Shape Memory of Human Red Blood Cells

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ABSTRACT The human red cell can be deformed by external forces but returns to the biconcave resting shape after removal of the forces. If after such shape excursions the rim is always formed by the same part of the membrane, the cell is said to have a memory of its biconcave shape. If the rim can form anywhere on the membrane, the cell would have no shape memory. The shape memory was probed by an experiment called go-and-stop. Locations on the membrane were marked by spontaneously adhering latex spheres. Shape excursions were induced by shear flow. In virtually all red cells, a shape memory was found. After stop of flow and during the return of the latex spheres to the original location, the red cell shape was biconcave. The return occurred by a tank-tread motion of the membrane. The memory could not be eliminated by deforming the red cells in shear flow up to 4 h at room temperature as well as at 37°C. It is suggested that 1), the characteristic time of stress relaxation is >80 min and 2), red cells in vivo also have a shape memory.

INTRODUCTION

From a mechanical perspective, the human red blood cell can be considered as an incompletely inflated bag, the plasma membrane, filled with a viscous fluid, the cytoplasm (Waugh and Hochmuth, 1995). Because of these properties, the resting shape of the red cell is determined by the elastic properties of the membrane, its surface area, and the enclosed volume. At rest the human red cell has the shape of a biconcave disk. Upon the action of external forces, the red cell deforms but returns to its resting shape when the forces are removed. This article deals with the question of whether after removal of the forces the dimples and the rim of the biconcave shape are always formed by the same parts of the membrane as before the deformation. In this case, the membrane is said to possess a memory of its biconcave shape, for shortness called shape memory in the following. In a membrane with no shape memory, one of the two dimples can form at any location on the membrane. Of course such a choice would determine the location of the other dimple and the rim.

For mechanical purposes, the red cell membrane can be considered as being laminated by two layers: 1), the phospholipid bilayer plus the attached glycocalyx at the plasmatic face of the bilayer and 2), the loose network of proteins fastened to the bilayer at its cytoplasmic face. The second is usually called membrane skeleton. For shortness we refer to it by skeleton. There is general agreement (Waugh and Hochmuth, 1995) that the bilayer provides bending elasticity and the skeleton shear elasticity to the membrane. Both layers have in addition an isotropic elasticity, i.e., a resistance against a change in surface area. The isotropic elasticity of the bilayer is larger by five orders of magnitude than that of the skeleton (Discher et al., 1994). During the deformations considered in this work, the isotropic elasticity of the bilayer is not challenged but serves to keep the surface area of the membrane constant. The isotropic elasticity of the skeleton is considered below.

Due to the lateral mobility of most of the constituents of the bilayer, they are in general well mixed. Therefore, on a continuum mechanical scale that averages over many molecules, the bilayer can be considered as being uniform in the plane of the membrane. As a consequence, its mechanical properties are uniform as well. (An exemption to this rule appears to occur after raising the cytoplasmic concentration of calcium; Fischer, 2003.) These properties are 1), the bending stiffness and 2), the reference curvature, also called spontaneous curvature. The uniformity in these two properties excludes a causal role of bending elasticity for a shape memory.

As to the skeleton, there is experimental evidence that, as long as the deformation of the red cell is small, the density of the skeleton is uniform in the plane of the membrane (Discher et al., 1994). As a consequence, it is likely that the shear stiffness is uniform as well. The analog to the reference curvature is the reference shear deformation. For shortness, we refer to it by reference deformation in the following. Since the skeleton does not behave like a two-dimensional fluid, as does the bilayer, the reference deformation has to be given at each location on the membrane. For a definition, we imagine increasing the cytoplasmic volume of the red cell until its shape has become spherical and define the local shear deformation of the skeleton as the reference deformation. In the following, the distribution of this reference deformation on the membrane is called the deformation state. On a molecular level, the deformation state depends on the manner the peripheral proteins constituting the skeleton are linked topologically.

If the reference deformation as defined above is zero everywhere on the membrane, the deformation state is called uniform. If the reference deformation is nonzero in some

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locations, the deformation state is called nonuniform. On a membrane with a uniform deformation state, all locations are indistinguishable from a continuum mechanical point of view. Such a membrane would have no shape memory. Vice versa, preferred locations of the dimples and the rim on the membrane, i.e., a shape memory, require a nonuniform deformation state.

The skeleton is anchored to the bilayer via binding to integral proteins. Since these proteins are laterally mobile, the distribution of the isotropic deformation of the skeleton is free to adjust to the prevailing shear deformation (Stokke et al., 1986; Fischer, 1992; Discher et al., 1994). Since this adjustment is passive, a causal role of the isotropic elasticity of the skeleton for the shape memory can be excluded. This means it is exclusively the deformation state that defines whether a shape memory exists or not.

Experimentally, the existence of preferred locations can be probed by attaching a marker to the skeleton and observing whether this marker returns to the same location on the biconcave shape after a deformation of the cell. Early experiments were performed by Bull (1972) on red cells bound to the bottom of a parallel-plate flow chamber. As markers of the membrane, this author used the spicules of a crenated red cell, a spot created by illumination of the membrane with a laser, or Heinz bodies. He found that by gentle flow a marker could be moved from the rim to the dimple with the overall shape of the cell remaining biconcave. This result was obtained when the red cell adhered to the flow chamber at several points that enclosed ~10% of the surface area of the membrane. Upon adhesion at a single point, the biconcave shape was moved by the flow, but a membrane marker at the rim remained at the rim. The last finding was interpreted as an indication of a shape memory by arguing that moving the attachment point laterally on the membrane required a smaller force than displacing the location of the dimple on the membrane.

