Release of \textsuperscript{14}C-Serotonin During Initial Platelet Changes Induced by Thrombin, Collagen, or A23187

By M. A. Packham, M. A. Guccione, J. P. Greenberg, R. L. Kinlough-Rathbone, and J. F. Mustard

Release of \textsuperscript{14}C-serotonin from prelabeled platelets during the lag phase before aggregation and during aggregation caused by low concentrations of thrombin, collagen, or A23187 was measured after stopping release instantaneously with paraformaldehyde. Imipramine was used to prevent reuptake of released serotonin. At the lowest point on the light transmission curve following the addition of thrombin, release of \textsuperscript{14}C-serotonin was barely detectable, although 5%-10% of the platelets were seen to be aggregated in pairs or triplets upon microscopic examination. The lag phase was slightly prolonged by creatine phosphate/creatine phosphokinase (CP/CPK), indomethacin, acetylsalicylic acid, or a combination of one of the drugs with CP/CPK; release at maximum aggregation was only partially reduced by these inhibitors. Thus thrombin appears to cause aggregation through at least three mechanisms: released adenosine diphosphate (ADP), formation of prostaglandin endoperoxides and thromboxane A\textsubscript{2}, and a third mechanism (not yet defined) which is the most dominant one. Collagen caused 1.9% release (representing 0.28 \textmu M ADP) at the lowest point on the light transmission curve. This concentration of ADP is insufficient to cause aggregation by itself. Studies with the inhibitors showed that the lag phase was prolonged by CP/CPK, indomethacin, or acetylsalicylic acid; collagen-induced shape change and aggregation were completely blocked by a combination of CP/CPK and one of these drugs. Thus collagen-induced shape change and aggregation are largely dependent on two mechanisms: released ADP and products formed from platelet arachidonate. The ionophore A23187 caused 1.2% release (representing 0.18 \textmu M ADP) at the point where aggregation began. Results with the inhibitors were similar to those with thrombin. Thus A23187 may also act through three mechanisms, the third probably caused by internal calcium shift(s). In no case was released ADP solely responsible for aggregation, but it acted synergistically with the other mechanisms to augment the extent of aggregation and release.

Platelet aggregation caused by low concentrations of collagen, thrombin, and several other agents occurs after a lag phase that may be as long as 60 sec or more.\textsuperscript{1,4} During the lag phase, light transmission through stirred platelet-rich plasma or a platelet suspension decreases slightly and the oscillations of light transmission decrease in amplitude, indicating that the platelets lose their normal disk shape. The delay had originally been assumed to be due to the time required for the release of sufficient adenosine diphosphate.
(ADP) from the amine storage granules to cause platelet aggregation.\textsuperscript{1,2} In the light of the recent demonstrations of the production of potent aggregating agents [prostaglandins (PG) G\textsubscript{2} and H\textsubscript{2} and thromboxane A\textsubscript{2}] when platelet arachidonate is mobilized by stimulation with collagen or thrombin,\textsuperscript{5,8} it is also possible that the lag phase may be related to the time required to produce aggregating concentrations of the prostaglandin endoperoxides and thromboxane A\textsubscript{2}. If, as has been suggested,\textsuperscript{9-11} there are other mechanisms also involved in causing aggregation, they too may require an appreciable time to come into play.

Several years ago we investigated the amounts of adenosine triphosphate (ATP), ADP, and \textsuperscript{14}C-serotonin released from prelabeled platelets during the lag phase and early aggregation caused by collagen or thrombin\textsuperscript{4} and showed that insufficient ADP was released during the lag phase to account for the extent of aggregation observed. At that time we were hampered by being unable to stop release instantaneously. We have now reinvestigated the amount of release occurring during lag phase using the technique developed by Costa and Murphy,\textsuperscript{12} in which the addition of paraformaldehyde completely blocks further release. Release of \textsuperscript{14}C-serotonin from prelabeled platelets was used to measure the extent of the release reaction. In a further refinement of the method, we have included imipramine in the platelet-suspending medium to prevent reuptake of small amounts of \textsuperscript{14}C-serotonin released from the platelets.

