On the Shear Rate Dependence of Red Cell Aggregation In Vitro

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ABSTRACT Non-Newtonian viscosity of blood, i.e., the rise in apparent viscosity at low flow, was believed to be a result of reversible aggregation of red cells at low velocity gradients (shear rate). By making a cone-plate viscometer transparent, direct observation was made possible of the blood flowing under defined shear rates. Red cell aggregates, occurring in all cases at low flow, were reversibly dispersed by increasing the shear rate. This behavior was independent of the addition of anticoagulants, but it could be altered by changing the plasma protein composition. Red cells in serum did not form aggregates; such nonaggregating samples did show an increase in viscosity at low shear rates. Since the sedimentation rate can be influenced by many parameters, it is not reliable in describing red cell aggregation. Aggregation of red cells is linked with a marked separation of plasma and cells. Such a separation is of considerable influence on cone-plate viscometry.

INTRODUCTION

Blood is known to be a non-Newtonian fluid, i.e., its viscosity depends on its flow conditions. Performing capillary viscometry, Hess (1) found the viscosity to increase with decreasing pressure head. He postulated that blood had, as he called it, an "elastic resistance against deformation," caused by coherence forces between particles. He suggested this might be related to the property of red cells to form aggregates (typical rouleaux or "Geldrollen"). Since Fahraeus (2), it has been known that aggregation or rouleaux formation is governed by shearing forces of the flow, irrespective of other factors such as the fibrinogen concentration.

The old concept of Hess has been revived successfully by Merrill, Cokelet, Britten, and Wells (3) who compared the rheological data of blood with data of better understood nonbiological suspensions. Finding a simple flow equation, given by Casson (4) for paints, applicable for blood, Merrill et al. postulated theoretically that red cells reversibly group into aggregates at low shear rates.

The purpose of this study is to examine the validity of these assumptions. Since, hitherto, aggregation could only be determined by its influence on the sedimentation rate of blood, direct observation of the blood cells under defined flow conditions will be necessary to study their behavior at different shear rates.

METHODS

By making the cone and plate of a Wells-Brookfield LVT-Microviscometer transparent, we succeeded in observing the flowing blood with a microscope. For a detailed description of the original Wells-Brookfield Viscometer, (Brookfield Engineering Laboratories, Stoughton, Mass.) see reference 5. The modified instrument, the details of which have been published elsewhere (6), is shown in Fig. 1. The cone and plate, as well as the water-jacket, are remodeled in Plexiglass without altering the geometrical features of the original instrument. Thus the shear rate of the flow is uniform in the entire measuring chamber. A rectangular prism is fixed to the bottom of the water-jacket and the entire observation chamber is transilluminated with a projection lamp (250 w). Thus, the streaming blood can be observed with a horizontally aimed microscope at magnifications from 16 to 100 ×.

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With maximum magnification of red blood cell smears, individual red cells will be clearly seen. The center of cone and plate is used for observation. This region proves to be most suitable because of the low angular speed and the narrow width of the blood layer. Nevertheless, the shear rate of the flow in this region is identical to that in all other parts of the measuring chamber.

Samples of arterial and venous blood were drawn from dogs with and without the use of heparin (Vetren,[1] 10 mg/kg). Human venous blood from apparently healthy students and members of the staff was in some cases heparinized with Liquemin[2] (0.2 ml/10 ml of blood). In other cases it was measured and observed without any anticoagulant before coagulation occurred.

Care was taken to avoid excessive venous stasis and mechanical suction while withdrawing the blood. The effect of changes in the plasma protein composition on aggregation was studied either by replacing increasing volumes of plasma with Ringer's solution or by adding 0.4 ml of a 3.8% Na-citrate solution to 1.6 ml of blood. The decrease in the plasma-protein concentration was then computed. In addition, canine red cells were washed three times in isotonic saline and then resuspended in Ringer's solution or serum.

