VISCOELASTICITY OF PACKED ERYTHROCYTE SUSPENSIONS SUBJECTED TO LOW AMPLITUDE OSCILLATORY DEFORMATION

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ABSTRACT Concentrated adult erythrocyte suspensions were subjected to low amplitude oscillatory shear in a Weissenberg rheogoniometer equipped with a cone-and-plate assembly. The dynamic viscoelastic properties of the suspension were measured over a broad range of frequency by a numerical solution that accounted for fluid inertia. Variation of shear amplitude and cell volume percent, and comparison of buffered saline, plasma, and dextran as suspending media showed that the cellular elements had undergone small bending and shearing deformations. Studies of normal adult erythrocytes, hypotonically swollen cells, temperature-altered cells, and erythrocyte ghosts suggested that the method was evaluating membrane material properties. The normal membrane was found to exhibit a shear rate dependent elastic modulus that increased by more than a factor of 20 over a frequency range from 0.0076 Hz to 60 Hz. The membrane viscosity showed a substantial drop with frequency indicative of a frequency thinning phenomenon. At high frequency of deformation the viscous response of normal erythrocytes was no longer indicative of a membrane property due to the dominant influence of the internal hemoglobin solution. The studies generally supported the ability of the method to quantify relative membrane material properties and detect changes in membrane structure.

INTRODUCTION

The viscoelastic character of a normal red blood cell suspension depends upon the intrinsic material properties of the suspension components as well as the nature of the imposed stress. The viscous response is derived from the internal hemoglobin solution, the suspending medium, and the viscous contribution from the membrane (1, 2). The elastic response is usually associated with the ability of the erythrocyte membrane to store energy reversibly (3, 4). Erythrocytes that are suspended in plasma also exhibit sustained viscoelastic character due to the presence of red cell aggregates (5, 6). The nature of the imposed stress affects the magnitude of deformation applied to the suspension and each individual red blood cell. Small deformations allow the erythrocyte to deform while maintaining constant surface area (3, 7). Large membrane deformations may require a change in membrane area to occur and can therefore depend upon the surface area to volume ratio of erythrocytes (8). Such areal deformations reflect membrane material properties that are several orders of magnitude larger than those observed at constant membrane area (9, 10).

Red blood cell suspensions have been exposed to oscillatory shear by Chien et al. using a Weissenberg rheogoniometer with a cone and plate fixture (11). Their work was limited to low frequency oscillations ranging from 0.01 to 1 Hz (12). The results showed that erythrocytes suspended in plasma at a physiological hematocrit exhibited large energy-storing capabilities, which could be attributed to the presence of rouleaux. Examination of the viscous response also illustrated the effect of rouleaux on the overall ability of the suspension to dissipate energy. At hematocrits above 80%, red cells suspended in salt solutions demonstrated the same viscoelasticity as plasma suspensions, suggesting a diminished role for adhesion in the response. Further work was necessary to quantitate the involvement of the erythrocyte membrane in the viscoelastic response observed under oscillatory shear.

In our study, the viscoelastic response of the erythrocyte membrane was evaluated by imposing low amplitude oscillatory shear deformation on a concentrated cell suspension. Erythrocytes were suspended at a very large volume percent to ensure intimate cell–cell contact and prohibit cellular sedimentation. The suspensions were exposed to a low amplitude oscillatory shear that imposed a small bending and/or stretching deformation and discouraged cellular slippage or area dilation upon deformation. The contribution of cell slippage and disruption of the adhesive membrane interactions to the viscoelastic response was evaluated by studying cells suspended in salt solution, plasma, and Dextran 40 solution. Examination of

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the viscoelastic response over a very large frequency range allowed the technique to be properly evaluated as a method of measuring membrane properties. Comparison of erythrocytes to ghosts made by both mild and harsh lysis procedures separated the contributions of the membrane and the internal hemoglobin solution. Temperature-altered cells and glutaraldehyde-hardened cells were also tested to determine the capability of detecting membrane and cellular alterations. In addition, cells were osmotically swollen to stomatocytes or spheres to explore the influence of shape alteration.

EXPERIMENTAL METHODS

Normal Erythrocyte Suspensions

Normal human erythrocytes were obtained from healthy adult controls seen at the Lipid Research Laboratory at the University of Minnesota in accordance with the Committee on the Use of Human Subjects in Research of the University of Minnesota. Blood was obtained by antecubital venipuncture into a Venojet vacuum centrifuge tube that contained EDTA as anticoagulant. Red cells were separated from leukocytes and platelets by differential centrifugation using a refrigerated centrifuge (DuPont Co., Sorvall Instruments Div., Newton, CT) operating at 1,000 *g* for 20 min. The red cell suspension was then washed three times with isotonic phosphate-buffered saline (PBS) containing 0.2 g% serum albumin and 0.1 g% glucose. Erythrocytes were adjusted to 85, 90, and 95 vol% for oscillatory viscometry testing.

Suspending Media

Viscometry was also performed on washed erythrocytes suspended in spun plasma and Dextran 40 (Sigma Chemical Co., St. Louis, MO) solution. EDTA plasma was spun at 2,000 *g* for 30 min in a Sorvall centrifuge to remove leukocytes and cellular debris. A 15 g% Dextran 40 solution, pH 7.4, was constituted according to the procedure of Abraham et al. (13). Washed, packed erythrocytes were diluted 1:20 in either autologous spun plasma or Dextran 40 solution, and then adjusted to 85 vol% in the new suspending media.

Hardened Erythrocytes

Erythrocytes were hardened with the bifunctional reagent glutaraldehyde because of its rapid reaction with cellular protein to form a rigid cellular structure while still maintaining normal cell shape. Washed erythrocytes suspended in PBS to a hematocrit of 50% were fixed by the sequential addition of 0.1% and then 3% glutaraldehyde according to the method of White (14). The hardened cells were then washed, suspended in PBS, and packed to their maximum of 65 vol% for oscillatory testing.

Washed erythrocytes were also altered by incubation at 47.8°C for a period of 10 min. This exposure has been shown to produce membrane stiffening in normal red blood cells without altering mean cell hemoglobin concentration or surface area to volume ratio (15, 16).