To assess the contribution of ADP, we have investigated the effects of the creatine phosphate/creatine phosphokinase (CP/CPK) system on the length of the lag phase, aggregation, and release of \textsuperscript{14}C-serotonin. This enzyme system converts any ADP in the suspending fluid to ATP. To assess the contribution of the active compounds formed from platelet arachidonate, we have used indomethacin or acetylsalicylic acid to block the conversion of arachidonate to PGG\textsubscript{2}, PGH\textsubscript{2}, and thromboxane A\textsubscript{2}.\textsuperscript{5,8}

**MATERIALS AND METHODS**

*Materials*

Creatine phosphate, creatine phosphokinase (salt-free powder), bovine tendon collagen, and indomethacin were from Sigma Chemical, St. Louis, Mo. Topical bovine thrombin was from Parke Davis, Detroit, Mich. Purified human thrombin (U.S. standard thrombin lot H-1, 2500 U/mg) was kindly supplied by Dr. D. L. Aronson, Division of Blood and Blood Products, Bureau of Biologics, Food and Drug Administration, Bethesda, Md.

Soluble reagents were dissolved and diluted in Tyrode solution (pH 7.35) unless otherwise noted. Indomethacin was prepared as a 20 mM solution in 95% ethanol and was diluted with 0.85% saline as required. Imipramine (Tofranil) was obtained from Geigy Canada and dissolved in 0.85% saline. Acetylsalicylic acid (ASA) from Matheson, Coleman and Bell, East Rutherford, N.J., was dissolved as described elsewhere.\textsuperscript{13} The divalent cation ionophore A23187 was a gift of the Eli Lilly Co., Indianapolis, Ind.; it was dissolved at a concentration of 10\textsuperscript{-6} M in dimethylsulfoxide (DMSO).

Collagen suspensions in Tyrode solution were prepared as described previously.\textsuperscript{14} Apyrase was prepared from potatoes by the method of Molnar and Lorand;\textsuperscript{15} it was used at a concentration capable of converting 0.25 \textmu M ATP to adenosine monophosphate (AMP) within 120 sec at 37\textdegree C.

Paraformaldehyde powder (Fisher Scientific, Fairlawn, N.J.) was dissolved in water at a concentration of 6% and heated to 60°–70° C with stirring; 5 N NaOH was added to produce a clear solution. The solution was adjusted to 1.2%, with Tyrode solution and to pH 7.35 by the addition of HCl.
PREPARATION OF PLATELET SUSPENSION

Suspensions of twice-washed rabbit platelets were prepared by the method of Ardlie et al. They were labeled in the first washing fluid by incubation with 0.007 μCi/ml of 14C-serotonin (5-hydroxytryptamine-3'-creatinine sulphate-14C, approximately 50 μCi/μmole; Amersham/Searle, Arlington Heights, Ill.) for 15 min at room temperature. The final resuspending medium was Tyrode solution containing 0.35% albumin (Pentex, Miles Laboratories, Kankakee, Ill.) and apyrase. The platelet count was adjusted to 700,000/cu mm and the suspensions were stored at 37°C.

AGGREGATION AND RELEASE STUDIES

Samples (2 ml) of platelet suspension were transferred to a turbidimetric device (Payton Aggregation Module, Payton Associates, Scarborough, Ont., Canada), kept at 37°C, and stirred at 1100 rpm. Imipramine (0.04 ml of 10⁻⁴ M) was added 15 sec before any other additions to the platelet suspension to give a final concentration of 1.7 μM after all additions. Imipramine has been shown to inhibit serotonin uptake; Born et al. have reported a Kᵣ of 0.3 μM. Imipramine was therefore included in the medium to inhibit reuptake of any serotonin that was released from the platelets. With the exception of ASA, inhibitors were added in 0.1-ml volumes at 60-sec intervals before the addition of the aggregating agent under study. ASA was incubated with the platelets for 5 min before the addition of an aggregating agent. Tyrode solution was used in place of inhibitors in the control studies to maintain constant volume.

Collagen or thrombin was added in 0.1-ml volumes. The ionophore A23187 in DMSO was added in 10-μl volumes. In control studies, this volume of DMSO was shown to have no effect on platelet aggregation or serotonin release. All concentrations given are the final concentrations in the platelet suspensions after all additions. ASA and indomethacin were used at concentrations that block malondialdehyde formation caused by 0.04 U/ml of thrombin or 0.4 μM A23187. The concentrations of creatine phosphate and creatine phosphokinase used prevented aggregation in response to 8 μM ADP in these platelet suspensions. (Although the suspensions contained apyrase, the activity of the CP/CPK system was much greater than that of the apyrase at the concentrations used.)

