

## DEMONSTRATION OF DEFECTIVE MAZE LEARNING AND ABNORMAL ELECTROENCEPHALOGRAMS IN PHENYLKETONURIC MICE

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**ABSTRACT.**—The effects of elevated levels of DL-Phenylalanine and L-Tyrosine in the diet of mice were studied. Litter mates of inbred albino mice were separated into experimental and control groups, equal in number and sex ratio. The experimental diets were supplemented with either 7% DL-Phenylalanine, 7% L-Tyrosine, or 3.5% of each of these amino acids. After three weeks, the animals were tested for the following responses: learning ability in a "T" maze, electroencephalogram patterns, body weight, and the presence of phenylpyruvic acid in urine samples. Deficient maze learning ability in experimental animals, a characteristically abnormal brain wave pattern, reduced body weight, and excretion of excess phenylpyruvic acid were noted in the experimental groups.

Phenylketonuria is an inherited metabolic disease appearing in the homozygous recessive condition and resulting from the inability to metabolize the amino acid phenylalanine. This inability is due to the inactivity of the liver enzyme phenylalanine hydroxylase which normally oxidizes phenylalanine to tyrosine. Phenylalanine then accumulates in the body and may reach near 30 times the normal level. The disease symptoms result from both the deprivation of the precursors necessary in the metabolic pathways, as well as, secondary effects relating to the inhibition of

other metabolic pathways due to the concentration of phenylalanine.

The most serious effect of phenylketonuria is the mental defect. Untreated the development of the central nervous will be permanently retarded. Most phenylketonurics have low I.Q. levels, usually below 50.

Treatment of phenylketonuria depends on an early administration of a low phenylalanine diet. The symptoms can be arrested and sometimes reversed, and, with proper treatment patients have developed with near normal mental abilities.

It has been demonstrated that an excess of phenylalanine and (or) tyrosine in the diet of experimental animals can induce many of the physiological manifestations of phenylketonuria. Since metabolic disorders are often associated with abnormal electroencephalograms (Condon et al., 1954), abnormal patterns could be expected in phenylketonuria. Indeed both a decrease in learning ability (Auerbach, 1958) and alterations in electroencephalogram patterns (Cadell, 1962) have been observed in experimental phenylketonuria; also investigations by Gibbs (1941) found electroencephal-

ogram abnormalities in human phenylketonuria and described them with the outstanding features being high voltage, wave and spike discharges similar to, but more irregular than those of petit mal.

It is noted, however, that within the literature there is very little work demonstrating EEG patterns and learning ability in the same group of animals. Furthermore, there is some controversy concerning the time at which excess amounts of phenylalanine and (or) tyrosine must be administered (Woolley, 1964). Finally, the proposal that excessive serum levels of phenylalanine and (or) tyrosine might inhibit active transport of various amino acids during absorption in the intestine (Linneweh, 1963) seemed to indicate that physical measurements, such as body weight, might have significance, since a lowered body weight might result if essential amino acids were not properly taken up in the intestine. It would seem that if phenylalanine and tyrosine concentrations (independent variables) were increased at some level of maturation (independent variable) certain changes from control values in learning ability, electroencephalogram patterns, body weight, and chemicals excreted in the urine (dependent variables) could be expected.

#### METHODS AND MATERIALS

Inbred strains of white mice were weaned at age 16 days, dividing the litter mates equally according to sex and number, into groups of controls and experimental. The controls were provided with stock diet (Wayne Lab-Blox, 4% fat, 4.5% ash and fiber, and 24% protein- 0.83% tyrosine and 1.17% phenylalanine). Experimental group I

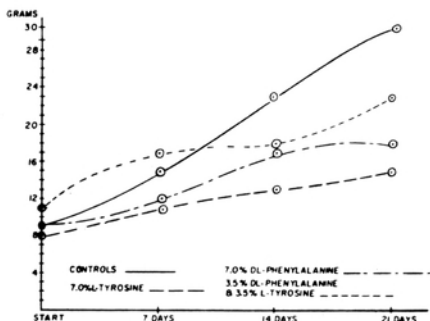


FIGURE 1. Weight during the period of experimental diet. Animals were placed on the selected diet at age 16 days.

