Tryptophan Hydroxylase Inhibition: the Mechanism by Which p-Chlorophenylalanine Depletes Rat Brain Serotonin

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SUMMARY

Administration of the specific serotonin depletor p-chlorophenylalanine to rats results in marked inhibition of tryptophan hydroxylase of the brain. The enzyme inhibition can be correlated with and is assumed to be responsible for brain serotonin depletion. Although *p*-chlorophenylalanine is a competitive inhibitor of tryptophan hydroxylase *in vitro*, it causes an irreversible inactivation of the enzyme *in vivo*. The findings also support the conclusion that tryptophan hydroxylation is the rate-limiting enzymic step in serotonin biosynthesis.

INTRODUCTION

It was demonstrated recently by Koe and Weissman (1) that *p*-chlorophenylalanine (PCP) is a potent and selective depletor of brain serotonin in mice, rats, and dogs. These workers also showed that PCP administered *in vivo* does not inhibit either aromatic-L-amino acid decarboxylase or monoamine oxidase, but that it is a potent inhibitor of liver phenylalanine hydroxylase. They suggested that PCP possibly decreased tissue serotonin levels by inhibition of tryptophan hydroxylase.

The development of a sensitive radioassay for tryptophan hydroxylase activity (2) has enabled us to show that inhibition of this enzyme by PCP is indeed responsible for serotonin depletion in rat brain. In addition to supporting the suggestion of Koe and Weissman (1) concerning the mechanism of action of PCP, the current work demonstrates the following. (a) PCP is a competitive inhibitor of rat brain tryptophan hydroxylase *in vitro*. (b) PCP causes a nearly complete loss of tryptophan hydroxylase activity in rat brain *in vivo* which is due to an irreversible inactivation of the enzyme. (c) Tryptophan hydroxylation is a rate-limiting reaction in serotonin biosynthesis.

MATERIALS AND METHODS

L-Tryptophan-3-^{14}C (30 μC/μmole) was obtained from Nuclear Chicago Corporation. Fifty microcuries (0.32 mg) was dissolved in 0.5 ml of deionized H_2O and passed through a 0.5 $\times$ 2 cm Permutit column (3) to remove impurities which would contribute to the blank in the hydroxylase assay. *p*-Chloro-DL-phenylalanine was a gift of Charles Pfizer and Co. The compound was prepared for intraperitoneal injection by suspension of 60 mg/ml in 1 N HCl, followed by adjustment to pH 2 with 5 N NaOH. Other materials were the best obtainable commercial grade. Animals used were N.I.H. strain male Sprague-Dawley rats (180–230 g).

For study of the effects of PCP *in vivo*, rats were injected with a single 300 mg/kg dose of the drug and sacrificed at various times by decapitation. For measurement of tryptophan hydroxylase activity, brain stems were removed and immediately

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placed on ice. A 30% homogenate was prepared with a conical glass homogenizer in ice cold 0.05 M Tris buffer pH 7.4. Using these conditions of homogenization, about 75% of the enzyme activity was found in the 30,000 *g* supernatant fraction. Thus, homogenates were routinely centrifuged at 30,000 *g* in a Servall RC2 centrifuge, and the supernatant fractions were used as enzyme. In some experiments the supernatant fraction was dialyzed overnight against 0.1 M 2-mercaptoethanol and 0.05 M Tris pH 7.4. This procedure was previously used by Hosada and Glick (4) to activate tryptophan hydroxylase from mouse mast cell tumors. Tryptophan hydroxylation was measured as reported recently (2) using 0.4 ml of enzyme in a total incubation volume of 0.5 ml. A single brain stem contained sufficient enzyme activity for duplicate analyses. Specific activity of the enzyme is expressed in millimicromoles of tryptophan hydroxylated per milligram of protein per hour. Protein was measured by a minor modification of the phenol-reagent assay (5).

Tissue levels of phenylalanine and PCP were determined as follows. Brain stems were homogenized in 2 volumes of 0.01 N HCl, and the proteins were removed by centrifugation after the addition of 1 volume of 25% trichloroacetic acid per 2 volumes of homogenate. The amino acids in the clear supernatant fraction were analyzed using a Beckman 120C Amino Acid Analyzer equipped for high sensitivity analyses. Aliquots of the supernatant fraction (0.05–0.30 ml) were applied to a 18 × 0.9 cm column of Beckman PA 35 resin, and the amino acids were chromatographed using 0.35 N sodium citrate buffer pH 5.28 at a flow rate of 60 ml per hour. The retention times for phenylalanine and PCP were 28 min and 54 min, respectively.

Serotonin in brain stem was assayed as described by Udenfriend *et al.* (6).

RESULTS

Under the standard assay conditions 10^{-4} M PCP was found to produce 30–50% inhibition of brain stem tryptophan hydroxylase *in vitro*. Examination of the kinetics of the inhibition with the crude enzyme preparation indicates that PCP is competitive with substrate for a binding site on the enzyme (Fig. 1). The K_m for tryptophan as determined in these experiments was approximately 3×10^{-4} M, and the K_i for PCP was found to be 3×10^{-4} M also.