The concentrations of aggregating agents were adjusted so that lag phases of 30-60 sec were obtained. (The lag phase was defined as the time required after the addition of the aggregating agent for light transmission to reach its lowest point.) After the addition of the aggregating agent 0.1-ml samples of the platelet suspension were removed with an Eppendorf pipette directly into 0.4 ml of 1.2% paraformaldehyde solution and mixed thoroughly with an omnimix. Samples were taken before the addition of the aggregating agent, at 10-sec intervals after its addition (for 1-2 min), and then at longer intervals (Fig. 1). The paraformaldehyde-platelet mixtures were centrifuged at 12,000 g for 1 min (Eppendorf centrifuge, Brinkmann, Rexdale, Ont.) and 0.1-ml samples of the supernatant fluids were removed for radioactivity determinations. The amount of 14C-serotonin in the suspending fluid was calculated as a percentage of the total radioactivity in the platelets. Appropriate corrections were made for dilution upon addition of reagents. Preliminary experiments showed that under these conditions the platelets did not release labeled serotonin when 0.1 ml of platelet suspension was added to 0.4 ml of 1.2% paraformaldehyde solution. Paraformaldehyde-treated platelets were incapable of taking up labeled serotonin.

RESULTS

Light microscopic examination of platelet suspensions fixed by the addition of paraformaldehyde showed that essentially all the platelets were present as single platelets before the addition of the aggregating agents. At the lowest point on the light transmission curves after the addition of an aggregating agent, 5%-10% of the platelets were in pairs; very few groups of three were apparent, and the remainder were present as single platelets. These observations were the same in the presence of the inhibitors, either singly or combined. As light transmission increased, the number of pairs and larger aggregates increased.
Fig. 1. Typical aggregation curve obtained with a low concentration of bovine thrombin (THR, 0.03 U/ml). Vertical lines indicate points at which samples were removed and added to paraformaldehyde solution to stop further release of granule contents. Percentages of total $^{14}$C-serotonin found in fluid surrounding platelets are shown in boxes. Some $^{14}$C-serotonin (6.6% in this experiment) was present in suspending fluid before addition of thrombin.

**Thrombin**

Figure 1 shows a typical aggregation curve obtained with a low concentration of thrombin. Very little $^{14}$C-serotonin was released as the platelets changed shape. (Shape change is indicated by reduction in light transmission and loss of oscillations of light transmission.) Appreciable release did not occur until aggregation (increase in light transmission) was well underway.

**Table 1. Effect of Thrombin or Collagen on Release of $^{14}$C-Serotonin From Prelabeled Rabbit Platelets**

<table>
<thead>
<tr>
<th>Addition to Platelet Suspension</th>
<th>No. of Exps.</th>
<th>20 sec Before Addition</th>
<th>10 sec Before Addition</th>
<th>At Lowest Point of Light Transmission Curve</th>
<th>10 sec After Low Point</th>
<th>20 sec After Low Point</th>
<th>30 sec After Low Point</th>
<th>At Maximum Aggregation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin (bovine)†</td>
<td>11</td>
<td>5.91</td>
<td>5.84</td>
<td>5.74</td>
<td>5.91</td>
<td>6.38</td>
<td>7.51</td>
<td>10.7</td>
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<td>5.06</td>
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<td>Collagen†</td>
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<td>5.27</td>
<td>5.85</td>
<td>6.50</td>
<td>7.66</td>
<td>10.5</td>
<td>18.2</td>
</tr>
</tbody>
</table>

*Maximum aggregation was assumed to have taken place when light transmission did not increase further. This effect occurred 2–3 min after the addition of thrombin and 3–4 min after the addition of collagen.

†Final concentrations of thrombin (0.02–0.04 U/ml) were chosen to give about 30 sec between the addition of thrombin and the lowest point on the light transmission curve. By paired difference analysis, there was no significant difference between the mean percentages of $^{14}$C-serotonin in the suspending medium before the addition of bovine thrombin and at this lowest point. With purified human thrombin, the difference was just statistically significant ($2p < 0.05$).