received a standard stock diet with 3.5% L-Tyrosine and 3.5% DL-Phenylalanine added. Experimental group II received the standard diet with 7.0% DL-Phenylalanine added, while experimental group III received the standard diet with 7% L-Tyrosine added. During the developmental period (weaning to the time of testing comprising approximately three weeks) weekly measurements of body weight were made for both experimental and controls. The amount of diet consumed was also recorded at the end of the growth period.

#### Maze Testing

When the animals were 6 weeks old they were tested in the maze (Fig 2).

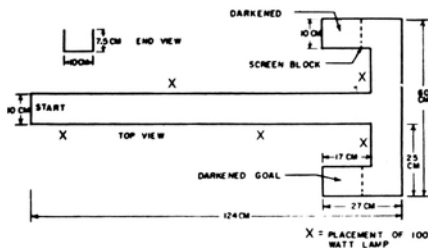


FIGURE 2. Diagram of maze used to test learning ability of mice. Material = 1/16" stainless steel.

Samples of urine were taken for analysis by Chromatographic methods. The maze was constructed of stainless steel, brightly illuminated, with one side providing a darkened hiding place, while the other side had a similar darkened hiding

place but the mouse was prevented from entering it by a wire mesh screen. The mouse entered the end of the channel and was given five minutes to acquire a spatial position habit consisting of a discrimination between the two equally darkened areas, one of which was closed to entrance. The stimulus was the bright light reflected from the steel surface into the non-pigmented eye of the albino animals. Rewards consisted of timed periods in the darkened hiding arm of the maze (goal). The maze was cleaned with 2% Lysol spray after the initial learning period to prevent scent from altering the results. For testing, the mouse was placed at the end of the channel and allowed to move down the channel to the cross of the "T", where a decision to move right or left was made. A positive response involved a movement toward the open darkened arm of the maze. The maze was cleaned between trials and process repeated ten times for each subject. Therefore random performance (lack of spacial habit acquisition) would have resulted in five positive responses in ten trials. Any

number of positive responses above the value of five represented learning as defined in this experiment.

#### *Electroencephalogram Method*

In this experiment the purpose of the electroencephalogram was to establish an indication of general brain activity. Monopolar EEG patterns were taken on an E&M Hi-Gain Preamplifier-E&M Physiograph Model #4 combination. A grounded steel box enclosing the animal and shielded leads to the recording instruments were necessary to minimize 60-cycle interference. Nembutal (.02cc) was administered intramuscularly to reduce muscle artifacts, simplify EEG interpretation, and facilitate electrode attachment. Mice were placed in a rack which held them in a pronated position, with their head held firmly in place and which allowed a nasopharyngeal electrode to be maintained in position. The other electrode to be maintained in position was attached to an electrically neutral region, the ear. EEG's were taken after the mice had been tested in the maze.

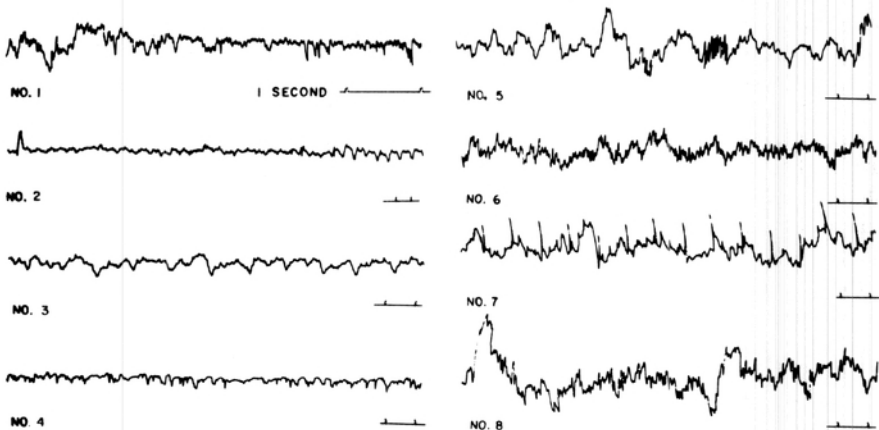


FIGURE 3. Characteristic electroencephalogram patterns shown with time intervals. No. 1-3—Controls; No. 4—Experiment 1; No. 5, 6—Experiment 2; No. 7, 8—Experiment 3.

