Intraplatelet Serotonin and Plasma 5-Hydroxyindoles in Health and Disease

By Robert D. Shuttleworth and John R. O'Brien

Using a fluorometric test sensitive to serotonin (5-HT) and to other 5-hydroxyindoles (5-HIs) it was shown that platelets take up 5-HT and that the added 5-HT and the inherent test-positive material in platelet lysate can be directly measured. However, platelets do not take up 5-hydroxyindole acetic acid or the 5-HIs from the plasma. Thus, 5-HT and the other 5-HIs can be distinguished. Various methods of liberating intraplatelet 5-HT were investigated. Several anticoagulants, temperatures, and speeds of centrifugation were employed, with no effect on the results. It was found that in healthy donors, with increasing age, there was a decrease in intraplatelet 5-HT and an increase in plasma 5-HIs. The quantities of these substances were inversely related. In acute myocardial infarction, the 5-HT was normal taking age into account, but the plasma 5-HIs were increased. In postoperative patients, the platelet 5-HT was low and the plasma 5-HIs were normal. In patients with chest pain but no myocardial infarction, both platelet 5-HT and plasma 5-HIs were normal. The relevance of these findings to 5-HT metabolism and the evidence for in vivo activation of platelets is discussed.

Choice of Subjects

Forty apparently healthy men and women aged 18-63 yr were studied as controls. Thirty-seven patients with acute myocardial infarction (MI) aged 45-74 yr volunteered and were studied either once or several times between the 3rd and 24th day after the event; in all, 66 MI observations were made. Nineteen postoperative patients (post-op) aged 23-77 yr were studied once between the 3rd and 10th day after major surgery. Single studies were carried out on 12 patients aged 40-65 yr who were admitted to hospital with severe chest pain but no MI—noninfarct chest pain (NICP). Many patients received subcutaneous heparin (5000 U/every 12 hr). This was noted, and blood was collected immediately before the next heparin administration. Any volunteers who had taken aspirin-like drugs other than paracetamol within 14 days of blood collection were excluded.

Blood Collection and Processing

Ten milliliters of venous blood were collected into chilled siliconized glass tubes containing 0.1 ml EDTA (15% w/v). The samples were mixed and placed in an ice-water mixture. Within 1 hr of blood collection the samples were centrifuged at 136 g for 10 min at 4°C to obtain platelet-rich plasma (PRP), which was separated and kept at 4°C. A platelet count on PRP was obtained using a Coulter Counter model FN. The infranatant blood was centrifuged further at 2500 g for 20 min at 4°C to produce platelet-poor plasma (PPP). To facilitate subsequent platelet resuspension, an equal volume of EDTA (4% w/v) was added to each PRP sample. These diluted PRP samples were then centrifuged at 1800 g for 20 min at 4°C, and the supernatant platelet-poor plasma (PPP) carefully decanted and discarded. The platelets were then lysed by adding distilled water to the platelet “pellet,” which was resuspended. To ensure lysis, the platelet resuspension was then left on the bench for 3 hr. Duplicate samples of lysate and PPP were stored in liquid nitrogen for up to 1 wk before assay.

Assay

The serotonin assay was that of Drummond and Gordon, somewhat modified. Essentially, the proteins were precipitated by strong trichloroacetic acid. O’thalaldehyde solution prepared by dissolving purified yellow/green crystals (Sigma Chemicals, Ltd.) in ethanol, and 8N HCl was added to supernatant samples, blanks, and 5-HT standards. The samples were then heated to 100°C to develop
the fluorophore. Excess trichloroacetic acid was extracted with chloroform and the fluorophore measured at predetermined wavelengths. After allowing for dilution, the results were read off the standard line and expressed in ng 5-HT/10^6 platelets and ng 5-HIS/ml plasma.

Statistical Analysis

Age regression analysis and partial correlation coefficients were calculated as indicated in the text. From evidence presented below, test-positive material in platelet lysate will be called 5-HT and that in plasma, 5-hydroxyindoles (5-HIS).

Before the technique described above was accepted for study of patients, 6 methodological problems were investigated. These concerned the influence of differences in technique and the release and uptake by intact platelets of pure 5-HT and platelet material (5-HT), of plasma constituents (5-HIS), and 5-hydroxyindole acetic acid (5-HIAA).

1) Effect of anticoagulant. Blood was collected from 16 controls, 7 MIs, and 4 post-op patients and put into both EDTA (final concentration 0.15% w/v) and citrate (final concentration 0.38% w/v) and processed at 4°C.

2) Effect of temperature and method of collection. Blood was collected from MIs patients into two vacutainers containing 1 EDTA and into a syringe and then transferred into 2 tubes containing 15% w/v EDTA. One tube from each collection method was plunged into ice-water and processed throughout at 4°C (a technique that even prevents the release of β-thromboglobulin), and the other tubes were processed at room temperature.

