The Enduring Behavioral Changes in Rats with Experimental Phenylketonuria*

(1-phenylalanine/p-chloro-DL-phenylalanine)

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ABSTRACT. The biochemical features of phenylketonuria have been reproduced in developing rat pups by administering to them a combination of p-chloro-DL-phenylalanine plus L-phenylalanine for the first 21 days after birth. During the treatment period, the experimental animals show delayed eye opening and decreased brain weight compared with controls given saline. Neuro-pathological examination of developing animals reveals deficient myelination and some inhibition of cerebellar maturation. When tested as adults, after a long recovery period, animals with phenylketonuria are hyperactive in activity wheels. Adult rats are deficient in reversing a position choice and demonstrate impaired performance in a Y-maze. Rats treated with p-chloro-DL-phenylalanine plus L-phenylalanine during the vulnerable period of rapid brain development thus have enduring behavioral changes that persist throughout life.

Phenylketonuria (PKU) is an inherited disorder of phenylalanine metabolism for which an adequate animal model has been sought. The well-defined biochemical changes resulting from this single gene defect cause the brain to develop abnormally, resulting in severe mental retardation, hyperactivity, increased seizure susceptibility, and muscular hypertonia (1).

The biochemical features of PKU have been most closely approximated by the use of p-chloro-DL-phenylalanine plus L-phenylalanine (ClPhe + Phe). Lipton et al. (2) first used ClPhe + Phe in adult rats to produce a lowered hepatic phenylalanine hydroxylase activity and an elevated blood phenylalanine level without associated tyrosinemia. When rats were treated with this regimen before they were weaned, during the critical first 21 days of life, they showed, during the treatment period, biochemical (3) and electrophysiological (4) abnormalities typical of PKU. Their behavioral abnormalities persisted throughout adult life (5). The present work is directed toward extending the previous investigations of model PKU in rats, especially in the area of permanent learning deficits which result from the early treatment with ClPhe + Phe. Other recent investigations using ClPhe + Phe as a method of PKU production include the behavioral studies of Vorhees et al. (6) and Butcher et al. (7, 8) and the biochemical studies of Edwards and Blau (9, 10).

METHODS

Model phenylketonuria production

This procedure is identical to that reported previously (5). After delivery, 12 litters were assigned to the control group given saline and 22 litters were assigned to the group with model PKU. Each litter was adjusted to a uniform size of 8 pups. Expressed as mg/kg, each pup in the experimental group received about 333 mg/kg of L-phenylalanine and 60 mg/kg of DL-ClPhe per injection. The subcutaneous injections were given daily for 21 consecutive days after birth.

Three additional litters were raised to serve as undernourished controls for weight and activity measurements. Additions were made to these three litters on their day of birth from other litters born the same day so that each of the three nursing mothers had 21 pups to raise.

Behavior testing

Body Weights. Animals were weighed daily from birth to 21 days of age, and the day of eye opening was recorded. The animals were also weighed on day 22 (the day after the conclusion of the treatment program), on day 39 (onset of puberty), and at 18 weeks of age.

Activity Measurements. Each subject was introduced into an activity wheel (Lafayette Instrument Company) at 5 p.m. and removed at 8 a.m. the next morning (15 hr). Male subjects were tested first: 16 that had been given saline and 16 that had been given ClPhe + Phe. At the time of their initial activity measurement they ranged in age from 26 to 32 weeks. To test the reproducibility of this activity test, a second test was administered to the males 2 weeks after the first. Females (22 that had been given saline and 31 that had been given ClPhe + Phe) ranged between 32 and 38 weeks of age at the time of activity testing. Their activity measurement was not repeated. No subjects having cataracts were included in any behavioral testing except as noted below.

Active Avoidance in a Two-Way Shuttlebox. Males (10 given saline and 10 given ClPhe + Phe) were tested at about 6 months of age; females (7 given saline and 6 given ClPhe + Phe) were tested at about 10 months of age.

Active Avoidance Learning with a Right-Left Choice in a Modified Shuttlebox. The two-way shuttlebox was modified for right-left choice experiments in the following way: one side, side A, was left unchanged. The other side, side B, was divided in two to form a right- and left-hand compartment. Testing took place over 4 days. On day 1, the subject was allowed to explore the apparatus for 15 min. On day 2, the subject's right-left preference was determined. On day 3, the animal was trained to choose the side opposite its natural preference. Criterion performance was defined as the number of trials required to make nine consecutive correct choices. Each trial was accomplished as follows: The subject was introduced...
into side A and allowed to rest for 10 sec. The central partition between side A and side B was raised. The subject had 5 sec in which to leave side A and enter the correct compartment of side B. If this was not accomplished, the subject received 0.5 sec of 0.7 mA shock through the grid floor every 3 sec until the correct compartment was entered. On day 4 each subject was trained to reverse the position choice learned on day 3. All subjects were female: 9 given saline and 9 given ClPhe + Phe, about 15 months of age.

