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INHIBITION OF RAT BRAIN TRYPTOPHAN HYDROXYLATION WITH *p*-CHLOROAMPHETAMINE

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Abstract—*p*-Chloroamphetamine (PCA) at high concentrations (0.0025 to 0·02 M) inhibited rat brainstem tryptophan hydroxylase and, to a lesser extent, rat corpus striatum tyrosine hydroxylase. Hog kidney aromatic L-amino acid decarboxylase was not affected. Inhibition of tryptophan hydroxylase by *p*-chloroamphetamine was competitive with tryptophan and noncompetitive with 6,7-dimethyl-5,6,7,8-tetrahydropterine ($DMPH_4$). In rats given *p*-chloroamphetamine, brainstem tryptophan-hydroxylating activity was only slightly reduced, whereas striatal tyrosine-hydroxylating activity was not altered. Synthesis rates of brain serotonin and dopamine in rats treated with *p*-chloroamphetamine, estimated by measuring the accumulation of 5-hydroxytryptophan and dopa, respectively, after blockade of decarboxylase, were both decreased. In view of the high concentrations of *p*-chloroamphetamine required to inhibit tryptophan and tyrosine hydroxylases *in vitro*, mechanisms other than inhibition of enzyme by PCA might be responsible for the decelerated synthesis of serotonin and dopamine in brain (e.g. blockade of monoamine re-uptake).

Investigations into the biochemical effects of *p*-chloroamphetamine (PCA) on serotonin (5-HT) levels in the central nervous system have suggested several sites of action for this compound [1–3]. These include the inhibition of tryptophan hydroxylase *in vivo* [4], membrane phenomena relative to 5-HT release [1, 5, 6] and blockade of 5-HT uptake [5], and monomine oxidase inhibition *in vitro* [7] and *in vivo* [8]. The question of mode of action of PCA is further complicated by differences between species [9, 10], variability of activity among organs [1, 9] and among different regions of the same organ [11], and the extreme duration of effects on brain 5-HT metabolism [12, 13]. The present study investigates one of the possible mechanisms—blockade of tryptophan hydroxylation—by examining some effects of PCA on brain tryptophan hydroxylase and the accumulation of 5-hydroxytryptophan (5-HTP) in brain after inhibition of aromatic L-amino acid decarboxylase.

MATERIALS AND METHODS

Animals. Sprague–Dawley male rats (180–220 g) were CD animals from Charles River Breeding Laboratories, Wilmington, Mass.

Chemicals. Commercial chemicals included DL-3,4-dihydroxyphenylalanine (dopa)-1-^{14}C (10 mCi/m-mole), L-tryptophan-1-^{14}C (8·98 mCi/m-mole) and L-tyrosine-1-^{14}C (54·7 mCi/m-mole) from New England Nuclear; DL-*p*-chloroamphetamine (PCA) hydrochloride from Regis Chemical Co.; and 6,7-dimethyl-5,6,7,8-tetrahydropterine ($DMPH_4$) from CalBiochem. α-*n*-Propyldopacetamide (H 22/54) was obtained courtesy of AB Hässle.

