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BEHAVIOUR OF ENDOGENOUS AND NEWLY ABSORBED SEROTONIN IN THE PLATELET RELEASE REACTION

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Abstract—The release of endogenous and newly absorbed radioactive serotonin (5-HT) from human platelets has been studied. Prior to release induction the platelets were allowed to absorb an amount of 5-HT being equal to or lower than that of endogenous 5-HT. Thrombin released from washed platelets 5-HT of the same specific radioactivity as 5-HT retained and present in control platelets. Thus, in this system the endogenous and absorbed 5-HT behaved identically. With platelet-rich plasma release was induced in an aggregeometer by ADP, adrenaline, collagen and thrombin as well as by recalcification. The specific activity of released 5-HT was the same or slightly higher than that of 5-HT in control platelets, indicating that newly absorbed 5-HT behaves like endogenous 5-HT, except for a slight tendency to be more easily released. Absorption of 5-HT caused a small reduction of the platelets' release capacity. It is concluded that under the conditions used, measurement of release of newly absorbed 5-HT is a reasonably good measure of the release of endogenous 5-HT.

ADENINE nucleotides, calcium and potassium are secreted from platelets during the platelet release reaction,[1–3] and have in common that they can not be labelled *in vitro* by external isotopic precursors, i.e. [^{3}H] or [^{14}C] adenine,[4] [^{45}Ca][5] and [^{42}K],[6] respectively. In contrast, serotonin (5-HT) which is located in platelet granules and also secreted, can rapidly be taken up by platelets *in vitro*, and then secreted during the release reaction.[7–13] This property has been utilized to measure 5-HT release from platelets, whereby the cells are allowed to take up radioactive 5-HT, and after addition of a release inducer the amounts released are determined by estimating the increase in extracellular or decrease in intracellular radioactivity. Thus, this method for assessing release differs from those commonly used for adenine nucleotides,[4] calcium,[5] fibrinogen[14] and acid hydrolases,[15] which all are measured as the platelets' own constituents.

Stacey[16] and Aledort *et al.*,[17] have questioned whether release of newly absorbed 5-HT is a measure for the release of the platelets' own 5-HT, and thereby, a proper parameter in the study of the platelet release reaction. To our knowledge, only Massini[18] has compared release of endogenous and newly absorbed 5-HT employing washed platelets and ADP as release inducer. He found the release of absorbed and endogenous 5-HT to be equal. The present paper describes the release of newly absorbed and endogenous 5-HT in platelet-rich plasma as induced by ADP, adrenaline,

collagen and thrombin. The release occurring in recalcified platelet-rich plasma and during thrombin action on washed platelets has also been studied.

MATERIALS

Platelet-rich plasma (*PRP*) was prepared from human blood (9 vol. of blood plus 1 vol. of 0·11 M disodium citrate) by centrifugation at $G_{max} = 190\ g$ and used immediately for experiments.

Suspensions of washed platelets were prepared from EDTA-anticoagulated human blood as described elsewhere.[15]

Radioactive 5-HT. (1) 2-[^{3}H]-*N*-5-hydroxytryptamine binoxalate (code NET 398, New England Nuclear, Boston), 1·34 Ci/m-mole, obtained as a 0·75 mM solution in 2% ethanol, was diluted with 2 vol. of 0·15 M Nacl. (2) [^{3}H]-*G*-5-hydroxytryptamine creatinine sulphate (code TRK 223, The Radiochemical Centre, Amersham), 2·2 Ci/m-mole, 0·455 mM in 5 ml of 2% ethanol was diluted with 0·15 M NaCl to 0·227 mM after most of the ethanol had been removed by concentrating the solution to 0·5 ml (N_2-jet). (3) 5-hydroxytryptamine-3-[^{14}C] creatinine sulphate (Code CFA 170, The Radiochemical Centre, Amersham), 39·6 mCi/m-mole obtained as the solid, was dissolved in 0·15 M NaCl to give a 1 mM solution.

The isotopes were stored in the concentrations given at −60° in small portions and diluted to desired concentrations immediately before use.