Osmotically Swollen Erythrocytes

Aliquots of washed adult erythrocytes were also packed in PBS and hypotonically swollen by dilution in large volumes (1:30) of 140 mOsmol and 190 mOsmol PBS buffers. The albumin and glucose concentrations were adjusted to 0.1 g%, and the cell suspensions were concentrated to 85 vol% by centrifugation and removal of supernatant. Viscometry was performed on the samples swollen in 140 mOsmol (osmotic 140) or 190 mOsmol (osmotic 190) buffer.

Ghost Suspensions

Erythrocyte ghosts were prepared by two procedures, one designed to preserve erythrocyte membrane structure and integrity, and the other in which the primary emphasis was removal of nearly all hemoglobin from the cell. The mild lysis procedure produced ghosts that had a slight pink cast and will be referred to as "red ghosts." The harsh lysis procedure resulted in "white ghosts." The mild lysis procedure was chosen to produce ghosts of comparable size, shape, and mechanical strength to normal erythrocytes and at the same time reduce the internal hemoglobin concentration to ~9% of the original hemoglobin level (17).

The mild lysis procedure involved a combination of the methods of Dodge and Goldsmith (18, 19). Washed red cells were added in a ratio of 1:9 to a stirred solution of ice-cold 20 mOsmol phosphate buffer containing 1.2 mM $MgCl_2$ and 0.1 g% ATP, at a pH of 7.35, and lysed for 5 min. The ghosts were then incubated for a period of 1 h at 37°C to promote membrane sealing (20, 21). The resealed ghosts were centrifuged at 10,000 *g* for 20 min and the supernatant hemoglobin was removed. The ghost suspension was washed in hypotonic phosphate buffer that was prepared by mixing isotonic phosphate buffer with water in a ratio of 1:6 with the addition of 0.2 g% human serum albumin. The return of the ghost suspension osmolarity to an isotonic level of 300 mOsmol was attained by adding concentrated 9% NaCl in a dropwise fashion. The ghosts were then centrifuged and washed in isotonic phosphate buffer containing 0.1 g% human serum albumin and placed in autologous human serum and allowed to stand for 2 h at 4°C. The suspension was centrifuged at 15,000 *g* to collect discocytic biconcave ghosts at the top and remove crenated ghosts that accumulated on the bottom. An additional dilution with isotonic phosphate buffer in the ratio of 20:1 followed by centrifugation at 10,000 *g* was necessary to remove the suspending serum. The ghost suspension was visualized by Nomarski optics and contained over 90% discocytes. The ghost suspension was then adjusted to the 85% vol% for testing.

A harsh lysis procedure was also used to ensure a more complete removal of hemoglobin. The general procedure was identical to the mild lysis technique except that the lysing solutions were lower in osmolarity and three separate lysing steps were performed instead of just one. The initial lysis involved the addition of packed cells in a ratio of 1:9 to hypotonic phosphate buffer with an osmolarity of 10 mOsmol. The suspension was incubated at 37°C for 1 h to allow membrane resealing as before and the resealed ghosts were then centrifuged to remove supernatant hemoglobin. The second lysis required that 5 mOsmol phosphate buffer be added in the ratio of one part ghosts to 50 parts phosphate buffer. The resealing and centrifugation steps were repeated before the final lysis involving a 1.0 mOsmol solution at the same 1:50 ratio. The remainder of the procedure was identical to that for mild lysis.

Hemoglobin Preparation

Stroma-free hemoglobin solutions were prepared from suspensions of fully washed erythrocytes that were packed to 99 vol% and subjected to five cycles of freezing and thawing. The samples were frozen at −20°C for 2 h. The amount of lysis induced by this slower crystallization procedure was equivalent to that obtained by rapid dry ice and ethanol freezing procedures (22). The frozen sample was thawed in a 37°C water bath with gentle agitation. After the five freeze–thaw cycles the sample was centrifuged at 27,000 *g* for 50 min to remove red cell stroma. Examination of the hemoglobin solution under a light microscope with Nomarski optics showed the solution to be free of red cells, membrane vesicles, and stroma.

Characterization of Suspensions

Hematocrit readings were measured by high speed centrifugation of capillary tubes using an Adams Readacrit micohematocrit centrifuge (Clay-Adams, Inc., New York, NY). The concentration of the internal hemoglobin solution was determined spectrophotometrically by the cyanomethemoglobin method (Sigma Chemical Co.). A Coulter counter

(Coulter Electronics Inc., Hialeah, FL) was used to obtain red cell counts used in the determination of mean corpuscular volume (MCV). Mean cell hemoglobin concentration (MCHC) was calculated from the hemoglobin concentration and hematocrit, and MCV was determined by dividing the red cell count by the hematocrit. The MCV of cells swollen in 140 mOsmol buffer was also calculated on the basis of osmotic equilibrium of an erythrocyte containing 65% osmotically active volume fraction (23). Comparison of the calculated and experimentally measured MCV showed a fluid trapping factor (f) of 0.83 (f = MCV × RBC/HCT) for 140 mOsmol cells; the calculated MCV was used in the study. The fluid trapping factors for isotonic cells and cells swollen in 190 mOsmol buffer were previously shown to be 0.99, and their experimentally measured MCV were not corrected for packing efficiency. Cell morphology was visualized by light microscopy and Nomarski interference optics.

Weissenberg Rheogoniometer

The oscillatory measurements were performed on a Weissenberg Rheogoniometer, model 18 (Sangamo Controls Limited, Sussex, England), equipped with a 0.328 cm^3 vol cone-and-plate fixture with a radius of 2.5 cm and angle of 0.01 radians (see Fig. 1).

