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# ANOMALOUS COMPARTMENTATION OF 5-HYDROXYTRYPTAMINE IN INTACT HUMAN PLATELETS

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**1** The movement of radiolabelled 5-hydroxytryptamine (5-HT) between the extracellular medium, the thrombin-releasable (vesicular) compartment, and a non-thrombin-releasable compartment has been investigated in washed human platelets.

**2** Appreciable amounts of extracellular 5-HT can accumulate in a non-releasable compartment. Depending on the incubation conditions and the amount of 5-HT already present inside the cell, non-releasable 5-HT can either remain non-releasable or rapidly migrate into the vesicular compartment.

**3** Measurable amounts of vesicular 5-HT can also enter a non-releasable compartment. However, vesicular 5-HT which becomes non-releasable does not appear to mix with 5-HT becoming non-releasable following uptake from the extracellular medium.

**4** Extracellular 5-HT can be added to vesicles and to the non-releasable compartment in the presence of appreciable quantities of 5-HT already in one or both compartments.

**5** The vesicular and non-releasable compartments appear to accumulate 5-HT independently of one another. Furthermore, most vesicular accumulation of 5-HT occurs from the extracellular medium, rather than by translocation of non-releasable 5-HT into the vesicular compartment.

## Introduction

We have recently developed techniques for the abrupt inhibition of 5-hydroxytryptamine (5-HT) uptake into platelets by formaldehyde fixation, and for the evaluation of 5-HT storage in vesicles by employing brief thrombin treatment for the exclusive release of vesicular 5-HT (Costa & Murphy, 1975; Costa, Murphy & Kafka, 1977a). When these methods are used to examine the accumulation of 5-HT in thrombin-releasable (vesicular) and non-thrombin releasable compartments of intact human platelets, the data reveal some unexpected features. For example, intracellular 5-HT can become non-releasable following its entry from the extracellular medium, and vesicular (releasable) 5-HT can become non-releasable under certain conditions (Costa *et al.*, 1977a; Costa, Murphy & Reveille, 1977b; Costa, Silber & Murphy, 1977c; Costa, Stark, Shafer, Corash, Smith & Murphy, 1978a; Costa, Murphy, Smith & Pettigrew, 1978b). In addition, under certain circumstances, non-releasable 5-HT can enter either the extracellular medium or the releasable compartment. The present experiments were designed to define in more detail the relationships between the non-releasable and releasable pools of 5-HT in intact human platelets.

## Methods

Whole blood was collected from volunteers (with no known history of haematological disorders or drug ingestion for at least two weeks before donation) into the citrate-EDTA medium of Detwiler & Feinman (1973). Platelet-rich plasma (PRP) was prepared by serial centrifugation at 4°C (Murphy, Colburn, Davis & Bunney, 1969). For all experiments, total, vesicular, and cytoplasmic 5-HT were measured by the use of formaldehyde fixative and brief thrombin treatment plus formaldehyde fixative as described previously (Costa & Murphy, 1975; Costa *et al.*, 1977a). To calculate the percentage release of vesicular 5-HT by brief thrombin treatment, 5-HT in the vesicular compartment was prelabelled in separate aliquots of the same PRP used for each experiment, by incubation for 30 min at 37°C with either [$^{14}$C]-5-HT (56 mCi/mmol; Amersham Corp., Arlington Hts., IL) or [$^{3}$H]-5-HT (25.1 Ci/mmol; New England Nuclear Corp., Boston, MA), at initial extracellular concentrations ranging from $10^{-8}$M to $10^{-6}$M as appropriate. Following this or other incubation conditions as described below, platelets were cooled to 0°C, centri-

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fuged at 1200 *g* for 15 min, and resuspended in NaCl-Tris-citrate buffer, pH 7.3, containing 0.35% bovine serum albumin (crystallized and lyophilized; Sigma Chemical Co., St. Louis, MO) and 5.9 mM glucose (Costa *et al.*, 1977a). At the end of all experimental protocols, labelled material remaining inside pelleted platelets was determined by digestion with 0.4 N $HClO_4$, mixing with Aquasol (New England Nuclear Corp., Boston, MA), and measurement in a liquid scintillation counter which performed automated calculation of the amount of $^3H$ and $^{14}C$ in each sample (Mark III Scintillation Counter, Searle Analytic, Des Plaines, IL). Platelet counts for all protocols were performed with an Electrozone Celloscope (Particle Data, Inc., Elmhurst, IL) with a logarithmic amplifier and a 19 μm orifice. The specific experimental protocol employed is shown in the footnotes to each Table.

## Results

Previous observations from this laboratory have suggested that under normal circumstances, essentially 100% of the intra-platelet 5-HT is located in the vesicular (thrombin-releasable) compartment (Costa *et al.*, 1977a). Incubation of such cells in PRP with $10^{-6}$ M labelled 5-HT results in the net addition of labelled 5-HT to the same compartment (Costa & Murphy, 1976; Costa *et al.*, 1978a). Since the amount of endogenous 5-HT present prior to any net addition can be determined, and the amount of label added also accurately measured, the labelled 5-HT can serve as a marker for the subsequent disposition of the entire 5-HT pool. When such prelabelled cells are incubated for consecutive time periods at 37°C in the absence of extracellular 5-HT, a portion (about 10%) of the vesicular 5-HT enters a non-releasable pool at between 60 and 90 min of incubation, and is lost from the cell after 90 min of incubation (Costa *et al.*, 1977a; 1977c).