The effects of PCP *in vivo* are shown in Fig. 2. After the injection of PCP, the

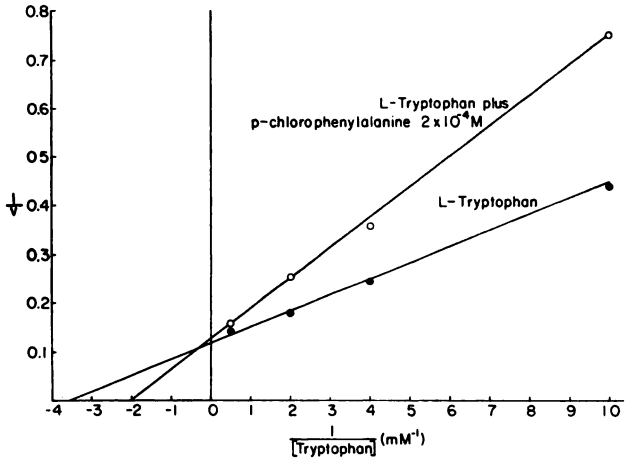

FIG. 1. *The effect of substrate concentration on the rate of tryptophan hydroxylation in the presence and absence of PCP*

The standard incubation mixture was used with 5.8 mg of brain stem protein.

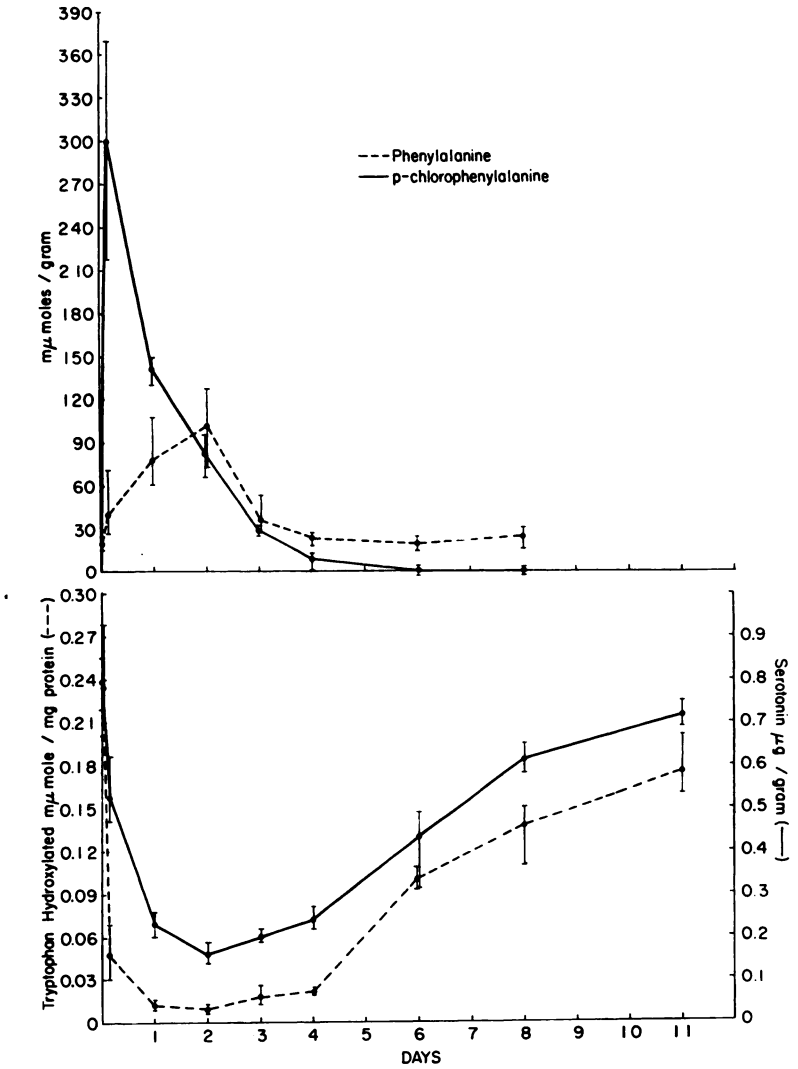

FIG. 2. *Brain stem levels of p-chlorophenylalanine (PCP), phenylalanine, serotonin, and tryptophan hydroxylase activity following administration of PCP (300 mg/kg, i.p.)*

Each value is the average of results in 4 to 6 animals, and the brackets indicate the range of values. Separate groups of animals were used for measuring the enzyme activity and serotonin content and for analysis of PCP and phenylalanine.

levels of PCP in the brain stem increased rapidly and then gradually decreased to undetectable levels over a 6-day period. Brain phenylalanine levels increased to a maximum value at 2 days and returned to control levels in about 6 days. Tryptophan hydroxylase activity and serotonin content decreased rapidly after the administration of drug, minimum levels being reached within 24 hours. Four days after treatment the tryptophan hydroxylase activity was still markedly reduced, as was the serotonin level. Thereafter, there was a slow and parallel return of tryptophan hydroxylase activity and serotonin level to control values which were reached by day 11.

