EFFECT OF HEAT TREATMENT ON THE ELASTICITY OF HUMAN ERYTHROCYTE MEMBRANE

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ABSTRACT A parallel plate flow channel is employed to study the effect of heat treatment on the elasticity of human red cell membrane. An irreversible transition between 46°C and 50°C results in an approximately 200% increase in an elastic constant measured at 25°C. This transition is attributable to irreversible protein denaturation which has been shown by others to occur at similar temperatures in calorimetric studies of red cell ghosts.

INTRODUCTION

In a previous study (1) it was determined that whole cells heated to 48.8°C for 2 min and then cooled to 25°C were less elastic than control cells maintained at 25°C. Cells heated to 47°C for the same time period and then cooled to 25°C had the same membrane elasticity as the control. The ATP levels of both the heated and control cells were identical and the rate of cooling had no effect on elasticity. This effect was attributed to irreversible protein denaturation which has been shown to occur at similar temperatures in calorimetric studies of red cell ghosts (2).

The present investigation was undertaken to study the kinetics of this irreversible transition in red cell membrane elasticity.

METHODS

Blood Preparation

Blood was collected from healthy adults in heparinized Vacutainers and centrifuged at 1,750 *g* for 15 min at 4°C. The plasma and buffy coat were then removed by aspiration. The red cells were washed twice in phosphate-buffered saline (PBS: 0.109 M NaCl, 0.022 M Na_2HPO_4, 0.005 M KH_2PO_4) and resuspended in PBS at an approximate hematocrit of 0.5%. Bovine serum albumin (BSA) and dextrose both at 0.1% concentration by weight were added to the suspension which had a pH 7.4 and an osmolarity 260 (mosmol/liter).

Heat Treatment

Glass tubes containing the cell suspension at room temperature were heated in a Forma constant temperature bath (±0.02°C) (Forma Scientific, Inc., Marietta, Ohio) which was preset at

a given temperature and the tubes were maintained at this temperature for different time periods. By using a precision thermometer immersed in the tube, the time necessary to reach equilibrium was determined. For example, it took 2 min to reach 48.8°C. In addition, measurements were made with a copper constantan thermocouple located at different positions in the tube. The thermocouple was connected to a Honeywell Elektronik 19 lab recorder (Honeywell, Inc., Denver, Colo.) through an Omega-C5 cold junction compensator (Omega Engineering, Inc., Stamford, Conn.) At most locations in the tube equilibrium was reached after 2 min, however, along the sides of the tube, in a thin layer, equilibrium was reached after 80 s. Agitation of the tube was necessary to insure a uniform temperature at a specific time.

After different time periods of equilibrium samples were removed from the bath and cooled to 25°C by swirling the tubes in a beaker of water. Here, equilibrium was reached after 1 min. Control samples were maintained at 25°C throughout the heat treatment experiments.

Flow Channel System

The method (3) used to study the mechanical properties of the membrane consists of allowing red cells to adhere to a glass coverslip, which is coated with BSA by adsorption, and observing shape changes as a function of the force applied to the attached cells. The force is created by a fluid (isotonic PBS at 260 mosmol/liter and 0.1% BSA) which flows over the cells.

This technique is carried out in a parallel plate flow channel which consists of a glass coverslip-Parafilm gasket-glass slide "sandwich" held between a base plate and a clamping plate. Fluid flows into and out of the channel through two holes drilled through the glass slide. The gasket is approximately 120 μm thick (micrometer measurement) and is cut to form a channel width of 0.95 cm and channel length of 3.5 cm. Flow is controlled by a Harvard variable-speed DC infusion pump (Harvard Apparatus Co., Inc., Millis, Mass.) which drives a 50 cm^3 glass syringe with Luer fitting. Since the flow is laminar, the shear stress τ_s at the coverslip surface is given by $\tau_s = 6\eta Q/Wh^2$ where Q is the volumetric flow rate, η is the fluid viscosity, W is the width of the flow channel, and h is the channel gap height (thickness of the Parafilm gasket). An estimate of the force exerted on a cell is obtained by multiplying the fluid shear stress at the surface (τ_s) by the area of the cell as projected onto the coverslip. This force is varied by changing the rate of flow.

