Effect of L-phenylalanine on central nervous system elements in tissue culture¹

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The histopathological findings in oligophrenia phenylpyruvica (phenylketonuria) are, in most cases, limited to the pallor of the myelin in the brain and spinal cord (Benda, 1952; Crome, 1962; Poser and van Bogaert, 1959; Baar, Grümer, Beverage, Gordon, and Lee, 1963), and occasionally, neuronal changes have been recorded in younger patients (Alvord, Stevenson, Vogel, and Engle, 1950; Corsellis, 1953). Evidence has been provided that a metabolic error, preventing the oxidation of phenylalanine to tyrosine, may result either in retarded myelin formation or in damage to the formed myelin. Such a metabolic disorder, in which excess of a known substance results in damage to the developing nervous system, can be investigated successfully in a tissue culture model.

MATERIAL AND METHODS

The cultures were derived from newborn puppies. The cerebral cortex was dissected into small particles not exceeding 1 mm.³ Three such explants were placed on each of the 12 × 50 mm. cover slips in a plasma clot. Two coverslips placed back to back in culture tubes were maintained in roller drums with a nutrient fluid containing 81% Eagle's basal medium, 15% foetal calf serum with the addition of 1% each of embryo extract and glutamine, as well as a total of 1% of penicillin and mycostatin and phenol red indicator. All cultures were divided into four groups of 25 each, and, while one served as the control, the remaining cultures were subjected from the start to three concentrations of L-phenylalanine (Sigma) in the nutrient fluid, namely, 25 mg. per 100 ml., 50 mg. per 100 ml., and 100 mg. per 100 ml. These concentrations were chosen in an attempt to duplicate the pathological condition occurring in the brains of patients with phenylketonuria. At various intervals, until the 53rd day when the material was exhausted, random samples from each group were incubated for the determination of glucose-6-phosphate dehydrogenase (G6PD) activity with D-glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate (NADPH);

for lactate dehydrogenase (LDH) activity with sodium lactate and nicotinamide adenine dinucleotide (NAD); and with reduced form of dihydronicotinamide adenine dinucleotide (NADH) for dehydrogenase activity (Pearse, 1960). The slides were also counterstained with oil red O for the detection of sudanophilia. The control cultures as well as the experimental cultures were evaluated in areas where the proliferation and maturation of the neuroectodermal system had occurred, and in the areas where fibroblastic activity was predominant, in order to evaluate the effect of the drug, not only on the neurones and glia, but also on the mesenchymal cells.

DESCRIPTION OF FINDINGS

The control cultures showed evidence of morphological differentiation of the neuroectodermal elements after 10 days *in vitro*. At this time, the activity of NADH was increased in the perikaria as well as in the processes. The absence of oil-red-O-positive material reflected the absence of degenerative changes (Fig. 1). The activity of LDH, although lower, showed after 10 days' activity both in the glial elements and in the parenchymal cells. The maturation of the cells occurred steadily until the last controls were evaluated on the 53rd day, and the activity of NADH remained high in the cells, perikaria, and in the processes. The marked thickening of the neuronal processes suggested at the time myelinization in the cultures. There was, at no time, evidence of degenerative changes either in parenchyma or in glia which resulted in negative oil red O staining (Fig. 2). The fibroblasts overgrew some parts of the culture and displayed the typical parallel arrangement of the spindle-shaped cells.

The cells which were maintained in the various concentrations of L-phenylalanine were evaluated on the basis of this baseline. In the cultures subjected to 25 mg. per 100 ml. of phenylalanine in the nutrient fluid, the growth was somewhat delayed, although the differentiation of the elements, without any evidence of degenerative changes, was observed on the seventeenth day (Fig. 3). The differentiation

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FIG. 1

FIG. 1. Pattern of growth and differentiation after 10 days of growth in nutrient fluid. NADH and oil red O $\times 300$.

FIG. 2

FIG. 2. Cells after 53 days in nutrient fluid. NADH and oil red O. $\times 300$.

FIG. 3

FIG. 3. Cellular population after 17 days of growth in 25 mg. of L-phenylalanine per 100 ml. of nutrient fluid. NADH and oil red O. $\times 300$.

FIG. 4

FIG. 4. The pattern of differentiation after 53 days of growth in 25 mg. of L-phenylalanine per 100 ml. of nutrient fluid. L.D.H. $\times 300$.

FIG. 5. After 39 days of growth in 50 mg. of L-phenylalanine per 100 ml. of nutrient fluid. Consistent accumulation of small sudanophilic droplets in the perinuclear zone. NADH and oil red O. $\times 300$.

FIG. 6. Cellular destruction with evidence of intra- and extracellular droplets of sudanophilic material. Fifty-three days of incubation in 100 mg. of L-phenylalanine per 100 ml. of nutrient fluid. NADH and oil red O. $\times 300$.

which was seen with LDH, progressed steadily, continuing slowly until the fifty-third day to the point when no major difference with the control culture was present from the standpoint of either differentiation and maturation of cells or of the lack of degenerative sudanophilic products (Fig. 4).

