A Study of the Kinetics of ADP-Triggered Platelet Shape Change

By Roy R. Hantgan

The rapid transformation of human blood platelets from inert discoid cells to spherocytocytes that is induced by adenosine diphosphate (ADP) has been followed by right-angle light scattering intensity measurements using a laser light source and a sensitive photomultiplier. Two steps have been observed, and their rate constants have been determined as a function of pH and [ADP] and in the presence and absence of calcium for both platelet-rich plasma and gel-filtered platelets. Both steps are significantly faster in the presence of physiologic levels of calcium. Platelets were fixed prior to and during activation, then examined by phase-contrast and scanning electron microscopy. The light scattering and morphological changes support a model in which, under physiologic conditions of pH, temperature, ionic strength, and calcium concentration, the initial rapid event in platelet shape change is the loss of discoid shape, with a decay time of two to three seconds, leading to an intermediate with short pseudopods. The slower extension of long pseudopods occurs next, with a time constant of approximately seven to eight seconds. These results may help to resolve the contradictory descriptions of the mechanism of platelet shape change that have recently appeared in the literature.

Human platelets circulate in blood primarily as disc-shaped cells, approximately 3 μm in diameter and 1 μm thick. Following injury to the blood vessel wall and exposure to physiologic activators, such as collagen, adenosine diphosphate (ADP), or thrombin, platelets undergo a rapid change in morphology, leading to an irregularly shaped cell with numerous pseudopodia. Cell–cell interactions, which require calcium and fibrinogen, result in the formation of platelet aggregates, forming a temporary hemostatic plug that is soon reinforced with fibrin fibers.

Platelet shape change has been extensively studied in vitro with platelet-rich plasma under physiologic as well as nonaggregating conditions, ie, in the absence of free calcium or without the rapid stirring that apparently accelerates aggregation by increasing the frequency of collisions between platelets. Changes in morphology have been assessed by light microscopy, as well as by transmission and scanning electron microscopy. These microscopic studies have led to a model of platelet shape change in which the initial event is the extension of short, blunt pseudopods from the periphery of the disc-shaped cell, while the formation of a roughly spherical cell body is thought to occur somewhat later.

Changes in the intensity of light transmitted through a suspension of platelets upon shape change and aggregation form the basis for the platelet aggregometer, which is widely used in clinical and research applications to study platelet function. Recently, this turbidimetric technique has been coupled with a stopped-flow mixing device, and the time course of ADP-triggered platelet shape change has been studied in detail. The observation of an initial increase in turbidity following ADP activation, followed by a decrease in turbidity of comparable rate and magnitude, has led to an alternate kinetic scheme in which the formation of a spherical platelet without pseudopods is postulated to be the first step in shape change, with the formation of pseudopods occurring next.

In this study, a sensitive technique has been developed that allows the measurement of light scattered at right angles to a dilute suspension of platelets that is stirred and thermostatted at 37°C. The time course of ADP-triggered platelet shape change has been studied as a function of pH over the range 6.8 to 7.8 and as a function of the concentration of ADP from 5 × 10^-8 mol/L to 1 × 10^-7 mol/L. Changes in light scattering intensity have been found to occur in two steps: an initial 10% (±3%) decrease in intensity is complete by seven to eight seconds, which is followed by a slower increase in 90° scattering over the next 30 to 60 seconds. The rate constants and their ADP concentration dependence have been determined for both steps over the pH range 6.8 to 7.8 for platelet-rich plasma, and at pH 7.2 for gel-filtered platelets; the effects of Ca^2+ and aggregation have been determined for both systems. Phase-contrast and scanning electron microscopic examination of the time course of activation has aided in the interpretation of the light scattering kinetic data.

Materials and Methods

Adenosine-5'-diphosphate (ADP), grade IX, was purchased from Sigma Chemical Co (St Louis), dissolved in pH 6.8 buffer and stored...
at \(-70^\circ\text{C}\) in aliquots at \(5 \times 10^{-2}\) mol/L. The concentration was determined from the absorbance at 259 nm, using an extinction coefficient of \(1.54 \times 10^3\) mol/L cm. Sepharose 2B was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). All other chemicals were reagent grade. Polyethylene or polypropylene labware was used for all platelet isolation procedures.

**Platelet Isolation**

Forty-three milliliters of whole blood was drawn from healthy adult volunteer donors (who had taken no aspirin for at least two weeks) into 7 mL of acid-citrate-dextrose anticoagulant (0.085 mol/L sodium citrate, 0.071 mol/L citric acid, 0.011 mol/L dextrose, pH 4.5 to 4.8). The procedures employed were fully examined and approved by the Committee on the Protection of the Rights of Human Subjects of the University of North Carolina at Chapel Hill School of Medicine, Edward Bishop, MD, Chairman. Platelet-rich plasma was obtained by centrifugation at 200 g for eight minutes at room temperature in a DuPont HS-4 rotor. The plasma was stored in polyethylene tubes under an atmosphere of 5% CO\(_2/95\%\) air to prevent CO\(_2\) loss and to help maintain the pH of the plasma.

