Studies of Cell Pellets: II. Osmotic Properties, Electroporation, and Related Phenomena: Membrane Interactions

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ABSTRACT Using the relations between pellet structure and electric properties derived from the preceding paper, the responses of rabbit erythrocyte pellets to osmotic or colloidal-osmotic effects from exchanged supernatants and from electroporation were investigated. Changing the ionic strength of the supernatant, or replacing it with dextran or poly(ethylene glycol) solutions, caused changes of R_p according to the osmotic behavior of the pellet. R_p was high and ohmic before electroporation, but dropped abruptly in the first few microseconds once the transmembrane voltage exceeded the membrane breakdown potential. After the initial drop, R_p increased as a result of the reduction of intercellular space. R_p increased regardless of whether the pellets were formed before or immediately after the pulse, indicating that porated cells experienced a slow colloidal-osmotic swelling. The intercellular or intermembrane distances between cells in a pellet, as a function of osmotic, colloidal-osmotic, and centrifugal pressures used to compress rabbit erythrocyte pellets, were deduced from the R_p measurement. This offered a unique opportunity to measure the intermembrane repulsive force in a disordered system including living cells. Electrohemolysis of pelleted cells was reduced because of limited swelling by the compactness of the pellet. Electrofusion was observed when the applied voltage per pellet membrane exceeded the breakdown voltage. The fusion yield was independent of pulse length greater than 10 μs, because after the breakdown of membrane resistance, voltage drop across the pellet became insignificant. Replacing the supernatant with poly(ethylene glycol) or dextran solutions, or coating pellets with unporated cell layers reduced the colloidal-osmotic swelling and hemolysis, but also reduced the electrofusion yield. These manipulations can be explored to increase electroloading and electrofusion efficiencies.

INTRODUCTION

In the preceding paper, we describe the rabbit erythrocyte pellets as porous bodies composed of closely packed soft particles. This is based on electrical resistance measurements, which indicate that the pellet porosity, ϵ, does not exceed several percent, whereas the intermembrane distance, d, is in the 100-Å scale. The pellet structure, however, is very sensitive to experimental conditions. Thus, both ϵ and d decrease when pellets are under acceleration, G, treated with a high-voltage electric pulse, or placed in a hypertonic environment.

This paper aims to expound the porous structure properties of erythrocyte pellets. In particular, their osmotic properties, their behavior in a strong electric field, and the field-induced effects such as swelling, electrohemolysis, and electrofusion are considered in detail. A unique opportunity to measure directly interacting forces between cell membranes is proposed.

MATERIALS AND METHODS

Cells, solutions, experimental setup, and procedures are described in the preceding paper. Only those details specific for this paper are added here.

Cells and solutions

Rabbit erythrocytes from fresh blood were prepared as described in the preceding paper. In electrofusion experiments, erythrocytes were labeled with DiI (Molecular Probes, Eugene, OR) by adding 10 μl of 3.5 mg/ml DiI in ethanol to 1 ml of cell suspensions.

Solutions of different tonicities were prepared by adding solutes to PBS. Both poly(ethylene glycol) (PEG) (8 kDa) and dextran (8, 40, and 500 kDa) (Sigma Chemical Co., St. Louis, MO) solutions contained at least 50 mM phosphate to provide a suitable electroconductivity. To follow the penetration of macromolecules in erythrocyte pellets, FITC-dextran of 8 or 40 kDa (Sigma) was used.

Chambers and equipment

Most experiments were performed using the centrifuge chamber described in the preceding paper. A similar chamber with a 6-mm diameter-hole and adjustable interelectrode distance was used to treat large (up to ~300 μl) volumes of cell suspensions with high-voltage pulses.

Diffusion of FITC-dextran in pellets was observed under a fluorescent microscope (Olympus IMT-2) using microchambers made from 2 mm glass tubes with one end sealed by a hematocrit capillary sealer (Clay-Adams, NY).

Eppendorf centrifuge and microtubes were used in some experiments on electrohemolysis.