Adenosine diphosphate (*ADP*) was obtained and stored as described elsewhere.[4]

Adrenaline (Norges Apotekerforening, Oslo), was a solution under N_2 in sealed ampoullas containing per 1000 ml: 1·82 g adrenaline bitartrate, 0·5 g sodium pyrosulphite, 0·1 g disodium EDTA, 1·5 g methyl-*p*-oxybenzoate and 8·5 g NaCl in sterile, distilled water. The ampoullas were stored at 6° and diluted 150-fold with 0·15 NaCl to 36·4 μM adrenaline immediately before use.

Collagen (from tendons, Sigma Chemical Co., St. Louis) was homogenized (1 g) in a MSE Atomix with 100 ml of 83·5 mM acetic acid for 30 sec at approx. 20,000 rev/min with ice cooling. One-hundred ml of H_2O was added, the mixture homogenized for another 30 sec and 800 ml of 16·7 mM acetic acid was finally added. The soluble collagen obtained was stored in 5 ml portions at −60°. No demonstrable change in platelet aggregating or releasing activity has been demonstrated during storage for the last 5 years. Immediately before use, one portion was thawed, lightly homogenized with a teflon pestle-glass homogenizer and used non-diluted.

Thrombin. Bovine thrombin (Hoffmann La Roche, Basle) and human thrombin were obtained and stored as described elsewhere,[19] and diluted with 0·15 M NaCl to desired concentrations immediately before use.

Working solutions of ADP, adrenaline, collagen and thrombin were kept in ice during an experiment and discarded afterwards.

Serotonin 5-hydroxytryptamine creatinine sulphate (Sigma Chemical Co., St. Louis) was stored as dry powder or 1 mM solution in 0·15 M NaCl at −60° and thawed immediately before use as standards in the 5-HT assays.

Triton X-100 (Liquid Scintillation Grade, Beckman Instruments, Calif.) was used for 5-HT solubilisation from platelets.

Triton X-100 (British Drug House Chemicals Ltd., Poole) was used for liquid scintillation counting. All other reagents were analytical grade.

METHODS

Release experiments with PRP. Two 50 ml portions of PRP were incubated at 37° for 15 min with 2·5 ml of 20 μM radioactive 5-HT (5-HT-PRP) and 0·15 M NaCl (NaCl-PRP), respectively. Portions (5 ml) of the PRP mixtures were then successively stirred at 37° with 1·25 ml of aggregating (releasing) agents and their solvents in an EEL titrator—aggregometer[4] using flat-bottomed glass cuvettes of 7 ml capacity and a Beckman 10 in. linear/log recorder. After a 4 min stirring period the contents of the cuvettes were poured into centrifuge tubes immersed in ice. They were centrifuged at $G_{max} = 25{,}000\,g$ for 10 min at 4° and the supernatants kept at −20° until determination of amounts and radioactivity of 5-HT. Since the releasing effect of ADP and adrenaline often disappears with time after preparation of PRP, these release inducers were always processed first. One sample of 5-HT-PRP was always followed by a sample of NaCl-PRP treated with the same release inducer. Two such sets of NaCl- and 5-HT-PRP (controls) were treated with 0·15 M NaCl and 16·7 M acetic acid, respectively. Samples of each PRP mixture were also stirred with 0·15 M NaCl and frozen *without* centrifugation. These were prepared for estimation of amounts and radioactivity of whole PRP. Clotting was performed by incubating 10·0 ml each of NaCl- and 5-HT-PRP with 2·5 ml of 25 mM $CaCl_2$ at 37°. The clot was detached, allowed to retract 80 per cent or more, and the serum decanted and kept at −20°.

Thrombin-induced release with washed platelets. To 9 ml suspension of washed platelets (1–4 × 10^6 cells/μl), prewarmed at 37°, was added 10 μl of 1 mM [^{14}C] 5-HT, and the mixture immediately distributed in eight 1-ml portions. At recorded times of incubation at 37° after 5-HT addition, 0·1 ml Tris–NaCl buffer[15] was added to one portion (control) and 0·1 ml of 50 NIH units/ml of human thrombin to another (test). Such pairs were incubated for another 40 sec, immersed in methanol–ice for 1 min and placed in ice. After all samples were in ice, they were centrifuged at $G_{max} = 17{,}500\,g$ for 10 min at 6°. The supernatants and cells (after resuspension in 1·1 ml Tris–NaCl buffer[14] containing 5 mM EDTA, pH 7·4) were kept at −20°.

