DEFORMATION AND FRAGMENTATION OF HUMAN RED BLOOD CELLS IN TURBULENT SHEAR FLOW

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ABSTRACT By means of glutaraldehyde fixation, human erythrocytes are "frozen" while suspended in turbulent shear flow. As the shearing stress is increased in steps from 100 to 2,500 dyn/cm^2, the deformed cells evolve gradually toward a smooth ellipsoidal shape. At stresses above 2,500 dyn/cm^2, approximately, fragmentation of the cells occurs with a concomitant increase in free hemoglobin content of the suspending medium. The photographic evidence suggests that the cells rupture in tension in the bulk flow.

INTRODUCTION

During the past decade the rapid growth in the technology of cardiovascular prostheses and blood-processing extracorporeal devices has stimulated a considerable amount of research on the problem of blood degradation associated with their use. The majority of this research has been focused on the red cell and the problem of mechanical hemolysis. An extensive review of past work in this area has been published by Blackshear (1).

Controlled studies of mechanical hemolysis have been carried out almost exclusively in vitro. Attempting to achieve simplified and well-defined flow fields, investigators have resorted to a variety of devices, such as the concentric cylinder or Couette viscometer, the cone-and-plate viscometer, and the capillary tube. In all of these systems the sheared suspensions are confined by various nonbiological surfaces such as steel, glass, and plastics, and sometimes gas interfaces; and the passages occupied by the suspensions are characteristically narrow (ranging from less than 100 μm to a few millimeters). These bounding surfaces are clearly accessible to the suspended cells and could therefore participate in the trauma observed in a shearing experiment. This possibility of a simultaneous surface influence has complicated attempts to interpret the effect of shear stress alone. A few experimental designs aimed at avoiding or minimizing the influence of foreign surfaces have been conceived. In this category, liquid jet action (2–4), a pulsating gas bubble (5), and an oscillating wire (6) have been used to hemolyze red cells.

In summary, one can say that the previous work on this subject leaves us with an

ambiguous picture of mechanical hemolysis in vitro. A central issue in the various interpretations presented is the extent to which the containing surfaces in a particular experimental device are responsible for the hemolysis observed. Some investigators have concluded that the hemolysis produced in their experiments was primarily surface related, and thus a characteristic of the particular apparatus, while others claim to have measured true "thresholds" for shear-induced, in-bulk hemolysis. The stress thresholds proposed vary considerably, from the order of 700 dyn/cm^2 (7) to 40,000 dyn/cm^2 (2, 4).

Blackshear (1) attempts to reconcile these different findings within the context of a general theory of mechanical hemolysis. He defines three classes of mechanical hemolysis: (*a*) surface-induced, i.e. dependent on the extent of the surface and proportional to the square of the shear rate in the boundary layer; (*b*) in-bulk, medium stress (1,000–2,000 dyn/cm^2); (*c*) in-bulk, high stress (order of 40,000 dyn/cm^2). The third class is presumed related to a nearly instantaneous tensile failure of the red cell membrane. It is suggested that the medium stress hemolysis is associated with long duration exposure to stress and gradual fragmentation of the erythrocytes.

In earlier studies of shear-induced hemolysis, at shear stresses from 0 to 2,500 dyn/cm^2 generated in a concentric cylinder viscometer, we concluded that the dominant hemolytic mechanism was surface related (8). However, we lacked direct evidence that this was, in fact, the case. Our subsequent experimental work has been aimed at clearly distinguishing between surface and so-called bulk stress effects. The results to be presented here are concerned primarily with the deformation and fragmentation of red cells in bulk. Independent studies of surface involvement are now being prepared.

A secondary purpose of the present experiments is to examine the additional effect of turbulence, if any, on red cell tolerance to shear flow. Although it is generally suspected that turbulent flow, if allowed, for example, in an extracorporeal circuit, would be prohibitively traumatic to blood, the tolerance of blood to turbulence is still to be thoroughly investigated. The question merits careful study because the effectiveness of mass-transfer devices could be greatly enhanced by their operation in the turbulent regime. Our previous experiments strongly suggest that hemolysis induced by shearing in a concentric cylinder viscometer at a particular stress is not accelerated by the presence of turbulent fluctuations.

