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Effect of p-Chlorophenylalanine on Hydroxylation of Tryptophan in Pineal and Brain of Rats

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SUMMARY

Tryptophan hydroxylase activity was measured *in vitro* in the pineal gland, brain stem, and cerebral cortex of rats after treatment with *p*-chlorophenylalanine. Enzyme activity in the brain stem or cortex, assayed at 10 μM tryptophan, decreased to 5% of control 1–3 days after treatment with *p*-chlorophenylalanine. Enzyme activity in the pineal remained at 60–75% of that in control rats. When the tryptophan concentration was greater than 100 μM, tryptophan hydroxylase activity in the pineal did not differ between control and *p*-chlorophenylalanine-treated rats. Tryptophan hydroxylase in the pineal is thus relatively resistant to inhibition with *p*-chlorophenylalanine.

p-Chlorophenylalanine depleted serotonin levels in the pineal as well as in the brain. Neither the pineal nor the brain could convert tracer doses of radioactive tryptophan to serotonin, indicating that the biosynthetic capacity was impaired by treatment of the rats with *p*-chlorophenylalanine. However, after injection of a large dose of L-tryptophan, the pineals of *p*-chlorophenylalanine-treated rats synthesized serotonin from tryptophan at almost the same rate as the pineals of control rats. Smaller doses of L-tryptophan were less effective in increasing serotonin levels. These results indicate that, although the pineals of *p*-chlorophenylalanine-treated rats retained tryptophan hydroxylase, the affinity of the enzyme for tryptophan was reduced.

The differential sensitivity to *p*-chlorophenylalanine of pineal tryptophan hydroxylase as compared to the enzyme from various brain regions suggests that the pineal enzyme has distinctive molecular properties.

INTRODUCTION

It has been demonstrated that *p*-chlorophenylalanine depletes serotonin in the brains of rat, mouse, and dog (1). CPA[3] also depletes serotonin in other tissues of the rat, including pineal (2, 3). Jéquier *et al.* (4) have shown that CPA is a competitive inhibitor of tryptophan hydroxylase of brain stem *in vitro*. However, CPA caused irreversible inactivation of tryptophan hydroxylase of brain stem *in vivo*. Gál *et al.* (5) proposed that incorporation of CPA into cerebral tryptophan hydroxylase could take place at

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[3] The abbreviation used is: CPA, *p*-chlorophenylalanine.

the catalytically active center to produce irreversible inhibition. The mechanism of the effect of CPA on hydroxylation of tryptophan, however, remains unclear. The present study shows that CPA does not inactivate tryptophan hydroxylase in rat pineal.

EXPERIMENTAL PROCEDURE

Chemicals. L-[*carboxyl*-^{14}C]Tryptophan (12 mCi/mmole) and L-[U-^{3}H]tryptophan (7.3 Ci/mmole) were purchased from New England Nuclear. CPA was purchased from Aldrich. 6,7-Dimethyl-5,6,7,8-tetrahydropterine, dithiothreitol, L-tryptophan (A grade), and pyridoxal phosphate were purchased from Calbiochem. Catalase from bovine liver (15,000 units/mg) was purchased from Sigma.

Preparation and administration of compounds. Male Sprague-Dawley rats weighing 180–200 g were supplied by Simonsen Laboratories, Gilroy, Cal. CPA was dissolved in 0.14 N HCl by gentle heating. The solution was cooled to room temperature and neutralized by the dropwise addition of 1 N NaOH. The concentration of the suspension of CPA was 15 mg/ml. The suspension of CPA (300 mg/kg of body weight) was injected intraperitoneally into rats once a day for 2 successive days. One or three days after the second injection of CPA the rats were killed by decapitation. In all experiments animals were killed between 1:00 and 3:00 p.m., because the serotonin level of rat pineal has been found to exhibit circadian variations (6). Although we found that tryptophan hydroxylase activity in rat pineal measured *in vitro* did not show any circadian change, the rats to be used for tryptophan hydroxylase assay were also killed during the same time period.

L-[U-^{3}H]Tryptophan was purified by passage through a Bio-Rex 70 (Na^+ form, 200–400 mesh) column (0.4 × 2.0 cm), and the effluent was lyophilized. L-[U-^{3}H]Tryptophan (130 μCi/rat) was dissolved in 0.5 ml of isotonic NaCl and injected into the left femoral vein of rats under slight ether anesthesia.