By using similar incubation conditions, we were able to compare the effects of the presence or absence of a non-releasable 5-HT pool on the *de novo* movement of extracellular [$^3H$]-5-HT into the platelet. Cells were prelabelled by adding to vesicles $1.5 \times 10^{-18}$ mol/platelet of [$^{14}C$]-5-HT to the approximately $5 \times 10^{-18}$ mol/platelet of endogenous 5-HT already present in this compartment. The cells were then resuspended, and the uptake of $10^{-8}$ M extracellular [$^3H$]-5-HT and the disposition of [$^{14}C$]-5-HT were measured at various times during a 90 min incubation period at 37°C (Table 1). As noted previously, during the first 60 min of incubation, no change was observed in the net amount of vesicular [$^{14}C$]-5-HT present. After 90 min of incubation at 37°C, although the total amount of [$^{14}C$]-5-HT in the

**Table 1.** Effect of incubation time at 37°C on human platelet vesicular and non-releasable [$^{14}C$]-5-hydroxytryptamine ([$^{14}C$]-5-HT) and *de novo* total platelet uptake of [$^3H$]-5-HT

| *Time of incubation at 37°C (min)* | *Amount of [$^{14}C$]-5-HT present* (mol/platelet × $10^{18}$) *Total* | *Non-releasable* | *Vesicular* | *Net uptake of [$^3H$]-5-HT in 10 s* (mol/platelet × $10^{21}$) |
|---|---|---|---|---|
| <1 (Control) | 1.456 ±0.025 | 0 | 1.441[2] ±0.013 | 1.710 ±0.054 |
| 30 | 1.464[1] ±0.021 | 0 | 1.472[2] ±0.064 | 1.679[1] ±0.145 |
| 60 | 1.502[1] ±0.038 | 0 | 1.559[2] ±0.024 | 1.854[1] ±0.064 |
| 90 | 1.474[1] ±0.025 | 0.135 ±0.034 | 1.339[3] ±0.034 | 1.896[1] ±0.038 |
| 120 | 1.309[4] ±0.022 | 0.186 ±0.016 | 1.123[4] ±0.016 | 1.812[1] ±0.121 |

Values are mean ± s.e. mean.
[1] Not significantly different from control (<1 min) value (Student's *t* test). [2] Value for amount of vesicular [$^{14}C$]-5-HT not significantly different from value for total [$^{14}C$]-5-HT (Student's *t* test). [3] Value for amount of vesicular [$^{14}C$]-5-HT significantly different from value for total [$^{14}C$]-5-HT, $P < 0.025$ (Student's *t* test), and from control value ($P < 0.025$). [4] Significantly different from control value, $P < 0.001$ (Student's *t* test).
Platelet vesicular 5-HT stores were labelled by incubation of PRP for 30 min at 37°C with $10^{-6}$ M [$^{14}C$]-5-HT. Cells were resuspended in Tris-citrate-NaCl buffer and incubated at 37°C. At varying times (up to 2 h) after platelet aliquots had reached 37°C, $10^{-8}$ M [$^3H$]-5HT was added and allowed to accumulate for 10 s before the addition of formaldehyde fixative or thrombin (with fixation 60 s after the addition of thrombin). Concentrations of radiolabelled 5-HT in vesicles and non-releasable pools were determined as described in Methods.

platelet remained unchanged, $1 \times 10^{-19}$ mol of [$^{14}$C]-5-HT per platelet (about 7% of the total) had become non-thrombin-releasable.

When the uptake of $10^{-8}$ M extracellular [$^{3}$H]-5-HT was examined in these cells, there were no significant differences in the initial (10 s) rate at which [$^{3}$H]-5-HT entered platelets immediately after warming to 37°C or at 30 min intervals thereafter. Thus the presence or absence of a non-releasable 5-HT pool had no apparent effect on the rate at which extracellular [$^{3}$H]-5-HT crossed the plasma membrane. It therefore seems unlikely that the non-releasable 5-HT created in this fashion is in equilibrium with extracellular 5-HT. Assuming an average extra-vesicular platelet volume not enclosed by membranes of $4 \times 10^{-15}$ litre (Corash, Tan & Gralnik, 1977; Morgenstern & Kho, 1977; Stahl, Themann & Dame, 1978), and that unlabelled vesicular 5-HT became non-releasable concomitantly with the vesicular [$^{14}$C]-5-HT, the total amount of extra-vesicular 5-HT (unlabelled plus [$^{14}$C]-5-HT) was $4.6 \times 10^{-19}$ mol/platelet, equalling a concentration of $10^{-4}$ M, after 90 min. Accordingly, if the $10^{-8}$ M extracellular [$^{3}$H]-5-HT entering the platelet were to achieve equilibrium with the non-releaseable platelet 5-HT, it would have to enter the cells from the medium against an apparent concentration gradient of approx. $10^{4}$ (with non-releasable 5-HT present) at the same rate as against no apparent gradient (no non-releasable 5-HT present).