The long-lasting inhibition of tryptophan hydroxylase in contrast to the disappearance of the drug suggested that PCP had

produced an irreversible inactivation of the enzyme. If such were the case, the inhibition should not be removable by dialysis. This was studied in enzyme preparations from control rats and from animals which had been treated 4 hours and 2 days previously with PCP, 300 mg/kg. The results are shown in Table 1. Dialysis of control brain extracts resulted in a 2- to 3-fold stimulation of hydroxylase activity and a complete reversal of the inhibition caused by the *in vitro* addition of PCP (5×10^{-4} M). About half the control activity was recovered by dialysis of the enzyme obtained from rats 4 hours after PCP treatment. However, the almost complete inhibition of tryptophan hydroxylase in rats sacrificed 2 days after treatment could not be removed at all, indicating irreversible inhibition of the enzyme.

TABLE 1

Effect of dialysis on the inhibition of tryptophan hydroxylase produced by PCP in vitro and in vivo

Enzyme source	*In vitro* addition	Tryptophan hydroxylated[a] (mμmoles)	
		Nondialyzed	Dialyzed
Untreated rats	None	0.81	2.80
		0.91	2.65
		1.04	2.98
		—	2.68
Untreated rats	5×10^{-4} M PCP	0.35	2.44
		0.25	2.18
PCP-Treated rats (4 hr)[b]	None	0.20	1.11
		0.20	0.94
PCP-Treated rats (2 days)[b]	None	0.11	0.41
		0.07	0.20

[a] The standard incubation was used with 5.6–5.8 mg of protein. The enzyme was the 30,000 *g* supernatant from a 30% brain stem homogenate in 0.05 M Tris pH 7.4. Dialysis was performed overnight against 0.1 M 2-mercaptoethanol and 0.05 M Tris pH 7.4.

[b] Sacrificed 4 hr and 2 days, respectively, after intraperitoneal injection of PCP, 300 mg/kg.

DISCUSSION

The finding of an excellent correlation between the degree of tryptophan hydroxylase inhibition and serotonin depletion by PCP supports the concept that serotonin depletion by the drug is the result of enzyme inhibition. It is of interest that the enzyme inhibition produced by PCP is completely different *in vivo* from *in vitro*. While PCP appears to be strictly a competitive inhibitor when added *in vitro*, administration of this compound to rats results rather quickly in an irreversible inhibition of the enzyme. Thus, while 4 hours after drug administration the inhibition is due to the competitive effects of PCP in tissue as well as irreversible enzyme inactivation, after 2 days the inhibition is due solely to enzyme inactivation. Since the drug has almost completely disappeared from tissue by 6 days, it is probable that the slow return to normal of tryptophan hydroxylase activity represents the rate of new enzyme synthesis.

The exact mechanism of enzyme inactivation by PCP *in vivo* remains to be determined. It has been possible to demonstrate irreversible inhibition *in vitro* even when brain stem extracts are preincubated at 37° for 1 hour, either with or without the pteridine cofactor. It is possible that a metabolite of PCP, which is formed outside of the brain, is causing the irreversible inhibition of tryptophan hydroxylase. Koe and Weissman (1) have shown that *p*-chlorophenylpyruvic acid, a major metabolite of PCP (7), is an effective serotonin

depletor and causes inhibition of liver phenylalanine hydroxylase.

As noted in Fig. 2 the administration of PCP also resulted in an elevation of brain phenylalanine. This is undoubtedly due to inhibition of liver phenylalanine hydroxylase which has been shown by Koe and Weissman (1) to be strongly inhibited for 2–3 days following a single dose of PCP and to return to normal by day 4 or 5. These results correlate well with the time course of the elevation of brain phenylalanine observed in the present study. Recent studies of Lipton *et al.* (8) have demonstrated that the inhibition of rat liver phenylalanine hydroxylase is analogous to that seen with brain stem tryptophan hydroxylase; i.e., the level of the inhibitor in the tissue cannot account for the inhibition of the enzyme observed and the enzyme inhibition cannot be reversed by dialysis. The much more rapid return to normal of liver phenylalanine hydroxylase in the rat suggests that this enzyme is synthesized at a more rapid rate than brain stem tryptophan hydroxylase.

Earlier studies (9) suggested that the rate of tryptophan hydroxylation in brain was too low to account for its serotonin content. The rates of hydroxylation in the present *in vitro* system are considerably higher (about 1 μg 5-HTP formed per gram of brain stem per hour) and appear sufficient to account for the serotonin levels normally formed in brain. Furthermore, the parallelism between serotonin content and tryptophan hydroxylase activity in brain after PCP administration suggests that brain serotonin is arising via tryptophan hydroxylase in the brain itself. This parallelism is also good evidence that tryptophan hydroxylation is the rate-limiting enzymic step in serotonin biosynthesis. The relatively high K_m observed for tryptophan indicates the enzyme may not be fully saturated with substrate normally and that the overall rate of serotonin synthesis may be partially dependent upon availability of the substrate. This is also apparent from recent studies of Weber (10) showing that loading doses of tryptophan in rats result in elevated brain serotonin levels.

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