Determination of amounts and radioactivity of 5-HT. (1) For release experiments with PRP the method of Udenfriend *et al.*[19] was used as described by Aledort *et al.*,[17] except that 5 ml sample (or standard) was used as starting material and 3·2 ml of 3·0 N HCl was added to the final 0·8 ml acid, aqueous phase. The fluorescence at 550 nm was measured using 4 ml cuvettes in a Farrand Spectrofluorometer with excitation at 295 nm.

The radioactivity of 5-HT was determined by counting 50 μl of the cuvette content in 10 ml of toluene/Triton X-100 (4:1 by volume) containing 2 g/l. of 2·5-diphenyloxazole in a Beckman LS 200B scintillation counter.

(2) For packed cells and release experiments with washed platelets 5-HT was determined as total 5-hydroxyindole.[20,21] Twenty-five μl of $ZnSO_4$–NaOH extracts was applied on small bits of Whatman filter paper No. 1 and counted as above, using 2 g diphenyloxazole per litre toluene as scintillation fluid. Platelets were counted in a 101 Celloscope.[22]

RESULTS

Comparison of methods for 5-HT estimation—effect of Triton X-100. In both methods used, 5-HT is set free from platelets as proteins are denaturated by $ZnSO_4$–NaOH. Determining 5-HT as total 5-hydroxyindole,[20] the sums of extra- and intracellular

5-HT in thrombin-treated platelet suspensions were always higher than corresponding values from control platelets (Table 1). The amounts released by thrombin alone were often higher than the content in control platelets. Seemingly, the release reaction induced by thrombin can solubilize more platelet-bound 5-HT than the $ZnSO_4$–NaOH extraction method.

TABLE 1. EFFECT OF THROMBIN ON 5-HT CONCENTRATION IN PLATELET SUSPENSION

Exp. no.	Pl. count ($\times 10^{-5}/\mu l$)		Total 5-hydroxyindole (μM) Buffer	Ca	Thrombin	Thrombin +Ca
		Cells	2·37	2·67	0·63	0·46
1	13·8	Medium	0·41	0·30	2·80	3·19
		Total	2·78	2·97	3·43	3·65
		Cells	3·55	2·94	1·50	1·31
2	11·8	Medium	0·46	0·65	3·42	3·97
		Total	4·01	3·59	4·92	5·82
		Cells	3·61	3·22	1·22	1·22
3	10·5	Medium	0·26	0·81	3·17	3·39
		Total	3·87	4·03	4·39	4·61

Four portions of platelet suspension were incubated for 5 min at 37° with, respectively: buffer, 3 mM $CaCl_2$, 5 units/ml of thrombin and 5 units/ml of thrombin +3 mM $CaCl_2$. The samples were centrifuged and total 5-hydroxyindole determined in supernatant and cells (after resuspension to original volume before centrifugation). Bovine thrombin was used. Further details are given elsewhere.[22]

The presence of 0·1% Triton X-100 in the medium used for suspending packed platelets isolated from PRP[23] markedly increased the fluorescence of total 5-hydroxyindole in acidified $ZnSO_4$–NaOH extracts (Table 2). The detergent did not influence* the fluorescence of 5-HT standards, or of supernatants from thrombin-treated platelet suspensions, and most likely increased the amounts of 5-HT extracted from the platelets. Use of Triton X-100 to improve 5-HT extraction in release experiments with washed platelets and thrombin, diminished, but did not eliminate the difference between released + retained 5-HT and 5-HT bound in control platelets (see Table 3). Hence, the requirement[1] that the sum of extra- and intracellular 5-HT in a thrombin-treated sample must equal that of the control sample was not entirely fulfilled by use of Triton X-100.

The two methods for 5-HT determination, of total 5-hydroxyindoles[20] and 5-hydroxyindole amines[19] (5-HT is the only amine in platelets[24]), differ in that the butanol/hexane extraction steps are omitted in the former procedure (Fig. 1). Presence of Triton X-100 in the latter method, however, caused most of the *n*-butanol layer to form wax-like structure from which too little liquid phase could be obtained (Fig. 1). Use of deoxycholate as detergent gave the same result. The "wax formation" could

* This was only true when Triton X-100 was specifically prepared for use in scintillation liquids (*scintanalyzed*). Triton X-100 obtained from Sigma Chemical Co. (St. Louis) contained large amounts of fluorescent material and could not be used in 5-HT determinations.

