ULTRASTRUCTURAL CHANGES IN RAT OPTIC NERVE ASSOCIATED WITH HYPERPHENYLALANINEMIA INDUCED BY PARA-CHLOROPHENYLALANINE AND PHENYLALANINE

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ABSTRACT

The morphologic effects of hyperphenylalaninemia induced by treatment with para-chlorophenylalanine (PCP) plus phenylalanine on optic nerve were studied in developing F344 rats. PCP and phenylalanine were injected daily between days 5 and 20 days of life. At 20 days optic nerve of treated animals, as compared with saline-injected controls, showed enhanced neuroglial activity with broad astrocytic septae and debris-laden oligodendrocytes. In specimens obtained long after treatment with PCP and phenylalanine, continuing gliosis with evidence of focally abnormal myelination and axonal degeneration were observed. The results are consistent with a metabolic insult sustained in early development by astrocytes and oligodendrocytes, and are considered in relation to other work in experimental hyperphenylalaninemia and to human phenylketonuria.

INTRODUCTION

The phenylalanine analogue para-chlorophenylalanine (PCP) inhibits phenylalanine hydroxylase irreversibly and, when administered along with phenylalanine to rats during early development, produces biochemical and behavioral abnormalities similar in many respects to those of human phenylketonuria (PKU) (1-3). Rats treated in this manner are hyperactive and exhibit learning disorders. These abnormalities persist long after treatment is stopped, the PCP disappears, and phenylalanine levels return to normal (1-3). Neuropathological changes in this experimental disorder have not been examined on the ultrastructural level, though light microscopic observations in both experimental hyperphenylalaninemia (2) and in PKU (9) suggest abnormalities in myelination. In this study we examined optic nerve in rats during, and some time after, treatment with PCP and phenylalanine. Optic nerve was chosen as an easily obtainable tissue in which to study myelination in the central nervous system and one for which normal early developmental morphology in the rat has been described previously (11).

METHODS

Litters of F344 rats were individually housed and received food pellets and water ad lib. Five days postnatally pups were marked and assigned within each litter to control or ex-

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perimental groups. Pups were injected daily for 15 days beginning at day 5. Control animals were injected subcutaneously with 20 \( \mu \)L of 0.5% saline per g body weight. Experimental animals received daily subcutaneous injections of 20 \( \mu \)L/g body weight from the supernatant of a sterile 1.5% suspension of PCP in 0.9% saline containing 1.7% phenylalanine (1). Litter groups were maintained until 70 days at which time surviving pups were separated by sex. Animals were sacrificed with litter-matched controls at 10, 15, 20, and 80 days of age. Tissue was also obtained from F344 rats treated in 1971 with the above regimen of PCP and phenylalanine and from age-matched controls.

**Preparation for electron microscopy.** The cranial vault was exposed under pentobarbital anesthesia and its contents immediately fixed in situ with 4% glutaraldehyde in 0.15 M phosphate buffer at pH 7.2. The brain was carefully removed, avoiding tension on the optic nerves. Specimens were immersed in fixative and quadrants of the nerve cylinder trimmed to less than 1 mm from approximately 2 mm proximal to the optic chiasm. These were fixed for a total of 40 minutes in glutaraldehyde and postfixed in 1% osmium tetroxide in the same buffer for 90 minutes. After dehydration in graded ethanol and propylene oxide, specimens were infiltrated and embedded in Epon 812. Thick (1 \( \mu \)) sections were stained with 1% toluidine blue for light microscopy. Thin sections were prepared on the LKB Ultratome, stained with uranyl acetate and lead citrate according to standard methods, and examined with the JEOL 100B transmission electron microscope.