For experiments with platelet-rich plasma, aliquots were (typical) diluted sixfold into 0.13 mol/L NaCl, 0.0026 mol/L KCl, 0.00042 mol/L NaH\(_2\)PO\(_4\), 0.01 mol/L HEPES, 0.0055 mol/L dextrose, and 0.002 mol/L EDTA (HEPES-Tyrode's-EDTA buffer), with pH adjusted to 6.8 with NaOH. The pH of the diluted plasma was then adjusted to 6.8 to 7.8, as required for a particular experiment, by addition of up to 0.1 vol of a buffer containing 0.1 mol/L HEPES, 0.13 mol/L NaCl, 0.0026 mol/L KCl, 0.00042 mol/L NaH\(_2\)PO\(_4\), 0.0055 mol/L dextrose, pH 8.3. The volume of concentrated HEPES-Tyrode's buffer required to achieve a particular pH was determined with samples used only for this purpose, so as to prevent platelet activation by the glass electrode with samples used for light scattering or morphological studies. As the plasma pH tended to rise slightly with time (less than 0.1 U/hr), the volume of concentrated HEPES buffer required to achieve a particular pH was redetermined on a freshly diluted aliquot of platelet-rich plasma at 30- to 45-minute intervals. Samples for light scattering or morphological studies were diluted into the appropriate buffer just before use, incubated for five minutes at 37°C without stirring, then transferred to an aggregometer cuvette. Approximately 30 seconds were allowed to establish a stable baseline, then the platelets were activated by the addition of an ADP solution. All experiments were completed within three hours of the time of venipuncture.

Gel-filtered platelets, prepared according to published procedures, were found to lose their ability to respond to ADP within 30 to 60 minutes of chromatography; therefore, a rapid small-scale procedure was developed to circumvent this difficulty. Polyethylene columns (1.5 \(\times\) 7 cm) were filled with acetone-washed Sepharose 2B and equilibrated with 0.13 mol/L NaCl, 0.0026 mol/L KCl, 0.00042 mol/L NaH\(_2\)PO\(_4\), 0.0055 mol/L dextrose, 0.01 mol/L HEPES, and 3 mg/mL bovine serum albumin (Centex, fraction V), pH 7.2 (HEPES-Tyrode's-albumin buffer). One milliliter of platelet-rich plasma was applied to the column, and the platelets were eluted with the albumin-containing buffer. One-milliliter fractions were collected, and the two tubes containing the maximum platelet count (determined visually) were combined for subsequent experiments; this procedure required approximately 10 to 15 minutes to complete. By repeating this procedure with a fresh plasma sample and a new column at 30- to 45-minute intervals, reproducible results were obtained.

**Platelet Count**

The concentration of platelets in platelet-rich plasma was determined by Coulter counter and used to calculate the concentration in diluted samples. Subsequently, a linear relationship was determined between the 90° scattering intensity and the platelet count for gel-filtered platelets; this calibration curve was used to establish the platelet concentration in experimental samples. Experiments with diluted platelet-rich plasma were carried out at platelet counts in the range 2.8 to 4.5 \(\times\) 10\(^7\)/mL; the data obtained exhibited no dependence on the platelet count over this range. Experiments with gel-filtered platelets verified that the rate constants exhibited no dependence on the platelet count over the range 3.3 \(\times\) 10\(^7\)/mL to 1.44 \(\times\) 10\(^8\)/mL, but most experiments were carried out at platelet counts under 10\(^7\)/mL, as the signal change was found to be greater at these lower cell concentrations, in agreement with the results of Born. Experiments in which the rate constants for platelet shape change were determined with gel-filtered platelets under aggregating conditions were carried out at platelet counts of 2.6 to 2.8 \(\times\) 10\(^7\)/mL.

**Light Scattering Instrumentation**

Kinetic studies of platelet shape change were carried out with a light scattering instrument equipped with a 5-mW He-Ne, 633 nm laser light source (Spectra-Physics model 120, Mountain View, Calif); light was detected at 90° to the incident beam with a photomultiplier tube (Hamamatsu, Middlesex, NJ) and digital photometer (Pacific Photometrics, model 124, Concord, Calif), and the analog output recorded on a Houston Instruments (Y-time, Austin, Tex) flatbed recorder. The cuvette temperature was maintained at 37 ± 0.1°C (measured with a Digilite model 5810 digital thermometer [United Systems Corp, Dayton, Ohio]) and a YSI [Yellow Springs, Ohio] model 702A thermistor probe) with a Haake (Saddlebrook, NJ) D3-G circulating water bath. The instrument was equipped with a magnetic stirring device that maintained a constant stirring rate of 1,100 ± 100 rpm (measured with a Power Instruments [Skokie, III] model 1891 digital tachometer). Samples of diluted platelet-rich plasma or gel-filtered platelets were incubated at 37°C without stirring for five minutes, then 0.5-mL aliquots were transferred to 7-mm diameter siliconized, cylindrical aggregometer cuvettes. After allowing 30 seconds to establish a stable baseline, platelet shape change was triggered by addition of 0.01 mL of activator at various concentrations via an Eppendorf pipette or microliter syringe.

**Data Collection**

The chart paper tracings of scattering intensity \(v\) time were converted to a set of (intensity, time) pairs with a Radio Shack digitizer, with a resolution of 0.025 cm or approximately 0.2% of the total signal change. Typically, 50 to 100 data points were obtained from a 60-second time course. The digitized intensities were normalized by dividing each point by the initial intensity, which was measured prior to any additions. The time required for completion of mixing was determined by addition of 0.01-mL aliquots of buffer to stirred platelet suspensions; mixing times of 1 to 1.5 seconds were determined by this procedure.