In all these samples the aggregation of red cells was observed in the transparent chamber, with shear stresses at shear rates between 5.8–230 sec^{-1} measured simultaneously at 37°C. The latter values were also measured in the original Wells-Brookfield LVT-Microviscometer. The viscosity values of the blood samples were computed from the shear stress/shear rate ratio: $\eta = \tau/\gamma$.

From the heparinized samples the sedimentation rate was determined in Westergren tubes at room temperature. The hematocrit was measured using the Wintrobe method; the values were not corrected for trapped plasma.

In all cases, the measurements were completed within 6 hr, in many cases within minutes after the withdrawal of the blood. Since the behavior of the red cells remained the same during periods of up to 6 hr, changes in pH and in plasma hemoglobin concentrations were not controlled.

RESULTS

A typical example of human blood flowing under different shear rates is shown in Fig. 2 of photographs taken with synchronized strobe light. At the lowest shear rate, groups of red cells with clear plasma between them are seen. These groups are made up of a three-dimensional cluster of red cells. No typical rouleaux of red cells can be distinguished at normal hematocrit. With increasing shear rate the size of the aggregates decreases, until at a shear rate of 46 sec^{-1} no more aggregates can

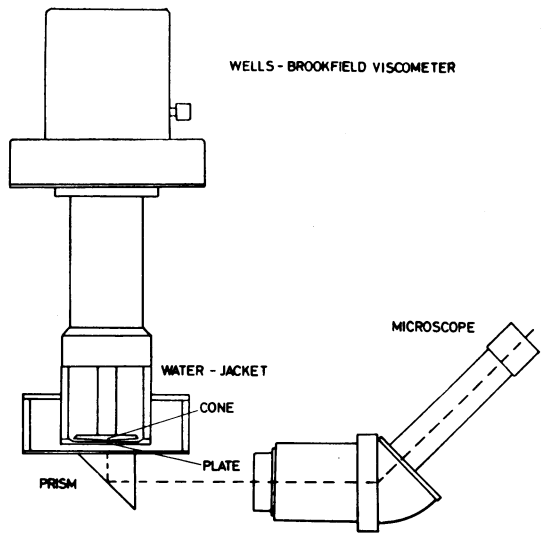

FIGURE 1 Schematic drawing of the remodeled viscometer. Water-jacket, cone, and plate are made of plexiglass. The flowing blood is observed at strong transillumination with a microscope.

be seen. Upon each reduction of the shear rate, the red cells immediately recombine into aggregates, which continue to increase with decreasing flow. This breakup or formation of red cell aggregates can be repeated at will by merely changing the shear rates. Even after 6 hr of increasing and decreasing the rotational speed, this dependence of aggregation upon shear rate was maintained. If rotation was stopped abruptly after maximum speed, no aggregates were formed. This happened only when rotation at low speed was started again. Immediately after filling the chamber with blood, no aggregation usually could be seen. Here, too, aggregation occurred only when the cone was rotating slowly.

The optical features of our system and of the stereomicroscopes used, however, limit the photographic recording. And, in addition, still photographs are not satisfactory for particles are projected into one plain while they are flowing in different fluid layers and are in relative motion to each other. Therefore, the size of the aggregates cannot be taken from still photographs, but must be measured directly while looking at the flowing blood. Measured parallel to the circular direction of flow, the size of the individual aggregates is approximately uniform over the whole field of vision, whereas in the radial direction their dimensions are not uniform. After each change in shear rate,

[1] Vetren (Chemische Fabrik Promonta, Hamburg Germany) is a heparin-like preparation with 145 IU of heparin activity per mg.

[2] Deutsche Hoffmann-La Roche, Grentach, Germany.

