until the subject would no longer respond, as was the case with two animals (see Table 1).

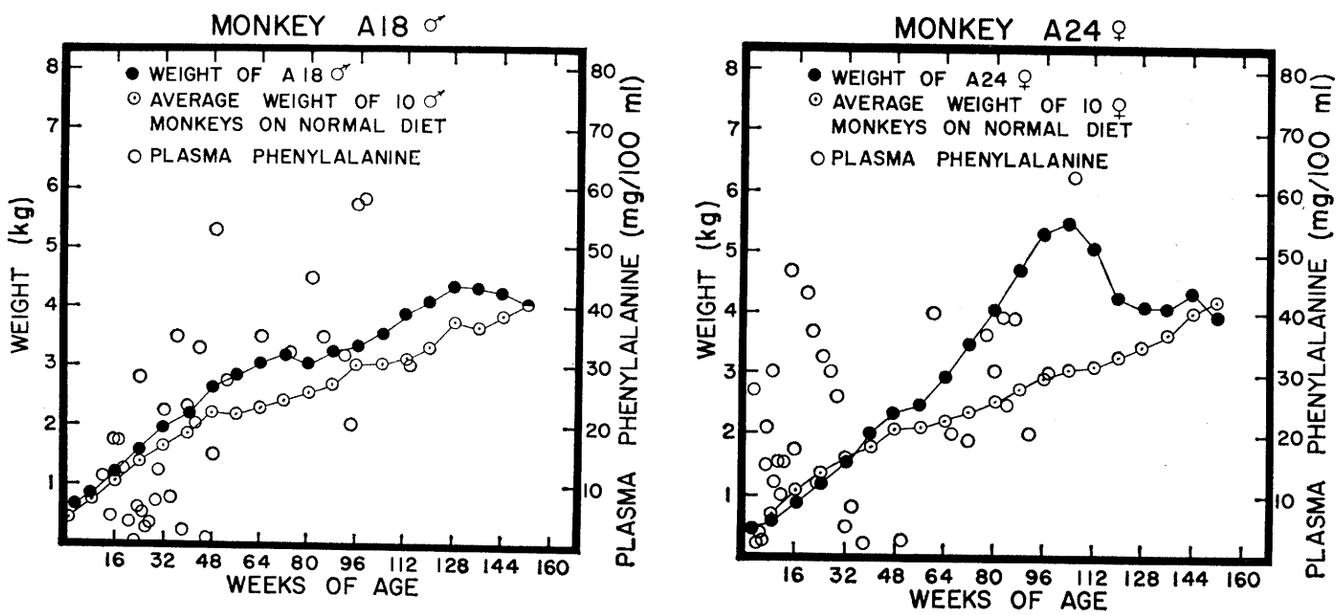
The animals were tested at approximately the same hour each day and were transported from their nursery-room cages to the test room in aluminum carrying boxes. A masking noise was maintained in the test room. A "noncorrection procedure" (*21*) was used throughout the testing.

Physical Development of Phenylketonuric Monkeys

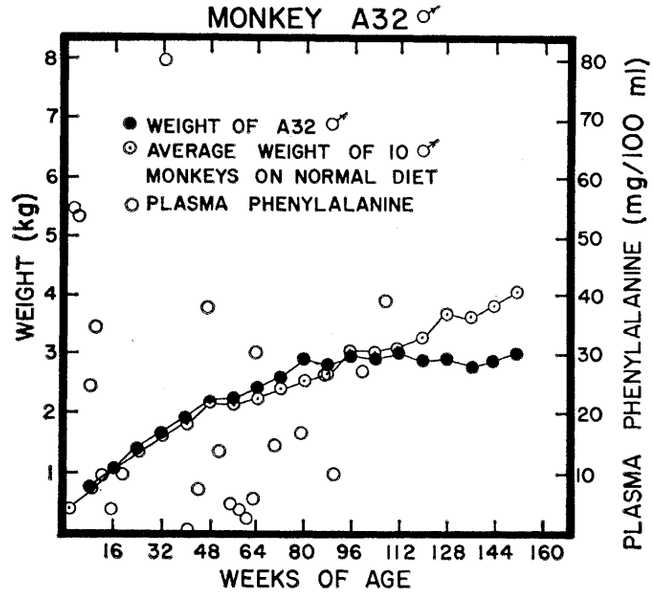
The six monkeys raised on a high phenylalanine diet from the first few days of infancy showed excretion of phenylketones in the urine when the plasma level of phenylalanine reached about 10 mg/100 ml. The urine had the typical musty odor of that of children with phenylketonuria. Figure 3 shows the growth and the blood levels of phenylalanine for monkey A08♂, typical of findings for all the monkeys on the diet of milk with phenylalanine. The weight during the first few months was slightly lower than the average for ten normal male animals fed apple, orange, lettuce, commercial monkey pellets, and lesser amounts of milk formula, but by the end of the first year the weight was as high as the average. The plasma levels of phenylalanine were usually above 10 mg/100 ml. As Fig. 3 also shows, for animal A08♂ the diet of milk with phenylalanine was discontinued after 2 years, and the animal has since remained on a normal diet except for the addition of small pieces of grapes or raisins used as rewards during the testing periods.