The experimental procedure consists of flushing the channel with PBS, containing 0.5% BSA, and then stopping the flow for 15 min to allow the BSA to coat the interior surfaces of the channel. This is followed by flushing with the cell suspension and inverting the channel for 10 min to obtain cell attachment. The channel is then placed on the working stage of a Zeiss Universal microscope (Carl Zeiss, Inc., New York) and the cells are viewed through a 5 × eyepiece with a dry phase-contrast objective. Deformation is recorded on 35 mm film. Experiments are performed at room temperature (25°C).

For each data point a sequence of three photographs is obtained: one before the initiation of flow when the cells are undeformed, one during steady flow, and finally one after flow is stopped. The "after flow" picture is necessary for following the recovery process, i.e. under certain conditions permanent deformation can occur and cells do not return to their normal biconcave-disc shape.

Data Analysis

Photographs showing the deformation of attached cells under steady flow conditions in the flow channel provide data on the elastic properties of the membrane (3, 4). Cell length before (l_o) and under flow (l) is measured with a computerized planimeter (9107 A Hewlett-Packard Digitizer, Hewlett-Packard Co., Palo Alto, Calif.) and the data statistically analyzed by a 9100 B Hewlett-Packard Calculator. Only point-attached cells (teardrop shape) are chosen in

this study (5). Each data point represents the average of a minimum of 20 individual cells and the extension ratio (l/l_o) is determined for *each individual cell*. For 20 cells the 95% confidence limits on the mean value of (l/l_o) are 0.46 $\bar{\sigma}$, where $\bar{\sigma}$ is the standard deviation.

The two-dimensional, incompressible material model of Evans (6), applied to fluid shear deformed cells, enables the calculation of an elastic constant from Evans's theoretically derived graph (4) of $\tau_s R_o/\mu$ vs. overall extension ratio l/l_o, where R_o is the radius of the undeformed disc and μ is the elastic constant. Evans's analytical results are linear in the region where the flow channel experiments were performed ($l/l_o \geq 1.2$) and, therefore, yield the following expression:

$$\tau_s R_o/\mu = m(l/l_o - 1.05), \qquad l/l_o \geq 1.2, \tag{1}$$

where m is the slope of the curve when l/l_o is greater than 1.2 and 1.05 is the value of l/l_o when the curve is extrapolated to $\tau_s R_o/\mu = 0$. Substitution of $R_o = l_o/2$ and rearrangement yields:

$$\mu = [\tau_s/2(l_o^2/l)1/m]/[1 - 1.05(l_o/l)], \qquad l/l_o \geq 1.2. \tag{2}$$

If measurements of l/l_o of heat-treated and control cells are made at the same shear stress, then the elastic constant in Eq. 2 can be normalized, i.e.,

$$\frac{\mu}{\mu_c} = \frac{(l_o^2/l)[1 - 1.05(l_o/l)_c]}{[(l_o^2/l)_c(1 - 1.05(l_o/l)]}, \quad l/l_o \text{ and } (l/l_o)_c \geq 1.2 \tag{3}$$

where the subscript c refers to the control. Thus, if heat-treated cells become stiffer relative to the control, the relative elastic constant μ/μ_c will be greater than 1.0. The relative elastic constant is a function of the ratio of cell diameters (l_o/l_{o_c}), but in these experiments there was never more than a 5% difference between l_o and l_{o_c}. Therefore, the expression for μ/μ_c in Eq. 3 can be further simplified:

$$\frac{\mu}{\mu_c} = \frac{(l/l_o)_c - 1.05}{(l/l_o) - 1.05}, \quad l/l_o \text{ and } (l/l_o)_c \geq 1.2. \tag{4}$$

RESULTS

Cells were heated to 47°C and 48.8°C and maintained at these temperatures for different periods of time. Upon cooling the overall extension ratios (l/l_o) were measured at a constant fluid shear stress. The results are shown in Figs. 1 and 2. The 0 min point represents cells that were heated to 47°C and 48.8°C and immediately cooled to 25°C after reaching equilibrium. At 47°C, a heat-treatment period of about 6 min is necessary before a constant l/l_o is achieved, and at 48.8°C about 3 min is required.

It was also found that cells heated to 45°C for 14 min and 46°C for 6 min did not differ from the control in extension ratio (l/l_o) at the same stress level (7.4 dyn/cm^2).