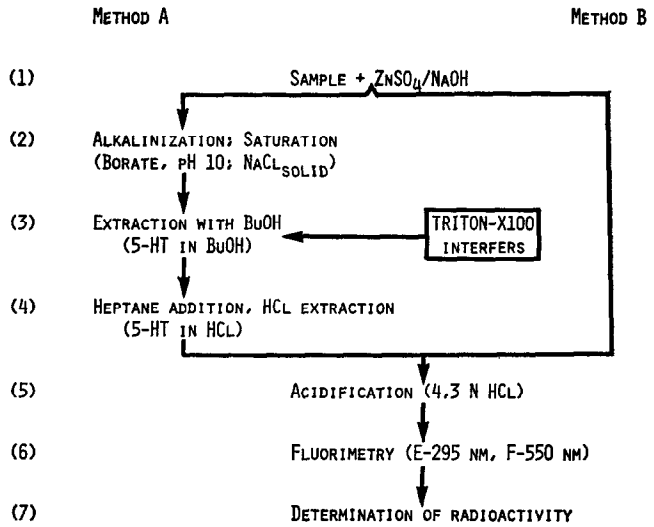

FIG. 1. Schematic presentation of the two methods used for determination of 5-HT. Method A = the 5-hydroxyindole base method of Udenfriend *et al.*[19] Method B = the total 5-hydroxyindole method of Crosti and Lucchelli.[20]

be prevented by use of saturating concentrations of $(NH_4)_2SO_4$ instead of NaCl. Unfortunately $(NH_4)_2SO_4$ reduced the recovery of 5-HT through the procedure from 95–100 per cent (with NaCl) to 15–30 per cent, which was also variable.

Using the specific amine method[19] unmodified (i.e. with NaCl) for PRP, serum or platelet suspension, the fluorescence spectrum (excitation at 295 nm) and excitation spectrum (fluorescence at 550 nm) of the substance(s) carried through the procedure were identical to that of standard 5-HT likewise treated. Extracts from plasma of control PRP gave no fluorescence (350–600 nm), showing that no substances from

TABLE 2. EFFECT OF TRITON X-100 (0·1 per cent) ON THE FLUORESCENCE FROM TOTAL PLATELET 5-HYDROXYINDOLE

	Fluorescence*	
Pair no.	−Triton X-100	+Triton X-100
1	150	230
2	150	220
3	140	240

Platelets from pairs of 2 ml portions of citrated PRP were isolated by centrifugation, the buttons freeze-thawed and resuspended in 1·0 ml of 0·15 M NaCl ± 0·1% Triton X-100 before extraction with $ZnSO_4$–NaOH and measurement of fluorescence.[25]

* Arbitrary units, corrected for blank readings which were: −Triton X-100 = 45; +Triton X-100 = 60.

plasma with the same fluorimetric characteristics as 5-HT were carried through the procedures. In contrast, when the extraction with butanol/hexane was omitted and fluorescence measured directly in (acidified) $ZnSO_4$–NaOH extracts of PRP, serum or plasma as in the total 5-hydroxyindole method (Fig. 1), great fluorescence was present in the 350–600 nm region, which at 550 nm (maximal fluorescence for 5-HT in acid medium) corresponded to 10–25 μM 5-HT in (5-HT-free) plasma. Since the range of 5-HT in PRP and hence, maximally released to plasma, is 0–1·5 μM, the total 5-hydroxyindole method was unsuitable for a plasma system. In a plasma-free system, however, such as washed platelets, this method gave fluorescence spectra ($\pm$ Triton X-100) which were identical to standard 5-HT. 5-HT added to plasma was quantitatively recovered by $ZnSO_4$–NaOH extraction (specific amine method).

