Deformability and Stability of Erythrocytes in High-Frequency Electric Fields Down to Subzero Temperatures

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ABSTRACT   High-frequency electric fields can be used to induce deformation of red blood cells. In the temperature domain $T = 0°$ to $-15°C$ (supercooled suspension) and for $25°C$ this paper examines for human erythrocytes (discocytes, young cell population suspended in a low ionic strength solution with conductivity $\sigma(25°) = 154$ $\mu S/cm$) in a sinusoidal electric field ($\nu = 1$ MHz, $E^0 = 0$–18 kV/cm) the following properties and effects as a function of field strength and temperature: 1) viscoelastic response, 2) (shear) deformation (steady-state value obtained from the viscoelastic response time), 3) stability (by experimentally observed breakdown of cell polarization and hemolysis), 4) electrical membrane breakdown and field-induced hemolysis (theoretical calculations for ellipsoidal particles), and 5) mechanical hemolysis. The items 2–4 were also examined for the frequency $\nu = 100$ kHz and for a nonionic solution of very low conductivity ($\sigma(25°) = 10$ $\mu S/cm$) to support our interpretations of the results for 1 MHz. Below $0°C$ with decreasing temperature the viscoelastic response time $\tau_{res}(T)$ for the cells to reach steady-state deformation values $d_{\infty, E}$ increases and the deformation $d_{\infty, E}(T)$ decreases strongly. Both effects are especially high for low field strengths. The longest response time of ~30 s was obtained for $-15°C$ and small deformations. For 1 MHz the cells can be highly elongated up to 2.3 times their initial diameter a_0 for $25°$ and $0°C$, $2.1a_0$ for $-10°C$ and still $1.95a_0$ for $-15°C$. For $T \geq 0°C$ the deformation is limited by hemolysis of the cells, which sets in for $E^0_{lysis}(25°)$ ≈ 8 kV/cm and $E^0_{lysis}(0°) \approx 14$ kV/cm. These values are approximately three times higher than the corresponding calculated critical field strengths for electrically induced pore formation. Nevertheless, the observed depolarization and hemolysis of the cells is provoked by electrical membrane breakdown rather than by mechanical forces due to the high deformation. For the nonionic solution, where no electrical breakdown is expected in the whole range for E^0, the cells can indeed be deformed to even higher values with a low hemolytic rate. Below $0°C$ we observe no hemolysis at all, not even for the frequency 100 kHz, where the cells hemolyze at $25°C$ for the much lower field strength $E^0_{lysis} \approx 2.5$ kV/cm. Obviously, pore formation and growth are weak for subzero temperatures.

INTRODUCTION

Mechanical properties of the human red blood cell (RBC) membrane have been intensively investigated at temperatures between 20° and 40°C, mainly because there is great interest in understanding the ability of the RBC to deform in in vivo blood microcirculation and under the influence of diseases. Extension of the well known deformation experiments to subzero temperatures (supercooled cell suspensions) and discussion of the effects of low temperatures on the deformability and stability of erythrocyte membranes can, on the other hand, give insight into the mechanism of membrane damage during freezing.

One of the theories about freezing injury of RBCs during cryopreservation (leading to cell hemolysis) deals with the possibility of mechanical membrane damage. P. Mazur (Mazur et al., 1981), for example, assumed that 1) the cells could be highly deformed in a freezing solution (also cf. Ishiguro and Rubinsky, 1994) and 2) the low temperature could render the cell membranes more liable to destruction under an applied mechanical stress (also cf. Muldrew and McGann, 1990).

Generally, the possible extent and rate of cellular deformation under an applied external force are determined by the elastic and viscous membrane properties and the cytoplasm viscosity (intrinsic properties) as well as the cell shape and the surface-area-to-volume ratio of the cell (extrinsic properties) (Hochmuth, 1987; Evans and Skalak, 1980). For temperatures between 5° and 50°C the intrinsic RBC properties such as elastic shear and compressibility moduli are only slightly temperature dependent, whereas the viscosities depend strongly on the temperature (Hochmuth, 1987; Engelhardt and Sackmann, 1988). For temperatures below 0°C our measurements exhibit strong temperature dependence both for elastic and viscous properties of the erythrocyte membrane.

The only practicable technique for cell deformation in supercooled solutions is the electric field method presented by Engelhardt et al. (1984) and Engelhardt and Sackmann (1988) and modified in our laboratory for subzero temperatures (Thom, 1988). A high-frequency electric field polarizes cells suspended in a low ionic strength solution in a frequency range where the Maxwell-Wagner polarization (Pohl, 1978) is effective ($\sim 10^5$–10^7 Hz) and thus generates within this frequency domain a constant uniaxial force in the direction of the electric field strength. The cells respond to that force by elongation, which is, up to high deformation values, mainly elastic shear deformation (deformation under constant cell volume and surface area). As the area com-

pressibility modulus of the RBC membrane (of a normal biconcave discocyte) exceeds the elastic shear modulus by four orders of magnitude (Evans and Skalak, 1980), very high forces are necessary for a membrane surface area dilation leading to destruction of the cell. However, application of high forces generated by an electric field is limited by electrical membrane breakdown (electrically induced pore formation and hemolysis of the blood cells). In direct current (DC) fields electrically induced hemolysis for human RBCs occurs for quite low critical electric field strengths ($E^0 \approx 2.5$ kV/cm; cf. Kinosita and Tsong, 1977a). Schwan's equation (Schwan, 1983) shows that both increasing frequency of an alternating current (AC) field and decreasing temperature will shift the critical field strength E_{crit} for hemolysis to higher values, whereas E_{crit} is lowered with increasing cell elongation (Bernhardt and Pauly, 1973). Thus, when discussing the mechanical stability of erythrocytes (i.e., no poration and hemolysis) during deformation as a function of temperature, the possible electrical effects have to be examined as well. This includes also an investigation of the sample heating by the electric field.

This paper investigates the effects of low temperatures on the deformation behavior and the stability of human erythrocytes of discoid shape.

EXPERIMENTAL METHODS

Cell preparation and measuring solution

Blood was drawn from the arm vein of a healthy donor (mostly the same person), heparinized, and then separated into young and old erythrocytes using the method of density separation by Nash and Meiselman (1983). The upper 10% of the cells (young cells) gained by this separation were then washed with the double amount of a buffered isotonic sodium citrate/glucose solution (cf. Meryman and Hornblower, 1991) and centrifuged for 10 min at 3000 $\times$ g. The washed erythrocytes were diluted by a factor of 1000 with the measuring solution. For most of the measurements, a low ionic strength solution (LISS) was used containing 280 mM/l sucrose, 5 mM/l glucose, and 0.9 mM/l Na$_2$HPO$_4$, with conductivity $\sigma = 154$ μS/cm (25°C), pH = 7.4, and osmolarity = 310 mosmol/kg. For some test measurements, a very low ionic strength solution (LISS2) was used containing 280 mM/l sucrose and 5 mM/l glucose, with $\sigma = 10$ μS/cm (25°C), pH = 7.5, and osmolarity = 305 mosmol/kg.

The very small amount of cells in the measuring solution does not change these values and, moreover, has the positive effect of avoiding dipole interaction between the cells. The cells were always measured on the day of venipuncture, and only erythrocytes of discoid shape were examined. Approximately 60% of the erythrocytes (after resuspension in LISS) were discocytes at $T = 25$°C; the rest were mainly echinocytes. The amount of discocytes was increased to 70–80% while cooling down the suspension to 0°C or below. For temperatures $T = 0$ to -15°C the discocytes did not exhibit shape changes within 30 min, whereas at $T =$

25°C the cells turned out to be less stable in shape; after 15 min most of the discocytes were changed to echinocytes, so a very short measuring time was required for reliable results.

The measuring procedure

A portion of 3 μl of the measuring solution containing 0.1% erythrocytes is brought into the measuring chamber. The chamber consists of a system of two parallel gold wires placed between an upper and lower coverslide. The wire diameter is 20 μm and their distance is $\sim$200 μm. The wires are renewed after every measurement to avoid contamination, and so their distance has to be measured with a micrometer before every measurement to calculate the field strength applied (the distance can be measured with an accuracy of $\sim$2%). The measuring chamber is mounted on a cryomicroscope table (see Thom and Matthes, 1988, for detailed description of the experimental equipment). Together with an electrically controlled heating element it allows the measurement of samples at any fixed temperature between 50°C and -180°C. Due to the composition of the solution and the small sample size our samples can be supercooled down to ~ -18°C. When the electric field is applied, ice nucleation often sets in for $T \leq -16$°C; thus measurements can be performed only down to -15°C.