The study of Bull (1972) suffered from the drawback that both the adhesion to the flow chamber as well as the membrane modification caused by marking the membrane may have introduced artifacts. In the work here, the red cells are freely suspended and membrane spots were marked by spontaneously adhering latex spheres. Furthermore, the number of cells observed was not reported by Bull (1972). In this work a large number of cells was studied to allow a statement on the whole population.

A relaxation of shear stresses has been reported to occur on the timescale of minutes to hours in micropipette experiments (Markle et al., 1983). On a molecular level, stress relaxation occurs by a change in the topology of the connections within the skeletal network. Such a change requires a spontaneous dissociation and reassociation of links within the network. A spontaneous dissociation of links has been probed by biochemical methods on red cell ghosts (An et al., 2002). An appreciable percentage of dissociation was found when the ghosts were either incubated at 37°C or were subjected to shear flow at room temperature. To test whether a shape memory can be made to fade by stress relaxation, the cells were deformed by shear flow at room temperature as well as at 37°C before probing the memory.

The instrument used in this work is the rheoscope (Schmid-Schönbein and Wells, 1969; Fischer et al., 1978). Red cells are suspended in media more viscous than blood plasma. The suspension is subjected to simple shear flow and the red cells are observed through a microscope. Above a threshold in shear rate, the red cells assume a steady shape and orientation in the shear field. In such cells, the membrane moves around the cell body and the cytoplasm is driven into an eddy-like flow (Fischer et al., 1978). This particular kind of membrane motion has been termed tank-tread motion (Schmid-Schönbein and Wells, 1969). The shape of the red cell during the tank-tread motion resembles a triaxial ellipsoid with the longest axis almost parallel to the direction of the undisturbed flow. The intermediate axis is parallel to the vorticity of the undisturbed flow. Upon stop of flow, the red cell regains its biconcave resting shape.

In an experiment called go-and-stop, the cells were first sheared to induce a tank-tread motion. Then the flow was stopped, and a subsequent motion of the red cell was taken as an evidence of a shape memory. It was found that virtually all cells tested showed a shape memory and that this memory was not eliminated even by continuous shearing of the red cells for 4 h. Preliminary results of the go-and-stop experiment were presented as an abstract (Fischer et al., 1981).

MATERIALS AND METHODS

Preparation of red cells

Blood was obtained on a voluntary basis from routinely appearing donors in the local blood bank. A total of 1–5 ml blood was aspirated in vacutainers containing 2 units heparin per milliliter of blood. After withdrawal, the blood was washed two times with HEPES-buffered salt solution (KNH). KNH contained (mM) 93 NaCl, 47 KCl, and 10 HEPES. The osmolality was measured by freezing point depression and adjusted to 290–300 mOsm. The pH was adjusted to 7.4. After the last centrifugation, the packed red cells had a hematocrit (hct) of ~85%. Packed cells that were not used immediately were stored in the refrigerator.

Preparation of dextran solutions

HEPES stock solution (Hstock) is a solution of HEPES in water. The concentration of HEPES and the pH of Hstock were adjusted in such a way that 2.5 g water plus 0.6722 g Hstock had an osmolality of 300 ± 3 mOsm and pH 7.3. HEPES solution (H) was prepared by adding 0.1 g Hstock to 10 g water. If Hstock is prepared correctly, the concentration of HEPES in H is 10 mM. Potassium-sodium stock-solution (KNstock) is a solution of KCl and NaCl (molar ratio, 2:1) in water. The concentrations were adjusted in such a way that 10 g H plus 0.9 g KNstock had an osmolality of 300 ± 3 mOsm. Dextran solution was prepared by layering dextran (molecular weight (MW) of 60,000 from Knoll, Ludwigshafen, Germany, or Serva, Heidelberg, Germany, or MW 2,000,000 from Pharmacia, Uppsala, Sweden) powder on water. When solvation was complete, water was added until the solution had the desired refractive index as measured with an Abbe

refractometer (Zeiss, Oberkochen, Germany). The refractive index was chosen to reproduce dextran concentrations because dextran is hygroscopic. This indirect method was calibrated with dextran from a freshly opened can (MW 60000 from Serva). A concentration of 15 g % (w/w) corresponded to a refractive index of 1.355 and 7.5 g % to 1.343. HEPES-buffered dextran solution was prepared by adding 0.1 g Hstock to 10 g D. HEPES-buffered dextran-salt solution (KNHD) was prepared by adding 0.673 g or 0.794 g KNstock to 10 g HEPES-buffered dextran solution when the refractive index was 1.355 or 1.343, respectively. The reduction in salt concentration compensated for the osmotic effect of dextran. The reduction was found to be independent of the MW of dextran (Fischer, 1999). KNHD was then titrated to a pH in which suspended red cells would have the same cytoplasmic pH as red cells suspended in KNH as calculated theoretically (Lew and Bookchin, 1986).

Rheoscopy

The rheoscope consists of a transparent cone-plate chamber adapted to a microscope equipped with interference contrast optics (Leitz 40 × /0.65, Wetzlar, Germany). Approximately 30 μl of a cell suspension is pipetted on the cone that is then put in place to form the final cone-plate geometry. Since cone and plate are counterrotating, a fluid layer exists within the chamber that does not rotate even during flow. Cells suspended in this layer are observed along the gradient of the undisturbed shear flow and videotaped. Cone and plate are either rotated by motor drive or after uncoupling the chamber from the motor by turning the drive shaft between motor and chamber by hand. Under microscopic control, the last operation allowed very precise movements of red cells. Rheoscopy was done at room temperature (23°C).

