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UPTAKE OF 6-FLUORO-5-HYDROXYTRYPTAMINE AND 4,6-DIFLUORO-5-HYDROXYTRYPTAMINE INTO RELEASABLE AND NON-RELEASABLE COMPARTMENTS OF HUMAN PLATELETS

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1 The ring-fluorinated compounds, 6-fluoro-5-hydroxytryptamine and 4,6-difluoro-5-hydroxytryptamine, are sterically similar to 5-hydroxytryptamine (5-HT) but have pK_as for their 5-hydroxyl groups (9.7 and 8.0 respectively) which are lower than that of 5-HT.

2 The rates at which [^{3}H]-5-HT, [^{3}H]-6-fluoro-5-HT, and [^{3}H]-4,6-difluoro-5-HT entered the releasable (vesicular) and non-releasable compartments of washed human platelets during a 1 min incubation period at 37°C were similar. These portions of the uptake process therefore appear to be relatively independent of the pK_a of the 5-hydroxyl group.

3 The [^{3}H]-4,6-difluoro-5-HT was unique since material accumulating in the non-releasable compartment during uptake did not migrate into the vesicular compartment under appropriate incubation conditions.

4 The data suggest that the zwitterionic form of 5-HT is not the transported species, but that the electronic configuration about the 5-hydroxyl group may be critical for translocation from a non-releasable to a releasable compartment.

Introduction

In general, the substitution of fluorine for an aromatic hydrogen introduces little significant steric alteration into the parent molecule (Goldman, 1969). Thus when differences in biological activity exist between the parent compound and its fluorinated isosteres, they can often be attributed to the electron-withdrawing effects of fluorine (Loncrini & Filler, 1970; Filler, 1976). A series of ring-fluorinated analogues of biogenic amines, for example, displays dramatic differences in biological behaviour, differences that may be due partly to the presence of phenolic hydroxyl groups with significantly lower pK_as than those of the parent amines (Kirk, Cantacuzene, Nimitkitpaisan, McCulloch, Padgett, Daly & Creveling, 1979; Cantacuzene, Kirk, McCulloch & Creveling, 1979).

An important physiological property of 5-hydroxytryptamine (5-HT) is its uptake by platelets. To explore the effects of ring fluorination on this biological activity of 5-HT, we have examined the uptake of tritium-labelled 6-fluoro-5-HT (6F-5-HT) and 4,6-difluoro-5-HT (4,6-diF-5-HT) by washed human platelets. It has been suggested that the transport of other phenolic amines requires zwitterionic structures (Bashford, Casey, Radda & Ritchie, 1976), whose formation at physiological pH is favoured when the pK_a of the phenolic hydroxyl group is lowered. Thus the significantly lowered pK_a of the 5-hydroxyl group in 6F-5-HT and 4,6-diF-5-HT assumes particular relevance in evaluating the participation of zwitterionic forms in the various states of the 5-HT uptake process.

Methods

The methoxy ethers of 6F-5-HT and 4,6-diF-5-HT were synthesized by the methods described previously (Kirk, 1976), and tritiated by performing the final decarboxylation in tritiated water (specific activity, 5 Ci/ml). Boron tribromide demethylation gave [^{3}H]-6F-5-HT and [^{3}H]-4,6,-diF-5-HT, both isolated as the creatinine sulphate complexes. On thin-layer chromatography (silica gel plates) with ethyl acetate: isopropanol: ammonium hydroxide (45:35:20) and butanol:ethanol:acetic acid:water (1:1:1:1), approximately 90% of the radioactivity co-chromatographed, respectively, with unlabelled 6F-5-HT or 4,6-diF-5-HT. The final specific activity for each labelled compound was approximately 30 mCi/mmol. Side-chain labelled [^{3}H]-5-HT (specific activity 25 Ci/mmol), obtained from the New England Nuclear Corp. (Boston, MA), was diluted with unlabelled 5-HT to a specific activity of 30mCi/mmol.