†With the concentrations of collagen chosen, 50–60 sec elapsed between addition of collagen and the lowest point on the light transmission curve. By paired difference analysis, there was a highly significant difference between the mean percentages of $^{14}$C-serotonin in the suspending medium before collagen addition and at this lowest point ($2p < 0.001$).
Table 1 shows that with bovine thrombin, the mean percentage of \(^{14}\text{C}\)-serotonin (5.9%) in the suspending fluid at the point where light transmission was at its lowest (approximately 30 sec after thrombin addition) was not significantly different from the mean percentage of \(^{14}\text{C}\)-serotonin in the suspending fluid before the addition of thrombin. With purified human thrombin, the mean percentage of \(^{14}\text{C}\)-serotonin released at the lowest point of the light transmission curve was 1.2% (6.05%−4.82%) (Table 1), which was just statistically significant \((2p < 0.05)\); 20 sec after light transmission began to increase, the mean value for \(^{14}\text{C}\)-serotonin released by bovine thrombin was 1.6% (7.51%−5.91%), and by purified human thrombin it was 4% (8.82%−4.82%). Maximum release at 2–3 min averaged 34% in 11 experiments with bovine thrombin and 24% in 11 experiments with human thrombin (Table 1).

Indomethacin (8 \(\mu\text{M}\)), added before thrombin, had little or no effect on the duration of the lag phase (Fig. 2); similar results were obtained with ASA (425 \(\mu\text{M}\)). At maximum aggregation the mean values for released \(^{14}\text{C}\)-serotonin with and without inhibitor were not significantly different (Table 2, mean values

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**Fig. 2.** Effect of CP/CPK and indomethacin (IND, 8 \(\mu\text{M}\)) on aggregation produced by purified human thrombin (THR, 0.04 U/ml). Concentration of CP, 0.44 mM; CPK, 4.4 U/ml. Tyrode solution (T) was added to sample without inhibitors to maintain constant volume. Values shown in boxes for percentage release of \(^{14}\text{C}\)-serotonin 3 min after addition of thrombin are mean values from six experiments; the percentage of \(^{14}\text{C}\)-serotonin in suspending fluid before addition of thrombin has been subtracted from these values. Paired difference analysis showed a significant difference \((2p < 0.02)\) between the amount of \(^{14}\text{C}\)-serotonin released in the absence of inhibitors and in the presence of the combination of indomethacin with CP/CPK.
from 4 experiments). A CP/CPK mixture had only a slight effect on the length of the lag phase (Fig. 2), but it did reduce the amount of $^{14}$C-serotonin that was released at 3 min (Table 2, mean of 8 experiments). The combination of indomethacin with CP/CPK had little further effect on the lag phase; in 4 experiments with bovine thrombin (Table 2) and 6 with purified human thrombin (Fig. 2) the lag phase was not prolonged by more than 10 sec. Similar results were obtained with ASA combined with CP/CPK. The combination of either of the nonsteroidal antiinflammatory drugs with CP/CPK inhibited $^{14}$C-serotonin release to some extent (Fig. 2 and Table 2).

Collagen

In contrast to the results with thrombin, the mean percentage of $^{14}$C-serotonin (6.5%) in the suspending fluid at the point where light transmission began to increase (50–60 sec after the addition of collagen) was significantly greater than the mean percentage of $^{14}$C-serotonin in the suspending fluid before the addition of collagen (4.65%; Table 1). This difference represented the release of 1.9% (6.5% - 4.6%) of the $^{14}$C-serotonin in the platelets. Twenty seconds later, 5.9% had been released. Although the time required to reach the lowest point of the light transmission curve was longer (50–60 sec) with the concentrations of collagen chosen than it was with the concentrations of thrombin used (approximately 30 sec), the collagen concentrations used caused an average release of 46% of the $^{14}$C-serotonin at maximum aggregation, whereas the concentrations of thrombin used caused only 34%, and 24% release.

