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EFFECT OF FENFLURAMINE ON 5-HYDROXYTRYPTAMINE UPTAKE AND RELEASE BY RAT BLOOD PLATELETS

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1 (+)-Fenfluramine reduces the central stores of 5-hydroxytryptamine (5-HT) by a poorly understood mechanism.

2 Rat blood platelets have been used in this study as a simple model for serotoninergic nerve endings.

3 (+)-Fenfluramine shows a dual effect: it inhibits the uptake of [^{14}C]-5-HT by platelets and it releases newly absorbed [^{14}C]-5-HT from platelets.

4 The inhibition of [^{14}C]-5-HT uptake induced by (+)-fenfluramine appears very rapidly, is concentration-dependent and seems not to be competitive. (+)-Fenfluramine is ten times less effective than chloroimipramine but ten times more effective than (+)-amphetamine; (+)-fenfluramine is more active than its (−)-isomer or its metabolite norfenfluramine ((+)- or (−)-form).

5 The release of [^{14}C]-5-HT from platelets induced by (+)-fenfluramine is concentration-dependent but increases with increased incubation time. Both chloroimipramine and (+)-amphetamine are in comparison very poor release inducers; (+)-fenfluramine is more active than its (−)-isomer or its metabolites.

6 The effect on [^{14}C]-5-HT uptake exerted by (+)-fenfluramine and chloroimipramine *in vitro* could not be observed *in vivo*.

7 The observed effect of fenfluramine on the uptake and release of 5-HT may explain the lowering action of fenfluramine on the brain 5-HT level, an effect considered of importance for the anorectic effect of this drug.

Introduction

Fenfluramine is a drug capable of suppressing food intake in animals (Le Douarec, Schmitt & Laubie, 1966) and in man (Munro, Seaton & Duncan, 1966), which unlike amphetamine, is devoid of central stimulant activity (Bizzi, Bonaccorsi, Jespersen, Jori & Garattini, 1970; Le Douarec & Neveu, 1970). A characteristic effect of fenfluramine on brain neurotransmitters is a long lasting, dose-dependent lowering of brain 5-hydroxytryptamine (5-HT) (Duhault & Verdavainne, 1967; Opitz, 1967; Costa, Groppetti & Revuelta, 1971) and 5-hydroxyindoleacetic acid (5-HIAA) (Opitz, 1967; Garattini, 1973). Since relatively little information is available on the mechanism by which fenfluramine reduces the central stores of 5-HT (Morgan, Cattabeni & Costa, 1972; Ghezzi, Samanin, Bernasconi, Tognoni, Gerna & Garattini, 1973) it was decided to investigate the effect of fenfluramine on the uptake or the release of 5-HT.

The study was carried out on blood platelets, considered by several authors as a simple model for nerve endings (Paasonen, 1965; Pletscher, 1968; Tuomisto, 1974). The selected animal species was the rat as most studies of the effects of fenfluramine on 5-HT have been carried out in this species; in addition, rat platelets are unable to metabolize 5-HT (Paasonen, 1965).

Methods

Preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP)

Blood was obtained from male Charles River rats (weight 170-200 g), anaesthetized with ether, by intracardiac puncture with a 10 ml plastic syringe containing 1 ml 3.13% trisodium citrate for 9 ml of blood. Throughout the experiments only

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disposable plastic material was used. Blood was centrifuged at 400 *g* for 15 min and the PRP was pipetted off. Platelets were counted by phase microscopy and adjusted to about 600,000/μl by appropriate dilutions with PPP from the same animal. PPP was obtained by further centrifugation of blood at 3,000 *g* for 20 minutes.

Preparation of plasma-free platelets (PFP)

PRP (10 ml) was gel filtered on a Sepharose 2 B column (2.0 x 20.0 cm) according to the method described by Tangen, Berman & Marfey (1971). The number of PFP was adjusted to about 600,000/μl by appropriate dilution with the elation buffer.