Procedures

Erythrocyte pellets

Typically, 1 ml of suspension containing a required number ($2–4 \times 10^7$) of erythrocytes was exposed to a constant acceleration G ($900 \times g$ unless otherwise noted) for at least 5 min to form a compact pellet in the regular centrifuge chamber. In some experiments, erythrocytes were pretreated in suspension with a high-voltage pulse in the large volume chamber. By adding cells into the chamber in steps, composite pellets containing differently treated cells (e.g., lower layers containing cells already subjected to high-voltage pulses, and higher layers containing unpulsed cells) could be

Received for publication 20 December 1993 and in final form 11 April 1994.

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Abbreviations used: DiI, 1,1′dioctadecyl-3,3,3′,3′tetramethylindocarbocyanine; FITC-dextran, fluorescein isthiocyanate dextran; PEG, poly(ethylene glycol).

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0006-3495/94/07/427/09 $2.00

made. Experiments were also performed with pellet systems where the original supernatant was gently replaced with one of a different ionic strength or osmolarity.

Response of erythrocyte pellets to different stimuli

This response was recorded as changes in the pellet resistance R_p, which was measured by using low-voltage rectangular waves as described in the preceding paper.

Processes developing in erythrocyte pellets

Processes developing in erythrocyte pellets during high-voltage pulses were followed similarly by current going through the pellet system. Currents recorded at given time points (usually at the end) of high-voltage pulses were used to plot "instant" $I(U)$-curves.

Hemolysis of red cells

Hemolysis of red cells in suspensions or in pellets, due to an osmotic or electric shock, was determined by the concentration of hemoglobin in the supernatant, measured spectroscopically at 415 nm. Pelleted cells were resuspended first before recentrifugation for hemoglobin determination. The percentage of hemolysis, F_h, is defined as the absorption measurement at 415 nm, as compared with that from 100% lysis by diluting the sample in distilled water.

Cell electrofusion in pellets

Cell electrofusion in pellets was performed using DiI-labeled cells. (Cells were labeled to make counting fused or unfused cells easier, especially when they became hemolyzed after high-voltage treatment.) Cells were usually subjected to one high-voltage pulse; then they were resuspended and counted under a fluorescent microscope. Usually, only unfused cells were counted to determine the fusion yield as

$$F = (1 - N_{uf}/N_o) \cdot 100\% \quad (1)$$

where N_{uf} is the number of cells remaining unfused after pulse treatment and N_o is the total number of cells.

Penetration of macromolecules in erythrocyte pellets

Penetration of macromolecules in erythrocyte pellets was also estimated by means of fluorescent microscopy. FITC-labeled dextran (8.8 or 39 kDa) was introduced directly into the supernatant in microchambers.

RESULTS

Effect of supernatant on erythrocyte pellets

As found in the preceding paper, erythrocyte pellets are very sensitive to the supernatant composition. Thus, when the NaCl concentration in the supernatant is changed after the pellet is formed, the pellet resistance R_p also changes from the initial value. There is a certain asymmetry in the behavior of R_p when the original PBS is replaced by either a more diluted or a more concentrated solution. If the original (e.g., 100 mM) solution (*curve* 1′, Fig. 1) is replaced by a more concentrated (e.g. 150 mM) solution (curve 2), R_p drops to a value typical for pellets formed directly in that (e.g. 150 mM) solution (compare minimal values for curves 2, 4 and 6′). However, if the supernatant is replaced by a more diluted solution, R_p first increases, sometimes by more then one order of magnitude, and then decreases slowly (curve 3) to a level even lower than that expected if the cell pellet were to be formed directly in that diluted solution (compare curves 3 with 1, 1′ and 5, 5′). It should be mentioned also that the thicker is the pellet, the slower the R_p changes after any manipulations of the supernatant.