The motor-driven oscillations of the lower plate displaced the outer perimeter of the plate a specified distance from its equilibrium position, providing a shear amplitude with respect to the outer perimeter of the cone. The distance from the cone to the plate at the outer perimeter was 250 μm; a plate displacement of 125 μm from equilibrium position therefore defined a dimensionless shear amplitude of 0.5 with reference to the cone. Shear amplitudes of 0.125, 0.250, and 0.750 were also tested. The frequency of oscillation of the plate was varied from 0.0076 Hz to 60 Hz. The plate fixture was aligned with respect to the cone with a total run-out of 0.0001 in. The positions of the cone and plate were monitored using a Boulton Paul aircraft transducer capable of detecting movements of less than a micrometer. The positional signals from both the cone and plate transducers were also fed into an ultraviolet chart recorder, type SE 2005 (S. E. Laboratories, Limited, Middlesex, England).

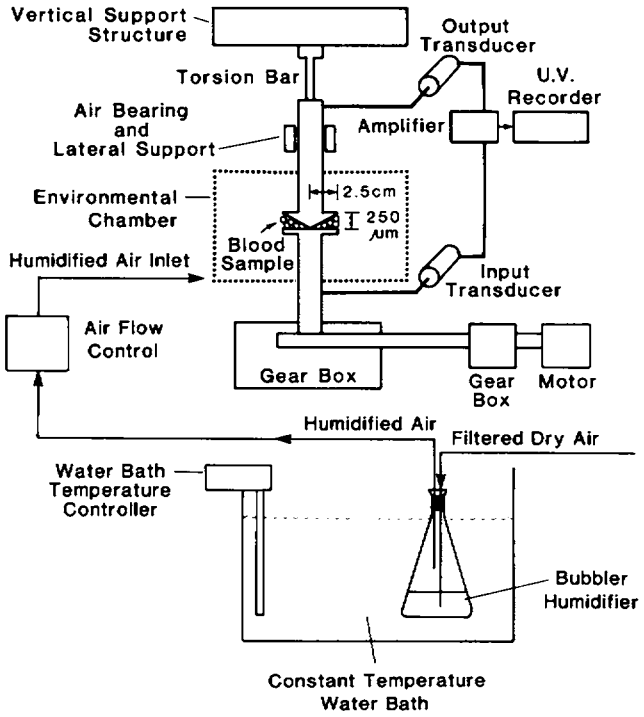

FIGURE 1 Weisenberg rheogoniometer equipped with a 0.4-ml cone-and-plate fixture. An environmental chamber was constructed to provide humidified air at 37°C to prevent drying of the sample and help maintain temperature.

The cone-and-plate fixture was completely enclosed in an environmental chamber that maintained the sample at 37°C in a relative humidity of 96 ± percent monitored by a Psychron hygrometer (Bendix Co., Baltimore, MD). A heating coil in contact with the metal of the Weissenberg frame within the chamber maintained the temperature of the cone-and-plate fixture at 37°C. Temperature control was further accomplished by adjusting the flow rate of warm humidified air bubbled into the environmental chamber. Each sample was tested over the entire frequency range and then the first frequency test was remeasured to ensure it had not changed with time or deformation.

The equipment was calibrated from steady shear and oscillatory measurements of three Newtonian fluids. The fluids were water, dioctylphalate, and a standard silicone oil having viscosities of 0.894 centipoise (cp), 0.560 poise, and 111 poise, respectively, at a temperature of 25°C.

Mathematical Analysis

The movement of the cone resulting from stresses transmitted through the sample differed in both amplitude and phase angle from the plate. Measurement of these two parameters provided the information necessary to determine the viscous and elastic component of the complex viscosity as defined by Bird et al. (24). For a fluid shear rate expressed as $\dot{\gamma} = Re\,[\dot{\gamma}_0 \exp(i\omega t)]$, where $\dot{\gamma}_0$ can be either a real or complex shear rate amplitude and ω is the frequency, and for a fluid shear stress given by $\tau = Re\,[\tau_0 \exp(i\omega t)]$, where τ_0 is the stress amplitude, the complex viscosity, η^*, is defined as

$$\tau_0 = -\eta^* \dot{\gamma}_0, \tag{1}$$

where $\eta^* = \eta' - i\eta''$, η' is the viscous component, and η'' is the elastic component of the complex viscosity. The use of complex viscosity then allows the fluid stresses to be divided into two components, one in phase with the shear rate and one out of phase. This general approach does not require that the measured fluid behave as a Maxwell or Voigt element but only that the fluid stress and shear rate are linear (25). One can similarly define a complex storage modulus, G^*, that is composed of a dynamic storage modulus, G', and a loss modulus, G'', that are related to the components of the complex viscosity by

$$G' = \omega\eta'' \tag{2a}$$

$$G'' = \omega\eta'. \tag{2b}$$

The viscous component, η', describes irreversible energy losses incurred by the sample during an oscillatory cycle and the storage modulus, G', is indicative of the amount of energy stored reversibly during the cycle. The

TABLE I
PHYSICAL PROPERTIES OF ERYTHROCYTES AND GHOSTS

	MCV	MCHC	$\eta'^{\ddagger}$ internal hemoglobin	Shape
	fl	*g/dl*	*cp*	
Normal adult	87 ± 2*	33.5 ± 0.5*	8.99 ± 0.51*	Disc
Heat 47.8°C	88 ± 6	33.6 ± 0.5	9.02 ± 0.65	Disc
Osmotic 190	119 ± 4	24.5 ± 0.5	3.5 ± 0.02	Stomatocyte
Osmotic 140	149 ± 5§	19.2 ± 0.6	1.6 ± 0.01	Sphere
Red ghost	87 ± 3	3.0 ± 0.2	0.89 ± 0.06	Disc
White ghost	84 ± 7	0.1 ± 0.02	0.75 ± 0.05	Disc

*Mean ± 1 SD.
‡Viscosity at 0.76 Hz, 37°C.
§Calculated MCV.

equations that describe the relationship between the dynamic viscoelastic properties η', G', and the experimentally measured amplitude ratio and phase angle of the cone-and-plate are found in Appendix A.

Statistics

Mean and standard deviation were determined by standard methods and differences between means were tested for significance to Student's t test. All data bars express the mean ± 1 SD.