Analysis of compounds. Pineals and brains were frozen on Dry Ice immediately after removal. For the serotonin assay, two pineals were homogenized in 0.5 ml of 75% ethanol in a small glass homogenizer. The homogenizer was rinsed three times with 0.5 ml of 75% ethanol for each sample. The homogenate and rinses were combined and centrifuged at 35,000 × *g* for 20 min. These operations were performed at 4°. The supernatant fluid was mixed with 2 ml of cold distilled water and passed through a Bio-Rex 70 (Na^+ form, 200–400 mesh) column (0.4 × 2.0 cm). The column was washed with 3 ml of 0.02 M sodium phosphate buffer (pH 6.5) and then with 2 ml of distilled water. Serotonin was eluted with 1.5 ml of 1.2 N HCl.

For the assay of serotonin from brain, a whole brain was homogenized with 10 ml of 0.4 N perchloric acid containing 0.2% EDTA. After centrifugation at 35,000 × *g* for 20 min, the supernatant fraction was neutralized to pH 6.0 by the dropwise addition of 2 N KOH. These operations were performed at 4°. The supernatant fluid was passed through a Bio-Rex 70 (Na^+ form, 200–400 mesh) column (0.6 × 2.5 cm). The column was washed with 10 ml of 0.02 M sodium phosphate buffer (pH 6.5) containing 0.2% EDTA and then with 3 ml of distilled water. Serotonin was eluted with 3 ml of 1.2 N HCl and measured fluorometrically in 3 N HCl in a Turner spectrophotofluorometer (7). The eluate (0.5–2.0 ml) was mixed with 10 ml of Bray's solution (8), and the radioactivity was measured by scintillation counting.

For the measurement of tryptophan, one or two pineals were homogenized in 0.5 ml of 5% trichloracetic acid in a small glass homogenizer. The homogenizer was rinsed twice with 0.5 ml of 5% trichloracetic acid with each sample. Homogenate and rinses were combined and centrifuged at 35,000 × *g* for 20 min. Plasma (50 μl) was homogenized in 2 ml of 5% trichloracetic acid and centrifuged at 35,000 × *g* for 20 min. These operations were performed at 4°. The tryptophan content of the supernatant fluid was determined by the method of Denckla and Dewey (9). Protein was determined by the method of Lowry *et al.* (10).

Assay of tryptophan hydroxylase. The cerebellum was removed, and the cerebral cortex and brain stem were dissected and frozen on Dry Ice. The cortex contained the cerebral

cortex, hippocampus, and striatum; the brain stem contained the medulla, pons, midbrain, and hypothalamus. Cortex or brain stem was homogenized in 2 or 4 volumes, respectively, of 0.02 M Tris-acetate buffer (pH 8.1) containing 1 mM dithiothreitol. We confirmed the report of other investigators (11, 12) that in brain all of the enzyme activity is present in the supernatant fraction after homogenization in hypotonic buffer and that an inhibitor of tryptophan hydroxylase is present in 105,000 × *g* precipitate fractions of brain. Consequently the supernatant fraction (100–200 μl) obtained by centrifugation at 105,000 × *g* for 30 min was used for the assay of tryptophan hydroxylase activity in brain.

Tryptophan hydroxylase of rat pineal is quite unstable compared to that of rat brain. Storing a pineal homogenate at 4° for 1 hr reduced the enzyme activity by 50%. Tryptophan hydroxylase activity therefore was measured immediately after obtaining and homogenizing the tissues. Two pineals (2.2–2.5 mg) were chilled in 0.15 ml of the buffer employed for brain, and homogenized in a small glass homogenizer. The entire homogenate was used for each assay of enzyme activity.

