Effects of Pyridoxine Deficiency and DL-*p*-Chlorophenylalanine Administration to Rats on 5-Hydroxytryptamine and Noradrenaline Concentrations in Brain and 5-Hydroxytryptamine Concentration in Blood

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Pyridoxine deficiency in post-weanling rats caused a marked decrease in body weight and a small but significant decrease in brain weight. Although the concentration of circulating 5-hydroxytryptamine was markedly decreased, the concentrations of 5-hydroxytryptamine and noradrenaline in the brain were not affected. *p*-Chlorophenylalanine, an inhibitor of 5-hydroxytryptamine synthesis, decreased the 5-hydroxytryptamine content of brain to very low values in both the deficient and control animals, whereas the noradrenaline contents were not appreciably affected. The concentration of 5-hydroxytryptamine in blood, the origin of which is primarily gastrointestinal, was decreased only in the controls but not in the deficient animals after *p*-chlorophenylalanine treatment. These results suggest that whereas L-tryptophan hydroxylase (EC 1.14.3.2) is rate-limiting in the brain as has been reported by others, the pyridoxal 5'-phosphate-dependent enzyme 5-hydroxytryptophan decarboxylase (EC 4.1.1.28) may be more important in the gastrointestinal tract in the regulation of 5-hydroxytryptamine synthesis.

Pyridoxine, as pyridoxal 5'-phosphate, functions as the coenzyme for aromatic L-amino acid decarboxylase (EC 4.1.1.26) and is thus directly involved in the synthesis of the putative neurotransmitters 5-hydroxytryptamine, dopamine and noradrenaline from tryptophan and tyrosine. The rate-limiting step in the synthesis of 5-hydroxytryptamine in brain, however, has been shown to be the site preceding the pyridoxal 5'-phosphate-dependent step (Jequier *et al.*, 1967), i.e. at the level of tryptophan hydroxylase (EC 1.14.3.2). It has been suggested that the synthesis of 5-hydroxytryptamine is controlled by a mechanism different from that for catecholamines in that it depends more on the concentration of the amino acid precursor (Moir & Eccleston, 1968; Fernstrom & Wurtman, 1971). The synthesis of 5-hydroxytryptamine is also readily affected by inhibitors of L-tryptophan hydroxylase (Koe, 1971). *p*-Chlorophenylalanine is one such inhibitor that has been widely used in recent years to deplete brain 5-hydroxytryptamine. In the present paper we describe studies on the interaction of pyridoxine deficiency and *p*-chlorophenylalanine treatment on the concentrations of 5-hydroxytryptamine in brain and blood, and of noradrenaline in brain.

Experimental

Animals

Sprague-Dawley male rats were used in the study. Weanling animals (21 days old) were placed on either

a pyridoxine-deficient or control diet (supplied by General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A.) of the composition described by Bhagavan *et al.* (1971). Animals not receiving pyridoxine showed all the typical signs of deficiency, which were quite marked after about 6 weeks on the diet. The animals were used in the study during the sixth and seventh weeks on the experimental diets.

Methods

***p*-Chlorophenylalanine treatment.** DL-*p*-Chlorophenylalanine suspension was prepared for injection by wetting 1.2 g with about eight drops of Tween 80 and was then mixed with water and made up to 20 ml (60 mg/ml). Pyridoxine-deficient and control rats (six per group) were given a single injection of *p*-chlorophenylalanine intraperitoneally (300 mg/kg body wt.). Uninjected animals (twelve per group) served as controls. The rats were killed by decapitation 48 h after the *p*-chlorophenylalanine injection (09:00–10:00 h). The time-interval of 48 h was chosen, since it has been shown that the maximum inhibition of 5-hydroxytryptamine synthesis in brain after *p*-chlorophenylalanine treatment occurs during this period (Jequier *et al.*, 1967; Brody, 1970; Koe, 1971). Blood was collected in chilled heparinized tubes and frozen. The brain was rapidly excised, homogenized in freshly prepared ice-cold 0.01 M-HCl containing 0.625% disodium EDTA (4 ml/g of brain) by the method of Welch & Welch (1969) and stored frozen.

Both blood and brain samples were analysed within a day.