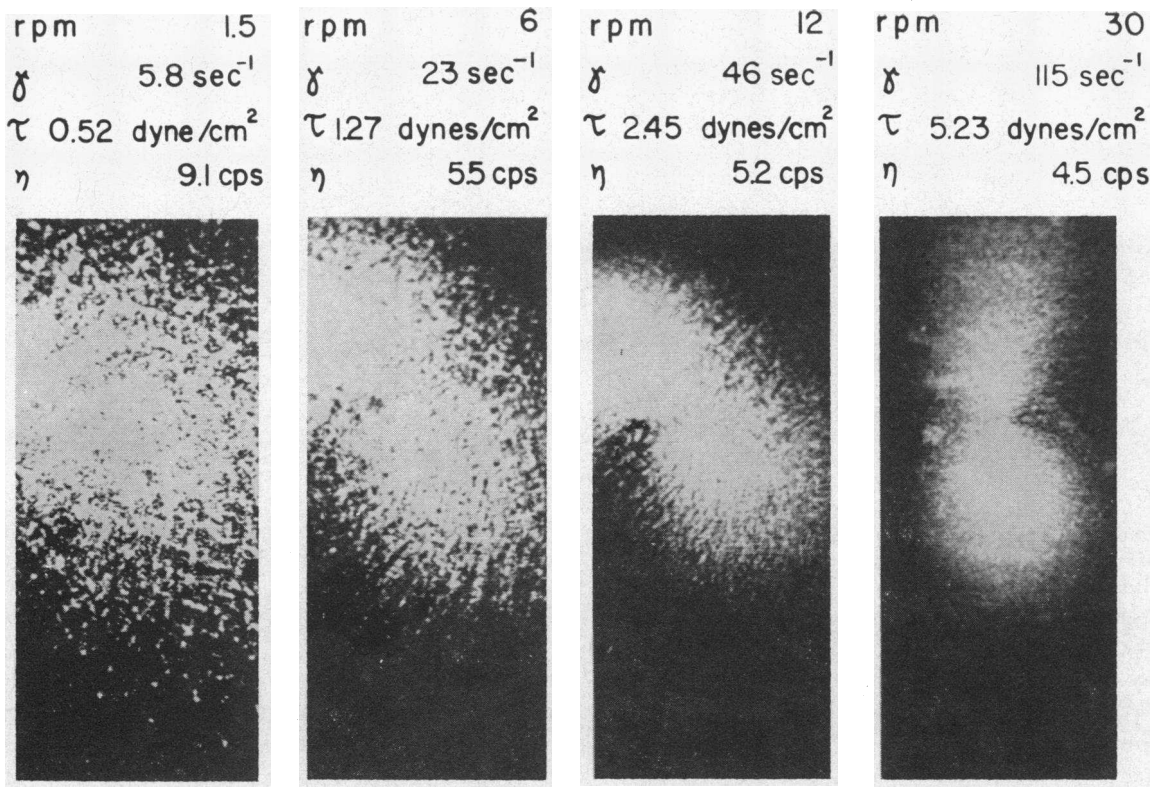

FIGURE 2 Human blood (heparinized) photographed while flowing under definite shear rates (γ). Observation restricted to the thinnest region between apex of cone and plate of viscometer. Each section represents one distinct shear rate: the size of the red cell aggregates (dark shadows) and of the clear plasma gaps (bright) decreases with increasing shear rate. Shear stress (τ) and viscosity (η) are given for each shear rate.

the dimensions of the aggregates are again relatively uniform after a short transient period.

In the region near the apex of the cone, single cells can be discerned even when the blood flows at maximum speed. The size of the particles in all other parts of the measuring chamber can be controlled immediately after a sudden stop of the rotation. Care must be taken, however, to avoid the effects of both gradual slowing and sedimentation, since both lead to an increase in aggregate size.

In this apical region it can also be seen that the streaming aggregates continuously lose individual cells and groups of cells while gaining others. The aggregate thus seems to be continuously changing its composition whereas its average total size remains constant in proportion to the shear rate that prevails. After each increase of the shear rate, the loss of cells predominates and a new size of the aggregates is reached. Aggregates of about 15 μ in diameter proved to be much less affected by further increase of shear rate. Even at much higher shear rates, they can be seen beside individual cells. For this reason, we chose the size of 15 μ as the arbitrary limit of what we call an aggregate.