Enzymes. Tryptophan hydroxylase was prepared by homogenizing fresh brainstems (medulla–pons region, 250 mg wet wt/rat) in 0·05 M Tris hydrochloride (Fisher Tris[hydroxymethyl]aminomethane + hydrochloric acid) buffer, pH 7·5, using 3·0 ml buffer/g of tissue; centrifuging the homogenate at 30,000 *g* for 15 min at 4°; and dialyzing the supernatant obtained against 3 liters of 0·05 M Tris–HCl, pH 7·5, containing 0·1 M 2-mercaptoethanol for 18–20 hr at 4° [14]. The tryptophan hydroxylase system consisted of: 200 μl enzyme (1·8 mg protein/200 μl); 200 μl hog kidney aromatic L-amino acid decarboxylase; 40 μl of 0·05 M Tris acetate buffer, pH 7·5, containing 0·05 M 2-mercaptoethanol and 2·9 mg $DMPH_4$/ml; 10 μl of 0·5 M pyridoxal phosphate; 10 μl of 4·0 mM $FeSO_4$; 20 μl L-tryptophan-1-^{14}C (60 nmoles); and inhibitor or H_2O to make 500 μl. All reactions were carried out for 1 hr at 37° with mild shaking. The $^{14}CO_2$ evolved from decarboxylation of the labeled 5-HTP product was trapped by a wick saturated with 0·2 ml NCS tissue solubilizer (Amersham/Searle) contained in a well within each stoppered vial. Reactions were terminated by injection of 0·5 ml of 10% trichloroacetic acid, and distillation of CO_2 proceeded for 2 hr at 37°. Wells were removed and counted in 15 ml Toluene–Omnifluor (New England Nuclear) in a Nuclear Chicago Mark I scintillation counter. Protein was assayed by the method of Lowry *et al.* [15]. The effects of standard inhibitors on this tryptophan hydroxylase preparation (10-min preincubation) expressed as mean per cent inhibition ±S.E. (five runs) were: α-*n*-propyldopacetamide 10^{-4} M, 85 ± 1 per cent; L-arterenol bitartrate 10^{-4} M, 47 ± 3 per cent; DL-*p*-chlorophenylalanine methyl ester hydrochloride 10^{-4} M, 74 ± 3 per cent; and DL-6-fluorotryptophan 10^{-3} M, 74 ± 2 per cent.

Tyrosine hydroxylase was prepared just prior to assay by homogenizing rat corpus striatum, which had been dissected free of adjacent cortex (90 mg wet wt/rat), in 10 ml water/g of tissue. The assay procedure was that described by Waymire *et al.* [16] and consisted of measuring the $^{14}CO_2$ evolved from labeled dopa derived enzymatically from L-tyrosine-1-^{14}C. This enzyme preparation was inhibited 64 and 91 per cent in the presence of α-*n*-propyldopacetamide 2×10^{-5} M and L-3-iodotyrosine 10^{-5} M, respectively.

Hog kidney aromatic L-amino acid decarboxylase was prepared and assayed by the method of Waymire *et al.* [16], using DL-dopa-1-^{14}C as substrate. This enzyme preparation was inhibited 94 per cent in the presence of NSD-1024 (3-hydroxy-*O*-benzylhydroxylamine hydrochloride) 10^{-4} M.

Determination of monoamines and monoamine precursors. Concentrations of 5-HT, tryptophan and dopamine (DA) in rat brain were estimated by the method of Neff *et al.* [17], using strong acid cation exchange for the preliminary separations. The latter method was also employed for recovering 5-HTP and dopa; the resin columns were washed with 60% methanol before eluting dopa or 5-HTP with 0·1 M Na acetate buffer, pH 6·0 [18, 19]. Dopa and 5-HTP were further purified by alumina adsorption and butanol extraction respectively. Measurement of dopa and DA via formation of fluorophores by oxidation with iodine was accomplished in the Technicon AutoAnalyzer by a modification of the method of Craig *et al.* [20]. 5-Hydroxytryptophan was determined by its native fluorescence in 3 N HCl.

Accumulation of dopa and 5-hydroxytryptophan in brain. After blockade of aromatic L-amino acid decarboxylase in brain, the monoamine precursors, dopa and 5-HTP, which are normally absent or present in very low concentrations, accumulate. The rate of dopa or 5-HTP accumulation has been shown to be an index of the synthesis rate of DA or 5-HT, respectively, by Carlsson *et al.* [21], who developed this method to investigate regulation of monoamine metabolism. Measurement of precursor accumulation is also a facile method for assessing the effects of drugs on the synthesis rate of brain DA or 5-HT [22, 23]. 3-Hydroxy-*O*-benzylhydroxylamine hydrochloride (NSD-1024), an effective 5-HTP/dopa decarboxylase inhibitor *in vivo* [24], was used to block this enzyme. After the appropriate pretreatment with the test compound, two intraperitoneal doses of NSD-1024, 1·0 m-mole/kg (176 mg/kg) and 0·5 m-mole/kg (88 mg/kg), were administered 30 min apart. Thirty min after the second NSD-1024 injection, rats were sacrificed. Dopa accumulation in the corpus striatum and 5-HTP accumulation in the midbrain + hindbrain region were determined as described above.