Tryptophan hydroxylase activity was measured according to the method of Ichiyama *et al.* (13). The assay mixture contained the following reagents in a total volume of 0.5 ml: Tris-acetate (pH 8.1), 50 μmoles; 6,7-dimethyl-5,6,7,8-tetrahydropterine, 1.0 μmole; dithiothreitol, 1.5 μmoles; pyridoxal phosphate, 0.1 μmole; catalase, 30 μg; ferrous ammonium sulfate, 10 nmoles; and L-[*carboxyl*-^{14}C]tryptophan (12 mCi/mmole), 5 nmoles. Aromatic L-amino acid decarboxylase purified from beef brain stem was added to the assay mixture (13). Ferrous ammonium sulfate was omitted from the assay mixture for brain because it did not activate the brain enzyme under the assay conditions employed in this study. Incubations were carried out at 37°. Incubation time was 60 min for brain stem or cortex, and 30 min for pineal.

RESULTS

Effect of CPA on tryptophan hydroxylase in vivo. Tryptophan hydroxylase activity was measured in brain stem, cerebral cortex, and pineal of rats after treatment with CPA. Tryptophan hydroxylase activity in brain stem decreased to 5% of the control value 1 day after injection of CPA, with a slight return 3 days after the injection (Table 1). Injection of CPA also decreased tryptophan hydroxylase activity in the cerebral cortex to almost zero. These results agree with the report by Jéquier *et al.* (4). However, CPA did not abolish tryptophan hydroxylase activity in the pineal. One or three days after the second injection of CPA, 60–65% of the enzyme activity in control rats was still present. Injection of CPA did not result in any change in pineal weight or in protein content of the gland. In another set of experiments CPA was administered to rats every day for 6 consecutive days at a dose of 100 mg/kg of body weight. One day after the last injection tryptophan hydroxylase activity was mea-

TABLE 1

Tryptophan hydroxylase activity after treatment with CPA

CPA was injected as described under EXPERIMENTAL PROCEDURE. One or three days after the second injection, the rats were killed and tryptophan hydroxylase activity was measured. Two groups of rats were chosen as controls: (a) rats without any injection and (b) rats receiving an injection of 0.9% NaCl 1 and 2 days before death. There was no difference in tryptophan hydroxylase activity between these two control groups, and the values given for control rats in this table are the mean of these two groups. Each group consisted of 6–10 samples. Results are expressed as means ± standard errors.

Treatment	Brain stem	Cortex	Pineal
	nmoles/g/hr	*nmole/g/hr*	*nmoles/g/hr*
Control	3.57 ± 0.17	0.797 ± 0.038	358 ± 21
CPA-treated (1 day)	0.18 ± 0.01	0.003 ± 0.002	222 ± 14
CPA-treated (3 days)	0.28 ± 0.01	0.005 ± 0.002	205 ± 14

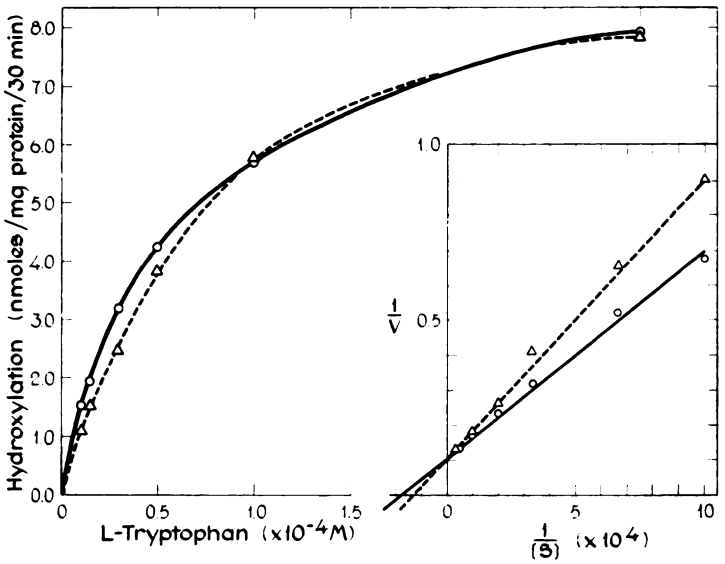

FIG. 1. *Tryptophan hydroxylase activity in pineals of CPA-treated and control rats*