The electric field is generated by a HAMEG function generator (HM 8130) with an external timer (HAMEG, HZ 830) and amplified by a specially designed high-voltage HF-amplifier (EMM21, Fa. Elektronik-Manufaktur Mahlsdorf, Berlin) that allows at the frequency 1 MHz an AC voltage output up to 100 V (effective value), controlled during the measurement by an AC voltmeter. From these measured output voltages U_{out} we calculated the applied electric field strength, although they differ slightly from the actual values for the voltage U between the electrodes in the measuring chamber filled with suspension. Using a symmetric high-resistance potentiometer (1:53, 2 $\times$ 100 kΩ) and an oscilloscope (RFT, EO 213) we measured this systematic deviation for U in the filled chamber from U_{out} to be at most -3% (for $U_{out} = 0$–100 V). Thus we neglected these small deviations and set $U \approx U_{out}$.

The frequency 1 MHz was chosen from the frequency regime where Maxwell-Wagner polarization becomes effective for the used suspension (Pohl, 1978; Engelhardt et al., 1984; Engelhardt, 1987; Engelhardt and Sackmann, 1988). It is a consequence of the different conductivities of the intracellular cytoplasm ($\sigma_i \approx 5$ mS/cm) and the external solution ($\sigma_e \approx 160$ μS/cm) in addition to the different values of the permittivity for both media. In an AC field this leads to a high charge density of opposite sign at the two poles of a cell that lie opposite on a line parallel to the direction of the electric field and perpendicular to the cell's rotational axis. The electric field force acting on this cellular dipole causes its transient deformation into long ellipsoids as the applied voltage is increased. For very high values of

the electric field strength, the cell membrane ruptures and hemolysis can be observed. The time-dependent deformation process of an ensemble of discocytes in a sample was recorded with a JVC system (CCD camera BY-10, providing 50 pictures per second, and editing recorder BR-S810E) in connection with a timer (type VTG-33, FOR-A Co., Tokyo, Japan). This was performed for several values of the applied AC voltage at constant temperature. The voltage was increased stepwise or a field jump method was used (rectangular pulse with a rise time that was small compared with the viscoelastic response time of the deformation). The gained images were analyzed by a computer-controlled measuring program (AMBA, Institut für Bildverarbeitung und Softwareentwicklung, Berlin, Germany), which determines the contour of a cell (i.e., its diffraction pattern obtained by phase contrast light microscopy) with the help of the displayed intensity profile of the membrane and then measures the long axis a of the elongated cell parallel to the direction of the electric field. Together with the measured diameter a_0 of the same cell in the unperturbed state we can define cell deformation as:

$$d = a/a_0$$

As the cell is a viscoelastic system (elastic and viscous properties of the membrane connected with the viscosity of the cytoplasm) the deformation amplitude is a characteristic (exponential) function of time for each value of the applied electric field strength E and the temperature T:

$$d(t)_{E,T} = a(t)_{E,T}/a_{0,T}$$

For field operation times exceeding the viscoelastic response time of a cell, equilibrium between the cellular viscoelastic forces and the external electric force and thus a constant (steady-state) value d_∞ is obtained. As the viscoelastic response time τ_{res} exhibits a strong temperature dependence due to the strongly increasing viscosity for subzero temperatures it is necessary to determine d_∞ for each measured point $d(E)_T$. We then recorded deformation curves

$$d_\infty(E)_T = a_\infty(E)_T/a_{0,T}$$

by increasing E stepwise with a step width $\Delta t > \tau_{res}$ for every chosen value of E. The above defined $d_\infty(E)_T$ then describes the elastic deformation behavior of the cell membrane because the influence of the viscosities is eliminated.

ELECTRIC FIELD STRENGTH

The electric field strength of a system (in vacuum) consisting of two parallel cylindrical electrodes has its maximal value in the plane midway between the two electrodes, so the polarized cells are always pulled toward this plane. It is

then sufficient to consider only one component of the field:

$$E = |\mathbf{E}(x, y)| = E_x$$

$$E_x = (Q/2\pi\epsilon_0 l)[(p - x)^{-1} + (p + x)^{-1}]$$

$$p = [(b/2)^2 - r_0^2]^{1/2}$$

where x is the distance from the center between the electrodes, l is the electrodes' length, r_0 is their radius, b their distance (i.e., of the cylinder axes), and Q their charge (cf. Küpfmüller, 1955). With the capacity of the system

$$C = (\pi\epsilon_0 l)/[\ln(b/2r_0 + ((b/2r_0)^2 - 1)^{1/2})]$$

it follows

$$E_x = Up/[(p^2 - x^2)\ln(b/2r_0 + ((b/2r_0)^2 - 1)^{1/2})]$$

For the measurement we take only cells close to the electrodes, because the electric field strength increases toward the electrodes and always moves the cells in these directions. Thus we take the value of the field strength at the center of a cell close to an electrode, that is, E_x at $x = b'/2 - 5$ μm ($b' = b - 2r_0$ is the measured electrode distance). For a distance b' of, for example, 200 μm one obtains (for an applied voltage U)

$$E = 119 \ U[V/cm]$$

As we measured the effective value for U instead of the peak value U^0, our deformation measurements generally refer to the effective field strengths $E^{eff} = E^0 \cdot \sqrt{2}$.

RESULTS AND DISCUSSION

1. Viscoelastic response

To attain an equilibrium state between the cellular viscoelastic forces and the external electric field force, the cell requires a characteristic response time τ_{res}. For an operation time of the applied field force $t = \tau_{res}$ the time-dependent deformation reaches its maximal value (steady-state value), which is constant for $t \geq \tau_{res}$ if the temperature and the field strength are constant during that time. The response time is a function of the surface viscosity η and the elastic shear modulus μ of the cell membrane: $\tau_{res} \sim \eta/\mu$. As every erythrocyte is an individual, the most outstanding problem of a measurement is the rather large variability of the measured values within an ensemble of cells. Above all, the deformation function $d(E, t)$ for erythrocytes depends on their in vivo age. For older cells τ_{res} is longer than for younger ones due to increased membrane viscosity η and also due to the increased internal viscosity caused by cell shrinkage (Nash and Meiselman, 1983). Furthermore, viscosity and shear modulus vary for cells of different donors (Engelhardt and Sackmann, 1988). These problems can be partly overcome by cell separation and restriction to the young cells of a single donor.

Differences in the mechanical behavior of cells are increased for measurements at temperatures below 0°C. Our

measurements show that the response time τ_{res} for a cell decreases with increasing E (Fig. 1) and it increases strongly with decreasing temperature (Fig. 2). These results are in agreement with the experimental results by Engelhardt and Sackmann (1988) for temperatures above 5°C. They found that the membrane viscosity increases remarkably with decreasing temperature below 20°C. From Fig. 1 and Fig. 2 it follows that the maximal response time for our experimental temperatures and applied field strengths will occur for low values of the field strength at $T = -15$°C. This time can be determined from Fig. 3, where the time-dependent deformation at -15°C is measured for a low value ($E^{eff} = 2000$ V/cm) of the stepwise increased field strength. This is the threshold value for E^{eff} from which deformation values exceeding the standard deviation of the deformation can be obtained. For the plot we chose the cell with the longest response time τ_{res}, which occurred within an ensemble of young cells. Consequently, if an operation time (step width) $\Delta t \geq \tau_{res} \simeq 30$ s is chosen for each step of the applied field strength in the whole temperature range of $T = 0$ to -15°C, it is ensured that every measured point $\langle E, d \rangle_T$ on the deformation curve is an equilibrium value.

In comparison with that, the response time for $T = 25$°C is only a few seconds. In our measurements we used operation times $\Delta t = 20$ s for $T = 25$°C and $\Delta t = 40$ s for $T \leq 0$°C for each value of the applied field strength.

