Mechanism of Erythrocyte Aggregation and Sedimentation

By Thomas L. Fabry

Unstirred suspensions of erythrocytes form stable spherical aggregates of uniform size. The radius of the spheres depends upon the suspending medium and the hematocrit. Erythrocyte suspensions will undergo sedimentation only after these aggregates are formed. Aggregation is a two-step process: first, erythrocytes associate in long chains (rouleau formation). Next, these chains form spheres of uniform size. The requirements for this well-defined process are an electrolyte and a neutral or negatively charged macromolecule in the solution and a metabolically active red cell. If these conditions are not met, red cells either will not aggregate at all or will form amorphous aggregates. Rouleau formation and sedimentation are inhibited by 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, an inhibitor of anion transport, but not by ouabain, a cation transport inhibitor. The kinetics of erythrocyte sedimentation reflects the aforementioned mechanism: no sedimentation occurs during rouleau formation. Once the spheres of uniform size are formed, they will settle according to the Einstein-Stokes equation. In this model, parameters of sedimentation kinetics are the delay before sedimentation starts, the rate of sedimentation in the steady state, and the radius of the sedimenting aggregate. The radius can be calculated from the rate of fall of the aggregates and agrees well with the microscopically observed radius. It is inversely proportional to the hematocrit, which explains the elevated sedimentation rates in anemia.

THE ERYTHROCYTE SEDIMENTATION RATE (ESR) is a useful qualitative empirical index of nonspecific disease activity.1 Its clinical usefulness is, however, limited by three major problems: (a) quantitative interpretation is not possible, (b) there is a poor correlation with measurements of other acute phase reactants, and (c) it is paradoxically low in several diseases (eg, certain forms of inflammatory bowel disease,2 sickle cell disease,3 and possibly in congestive heart failure4). Increased ESR has been attributed to increased amounts of some plasma proteins (fibrinogen,5 etc), but again there is only very poor quantitative correlation. Expressing the sedimentation rate as the amount of sedimentation at one hour is only meaningful if this process occurs uniformly over time. Any deviation from zero-order kinetics means that more than one parameter must be used to describe the process.

Chien6 laid the foundation for interpreting the rheology of red cell suspensions. He established the need for electrolytes and macromolecules to be present in the suspending medium for rouleau formation to occur. Perelson and Wiegel,7 using techniques of statistical mechanics, showed that the average rouleau size increases as the adhesion energy between erythrocytes increases. Samsel and Perelson8 developed an elegant kinetic model for rouleau formation and pointed out that as rouleaux grow large they form rings and branching aggregates. Evans and Buxbaum9 and Buxbaum et al10 quantified the surface affinities of erythrocyte membranes for macromolecules and related them to aggregation.

Quantitative interpretation of the sedimentation rate is possible by developing a kinetic model based on the underlying mechanism. This may make erythrocyte sedimentation a useful tool for investigating the pathophysiology of some diseases. Furthermore, quantitative interpretation of sedimentation may provide an inexpensive screening test of plasma protein and erythrocyte membrane abnormalities in some less common disorders.

MATERIALS AND METHODS

Blood specimens anticoagulated with K3EDTA were obtained from healthy volunteers and were processed immediately after collection. Red cells were suspended in autologous plasma to obtain different final hematocrit values (Hct). For the inhibition experiments, washed red cells were incubated with 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) (0.1 mg/mL) for 30 minutes at 37°C. This is the same concentration of DIDS that is used to inhibit the chloride/bicarbonate antiporter of the erythrocyte membrane. After incubation the cells were washed with isotonic saline containing 10 mmol/L glucose and then reconstituted with plasma.

Ouabain (Sigma Chemical Co, St Louis) was dissolved in water (0.01 mol/L stock solution; final concentration of incubation mixtures, 0.1 mmol/L). DIDS was prepared in HEPES buffer immediately before use. Other chemicals were obtained from standard sources.