Determination of 5-hydroxytryptamine and noradrenaline in brain homogenates. The reagents used were of highest purity and of fluorimetric grade wherever possible. All operations were carried out at 0–4°C unless otherwise indicated. Brain homogenate (1.5 ml) was pipetted into a tube containing 2 g of NaCl and 10 ml of butan-1-ol (water-saturated). The tubes were stoppered, shaken mechanically for 10 min and centrifuged at 2000g for 10 min. A portion (3 ml) of the butan-1-ol extract was pipetted into another tube, then 2 ml of 0.1 M-HCl and 5 ml of cyclohexane were added and the tubes shaken for 10 min and centrifuged as before. The organic phase was then carefully aspirated and the HCl extract was treated as follows at room temperature.

For determination of 5-hydroxytryptamine: 1 ml of the acid extract was treated with 0.4 ml of 10 M-HCl containing disodium EDTA (1 µg/ml). The fluorescence was read at 540 nm (uncorrected) with activation at 295 nm (uncorrected) on an Aminco-Bowman spectrophotofluorimeter at maximum sensitivity and 0.001 photomultiplier microphotometer setting.

For determination of noradrenaline: 0.5 ml of the acid extract was treated with 0.25 ml of 0.1 M-NaOH followed by 0.6 ml of 0.1 M-phosphate buffer ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$), pH 7.0, containing 0.9% disodium EDTA so that the pH of the resulting mixture was 6.5 ± 0.2 . The procedure of Shellenberger & Gordon (1971) was then used, with the following modifications: noradrenaline was oxidized in 0.1 ml of iodine reagent [5% (w/v) KI and 1.25% (w/v) I_2 in water]. After 2 min, 0.25 ml of Na_2SO_3 [2.5% (w/v) in 5 M-NaOH] was added and the mixture was left for 2 min before being acidified with 0.5 ml of acetic acid (33%, v/v) to pH 4.6 ± 0.2 . It was then heated in a boiling-water bath for 4 min and chilled in ice for 5 min. Fluorescence was then determined at 495 nm (uncorrected) with activation at 380 nm (uncorrected) at maximum sensitivity and 0.003 photomultiplier microphotometer setting.

Standards. Stock solutions of 5-hydroxytryptamine creatinine sulphate monohydrate (4.6016 mg/ml) and noradrenaline bitartrate monohydrate (400 µg/ml) were prepared in deionized double-distilled water and stored frozen in small portions (up to 4 weeks). Working standard for the combined assay was prepared as follows. Stock 5-hydroxytryptamine solution was diluted 1:10 in 3% (w/v) ascorbic acid saturated with disodium EDTA and KCl. This solution (200 µl) was mixed with 100 µl of stock noradrenaline solution and made up to 10 ml with 0.01 M-HCl containing disodium EDTA (0.625%), and 25–100 µl of this mixture was used in the assay (made up to 1.5 ml with 0.01 M-HCl containing EDTA and then treated exactly as the unknowns).

Determination of 5-hydroxytryptamine in blood. As the method for the combined assay of 5-hydroxytryptamine and noradrenaline was found unsuitable for blood, only 5-hydroxytryptamine was determined in blood, essentially by the method of Yuwiler *et al.* (1970).

Results

Pyridoxine deficiency markedly retarded the growth of the rats as shown by their body weights. Although the deficiency was induced in the post-weanling period when the growth of the brain is known to be essentially complete, the brain weights also showed a slight, but significant difference, as reported previously (Bhagavan & Coursin, 1971a,b). *p*-Chlorophenylalanine treatment resulted in a net loss of body weight in the control animals over the 2-day period after its administration, which may possibly be caused by decreased food intake. However, this effect of *p*-chlorophenylalanine was not conspicuous in the case of the deficient animals, whose growth rate was already constant at the time of treatment, unlike the controls which were still in an active period of growth.

Pyridoxine deficiency resulted in a marked decrease in the 5-hydroxytryptamine content of blood. However, the deficiency had no appreciable effect on the

Table 1. *Effect of pyridoxine deficiency on 5-hydroxytryptamine and noradrenaline contents in brain and 5-hydroxytryptamine content in blood of rats*

Pyridoxine deficiency was induced in post-weanling rats. Deficient and control animals (twelve per group) were killed by decapitation, and the 5-hydroxytryptamine and noradrenaline contents were analysed as described in the text. The values are given $\pm$ S.E.M.

