Platelet Antiheparin Activity: Storage Site and Release Mechanism

By Peter N. Walsh and Giovanna Gagnatelli

The platelet storage and release mechanisms for the heparin-neutralizing activity (HNA), adenosine diphosphate (ADP), serotonin, and lysosomal enzymes were investigated in normal human platelets and in platelets with defective storage or release of ADP and serotonin. The time course of release of HNA from normal washed platelets by thrombin and collagen was slower than that of serotonin. Lysosomal enzymes were not released by collagen from normal washed platelets, whereas under the same conditions HNA was released. In four of six patients with storage pool deficiency, the platelets contained normal amounts of HNA but definitely decreased amounts of ADP and serotonin, whereas in the remaining two patients the total contents of ADP, serotonin, and HNA were all definitely lower than normal. In four of six patients with storage pool deficiency, the amounts and per cents of total HNA released by collagen were normal, whereas the amounts and per cents of total ADP released were diminished compared with normal. Platelets from patients with the aspirinlike platelet release defect and from aspirin-treated normal subjects contained normal quantities of ADP, serotonin, and HNA, but HNA and ADP were not released in response to collagen. It is concluded that either HNA is stored in and released from dense granules by mechanisms different from those for ADP and serotonin, or that HNA is stored in and released from granules other than the dense granules, which contain ADP and serotonin and the α-granules, which contain lysosomal enzymes.

The heparin-neutralizing activity (HNA) of platelets, termed platelet factor 4 (PF4), has been attributed to a protein of low molecular weight, which has been partially purified and characterized and found to be released with other contents of platelet granules when platelet-rich plasma (PRP) is stirred with adenosine diphosphate (ADP), adrenaline, thrombin, and collagen.

Because of reports that the release of HNA parallels that of ADP and serotonin, which are stored in and released from the dense granules, it has been suggested that HNA is also stored in the dense granules. This suggestion is supported by direct studies of subcellular localization of HNA. In conflict with this view are the findings of Weiss and Rogers, who found a normal quantity of HNA in platelets deficient in storage pools of ADP and serotonin, i.e., platelets from patients with so-called storage pool deficiency. Although we have found decreased amounts of HNA in platelets from two patients with storage pool deficiency, our findings are not necessarily in conflict with those of Weiss and Rogers, since our patients may represent a different subgroup of the general class of patients with storage pool deficiency.

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The purpose of the investigations reported here is to acquire additional information about the storage sites and release mechanisms for HNA, ADP, serotonin, and lysosomal enzymes by studying their release from platelets washed by albumin density gradient separation and by investigating the distribution and release of these substances in platelet suspensions from other individuals with various defects of storage and release mechanisms.

MATERIALS AND METHODS

General

Human blood was collected into 3.8% trisodium citrate, and platelet-rich plasma (PRP) was prepared as previously described. Rimless 10 x 75-mm soft soda glass test tubes were used for clotting assays. Siliconized glassware or nonwettable plastic equipment was used except where indicated. Calibrated Eppendorf pipettes (Brinkman Instruments, Westburg, N.Y.) with disposable polypropylene tips were used for many experiments.

Platelet Counts

Platelets were counted and their morphology evaluated by phase contrast microscopy. For some experiments platelets were counted with a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, Fla.), and the results were averaged with those of the visual counts.

Reagents

Bovine factor X, prepared by DEAE-cellulose column chromatography, and kindly given by Dr. K. W. E. Denson, was activated with Russell's viper venom, diluted in citrate-albumin-imidazole buffer and stored in aliquots at -40°C as previously reported. The antifactor Xa preparation used was pooled human plasma heated for 15 min at 56°C, diluted in citrate-albumin-imidazole buffer, and stored at -40°C as previously described. Bovine factor-X deficient substrate plasma was obtained from Diagnostic Reagents Ltd., Thame, Oxon, England or from Sigma Chemical Co., St. Louis, Mo. Chloroform extract of rabbit brain was obtained from Sigma. Aqueous heparin (sodium salt) was obtained from Riker Laboratories, Northridge, Calif. Imidazole buffer was used to prepare citrate-albumin-imidazole. Triton X-100, obtained from Rohm and Haas, Philadelphia, Pa., was added to platelet suspensions to effect total cell lysis as previously described. Calcium-free Tyrode's solution was prepared and used at pH 6.5 or 7.3 as previously described. Bovine albumin (obtained from Sigma or from Reheis Chemical Co., Division of Armour Pharmaceutical Co., Kankakee, Ill., as fraction V from bovine plasma) was prepared and adjusted to pH 6.5 or 7.3, 300 milliosmols/liter, specific gravity 1.1300-1.1400, as previously described. Acid-soluble collagen was bovine achilles tendon in powdered form (Sigma), prepared as previously described to a concentration of approximately 1 g/ml and used at a final dilution of 1 in 50 (approximately 20 μg/ml). Adenosine diphosphate (Sigma) was stored at -60°C as a 1 mM solution in imidazole buffer, pH 7.3. Human thrombin was obtained as Fibrindex from Ortho Pharmaceuticals. Experiments with labeled serotonin were done with 18-14C-5-hydroxytryptamine creatinine sulfate (5 HT), 26-57 mCi/mM, obtained from Amersham/Searle. Imipramine (Tofranil, Geigy) was obtained in powdered form and was diluted appropriately from a stock solution of 5 mM in Tris saline, pH 7.3.