CPA-treated rats received CPA (300 mg/kg) 1 and 2 days before death. Control rats received 0.9% NaCl. Tryptophan hydroxylase activity was measured as described under EXPERIMENTAL PROCEDURE. Incubation was carried out for 30 min at the different concentrations of L-tryptophan indicated. The amount of aromatic L-amino acid decarboxylase added in this reaction mixture was enough to decarboxylate 5 nmoles of L-5-hydroxytryptophan in 30 min. The reaction mixture without pineal homogenate was run as a blank. Blank values have been subtracted from the enzyme activities given in the figure. O——O, control rats; △- - -△, CPA-treated rats.

sured. Between 70 and 75% of the control level of tryptophan hydroxylase activity was found in the pineals of CPA-treated rats, whereas tryptophan hydroxylase activity in either the brain stem or cortex was less than 5% of that in control rats.

The concentration of L-tryptophan employed in the routine assay mixture was 10 μM, which is far below the K_m value for tryptophan hydroxylase (see below). Enzyme activity therefore was assayed at various concentrations of substrate from 10 to 300 μM. As shown in Fig. 1, tryptophan hydroxylase activity in the pineals of CPA-treated rats was 75% of that in control rats when L-tryptophan was at 10 μM. At concentrations higher than 100 μM L-tryptophan, however, enzyme activity reached the same value in both control and CPA-treated rats. Double-reciprocal plots showed that the V_{max} was the same with pineal homogenates from both control and CPA-treated rats. The V_{max} was calculated to be 9 nmoles/mg/30 min, or 1.3 nmoles/pineal/30 min. The K_m values of tryptophan were calculated to be 60 μM for tryptophan hydroxylase from control rats and 80 μM for the enzyme from CPA-treated rats. These results indicate that total tryptophan hydroxylase activity is the same in the pineals of both groups of rats. Lovenberg *et al.* (14) reported a high K_m value (400 μM) of tryptophan for tryptophan hydroxylase of beef pineal, whereas our value with rat pineal was 60 μM. Although we repeated the experiments with the supernatant enzyme obtained by centrifugation at 105,000 × *g*, the K_m value was the same as with the homogenate. The differences in the K_m values might represent species differences.

The supernatant fraction from brain stem of CPA-treated rats showed almost no tryptophan hydroxylase activity at concentrations of L-tryptophan from 10 to 500 μM. Brain homogenates of CPA-treated rats also showed no tryptophan hydroxylase activity. The K_m value of L-tryptophan was 250 μM for tryptophan hydroxylase from brain stem

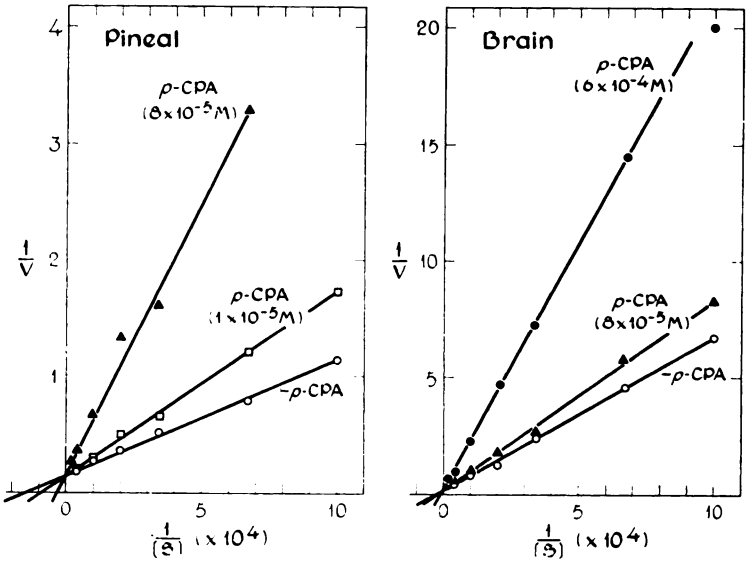

FIG. 2. *Effect of CPA on tryptophan hydroxylase in vitro*

Double-reciprocal plots of tryptophan hydroxylase in pineal and brain stem. Tryptophan hydroxylase activity was measured as described in Fig. 1 in the absence or in the presence of CPA. V = nanomoles per milligram of protein per 30 min; S = moles per liter.