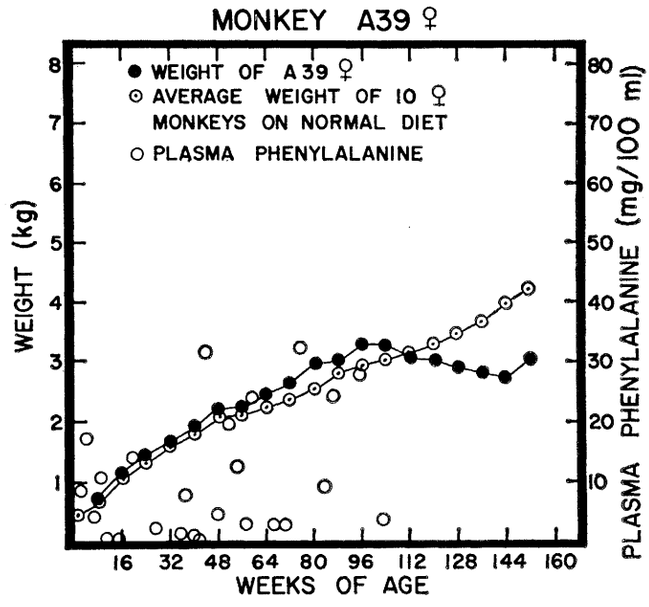
When the phenylketonuric monkeys were about 1 year old, the weights of five of the six animals had reached or surpassed the average for normal monkeys, and the weights of these five monkeys (Figs. 4–8) remained above this average throughout most of the second year. During the third year the growth of the four monkeys that were continued on the diet of milk with phenylalanine was very slow (Figs. 5–8), and there were at least a few weeks in which there was a loss of weight. At the end of 3 years, two of these monkeys (Figs. 5 and 6) were of average weight and two (Figs. 7 and 8) were of less than average weight. The bowel movements of these milk-fed animals were soft and semiliquid, probably reflecting the lack of roughage in the diet. Diarrhea was not a problem, and, on repeated cultures of the stool, no intestinal infections were observed. The plasma levels of phenylalanine varied considerably from week to week but were between 10 and 45 mg/100 ml in more than half the determinations. When the milk intake was less than usual, the plasma level was lower, as might be expected. Tests (Phenistix or 5 percent ferric chloride) for phenylketones in the urine were always strongly positive.

The weight of monkey A23♀ (Fig.



Figs. 5 and 6. Plots of weights and of plasma levels of phenylalanine for monkey A18 ♂ (left) and monkey A24 ♀ (right).





Figs. 7 and 8. Plots of weights and of plasma levels of phenylalanine for monkey A32 ♂ (left) and monkey A39 ♀ (right).

4) was below the average throughout the first 2 years, and plasma levels of phenylalanine were consistently elevated and seldom dropped below 10 mg/100 ml after the first 18 weeks.

The diet of milk with phenylalanine was discontinued at the end of 2 years for monkeys A08♂ and A23♀, to see if there would be signs of improvement in learning ability or changes in the rate of weight gain. The plasma levels of phenylalanine promptly dropped to normal the day after the phenylalanine was omitted from the milk. The monkeys then showed an increase in rate of growth throughout the next year, doubtless due to an increased intake of milk, which probably resulted from the fact that the milk now had a better taste. The animals which were continued on the diet of milk with phenylalanine grew very slowly during the third year. It should be again noted, however, that until they were 2 years old their growth was nearly comparable to that of the controls, indicating no serious nutritional defect.

Infant A18♂ (Fig. 5) grew at a rate higher than the average for ten control males fed a normal diet. Plasma levels of phenylalanine were above 10 mg/100 ml in about half the determinations for this monkey during the first year, and they were always above 20 mg/100 ml during the second year.

Monkey A24♀ (Fig. 6) has remained on the diet of milk with phenylalanine for more than 3 years and has shown good growth. For this monkey, plasma levels of phenylalanine have seldom dropped below 10 mg/100 ml.

Monkey A32♂ has been on the diet of milk with phenylalanine for more than 3 years, and its growth and maturation have been very satisfactory (Fig. 7). This animal was fed α-methyl dihydroxyphenylalanine, a known decarboxylase inhibitor (*22*), for the first year in the hope that the large amount of phenylalanine circulating in the brain would not be decarboxylated to phenylethylamine *in situ* (*23*). While it has never been proved that brain damage results if phenylethylamine is present, the possible effects of feeding this inhibitor with the milk and phenylalanine to one monkey were of interest. This substance was not harmful to the animal, but, as it turned out, it did not give the animal any superiority over the other phenylketonuric animals in behavioral testing. It may be that the quantity of inhibitor given the monkey (approximately 150 mg/day, beginning on the 12th day) was too low to give an adequate pharmacologic effect, despite a year-long feeding. The plasma levels of phenylalanine in this animal were between 10 and 60 mg/100 ml in more than half the determinations, and in one sample they reached 80 mg/100 ml.

