THERMOELASTICITY OF RED BLOOD CELL MEMBRANE

R. WAUGH AND E. A. EVANS, *Departments of Biomedical Engineering and Physiology, Duke University, Durham, North Carolina 27706 U.S.A.*

ABSTRACT The elastic properties of the human red blood cell membrane have been measured as functions of temperature. The area compressibility modulus and the elastic shear modulus, which together characterize the surface elastic behavior of the membrane, have been measured over the temperature range of 2–50°C with micropipette aspiration of flaccid and osmotically swollen red cells. In addition, the fractional increase in membrane surface area from 2–50°C has been measured to give a value for the thermal area expansivity. The value of the elastic shear modulus at 25°C was measured to be 6.6×10^{-3} dyne/cm. The change in the elastic shear modulus with temperature was -6×10^{-5} dyne/cm°C. Frictional forces were shown to be only on the order of 10–15%. The area compressibility modulus at 25°C was measured to be 450 dyne/cm. The change in the area compressibility modulus with temperature was −6 dyne/cm°C. The thermal area expansivity for red cell membrane was measured to be 1.2×10^{-3}/°C. With this data and thermoelastic relations, the heat of expansion is determined to be 110–200 ergs/cm^2; the heat of extension is $\hat{2} \times 10^{-2}$ ergs/cm^2 for unit extension of the red cell membrane. The heat of expansion is of the order anticipated for a lipid bilayer idealized as twice the behavior of a monolayer at an oil-water interface. The observation that the heat of extension is positive demonstrates that the entropy of the material increases with extension, and that the dominant mechanism of elastic energy storage is energetic. Assuming that the red cell membrane shear rigidity is associated with "spectrin," unit extension of the membrane increases the configurational entropy of spectrin by 500 cal/mol.

INTRODUCTION

The mechanical properties of red cell membranes have been of interest to investigators for some time; however, constitutive relations have only recently been developed that accurately model the elastic behavior of the membrane, independent of the particular experiment (Skalak et al. 1973; Evans, 1973*a*, *b*; Evans and Hochmuth, 1978; Evans and Skalak, 1979). The red cell membrane is a thin material, only a few molecules thick, and essentially lamellar in structure. It can only be considered as a continuous substance in the two dimensions that describe its surface. Consequently, the surface elastic behavior of the membrane is characterized by two constants: the area compressibility modulus, K, which represents the elastic energy storage produced by area dilation or compression and the shear modulus, μ, which represents the elastic energy storage produced by extension of the membrane in the surface plane without change in membrane area. These surface elastic moduli, with units of force per length, are the primary determinants of intrinsic membrane deformability; the additional

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Dr. Waugh's present address is Department of Radiation Biology and Biophysics, University of Rochester, Rochester, N.Y. 14642.

effects of curvature elasticity or bending rigidity usually contribute negligibly to membrane resistance to deformation (Evans, 1974; Zarda et al. 1977; Evans and Hochmuth, 1978).

Another area that has been of interest to investigators is the effect of temperature on biological membranes. The effect of high temperatures on red cells was observed as long ago as 1865 (Schultze). More recently Hamm et al. (1948) have shown that above 48–50°C red cells lose their ability to maintain their shape and undergo spontaneous budding and fragmentation. Rakow and Hochmuth (1975) measured an increase in membrane resistance to extension when cells were briefly incubated above 48°C and returned to room temperature. Similar but smaller increases in red cell membrane resistance to extension have also been observed at low temperatures. Williamson et al. (1975) used fluid shear to extend red cells at several temperatures and measured a 23% increase in extensional resistance from 25 to 2°C.

Values for the elastic moduli of the red cell membrane at 25°C have been reported in several publications (Evans, 1973*b*; Hochmuth et al., 1973; Evans and LaCelle, 1975; Waugh and Evans, 1976; Evans and Waugh, 1977*b*). In this paper we will present measurements of the elastic area compressibility modulus and surface shear modulus as a function of temperature over the range of 2–45°C. In addition we will present measurements of the thermal area expansivity of the red cell membrane; the thermal area expansivity is the fractional change in membrane surface area per degree temperature increase. The data are determined from micropipette aspiration experiments. In addition to the contribution to the knowledge of the mechanical properties of biological membranes, this work provides an essential link between the continuum mechanics and thermodynamics of membrane *in situ*. The temperature gradients of the elastic moduli and membrane surface area will be used to decompose the reversible work of deformation at constant temperature into internal energies and heats of deformation (Evans and Waugh, 1977*a*; Evans and Skalak, 1979). The heats of deformation establish the nature of configurational entropy change that occurs as a result of deformation of the material structure. Also, the heat of expansion, which represents thermal repulsive forces in the membrane plane, is a direct measure of the cohesive energy density of the membrane surface.

CELL PREPARATION METHODS

Blood samples are drawn from healthy adult donors, with heparin as anticoagulant. Cells are washed two or three times in phosphate-buffered saline (PBS-30), which contains 121.5 mM NaCl, 25.2 mM Na_2HPO_4, and 4.8 mM KH_2PO_4. The pH is adjusted to be in the range 7.36–7.44 at 25°C. White cells and platelets are removed. All the solutions used in the experiments contain penicillin (100 u/ml) and streptomycin (110 μg/ml) to retard bacterial growth. Millipore GS-grade filters (Millipore Corp., Bedford, Mass.) are used to remove bacteria and debris. Osmolarities are measured by freezing point depression. In preparation for elastic shear modulus measurements, cells are washed in a solution of human serum albumin (0.75 g/100 ml) and PBS-30. The osmolarity is adjusted to the range 295–305 mosmol so that the cells remain flaccid, bioconcave disks. The pH is adjusted to the range 7.36–7.42. After washing, a small drop of cells is resuspended at very low hematocrit in filtered solution. Cell preparations for area compressibility and thermal area expansivity measurements differ in that the ionic strength of the cell cytoplasm is adjusted when necessary to produce spheroidal cells at various osmotic strengths. The method employed to change internal ion content is to incubate cells with an ionophore, nystatin, in buffer solutions that contain specific potassium chloride concentrations (see Evans and Waugh, 1977*b* for details). Nearly spherical cells are produced by resuspension in a medium hypotonic relative to the cell contents with negligible lysis of the cells in the suspension. All solutions contain bovine

serum albumin (BSA) at 0.5 g/100 ml final concentration. After the cells have been washed and resuspended in the appropriate solutions, they are briefly kept in a syringe at room temperature until the time of measurement. Then, they are placed in the temperature-controlled chamber and brought to the desired temperature.