In 123 canine and in 83 human blood samples there was not a single one that did not show the typical picture of aggregation. Before the onset of coagulation, this behavior could also be observed in freshly drawn blood which had not been anticoagulated. The human samples showed aggregates up to a maximum shear rate (γ_{max}) of 46 sec^{-1} (Fig. 3). In the canine samples, however, the distribution of the maximum shear rates was wider. Some of these samples showed aggregates even at shear rates of 230 sec^{-1}. The picture of aggregation, as seen in the observation chamber, was identical in both human and canine blood samples. Furthermore, even in cases of strong or weak aggregation

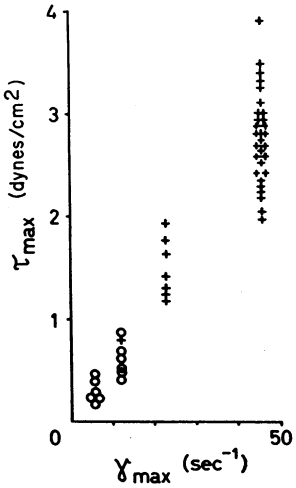

FIGURE 3 Maximum shear rates at which aggregates are seen (γ_{max}) plotted against the shear stress prevailing (τ_{max}). Heparinized (+) and citrated (○) human venous blood of apparently healthy subjects. The majority of the undiluted blood samples show aggregates up to shear rates of 46 sec^{-1} and shear stresses of 4 dynes/cm^2.

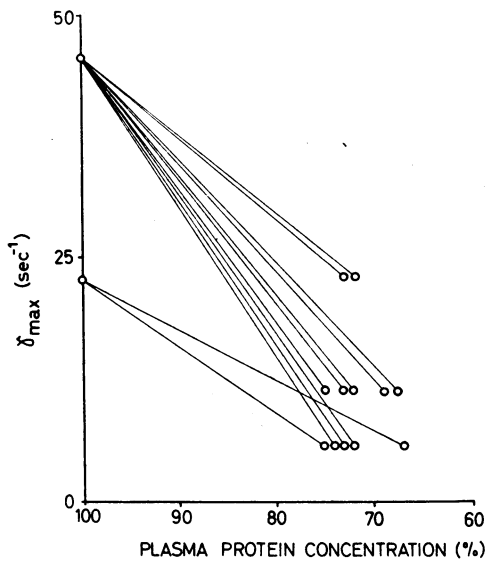

FIGURE 5 Effect of plasma protein dilution by Na-citrate on the tendency to aggregation. Venous blood of 12 apparently healthy subjects. In all cases the γ_{max} decreases with the addition of Na-citrate.

in canine blood, the aggregation was fully reversible by merely changing the shear rate.

Whenever strong aggregation prevailed, cell-free plasma gaps were observed. The size of these gaps increased with decreasing shear rates. Whenever this happened, we felt that the network of aggregates less and less followed the rotation of the cone. In spite of constant rotational speed of the cone the rotational speed of this network decreased, with the size of the aggregates increasing and leading to a tightly-woven network of red cells. If this condition of flow was maintained for more than 2 min, clear plasma could be seen in the drop

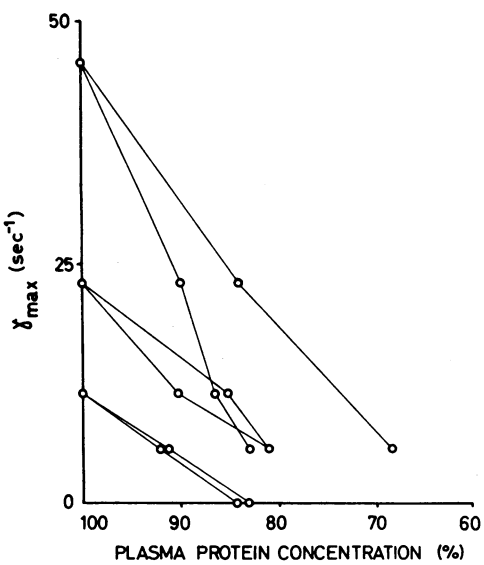

FIGURE 4 Effect of plasma protein dilution by increasing volumes of Ringer's solution on the tendency to aggregation. Six canine blood samples. The maximum shear rate at which aggregates can be seen in the blood (γ_{max}) decreases with increasing dilution of plasma proteins.