A sixth infant, monkey A39♀ (Fig. 8), has been fed the milk with phenylalanine for 3 years and has grown satisfactorily. The plasma levels of phenylalanine were less high than had been hoped for; slightly more than half the values were below 10 mg/100 ml. The low values could usually be correlated with decreased intake of the milk with phenylalanine during the previous 24 hours.

The fur of these phenylketonuric animals was essentially normal during the first year, but thereafter it lacked sheen, was frequently "fuzzy," and was brownish-gray instead of the bronze characteristic of macaques fed a standard diet. The teeth were in good condition, although some deciduous teeth failed to drop out when the permanent teeth appeared. The "baby teeth" were easily extracted.

Tests on the urine demonstrated large quantities of phenylalanine, phenylpyruvic acid, *o*(OH)-phenylacetic acid, *p*(OH)-phenyllactic acid, indolelactic acid, and phenylacetylglutamine. Quantitative analysis of urine for phenylpyruvic acid from normal and phenylalanine-fed monkeys provided the data of Table 2. Normal milk-fed animals do not excrete phenylpyruvic acid at any age, but the high-phenylalanine diet caused excretion of remarkable quantities of this acid. Attempts were made to correlate the amounts excreted with the quantity of phenylalanine consumed, and with the weight of the animal, but the results were inconclusive.

Complete blood counts were made at intervals throughout the experimental periods; all the phenylketonuric monkeys had normal hemoglobin values of 12.1 to 15.1 g/100 ml, normal white cell counts of 8200 to 12,000 per cubic millimeter, and normal differential

counts. Fasting blood sugar determinations showed normal values of 90 to 118 milligrams of glucose per 100 milliliters.

The phenylketonuric monkeys had convulsive seizures which began at various intervals after they were first fed the high-phenylalanine diet. Convulsions were first observed in monkeys A24♀ and A39♀ at 4 months; monkey A08♂ had its first observed grand-mal seizure at 8 months. The three other animals had their first observed seizures at various ages between these extremes. The tonic-clonic contractions of all extremities, salivation, and involuntary defecation were followed by the usual postictal sleep for several minutes. The electroencephalographic tracings for the phenylketonuric monkeys (*24*) were typical of the spike-and slow-wave activity seen in some human epileptic and phenylketonuric patients. No residual motor defects were observed between seizures.

Table 2. Phenylpyruvic acid excretion in 24-hour urine collections.

Animal	Urine (ml)	Phenylpyruvic acid (mg)
Phenylketonuric animals		
A08	47–85	113–257
A18	224–250	79–465
Controls		
A09	304–345	0
A15	260–292	0

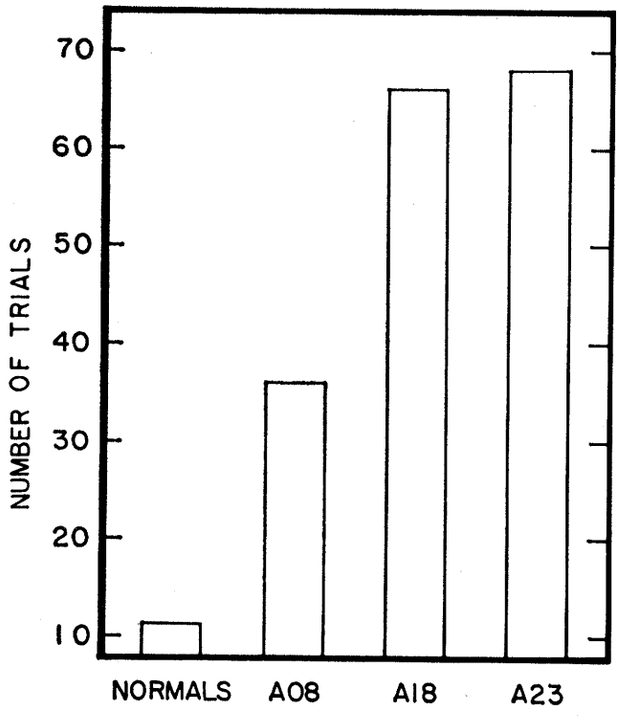
Results of Behavioral Tests

The results of the behavioral tests are illustrated in Figs. 9 to 17. The phenylketonuric animals adapted to the testing procedure more slowly than the controls did. It should be noted here that, with one exception (A32♂), no gross neurological abnormalities such as incoordination in walking, climbing, or prehension were observed in these animals during the first 2 years, although all showed some awkwardness in prehension and in avoiding obstacles. Convulsions of the grand-mal type, which were observed in most of the phenylketonuric animals during the first 2 years, were not debilitating or so incapacitating that they interfered with the behavioral testing program. The animals recovered spontaneously after the postictal period.

They did show frequent evidence of disturbance during testing, such as strong fear responses, hyperactivity involving circling the cage or head-banging or self-biting, occasional vomiting, and reluctance to respond at all to the test stimuli. Some animals sat in the back of the test cage or lay down, instead of remaining at the front to make the necessary choice. Early in the testing, in particular, they dropped the rewards or played with the food instead of eating it, and dropping was frequent even late in the testing. In delayed-response and learning-set testing there was frequently a latency of 7 or 8 minutes between presentation of the stimulus and the subjects's response. Phenylketonuric monkeys took at least four times as long to complete a session as normal animals, and occasionally they took 3 or 4 hours to complete a session which normal animals of the same age complete in 10 or 15 minutes.