ELASTIC AREA COMPRESSIBILITY MODULUS AT DIFFERENT TEMPERATURES

The area compressibility modulus of red cell membrane is measured by aspiration of a pre-swollen cell into a micropipette schematically illustrated in Fig. 1 *a* (Evans and Waugh, 1977*b*). At a small initial pressure of 8.0–13.0 × 10^3 dyne/cm^2 (6.0–10.0 mm Hg) the outer portion of the cell is spherical, which shows the dominance of isotropic tension. The pressure is

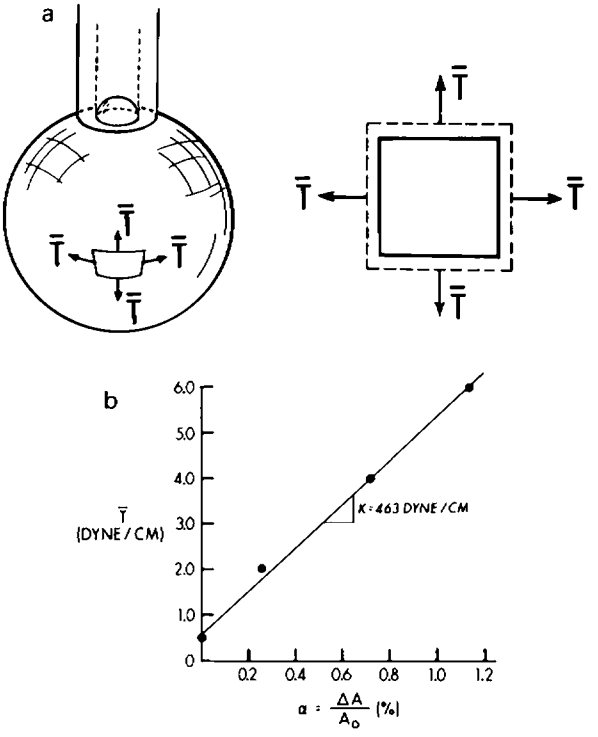

FIGURE 1

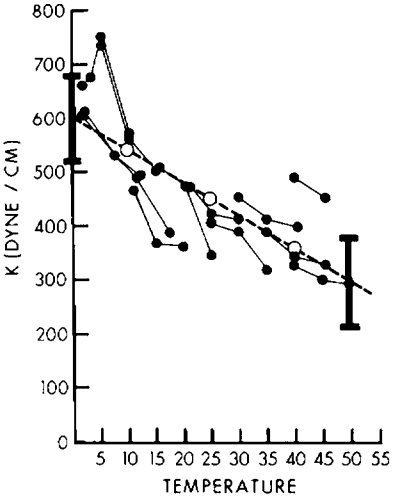

FIGURE 2

FIGURE 1 (*a*) Schematic illustration of isotropic membrane tension produced by micropipette aspiration of osmotically swollen red cells. (*b*) Isotropic tension, $\bar{T}$, vs. the fractional change in membrane area, α (given in percent), for a single cell experiment. The straight line is the linear regression to the data. The slope is the area compressibility modulus. The nonzero intercept is determined by the initial pipette suction pressure necessary to draw the cell into a sphere.

FIGURE 2 Area compressibility modulus of red cell membranes as a function of temperature. Solid points are initial data that were taken on swollen cells with normal internal ion composition (Waugh, 1977). Each dot is the average of $\simeq$ 20 single cell measurements. The dotted line is the linear regression to this initial data. Open circles are data from subsequent experiments where cells were swollen with increased internal ion concentrations (Evans and Waugh, 1977*b*). The open circles are the average of $\simeq$60 cell measurements at each temperature, with three different osmotic strengths, extrapolated to infinite internal osmotic strength.

then increased by increments to a maximum value of 5.0–9.0 × 10^4 dyne/cm^2 (40.0–70.0 mm Hg). After the cell is released from the pipette, it is reaspirated at the initial pressure to check for possible irrecoverable volume loss during the measurement. The experiment is recorded on videotape along with the time, temperature, and pressure for subsequent analysis. Hysteresis checks have been made, but none was detected within the limitations of experimental resolution. Each addition to the applied pressure, ΔP, produces a change in the projection length, ΔL. The area compressibility modulus, K, is obtained from this data by calculating the slope of the isotropic tension, $\bar{T}$, vs. the fractional area change, α, for the data set, (ΔP, ΔL).

The tension in the surface is obtained from the balance of forces on the surface. Provided that the frictional interaction between the cell and the pipette is negligible, the tension will be uniform throughout the surface. $\bar{T}$ can be calculated from the relationship,

$$\bar{T} = \frac{\Delta P \cdot R_p}{2}\Big/\left(1 - \frac{R_p}{R}\right), \tag{1}$$

where ΔP is the pressure difference between the pipette and the suspending medium, R_p is the radius of the pipette, and R is the radius of the outer spherical portion of the cell.

The fractional change in membrane area, $\Delta A/A_0$, is related to the movement of the cell projection in the pipette, ΔL, and the change in cell volume, ΔV. Because the changes are small, the fractional area change is given by the first order term in a Taylor series expansion,

$$\frac{\Delta A}{A_0} \simeq 2\pi\left[R_p \cdot \Delta L \cdot \left(1 - \frac{R_p}{R}\right) + \frac{\Delta V}{\pi R}\right]\Big/A_0. \tag{2}$$

All of the variables in Eq. 2 can be measured directly in the experiment, except for ΔV. The change in volume must be determined by an independent relation because it is too small to be accurately measured with an optical system.