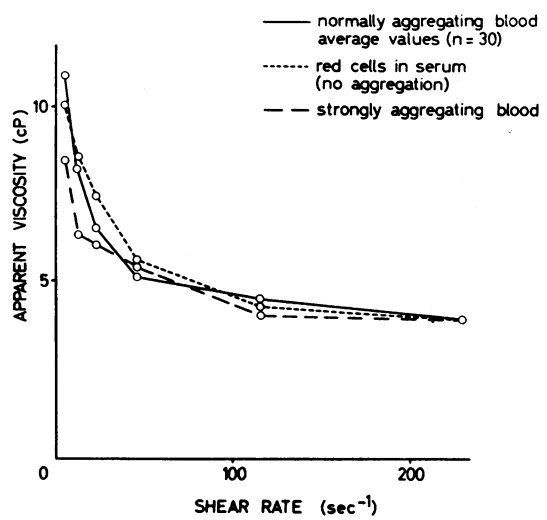

FIGURE 6 Viscosity profiles of canine blood samples with different tendency to aggregation and identical asymptotic viscosity at high shear rates. The viscosity profiles show no significant difference.

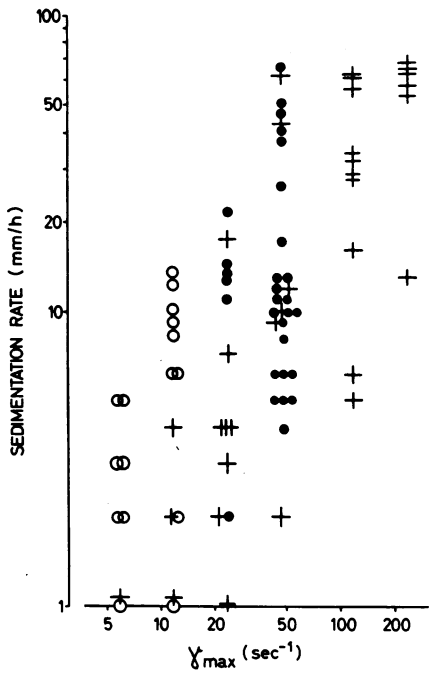

FIGURE 7 Maximum shear rates at which aggregation was observed (γ_{max}) plotted against the sedimentation rate. + = heparinized canine blood samples; ● = heparinized human blood samples; ○ = citrated human blood samples. A strong correlation between sedimentation rate and tendency to aggregation cannot be deduced from this figure.

hanging under the cone after the instrument had been taken apart. This sedimentation process, originally observed in the transparent chamber, was also found in the metal cone-plate viscometer under identical experimental conditions.

The tendency of red cells to form aggregates could easily be altered. Suspensions of red cells in Ringer's solution showed no aggregation at all. Dilution of the plasma proteins with added increasing volumes of Ringer's solution reduced the tendency to aggregation (Fig. 4). The dilution did not affect the tendency to aggregation in all samples alike. A similar reduction of this tendency could be observed when the plasma was diluted with an isotonic solution of Na-citrate, as is customary in the Westergren method (Fig. 5). The canine red cells suspended in serum showed no tendency to aggregation.

The viscosity profiles of three different groups of samples are plotted in Fig. 6. All have the same asymptotic viscosity at high shear rates. Curve *A* shows a group of canine blood samples with an average γ_{max} (maximum shear rate of aggregation) of 46 sec^{-1}. Curve *B* shows a group of samples in which aggregation was completely abolished by suspending the red cells in serum. Curve *C* shows the values of strongly aggregating blood samples taken from diseased dogs. The slopes of these three profiles show no significant difference within a range of shear rates between 5.8–230 sec^{-1}.

The maximum shear rate at which aggregation could be seen (γ_{max}) and the sedimentation rate of blood samples are only loosely correlated (Fig. 7). Weakly aggregating blood samples in most cases have a lower sedimentation rate than that of strongly aggregating blood samples.