<table>
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<tr>
<th>Inhibitor†</th>
<th>No. of Exp.</th>
<th>Before Thrombin Addition</th>
<th>At Lowest Point of Light Transmission Curve</th>
<th>10 sec After Low Point</th>
<th>20 sec After Low Point</th>
<th>30 sec After Low Point</th>
<th>At Maximum Aggregation*</th>
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<td>6.0</td>
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<td>7.7</td>
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*See footnotes to Table 1. Bovine thrombin was used in these experiments.
†Concentrations: Creatine phosphate (CP) 0.44 mM; creatine phosphokinase (CPK) 4.4 U/ml; indomethacin (Indo) 8 µM; acetylsalicylic acid (ASA) 425 µM. ASA was incubated with the platelets for 5 min at 37°C before thrombin was added.
Fig. 3. Effect of CP/CPK and indomethacin (see Fig. 2 legend for concentrations) on aggregation curve produced by (A) collagen at a concentration that gave a maximum release of 32% of $^{14}$C-serotonin in the absence of inhibitors, or (B) collagen at a concentration that gave a maximum release of 77% of $^{14}$C-serotonin in the absence of inhibitors. Similar results were obtained with ASA (425 $\mu$M). Percentage of $^{14}$C-serotonin released 4 or 3 min after addition of collagen is shown in boxes beside curves.

The presence of CP/CPK prolonged the time required to reach the low point of the light transmission curve from 60 sec after the addition of collagen to 92 sec (mean values from 6 experiments; Fig. 3). However, in all cases, collagen-induced aggregation occurred in the presence of CP/CPK. The amount of $^{14}$C-serotonin in the suspending fluid when light transmission began to increase was 2.2% greater than before the addition of collagen (Table 3). CP/CPK partially inhibited the amount of $^{14}$C-serotonin released at 4 min.

Indomethacin (8 $\mu$M) or ASA (425 $\mu$M) had different effects depending on the strength of the collagen stimulus. With collagen concentrations which gave a maximum release of up to 50%, these drugs prevented platelet aggregation but did not prevent collagen-induced shape change (Fig. 3A). With collagen concentrations which gave a maximum release of 50%-75%, the drugs did not prevent aggregation, but they prolonged the time required to reach the low point of the light transmission curve to 95 sec (Fig. 3B).

CP/CPK had some effect on the maximum amount of $^{14}$C-serotonin released by collagen. In contrast, indomethacin or ASA markedly reduced the amount of release (Table 3).

With the stronger collagen suspensions, the combination of either one of the drugs with CP/CPK prevented aggregation but did not prevent shape change (Fig. 3B). With the weaker collagen suspensions, these combinations blocked both aggregation and platelet shape change (Fig. 3A). Low amounts of $^{14}$C-
serotonin, however, were released even when the platelets did not change shape (Table 3).

Ionophore A23187

The mean percentage of $^{14}$C-serotonin in the suspending fluid at the point where light transmission began to increase (10–20 sec after the addition of the ionophore) was significantly greater than the mean percentage of $^{14}$C-sero-

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**Table 3. Effect of CP/CPK, Indomethacin, and ASA on Collagen-induced Release of $^{14}$C-Serotonin From Prelabeled Platelets**

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>No. of Exp.</th>
<th>Before Collagen Addition</th>
<th>At Lowest Point of Light Transmission Curve</th>
<th>10 sec After Low Point</th>
<th>20 sec After Low Point</th>
<th>30 sec After Low Point</th>
<th>At Maximum Aggregation</th>
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NA, no aggregation; NSC, no shape change.

* See footnote to Table 2 for concentrations.
† See footnote to Table 1.
§ In one of these experiments the platelets changed shape but did not aggregate.
|| When the platelets did not aggregate, this sample was taken at the same time after collagen addition as the sample taken at maximum aggregation without inhibitor.
\| In one of these experiments the platelets did not change shape.

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**Table 4. Effect of CP/CPK and Indomethacin on Ionophore-induced Release of $^{14}$C-Serotonin From Prelabeled Platelets**

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>Before Ionophore Addition</th>
<th>At Lowest Point of Light Transmission Curve</th>
<th>10 sec After Low Point</th>
<th>20 sec After Low Point</th>
<th>30 sec After Low Point</th>
<th>At Maximum Aggregation</th>
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</thead>
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</tr>
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<td>CP/CPK</td>
<td>4.0</td>
<td>5.4</td>
<td>5.4</td>
<td>5.2</td>
<td>7.0</td>
<td>54</td>
</tr>
<tr>
<td>Indo</td>
<td>3.8</td>
<td>5.0</td>
<td>5.9</td>
<td>5.9</td>
<td>6.7</td>
<td>34</td>
</tr>
<tr>
<td>CP/CPK + Indo</td>
<td></td>
<td>3.7</td>
<td>5.5</td>
<td>5.6</td>
<td>5.2</td>
<td>6.7</td>
</tr>
</tbody>
</table>