Evans and Waugh (1977*b*) have shown that the recoverable change in the cell volume is a result of the pressure imposed on the cell by micropipette suction. Water is filtered out of the cell, but the number of ions in the cell remains essentially constant. The osmotic concentration within the cell increases to oppose water filtration. Steady state is reached when the net flow of water out of the cell is zero. The fractional change in volume resulting from this water loss is proportional to the isotropic tension and inversely proportional to the osmotic concentration, c_i, inside the cell (Evans and Waugh, 1977*b*),

$$\frac{\Delta V}{V_0} \sim -\frac{\bar{T}}{c_i}, \tag{3}$$

where V_0 is the initial volume of the cell. This volume change is recovered as the cell is released and can be reduced in proportion to the increase in osmotic strength of the cell contents. In addition to recoverable exchanges of water from the cell, sometimes an irrecoverable change in volume occurs during the measurement. The irrecoverable changes are long-term transients (Waugh, 1977) that can be essentially eliminated by closely matching the tonicity of the pipette and suspending solutions. This effect is also minimized at higher osmotic concentrations of the suspending media. The ionophore nystatin is used to load the red cells with high ionic strength potassium chloride (up to 300 mM). With these methods for calculating volume changes and by varying the osmotic concentrations at which the cells

are in the swollen state, it has been demonstrated that the effects of volume alterations can be eliminated (Evans and Waugh, 1977*b*).

The fractional change in membrane areas, given by Eq. 2, is a linear function of the membrane isotropic tension for small dilations (Fig. 1 *b*). The slope of this line is the isothermal area compressibility modulus. The nonzero intercept is a consequence of using an initial applied pressure to insure that isotropic tension dominates the balance of membrane forces before the fractional change in area is measured. The area compressibility modulus is calculated by linear regression.

An initial set of area compressibility experiments at different temperatures took place on 12 different days over a 19-wk period. On most days, 60 individual cells were measured. Cells were measured in batches of 10, and each batch was measured at a specific temperature. Measurements were made at three different temperatures in ascending and descending order. For example, the order of the six batch temperatures might have been 15, 25, 35, 35, 25, 15°C; or 40, 30, 20, 20, 30, 40°C. The initial data is summarized in Fig. 2 as the solid points (Waugh, 1977). Each solid point represents the average area compressibility moduli of $\simeq 20$ cells. Points that are connected correspond to the data taken in the sequence described above. The dashed line is the least-squares fit to the data represented by the solid points. The standard deviation of the linear regression is represented by the brackets at each end of the line. The slope shows a decrease in the area compressibility modulus with temperature of 6.0 dyne/cm°C. The compressibility modulus at 37°C is 375.0 ± 60.0 dyne/cm. Subsequently, a second set of experiments was performed with cells swollen at three different osmolarities. The large open circles in Fig. 2 represent the average values measured on 60 cells swollen at 3 different osmolarities for each temperature. They were not included in the original linear regression, but fall exactly on the calculated regression line.

The scatter in the area compressibility data is large. It appeared to be associated with irrecoverable volume changes in the initial set of experiments; subsequently, when the cells were swollen with increased internal osmotic strengths, the scatter corresponded to measurement error. The observation (Fig. 2) that the data for cells swollen at higher internal osmotic strengths fall on the regression line for the initial experiments shows that errors as a result of transient volume change are essentially averaged out. To assess measurement errors, calculations were carried out for a model cell; each parameter was varied within the limits of our measuring capability. The critical parameters are the change in the length of the cell projection in the pipette and the irrecoverable volume change. An error of 0.06 μm in the length change causes a difference of $\simeq 15.0\%$ in the calculated compressibility modulus. An error in the irrecoverable volume change equal to 0.2% of the cell volume causes an error of $\simeq 13\%$ in the calculated modulus. These values correspond to the detection limits of the optical measurement (Evans et al., 1976); thus, standard deviations of 15% are consistent with the observed data spread.

THERMAL AREA EXPANSIVITY

The fractional change in red cell membrane area with temperature is determined by a procedure similar to that employed in the area dilation experiment. With micropipette aspiration of an osmotically swollen, nearly spherical red cell, the isotropic tension is easily controlled by the suction pressure. The cell is held at a constant suction pressure; the

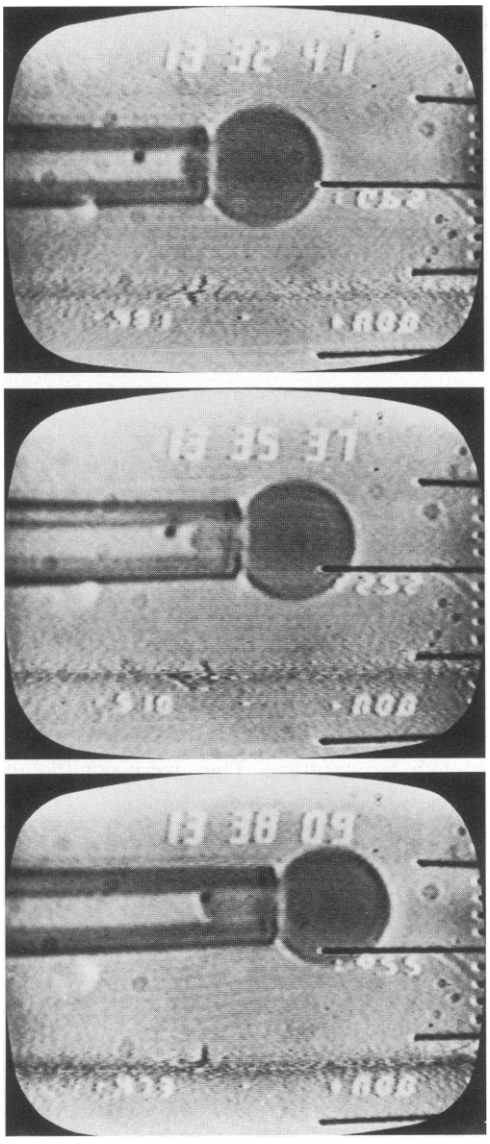

FIGURE 3 Photographs of videorecordings for a single red cell aspirated in a micropipette at constant suction pressure; each photograph corresponds to a specific temperature: top, 6°C; middle, 25°C; and bottom, 45°C. This cell was swollen in a solution with osmotic strength of 575 mosmol. Consequently, 85% of the change in aspirated length is due to surface area change; water displacement contributes $< 15\%$ of the total length change.

temperature change produces a reversible change in the length of the aspirated cell projection, Fig. 3. The length increase is proportional to the increase in area of the cell membrane. There is also a contribution to the movement of the cell projection as a result of recoverable water exchange across the cell membrane. As discussed previously, the water exchange is inversely proportional to the osmotic strength of the red cell contents and, therefore, can be minimized