Human platelets were collected into the citrate

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disodium edetate (EDTA) medium of Detwiler & Feinman (1973). Platelet-rich plasma (PRP) was prepared by differential centrifugation at 4°C (Murphy, Colburn, Davis & Bunney, 1969), and platelets were pelleted and resuspended to a final concentration of $1-2 \times 10^8$/ml in Tris-citrate buffer, pH 7.35, containing 6 mM glucose and 0.35% bovine serum albumin (crystallized and lyophilized; Sigma Chemical Co., St. Louis, MO) (Costa, Murphy & Kafka, 1977a). Formaldehyde fixative (Costa & Murphy, 1975) was used to stop uptake, and human thrombin (final concentration 4 units/ml) was used to release platelet storage vesicles (Costa *et al.*, 1977a). After fixation, cells were cooled to 4°C and pelleted; intracellular radioactivity was mobilized with 0.4 N $HClO_4$; scintillation counts were measured in a Tracor Analytic Mark III instrument (Tracor Analytic, Des Plaines, IL). Where appropriate, the percentage release of vesicles by thrombin was measured as described previously and this information was used to estimate the amount of labelled material accumulating in the vesicular (thrombin-releasable) and non-releasable compartments (Costa *et al.*, 1977a). Platelet counts were performed with an Electrozone Celloscope (Particle Data, Inc., Elmhurst, IL) equipped with a 19 μm orifice and a logarthmic amplifier.

Specific experimental protocols are given in the legends to each Table.

Results

Accumulation of tritiated hydroxytryptamine in releasable and non-releasable compartments during 1 min incubation periods

Previous work has shown that human platelets accumulate 5-HT in both releasable (vesicular) and non-releasable (cytoplasmic) compartments (Costa *et al.*, 1977a), and that uptake of extracellular 5-HT into each compartment may proceed independently (Costa, Kirk, Murphy & Stark, 1981). These processes can be evaluated by monitoring the rate at which labelled 5-HT, present initially at an extracellular

Table 1 Comparison of the uptake of 10^{-6} M [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT), [^{3}H]-6-fluoro-5-HT ([^{3}H]-6F-5-HT) and [^{3}H]-4,6-difluoro-5-HT ([^{3}H]-diF-5-HT) into releasable and non-releasable compartments over a 1 min period

	Amount of label accumulating after various incubation times (mol/platelet × 10^{19})					
	10s	20s	30s	40s	50s	60s
Total in platelets						
[^{3}H]-5-HT	0.73 ± 0.11	1.91 ± 0.09	2.86 ± 0.17	4.26 ± 0.10	—	6.18 ± 0.23
[^{3}H]-6F-5-HT	1.28 ± 0.13	2.89 ± 0.17	3.53 ± 0.04	4.73 ± 0.22	—	6.70 ± 0.36
[^{3}H]-diF-5-HT	0.64 ± 0.02	1.68 ± 0.03	2.08 ± 0.03	3.20 ± 0.06	3.75 ± 0.16	4.73 ± 0.10
Non-releasable						
[^{3}H]-5-HT	0.18 ± 0.02	0.55 ± 0.03	0.92 ± 0.16	0.99 ± 0.14	—	1.07 ± 0.28
[^{3}H]-6F-5-HT	0.97 ± 0.06	1.09 ± 0.15	0.95 ± 0.14	0.73 ± 0.10	—	0.61 ± 0.17
[^{3}H]-diF-5-HT	0.25 ± 0.03	0.54 ± 0.11	0.77 ± 0.06	0.79 ± 0.05	0.86 ± 0.09	1.25 ± 0.15
Releasable						
[^{3}H]-5-HT	0.55 ± 0.02	1.36 ± 0.03	1.94 ± 0.16	3.26 ± 0.14	—	5.12 ± 0.28
[^{3}H]-6F-5-HT	0.31 ± 0.04	1.80 ± 0.16	2.58 ± 0.17	4.00 ± 0.08	—	6.09 ± 0.18
[^{3}H]-diF-5-HT	0.39 ± 0.03	1.14 ± 0.10	1.31 ± 0.06	2.41 ± 0.04	2.89 ± 0.10	3.48 ± 0.22

