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# Effect of Phenylalanine and *p*-Chlorophenylalanine Administration on the Development of Rat Brain 2′,3′-Cyclic Nucleotide 3′-Phosphohydrolase[1] (38388)

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(Introduced by R. W. Luecke)
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Attempts to explain the mental defect associated with the untreated phenylketonuric patient have encompassed a wide range of proposals, but as yet, a clear explanation has failed to emerge. Alvord *et al.* (1) first suggested that the neuropathology of phenylketonuria (PKU) was characterized by an arrest of myelination in young patients; chemical evidence (2, 3) indicating lower concentrations of cholesterol, cerebrosides, and proteolipid protein supported this view. However, other theories have been suggested involving demyelination in the adult or absence of pathological changes of myelin [for a review, see (4)].

Prior work on the effects of experimental PKU on the developing rat brain have emphasized the reduction in myelin lipids (5–8). This report deals with the development of the myelin-associated protein, 2′,3′-cyclic nucleotide 3′-phosphohydrolase (EC 3.1.4.16b) and of the mitochondrial proteins, hexokinase (EC 2.7.1.1) and cytochrome *c* oxidase (EC 1.9.3.1), under conditions employing injection of both phenylalanine and *p*-chlorophenylalanine (9), since neuronal protein synthesis is disrupted by this treatment (10).

*Materials and Methods.* Pregnant rats were obtained from the Holtzman Co. (Madison, WI) and were fed either a synthetic control diet described previously (11) or a commercial pellet diet purchased from Allied Mills, Inc. (Chicago, IL). DL-*p*-Chlorophenylalanine, L-phenylalanine, and L-tyrosine were the products of Sigma Chemical Co. (St. Louis, MO). 1-Nitroso-2-naphthol was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Treatment was induced in rat pups by sc injection of L-phenylalanine (30 mg/ml, pH 7.4) at a dose of 1 mg/g body weight (12). The injection solution also contained DL-*p*-chlorophenylalanine (3 mg/ml) so that a dose of 0.1 mg/g body weight was achieved. Pups receiving 33 μl/g body weight of 0.2 *M* NaCl, pH 7.4, served as injection controls. In injection experiment I, dams were fed the synthetic diet and pups were injected twice daily from age 6–20 days. In injection experiments II and III, dams were fed the pellet diet and pups were injected once daily between ages 6–20 (Expt II) and ages 3-13 (Expt III). These pups were weaned at 21 days of age and were fed the pellet diet until 40 days of age. In all injection experiments, pups were randomized at 1 day of age and distributed at eight per litter.

Pups were killed either by exsanguination (Expts I and II) or by decapitation (Expt III). In Expt I, plasma tyrosine was fluorometrically determined by the method of Ambrose *et al.* (13). Brain 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP) (14) and cytochrome *c* oxidase (11) were measured on tissue stored for various periods at −90°. CNP activity was stable to storage; however, loss of cytochrome *c* oxidase activity on storage is sufficient to permit only relative comparisons between experiments. For injection Expt III, cerebellar hexokinase was measured on crude homogenates of fresh tissue according to the procedure of Knull *et al.* (15). Details of these procedures including statistical methods are reported elsewhere (11). Enzyme activities were defined on a unit/g fresh weight tissue basis with 1 unit defined as 1 μmole of

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product formed/min.

*Results*. During injection Expt I, measured tyrosine ratios indicated an initial rise in plasma tyrosine of the treated pups, which was followed by a drop to levels found in pups injected with saline (Table I). Pups which received no injections and whose dams were on a commercial diet had plasma tyrosine levels equivalent to the saline-injected pups (Table I). Attempts to quantify plasma phenylalanine levels by the fluorometric method of McCaman and Robins (16) were unsuccessful due to interference by DL-*p*-chlorophenylalanine, which when analyzed under optimal conditions for L-phenylalanine, resulted in an increased relative fluorescence.