We considered that after 90 min of incubation at 37°C, [$^{14}$C]-5-HT could have accumulated in a non-releasable pool following movement directly from vesicles to the extracellular medium and then back into the cell. To evaluate this possibility, $10^{-6}$ M imipramine was added to the prelabelled, resuspended cells prior to incubation at 37°C. When the imipramine-treated cells were then incubated for 90 min at 37°C, the prelabelling [$^{14}$C]-5-HT remained in vesicles during the first 60 min. After 90 min, the total amount of [$^{14}$C]-5-HT in the cell was unchanged, but 10% of the total was not releasable (an amount comparable to that found above). At all time points, we checked the ability of the imipramine to inhibit the *de novo* uptake of extracellular 5-HT by adding $10^{-8}$ M [$^{3}$H]-5-HT and measuring its uptake over a 10 s period. [$^{3}$H]-5-HT uptake was consistently only 2% of the control value (see below). Although imipramine effectively blocked the movement of extracellular 5-HT into the platelet, it failed to prevent the movement of vesicular [$^{14}$C]-5-HT into a non-releasable compartment. Thus the non-releasable [$^{14}$C]-5-HT present appeared to represent material lost from vesicles.

The data presented in Table 1 thus suggested that 5-HT entering a non-releasable pool from vesicles appeared not to re-enter vesicles from this pool, and that the presence of this material in the cell did not alter the *de novo* entry of extracellular 5-HT into platelets. To continue our investigation of these phenomena, we next examined the effects of a pool of non-releasable 5-HT, newly added to the cells from the extracellular medium, on the entry of extracellular 5-HT into the platelet.

Previous work has shown that in platelets not loaded to their maximal storage capacity with 5-HT, 5-HT newly added to a non-releasable compartment from the extracellular medium can migrate rapidly into vesicles when the source of extracellular 5-HT is removed (Costa *et al.*, 1977a; 1977c). This occurs during resuspension of cells in amine-free buffer, or by allowing uptake of essentially 100% of the extracellular 5-HT into platelets. To take advantage of this fact, cells were incubated first with a relatively high concentration of extracellular [$^{14}$C]-5-HT ($10^{-6}$ M). After incubation for 5 min, followed by formaldehyde fixation, there were $53 \times 10^{-20}$ mol/platelet of [$^{14}$C]-5-HT in a non-releasable compartment (Table 2). Duplicate cell aliquots were incubated for 5 min at 37°C with or without $10^{-6}$ M [$^{14}$C]-5-HT, cooled to 0°C, pelleted at 4°C, and resuspended in buffer at 0°C. Unlabelled cells and [$^{14}$C]-5-HT prelabelled cells were then incubated for 1 min at 37°C with a 100 fold lower concentration of [$^{3}$H]-5-HT ($10^{-8}$ M). While the [$^{14}$C]-5-HT prelabelled cells were cooling to 0°C, they accumulated additional [$^{14}$C]-5-HT; following resuspension and a second incubation period at 37°C, all the [$^{14}$C]-5-HT present in these cells became thrombin-releasable. Simultaneously, $2.51 \times 10^{-20}$ mol/platelet of [$^{3}$H]-5-HT entered the vesicles from the extracellular medium. In comparison, the unlabelled cells accumulated $2.62 \times 10^{-20}$ mol/platelet of [$^{3}$H]-5-HT. Cells were also incubated first for 1 min with a relatively low concentration of [$^{3}$H]-5-HT ($10^{-8}$ M), cooled, resuspended, and incubated for 5 min at 37°C with or without a 100-fold higher concentration of [$^{14}$C]-5-HT ($10^{-6}$ M). Prior to cooling, these cells contained $1.41 \times 10^{-20}$ mol/platelet of [$^{3}$H]-5-HT, of which $0.59 \times 10^{-20}$ mol/platelet was non-releasable. After resuspension and incubation at 37°C, cells not exposed to [$^{14}$C]-5-HT contained $2.10 \times 10^{-20}$ mol/platelet of [$^{3}$H]-5-HT, of which essentially 100% was vesicular. Similarly, cells exposed to [$^{14}$C]-5-HT contained $1.97 \times 10^{-20}$ mol/platelet of [$^{3}$H]-5-HT in their vesicles, and essentially none in a non-releasable compartment, and had in addition added $66.8 \times 10^{-20}$ mol/platelet of [$^{14}$C]-5-HT to the vesicles and $19.3 \times 10^{-20}$ mol/platelet of [$^{14}$C]-5-HT to a non-releasable compartment.