Sedimentation experiments were performed in Kimble disposable 200-mm Westergren tubes at pH 7.4 and at 25 ± 2°C. Experiments were completed within three hours of sample preparation to avoid artifacts from irreversible aggregation of red cells. To obtain the photomicrographs, erythrocyte suspensions were allowed to sediment in rectangular cross-section tubes 0.2 × 5 or 0.3 × 5 mm and 100 mm long (Vitro Dynamics, Rockaway, NJ). The kinetics of sedimentation in this tube is the same as in a Westergren tube filled to 100 mm. The tubes were placed flat side down on the microscope stage in different phases of the sedimentation, and photographs were taken at 4× and 10× magnification.

Curve fitting and statistical calculations were carried out by using the computer programs SAS11 and Statgraphics.12 The Einstein-Stokes equation was used as derived by Hiemenz.13

RESULTS

Kinetics of erythrocyte sedimentation and the effect of Hct. Erythrocyte sedimentation follows an S-shaped curve with time (Fig 1 and Table 1). There is a delay in the start of sedimentation; during this time the rate of sedimentation is
zero (phase 1 of the curve). The next segment (phase 2) is linear; sedimentation continues at a constant rate. Finally, in phase 3 the rate levels off to zero; sedimentation is completed.

At different red cell concentrations (Hct range, 10 to 50) the shape of the curve is qualitatively the same; however, there is a shift to the right as the Hct increases (Fig 1): the delay (phase 1) increases and the slope in phase 2 decreases. This results in slower sedimentation at a higher Hct value.

**Effect of membrane transport inhibitors on sedimentation.** DIDS, an irreversible inhibitor of anion transport, inhibits both rouleau formation and sedimentation in a dose-dependent manner. Ouabain (0.1 mmol/L) has no effect on the sedimentation rate. Table 2 shows the slope of the sedimentation curve and the ESR as a function of inhibitor concentration.

**Direct observation of aggregation during sedimentation.** In phase 1 the homogeneous red cell suspension forms a three-dimensional network (Fig 2) that, late in this phase, rearranges to form spheres of equal size. This is a sudden change. It can be observed with the naked eye in the Westergren sedimentation tube: the homogeneous cell suspension suddenly becomes granular in appearance, with the spheres visible at the sides of the tube. In phase 2 these spheres sediment at a steady rate (Fig 3). Figure 4 is a photomicrograph of the interface between the sedimented cells and the plasma. The sedimenting entities are the spheres formed from the rouleaux. The extent of aggregation does not change during phase 2. To demonstrate that the red cells form fairly stable aggregates during the steady phase of sedimentation (ie, after the delay period) the ESR tube was gently inverted (ie, turned upside down). Sedimentation continued at the same steady rate without a second delay period.

**DISCUSSION**

The delay in red cell suspension sedimentation suggests either aggregation (ie, red cells will not settle until aggregates of sufficient size are formed) or cooperatively (ie, the rate constant for the sedimentation increases with time). Red cell sedimentation kinetics is not compatible with either. Simple aggregation would require the sedimentation rate to increase with an increasing concentration of erythrocytes (Hct); in fact, the opposite occurs. As shown in the Appendix, the equation describing sedimentation kinetics does not fulfill the criteria for cooperativity.

**Aggregation of red cells to form uniform spheres.** The mechanism of rouleau formation has been extensively investigated. Our data allow further elaboration of the mechanism of aggregation.

Red cells repel each other because of their negative surface charge from sialic acid residues. At the same time they are attracted by the van der Waals forces, which are electrodynamic in nature. The balance of these two forces determines the most stable arrangement of red cells in an electrolyte solution in the absence of other forces. Assuming a discoid shape for red cells, van Oss and Absolom calculated that at an intercellular distance of 103 nm there is a minimum of $-4.6 \times 10^{-16}$ ergs/cell in the potential energy distribution of erythrocytes suspended in isotonic saline. The distance of the closest approach for erythrocytes increases with decreasing electrolyte concentration because the repulsive force ($\hat{F}$ potential) increases with decreasing ionic strength and the van der Waals forces are unchanged. (This explains why there is no rouleau formation in the absence of electrolytes, eg, in sucrose solutions.) Therefore, in the presence of electrolytes, the most stable configuration of erythrocyte suspensions is a chain of cells attached by their flat surfaces. However, this energy is small relative to the energy from Brownian motion ($kT$); therefore, cells would align only transiently but would not form stable structures. For this reason red cells will not form rouleaux if the suspending solution contains electrolytes only.