TABLE 3. SPECIFIC RADIOACTIVITY OF 5-HT RELEASED AND RETAINED DURING THE INTERACTION BETWEEN THROMBIN AND WASHED PLATELETS

Time after 5-HT addition (min)		Amounts (μmole/10^{11} cells)			Specific radioactivity (counts/min/nmole $\times$ 10^{-6})	
		Cells	Medium	Sum	Cells	Released*
10	Control	0·63	0·05	0·68	3·19	—
20	Control	0·60	0·06	0·68	3·49	—
30	Control	0·52	0·09	0·61	3·23	—
10	Test	0·20	0·60	0·80	3·25	3·24
20	Test	0·23	0·55	0·78	3·42	3·15
30	Test	0·25	0·47	0·72	3·13	3·24

Washed platelets suspended in Tris–NaCl buffer containing 3 mM EDTA, ph 7·4 ($2{\cdot}65 \times 10^6$ cell/μl) were incubated with 1 μM[^{14}C]-5-HT at 37°. At the times indicated samples were treated with thrombin (test) (4·5 NIH units/ml) or Tris–NaCl buffer (control) for 40 sec at 37°, then cooled, and amounts (total 5-hydroxyindole) and radioactivity of 5-HT determined in cells and medium after their separation by centrifugation. Triton X-100 (0·1 per cent) was used in the $ZnSO_4$–NaOH extraction step. For further details, see methods.

* The specific radioactivity of released 5-HT is calculated as $R_{snR} - R_{snC}/A_{snR} - A_{snC}$ where R is radioactivity (counts/min/10^{11} platelets), A = amounts (μmole/10^{11} platelets) and the subscript snR and snC indicate supernatants from sample treated with release inducer and its solvent control, respectively.

Specific radioactivity of 5-HT released by thrombin from washed platelets. After incubation of suspensions of washed platelets with [^{14}C]-5-HT for 10 min at 37°, no change in specific radioactivity (s.r.a.) occurred in control platelets (Table 3), indicating that an equilibrium between exogenous and endogenous 5-HT had been reached. Addition of thrombin at this stage gave release of 5-HT with the same s.r.a. as 5-HT both in control platelets and remaining in the thrombin treated platelets (Table 3). The endogenous/absorbed 5-HT ratio was 10–20 (17 in Table 3) in these experiments.

Specific radioactivity of 5-HT released by different release inducers in PRP. Figure 2 shows typical aggregometer tracings obtained when samples of 5-HT-PRP or NaCl-PRP were stirred for 4 min with adrenaline, ADP, collagen and thrombin, respectively. Table 4 shows the corresponding amounts and radioactivities of 5-HT in extracellular

TABLE 4. AMOUNTS AND SPECIFIC RADIOACTIVITY OF 5-HT RELEASED BY VARIOUS STIMULI AND PRESENT IN CONTROL PLATELETS

	Control cells	Treatment				
		ADP	Adren.	Collag.	Thromb.	Clotting
				Release (%)		
Without [^{3}H]-5-HT (Amounts*)	0·270	38·3	67·8	47·2	76·8	67·9
With [^{3}H]-5-HT (Amounts*)	0·446	34·4	39·1	43·7	54·5	78·1
With [^{3}H]-5-HT (Radioactive 5-HT†)	19,340	42·1	42·3	56·0	68·9	88·9
		Specific radioactivity (counts/min/nmole $\times 10^{-3}$)				
	Control cells	Released				
With [^{3}H]-5-HT	44·3	55·0	48·4	57·4	56·4	51·3

Two portions of PRP ($3{\cdot}0 \times 10^5$ cells/μl) were incubated for 30 min at 37° with and without 1 μM [^{3}H]-5-HT, respectively. Aliquots of each portion were stirred with 10 μM adrenaline, 10 μM ADP, collagen and 5 units/ml of thrombin for 4 min in the aggregometer (tracings, see Fig. 2). Some aliquots were stirred with solvents of the aggregation inducers and others were clotted by recalcification. Released and platelet-bound amounts and radioactivity of 5-HT was determined as described in Methods. Amounts (a) released is calculated $A_{snR} - A_{snC}$, radioactivity (r) released as $R_{snR} - R_{snC}$ and specific radioactivity as r/a, where A, R, snR and snC are defined in Table 3. Platelet bound amounts (ba) and radioactivity (br) is calculated as $A_{PRP} - A_{snC}$ and $R_{PRP} - R_{snC}$, respectively, and specific radioactivity = br/ba, where PRP indicates extracts of non-centrifuged PRP. Per cent release is related to platelet bound amounts or radioactivity.

* μmoles/10^{11} platelets.