EXPERIMENTAL PROCEDURE

All experiments were performed on dilute suspensions of human erythrocytes collected from healthy, fasted adult donors. Venous blood samples were collected into heparin-coated 20-ml Vacutainer tubes and the red cells separated by centrifugation at 1,550 *g* for approximately 10 min at a temperature between 0° and 10°C. The plasma and buffy layer were discarded and the red cells washed by resuspending in 0.05 M phosphate-buffered saline (270 mosm, pH 7.4, 90 mg/100 ml dextrose). After another centrifugation the cells are resuspended in the phosphate-buffered saline. This suspension was then diluted to a hematocrit of 0.2% for the shearing test. Generally, all tests were completed within 6 h of the drawing of the blood.

The suspensions were sheared in a concentric cylinder viscometer with rotating inner cylinder. The rotor, 10 cm in diameter and 5 cm long, is of stainless steel. A liquid solution circulating through the rotor maintained the system at 37°C. The gap between cylinders is variable but the results presented here were all obtained with a gap of 2.07 mm. This configuration can generate a maximum shear stress of 4,500 dyn/cm^2 in a dilute aqueous suspension. In the present case of turbulent flow, numerical values of shear stress are to be interpreted as *time-averaged* values. Owing to the narrowness of the gap relative to the rotor radius, the stress transmitted through the suspension in the gap is practically uniform across the gap. The gap stress at any given rotor speed is determined from direct measurements of torque transmitted from the rotor to the outer cylinder corrected for the contributions of the low shear end-zones. In this manner the evaluation of the gap stress requires no assumption as to the effective or "eddy" viscosity in the turbulent shear flow.

In a typical experiment the viscometer is charged with the test suspension while the rotor turns slowly to maintain good mixing and prevent sedimentation. Upon completion of filling the rotor is accelerated to the desired speed in about 5 s. After 5 min of shearing about 2 ml of a 1% glutaraldehyde solution is added through a narrow annular opening at the top of the rotor. When the flow is turbulent the glutaraldehyde solution diffuses thoroughly through the red cell suspension within a fraction of a second. The volume of the test suspension initially contained in the viscometer is about 40 ml, so that the final (fully mixed) glutaraldehyde concentration is approximately 0.05%. The contact of the glutaraldehyde with the red cell membrane initiates a polymerization reaction which ends in the permanent fixation and solidification of the entire cell. Such a reaction is not instantaneous, of course, but it is quite rapid.[1] 15–20 s after introduction of the fixative the rotor is stopped and the cells withdrawn for microscopic examination. In similar tests the recoverability of the cells after 5 min of shearing is recorded by adding the glutaraldehyde after the rotor is brought to rest and the suspension is removed from the viscometer.

Free hemoglobin in the suspending medium is measured in independent runs in which all steps are duplicated except fixation. This gives a measure of hemolysis and/or leakage of intracellular hemoglobin due to membrane deformation at sub-hemolytic stresses. Free hemoglobin is determined by measuring the optical density of the supernate in a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at a wavelength of 0.54 μm.

To prepare a sample of the fixed cells for the scanning electron microscope (SEM), 1–2 ml of the suspension was centrifuged. The fixed cells were then washed twice with distilled water. A drop of distilled water is placed on a 12-mm diameter glass cover

[1] Independent experiments performed in our laboratory by R. M. Hochmuth indicate that fixation may be complete within 1 s after the fixative contacts a red cell. In his experiments, individual red cells attached to a glass surface in a parallel plate flow channel were subjected to a pulsatile flow with a frequency of about 1 Hz. With the cells under constant microscopic observation, a sharp "front" of glutaraldehyde solution was admitted to the channel. The cells were seen to stop responding to the flow pulsations within one cycle, i.e. in less than 1 s.

slip mounted on a SEM stub. A small amount of the fixed cells were added to the drop and the stub was vacuum-dried and finally coated with chromium in a vacuum evaporator.

To evaluate quantitatively the deformations observed in the cells fixed under shear, particularly in those cases where the cells had assumed a pronounced ellipsoidal shape, histograms of the maximum cell dimension were developed. Individual cell lengths were obtained from an enlarged photograph of a wet preparation of the fixed cells with a magnetic board digitizer interfaced to a Hewlett-Packard desk calculator (Model 9001 B, Hewlett-Packard Co., Palo Alto, Calif.). The calculator was programmed to compute and classify all the cell lengths over 20 equal length increments. Also programmed was the calculation of the mean length and SD for the whole population.