3) Effect of centrifugation. One-milliliter aliquots of EDTA PRP prepared from 2 controls and 2 MI patients were centrifuged at 185, 739, 1663, or 2957 g for 20 min at 4°C to produce a platelet “pellet” and PPP. These samples were then processed and stored as outlined in Blood Collection and Processing.

4) Effect of different methods of lysing platelets. The 5-HT content of platelets in PRP prepared from 5 controls and 4 arbitrarily chosen patients with vascular disease (arteriopathtas) was liberated by the following techniques: (A) spinning the platelets to a “pellet” and removing the plasma and adding back an equal volume of distilled water, (B) by freezing and thawing twice, and (C) by treating the PRP with Triton X20. In each case, the proteins were removed by precipitation with trichloroacetic acid and centrifuging. The 5-HT in the supernatant and 5-HIS in the decanted PPP was determined after storage in liquid nitrogen at −70°C.

5) Leakage of 5-HT/5-HIS from platelets at 37°C and 5-HT uptake. To aliquots of PRP from each of 5 controls and 4 MIs patients was added either saline or 100 ng pure 5-HT or 1000 ng 5-HT. These were incubated at 37°C. The reaction was stopped after a few seconds (zero time) or after 15 min or 4 hr by plunging the tubes into an ice-water mixture at 4°C and adding an equal volume of 0.4% EDTA. All these samples were then processed and stored as outlined above.

6) Platelet uptake of platelet lysate material. 5-HT, 5-HIAA, and plasma 5-HIS. Aliquots of substrate-citrated PRP prepared from 3 controls were incubated at 37°C with additions of either saline or pure 5-HT, or 5-HIAA or lysate prepared from normal platelets. The final concentration of the added 5-HT and 5-HIAA in the PRP was 1000 ng/ml and of the added platelet 5-HIS lysate 150 ng/ml. The reaction in each aliquot was stopped after a few seconds (zero time) or after 15 min or 90 min, and thereafter they were treated as in method 5 above.

RESULTS

Reproducibility

The mean coefficient of variation for intraplatelet 5-HT of 10 controls studied up to 5 times within a period of 8 wk was 14%, and for the plasma 5-HIS it was 10%. Seven MI patients studied 4 times between day 1 and day 14 after the infarct showed more variation, the mean coefficient of variation for the intraplatelet 5-HT and plasma 5-HIS was 26% and 18%, respectively. No sex difference was found in any of the measurements. Duplicate samples for plasma 5-HIS showed a coefficient of variation of 15%, where n = 22 controls.

1) Effect of anticoagulant. Although there were significantly different values between the clinical groups, there was no significant difference in the results when the anticoagulants were compared.

2) Effect of temperature and method of collection. Table 1 shows that the platelet 5-HT and plasma 5-HIS were not significantly affected by these four preparative methods.

3) Effect of centrifugation. Each centrifugation

| Table 1. Effect of Different Methods of Blood Collection on Platelet 5-HT Level and Plasma 5-HIS |
|---------------------------------|------------------------|------------------------|
| Temperature | Method | Platelet 5-HT (ng/10^6 platelets) | Plasma 5-HIS (ng/ml^-1) |
| 22°C | Vacutainer | 716 | 190 |
| 22°C | Syringe | 735 | 188 |
| 4°C | Vacutainer | 713 | 170 |
| 4°C | Syringe | 755 | 169 |

(6) Platelet uptake of platelet lysate material, of 5-HT, 5-HIAA, and plasma 5-HIS. Aliquots of substrate-citrated PRP prepared from 3 controls were incubated at 37°C with additions of either saline or pure 5-HT, or 5-HIAA or lysate prepared from normal platelets. The final concentration of the added 5-HT and 5-HIAA in the PRP was 1000 ng/ml and of the added platelet 5-HIS lysate 150 ng/ml. The reaction in each aliquot was stopped after a few seconds (zero time) or after 15 min or 90 min, and thereafter they were treated as in method 5 above.

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| Table 2. Distribution of Added 5-HT Between Platelet Lysate and Plasma on Incubation at 37°C of 1 ml PRP (2.5 × 10^9 Platelets) |
|---------------------------------|------------------------|------------------------|
| Volunteers | Incubation Time | + Saline | + 100 ng 5-HT | + 1000 ng 5-HT |
| 0 Time | 4 hr | 0 Time | 15 min | 4 hr | 0 Time | 15 min | 4 hr |

Each figure is the mean for the group ± 1 SD. “Total” is the sum of the 5-HIS in the two compartments.
speed gave similar results for the plasma 5-HIs and there was no decrease in the intraplatelet 5-HT, so evidently no release occurred.

(4) Effect of different methods of lysing platelets. There was no significant difference in the level of 5-HT liberated by the three methods studied.