**Light Microscopy**

Aliquots of platelet-rich plasma were diluted sixfold with 0.13 mol/L NaCl, 0.0026 mol/L KCl, 0.00042 mol/L NaH\(_2\)PO\(_4\), 0.01 mol/L HEPES, 0.0055 mol/L dextrose, and 0.002 mol/L EDTA and the pH adjusted to 7.0 or 7.8, as required, by the addition of concentrated HEPES-Tyrode's buffer, pH 8.3 to 8.5. Gel-filtered platelets were isolated as previously described. Samples were incubated for five minutes at 37°C, then fixed by the addition of an equal volume of 4% formaldehyde or 4% glutaraldehyde, buffered at pH 7.4 with 0.1 mol/L phosphate, prior to activation or at 7.10, 30, or 60 seconds after activation by saturating ADP concentrations (5 \(\times\) 10\(^{-4}\) mol/L or 1 \(\times\) 10\(^{-3}\) mol/L).
The fixed platelet samples were examined by phase-contrast microscopy with a Leitz (Rockleigh, NJ) Laborlux 11 microscope equipped with a 50× and 100× objective and green filter. Photographs were taken with high resolution Kodak (Rochester, NY) recording film 2415.

**Scanning Electron Microscopy**

Samples of gel-filtered platelets in HEPES-Tyrode's-albumin buffer, pH 7.2, were incubated for five minutes at 37°C, then fixed by the addition of an equal volume of 2.5% glutaraldehyde buffered at pH 7.2 with 0.1 mol/L phosphate. Incubation with the fixative was continued for at least 30 minutes at 37°C, then the fixed samples were treated with phosphate-buffered 1% osmium tetroxide, dehydrated through a graded alcohol series, and dried from CO₂ by the critical point method. The dried samples were sputter coated with gold:palladium (60:40) and observed at 15 kV in a Philips (Mahwah, NJ) EM 507 scanning microscope.

**RESULTS**

**Light Scattering Kinetic Experiments**

Figure 1 shows typical time-dependent changes in the 90° scattering intensity, which result from the addition of 0.01-mL aliquots of buffer (upper curve) or ADP (final concentration 5.6 x 10⁻⁶ mol/L) to stirred thermostatted (37°C) suspensions of platelet-rich plasma, diluted to a platelet count of 6 x 10⁹/mL with HEPES-Tyrode’s buffer, pH 7.4, containing 2.0 x 10⁻³ mol/L EDTA. The points were obtained by digitizing the chart paper recordings, which are shown as an inset to Fig 1. The addition of buffer results in a rapid drop in intensity to a plateau value close to that calculated by correcting the initial scattering intensity for the subsequent 2% dilution. Addition of the platelet activator, ADP, causes a further decrease of intensity, which reaches a minimum seven to eight seconds later and is followed by a slower increase to an intensity comparable to that caused by dilution alone.

**Data Analysis**

The following generalized reaction sequence can be employed to analyze the kinetics of platelet shape change:

\[
P_0 + \text{ADP} \xrightarrow{k_1} P^*_0 \xrightarrow{k_2} P_1 \xrightarrow{k_3} P_2
\]

\[P_0\] is the resting cell, \(P^*_0\) has bound ADP, \(P_1\) is an intermediate, and \(P_2\) is the fully activated platelet. Frojmovic and Milton and Milton et al have analyzed the initial rate of increased turbidity during ADP-triggered shape change in terms of a similar model and have found this parameter to be a saturable function of the activator concentration. Deranleau et al have determined \(k_2\) and \(k_3\) from the two-step turbidity changes observed at saturating ADP concentrations by fitting their data to the two-exponential model that results from this scheme.

The data presented in Fig 1 are representative of the scattering changes that have been observed at saturating ADP concentrations, ie, in the range 5 x 10⁻⁷ mol/L to 1 x 10⁻⁴ mol/L. The data have been analyzed with a nonlinear least-squares procedure, based on the method of Marquardt in order to determine the kinetic parameters for platelet shape change by the following equation:

\[\frac{I(t) - I(t_m)}{I(0)} = I(1)[e^{-k_2(t-t_m)} - 1] + I(2)[1 - e^{-k_3(t-t_m)}] \quad (2)\]

In this equation, the rate constants, \(k_2\) and \(k_3\), and the amplitudes, \(I(1)\), \(I(2)\), are variables to be determined by curve fitting; \(t_m\) is defined as the time at which mixing is complete; \(I(t_m)\) is the corresponding intensity; and \(I(0)\) is a normalization constant, the initial intensity. The changes in scattering intensity due to dilution alone are rapid, compared to the subsequent morphological events; therefore points obtained during the mixing time, one to two seconds were omitted from the analysis. The validity of this approach is confirmed by the observation that the fitted parameters are relatively insensitive to the choice of \(t_m\). The solid line in Fig 1 was calculated with \(k_2 = 0.249 \text{ sec}^{-1}\), \(k_3 = 0.095 \text{ sec}^{-1}\), \(I(1) = 0.122\), and \(I(2) = 0.136\) — values obtained by the least-squares analysis. The agreement between the fitted line and the experimental approach is quite good, indicating that a suitable model has been chosen. Note that the biphasic changes in scattering...
require a model with at least two rate constants and two amplitude parameters. (For each kinetic experiment, fits were carried out with at least two values of \( t_m \) chosen between one and two seconds and the parameters averaged; the uncertainty in the rate constants derived in this manner was typically 5% to 15%.)

