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5-HYDROXYTRYPTAMINE: THE EFFECTS OF IMPAIRED SYNTHESIS ON ITS METABOLISM AND RELEASE IN RAT

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1 Control rats given L-tryptophan (100 mg/kg) showed a smaller increase of brain 5-hydroxytryptamine (5-HT) than its metabolite 5-hydroxyindoleacetic acid (5-HIAA). However, when brain 5-HT concentrations were depleted by 40–50% after treatment with the synthesis inhibitor *p*-chlorophenylalanine (PCPA) (150 mg/kg) L-tryptophan caused a considerable increase in 5-HT but no change in 5-HIAA. Similar results were obtained following depletion of brain 5-HT by pretreatment with *p*-chloroamphetamine (10 mg/kg).

2 Electrical stimulation of the median raphe nucleus of control rats significantly increased 5-HIAA in the hypothalamus, hippocampus and striatum. However, stimulation of PCPA (200 mg/kg) pretreated animals did not significantly increase 5-HIAA either 24 or 72 h after administration of the drug.

3 Pretreatment of rats with PCPA (200 mg/kg) increased striatal synaptosomal uptake of [^{3}H]-5HT by 30% and reduced 5-HT concentration in the rest of the brain by 62%.

4 PCPA (150 mg/kg) markedly reduced the acute behavioural response (−76%) to *p*-chloroamphetamine (10 mg/kg) although brain 5-HT was only moderately reduced (−36%). L-Tryptophan (100 mg/kg) given 15 min before *p*-chloroamphetamine restored both brain 5-HT and the behavioural effects of *p*-chloroamphetamine in PCPA pretreated rats and enhanced the behavioural response to *p*-chloroamphetamine in control rats.

5 The results suggest that newly synthesized 5-HT is less rapidly metabolized in rats with low brain 5-HT. The possible reasons for this and the relevance of the results to the use of L-tryptophan in the treatment of depressive illness are discussed.

Introduction

Most investigations of the biochemical and behavioural effects of inhibiting the synthesis of brain 5-hydroxytryptamine (5-HT) have involved administration of large doses of inhibitors of tryptophan hydroxylase so that synthesis of 5-HT was largely prevented. While this may have led to striking behavioural changes, the consequences of partial inhibition of 5-HT synthesis are likely to be of greater relevance to physiological mechanisms. Furthermore the lower drug doses required for partial inhibition are less likely to have non-specific effects.

Therefore in previous work (Marsden & Curzon, 1976) we studied the behavioural effects of partial depletion of brain 5-HT by the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (PCPA). These effects can be reversed by a dose of L-tryptophan which leads after 1 h to a marked increase in the concentration of 5-HT in the brain but only a small increase in the concentration of its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) (Marsden & Curzon, 1976). This is in contrast to the greater increase in the concentration of 5-HIAA rather than 5-HT when the same dose of L-tryptophan is given to rats with normal brain 5-HT concentration (Curzon & Marsden, 1975). These differences in metabolism of 5-HT may indicate a presynaptic adaptation of 5-HT neurones to reduced transmitter availability. Evidence is against postsynaptic adaptation to the reduction of brain 5-HT following PCPA treatment (Stewart, Growdon, Cancian & Baldessarini, 1976; Trulson, Eubanks & Jacobs, 1976).

The above mechanisms may be of relevance to depressive illness when brain 5-HT metabolism may be defective and tryptophan has been reported to have beneficial effects (Coppen, Shaw, Herzberg & Maggs, 1967; Moller, Kirk & Fremming, 1976).

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In the present work we have studied the metabolism and availability of 5-HT in the brains of rats, after partial inhibition of the synthesis of this amine. Three procedures were used: tryptophan loading, to investigate the fate of newly synthesized 5-HT; electrical stimulation of cell bodies (Dahlström & Fuxe, 1964) to investigate impulse-dependent releasability of residual 5-HT stores and *p*-chloroamphetamine administration (Sanders-Bush & Massari, 1977), to investigate non-impulse dependent releasability of residual 5-HT stores.

Methods

Male Sprague-Dawley rats (Anglia Laboratory Animals, Alconbury, Cambs. 200 to 250 g) were housed in groups of three per cage under a 12 h light-dark schedule (06 h 00 min to 18 h 00 min) at 23°C ± 3 in a quiet room and given food (Grain Harvesters Rodent Diet) and water *ad libitum*.

