DEFORMATION OF HUMAN ERYTHROCYTES IN A CENTRIFUGAL FIELD

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ABSTRACT A new method for altering red cell morphology by high-speed centrifugation of cells through a physiological medium is described. Cell shape is preserved for microscopic analysis by allowing the sedimenting cells to pass from the physiological medium into a glutaraldehyde fixative solution. Examination of the deformed, fixed cells indicates that the vast majority resemble spheres with a flat, triangular tail. Measurements of the overall length of deformed cells show a nearly linear relationship between cell length and centrifugal force; average cell length increased from 8 to 11 μm as the centrifugal field was increased from 2,000 to 15,000 g. These data suggest that this centrifugal technique may be useful for evaluating cellular deformability and, potentially, the material properties of red cells.

INTRODUCTION

Interest in blood physiology and the structure of red cells has prompted a number of studies (Hochmuth and Mohandas, 1972; Bull and Brailsford, 1975, 1976; Evans, 1973) concerned with elucidating the mechanical properties of erythrocytes. These studies typically measure the extent of cell deformation when the cells are stressed by a known force. These techniques are time-consuming and frequently quite difficult to analyze, as fluid shear stresses are often used to deform the cell. Calculation of the fluid forces over an immobilized red cell can involve mathematical problems which are intractable without the use of simplifying assumptions that may vitiate the analysis. Furthermore, these techniques often require that the cells studied be in contact with or adhere to a foreign surface, a procedure that risks altering cellular properties or preselecting a certain population of cells.

Cell centrifugation presents an alternative method of studying the deformation of red cells stressed by known forces. The potential of centrifugation as an analytical tool in investigating the mechanical properties of cells has been demonstrated by Shapiro (1941). Centrifugation provides a means of applying a force that is well-defined, unidirectional, and of a known duration, and it can be used with or without fluid drag. The centrifugal force is simultaneously applied to all cellular components with little risk of altering the integrity of the cellular membrane by its association with foreign surfaces.

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Two types of centrifugal analysis are potentially useful in the study of cell structure: sedimentation equilibrium and sedimentation velocity studies. The former involves the sedimentation of the cells through a density gradient into a neutrally buoyant zone. When appropriate optical systems are available, this system provides an opportunity for examining the shape of the cell under the influence of a known force, in the absence of fluid drag. Unfortunately, density gradients inevitably place cells in a hyperosmotic medium, which may substantially alter the properties of the cell.

Sedimentation velocity studies, in which cells are rapidly centrifuged through a fluid medium, present an alternative method of investigating the mechanical properties of the cell. This technique has most of the assets of a sedimentation equilibrium experiment and, in addition, allows the cells to be centrifuged in a physiological medium. These advantages are somewhat offset by the presence of fluid drag forces that complicate analysis of the net forces acting on the cell. Furthermore, characterization of cell morphology during the sedimentation process is difficult, because the construction of a centrifuge and optical system for photographing moving cells is technically very demanding and makes no provision for subsequent investigation of cellular ultrastructure.

A number of studies on the use of glutaraldehyde as a fixative suggest a means of circumventing these difficulties. Morel et al. (1971) and Gordon et al. (1963) have demonstrated that the fixation of cells with isotonic buffer solutions containing 1 and 2% glutaraldehyde does not alter the morphology of the cells. Sutera and co-workers (1975) have shown that the fixation process takes on the order of 1 s. Thus, any transformations of cell shape which occur as a result of fluid drag and centrifugal forces on the cell might be preserved by allowing the deformed cell to centrifuge into a glutaraldehyde solution.

The intent of this study was: *a*) to assess the optimal conditions for preserving red cell shape when under the influence of a centrifugal field; *b*) to determine the variables which control cell shape in a centrifugal field; *c*) to examine red cell morphology as a function of the forces which deformed the cell.

METHODS AND MATERIALS

Materials

CENTRIFUGE A Beckman 152 microfuge, stripped of its outside metal housing cover and cylindrical metal basket, was mounted horizontally in a bracket designed to dampen vibration (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.). The speed of the centrifuge was controlled by a Variac transformer (Gen Rad, Inc., Concord, Mass.), whose output could be varied from 0 to 140 V. The metal microfuge tube holding strips were replaced with two sturdier (66 × 12 × 2 mm) steel plates with a single 6-mm hole, 6 mm from one end of the plates.

MICROFUGE TUBES 550-μl round-bottom microfuge tubes, 4.7 cm long with a 4.2 mm inside diameter, were obtained from Bolab, Inc., Derry, N.H.

MICROFUGE TUBE INSERTS Teflon microfuge tube inserts were designed to prevent vibrations from mixing fixative and suspending fluids in the centrifuge tube during the course of a run and to prevent the centrifuged cells from spreading over a large area on the bottom of the tube. A schematic drawing of the microfuge tube and Teflon tube insert is shown in Fig. 1.

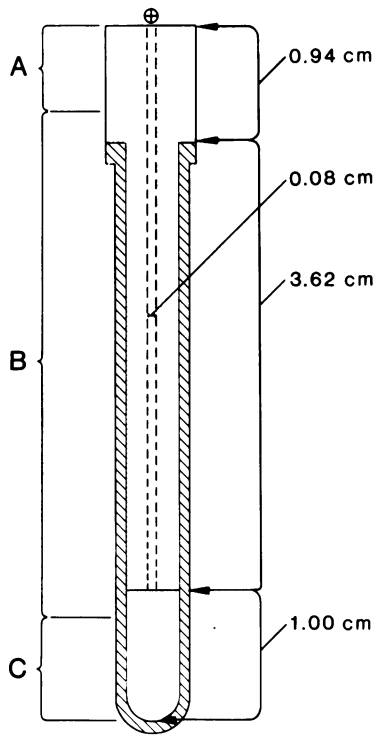


FIGURE 1 Microfuge tube with Teflon insert. Head of Teflon insert is 0.08 cm from the center of rotation (center of rotation indicated by ⊗). The regions to the left of the microfuge tube indicate the locations of the various fluids immediately before centrifugation: *A* = erythrocytes in albuminated PBS in head of Teflon insert; *B* = albumin-free PBS; *C* = 2% glutaraldehyde in albumin-free PBS.