TABLE 2

Conversion of L-[^3H]*tryptophan to* [^3H]*serotonin in pineal*

L-[U-^{3}H]Tryptophan was injected intravenously, and the tritiated serotonin formed was analyzed as described under EXPERIMENTAL PROCEDURE. The radioactive compound in the serotonin fraction was identified as serotonin by paper chromatography in 1-butanol–acetic acid–water (12:3:5). Benzene did not extract the radioactivity in the serotonin fraction at pH 12, indicating that radioactive tryptamine or 5-methoxytryptamine was not produced. Most of the radioactive compound in the effluent from the Bio-Rex 70 column was identified as tryptophan by paper chromatography. Each group consisted of 10 rats, and two pineals were pooled for each analysis. Results are expressed as means ± standard errors.

Time after injection of tryptophan	Treatment of rat	Total radioactivity in supernatant	Radioactivity in serotonin fraction	Radioactivity in tryptophan fraction
min		*dpm/pineal*	*dpm/pineal*	*dpm/pineal*
10	Control	14,250 ± 453	12,596 ± 380	1,402 ± 145
	CPA-treated	2,328 ± 120	614 ± 37	1,453 ± 102
30	Control	6,478 ± 387	5,558 ± 355	918 ± 44
	CPA-treated	1,076 ± 83	279 ± 39	797 ± 59

of normal rats, which agrees with the value reported by other investigators (5, 13, 14).

Effect of CPA on tryptophan hydroxylase in vitro. The effect of CPA on tryptophan hydroxylase *in vitro* is shown in Fig. 2. CPA was a competitive inhibitor of tryptophan hydroxylase in both pineal and brain stem. The K_i value is 20 μM for the pineal enzyme and 300 μM for the brain stem enzyme.

Effect of CPA on levels of serotonin. In confirmation of previous investigations, serotonin levels in the brain decreased to less than 10% of that of control rats 1 or 3 days after injection of CPA. Serotonin levels in the

pineal also decreased to 5% of control rats 1 day after injection of CPA. Pargyline, a monoamine oxidase inhibitor, did not increase serotonin levels in the pineal or brain of CPA-treated rats when injected at a dose of 75 mg/kg 1 day after the second injection of CPA.

Conversion of L-[^3H]*tryptophan to* [^3H]*serotonin.* The conversion of tryptophan to serotonin was studied by injecting a tracer dose of tryptophan. As shown in Table 2, more than 80% of the tritiated tryptophan was converted to serotonin in the pineals of control rats within 10 min. Thirty minutes after administration of tritiated tryptophan, radioactive serotonin decreased by more than 50%, indicating a rapid turnover of serotonin in the pineal. In the pineals of CPA-treated rats, however, almost no radioactivity was detected in the serotonin fraction, indicating that the conversion of a tracer dose of tryptophan to serotonin was blocked. Although the total radioactivities in the supernatant fractions were different, the amounts of radioactivity found in the tryptophan fraction were the same in both groups of rats.

Radioactive serotonin was detected in the brains of control rats, whereas no radioactivity was detected in the serotonin fraction of CPA-treated rats, suggesting that CPA completely blocked the conversion of tryptophan to serotonin.

Increase of serotonin after loading of L-*tryptophan.* A large dose of L-tryptophan (500 mg/kg) was injected intraperitoneally into rats, and the serotonin level in the pineal was measured. As shown in Fig. 3, the serotonin level in the pineals of control rats increased 3-fold above the initial level in 30 min and remained at the increased level for 4 hr, returning to the initial level 7 hr after the injection. In the pineals of CPA-treated rats the level of serotonin also increased, at almost the same rate as in control rats, reaching a maximum 30 min after injection. Serotonin levels in the pineals of CPA-treated rats, however, decreased more rapidly than in control rats. Four hours after injection of L-tryptophan the serotonin level decreased to 40% of the maximum level, returning to zero 7 hr after the injection. These results clearly show that the pineals of CPA-treated rats can synthesize serotonin from L-tryptophan at almost the same rate as in control rats, after loading with a large amount of L-tryptophan.