RESULTS

Inhibition in vitro. Although prior reports have shown no activity *in vitro* of PCA against tryptophan hydroxylase, concentrations have not exceeded 10^{-3} M [4]. In the present study, increasing inhibition of brain tryptophan hydroxylase was observed as concentrations of PCA were raised from 10^{-3} to 2×10^{-2} M (Fig. 1). Under similar assay conditions with DL-dopa-1-^{14}C as substrate, aromatic L-amino acid decarboxylase was not affected by PCA in this concentration range. Tyrosine hydroxylase (particulate preparation) was also inhibited but to a lesser extent than tryptophan hydroxylase.

Kinetic studies. Further studies were undertaken to determine the nature of the blockade of tryptophan hydroxylase. Variation of tryptophan concentration suggested competitive inhibition of the enzyme by PCA (Fig. 2A). The apparent K_m is $3{\cdot}8 \times 10^{-4}$ M for tryptophan. The common V_{max} value is 2·0 nmoles/mg of protein/hr. Variation of $DMPH_4$ cofactor concentration over a 10-fold range showed a noncompetitive pattern of inhibition by PCA (Fig. 2B). The apparent K_m for DMPH is $1{\cdot}7 \times 10^{-4}$ M. The respective V_{max} values are 0·71 and 0·36 nmoles/mg of protein/hr for control enzyme and enzyme containing 10^{-2} M PCA.

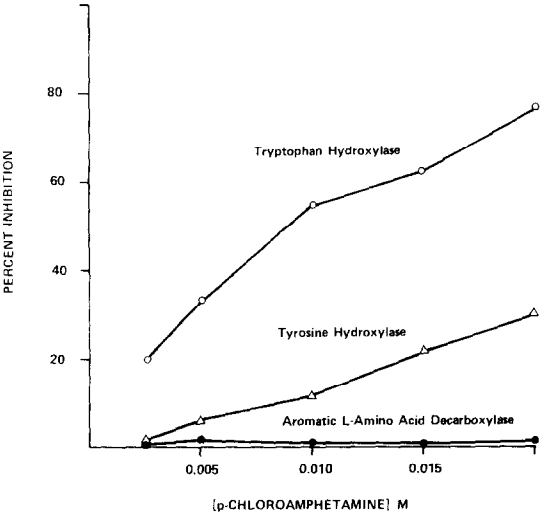

Fig. 1. Inhibition of rat brainstem tryptophan hydroxylase, rat corpus striatum tyrosine hydroxylase, and hog kidney aromatic L-amino acid decarboxylase as a function of *p*-chloroamphetamine concentration. Each point is the mean of duplicate determinations.

Activity of brain enzymes of rats treated with p-chloroamphetamine. Tryptophan-hydroxylating activity of brainstem extracts from rats treated with PCA (73 μmoles/kg) 18 hr before sacrifice was 23 per cent lower than the corresponding enzyme preparation from control rats (Table 1, part A). The undialyzed enzyme from PCA-treated rats also showed the same degree of loss inactivity. We observed less than 20 per cent decrease in control enzyme activity on dialysis, in contrast to the greater loss of activity reported by other workers [4, 25]. Tyrosine-hydroxylating activity of corpus striatum preparations from rats treated 18 hr prior to sacrifice with PCA (73 μmoles/kg, i.p.) was virtually the same as that of the corresponding enzyme from control rats (10 rats/treatment). Mean tyrosine hydroxylase activities (nmoles/g wet wt/hr ± S.E.) were: controls (N = 5), 594 ± 11; PCA-treated rats (N = 5), 644 ± 11 ($P > 0{\cdot}05$).

Activity of tryptophan hydroxylase after incubation with p-chloroamphetamine. Brainstem tryptophan hydroxylase preincubated for 6 hr (0°) in the presence of 10^{-2} M PCA and untreated (control) enzyme was handled in two ways: one portion of each was dialyzed overnight, while the remainder was kept at 0° overnight without dialysis. The dialyzed enzyme showed no effects of the preincubation with PCA, whereas the preparation standing for 6 hr in addition to overnight at 0° with PCA was inhibited by 39 per cent (Table 1, part B).