Twice-daily injections in Expt I were accompanied by high mortality and marked differences in growth of whole body and brain (Fig. 1). In comparison (Table II), although pups from Expt II, in which injections were halved, demonstrated differences in body and brain weights even after 20 days of recovery, absolute differences between groups were less than in Expt I. When pups were allowed to recover after day 13, Expt III, only cerebellar weights were different at 40 days of age. Differences between the groups were also less reduced than in Expt I.

In Expt I, the concentration of whole brain 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNP), an enzyme enriched in CNS myelin (17),

TABLE I. Plasma Tyrosine Ratios in Developing Rats After Injection of L-Phenylalanine and DL-*p*-Chlorophenylalanine.[a]

| Age | Phenylalanine *p*-chlorophenylalanine | Control[b] |
|---|---|---|
| (days) | Saline | Saline |
| 6 | 1.0 | — |
| 8 | 1.92 | — |
| 10 | 2.72 | — |
| 12 | 4.31 | — |
| 14 | 1.75 | 1.07 |
| 16 | 0.99 | 1.07 |
| 18 | 0.81 | 0.97 |
| 20 | 0.99 | 1.14 |
| 22 | — | 0.99 |

[a] Ratios were determined on mean tyrosine values of duplicate determinations on pools of at least four pups.

[b] Control pups refer to rats given no injections and whose dams were fed the commercial pellet diet.

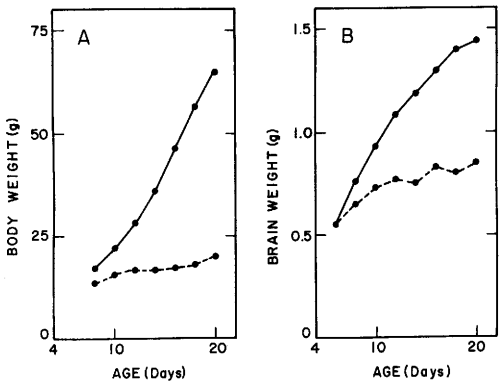


FIG. 1. The postnatal growth [body weight (A) and whole brain weight (B)] of rats injected twice daily from 6 days of age with either saline (●——●) or phenylalanine and *p*-chlorophenylalanine (●---●), Expt I. Plotted points are the means of pools of pups (A) or of pools of five brains (B). Injection procedures can be found detailed under Materials and Methods.

was much lower in animals receiving the L-phenylalanine DL-*p*-chlorophenylalanine; mean differences were highly significant on days 14, 16, 18, and 20 (Fig. 2A). In Expt II in which injections were given once daily, whole brain CNP concentration was reduced at day 20 but equivalent at day 40 after recovery (Table III). At this age, however, CNP content per brain was significantly higher in the saline-injected rats [682 ± 32.4 units versus 596 ± 49.8 units ($\alpha$ = 0.05)]. For injection Expt III in which treatment began earlier, day 3 versus day 6, but was terminated earlier, day 13 versus day 20, CNP concentration in the cerebellum and in the whole brain less the cerebellum demonstrated a different pattern (Table III). At 7 days of age, there was a significant decrease in both cerebellar and whole brain (less cerebellum) CNP concentrations. At 20 days of age, a week after treatment, only whole brain (less cerebellum) CNP concentration was higher in the saline injection pups; at 40 days of age, no differences were measureable in either region.

Like CNP, both hexokinase and cytochrome *c* oxidase increased with increasing age (Fig. 2B and Table IV). However, there were no consistent differences in concentration of cytochrome *c* oxidase between saline injected and L-phenylalanine DL-*p*-chlorophenylalanine-injected pups for either Expt I or III. Cerebellar hexokinase also failed to show a difference between groups in injection Expt III.