The data summarized in Table 2 indicated that over a 5 min period at 37°C, the same amount of extracellular 5-HT entered vesicles in the presence and absence of the concomitant translocation of much smaller or much larger amounts of labelled

**Table 2** Concomitant movement of non-releasable and extracellular 5-hydroxytryptamine (5-HT) into vesicles of intact human platelets

| | *Experimental protocol* | *Radio-label used* | *First incubation* *Intracellular disposition of* first 5-*HT label* (mol/platelet × $10^{20}$) | | | *Resuspension and second incubation* *Intracellular disposition of* first 5-*HT label* (mol/platelet × $10^{20}$) | | | *Intracellular disposition of* second 5-*HT label* (mol/platelet × $10^{20}$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Total* | *Non-releasable* | *Vesicular* | *Total* | *Non-releasable* | *Vesicular* | *Total* | *Non-releasable* | *Vesicular* |
| I | 5 min $10^{-6}$ M[$^{14}$C]-5-HT; then | [$^{14}$C]-5-HT | 154 ± 4 | 53.3 ± 4.3 | 101 ± 4 | 192 ± 3 | 0 | 191 ± 1 | | | |
| | 5 min $10^{-8}$ M[$^{3}$H]-5-HT | [$^{3}$H]-5-HT | | | | | | | 2.51[1] ± 0.03 | 0 | 2.45[1] ± 0.06 |
| II | 1 min $10^{-8}$ M[$^{3}$H]-5-HT; then | [$^{3}$H]-5-HT | 1.41 ± 0.03 | 0.59 ± 0.01 | 0.82 ± 0.01 | 1.97[2] ± 0.03 | 0 | 1.94[2] ± 0.03 | | | |
| | 1 min $10^{-6}$ M[$^{14}$C]-5-HT | [$^{14}$C]-5-HT | | | | | | | 86.0 ± 0.9 | 19.3 ± 2.7 | 66.8 ± 2.7 |

Values are mean ± s.e. mean.

[1] To be compared to a value of 2.62 ± 0.07 × $10^{-20}$ mol/platelet of [$^{3}$H]-5-HT (100% vesicular) in cells not previously exposed to [$^{14}$C]-5-HT. [2] To be compared to a value of 2.10 ± 0.02 × $10^{-20}$ mol/platelet of [$^{3}$H]-5-HT (100% vesicular) in cells not exposed to [$^{14}$C]-5-HT.

This experiment was done in two parts. First, unlabelled, resuspended platelets were incubated for 5 min at 37°C with $10^{-6}$ M [$^{14}$C]-5-HT. Control cells were incubated for a similar period with the vehicle. At the end of this period, aliquots were either (a) fixed or treated with thrombin and then fixed (to determine the amount of [$^{14}$C]-5-HT in releasable and non-releasable compartments), or (b) cooled to 0°C, pelleted, resuspended in fresh buffer, and held at 0°C. While the resuspended cells were held at 0°C, $10^{-8}$ M [$^{3}$H]-5-HT was added; cells were then warmed to 37°C, held at this temperature for 5 min, and either fixed or treated with thrombin and then fixed (to determine non-releasable and vesicular [$^{14}$C]-5-HT and [$^{3}$H]-5-HT). Second, cells were manipulated with the same protocol except that the first incubation was for 1 min at 37°C with $10^{-8}$ M [$^{3}$H]-5-HT, and the second (after resuspension) incubation with $10^{-6}$ M [$^{14}$C]-5-HT for 1 min after the cells reached 37°C.

5-HT from a non-releasable pool into the vesicles. (Note that under the conditions described here, there was no change in the amount of endogenous 5-HT present in vesicles.) The data thus suggested that extracellular 5-HT entered vesicles by a pathway separate from that by which non-releasable 5-HT did so. This hypothesis would be proven if the following condition could be shown to apply: movement of the 5-HT added during the 'first incubation' from a non-releasable to a releasable pool must have occurred (a) after the cells were resuspended and warmed to 37°C and (b) at approximately the same rate and over a similar time period (1 to 5 min) as the extracellular 5-HT entered vesicles. To investigate the time at which 'first incubation' non-releasable 5-HT became releasable, we conducted experiments with $10^{-6}$ M imipramine. As also noted previously (Campbell & Todrick, 1976; Costa *et al.*, 1977c), $10^{-6}$ M imipramine added to cells containing non-releasable 5-HT completely inhibited the non-releasable to releasable movement of 5-HT until the cells were resuspended in fresh buffer and warmed to 37°C. (This procedure presumably diluted out the accumulated imipramine sufficiently to permit movement of 5-HT into platelet cytoplasm and from cytoplasm to vesicles.) Thus platelets were incubated for 5 min at 37°C with $10^{-6}$ M [$^{14}$C]-5-HT, and were either (1) treated immediately with $10^{-6}$ M imipramine, cooled to 0°C, resuspended and held at 0°C, or (2) cooled to 0°C, treated with $10^{-6}$ M imipramine , resuspended, and held at 0°C, or (3) cooled to 0°C, resuspended, and treated with $10^{-6}$ M imipramine. All cell aliquots were then incubated for 5 min at 37°C, and the intracellular disposition of the [$^{14}$C]-5-HT was examined. [$^{14}$C]-5-HT in both sets of cells treated with imipramine before resuspension was all in vesicles; in cells treated with imipramine after resuspension, 30% of the total [$^{14}$C]-5-HT was non-releasable. Thus 'first incubation' non-releasable 5-HT apparently entered vesicles only after resuspension and incubation at 37°C (during the 'second incubation'). Since 'second incubation' 5-HT was added extracellularly to the resuspended cells while they were at 0°C, a temperature at which 5-HT uptake is negligible (Costa & Murphy, 1975), it must also have entered vesicles during incubation at 37°C. In addition, the fact that regardless of its size, 100% of the non-releasable pool entered the vesicles over a 5 min period, suggests, but does not prove, that the rate of vesicular entry is the same for all pools of labelled 5-HT.