Drugs

p-Chlorophenylalanine ethyl ester (Aldrich) and *p*-chloroamphetamine hydrochloride (Regis) were dissolved in 0.9% w/v NaCl solution(saline). L-Tryptophan (Cambrian Chemicals) was suspended in 0.5% polyoxyethylene lauryl ether (BDH) in saline. Probenecid (Merck, Sharpe & Dohme) was dissolved in the minimum volume required of 1 N NaOH, diluted with saline and adjusted to pH 9. Drugs were administered intraperitoneally at 2 ml/kg body weight. Control animals were injected with the appropriate vehicle.

Stimulation

The median raphe nucleus was electrically stimulated in anaesthetized rats sodium pentobarbitone, 40 mg/kg, i.p.) with bipolar steel electrode (Plastic Products, Roanoke, Virginia) and a 10/s, 6 volt (peak to peak) square wave pulse. The electrode was inserted by use of a Stoelting Stereotaxic frame (Stoelting, Chicago, Illinois) using the following coordinates (Pellegrino & Cushman, 1967): sagittal 0.0 mm; frontal −6.0 mm posterior to bregma; horizontal −4.6 mm below the horizontal zero plane. In the sham-stimulated rats an electrode was placed but no current passed. Rats were studied in pairs (one stimulated, one sham-stimulated) during the light period, between 9 h 30 min and 15 h 00 min. PCPA (200 mg/kg) was administered either 24 h or 72 h before stimulation and sham-stimulated controls were injected with saline. Following stimulation or sham-stimulation for 45 min the rats were immediately killed, their brains removed and the hypothalamus, hippocampus, striatum, cortex and spinal cord separated, frozen over dry ice and stored at −25°C. The brain stem was sectioned at 30 mμ on a cryostat to check the placement of the electrodes.

Biochemical determinations

Tryptophan, 5-HT and 5-HIAA were determined by the method of Curzon, Joseph & Knott (1972) as modified by Knott & Curzon (1974) when determinations were made on brain regions.

Uptake of [^{3}H]-5-*hydroxytryptamine by synaptosomes*

Synaptosomal uptake was measured by a slight modification of the method of Sanders-Bush, Bushing & Sulser (1975). Rats given saline, PCPA (200 mg/kg) or *p*-chloroamphetamine (10 mg/kg) 48 h previously were killed, their brains removed and the striata dissected out and kept over ice. The rest of the brain was frozen over dry ice and stored at −25°C until subsequent determination of 5-HT and 5-HIAA. The striata of each rat were weighed and homogenized in 20 vols/w of ice cold 0.25 M sucrose with a Potter-Elvehjem homogenizer and an ice cold Teflon pestle. The homogenate was centrifuged at 1,000 *g* at 5°C for 10 min, the supernatant (crude synaptosomal fraction) kept over ice and 0.2 ml samples preincubated, at 37°C for 5 min with 3.8 ml of pre-oxygenated medium in small vials. The medium contained: glucose (2 mg/ml), ascorbic acid (0.2 mg/ml), ethylenediamine tetraacetic acid disodium salt (0.06 mg/ml) and pargyline hydrochloride (0.12 mg/ml) in Krebs phosphate buffer pH 6.8. After preincubation, [^{3}H]-5-HT (14 Ci/mmol; Radiochemical Centre, Amersham) was added (12×10^{-9} M) and 4 min later the samples were filtered through Millipore filters (AAWPO 2500) under vacuum. The filters were washed with 30 ml saline and then dissolved in 2 ml ethoxy ethanol (BDH Scintillation grade) overnight at 4°C and counted after the addition of 10 ml of scintillant (2,5-diphenyloxazole 6 g in 500 ml Triton X-100 + 1 litre toluene).

Assays were performed in duplicate together with blanks obtained by incubation at 2°C. Uptake of [^{3}H]-5-HT into the synaptosomal fraction was completely prevented by either freezing and thawing three times before incubation or by addition of the 5-HT uptake inhibitor chlorimipramine (0.1 mM) to the incubation mixture.

Behaviour

Locomotor activity and various components of behaviour were measured in groups of 3 rats per cage. Locomotor activity was recorded with 'Animex' activity meters (LKB) tuned at 40 μA, with sensitivity

set at 30 μA. Components of the behavioural effect of *p*-chloroamphetamine were scored at various times after injection i.e. reciprocal forepaw treading, lateral head weaving, straub tail, hindlimb abduction and tremor. The rating scale used was: 0 = absent; 1 = occasional; 2 = frequent and 3 = continuous. Results of the behaviour scores are given as means of summated scores and statistical significance determined by the Mann-Whitney U test. The results of the activity counts and biochemical determinations are given as means ± s.d. and significance determined by Student's *t* test.