Initially the three phenylketonuric monkeys given the Hebb-Williams test took more than an hour to complete the session, and frequently a session lasted more than 2 hours; therefore, an arbitrary limit of 5 minutes was placed on each trial. The monkeys had to be prodded to complete a trial or they would "freeze" or lie in the maze. Because of the balking it was necessary to rerun the trials later, when the monkeys had reached an age greater than that at which the normal monkeys were given this test. The number of such reruns is shown in Fig. 9.

Figure 10 shows the data from the object-discrimination learning tests for both phenylketonuric and control monkeys. The performance of monkey A23♀ was significantly below that of normal monkeys during the first 100 trials. This was of interest because in A23♀ the plasma concentrations of phenylalanine were usually about 10 mg/100 ml and ranged up to 50 mg/



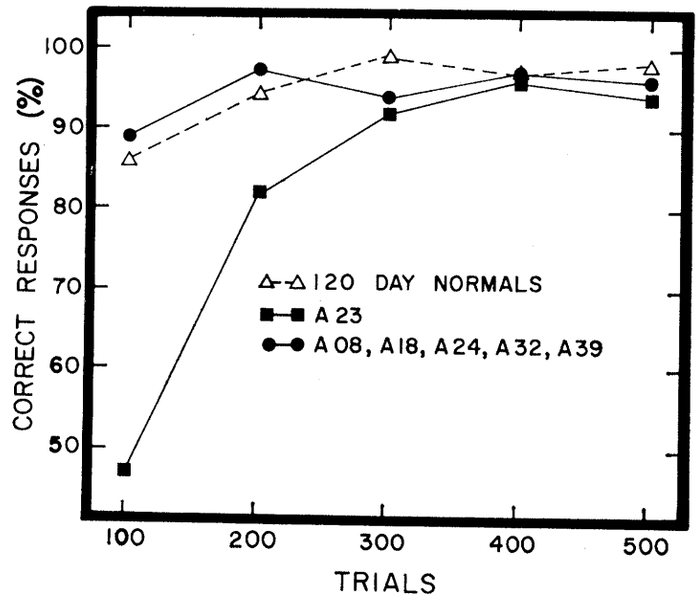
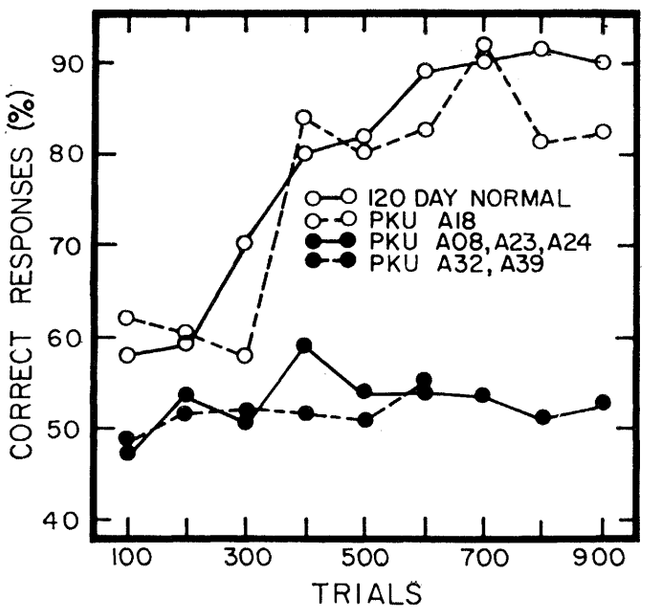
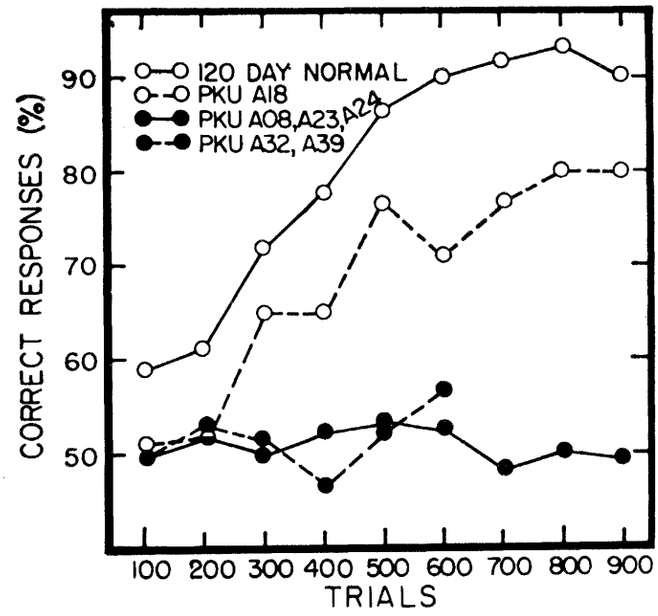


Fig. 9 (left). Number of rerun trials in a maze of Hebb-Williams type for normal and for phenylketonuric monkeys. Fig. 10 (right). Performance on the object-discrimination test by normal and by phenylketonuric monkeys.