Group	Body weight (g)	Brain weight (g)	5-Hydroxytryptamine in brain (µg/g)	Noradrenaline in brain (µg/g)	5-Hydroxytryptamine in blood (µg/ml)
Deficient	82 $\pm$ 3.1	1.635 $\pm$ 0.015	0.544 $\pm$ 0.026	0.436 $\pm$ 0.010	0.519 $\pm$ 0.058
Control	335 $\pm$ 13.5	1.879 $\pm$ 0.023	0.550 $\pm$ 0.022	0.405 $\pm$ 0.010	1.464 $\pm$ 0.102
<i>P</i>	<0.005	<0.005	Not significant	<0.01	<0.005

Table 2. Effect of *p*-chlorophenylalanine on 5-hydroxytryptamine and noradrenaline contents in brain and 5-hydroxytryptamine content in blood in pyridoxine-deficient and control rats

Pyridoxine-deficient and control rats (six per group) were injected intraperitoneally with *p*-chlorophenylalanine (300 mg/kg). The animals were killed 48 h after the injection and the analyses of 5-hydroxytryptamine and noradrenaline in tissues were carried out as described in the text. Values in parentheses indicate the number of animals. Values are given $\pm$ S.E.M.

Group	Body weights (g)		Brain weight (g)	5-Hydroxytryptamine in brain (μ g/g)	Noradrenaline in brain (μ g/g)	5-Hydroxytryptamine in blood (μ g/ml)
	Before treatment	After <i>p</i> -chlorophenylalanine treatment				
Deficient (6)	105 $\pm$ 4.9	101 $\pm$ 4.4	1.743 $\pm$ 0.020	0.147*	0.387 $\pm$ 0.020	0.445 $\pm$ 0.067
Control (6)	330 $\pm$ 13.6	318 $\pm$ 12.3	1.930 $\pm$ 0.027	0.211*	0.433 $\pm$ 0.010	0.507 $\pm$ 0.055
P	<0.005		<0.005	<0.05		
				Not significant		

* Results given are only for two animals in each group. The 5-hydroxytryptamine contents of the brain of the other animals were very low and could not be determined with a reasonable degree of accuracy. No comparison could therefore be made between the two groups.

concentrations of 5-hydroxytryptamine or noradrenaline in the brain (Table 1). *p*-Chlorophenylalanine treatment did not alter the concentration of noradrenaline in brain in either the deficient or control animals, but there was a striking decrease in the content of 5-hydroxytryptamine (Table 2). These results are for two rats only per group, as the 5-hydroxytryptamine content of the rest of the brains was so low that it could not be determined with any reasonable degree of accuracy. No difference between the groups is seen with this sample size.

p-Chlorophenylalanine treatment had a differential effect in the case of blood 5-hydroxytryptamine. It resulted in a marked decrease in the 5-hydroxytryptamine content of blood in the control animals, the values found being comparable with those of untreated deficient animals. The low content of 5-hydroxytryptamine in blood in the deficient rats was not decreased further by administration of *p*-chlorophenylalanine (Table 2).

Discussion

The present results show that pyridoxine deficiency has no appreciable effect on the concentrations of 5-hydroxytryptamine and noradrenaline in brain in rats. This is consistent with the findings of Schlesinger & Schreiber (1969) in mice, and of Sourkes *et al.* (1960) and Sourkes & Missala (1969) for noradrenaline in rats. However, there is a striking decrease in the concentration of 5-hydroxytryptamine in blood in pyridoxine deficiency. The origin of the circulating 5-hydroxytryptamine is known to be mostly gastrointestinal. As pyridoxine is directly involved in the synthesis of the biogenic amines, this effect of the deficiency may be attributed to a differential depletion of the coenzyme pyridoxal 5'-phosphate from various tissues. Previous reports (Maruyama & Coursin, 1968*a,b*; Sundaresan & Coursin, 1969) and unpublished work (H. N. Bhagavan & D. B. Coursin) from this laboratory show that whereas the depletion of pyridoxal 5'-phosphate from the brain is only about 50%, liver loses about 75% and blood concentrations are almost negligible (over 95% loss) after 6-8 weeks on the deficient diet. Although the pyridoxal 5'-phosphate content of the gastrointestinal tract was not assayed in this study, it seems likely that the extent of depletion of pyridoxal 5'-phosphate was great enough to decrease the synthesis of 5-hydroxytryptamine in the deficient animals. On the other hand, pyridoxal 5'-phosphate-dependent enzyme systems involved in the synthesis of 5-hydroxytryptamine and noradrenaline are apparently not rate-limiting with the depletion of about 50% of pyridoxal 5'-phosphate from the brain. Weber & Wiss (1963) and Davis (1963) found little change in the activity of 5-hydroxytryptophan decarboxylase (EC