Results

Effects of L-*tryptophan on brain tryptophan, 5-HT and 5-HIAA concentrations of saline, PCPA and* p-*chloroamphetamine pretreated rats*

Tryptophan, 5-HT and 5-HIAA were determined 30, 90 and 150 min after L-tryptophan injection in control and PCPA pretreated rats. L-Tryptophan (100 mg/kg) significantly increased brain 5-HT at 30 and 90 min in saline pretreated rats. Increases in brain 5-HIAA were more marked than those of 5-HT and were significant at all three times after injection (Figure 1). When rats had been pretreated with PCPA (150 mg/kg, 24 h beforehand) there was a marked increase in 5-HT at all three times after tryptophan injection while 5-HIAA did not rise significantly at any of these times and showed a small but significant decrease at 30 min after tryptophan injection. Brain tryptophan concentrations of the saline and PCPA pretreated rats (Figure 1) were comparably raised 30 min after tryptophan injection but subsequently fell more rapidly in the PCPA pretreated animals (Figure 1). When egress of 5-HIAA from the brain was blocked by administration of probenecid (150 mg/kg i.p.) 60 min before L-tryptophan (50 mg/kg), 5-HIAA still did not increase in PCPA pretreated rats, while there was a significant increase in 5-HT (Figure 2). Therefore the failure of brain 5-HIAA to rise in PCPA pretreated rats appeared to be due to reduced 5-HT breakdown and not to increased removal of 5-HIAA from the brain.

L-Tryptophan (100 mg/kg) given to *p*-chloroamphetamine pretreated rats (10 mg/kg, 4 h earlier) produced similar changes in 5-HT and 5-HIAA to those seen in PCPA pretreated rats (Figure 3).

Effect of electrical stimulation of the median raphe nucleus on 5-HIAA concentrations in brain regions

In normal rats electrical stimulation of the median raphe causes release of 5-HT from terminals and this is associated with increased 5-HIAA in terminal-rich

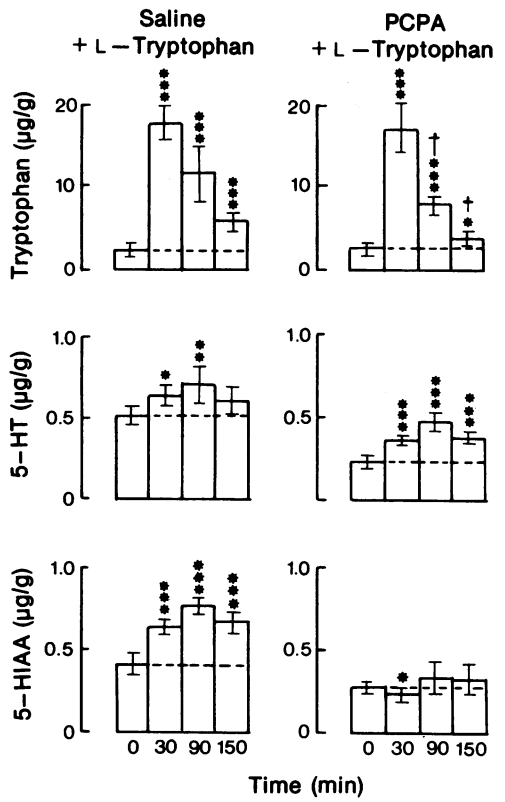

Figure 1 Effects of L-tryptophan (100 mg/kg) on brain tryptophan, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) 30, 90 and 150 min after administration to saline and *p*-chlorophenylalanine (PCPA, 150 mg/kg) pretreated rats. PCPA was given 24 h before L-tryptophan. Results are shown as means of 6 determinations; vertical lines show ±s.d. Differences from values at 0 min: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Differences from corresponding values in saline-treated rats: $\dagger P < 0.05$.

regions (Sheard & Aghajanian, 1968; Collard & Roberts, 1974). In the present study stimulation of the median raphe for 45 min significantly increased 5-HIAA concentrations in the hypothalamus, hippocampus and striatum (Figure 4). Significant changes did not occur in the spinal cord (not shown) or cortex. However, in rats pretreated with PCPA (150 mg/kg) 24 h previously (so that the brain 5-HT concentration of unstimulated animals was reduced by 38%) the 5-HIAA concentration did not alter significantly in any of the above brain regions after stimulation, when compared with sham-stimulated PCPA treated animals.