#### RESULTS

The results of the maze learning test are shown in Table 1. Control animals were rather consistent in their performance with 7.5 (S.D. 0.4) positive responses in 10 trials.

The group of animals receiving 3.5% DL-Phenylalanine and 3.5% L-Tyrosine demonstrated approximately the same results, while both groups of experimental animals receiving 7.0% L-Tyrosine and 7.0% DL-Phenylalanine respectively, showed signifi-

TABLE 1. Learning ability of mice as measured in maze learning test. Duration of diet: 3 weeks.

| Treatment             | No. of Mice | Maze Score   |
|-----------------------|-------------|--------------|
| Controls              | 66          | 7.5 $\pm$ .4 |
| 3.5%DL—P+3.5%L—T      | 24          | 7.6 $\pm$ .8 |
| 7.0%DL—P              | 26          | 5.8 $\pm$ .6 |
| 7.0%L—T               | 25          | 5.5 $\pm$ .8 |
| DL—P=DL—Phenylalanine |             |              |
| L—T=L—Tyrosine        |             |              |

cant reduction in maze performance. Analysis of control animals compared to the experimental diet groups consisting of 7% DL-Phenylalanine and 7% L-Tyrosine shows significantly reduced performance in the experimental group ( $p$  less than 0.28). There was no significant difference in performance of the control group when compared to the experimentals on the 3.5% DL-Phenylalanine plus 3.5% L-Tyrosine supplemental diet. Correlation between the group on the 3.5% DL-Phe plus 3.5% L-Ty diet and the other two experimental groups showed significant differences ( $p$  less than 0.27).

It was found that the representative EEG patterns of Experiment I (pattern number 4) appear essentially similar to the control EEG's (patterns 1, 2, and 3). In the groups receiving 7.0% L-Tyrosine (patterns 7 and 8) and 7.0% DL-Phenylalanine (patterns 5 and 6) respectively, characteristic large wave complexes were observed superimposed on a background pattern very similar to the controls. Occasional irregularities of rhythm were noted as shown in example 5 of the EEG diagrams. The above observations represent qualitative clinical judgments and

are not meant to imply quantitative analysis.

The average weight of the animals at age six weeks is shown in Table 2.

TABLE 2. Average weight of mice at six weeks. Animals were placed on diet when 16 days old.

| Treatment             | No. of Mice | Average Weight (grams) |
|-----------------------|-------------|------------------------|
| Controls              | 66          | 30                     |
| 3.5%DL—P+3.5%L—T      | 24          | 23                     |
| 7.0%DL—P              | 26          | 18                     |
| 7.0%L—T               | 25          | 15                     |
| DL—P=DL—Phenylalanine |             |                        |
| L—T=L—Tyrosine        |             |                        |

Controls were highly consistent with an average weight of 30 grams. The experimental groups all showed a reduced body weight with the lowest average being 15 grams for the 7.0% L-Tyrosine diet. Figure 1. shows the growth curves for the various experimental diets. All the curves are similar in slope for the first week. Beginning at 7 days the curves of the experimentals become flattened, while the growth curve for the controls has its greatest slope. The curves end with the controls significantly heavier than any of the experimental groups. This is in spite of the fact that equal amounts of diet per gram body weight were eaten by all animals. Analysis of experimental group body weights shows significant differences from control values ( $p$  less than 0.01). Furthermore the differences between groups receiving 7% DL-Phe and 7% L-Ty compared to the group receiving a mixture of 3.5% DL-Phe plus 3.5% L-Ty is significant ( $p$  less than 0.02).