Figs. 11 and 12. Performances by normal and by phenylketonuric monkeys on the delayed-response test, with delay interval of 0 seconds (left) and delay interval of 5 seconds (right).

100 ml. If brain damage can be correlated with high plasma levels of phenylalanine, one would expect this animal's performance to be inferior. However, since there are wide individual differences in the performance of normal monkeys, there is only suggestive evidence of impaired performance of the object-discrimination learning test on the part of phenylketonuric monkeys.

In the delayed-response tests, delay intervals (intervals between baiting of the food well and presentation of the stimuli) of 0 seconds and 5 seconds were intermixed. Figure 11 illustrates the data for the trials with 0-second delay interval. Five of the six phenylketonuric subjects failed to learn, but animal A18♂ learned at a normal rate. It should be noted that the blood levels of phenylalanine in this animal were not so high during the first 30 weeks as was desired. The graph for A32♂ and A39♀ terminates after 600 trials, when testing was discontinued because neither subject was performing at a level significantly different from chance. On the 5-second delayed-response trials, all six phenylketonuric monkeys showed impairment, although A18♂ performed more nearly like the control animals than like the other five experimental animals (see Fig. 12).

It was necessary to determine whether the long latency observed between presentation of the stimuli, at which time the subject had the opportunity to choose a stimulus, and the subject's response was adversely affecting the performance. Accordingly, the percentages of correct responses were analyzed for length of periods of latency between stimulus and response for both the 0-second and the 5-second delay intervals. This analysis was made to test the possibility that a defect in response motivation was being measured and that possibly forgetting occurred during the animal's self-imposed delay. From Fig. 13 it is apparent that there were no significant differences in performance associated with minimum and maximum periods of response latency in trials on which the monkeys tarried. Thus it appears that poor performance was not a result of delay in responding but that the generally low performance level was a function of failure prior to the moment the animal was permitted to make its choice. This could stem from failure to learn or failure to attend during the baiting process.

In the object-discrimination learning-set testing, monkeys A08♂ and A18♂ completed 400 problems, and monkey A23♀ completed 300 problems. In Fig. 14 their learning curves are compared with those for groups of normal animals started on these tests at 150 and 365 days of age. The phenylketonuric animals were 250 to 295 days old when these tests were begun; the curves demonstrate that A08♂ and A23♀ performed at chance levels. Monkey A18♂ performed at a better than chance level, but its performance, instead of improving, deteriorated after the first 300 problems. There was no overlap in the performance of the 360-day-old normal monkeys and the phenylketonuric monkeys.

The data for the successive discrimination reversals are presented in Fig. 15, which shows the number of days needed by normal monkeys and by the phenylketonuric animals to complete each group of four reversals. The data demonstrate that subjects A23♀ and A24♀ never made an adequate adaptation to the test; data for these animals were incomplete when the experiment was discontinued. The overall performance of the other four phenylketonuric monkeys (mean age at start of the tests, 334 days) was not significantly inferior to the mean performance of the normal subjects (mean age, 214 days). The 4-month age advantage of the experimental animals should have enabled them to perform at a higher level.

The data for normal animals and for the six phenylketonuric animals (mean age, 344 days) were also examined by plotting the mean for number of trials necessary to meet the criterion against the number of reversals; the plot yielded the curves shown in Fig. 16. The performance of the phenylketonuric subjects was found to be independent of age, and their mean performance was substantially below the mean for the normal controls.

Of the six phenylketonuric animals, only three adapted to the string test. It was impossible to test A18♂, A23♀, and A24♀, although they had been given 31, 18, and 20 days of adaptation

trials, respectively. Figure 17 shows the results for A08♂, A32♂, and A39♀; in each case, mean performance for the first 200 trials was below that of normal monkeys but within the range of individual normal monkeys of the same age. That three phenylketonuric animals failed to adapt is more indicative of the abnormality of the group than is the slightly depressed performance of the three monkeys that did adapt. In scores of normal and brain-damaged monkeys tested at the laboratory on string tests over the years, only one animal prior to that time had failed to adapt to the single string.

For A08♂ and A23♀, preliminary data were obtained bearing on the question of how much brain damage is caused by the high-phenylalanine diet in 2 years and how much, if any, recovery takes place when the phenylalanine is discontinued. Observations of behavior and tests of learning ability were continued for 2 additional years after discontinuance of the phenylalanine; it is clear that the brain damage caused during the first 2 years is severe enough to interfere permanently with performance of the tests. Additional testing is needed to provide quantitative data on the influence of 3-month and 6-month feeding of the high-phenylalanine diet.