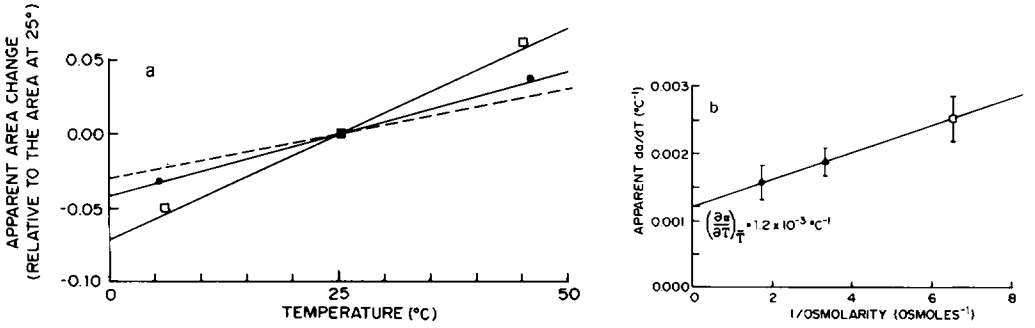

FIGURE 4 (*a*) Apparent area change of a single red cell relative to its area at 25°C vs. temperature. The apparent area change is calculated with the assumption that the cell volume is constant. Data given by the open squares are for a cell swollen in 150 mosmol salt solution; data shown by solid circles are for another cell swollen in ≃ 575 mosmol solution. The dashed line is the calculated behavior for relative changes in cell area at infinitely large osmotic strengths, i.e., in the absence of volume changes due to water displacement. (*b*) Slopes of the apparent area change with respect to temperature vs. one over the osmotic strength. Each point is the average value from data for ≃ 50 cells swollen at a specific salt concentration: the open squares are for 150 mosmol; the solid triangles are for 300 mosmol; and the solid circles are for 575 mosmol. The straight line and intercept are determined by linear regression to the apparent area change gradients vs. the inverse osmotic strength. The intercept is the fractional change in membrane area per degree centigrade, $(\partial\alpha/\partial T)_{\bar{\tau}}$. The bars represent the standard deviation.

by increasing the intracellular osmotic strength. This is accomplished by treating the cells within nystatin, loading them with potassium chloride, and washing away the nystatin, leaving red cells with high internal osmotic strength. If the change in volume as a result of water movement is eliminated, the change in area of the red cell is simply a linear function of the projection length for small area changes; thus, Eq. 2 becomes

$$\Delta A \cong 2\pi R_p \cdot \Delta L \cdot \left(1 - \frac{R_p}{R}\right). \tag{4}$$

From this relation, the change in membrane area is obtained as a function of temperature at constant membrane tension. Fig. 4 *a* is an example of the area change obtained for single cell experiments at different cellular osmotic strengths. The fractional change in area is obtained by division of each experimental result by the initial cell area at 25°C. The results for specific osmotic strengths are the apparent fractional change in membrane area with temperature. The apparent fractional change in membrane area is measured at different internal osmotic strengths; thus, it is possible to obtain the actual fractional change in membrane area with respect to temperature from the extrapolation to infinite internal osmotic strength (i.e., zero water movement). Fig. 4 *b* is the plot of the temperature gradients of the apparent fractional area change vs. one over the osmotic strength of the swollen cells. The intercept in Fig. 4 *b* is the thermal area expansivity of the red cell membrane at zero membrane isotropic tension. (The membrane tension as a result of the applied suction pressure is small and is corrected with the measured area compressibility modulus of red cell membrane.)

The thermal area expansivity of red cell membrane is found to be 1.2×10^{-3} °C^{-1} with a standard deviation of $\pm$ 0.2×10^{-3} °C^{-1}, i.e.,

$$\left(\frac{\partial \alpha}{\partial T}\right)_{\bar{T}=0} = 1.2 \times 10^{-3}\,^{\circ}\mathrm{C}^{-1}.$$

With the measured values for the elastic area compressibility modulus of the red cell membrane, it is now possible to determine the reversible heat of expansion for the membrane (Evans and Waugh, 1977*a*; Evans and Skalak, 1979).

ELASTIC SHEAR MODULUS AT DIFFERENT TEMPERATURES

The elastic shear modulus of the membrane is measured by aspirating a flaccid, unswollen cell in the dimple region, schematically illustrated in Fig. 5 *a* (Evans and LaCelle, 1975; Waugh and Evans, 1976; and Waugh, 1977). From an initial pressure of 200–300 dyne/cm^2 ($\simeq$2–3 mm H_2O), the pressure is increased in small increments. If the cell begins to fold or buckle, the measurement is terminated. Cells are then tested for hysteresis as the suction in the pipette is decreased in several increments to the initial pressure. The test is recorded on videotape with the temperature and pressures. Frictional effects from the pipette wall are shown to be on the order of only 10–15%, within the limits of experimental resolution. These effects are further minimized by averaging the loading and unloading phases of the aspiration experiment. The aspiration of the flaccid cell membrane requires suction pressures two orders of magnitude smaller than for the spheroidal cell experiments; consequently, the membrane area can be considered as constant, i.e., an incompressible surface. Thus, this experiment produces membrane extension at constant area.

Membrane tensions produced by a small caliber pipette are concentrated in, and near, the pipette mouth (Evans, 1973*b*). These tensions drop off inversely as the square of the distance