ELECTRONIC CELL COUNTER An Electrozone-celloscope, equipped with a 76-μm orifice, was purchased from Particle Data Inc., Elmhurst, Ill.

REAGENTS All salts and other reagents were of reagent grade. All water was treated with ion exchange resins before distillation.

Electron microscope grade glutaraldehyde (50 g/100 ml) was obtained from Electron Microscopy Sciences, Fort Washington, Pa. Phosphate-buffered saline (PBS) for red cell suspensions was made up with a pH of 7.42 ± 0.02 and a total PO_4 concentration of 0.03 M. The osmolality of the PBS was adjusted to 290 ± 3 mosmol/kg by the addition of NaCl. The PBS was albuminated with salt-poor human serum albumin (American Red Cross) to a concentration of 0.2%.

Methods

PREPARATION OF RED CELLS Venous blood was taken from healthy donors into a heparinized syringe (0.05 mg heparin/ml blood). The blood was centrifuged, the plasma and buffy coat were removed, and the packed cells were washed twice in albuminated PBS. All centrifugations were at 20 – 24°C for 10 min at 2,000 *g*. After the second wash, the cells were suspended in albuminated PBS to a predetermined cell concentration. Cell concentrations were determined with an electronic cell counter. All studies on cell deformation were completed within 6 h of blood donation.

DEFORMATION OF RED CELLS Red cells were deformed by centrifuging them into a PBS solution containing 2% glutaraldehyde. To accomplish this, 75 μl, unless stated otherwise, of the fixative was placed in 550-μl microfuge tubes and centrifuged to the bottom of the tube. The fixative solution was prepared by adding enough glutaraldehyde to nonalbuminated PBS to make its final glutaraldehyde concentration 2%. PBS was then layered on to the fixative solution with a Harvard syringe pump at a rate of 0.1 ml/min (Harvard Apparatus Co., Inc., Millis, Mass.). Dye studies showed that this procedure produced a minimum of mixing between the fixative and suspending fluid layers. A Teflon insert was then pushed into the tube after being rinsed three to four times with albuminated PBS. The cells were introduced into the bore of the insert by inserting a 25-gauge needle 5–7 mm down the bore and then placing 0.2 ml of the red cell suspension on the top of the insert. A 1-ml syringe was used to pull all but approximately 3 μl of the red cell suspension into the insert and up into the needle. Immediately thereafter, the entire assembly was centrifuged for a minimum of 5 s.

MEASUREMENT OF CELL LENGTH The greatest dimension of a cell was recorded as a measure of its length. A 100× or 95× oil immersion objective was used in conjunction with a 12.5× ocular for sizing. The 140-division reticle of the microscope was calibrated with a metric stage micrometer. Cells were sized only when one end of their greatest dimension lay within 2 μm of the focal plane in which the other end lay.

SCANNING ELECTRON MICROSCOPY Cells removed from the bottom of the microfuge tube were critical point dried with CO_2, coated with a gold-palladium alloy, and viewed in an AMR-1000 scanning electron microscope (Advanced Metals Research Corp., Bedford, Mass.) operating in the secondary emission mode at an accelerating voltage of 20 kV. No discrepancies in cell morphology between light and scanning electron microscopy were observed.

TRANSMISSION ELECTRON MICROSCOPY Cells removed from the 2% glutaraldehyde-PBS solution in the microfuge tubes were post-fixed in a solution of 1% osmium tetroxide in PBS. After post-fixation, the cells were washed three times in nonalbuminated PBS buffer and the cell pellet was suspended in a drop of 2% agarose at 50°C. The agarose was subsequently allowed to jell at 4°C, then dehydrated with a graded acetone series. Cells were then embedded in Epon 812 (Shell Chemical Co., Houston, Tex.). Thin sections, silver or gold in appearance, were cut with either a glass or diamond knife, stained with uranyl acetate and lead citrate, and photographed in a Philips EM 300 electron microscope (Philips Electronics Instruments, Inc., Mount Vernon, N.Y.).

CALIBRATION OF MICROFUGE SPEED The terminal speed of the microfuge was determined with a strobe lamp (Strobotac, type 1531-A, Gen Rad, Concord, Mass.). The rate of acceleration of the microfuge was measured with an optical tachometer interfaced to a strip chart recorder. The tachometer consisted of a photoelectric cell whose output was altered to a voltage signal by a high-frequency response (<0.001 s) frequency to voltage converter.

RESULTS

Centrifugation of normal erythrocytes into PBS containing 2% glutaraldehyde alters the morphology of the cells. The morphology of the deformed cells depends on the conditions under which the cells were deformed and is influenced by the concentration of cells centrifuged, the height of the glutaraldehyde column, and speed of centrifugation. The effects of these variables are discussed below.

Cell Concentration

When the concentration of cells loaded into the bore of the Teflon insert is less than 2×10^7 cells/ml, at least 95% of the cells have shapes similar to the cells shown in Figs. 2 *a* and *b*. These cells consist of a rounded head approximately 5 μm in diameter

