

Effect of Wheat Germ Agglutinin on the Viscoelastic Properties of Erythrocyte Membrane

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ABSTRACT The elasticity and viscosity of the human erythrocyte membrane were measured as a function of the concentration of wheat germ agglutinin (WGA) in a suspending solution containing 1 mg/ml albumin, $\sim 5 \times 10^5$ cells/ml and between 0.0 and 0.2 $\mu\text{g/ml}$ WGA. Membrane elasticity was characterized by the elastic shear modulus, which provided a measure of the resistance of the membrane to constant-area elastic deformations that occurred in the membrane plane. The elastic shear modulus was determined by aspirating a portion of the membrane into a micropipette and measuring the extension of the membrane into the pipette as a function of the suction pressure. The results indicated no significant change in shear modulus for concentrations of WGA between 0.0 and 0.2 $\mu\text{g/ml}$. Membrane viscosity was characterized by the coefficient of surface viscosity, which, in effect, was a measure of the membrane's resistance to rates of deformation. This coefficient was determined from the time required for an erythrocyte to recover its undeformed shape after it had been elongated by the application of an equal and opposite force applied at diametrically opposite points on the erythrocyte rim. The value for the coefficient of surface viscosity was found to increase by a factor of almost three when the WGA concentration was increased from 0.0 to 0.2 $\mu\text{g/ml}$. These results indicated that, in the presence of albumin, WGA can increase membrane dissipation (viscosity) without altering the structural rigidity (elasticity) of the membrane.

The erythrocyte membrane is a composite material with both solid and liquid components. Information about the composite structure and possible alterations in the structure is provided through the study of the mechanical behavior of the membrane. By direct mechanical experiments, the intrinsic material properties that characterize the "deformability" of the membrane are measured. In particular, the measurement of material properties characterizing the elastic and viscous behavior of the erythrocyte membrane (5) has led to a search for physical and chemical factors that would change these properties.

The use of the lectin wheat germ agglutinin (WGA) as an agent to alter membrane properties was suggested by the work of Anderson and Lovrien (2, 7), which showed that WGA can stabilize and preserve certain erythrocyte morphologies. The lectin interacts with the erythrocyte membrane through the transmembrane protein glycophorin (1). Specifically, WGA is able to block the reversible transformation occurring between the normal biconcave shape (discocyte) and the tightly crenated spherical shape (echinocyte) under conditions that would normally drive the reaction to completion in either direction. Lovrien and Anderson (7) suggest that the intramolecular binding of WGA to glycophorin on the outside of the mem-

brane influences the cytoskeleton network that lies on the inner face of the membrane. This network plays a fundamental role in determining the mechanical behavior of the membrane. In particular, it is believed to be largely responsible for the solid elastic properties of the membrane (4, 5).

The purpose of this study is to determine the effect of WGA on membrane mechanical behavior as it is reflected in the measurement of two specific membrane material properties: the elastic shear modulus (μ) and the coefficient of surface viscosity (η). Experimentally, we are able to determine these properties by applying known forces to the membrane and observing the resulting deformation and rate of deformation.

The erythrocyte membrane exhibits elastic behavior as evidenced by the ability of the erythrocyte to recover its initial shape after being deformed. The elastic shear modulus characterizes membrane elasticity by relating the force on the membrane to the constant-area deformation that the membrane undergoes in response to the force. In effect, this modulus is a measure of the resistance of the membrane to constant-area deformation occurring in the plane of the membrane. We have determined μ with the method of Evans (4) and Waugh and Evans (8). This method involves the aspiration of a small

portion of an erythrocyte membrane into a micropipette (Fig. 1). The membrane is deformed as it is drawn from a flat surface (i.e., the essentially flat portion of the cell perpendicular to the pipette) into the cylindrical pipette by the suction force. The state of membrane deformation is determined as a function of the length of the aspirated portion of the cell, and the force applied to the membrane is known from the pressure in the pipette and the pipette radius. From the force-deformation data obtained during the aspiration of the cell into the pipette, a value for μ is calculated (3, 4, 8).

Even though the membrane will return to its initial shape when all external forces are removed, it does not do so instantaneously. The rate at which it recovers is limited by viscous energy dissipation in the membrane. This dissipation in the material produced by finite rates of deformation is a measure of membrane viscosity, which is characterized by the coefficient of surface viscosity η .