Platelet aliquots at 37°C were incubated for various time periods with [^{3}H]-5-HT, [^{3}H]-6F-5-HT, or [^{3}H]-4,6-diF-5-HT (final concentration of each, 10^{-6} M). To ensure accurate timing, amines were added to platelet aliquots with plumpers (Calbiochem-Behring, La Jolla, CA). At the end of each incubation period, uptake was stopped either by the addition of formaldehyde (1.5%, final concentration; Costa & Murphy, 1975) plus imipramine (10^{-6} M, final concentration), or by imipramine (10^{-6} M) plus human thombin (4 units/ml). Imipramine (the generous gift of the Ciba-Geigy Corp., Summit, NJ) was used to stop uptake immediately after thrombin addition. Releasable and non-releasable material was estimated as described in methods. Values are mean ± s.e.mean.

concentration of 10^{-6} M, enters each compartment during the course of a 1 min incubation period at 37°C. Comparison of the accumulation uptake of [^{3}H]-5-HT with that of [^{3}H]-6F-5-HT and [^{3}H]-4,6,diF-5-HT revealed that during the entire 60 s of incubation, all three amines accumulated approximately linearly in the releasable compartment, and in a similar fashion in the non-releasable pool (Table 1). The rate of accumulation of [^{3}H]-6F-5-HT in the releasable compartment was somewhat higher than that of [^{3}H]-5-HT, and that of [^{3}H]-4,6-diF-5-HT somewhat lower than that of [^{3}H]-5-HT.

Evaluation of the movement of tritiated hydroxytryptamine from the non-releasable to the releasable compartment

When resuspended platelets at a density of $1-2 \times 10^8$/ml are incubated at 37°C with 2×10^{-7} M [^{3}H]-5-HT, the cells accumulate essentially 100% of the labelled 5-HT during a 30 min period (Costa *et al.*, 1977a; 1981). Non-releasable [^{3}H]-5-HT accumulated early in the incubation period moves from the non-releasable to the vesicular compartment as the extracellular concentration falls toward zero, so that at the end of the incubation period, all the intra-platelet [^{3}H]-5-HT is vesicular. A similar translocation of non-releasable [^{3}H]-5-HT into the vesicular compartment also occurs when platelets containing non-releasable [^{3}H]-5-HT are resuspended in amine-free buffer and warmed to 37°C (Costa, Silber & Murphy, 1977b; Costa *et al.*, 1981).

To examine the effects of ring fluorination on the ability of the 5-HT moiety to make a non-releasable to releasable transition, we incubated platelets for 30 min at 37°C with an initial concentration of 2×10^{-7} M [^{3}H]-5-HT, [^{3}H]-6F-5-HT, or [^{3}H]-4,6-diF-5-HT (Table 2). During the first 2 min of incubation, appreciable quantities of all three amines accumulated in the non-releasable compartment. After 5 to 10 min of incubation, the amount of non-releasable [^{3}H]-5-HT or [^{3}H]-6F-5-HT had declined to essentially unmeasurable levels, although the amount of vesicular amine continued to increase in both cases. Non-releasable [^{3}H]-5-HT and [^{3}H]-6F-5-HT remained unmeasurable after incubation for 30 min, despite a small (about 12%) increase in the amount of releasable amine present. Approximately one-third of the intracellular [^{3}H]-4,6-diF-5-HT remained non-releasable, however, during the entire incubation period at 37°C. This non-releasable amine did not become releasable when the cells were resuspended in fresh buffer and warmed to 37°C (see below).