*Discussion*. Experimental animal models of

TABLE II. Body, Whole Brain, and Cerebellar Weights After Injection of L-Phenylalanine DL-*p*-Chlorophenylalanine.[a]

| Experiment no. | Age (days) | Body weight (g) | | Brain weight (g) | | Cerebellar weight (mg) | |
|---|---|---|---|---|---|---|---|
| | | Saline | L-phe *p*-Clphe | Saline | L-phe *p*-Clphe | Saline | L-phe *p*-Clphe |
| II | 13 | 33 ± 5.7 | 23 ± 3.2[b] | 1.13 ± 0.07 | 0.952 ± 0.065[b] | — | — |
| | 20 | 46 ± 5.1 | 30 ± 4.3[c] | 1.35 ± 0.06 | 1.08 ± 0.10[c] | — | — |
| | 40 | 151 ± 4.3 | 115 ± 20.6[b] | 1.59 ± 0.05 | 1.37 ± 0.07[c] | — | — |
| III | 7 | 19.5 ± 0.74 | 14.2 ± 1.6[c] | 0.72 ± 0.02 | 0.61 ± 0.03[c] | 53 ± 3.2 | 41.4 ± 2.5[c] |
| | 20 | 59.5 ± 4.4 | 33.2 ± 8.5[c] | 1.45 ± 0.05 | 1.18 ± 0.11[c] | 196 ± 16.0 | 139 ± 11.3[c] |
| | 40 | 174 ± 18.2 | 180 ± 18.8 | 1.62 ± 0.09 | 1.59 ± 0.03 | 234 ± 21.3 | 201 ± 15.4[b] |

[a] Values listed are means ± SD for four animals; tissue weights are on a wet weight basis. Animals were injected sc daily between 6 and 20 days of age (Expt. II) or 3 and 13 days of age (Expt. III), after which time they were allowed to develop without treatment. Means were compared by use of the *F*-variance ratio and Student's two-tailed *t* test at levels of $\alpha$ [0.05 (significant), 0.01 (highly significant)].

[b] Means different from saline controls when tested at $\alpha = 0.05$.

[c] Means different from saline controls when tested at $\alpha = 0.01$.

PKU in which administration of excess phenylalanine was used have been criticized (9) for failing to duplicate the high ratio of phenylalanine/tyrosine found in PKU patients. These authors have developed a model in which *p*-chlorophenylalanine, an *in vivo* inhibitor of rat liver phenylalanine hydroxylase (18), is administered in conjunction with phenylalanine. This approximates the plasma phenylalanine/tyrosine ratios found in PKU (19). Since injection of either phenylalanine or tyrosine into neonatal rats produced equivalent effects on reduced body weight, brain weight, total brain lipid, and incorporation of $^{35}$S-sulfate into brain (6), the model system of Andersen and Guroff (9) employing both phenylalanine and *p*-chlorophenylalanine was used in these experiments. The absence of elevated plasma tyrosine levels during the last week of injections in Expt I would imply reduction in phenylalanine hydroxylase activity. The injection of *p*-chlorophenylalanine alone causes no changes in serum tyrosine levels (20).

The chemical composition of isolated myelin from experimental PKU rat brain was reported to be normal although less myelin was present (8). These same conclusions were reached when comparing myelin from four PKU and five nor-

TABLE III. Effect of Injections of L-Phenylalanine and DL-*p*-Chlorophenylalanine on Development of 2′, 3′-Cyclic Nucleotide 3′-Phosphohydrolase in Rat Brain. [a,b]

| Experiment no. | Age (days) | Whole brain activity (units/g) | | Cerebellar activity (units/g) | |
|---|---|---|---|---|---|
| | | Saline | L-phe *p*-Clphe | Saline | L-phe *p*-Clphe |
| II | 13 | 83.5 ± 13.1 | 81.4 ± 11.5 | — | — |
| | 20 | 269 ± 22.9 | 217 ± 31.4[c] | — | — |
| | 40 | 429 ± 9.6 | 436 ± 19.1 | — | — |
| III | 7 | 16.7 ± 0.34 | 15.7 ± 0.63[c] | 26.0 ± 0.42 | 21.3 ± 3.34[c] |
| | 20 | 211 ± 4.7 | 181 ± 13.6[d] | 246 ± 14.2 | 234 ± 15.4 |
| | 40 | 396 ± 20.2 | 393 ± 19.9 | 324 ± 13.2 | 319 ± 22.0 |

[a] Whole brain less cerebella for Experiment III.