Measurements of η were made with the method of whole-cell extensional recovery developed by Hochmuth et al. (6). In this experiment, an erythrocyte that is attached to a glass cover slip at one "point" is extended by pulling at a diametrically opposite point on the cell with a small micropipette (Fig. 2). When the cell is released from the pipette, it recovers its initial shape within a characteristic time period that is proportional to the viscosity of the membrane: $t_c = \eta/\mu$, where t_c is the measured time constant of recovery, η is the coefficient of surface viscosity, and μ is the elastic shear modulus (5). Thus, from a value of t_c measured in a recovery experiment and an independent measurement of the elastic shear modulus, a value for the surface viscosity is calculated.

MATERIALS AND METHODS

A drop of fresh human blood was suspended in 10 ml of phosphate-buffered saline (7.10 g/l NaCl, 3.58 g/l Na_2HPO_4 , and 0.654 g/l KH_2PO_4 ; pH adjusted to 7.4; and osmolarity adjusted to 295 mosM) containing WGA at a concentration of 0.0, 0.1, or 0.2 $\mu\text{g}/\text{ml}$. This procedure provided a cell concentration of $\sim 4\text{--}5 \times 10^9$ cells/ml. The solution also contained human serum albumin at a concentration of 0.1 g/100 ml (1 mg/ml). During the preparation of the suspending

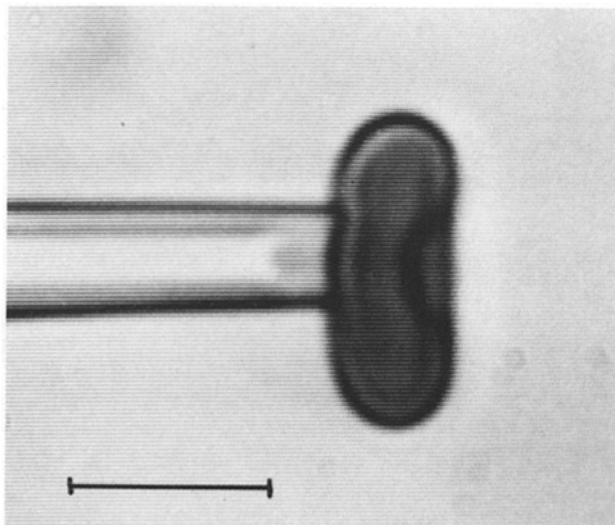


FIGURE 1 Photograph of the aspiration of a flaccid erythrocyte into a micropipette. As the suction pressure is increased, the increase in the length of the cell membrane projection in the pipette is measured. The ratio of the suction pressure to the membrane projection is a measure of the elastic shear modulus of the membrane. Bar, 5 μm . $\times 5400$.

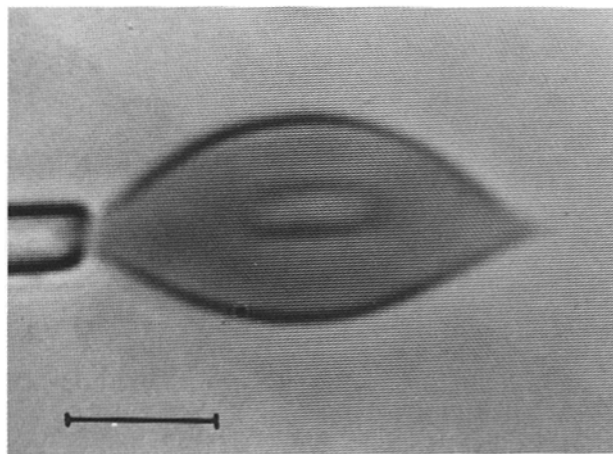


FIGURE 2 Photograph of an erythrocyte as it is extended by aspirating a small portion of the rim of the cell with a micropipette and then gently withdrawing the micropipette. The cell remains attached to the surface at a "point" diametrically opposite the micropipette position. Upon release from the pipette, the cell recovers its original shape. During recovery, the length and width of the cell are measured as functions of time. Bar, 5 μm . $\times 4000$.

solution, exposure of the solution to glassware with relatively large surface-to-volume ratios was avoided in order to prevent changes in WGA concentration that could occur if WGA were to bind to the glass surfaces.