RESULTS

The technique of fixation of red cells while they are immersed in a shear flow appears capable of capturing the deformed shapes of the entire cell population. The deformations can then be permanently recorded photographically. With this technique, microphotographs, both optical and SEM, were obtained showing a regular progression in the deformations with stress.

The observations reported here cover a range of average shearing stress in the gap from 100 to 4,500 dyn/cm^2. Throughout this stress range the flow regime in the 2.07-mm gap is always turbulent. This is verified by a check of the Taylor number, a dimensionless parameter well known to characterize the various flow regimes that occur between concentric cylinders. For the case of inner cylinder rotating, the Taylor number is given by

$$Ta = \Omega_i R_i^{1/2} h^{3/2} / \nu,$$

where Ω_i is the angular speed of the inner cylinder, R_i its radius, h the gap width, and ν the kinematic viscosity of the fluid. Experience has shown that the flow becomes turbulent when $Ta > 400$ (9). For the aforementioned stress range Ta ranges from 1,500 to 12,000.

As seen in Fig. 1 *a*, showing cells "frozen" at 100 dyn/cm^2, many of the cells have taken a bulbous shape similar to the trilobular form observed by Goldsmith and Marlow (10). Qualitatively similar deformations have also been recorded by Hochmuth et al. (11), using large scale, deformable model cells. This form was found to be very common at low stress. Note that the natural concavities of the cells are still easily recognizable.

The cells began to lose their biconcavity in the neighborhood of 500 dyn/cm^2 and a majority of them assumed an essentially prolate ellipsoidal shape when the stress exceeded about 1,500 dyn/cm^2. Fig. 1 *b* shows cells fixed at 2,000 dyn/cm^2. As the stress was increased the ellipsoidal shapes gradually became predominant and the ratio of major to minor axes increased slowly. It was not until a stress of about 2,500 dyn/cm^2 was exceeded that fragmentation became apparent. See Fig. 1 *c* taken at