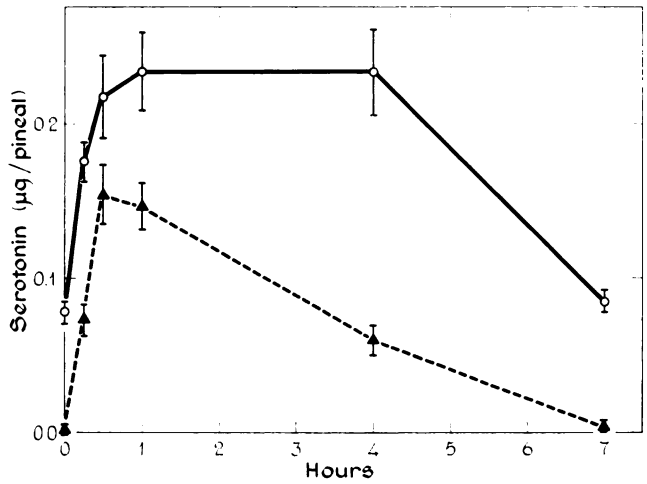

FIG. 3. *Increase of serotonin level in pineal after injection of* L-*tryptophan*

L-Tryptophan (500 mg/kg) was injected intraperitoneally, and serotonin levels in the pineal were measured. 5-Methoxytryptamine in the serotonin fraction was extracted into chloroform at pH 12 and measured fluorometrically. Fluorescence due to 5-methoxytryptamine was less than 3% of the total fluorescence. At each time point 10 rats were used, and two pineals were pooled for each analysis. Vertical bars indicate standard errors of the mean. O——O, control rats; ▲- - -▲, CPA-treated rats.

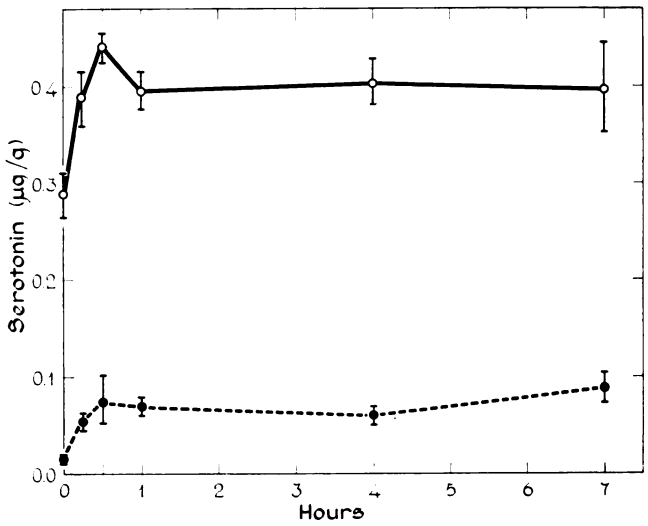

FIG. 4. *Increase of serotonin level in brain after injection of* L-*tryptophan*

The experimental design was the same as that in Fig. 3. A whole brain was analyzed for serotonin, and each group consisted of five rats. Vertical bars indicate standard errors of the mean. O——O, control rats; ●- - -●, CPA-treated rats.

TABLE 3

Dose response of pineal serotonin to L-*tryptophan*

L-Tryptophan was injected intraperitoneally at the doses indicated, and the rats were killed 30 min later. Each group consisted of 10 rats, and two pineals were pooled for each analysis. Results are expressed as means ± standard errors.

Dose of L-tryptophan	Control rats		CPA-treated rats	
	Serotonin level	Increase of serotonin	Serotonin level	Increase of serotonin
mg/kg	*ng/pineal*	*ng/pineal/30 min*	*ng/pineal*	*ng/pineal/30 min*
0	75 ± 4		3 ± 1	
20	195 ± 17	120	18 ± 3	15
40	232 ± 36	157	26 ± 2	23
100	257 ± 26	182	70 ± 4	67
300	215 ± 13	140	92 ± 5	89
500	267 ± 41	192	154 ± 20	151

The result with brain is shown in Fig. 4. The serotonin level in the brains of control rats increased by 40 % 30 min after injection of tryptophan. In the CPA-treated rats brain serotonin also increased significantly and remained at this level for 7 hr. The amount of increase, however, was less than half that in control rats. The serotonin level was far lower in the brains of CPA-treated rats than in controls, indicating that biosynthetic capacity was impaired in the brains of CPA-treated rats.