RESULTS

The mean cell size, cell shape, and internal hemoglobin concentration and viscosity of normal and experimentally altered erythrocytes are listed in Table I. The MCV and shape of heat-treated cells and red and white ghosts were similar to that of native erythrocytes. On the contrary, osmotically swollen cells were stomatocytic in shape in 190 mOsmol buffer, and were nearly spherocytic at 140 mOsmol. The mean cell hemoglobin concentrations of all the altered cells were lower than normal except for heat-treated cells. The viscosities of the internal hemoglobin solutions were exponentially related to their respective hemoglobin concentrations and were nearly constant over the frequency range of 0.0076 Hz to 60 Hz. The viscous response of a 33 g% stroma free internal hemoglobin solution is shown as a function of frequency in Fig. 5. The elastic modulus, G', of all the hemolysates treated was too low for accurate measurement (<0.001 poise/s) over the entire frequency range. Erythrocytes hardened in glutaraldehyde retained a biconcave shape. The MCV of the crosslinked cells could not be adequately measured by the methods used in the study because of poor packing efficiency of 65%. The cells were unbreakable by standard lysis technique and internal characteristics were also not studied.

Normal erythrocyte suspensions adjusted to 85 vol% in PBS exhibited the frequency dependent viscous response shown in the top of Fig. 2 in response to a shear amplitude of 0.5. The η' of the erythrocyte suspension was over 50 times greater than the viscosity of the internal hemoglobin solution at the lowest frequency of oscillation. For frequencies above 10 Hz the viscous component remained nearly constant at 7.5 cp, similar in magnitude to the internal hemoglobin solution viscosity. The elastic modulus, G', exhibited a steady increase over the frequency range as shown in the bottom of Fig. 2.

Several shear amplitudes were examined to determine if they imposed sufficiently low shear deformations to potentially measure membrane material properties. If cellular slippage between neighboring cells were occurring or if cellular elements were slipping at the boundary surfaces, then the viscoelastic response of the suspension would be dependent upon the amplitude of oscillation. The dynamic viscosity and elastic modulus measured with imposition of shear amplitudes of 0.125, 0.250, 0.5, and 0.75 on an 85 vol% suspension of erythrocytes were all similar to each other ($P > 0.90$) over the entire frequency range. This

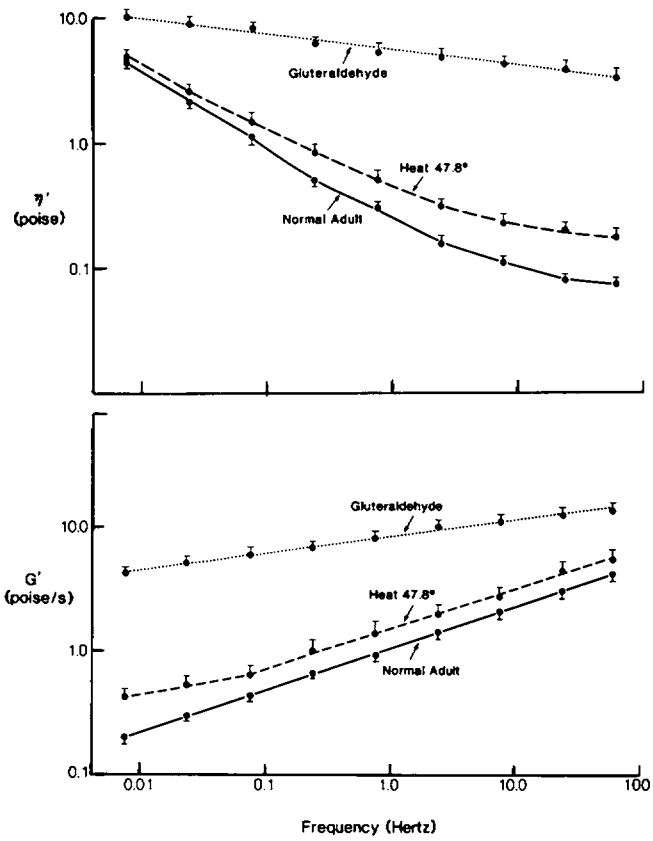

FIGURE 2 The dynamic viscosity, η', and elastic modulus, G', of normal, glutaraldehyde-hardened, and temperature-altered adult erythrocytes are contrasted over the range of frequency of deformation. Incubation at 47.8°C for 10 min increased membrane viscosity by more than 100% at high frequency. Temperature-altered and glutaraldehyde-hardened cells exhibited enhanced energy storage capacity at low frequency. The viscoelastic data are presented as log–log plots with standard deviation bars; the data were statistically different at all frequencies ($P < 0.005$). The sample size for the normal erythrocyte suspensions was 29 and 12 samples of the other suspensions were tested.

suggested that the low amplitude shear was not causing slippage between cells and that the fluid and cellular elements in contact with the cone-and-plate surfaces were moving at the velocity of the surface. All further viscoelastic data reported in the study are from tests performed at a shear amplitude of 0.5.

More concentrated erythrocyte suspensions of 90 and 95 vol% in PBS were also subjected to oscillatory shear and the results are shown in Table II. The viscoelastic response increased linearly with increasing hematocrit at these very high hematocrits. The behavior was similar over the entire frequency range.

Additional experiments were conducted to evaluate the role of suspending medium, intercellular gaps, and cellular adhesion on the material properties of 85 vol% erythrocyte suspensions subjected to small amplitude deformation. Erythrocyte adhesivity has been shown to increase due to the presence of Dextran 40 (26) and by the proteins present in plasma (5). Suspensions of packed erythrocytes

TABLE II
VISCOELASTIC PROPERTIES OF NORMAL ADULT ERYTHROCYTE SUSPENSION IN SALINE AT DIFFERENT HEMATOCRITS AND IN DEXTRAN AND PLASMA

	Hct.	η'	G'
	%	*poise*	*poise/s*
In saline	85	0.302 ± 0.025*	0.933 ± 0.100*
	90	0.323 ± 0.032	0.992 ± 0.107
	95	0.340 ± 0.038	1.06 ± 0.101
In dextran	85	0.294 ± 0.027	0.940 ± 0.113
In plasma	85	0.317 ± 0.035	0.957 ± 0.108

Frequency = 0.76 Hz, n = 12.
*Mean ± 1 SD.

were adjusted to hematocrits of 85% in iso-osmotic 15 g% Dextran 40 solution or autologous spun plasma. The viscosities of the suspending media varied greatly and were 0.0072 poise for PBS, 0.013 poise for plasma, and 0.10 poise for the dextran solution. As shown in Table II, no differences in the dynamic viscosity or elastic modulus of erythrocytes packed in dextran, plasma, or buffered salt solution were found.