Albumin Density Gradient Separation (ADGS) and Washing of Platelets—Modified Method

Platelets were washed four times by a modification of the original method of ADGS.

Assay for Platelet HNA

The method for assaying the antiheparin activity of a suspension of washed platelets is based on the potentiation by residual heparin (i.e., not neutralized by HNA) of factor Xa inactivation by antifactor Xa. The details of this highly specific and reproducible assay, which is capable of measuring heparin in concentrations as low as 0.0005 U/ml (5 ng/ml) were previously reported.
PLATELET ANTIHEPARIN ACTIVITY

Determination of Lysosomal Enzyme Activities

Assays for lysosomal enzyme activities on supernatants of platelet suspensions washed four times by ADGS were kindly performed by Miss Carol Setkowsky as previously described.28 The following enzyme activities were assayed: acid phosphatase in the form of acid p-nitrophenyl-phosphatase, determined in citrate buffer, pH 4.8; fl-glucuronidase as phenolphthalein f-D-glucuronidase; and 3-galactosidase.

Patients

The clinical details and laboratory findings for the patients with disorders of storage and release mechanisms are reported in a separate communication,29 and the serotonin and adenine nucleotide values are shown in Table 2.

Storage-pool deficiency without albinism. Three patients similar to those reported by Holmen and Weiss17,18 were studied. Platelets from these patients were deficient in storage pools of adenine nucleotides and serotonin. These patients had moderate hemostatic defects, prolonged bleeding times, normal platelet counts, deficient collagen-induced platelet aggregation, and absent secondary aggregation in response to ADP and epinephrine. Patient 1 (F.S.) is the mother of patient 2 (L.S.). The total platelet HNA of these two patients was previously reported.19

Storage-pool deficiency with albinism. Three albino patients were studied. All had deficient storage pools of adenine nucleotides and serotonin.20 These patients are similar to those originally reported by Hermansky and Pudlak.31 They had moderate hemostatic defects, long bleeding times, normal platelet counts, absent secondary aggregation in response to ADP and epinephrine, and deficient collagen-induced platelet aggregation. Patients 5 (M.L.B.) and 6 (M.T.B.) are sisters.

"Aspirin-like" platelet release defect.16,18 Three unrelated patients with normal storage pools but defective release of adenine nucleotides and serotonin were studied. These patients had mild bleeding tendencies, long bleeding times, normal platelet counts, impaired collagen-induced platelet aggregation, and a diminished second wave of epinephrine- and ADP-induced platelet aggregation.

Aspirin-treated normal subjects. Three normal subjects were studied first before and then after receiving 300 mg of aspirin four times daily for 2 days. Platelets from these aspirin-treated subjects uniformly lacked secondary aggregation in response to epinephrine and ADP, and when determinations were made, the storage pools of serotonin and ADP were normal, but the release of serotonin and ADP in response to collagen was absent or severely deficient.

RESULTS

Release Experiments with Normal Platelets

Incubations with ADP. When normal platelets washed four times by ADGS were stirred at 37°C for 1-10 min with 1-50 μM ADP (final concentration), platelet aggregation did not occur, and little or no release of serotonin or HNA occurred. The calcium-free Tyrode's solution in which platelets were suspended contained no added fibrinogen or other plasma proteins. When fibrinogen was added to the suspending medium, ADP-induced platelet aggregation and release of HNA and serotonin were observed. Experiments beyond the scope of the present report, currently in progress, are designed to determine the optimal conditions for aggregation of and release from platelet suspensions washed by ADGS.