Table 2 Comparison of the uptake of 2×10^{-7} M [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT), [^{3}H]-6-fluoro-5-HT ([^{3}H]-6F-5-HT) and [^{3}H]-4,6-difluoro-5-HT ([^{3}H]-diF-5-HT) into releasable and non-releasable compartments over a 30 min period

	Amount of label accumulating after various incubation times (mol/platelet $\times 10^{19}$)				
Total in platelets	1 min	2 min	5 min	10 min	30 min
[^{3}H]-5-HT	1.83 ±0.03	2.88 ±0.05	4.35 ±0.03	4.43 ±0.08	4.95 ±0.03
[^{3}H]-6F-5-HT	2.66 ±0.05	3.50 ±0.27	5.89 ±0.08	7.36 ±0.22	8.19 ±0.19
[^{3}H]-diF-5-HT	1.57 ±0.09	2.73 ±0.07	4.61 ±0.11	5.78 ±0.17	5.71 ±0.17
Non-releasable					
[^{3}H]-5-HT	0.48 ±0.08	0.29 ±0.06	0.17 ±0.03	<0.1	<0.1
[^{3}H]-6F-5-HT	0.75 ±0.14	1.16 ±0.24	<0.1	<0.1	<0.1
[^{3}H]-diF-5-HT	0.48 ±0.10	1.07 ±0.11	1.54 ±0.20	1.33 ±0.17	1.33 ±0.16
Releasable					
[^{3}H]-5-HT	1.35 ±0.09	2.59 ±0.07	4.18 ±0.03	4.43 ±0.13	4.95 ±0.12
[^{3}H]-6F-5-HT	1.91 ±0.15	2.34 ±0.24	5.87 ±0.17	7.35 ±0.21	8.19 ±0.13
[^{3}H]-diF-5-HT	1.09 ±0.10	1.66 ±0.13	3.07 ±0.21	4.45 ±0.19	4.38 ±0.18

Platelets at 37°C were incubated for various times with 2×10^{-7} M [^{3}H]-5-HT, [^{3}H]-6F-5-HT, or [^{3}H]-4,6-diF-5-HT. After 1, 2, 5, 10 or 30 min of incubation, cells were either fixed with formaldehyde or treated with human thrombin for 60 s and then fixed. Releasable and non-releasable material was estimated as described in Methods. Values are mean ± s.e.mean.

Effect of non-releasable 4,6-difluoro-5-hydroxytryptamine on the uptake of [^{3}H]-5-HT into non-releasable and vesicular compartments

It seemed apparent that newly taken up 4,6-diF-5-HT, unlike 5-HT or 6F-5-HT, could accumulate in an intra-platelet pool which was both extra-vesicular and not capable of translocation into the vesicular pool. We therefore designed an experiment to explore the relationship of this non-releasable 4,6-diF-5-HT to the uptake of 5-HT into two other pools known to occur in platelets, the vesicular 5-HT pool and the non-releasable 5-HT pool I (Costa *et al.*, 1981). When platelets were incubated for 5 min at 37°C with 2×10^{-7} M 4,6-diF-5-HT, resuspended in fresh buffer, and warmed to 37°C, approximately 1×10^{-20} mol/platelet of 4,6-diF-5-HT remained non-releasable (Table 3). Nevertheless, extracellular [^{3}H]-5-HT present initially at 10^{-8} M entered both releasable and non-releasable compartments at the same rate as in cells containing no 4,6-diF-5-HT. After both 10 s and 1 min of incubation, the amounts of [^{3}H]-5-HT taken up in each compartment were small relative to the total amounts of 4,6-diF-5-HT present in those compartments (3% to 10% of the amount of 4,6-diF-5-HT in the non-releasable compartment, for example).