DISCUSSION

As first shown by Brundage (7), the apparent viscosity of blood depends on the shear rate of the flow. This shear rate dependence has lately been confirmed by many authors (5, 8–11). Casson (4), performing viscometry on non-Newtonian paints, established a linear correlation between the square roots of shear stress (τ) and shear rate (γ). He deduced the following equation from the assumption that the dye particles of the paints form reversible aggregates at low shear rates, k_0 and k_1 being two constants expressing the flow properties of the suspension:

$$\sqrt{\tau} = k_0 + k_1\sqrt{\gamma}.$$

Employing their blood measurements, Merrill et al. (3) found that their values fitted the Casson equation. They concluded that Casson's assumption also held true for the red cells of blood and postulated that erythrocytes form aggregates at low shear flow. Using viscometers of great sensitivity, Merrill, Gilliland, Cokelet, Shin, Britten, and Wells (8) were able to extend their viscosity measurements down to shear rates of 0.1 sec^{-1}. When the linear relation between the square roots of shear rate and shear stress was extrapolated to zero shear rate, a positive intercept with the shear stress axis resulted, termed "yield shear stress." They concluded from the existence of such a yield shear stress that the red cell aggregates could withstand finite shearing stresses: in other words that near zero shear rate blood behaved like a solid.

The results described above show that the red cells of human and canine blood do indeed form aggregates when flowing at low shear rates. Be-

cause this phenomenon was observed in all blood samples without exception we conclude that red cell aggregation is a normal feature of normal red cells suspended in plasma of normal protein composition. "Aggregation" is seen as a reversible grouping of red cells suspended in plasma, the formation or breakup of the aggregates depending on the flow conditions. This "aggregation" is to be distinguished from the so-called "agglutination," i.e. a massing of red cells due to any kind of antigen-antibody reaction involving the erythrocytes, which also occurs in serum, and which does not depend on the flow conditions. Such agglutination can easily be produced by mixing incompatible blood samples. The agglutinates, in contrast to the aggregates, are very dense. They do not form any networks and their size is not uniform and it does not alter with changes in the shear rate.

These findings confirm many of the ideas and results of Fahraeus (2, 12), who repeatedly stressed that rouleaux formation is a normal property of human red cells. He discovered that the formation of rouleaux depended not only on fibrinogen and globulin concentration, but also on the blood flow conditions. The aggregates described by Fahraeus mostly had chain-like forms and consisted of typical rouleaux or "Geldrollen." He indicated, however, that this was not the only possible form of aggregation. This point was reemphasized by Ditzel (13), among others.

With the technique described, we are unable to decide whether the aggregates observed are made up of typical rouleaux, even though this is very probable. One might therefore speculate that a rouleaux is the remainder of a three-dimensional aggregate, produced by a shear stress that acts largely in one direction and which pulls the aggregates apart.

Fahraeus also emphasized that the suspension stability of blood was governed by the rouleaux formation. The sedimentation rate of a blood sample, however, cannot be taken as a valid parameter describing aggregation, since the size of a particle is only one factor governing the sedimentation process. Correspondingly, there is a rather wide variation between data in Fig. 7. In addition, it must be noted that in the sedimentation tube only low shear rates and consequently large aggregates are to be expected, whereas in the viscometer aggregates are examined under a wide range of shear rates, including high ones.

Furthermore, our findings and conclusions are fully consistent with the conclusions drawn by Merrill et al. (3) from their viscosity measurements. In contrast to this, Knisely and Bloch (14, 15), studying the phenomenon of intravascular aggregation (sludged blood), implied that normal erythrocytes repel each other. These authors consequently regard red cell aggregation as occurring solely under pathological conditions. We, on the other hand, consider the aggregation found under pathological conditions to be a mere intensification of the physiological aggregating process. Beyond that, it must be concluded from our findings that there is not only a lack of repulsion between red cells, but that energy must be dissipated to keep the red cells flowing as individual cells, Whenever this energy is not sufficient, the red cells will group into aggregates.