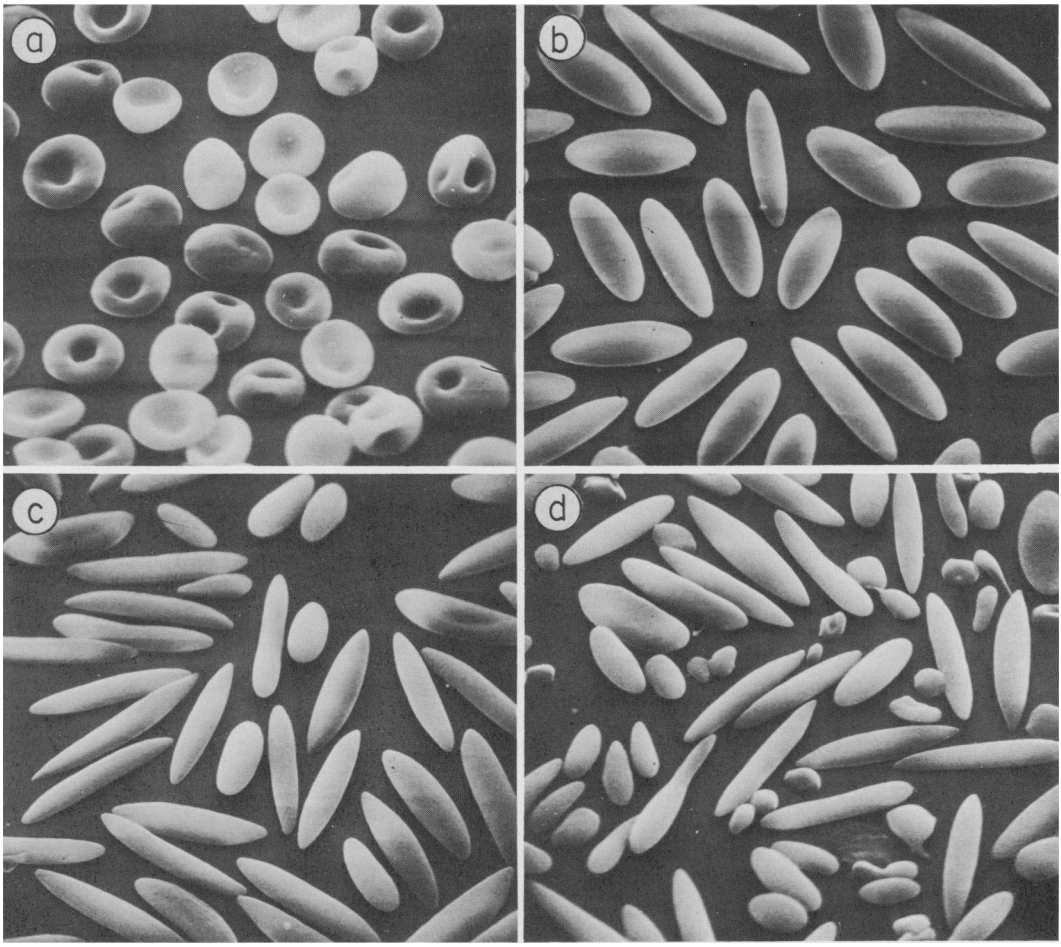

FIGURE 1 Human red cells fixed while immersed in shear flow in concentric cylinder viscometer. Applied shearing stress: (*a*) 100, (*b*) 2,000, (*c*) 3,500, (*d*) 4,500 dyn/cm^2. Cells were fixed after 4 min shearing at 37°C. Initial cell concentration: 0.2% by volume. 45° oblique views.

3,500 dyn/cm^2. At 4,500 dyn/cm^2, the highest stress applied, Fig. 1 *d*, many more and smaller fragments are in evidence. In each of the cases seen in Fig. 1, the suspensions were maintained at 37°C during shear and the average time of exposure to the shear in the gap between cylinders was kept at 4 min. This exposure time is less than the total running time (5 min) because the cells are free to circulate between the gap and adjoining end zones where the average stress is much lower than the gap stress. The ratio of average time spent in the gap flow to total running time is estimated from a simple two-chamber mixing hypothesis.

To investigate the degree to which these deformations may be irreversible, we performed experiments in which the cells were first exposed to stress for 4 min at 37°C, then fixed subsequent to the cessation of shearing and removal from the viscometer.

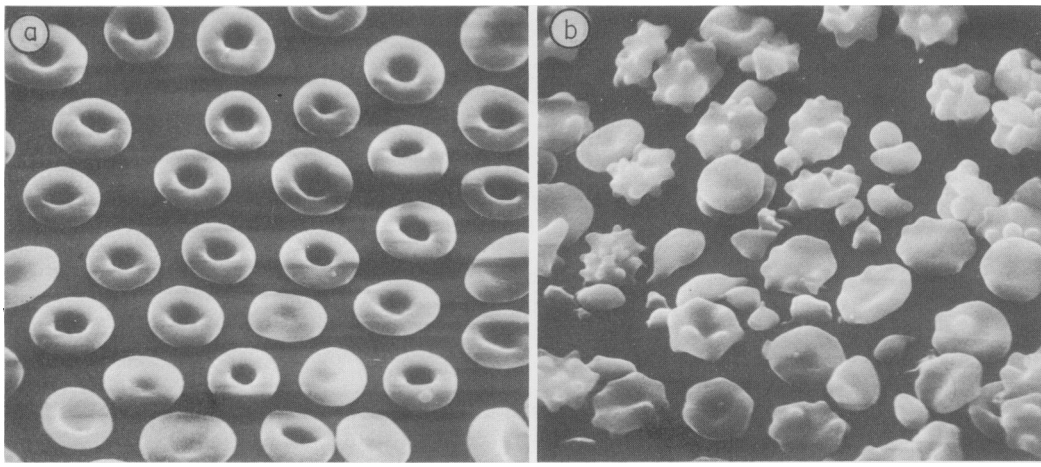

FIGURE 2 Human red cells sheared for 4 min at (*a*) 2,000, (*b*) 3,500 dyn/cm^2, then fixed after cessation of shear and removal from viscometer. The cells sheared at 2,000 dyn/cm^2 show nearly complete recovery of normal shape. 45° oblique views.

Altogether the cells were allowed a period of about 1 min for recovery of elastic strain. Typical results are shown in Fig. 2. In Fig. 2*a* we see that cells sheared at 2,000 dyn/cm^2 have very nearly recovered their normal shape. On the other hand, the cells sheared at 3,500 dyn/cm^2, a stress known to cause fragmentation, are seen in Fig. 2*b* to be badly crenated as well as fragmented.