† Counts/min/10^8 platelets.

phases, serum and non-centrifuged PRP. After incubation with [^{3}H]-5-HT the amount of platelet 5-HT is increased by 63 per cent, giving an endogenous/absorbed 5-HT ratio of 1·8. The amounts of 5-HT released from platelets into plasma (Table 4) are directly comparable, as they represent direct measurements in plasma or serum

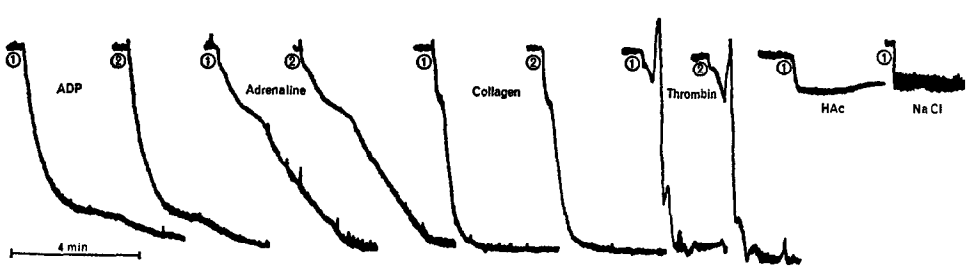

FIG. 2. Aggregometer tracings from a release experiment with PRP. The curves marked (1) and (2) represent NaCl-PRP and 5-HT-PRP, respectively. Controls with HAc (acetic acid) and NaCl were also performed with 5-HT-PRP. The concentrations of release inducers and results of 5-HT determinations from this experiment are given in Table 4. For experimental details, see Methods.

after release. Because 5-HT is not sufficiently extracted from cells in PRP, the per cent release values are somewhat overestimated. Assuming an equal degree of 5-HT extraction in the two PRP incubation mixtures, the cells in 5-HT-PRP release generally smaller amounts (per cent-wise) of 5-HT than cells in NaCl-PRP (Table 4). The release of absorbed [^{3}H]-5-HT was generally (per cent wise) greater than that of endogenous 5-HT in corresponding NaCl-PRP (Table 5, with the exception of adrenaline). These differences varied from experiment to experiment (eight experiments were performed), but there was always the same trend: 5-HT-PRP had equal or lower per cent-wise release capacity of 5-HT amounts than NaCl-PRP, and the per cent release of radioactive 5-HT was equal or higher than release of amounts in both 5-HT-PRP and NaCl-PRP. Consequently, the specific radioactivity of released 5-HT was either equal or higher (< 25 per cent), but never lower, than that of 5-HT in the control platelets (Table 4).

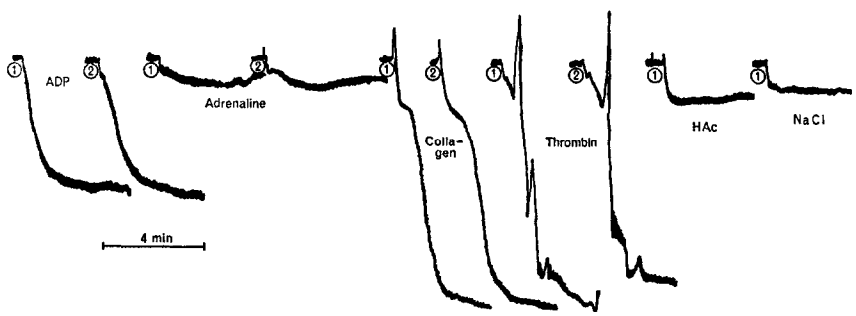

FIG. 3. Aggregometer tracings from a release experiment with PRP in which ADP and adrenaline fail to give secondary aggregation. Controls with HAc and NaCl were also performed with 5-HT-PRP. The concentrations of release inducers are given in Table 4, and the results of 5-HT determinations in this experiment are given in Table 5.

TABLE 5. FAILURE OF ADP AND ADRENALINE TO PRODUCE SECONDARY AGGREGATION AND RELEASE

	Control cells	Treatment				
		ADP	Adren.	Collag.	Thromb.	Clotting
				Release (%)		
Amounts*	0·411	0·1	0·7	25·7	65·0	88
Radioactivity†	10400	0	0·3	26·0	66·7	86
		Specific radioactivity (counts/min per nmole $\times 10^{-3}$)				
	Control cells			Released		
	24·1	—	—	24·9	25·7	23·9

The experiment was performed exactly as described in Table 4, but adrenaline and ADP did not give secondary aggregation, as shown in Fig. 3. The values represent PRP ($2{\cdot}55 \times 10^5$ cells/μl) incubated with [^{3}H]-5-HT.