**Dependence of the Kinetic Parameters on Solution Conditions**

**ADP concentration.** The data presented in Fig 2 were obtained in a series of experiments in which separate aliquots of platelet-rich plasma (obtained from one donor on a single occasion) were diluted into HEPES-Tyrode’s-EDTA buffer, pH 7.2. Each aliquot of diluted platelet-rich plasma was activated with a specific concentration of ADP, and the time course of platelet shape change was measured as previously described. Each trace was digitized, and the data points fit to equation 2 with the nonlinear least squares fitting the data to equation 2, as described in the text. (Inset) Dependence of the rate constants \( k_2 \) and \( k_3 \), as well as the corresponding amplitude parameters \([I(1)]\) and \([I(2)]\). The solid lines were then calculated from these four parameters and equation 2.

The bottom curve in Fig 2 (circles) was obtained with a saturating ADP concentration, and the other two curves (squares and triangles) were obtained with lower, ie, rate-limiting, activator concentrations. In each case, the data are well described by the two-exponential model. It is interesting to note, however, that the apparent first-order rate constant, \( k_3 \), is a function of the ADP concentration, whereas \( k_1 \) was found to be constant over the 2,000-fold range of concentrations studied.

This behavior follows from the model presented in equation 1, which involves the reversible formation of a platelet–ADP complex prior to any overall morphological changes. The similarity of this scheme to receptor–effector interactions and the Michaelis-Menten kinetic scheme, employed by Milton et al to study the initial rates of ADP-triggered platelet shape change,\(^{12}\) suggests that this concentration dependence can be quantitated by fitting the rate constants \([k_2]_0\) obtained as a function of the ADP concentration to this equation:

\[
k_3(\text{ADP}) = k_3(\text{max}) \cdot \frac{[\text{ADP}]/([\text{ADP}]_{1/2} + [\text{ADP}])}{1}
\]

The inset to Fig 2 shows the results of this procedure, in which the rate constants measured in three complete ADP concentration-dependence studies (each carried out with plasma drawn from a separate donor) at pH 7.2 were combined into one data set and plotted vs the [ADP], on a logarithmic concentration scale. A nonlinear least-squares curve-fitting procedure was employed to determine the parameters \( k_3(\text{max}) \) and \([\text{ADP}]_{1/2}\), the [ADP] at which \( k_2 \) is one-half maximum. The solid line in the inset figure was calculated from these parameters and equation 3.

As \( k_3 \) was observed to be independent of the [ADP], within experimental error, over the concentration range \( 5 \times 10^{-8} \) to \( 1 \times 10^{-4} \) mol/L, the values of \( k_3 \) were simply averaged. The dashed line drawn through the squares corresponds to this average value. These observations place an upper bound of \( 5 \times 10^{-8} \) mol/L on the [ADP]_{1/2} for this step.

The relevant kinetic parameters are cited in the figure legend, and the values obtained in these, and a number of other experiments, are summarized in Table 1. The values of \( k_3(\text{max}) \) are well determined by the curve-fitting procedure (standard deviation typically 5% to 10%), but the value of \([\text{ADP}]_{1/2}\) is subject to a larger error (30% to 50%), probably due to the smaller signal changes observed at the lower activator concentrations. The uncertainty in the average value of \( k_3 \) is typically 20% to 30%.

**pH**

The rate constants \( k_1 \) and \( k_2 \) and their dependence on the ADP concentration were determined as a function of pH over the range 6.8 to 7.8; the data are listed in Table 1. Each set of four kinetic parameters in that

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dependence study; the rate constants $k_2$ have been plotted on a logarithmic scale. The solid line was determined by fitting the data for equation 2, as described in the text. (Inset) Dependence of the rate constants $k_2$ (circles) and $k_3$ (squares) on the concentration of ADP. The solid lines were determined by fitting the data for equation 2, as described in the text. (Inset) Dependence of the rate constants $k_2$ (circles) and $k_3$ (squares) on the concentration of ADP, logarithmic scale. The data were obtained with gel-filtered platelets at a count of 3 x 10^7/mL in HEPS-Tyrode's-albumin buffer, pH 7.2. The solid line corresponds to an average value of $k_2 = 0.105 \pm 0.002$ sec$^{-1}$. The dashed line corresponds to an average value of $k_1$. The kinetic parameters measured in three experiments carried out with gel-filtered platelets (obtained from separate donors) are cited in Table 2. The kinetic parameters measured with gel-filtered platelets at pH 7.2 in the absence of externally added calcium are in excellent agreement with those values determined with platelet-rich plasma in HEPS-Tyrode's-EDTA buffer over the pH range 6.8 to 7.8. This confirms that brief exposure to EDTA did not affect the results obtained with platelet-rich plasma.