For rouleau formation to occur, macromolecules must be present in the suspending medium. Chien and Jan showed...
that increasing the length of a macromolecule (dextran) will increase the intercellular spacing of the red cells in the rouleau. He concluded that the macromolecule actually bridges two erythrocytes and only elongated macromolecules of sufficient length can serve this function. For example, fibrinogen but not albumin meets the shape requirements to cross-link erythrocytes. Therefore, sedimentation will not take place in serum. It has not been made clear until now, however, that the macromolecule has to be either neutral or negatively charged (as opposed to positively charged).

The receptor (binding) site for the macromolecule on the erythrocyte membrane has not been identified. We have shown that DIDS, an inhibitor of anion transport, interferes with formation of the aggregates in either plasma or dextran. DIDS at similar concentrations inhibits the chloride-bicarbonate exchange across the red cell membrane. This suggests that the macromolecule binds at the anion transport site. DIDS also reacts with lysine residues on the membrane surface. If the fibrinogen binding involves these lysine residues, then DIDS would interfere at this point. Against this latter possibility is the fact that there are $10^6$ anion transport sites (band 3 protein) per erythrocyte and about $10^4$ fibrinogen molecules are bound to each cell at equilibrium. Because fibrinogen binding is weak, the 100:1 ratio of binding sites to bound molecules is the right order of magnitude. In the case of rouleau formation, the two ends of the macromolecule bind to adjoining erythrocytes to form the aggregates.

In contrast to the aforementioned mechanism, positively charged macromolecules are not able to bridge the erythrocytes in this fashion; they react with the sialic acid residues and form amorphous aggregates. This process is not inhibited by DIDS.

After the formation of the rouleaux, the chains form spheres of uniform size (Fig 3). The driving force may be the reduction of the surface free energy. Uniform size is probably the result of competition between the attractive forces between cells and the hydrodynamic forces that tend to reduce the size of the spheres.

The spheres contain not only erythrocytes but also the macromolecules binding them together. This is in agreement with the findings of Janzen et al who demonstrated binding of fibrinogen to red cell surfaces. It also explains why increased Hct values do not increase the rate of sedimentation: the formation of the spherical aggregates is limited by the availability of fibrinogen or other plasma macromolecules linking the erythrocytes.

In summary, the mechanism of red cell aggregation to spheres of uniform size proceeds via the following steps: (a) electrostatic forces draw erythrocytes together to about 100 nm, with the discoid shapes lining up parallel to one another. This requires the presence of electrolytes. (b) If a neutral or negatively charged macromolecule of length greater than
ERYTHROCYTE AGGREGATION AND SEDIMENTATION

100 nm is present, it will bind to the anion transport sites of adjoining erythrocytes. The energy of this binding will stabilize the parallel configuration of red cells, and rouleaux are formed. (c) Rouleaux rearrange to form spheres, which reduces the surface free energy. (d) The kinetics of sedimentation must be described by at least two independent parameters (e.g., delay before sedimentation starts and steady-state rate of sedimentation). For a mathematical analysis see the Appendix.