Erythrocyte suspensions exposed to 47.8°C for 10 min showed a larger magnitude for both η' and G' and less frequency dependence than normal red blood cell suspensions. At 0.0076 Hz the viscous component for the heated suspension was only 10% larger than that of normal cells, but at high frequency η' was more than doubled (Fig. 2, *top*). The elastic modulus for the temperature altered suspension was 40% larger than normal over most of the frequency range (Fig. 2, *bottom*) and was more than double that for the normal adult erythrocyte suspension at low frequency. The glutaraldehyde-hardened erythrocyte suspensions were the only samples tested at 65% due to inability to compress them further. The results again showed a larger magnitude for both η' and G' and considerably less frequency dependence than other cell suspensions. The dynamic viscosity for the gluteraldehyde-hardened suspension ranged from twice that of normal erythrocytes at 0.0076 Hz to over 44 times that of normals at high frequency. The elastic storage modulus for the glutaraldehyde cell suspension was 21 times larger than that for the normal cell suspension.

Osmotically swollen cells were studied to evaluate the contributions of altered cell shape and availability of surface area for deformation on the viscoelastic response of the cell pack. The dynamic viscosities of the osmotically swollen erythrocyte suspensions are contrasted with isotonic cells in the top of Fig. 3. The osmotically swollen erythrocytes had lower η' than isotonic adult suspensions throughout the entire frequency range, although differences were more marked at high frequency.

The number of cells and total amount of cell membrane present in osmotically swollen and normal isotonic 85 vol% suspensions differed because of variations in cell size. A more appropriate comparison of the hypotonic and isotonic samples was obtained by numerically correcting the η' of the osmotically swollen samples to account for disparities from the quantity of cell membrane present in the isotonic sample. The MCVs of the osmotically swollen adult cells were larger than isotonic adult, but the membrane content of each cell was identical. The membrane content of 85 vol% suspensions of isotonic cells was greater than osmotically swollen samples by factors equaling the ratio of the MCVs, 1.39 for osmotic 190, and 1.72 for osmotic 140. The η' of the osmotic samples was multiplied by these factors to obtain η'_c and allow the membrane contributions to the viscoelastic response to be compared directly.

The η'_c of the hypotonically swollen cells are shown in the bottom of Fig. 3 in comparison to the η' of isotonic cells. The η'_c of the osmotic 190 and osmotic 140 samples were virtually identical to the isotonic cell viscosity throughout

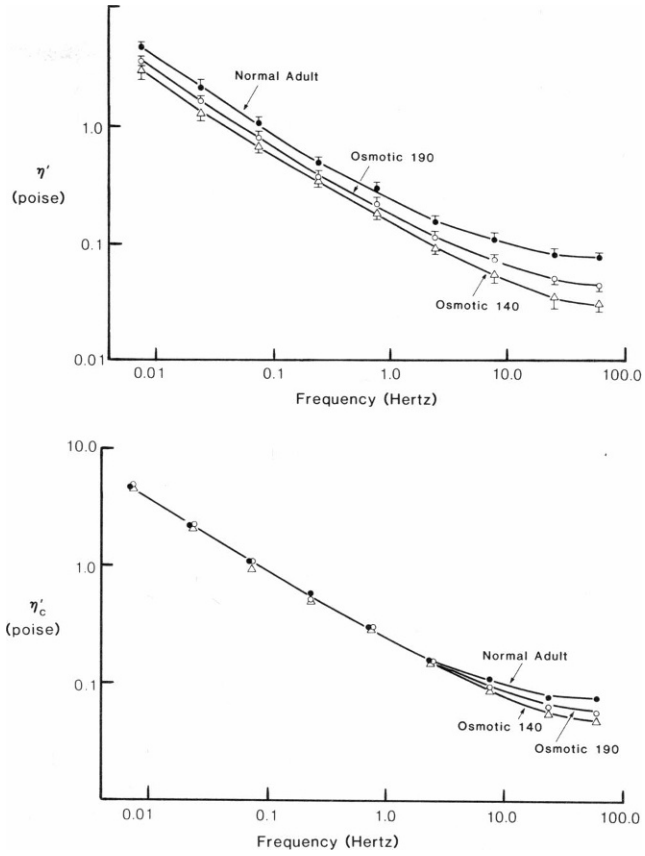

FIGURE 3 The η' of hypotonically swollen (osmotic 190, osmotic 140) and isotonic adult erythrocytes are shown over the range of frequency in the top graph. The viscosities of the samples standardized to comparable membrane content (η'_c) are shown in the bottom graph (see text for details). The hypotonically swollen cells showed differences from isotonic cells only at high frequency deformation after correction for the disparity in membrane content. Both graphs are log–log plots and the error bars shown in the top graph represent one standard deviation. The η'_c of the osmotically swollen adult samples are significantly different from isotonic adult at 24 and 60 Hz ($P < 0.01$). Studies represent 29 isotonic and 12 hypotonically swollen samples.

most of the frequency range. The corrected viscosities showed differences at high frequency compatible with the hierarchy of internal hemoglobin solution viscosities.

The variation of elastic storage modulus, G', with frequency is shown in the top of Fig. 4. The elastic storage modulus of the hypotonically swollen adult samples was lower than isotonic cells, but the frequency dependence was similar. Adjustment of the G' of hypotonic suspensions by their respective correction factors for membrane area disparity led to the relationships shown in the bottom of Fig. 4. The G'_c of hypotonically swollen cells was indistinguishable from isotonic cells.