A more conclusive result was obtained by studying the 5-HT interrelationships in the presence of a non-releasable pool of 5-HT *not* capable of translocation into vesicles (see also references Costa *et al.*, 1977b and Costa *et al.*, 1978a). To demonstrate the creation of such a 'stationary' non-releasable pool, platelets were allowed to accumulate a large amount of 5-HT by incubation for 30 min in PRP at 37°C with $10^{-5}$ M [$^{14}$C]-5-HT, resuspension in fresh buffer, and a second incubation for 30 min at 37°C with $10^{-5}$ M [$^{3}$H]-5-HT (Table 3). In these doubly-labelled cells, appreciable amounts of each isotope were not thrombin-releasable; when they were again pelleted, resuspended in amine-free buffer, and warmed to 37°C, no significant change was noted in the amount of non-releasable or vesicular labelled 5-HT. Furthermore, no change occurred on continued incubation at 37°C for 20 min.

To evaluate what would happen to small amounts of extracellular [$^{3}$H]-5-HT in the presence of a large 'stationary' pool of non-releasable [$^{14}$C]-5-HT, cells were first loaded in PRP with $10^{-5}$ M unlabelled 5-HT (30 min at 37°C), resuspended, and then incubated with $10^{-5}$ M [$^{14}$C]-5-HT (30 min at 37°C) (Table 4). The approx. $8 \times 10^{-19}$ mol/platelet of [$^{14}$C]-5-HT in the non-releasable compartment remained in this pool when cells were resuspended again and incubated for 1 min at 37°C in a 5-HT-free medium, or for 1 min at 37°C with either $10^{-8}$ M or $2 \times 10^{-7}$M [$^{3}$H]-5-HT. Intracellular compartmentation of label under these conditions was compared with that seen when the unlabelled, resuspended cells (without prior exposure to extracellular 5-HT) were incubated for 1 min at 37°C with [$^{3}$H]-5-HT ($10^{-8}$ M or $2 \times 10^{-7}$ M). In the presence of a stationary non-releasable [$^{14}$C]-5-HT pool, total uptake of [$^{3}$H]-5-HT at each initial concentration was inhibited by about 50%, but in each case the amount of [$^{3}$H]-5-HT accumulating in the non-releasable compartment was approximately doubled. Furthermore, extracellular [$^{3}$H]-5-HT entered vesicles *despite no statistically significant change in the amount of* [$^{14}$C]-*5-HT present in the non-releasable and releasable compartments.* With $10^{-8}$ M [$^{3}$H]-5-HT, the $2 \times 10^{-21}$ mol/platelet of [$^{3}$H]-5-HT in vesicles after 1 min represented only 0.3% of the amount of [$^{14}$C]-5-HT in the non-releasable pool. If this vesicular [$^{3}$H]-5-HT had mixed with the non-releasable [$^{14}$C]-5-HT prior to entry into vesicles, approx $667 \times 10^{-21}$ mol of [$^{14}$C]-5-HT would have become releasable concomitantly with the [$^{3}$H]-5-HT. Similarly, with a 20 fold higher initial concentration of extracellular [$^{3}$H]-5-HT, about 20 fold more [$^{3}$H]-5-HT entered vesicles (5.2% of the amount of [$^{14}$C]-5-HT in cytoplasm). If this amount of [$^{3}$H]-5-HT had mixed with non-releasable [$^{14}$C]-5-HT, it would have moved into vesicles together with $750 \times 10^{-21}$ mol/platelet of [$^{14}$C]-5-HT. Since no net loss of non-releasable [$^{14}$C]-5-HT was observed, it seems that [$^{3}$H]-5-HT must have entered vesicles by some mechanism which permitted it to bypass the 'stationary' pool of non-releasable [$^{14}$C]-5-HT.

One possible explanation for the apparent nonmixing of non-releasable 5-HT pools might be preferen-

**Table 3** Effect of resuspension in amine-free medium and subsequent incubation at 37°C on the distribution of labelled 5-hydroxytryptamine (5-HT) in human platelets

| | | *Amount of labelled 5-HT present* (mol/platelet × $10^{18}$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | [$^3$H]-5-HT | | | [$^{14}$C]-5-HT | | |
| | *Experimental* | *Total* | *Non-releasable* | *Vesicular* | *Total* | *Non-releasable* | *Vesicular* |
| (1) | Incubation for 30 min with [$^3$H]-5-HT | 10.55 ±0.27 | 2.44 ±0.11 | 8.10 ±0.11 | 13.51 ±0.50 | 1.56 ±0.10 | 11.95 ±0.10 |
| (2) | Incubation for 30 min with [$^3$H]-5-HT and resuspension | 10.38 ±0.27 | 1.76 ±0.26 | 8.62 ±0.26 | 12.96 ±0.48 | 1.55 ±0.35 | 11.41 ±0.35 |
| (3) | Incubation for 30 min with [$^3$H]-5-HT and resuspension; incubation for 20 min in amine-free media | 10.34 ±0.15 | 1.80 ±0.21 | 8.54 ±0.21 | 12.34 ±0.27 | 0.76 ±0.27 | 11.58 ±0.27 |