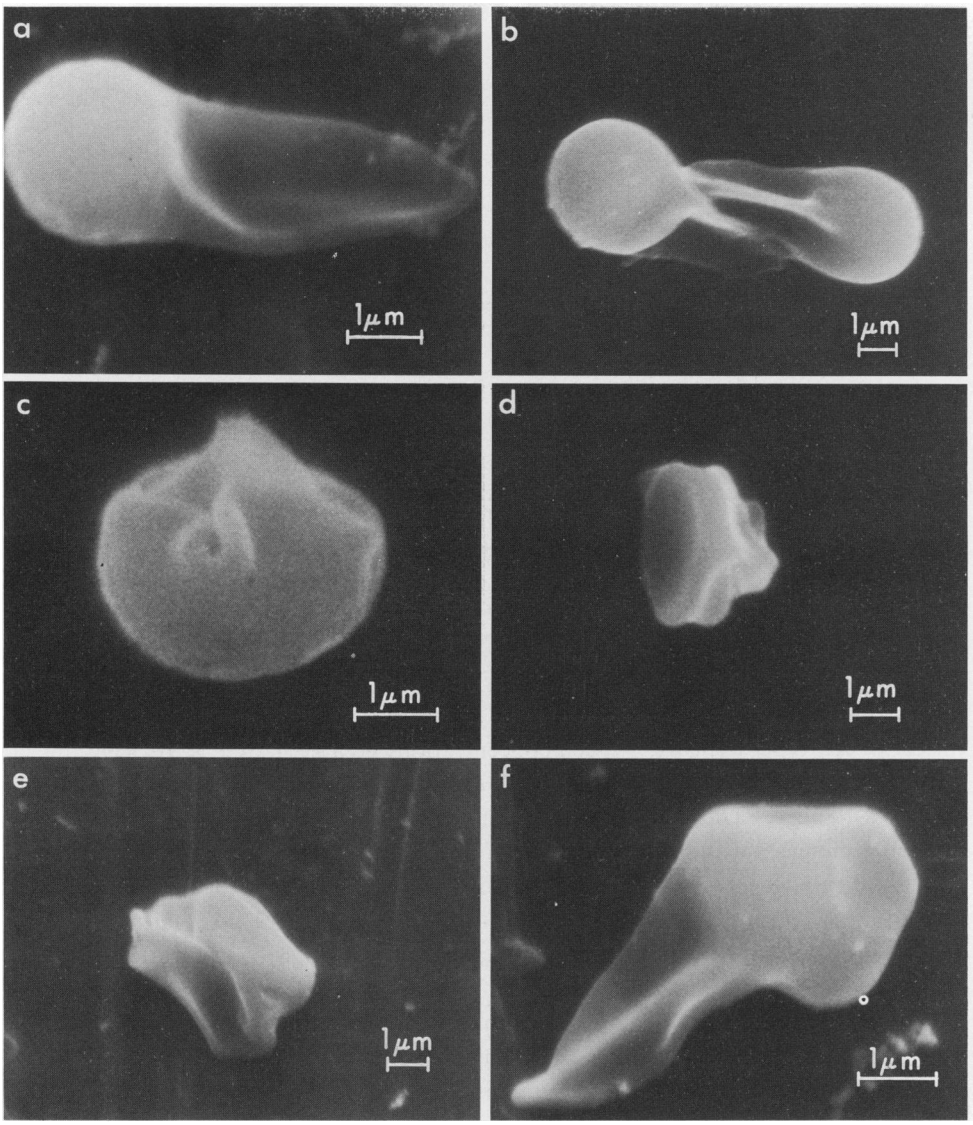


FIGURE 2 Typical cell types observed when cells are deformed under varying conditions. The concentration of cells loaded into the Teflon insert (cells per milliliter) and the length of the glutaraldehyde zone are as follows: *a*,*b*,*c* 2×10^7, 8.0 mm; *d*,*e*,*f* $> 1 \times 10^8$ cells/ml or nonzero glutaraldehyde zones less than 7.0 mm in length. All centrifugations were at the maximum microfuge speed, 16,000 rpm.

and a long triangular tail. The overall length of the cell varies between 7 and 15 μm. The tail of the cell appears either flat or Y-shaped. The remaining 3–5% of the cell population which have no tails have the appearance of the cell pictured in Fig. 2 *c*. These cells are roughly spherical and smooth over approximately 70% of their surface.

As the concentration of cells loaded into the microfuge tube bore increases, a

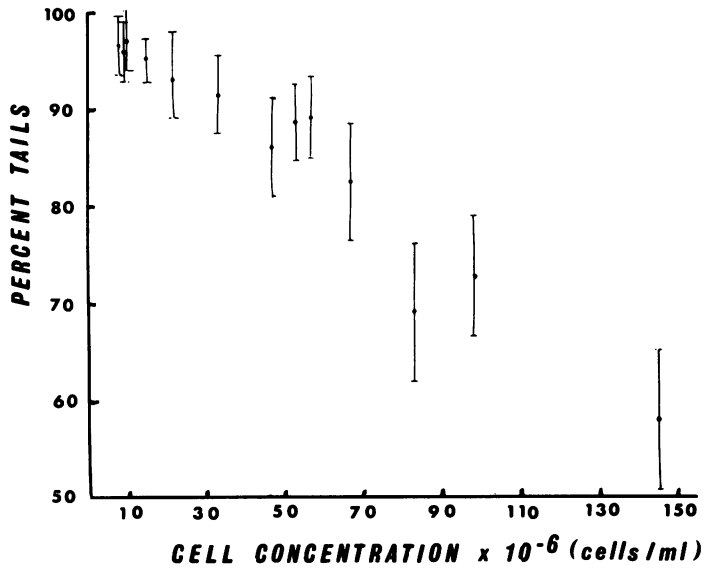


FIGURE 3 Relationship between percentage of cells having tails and the concentration of cells loaded into Teflon insert. Deforming conditions were: RCF = 15,000 *g*, 2 × 10^7 cells/ml, 8.0 mm = glutaraldehyde zone length. Erro bars represent the standard deviation of at least 30 cells.

variety of cell types appears. At cell concentrations greater than 1 × 10^8 cells/ml, a substantial number (>10%) of cells have an ill-defined shape similar to those portrayed in Figs. 2*d* and *e*. These cells have no tails or characteristic morphology and their frequency increases with the concentration of cells centrifuged. The last major cell type is pictured in Fig. 2*f*. Morphologically, these cells are similar to those pictured in Figs. 2*a* and 2*b*, except that their heads are often dented and their tails frequently bent in irregular shapes. These cells appear when very high concentrations of cells (>1 × 10^8 cells/ml) are centrifuged.

The effect of cell concentration on cell morphology is summarized in Fig. 3, where the percentage of cells morphologically similar to those pictured in Figs. 2*a* and *b* is plotted as a function of the concentration of red cells in the suspension loaded into the Teflon insert. The percentage of cells forming tails approaches 98% as the cell concentration nears zero and gradually decreases with increasing cell concentration. At a concentration of 5 × 10^8 cells/ml approximately 10% of the cells have tails.