Viscometry

The viscometer consists of a custom made chamber with Mooney configuration adapted to a commercial measuring unit (Rheomat 15, Contraves, Zürich, Switzerland). The outer surface of the chamber is stationary; the inner one rotates at preselected rates. The chamber is waterjacketed for temperature control. Viscosity measurements were done at 23°C. KNHD prepared with 7.5 or 15 g % dextran (MW 60,000) had a viscosity of 6.4 or 12.9 mNs/m^2, respectively. KNHD from 15 g % dextran (MW 2,000,000) gave 110 mNs/m^2.

Go-and-stop

Latex spheres (diameter 0.7 μm) were suspended in KNH at a volume concentration of ~1%. Packed cells (5μl) were added to 100 μl latex suspension. After thorough mixing, 7 μl of this mixture were added to 200 μl KNHD (12.9 mNs/m^2) to which 4 μl 4 g % solution of bovine albumin (fraction V, Sigma, Deisenhofen, Germany) had been added. The resulting cell suspension was put into the cone-plate chamber of the rheoscope.

Red cells were tested in turn. After each test the field of microscopic observation was shifted to avoid evaluating a cell twice. All biconcave red cells having between one and three latex spheres attached to the membrane were tested. By turning the cone-plate chamber slowly by hand, the cell in question was rotated to recognize the location where the latex adhered on the biconcave shape. Then the cone-plate chamber was rotated (again by hand operation) fast enough as to induce a tank-tread motion (go). The rotation was stopped (stop) when the position of the latex indicated that the membrane parts forming the dimples at rest were at the rim of the tank-treading cell.

After stop (labeled *0 s* in Figs. 1 and 2), the red cell relaxes to a biconcave shape that is oriented approximately face on. In the first two rows of Figs. 1 and 2, respectively, the rim of the biconcave shape at 0 s is formed by those parts of the membrane that formed the rim of the tank-treading cell in the moment of flow stop but not the rim of the biconcave shape before go. If a shape memory exists, the membrane returns to its original position in a transient lasting 5–20 s. The return can be recognized by two observations:

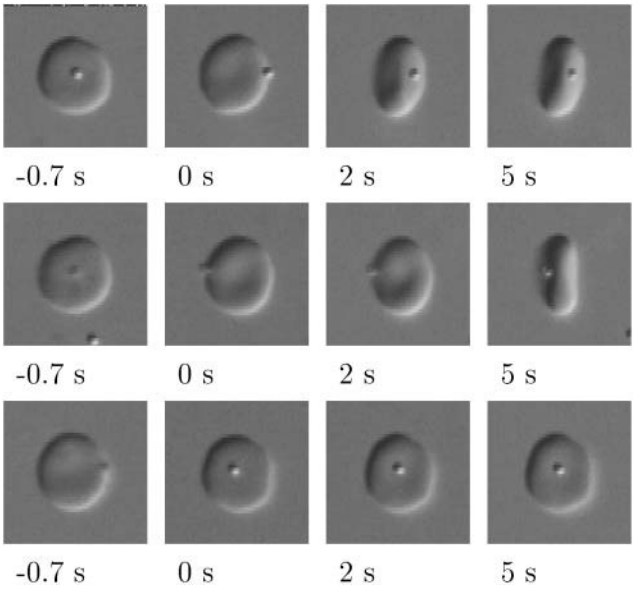

FIGURE 1 Three (*top*, *middle*, and *bottom rows*) go-and-stop experiments performed on the same red cell. Shown are selected frames (each 16.6 μm wide) from a video recording through the rheoscope. The numbers indicate relative times. In the first column, the red cell is shown tank treading in shear flow (flow direction horizontal, shear rate = 10/s). Shearing was done by motor drive of the cone-plate chamber. The second column shows the red cell after the shear flow had stopped. In the last two columns the subsequent transient is shown. The difference between the three experiments, rows respectively, is the location of the latex sphere after stop of flow (*top row*, right edge; *middle row*, left edge; *bottom row*, middle).

1. A slow motion of the latex relative to the outline of the projected silhouette of the cell. This motion is most prominent in the first part of the transient (compare the frames labeled *0 s* with the following ones in the *first two rows* of Fig. 2).
2. A shape change of the projected silhouette (compare the frames labeled *0 s* with the following ones in the *first two rows* in Figs. 1 and 2). This shape change becomes most obvious in the late stages of the transient.

If the first observation was clearly made, the go-and-stop experiment was classified as successful and another cell was selected. If the first observation was not clearly made, the second observation was checked. If the second observation was made, the go-and-stop experiment was also classified as successful. If neither of the two observations could be made or when the outcome was doubtful, the experiment was classified as unsuccessful. In the absence of a shape memory, an unsuccessful outcome is always expected. But even in the presence of a shape memory an unsuccessful outcome is expected if tank treading is stopped when those membrane parts forming the dimples at rest are at the flat sides of the tank-treading cell, e.g., Figs. 1 and 2, *bottom rows*. To avoid false-negative results, an unsuccessful experiment was repeated up to three times. This number was chosen pragmatically. Testing a cell more often would have wasted the limited time.