2. Viscoelastic relaxation and irreversibility

Completely reversible deformation of cells for temperatures below 0°C is possible only for very short operation times $\Delta t \ll \tau_{res}$ and for low values of the applied field strength. For increasing operation time Δt, the viscoelastic relaxation

FIGURE 2   Temperature dependence of the viscoelastic response function measured with field jumps for a constant value of the electric field strength. The response time increases with decreasing temperature. For $T = -10$°C and $T = -15$°C, the cells need more than 20 s to reach the equilibrium value for the deformation.

time τ_{rel} is also increased, and thus, due to the high response times required for subzero temperatures, the deformed cell will never relax to its original shape when the field strength is turned off. The question is how the cell might be altered during such a measurement and whether it makes sense to perform measurements for several values $d_i = f(E_i)_T$ on the same cell. So the deformations (equilibrium values) that were obtained after applying a stepwise deformation procedure for increasing values of the electric field strength were compared with single measurements, where the cells of a sample were measured only once for a given value of the field strength. The latter method was performed for several
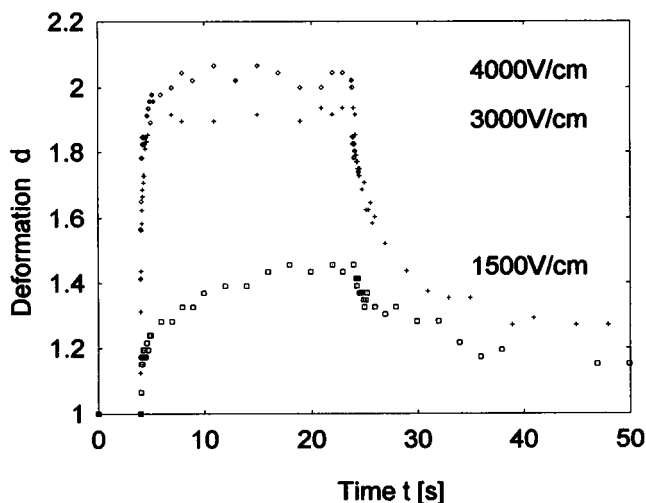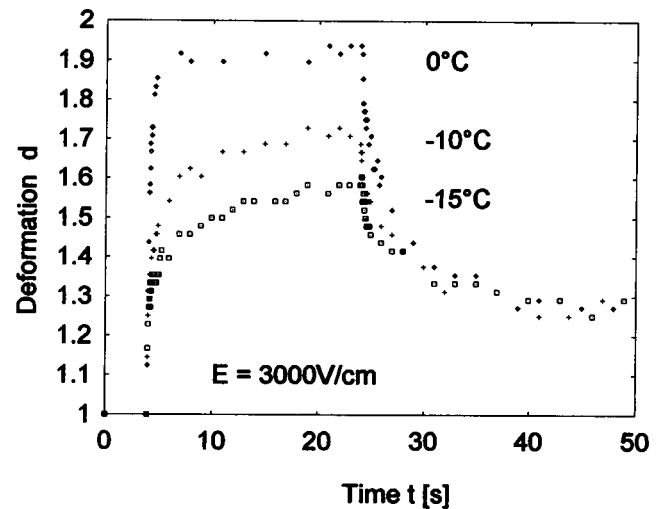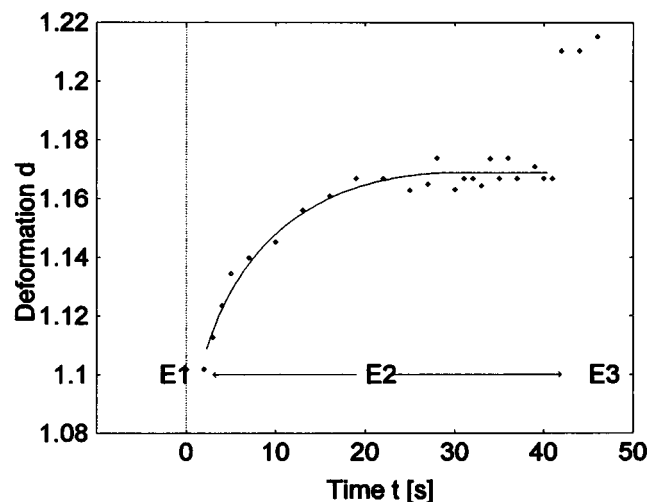

FIGURE 1   Response and relaxation functions measured by the field jump method for different values of the electric field strength E^{eff} at $T = 0$°C. The response time τ_{res} increases with decreasing deformation. For $E^{eff} = 1500$ V/cm, the deformation has not yet reached its maximal amplitude. This means that the field has to be applied to the cell for times longer than 20 s to attain an equilibrium state.

FIGURE 3   Determination of the response time for the equilibrium deformation amplitude at $T = -15$°C measured for a low field strength E_2^{eff} of the stepwise increased field strength, that is, $E_2^{eff} = 2000$ V/cm between $E_1^{eff} = 1000$ V/cm (deformation is too small) and $E_3^{eff} = 3000$ V/cm.

values of E in the whole range up to 13 kV/cm and for different temperatures. The results for the deformation from both methods were in good agreement. It turned out that even repetitions of a single measurement at the same value of E always lead to the same steady-state deformation value. However, the step operation method is restricted to increasing values of E and leads to mistakes if E is decreased stepwise (due to the long relaxation times).

3. Cell alteration during the measurement

If the erythrocytes are removed from the plasma and brought into LISS, the efflux of potassium ions is increased. Thus investigations were necessary to prove that this increasing amount of ions within the LISS has no effect on the polarization mechanism during the time of measurement. Deformation measurements were taken from cells that have been incubated in LISS for $\sim$30 min before measuring and were compared with measurements of cells that were performed immediately after the resuspension in LISS. Results from both methods agreed well. The reason is probably the very small amount of cells within the measuring solution. From separate measurements we found that, when the cells are diluted 20-fold with LISS, the K^+ concentration rises by 10% after 30 min of incubation and by 20% after 60 min ($T = 25°C$). For dilution by a factor of 1000 (which corresponds to our measurement conditions) the increase of potassium ions within the sample after 60 min is below the detection limit.

On the other hand, strong deformation of the cells and high electric fields applied for long periods of time may lead to an increased efflux of ions or change the intracellular amount of water. Engelhardt (1987) found that the applied (Maxwell-Wagner) force is decreased by 20% during a field operating time of 15 min. Our test measurements (mentioned in section 2) exhibited no significant changes for the deformation during a measurement time of 10 min.

4. Deformation $d_\infty(E)_T$

Fig. 4 shows the deformation of cells as a function of the applied vacuum field strength (effective value) for several temperatures from 25° to $-15°C$. Each measured point on the deformation curves represents the maximal value of the deformation (steady-state deformation), d_∞, at the given field strength, which means that the cells are in equilibrium with the external force. Thus d_∞ describes the elastic behavior of the membrane determined mainly by the elastic shear modulus. Viscoelastic parameters such as viscosities of the membrane, cytoplasm, and external solution do not have any influence in this state.

Most of the measured points are mean values of the deformation taken from two to seven different cells of a sample. Some of them represent measurements of single cells. The solid lines are averages over all measured cells from different samples obtained by the method of least

squares. Roughly, the deformation functions exhibit four different regimes: I) $d \sim E^2$ for very small values of E; II) $d \sim E$ for medium values of E; and III) $d \sim E^{1/2}$ for high values of E. For the highest field strengths the deformation increases only weakly with increasing E (IV). Generally, this is valid for the whole temperature range, but for decreasing temperature the regimes I and II are broadened over a wider range of E. For $T = 25°$ and $0°C$ the functions $d(E)_T = 1 + A_T\exp(-\alpha_T E_0/E)$ (with appropriate values for A_T and α_T and $E_0 = 1$ kV/cm) represent the data fairly well in the whole range for E. Below $0°C$ the data cannot be approximated by an exponential function alone due to the more distinct parabolic (for small elongations) and linear (for medium elongations) behavior of the deformation curves.

For 25°C the results for the first and second deformation regime (up to $d \approx 1.4$) are in good agreement with measurements carried out by similar techniques (Engelhardt and Sackmann, 1988) and with flow-channel data (Nash and Meiselman, 1983). For higher deformation (regimes III and IV) there exist only data from experiments where mechanically generated forces have been applied to the cells. In the case of imposed uniaxial mechanical stresses (e.g., laminar shear flow) the resulting cell deformations can be qualitatively compared with our results (for example, see Pfafferott et al., 1985; Johnson, 1994). It turns out that the elastic response of the RBCs to our electrically induced force (in the whole range of E) is very similar to the cell behavior under an applied mechanical shear force. This is especially important for the interpretation of the fourth deformation regime. Johnson (1994) found that the permeability of RBCs to cations increases when they are highly deformed by mechanical forces up to ellipticities $EI \geq 0.75$, with the definition $EI = (L - W)/(L + W)$, where L and W are the length and width of the ellipsoidally elongated cell (projection into the plane parallel to the long axis). The maximal value of the above defined ellipticity index that could be attained in our experiments was $EI = 0.66 \pm 0.04$ (for the highest deformation at 25°C and 0°C). In our case an increased ion permeability caused either by high deformation or by pore formation induced directly by the electric field (see section 6) could mean a decreased electric force for high field strengths due to the progressive reduction of the Maxwell-Wagner polarization leading to flattened deformation curves. But this effect cannot play a predominant role in our measurements as the flattening of the deformation curves for high shear stresses can also be observed under mechanically generated forces.