TABLE 3. Relationship between the score in the maze test, excess phenylalanine, Tyrosine, and Phenylpyruvic acid in the urine, and irregular Electroencephalograms.

| Treatment             | Score in Maze | Chemicals in Urine | EEG Patterns*          |
|-----------------------|---------------|--------------------|------------------------|
| Controls              | 7.5 $\pm$ .4  | None               | Regular                |
| DL-P + L-T            | 7.6 $\pm$ .8  | None               | Regular                |
| DL-P                  | 5.8 $\pm$ .6  | DL-P, L-T, PPA     | Irregularities Present |
| L-T                   | 5.5 $\pm$ .8  | L-T                | Irregularities Present |
| DL-P=DL-Phenylalanine |               | L-T=L-Tyrosine     | PPA=Phenylpyruvic Acid |

\* Patterns of controls were studied to determine regularity of EEG; major deviations from controls as found in experiments are explained in text.

tions from controls as found in experiments are explained in text.

The urine analysis (Table 3) shows that in the case of experimentals on the 7.0% L-Tyrosine diet, tyrosine was excreted in the urine. Animals on the 7.0% DL-Phenylalanine diet were found to have large amounts of phenylalanine, tyrosine, and phenylpyruvic acid in the urine. The appearance of phenylpyruvic acid and phenylalanine in the urine of all the animals receiving 7% DL-Phenylalanine was to be expected as such excretion has long been known to be a clinical result of such dietary treatment. Such excretion was observed in all cases of the 7% DL-Phe diet. Likewise the excretion of tyrosine in the experimental group receiving 7.0% L-Tyrosine supplement was to be expected and was observed. Tyrosine was expected in the urine of the experimental animals receiving 7.0% DL-Phenylalanine, since a large portion of the phenylalanine is transformed into tyrosine (Duncan, 1964) thus elevating serum levels to a degree exceeding the renal threshold. Such excretion was observed in 68% of all animals on DL-Phenylalanine 7% diet. (p less than 0.01)

#### CONCLUSIONS

It would seem from the results of

the maze learning test (Table 1.) that excessive amounts of either DL-Phenylalanine or L-Tyrosine can cause significant reductions in learning ability in experimental animals. Excessive amounts of these metabolites also appear to affect the electroencephalogram pattern as noted by the characteristic differences in EEG observed between the experimental animals receiving 7.0% DL-Phenylalanine or 7.0% L-Tyrosine, and those receiving 3.5% DL-Phenylalanine plus 3.5% L-Tyrosine, or the standard diet. Table three summarizes the primary differences noted in maze learning, urine excretions, and EEG irregularities. It is noted when both DL-P and L-T are fed in excess, but in moderate amounts and in relative balance, that there is no significant change in any of the parameters measured when compared to the controls. However, when either DL-P or L-T is fed alone in great excess, the significant decrease in maze learning ability is accompanied by urine excretion of the compound or metabolite, as well as the appearance of abnormal EEG patterns.

The rather extreme reduction in body weight among the experimentals substantiates the hypothesis that physical factors such as body weight should be studied in experiments in-

volving special diets, and it is felt that the shape of the growth curves of the experimental animals from the seventh day to the fourteenth day on the diet may have significance. It is hypothesized that some time between the seventh and the ninth day serum levels of phenylalanine and (or) tyrosine reach levels which can inhibit absorption of essential amino acids in the intestine, thus causing a mild form of malnutrition. However, further research is needed to substantiate this point. No definite explanation can be offered at this time for the reduced maze learning or the abnormal electroencephalograms. However, if tryptophan absorption in the intestine, or across cell membranes were inhibited, this might result in a reduction in cerebral serotonin since tryptophan is a precursor for serotonin. Woolley (1964) feels that such a reduction from birth leads to decreased learning ability. Perhaps a similar reduction in slightly older mice will also reduce learning ability. Research is planned using specific agents (serotonin, its precursors, and inhibitors) which should help clarify this point.

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