A 50 mg. per 100 ml. concentration of L-phenylalanine caused delay in the migration and differentiation of the neuroectodermal cells. This effect was accompanied by the appearance of breakdown materials after three weeks. In some of the areas, the differentiation of the cells was distinct after 29 days. The overall NADH dehydrogenase activity was low, while some cells showed numerous sudanophilic droplets indicating degenerative changes. This finding was augmented by more specific degenerative changes in other areas, where apparently the maturation had progressed at a normal pace. In these cells an abundance of small sudanophilic droplets was present arranged concentrically around the nucleus. The droplets were found both in cells with normal or elevated NADH dehydrogenase activity and also in numerous cells where this activity was reduced or had disappeared completely. In the latter cells, the presence of the circular pattern of accumulation of the sudanophilic material was the only indication of the presence of the nucleus, indicating functional damage of severe degree, although morphological disintegration did not take place (Fig. 5). This particular type of degeneration was not observed in any of the examined cultures with either lower or higher concentrations of L-phenylalanine. The fibroblasts which were also observed in this culture did not exhibit any pathological changes. As the culture aged, the number of areas in which this pathological finding was observed increased, and more cells showed that NADH dehydrogenase activity had completely disappeared.

The culture, which was maintained at the L-phenylalanine level of 100 mg. per 100 ml. of nutrient fluid, showed normal fibroblastic growth without any evidence of degenerative changes or slowing down of the cell proliferation. In contrast, the neuroectodermal elements were severely affected. The migration of the cells from the explant was retarded and the culture never reached the level of maturity found in the control. The cells in numerous areas showed evidence of pronounced morphological damage. The activity of NADH was low or non-existent. In many areas, the disintegration of the processes and cell bodies could be observed. This disintegration was accompanied by the presence of a sudanophilic substance both within the damaged and degenerating parenchymal cells and free, apparently following breakdown of the cell continuity. These changes, after 53 days *in vitro*,

resulted, in many areas, in almost complete disintegration of all neuroectodermal elements (Fig. 6).

DISCUSSION

The results of the experiment indicate that concentrations of L-phenylalanine up to 100 mg. per 100 ml. ($6.06 \times 10^{-3}M$) *in vitro* do not affect the rate of growth or the maintenance of mesenchymal elements. In contrast, the effect on the neuroectodermal elements, and especially on the parenchymal, cells is obvious. The changes in concentration of 25 mg. per 100 ml. ($1.52 \times 10^{-3}M$) are of a mild character. The slowing of outgrowth and of differentiation is difficult to quantitate and the fact that no degenerative changes were present after 53 days *in vitro* indicates only a minor effect of phenylalanine at this concentration. The doubling of the amount of L-phenylalanine to 50 mg. per 100 ml. ($3.03 \times 10^{-3}M$) results in a definite retardation of the growth of neuroectodermal elements with evidence of degenerative changes, which are indicated by the appearance of the sudanophilic substance in the pericarium of the parenchymal cells. We have not observed any degenerative changes in the oligodendroglia and the occasional evidence of sudanophilic material in the astrocytes could be the expression either of the damage to the astroglia or the result of phagocytic activity of the astroglia *in vitro*. It should be noted that the distribution of the sudanophilic material in the cytoplasmic area surrounding the nucleus suggests a specific topographical target which is damaged by L-phenylalanine *in vitro*. The degree of damage observed at 100 mg. per 100 ml. ($6.06 \times 10^{-3}M$) cannot be evaluated with the same accuracy since the evidence of destruction is overwhelming at this concentration. It should be noted, however, that although the number of differentiated neuroectodermal cells, especially the parenchymal elements, was lower than in the lesser concentrations, some of the cells, despite the presence of a high concentration of phenylalanine, did succeed in attaining morphological differentiation, although no evidence of myelinization was observed in any of the cultures.

In correlating these findings with the symptoms of brain damage and the known pathological

findings in patients with phenylketonuria, the experimental model suggests that the target prone to damage in phenylketonuria is the neurone. The neuronal damage, which may be reversible in the initial stages, eliminates, at least temporarily, one of the elements essential for formation of myelin. Therefore, the retardation of myelinization which is observed in the brains of patients with phenylketonuria should be regarded as secondary to neuronal damage. Also, the pathological changes in neurones as reported in the literature are substantiated by this experiment. The seizures which are observed in these patients, especially in early, rapidly progressing cases, should be regarded as aetiologically precipitated by the neuronal damage. The concentration up to 100 mg. per 100 ml. shows an exaggerated picture which cannot be correlated with the pathology *in vivo* since the overwhelming neuronal destruction could not be compatible with life.

SUMMARY

The effect of L-phenylalanine on mammalian cells was evaluated *in vitro* in concentrations of 25 mg. per 100 ml. to 100 mg. per 100 ml. of nutrient fluid. The lowest concentration did not interfere visibly with cell differentiation, while the highest concentration was toxic and resulted in destruction of the cells. The intermediate concentration (50 mg. per 100 ml.) produced neuronal damage with the implication that the perikaryon zone is the target area.

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