The behavior for high forces (or high field strengths) seems to be a property of the erythrocyte mainly caused by its geometry, as has been mentioned by many authors. As the shear modulus for the RBC is approximately four orders of magnitude lower than the elastic area compressibility modulus (Evans and Skalak, 1980) the cells can be deformed easily into prolate ellipsoids as long as their surface area and volume remain constant (shear deformation), but much higher forces (correlated to the area compressibility
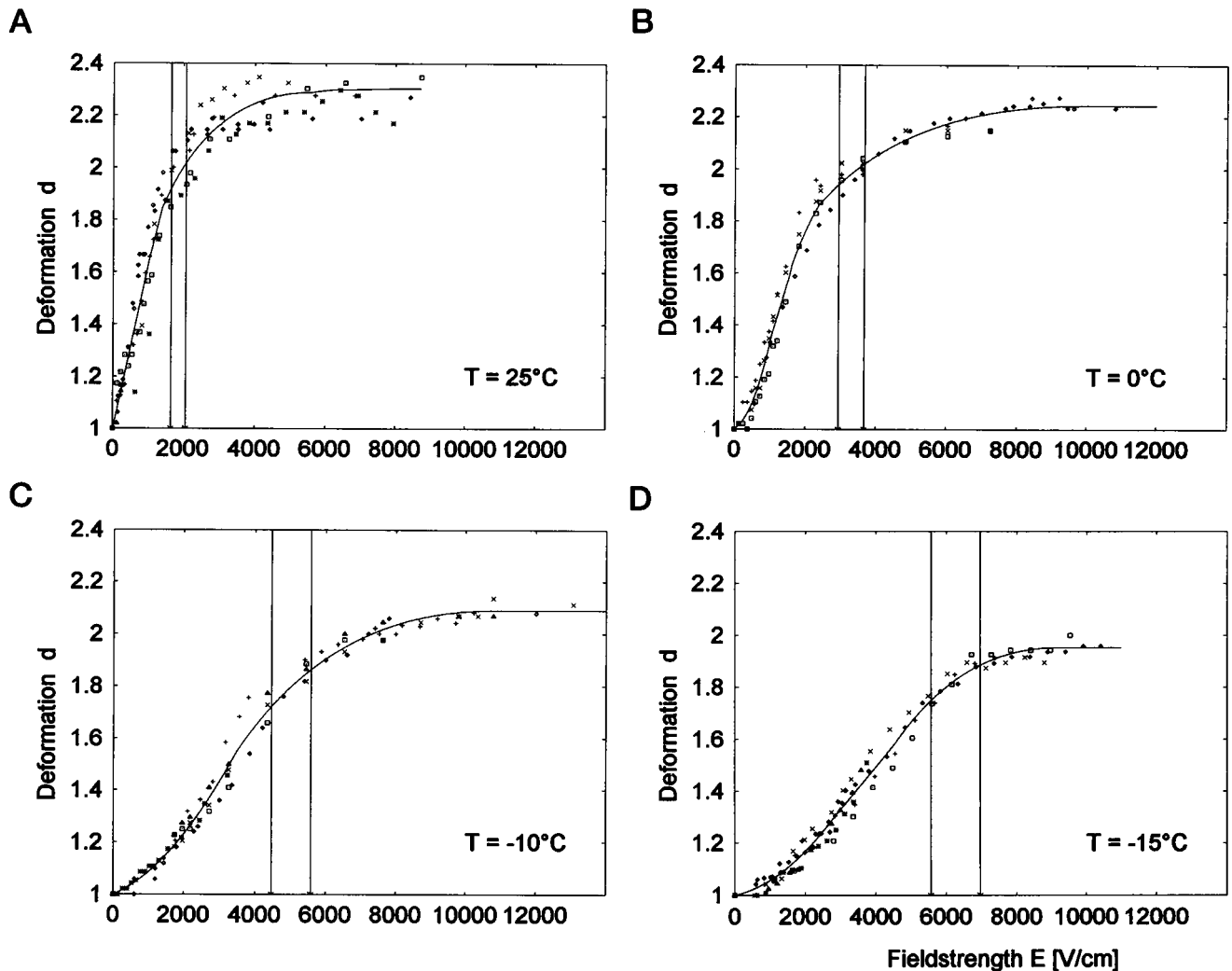
A

B

C

D

Fieldstrength E [V/cm]

FIGURE 4 Deformation of human RBC discocytes as a function of the vacuum field strength (effective value) for 25°C, 0°C, −10°C, and −15°C. The measured points are equilibrium values of the deformation. Solid lines are averages over all measured cells from different samples (four to seven different samples for every value of T). The vertical lines indicate the critical interval for E^{eff} where electrically induced pore formation is expected (see section 6).

modulus) are to be applied to increase the surface area. Thus if the applied electric force, generated by increasing E linearly, remains too low to increase the surface area of the cells markedly, the result for even high field strengths will be an only marginal increase in cell elongation leading to the asymptotic behavior of the deformation in regime IV. In our model calculation (see Appendix) for the deformation of a simple oblate ellipsoid with a given volume and semi-axes $f = g > h$ into a prolate ellipsoid $f > g \geq h$ the surface area remains constant for increasing elongation in direction of $f(= a/2)$ up to a maximal value f_{max}. For $f = f_{\text{max}}$ the prolate ellipsoid has obtained rotational symmetry ($g = h$). For $f > f_{\text{max}}$ the surface area has to be increased (with fixed volume). Independent of the initial size of the ellipsoid, f_{max} leads to $a_{\text{max}}/a_0 \approx 1.7$ (or $EI_{\text{max}} \approx 0.6$). The surface area of a human RBC cannot be increased by more than ~6% without hemolysis (Blackshear and Blackshear, 1987), which gives $a_{\text{lysis}}/a_0 \approx 2$ (cf. Table 2, Appendix). The true

value for a_{lysis}/a_0 may be higher due to the biconcave shape of the erythrocyte resulting in a higher surface area for the same initial volume and axes, so it is not surprising that we could measure cells up to $a_{\text{lysis}}/a_0 = 2.3 \pm 0.2$ (for 25° and 0°C).

For the temperatures below 0°C these high values for a/a_0 and EI could not be reached. For the maximal field strengths that could be obtained in the measurements we found for −10°C, $a/a_0 = 2.1 \pm 0.1$ and $EI = 0.59 \pm 0.03$, and for −15°C, $a/a_0 = 1.95 \pm 0.1$ and $EI = 0.57 \pm 0.03$. In the whole range for E the deformation is decreased for decreasing temperature, as can be clearly seen from Fig. 5 for several discrete values of E. Fig. 5 shows mean values for $d(E)$ taken from Fig. 4 A–D at temperatures $T + \Delta T$, where ΔT denotes the temperature rise of the solution due to heating by the electric field, which becomes evident for $E \geq 2000$ V/cm (cf. section 8). Engelhardt and Sackmann (1988) found that cell elongation decreases (or the elastic
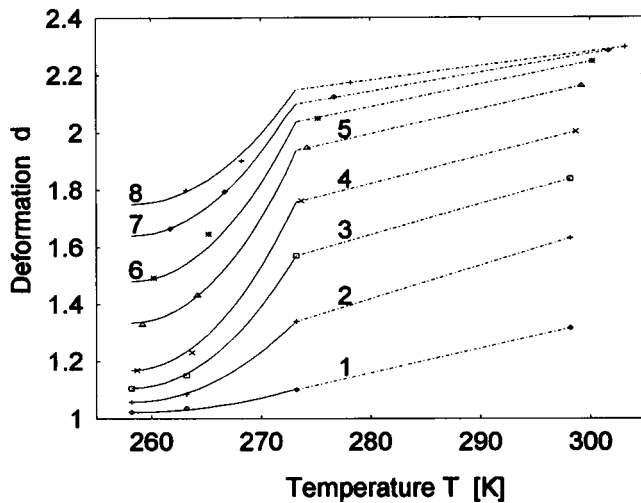
FIGURE 5   Temperature dependence of the cell deformation (mean values from Fig. 4) for different values of the field strength (effective values): curve 1 = 500 V/cm ($\Delta T \approx 0$); 2 = 1000 ($\Delta T \approx 0$); 3 = 1500 ($\Delta T \approx 0$); 4 = 2000 ($\Delta T = +0.5K$); 5 = 3000 ($\Delta T = +1K$); 6 = 4000 ($\Delta T = +2K$); 7 = 5000 ($\Delta T = +3.5K$); 8 = 6000 ($\Delta T = +5K$), where ΔT is the temperature rise due to specimen heating by the electric field (see section 8), which has been taken into account in the plot.
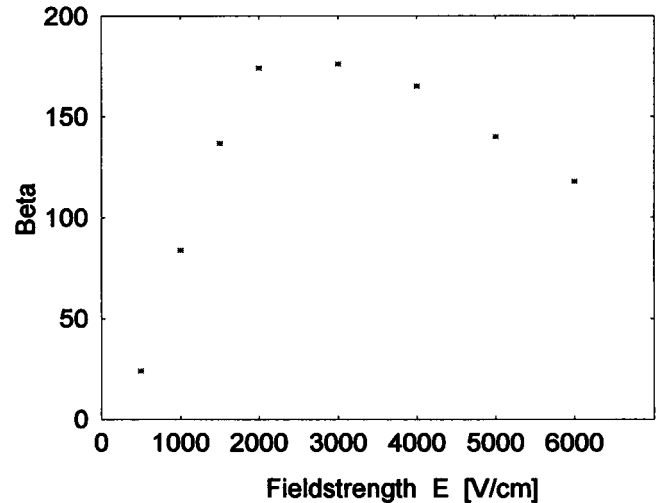
FIGURE 6   The slope $[\beta]_E$ of the functions $d_E(T) = d_E(T_0) + [\beta]_E \cdot [(T - T_0)/T_0]^2$ that have been used in Fig. 5 to approximate the data in the temperature interval $-15°C \ldots 0°C$.

shear modulus increases) only moderately with decreasing temperature in the interval 25° to 5°C. This agrees with our results down to 0°C for all $E_i^{eff} = 500\text{-}6000$ V/cm ($i = 1\text{-}8$; see Fig. 5). For 25°C and 0°C the chosen values of E are in the same deformation regimes for both temperature values. Thus, if the data are approximated linearly in this interval, the functions $d_{Ei} = f(T)_{Ei}$ are nearly parallel for all values of the field strength, except for $E_8 = 6000$ V/cm. This is because d_{E8} ($T = 25°C$) is not very reliable, for it is averaged over different cells, even those that exhibit shape changes during the measurement time. For the same reason the standard deviation from the mean values of the deformation is larger for $T = 25°C$ than for the other temperatures.