The viscoelastic response of normal erythrocyte ghosts prepared by gentle lysis is shown in Fig. 5. The dynamic viscosity of the red ghosts was very similar to normal intact erythrocytes until frequencies rose above 1 Hz. For frequencies of 0.0076–0.076 Hz the viscous component of the ghost suspension was over 80% of the magnitude of the normal erythrocyte suspension and followed a similar slope. At higher frequencies, however, the viscous component of the ghost suspension continued to decline in contrast to the behavior of the intact erythrocytes. The mild lysis procedure left ghosts with an internal hemoglobin solution concentration that was only 9% of the intact erythrocyte hemoglobin concentration and had a viscosity of 0.90 cp. The elastic modulus of red ghosts maintained a magnitude of 85% of the normal suspension storage modulus.

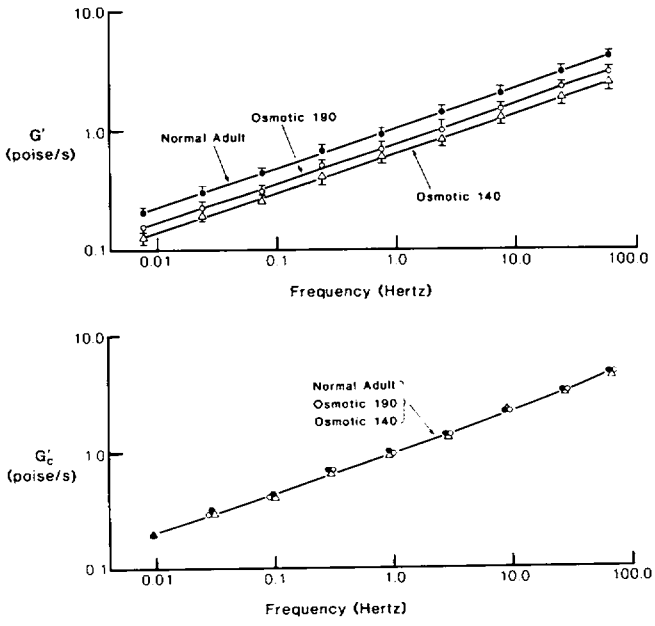

FIGURE 4 The G' of hypotonically swollen (osmotic 190, osmotic 140) and isotonic adult erythrocytes are shown over the range of frequency in the top graph. The elastic moduli of the samples standardized to comparable membrane content (G'_c) are shown in the bottom graph. No differences were observed between hypotonic and isotonic cells after correction for the disparity in membrane content. Both graphs are log–log plots and error bars represent one standard deviation. Studies were performed on 29 isotonic and hypotonically swollen samples.

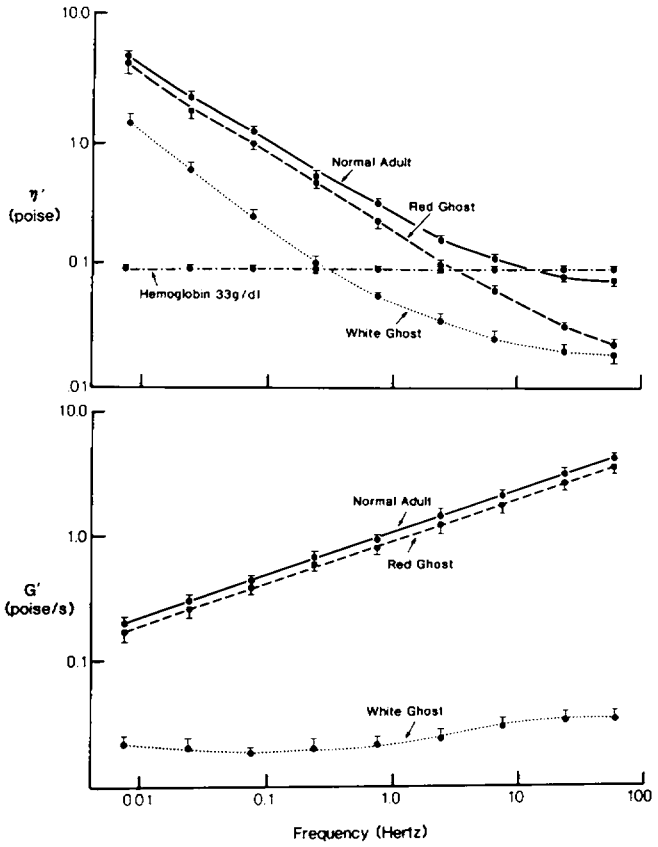

FIGURE 5 The η' and G' of erythrocyte ghosts prepared by two procedures (red and white ghosts) are compared to the frequency dependent viscoelastic behavior of intact cells. Red ghosts made by a mild lysis procedure exhibited a continued decline in viscosity at high frequency due to low internal hemoglobin concentration; the frequency-independent η' of a 33 g% hemolysate is shown for comparison.

The viscoelastic response of white erythrocyte ghosts made by the harsh lysis procedure was markedly different, both qualitatively and quantitatively, from normal erythrocyte suspensions. The magnitude of η' for white ghosts was <27% of the normal erythrocyte viscous component over the entire frequency range. The elastic modulus response was distinctly unusual as it remained relatively constant around 0.02 poise/s from 0.0076–0.76 Hz and then slowly increased to only 0.34 poise/s at 60 Hz.

DISCUSSION

Low amplitude oscillatory shear was applied to concentrated red cell suspensions over a broad range of frequency in a Weissenberg rheogoniometer equipped with cone-and-plate geometry. The visco-elastic behavior of the normal erythrocyte suspension exhibited a marked frequency dependence covering more than an order of magnitude over the frequency range tested. The dynamic viscosity of the concentrated suspensions tended to plateau at high frequency and reflected the viscosity of the internal hemoglobin solution. Chien et al. (11) reported similar findings on concentrated cell suspensions at low frequencies of oscilla-

tion, but did not extend the observations beyond 1 Hz. The mathematical analysis and solution of the velocity profile within the pack at high frequencies required consideration of an inertial term in the equation of motion (see Appendix A).

Chien also showed that at cell concentrations at or above 80 vol%, the elastic response of the pack was identical in plasma or buffered saline, and hypothesized that the pack's mechanical behavior was reflecting the elastic modulus of the cell membrane. Our studies confirmed the lack of influence of suspending media on the dynamic storage modulus of cell suspensions concentrated above 80 vol%. In contrast to their work, we found that the viscous behavior of the cell pack at low shear frequencies was also not affected by suspending media. Further studies provided evidence that the material properties of the red cell membrane were reflected under conditions of small deformation and low frequency of oscillation.