To study whether the residual brain 5-HT was subsequently mobilised for release, the above experiment

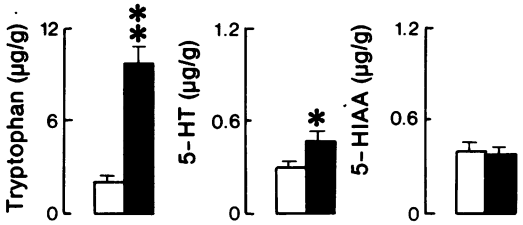

Figure 2 Effects of L-tryptophan (50 mg/kg) on brain tryptophan, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) of rats pretreated with *p*-chlorophenylalanine (PCPA, 150 mg/kg) and probenecid (150 mg/kg). PCPA was given 24 h before probenecid which was given 1 h before L-tryptophan (solid columns) or saline (open columns) and the rats killed 1 h later. Results are shown as means of 6 determinations; vertical lines show ±s.d. Differences from saline-treated rats: $^{*}P < 0.05$; $^{**}P < 0.01$.

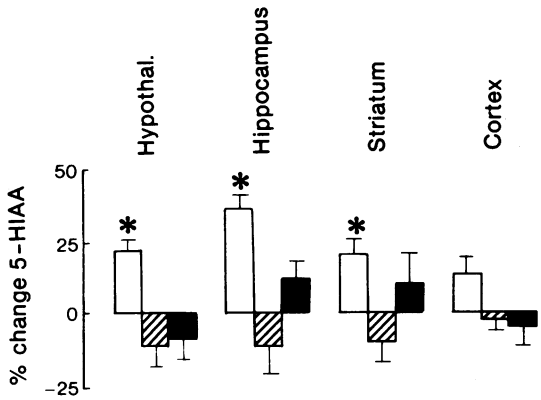

Figure 4 Effect of 45 min electrical stimulation of the median raphe nucleus of anaesthetized rats on 5-hydroxyindoleacetic acid (5-HIAA) in brain regions of rats treated 24 h previously with saline (open columns) and 24 (hatched columns) or 72 h (solid columns) previously with *p*-chlorophenylalanine (PCPA, 200 mg/kg). Results are given as % change from the respective sham-stimulated group; vertical lines show s.e. means. All stimulations were carried out using time and drug matched pairs with one rat sham-stimulated and the other stimulated. Results are shown as means of values obtained on 8 rats. Differences from values for saline pretreated sham stimulated rats: $^{*}P < 0.05$. In the sham-stimulated groups 5-hydroxytryptamine (5-HT) was reduced by 38% 24 h after PCPA and 47% 72 h after PCPA when compared with sham-stimulated saline pretreated rats.

was repeated 72 h after PCPA treatment when the brain 5-HT concentration of unstimulated rats was reduced by 47%. As before, 5-HIAA did not increase significantly on stimulation.

These results suggested that when the brain 5-HT concentration was reduced to about 60% of normal values by PCPA pretreatment, either the amine was not released by stimulation or was released, but more efficiently conserved and consequently turned over more slowly. To investigate the possibility that conservation was occurring through enhanced neuronal

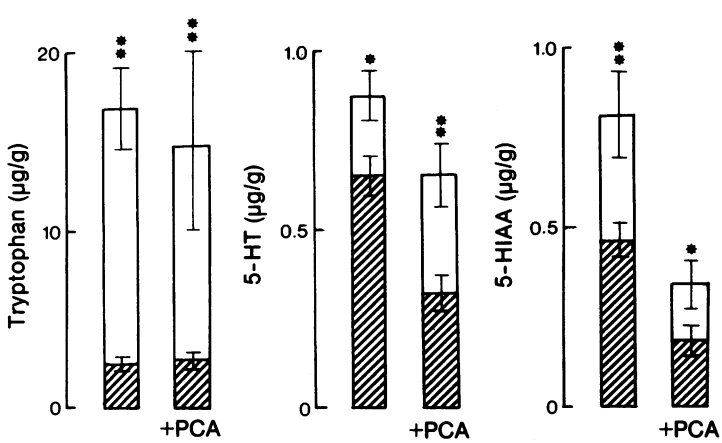

Figure 3 Effects of L-tryptophan (100 mg/kg) on brain tryptophan, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) 60 min after administration to saline and *p*-chloroamphetamine (PCA) (10 mg/kg) pretreated rats. *p*-Chloroamphetamine was given 4 h before L-tryptophan. The hatched columns are values obtained without L-tryptophan administration and the open column shows the increase obtained after giving L-tryptophan. Results are shown as means of 6 determinations; vertical lines show ±s.d. Differences from values without L-tryptophan; $^{*}P < 0.01$; $^{**}P < 0.001$.