Oxidative deamination of tritiated hydroxytryptamines by platelet sonicates

Human platelet sonicates fail to oxidatively deaminate 5-HT (Donnelly & Murphy, 1977), and intact platelets do not appear to sequester deaminated 5-HT metabolites in their non-releasable compartments (Costa *et al.*, 1981). Because of the apparently anomalous behaviour of non-releasable 4,6-diF-5-HT, we examined the possibility that this amine might represent a deaminated metabolite of the parent amine (Table 4). Sonicates of human platelets incubated for 30 min at 37°C with 10^{-3} M [^{3}H]-5-HT produced negligible amounts of deaminated [^{3}H]-5-HT (< 0.01% of the amount available). Although with both [^{3}H]-6F-5-HT and [^{3}H]-4,6-diF-5-HT platelet sonicates produced approximately 15 fold more metabolite, this amount still represented a very small fraction of the total amine present (< 0.2%).

Discussion

The pK_a of the ring hydroxyl group in the present series of substituted 5-hydroxytryptamines decreases from 10.73 for 5-HT to 9.07 for 6F-5-HT, and to 7.97 for 4,6-diF-5-HT (Kirk, 1976). Although less

Table 3 Comparison of the uptake of 10^{-8} M [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) in the presence and absence of non-releasable and releasable (vesicular) 4,6-difluoro-5-HT (diF-5-HT)

Time of Incubation With 10^{-8} M [^{3}H]-5-HT	*Amount of [^{3}H]-5-HT accumulated* (mol/platelet × 10^{20})			*Amount of diF-5-HT present during uptake of [^{3}H]-5-HT* (mol/platelet × 10^{20})		
	Total	*Non-Releasable*	*Releasable (Vesicular)*	*Total*	*Non-Releasable*	*Releasable (Vesicular)*
Control						
10 s	0.23 ± 0.04	0.03 ± 0.01	0.20 ± 0.01	0	0	0
1 min	1.04 ± 0.07	0.10 ± 0.02	0.94 ± 0.02	0	0	0
Pre-incubation with diF-5-HT						
10 s	0.24 ± 0.02	0.04 ± 0.01	0.20 ± 0.01	53.2 ± 0.1	10.0 ± 0.4	43.2 ± 0.5
1 min	10.6 ± 0.7	0.76 ± 0.12	9.82 ± 0.13	53.2 ± 0.1	10.0 ± 0.4	43.2 ± 0.5

Platelets at 37°C were incubated for 5 min with either no additions (control cells) or with 2×10^{-7} M 4,6-diF-5-HT. Cells were then pelleted, resuspended in fresh buffer, and warmed to 37°C. Control cells and cells loaded with 4,6-diF-5-HT were allowed to accumulate [^{3}H]-5-HT (initial concentration, 10^{-8} M) for 10 s or 1 min and either fixed or treated with thrombin and then fixed. The amount of releasable and non-releasable label was determined as described in Methods. The amount of unlabelled 4,6-diF-5-HT in cells was estimated by incubating platelets at 37°C for 5 min with 2×10^{-7} M [^{3}H]-4,6-diF-5-HT, resuspension of cells in fresh buffer, and warming to 37°C prior to the addition of fixative or thrombin and then fixative.
Values are mean ± s.e.mean.

Table 4 Production of deaminated metabolites by platelet sonicates during a 20 min incubation with 10^{-3} M [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT), [^{3}H]-6-fluoro-5-HT ([^{3}H]-6F-5-HT) or [^{3}H]-4,6-difluoro-5-HT ([^{3}H]-4,6-diF-5-HT)

Substrate	*Amount of metabolite produced* (nmol h^{-1} mg^{-1} protein)
[^{3}H]-5-HT	0.85 ± 0.08
[^{3}H]-6F-5-HT	13.20 ± 1.60
[^{3}H]-4,6-diF-5-HT	14.20 ± 0.90