The histograms of cell-length distribution obtained for four stress levels, 500, 1,000, 2,000, 3,500 dyn/cm^2, are presented in Fig. 3. On each histogram the mean length of the measured population is indicated. At a gap stress τ_g = 3,500 dyn/cm^2, Fig. 3*d*, the occurrence of fragmentation is apparent from the significant short length "tail" of the histogram. With regard to the mean lengths indicated, it should be pointed out that glutaraldehyde fixation normally causes a uniform shrinkage of the fixed cell relative to its normal size. Thus, normal erythrocytes, when fixed with glutaraldehyde, would probably have a mean diameter in the neighborhood of 7–7.5 μm instead of the normal 8–8.5.

The mean elongation of the fixed cells relative to the mean diameter of an unstressed control group was defined as

$$\bar{e} = (\bar{l}/\bar{l}_c) - 1$$

and calculated for each run. A plot of the percent mean elongation versus the applied stress is given in Fig. 4. The relationship is approximately linear in the range $0 < \tau_g < 2{,}500$ dyn/cm^2 and the elongation reaches 100%. It should be noted that the calculated mean elongation is meaningful only for a population of intact cells. The presence of significant numbers of fragments, as in Fig. 3*d*, will reduce the calculated mean elongation until, when fragments outnumber intact cells, $\bar{e}$ could become negative.