**Method of Platelet Isolation**

Platelets have been isolated from plasma by a rapid, small-scale gel-filtration procedure in HEPS-Tyrode's buffer containing a low albumin concentration. These preparations are substantially free of calcium and fibrinogen, which are both required cofactors for platelet aggregation. Therefore, platelet shape change can be studied separately from the aggregation response without the necessity of chelation of the calcium with EDTA, which has been reported to interfere with measurements of platelet aggregation (but not shape change) and to cause partial blocking of the platelet volume changes induced by ADP. A representative data set, obtained with gel-filtered platelets isolated from blood obtained from a single donor, is presented in Fig 3. This figure depicts three kinetic experiments in which gel-filtered platelets were activated with increasing concentrations of ADP; the solid lines were determined by the previously described curve-fitting procedure in which the digitized data points were fit to equation 2. The inset to Fig 3 shows the results of a complete activator concentration-dependence study; the rate constants $k_2$ and $k_3$ have been plotted vs the ADP concentration (logarithmic scale). The solid line was determined by fitting $k_2$ to equation 3, whereas the dashed line corresponds to an average value of $k_1$. The kinetic parameters measured in three experiments carried out with gel-filtered platelets (obtained from separate donors) are cited in Table 2. The kinetic parameters measured with gel-filtered platelets at pH 7.2 in the absence of externally added calcium are in excellent agreement with those values determined with platelet-rich plasma in HEPS-Tyrode's-EDTA buffer over the pH range 6.8 to 7.8. This confirms that brief exposure to EDTA did not affect the results obtained with platelet-rich plasma.

**Table 1. Kinetic Parameters Measured With Platelet-Rich Plasma Diluted Into HEPS-Tyrode's-EDTA Buffer at the pH Indicated**

<table>
<thead>
<tr>
<th>Donor No.</th>
<th>pH</th>
<th>$k_2$ (sec$^{-1}$)</th>
<th>$[ADP]_{1/2}$ (mol/L)</th>
<th>$k_3$ (sec$^{-1}$)</th>
<th>$[ADP]_{1/2}$ (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.8</td>
<td>0.325</td>
<td>1.2 x 10$^{-7}$</td>
<td>0.14</td>
<td>&lt; 5 x 10$^{-8}$</td>
</tr>
<tr>
<td>2</td>
<td>7.0</td>
<td>0.301</td>
<td>&lt; 10$^{-7}$</td>
<td>0.069</td>
<td>&lt; 5 x 10$^{-8}$</td>
</tr>
<tr>
<td>3</td>
<td>7.2</td>
<td>0.306</td>
<td>9.4 x 10$^{-8}$</td>
<td>0.11</td>
<td>&lt; 5 x 10$^{-8}$</td>
</tr>
<tr>
<td>4</td>
<td>7.2</td>
<td>0.309</td>
<td>1.0 x 10$^{-7}$</td>
<td>0.096</td>
<td>&lt; 5 x 10$^{-8}$</td>
</tr>
<tr>
<td>5</td>
<td>7.2</td>
<td>0.298</td>
<td>8.6 x 10$^{-8}$</td>
<td>0.088</td>
<td>&lt; 5 x 10$^{-8}$</td>
</tr>
<tr>
<td>6</td>
<td>7.4</td>
<td>0.266</td>
<td>9.5 x 10$^{-8}$</td>
<td>0.10</td>
<td>&lt; 5 x 10$^{-8}$</td>
</tr>
<tr>
<td>7</td>
<td>7.8</td>
<td>0.293</td>
<td>&lt; 10$^{-7}$</td>
<td>0.093</td>
<td>&lt; 5 x 10$^{-8}$</td>
</tr>
</tbody>
</table>

The kinetic parameters cited here were determined by fitting the rate constants $k_2$ and $k_3$, which had been measured as a function of ADP concentration at constant pH, equation 3. As described in the text, this procedure yields values of the maximum rate constant (at saturating activator concentrations) as well as $[ADP]_{1/2}$, the concentration at which the rate constant reaches one half its maximum value. Each individual row represents data obtained with platelets from a single donor (identified by the donor number), buffered at a particular pH, with rates determined at five to nine ADP concentrations ranging from 5 x 10$^{-7}$ mol/L to 1 x 10$^{-4}$ mol/L. Multiple entries at pH 7.2 and 7.4 are included to show the precision of the data. As the values of $k_2$ exhibited no dependence on the [ADP], only an upper bound can be placed on the [ADP]$_{1/2}$ for this step.

**Table 2. Kinetic Parameters Measured With Gel-Filtered Platelets in HEPES-Tyrode's-Albumin Buffer**

<table>
<thead>
<tr>
<th>Donor No.</th>
<th>pH</th>
<th>$k_2$ (sec$^{-1}$)</th>
<th>$[ADP]_{1/2}$ (mol/L)</th>
<th>$k_3$ (sec$^{-1}$)</th>
<th>$[ADP]_{1/2}$ (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>7.2</td>
<td>0.287</td>
<td>3.8 x 10$^{-8}$</td>
<td>0.11</td>
<td>&lt; 5 x 10$^{-8}$</td>
</tr>
<tr>
<td>4</td>
<td>7.2</td>
<td>0.305</td>
<td>3.9 x 10$^{-8}$</td>
<td>0.10</td>
<td>&lt; 5 x 10$^{-8}$</td>
</tr>
<tr>
<td>5</td>
<td>7.2</td>
<td>0.172</td>
<td>9.4 x 10$^{-8}$</td>
<td>0.071</td>
<td>&lt; 5 x 10$^{-8}$</td>
</tr>
</tbody>
</table>