The viscosity of the dextran solution chosen for the go-and-stop experiment (12.9 mNs/m^2) allowed testing most red cells of the population. In an estimated few percent, the maximal shear rate achievable by hand operation was not sufficient to induce tank treading. This by itself indicates the presence of a strong shape memory. Nevertheless such cells were excluded from the study. A much smaller portion of cells on the other end of the scale could hardly be turned into an edge-on orientation, because they

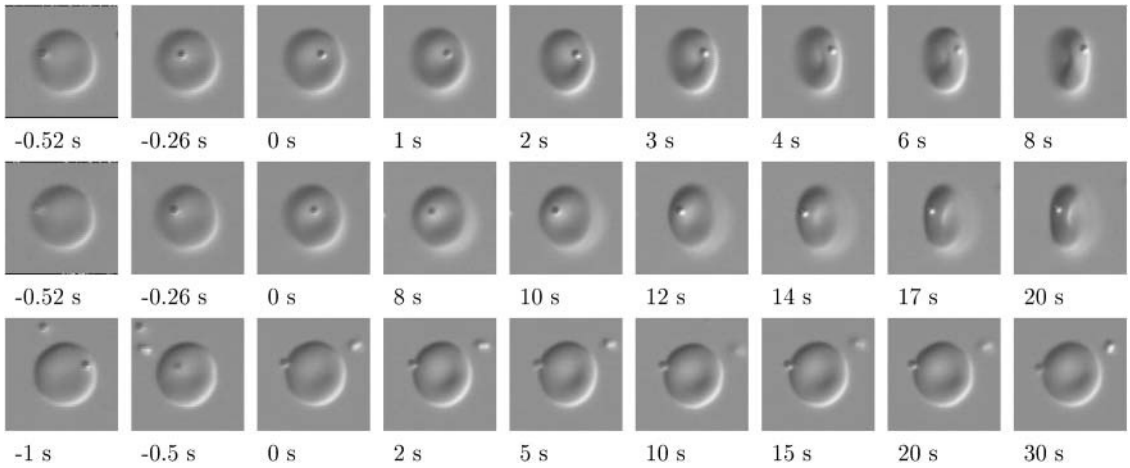

FIGURE 2 Three (*top*, *middle*, and *bottom rows*) go-and-stop experiments performed on the same red cell. Shown are selected frames (each 16.6 μm wide) from a video recording through the rheoscope. The numbers indicate relative times. In the first two columns, the red cell is shown tank treading in shear flow (flow direction horizontal, shear rate = 10/s). Shearing was done by motor drive of the cone-plate chamber. The third column shows the red cell after flow stop. In the following columns, the subsequent transient is shown. The difference between the three experiments, rows respectively, is the direction of motion of the latex sphere during the transient. In the top row, the latex moves to the right. In the middle row, it moves to the left. In the bottom row, it hardly moves at all.

reacted by tank treading to the smallest shear rate that could be imposed by hand operation. In such cells, it occurred that the go-and-stop test was unsuccessful four times. This, however, does not mean that they did not have a shape memory. It does indicate that the memory was comparatively weak, i.e., the difference in elastic energy driving the transient was comparatively low.

RESULTS

Absence of a rigid body rotation

Fig. 1 shows selected frames from three go-and-stop experiments performed with the same red cell. The indicated times are relative to the frame when the shear flow had come to a complete stop. In the first column, the red cell is tank treading. The second column shows the cell right after stop of flow. In the third and fourth columns, the cell is shown 2 s and 5 s after stop of flow.

The bottom row shows an unsuccessful go-and-stop experiment. There is practically no motion of the latex sphere and practically no change in aspect ratio in the frames labeled 2 s and 5 s compared to the frame labeled 0 s. In the top and middle rows, on the other hand, the cell starts in a face-on orientation at 0 s and ends in an almost edge-on orientation at 5 s. The interpretation of the projected outline in the frames labeled 2 s is postponed to the next subsection. In the top and middle rows of Fig. 1, the existence of a shape memory is indicated by the shape change of the projected silhouette of the red cell. A motion of the latex sphere relative to the projected cell outline is less noticeable for reasons detailed in the next subsection.

In the top and middle rows of Fig. 1, it takes ~5 s to reach static equilibrium after stop of flow. It can be appreciated that the latex is then right in the middle of the dimple. In the frame labeled 0 s, the latex is left of the cell center in the top row and right of it in the middle row. In the frames labeled 5 s, the orientation of the latex sphere relative to the cell center is the same as in the frames labeled 0 s. This demonstrates an absence of rigid body rotation that was to be expected since no turning moments act on the red cell after stop of shear flow.

Decision between two modes

Two possible modes are envisaged for the return of the membrane toward static equilibrium that are compatible with the absence of a rigid body rotation. These are a folding mode and a tank-treading mode. They are sketched schematically in a side view in Fig. 3. Fig. 3 *A* depicts a tank-treading red cell with a latex sphere bound to the membrane and superimposed the profile of the undisturbed shear flow. Fig. 3 *B* shows the cell right after stop of flow and relaxation to a biconcave shape. The two modes are indicated by the split arrow. In the folding mode (*bottom row*), the rim of the red cell begins to form at its final position. During the transient, the shape is not biconcave. In the tank-treading mode (*top row*), the cell preserves its biconcave shape and the membrane returns toward static equilibrium by tank treading as indicated by the arrows. The tank-treading membrane exerts a torque (Keller and Skalak, 1982) on the cell that causes an apparent rotation of the biconcave shape. (The same torque is responsible for the angle of inclination during steady-state tank treading; see Fig. 3 *A*. The value of this angle depends on the balance with the torque caused by the vorticity of the shear flow; see Keller and Skalak, 1982.)