As described above, aggregates are formed only when the individual cells come into contact with one another. If a rapid flow is stopped abruptly, the cells remain apart. This behavior calls to mind an observation made by Knisely (15): "Whenever the flow in a small arteriole or venule stops, the red cells do not come together forming masses. . . . Whenever by chance the pressure becomes the same at each end of the interconnecting venule the flow through it stops. The unagglutinated red cells in it remain in the position they occupied at the moment the flow stops. . . ." From this and other evidence Knisely draws the conclusion that a repulsion between red cells takes place. Our results, however, suggest that only an abrupt flow stop leads to such an occurrence, whereas any gradual slowing of the flow always results in red cell aggregation.

The results of our dilution experiments show that the aggregation of red cells is influenced by the blood protein composition. If the plasma is fully replaced by Ringer's solution, no aggregation occurs. From the unequivocal results of many investigators (15–17) it has been concluded that high molecular weight proteins are mainly responsible for aggregation. Fahraeus (2) and recently Merrill et al. (3) and Wells et al. (18) showed that it is the fibrinogen fraction which has the greatest effect on the tendency to aggregation. This is fully consistent with the fact that no aggre-

gation could be seen in red cell serum suspensions with our instruments, the sedimentation rates of these samples being minimal. In view of these facts, all blood samples were heparinized, since the customary 4:1 dilution with isotonic Na-citrate solution proved to diminish the tendency to aggregation (Fig. 5).

The very fact that aggregation and sedimentation are linked has a considerable influence on the viscometry of blood. As described above, clear plasma gaps are always found between aggregates. This leads to a separation of plasma and cells in the viscometer. Whenever large aggregates are present, they tend to settle to the bottom of the measuring chamber. There they participate less and less in the streaming of the blood. This retardation reduces their actual rate of shear with a consequent increase in the size of the aggregates. This seems to be a self-increasing phenomenon, which destroys both the uniform shear field and the homogeneity of the suspension.

A similar phenomenon has been observed by Merrill et al. (5) who called it the "second heterophase effect." We observed that it consistently accompanied pronounced aggregation in the transparent chamber. Indirectly, the consequences of this effect could be determined by measuring the hematocrit of the drop hanging under the cone after the instrument had been taken apart, immediately after a measurement at low shear rate. In cases of strong aggregation, this drop consisted almost entirely of clear plasma. This separation of cells and plasma was seen both in the transparent and in the original cone-plate viscometer. This effect, which could also be called "plasma skimming," is being enhanced by settling, and corresponds to the degree of aggregation.

Consequently the separation leads to viscosity values that are lower than would be expected, as Cokelet et al. (19) have shown with their GDM-Viscometer. The reason for this seems to be that the hematocrit of those layers that are actually being sheared is progressively decreasing. Similar phenomena have been described by Rand et al. (20). Therefore, it seems advisable that blood measurements with cone-plate viscometers should begin at high rotational speeds and pass on to lower ones without much delay.

Within a range of shear rates between 230–5.8 sec^{-1}, it would appear there is no difference in the slope of viscosity profiles of normally aggregating, strongly aggregating, and nonaggregating blood samples (Fig. 6). The viscosity profile of strongly aggregating blood samples is very probably influenced by the settling effects described above.

Blood which shows no optically detectable aggregation shows non-Newtonian viscosity, nevertheless. According to Merrill (3), differences in viscosity of aggregating and nonaggregating blood samples can only be expected at shear rates well below those measured by us. On the other hand, our results show that aggregation is a physiological phenomenon not only at these extremely low shear rates, but that it is present at shear rates up to about 50 sec^{-1} at normal, and at even higher shear rates in cases of disease. These profiles therefore show that, only a combination of direct observation with the viscosity measurements will reveal the existence of red cell aggregation and its behavior in flow. Our findings show that on the basis of traditional cone-plate viscometry, only limited predictions are possible on the influence of red cell aggregation and its consequences on the rheological behavior of whole blood.

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