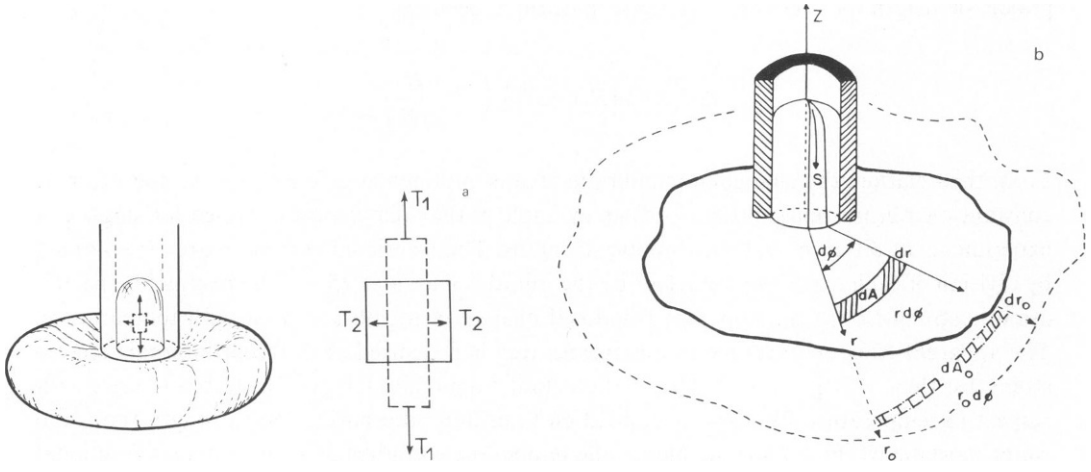

FIGURE 5 (*a*) Schematic illustration of the principal membrane tensions produced by micropipette aspiration of flaccid red cells. The maximum surface shear resultant, T_s, is the deviator, $(T_1 - T_2)/2$. (*b*) Diagram of the membrane surface local to the pipette entrance. The axisymmetric deformation is determined from the ratio of dimensions of the shaded membrane surface elements; r is the radial coordinate in the deformed state and r_0 is the radial coordinate that describes the undeformed material. The extension ratio along a meridian, defined by the curvilinear coordinate s, is given by the ratio of radial coordinates in the outer surface, i.e., $\lambda_m = (r_0)/r$.

from the pipette center, normalized by the pipette radius. Aspiration of the cell to such an extent that the membrane tensions at the cell rim or periphery are appreciable results in curvature changes at the cell rim that are directly observable, which thereby provides an experimental indicator for the limitation of the analysis.

The balance of forces and equations of equilibrium for the membrane require two critical assumptions: (*a*) the membrane is pushed tightly against the glass wall of the pipette, without wall friction and (*b*) only force resultants in the plane of the membrane are important in the equations of equilibrium, i.e., contributions from bending moments are negligible. These assumptions are verified by experimental observations.[1] The two assumptions permit the treatment of the principal force resultants (tensions) in the membrane as continuous from the aspirated region to the outer membrane surface. The exterior membrane surface is conceptualized as infinitely large in comparison to the pipette dimension; thus, the equations of equilibrium are integrated in the plane of the membrane from the pipette tip outward to give

$$\Delta P = \frac{4}{R_p} \int_{R_p}^{\infty} \frac{T_s}{r} dr, \tag{5}$$

where ΔP is the pipette suction pressure and T_s is the maximum membrane shear resultant given by the deviator, $(T_1 - T_2)/2$, of the principal tensions shown in Fig. 5 *a* (Evans and Hochmuth, 1978; Evans and Skalak, 1979). The force resultants are negligible at distances far from the pipette (i.e., at the periphery of the cell; this is consistent with the flaccid, essentially unchanged shape of the equatorial region of the red cell during the experiment). The intensive membrane shear force resultant is related to the pipette suction pressure by the integral Eq. 5.

The state of deformation in the membrane surface must be determined as a function of the length of the cell projection that is aspirated into the pipette. The material extension ratio is a function of radial position and is used with an elastic constitutive relation in Eq. 5 to predict the suction pressure that is required to aspirate a membrane projection of length, L. Correlation of the prediction with the observed relation between pressure and length provides

[1]If the membrane is not held tightly against the pipette inner wall (by the hydrostatic pressure excess of the cell over the effect of the circumferential tension), the membrane will pull away from the wall and the projection will neck down. Such behavior is observed with a long aspirated membrane projection when the outside portion of the cell becomes spherical and isotropic tension increases (see Evans 1973*b*, for discussion). Data in this paper will show that frictional effects are on the order of only 10%; therefore, the first assumption can be taken as valid. The second assumption concerning bending moments is more difficult to evaluate. Elastic energy storage resulting from membrane curvature changes or bending will be largest at the entrance of the pipette (where the membrane bends around the edge of the entrance) and over the spheroidal cap of the aspirated projection. Such bending energy changes occur during the initial aspiration of a small spheroidal bump (the order of a pipette radius); further aspiration will not appreciably change the initial contribution. Additional bending energy is produced as the cylindrical portion of the projection is formed. This contribution to bending energy increases linearly with the projection length; thus, it does not contribute to an increased pipette suction pressure as the projection increases. Therefore, the curvature or bending elastic effects are primarily an initial bias in the force required to aspirate the membrane projection into the pipet. Experimentally, it has not been possible to detect this bias indicating that it is negligible. Also, Evans and Hochmuth (1978) have estimated that the bending or curvature elastic energy contribution would be only $\simeq 20\%$ for the bump formation and would be progressively less with further aspiration and extension of the membrane surface. From an intuitive viewpoint, if the bending resistance of the membrane is large, then the membrane region just outside the entrance of the pipette would gradually bend away from the tip of the pipette; this is not observed. Consequently, the second assumption appears to be reasonable.

the value for the elastic shear modulus, μ. From the previous data for resistance to area dilation, it is recognized that the red cell membrane greatly resists changes in local surface area; therefore, only a single principal extension ratio, λ_m, along the radial direction need be considered inasmuch as the other extension ratio for the surface deformation is given by its reciprocal. Incompressibility or constant area implies that each element of area of the membrane surface remains constant in magnitude when deformed even though its shape has changed. Fig. 5 *b* illustrates the displacement and constant area deformation of an annular ring that results from the pipette aspiration. The square of the material extension ratio drops off as $1/r^2$ from the pipette entrance. The exact shape of the spheroidal cap inside the pipette can only be established by satisfying the equations of equilibrium for the membrane surface in this region subject to a hydrostatic pressure difference; this is not possible in closed form but can be done on a digital computer. However, this region contributes minimally to the maximum degree of extension as the projection increases. Therefore, we simply use spherical surface segments to approximate the cap geometry as the projection length goes from $0 \leq L \leq R_p$ and consider the cap as hemispherical for projection lengths greater than a pipette radius. With this approach, the extension ratio at a point outside the pipette entrance is given by

$$\lambda_m^2 = 1 + \left(\frac{R_p}{r}\right)^2 \cdot \left(\frac{2L}{R_p} - 1\right) \tag{6}$$

for projection lengths greater than one pipette radius, $L \geq R_p$. The maximum extension ratio in the membrane occurs at the pipette entrance and is equal to $\hat{\lambda} = \sqrt{2L/R_p}$.