Incubations with thrombin. The time course of thrombin-induced release of HNA and 14C-serotonin was determined (Fig. 1). It is evident that 85%-90% of the total amounts of both serotonin and HNA were released after stirring of washed platelet suspensions for 6 min with thrombin, 0.5 U/ml. The unexpected finding, in view of demonstrations of simultaneous and parallel release of serotonin and HNA from PRP,11 was the striking difference in time course
Fig. 1. Thrombin-induced release of HNA (○–○) and 14C-serotonin (△–△) from washed platelets. PRP was incubated for 20 min at 37°C with 14C-labeled 5-HT at a concentration of 1 μM. The platelets were then washed four times by ADGS, and a 0.5 ml aliquot of the platelet suspension was pipetted into a siliconized glass cuvette which was placed in a metal heating block and prewarmed to 37°C for 1 min. A 50 μl quantity of thrombin (final concentration 0.5 N.I.H. units/ml) or imidazole buffer was added to the cuvette. After stirring magnetically with a metal stirring bar for the required time, the sample was immediately transferred to an Eppendorf centrifuge and centrifuged for 10 sec at 15,000 g. The supernatant was removed and assayed for HNA and serotonin, and the sediment was resuspended in Triton X-100, 0.5% final concentration to solubilize the pellet, which was similarly assayed for HNA and serotonin. In addition, the total platelet contents of HNA and serotonin were determined after stirring a 0.5 ml aliquot of the platelet suspension with 0.5% Triton X-100, final concentration for 4 min. The sum of the amount of serotonin or HNA released (i.e., in the supernatant) and the amount retained (i.e., in the sediment) was within 5% of the total (i.e., the whole sample). Per cent release was calculated according to the formula: $\frac{a - b}{c - b} \times 100$, where $a = $ amount in supernatant of treated sample; $b = $ amount in supernatant of control sample; and $c = $ amount in whole treated sample. The results represent the means (and standard error of the mean) of four separate experiments. The mean per cent release of serotonin is significantly different from per cent release of HNA ($p < 0.001$) where indicated by an asterisk (*).

of thrombin-induced release of HNA and 14C-serotonin from washed platelets. Whereas nearly all the releasable serotonin was released in the first 30 sec of incubation with thrombin, the plateau of HNA release occurred only after 4–6 min. This delay in HNA release compared with serotonin suggests the possibility of different storage sites or release mechanisms for serotonin and HNA.

Incubations with collagen. To compare further the release of HNA and 14C-serotonin from washed platelets, a total of nine separate experiments was done with collagen as the release inducer (Fig. 2). A progressive release of HNA occurred to approximately 40% of the total, with a plateau occurring after 4–5 min of stirring with collagen. In contrast, there was an initial rapid appearance of 14C-serotonin in the supernatant followed by a subsequent decrease with a maximum release of only 20% at 2 min stirring with collagen. It was postulated
Fig. 2. Collagen-induced release of HNA (○ - ○) and 14C-serotonin (△ - △) from washed platelets. Exactly as detailed in Fig. 1, a 0.5-ml aliquot of normal human platelets, washed four times by ADGS, was stirred at 37°C for the incubation time indicated with acid-soluble collagen, final concentration approximately 20 μg/ml, or with 0.1% acetic acid. The sample was then immediately centrifuged and determinations made of the amount of HNA (○ - ○) and serotonin (△ - △) in the supernatant and in the sediment. The points represent the means (and standard error of the mean) of the amount of HNA and serotonin in the supernatant and in the sediment. The points represent the means (and standard error of the mean) of nine separate experiments, per cent release calculated as in Fig. 1. Less than 5% release occurred in incubations with acetic acid. The mean per cent release of serotonin is significantly different from per cent release of HNA \( (p < 0.005) \) where indicated by an asterisk (*).

That the results with serotonin were due to reuptake of serotonin by the platelets.