Our studies suggest that low amplitude oscillatory shear testing of concentrated cell suspensions was capable of detecting alterations in membrane properties. Shear amplitudes ranging from 0.125 to 0.750 all resulted in the same measured η' and G'. These findings are consistent with the notion that the cells are not slipping past each other, nor are they slipping at the interface of the cone or plate. The results indicate that the cells are being deformed in a manner that requires some combination of membrane bending, shear, and extension. Finally, we found that the use of suspending media known to increase cell adhesion did not alter high hematocrit cell pack viscoelasticity. It is well established that plasma promotes cell aggregation in loose cell suspensions (5, 27), and recent elegant micropipette studies have shown that more force is required to separate erythrocytes from endothelial cells when the cell surfaces are opposed in the presence of plasma (28). Dextran has been shown to increase cell aggregation (26), although the molecular weight and concentration required for aggregation is under investigation (29). The failure of more adhesive cell packs to yield higher viscoelasticity strongly argues that relative cell motion was not present and that the organization of the packs was preserved during the oscillatory deformations.

The fluid filled intercellular gaps within the cell pack did not contribute significantly to the viscoelastic response at high cell concentrations. The use of suspending media with different viscosities ranging from 0.0072 to 0.100 poise did not alter the viscoelastic response of 85 vol% cell suspensions. This was true at both low and high frequencies of oscillatory deformation. The linear increase in pack viscoelasticity with cell concentration at high hematocrit was further support for the dominance of cell constituents in the response.

Moderate variations in cell shape and size altered the viscoelastic response of the pack only to the degree that they altered the amount of cell material in 85 vol% cell suspensions. Osmotically swollen erythrocytes with stomatocytic bowl shape or nearly spherical configuration demonstrated essentially identical viscoelasticity as normal cells at low frequencies of oscillation after correction for the discrepancy in cell material in the compared 85 vol% cell suspensions. These alterations in shape did not appear to facilitate disruption of the cellular interactions of the pack under the experimental conditions of deformation, and hence suggests that this technique is indeed measuring a membrane property. More dramatic shape alterations could conceivably alter the stability of pack organization, thereby adding the work of peeling cell surfaces apart to the measured viscoelastic behavior. The studies also suggest that the shear deformation was small enough that cell extensional properties were being measured. If availability of cell surface area for deformation were constraining the pack's movement, the osmotically swollen cells would have yielded greater apparent viscoelasticity. These conclusions are further supported by the independence of pack viscoelasticity with variations in shear amplitude at small magnitudes of deformation.

Hardening of red cells by glutaraldehyde fixation and heat treatment at 47.8°C significantly increased the viscoelastic magnitude of the cell pack and reduced the frequency dependence. The large modulus, G', at low frequency reflected a cellular structure that was more capable of storing energy than normal erythrocytes. The relatively larger dynamic viscosity throughout the high frequency range suggested a less deformable cell than the normal erythrocyte.

At low frequencies of oscillation, the mechanical behavior of the pack of heat-treated cells was similar to that previously reported for the membrane of heat-treated cells. Rheological investigations using different methodologies have confirmed that heat treatment at 47.8°C for 10 min doubles red cell membrane shear elastic modulus with a smaller increment in membrane viscosity (16).

Studies of erythrocyte ghosts made by a gentle lysis procedure further confirmed the dominant role of the red cell membrane in the viscoelastic response of the cell pack at low frequency of oscillation. Packs of red ghosts manifested 80–90% of the viscoelasticity of intact cells at low frequency. The dynamic viscosity of the red ghosts diverged from the normal red cell at high frequency of oscillation consistent with the 90% reduction in internal hemoglobin solution viscosity. The notion that low frequency viscoelasticity was attributable to the membrane, and that high frequency viscous response was governed by the cell interior in normal red cell packs was also supported by the osmotic swelling experiments. The erythrocyte ghost suspensions made with the harsh lysis procedure showed a much lower dynamic viscosity and elastic modulus over the entire frequency range. This is consistent with the notion of a damaged membrane that was stripped of a significant molecular fraction during the lysing and washing procedure.

The viscoelastic studies of normal and altered concen-

trated cell suspensions indicated that the material properties of the red cell membrane are frequency dependent. The erythrocyte membrane dominated the viscous response of the cell pack over most of the frequency range of deformation, and the elastic response over the entire range of oscillation. The membrane demonstrated shear thinning behavior, and an increase in dynamic elastic modulus with higher frequency. The study lends support to the concept that the rheological properties of the red cell membrane undergo dynamic changes depending on the extent and duration of deformation. Membrane extensional shear viscosity varied inversely with the degree of deformation in the micropipette aspiration studies of Chien et al. (30). Membrane shear thinning behavior was also clearly shown by Tran-Son-Tay and Sutera (31) in determinations of surface shear viscosity deduced from measurements of the membrane rotational frequency of tank-treading cells. Their studies are consistent with an increase in extension elastic modulus with large shear deformations. The observations are consistent with the current structural models of the red cell membrane. On a molecular level, erythrocyte membrane energy storage may be associated with the cytoskeletal lattice of proteins that forms an interconnected structure occupying about one-third of the inner membrane surface (32, 33). The network of interwoven and branched segments may allow small movements relative to each other at low frequency without substantial energy storage. However, rapid shearing movements may bring about entanglement couplings of the molecules and may be responsible for the increased capacity for energy storage found at high frequency (34). The viscous character of the erythrocyte membrane may be associated with the lipid bilayer and associated proteins not intimately connected with the elastic cytoskeleton. At low frequency the shearing motion may result in large energy losses due to movement of membrane viscous elements. With increasing frequency, however, molecular alignment of these viscous molecules may effect a reduction in membrane viscosity.