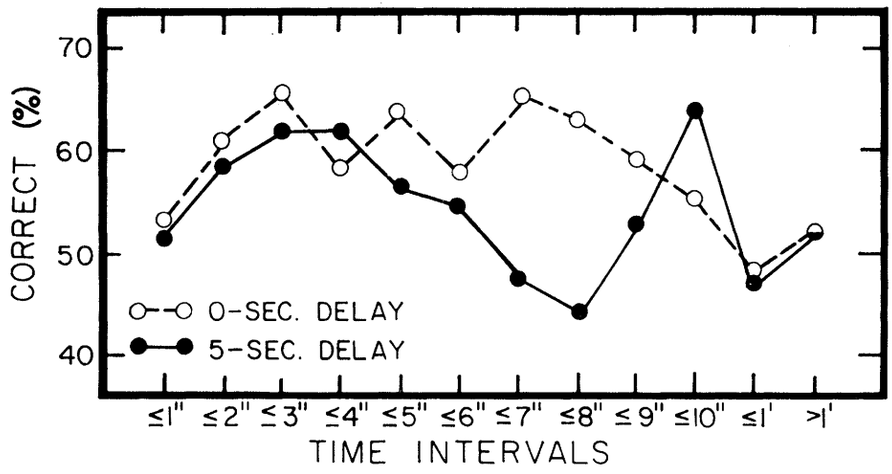


Fig. 13. Latency of response of phenylketonuric monkeys on the delayed-response test.

Discussion

There are a number of well-established clinical criteria for diagnosing phenylketonuria in children, although signs and symptoms of the disease vary in severity in individual patients. Gross mental retardation, elevated plasma levels of phenylalanine, excretion of phenylketones in the urine, convulsions, eczema, decrease in normal pigmentation, and abnormal electroencephalographic tracings are the major findings in these patients, who lack a certain gene or have a defective gene as part of their hereditary abnormality.

The ability to produce experimental phenylketonuria in monkeys by biochemical means depends on the ability to elevate the plasma levels of phenylalanine to levels found in human patients with the disease. Theoretically it seemed that this could be done either by decreasing the activity of the phenylalanine hydroxylase enzyme, through which phenylalanine is normally converted to tyrosine, or by overloading with so much substrate (in this case, phenylalanine) that the activity of the enzyme would be inadequate to deal with the load. Experiments in the laboratory demonstrated that the activity of the phenylalanine hydroxylase in rat liver could be lowered by injecting or by feeding (*4*) a number of compounds, among them phenylalanine. Now it is known that rat liver has more phenylalanine hydroxylase activity than human liver (*25*) and that rat liver (*26*) has 10 to 20 times as much activity per gram as monkey liver. Therefore, two factors which may work to elevate the plasma level of phenylalanine in the monkey are (i) the normally low hydroxylase activity, and (ii) decreased enzyme activity in the presence of excess phenylalanine in the diet.

The initial results on experimental phenylketonuria in the adolescent monkey (*12*) showed that it was necessary to increase the dietary L-phenylalanine to approximately 1 gram per kilogram of body weight per day before a significant rise in plasma levels of phenylalanine in fasting monkeys could be obtained. This plasma level was not sustained, and maintenance of consistently elevated blood levels of L-phenylalanine required an increase in the quantity of dietary L-phenylalanine to 3 grams per kilogram per day. It was also found that two of the adolescent monkeys fed 3 grams per kilogram per day of both L-phenylalanine and L-tyrosine had plasma levels of phenylalanine no higher than the levels obtained when they were fed phenylala-

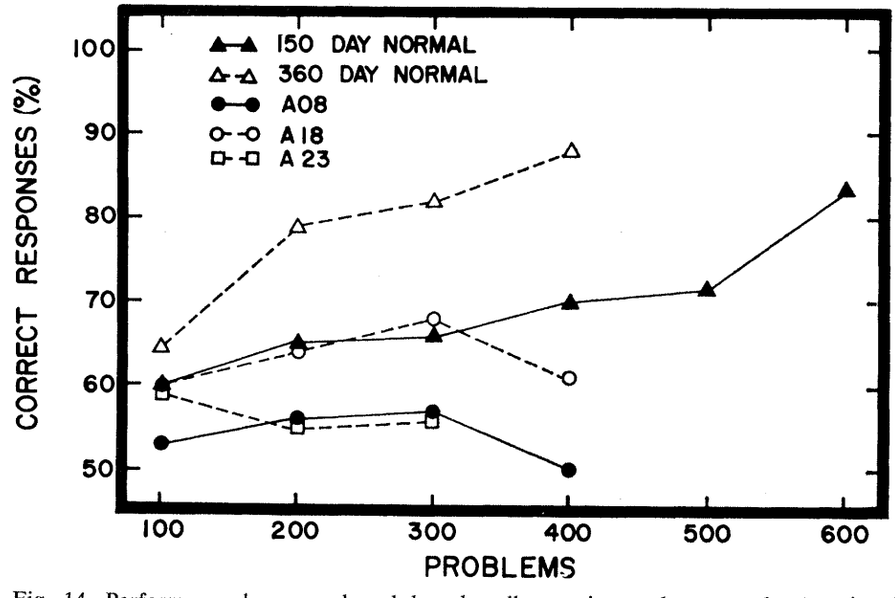


Fig. 14. Performance by normal and by phenylketonuric monkeys on the learning-set test.

nine alone. Feeding L-tyrosine alone was also ineffective in raising the plasma levels of phenylalanine in the adolescents, and it appeared that the theory of product inhibition or feedback (*3*) as a mechanism for suppressing phenylalanine hydroxylase activity was unsatisfactory. In preliminary experiments, feeding greater quantities of tyrosine to infant monkeys (*27*) has proved no more effective, and it must be concluded that tyrosine feedback plays no role in raising plasma levels of phenylalanine under the conditions described. Plasma levels of tyrosine were consistently elevated, as would be expected when either phenylalanine or tyrosine is included in the diet.