To investigate this question, the reuptake of serotonin was inhibited by imipramine at a final concentration of 1 μM.\(^{32-34}\) The results (Fig. 3) indicate that the release of HNA was not affected by imipramine. In contrast, a rapid release of 14C-serotonin occurred in the presence of imipramine to a maximum of 48% of the total after 2 min incubation with collagen. Approximately the same per cent release of HNA occurred, but in similar fashion to the results with thrombin, the time course of HNA release was considerably slower than that of...
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1. Release of HNA and Lysosomal Enzymes From Washed Platelet Suspensions by Collagen

Table 1. Release of HNA and Lysosomal Enzymes From Washed Platelet Suspensions by Collagen

<table>
<thead>
<tr>
<th>Activity Tested</th>
<th>Per cent Release After Incubation Time (min) of</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin-neutralizing activity</td>
<td>11.1</td>
<td>20.2</td>
<td>28.8</td>
<td>37.8</td>
<td>42.4</td>
<td></td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>1.3</td>
<td>1.1</td>
<td>1.7</td>
<td>2.3</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>0.2</td>
<td>0.2</td>
<td>1.0</td>
<td>1.9</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>0.5</td>
<td>0.6</td>
<td>0.3</td>
<td>0.8</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

The experimental design and method of calculating per cent release was the same as for Figs. 1–3, except that the PRP was not preincubated with 14C-labeled serotonin.

Percentage figures represent the means of four separate experiments.

14C-serotonin. Approximately 40%–50% of both serotonin and HNA were released by collagen compared with approximately 90% of each in response to thrombin.

The slower time course of release of HNA compared with serotonin suggested the possibility that HNA is not stored in and released from the dense granules as is serotonin. Another possibility is that HNA is stored in and released from α-granules, which is presumably the site of storage of various lysosomal enzymes. To investigate this possibility, four experiments were done in which HNA release and lysosomal enzyme release by collagen were studied. The results (Table 1) indicate that none of the lysosomal enzymes was released from washed platelets by collagen, whereas under the same conditions approximately 40% release of HNA occurred over 4–5 min. This suggests that the storage site or release mechanism for HNA is different than for lysosomal enzymes.

Investigations in Patients

To explore further the storage and release of HNA and to compare them to the conditions required for storage and release of serotonin and ADP, a study was made of the platelets from patients with various disorders of the storage and release mechanisms (Table 2).

Storage pool deficiency with albinism. All three of these patients had decreased storage pools of serotonin and ADP, and the amount and the per cent of total ADP released were diminished. Two of these patients, F.S. and her daughter L.S., had definitely and repeatedly low levels of total HNA compared with normal and no detectable release of HNA in response to collagen. The third patient (C.F.) had normal total platelet HNA, and a normal amount and per cent of the total was released by collagen.

Storage-pool deficiency with albinism. All three of these unrelated patients had lower than normal total platelet serotonin and ADP contents, and the amount and per cent of total ADP released by collagen, for the two patients in whom the determination was made, were definitely diminished compared with normal. In contrast, the total platelet HNA and the amount and per cent of total HNA released by collagen were entirely normal for all three patients.

"Aspirin-like" platelet release defect. All three of these unrelated patients had normal storage pools of serotonin and ADP, but decreased amounts and percents of total ADP compared with normal were released by collagen. Simi-
Table 2. Serotonin, ADP, and HNA Storage and Release by Collagen in Patients With Storage and Release Defects

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Patient</th>
<th>Serotonin Content*</th>
<th>ADP Released by Collagen</th>
<th>Heparin-neutralizing Activity Released by Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total* Amount* %</td>
<td>Total Amount* Per cent</td>
<td></td>
</tr>
<tr>
<td>Storage-pool deficiency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>without albinism</td>
<td>1 F.S.</td>
<td>&lt;0.1 1.02 0.14 14</td>
<td>3.11 &lt;0.01 &lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 L.S.</td>
<td>&lt;0.1 1.27 0.10 8</td>
<td>4.10 &lt;0.01 &lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 C.F.</td>
<td>0.06 0.85 0.07 8</td>
<td>15.50 3.72 24</td>
<td></td>
</tr>
<tr>
<td>Storage-pool deficiency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with albinism</td>
<td>4 M.R.</td>
<td>&lt;0.05 0.72 0.08 11</td>
<td>13.12 2.68 20.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 M.L.B.</td>
<td>&lt;0.05 0.98</td>
<td>16.30 5.27 32.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 M.T.B.</td>
<td>&lt;0.05 1.41 &lt;0.01 &lt;1</td>
<td>18.30 5.60 30.6</td>
<td></td>
</tr>
<tr>
<td>“Aspirin-like” platelet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>release defect</td>
<td>7 V.K.</td>
<td>0.24 3.02 0.13 4</td>
<td>16.9 0.10 0.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 B.L.</td>
<td>0.36 3.50 0.24 7</td>
<td>17.1 &lt;0.01 &lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 P.P.</td>
<td>0.31 4.50 0.45 10</td>
<td>15.4 0.80 5.2</td>
<td></td>
</tr>
<tr>
<td>Normal Values†</td>
<td>0.39</td>
<td>3.81 1.55 40.6</td>
<td>16.09 4.84 30.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>±0.13</td>
<td>±0.41 ±0.51</td>
<td>±2.84 ±1.12</td>
<td></td>
</tr>
</tbody>
</table>

*nmol/10^8 platelets.
†Units of heparin neutralized per 10^10 platelets.
‡Values represent means ± SD.