re-uptake the uptake of [^{3}H]-5-HT by a crude synaptosomal fraction prepared from the brains of saline and PCPA pretreated rats was determined.

Uptake of [^{3}H]-5-HT by striatal synaptosomes

When PCPA (200 mg/kg) was given 48 h before killing, the uptake of [^{3}H]-5-HT by striatal synaptosomes was significantly increased (+30%, Figure 5) and 5-HT in the rest of brain reduced (−62%). *p*-Chloroamphetamine (100 mg/kg) also decreased brain 5-HT (−50%) but reduced 5-HT uptake (−47%) in agreement with the results of Sanders-Bush *et al.*, (1975).

Effects of p*-chloroamphetamine on the behaviour of saline and PCPA pretreated rats*

To investigate whether 5-HT can be released to receptors from partly depleted 5-HT terminals by means other than raphe stimulation, use was made of the behavioural syndrome which rapidly follows *p*-chloroamphetamine treatment and is thought to be due to central 5-HT release (Trulson & Jacobs, 1976). *p*-Chloroamphetamine (10 mg/kg) given to saline pretreated (24 h) rats caused hyperactivity, hyper-reactivity, lateral head weaving, reciprocal forepaw treading, straub tail, hindlimb abduction, tremor and pyrexia. These effects were apparent 3 to 5 min after injection and maximal after 10 to 20 minutes. Apart from some hyperactivity and hyper-reactivity, the behaviour was almost normal 90 min after injection.

In rats treated 24 h previously with PCPA (150 mg/kg) the behavioural score after *p*-chloroamphetamine injection was greatly reduced (Figure 6) but activity counts only moderately decreased. These animals showed some components of the *p*-chloroamphetamine behavioural syndrome (i.e. forepaw treading and hindlimb abduction) but also showed abnormalities of response (i.e. rearing against the cage walls, gnawing and increased locomotion). Similar changes were noted by Crow & Deakin, (1977) on giving *p*-chloroamphetamine to rats previously treated with the 5-HT antagonist, metergoline.

When L-tryptophan (100 mg/kg) was given 15 min before *p*-chloroamphetamine (10 mg/kg) the behavioural score, was significantly increased but not the activity counts (Figure 6) and the animals showed marked tremor. The L-tryptophan pretreatment also caused a significant increase of brain 5-HT in rats killed 1 h after *p*-chloroamphetamine. Similarly, when rats pretreated with PCPA (150 mg/kg) were given L-tryptophan (100 mg/kg) followed by *p*-chloroamphetamine, there was a significant reversal of the effects of PCPA on brain 5-HT concentration and the behavioural response to *p*-chloroamphetamine (Figure 6).

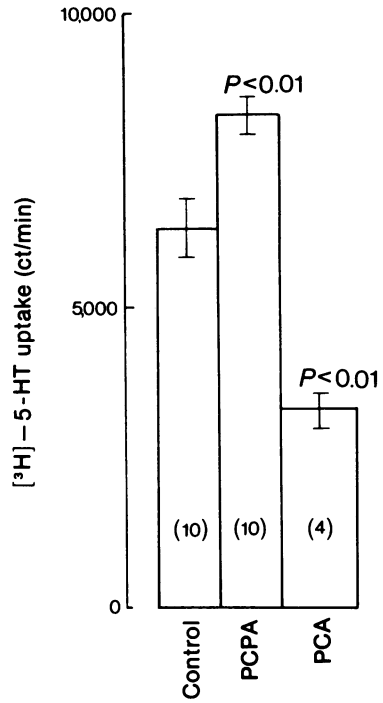

Figure 5 Effects of *p*-chlorophenylalanine (PCPA, 200 mg/kg) and *p*-chloroamphetamine (PCA, 5 mg/kg) pretreatment on the uptake of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) into a crude synaptosomal preparation. Drugs were administered 48 h before killing the rats. Results are shown as mean ct/min (after subtraction of blank values); vertical lines show ±s.d. Nos of experiment are shown in parentheses. Significance of the differences between control and drug-treated groups are indicated.