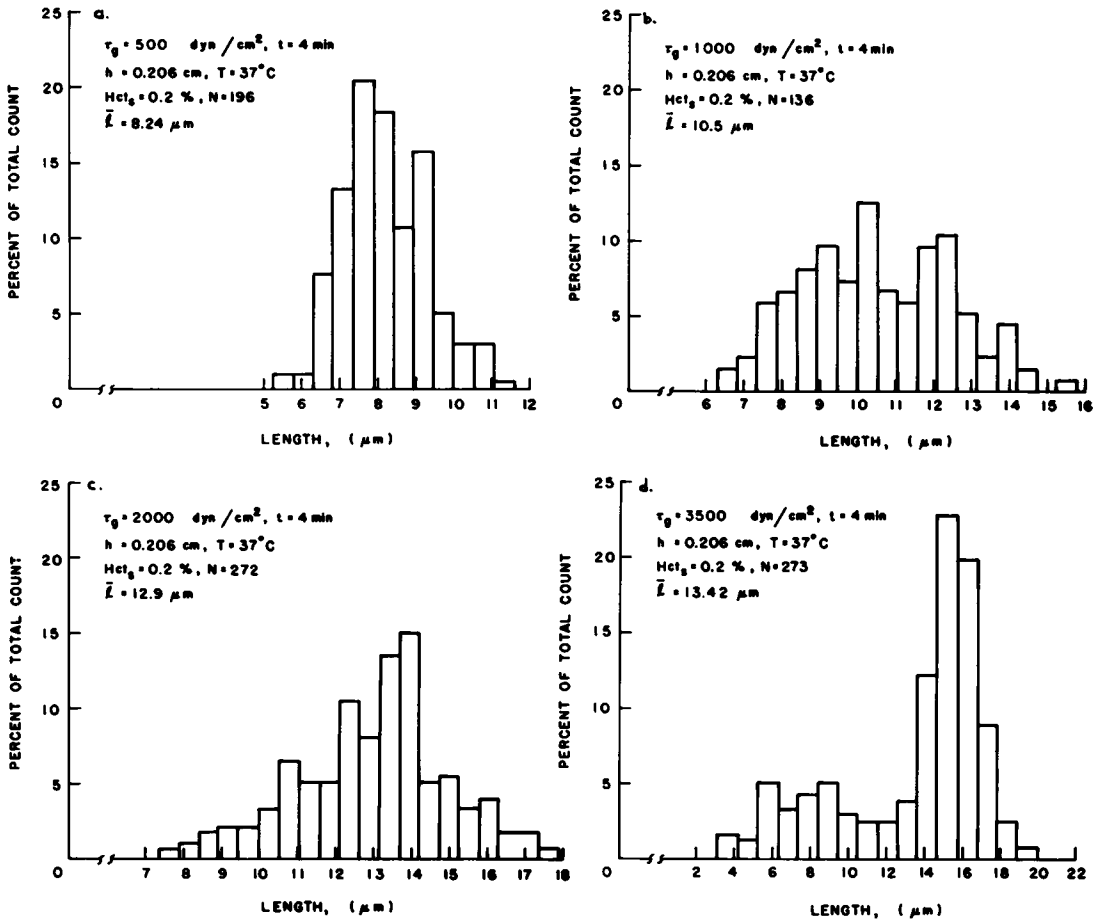

FIGURE 3 Histograms showing distributions of maximum cell dimension in human red cells fixed under shear at gap stresses (τ_g) of (*a*) 500, (*b*) 1,000, (*c*) 2,000, (*d*) 3,500 dyn/cm^2. Cells were fixed after 4 min of shearing at 37°C. Initial cell concentration: 0.2% by volume. On each histogram the corresponding mean length, $\bar{l}$, is given.

The results of the free hemoglobin determinations are summarized in Fig. 5. The data are presented as percent hemolysis, defined as the ratio of the free hemoglobin concentration to that which would obtain if 100% of the cells present were osmotically lysed. We see that the concentration of hemoglobin in the suspending medium is quite small until the stress exceeds 2,500 dyn/cm^2 or so. It then rises sharply to nearly 90% at 4,500 dyn/cm^2. Recall that this is also the range wherein cell fragmentation became strongly apparent.

DISCUSSION AND CONCLUSIONS

The technique of fixing red cells freely suspended in flow may prove to be a convenient and inexpensive tool for studying changes in cell shape in complex flows. However, a

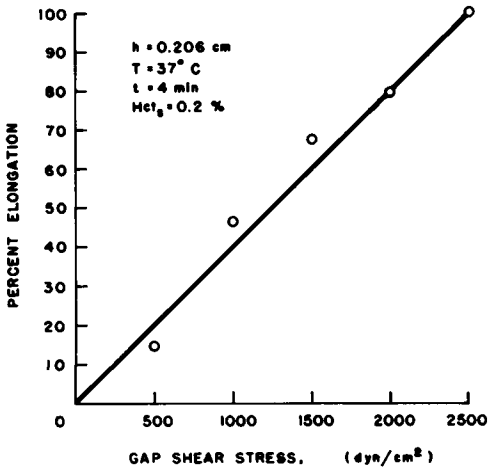

FIGURE 4

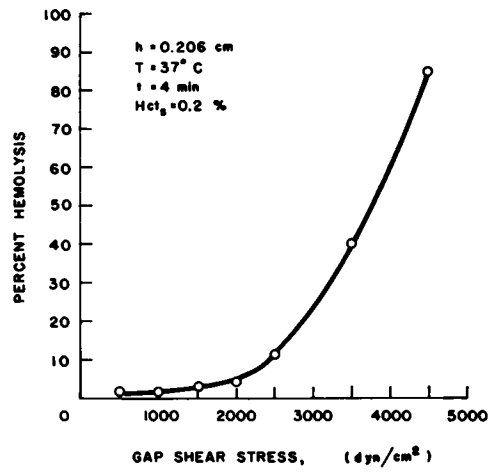

FIGURE 5

FIGURE 4 Percent elongation based on $\bar{l}$ and mean diameter of a control sample versus applied shear stress. Duration of shearing standardized at 4 min.

FIGURE 5 Percent hemolysis as a function of applied stress, after 4 min shearing at 37°C. Based on hemoglobin release.

number of fundamental questions must first be answered concerning the relationship of the final shape observed for a cell to that which it had at the instant the polymerization began. The two shapes would be identical only if the cell is in a steady state of deformation or if the typical rate of cell deformation is much smaller than the rate of the polymerization reaction.

The turbulent Couette flows used in this work are steady and homogeneous only on the average and only within the gap region. Nevertheless, at sufficiently high stress, there is a remarkable regularity and uniformity of deformations throughout the entire cell population. (See, for example, Fig. 1*b*.) This could be interpreted to mean that the random fluctuations present in the flow are not significant at the length scale of the cell. In other words, perhaps the scale of the turbulent eddies is much larger than the cells, so that the cells feel themselves in a local flow which is nonfluctuating and well correlated, i.e., laminar.

Some idea of the scale and intensity of the turbulent fluctuations in this particular flow can be gained from the data of Pai (12) and McPhail (13). According to those data, the turbulence intensity (the ratio of the fluctuating components of velocity to the speed of the rotor surface) is of the order of 10% and most of the turbulent energy resides in eddies about $^1/_{10}$ the gap width in diameter or larger. In the present device the gap is nominally 2 mm, and eddies 0.2 mm (= 200 μm) in diameter are clearly much larger than a red cell. Of course, smaller eddies must also be present, even though their energy content may not be appreciable, so that the scale argument is not entirely convincing.