Dose response of pineal serotonin to L-*tryptophan*. Since the amount of serotonin increased linearly for 30 min, serotonin was measured 30 min after the injection of various doses of L-tryptophan. Serotonin levels in the pineals of control rats reached almost the maximum level 30 min after a dose of 20 mg/kg (Table 3). In contrast, serotonin levels in the pineals of CPA-treated rats gradually increased with increasing doses of L-tryptophan. Double-reciprocal plots of the increase in serotonin as a function of the dose of L-tryptophan are shown in Fig. 5. From the intercept, the V_{max} value was calculated to be 220 ng/pineal/30 min, or 1.25 nmoles/pineal/30 min for both control and CPA-treated rats, which is identical with the V_{max} value obtained from the study *in vitro*. We also calculated that roughly half the V_{max} was obtained at the L-tryptophan dose of 300 mg/kg for CPA-treated rats and at 10 mg/kg for control rats. The result indicates that the maximum amount of hydroxylation was the same in pineals of both groups of rats. The rate of hydroxylation of tryptophan, however, was slower in the pineals of CPA-treated rats than in controls after load-

TABLE 4

Concentration of tryptophan in pineal and plasma of rats

The experimental design was the same as that in Table 3. Tryptophan levels in the pineal and plasma were measured as described under EXPERIMENTAL PROCEDURE. Results are expressed as means ± standard errors.

Dose of L-tryptophan	Pineal		Plasma	
	Control	CPA-treated	Control	CPA-treated
mg/kg	*ng/mg*		*μg/ml*	
0	10.6 ± 1.1	12.6 ± 0.6	16.6 ± 1.9	12.4 ± 1.1
20	30.8 ± 4.6	34.7 ± 2.2	24.7 ± 0.8	20.5 ± 0.9
100	112 ± 11	135 ± 20	77.8 ± 8.9	87.6 ± 4.5
500	646 ± 28	741 ± 37	368 ± 24	439 ± 39

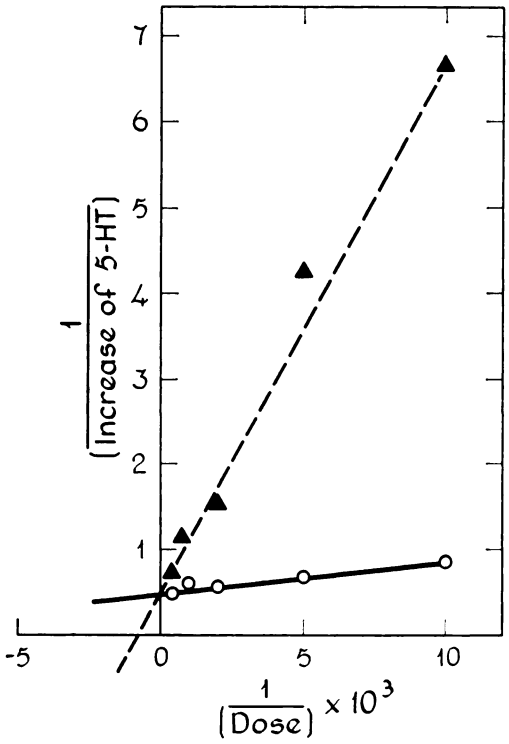

FIG. 5. *Double-reciprocal plots of increase in pineal serotonin as a function of dose of L-tryptophan*

The values are calculated from Table 3. O——O, control rats; ▲- - -▲, CPA-treated rats. Increase in serotonin is expressed as nanograms per pineal per 30 min. Dose is expressed in moles/kg.

ing with lower doses of L-tryptophan. The difference might be due either to a lower affinity of tryptophan hydroxylase for L-tryptophan or to a lower concentration of tryptophan in the pineals of CPA-treated rats than in controls.

To investigate the second possibility, L-tryptophan levels in the pineal and plasma were measured (Table 4). Tryptophan was not significantly higher in the pineals of CPA-treated rats than in controls. There was no statistically significant difference in the tryptophan level in plasma between control and CPA-treated rats.

The results suggest that the difference in the rate of hydroxylation of tryptophan in the pineals of control and CPA-treated rats is not due to a difference in the level of tryptophan but to a difference in affinity of the enzyme for tryptophan.