Discussion

The results show that when L-tryptophan (100 mg/kg) is given to rats pretreated with PCPA (150 mg/kg), although brain 5-HT concentration is decreased its synthesis is not completely impaired and the concentration of brain 5-HT but not its metabolite 5-HIAA is elevated over the next 150 minutes. This is strikingly different from the effect of the same dose of L-tryptophan in normal rats where there is a much greater accumulation of 5-HIAA than 5-HT. As brain 5-HIAA still did not increase when tryptophan was given to rats pretreated with PCPA and probenecid its failure to rise is not explicable by an effect of PCPA on the egress of 5-HIAA from the brain. Therefore newly synthesized 5-HT appears to be less readily metabolized to 5-HIAA in the brains of rats with depleted brain 5-HT. This conclusion is supported by the finding that in PCPA pretreated rats, radio-

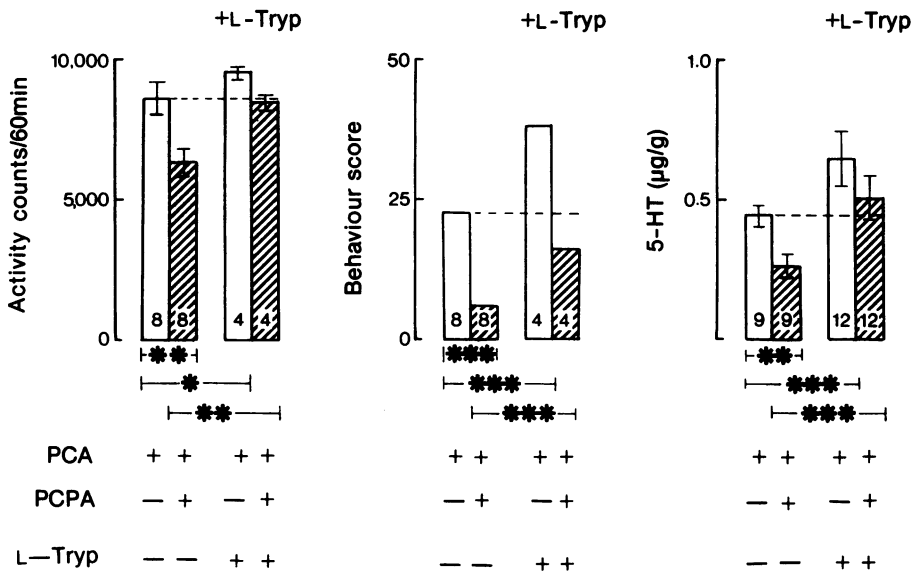

Figure 6 Effects of *p*-chlorophenylalanine (PCPA, 150 mg/kg) and L-tryptophan (L-Tryp, 100 mg/kg) on *p*-chloroamphetamine (PCA) (10 mg/kg)-induced changes in activity, behaviour score and brain 5-hydroxytryptamine (5-HT). PCPA was administered 24 h and L-tryptophan 15 min before *p*-chloroamphetamine. Activity was counted for 60 min after *p*-chloroamphetamine injection. Behavior was scored 5, 10, 20, 40 and 60 min after injection and the values summated. Rats were killed 60 min after giving *p*-chloroamphetamine and brain 5-HT determined. Nos. of experiments done are shown in the columns. Activity and behaviour were measured with 3 rats/cage. 5-HT was determined on individual brains. Differences between groups indicated in the figure: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Differences were determined by Student's *t* test for the activity and 5-HT values and Mann Witney U test for the behavioural scores.

labelled 5-HT disappears more slowly from the brain and also that 5-HT formed from exogenous 5-hydroxytryptophan declines more slowly and 5-HIAA rises less markedly than in normal rats (Koe & Weissman, 1966).