Kinetic parameters determined with gel-filtered platelets from three donors (identified by the donor number), buffered at pH 7.2, by the procedure described in the text and in the legend to Table 1. Multiple entries are included to show the precision of the data. The resultant parameters agree, within experimental error, with those cited in Table 1 for diluted platelet-rich plasma.
characteristic two-step changes in right-angle light scattering intensity that have been described for the calcium-free systems. ADP stimulation in the presence of both calcium and fibrinogen resulted in shape change, followed by aggregation, as evidenced by the light scattering kinetic data presented in Fig 4. In the absence of aggregation, the kinetic parameters for platelet shape change were determined by fitting the data to equation 2, as previously described. The presence of aggregation complicates the kinetic analysis; however, to a first approximation, the rate constants for platelet shape change could be determined by fitting only the data obtained during the first 15 to 20 seconds following ADP activation. As can be seen in Fig 4, the fitted line determined in this manner shows good agreement with the experimental points, up to the onset of aggregation.

When platelet-rich plasma was diluted into HEPES-Tyrode’s buffer at pH 6.8 or 7.2 (in the absence of EDTA) or the same buffer containing 2 mmol/L Ca<sup>2+</sup> at pH 7.2 to 7.4, and the platelets stimulated by ADP, a similar pattern of platelet shape change and aggregation was observed.

The rate constants $k_2$ and $k_3$ are summarized in Table 3 for both gel-filtered platelets and platelet-rich plasma under aggregating and nonaggregating conditions. The results demonstrate that Ca<sup>2+</sup> alone causes an approximately 1.5-fold increase in the values of $k_2$ and a smaller increase in the value of $k_3$ for gel-filtered platelets. Similar values were obtained in the presence of both calcium and fibrinogen, although the data display less precision, due to the necessity to extract the shape change signal from the larger, but slower, scattering changes due to aggregation. Moderate increases were found for both rate constants with platelet-rich plasma in buffer alone, and in buffer containing 2 mmol/L Ca<sup>2+</sup>.

Phase-Contrast Microscopy

Phase-contrast microscopy has been employed to establish a correlation between the light scattering kinetic data and the sequence of morphological steps in

Table 3. Kinetic Parameters Measured With Gel-Filtered Platelets and Platelet-Rich Plasma in the Presence of Ca<sup>2+</sup> or Ca<sup>2+</sup> Plus Fibrinogen

<table>
<thead>
<tr>
<th>Buffer Conditions</th>
<th>$k_2$ (sec&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>$k_3$ (sec&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel-filtered platelets, pH 7.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES-Tyrode’s-albumin buffer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer with $2 \times 10^{-3}$ mol/L Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.288 ± 0.048</td>
<td>0.110 ± 0.031</td>
<td>No</td>
</tr>
<tr>
<td>Buffer with $2 \times 10^{-3}$ mol/L Ca&lt;sup&gt;2+&lt;/sup&gt; and $4 \times 10^{-8}$ mol/L fibrinogen</td>
<td>0.418 ± 0.058</td>
<td>0.129 ± 0.053</td>
<td>No</td>
</tr>
<tr>
<td>Platelet-rich plasma diluted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES-Tyrode’s buffer, pH 7.2 to 7.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES-Tyrode’s buffer with $2 \times 10^{-3}$ mol/L EDTA</td>
<td>0.279 ± 0.039</td>
<td>0.099 ± 0.008</td>
<td>No</td>
</tr>
<tr>
<td>HEPES-Tyrode’s buffer</td>
<td>0.501</td>
<td>0.168</td>
<td>Yes</td>
</tr>
<tr>
<td>HEPES-Tyrode’s buffer with $2 \times 10^{-3}$ mol/L Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.433 ± 0.066</td>
<td>0.194 ± 0.026</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Platelets were stimulated with saturating concentrations of ADP ($7.4 \times 10^{-5}$ mol/L) and the changes in 90° scattering followed. The data were digitized and fitted to equation 2 to determine the rate constants $k_2$ and $k_3$, ad described in the text. The values cited are (with one exception) averages of three to six determinations, with the standard deviations listed. The experiment with platelet-rich plasma diluted into buffer with no additions was repeated at pH 6.8 with similar results.
platelet shape change. Experiments carried out at pH 7.0 and 7.8 with platelet-rich plasma and at pH 7.2 with gel-filtered platelets, activated with saturating ADP concentrations then fixed with either formaldehyde or glutaraldehyde, yielded comparable results. The results of Milton et al.\(^2\) indicate that this fixation procedure rapidly and irreversibly blocks further morphological changes in partially activated platelets. Representative photomicrographs of samples fixed with formaldehyde (a) prior to, (b) seven to ten seconds, and (c) 30 to 60 seconds following addition of ADP are shown in Fig 5 for experiments carried out at pH 7.0 and 7.8. Platelets fixed at \(t = 0\) usually exhibited the discoid shape characteristic of resting platelets, although some cells exhibited one or two long pseudopodia extending from a disc-shaped cell body. Samples fixed at the minimum in the scattering curve (seven to ten seconds), displayed a more irregular shape characterized by a ruffled surface with short blunt pseudopods. By 30 to 60 seconds, when the scattering intensity is nearing a plateau, longer pseudopods were considerably more frequent. Disc-shaped cells with both blunt and extended pseudopods were also observed at these time points.