Feeding a high-phenylalanine diet elevates the plasma levels of this amino acid, and brain damage results from an excess of phenylalanine and its metabolites, or from disruption of the balance of other amino acids crossing the blood-brain barrier, or from an adverse effect on important enzyme systems necessary for the intricate development of the intellectual processes.

The most outstanding behavioral finding in the phenylketonuric animals is their low motivational level during testing, as evidenced by a high incidence of balking, long latencies before responding, failure to orient themselves to the test stimuli, and frequent lack of interest in the reward foods. The frequent occurrence of hyperactivity and the somewhat less frequent occurrence of psychological withdrawal, as seen in body-clasping, lying down in the test cage, and huddling in a corner, may also be functions of the motivational deficit, or they may be independent of it (for example, indicators of fear) but contributory to the difficulty encountered in testing these subjects.

To what extent the motivational defect stemmed from the failure of the animals to adapt to the solid-food rewards and to what extent it resulted from other conditions cannot be determined. However, the reward for completing the maze test was the milk to which the monkey was already adapted. When the monkeys were hand-fed they were usually encouraged to take milk after they had rejected the bottle, so that ingestion of phenylalanine would be high. Vomiting during hand-feeding was common in some animals. Since vomiting occurred from time to time during early testing, one can conjecture that sometimes, at least, the animals

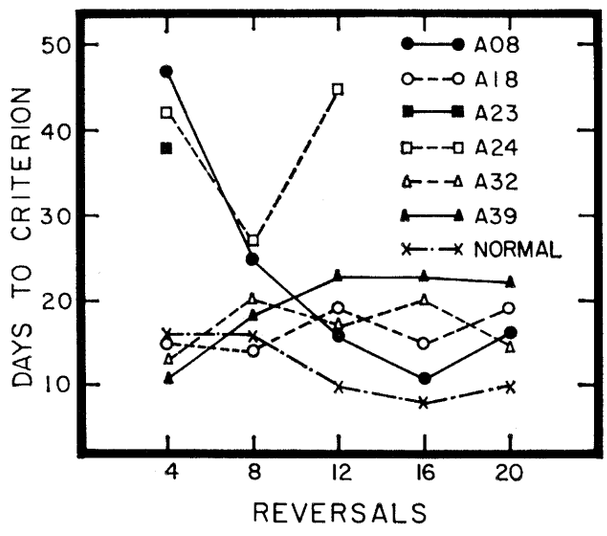
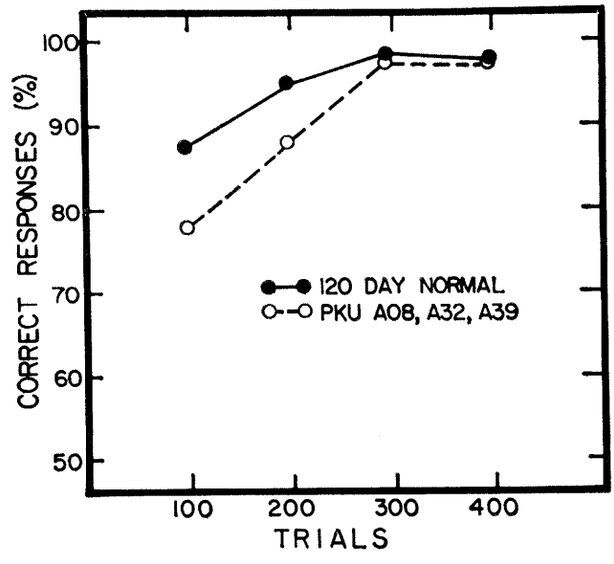


Fig. 15. Performance (in days to criterion) by normal and by phenylketonuric monkeys on the successive discrimination reversal tests.