Length of Glutaraldehyde Zone

Studies of the amount and concentration of glutaraldehyde to be used in fixing the cells were necessitated by the nature of the centrifugation process. Since red cells traverse the length of the microfuge tube in less than 5 s at maximal microfuge speeds, a high concentration of glutaraldehyde was necessary to ensure complete fixation of the cells before they reach the bottom of the tube. Here fixation refers to the ability of

the cell to withstand impact on the bottom of the microfuge tube without a change in cell morphology. However, Morel and co-workers (1971) demonstrated that the fixation of red cells with PBS containing 8% glutaraldehyde caused changes in cell morphology. Although preliminary studies at glutaraldehyde concentrations up to 8% indicated no substantive changes in the appearance of the deformed cells, a concentration of 2% glutaraldehyde was chosen, as it seemed probable that it would rapidly fix erythrocytes without the possibility of subtle morphologic alterations.

The minimum length of a zone containing 2% glutaraldehyde which would fix cells before they reached the bottom of the tube was determined by centrifuging cells (2×10^7 cells/ml) into zones of fixative solution of varying lengths. These results are shown in Fig. 4, where the mean length of the deformed cells is plotted as a function of the length of the zone containing glutaraldehyde. When the zone was 20.0 mm long, the average length of the deformed cells, $\bar{x}$, was only 8 μm. Decreases in the length of the fixative zone up to 10.8 mm produced minor increments in $\bar{x}$. Mean cell length increased rapidly as the length of the fixative zone was decreased from 10.8 to 8.0 mm. A decrease in the length of the fixative zone from 8.0 to 7.2 mm, however, produced a

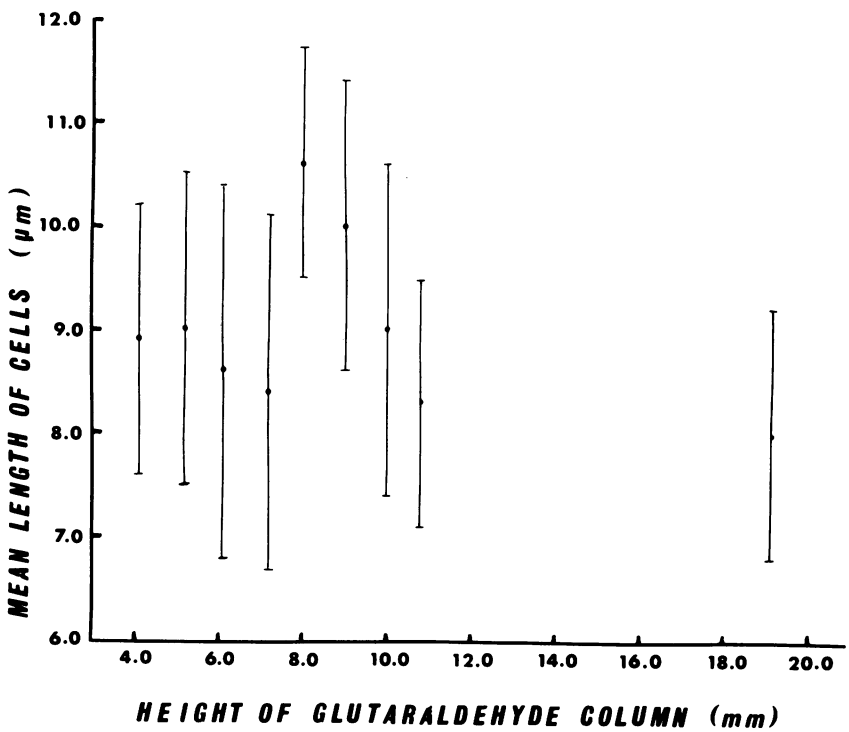


FIGURE 4 Relationship between mean length of deformed cells and the length of the glutaraldehyde zone used to fix the cells. All data were obtained at a microfuge speed of 16,000 rpm and a cell concentration of 1×10^7 cells/ml. Each point represents the mean and standard deviation of at least 30 cells.

significant decrease in $\bar{x}$. Further decreases in the length of the fixative zone did not result in significant ($P > 0.05$) changes in $\bar{x}$.

While changes in the overall length of the deformed cell provided a quantitative measure of the effects of changing the length of the fixative zone, definite changes in the morphology of the fixed cell were also observed. As zone lengths approached 20.0 mm, most cells formed only very short tails, with an increasing percentage failing to form tails entirely. Decreases in the height of the fixative zone allowed cell morphology to approximate that of the cells pictured in Figs. 2 *a* and *b*. When the length of the column decreased from 8.0 to 7.2 mm, the sudden decrease in cell length was accompanied by an abrupt change in morphology. Most cells assumed a crumpled shape similar to that shown in Fig. 2 *f*. Further shortening of the glutaraldehyde column brought no major alterations to the appearance of the cell, until no glutaraldehyde was present in the column. In the absence of glutaraldehyde, the cells assumed the shape of discocytes or echinocytes I when resuspended in PBS.

Relative Centrifugal Field

The effect of the relative centrifugal field on cell morphology was investigated by altering the speed at which the cells were centrifuged. Auxiliary studies showing that the centrifuge was always at its maximum speed when the cells reached the fixative solution are given in Appendix I.

At low speeds (500 *g*) minimal cell deformation was observed, with the majority of cells having very short tails (Fig. 5). On edge, the cells appeared as teardrops and from

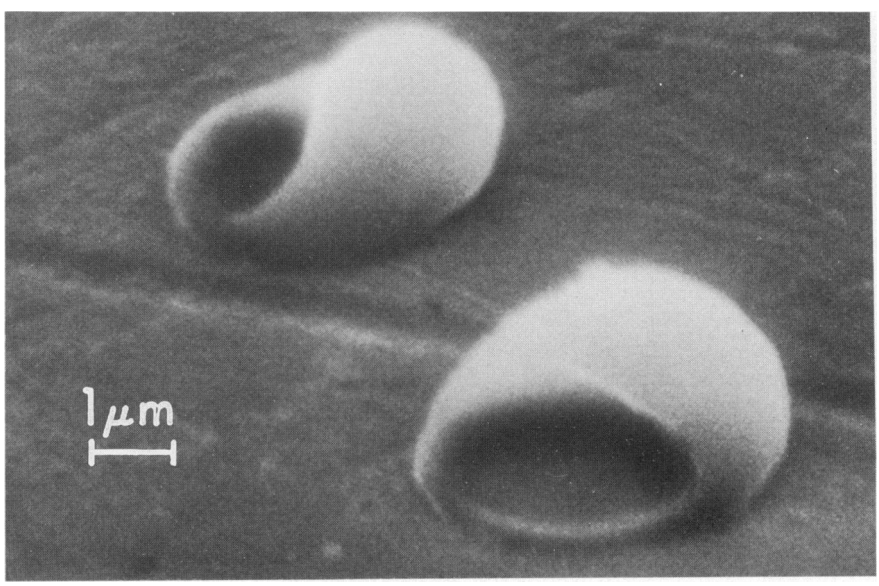


FIGURE 5 Morphology of cells deformed at 500 *g*.