* Concentration of ionophore A23187: 0.39–0.42 μM. Mean values from five independent experiments are listed.
† See footnote to Table 2 for concentrations.
‡ See footnote to Table 1. Maximum aggregation occurred 3 min after the addition of the ionophore.
tonin in the suspending fluid before the addition of the ionophore (Table 4). This difference was observed in the control experiments and in the presence of the inhibitors CP/CPK and indomethacin (singly or combined; Table 4). Paired difference analysis showed that the values without inhibitors were significantly different from those with the inhibitors (singly or combined; \( p < 0.005 \) in all cases). If lower concentrations of the ionophore were used, the platelets changed shape but did not aggregate in the presence of the inhibitors (singly or combined).

Although indomethacin reduced the amount of \( ^{14} \)C-serotonin released by the ionophore in 3 min, CP/CPK had less effect either alone or combined with indomethacin (Table 4).

**DISCUSSION**

In interpreting the results of these studies, observations from other investigations will be used: (1) release of labeled serotonin from the amine storage granules of prelabeled platelets parallels the release of ATP and ADP,

\[ \text{release of ATP and ADP} \]

and (2) the initial fall in light transmission through the stirred platelet suspension and the narrowing of the oscillations of light transmission represent the change of the shape of the platelets from disks to spheres with pseudopods,

\[ \text{change of shape} \]

whereas the following increase in light transmission represents the formation of platelet aggregates involving larger and larger numbers of platelets.

\[ \text{formation of aggregates} \]

In agreement with the observations of Born and Hume,

\[ \text{observations of Born and Hume} \]

who used a similar technique to study ADP-induced aggregation, we found that at the lowest point on the light transmission curve, aggregation had already begun and 5\%–10\% of the platelets were in pairs.

The amounts of ADP released at the lowest point on the aggregation curves were insufficient to cause aggregation by themselves. From earlier experiments

\[ \text{earlier experiments} \]

in which released \( ^{14} \)C-serotonin, ATP, and ADP were measured in the same samples, it could be calculated that the release of 1\% of the labeled serotonin from rabbit platelets represented the appearance of ADP at a final concentration of 0.15 \( \mu M \) in the platelet suspending fluid. Thus the mean release of 1.2\% of \( ^{14} \)C-serotonin by purified human thrombin at this point represented 0.18 \( \mu M \) ADP; bovine thrombin did not cause the release of \( ^{14} \)C-serotonin at this point.

The release of 1.9\% of \( ^{14} \)C-serotonin by collagen at the lowest point on the light transmission curve represented 0.28 \( \mu M \) ADP; the mean release of 1.2\% by A23187 represented 0.18 \( \mu M \). Washed rabbit platelets prepared in this way require more than 1 \( \mu M \) ADP to produce the extent of aggregation observed with thrombin, collagen, or the ionophore. Therefore aggregation caused by these agents could not be attributed solely to the release of ADP.

Experiments with indomethacin or ASA were done to assess the role of prostaglandin endoperoxides and thromboxane \( \text{A}_{2}\)

\[ \text{prostaglandin endoperoxides and thromboxane} \]

in aggregation produced by thrombin, collagen, or A23187. The nonsteroidal antiinflammatory drugs were used at concentrations which we have previously shown block aggregation of rabbit platelets by 41 \( \mu M \) sodium arachidonate and prevent malondialdehyde formation caused by 0.04 U/ml of thrombin or 0.4 \( \mu M \) A23187.

\[ \text{formation of malondialdehyde} \]

With thrombin, these drugs had little or no effect on the time that elapsed before light transmission began to increase or on the amount of \( ^{14} \)C-serotonin released
at maximum aggregation. Thus thrombin caused extensive aggregation when
the formation of prostaglandin endoperoxides and thromboxane A₂ from plate-
et arachidonate was blocked and the release of ADP was insufficient to cause
the extent of aggregation observed. It seems probable, therefore, that thrombin
can act to produce aggregation by another mechanism (or mechanisms) in
addition to ADP release and thromboxane A₂ formation.