(5) Leakage of 5-HT/5-Hls from platelets at 37°C and 5-HT uptake. On incubation with saline for 4 hr there was no transfer of 5-Hls into the platelets from the plasma and no loss of intraplatelet 5-HT. (see Table 2). After the addition of 100 ng of 5-HT, even at “zero” time, the mean platelet 5-HT level was increased relative to the function, zero time + saline; thus, uptake was very rapid, and uptake was complete after 15-min incubation. The total uptake of 1000 ng 5-HT took longer, but in other experiments it was shown to be complete at 1.5 hr. The total of all 5-Hls in the two compartments, i.e., platelet + PPP added together, remains almost constant in every case, thus validating the method and showing that the 5-HT taken up by the platelets is recovered and assayable after platelet lysis with water. Evidently, little or no enzymatic degradation occurs to render the product insensitive to the assay.

(6) Platelet uptake of platelet lysate material, of 5-HT, 5-HIAA, and of plasma 5-Hls. No change in platelet 5-HT and plasma 5-Hl levels were observed in PRP samples incubated with saline for 90 min in agreement with study 5 above. Pure 5-HT and platelet lysate material were both readily taken up by platelets incubated at 37°C for 90 min. In contrast, 5-HIAA detectable by this assay procedure was not taken up by the platelets (the small change in 5-Hls after 15 min is unexplained), and the levels of 5-Hls in the plasma remained constant during the incubation period, as none was taken up into the substrate platelets (see Fig. 1).

Patients and Controls

Platelet Serotonin

From a study of 40 controls, the intraplatelet 5-HT was found to decrease significantly with increasing age; see Table 3 for age regression analysis and Table 4 for correlation coefficients. In the MI group, a similar relationship between intraplatelet 5-HT and age was observed; the level decreased significantly with increasing age and was not significantly different to age-matched controls. In the post-op group, no relationship between intraplatelet 5-HT and age was found, and the intraplatelet 5-HT level was significantly lower than in age-matched controls. In the noninfarct chest pain group (NICP), the intraplatelet 5-HT was not significantly different to age-matched controls.

Plasma 5-Hydroxyindoles

In the controls, the plasma 5-Hl levels increased significantly with age (see Table 5 for age regression analysis and Table 4 for correlation coefficients). The plasma 5-Hl levels in the three groups of patients studied in relation to age are also shown in these
tables. In the MI patients, the plasma 5-HI levels were significantly increased. In contrast in the post-op patients, the plasma 5-HI levels increased significantly with age and were not significantly different from the levels in age-matched controls. In the NICP group, taking age into account, the plasma 5-HI levels were similar to the controls.

**Relationship Between Platelet 5-HT and Plasma 5-HIs**

In the control volunteers, a significant negative correlation between plasma 5-HIs and the intraplatelet 5-HT was observed; the lower the platelet 5-HT, the higher the plasma 5-HIs (see Table 4 for correlation coefficients). In the post-op and MI groups, this relationship was broken. However, in MI patients studied sequentially there appeared to be a loose inverse relationship within each patient, which may differ between patients. In the noninfarct chest pain group, an inverse relationship between these two measurements was again demonstrated.

Partial correlation coefficients were calculated to remove mathematically the effect of age on the platelet 5-HT and the plasma 5-HIs relationship. In the controls, the partial correlation coefficient was -0.188, which was not significant.

**DISCUSSION**

The methodological studies presented show that ex vivo, the intraplatelet 5-HT is remarkably stable, and providing there is no aggregation or lysis, the 5-HT remains within the platelet; even incubation at 37°C for 1 hr does not cause leakage or release, and this is in emphatic contrast to the ease of escape of other platelet constituents such as β-thromboglobulin. The reproducibility and the almost full recovery of the 5-HT taken up ex vivo further validates the method. This method of estimating 5-HT does not itself distinguish between 5-HT and the other 5-HIs. However, added pure 5-HT and platelet lysate are avidly taken up by substrate platelets, but added 5-HIAA and the plasma 5-HIs are not taken up. This distinguishes between 5-HT and the other 5-HIs and is in line with the many reports that there is only 5-HT in the platelet and little or no 5-HT in the plasma.

In 1967 Crawford7 reported that “platelet 5-HT tends to decrease with increasing age.” We have now confirmed (n = 40, r = -0.711, p < 0.001) and extended this finding in health when drugs could not influence the results. Furthermore, plasma 5-HIs increase with age and these two measurements—intraplatelet 5-HT and plasma 5-HIs—are inversely correlated (r = -0.610). In renal failure, the serum creatinine is high and so the plasma 5-HIs, suggesting a parallel failure of renal excretion.5 However, in 20 controls aged 18–63 yr, it was shown that the plasma 5-HIs were not correlated to the creatinine level, so a failure of excretion of 5-HIs with increasing age is unlikely.