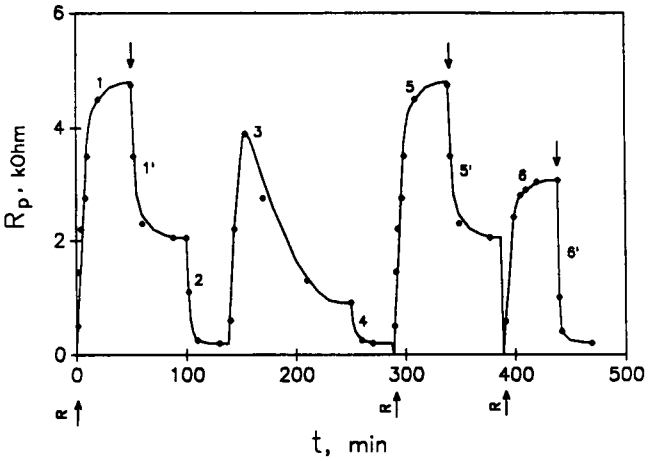

FIGURE 1 Effect of PBS concentration on the pellet resistance. The pellet was formed from a suspension of 4×10^7 erythrocytes in hypotonic (100 mM NaCl) PBS at $900 \times g$ (*curve* 1). Then, after stopping the centrifuge and pellet relaxation (*curve* 1′), the supernatant was replaced with isotonic (150 mM) PBS (*curve* 2). Further manipulations (*curves* 3–6′) included supernatant replacements with hypotonic (100 mM, *curve* 3) or isotonic PBS (150 mM, *curve* 4) as well as resuspending and repelleting of erythrocytes in hypotonic (100 mM, *curves* 5 and 5′) and isotonic PBS (150 mM, *curves* 6 and 6′) as shown by arrows. Up and down arrows mean starts and stops of centrifugation; R shows resuspension by shaking before pelleting.

R_p increase is observed also when isotonic PBS is replaced with hypertonic solutions containing macromolecules like PEG or dextran (Fig. 2). In such cases R_p increases with the supernatant tonicity, but the final result depends more on the nature and molecular weight of the polymers than on their

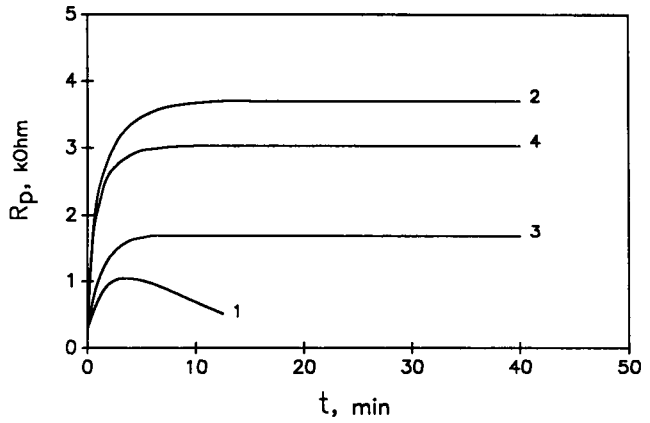

FIGURE 2 Effect of adding PEG and dextran in supernatant on the pellet resistance. Pellets were formed from 1.5×10^7 erythrocytes in isotonic PBS (300 mOsm); then, to the supernatant were added: (*curves* 1–3) dextran 8 kDa, 30%; 40 kDa, 40%; 500 kDa, 20%; (*curve*) 4 (PEG 8 kDa, 30%).

molar concentration. For instance, for supernatants containing such macromolecules as PEG (8 kDa) and dextran (40 and 500 kDa), R_p reaches higher levels in ~ 5 min and stays there for hours. However, unlike PEG (8 kDa), dextran (8 kDa) induces only a transient increase of R_p (compare curves 1 and 4, Fig. 2). A similar short transient effect was also observed with sucrose (0.3 kDa) (data not shown here).