Uptake of [^{14}C]-5-hydroxytryptamine by platelets

In vitro *experiments* PRP (1.9 ml) or PFP was first preincubated at 37° C with 0.1 ml of 0.9% w/v NaCl solution (saline) or drug solution, then with either 0.05 μg, 0.1 μg or 0.2 μg [^{14}C]-5-HT, corresponding to 0.007 μCi/ml, 0.014 μCi/ml and 0.028 μCi/ml respectively. The mixtures were gently shaken during the incubation period. Incubation was stopped by cooling the tubes in a melting ice-bath; afterwards the samples were centrifuged at 4° C at 3,000 *g* for 20 min and the supernatant carefully collected.

Radioactivity (ct/min) was counted in duplicated samples for 1 min in a liquid scintillation counter (Beckman LS 250) after addition of 0.2 ml uncentrifuged PRP or PFP or supernatant to 15 ml of a dioxane-naphthalene scintillation mixture. As rat blood platelets do not contain monoamine oxidase (Paasonen, 1965), it was assumed that radioactivity was a measure of 5-HT uptake. Uptake of [^{14}C]-5-HT was calculated as described by David & Hérion (1972). Preliminary experiments showed no difference in radioactivity counting when a given amount of [^{14}C]-5-HT was added to equal volumes of distilled water, PRP or PPP, both in presence and in absence of (+)-fenfluramine. At any time, the sum of radioactivity counted in the supernatant and in the platelet pellet (the latter carefully resuspended in saline) corresponded to the amount (97-98%) of the added radioactivity.

In vivo *experiments* Rats were injected intraperitoneally with one of the following drugs: (+)-fenfluramine (either 15 or 30 mg/kg body wt) and chloroimipramine (10 or 20 mg/kg body wt.). The animals were killed 15, 30, 60 and 120 min after drug administration.

In other experiments rats received (+)-fenfluramine (5 mg/kg, i.p.) or chloroimipramine (10 mg/kg, i.p.) twice a day for 6 days; 12 h after the last injection the animals were killed and blood collected as usual. Uptake of [^{14}C]-5-HT by platelets was studied in PRP as described above.

Release of [^{14}C]-5-hydroxytryptamine by platelets

Samples of 1.9 ml PRP were incubated with 0.2 μg [^{14}C]-5-HT (corresponding to 0.028 μCi/ml) for 15 min; to induce release of the accumulated 5-HT, 0.1 ml of different drug solutions was added to the samples which were further incubated at 37° C for 15, 60 or 120 minutes. The radioactivity in the supernatant was counted as described above. Release induced by the different drugs was then calculated from the difference between samples incubated with and without addition of the drugs under the same experimental conditions (David & Hérion, 1972).

Evaluation of other platelet functions

Changes in platelet shape and aggregation were studied at 37° C in a Born-Michal MK IV Aggregation and Shape-Change Monitor (Pharmacological Research, England) connected to a two-channel chart recorder (Servoscribe 2, type RE 520, Smith's Industries Ltd., England) (Born, 1970). A sample (0.7 ml) of PRP was preincubated for 15 min with 0.1 ml saline with or without (+)-fenfluramine (10^{-4} M); then 0.2 ml of one of the following substances was added: adenosine-5′-diphosphate, disodium salt (ADP) (Sigma, USA) (at concentrations ranging between 2.10^{-4} and 10^{-7} M), undiluted Thrombofax (a commercial cephalin prepared as an ether extract of acetone-dried bovine brain by Ortho, U.S.A.) or collagen (acid soluble, Hormon; Germany) 5-20 μg/ml.

Drugs

5-Hydroxytryptamine-[3′-^{14}C] creatinine sulphate (55 mCi/mmol) was obtained from Radiochemical Centre, Amersham, dissolved in 70% ethanol and stored at −20° C. 5-Hydroxytryptamine creatinine sulphate (Farmitalia, Italy), (+)-fenfluramine hydrochloride, (−)-fenfluramine hydrochloride (+)-norfenfluramine hydrochloride and (−)-norfenfluramine hydrochloride (Servier, France), chloroimipramine (Ciba Geigy, Switzerland) and (+)-amphetamine sulphate (Recordati, Italy) were dissolved in saline. Final concentrations are indicated throughout.