The suspension of cells was gently stirred for 10 min before an experiment. After this time, a small portion (~ 0.2 ml) of the suspension was injected into a chamber consisting of a glass slide and cover slip separated by a U-shaped plastic insert. The chamber, which was open at one end to allow for insertion of the pipette, was placed on the stage of an inverted microscope.

To minimize any decrease in the WGA concentration of the suspending solution due to the possible binding of WGA to the chamber surfaces, we prepared the chamber with a preflush procedure before the injection of the cells. This procedure consisted of slowly flushing 10 ml of suspending solution through the chamber, using two syringes, one for infusion and one for withdrawal. Several times during the flushing procedure, the flow was stopped and the chamber allowed to sit undisturbed for 30 s while filled with solution. The preflush was intended to allow the chamber surfaces to absorb WGA in amounts sufficient to minimize any tendency for WGA to be depleted from the cell suspension injected after the preflush.

The micropipette was filled by boiling under a vacuum in phosphate-buffered saline at an osmolarity of 295 mosM. Once filled, it was connected via a water-filled plastic tube to a manometer and mounted in a DeFonbrune micromanipulator (Arenberg Ultrasonic Lab, Inc., Jamaica Plain, MA). The pressure in the pipette was nulled by adjusting the water level in the manometer until the flow of water in the pipette stopped. This null point was detected by observing the movement of cells near the entrance of the pipette. The pipette pressure was decreased relative to "zero" by lowering a water-filled reservoir below the level of the microscope stage. A pressure transducer (Validyne Engineering Corp., Northridge, CA) coupled to the pipette by a continuous water connection measured changes in pipette pressure.

Cells were observed through a Leitz Diavert microscope equipped with $\times 25$ eyepieces and a $\times 40$, 0.65 N.A., long working-distance Nikon objective. Experiments were recorded on videotape through a video camera (Dage-MTI, Michigan City, IN) mounted above one of the eyepieces. Pressure and time were also displayed on the videoscreen with a "data mixer" (Vista Electronics, La Mesa, CA). The combined signal, consisting of camera image, pipette pressure, and time, was recorded with a $\frac{3}{4}$ inch videocassette recorder (Sony Corp. of America, New York). For data analysis, the tape was played through a video monitor (Setchel-Carlson Electronics, Inc., New Brighton, MN) equipped with a position analyzer (Vista Electronics). The position analyzer placed two movable cursors on the video screen and provided a digital readout proportional to the distance between the cursors.

Elasticity

The elastic shear modulus was measured with a micropipette (~ 1 μm ID), which was used to aspirate the cell in its dimple region at an initial aspiration pressure of 200–300 dyn/cm^2 (Fig. 1). The aspiration pressure was increased in four or five small increments up to a final pressure of ~ 500 dyn/cm^2 . After each step increase in aspiration pressure, the length of the cell projection in the pipette

was measured. In this manner, four or five data points were obtained for each cell tested. Data for a typical control cell (zero concentration of WGA) are shown in Fig. 3. After reaching a maximum value, the aspiration pressure was decreased in several increments to the initial pressure in order to check for possible adhesion of the cell to the pipette. From start to finish the experiment took ~1 min.

The theoretical relationship between the applied pressure P and the length, L , of the cell projection in the pipette is given by Waugh and Evans (8):

$$\frac{P \cdot R_p}{\mu} = \left(\frac{2L}{R_p} - 1 + \ln \frac{2L}{R_p} \right), \quad L/R_p \geq 1, \quad (1)$$

where R_p is the pipette radius.

For L/R_p over a range of 1–4, the above theoretical relationship is closely approximated by a linear equation (3):

$$\frac{P \cdot R_p}{\mu} = C_1 \left(\frac{L}{R_p} \right) + C_2 \quad (2)$$

where $C_1 = 2.45$ and $C_2 = -0.603$. The elastic shear modulus μ given in Eq. 2 is calculated by linear regression given the pipette radius R_p and the pressure-length data pairs (P , L).