Effect of high-voltage pulses on the pellet resistance

The resistance of multilayer pellets, R_p, is usually much higher than the resistance of the chamber filled with pure PBS or erythrocyte suspension, R_{ch}; therefore, the voltage applied to the chamber drops mostly across the pellet. Treatment of cell pellets with high-voltage pulses leads to a drastic decrease of R_p, reflecting an electrical breakdown of cell membranes. Current responses of the pellet system to 30-μs pulses with variable high-voltage, U_o, are shown in Fig. 3 *a*.

Fig. 3 *b* shows "instant" $I(U_o)$ curves measured at the ends of pulses with chambers containing no pellet (*curve* 1) and with pellets containing 4 or 8 × 10^7 cells (*curves* 2 and 3). For the pellet-free chamber, $I(U_o)$ curve obeys Ohm's law, i.e., this is a straight line with a slope $1/(R_{ch} + R_L)$. Curves 2 and 3 are more complicated: their initial slopes are lower and equal to $1/(R_p + R_{ch} + R_L)$ at low voltages, then increase to $1/(R_{ch} + R_L)$ at higher U_o. The deflection points of the curves correspond to "critical" breakdown voltages for given cell pellets. Note that to the right of the deflection point, curves 1–3 are almost parallel. It means that, at high U_o, the voltage drop on the pellet U_p remains almost constant, giving U_m ~ 0.8 V per membrane as calculated from the number of cell layers (see Discussion).

The behavior of the pellet resistance in a longer time scale is shown in Fig. 4 *a*. In these experiments, 2 × 10^7 erythrocytes were pelleted in isotonic PBS at 900 × *g* for 10 min while R_p continuously increased up to ~1 kOhm. After the application of a 250 V, 10-μs pulse, R_p dropped at least 90% within the first microsecond, then rose to over ~4 times the initial value. However, ~5 min later, R_p started to decrease slowly, dropping ~4 times in 40 min. Stopping the centrifuge (shown by an *arrow*) had no effect on the decrease of R_p. The

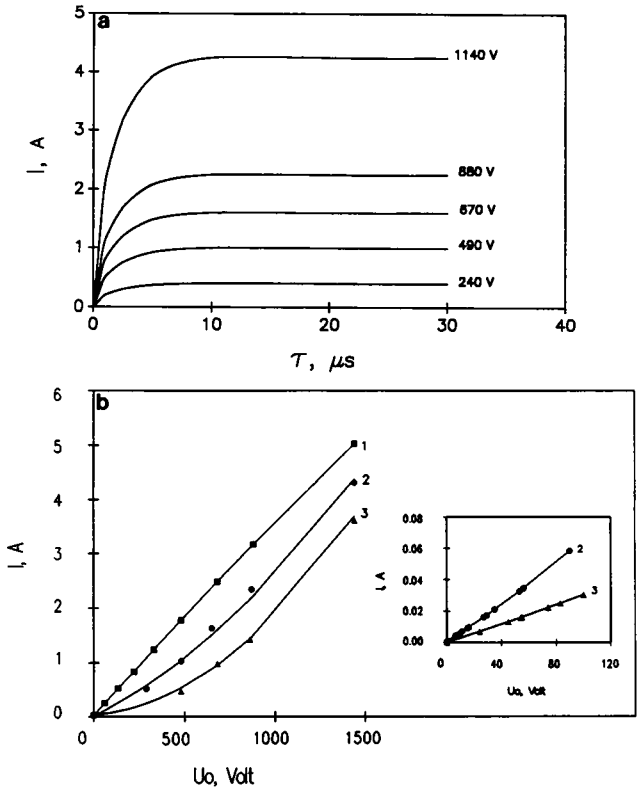

FIGURE 3 (*a*) Current through a pellet formed from 2 × 10^7 cells in isotonic PBS. 30-μs pulses of increasing amplitude (as indicated for each trace) were applied to the pellet system in 5-s intervals. (*b*) "Instant" current-voltage curves measured at the ends of 30-μs pulses of different amplitudes for the chamber without (*curve* 1) and with pellets formed from 2 × 10^7 (*curve* 2) and 4 × 10^7 cells (*curve* 3) in isotonic PBS. (*b, inset*) Expanded low voltage regions of the lower two curves in *b* showing linear (ohmic) relationship.