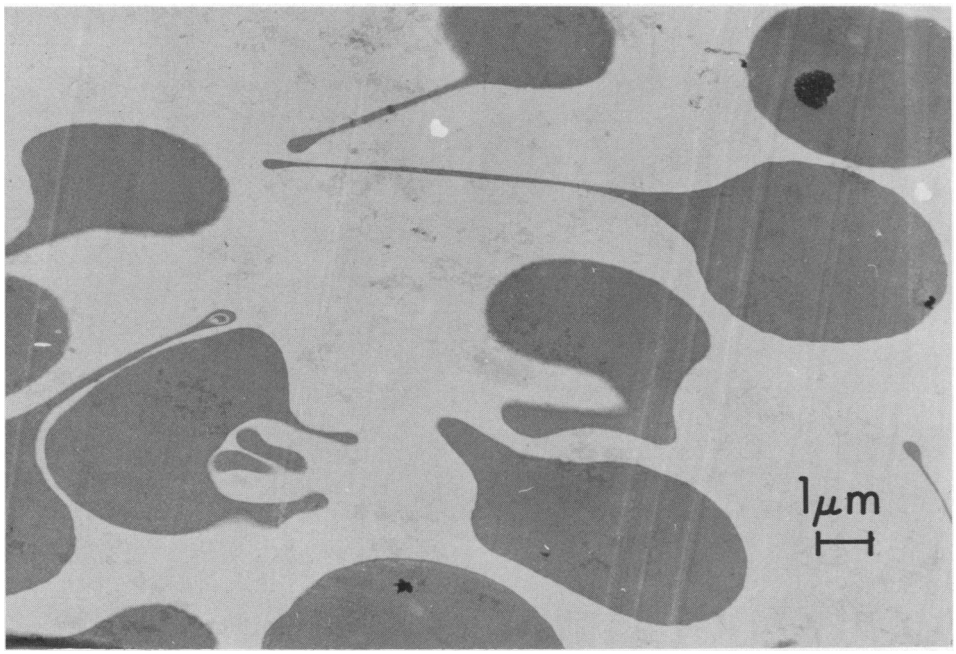
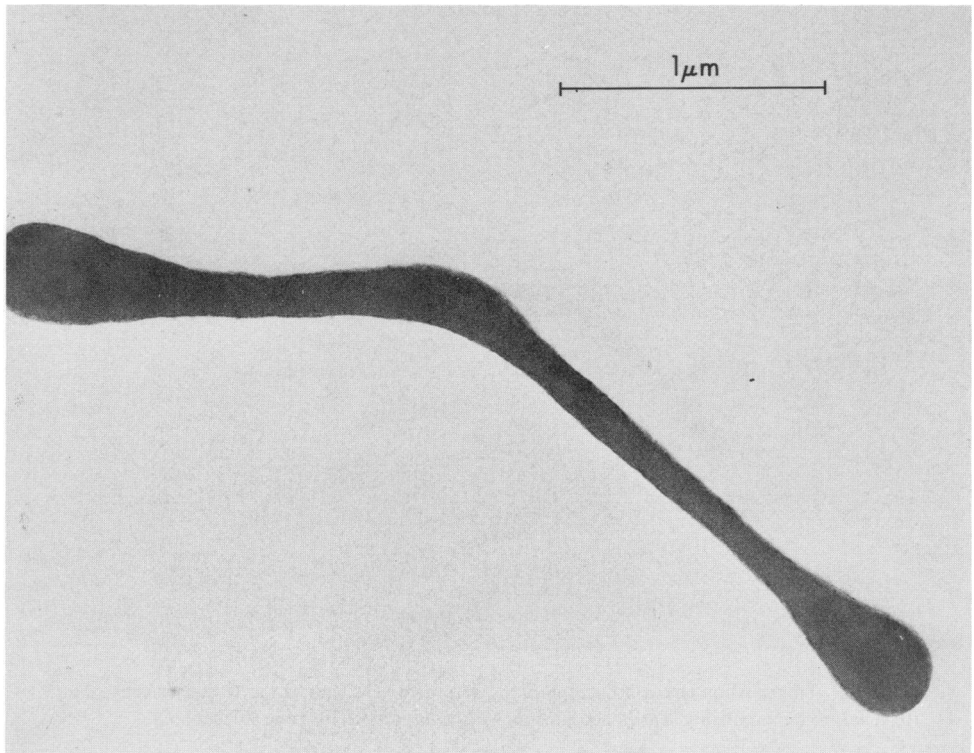