Viscosity

Before a measurement of the coefficient of surface viscosity, cells were injected into the chamber and allowed to settle onto the lower surface (i.e., the cover slip). Only those cells attached to the surface at a single point were tested. A small portion of the rim of the cell was aspirated into the tip of a small micropipette (~0.5 μm ID) at a point opposite the attachment site (Fig. 2). Withdrawal of the pipette caused the cell to elongate until the cell reached a maximum length-to-width ratio of ~2:1, at which point it pulled free from the pipette and recovered its original biconcave shape. A stop-action videotape recorder with a scanning rate of 60 frames/s was used to record the cell recovery process. During analysis, the length and width of the cell were measured at each frame, and this produced a datum point, in the form of a length-to-width ratio (L/W), every 1/60 s.

A viscoelastic model has been developed that describes the time-dependent recovery of the membrane in terms of the time constant t_c , and the undeformed length-to-width ratio (L/W) $_{\infty}$ (6). (The cells have a slight "residual" deformation produced during the extension process and, thus, (L/W) $_{\infty}$ is always slightly >1.) For an initial (maximum) length-to-width ratio of (L/W) $_m$, the viscoelastic recovery as a function of time is given by the following expression for L/W :

$$\left(\frac{L}{W} \right) = \left(\frac{L}{W} \right)_{\infty} \cdot \frac{\Lambda + \exp(-t/t_c)}{\Lambda - \exp(-t/t_c)}, \quad (3)$$

where

$$\Lambda = \frac{(L/W)_m + (L/W)_{\infty}}{(L/W)_m - (L/W)_{\infty}},$$

and

$$t_c = \eta/\mu. \quad (4)$$

Values for the two parameters, (L/W) $_{\infty}$ and t_c , were chosen to minimize the sum of the squares of the errors between the viscoelastic model (Eq. 3) and experimental values for L/W . Thus, for a cell recovery with N data points, the values of (L/W) $_{\infty}$ and t_c are found that minimize the following functional:

$$\epsilon^2 = \sum_1^N [(L/W)_{\text{expr}} - (L/W)]^2/N, \quad (5)$$

where ϵ is the root mean square error per datum point.

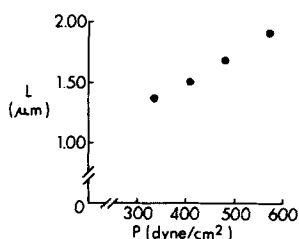


FIGURE 3 The length (L) of the cell projection vs. the applied pressure P for the micropipette aspiration of a normal flaccid erythrocyte. The slope of the straight line through the data points is proportional to the elastic shear modulus μ . The shear modulus for this control cell (0.0 $\mu\text{g/ml}$ WGA) is 5.1×10^{-3} dyn/cm.

RESULTS

The results of the elastic shear modulus experiments performed at three different concentrations of WGA are summarized in Table I. Values of μ are calculated only from data taken as the cell moves into the pipette with increasing aspiration pressure, that is, during the loading phase. Application of the Student's t test to the data shows that the differences in means at the different WGA concentrations are insignificant. The average elastic shear modulus obtained from all the data is 5.1×10^{-3} dyn/cm.

Due to the problem of adhesion of the cell to the pipette, no shear modulus experiments were performed for a WGA concentration >0.2 $\mu\text{g/ml}$. At the highest WGA concentration tested, 0.2 $\mu\text{g/ml}$, some cells did not move freely out of the pipette as the aspiration pressure was decreased. However, values of μ were calculated for those cells if the data taken in the loading phase were linear. It is conceivable that significant adhesive interaction could be occurring due to WGA despite the linearity of the pressure vs. length data. If this were the case, the calculated modulus would tend to be artificially increased. Since our results show no increase in μ , the effect of enhanced adhesion to the glass due to WGA is assumed to be negligible in our experiments.

Although there is no measurable change in μ at the WGA concentrations tested, there are observable differences between the cells treated with WGA and the untreated control cells. As mentioned above, cells suspended in 0.2 $\mu\text{g/ml}$ WGA have an increased tendency to adhere to the glass as they move out of the pipette during the unloading phase, although there appeared to be no increased tendency for these cells to adhere during the loading phase. In addition, when a cell suspended in 0.2 $\mu\text{g/ml}$ WGA is expelled from the pipette at the end of a shear modulus experiment, in some instances a small, residual, membrane "bump" is present at the point where the membrane was aspirated into the pipette. The bump, ~0.25 μm in height on average, slowly retracts, and the cell regains its initial biconcave shape. The bump retraction time is on the order of 1 min in most cases. Cells suspended in 0.1 $\mu\text{g/ml}$ WGA form much smaller bumps which recover quickly. Control cells do not form bumps.