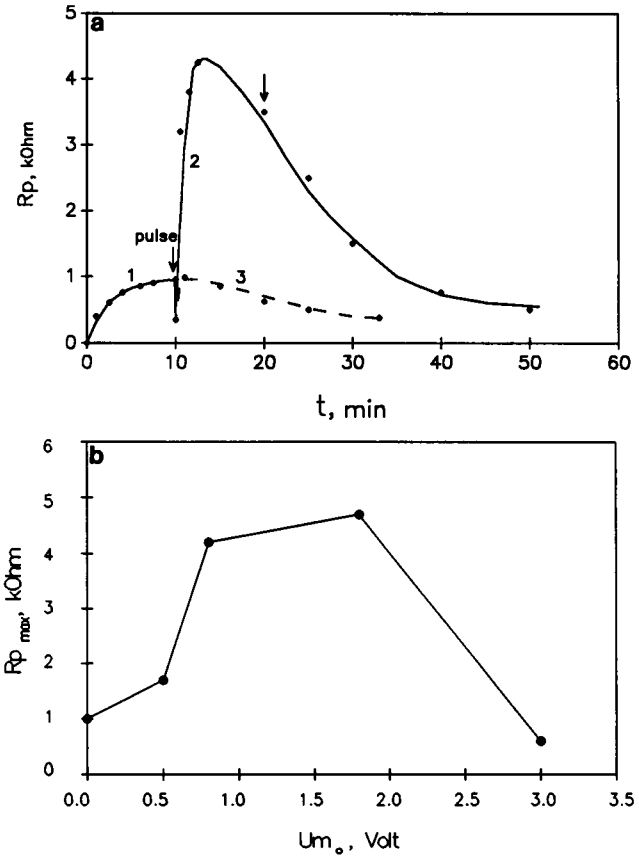

FIGURE 4 (*a*) Effect of high-voltage pulse treatment on the pellet resistance. 2 × 10^7 erythrocytes were pelleted in isotonic PBS at 900 × *g* for 10 min while R_p continuously increased up to ~1 kOhm (curve 1). After application of a 250 V, 10-μs pulse, R_p drops very rapidly (within a microsecond scale) then rises again exceeding initial value. However, about 5 min later, R_p starts to decrease slowly, dropping about 4 times in 40 min. Stopping the centrifuge (shown by an *arrow*) has no effect on the decrease of R_p (*curve* 2). In 20 mM dextran-containing media (M_r 8800), the pellet resistance increase after pulse treatment is almost unnoticeable (*curve* 3). (*b*) Maximal values of R_p after 10-μs pulses as a function of the equivalent voltage across each cell membrane U_{mo}, regardless of breakdown.

maximal values of R_p depended on the applied voltage U_o, as seen from Fig. 4 *b*. The value of U_o that causes the maximum increase of R_p corresponds to an equivalent applied voltage per membrane of about 1.8 V. A similar effect was observed earlier with L-cells (Abidor et al., 1993): perhaps higher electric field damages cell membranes irreversibly, such that cell swelling is less pronounced.

The behavior of erythrocyte pellets after the application of high-voltage pulses depends highly on the pellet history. As seen from Fig. 5 *a*, for pellets formed from erythrocytes already subjected to a high-voltage pulse in suspension (*curve* 2), R_p is much higher than that for pellets formed from intact erythrocytes (*curve* 1). Moreover, R_p increases continuously even after stopping the centrifuge (*curve* 3). Furthermore, if a pellet subjected to pulse treatment is coated with a layer of intact erythrocytes, R_p stays at the maximal level for a long time (compare *curves* 3 and 4 in Fig. 5 *b*). It is noteworthy also that for pellets formed from 1, 2, and 5×10^7 erythrocytes, maximal values of R_p after pulse treatment increase with the pellet thickness, about 2, 4, and 7 times the initial value (not shown).