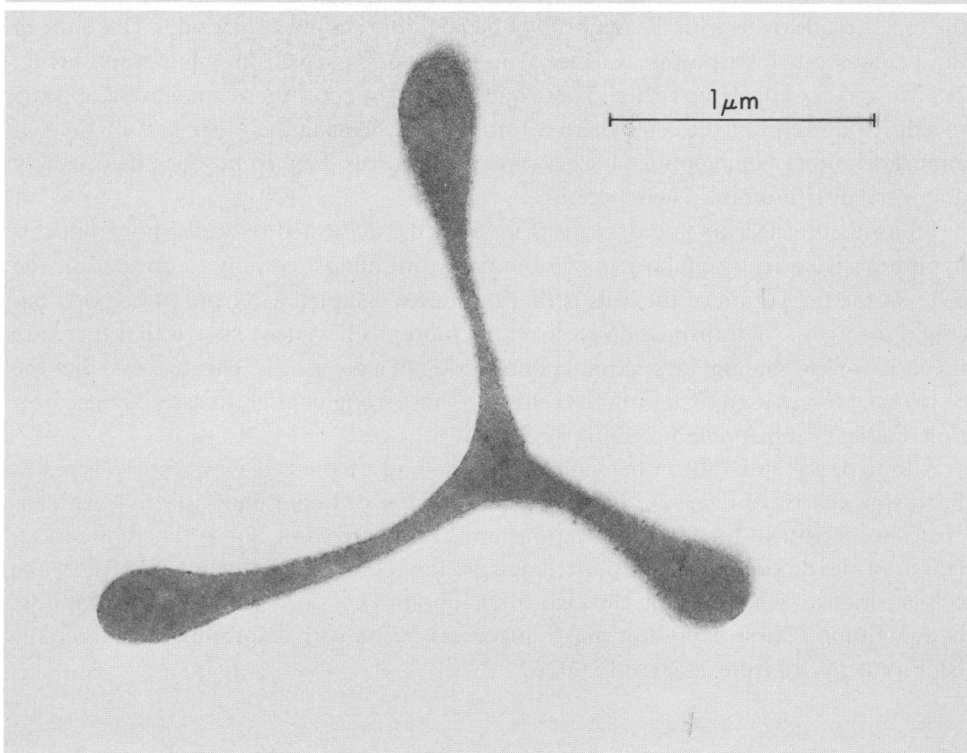
FIGURE 6 Transmission electron micrograph of deformed cells. Erythrocyte suspensions (2×10^7 cells/ml) were centrifuged at 14,800 *g* through a 2% glutaraldehyde zone 8.0 mm long.

the top as crude ovals with the majority of hemoglobin shifted to one side. This shift in the hemoglobin distribution was seen in all scanning electron micrographs (Fig. 2 *a*, 2 *b*, and 5) of deformed cells. These micrographs suggest that hemoglobin appears to shift to one end of the cell, where it forms a ball, leaving the other end of the cell almost devoid of hemoglobin. Larger centrifugal forces seem to produce increasingly abnormal distributions of hemoglobin.

Transmission electron micrographs (Fig. 6) of the deformed red cells show that this hypothesis is correct; cellular hemoglobin does shift almost entirely to one end of the cell and the tail region of the cells is thin but never completely devoid of hemoglobin (Fig. 7 *a* and *b*). All transmission electron micrographs have also shown that there are no variations in shading throughout a thin section of a given cell. This suggests that the centrifugal forces used to deform the cells were not strong enough to set up concentration gradients of hemoglobin within a cell.

Although a major shift in hemoglobin to one end of the cell was accomplished at fairly low centrifugal forces, the total length of the cell continued to grow with increasing centrifugal force. This relationship is summarized in Fig. 8, where the mean length of the deformed cells is plotted against the relative centrifugal field (RCF) the cell experiences while passing through the midpoint (r = 5.24 cm) of the glutaraldehyde solution. These data show that $\bar{x}$ increases linearly with deforming force when the RCF is increased from 2,000 to 15,000 *g*.


1μm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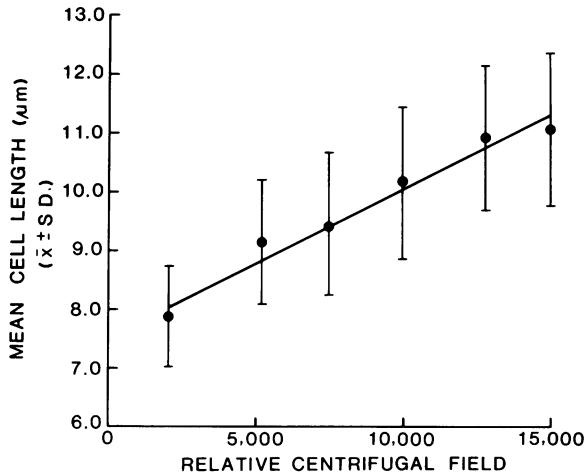

FIGURE 8 Relationship between mean length of deformed cell and average relative centrifugal field in glutaraldehyde zone 8.0 mm in length; cell concentrations were kept between 1–2 × 10^7 cell/ml. Error bars represent the standard deviation of at least 90 cells.

DISCUSSION

Inspection of the cells in Fig. 2 indicates that centrifugation of red cells can cause major changes in cell morphology. The extent of deformation is linearly related to how much centrifugal force the cell is subjected to and, apparently, the nature of the fluid dynamic forces the cell experiences.

In very dilute cell suspensions, high-speed centrifugations (15,000 *g*) transform red cells into particles with rounded heads and long, flat or Y-shaped tails. A cursory analysis of the forces acting on the cell suggests at least two mechanisms by which cell shape may be altered. The first is due to the difference in the densities of the cell membrane and hemoglobin solution. In addition, the drag of the suspending fluid on the membrane of the cell will cause a further difference in the accelerations that the cell contents and membrane experience. These two effects should allow the hemoglobin to collect at the head of the cell. This analysis is corroborated by the transmission electron micrograph (Fig. 6) that shows that the normal distribution of intracellular hemoglobin has radically changed.

Although all of the red cells are exposed to the deforming effects of large centrifugal forces, a small percentage of the cells fail to form tails (Fig. 2 *c*). Two hypotheses for the mechanism by which tailless cells are formed seem plausible. First, these cells constitute a special population that does not form tails under the forces they experience in the microfuge. This hypothesis is supported by studies (Leblond, 1973; LeCelle, 1969)

FIGURE 7 Transmission electron micrographs of tail sections of cells. Sections are cut perpendicular to the axis of the cell. Upper panel: cell with flat tail; lower panel: cell with Y-shaped tail. All cells were deformed with an average RCF of 15,000 *g*.

on the deformability of red cells that show that in a normal population of red cells, a wide range of cellular deformabilities exist. An equally plausible hypothesis is that a small random percentage of the cell population never stays oriented within the centrifugal field long enough for the hemoglobin to migrate to one end of the cell before it is fixed. A method of determining which hypothesis is correct has not presented itself.