Accumulation of brain dopa and 5-hydroxytryptophan in rats treated with p-chloroamphetamine. After blockade of aromatic L-amino acid decarboxylase with NSD-1024, 5-HTP accumulation (i.e. 5-HT synthesis rate)

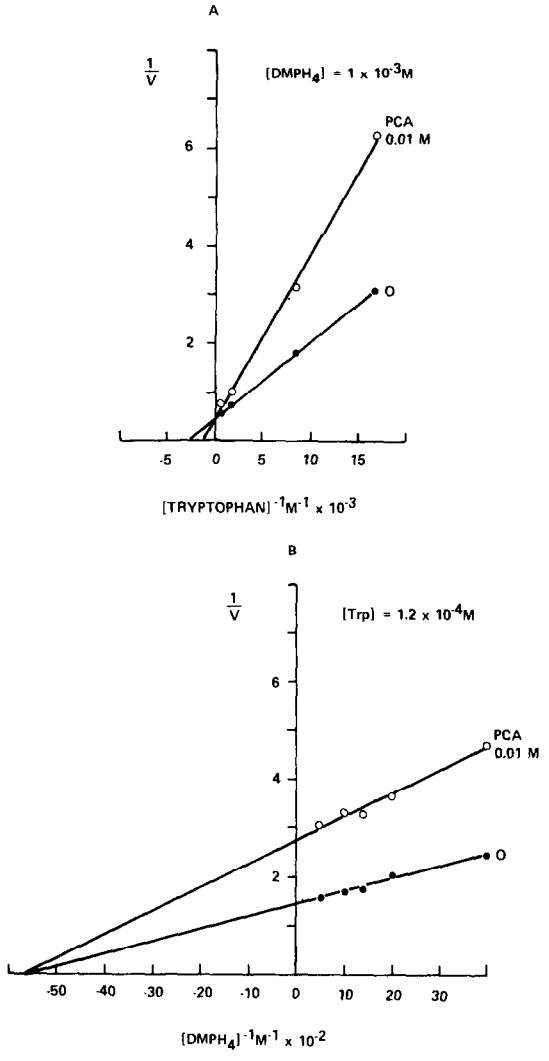

Fig. 2. Kinetics of inhibition of rat brainstem tryptophan hydroxylase by *p*-chloroamphetamine (0·01 M). (A) Inhibition competitive with tryptophan. K_m is $3{\cdot}8 \times 10^{-4}$ M; V_{max} is 2·0 nmoles/mg of protein/hr. (B) Inhibition noncompetitive with $DMPH_4$. K_m is $1{\cdot}7 \times 10^{-4}$ M; V_{max} in nmoles/mg of protein/hr: control, 0·71; PCA, 0·36. Each point is the mean of duplicate determinations.

in midbrain + hindbrain region decreased by 50 per cent in rats treated 16 hr earlier with PCA (73 μmoles/kg, i.p.). This drop was in good agreement with the 53 per cent decrease in 5-HT concentration elicited by PCA (Table 2A). The tryptophan content of the same brain region was not altered by the PCA treatment. Dopa accumulation (i.e. DA synthesis rate) in corpus striatum after blockade of decarboxylase decreased by 30 per cent in the PCA-treated rats. In contrast, striatal DA concentration was unaffected by this dose of PCA (Table 2B).

DISCUSSION

The mechanism of action of *p*-chloroamphetamine—of considerable interest because of the selective, long-term lowering of brain serotonin content—so far remains unresolved, possibly because of the multiple neurochemical actions exerted by this drug [1–3]. One mechanism suggested is the specific inhibition of brain tryptophan hydroxylase *in vivo* [4]. This proposal is intriguing because of the failure to demonstrate effects of PCA *in vitro* on this enzyme or any antagonism, *in vitro* or *in vivo*, of PCA on the somewhat similar enzymes, tyrosine hydroxylase [26], phenylalanine hydroxylase [9] and intestinal tryptophan hydroxylase [1]. In the present work, we have investigated the effect of high concentrations of PCA on brainstem tryptophan hydroxylase by means of an assay system which does not require isolating and purifying serotonin formed from labeled tryptophan [27, 28]. The lack of inhibition of tryptophan hydroxylase by 10^{-3} M PCA confirms previous findings in the literature. However, at higher concentrations, PCA exhibits dose-related inhibition which apparently is competitive with tryptophan and noncompetitive with $DMPH_4$. Aromatic L-amino acid decarboxylase is not inhibited up to 0·02 M PCA. Because inhibition of tryptophan is competitive with tryptophan, one might speculate that at very high PCA concentrations, this α-methylphenethylamine is mimicking aromatic α-amino acid inhibitors, such as *p*-chlorophenylalanine, which competes with substrate *in vitro* [28, 29]. Tyrosine hydroxylase of corpus striatum is also blocked by high PCA concentrations, although to a much lesser degree. The antagonism of both hydroxylases *in vitro* suggests that PCA is a weak nonspecific inhibitor.