Active Avoidance Learning in a Y-Maze. All subjects were mature males (About 17 months of age): 12 given saline and 13 given ClPhe + Phe. A reinforcement of about 0.75 mA was chosen, 0.1 mA above the jump threshold of these animals in this apparatus. Since 10 of the 13 rats given ClPhe + Phe had cataracts at this age, the entire experiment was conducted in the dark, except for a small red light. On day 1, each animal received a 15-min exploration period in the maze. On day 2, position preference was determined. On day 3, the animal was trained to a criterion level of nine consecutive correct choices to choose the arm opposite to its natural preference. On day 4, the animal was trained to choose the side opposite that learned on day 3.

Appetitive Testing in a Y-Maze was conducted at the Bourne Laboratory of the New York Hospital, Westchester Division, by Dr. Jeri Sechzer. A pilot study with 3 males given saline and 3 males given ClPhe + Phe, about 1 year old, was conducted in which the subjects were trained in a Y-maze to run for a food reward. The animals’ weights were gradually decreased to 70% of the initial weight by food deprivation; they were then trained to obtain food reward in the Y-maze. After position preference testing, they were trained to a criterion level of nine consecutive correct choices opposite to their natural position preference.

Cataracts. Animals were inspected at 22 days, 39 days, 5-7 months of age, and 10-12 months of age for cataracts.

Brain weights and neuropathology

Several litters were raised separately for neuropathological examination. Within each litter, some pups received saline injections and some received ClPhe + Phe. Subjects were prepared at 10, 20, and 39 days of age for neuropathological examination (11). After the rats were decapitated, the brain was exposed and the head immersed in formalin–saline for 48 hr. The brain was then removed, weighed, and submitted for 8- or 10-step serial sections. At each step one slide was prepared with H&E, one with Heidenhain’s myelin stain, and, in the 10-day-old litter, one with thionine. This procedure was slightly modified for the preparation at 1 year of age of 3 females given saline and 3 females given ClPhe + Phe (12).

Statistics

Probability figures refer to the Student’s t-test. Where more than one comparison of means is possible the conservative F.S.D. procedure is used (13). In this procedure, the significance level is divided by the number of comparisons of interest. For example, if there are two comparisons of interest, e.g., saline compared with ClPhe + Phe and saline compared with undernourished, the significance level becomes the t-value which gives a probability of 5% divided by two comparisons; in other words, a probability of < 0.025 is now required.

RESULTS

Body Weights (Table 1). At the end of the treatment period, the group given ClPhe + Phe was significantly lower in weight than the controls given saline, about two-thirds control weight. By day 39 all the animals given ClPhe + Phe had attained the weight of the controls given saline and remained so throughout these experiments. The undernourished animals weighed about the same as the animals given ClPhe + Phe at the end of the treatment period. However, the male undernourished animals continued to be significantly lower in weight at 39 days (P < 0.01) and 18 weeks (P < 0.0025). The undernourished females were also lower in weight at 39 days and at 18 weeks (P < 0.1). The F.S.D. level of significance here is P < 0.025.

Eye Opening. Controls given saline opened their eyes at 16.8 days of age on the average, significantly before the group given ClPhe + Phe, which opened their eyes at 18.0 days (P < 0.001). Undernourished controls opened their eyes at 17.0 days, not significantly different from controls given saline. F.S.D. level of significance is again P < 0.025.

Activity Wheel (Fig. 1). The ClPhe + Phe-treated subjects, both males and females, were more active than their controls given saline (P < 0.025 for males, P < 0.01 for females). The repeated measure of the activity of the males confirmed the initial results (P < 0.025). The activity of 17 undernourished females tested at 29 weeks of age was not significantly different from that of 7 well-nourished females raised simultaneously and tested at the same age.

Activity Avoidance in a Two-Way Shuttlebox. There was no difference between saline- and ClPhe + Phe-treated animals in simple two-way shuttlebox active avoidance.

Activity Avoidance with a Right-Left Position Choice (Fig. 2). There was no significant difference between saline-treated and

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**Table 1. Body weights ±SEM**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Sex</th>
<th>22 days</th>
<th>30 days</th>
<th>18 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weights (in grams) at</td>
<td></td>
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<tr>
<td>Saline</td>
<td>Males</td>
<td>28.5 ± 0.7 (74)</td>
<td>77.5 ± 2.7 (30)</td>
<td>266 ± 3.0 (29)</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>28.5 ± 0.7 (74)</td>
<td>77.5 ± 2.7 (30)</td>
<td>266 ± 3.0 (29)</td>
</tr>
<tr>
<td>ClPhe + Phe</td>
<td>Males</td>
<td>*20.4 ± 0.4 (76)</td>
<td>68.2 ± 1.1 (55)</td>
<td>154 ± 1.6 (46)</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>*20.4 ± 0.4 (76)</td>
<td>68.2 ± 1.1 (55)</td>
<td>154 ± 1.6 (46)</td>
</tr>
<tr>
<td>Undernourished</td>
<td>Males</td>
<td>*19.2 ± 0.5 (40)</td>
<td>168.5 ± 1.9 (20)</td>
<td>1253 ± 2.5 (19)</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>*19.2 ± 0.5 (40)</td>
<td>168.5 ± 1.9 (20)</td>
<td>1253 ± 2.5 (19)</td>
</tr>
</tbody>
</table>