In the rheoscope, the observation is along the gradient of the undisturbed flow (vertical in Fig. 3). In this view, the motion of the latex spheres during the transient is only suggestive of the tank-treading mode. A safe distinction

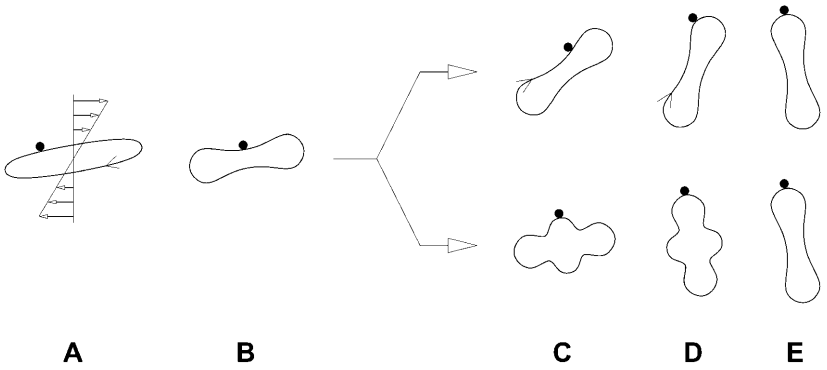

FIGURE 3 Schematic of a go-and-stop experiment. The view is along the vorticity of the undisturbed shear flow. (*A*) Profile of the undisturbed flow velocity and an elongated and tank-treading (*arrow*) red cell. (*B*) Shape after stop with a phase difference of $\pi/2$ relative to the static equilibrium. (*C* and *D*) Intermediate stages of the transient in a tank-treading mode (*top row*) and in a folding mode (*bottom row*). (*E*) Cell in static equilibrium.

between both modes is possible by the following arguments. If after stop the phase difference in tank-tread motion from the equilibrium position is exactly $\pi/2$, the membrane motion may start in one direction or the other. This bifurcating behavior is shown schematically in Fig. 4. Fig. 4, *A* and *B*, depicts again a red cell during tank treading and after stop of shear flow, respectively. The split arrow indicates the bifurcation. In the top row, tank treading is clockwise and the apparent rotation of the cell is anticlockwise. In the bottom row, rotations are opposite. In contrast, the folding mode is not expected to show such a bifurcating behavior.

Fig. 2 shows selected frames from three go-and-stop experiments performed with the same red cell. The indicated times are relative to the frame when the shear flow had come to a complete stop. In the first two columns, the red cell is tank treading. The third column shows the cell right after stop of flow. In the following columns, the cell is shown at selected times after stop of flow.

The bottom row shows an unsuccessful go-and-stop experiment that indicates that in static equilibrium the latex sphere is somewhere at the rim. This means that at a phase difference of $\pi/2$ from the equilibrium position, the latex is in the dimple. This is the case in the top and middle rows of Fig. 2 in the frames labeled 0 s. In the top row, the latex moves to the right during return. This corresponds to the top row of Fig. 4. In the middle row of Fig. 2, the latex position at 0 s is slightly more left than in the top row. The following frames show that it moves to the left corresponding to the bottom row in Fig. 4. As in Fig. 4 *A*, the latex moved from left to right just before stop of shear flow (Fig. 2, *top* and *middle rows*, the *first three columns*). The complete correspondence of Figs. 2 and 4 demonstrates experimentally the existence of a bifurcating behavior and therefore of the tank-treading mode.

In Fig. 1, the motion of the latex sphere relative to the projected cell outline during the early phase of the return is less noticeable than in Fig. 2. The reason is the location of the latex in static equilibrium. The early motion is most obvious when in equilibrium the latex sphere is located close to the rim.

Comparison of the frames in the fourth column of Fig. 2 shows that in the top row it takes 1 s and in the middle row 8 s for the latex sphere to move about the same distance since stop of flow. This big difference in velocity is interpreted as follows. The forces driving the motion during the transient are proportional to the derivative of the elastic energy with respect to the phase difference. At a value of $\pi/2$, the elastic energy is expected to have a maximum. The closer the phase difference to $\pi/2$, the smaller is the derivative and therefore the tank-treading speed. In other words, the latex in the middle row appears to reside for a few seconds almost in labile equilibrium. After this period, the further motion proceeds about at the same speed as in the top row.

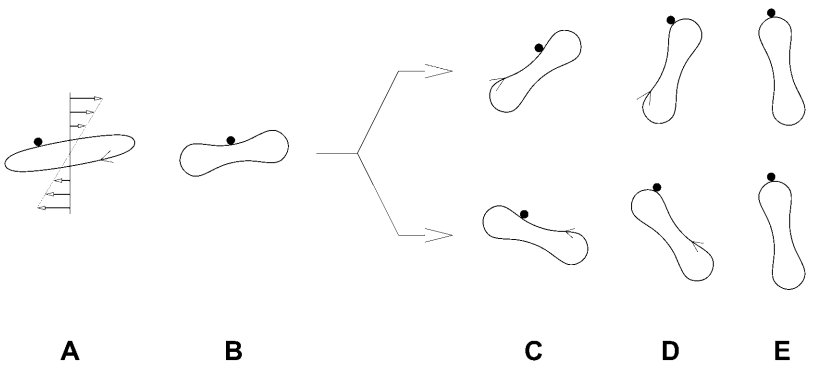

FIGURE 4 Schematic of a go-and-stop experiment. The view is along the vorticity of the undisturbed shear flow. (*A*) Profile of the undisturbed flow velocity and an elongated and tank-treading (*arrow*) red cell. (*B*) Shape after stop with a phase difference of $\pi/2$ relative to the static equilibrium. (*C* and *D*) Intermediate stages of the transient in a tank-treading mode (*top row*, tank-treading clockwise; *bottom row*, tank-treading anticlockwise). (*E*) Cell in static equilibrium.

Tests without preshear

In these tests, the preparation of red cells for the go-and-stop test was performed as quickly as possible. Nevertheless, the procedure lasted 15 min because without washing latex spheres do not adhere spontaneously to the red cell membrane. Go-and-stop tests were performed up to 30 min after blood withdrawal. The results are shown in Table 1. In most cells, a memory could be demonstrated in the first trial. Less than 10% required a second trial. A third, fourth, or an unsuccessful trial happened only rarely. These results appear to indicate that the red cells in the circulation also have a shape memory. However, the characteristic time of stress relaxation (τ) is not known. Therefore the results of Table 1 could be alternatively interpreted as follows. Red cells in the circulation have no memory due to their continuous deformation. But they acquire a memory by stress relaxation during the preparation where they are most of the time biconcave.