Results

In vitro *experiments*

Uptake of [^{14}C]-5-hydroxytryptamine The percent uptake of [^{14}C]-5-HT by platelets was very rapid; it reached its maximum within 1 min, was unrelated to the concentration of [^{14}C]-5-HT used and did not increase after 1 h of incubation (Table 1). When platelets were first preincubated for either one or 15 min with (+)-fenfluramine (2.5×10^{-5} M) and subsequently with [^{14}C]-5-HT, a marked inhibition of the uptake of the radioactive amine was observed; this uptake was almost completely inhibited during the first minute after the addition of the labelled material, then slowly increased and reached a plateau after 15 min (Table 1). Similar results were obtained when platelets were preincubated for 15 min with chloroimipramine (Table 1). The inhibition of platelet uptake of [^{14}C]-5-HT was linearly related to the concentration of the drugs used and was not competitive; compared to (+)-fenfluramine, chloroimipramine was ten times more effective; in contrast, (+)-amphetamine had ten times less inhibitory effect than (+)-fenfluramine (Figure 1). Prolongation of the preincubation time to 4 h only slightly increased the inhibitory effect of (+)-fenfluramine.

The inhibitory activity of (+)-fenfluramine on [^{14}C]-5-HT uptake was compared with that of (+)-norfenfluramine, (−)-fenfluramine and (−)-nor-fenfluramine both in PRP and PFP; these compounds were used at the same final concentration as (+)-fenfluramine (2.5×10^{-5} M) which gave 50% inhibition. The results shown in Table 2, indicate that in PRP (+)-fenfluramine was the most effective substance, whereas in PFP no difference could be observed between (+)-fenfluramine and (+)-norfenfluramine; in both systems, the (+)-compounds were more active than the (−)-compounds.

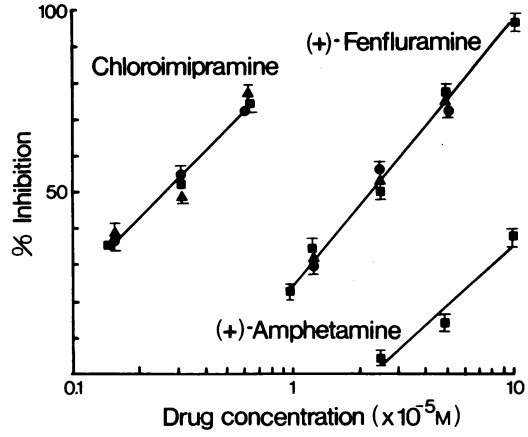

Figure 1 Dose-response curves for the inhibitory activity of chloroimipramine, (+)-fenfluramine and (+)-amphetamine on [^{14}C]-5-hydroxytryptamine (5-HT) uptake by rat platelets. Each point represents the mean of four duplicated experiments. Vertical bars show s.e.mean. Drugs were preincubated with platelet rich plasma for 15 min before an additional incubation period of 15 min with [^{14}C]-5-HT (0.2 μg (■); 0.1 μg (▲) or 0.05 μg (●)).

Release of [^{14}C]-5-hydroxytryptamine Incubation of labelled platelets with (+)-fenfluramine for 15 min provoked a release of [^{14}C]-5-HT which was concentration-dependent. When the incubation was prolonged up to 2 h, the release of [^{14}C]-5-HT was greatly increased. Both (+)-norfenfluramine and (−)-fenfluramine were less effective than (+)-fenfluramine in inducing [^{14}C]-5-HT release whereas (−)-norfenfluramine was almost ineffective. Chloroimipramine even at a concentration (5×10^{-5} M) which completely inhibited the uptake of [^{14}C]-5-HT, provoked a small release of radioactivity. Similarly (+)-amphetamine was poorly active in releasing 5-HT from platelets (Table 3).