A decrease of 60 per cent in tryptophan-hydroxylating activity of brain extracts has been reported for rats given PCA (10 mg/kg, i.p.) and sacrificed 16 hr later [4]. Our results with PCA confirm a diminution in tryptophan-hydroxylating activity, but this reduction is much less (23 per cent). This difference probably reflects the fact that our preparations are derived from brainstem (medulla-pons), a region more resistant to the effects of PCA on 5-HT metabolism [11]

Table 1. Rat brainstem trytophan hydroxylase activity

Treatment	Dialyzed (nmoles/mg protein/hr)	Nondialyzed (nmoles/mg protein/hr)
Part A. Brain extracts of rats treated with *p*-chloroamphetamine*		
Control	0·561 ± 0·028 (4)	0·682 ± 0·036 (5)
p-Chloroamphetamine	0·428 ± 0·029 (4)†	0·525 ± 0·027 (5)†
% Decrease	23·7	23·2
Part B. Brain extracts preincubated with *p*-chloroamphetamine‡		
Control	0·433 ± 0·009 (4)	0·471 ± 0·006 (4)
p-Chloroamphetamine	0·431 ± 0·010 (4)	0·287 ± 0·014 (4)§
% Decrease	< 1	39·1

* Rats in groups of 30 received water (control) or *p*-chloroamphetamine, 73 μmoles/kg (15 mg PCA-HCl/kg) i.p., 18 hr before sacrifice. Six brainstems were pooled per enzyme preparation. Part of each brainstem extract was dialyzed overnight as usual before determining tryptophan-hydroxylating activity. The remainder was assayed without the dialysis step. Entries are mean activity ± S. E.; number of brainstem extracts is given in parentheses. Each extract was assayed in duplicate.

† $P < 0{\cdot}05$ compared to controls.

‡ Rat brainstem tryptophan hydroxylase (before dialysis) was incubated for 6 hr with *p*-chloroamphetamine (0·01 M). One portion of this preparation was dialyzed overnight, while the remainder stood overnight at the same temperature. The control enzyme was subjected to the same preincubation (no inhibitor) followed by dialysis or standing overnight.

§ $P < 0{\cdot}001$ compared to controls.

Table 2. Effect of *p*-chloroamphetamine on accumulation of 5-hydroxytryptophan and DOPA and on concentration of serotonin and dopamine in rat brain

Part A. 5-Hydroxytryptophan accumulation and serotonin content in midbrain + hindbrain

Treatment	5-HTP accumulation* (ng/g)	5-HT content† (ng/g)
Control	303 ± 11 (10)	288 ± 21 (5)
p-Chloroamphetamine	151 ± 5 (10)‡	134 ± 5 (5)‡
% Control	50·0	46·5

Part B. Dopa accumulation and dopamine content in corpus striatum

Treatment	Dopa accumulation* (ng/g)	DA content† (ng/g)
Control	514 ± 15 (10)	2956 ± 54 (5)
p-Chloroamphetamine	358 ± 15 (10)‡	2961 ± 28 (5)
% Control	69·6	100·2

* Rats received water (control) or *p*-chloroamphetamine, 73 μmoles/kg (15 mg PCA-HCl/kg) i.p. Sixteen hr later, two intraperitoneal doses of NSD-1024 (3-hydroxy-*O*-benzylhydroxylamine hydrochloride) were administered: 1·0 m-mole/kg (176 mg/kg) and 0·5 m-mole/kg (88 mg/kg) 60 and 30 min before sacrifice respectively. Midbrain + hindbrain for 5-HTP determinations consisted of regions left after removing cerebellum, cortex and corpus striatum from whole brain. The corpus striatum of the same rats was assayed for dopa. Tissues from two rats were pooled for assays; mean wet wt ± S. E. of pairs: midbrain + hindbrain, 1·007 ± 0·007 g; corpus striatum, 0·677 ± 0·005 g (N = 45). Entries are mean concentrations ± S. E.; number of assays is given in parentheses.