The results of the whole-cell recovery experiments are presented also in Table I. In these experiments, the WGA concentration of the suspending medium (C_{WGA}) was limited to 0.2 $\mu\text{g/ml}$ due to the difficulty in finding cells with single-point attachments to the surface at higher WGA concentrations. Cells suspended in WGA at a concentration of 0.2 $\mu\text{g/ml}$ take

TABLE I

Calculation of the Surface Viscosity from Measurements of the Elastic Shear Modulus and the Recovery Time Constant

C_{WGA} $\mu\text{g/ml}$	$\mu \pm \text{SD}$ (no. cells) 10^{-3} dyn/cm	$t_c \pm \text{SD}$ (no. cells) s	$\eta \pm \text{SD}$ 10^{-3} dyn·s/cm
0.0	5.14 ± 0.46 (14)	0.100 ± 0.018 (20)	0.51 ± 0.1
0.10	5.09 ± 0.76 (18)	0.201 ± 0.032 (23)	1.0 ± 0.2
0.20	5.01 ± 0.37 (19)	0.263 ± 0.054 (20)	1.3 ± 0.3

This table gives the averages and standard deviations of the elastic moduli μ and recovery time constants t_c measured at each WGA concentration C_{WGA} . The number of cells measured at each concentration is given in parentheses beside the average value. Values of the surface viscosity η are calculated from the formula $\eta = t_c \cdot \mu$. The standard deviations of the η values are calculated knowing the averages and standard deviations of the measured quantities, μ and t_c . The increase in η with C_{WGA} is statistically significant, whereas the differences in the values of μ at the three WGA concentrations tested are not significant.

~2.5 times longer than the control cells to recover their undeformed shapes. The actual recovery curves for a control cell and a cell at 0.2 $\mu\text{g/ml}$ of WGA are shown in Fig. 4. In both cases, $0 \leq t \leq 4t_c$, i.e., data are taken and plotted only over a range of four recovery time constants. Clearly, individual data points in Fig. 4 deviate from the "best fit" of the theoretical curve given by Eq. 3. However, these deviations are within experimental accuracy. In all cases, values of ϵ (Eq. 5) were obtained that correspond to errors in length and width measurements that are within the limits of optical resolution of the measuring system. For the results shown in Table I, values of ϵ range from 0.01 to 0.05. Values of $(L/W)_\infty$ (Eq. 3) range from

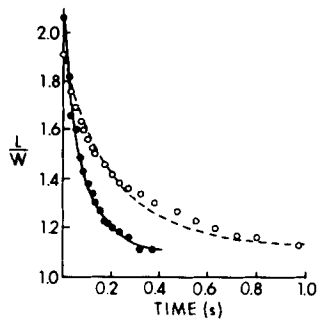


FIGURE 4 Experimental and theoretical results for the extensional recovery of two cells, one suspended in 0.0 $\mu\text{g/ml}$ WGA (solid circles and solid line; $t_c = 0.10$ s) and the other in 0.2 $\mu\text{g/ml}$ WGA (open circles and dashed line; $t_c = 0.25$ s). For the two sets of results shown, the values for $(L/W)_\infty$ are nearly identical. The experimental data are seen

to correlate closely with the theory (Eq. 3) for times less than approximately four recovery time constants (0.4 s at 0.0 $\mu\text{g/ml}$ WGA and 1.0 s at 0.2 $\mu\text{g/ml}$ WGA).

1.01 to 1.28 and show no correlation with WGA concentration. The values for $(L/W)_\infty$ are virtually identical for the two cases shown Fig. 4.

From measurements of t_c as a function of C_{WGA} and independent measurements of μ as a function of C_{WGA} , values for η are calculated with Eq. 4 (Table I). The membrane surface viscosity η increased as a function of C_{WGA} . The t test was applied to the data to determine the significance of the changes in η with WGA concentration. The difference in η at $C_{\text{WGA}} = 0.0$ $\mu\text{g/ml}$ and $C_{\text{WGA}} = 0.1$ $\mu\text{g/ml}$ is significant at the 0.001 level. The difference in η at $C_{\text{WGA}} = 0.1$ $\mu\text{g/ml}$ and $C_{\text{WGA}} = 0.2$ $\mu\text{g/ml}$ is significant at the 0.01 level. The effect of the increase in t_c (and η) with C_{WGA} is clearly demonstrated by the photographs shown in Fig. 5, which compare the recovery of a control cell to the recovery of a cell suspended in 0.2 $\mu\text{g/ml}$ WGA at 0.25 s after the release of each cell from the micropipette.