Preshear at room temperature

To find out whether a deformation resembling that within the circulation can eliminate the memory, red cells were sheared before performing the go-and-stop test. Since τ was not known, preshear was done in several time regimes in a hierarchical fashion as follows. In the first regime, a red cell was selected (see Go-and-Stop). For preshear, the cell suspension in the rheoscope was sheared at a rate of 100/s for 30 s. During this period, the direction of flow was reversed whenever the selected cell approached the edge of the field that could be observed through the microscope. After 30 s shear, the flow was stopped and the transient was observed. If the test was successful, another cell was selected. If it was not successful, preshear and stop were repeated (at most three times) with the same cell. Table 2 shows that in most cells a memory could be demonstrated in the first trial. In only two out of 88 cells, four successive go-and-stop tests were unsuccessful. This finding indicates that in most cells a stress relaxation was not fast enough to eliminate a shape memory within 30 s.

In the second regime, the cells in the rheoscope were first sheared for 1 min at a rate of 100/s. After this period, the flow was stopped, and a time window of 20 s was allowed to select cells and to perform go-and-stop experiments (see Go-and-Stop) with as many cells as possible. Again a maximum of four trials were allowed on each cell.

TABLE 1 The shape memory of red cells was tested by go-and-stop experiments up to 30 min after blood withdrawal ($n = 6$)

Number of go-and-stop tests				
Successful after number of trials				Not successful
1	2	3	4	
116	8	3	3	2

In each of the following regimes, the preshear time was increased by a factor of two (Table 2). The time window was taken as one-third of the preshear time. For preshear times of 30 min and longer, shearing was not performed in the rheoscope. Rather, the cells were suspended in KNHD (6.4 mNs/m^2) at a hct of 45% and sheared in the viscometer at a rate of 110/s. The red cell elongation, as checked by observing the high hct suspension in the rheoscope, was comparable to that during preshear in the rheoscope (Fig. 5, *B* and *C*). As can be seen in Table 2, the result was about the same in all time regimes. As observed without preshear, essentially all cells tested displayed a shape memory.

Preshear at 37°C

Since it has been shown that at 37°C an appreciable dissociation of links within the skeleton occurred that was not observed at room temperature (An et al., 2002), preshear was also performed at 37°C. Two conditions were employed. In the first, the suspension and the shear rate were the same as in preshear at room temperature. The second condition was close to those of An et al. (2002). The viscosity of the suspending medium at room temperature was 110 mNs/m^2. The shear rate and the hct were 200/s and 25%, respectively. The red cell elongation in the second condition is much higher than in the first one (Fig. 5, *C* and *D*). For comparison with in vivo conditions, Fig. 5 *A* shows red cell elongation as it would occur close to the wall of rapidly perfused arterioles. It is obvious that the first preshear condition is much closer to in vivo situations than the second one.

At the end of the preshear period, part of the red cell suspension was pipetted in a much larger volume of cooled KNH to freeze the topology of the skeleton. The red cells were kept cold until they were used for the go-and-stop experiment. After the second condition, the cells were washed free of the high MW dextran to avoid red cell aggregation. The period allowed for testing was again taken as one-third of the preshear time.

The results of the go-and-stop tests are shown in Table 3. There is no essential difference to the results obtained with preshear at room temperature.

In an exponential process it takes ~3 characteristic times to reach the final state within experimental error. The final state approached during preshear corresponds to a loss of the shape memory. The absence of such a loss after 4 h preshear indicates $\tau > 80$ min. This concerns stress relaxation under dynamic deformations at room temperature or at 37°C.

Nonequilibrium biconcave shape

The existence of a shape memory implies that the elastic energy has a minimum when the membrane is in static equilibrium. Any disturbance from the equilibrium increases the elastic energy and might be expected to lead to a change

TABLE 2 Red cells suspended in dextran solutions were presheared at room temperature under two different conditions (see Preshear at Room Temperature); subsequently the shape memory was tested by go-and-stop experiments for one-third of the preshear period

Period of preshear	Number of blood donors	Number of go-and-stop tests				
		Successful after number of trials				Not successful
		1	2	3	4	
30 s	10	85	1			2
1 min	9	83	7	1		2
2 min	9	84	5	1		
4 min	9	92	8	3	1	1
8 min	9	106	9	2		
15 min	9	128	9	1		
30 min	4	123	13			
1 h	3	105	9	1	1	
2 h	3	125	7		1	
4 h	3	121	16	4		
Altogether	20	1052	84	13	3	5

in cell shape. However, after stop a biconcave shape formed that looked the same, irrespective of whether the cell was close to (Figs. 1 or 2, *last rows*, frames labeled *0 s*) or far from static equilibrium (Figs. 1 or 2, *top* and *middle rows*, frames labeled *0 s*). An explanation for the absence of an observable shape change probably requires accounting for the elastic contributions in shear as well as in bending. Such a consideration is outside the scope of this work.