† In a separate experiment, rats receiving water (control) or the same dose of *p*-chloroamphetamine were sacrificed 16 hr later for the 5-HT and DA determinations. Entries are mean concentrations ± S. E. with the number of assays in parentheses. The tryptophan content of the midbrain + hindbrain region from the PCA-treated rats (2704 ± 83 ng/g) did not differ from that of controls (2466 ± 77 ng/g) ($P > 0·05$).

‡ $P < 0·001$ compared to controls.

than is the tissue (brain minus cerebral hemispheres and cerebellum) used in the former study [4]. The decrease in brain tryptophan-hydroxylating activity of PCA-treated rats in our experiments is detectable with or without dialysis of the enzyme extracts, an observation in agreement with that reported by Sanders-Bush *et al.* [4]. However, we have not encountered the large loss in enzyme activity on dialysis found by others [4, 25]. The persistence of inhibition after dialysis in brain extracts from PCA-treated rats indicates that the diminished hydroxylating activity is not caused by the type of inhibition *in vitro* described in our present study. Possibly, irreversible inactivation of tryptophan hydroxylase is induced by PCA as proposed by Sanders-Bush *et al.* [4]. However, the relatively modest antagonism of tryptophan hydroxylase activity observed in our experiments *in vivo* and the fact that tryptophan-hydroxylating capability *in vivo* with a tryptophan challenge is not altered by PCA treatment [9, 13] suggest that PCA might induce a decrease in brain tryptophan hydroxylation by a mechanism other than specific inhibition of the enzyme in the manner of *p*-chlorophenylalanine. Our 5-HTP accumulation studies indicate that the rate of tryptophan hydroxylation in brain is reduced by 50 per cent 16 hr after 73 μmoles/kg of PCA. Similar decreases in tryptophan-hydroxylating activity [4] and 5-HT turnover rate [11] have been detected in comparable brain regions of rats treated with PCA. As pointed out by several authors, tryptophan hydroxylation may be decreased because PCA is a strong blocker of neuronal 5-HT re-uptake [1, 2, 5]. The resulting activation of serotoninergic function would be expected to induce a compensatory negative feedback deceleration of 5-HT synthesis and turnover as in the case of imipramine and chlorimipramine [30]. Support for this view is provided by the findings of Sheard [31] on the effects of PCA on firing rates of single cells in the rat raphe. *p*-Chloroamphetamine decreases the firing rate and thus resembles chlorimipramine, a potent 5-HT uptake blocker. The concentrations of PCA, imipramine and chlorimipramine inhibiting 5-HT (0·1 μM) uptake into rat striatal synaptosomes by 50 per cent are 0·6, 0·8 and 0·1 μM respectively (unpublished data). On the other hand, the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine does not affect the firing rate [31].

In the present study, the tyrosine-hydroxylating activity of corpus striatum of rats given PCA (73 μmoles/kg) 18 hr earlier is not different from that of control rats. Nevertheless, dopamine synthesis in corpus striatum is decelerated by PCA (16 hr after the same dose), as indicated by the decreased rate of dopa accumulation after blockade of decarboxylase. It is noteworthy that 15 min after a PCA dose (3·5 μmoles/kg, i.v.) which increases motor activity, the turnover rate of DA in rat striatum is reported to increase [30]. Possibly, the slower synthesis of DA after PCA (Table 2B) is a compensatory reaction to the initial activation of dopaminergic function (leading to increased motor activity [26, 32]) elicited by *p*-chloroamphetamine's release and blockade of re-uptake of DA. The concentration of PCA inhibiting DA (0·1 μM) uptake into rat striatal synaptosomes by 50 per cent is 4 μM (unpublished data).

Although blockade of neuronal re-uptake of monoamines by PCA might contribute to the diminished tryptophan-hydroxylating activity and the lower synthesis rates of 5-HT and DA observed in rat brain, it is possible that these effects are also elicited in part by the ability of PCA to enhance monoaminergic function at synapses by other mechanisms, such as release of monoamines and blockade of monoamine oxidase [3].

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