The apparent decrease in metabolism of newly formed 5-HT may reflect either increased storage in the unfilled 5-HT vesicles (Segawa & Fujisawa, 1972; Taber & Anderson, 1973) or a presynaptic neuronal adaptation to low brain 5-HT involving enhanced neuronal reuptake so that the available 5-HT is used more efficiently. It is relevant that in the present work 5-HIAA failed to rise significantly in the hippocampus, hypothalamus and striatum when the 5-HT neuronal cell bodies in the mediun raphe were stimulated 24 or 72 h after PCPA pretreatment even though brain 5-HT was reduced by only about 40%. In agreement with Sheard & Aghajanian, (1968) 5-HIAA rose when normal rats were stimulated; these results may indicate either that 5-HT is not released when partially depleted neurones are stimulated or that it is released but more readily taken up again by presynaptic terminals and re-used. The releasability of 5-HT when partly depleted neurones are stimulated is suggested by the increased rate of 5-HT synthesis after raphe stimulation of rats given a large dose of PCPA (Shields & Eccleston, 1972). However, this assumption depends on the increased synthesis resulting from neuronal release of 5-HT and not more directly from the stimulation of the cell bodies. Release of 5-HT from neurones partly depleted of 5-HT is also indicated by the finding that the inhibitory responses of hippocampal neurones to raphe stimulation were still detectable in rats previously given PCPA (3 × 100 mg/kg) (Segal, 1975). On the other hand the 76% decrease of the acute behavioural response to *p*-chloroamphetamine in PCPA-treated rats, although brain 5-HT was only reduced by 36%, suggests that the residual 5-HT is less releasable by *p*-chloroamphetamine. This drug releases 5-HT (Sanders-Bush, Gallager & Sulser, 1974), though unlike the release resulting from nerve stimulation it does not occur by exocytosis (Ross & Kelder, 1977). The intensity of the behavioural syndrome that appears very shortly after injection of *p*-chloroamphetamine is directly proportional to the dose given (Trulson & Jacobs, 1976) and so may also be proportional to the amount of 5-HT released. That the initial behavioural effects of *p*-chloroamphetamine are associated with 5-HT release is also suggested by the restoration

by pretreatment with L-tryptophan of both the brain 5-HT levels and the behavioural response to *p*-chloroamphetamine of PCPA pretreated rats. Furthermore L-tryptophan also enhanced the response in normal rats when it was given before *p*-chloroamphetamine.

Increased reuptake of 5-HT which may have been released from terminals of PCPA treated rats is suggested by the enhanced uptake of [^{3}H]-5-HT by synaptosomal preparations from such animals. Increased 5-HT reuptake however is not necessarily responsible for the apparent conservation of 5-HT formed from a tryptophan load in PCPA treated rats, since evidence of increased conservation was also obtained when tryptophan was given to *p*-chloroamphetamine pretreated rats, a procedure which rapidly decreases 5-HT reuptake (Wong, Horng & Fuller, 1973). As L-tryptophan was given only 4 h after *p*-chloroamphetamine administration it is unlikely that the long-term neurotoxic action of *p*-chloroamphetamine (Sanders-Bush & Massari, 1971) influenced the results.

Conclusive evidence on the availability of 5-HT for release from partly depleted neurones demands the measurement of transmitter release *in vivo*. An approach to this problem is the use of *in vivo* chronoamperometry in which a potential is applied to an electrode placed in a ventricle (Wightman, Strope, Plotsky & Adams, 1976) or in a brain region (Adams, Conti & Strope, unpublished observations). The current produced during the oxidation of substances in the region of the electrode is proportional to their concentration. Preliminary experiments indicate that this method can measure 5-HT released when either the raphe is stimulated or *p*-chloroamphetamine is given and that release is strikingly reduced or undetectable after pretreatment with PCPA (150 mg/kg) (Adams, Conti, Curzon, Marsden and Strope, unpublished observations). These findings suggest that residual 5-HT in partially depleted neurones has little availability for release.

The present findings have some relevance to the understanding of the behavioural and biochemical effects of treating depressive illness with L-tryptophan. If the releasability of brain 5-HT as well as its concentration (Coppen, 1972) is diminished in depressives, then L-tryptophan may only confer appreciable benefit at doses which bring brain 5-HT to near normal concentrations. Furthermore the negligible increase in brain 5-HIAA found when tryptophan was given to rats with decreased brain 5-HT may explain why the concentration of 5-HIAA in the CSF did not rise when L-tryptophan (2 to 3 g in divided doses) was given to a small group of depressives but did in other subjects (Bowers, 1970). This difference was not found when a somewhat larger single dose was given in another study (Ashcroft, Crawford, Cundall, Davidson, Dobson, Dow, Eccleston, Loose & Pullar, 1973).

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