Echinocytes and stomatocytes

Red cells suspended in buffered salt solutions become echinocytes when they come close to a glass surface (Eriksson, 1990). To counteract this effect, albumin was added to KNHD (see Go-and-Stop). If the albumin concentration was chosen too low, the red cells in the rheoscope were echinocytes. If it was chosen too high, the shape was stomatocytic (Bessis, 1972). Go-and-stop tests were performed also with such nonbiconcave cells, although not systematically. Echinocytes I (biconcave but with irregular contours when viewed face on) behaved as discocytes. Moderate stomatocytes still show rotational symmetry. Such cells did show motion after flow stop indicating a nonuniformity of the deformation state. However, when the invagination was deep enough, a loss of uniqueness of the location of a latex sphere could be observed. Fig. 6 shows the same red cell in static equilibrium after two consecutive go-and-stop experiments (*top* and *bottom rows*). The first and second columns show the cell face on and edge on, respectively. It is obvious that in the top row the latex is at the rim, whereas in the bottom row it is in the middle of the convexity. This finding is not surprising because the arc length from the rim to the inside bottom of the cup is much shorter than to the outside bottom. Therefore there is no way for the rim to form at the same location as in the original biconcave cell.

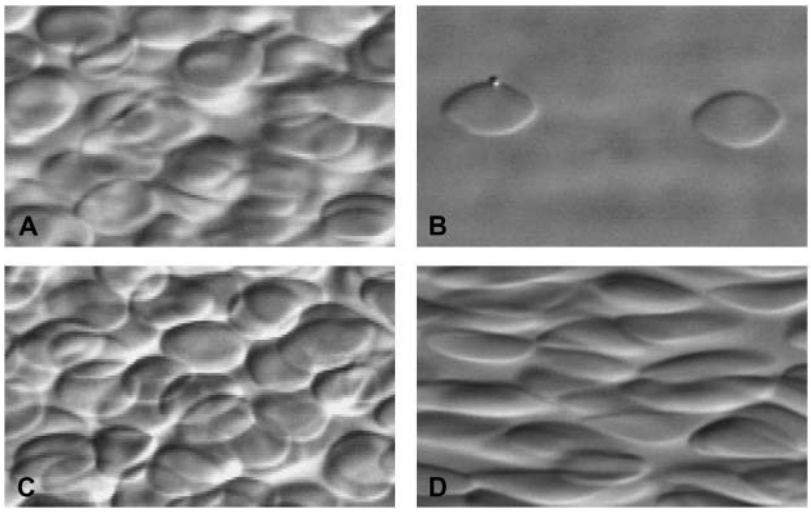

FIGURE 5 Elongation of red cells in the rheoscope at room temperature. Shown are selected frames (each 50.0 μm wide) from a video recording through the rheoscope. (*A*) Red cells in autologous plasma, hct $\approx$ 45%, shear rate 800/s. (*B*) During preshear in the rheoscope, hct $\approx$ 0.1%, medium viscosity 12.9 mNs/m^2, and shear rate 100/s. (*C*) Under the same shear rate as during preshear in the viscometer (first condition), hct $\approx$ 45%, medium viscosity 6.4 mNs/m^2, and shear rate 110/s. (*D*) Under the same shear rate as during preshear in the viscometer (second condition), hct $\approx$ 25%, medium viscosity 110 mNs/m^2, and shear rate 200/s.

TABLE 3 Red cells suspended in dextran solutions were presheared at 37°C under two different conditions (see Preshear at 37°C); subsequently the shape memory was tested by go-and-stop experiments for one-third of the preshear period

			Number of go-and-stop tests				
			Successful after number of trials				
Red cell elongation	Period of preshear	Number of blood donors	1	2	3	4	Not successful
Moderate	2 h	3	63	11	6	2	2
Moderate	4 h	3	241	13	9	3	2
High	4 h	3	241	8	2	4	2
	Altogether	9	545	32	17	9	6

DISCUSSION

Adhesion of latex spheres

In this work, it has been assumed that the latex spheres mark a specific location of the skeleton. Two arguments support this assumption. First, it is plausible considering the mere size (0.7 μm in diameter) of the latex spheres that they adhere to extracellular carbohydrate residues of many transmembrane proteins to which in turn the skeleton is fastened. Even if these bonds were in a state of dynamic dissociation and reformation, it is unlikely that a latex would move to an adjacent location of the skeleton because all bonds would have to dissociate at the same time. Second, it was checked experimentally that no lateral diffusion of latex spheres occurs. Red cells with several latex bound to the membrane were videotaped for 2 h. To avoid a possible artifact by a close foreign surface, these cells were suspended in dextran solutions to prevent sedimentation. Upon replay of the tape, no change of the pattern formed by the latex was observed (not shown). As a negative control it was observed that, after denaturation of spectrin with urea, latex spheres attached to the resulting cell fragments changed their pattern rather quickly due to lateral diffusion (not shown).

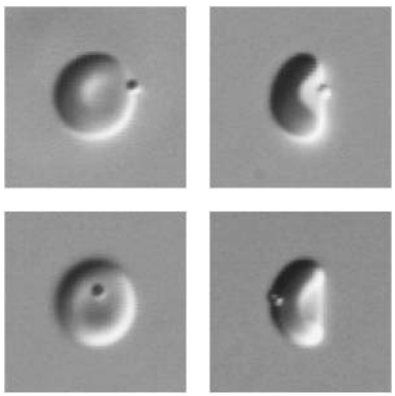

FIGURE 6 Two equilibrium configurations of a stomatocyte after two go-and-stop experiments (*top* and *bottom rows*). Shown are selected frames (each 16.6 μm wide) from a video recording through the rheoscope. Rotation of the cell was done by hand operation of the cone-plate chamber. In the left column, the orientation is face on. In the right column, the orientation is edge on. In the top row, the latex sphere is at the rim. In the bottom row, the latex sphere is in the middle of the convexity.