Monoamine oxidase activity in platelet sonicates was measured as described by Murphy, Donnelly & Richelson (1976). Human platelets collected in ACD anticoagulant were pelleted, suspended in distilled water, and sonicated. Aliquots of sonicate containing 0.2 mg of protein were diluted with 0.08 M potassium phosphate buffer, pH 7.2, and incubated for 30 min at 37°C with 10^{-3} M [^{3}H]-5-HT, [^{3}H]-6F-5-HT, or [^{3}H]-4,6-diF-5-HT. Deaminated metabolites were separated by passage through a column containing Amberlite CG-50 resin. Aliquots of boiled platelets, incubated as described above, were used as controls.
Values are mean ± s.e.mean.

than 0.05% of the 5-HT is present at pH 7.4 in a zwitterionic state (i.e., with a protonated amine terminus and a deprotonated hydroxyl group), approximately 0.5% of the 6F-5-HT and more than 5% of the 4,6-diF-5-HT exist as zwitterions. Since all three 5-hydroxytryptamines are sterically essentially identical, one would expect a platelet uptake system selecting for or producing the zwitterionic amine to favour the fluorinated derivatives, especially 4,6-diF-5-HT, over 5-HT itself. In fact, there exists no large difference between the three species in the rate of uptake into either vesicles or a non-releasable pool. Thus if the neutral 5-HT molecule is the transported species, it seems likely to be a molecule protonated on its hydroxyl group and deprotonated on its amine group, rather than the zwitterionic form. It should be possible to test this hypothesis with a series of tritiated 5-HT analogues with altered pK_as for deprotonation of the terminal amine group.

Despite the similar uptake of all three 5-hydroxytryptamines, the behaviour of 4,6-diF-5-HT differs strikingly in one respect from that of both 5-HT and 6F-5-HT. Non-releasable 4,6-diF-5-HT newly taken up from the extracellular medium remains non-releasable under conditions which permit migration of both 5-HT and 6F-5-HT into the vesicular compartment. The non-releasable 4,6-diF-5-HT thus behaves as if it is not sequestered in non-releasable pool I, in which newly-accumulated 5-HT usually resides, but rather as if it is a component of non-releasable pool II, which is usually reserved for 5-HT lost from vesicles and destined to leave the platelet (Costa *et al.*, 1981). The possible assignment of non-releasable 4,6-diF-5-HT to non-releasable pool II is further supported by the fact that this non-releasable amine has no effect on the subsequent uptake of [^{3}H]-5-HT into either non-releasable pool I or the vesicles.

Regardless of its location within the platelet, non-releasable 4,6-diF-5-HT does not appear to represent a deaminated metabolite of the amine. Although both 6F-5-HT and 4,6-diF-5-HT are slightly better substrates than 5-HT for platelet monoamine oxidase, neither is as good a substrate as tyramine or dopamine. Relatively large amounts of the latter two amines also accumulate in a non-releasable compartment (pool II?) which does not affect subsequent 5-HT uptake regardless of whether or not monoamine oxidase is inhibited by prior treatment with deprenyl (Costa *et al.*, 1977c). At the maximal metabolic rate of 2×10^{-21} mol of 6F-5-HT or 4,6-diF-5-HT per platelet per min (estimated to occur from the metabolic rate observed in platelet sonicates), the amount of 'metabolite' expected would be too small to account for the observed accumulation of non-releasable material. In addition, the metabolism of 6F-5-HT, which immediately after uptake does not appear to enter the non-releasable pool II, proceeds at the same rate as that of 4,6-diF-5-HT.

The reasons for the apparently anomalous behaviour of 4,6-diF-5-HT are not clear at present. Since *de novo* the amine enters vesicles at approximately the same rate as do 5-HT and 6F-5-HT, it seems possible that the altered step involves an inability to enter non-releasable pool I or to move from this pool into vesicles. Sterically all three amines are essentially identical, and the introduction of a single ring fluorine in 6F-5-HT fails to produce any dramatic alteration of behaviour. Thus one or both of these portions of intra-platelet amine translocation process appear to be exquisitely sensitive to the electronic configuration around the ring hydroxyl group.

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