For subzero temperatures d decreases strongly with decreasing T. Moreover, the decrease depends on the field strength. The data for $T = -15°$ to 0°C can be fitted by functions

$$d_E(T) = d_E(T_0) + \beta_E \cdot [(T - T_0)/T_0]^2$$

with T in Kelvin and $T_0 = 258.2$ K). The field-strength-dependent slope β_E (in the interval $[-15, 0]°C$), determined by numerical approximation, is plotted in Fig. 6. This is, of course, only a rough approximation. Detailed measurements in the interval $[-5, +5]°C$ will probably lead to smoother functions.

Summarizing the elastic behavior of the erythrocyte membrane for temperatures below 0°C, we can say that it remains deformable down to $-15°C$ when high electric fields are applied, but it exhibits high shear rigidity under low field strengths (i.e., in the deformation regimes I and II; notice, for example, the high slope of curve 4 in Fig. 5

between $-10°$ and 0°C). This effect can be explained neither by the temperature rise due to specimen heating for high field strengths (cf. section 8) nor by a decreased Maxwell-Wagner force due to the decreased conductivities of external solution and cytoplasm for subzero temperatures. The latter follows from calculations by Engelhardt (1987) and Engelhardt and Sackmann (1988) for the theoretical frequency dependence of the electric force acting on the human RBC due to Maxwell-Wagner polarization. For $\sigma_e \leq 10^{-4}$ S/cm the force is independent of the conductivity of the external and internal medium within a frequency interval around 1 MHz even if the internal conductivity is lowered from 10^{-2} to 10^{-3} S/cm (the actual values from our measurements are for the cytoplasm: $\sigma_i = 4.9 \times 10^{-3}$ S/cm for 25°C, $\sigma_i = 1.5 \times 10^{-3}$ S/cm for $-15°C$ (F. Thom and F. Wolter, in preparation; see Table 1). Thus, within the meaning of the above cited model, for a given field strength the electric force is constant in the temperature interval $-15°$ to $+25°C$. However, the model calculation has been performed for spherical cells. As it is known, the electric force acting on the ellipsoidal cell increases with increasing ellipticity. For our highly deformed cells this could become an important fact that should be investigated. Furthermore, Engelhardt used for his calculation the relative permittivity for the cytoplasm $\epsilon_i = 60$ ($\sim 20°C$), which is unknown for subzero temperatures.

5. Hemolysis during the deformation experiments

The hemolytic process for individual cells during deformation in a strong electric field can be observed in detail in a light microscope (see Fig. 7, elongated and hemolyzing cells); when a certain critical field strength is reached, the deformation suddenly decreases and the cells quickly relax to a spherical shape. Those that were pulled previously to an
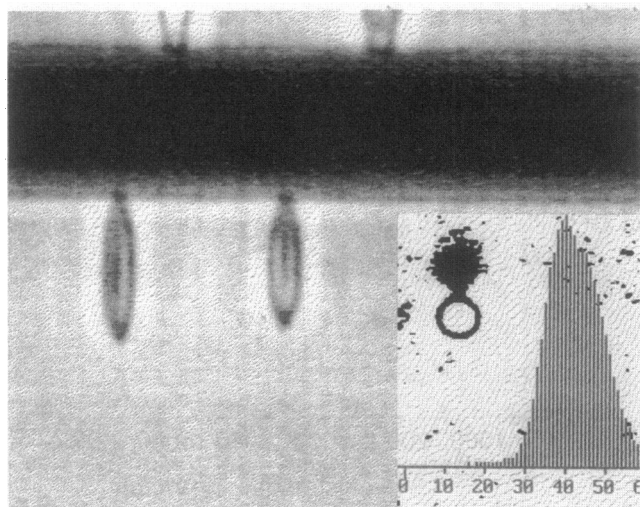
FIGURE 7 On the left are erythrocytes highly elongated in a 1 MHz electric field as it can be observed in a light microscope (phase contrast). On the right is a hemolyzing erythrocyte during application of a critical field strength for 25°C (enhanced contrast).

electrode or to other cells are now moving freely. This process can be interpreted as depolarization of the cells that should be caused by the efflux of ions from the cell interior. Immediately after is the release of hemoglobin, which is often ejaculated from one of the poles of the former ellipsoidal cell in a direction parallel to the electric field, subsequently turning the cell into a ghost. From the onset of cell relaxation until the beginning of hemolysis it takes $\sim 1-4$ s (note that the field is applied to the cells all the time at a constant field strength).

For 1 MHz hemolysis of the cells can be observed only for high field strengths. The maximal value of the field strength that can be obtained for the applied voltage $U_{max}^{eff} = 100$ V depends on the electrodes' distance in the measuring chamber. For a few measurements the distance happened to be <100 μm, so a very high field strength up to 13 kV/cm (for $T = -10°C$) could be reached. Thus the cutoff for the measured deformation curves for $T = 25$ to $-15°C$ occurred for different reasons. For 25°C the cells could never be measured for extremely high field strengths, because hemolysis regularly sets in for field strengths $E_{lysis}^{eff} = 5-8$ kV/cm. For 0°C the cells were damaged only at the highest values of E^{eff} (>10 kV/cm). Contrary to this, hemolysis was almost never observed for $-10°C$ and $-15°C$, not even for very high field strengths. For temperatures below 0°C cells were damaged only due to unusual situations, e.g., when the field strength got so high that the electrodes were pulled together. The onset of hemolysis did not depend on the rise time of the applied field. E_{lysis} had the same value no matter whether E was stepwise or gradually increased or a field jump method was applied.

These results were also obtained for the frequency $\nu = 100$ kHz, where hemolysis was observed for much lower field strengths for 25°C ($E_{lysis}^{eff} \cdot \sqrt{2} \approx 2.3$ kV/cm; see next

section). Tests for $T = -10°$ and $-15°C$ again showed no hemolysis, not even for the highest field strengths ($E^{eff} \cdot \sqrt{2} = 17$ kV/cm). If there is pore formation at all for subzero temperatures, the pore size is obviously too small to allow hemolysis.

The low temperature behavior is not a result of the increased viscosity of the hemoglobin (cytoplasm) that might prevent its outflow. This could be proved indirectly by heating up the suspension slowly to 25°C after the field had been turned off. When $T = 0°$ to $+4°C$ was reached, the residual deformation that had remained from the deformation at $-10°$ or $-15°$ disappeared completely and the cells relaxed to their initial discocytic shape, but no hemolysis could be observed. On the other hand, an eventual resealing of pores formed by the electric field at subzero temperatures is not expected at temperatures 0° to $+3°C$. Kinosita and Tsong (1977b) discussed the temperature dependence of the resealing process for electric-pulse-treated erythrocytes. At 3°C the porated cells remained highly permeable even after 20 h. Thus if pores large enough to allow hemolysis were built at subzero temperatures, the hemolytic reaction would be visible at 0°C, because for this temperature we normally get hemolysis in the electric field (for $\nu = 100$ kHz even at low field strengths). For $T > 5°C$ most of the cells became echinocytes, but only a few hemolyzed. When the field was turned on again at 25°C the cells could be polarized and deformed again and showed hemolysis when $E \approx E_{lysis}$ (25°C) was reached. This would not have been possible if the cells had been highly permeable to ions before.

6. Electrical breakdown and field-induced hemolysis

In the following we will discuss the possibility that the membrane damage observed in our experiments is directly induced by the electric field. Electroporation and irreversible electrical breakdown of the cellular membrane in DC and AC fields, depending on frequency and pulse duration, have been extensively investigated for human RBCs for temperatures above 0°C. The interfacial polarization at the membrane (charge accumulations at the membrane inside and to some extent also outside the cell) caused by the electric field generates a potential difference across the membrane that might exceed the biological transmembrane potential to such a degree that poration sets in.