4.1.1.28) in the brain, whereas the enzyme activity in the liver was markedly decreased in rats subjected to pyridoxine deficiency after weaning. The developing brain, however, appears to be more susceptible as the enzyme activity was significantly diminished in neonatal rats when the deficiency was induced either during gestation and/or lactation (Eberle & Eiduson, 1968; Wiss & Weber, 1964). This may be attributed to a much greater depletion of pyridoxal 5'-phosphate from the neonatal brain (Dakshinamurti & Stephens, 1969; H. N. Bhagavan & D. B. Coursin, unpublished work).

The inhibitory effect of *p*-chlorophenylalanine on 5-hydroxytryptamine synthesis appears to be influenced by the pyridoxine status of the animals. This may be expected as pyridoxine is also directly involved in the synthesis of 5-hydroxytryptamine at the 5-hydroxytryptophan decarboxylase step, which follows the site of *p*-chlorophenylalanine inhibition at the level of tryptophan hydroxylase. As *p*-chlorophenylalanine blocked the synthesis of 5-hydroxytryptamine to very low values in the brain, differences, if any, between the deficient and control rats could not be discerned under the experimental conditions. This may have been possible at lower dosages of *p*-chlorophenylalanine. However, with circulating 5-hydroxytryptamine, *p*-chlorophenylalanine produced a marked decrease in the control animals, the values found being comparable with those in the untreated deficient animals. Pyridoxine deficiency *per se* produced a marked diminution in the blood 5-hydroxytryptamine content, but this was not further lowered by *p*-chlorophenylalanine treatment. This suggests that for the circulating 5-hydroxytryptamine, which is primarily of gastrointestinal origin, the pyridoxal 5'-phosphate-dependent enzyme 5-hydroxytryptophan decarboxylase may be more important in the control of 5-hydroxytryptamine synthesis under experimental conditions. This, then, is in contrast with the brain 5-hydroxytryptamine, which is not affected by pyridoxine deficiency but by *p*-chlorophenylalanine treatment, thus suggesting that the rate-limiting step in this case is at the tryptophan hydroxylase site, which is in support of previous findings (Jequier *et al.*, 1967; Moir & Eccleston, 1968).

In addition to its inhibitory effect on tryptophan hydroxylase, *p*-chlorophenylalanine decreases the tryptophan content in brain (Tagliamonte *et al.*, 1971). Since the normal concentration of L-tryptophan in the mammalian brain is below the K_m value of tryptophan hydroxylase (Jequier *et al.*, 1967; McGeer *et al.*, 1968), changes in the intraneuronal concentration of L-tryptophan may also be expected to affect the rate of 5-hydroxytryptamine synthesis (Moir & Eccleston, 1968; Fernstrom & Wurtman, 1971). Although the tryptophan concentration of the brain in pyridoxine-deficient animals was not determined in the present study, it seems likely that there

were no significant changes, since no differences were observed in the content of 5-hydroxytryptamine in brain between the deficient and control animals with or without *p*-chlorophenylalanine treatment.

The results on noradrenaline show that pyridoxine deficiency has no appreciable effect on its concentration in brain. This indicates that 3,4-dihydroxyphenylalanine decarboxylase (EC 4.1.1.26) activity does not become rate-limiting under the experimental conditions. Further, *p*-chlorophenylalanine administration at the dosage employed has very little effect on the concentration of noradrenaline in brain in either the control or deficient animals, which indicates no appreciable interaction between the pyridoxine status of the animals and *p*-chlorophenylalanine treatment with respect to brain noradrenaline. The result that *p*-chlorophenylalanine had no appreciable effect on the brain noradrenaline content in control rats is consistent with previous reports (Koe & Weissman, 1966; Brody, 1970).

Although the overall concentrations of 5-hydroxytryptamine and noradrenaline in the brain were not affected by pyridoxine deficiency, possible differences in turnover rates or in the regional distribution should be considered. Whether the turnover of 5-hydroxytryptamine and the activity of 5-hydroxytryptophan decarboxylase in the gastrointestinal tract are affected in pyridoxine deficiency is not known. It will also be of interest to investigate kinetic differences, if any, between the brain and intestinal tryptophan hydroxylase and 5-hydroxytryptophan decarboxylase.

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