DISCUSSION

The erythrocyte membrane exhibits different material behavior according to the magnitude and duration of the applied forces. For the range of forces and times in the present experiments, the membrane behaves as a viscoelastic solid, that is, it can undergo large recoverable deformations with internal viscous dissipation. As a viscoelastic solid, the shear behavior of the membrane is characterized by two constants: the elastic shear

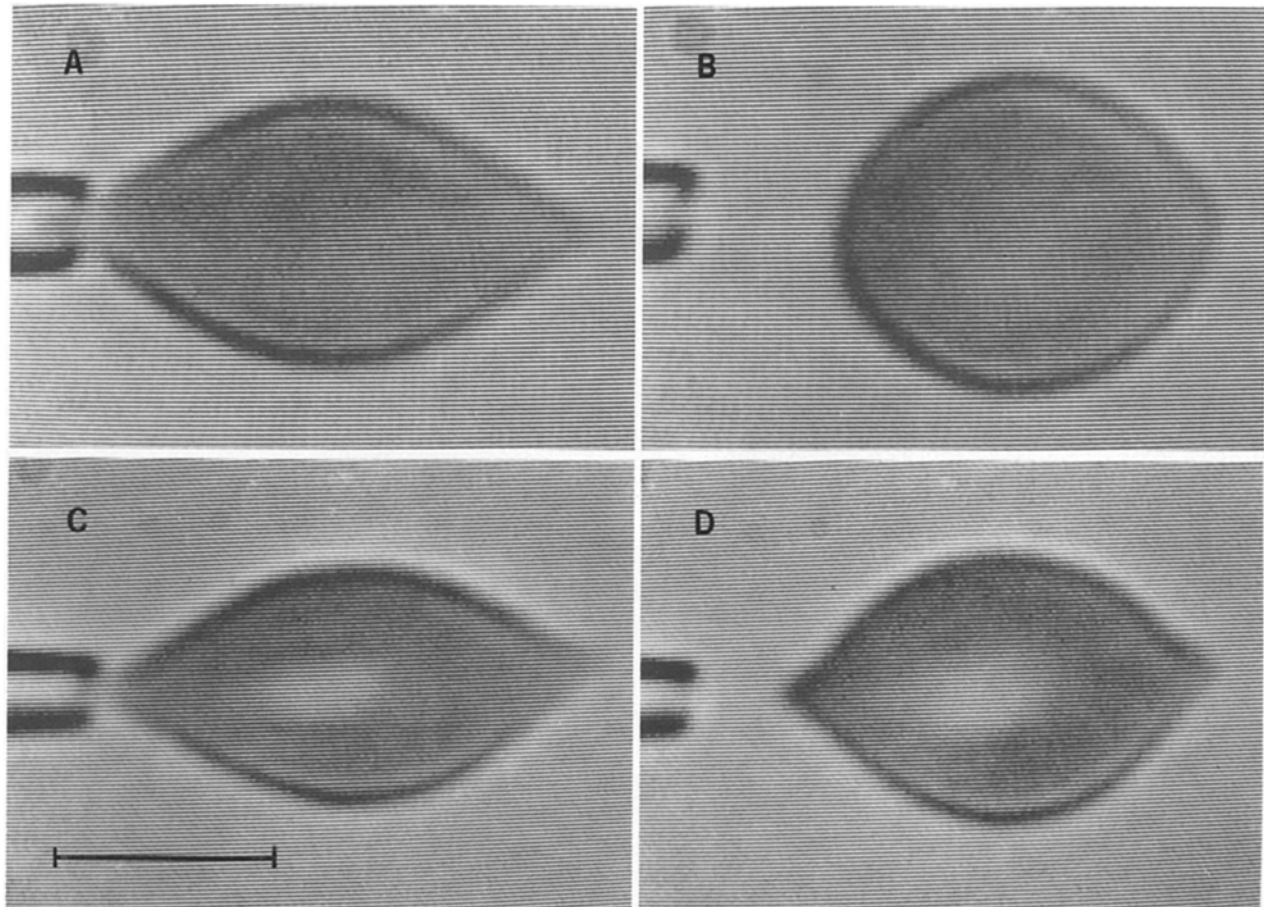


FIGURE 5 Comparison of the viscoelastic recovery of a cell suspended in a solution containing WGA vs. that of a control cell. The upper photographs show a cell suspended in a solution containing no WGA just as it released from the pipette (A) and 0.25 s after release (B). The time constant for this cell is 0.08 s. The lower photographs show another cell suspended in 0.2 $\mu\text{g/ml}$ WGA just after release (C) and 0.25 s after release (D). The time constant for this cell is 0.25 s. Bar, 5 μm . $\times 5800$.

modulus μ , which represents elastic energy storage produced by constant area deformation in the plane of the membrane, and the coefficient of surface viscosity η , which represents viscous dissipation (5).

In our experiments, a relatively small concentration of cells ($\sim 5 \times 10^5$ cells/ml) and WGA ($\sim 0.1 \mu\text{g/ml} = 2.8 \times 10^{-9}$ M) are studied in conjunction with a relatively large concentration of albumin ($1 \text{ mg/ml} = 1.5 \times 10^{-5}$ M). Albumin alone affects neither membrane elasticity nor viscosity over a wide range of concentrations (our unpublished observations). On the other hand, WGA alone at $0.1\text{--}0.2 \mu\text{g/ml}$ and 5×10^5 cells/ml completely inhibits the movement of the membrane into the micropipette—the membrane appears to be rigid—until the cell is subjected to aspiration pressures that are more than an order of magnitude larger than those used in the present experiments.¹ In the absence of albumin, Lovrien and Anderson² estimate that, at a cell concentration of 5×10^5 cells/ml and a WGA concentration of $0.1 \mu\text{g/ml}$, $\sim 1.8 \times 10^6$ molecules/cell will bind to the membrane, presumably through intramolecular binding to individual glycoporphin dimers (2, 7). Since there are $3\text{--}5 \times 10^5$ copies of glycoporphin per cell, 1.8×10^6 molecules/cell represents about five WGA molecules per