Adding macromolecules like PEG and dextran after pulse application leads to an even higher increase of R_p. However, final results are practically the same as for pellets without pulse treatment. Effects of macromolecules alone on the pellet resistance are already given in Figs. 2 and 4 of the preceding paper.

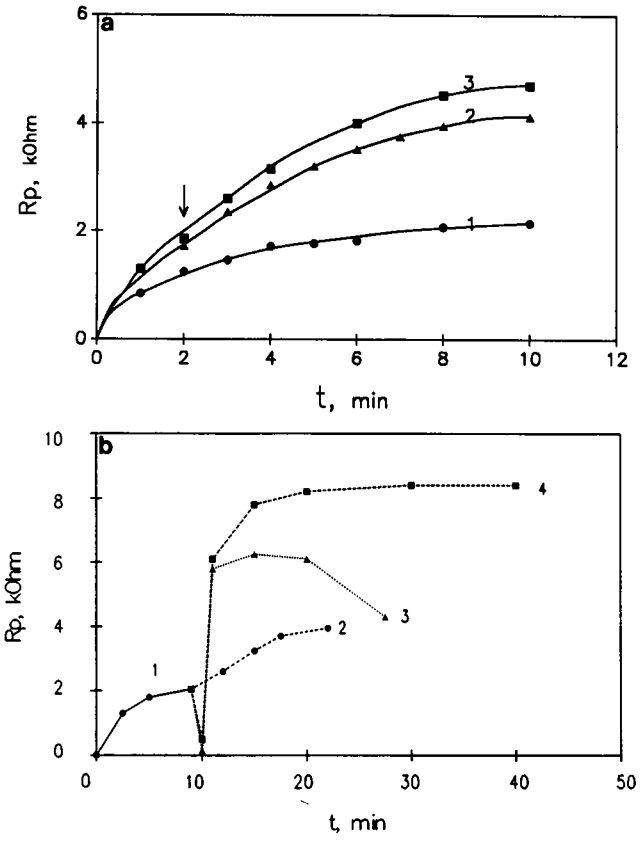

FIGURE 5 (*a*) Effect of a prior pulse treatment in suspension on R_p for pellets formed from 5×10^7 erythrocytes in isotonic PBS at $900 \times g$. (Curve 1 (control)) The pellet was formed from intact erythrocytes without prior pulse treatment. (Curve 2) Erythrocytes in suspension were treated with a 2.3 kV/cm, 40-μs pulse and then were immediately spun-down at $900 \times g$. (Curve 3) Same conditions as for curve 2, except that after 2 min the centrifuge was stopped as shown by an arrow. (*b*) Effect of pellet coating with intact erythrocytes on the pellet resistance. Pellets were formed from 5×10^7 erythrocytes in PBS at $900 \times g$ for 10 min (*curve* 1). For control (curve 2), one pellet was covered with the same number of erythrocytes at continued centrifugation. As expected, R_p increases twice (*curve* 2). If, after formation, the pellet was treated with a 450 V, 30-μs pulse (*curve* 3) R_p first dropped to a very low level rises to a maximal value of 6.5 kOhm, followed by a slow decrease (comparable with *curve* 2 in Fig. 4 *a*). Coating of the pellet immediately after pulse led to an increase of the total pellet resistance (*curve* 4, more than 4 times comparing with control curve 2); moreover, R_p stayed at this level for a long time.

Electrohemolysis of red cells in suspensions and pellets

Data on the extent of electrohemolysis F_h are given in Fig. 6 *a*. Suspensions and pellets formed from 5×10^7 eryth-