* μmoles/10^{11} platelets.

† Counts/min/10^8 platelets.

In some PRP, ADP and adrenaline failed to give biphasic aggregation, although collagen and thrombin gave maximal aggregation. Aggregometer tracings from such an experiment are shown in Fig. 3, and the corresponding analytical results given in Table 5. Clearly, 5-HT was not released by ADP or adrenaline under these circumstances. Table 5 exemplifies a situation with equal specific radioactivity of released and platelet-bound 5-HT.

DISCUSSION

5-HT released from platelets in PRP had equal or slightly higher specific radioactivity than 5-HT in corresponding platelets which had not undergone release. Thus, for the inducers studied, ADP, adrenaline, collagen, thrombin and clotting of PRP, newly absorbed 5-HT behaves usually as endogenous 5-HT, although with a tendency to be somewhat preferentially released. It is uncertain whether this is due to (1) preferential binding of absorbed 5-HT to sites within one platelet from which 5-HT is more easily released than (endogenous) 5-HT bound to other sites, or (2) that some platelets take up and release 5-HT more easily than other platelets. Thrombin always released 5-HT from washed platelets with the same specific radioactivity as 5-HT in control platelets and retained by the thrombin-treated cells. The method used for isolating washed platelets tends to isolate large, heavy platelets (unpublished results) that have been shown to be metabolically different from smaller, lighter platelets.[25-29] Our results conform with washed platelets as opposed to results using PRP, which favours view (2) above.

The term "newly absorbed" 5-HT refers to washed platelets and platelets in PRP after 10 and 15 min, respectively, of incubation with radioactive 5-HT at 37°. Since 5-HT uptake is almost totally inhibited when release is initiated,[30] shorter incubation times might be sufficient (i.e. absorbed and endogenous 5-HT might even behave equally during 5-HT uptake). The conditions used here for PRP are similar to those used in laboratories measuring 5-HT release as release of newly absorbed 5-HT.[7-13] The conditions used for interaction between thrombin and washed platelets are those used in our own previous experiments.[15,31,32]

Aledort *et al.*[17] failed to demonstrate release of endogenous 5-HT in PRP with ADP and thrombin, and obtained less release with collagen than found by others[7-9] measuring release with absorbed radioactive 5-HT. In their study Aledort *et al.*[17] recorded aggregation by visual examination of a PRP in tube, "using gentle tipping as means of producing aggregation". In our study, aggregation was followed in the aggregometer, and for ADP and adrenaline it was clear that aggregation could occur without release. In such cases release of *both* endogenous and absorbed 5-HT was absent. It is thus possible that the rather weak release/aggregation stimuli used by Aledort *et al.*[17] just brought about aggregation, but not release. Moreover, since the behaviour of absorbed, (radioactive) and endogenous 5-HT was not studied together, the failure of release of endogenous 5-HT alone is not a valid argument for criticizing the use of absorbed 5-HT in release studies.

In his study, Stacey[16] failed to demonstrate loss of 5-HT in "unloaded" platelets stirred with ADP, although ATP disappeared in the cells. This ATP disappearance might have been due to direct release of nonmetabolic ATP to the extracellular space and/or intracellular ATP-hypoxanthine conversion,[4] both indicating that release had occurred. Stacey[16] explains this by suggesting that ATP platelets normally

have more ATP than necessary for 5-HT binding, and a considerable amount of ATP can be lost from the platelet before the storage mechanism for 5-HT is suffering. Since we no not know what the intracellular ATP-loss actually means, it is hard to reconcile this view with ours[1]: the release of a "package" of adenine nucleotides, 5-HT and metals directly to the surroundings. Under our conditions the releasable substances are always released *together*; we never saw *one* component being released alone.

Incubation of platelets with radioactive 5-HT usually decreased the platelets ability to release (both endogenous and absorbed) 5-HT. We have earlier noted[1] that after uptake of nonradioactive 5-HT, the ability to release ATP + ADP with collagen is reduced, and Baumgartner and Born[33] have shown that aggregation is impaired by allowing platelets to absorb 5-HT. The present work shows that although the release capacity is slightly decreased after absorption of 5-HT, the absorbed and endogenous amine behave almost identically during release under the conditions used.