If the formation of tails by red cells is due to the combined action of centrifugal and fluid drag forces, the effects of centrifugal force, cell concentration, and the length of the fixation zone on cell morphology can be accounted for. To facilitate a discussion of the effects of cell concentration on cellular morphology, consider the passage of a concentrated red cell suspension (5×10^8 cells/ml) into the glutaraldehyde-PBS solution. At the highest microfuge speeds, the cells pass through a zone of 2% glutaraldehyde 8 mm long and 0.8 mm in diameter (the diameter of the bore of the Teflon insert). If the passage of the cells through the glutaraldehyde solution is so rapid that any glutaraldehyde removed by the cells of the suspension is not replaced by diffusion, the cells are exposed to 5×10^{17} molecules of glutaraldehyde. Thus, when the concentration of cells in the suspension loaded into the centrifuge is 5×10^8 cells/ml, 4×10^{11} molecules of glutaraldehyde are present for each red cell. This is several orders of magnitude more glutaraldehyde than is necessary to render the cell rigid (Heusinkveld, 1973) and roughly eight times the amount required to occupy every available glutaraldehyde binding site in the cell (Morel et al., 1971). These data suggest that at high cell concentrations, the cell suspension acts as a single nonporous object that allows very little fluid to flow between the cells. Under these conditions, most cells would not be fixed until they had come to rest on the bottom of the tube. This would account for the shape of the cells in Figs. 2 *d* and *e*. Cells that form tails at high cell concentrations are presumably located at the periphery of the pack, where the flow of fixative through the pack is sufficient to fix the cells before they reach the bottom of the microfuge tube. The effects of cell crowding vanish at low cell concentrations and the percent of cells that form tails approaches 98%.

Changes in cell morphology and length associated with alterations in the length of the zone containing glutaraldehyde are caused by variations in the length of time the cells are exposed to the glutaraldehyde and the force the cells experience before they are fixed. When the zone length nears 20 mm, centrifuged cells travel only a short distance from their center of rotation before fixation. As such, they will not have encountered a large deforming force and will exhibit only minimal morphological changes. This is readily deduced from the cells pictured in Fig. 5, which have been subjected to much smaller forces than the cells depicted in Figs. 2 *a* and 2 *b*. As the height of the glutaraldehyde column continued to decrease, deforming forces and alterations in morphology continue to increase. The effect of increasing the centrifugal forces a cell experiences before it is fixed is shown in Fig. 4, where $\bar{x}$ is shown to rapidly increase with decreases in the length of the fixation zone. However, when the length of the fixation zone is decreased to 7.2 mm, the abrupt change in cell morphology suggests that the cells reach the bottom of the tube before they are completely fixed. Presumably, cell shape is transformed on contact with the bottom of the tube from the classic morphology (Fig. 2 *a* and *b*) to a buckled configuration (Fig. 2 *f*) and then fixed.

This observation provides a means of estimating the length of time required to fix a cell in 2% glutaraldehyde. If fixation is defined in terms of the cell's ability to resist gross morphological changes on impact with the bottom of the microfuge tube, the fixation time of the cell roughly equals its transit time through the fixation zone. At 15,000 *g*, this has been estimated at 1 s by treating the cell as a hydrodynamic sphere of radius 2.5 μm, and buoyant density 0.1 g/ml (Appendix I). This estimate agrees with the work of Sutera and co-workers (1975), but neglect of the hydrodynamic effects of the cell's tail causes an underestimate of its fixation time.

The effects of centrifugal force on cell length are shown in Fig. 8, where the average length of the deformed cells is shown to be linearly related to the applied relative centrifugal field between field strengths of 2,000 and 15,000 *g*. Since the total length of the cells changed by only 40% at maximal deforming forces, it is not possible to predict whether the length vs. force curve will change exponentially, as claimed by Bull and Brailsford (1975), or continue to change linearly. Beams and Kessel's (1966) photographs of rat cells, deformed in a centrifugal field of 400,000 *g*, suggest that the linear relationship is not obeyed up to these extremely high deforming forces. However, their method of deforming the cells did not allow the erythrocytes, enclosed in pieces of spleen, to experience fluid drag forces and as such, it is very difficult to calculate the actual deforming force the cells experienced.

These experiments have demonstrated that under well-defined conditions, centrifugal forces can be used to deform red cells consistently. The nature of cellular deformation can be studied by fixing the red cells with glutaraldehyde. Measurements of the extent of red cell deformation as a function of the centrifugal field experienced by the cells have shown the two variables to be linearly related. Since the deforming forces on a sedimenting red cell are fluid drag and centrifugal forces, which can be estimated from easily measured parameters of this system, it is theoretically possible to relate the extent of cell deformation to the deforming forces on the cell. Thus, it is probable that the technique of centrifugally deforming cells may be used as a means of investigating the material properties of red cells or, if properly defined, cell deformability. An initial attempt at estimating the material properties of red cells based upon this centrifugal technique is presented in Appendix II.

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APPENDIX I

Calculation of Transit Times of a Spherical Particle in a Centrifugal Field

The length of time a spherical particle takes to travel between two points in a centrifuge tube is calculated by a simple integration of Stokes' equation. This equation states that the velocity,

V, of a spherical particle of radius a, and density, ρ_2, traveling through a fluid of density ρ_1 and viscosity η, under a relative centrifugal field, P, is given by:

$$V = 2(\rho_2 - \rho_1) Pga^2/9\eta, \tag{1}$$

where g is the gravitational constant. However, applicability of the equation is limited to situations characterized by low Reynolds numbers. The maximum Reynolds number for red cells in the microfuge is on the order of 0.1.

The relative centrifugal field, P, a particle experiences in a centrifuge is given by:

$$P = \omega^2 r/g, \tag{2}$$

where r is the distance of the particle in centimeters from the center of rotation and ω is the angular velocity of the rotor (radians per second).

Substituting Eq. 2 into Eq. 1 gives:

$$V = dr/dt = 2\omega^2 a^2 r(\rho_2 - \rho_1)/9\eta. \tag{3}$$

Although Stokes' equation is strictly applicable only in situations where the particle has reached a terminal velocity, calculations of the velocity of an idealized red cell in PBS in a uniform centrifugal field show that it will reach terminal velocity within microseconds. Thus, the red cell is essentially always traveling at terminal velocity and the use of Stokes' equation in Eq. 3 is justified.