With the first order elastic constitutive relation for the shear resultant (Evans, 1973*a*; Evans and Hochmuth, 1977 and 1978),

$$T_s = \frac{\mu}{2} \cdot (\lambda_m^2 - \lambda_m^{-2}), \tag{7}$$

the integral relation (Eq. 5) predicts the relation between the pipette suction pressure and the aspirated projection length.[2]

$$\Delta P = \frac{2\mu}{R_p} \int_{R_p}^{\infty} (\lambda_m^2 - \lambda_m^{-2}) \frac{dr}{r}$$

or,

$$\Delta P = \left(\frac{\mu}{R_p}\right) \cdot \left[\left(\frac{2L}{2_p} - 1\right) + ln\left(\frac{2L}{R_p}\right)\right]. \tag{8}$$

The predicted relationship between between ΔP and L is shown in Fig. 6, along with data from a typical cell. Because of the biconcave shape of the cell, the mouth of the pipette is often obscured from view by the cell's outer rim. Careful inspection of the diffraction pattern permits accurate measurements of the change in the projection length to be made, although

[2]It is important to recognize that the shear modulus, μ, which appears in Eq. 7 is half the value defined originally by Evans (1973*b*). The factor of two has been introduced as the result of the thermodynamic development of membrane elasticity (see Evans and Waugh, 1977*a*; Evans and Hochmuth, 1978; or Evans and Skalak, 1979, for the thermodynamic development).

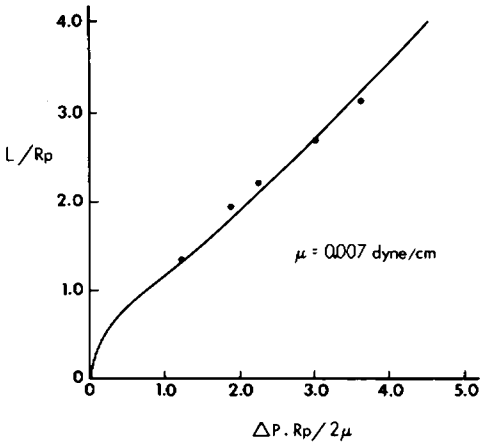

FIGURE 6

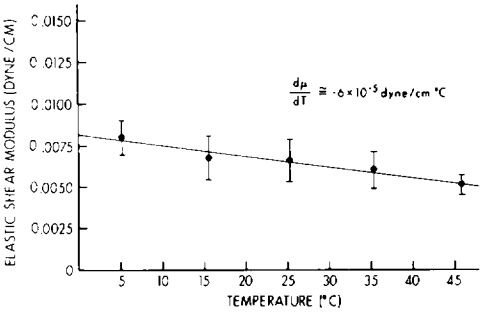

FIGURE 7

FIGURE 6 Pipette suction pressure vs. length of the cell projection. The solid line is the theoretical prediction for first order elastic behavior (Evans, 1973*b*; Evans and Hochmuth, 1978). Dots are data for a single cell experiment correlated with a membrane shear modulus equal to 7×10^{-3} dyne/cm.

FIGURE 7 The elastic shear modulus as a function of temperature. Points are the average of $\simeq 25$ cell moduli at each temperature; brackets are ± 1 SD. The solid line is the linear regression to the data (Waugh, 1977).

the exact location of the pipette tip is uncertain. A least-squares routine is used to calculate a correction to the apparent location of the tip. These corrections are always within the uncertainty of the tip location (Waugh and Evans, 1976).

The elastic shear modulus experiments were performed on 5 different days. A total of 136 cells were measured at 5, 15, 25, 35, and 45°C. The averages and standard deviations of all the moduli measured at each temperature are listed in Table I (Waugh, 1977). Fig. 7 shows the average moduli plotted vs. temperature. Brackets in the figure correspond to standard deviations. The change in the elastic shear modulus with temperature was found by linear regression to be -6.3×10^{-5} dyne/cm°C. Within the resolution of the data, the elastic shear modulus appears to change linearly with temperature from 5 to 45°C. Fractionally, the change is the same magnitude as the change in extensibility of whole red cells observed by Williamson et al. (1975). The average elastic shear modulus at 25°C is 6.6×10^{-3} dyne/cm.

TABLE I

ELASTIC SHEAR MODULUS[2] AS A FUNCTION OF TEMPERATURE*

Average temperature	μ	SD	Cells measured
°C	10^{-3} dyne/cm		n
45.8	5.16	0.62	29
35.3	6.07	1.08	23
24.8	6.61	1.24	29
15.5	6.78	1.30	23
5.2	7.99	1.08	33

*Waugh, 1977.

This value is in good agreement with the results of Evans (1973*b*) and Waugh and Evans (1976).[2]

Frictionless movement of the cell inside the pipette is an important assumption in the calculation of the elastic shear modulus. The validity of this assumption can be tested by measuring the differences in the apparent moduli calculated from data taken as the cell moves in, $\hat{\mu}_{in}$, and as the cell moves out of the pipet, $\hat{\mu}_{out}$. (The $\hat{}$ symbol indicates that the modulus is apparent; i.e., it includes possible frictional force effects.) A small difference was observed between measurements taken in the loading and unloading phases. Only frictional interactions between the membrane and the pipette are plausible explanations for the differences because the membrane was in static equilibrium with the applied pressure (i.e., no viscous force resultants) and the cells returned completely to the biconcave shape without plastic or irrecoverable deformation.

The exact mechanism of the small frictional force is not perceptible from the data, but it is possible to estimate the maximum error that can be introduced by frictional forces. A constant frictional force on the cell, e.g., at the pipette entrance, would not change the slope in the data, and so would not affect the calculated modulus (Fig. 8 *b*). However, if the friction were

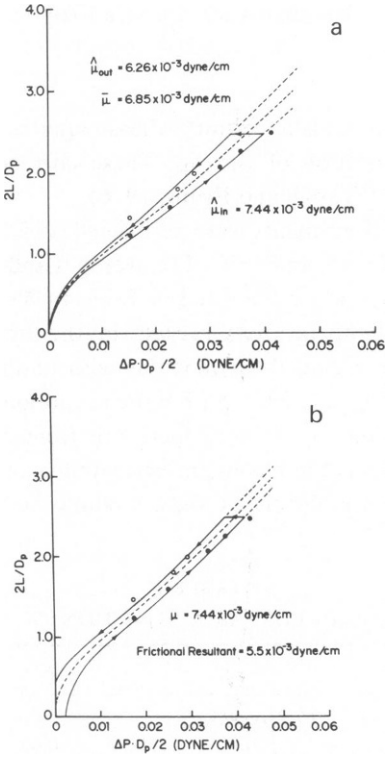

FIGURE 8 (*a*) Hysteresis behavior of a single cell experiment modeled by a length-dependent frictional force. The maximum possible error in the calculated modulus due to such a force is 10–13% (Waugh, 1977). (*b*) Hysteresis behavior of the single cell experiment modeled by a constant frictional force, such as edge friction at the pipette entrance. Such a force would have no effect on the calculated modulus (Waugh, 1977).