Values are mean ± s.e. mean.
Cells were labelled in PRP by incubation for 30 min at 37°C with $10^{-5}$ M [$^{14}$C]-5-HT, resuspended in buffer, and incubated for 30 min at 37°C with $10^{-5}$ M [$^3$H]-5-HT. At the end of the incubation period with [$^3$H]-5-HT, one set of aliquots was fixed or treated with thrombin to evaluate the non-releasable and vesicular distribution of [$^{14}$C]-5-HT and [$^3$H]-5-HT. A second set was cooled to 0°C, resuspended in fresh buffer, and incubated at 37°C for either 4 or 20 min. Vesicular and non-releasable amounts of [$^{14}$C]-5-HT and [$^3$H]-5-HT were then determined as described in Methods.

**Table 4** Intracellular distribution of platelet [$^{14}$C]-5-hydroxytryptamine ([$^{14}$C]-5-HT) during the *de novo* entry of extracellular [$^3$H]-5-HT into vesicles and a non-releasable pool

| *Initial extracellular [$^3$H]-5-HT concentration* (M) | *Amount of [$^{14}$C]-5-HT present in each compartment* (mol/platelet × $10^{21}$) | | | *Amount of [$^3$H]-5-HT added to each compartment during a 1 min incubation* (mol/platelet × $10^{21}$) | | |
|---|---|---|---|---|---|---|
| | *Total* | *Non-releasable* | *Vesicular* | *Total* | *Non-releasable* | *Vesicular* |
| None | 4947 ±71 | 809 ±59 | 4138 ±59 | 0 | 0 | 0 |
| $1 \times 10^{-8}$ | 4851 ±71 | 751 ±66 | 4100 ±66 | 4.528 ±0.058 | 2.574 ±0.116 | 1.954 ±0.116 |
| $2 \times 10^{-7}$ | 4858 ±64 | 662 ±141 | 4196 ±141 | 80.47 ±1.14 | 41.63 ±2.82 | 38.85 ±2.82 |
| $1 \times 10^{-8}$ | 0 | 0 | 0 | 8.990 ±0.255 | 1.068 ±0.262 | 7.922 ±0.262 |
| $2 \times 10^{-7}$ | 0 | 0 | 0 | 158.9 ±4.5 | 27.1 ±2.5 | 131.7 ±2.4 |

Values are mean ± s.e. mean.
In order to obtain platelets containing a large stationary pool of non-releasable [$^{14}$C]-5-HT, cells were first loaded with unlabelled 5-HT (incubation of PRP for 30 min at 37°C with $10^{-5}$ M 5-HT), resuspended in buffer as above and incubated with $10^{-5}$ M [$^{14}$C]-5-HT for 30 min at 37°C. These cells were then pelleted, resuspended in fresh buffer, warmed to 37°C, and allowed to accumulate [$^3$H]-5-HT (25.1 Ci/mmol), added to the extracellular medium at an initial concentration of either $10^{-8}$ M or $2 \times 10^{-7}$ M, for 1 min before the addition of fixative or thrombin (to evaluate [$^3$H]-5-HT distribution between vesicles and the non-releasable pool). Control cells were incubated, resuspended, incubated, and resuspended in the same fashion but without the addition of 5-HT; the uptake of [$^3$H]-5-HT and its releasable/non-releasable distribution was then measured.

**Table 5** Does oxidative metabolism of [$^3$H]-5-hydroxytryptamine ([$^3$H]-5-HT) occur during its addition to or loss from vesicles?

| *Experimental protocol* | *Net amount of deaminated metabolites detected* (mol/platelet × $10^{18}$) | *Net amount of [$^3$H]-5-HT added to or lost from platelet vesicles* (mol/platelet × $10^{18}$) | *Amount of metabolites as % of [$^3$H]-5-HT added to or lost from platelets* | *Amount of metabolites as % of [$^3$H]-5-HT present in extracellular medium* |
|---|---|---|---|---|
| Incubate for 30 min at 37°C with $10^{-5}$ M [$^3$H]-5-HT | 0.0346 ± 0.0027 | 15.45 ± 0.19 | 0.22% | 0.61% |
| Label vesicles with $10^{-5}$ M [$^3$H]-5-HT then incubate 30 min at 37°C with $10^{-5}$ M cold 5-HT | 0.0195 ± 0.0009 | 6.05 ± 0.12 | 0.32% | 0.82% |

Values are mean ± s.e. mean.