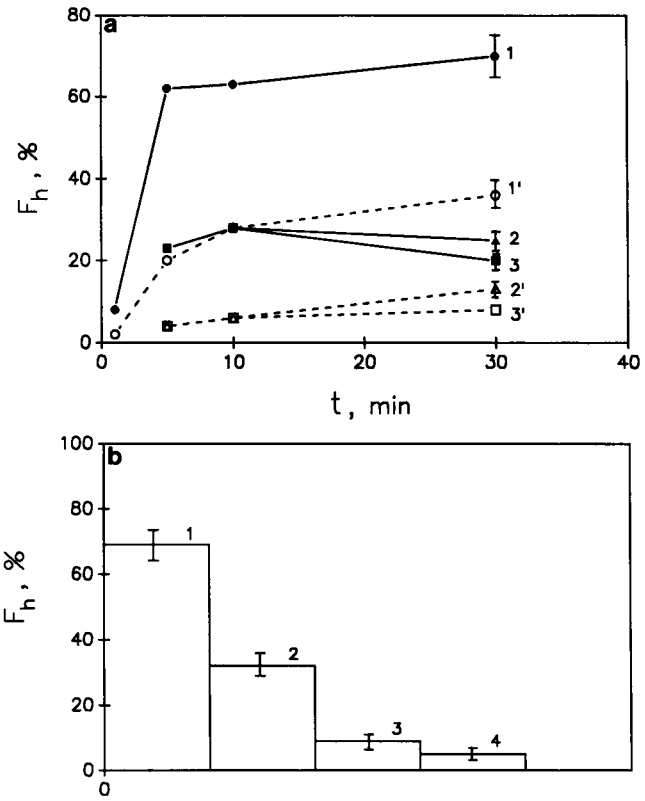

FIGURE 6 (*a*) Extent of electrohemolysis F_h of 5×10^7. erythrocytes exposed to 30-μs pulses of 3.3 (*curves* 1–3) and 2.3 kV/cm amplitude (*curves* 1′–3′) in PBS suspensions (*curves* 1 and 1′) and in pellets either formed from suspensions immediately after pulse treatment (*curves* 2 and 2′) or subjected to electric pulses after pellet formation (*curves* 3 and 3′). (*b*) Effect of resuspension and coating on F_h for pellets formed from 5×10^7 erythrocytes in PBS. (Column 4 (control)) Pellets formed from PBS suspension at $900 \times g$ for 20 min were treated with a 1 kV, 30-μs pulse; 30 min later they were resuspended to measure F_h. (Column 1) The same pellets were resuspended immediately after pulse treatment. (Column 2) After resuspension, pellets were pelleted again for 30 min. (Column 3) Repelleted samples treated as in column 2 were additionally coated with 5×10^7 intact erythrocytes.

rocytes in PBS were exposed to 30-μs pulses of 3.3 and 2.3-kV/cm amplitude. As expected, 3.3-kV/cm pulses cause more extensive hemolysis (*curves* 1–3) than 2.3-kV/cm pulses (*curves* 1′–3′), but results are qualitatively similar. In comparison, after subjected to electric pulses in suspension, erythrocytes suffer massive and fast hemolysis (*curves* 1 and 1′). Pelleting cells just after pulse treatment with continuous centrifugation (*curves* 2 and 2′) or with only 1 min centrifugation (*curves* 3 and 3′) notably reduces the extent of hemolysis.

The extent of hemolysis in pellets, however, depends highly on the pellet history, as illustrated by Fig. 6 *b*. Control (*column* 4) pellets formed from 5×10^7 erythrocytes in PBS were treated with a 1 kV, 30-μs pulse, and 30 min later pellets were resuspended. A moderate percentage (~6%) of released hemoglobin was observed. When pellets were resuspended immediately after pulse and 30 min later, F_h increased to 70% showing massive hemolysis (*column* 1). If, however, erythrocytes resuspended after the pulse were immediately repelleted, F_h dropped to 30% (*column* 2). The extent of hemolysis in pellets decreased even more when repelleted samples treated as in the previous case were coated immediately with 5×10^7 intact erythrocytes (*column* 3).

Electrofusion in erythrocyte pellets

As seen from Fig. 7 *a*, the fusion yield F of rabbit erythrocytes in pellets increases up to ~50% with the amplitude of fusogenic pulses. However, for cells in the pellet form, F does not depend on the pulse duration (Fig. 7 *b*), as was expected, because membrane electroporation occurs within the first few microseconds of pulse application (Fig. 3 *b*).