TABLE II

EFFECT OF FRICTIONAL FORCES ON THE CALCULATED VALUE OF THE ELASTIC SHEAR MODULUS*

Temperature	Cells	$\hat{\mu}_{in} \pm SD$	$\hat{\mu}_{out} \pm SD$	$\bar{\mu}$	$\frac{\hat{\mu}_{in}}{\bar{\mu}} - 1$
°C	n	10^{-3} dyne/cm	10^{-3} dyne/cm	10^{-3} dyne/cm	%
5	13	8.43 ± 0.76	6.54 ± 0.63	7.49	13
15	9	7.28 ± 1.33	5.99 ± 1.73	6.64	10
25	9	6.67 ± 1.21	5.45 ± 1.04	6.06	10
35	9	6.25 ± 1.40	5.09 ± 1.11	5.67	10
45	6	4.83 ± 0.47	3.64 ± 0.66	4.24	14

*Waugh, 1977.

proportional to the area of contact between the cell and the interior of the pipette, the calculated modulus would be affected (Fig. 8 *a*). As can be seen from Fig. 8, the resolution in the data is not sufficient to distinguish between these two mechanisms. The maximum error that might be introduced by friction can be obtained by assuming that the frictional force depends on contact area and then calculating apparent moduli for data taken in the loading and unloading phases. The actual modulus of the material would be the average of the two apparent moduli. 46 of 137 cells were measured both moving in and moving out of the pipette. The apparent moduli and the expected value of the actual modulus are tabulated as functions of temperature in Table II (Waugh, 1977). The results show that, at worst, the apparent modulus, $\hat{\mu}_{in}$ is 10–15% larger than the modulus of the material as determined by $(\hat{\mu}_{in} + \hat{\mu}_{out})/2$. Only data taken with increasing tongue length (loading phase) have been included in Table I and used in the calculation of $d\mu/dT$. The frictional effects did not vary with temperature (Table II). Consequently, the magnitude of $d\mu/dT$ is possibly too large by only 10–15%.

THERMOELASTICITY AND HEATS OF DEFORMATION

Thermoelastic behavior is predicted by the interrelation of the equations of state for the material with the combined first and second laws of thermodynamics for the closed material system (Evans and Waugh, 1977*a*; Evans and Skalak, 1979). The first equation of state is isotropic in the surface, i.e., independent of surface coordinates; this equation relates the mean or isotropic tension (dyne per centimeter) to changes in temperature and fractional change in area. The thermoelastic effect is the isotropic tension change that is produced by a change in temperature at constant surface area; this is given by the equation

$$\left(\frac{\partial \bar{T}}{\partial T}\right)_{\alpha} = -K \cdot \left(\frac{\partial \alpha}{\partial T}\right)_{\bar{T}}, \tag{9}$$

in terms of the thermal area expansivity, $(\partial\alpha/\partial T)_T$ ($°C^{-1}$). For a solid membrane material (like red cell membrane), there exists an additional surface equation of state that relates the shear force resultant in the membrane to extensional deformation at constant surface area and temperature. The thermoelastic change in shear resultant at constant membrane extension

(and area) is given by

$$\left(\frac{\partial T_s}{\partial T}\right)_{\lambda,\alpha} = \left(\frac{d\mu}{dT}\right) \cdot \frac{(\lambda^2 - \lambda^{-2})}{2},$$

where the surface elastic shear modulus, μ (dyne per centimeter), is assumed to be independent of the membrane extension.

The reversible heats of deformation at constant temperature are obtained from the thermoelastic relations. The heat of expansion is

$$T\left(\frac{\partial \tilde{S}}{\partial \alpha}\right)_T = T \cdot K \cdot \left(\frac{\partial \alpha}{\partial T}\right)_{\bar{T}}, \tag{11}$$

where $\tilde{S}$ is the entropy per unit undeformed area (ergs per squared centimeter − °C). The heat of extension (at constant area) is given by

$$T\left(\frac{\partial \tilde{S}}{\partial \lambda}\right)_{T,\alpha} = -T \cdot \frac{d\mu}{dT} \cdot (\lambda - \lambda^{-3}), \tag{12}$$

where λ is the surface extension ratio at constant area. The internal energy density changes that are produced by deformation have similar relations:

$$\left(\frac{\partial \tilde{E}}{\partial \alpha}\right)_T = \bar{T} + T \cdot K \cdot \left(\frac{\partial \alpha}{\partial T}\right)_{\bar{T}}, \tag{13}$$

and

$$\left(\frac{\partial \tilde{E}}{\partial \lambda}\right)_{T,\alpha} = \left(\mu - T \cdot \frac{d\mu}{dT}\right) \cdot (\lambda - \lambda^{-3}), \tag{14}$$

where $\tilde{E}$ is the internal energy per unit undeformed area (ergs per squared centimeter).

In the natural state, free of applied forces, the membrane force resultants are zero and the extension ratio is identically one. Therefore, the free energy density at constant temperature is a minimum with

$$\left(\frac{\partial \tilde{E}}{\partial \lambda}\right)_{T,\alpha} = T\left(\frac{\partial \tilde{S}}{\partial \lambda}\right)_{T,\alpha} = 0$$

and

$$\left(\frac{\partial \tilde{E}}{\partial \alpha}\right)_T - T\left(\frac{\partial \tilde{S}}{\partial \alpha}\right)_T = 0.$$

Hence, the heat of expansion at zero tension, $T \cdot K \cdot [\partial\alpha/\partial T]_{\bar{T}=0}$, is opposed by the potential increase in internal energy density with expansion. The heat of expansion is a direct measure of the surface cohesion that resists thermal expansion. The heat of expansion is calculated using the measured values for elastic area compressibility modulus and thermal area expansivity. The results for red cell membrane yield

$$T\left(\frac{\partial \tilde{S}}{\partial \alpha}\right)_T = T \cdot K \cdot \left(\frac{\partial \alpha}{\partial T}\right)_{\bar{T}=0} = 197 \pm 47 \text{ ergs/cm}^2 \text{ (at 0°C)}$$

$$= 116 \pm 27 \text{ ergs/cm}^2 \text{ (at 50°C)}.$$

(The standard deviations in the parentheses are deduced from the combined standard deviations for the area compressibility and expansivity data.)