Cells from PRP were resuspended in buffer, incubated for 30 min at 37°C with $10^{-5}$ M [$^3$H]-5-HT and treated as follows: (a) as a control for the evaluation of [$^3$H]-5-HT deamination, aliquots were incubated for 30 min at 37°C with either cells plus $10^{-5}$ M [$^3$H]-5-HT and $10^{-6}$ M imipramine or with cell-free buffer plus $10^{-5}$ M [$^3$H]-5-HT. At the end of the incubation period, control and uptake aliquots were either fixed with formaldehyde or treated with thrombin and then fixed. Cells were pelleted as above (for measurement of total and vesicular [$^3$H]-5-HT content) or cooled to 0°C and pelleted (for measurement of deaminated [$^3$H]-5-HT by the column procedure of Murphy, Donnelly & Richelson (1976)). In the supernates from aliquots incubated to provide control (blank) values, the largest amount of tritium coming through the column occurred in aliquots of cells incubated with [$^3$H]-5-HT and imipramine, and this was used as the blank value. (b) Cells were cooled to 0°C, resuspended in buffer, warmed to 37°C, and incubated for 30 min with $10^{-5}$ M unlabelled 5-HT. As a control for the evaluation of [$^3$H]-5-HT deamination, cells were incubated for 30 min at 37°C. At the end of the incubation period, [$^3$H]-5-HT in vesicles and cytoplasm was measured (see Methods), or cells were cooled to 0°C and pelleted. Deaminated [$^3$H]-5-HT in the supernates was measured by the column procedure as described above. In both experimental protocols, extracellular labelled material was evaluated by thin-layer chromatography with two solvent systems (butanol, ethanol, acetic acid, and water, 1:1:1:1; and ethyl acetate, isopropanol, and ammonium hydroxide, 45:35:20).

tial binding or chemical change of 5-HT in either or both of the pools. We looked for such alterations by two methods, and the results are summarized in Tables 5 and 6. (a) Intact platelets did not appreciably deaminate [$^3$H]-5-HT during its *de novo* uptake and addition to vesicles (less than 1% metabolism), or during its displacement from vesicles to the extracellular medium (less than 1% metabolism) (Table 5). Thin-layer chromatography of the tritiated extracellular material in each case revealed that at least 90% of the radioactivity co-migrated with authentic 5-HT. (b) The cation ionophore, X537A (Lasalocid), has been shown in other work to remove about 90% of intravesicular 5-HT by a non-exocytotic, ionophoric mechanism (Murer, Davenport & Day, 1976; Costa, Murphy & Smith, 1978c; Costa, Pettigrew & Murphy, 1979). In two separate experiments, X537A (25 μM) removed respectively 96% and 90% of the total intracellular [$^3$H]-5-HT residing in vesicles (Table 6). When vesicular [$^3$H]-5-HT became non-releasable following a long incubation at 37°C, X537A removed significantly less of the total intracellular [$^3$H]-5-HT (about 80% in each case). Platelets were also allowed to accumulate varying amounts of non-releasable (but not vesicular) [$^3$H]-5-HT following *de novo* addition from the extracellular medium by incubation with varying concentrations of [$^3$H]-5-HT in the presence of $10^{-6}$ M reserpine (Costa *et al.*, 1977c). Under these conditions, 25 μM X537A removed 90 to 95% of the non-releasable [$^3$H]-5-HT regardless of the total amount of [$^3$H]-5-HT present (range from $1 \times 10^{-19}$ to $30 \times 10^{-19}$ mol/platelet, depending on initial extracellular [$^3$H]-5-HT concentration and time of incubation at 37°C). These data suggested that vesicu-

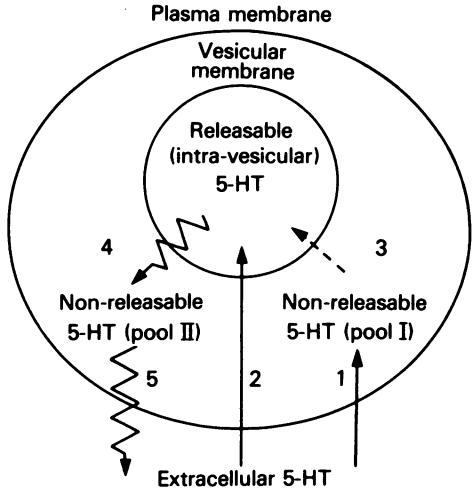


**Figure 1** Proposed model for the uptake pathways, compartmentation, and inter-compartment translocations of 5-hydroxytryptamine (5-HT) in intact human platelets. See text and Table 7 for details.

**Table 6** X537A-mediated removal of vesicular or non-releasable plus vesicular [$^3$H]-5-hydroxytryptamine ([$^3$H]-5-HT) in intact human platelets

| | *Experimental protocol* | *Amount of [$^3$H]-5-HT present* (mol/platelet × $10^{19}$) *Total* | *Vesicular* | *After X537A*[1] | |
|---|---|---|---|---|---|
| (1) | Experiment No. 1 | | | | |
| | 5 min at 37°C (control) | 1.878 ±0.068 | 1.878 ±0.068 | 0.082 ±0.013 | (4.4%) |
| | 4 h at 37°C | 2.031 ±0.027 | 1.419[2] ±0.066 | 0.432[3] ±0.034 | (21.3%) |
| (2) | Experiment No. 2 | | | | |
| | 5 min at 37°C (control) | 1.403 ±0.024 | 1.403 ±0.024 | 0.145 ±0.008 | (10.3%) |
| | 2 h at 37°C | 1.418 ±0.018 | 1.201[3] ±0.036 | 0.273[2] ±0.016 | (19.2%) |

[1] Percentage of total amount of [$^3$H]-5-HT present remaining after X537A treatment is given in parentheses. [2] Significantly different from control value, $P < 0.005$ (Student's $t$ test). [3] Significantly different from control value, $P < 0.001$ (Student's $t$ test).