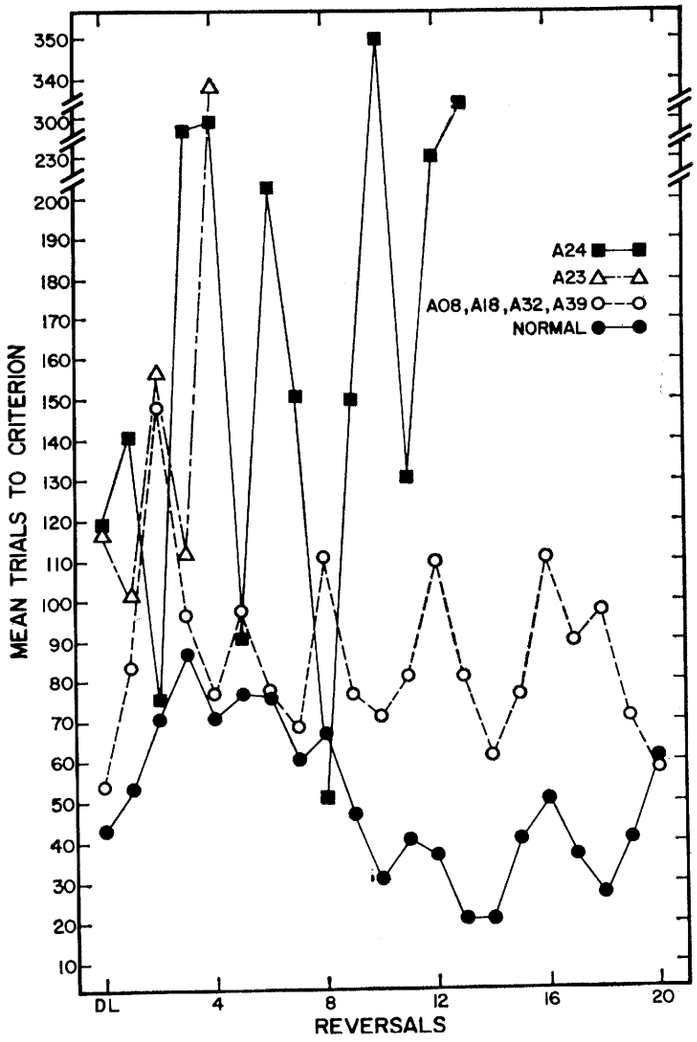


Fig. 16 (above). Performance (in mean number of trials to criterion) by normal and by phenylketonuric monkeys on the successive discrimination reversal tests.

Fig. 17 (bottom left). Performance by normal and by phenylketonuric monkeys on the parallel-strings test.

did not want food. Even more important, perhaps, is the depression of motivation that comes with frustration. A behavioral test that makes demands upon a subject that it is intellectually unable to meet leaves even normal monkeys frustrated and balky. The tasks presented to the experimental subjects in this experiment were within the range of mastery of normal monkeys of the same age or younger, but they may have severely challenged the phenylketonuric animals. Where performance deteriorates with time (as, for example, in learning-set performance), one suspects frustration as a factor. It seems reasonable to expect that lack of interest in the reward and, possibly, bodily discomfort and fear were important early in testing with the Wisconsin General Test Apparatus and that frustration was important later. Fear is normal in monkeys put in new situations at 3 months of age, but it normally subsides within a few weeks. That it never subsided in three of the six animals given adaptation training for the parallel-strings test is especially noteworthy, for none of the three would touch either the metal chain or a cotton string that was substituted for it.

The experimental animals that completed the tests were probably superior in intellect and motivation to the other animals in the group, yet their performance, except for that of A18♂, was consistently below the performance of normal animals on delayed-response, learning-set, and discrimination-reversal tasks—the tasks in which performance is known to be highly correlated with age in the first year of life in the normal monkey. Of the six phenylketonuric monkeys, A18♂ had the best growth record and, together with A39♀, the lowest concentrations of blood phenylalanine in the first year. It is probable that mental retardation did play a major role in the generally depressed performance of the phenylketonuric animals on the more complicated tasks, although it is recognized that low motivation played some role.

The behavioral studies indicate that monkeys first fed phenylalanine in adolescence are retarded (*28*) but probably less retarded than monkeys fed phenylalanine from infancy. Results of feeding phenylketonuric children a low-phenylalanine diet (*29*) support this premise; the earlier the diagnosis is made and the earlier the treatment is begun, the higher the intellectual attainment of these patients is.

Summary

Experimental phenylketonuria can be produced in infant monkeys by feeding excessive quantities of L-phenylalanine soon after birth. Both the phenylketonuric monkey and the phenylketonuric human patient have elevated plasma levels of phenylalanine, and monkey and human excrete almost the same phenylalanine metabolites in the urine. Grand-mal convulsions, observed in some children with phenylketonuria, were also observed in the experimental animals. The biochemical evidence was supported by the learning data. The observed slowness in adapting to testing procedures, or even failure to adapt, and the inadequate performance suggest an intellectual deficit.

The mentally retarded monkey can serve as a valuable experimental model for further studies on phenylketonuria. It will be possible to perform experiments with these animals that cannot be performed with phenylketonuric children. Labeled amino acids can be administered, brain biopsies can be performed for enzyme determination, and various drugs and enzyme inhibitors can be administered to determine the exact mechanisms by which the brain is damaged in phenylketonuria. The experiments described can be followed by others in which mental retardation is produced in monkeys by feeding them other amino acids which are involved in diseases due to inborn errors of metabolism.

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30. We thank Hwa Li Wang and Gail Palmer for their assistance with this project. This article is one of a series of reports from these laboratories in which the behavior and learning ability of animals are correlated with biochemically induced mental retardation. The project was supported in part by U.S. Public Health Service grant FR-0167.