**Scanning Electron Microscopy**

Samples of gel-filtered platelets in HEPES-Tyrode's-albumin buffer, pH 7.2, were fixed with glutaraldehyde prior to ADP stimulation \((t = 0)\) and at seven and 60 seconds following activation, then processed for scanning electron microscopy. As illustrated by the micrographs in Fig 6A, the majority of the unstimulated cells exhibited a smooth discoid shape, although some activated platelets were present. Substantial morphological changes have occurred at the earliest time point examined. As illustrated in Fig 6B, seven seconds after addition of ADP, the surface of most of the cells was no longer smooth, but was ruffled and irregular with the presence of numerous pseudopods. Comparison to micrographs of samples fixed at 60 seconds following activation (Fig 6C) shows that the pseudopods continued to grow during this period and that discoechinocytes as well as spherocinocytes were present. Measurements of pseudopod length from 77 individual cells fixed at seven seconds indicated an average value of 0.98 ± 0.43 \(\mu\); similar analysis of 74 platelets fixed at 60 seconds after activation yielded an average pseudopod length of 1.47 ± 0.54 \(\mu\). This difference, which is statistically significant \((P < .005)\), confirms the phase-contrast microscopy result that pseudopod elongation continues during the period from seven to ten seconds to 60 seconds following ADP activation.

**DISCUSSION**

The simplest kinetic scheme consistent with the light scattering and phase-contrast microscopic data obtained in this study is the following:

\[
D + ADP \stackrel{k_1}{\longrightarrow} D^* \stackrel{k_2}{\longrightarrow} I \stackrel{k_3}{\longrightarrow} SE
\]

\(D\) is the discocyte or disc-shaped cell, prior to activation; \(I\) is an intermediate, observed by phase-contrast and scanning electron microscopy of samples fixed at the minimum in the scattering intensity \(v\) time curve,
to exhibit an irregular shape with short pseudopods; and SE is the spherocynocyte or spiny sphere that results from exposure to ADP.1

The rate constants $k_2$ and $k_3$, and their pH, ADP, and calcium concentration dependence have been determined in this study with a sensitive, noninvasive light scattering technique. In addition, a rapid, reproducible, small-scale gel-filtration procedure has been developed that has enabled this investigator to measure the kinetic parameters for platelet shape change with gel-filtered platelets. The agreement between the data obtained with platelet-rich plasma and that with gel-filtered platelets is good, confirming the validity of the experimental approach.

The results indicate that under physiologic conditions of pH, temperature, ionic strength, and calcium ion concentration, platelets activated with concentrations of ADP in excess of $5 \times 10^{-7}$ mol/L will lose their discoid shape with an exponential decay time, $r$, of two to three seconds and form spherocynocytes with a time constant of seven to eight seconds. The data obtained by light scattering measurements indicate that five seconds after stimulation, nearly 90% of the platelets will have lost their discoid shape to form partially activated intermediates and that almost 50% of the cells will be present as fully activated spherocynocytes. Although both steps will be essentially complete by 15 seconds, at which time the light scattering signal is dominated by aggregation, it may well be that aggregation begins with the partially activated platelets and that long pseudopods are not essential. The data presented here have also demonstrated that extracellular calcium plays a role in platelet shape change as well as aggregation, as both loss of discoid shape and pseudopod formation have been found to be significantly faster in the presence of physiologic levels of calcium.

These observations are especially interesting in light of recent publications by Gear16,34 and Milton and Frojmovic35 concerning the rates of platelet activation and aggregation. Gear has employed scanning electron microscopy and single particle counting, coupled with quenched flow techniques, to determine the time course of ADP-induced platelet shape change and aggregation. His data indicate that platelet activation is substantially complete by two seconds after exposure to ADP,16 and that after five seconds, more than 50% of the platelets will have formed aggregates.34 However, Milton and Frojmovic have presented data based on phase-contrast microscopy that show a maximum concentration of partially activated discocynocytes at ten seconds after exposure to ADP, and a slower increase in the fraction of fully activated spherocynocytes over a 60-second period.35 These authors have also concluded that platelet aggregation is significantly faster than conventional platelet aggregometry measurements indicate.35

The data obtained in this study further indicate that the concentration of ADP yielding one-half maximum rates of activation is (approximately) $1 \times 10^{-7}$ mol/L, in reasonable agreement with literature values derived from turbidimetric12,23 and microscopic studies of platelet shape change. This value of [ADP]$_{1/2}$ is comparable to the dissociation constant, $K_d = 1.5 \times 10^{-3}$, measured by Nachman and Ferris36 for the binding of ADP to isolated platelet membranes. Both parameters are related to the ratio of $k_{-1}/k_1$, although the former is a kinetic and the latter an equilibrium result.