X537A (Lasolocid) was dissolved in absolute ethanol so that a final concentration in platelet aliquots of 25 μM X537A was obtained at an ethanol concentration of 1%. This concentration of ethanol alone had no effect on labelled 5-HT in platelets. PRP was incubated for 30 min at 37°C with $10^{-7}$ M [$^3$H]-5-HT; cells were then resuspended in buffer and warmed to 37°C. After an incubation period of 5 min (control incubation) or of 120 or 240 min, releasable and non-releasable [$^3$H]-5-HT was measured as outlined in Methods. Other aliquots were treated with 25 μM X537A for 1 min before fixation and measurement of the [$^3$H]-5-HT remaining inside the cells.

**Table 7** Characteristics of thrombin-releasable (vesicular) and non-thrombin-releasable pools of 5-hydroxytryptamine (5-HT) in intact human platelets

| | | *Thrombin-releasable pool* (vesicular) | *Non-thrombin-releasable pools* (I and II) | *Reference* |
|---|---|---|---|---|
| (1) | Any evidence for separate 5-HT pools? | No | Yes (I and II) | Costa *et al.*, 1977a, 1977c & 1978a |
| (2) | Approximate maximal capacity for 5-HT | $3 \times 10^{-17}$ mol/platelet | $0.3 \times 10^{-17}$ mol/platelet (sum of I plus II) | Costa & Murphy, 1976; Costa *et al.*, 1977b, 1978a; and Table 3 |
| (3) | Other amines besides 5-HT? | Yes | Yes | Costa *et al.*, 1977b |
| (4) | 5-HT metabolism? | No | No | Table 6 |
| (5) | Removal of 5-HT by X537A | Yes ($\sim 90\%$) | Pool I : Yes ($\sim 90\%$)<br>Pool II: No ($< 20\%$) | Costa *et al.*, 1978c and Table 7 |
| (6) | Potential for translocation of 5-HT to another compartment | Slow movement to non-releasable pool II ($< 60$ min) | Pool I : rapid movement to releasable pool ($< 5$ min); not to extracellular medium<br>Pool II: slow movement to extracellular medium (30 min); not to releasable pool<br>No movement from I → II<br>No movement if large amount present | Costa *et al.*, 1977a, 1977c, and Tables 1, 2 & 3 |
| (7) | Effect on uptake of 5-HT from extracellular medium | None | None (unless large amounts present) | Tables 1 & 4 |
| (8) | Effect on translocation of 5-HT between pools | None | None (unless large amounts present) | Tables 1, 2 & 4 |

lar and non-releasable 5-HT recently added from the extracellular medium could be almost completely removed from platelets by X537A-mediated ionophoresis, whereas 5-HT becoming non-releasable after residence in vesicles appeared to resist this type of removal.

## Discussion

Based on the data presented and reviewed here, we can postulate a model to describe the uptake pathways, compartmentation, and possible inter-compartment movements of 5-HT in intact platelets (Figure 1 and Table 7). As far as we have been able to determine, all intra-platelet movement of 5-HT appears to be in one direction only. The non-releasable 5-HT newly added from the extracellular medium (termed non-releasable pool I) can move only into vesicles, while 5-HT becoming non-releasable following its residence in vesicles (pool II) will enter the extracellular medium (but not the vesicles or pool I). Furthermore, our data suggest that vesicles generally do not accumulate 5-HT from the extracellular medium via uptake into an intra-platelet, non-releasable 5-HT compartment. Rather, both the releasable and pool I non-releasable compartments behave as if they have direct access to 5-HT present in the extracellular medium. It seems possible that the vesicular membrane may lie in close juxtaposition to the plasma membrane, so that extracellular 5-HT can move in some sort of coupled sequence across specific channels in each membrane and into the vesicle interior without ever being truly 'free' in the intracellular matrix.

A number of platelet-derived molecules (proteins, glycolipids, and sialic-acid-bearing complex carbohydrates) are known to bind 5-HT with a high degree of affinity and specificity (Pignatti & Cavalli-Sforza, 1975; Krishnan & Balaram, 1976; Ochoa & Bangham, 1976; Jonakait, Tamir, Rapport & Gershon, 1977). If one or all of these substances were to bind pool II non-releasable 5-HT, and exclude pool I non-releasable 5-HT, the two pools might fail to mix, and might show different susceptibility to translocation by X537A. At present, however, it is not known by what means the cell can assign its intracellular 5-HT stores to such a variety of compartments, how translation between pools is mediated and controlled, and what role this complex hierarchy plays in normal platelet function.

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