Clinical correlation: correction for Hct. In clinical practice there are two reasons for variations in the sedimentation rate: (a) its dependence on Hct and (b) its dependence on the plasma protein concentration. The latter makes it useful as an empirical index of disease activity. In Fig 5 the radius of the sedimenting sphere (which is directly proportional to the true rate of sedimentation) is shown as a function of Hct and plasma protein concentration. The radius is increased by either decreasing the Hct or increasing the plasma protein concentration. Therefore, a change in Hct masks the effect of changing plasma protein concentration and composition. Because the steady-state sedimentation (slope in phase 2) in the range of Hct 10 to 50 is a linear function of Hct (Fig 6), correction can be made by interpolating to a set value, e.g., to an Hct of 40. This eliminates the variations in the sedimentation rate due to changes in Hct. In practice a good estimation of the steady-state sedimentation can be obtained by measuring the amount of sedimentation at 30 and 60 minutes and multiplying their difference by 2 (this way the sedimentation will have a value that is similar to the ESR).

Because the value of the slope approaches 0 when Hct = 55, one can obtain the value at an Hct of 40 from the value measured at any other Hct by the relationship: \( \text{Sed (Hct = 40)} - \text{Sed (observed)} \times (55 - 40)/(55 - \text{Hct}) \) (equation 1), where Sed (observed) is the sedimentation observed at the given Hct and Sed (Hct = 40) is its value corrected to Hct 40.

Table 3 shows both Sed (observed) and ESR and their values corrected to Hct 40 by using equation 1. In a larger series of patients with inflammatory bowel disease we obtained identical results. Clearly Sed (Hct = 40) provides an index of disease activity that is fairly independent of Hct.

Additionally, these can be directly measured from the plot of the curves in Fig 1. Table 1 gives the values for these parameters in equation 1. The first is the delay before sedimentation starts, i.e., the length of phase 1. The second is the slope of the linear part of phase 2. These two entities are related to the parameters in equation 2: the slope is the value of the first derivative of equation 2 when the second derivative is equal to zero and the delay is the x-intercept of the straight line originating from the inflection point of this curve and having the calculated slope. Alternatively, these can be directly measured from the plot of the curves in equation 2.

The linear part (phase 2) of the sedimentation results as the spheres formed from the rouleaux fall in a viscous medium. The radius \( R \) of this sphere can be calculated from the Einstein-Stokes equation: \( R^2 = 9 \eta \nu t / 2(\sigma_2 - \sigma_1)g \) (equation 3), where \( \eta \) is the plasma viscosity, \( \sigma_1 \) is the plasma density, and \( \sigma_2 \) is the red cell density. The densities and viscosities are literature values, and \( \nu \) is the measured rate of sedimentation in the linear phase (phase 2).

These radii can also be measured in the photomicrographs. Figure 6 shows both the calculated and measured values for the radii as a function of Hct for seven samples for a series of different Hct values. Because the concentration of the bridging macromolecules was different in each series, the radius is expressed relative to that at Hct 40. There is an excellent agreement between calculated and measured values.

ACKNOWLEDGMENT

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APPENDIX

By using SAS for modeling, the best functional form of the kinetics of erythrocyte sedimentation is as follows: sedimentation = \( a_0 \times t^2 / (a_1 + a_2 \times t + t^2) \) (equation 2), where \( t \) is the sedimentation time and the \( a \)'s are experimental parameters. For a cooperative phenomenon \( a_2 = 0 \).

Two additional descriptive parameters can be derived from the three parameters in equation 1. The first is the delay before sedimentation starts, i.e., the length of phase 1. The second is the slope of the linear part of phase 2. These two entities are related to the parameters in equation 2: the slope is the value of the first derivative of equation 2 when the second derivative is equal to zero and the delay is the x-intercept of the straight line originating from the inflection point of this curve and having the calculated slope. Alternatively, these can be directly measured from the plot of the curves in equation 1. Table 1 gives the values for these parameters for the curves from Fig 1.

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Table 3. Hct Correction of Sedimentation by Using Equation 1

<table>
<thead>
<tr>
<th>Hct (observed)</th>
<th>ESR (Hct = 40)</th>
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<td>25.8</td>
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Sed (observed) and ESR are experimentally determined (see the text). Sed (Hct = 40) and ESR (Hct = 40) are the calculated corrected values.
REFERENCES

Mechanism of erythrocyte aggregation and sedimentation

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