Similarly, the reversible heat of extension can be calculated using the temperature gradient of the surface elastic shear modulus, $d\mu/dT$; the results for red cell membrane give

$$T\left(\frac{\partial \tilde{S}}{\partial \lambda}\right)_{T,\alpha} = 2 \pm 1 \times 10^{-2} \cdot (\lambda - \lambda^{-3}) \text{ ergs/cm}^2$$

at 25°C. The change in internal energy density with extension at constant area is calculated to be

$$\left(\frac{\partial \tilde{E}}{\partial \lambda}\right)_{T,\alpha} = 2.6 \pm 1 \times 10^{-2} \cdot (\lambda - \lambda^{-3}) \text{ ergs/cm}^2.$$

(The corresponding limits for the internal energy and heat of extension are given by the plus/minus increments in the parentheses. These bounds are estimated from temperature gradients of the possible lines that lie within the error brackets for the elastic shear modulus data shown in Fig. 7.) The difference between these two relations is the free energy density change at constant temperature and density that is produced by extension:

$$\left(\frac{\partial \tilde{F}}{\partial \lambda}\right)_{T,\alpha} = \left(\frac{\partial \tilde{E}}{\partial \lambda}\right)_{T,\alpha} - T\left(\frac{\partial \tilde{S}}{\partial \lambda}\right)_{T,\alpha} = \mu(\lambda - \lambda^{-3}),$$

where the coefficient is the surface elastic shear modulus. It is apparent that the small shear modulus is the difference between two contributions of nearly equal magnitude; consequently, the resolution of the temperature dependence of the shear modulus is critical in the determination of the free energy decomposition.

DISCUSSION

The heat of expansion, thermal area expansivity, and elastic area compressibility modulus are isotropic surface properties of a closed membrane system. As such, they do not distinguish between solid or liquid membrane materials (e.g., red cell membrane or phospholipid bilayer); these properties characterize the changes in state of the membrane composite with changes in surface area. Thus, it is desirable and informative to compare the measured properties of red cell membranes with phospholipid bilayers. However, essentially no data of this type exists as yet for closed phospholipid bilayer systems, e.g., single-walled vesicles. Unpublished x-ray data by Dr. Costello and Dr. Gulik-Krzywicki show that lamellar phospholipid (lecithin) phases have a fractional change in thickness of $\simeq 2 - 2.7 \times 10^{-3}$ per degree centigrade. Inasmuch as the volumetric thermal expansivity is probably much smaller, the thermal area expansivity for a phospholipid bilayer is at least this large but of opposite sign (Evans and Waugh, 1977*a*). This value is twice the measured thermal area expansivity of red cell membrane. Unfortunately, the elastic area compressibility modulus has not been measured for lipid bilayer membranes, but it is possible to estimate the area compressibility by idealizing the bilayer as two lipid monolayers above the phase transition for ordered acyl chains. If data is chosen from measurements for lecithin monolayers (Yue et al., 1975), the elastic area compressibility for a lecithin bilayer is estimated to range from 220 dyne/cm at 0°C to 150 dyne/cm at 50°C (Evans and Skalak, 1979). These values are approximately

one-half the size of the elastic area compressibility modulus that has been measured for red cell membrane. The heat of expansion for the lecithin bilayer is calculated to be 125 ergs/cm^2. Above 45–50°C, the red cell membrane loses its structural rigidity and can be considered as a two dimensional liquid structure. It appears to have a heat of expansion that is expected for a lipid bilayer. Investigations are underway to obtain direct experimental evidence on the state of pure lipid vesicle systems and lipid mixtures.

Unlike the phospholipid component of a biological membrane, the composite red cell membrane exhibits solid elastic properties, i.e., the ability to support membrane shear force resultants in proportion to static deformation or uniaxial extension in the membrane plane. Because of the red cell membrane exhibits first order hyperelastic behavior in shear, it was anticipated that the hyperelasticity was due to the negative configurational entropy changes when the material was deformed, i.e., ordering of the structural component. However, the thermoelastic experiments show that the red cell membrane is not this type of simple entropic elastomer. The membrane elastic shear modulus decreases with temperature. In other words, the membrane configurational entropy is lowest in the undeformed state and increases with extension. Thus, the dominant change in extension of the membrane is energetic.

Biochemical and ultrastructural evidence strongly support the thesis that the spectrin protein material (adsorbed on or bound to the cytoplasmic face of the red cell membrane) is responsible for the solid elastic behavior of the red cell membrane (Marchesi et al., 1969; Steck, 1974; Bennett and Branton, 1977). The likelihood of spectrin as the primary structural element for surface shear rigidity indicates that the heat of extension and internal energy density change should be considered on a per-mole basis of spectrin material. The surface density of spectrin for the red cell membrane is on the order of 10^{-7} g/cm^2, and its molecular weight is on the order of 10^5 daltons; therefore, the molar density is calculated to be 10^{-12} mol/cm^2. With this value and the measurements for the heat of extension of the red cell membrane and internal energy density change produced by extension, the values distributed per mole of spectrin are obtained:

$$\left(T\frac{\partial \tilde{S}}{\partial \lambda}\right)_T \sim 2 \times 10^{-10} \cdot (\lambda - \lambda^{-3}) \text{ ergs/mol},$$

$$\left(\frac{\partial \tilde{E}}{\partial \lambda}\right)_T \sim 2.5 - 2.8 \times 10^{-10} \cdot (\lambda - \lambda^{-3}) \text{ ergs/mol},$$

or in terms of calories per mole,

$$\left(T\frac{\partial \tilde{S}}{\partial \lambda}\right)_T \sim 500 \cdot (\lambda - \lambda^{-3}) \text{ cal/mol},$$

$$\left(\frac{\partial \tilde{E}}{\partial \lambda}\right)_T \sim 600 - 700 \cdot (\lambda - \lambda^{-3}) \text{ cal/mol}.$$

These values are only on the order of thermal energies, i.e., the gas constant times temperature. As such, the values are much lower then energies associated with hydrophobic, electrostatic, and metabolic effects. This illustrates that the elastic extension of red cell membrane involves small thermodynamic changes in chemical equilibrium.

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