[b] Values represent means ± SD for four animals and are based on fresh weight of tissue. Treatment means were compared by the *F*-variance ratio and Student's *t* test at two levels of $\alpha$ [0.05 (significant); 0.01 (highly significant)].

[c] Means were different from saline controls ($a = 0.05$).

[d] Mean was different from saline control ($^{a} = 0.01$).

mal human brains (21). However, other evidence suggests that the myelin lipid from experimental PKU rat brain appears to be different, *i.e.*, there is a reduction in unsaturated and long-chain fatty acids (22). Evidence of abnormal myelin based on whole brain or white matter specimens from PKU patients also includes reductions in unsaturated and long-chain fatty acids (23) and changes in proteolipid fractions (24).

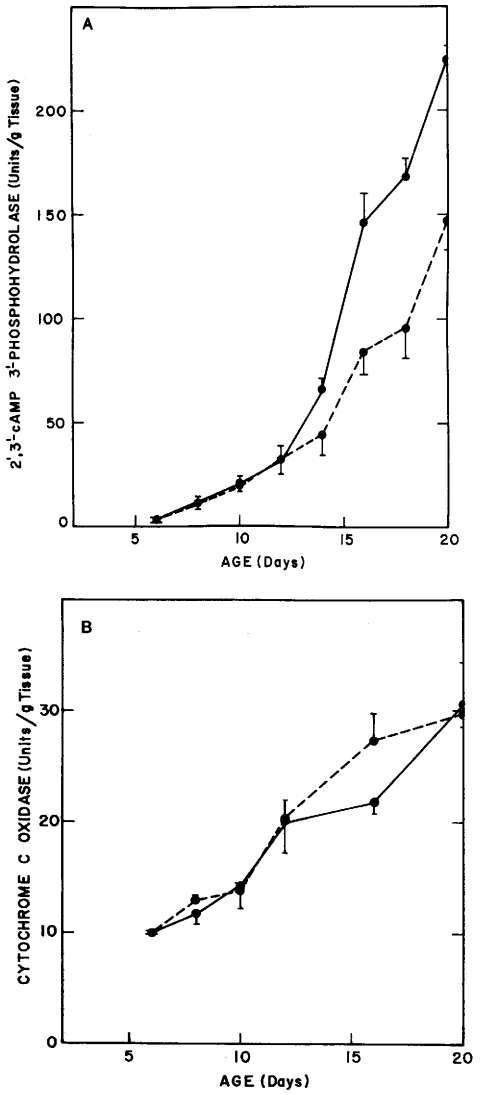


FIG. 2. A. The postnatal development of whole brain 2',3'- cAMP 3'-phosphohydrolase in saline-injected (●—●) and in phenylalanine, *p*-chlorophenylalanine-injected (●---●) rats. Injections were started at 6 days of age and were given twice daily. On alternate days, pups were selected from a random pool, decapitated, and brains were frozen (−90°) until enzyme activity was measured spectrophotometrically. Details of this injection procedure (Expt I) as well as the enzyme assay are described in Materials and Methods. Plotted points represent means ± SD for four brains based on wet weight of tissue. B. The postnatal development of whole brain cytochrome *c* oxidase in saline-injected (●—●) and in phenylalanine, *p*-chlorophenylalanine-injected (●---●) rats. Injections were started at 6 days of age and were given twice daily. On alternate days, pups were selected from a random pool, decapitated, and brains were frozen (−90°) until enzyme activity was measured spectrophotometrically. Details of this injection procedure (Expt I) as well as the enzyme assay are described in Materials and Methods. Plotted points represent means ± SD for four brains based on wet weight of tissue.