Stress relaxation

In previous experiments probing stress relaxation, red cells or resealed ghosts were aspirated into micropipettes. A permanent deformation after release from the pipette (Markle et al., 1983) or a change in the lateral distribution of the density of skeletal components (Discher et al., 1994) were taken as indications of stress relaxation. In these experiments, a static deformation was induced. In the work here, the deformations imposed on the red cells oscillate between stretch and compression every half tank-tread cycle. However, arguments based on molecular mechanisms are presented that show that stress relaxation can also occur in dynamic deformations.

As mentioned earlier, stress relaxation builds on a spontaneous dissociation and reassociation of links within the skeletal network. First, static deformations are considered. Upon dissociation of a stressed link, the network will rearrange. As a consequence, the distance of the original partners may change in such a way that the original link cannot reform, and a new link will form that is not stressed. This way the shear stress supported by the skeleton will vanish in an exponential fashion with a characteristic time called τ.

Tank-tread motion during preshear subjects each membrane patch to oscillatory shear deformations changing much faster than τ. In addition, the cell orientation within the shear field varies in a random fashion due to cell-cell interactions when the hct is large (25% or 45%) as during preshear in the viscometer. Therefore the probability density of membrane deformation during the whole preshear period mapped on a sphere having the surface area of the membrane is expected 1), to be uniform on the sphere and 2), to show a symmetric distribution centered about zero provided the deformation state is uniform. If, however, the reference deformation of a particular membrane patch is nonzero, the distribution of its deformations and thus of stresses is not centered about zero. By analogy to the static case, stress relaxation is expected to minimize the stresses. This in turn means that the reference

deformation of this particular membrane patch becomes zero and the deformation state of the whole membrane becomes uniform.

Preshear in the rheoscope was induced at very low hct with negligible cell-cell interactions. Therefore each patch of the skeleton remains on its specific flow line on the deformed membrane. Provided the spontaneous dissociation and reassociation of links within the network do not occur in exact synchrony with the tank-tread motion, the reference deformation eventually approached by each patch of the skeleton is constant on each flow line but may be different on different flow lines. This in turn means that the deformation state might not be uniform. Nevertheless, if stress relaxation was to occur, the outcome of a go-and-stop experiment would be unsuccessful since preshear and go-and-stop tests were performed at the same orientation of the red cell within the shear field.

In the pipette experiments mentioned above, the presence or absence of stress relaxation was observed during the deformation or immediately afterwards. In this work, this is not possible. Except for the shortest preshear period (30 s), red cells with the same preshear history were tested in turn. Since the go-and-stop test takes time the deformation history for the first red cell tested and the last one are different. This disadvantage was overcome by a protocol of hierarchical preshear periods (see Preshear at Room Temperature). Furthermore, no difference in behavior was found between cells tested immediately after preshear and cells tested at the end of the time window allowed.

In this work, $\tau > 80$ min was found. This is in agreement with pipette experiments where $\tau = 120$ min (Markle et al., 1983) and $\tau > 30$ min (Discher et al., 1994) were reported when the suspending medium contained concentrations of albumin $\geq 1\%$.

The rate of spontaneous dissociation of constituents of the skeleton has been measured by An et al. (2002). In static cells, they found no effect at room temperature whereas the rate became clearly measurable either after preshear for 30 min at room temperature or in static cells at 37°C. In contrast, in this work, elevation of the temperature to 37°C during preshear did not eliminate the memory under conditions imposing either moderate or high red cell elongation. A possible reason could be a different behavior of red cells as studied in this work and red cell ghosts (An et al., 2002).

Shape memory in vivo

It was shown in vitro that $\tau > 80$ min. In the experiments without preshear, 15 min elapsed from blood withdrawal to the begin of the go-and-stop tests. Since this is well below 80 min, it seems to indicate that red cells in vivo also possess a shape memory. However, although it takes $\sim 3\tau$ to reach the final state, a fraction of τ suffices for a small change. The possibility that a shape memory is induced by the handling of the blood after withdrawal can therefore not be absolutely ruled out. However, the consistent nature of the observed behavior, independent of the time lag, whether it was stored as packed cells or used as quickly as possible, suggests that it is an intrinsic property that would be present also in vivo.

Reference deformation and deformation state

In theoretical models of the mechanical behavior of red cells, membrane shear deformations are usually measured relative to a stress-free shape of the skeleton. However, the variety of shapes assumed by red cells during passage through the circulation makes it likely that due to stress relaxation no stress free shape of the skeleton exists at all. Therefore a more general approach was adopted by defining a reference deformation. The sphere was chosen as the shape on which the reference deformation was measured. In principle any other shape could have been used, but this choice is particularly suited to interpret the go-and-stop experiment as detailed below.

A uniform distribution of the reference deformation on the red cell membrane indicates the absence of a shape memory. A nonzero shear deformation defines a direction in the plane of the membrane that cannot be uniform on a spherical surface. Therefore the only uniform value that is possible is zero. Accordingly, a reference deformation of zero everywhere on the membrane was abbreviated as a uniform deformation state. Complementarily, a nonzero reference deformation anywhere on the membrane defined a nonuniform deformation state.

The go-and-stop experiment provides a yes or no answer. Both answers are defined by the deformation state being either uniform or nonuniform. A uniform deformation state implies the absence of a shape memory that is indicated by an unsuccessful go-and-stop experiment. A successful go-and-stop experiment, on the other hand, implies the presence of a shape memory or a nonuniform deformation state.

The go-and-stop experiments demonstrated the prevalence of a nonuniform deformation state of the red cell membrane. The actual deformation state is not known. In consideration of stress relaxation within the circulation, it could vary between two extremes, a stress free sphere and a stress free biconcave disk. A localization of the actual deformation state within this continuum could be achieved by modeling the transient after stop of flow. The collection of data describing the transient is planned.

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