In contrast, the nonsteroidal antiinflammatory drugs either prevented col-
gen-induced aggregation, or (with the higher concentrations of collagen)
delayed the time before aggregation began and markedly reduced the amount
of ¹⁴C-serotonin released at maximum aggregation. Thus it appears that the
products formed from platelet arachidonate in response to collagen play a large
role in collagen-induced aggregation. It should be pointed out, however, that the
light transmission system mainly records the behavior of platelets that are not
adherent to collagen, but are affected indirectly by prostaglandin endoperox-
ides, thromboxane A₂, and ADP that are lost or released from platelets ad-
derent to collagen. We have shown elsewhere that the nonsteroidal antiinflam-
matory drugs inhibit the adherence to collagen of platelets in this medium. It
seems likely that the prolongation of the lag phase that they cause may be
attributable to this inhibitory effect as well as to inhibition of the arachidonate
pathway.

In keeping with earlier observations, the nonsteroidal antiinflammatory
drugs reduced the amount of ¹⁴C-serotonin released by the ionophore A₂₃₁₈₇
at maximum aggregation. Thus it is probable that the formation of prostaglan-
din endoperoxides and thromboxane A₂ contributes to the aggregation and re-
lease responses to this compound.

Conversion of released ADP to ATP with the CP/CPK system (in concen-
trations that blocked aggregation caused by 8 μM ADP) prolonged the lag
phase to a slight extent with thrombin, but this effect was more apparent with
collagen. In no instance, however, did CP/CPK prevent aggregation or release
of ¹⁴C-serotonin. This finding is another indication that collagen, thrombin,
and A₂₃₁₈₇ can cause aggregation that is independent of released ADP. Using
platelets devoid of granule contents, we previously demonstrated that collagen
and A₂₃₁₈₇ can cause aggregation when no ADP is available for release. Sodium arachidonate also causes the degranulated platelets to aggregate be-
cause it gives rise to the prostaglandin endoperoxides and thromboxane A₂.²⁸
It seems likely that in the presence of CP/CPK, or with degranulated platelets,
the labile products formed from platelet arachidonate in response to thrombin,
collagen, or A₂₃₁₈₇ are at least partly responsible for aggregation.¹¹

Combinations of an antiinflammatory drug with the CP/CPK system pro-
longed the lag phase with thrombin or collagen and reduced the extent of re-
lease of ¹⁴C-serotonin with all of the aggregating agents. Only with collagen,
however, (at the low concentrations used), was aggregation completely in-
hibited. This observation may indicate that aggregation of the platelets not
adherent to collagen is primarily mediated through released ADP and the
products formed from platelet arachidonate and that if other mechanisms of
collagen-induced aggregation exist, they are of minor importance. In contrast,
thrombin can cause aggregation through a third mechanism (or mechanisms)
that is apparently independent of released ADP or thromboxane A₂ formation, since the combination of inhibitors was only partially inhibitory.

The ionophore A23187 is thought to act by mobilizing internal platelet calcium. Feinman and Detwiler have suggested that the mechanism of the actual secretion step may be similar for thrombin and A23187. Previously, we have established that this ionophore can act through a mechanism that is independent of released ADP and the formation of products from platelet arachidonate, and the present results are in keeping with this conclusion.

Thus all the agents used can cause aggregation through more than one mechanism, but it should be emphasized that these mechanisms act synergistically with each other. Synergistic effects have been demonstrated with combinations of low concentrations of ADP and thrombin, ADP and collagen, and ADP and arachidonate. If an agent causes the platelets to release ADP, the released ADP increases the effect of other mechanisms that can cause aggregation and the release reaction (e.g., the prostaglandin endoperoxides and thromboxane A₂). With thrombin or A23187, the other mechanism(s) probably acts synergistically with released ADP and the labile compounds formed from platelet arachidonate.

The third mechanism(s) responsible for thrombin-induced aggregation appears to act more rapidly than ADP release or the formation of prostaglandin endoperoxides and thromboxane A₂. This conclusion is based on the observations that (1) lag phases following thrombin addition were shorter than lag phases following collagen, although the mean percentage release at maximum aggregation in the collagen experiments was greater than in the thrombin experiments; and (2) the combination of CP/CPK with indomethacin or acetylsalicylic acid caused only a slight prolongation of the time before thrombin-induced aggregation began, indicating that the third mechanism(s) of thrombin-induced aggregation is dominant.

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Release of 14C-serotonin during initial platelet changes induced by thrombin, collagen, or A23187

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