In comparison with controls given saline, P < 0.001 (*), P < 0.0025 (†), or P < 0.01 (‡). Figures in parentheses indicate number of animals in each group. At 22 days the animals had not been separated by sex.
CIPhe + Phe-treated subjects in acquiring an initial position choice opposite to their natural preference although the CIPhe + Phe-treated animals took, on the average, longer to learn. When subjects were required to reverse their initial position choice, the CIPhe + Phe-treated animals took significantly longer ($P < 0.01$).

**Y-Maze.** CIPhe + Phe-treated subjects take 15.1 trials to achieve criterion performance while saline-treated controls require 10.4 trials. These results are not statistically significant ($P < 0.08$). If the saline-treated rats are compared with the 10 of the 13 CIPhe + Phe-treated subjects who have cataracts, there is a statistically significant difference between these groups ($P < 0.02$) on initial position learning.

**Appetitive Learning.** The numbers involved are too small to be statistically significant ($P < 0.06$) but the observations are interesting. One CIPhe + Phe-treated animal died before fully trained to work in the Y-maze. The other two took an average of 475 trials to become trained for actual appetitive learning testing (see Methods) while the saline-treated controls required 260 trials to work to a uniform level in the Y-maze. The CIPhe + Phe-treated subject required 30% more trials during the actual testing to reach criterion, and retained the position learning less than half as well as the saline-treated rats when retested 2 weeks later. The CIPhe + Phe-treated subjects were described as being less motivated to learn than the saline-treated controls during all phases of the training.

**Cataracts.** Less than 10% of CIPhe + Phe-treated animals developed cataracts before 39 days of age. Between 39 days and 6 months of age, fewer than 5% of the remaining animals developed cataracts. Between 6 and 12 months of age, however, 40% of animals not having cataracts at 6 months, developed cataracts. Detailed examination of the biochemistry of these late-appearing cataracts is presented elsewhere (14).

**Neuropathology and Brain Weights (Fig. 3).** Procedures were conducted by Dr. Anatole Dekaban, Section on Neurological Development, National Institute of Neurological Diseases and Stroke. The cerebellum of the 10-day-old CIPhe + Phe-treated animals was smaller than those of the saline-treated controls. Additionally, the cells in the external granular layer of the cerebellum were reduced in number. The 20-day-old experimental animals showed a slightly thinner white-matter area, but there were no focal lesions and no obvious myelin deficits. In the 30-day-old CIPhe + Phe-treated animals, the cerebellum and brainstem were about 20% smaller in diameter than those of the saline-treated controls. The myelin in the CIPhe + Phe-treated animals stained considerably lighter than that of the saline-treated animals. The brain weights of the CIPhe + Phe-treated animals averaged 76% of controls on day 10 and 86% of controls on day 20.

**DISCUSSION**

This paper reports further observations on the enduring behavioral deficits found in rats with model PKU treated with CIPhe + Phe during the first 21 days of life. By use of a new set of litters and different methods of testing, the results reported previously (5), i.e., hyperactivity and learning deficits in adult animals, have been extended. In addition, data suggesting a delay in neural development are presented.

A control group of moderately undernourished animals was included to assess the contribution of the temporary lowering in weight during the treatment period to the total deficit of the phenylketonuric animals. The data indicate that moderate undernutrition does not delay eye opening significantly and suggests that CIPhe + Phe retards cerebral development be-
yond that caused by weight deficiency alone. Smart and Dobbing's undernourished animals (15) showed a significant delay in eye opening, but their experimental undernourished animals weighed only one-third that of controls. In contrast, the moderately undernourished animals in these experiments weighed two-thirds that of controls at the end of the treatment period, about the same, happily, as the group with PKU.

The data showing that all the subjects with PKU achieved the weight of the saline-treated controls by day 39, whereas, the male undernourished animals did not, suggest another difference between these groups. This difference is consistent with published reports that the decreased body weight resulting from early malnutrition is a permanent effect (16). Finally, the activity of the undernourished animals was not different from that of normally nourished, uninjected controls, when studied with the activity wheel. Thus, it appears that the effects of the ClPhe + Phe treatment are not primarily due to the moderate decrease in body weight occurring during the treatment period.