Table 1 Kinetics of the uptake of [^{14}C]-5-hydroxytryptamine (5-HT) by rat blood platelets. Effects of (+)-fenfluramine and chloroimipramine.

Time (min) after addition of 0.05 μg [^{14}C]-5-HT	*% uptake of [^{14}C]-5-HT*			
	Control	*(+)-Fenfluramine (2.5×10^{-5} M)*		*Chloroimipramine (3.1×10^{-6} M)*
1	95.0 ± 2.3	8.2 ± 2.6*	4.6 ± 1.0**	2.0 ± 1.1**
2	–	17.0 ± 1.0	9.7 ± 0.7	4.5 ± 2.6
3	–	16.2 ± 2.4	15.5 ± 1.5	12.5 ± 1.8
5	–	24.7 ± 3.1	20.0 ± 1.9	17.0 ± 1.9
10	–	35.7 ± 4.6	34.7 ± 1.2	33.5 ± 3.6
15	95.7 ± 1.0	45.5 ± 5.2	43.1 ± 6.0	42.2 ± 3.3

Results are mean ± s.e.mean of four duplicated experiments.
* Drug added to PRP 1 min before [^{14}C]-5-HT. ** Drug added to PRP 15 min before [^{14}C]-5-HT.

Table 2 Comparison of the inhibitory effect of (+)-fenfluramine, (−)-fenfluramine and their metabolites on [^{14}C]-5-hydroxytryptamine (5-HT) uptake by rat platelets, suspended either in plasma (PRP) or in a plasma-free medium (PFP).

Drug (2.5×10^{-5} M)	*% Inhibition* PRP	PFP
(+)-Fenfluramine	52.0 ± 2.8	40.1 ± 2.9
(+)-Norfenfluramine	27.0 ± 1.5	39.0 ± 3.1
(−)-Fenfluramine	26.3 ± 2.1	16.4 ± 0.7
(−)-Norfenfluramine	3.1 ± 0.2	4.3 ± 0.3

Results are mean ± s.e.mean of four duplicated experiments.

Table 3 Percent release of platelet-bound [^{14}C]-5-hydroxytryptamine (5-HT) induced by incubation of PRP with different drugs for 2 hours

Drugs (5×10^{-5} M)	*% Release*
Chloroimipramine	14.3 ± 0.3
(+)-Amphetamine	16.7 ± 1.4
(+)-Fenfluramine	52.7 ± 3.0
(+)-Norfenfluramine	41.7 ± 0.3
(−)-Fenfluramine	39.7 ± 0.3
(−)-Norfenfluramine	6.3 ± 0.3
Saline	<2.0

Results are mean ± s.e.mean of four duplicated experiments.

In vivo *experiments*

The influence of (+)-fenfluramine given intraperitoneally to rats on the subsequent *in vitro* uptake of [^{14}C]-5-HT by platelets was studied after both acute and chronic treatments and compared with the results of analogous experiments performed with chloroimipramine; an appreciable inhibition of the uptake (23.8% ± 4.1 and 14.0% ± 7.9 respectively) was only found 15 min after a single injection of either drug. No inhibition was observed at 120 min or after a repeated treatment (6 days) with either drug.

Other platelet functions

In vitro shape change and platelet aggregation induced by ADP, collagen and Thrombofax were not modified by (+)-fenfluramine. This drug even at high concentrations (10^{-4} M) neither provoked platelet aggregation *in vitro* (up to 4 h incubation) nor influenced the platelet count after acute or repeated *in vivo* treatments.

Discussion

The experiments described here indicate that fenfluramine interferes with the storage and/or the transfer of 5-HT in rat blood platelets, used in this study as a simple model of serotoninergic nerve terminals. In this *in vitro* model fenfluramine has a dual effect: it inhibits 5-HT uptake and it induces release of 5-HT. No other effect of fenfluramine on platelet number and function could be detected either *in vitro* or *in vivo*, indicating that the drug does not cause nonspecific damage to the platelet membrane.