**RESULTS**

**Light microscope observations.** The effects of PCP and phenylalanine administration were apparent at this level of magnification in animals sacrificed at 80 days and at two years of age, at which times changes were similar. Optic nerve from saline-injected controls is fully myelinated at two years, with fine astrocytic processes dividing the nerve into densely packed axon bundles (Fig. 1). Neuroglial elements are not prominent, nor is there evidence of cell death. Optic nerve from PCP and phenylalanine animals also appears well myelinated (Fig. 2). Abnormal axon profiles are occasionally noted, representing pathology of the axon itself, the myelin sheath, or some combination of both. More striking, however, is the prominence of broad astrocytic processes dividing the nerve into smaller axon bundles. Twice the number of neuroglial nuclei are present in Figure 2 as in its saline-injected control; this impression of increased neuroglial cellularity was consistent in numerous specimens examined both at 80 days and two years of age.

**Electron microscope observations.** The first consistent abnormalities in the experimental group were noted in animals 20 days of age, after 15 days of PCP and phenylalanine administration.

Representative tissue from a 20-day control is shown in Figure 3. At this stage roughly half the axon population is involved to some extent in the process of myelination. This is in accordance with Rawlins' quantitative description of optic nerve myelination in the rat (11). Astrocytes contain scant endoplasmic reticulum, and their processes are long and delicate. Oligodendrocytes are seen as narrow tongues of cytoplasm enveloping myelinating axons.

Myelination is also well advanced in PCP and phenylalanine-treated specimens of the same age, and axonal profiles seem normal (Fig. 4). Astrocytes, however, appear more active, with abundant free ribosomes and broad "fibrous" processes occupying the increased inter-axonal space. Oligodendrocytes are laden
Fig. 1. Light micrograph of optic nerve from 2 year old control animal. Toluidine blue stain, × 1100.

Fig. 2. Optic nerve of PCP + phenylalanine-treated animal at 2 years. Note prominent astrocytic processes (ap), increased number of glial cell nuclei (small arrow) and degenerating axon (large arrow). Toluidine blue stain, × 1100.
FIG. 3. Electron micrograph of normally myelinating optic nerve, 20 day control animal. Astrocyte (As) at upper left.

FIG. 4. Optic nerve of PCP + phenylalanine-treated animal at 20 days. Oligodendroglial cell in center (OI), containing phagosomes (p), necrotic material (arrow), and mitochondria with electron dense matrix (m). "Active" astrocyte (As) at upper left.
with dense bodies and debris, consistent perhaps with diversion from their usual role in myelin formation to phageotic activity.

At 80 days a control specimen is fully myelinated with densely packed axon bundles (Fig. 5). Oligodendroglial cytoplasm is again generally restricted to a narrow tongue within either innermost or outer lamellae of the myelin sheath, the usual "type A" configuration described by Hirano and Dembitzer (6).

Specimens from experimental animals at 80 days demonstrate abnormalities in both axonal and glial elements. Certain axons appear grossly swollen with microtubules decreased in number (Fig. 6). Myelin sheaths are occasionally abnormal, with persistence of oligodendroglial cytoplasm within loosely packed lamellae (Fig. 7). The much increased inter-axonal space is occupied by broad interdigitating processes of fibrous astrocytes. Oligodendrocytes, seen apart from their usual association with myelinating axons, contain abundant rough endoplasmic reticulum, free ribosomes, Golgi apparatus, and numerous dense bodies (Fig. 8) similar to those noted previously in the 20 day experimental group (Fig. 4).

Control specimens at two years again show densely packed axonal bundles in which only very rare profiles remain unmyelinated (Fig. 9). Oligodendroglial cytoplasm remains confined to the innermost or outer lamellae (Figs. 9, 10). With very high magnification the periodicity within the myelin sheath is the expected 125 Å. Sheaths of adjacent axons occasionally fuse, and the two outer membranes condense to form an intraperiod line (arrow, Fig. 10).

Extreme pathological changes noted at two years in PCP and phenylalanine-treated animals are seen in Figures 11 and 12. Included are degenerating axons (Fig. 11), amorphous electron-dense debris (Fig. 11), and whorls of membranes (Fig. 12) apparently contained within fibrous astrocytes. It should be emphasized that the changes with lower magnification at this age as at 80 days consist mainly of prominent astrocytic septae with scattered necrotic material.