glycoporphin. This concentration of WGA blocks the diskocyte \rightleftharpoons echinocyte conversion (2, 7) and the mechanical deformation of the membrane. However, in our experiments, the presence of albumin greatly diminishes the effect of WGA to the point where the elasticity is normal while the viscosity is two to three times that of the control at WGA concentrations of $0.1\text{--}0.2 \mu\text{g/ml}$. It should be noted that the elasticity measurements were made only for WGA concentrations of $0.2 \mu\text{g/ml}$ or less. It is probable that higher concentrations of WGA would alter the elasticity of the membrane, although this would be very difficult to determine since the membrane becomes highly adhesive at WGA concentrations $>0.2 \mu\text{g/ml}$.

It is interesting to speculate about how WGA in the presence of large concentrations of albumin is able to alter the viscosity of the membrane but not the elasticity. We suggest that large concentrations of albumin shift the spectrin-glycoporphin reaction significantly to the left, i.e., to higher concentrations of the

individual “reactants” rather than the WGA-glycoporphin “product.” Thus, fewer WGA-glycoporphin complexes are formed and, as a consequence, the membrane’s resistance to mechanical deformation is not altered. However, what little WGA still binds to the membrane is sufficient for the creation of an additional “viscous drag” of the membrane on the surrounding aqueous phase. For example, 10^5 molecules (say) of WGA at a diameter of 40 \AA and a velocity of $10 \mu\text{m/s}$ (comparable to the velocity of the membrane during the recovery phase) will experience a drag force of 4×10^{-6} dyn if the nonmembrane aqueous phase has the viscosity of water. Since this drag force has the same order of magnitude as the elastic restorative force in the membrane (calculated as the product of μ , (L/W) , and W), the drag of a relatively small number of glycoporphin-attached WGA molecules on the surrounding extracellular solution could significantly increase the recovery time and, thus, the membrane viscosity, without affecting membrane elasticity. Clearly, further research is needed to clarify the role of serum albumin in mediating the effect of WGA on membrane material properties.

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² R. Lovrien and R. A. Anderson, University of Minnesota, personal communication.