Table I shows the relative elastic constant, μ/μ_c measured at room temperature (25°C), as a function of temperature and time of heat treatment. A transition occurs over a narrow range of temperature since μ/μ_c is 1.0 for cells heated to 45°C for

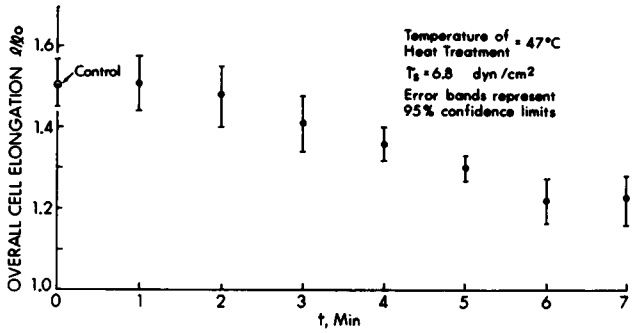

FIGURE 1 Overall cell elongation vs. time of heat treatment at 47°C.

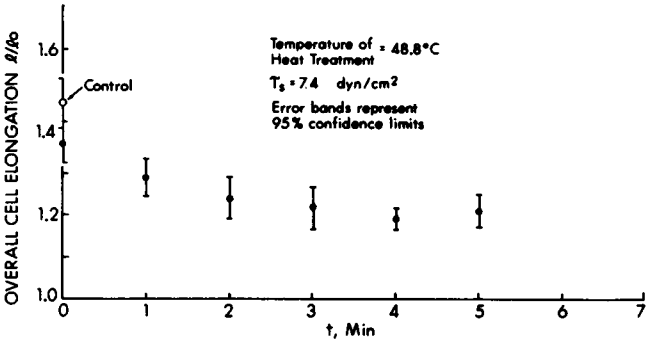

FIGURE 2 Overall cell elongation vs. time of heat treatment at 48.8°C.

TABLE I

RELATIVE ELASTIC CONSTANT, μ/μ_c, FOR INCUBATIONS OF GIVEN LENGTHS AND TEMPERATURES

Time	T(°C):	45	46	47	48.8
min					
0		-	-	1.0	1.4
1		-	-	1.0	1.8
2		-	-	1.0	2.4
3		-	-	1.3	2.7
4		-	-	1.5	3.1
5		-	-	1.8	2.9
6		-	1.0	2.8	-
7		-	-	2.8	-
14		1.0	-	-	-

14 min and the values for μ/μ_c after 6 min of heat treatment at 47°C and 48.8°C are not significantly different.

It is impossible to determine the nature of the transition for higher temperatures since dramatic morphological changes that occur almost instantly at 50°C (7) obviate flow channel experiments which are limited to normal biconcave disc shapes.

DISCUSSION

The human red cell membrane consists of approximately 50% protein and 40% lipid, with the lipid forming a fluid-like bilayer. Because the lipid is in a fluid rather than a crystalline state, it is likely that the membrane obtains its unique elastic behavior from a structural membrane protein or proteins which form a kind of "meshwork."

In the present study a dramatic alteration in cell membrane elasticity has been shown to occur over a narrow temperature range. Williamson et al. (8) have also observed a change in elasticity of heat-treated cells using a rotating disc and glutaraldehyde fixation technique. It is felt that this irreversible change in membrane elasticity reflects an actual denaturation of the structural membrane protein. In differential scanning calorimetry studies of ghost membranes, Jackson et al. (2) have shown that a portion of the membrane protein will begin irreversible denaturation at approximately 45°C, with a maximum rate of change in heat capacity at 50°C. Because of the similarity between the temperatures and times in our experiments and those of Jackson, we feel that the same protein is involved in both cases. However, much work remains to be done before this membrane structural protein(s) is identified.

It is likely that spectrin is "an important structural component, since the intact ghosts become fragmented during the removal of spectrin, and lose a coating of filamentous material normally present on the inner surface" (9). If spectrin is the protein responsible for the elastic behavior of the membrane and comprises a weak elastomer network at the inner surface it is possible that heat treatment results in a greater number of network cross-links.

According to the theory for the elasticity of a molecular network, the elastic constant for this network is inversely proportional to the first power of the number average molecular weight of a subchain (10). Therefore, an increase by a factor of 3 in the elastic constant after heat treatment (Table I) might correspond to a 1/3 reduction in subchain molecular weight resulting from the formation of cross-links.

Protein conformational change could also explain the increased membrane stiffness. An irreversible change from a predominantly α-helical spectrin chain to an extended conformation would alter the elastic properties of the network regardless of the occurrence of cross-linking.

Another possible explanation for increased stiffness resulting from heat treatment is irreversible aggregation or precipitation of the spectrin meshwork with or without accompanying secondary conformational changes. Preliminary experiments indicate that this explanation is the most likely (11).

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