**DISCUSSION**

Administration of PCP and phenylalanine to rats between days 5 and 20 of life results in significant hyperphenylalaninemia during a period of rapid brain development (3). After cessation of treatment at 20 days, phenylalanine levels return to normal due to the hepatic synthesis of phenylalanine hydroxylase (5). This permits an assessment of both acute and chronic morphological changes in the induced hyperphenylalaninemia.

Prominent among the earliest abnormalities noted at 20 days in treated animals was enhanced neurogiial cell activity. In particular, unusually broad astrocytic processes were noted. Oligodendrocytes, while seen in their usual role in myelination, were observed laden with dense bodies and debris. Their dense cytoplasm, abundant free ribosomes, and lack of microtubules appear to differentiate these cells from the multipotential glia described by Vaughn and his co-workers (12). There appeared to be no general arrest or retardation in the process of myelination.
FIG. 5. Control specimen of optic nerve at 80 days, with narrow tongues of oligodendroglial cytoplasm (arrows) restricted to innermost or outer myelin lamellae. X 17,800.

FIG. 6. Optic nerve of PCP + phenylalanine-treated animal, 80 days. Swollen, degenerating axon in center, with smaller, more completely degenerated axon at right (arrow). X 17,800.
Fig. 7. Optic nerve of PCP + phenylalanine-treated animal, 80 days. Myelin sheath of axon at lower right is abnormally formed, containing glial cytoplasm within the sheath (arrow). Prominent astrocytic and collagen matrix. × 17,500.

Fig. 8. Optic nerve of PCP + phenylalanine-treated animal, 89 days. "Active" oligodendrocytes (01) with prominent rough endoplasmic reticulum, Golgi apparatus (g), and phagosomes (p). × 17,500.

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Fig. 9. Control specimen of optic nerve at 2 years. $\times 26,000$.

Fig. 10. Detail of Fig. 9, showing 125 Å periodicity of myelin sheath, with intraperiod line forming at arrow. $\times 220,000$. 

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Fig. 11. Optic nerve of PCP + phenylalanine-treated animal, 2 years. Degenerating axons and myelin fragments. × 20,000.
Fig. 12. Optic nerve of PCP + phenylalanine-treated animal, 2 years. Myelin remnants engulfed by astrocyte (As). × 26,000.
In mature animals examined long after phenylalanine levels had returned to normal, gliosis was more pronounced, with evidence of continuing phagocytic activity. Loosely wrapped myelin sheaths contained persistent islands of oligodendroglial cytoplasm, similar to the "type C" sheath described after dibenzanthracene and cyanide administration (6). These abnormal profiles, seen together with evidence of axonal degeneration, membrane inclusions, and phagocytosis, suggest the possibility of a metabolic insult to oligodendrocytes resulting in a population of unstable myelin sheaths with ensuing cell death, phagocytosis, and gliosis. Since even greater damage was evident in the two-year old rats than in the 35-day old animals, the pathologic changes appear to be progressive and continue long after the end of the insult.

It seems unlikely that these abnormalities were the result solely of PCP toxicity. PCP administered alone, in the dosage range used in this experiment, causes neither marked hyperphenylalaninemia (3) nor significant behavioral changes in the developing rat (10). On the other hand abnormalities similar to those described here have resulted from oral phenylalanine loading alone, namely gliosis with "immature" myelination in which glial cytoplasm persists within the myelin sheath (13).

The morphological changes reported are interesting in relation to current understanding of neuropathological changes in human PKU. Crome and Pare, reviewing 24 cases of PKU at postmortem, reported negligible deficits in myelination but distinct gliosis at the light microscope level (4). Others, observing gliosis in brain and optic nerve, hypothesized that decreased availability of myelin precursors in early development may result in "misdirected" glial activity (7, 9). Consistent with this is the finding of deficient myelin proteolipids in brain biopsies from human phenylketonurics (8). The observations presented here lend support to this early formulation of biochemical and morphological abnormalities in hyperphenylalaninemia.

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