The concomitant increase in 2',3'-cyclic nucleotide 3'- phosphohydrolase concentration in rat whole brains with the period of myelination (Fig. 2A) agrees with the work of Olafson *et al.* (25). The decrease in enzyme content in treated pup brain from Expt I suggests a deficit in myelin protein concentration. Others have also suggested a decrease in myelin levels, in studies in which pups were injected with L-phenylalanine alone, based on reduced cholesterol and galactolipid (5), sulfatide (6), or proteolipid (7). Furthermore, Clarke and Lowden (5) found lower concentrations of cholesterol and galactolipid from brains of rats 40 days of age. These animals had been injected twice daily between ages 2 to 20 days before recovery until age 40 days. In the present study, experiment II, when pups injected between ages 6 to 20 days were allowed to recover, although body and brain weight differences persisted, the concentration of the myelin protein CNP showed no differences at 40 days. However, the amount of CNP per brain was higher in the saline-injected rats. When recovery was begun earlier, Expt III, body and brain weight differences were not observed at 40 days of age. The degree and duration of the initial insult seem to dictate whether recovery will occur. Fish and Winick (26) reported that DNA synthesis continues until 17 days of age in various regions of rat brain; thus, potential for recovery would be reduced if the abuse were carried beyond this period. Geison and Waisman (27) found little change in lipid distribution in rats that were fed excess phenylalanine beginning at 3 weeks of age.

Recently, Adelman *et al.* (28) reported that

TABLE IV. Effect of Injections of L-Phenylalanine and DL-*p*-Chlorophenylalanine on Development of Hexokinase and Cytochrome *c* Oxidase in Rat Brain, Expt. III.[a]

| Age (days) | Activity (units/g) | | | | | |
|---|---|---|---|---|---|---|
| | Cerebellar | | | | Whole brain[b] | |
| | Hexokinase | | Cytochrome *c* oxidase | | Cytochrome *c* oxidase | |
| | Saline | L-phe *p*-Clphe | Saline | L-phe *p*-Clphe | Saline | L-phe *p*-Clphe |
| 7 | 2.03 ± 0.11 | 2.16 ± 0.14 | 12.5 ± 1.2 | 13.3 ± 1.7 | 5.94 ± 2.0 | 5.07 ± 0.61 |
| 20 | 5.99 ± 0.33 | 6.05 ± 0.29 | 43.9 ± 3.5 | 38.9 ± 7.7 | 13.3 ± 3.2 | 16.3 ± 4.3 |
| 40 | 8.77 ± 0.24 | 9.10 ± 0.53 | 61.0 ± 6.5 | 62.8 ± 6.3 | 23.4 ± 13.0 | 29.9 ± 13.4 |

[a] Values are means ± SD based on four animals and wet weight of tissue.
[b] Whole brain less cerebella.

injections of phenylalanine to neonatal rats resulted in lesions to both cerebellar Purkinje and granule cells. Although we found no decreases in cerebellar cytochrome *c* oxidase or hexokinase, two mitochondrial proteins presumably enriched in neuronal cells (29), neuronal cell dysfunction may exist and contribute to the reduced myelin level. The molecular events giving rise to the neuropathology of PKU, hypomyelination and demyelination, remain an area for further inquiry.

*Summary.* Subcutaneous injections of L-phenylalanine and DL-*p*-chlorophenylalanine in developing rats resulted in reduced body weight, brain weight, and concentration of 2′,3′-cyclic nucleotide 3′-phosphohydrolase—a myelin-enriched protein. These reductions were less marked when injections were reduced by half; when pups were allowed to recover after age 13 days, no differences were observed at 40 days of age. Brain hexokinase and cytochrome *c* oxidase development was not affected by the injection treatments.

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