Small amplitude oscillatory deformation of concentrated cell suspensions appears to be a useful means of evaluating dynamic membrane viscosity and shear modulus relative to the material properties of the normal erythrocyte membrane. The method has severe limitations in an attempt to extract an absolute membrane viscoelasticity from the rheological measurements of the cell suspension. The exact nature of the deformation that occurs during a single oscillatory cycle is difficult to define, but may consist of a combination of membrane bending and extension deformation. The method, however, provides a means for detecting an altered membrane property and quantifying the magnitude of the membrane change through comparison with normal cells.

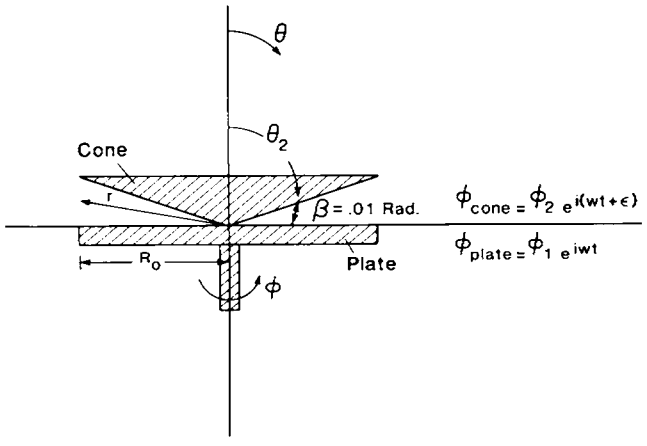

FIGURE 6 Spherical coordinate system for cone-and-plate geometry. Cone-and-plate position, ϕ-cone, and ϕ-plate are monitored to determine phase angle ϵ, and amplitude ratio, ϕ-cone/ϕ-plate.

APPENDIX A

Mathematical Development

Applying the definitions outlined in the Mathematical Section of this paper to the cone-and-plate geometry with the plate oscillating with position, $\phi_p = \phi_1 \exp(i\omega t)$ (see Fig. 6) allowed the development of a relation from which η' and η'' could be obtained from measurements of the cone/plate amplitude ratio, ϕ_2/ϕ_1, and phase angle ϵ.

The velocity profile in the fluid sample was determined by application of the analysis of Nally (35) that included inertial terms. At frequencies higher than 1 Hz the inertia of the fluid sample became significant and required a more rigorous treatment of the equation of motion in spherical coordinates than offered by Maude and Walters (12). The more rigorous treatment involves the component of the equation of motion shown in Eq. 3:

$$\rho \frac{\partial V}{\partial t} = \eta^* \frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2 \frac{\partial V_\phi}{\partial r}\right) + \frac{1}{r^2 \sin_\theta}\frac{\partial}{\partial \theta}\left(\sin\theta \frac{\partial V_\phi}{\partial \theta}\right) - \frac{V_\phi}{r^2 \sin_\theta}, \quad (3)$$

where V_ϕ is the velocity of fluid in the ϕ-direction and ρ is the fluid density. The velocity distribution was assumed to consist of time-dependent and position-dependent parts:

$$V_\phi = Re[F(r, \theta) \exp(i\omega t)]. \quad (4)$$

Upon substitution into Eq. 3 and letting $\alpha^2 = i\omega\rho/\eta^*$, the equation of motion takes the form:

$$-\alpha^2 F = \frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2 \frac{\partial F}{\partial r}\right) + \frac{1}{r^2 \sin\theta}\frac{\partial}{\partial \theta}\left(\sin\theta \frac{\partial F}{\partial \theta}\right) - \frac{F}{r^2 \sin^2\theta}. \quad (5)$$

The boundary conditions provide that the fluid velocity at each surface is assumed to be equal to the velocity of the surface with which it is in contact. The no slip condition is an assumption that has not been conclusively verified by experimental observation. In addition, the fluid velocity must be finite at the origin, the momentum flux in the r-direction must be equal to zero at the fluid/air interface, and secondary flows have been neglected (35). After applying appropriate boundary conditions, the solution to Eq. 5 can be obtained by using the method of separation of variables. The fluid velocity profile has been found by Nally (35) and is given by the following summation:

$$V_\phi = \exp(i\omega t) r^{-1/2} \sum_{n=1,3}^{\infty} \cdot J_{n+1/2}(\alpha r)[A_n P'_n(\cos\theta) + B_n Q'_n(\cos\theta)], \quad (6)$$

where $J_{n+1/2}$ is a Bessel function of the first kind of order $n + \frac{1}{2}$, and $P'_n(\cos\theta)$, $Q'_n(\cos\theta)$ are Legendre's associated functions of the first and second kind, respectively, of order unity and degree n. The velocity profile continues as $r \to \infty$ and is unbounded. A_n and B_n are constants expressed in terms of known Legendre functions. The final working equation developed by Nally (35) that must be solved to determine the dynamic viscosity, η', and the dynamic modulus, G', for the red cell suspension is

$$\frac{\phi_2}{\phi_1} e^{i\epsilon}\left[\sum_{n=1,3}^{\infty} a_n A'_n I_n - \frac{(K - I\omega^2) - \alpha^{3/2}}{(2\pi \sin^2\theta_2)\, i\omega\eta^*}\right] = \sum_{n=1,3\ldots}^{\infty} a_n B'_n I_n, \quad (7)$$

where A'_n, B'_n, and a_n are constants, K is the torsion spring constant, and I is the moment of inertia of the cone assembly. I_n is given by

$$I_n = \int_0^{R_0} r^{1/2} J_{n+1/2}(\alpha r)\, dr, \quad (8)$$

where $J_{n+1/1}(\alpha r)$ is a Bessel function of the first kind of order $n + \frac{1}{2}$. In Eq. 7, ϕ_2/ϕ_1 and ϵ are experimentally measured. For a particular experiment the frequency, ω, and the sample density, ρ, were also known. Recursion relations were developed to calculate the terms under the summation found in Eq. 6 (36). A computer program was written that expanded this equation in like powers of α^2, and a subroutine was used to solve the polynomial equation although many of the roots resulted in negative values for η', η'', or for both. An additional program was written that calculated the value of V_ϕ from Eq. 6 and checked to verify that the boundary conditions at the cone-and-plate surfaces were met and a single unique solution was chosen on that basis.

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