If the effect of age is removed mathematically, then the platelet 5-HT level is no longer related to the plasma 5-HIs. Thus, some age-dependent factor has a profound influence on one or both the platelet 5-HT and the plasma 5-HIs. It may be relevant that we have also found that the intraplatelet PF4 level also decreases with age,* which supports the concept that platelet “activation” and partial release1 occurs with increasing age. A possible alternative is a failure of platelet 5-HT uptake with age. A decrease of dietary

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**Table 4. Correlation Coefficients (r) Between Platelet 5-HT, Plasma 5-HIs, and Age**

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 40)</th>
<th>MIs (n = 66)</th>
<th>NICP (n = 12)</th>
<th>Post-Op (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelet 5-HT versus age</strong></td>
<td>-0.711 0.001</td>
<td>-0.308 0.02</td>
<td>-0.263 NS</td>
<td>0.016 NS</td>
</tr>
<tr>
<td><strong>Plasma 5-HIs versus age</strong></td>
<td>0.711 0.001</td>
<td>0.056 NS</td>
<td>0.059 NS</td>
<td>0.550 0.02</td>
</tr>
<tr>
<td><strong>Platelet 5-HT versus plasma 5-HIs</strong></td>
<td>-0.610 0.001</td>
<td>-0.154 NS</td>
<td>-0.273 NS</td>
<td>0.234 NS</td>
</tr>
</tbody>
</table>

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**Table 5. Mean Plasma 5-HI Values Calculated From Age Regression Analysis**

<table>
<thead>
<tr>
<th>Age (Decades)</th>
<th>Controls (n = 40)</th>
<th>MIs (n = 66)</th>
<th>NICP (n = 12)</th>
<th>Post-Op (n = 19)</th>
<th>Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>C vs. MIs</td>
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<td>C vs. NICP</td>
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<td></td>
<td></td>
<td></td>
<td>C vs. PO</td>
</tr>
<tr>
<td>20–29</td>
<td>73.0</td>
<td>—</td>
<td>—</td>
<td>81.0</td>
<td>—</td>
</tr>
<tr>
<td>30–39</td>
<td>86.3</td>
<td>—</td>
<td>—</td>
<td>93.9</td>
<td>—</td>
</tr>
<tr>
<td>40–49</td>
<td>99.6</td>
<td>233.7</td>
<td>116.5</td>
<td>106.7</td>
<td>0.01</td>
</tr>
<tr>
<td>50–59</td>
<td>112.9</td>
<td>241.0</td>
<td>119.7</td>
<td>119.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>60–69</td>
<td>126.3</td>
<td>248.3</td>
<td>123.0</td>
<td>132.5</td>
<td>*</td>
</tr>
</tbody>
</table>

*Control group too small for Student t test.

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*A recent study has shown a significant correlation between intraplatelet PF4 and age (r = 0.64; p < 0.001, n = 20).
tryptophane or its assimilation with increasing age seems unlikely. Thus, the effect of increasing age cannot be accounted for.

In acute MI, the inverse relation between intraplatelet 5-HT and plasma 5-HIs is broken. The platelet 5-HT is normal in spite of reports that other platelet function tests are abnormal. The high plasma 5-HIs with normal renal function are thus anomalous and unexplained.

In post-op patients studied between the 3rd and 10th day, the relation is again broken. These patients have low platelet 5-HT but normal plasma 5-HIs. The low 5-HT is compatible with the suggestion of Harbury and Schrier that platelets undergo a thrombin-induced release reaction during surgery. The studies of Reimers et al. may also be relevant to the persistance in the circulation of platelets relatively empty of 5-HT. They showed that thrombin-treated rabbit platelets were degranulated and did not take up 5-HT, but on reinfusion into the rabbit, they continued to circulate “empty of 5-HT for a normal life span.”

Our preliminary studies of post-op patients and controls agree with previous reports that human platelets with low 5-HT content do not readily take up 5-HT.

The level of plasma 5-HIs is presumably influenced by the rate of synthesis of 5-HT, the rate of 5-HT metabolism in the various pools, and its excretion as 5-HIs. The tight inverse relation in health of platelet 5-HT to plasma 5-HIs is therefore remarkable and particularly so since the changes in both with increasing age do not alter their apparent interdependence. Why this correlation breaks down in acute MI and post-op patients is not clear. The normality of the “non-MI chest pain” patients serves to emphasise that bed rest, food, and some drugs do not upset the level of platelet 5-HT and the plasma 5-HIs.

These studies record a number of interrelated observations that must be relevant to platelet 5-HT metabolism in vivo, but the processes involved are poorly understood. It is at least established that before platelet 5-HT levels can be evaluated, the patient’s age must be known. Further work should contribute to better understanding of platelet physiology in health and disease.

ACKNOWLEDGMENT

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REFERENCES

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