An AC-field-induced transmembrane potential U_m for a frequency $\nu = 1$ MHz depends on the frequency of the applied field, as $1/2\pi\nu$ approaches the electrical relaxation time of the membrane, τ_m (nonstationary conditions). For ellipsoidal particles (homogeneous interior) that have a thin membrane ($\Delta r \ll r_{cell}$) with capacitance C_m and conductance G_m (per unit area) and that have major semi-axes $f > g >$ minor semi-axis h, the maximal amplitude of the transmembrane potential at the ellipsoidal poles (the longest semi-axis $f = a/2$ is parallel to E) can be expressed as (Schwan, 1983; Bernhardt and Pauly, 1973; Jeltsch and

Zimmermann, 1979; Holzapfel et al., 1982; Marszalek et al., 1990; Stenger et al., 1991; Tsong, 1991)

$$U_m^0 = \frac{\mathbf{f}_s f E_f^0}{(1 + \alpha)} \bigg/ \sqrt{1 + (\omega \tau_m)^2} \qquad (1)$$

with the electrical relaxation times of the cell membrane τ_m and those describing the external and internal relaxation processes, τ_e and τ_i

$$\tau_m^{-1} = \tau_e^{-1} + \tau_i^{-1} \qquad (2)$$

$$\tau_e = f C_m (\sigma_i^{-1} + (\sigma_e \gamma)^{-1}) \qquad (3)$$

$$\tau_i = C_m / G_m \qquad (4)$$

with shape factor $\mathbf{f}_s$ and geometric parameter γ for ellipsoidal bodies

$$\mathbf{f}_s = 1 + \gamma^{-1} = (1 - L_f)^{-1}$$

where L_f is the elliptic integral along the f direction

$$L_f = (fgh/2) \int_0^\infty (u + f^2)^{-1} \qquad (5)$$

$$[(u + f^2)(u + g^2)(u + h^2)]^{-1/2} du$$

σ_i, σ_e are the conductivities of the internal and external medium, respectively, $\omega = 2\pi\nu$ is the angular frequency of the applied voltage $U = U^0 \sin\omega t$ and

$$\alpha = \tau_e G_m / C_m \qquad (6)$$

where $\alpha = 0$ for nonconducting membranes.

Formation of pores within the cell membrane sets in when the field-induced transmembrane potential reaches a certain critical value. For human erythrocytes this value has been determined to be 0.8 V (for $T = 25°$ to 37°C) and 0.87 V ($T = 5°C$) in experiments measuring the increase in the conductivity of the erythrocyte suspension under an 80-μs electric pulse (Kinosita and Tsong, 1979), and ~1 V in potassium release experiments after applying a 20-μs pulse (Kinosita and Tsong, 1977a).

In the following we calculate an interval for the critical electric field strength from Eq. 1 using $U_m^0 = U_{pore}^0$ between 0.8 and 1 V, assuming that for temperatures below 0°C U_{pore}^0 will not exceed 1 V. The membrane capacitance C_m was measured to be ~0.7 μF/cm^2 for $\nu = 10$–200 kHz (Takashima et al., 1988). We assume that this value will not vary strongly with increasing frequency up to 1 MHz.

As the cell subsequently elongates for increasing field strengths, the ellipsoidal semi-axes f, g, and h change their values. We measured the minor semi-axis $h = 1.5$ μm to be nearly independent of deformation, and the major semi-axis f increases and the major semi-axis g decreases with increasing E. With increasing elongation of the cell the critical field strength for pore formation is shifted to lower values (for constant U_{pore}^0) mainly due to the increasing geometric factor γ. For calculating $E_{pore}^0 = E_f^0(U_{pore}^0)$ Eq. 1 requires

the actual values $f(E_{pore}^0)$ and $g(E_{pore}^0)$ of the ellipsoidal cell, so E_{pore}^0 is obtained by successive approximation.

As long as the transmembrane potential is below U_{pore}^0 the membrane should be intact and, therefore, almost perfectly insulating. Then $G_m = 0$, $\alpha = 0$ and $\tau_m = \tau_e$ (cf. Eqs. 2, 4, and 6). Using this in Eq. 1 the calculated E_{pore}^0 then denotes the applied field strength (maximal amplitude at the poles of the elongated cell) for which pore formation in a 1 MHz AC field sets in. These results and our input data are summarized in Table 1. The conductivities of external (LISS) and internal (cytoplasm) media have been taken from impedance measurements for $\nu = 1$ MHz (Wolter and Thom, 1996; Thom and Wolter, in preparation). $E_{pore,1}^0$ and $E_{pore,2}^0$ are the critical field strengths for $U_{pore}^0 = 1$ and 0.8 V, respectively, and the geometric parameters were calculated for a mean size of an elongated cell within this critical interval by numerical integration of Eq. 5.

The critical field intervals for pore formation are displayed in Fig. 4 in terms of the effective field strength $E_{pore}^{eff} = E_{pore}^0/\sqrt{2}$, calculated from the measured effective voltage U^{eff} of the applied field. It turns out that for all temperatures examined here the calculated critical field strengths for pore formation lie at the beginning of the third deformation regime $d \sim \sqrt{E}$, but are still well below those field strengths E_{lysis} for which the breakdown of the cell polarization and the immediately following hemolysis can be observed. For 25° and 0°C the experimentally measured E_{lysis}^0 is approximately three times larger than the calculated E_{pore}^0 (for $U_{pore}^0 = 1$ V). If this is also true for $-10°$ and $-15°C$, this will explain why we cannot observe hemolysis for these temperatures. In the following we will discuss some effects that could account for the large difference between E_{pore}^0 and E_{lysis}^0. Beside these effects one has of course to bear in mind that Eq. 1 is strictly valid only for homogeneous electric fields and thus in our case can serve only as an approximation.

TABLE 1 Electrical and geometrical parameters and critical field strength (mean values of cells from Fig. 4 A–D)

Parameter	25°C	0°C	−10°C	−15°C
f_{pore} (μm)	9.2	9.4	8.6	8.6
g_{pore} (μm)	3.1	2.5	2.5	3.0
$L_f \times 10^2$	6.69	5.63	6.41	7.17
γ	13.94	16.76	14.59	12.94
f_s	1.07	1.06	1.07	1.08
$\sigma_e \times 10^4$ (S/cm)	1.54	0.74	0.52	0.45
$\sigma_i \times 10^3$ (S/cm)	4.92	2.37	1.72	1.47
$\tau_m \times 10^7$ (s)	4.32	8.09	11.47	14.49
$E_{pore,1}^0$ (kV/cm)	2.928	5.198	7.893	9.856
$E_{pore,2}^0$ (kV/cm)	2.342	4.158	6.315	7.885
E_{max}^0 (kV/cm)	9.269	15.292	18.487	14.713
U_{max}^0 (kV/cm)	3.75	3.4	2.52	1.71

E_{max}^0 for 25° and 0°C is the field strength (peak value) for which most of the cells hemolyze, for $-10°$ and $-15°C$ it denotes the maximal field strength reached in the experiment (no lysis); U_{max}^0 is the corresponding membrane potential calculated from Eq 1.

Pulse length and pore size

The calculated E^0_{pore} denotes the beginning of pore formation that can be measured as an increase of the conductance and possibly also of the permeability of cell membranes. This has been called electrical (or dielectric) breakdown, which is reversible, because the pores reseal when the electric field is turned off. To rupture the membrane (irreversible breakdown), the pore size has to be increased to an amount large enough to discharge the membrane completely (which should be visible as depolarization of the cell) and finally to allow the release of hemoglobin. This can be obtained by increasing the field intensity or the pulse duration. Kinosita and Tsong (1977a) found that the hemolysis reaction is very slow for applied electric pulses <5 μs. Zimmermann (1982), using the theory of inverted pores developed by Chizmadzhev et al. (see Refs. 265–273 in Zimmermann's review article, 1982), showed that the formation of these pores leading to irreversible membrane breakdown cannot be expected for charging times less than 1 μs (for artificial bilayer membranes). Although the field duration is quite long in our experiments (several seconds for 25°C) one half-period of the applied sine-wave for 1 MHz lasts only $\Delta t = 0.5$ μs. After carrying out our deformation experiments for $\nu = 100$ kHz it could be proved that the frequency 1 MHz is indeed the main cause for shifting E^0_{lysis} to such high values. Exposing our cell suspension at 25°C to a sine-wave pulse at $\nu = 100$ kHz for some seconds, we observed hemolysis for field strengths E^0_{lysis} between 2260 V/cm (threshold value) and 3000 V/cm (100% hemolysis), which is in good agreement with data from literature obtained from DC pulse experiments. The cell deformations for 100 kHz and 1 MHz are of the same order of magnitude, so that the electric forces stretching the cell are constant within this frequency interval. For σ_i (25°C, 100 kHz) = 4.5 $\times$ 10^{-3} S/cm, $\sigma_e = 1.54 \times 10^{-4}$ S/cm (Wolter and Thom, 1996; F. Thom, in preparation) and $U^0_{pore} = 1$ V we obtain from Eq. 1 $E^0_{pore} = 1640$ V/cm, which is very close to the measured value for E^0_{lysis}.