Another possible implication of the uniformity of the observed deformations is that the cells have assumed a steady state orientation with respect to the streamlines of the average shear flow, or else they are rotating at a rate very much less than the rate of polymerization. In contrast, the shapes recorded in Fig. 1*a*, taken at the relatively low stress of 100 dyn/cm^2, are much more random in appearance.

The difference between the low and high stress cases appears to be consistent with the observations of Schmid-Schonbein and Wells (14) and Goldsmith and Marlow (10). They reported that at low rates of shear, red cells behaved more or less like solid particles, tumbling, bending, etc., as they were carried along by the flow. On the other hand, at sufficiently high shear rates, the cells behaved more like liquid drops, deforming into ellipsoids, the ellipsoids holding a constant orientation in the flow, and the membranes rotating about the liquid interior (a kind of motion dubbed "tank-treading").

The evidence that rapid hemolysis and fragmentation go hand in hand beyond some critical stress is interesting, for it indicates that the fragments are not immediately self-sealing and that a substantial part of the intracellular hemoglobin escapes before they do seal. Although the hemolysis-stress curve of Fig. 5 is qualitatively similar to those presented by Leverett et al. (15), we should emphasize that our data were obtained in 4-min *turbulent* runs, whereas they operated in the laminar regime with a standard 2-min shearing time.

Our experience also indicates that the hemolysis-stress relationship is temperature-dependent (the data of Fig. 5 were obtained at 37°C) and, moreover, dependent on the kinematics of the shear flow. Experiments performed in our viscometer with smaller

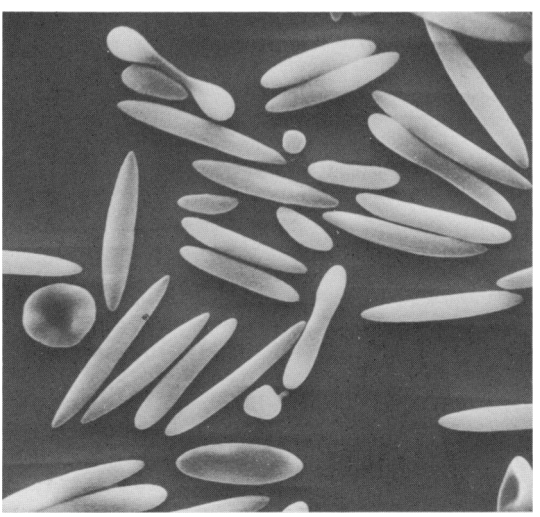

FIGURE 6 Human red cells fixed under shear at 4,500 dyn/cm^2 after 2 min of shearing at 37°C. One cell has been stretched into a dumbbell shape, possibly before separating into two fragments.

gaps indicate a shifted curve. In these smaller gap experiments the average *rates* of shear are higher over the same range of shear stress, and the transitions from the laminar to Taylor regimes and from the Taylor to turbulent regimes occur at higher stresses. It must also be expected that the microstructure of the turbulence changes with the size of the gap. To summarize, it seems to be inadequate to discuss the deformation (or destruction) or red cells as a function of shear stress or average shear rate alone. Rather, it is necessary to specify completely the shear flow imposed on the suspended cells, both kinematically and kinetically.

Finally, in regard to the important question of whether the trauma observed is occurring at the walls of the viscometer or in the shear flow away from the walls in bulk, or both, it can only be said that the photographic evidence strongly suggests that the cells are being ruptured in the bulk flow. For example, we see in Fig. 6 one cell which has been stretched into a dumbbell shape. This would be the stage of deformation logically preceding rupture into two teardrop-shaped fragments. Such fragments, featuring one sharply pointed tail, are very much in evidence in some of the photographs we have obtained at stresses beyond the fragmentation threshold. On the other hand, a wall-encounter mechanism cannot be definitively ruled out as a factor in the cell fragmentation.

The technique of cell fixation during flow in the Couette viscometer was first proposed and tested in our laboratory at Washington University by Dr. N. Mohandas, who is presently associated with the Institut de Pathologie Cellulaire, Hôpital de Bicêtre, Paris, France.
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