Integration of Eq. 3 provides the relationship for calculating the time required to travel between two points in a centrifugal field whose distance from the center of rotation is r_1 and r_2. Rearranging Eq. 3:

$$\int_0^t dt = \int_{r_1}^{r_2} [9\eta/2\omega^2 a^2(\rho_2 - \rho_1)](dr/r), \tag{4}$$

$$t = [9\eta/2\omega^2 a^2(\rho_2 - \rho_1) \ln (r_2/r_1). \tag{5}$$

This equation can be used to estimate the length of time a red cell takes to cross the zone containing glutaraldehyde, its fixation time, or to show that the centrifuge is always at its terminal speed when the red cells reach the glutaraldehyde zone.

In demonstrating that the centrifuge was always at its terminal speed before the red cells were fixed, the red cells are assumed to be hydrodynamically equivalent to spheres of diameter 5 μm,

TABLE I

Output voltage	Terminal speed	Average RCF*	Time to 99% terminal speed	Cell transit time
	rpm		*s*	*s*
140	16,000	15,000	0.69	0.83
120	14,800	12,800	0.77	0.94
100	13,100	10,050	0.90	1.2
80	11,300	7,500	1.4	1.6
60	9,400	5,200	2.1	2.3
40	6,000	2,100	3.1	5.8

*The average RCF a cell experiences while passing through an 8.0-mm-long fixative zone (r = 5.24 cm).

(the approximate diameter of the hemoglobin mass on a cell deformed at 15,000 g), with a buoyant density of 0.10 g/ml. Furthermore, it was assumed that their position in the Teflon insert when the microfuge was started was 8 mm from the microfuge's center of rotation and that they encountered glutaraldehyde at the end of the Teflon insert (r_2 = 4.64 cm). The microfuge was treated as if it had instantaneously reached terminal velocity. Given these assumptions, the length of time a red cell took to travel to the glutaraldehyde, the cell transit time, was calculated for various centrifuge speeds. The results of these calculations are presented in Table I. The table also lists the length of time the centrifuge took to reach 99% of its terminal velocity, the output voltage of the Variac transformer necessary to drive the microfuge at a given speed, and the average relative centrifugal field a cell experiences when passing through a glutaraldehyde zone 8.0 mm long at the various speeds. It is apparent from Table I that the microfuge is at maximum speed when the cells encounter the glutaraldehyde fixative. This conclusion is even more evident when the assumptions concerning cell shape, microfuge acceleration rate, and the positions of the cell at the start of centrifugation and when it encounters the glutaraldehyde are noted, as they produce underestimates of the actual cell transit time.

The minimal length of time required to fix a cell is estimated from Eq. 5 by making the aforementioned assumptions about cell size, shape, and buoyant density and using 16,000 rpm with r_1 = 4.84 cm and r_2 = 5.64 cm as the limits of integration. This procedure again underestimates the actual fixation time but provides a lower estimate of 1 s for the time required to fix cells in 2% glutaraldehyde.

APPENDIX II

Estimation of Material Constants of the Red Cell Membrane

An estimation of the material constants of the red cell membrane requires an analysis of the stresses on the cell and measurement of the strains produced. To analyze stresses on an erythrocyte in a centrifugal field, it is convenient to envisage the cell as a mass of fluid enclosed by an incompressible membrane. Under these circumstances, two forces act to distort the shape of the cell: (*a*) Fluid drag forces: As the cell moves through the fluid, fluid drag will retard the membrane of the cell with respect to the cellular contents and thereby elongate the cell. At the low particle Reynolds numbers which prevail in the present situation, flow should be laminar and the fluid should not separate from the cell (Jensen, 1959). The total drag on the cell will be equivalent to the centrifugal force on the cell. To evaluate the elastic modulus, μ, of the cellular membrane, the theory of Evans and Hochmuth (1977) was applied to the tail portion of the cell. This theory states that for a rectangular strip of material, the elastic modulus of the material is related to the tension, T, and the extension ratio, λ, of the material by the following equation: $T = (\mu/2)(\lambda^2 - \lambda^{-2})$, where the extension ratio is defined as the ratio of the length of the strip to its original undeformed length. To apply this theory to the red cell, the tension in the membrane of the tail was calculated in a plane normal to the axis of the cell and tangential to the head, by assuming that the width of the tail was equivalent to that of the head; the percent of the total drag experienced by the tail was proportional to the surface area of the tail; the extension ratio of the tail could be calculated from the ratio of the length of the tail to the extrapolated length of the tail at 0 g. The mean length of the tail was defined as the average length of the cell minus the average diameter of the head (5 μm). The extrapolated length of the tail at 0 g was obtained from a plot of mean cell length vs. the centrifugal field the cell experienced midway through the glutaraldehyde. Calculations of the amount of membrane required to enclose a sphere of 5 μm diameter suggested that 50% of the cell's membrane would be available for tail formation. On this basis, 50% of the total drag on the cell was assumed to be acting on the tail. Using these assumptions, an average elastic modulus of ~0.1 dyn/cm

was calculated for a red cell deformed by RCF of 3,000–15,000 *g*. An alternative method of calculating the elastic modulus, in which the calculated extension ratio of the entire cell is applied to Evans' theory (Evans, 1973) for point-attached disks, yields an elastic modulus of ~0.1 dyn/cm. These values are approximately five times the accepted value for the red cell membrane but represent a reasonable agreement when the nature of the approximations used to make the calculations are considered. From these estimates, it seems likely that with appropriate model studies to determine drag coefficients of the deformed red cell, better estimates of membrane properties can be made.

(*b*) Differential accelerative forces: The centrifugal force on any element of a cell is proportional to the product of the buoyant density and volume of the element. Thus, isovolumic cellular elements of nonidentical densities will be subjected to forces of different magnitudes, which will tend to separate them. Since the red cell membrane and hemoglobin contents have different densities, approximately 1.19 (Lim et al., 1975) and 1.10, respectively, they will be subjected to different rates of acceleration. Since they are physically joined and accelerate at the same rate, a membrane tension must exist. An estimate of the tension in the membrane of the tail in a plane normal to the axis of the cell and tangential to the head of the cell yields a value of -2×10^{-3} dyn/cm. This value suggests that differential accelerative forces reduce the tension produced by fluid drag forces. However, this effect is negligible compared to the tensions produced by fluid drag forces (0.1 dyn/cm) and can be ignored.

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