Underestimation of U^0_{pore}

The critical transmembrane potential $U^0_{pore} \approx 1$ V has been obtained from experiments measuring the critical (external) field strength for dielectric breakdown by applying a rectangular DC pulse of 1 μs to 1 ms duration and using the equation $U^0_{pore} = f_s f E^0_{pore}$ with $f_s = 1.5$ (spheres) and $f = 3$ μm. However, many experiments have shown that U^0_{pore} itself is strongly dependent on the pulse duration. For decreasing pulse lengths from 20 μs to 0.8 μs, U^0_{pore} was measured to increase by a factor of ~2 for algal cells (Zimmermann and Benz, 1980). On the other hand, Marszalek et al. (1990) found $U^0_{pore} \approx 0.5$ V for a 200-ms sine-wave pulse at $\nu = 0.1$–300 kHz, whereas using a 1-ms square wave DC pulse gave a $U^0_{pore} = 0.95$ V. Although these results cannot be transferred directly to the erythrocyte membrane, it is possible that for 1-MHz pulses U^0_{pore} can

exceed 1 V. If for 25° and 0°C the transmembrane potential U^0_m is calculated from Eq. 1 by using $E^0_f = E^0_{lysis}$ (see Table 1) one obtains $U^0_{lysis} = 3.75$ V (25°C) and $U^0_{lysis} = 3.4$ V (0°C). It seems to be quite improbable that a cell membrane can be polarized to such high potentials without being destroyed, so that the pulse length dependence of U^0_{pore} alone cannot account for shifting E^0_{lysis} to the observed high values for 1 MHz.

Sucrose

Hemolysis provoked by an electric field can be explained by a colloid-osmotic swelling of the cells due to the permeability increase of the membrane (uptake of water and sodium and release of potassium) induced above a critical value of the field strength. It is known that in isotonic solutions containing increasing concentrations of impermeable molecules such as sucrose the cell swelling is blocked as long as the pore size is too small to permit influx of sucrose. Thus the hemolytic reaction is slowed down, shifting the onset of hemolysis to higher field strengths, although the critical membrane potential of ~1 V remains unaffected (Zimmermann et al, 1976; Kinosita and Tsong, 1977c). Zimmermann et al. (1976) found that an amount of 30 mM sucrose added to an isotonic phosphate buffer solution already leads to a 1.5-fold higher value for E^0_{lysis}. As our external solution contains 280 mM sucrose this effect could be much stronger for our measurements. Additional investigations will show whether this concentration allows field-induced hemolysis at all for the field strengths investigated here. Comparing the results for E^0_{lysis} and E^0_{pore} for 1 MHz and 100 kHz the sucrose effect must be strongly frequency dependent if it is to account for the large shift of E^0_{lysis} for 1 MHz. Perhaps the pore size induced by the 100 kHz field already permits the influx of sucrose for low field strengths.

Membrane conductance

Another aspect of the problem is that pore formation leads to an increase of the membrane conductance. As can be seen from Eq. 1) for increasing conductance G_m the factor α can no longer be neglected, and thus the membrane potential U^0_m is lowered. Therefore the external field strength has to be increased to reach the critical transmembrane potential U^0_{pore} again. For high-frequency pulses this process can possibly retard the hemolytic reaction. Assuming that when hemolysis sets in, the transmembrane potential is ~1 V and using $E^0_f = E^0_{lysis}$ (25°C) = 9270 V/cm we calculated that the membrane conductance just before the observed breakdown of the cell polarization should be G_m (25°C) = 15.5 S/cm^2 (or $\sigma_m = 1.55 \times 10^{-5}$ S/cm). This value is not extraordinarily high ($\sigma_m = 0.1\sigma_e$) and lies in a possible range for the conductance of the perforated membrane that was estimated by Kinosita and Tsong (1979) from conduc-

tivity measurements of a cell suspension (containing 90% sucrose).

7. Hemolysis provoked by mechanical forces?

There is no doubt that for our test measurements at 100 kHz and 25°C the observed hemolysis is induced directly by the electric field. Here the cells were damaged (for $d \approx 1.9$ at $E_{lysis}^{eff} \approx 1.6$ kV/cm) before they could reach these high deformations that could be attained in the 1 MHz measurements ($d \approx 2.3$ at $E_{lysis}^{eff} \approx 8$ kV/cm). But for the 1 MHz measurements it could not be excluded from the outset that hemolysis was caused by the mechanical forces generated by the AC field. The deformation behavior of the cells at high field strengths (fourth deformation regime) leads to the assumption that, in addition to the shear deformation, the surface area of the cells might be already increased critically by the applied force. To separate the effects of mechanical and electrical breakdown we performed test measurements for RBCs in a LISS2 without Na_2HPO_4 ions giving a very low conductivity $\sigma_e = 10$ $\mu S/cm$ (25°C). If the critical transmembrane potential for pore formation in this solution is assumed to be 1 V, the critical field strength can be calculated from Eq. 1 (see section 6) to be $E_{pore}^{eff} = 11.9$ kV/cm (for 25°C and 1 MHz). Thus for our field strengths electrically induced hemolysis can be excluded for the LISS2. As Fig. 8 shows, the deformation for 25°C can be measured up to values for E that are limited only by our technical equipment. The hemolysis rate is below 20% for $E^{eff} \approx 12$ kV/cm whereas a maximal deformation $d \approx 2.4$ (or $EI \approx 0.73$) could be obtained.

For our measurements in the LISS with $\sigma_e = 154$ $\mu S/cm$ (displayed in Fig. 4) the observed hemolysis for 25° and
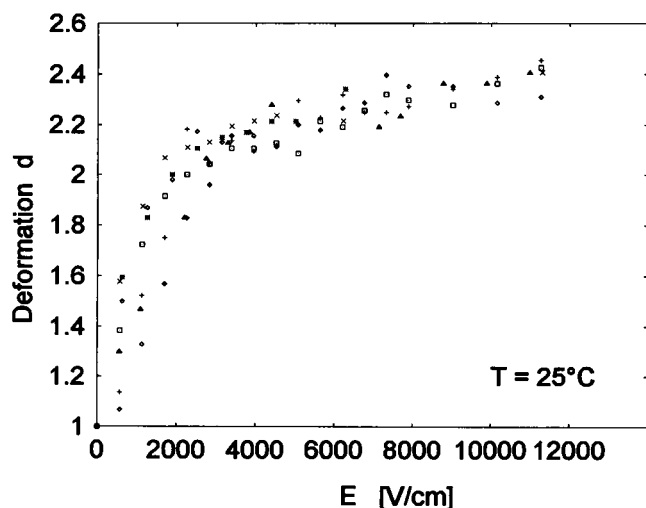

FIGURE 8 Deformation of erythrocytes as a function of the applied vacuum field strength (effective value) in a very low conductive external solution LISS2 ($\sigma_e = 10$ $\mu S/cm$). Even for $E^{eff} \approx 12$ kV/cm, the rate of hemolysis is below 20%.

0°C therefore is mainly caused by electrical breakdown of the membrane.

8. Specimen heating in the electric field

Our experiments were performed under conditions where heating effects for suspensions in electric fields are not negligible, i.e., high frequency, high field strengths, and long pulse durations. Generally, the temperature rise due to electrical heating is much smaller in low ionic solutions than, for example, in an isotonic NaCl solution. Kinosita and Tsong (1977c) obtained temperature jumps $\Delta T \approx 0.1$°C for low ionic NaCl/sucrose solutions (containing 3% isotonic NaCl) and $\Delta T \approx 3$°C for an isotonic NaCl (100%) solution (both for field strengths $E^0 \approx 4$ kV/cm). Also, Zimmermann et al. (1974) obtained a temperature rise in the NaCl solution of 3–5°C at $E^0 \approx 4.5$ kV/cm. For these experiments the influence of Joule heating on the field-induced hemolysis could be excluded. However, these measurements were performed by applying a short DC pulse to the sample.

We examined the heating of our external (LISS) solution in the alternating electric field (1 MHz) using the technique of liquid-crystal thermography (unpublished). The basic temperature of the sample was fixed at 9°C. A defined field strength was then applied, and the time-dependent temperature rise in the solution between the electrodes of the measuring chamber was recorded by a video camera. The value for the temperature rise, ΔT, leading to a new local steady-state temperature between the electrodes was then determined spectroscopically. The results are that 1) the temperature rise ΔT increases linearly with $(E^{eff})^2$ and 2) for the temperature rise for several applied (effective) field strengths, $\Delta T \leq +1$°C (for $E^{eff} \leq 3$ kV/cm), +2°C (4 kV/cm), +3.5°C (5 kV/cm), and +6.5° (7.4 kV/cm), with an accuracy of ±1°C.