The inhibition of 5-HT uptake appears very rapidly and is concentration-dependent (fenfluramine 1-10 x 10^{-5} M). The effect of fenfluramine is not inhibited by increasing the concentration of 5-HT four-fold, suggesting that the inhibition is not of a competitive nature. Fenfluramine is about ten times less active than a classic inhibitor of 5-HT uptake such as chloroimipramine, but it is at least ten times more active than its analogue. (+)-amphetamine. (+)-Fenfluramine appears to be more active than its (−)-isomer or its metabolite, norfenfluramine ((−)- or (+)-form). This finding further supports the suggestion that fenfluramine acts on the serotoninergic system *per se* rather than through the formation of norfenfluramine (Morgan *et al.*, 1972; Garattini, Buczko, Jori & Samanin, 1974). In a plasma-free medium, however, no differences were observed between (+)-fenfluramine and (+)-norfenfluramine; this could be explained by a stronger binding of norfenfluramine to plasma proteins; however the role of plasma proteins in our system requires further investigation.

As far as the release is concerned, fenfluramine, at concentrations comparable to those which inhibit uptake, is effective in decreasing the levels of labelled 5-HT in platelets. The effect of fenfluramine on 5-HT release is concentration-dependent and increases with the time of incubation. As was seen in the uptake experiments, (+)-fenfluramine is also more active than its (−)-isomer or its metabolite in inducing [^{14}C]-5-HT release from platelets. In contrast, chloroimipramine, which is much more effective than (+)-fenfluramine as an inhibitor of uptake, is a very poor release inducer. Amphetamine is also much less effective than (+)-fenfluramine in releasing 5-HT from platelets. It is remarkable that the effect on 5-HT uptake exerted by fenfluramine and chloroimipramine *in vitro* could not be observed *in vivo* during acute or repeated treatments. This may be due to the fact that both

drugs penetrate very rapidly into tissues and so only very low concentrations are found in plasma (Ghezzi *et al.*, 1973; Bizzi, Tacconi, Tognoni, Morselli & Garattini, unpublished observations).

The observed effect of fenfluramine on the uptake and on the release of 5-HT may help to explain the reduction in brain 5-HT levels caused by fenfluramine (Duhault & Verdavainne, 1967; Opitz, 1967; Costa *et al.*, 1971), an effect considered of importance for the anorectic effect of this drug (Jespersen & Scheel-Krüger, 1970; Samanin, Ghezzi, Valzelli & Garattini, 1972). In fact the inhibitory effect on uptake together with the releasing action may also explain the reduction in brain 5-HIAA levels caused by fenfluramine (Duhault & Verdavainne, 1967), since it is believed that 5-HT is chiefly deaminated intraneuronally (Blaschko & Levine, 1966). The inhibition of 5-HT uptake is not sufficient alone to lower brain 5-HT or 5-HIAA (Carlsson, Corrodi, Fuxe & Hökfelt, 1969; Ghezzi *et al.*, 1973), unless there is a concomitant release of 5-HT, as for instance during electrical stimulation of the midbrain raphe (Samanin, Ghezzi & Garattini, 1972). The possibility that the inhibition of 5-HT uptake and the release of 5-HT may be important factors in the reduction of brain 5-HT produced by fenfluamine is, in any case, reinforced by other experimental studies showing that this anorectic drug does not inhibit tryptophan hydroxylase, monoamine oxidase (Morgan, Löfstrandh & Costa, 1972) or 5-HT synthesis (Costa *et al.*, 1971). On the contrary, there may be an increase in 5-HT synthesis which may depend on an increased level of tryptophan in the brain after administration of fenfluramine (Costa *et al.*, 1971). It may be added that recently Fuxe (1974) working with brain slices *in vitro* has also observed a dual effect of fenfluramine on uptake and release of 5-HT.

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