The activity wheels were chosen instead of the open field, which was used previously (5), to test activity for several reasons. (i) Testing is done at night when the animals are more active; (ii) the activity-monitoring period is 15 hr instead of 5 min; and (iii) the activity wheels are less subject to the effects of pretesting variables such as handling, which may affect results on the open field test. In this study, both treated males and females were more active than their controls. These data agree with the clinical reports of restlessness and hyperactivity among children with PKU. Previous studies with ClPhe + Phe have found either hypoactivity (6) or no difference between experimental and control subjects (7, 8). The time of treatment in those experiments, however, was not during the birth-to-21-day period; thus the treatment did not occur during the most vulnerable period.

The results of the performance on a position choice task in the modified shuttlebox indicate that the ClPhe + Phe-treated adult females have a perseverative defect which appears on reversal training. Apparently, the initial task of a right-left position choice is not sufficiently complex to differentiate between the groups. Reversal learning is more difficult and is subject to errors of perseveration from the initial learning experience. The result is similar to that found in our previous studies (5), showing that treated animals are equal to control animals on simple avoidance but have difficulty in reversal learning of an active avoidance task. An adequate animal model of PKU should demonstrate a permanent deficit in learning. One investigator reported a deficit in water-maze learning (7, 8). Most studies, however, have shown only a reversible learning deficit due to the nonspecific effects of the treatment (17, 18), or no deficit at all (19). These last three experiments differed from ours in not supplementing ClPhe with phenylalanine. Thus, the blood levels of phenylalanine in these experiments did not approximate the clinical state.

In the Y-maze avoidance experiment, the difference between saline-treated and ClPhe + Phe-treated groups becomes statistically significant only when the three noncataractous animals are not included in the results. This result suggests that perhaps the cataractous and noncataractous animals differ in the severity of the underlying brain damage, the cataractous animals having suffered greater brain damage, an external sign of which is the associated cataract. The information on the appetitive testing is included because it is the first experiment involving food deprivation and appetitive learning in this model. These first results suggest that a learning deficit is present on appetitive as well as aversive conditioning.

The data on performance in the learning tests are still preliminary for several reasons. Learning is a complex activity, including memory, motivation, and activity. The relative contribution of differences in these variables to each learning test still needs to be carefully assessed. The fact that ClPhe +
Phe-treated subjects do well on two-way active avoidance, a test in which the learning task is simple and a high degree of activity is involved, suggests that differences in activity do not explain differences in performance on more complex learning tasks. Nevertheless, specific measures of starting times and running speeds would be desirable. Early hyperphenylalaninemia causes a global change in the development of the brain, and motivational differences probably also contribute to the overall learning deficit. The data in these experiments do not explain why there is a difference in the impairment profile on the results of the two active avoidance tests.

The most consistent neuropathological findings in patients with PKU have been decreased brain weight, increased gliosis, and a variable myelin deficit (20). The disparity between the moderate neuropathological changes and the severe intellectual deficit is impressive. It is clear that we don’t understand in detail how the hyperphenylalaninemia of PKU causes mental retardation. An important fact to consider is that in addition to the contribution of an enduring structural neuropathology to mental retardation, a brain that develops too slowly will leave permanent functional changes even after the brain “catches up” in appearance because the necessary physical substrate for some learning was not present at the critical period when it was required. The present data show that the brains of treated animals range from 76 to 86% of controls during the treatment period. This difference is greater than the 5% difference found previously for mature adult animals (5) who have shown complete body weight recovery, indicating that partial recovery of the deficit in brain weight occurs as the animal matures. Hole (19), treating rat pups intraperitoneally with ClPhe alone, noted a 5.5% decrease in brain weight on day 15.

The two consistent findings on neuropathological examination of our treated rats during the development phase have been a reduction in the size of the brain compared with controls and some general defect in myelin staining. The defect is diffuse, not focal. There is an accompanying reduction in the number of cells in the external granular layer of the cerebellar cortex, an area that undergoes considerable development postnatally and is subject to effects that inhibit or retard cell division (21). The differences in staining between the ClPhe + Phe-treated and the control animals may be a reflection of a defect similar to that found upon comparing the biochemistry of normal myelin and myelin from subjects with PKU. Such studies show a markedly lower cerebroside content of the white matter in phenylketonurics (22).

In summary, the treatment of rat pups with ClPhe + Phe during the critical period of rapid brain growth between birth and 21 days of age results in a delay before weaning in neural development and enduring behavioral changes in activity and performance on learning tests in adult animals. Associated with these behavioral abnormalities is a lower than normal brain weight and a deficit in myelination.

We feel the use of ClPhe + Phe in rat pups before they are weaned results in reproducible and enduring changes in adult behavior which have many similarities to the behavioral disorder associated with clinical phenylketonuria.