The total amount of platelet HNA was determined by assaying for NHA a platelet suspension washed four times by ADGS and solubilized with Triton X-100. The amount of HNA released was determined on the supernatants and solubilized sediments of platelet suspensions stirred magnetically at 37°C for 4 min with acid-soluble collagen, final concentration approximately 20 µg/ml or with 0.1% acetic acid in similar fashion to the release experiments in Figs. 1-3. The sum of the released (supernatant) and retained (sediment) HNA was within 5% of the total (tritonized whole sample). The amount of HNA "spontaneously released," i.e., present in the supernatant of samples stirred with 0.1% acetic acid, was uniformly less than 5%. Per cent release was calculated as described in Fig. 1. Serotonin, total ADP, and ADP released by collagen are reported in a separate communication (Pareti et al., 1973) and are reprinted here to allow comparison with the results for HNA.

Normal values for storage pools and amounts released for HNA, serotonin, and ADP were obtained by always studying platelets from a normal donor in parallel with the patient study. Each patient (with a normal control) was studied on at least two separate occasions, and the results for a given patient are expressed as the mean of two to four separate determinations.

Aspirin-treated normal subjects (Table 3). All three of these normal subjects had platelets containing a normal amount of HNA both before and after they had received aspirin, and the amount and percentage of total HNA released by collagen was normal before aspirin. However, after aspirin treatment collagen had no detectable effect in releasing HNA from platelets obtained from the same three subjects.

DISCUSSION

Since thrombin was originally shown to release serotonin from platelets, the platelet release reaction has been studied extensively, and the concept has emerged that a variety of granular constituents are selectively extruded in re-
Table 3. Effect of Treatment With Aspirin on Total HNA and Release of HNA by Collagen

<table>
<thead>
<tr>
<th>Conditions of</th>
<th>Conditions of</th>
<th>Subject</th>
<th>Total*</th>
<th>Amount*</th>
<th>Per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before aspirin</td>
<td>10</td>
<td>18.2</td>
<td>5.9</td>
<td>32.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>15.7</td>
<td>4.7</td>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>17.2</td>
<td>6.3</td>
<td>36.6</td>
<td></td>
</tr>
<tr>
<td>After aspirin</td>
<td>10</td>
<td>17.7</td>
<td>&lt;0.1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>16.8</td>
<td>&lt;0.1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>16.0</td>
<td>&lt;0.1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>Normal values</td>
<td></td>
<td>16.09</td>
<td>4.84</td>
<td>30.1</td>
<td></td>
</tr>
</tbody>
</table>

*Units of heparin neutralized per 10^10 platelets.
Values represent means ± SD.
Determinations made as described in caption to Table 2.

Response to a number of agents including ADP, thrombin, collagen, and epinephrine by a mechanism similar to cell secretion. There is good evidence that substances such as ADP and serotonin are stored in the dense granules and are released rapidly, whereas others, such as lysosomal enzymes are stored in α-granules and are released more slowly. Investigations of the subcellular localization of HNA suggest a granular storage site, but it is unclear which granules contain HNA. Most of the studies which have been done to investigate the mechanism of release of HNA have been done with PRP and suggest that HNA is released in parallel with ADP and serotonin. It has therefore been suggested that HNA is stored in and released from the dense granules.

The observations presented here were made using platelets washed by ADGS and suggest that the storage site or release mechanism for HNA is different from that for ADP, serotonin, and lysosomal enzymes. The evidence can be summarized as follows: First, the time course of release of HNA from normal platelets by thrombin and collagen was considerably slower than that of serotonin. Second, HNA was released from normal washed platelets by collagen, whereas under the same conditions lysosomal enzymes were not. Third, in four of six patients with storage pool deficiency, the platelets contained normal amounts of HNA but definitely decreased amounts of ADP and serotonin, whereas in the remaining two patients the total contents of ADP, serotonin, and HNA were all definitely lower than normal. Finally, in four of six patients with storage pool deficiency the amounts and per cents of total HNA released by collagen were entirely normal, whereas the amounts and per cents of total ADP released were diminished compared with normal. Weiss and Rogers found normal amounts but diminished release of HNA from platelets obtained from patients with storage pool deficiency. This apparent divergence of results might be explained by methodologic differences, since Weiss and Rogers studied release using PRP, whereas we have studied release using washed platelets.