Further interpretation of the results requires a detailed consideration of the assignment of the initial
rapid decrease and subsequent slower increase in (90°)
light scattering intensity to specific morphological
changes. Although an initial decrease in light scatter-
ing intensity during ADP-triggered platelet activation
has been previously reported,21,37 most literature
reports have concentrated on the increased turbidity
that occurs under similar experimental condi-
tions.12,21,22,24,25,29,30 In addition, Stolz39 has reported
that the magnitude of the changes in light scattering
intensity during ADP-induced aggregation of platelet-
rich plasma exhibits a complex dependence on the
angle of observation. This initial increase in turbidity
during shape change has frequently been interpreted
as evidence of a change from a disc (or cylindrical) cell
to a spherical shape.25,38,40 For particles with dimen-
sions small compared to the wavelength of scattered
light, such a disc to sphere transition should increase
both the turbidity and the scattering intensity at all
angles. A consideration of the form of the angular
dependence of the scattering curve for larger particles
may help to resolve this problem.

\[ I(\theta) = \left( \frac{k^4 V^2}{4 \pi^2} \right) (\Delta n)^2 P(\theta) \]  

(5)

The intensity of light reaching the detector, \( I(\theta) \), is a
function of the wave number, \( k = 2 \pi n / \lambda \), \( V \) (the
particle volume), \( \Delta n \), the refractive index of the par-
ticle relative to its surroundings, and \( P(\theta) \), the particle
scattering function (averaged over all orientations of
the particle, relative to the primary beam), which
depends on the size and shape of the particle as well as
the angle of observation \( (\theta) \).38,40

Calculation of \( I(\theta) \), even for the resting discoid
platelet, is complicated by several factors. First, esti-
mation of the true platelet volume from, eg, phase-
contrast microscopic data, is ambiguous due to diffrac-
tion effects and variations in platelet size.1 Second, the
distribution of scattering mass within the platelet is not
uniform, which presents a problem in calculating the
true \( \Delta n \). Finally, calculations of \( P(\theta) \) is not straightfor-
dward for particles the size of platelets, the dimensions
of which exceed the wavelength of scattered light.
Deviations from a regular geometric shape add to the
complexity of the problem.

Latimer41 and Latimer et al32 have modeled the
resting platelet as an oblate ellipsoid, considered orient-
tation effects caused by stirring, and predicted an
increase in turbidity for the disc to sphere transition, as
well as a smaller decrease due to pseudopod formation.
However, the scattering curves presented by Chang
and Robertson,38 in which the platelet is modeled as
either a (randomly oriented) cylinder (radius = 1.2
\( \mu m \), length = 1.46 \( \mu m \)) or a sphere (radius = 1.6 \( \mu m \)),
show a series of deep minima at different positions for
the two models. This suggests that a transition from a
cylinder to a sphere could result in a uniform increase
of turbidity and an increase of scattering at low angles
(\( \theta < 15^\circ \)), but a decrease in scattering could result at
some higher angles, where the two calculated scatter-
ing curves cross repeatedly.

The disc → sphere → spiny sphere model of platelet
shape change25 may not adequately account for the
more subtle changes in platelet morphology observed
by light2,11–13 and electron microscopic tech-
niques.5,13,14,16,17 The principal difficulty concerns the
nature of the intermediate, observed seven to ten
seconds after ADP stimulation (the minimum in the
90° scattering kinetic curves). The partially activated
platelets in Figs 5, B and E (phase-contrast micro-
scopy), and 6B (scanning electron microscopy) appear
to fit the definition of the discoechinocyte observed by
Frojmovic and Milton1 as the intermediate on the
activation pathway. The presence of an irregular sur-
face with short blunt pseudopods probably contributes
to the complexity of the scattering and turbidity
changes.

The presence of longer pseudopods and an irregular
cell shape (Figs 5, C and F, and 6C) near the plateau in
scattering intensity (30 to 60 seconds after activation)
is also consistent with the report by Frojmovic and
Milton1 of a maximum concentration of spheroechin-
cytes at the later stages of shape change. Again,
devising a model that can predict the changes in
scattering and turbidity is complex, as the formation
of a spherical body is expected to cause an increase in
turbidity and scattering, while a decreased intensity
should result from the formation of long pseudo-
ods.38,41,42

Resolution of this problem has not been possible
with existing light scattering instrumentation. An
attempt to measure the angular dependence of the
intensity of light scattered from resting, partially acti-
ated, and fully activated platelet samples yielded
ambiguous results, probably due to variations in the
platelet count in the three samples. A more sophisti-
cated approach would be to obtain simultaneous
recordings of the intensity at multiple scattering angles
in order to extract the information available in the
scattering envelope.

Deranleau et al33 have reported two first-order rate
constants, both equal to 0.16 sec−1, for the two steps in
platelet shape change, based on an analysis of their
stopped-flow turbidity data. Here, a two-exponential
model of the same form has been employed to deter-
mine the rate constants for the 90° scattering changes.
The parameters summarized in Table 3 are signifi-
cantly different from their values. Whether this is a true difference resulting from the different experimental approaches employed, or a difference in curve-fitting routines remains to be resolved. Based on the observation of a single rate constant, Deranleau et al.\textsuperscript{25} have suggested that a single rate-limiting step controls platelet shape change. The observations presented here, of two distinct steps with different rate constants, indicate that the situation is more complex and that a more detailed understanding of a number of biochemical processes, including calcium uptake, actin polymerization, microtubule assembly/disassembly, and intracellular viscosity changes, is required in order to accurately model the transition of the resting platelet to a hemostatically active spherocinocyte.

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RR Hantgan