DISCUSSION

It has been proposed that CPA inactivates phenylalanine hydroxylase in the liver (2) and tryptophan hydroxylase in the brain (4). The results in this study show that CPA does not inactivate tryptophan hydroxylase in the pineals of rats. In the brains of CPA-treated rats, however, tryptophan hydroxylase activity decreased to undetectable levels under any assay conditions employed. This observation indicates that tryptophan hydroxylase in pineal may have markedly different properties from brain tryptophan hydroxylase.

In addition to the difference in susceptibility to CPA, there are several other properties which differ between brain and pineal tryptophan hydroxylase. Both the K_m values of tryptophan and the K_i values of CPA are different for these two enzymes. Lovenberg

et al. (14) demonstrated that CPA does not inactivate tryptophan hydroxylase in mast cell tumors of mice, although it depletes serotonin levels to about 30%. This phenomenon is similar to that observed in rat pineal. The K_m value for tryptophan hydroxylase in mast cell tumor was shown to be 40 μM by these investigators, which is almost identical with the K_m for tryptophan hydroxylase of rat pineal. Jéquier *et al.* (15) suggested that the molecular nature of the beef pineal and rat brain stem enzymes may be different, because the pineal enzyme preparation hydroxylates both tryptophan and phenylalanine, whereas the brain stem enzyme preparation has negligible phenylalanine hydroxylase activity. For several reasons mentioned above, it might prove true that pineal tryptophan hydroxylase is the same enzyme as that in mast cell tumor, but different from brain tryptophan hydroxylase.

The studies *in vivo* show that CPA depletes serotonin in rat pineal. CPA also blocks the conversion of tracer doses of tryptophan to serotonin. After loading with a large amount of L-tryptophan, however, the CPA-treated rats can synthesize serotonin at almost the same rate as the control rats. These observations support the conclusion, obtained with studies *in vitro*, that CPA does not inactivate tryptophan hydroxylase in rat pineal. However, CPA lowers the affinity of tryptophan hydroxylase for tryptophan. Under normal conditions, therefore, the pineals of CPA-treated rats cannot synthesize serotonin, because the concentration of tryptophan is far lower than the apparent K_m value for tryptophan hydroxylase in the pineals of CPA-treated rats. Thus the serotonin in the pineal was depleted by CPA.

The affinity of the pineal enzyme for tryptophan measured *in vitro* was slightly lower in CPA-treated rats than in control rats, whereas the effect of CPA in lowering the affinity for tryptophan was striking *in vivo*. The difference would be due to dilution of the tissue in the assay *in vitro*. Since each homogenate of two pineals was assayed in a reaction volume of 0.5 ml, the inhibitor present in the gland might have been diluted to the extent that it exerted almost no inhibitory effect on the enzyme activity. In contrast to the proposal of Gál *et al.* (5), these observations indicate that CPA might not be incorporated into the catalytically active site of the enzyme protein of rat pineal. It is more likely that CPA or one of its metabolites attaches reversibly to a site on the enzyme, resulting in competitive inhibition *in vivo*.

The brains of CPA-treated rats could synthesize serotonin from tryptophan after loading with a large amount of tryptophan, although the amount of increase was far less than that of controls. This observation suggests that the brains of CPA-treated rats retained tryptophan hydroxylase activity to some extent *in vivo*. It remains questionable whether CPA induces total inactivation of cerebral tryptophan hydroxylase, although tryptophan hydroxylase activity of brain measured *in vitro* decreased to an undetectable level after treatment with CPA. Recently Aghajanian and Asher (16) reported that a large dose of L-tryptophan increased yellow fluorescence (which might be due to serotonin) in raphe neurons of CPA-treated rats. Tryptophan hydroxylase in raphe nuclei of rats may respond in the same way as the pineal enzyme to CPA.

CPA is a unique pharmacological agent because it alters sleep patterns and behavior in cats, rats, and other mammals (17–19). These changes are thought to be due to depletion of brain serotonin. Our results suggest that the biochemical mechanism of depletion of serotonin may be different from tissue to tissue. It would be interesting to determine whether or not some of the behavioral changes induced by CPA can be restored by administration of a large dose of L-tryptophan.

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