These moderate values for ΔT even at high field strengths result mainly from the high heat conduction within the aqueous (LISS) solution driven by the temperature gradient between the region of heat formation and the cooled basis of the experimental system. The heat conductivity of water is decreased by 7% with decreasing temperature between 25° and 0°C (Lax, 1967). This would mean that ΔT is slightly higher at 0°C compared with our measured values at 9°C. But in the interval $T = 25$° to 0°C a temperature rise of maximally 2°C (for $E^{eff} \leq 4$ kV/cm) would lead to a negligible increase for the deformation. Even for field strengths exceeding 4 kV/cm a severe increase for the deformation is not possible as the deformation generally increases only weakly in the fourth deformation regime due to the limited cell geometry. It is also quite improbable that a temperature jump of ~6°C could be responsible for the observed hemolysis at 25°C.

For subzero temperatures (that means for supercooled systems) there are, to the authors' knowledge, no data available for the heat conductivity.

CONCLUSIONS

At 25°C the deformation function $d_\infty(E)_T$ for human discocytic RBCs obtained by the electric field method qualitatively agrees with results from mechanical deformation techniques. The cells can be highly elongated (up to 2.3 times their initial diameter) without hemolysis.

Below 0°C, both the deformation behavior and the stability of erythrocytes exhibit distinct deviations from the behavior observed between 25° and 0°C. Whereas the viscoelastic response time τ_{res} (determined mainly by the membrane/cytoplasm viscosities) and the steady-state deformation value d_∞ (correlated to the membrane elastic shear modulus) change only moderately between 25° and 0°C, τ_{res} increases and d_∞ decreases strongly below 0°C. Both the increase of τ_{res} and the decrease of $d_{\infty,E}(T)$ between 0° and -15°C proved to be especially high for low deformations (at low field strengths). In addition to altered membrane properties at subzero temperatures the following items that could have an influence on the applied electric force should also be taken into consideration: 1) for a given field strength (in a homogeneous field) the electric force acting on the ellipsoidal cell increases linearly with increasing elongation (Landau and Lifschitz, 1967; and unpublished data), 2) the field strength in our inhomogeneous field decreases with the distance from an electrode; i.e., with increasing elongation the electric force acting on the cell ($\sim E^2$) decreases continually, and 3) the conductivities of the cell cytoplasm and the external solution are decreased by 70% between 25° and -15°C. Thus the interval for which the Maxwell tension acting on the cell is constant is shifted to lower frequencies. For the frequency 1 MHz therefore the acting electric force could be lowered for subzero temperatures, leading to a decreased deformation for the same field strength. Items 1 and 2 are counteracting, but the decrease of the force in 2 with the elongation is stronger than the linear increase in 1, which can be determined from the equation for the field strength. Thus, for a fixed voltage U the resulting force would be even higher for the low defor-

mations at subzero temperatures. The contribution of 3 cannot be discussed up to now because it should be determined by calculating the Maxwell tension using the permittivities for subzero temperatures.

However, the most important result of our measurements is that erythrocytes can be deformed to appreciable values ($d_\infty \approx 2$) even at temperatures below 0°C (for high field strengths) without destruction. For $T < 0$°C we observed no cell hemolysis.

For the frequency 1 MHz the values for the critical field strength, E^0_{lysis}, for which the polarization breakdown and hemolysis of the cells were observed for $T \geq 0$°C are three times higher than the calculated critical field strengths for electrically induced pore formation, E^0_{pore}. As E^0_{pore} is shifted to higher field strengths with decreasing temperature, the expected values for E^0_{lysis} at $T < 0$°C exceed our possible maximal field strength, and thus hemolysis could not be observed for 1 MHz.

However, the measurements for 100 kHz lead to the conclusion that there must be another mechanism for retarding pore growth at subzero temperatures. For this frequency the observed E^0_{lysis} at 25°C lies at low field strengths and close to the calculated E^0_{pore}. But nevertheless no polarization breakdown or hemolysis occurred for test measurements at -10° and -15°C.

The fact that the maximal deformation $d \approx 2.3$ that could be obtained for 25° and 0°C before hemolysis sets in could not be reached for the temperatures below 0°C can at first sight lead to the assumption that hemolysis for $T \geq 0$°C was caused by mechanical forces arising from the high deformation. But our results show that the observed hemolysis is mainly caused by electrical membrane breakdown, because even higher deformations ($d \approx 2.4$) nearly without hemolytic reactions could be obtained in an external solution of lowered conductivity, for which the onset of electrically induced pore formation is shifted to very high field strengths.

For E^{eff} below 4 kV/cm in the interval $T = 25$° to 0°C the temperature rise in the external medium due to electrical

TABLE 2   Computed geometrical parameters for gradually elongated ellipsoids under constant volume (two representative values)

V = 96 μm³					V = 139 μm³				
f	g	h	S	a/a₀	f	g	h	S	a/a₀
4.2	4.20	1.30	131.32	1.00	4.7	4.71	1.50	166.00	1.00
4.5	3.92	1.30	131.35	1.07	5.0	4.42	1.50	166.10	1.06
5.0	3.48	1.32	131.30	1.19	6.0	3.60	1.54	165.90	1.28
5.5	3.11	1.34	131.30	1.31	7.0	2.93	1.62	166.00	1.49
6.0	2.78	1.37	131.39	1.43	8.0	2.21	1.87	166.00	1.70
7.0	2.15	1.52	131.30	1.67	**8.1**	**2.20**	**2.20**	**165.98**	**1.72**
7.37	**1.76**	**1.76**	**131.32**	**1.76**	8.3	2.00	2.00	167.74	1.76
7.7	1.72	1.73	133.96	1.83	8.5	1.95	2.00	169.52	1.80
8.0	1.68	1.70	136.18	1.90	9.0	1.94	1.90	173.86	1.91
8.4	1.67	1.63	139.24	2.00	10	1.84	1.80	182.39	2.13
9.0	1.59	1.60	143.68	2.14	11	1.77	1.70	190.70	2.34
10	1.53	1.50	150.94	2.38					
11	1.44	1.45	158.06	2.62					

Semi-axes f, g, h in μm, surface area S in μm². V is the cell volume, and $a/a_0 = f/f_0$ represents the deformation.

heating is below 2°C, for higher field strengths d increases only weakly in any case. Thus the deformation curves will not be falsified at least for $T \geq 0°C$. A temperature jump of 6° to 7°C measured for $\sim7-8$ kV/cm will not be the cause for the observed hemolysis for $T \geq 0°C$.

APPENDIX

In the following the elongation of an oblate ellipsoid with semi-axes $f = g > h$ along the f axis resulting in a prolate ellipsoidal body $f > g \geq h$ will serve as a simplified model to describe the deformation of an erythrocyte by a uniaxial force parallel to f. Starting with initial axes f_0, g_0, h_0 corresponding to a given cell volume V and an initial surface area S_0, the major semi-axis f was stepwise increased and then the solutions for g,h and S satisfying the equation for the surface area of an ellipsoidal body with the boundary conditions of 1) constant volume, 2) constant surface area $S = S_0$, and 3) $g \geq h$ (there exist symmetrical solutions $h \geq g$) were calculated numerically. The aim was to determine the maximal value for f, f_{max} to fulfill the conditions of constant surface area and volume. For $f > f_{max}$ the surface area of the ellipsoid has to be increased to obtain solutions for g and h fulfilling the condition of constant volume.

These calculations have been performed for two different cell volumes. $V = 96 \pm 2$ μm^3 and $f_0 = g_0 = 4.2 \pm 0.13$ μm represent mean values from the top fraction of age-fractionated human erythrocytes (young cells) measured by Nash and Meiselman (1983). The value for h_0 then follows from $V = 4\pi fgh/3$. The second volume $V = 139$ μm^3 follows from the mean values $f_0 = g_0 = 4.7 \pm 0.5$ μm and $h_0 = 1.5 \pm 0.25$ μm obtained from our measurements of the unstressed cells. The configuration for the maximal value f_{max} in Table 2 is printed in bold numbers. The values for the maximal deformation under constant surface area and volume $d_{max} = a_{max}/a_0 = f_{max}/f_0$ for the two different volumes differ only by 2%; thus a mean value $d_{max} = 1.74$ can be obtained.

If the surface area of a human RBC is increased by more than 6%, hemolysis will occur (Blackshear, 1987). For both values of V this would lead to $d_{lysis} \approx 2$ in our simple model. The true value for d_{lysis} will be higher due to the biconcave shape of the erythrocyte resulting in a higher surface area for the same volume.

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