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REFERENCES

1. H. HOLMSEN, H. J. DAY and H. STORMORKEN, *Scand. J. Haemat.* Suppl. 8 (1969).
2. J. F. MUSTARD and M. A. PACKHAM, *Pharmac. Rev.* **22,** 97 (1970).
3. H. J. DAY and H. HOLMSEN, *Ser. Haemat.* **4,** 3 (1971).
4. H. HOLMSEN, H. J. DAY and C. A. SETKOWSKY, *Biochem. J.* **129,** 68 (1972).
5. E. H. MÜRER and R. HOLME, *Biochim. biophys. Acta* **222,** 197 (1970).
6. P. D. ZIEVE, J. L. GAMBLE and D. P. JACKSON, *J. clin. Invest.* **43,** 2063 (1964).
7. T. H. SPAET and M. B. ZUCKER, *Am. J. Physiol.* **206,** 1267 (1964).
8. J. F. VALDORF-HANSEN and M. B. ZUCKER, *Am. J. Physiol.* **220,** 105 (1971).
9. D. C. B. MILLS, I. A. ROBB and G. C. K. ROBERTS, *J. Physiol.* **185,** 715 (1968).
10. E. A. CASPARY and E. J. FIELD, *Deutsche Z. Nervenheilkunde* **190,** 267 (1967).
11. J. J. SIXMA and J. G. NIJESSEN, *Thromb. Diath. haem.* **24,** 206 (1970).
12. P. MASSINI and E. F. LÜSHER, *Thromb. Diath. haem.* **25,** 13 (1971).
13. H. E. KATTLOVE and T. H. SPAET, *Blood* **35,** 659 (1970).
14. J. P. KEENAN and N. O. SOLUM, *Br. J. Haematol.* **23,** 461 (1972).
15. H. HOLMSEN and H. J. DAY, *J. Lab. clin. Med.* **75,** 840 (1970).
16. R. S. STACEY, *Experientia* **24,** 908 (1968).
17. L. M. ALEDORT, H. S. GILBERT and E. PUSZKIN, *Transfusion* **11,** 266 (1971).
18. P. MASSINI, *Experientia* **26,** 87 (1970).
19. S. UDENFRIEND, H. WEISSBACH and B. B. BRODIE, *Meth. Biochem. Anal.* **6,** 95 (1958).
20. P. F. CROSTI and P. E. LUCCHELLI, *J. clin. Path.* **15,** 191 (1962).
21. H. J. DAY, H. HOLMSEN and T. HOVIG, *Scand. J. Haematol.* Suppl. 7 (1969).
22. H. STORMORKEN, Å. LUND-RIISE and T. O. RØRVIK, *Scand. J. clin. Lab. Invest.* **17,** Suppl. **84,** 183 (1965).
23. H. HOLMSEN and H. J. WEISS, *Blood* **39,** 197 (1972).
24. C. BARTHOLINI and A. PLETSCHER, *Experientia* **20,** 376 (1964).
25. T. P. MCDONALD, T. T. ODELL and D. G. GOSSLEE, *Proc. Soc. exp. Biol. Med.* **115,** 684 (1964).
26. S. KARPATKIN, *J. clin. Invest.* **48,** 1073 (1969).
27. S. KARPATKIN, *J. clin. Invest.* **48,** 1083 (1969).
28. F. U. KNUDSEN, *Scand. J. Haematol.* **8,** 189 (1971).
29. E. AMOROSI, S. K. GARG and S. KARPATKIN, *Br. J. Haematol.* **21,** 227 (1971).
30. H. HOLMSEN, H. J. DAY, C. A. SETKOWSKY, M. C. BIBRO and L. ROBKIN, to be published.
31. H. HOLMSEN and H. J. DAY, *Nature, Lond.* **219,** 760 (1968).
32. H. HOLMSEN, H. J. DAY and E. STORM, *Biochim. biophys. Acta* **186,** 254 (1969).
33. H. R. BAUMGARTNER and G. V. R. BORN, *Nature, Lond.* **218,** 137 (1968).