A number of possible explanations might be proposed to account for these observations. The difference in time course of release of serotonin and HNA...
might be explained if the release process consists of a sudden reversible change in membrane permeability,\textsuperscript{41} which might allow more rapid diffusion of low-molecular-weight substances (ADP and serotonin) than of higher-molecular-weight substances (HNA). However, amino acids,\textsuperscript{42} cytoplasmic enzymes,\textsuperscript{43} and acid phosphatases\textsuperscript{35} can be retained by platelets from which other lysosomal enzymes, ADP, and serotonin are released. It has therefore been suggested\textsuperscript{35,37} that platelets extrude the entire contents of certain granules in response to specific stimuli. If this concept is valid, another explanation must account for the observation that serotonin was released rapidly, HNA slowly, and lysosomal enzymes not at all under the same conditions.

It is possible that HNA is stored in granules other than the dense granules, as suggested by Weiss and Rogers,\textsuperscript{16} who found normal platelet contents of HNA in patients with storage pool deficiency. Our findings confirm their observations, since four of our six patients with storage pool deficiency had normal platelet HNA contents. The fact that patients 1 and 2 had platelets deficient in HNA, serotonin, and ADP suggests the possibility that these two related patients may have inherited two distinct abnormalities, one involving the storage granule or mechanism for ADP and serotonin and the other involving the storage granule or mechanism for HNA. Alternatively, it is also possible, as pointed out by Weiss and Rogers,\textsuperscript{16} that HNA is stored in dense granules which might have selective and independent mechanisms for storage of HNA on the one hand and ADP and serotonin on the other. One question which remains is how these alternative explanations might account for the observed differences in rates of release of HNA and serotonin.

These two alternative mechanisms for storage and release of HNA, ADP, and serotonin are presented in diagrammatic form (Fig. 4). According to the

![Diagram](image-url)

**Fig. 4.** Two alternative storage and release mechanisms for HNA, ADP, and serotonin. The platelet membrane is shown with storage granules containing either (in mechanism 1) ADP and serotonin (dense granules) or HNA (? granules), or (in mechanism 2) ADP, serotonin, and HNA (dense granules). For simplicity the α-granules which contain lysosomal enzymes are not shown. Mechanism 2 was suggested by Dr. D. C. B. Mills.
first possibility, ADP and serotonin are stored in dense granules, the contents of which are released rapidly, and HNA is stored in a more slowly released granule which is neither a dense granule nor the type of α-granule in which lysosomal enzymes are stored. This suggestion implies that the platelet release reaction consists of a sequential series of extrusions of different types of granules, whose contents are stored and released independently.

An alternative mechanism, suggested by Dr. D. C. B. Mills, requires that ADP, serotonin, and HNA are all stored in dense granules which extrude HNA less rapidly than ADP and serotonin. This possibility is intriguing in the light of recent evidence that HNA is released in conjunction with a high-molecular-weight proteoglycan carrier. It is tempting to wonder whether this carrier molecule might be a constituent of the granular membrane, which is thereby released more slowly than other soluble granular contents (ADP and serotonin).

Our observations in the patients with storage-pool deficiency are easier to explain on the basis of the second mechanism than the first. If there are two separate storage sites for HNA and for ADP and serotonin (mechanism I), the inference is that patients 1 and 2 must have inherited two separate storage defects, i.e., they must lack two different types of granules. In contrast, if the dense granules serve as one storage site for HNA, serotonin, and ADP, one might explain the findings in patients 3–6 on the basis of a defect in ADP and serotonin storage, whereas the deficiency of HNA, serotonin, and ADP found in patients 1 and 2 could be explained by an absence or deficiency of dense granules. Our findings do not prove the validity of either of the two suggested mechanisms.

The observations presented here of defective release of HNA from platelets obtained from aspirin-treated normals and from patients with the "aspirin-like" platelet release defect confirm previous observations. These findings are consistent with the view that storage mechanisms are normal under these clinical conditions, but the triggering mechanism for the entire platelet release reaction is inoperative.

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