To study the effects of osmotic compression of cell pellets on electrofusion, special experiments were performed. Pellets were formed from 2×10^7 erythrocytes in isotonic PBS (Fig. 8, *columns* 1–4) or in 300 mM sucrose (+5 mM NaP_i) solution, sedimented at $900 \times g$ for 20 min and then were treated with 1 kV, 30-μs pulses. In the control sample (*column* 1), for pellets exposed to electric pulses immediately after formation, the fusion yield F was very high (up to ~45%). Adding 40% dextran (8.8 kDa), or especially 40% PEG (8 kDa) before pulse application (*columns* 3 and 2) led to a drastic decrease in F. If, however, before pulse treatment, the supernatant containing dextran was replaced with PBS (*column* 4), the fusion yield became even higher than that of the control (reaching ~60%). Finally, when pellets were formed in sucrose solution, but after pulse treatment, the supernatant was replaced with PBS (*column* 5), the electrofusion efficiency was virtually the same as in control samples.

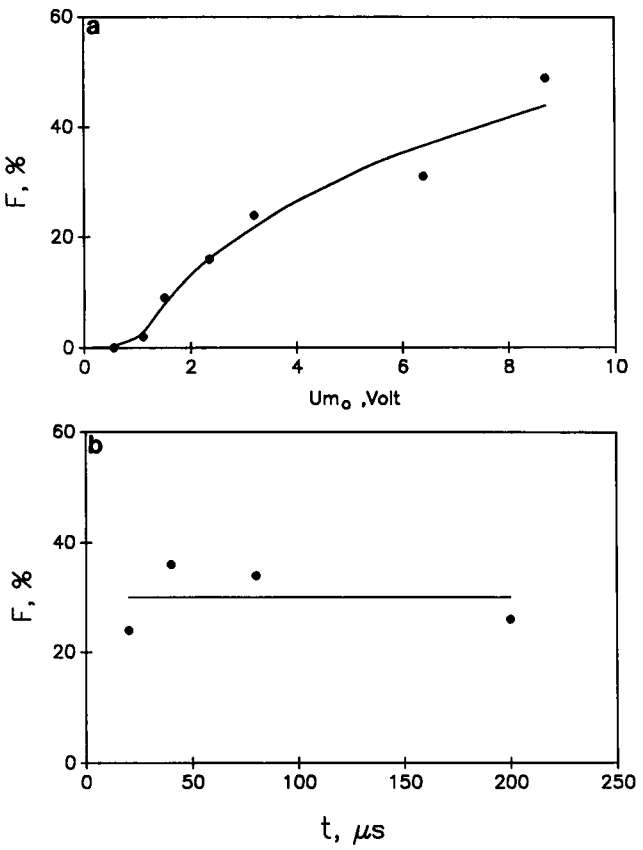

FIGURE 7 Effect of the pulse amplitude/membrane U_{mo} (*a*) and pulse duration t (*b*) on the fusion yield F for pellets formed from 2×10^7 erythrocytes in PBS.

DISCUSSION

Electroporation in cell suspensions and pellets

Electroporation in cell pellets is considerably different from the traditional concept of electroporation of cells in suspension. Electropore formation and resealing have been studied comprehensively in cell suspensions (Kinosita and Tsong, 1977) and in single cells. The transmembrane potential U_m imposed by a pulse on a single cell in suspension can be calculated precisely. However, finding U_m induced in cell pellets is a more difficult task. Because the pellet resistance R_p is comparable with or often even higher than the chamber resistance R_{ch}, a considerable part of the applied voltage U_o drops across the pellet. This voltage drop U_p can be expressed as

$$U_p = U_o \cdot R_p/(R_p + R_{ch} + R_L). \quad (2)$$

The relation between U_m and U_p is less precise. U_m varies over the cell membrane: it is maximal on the areas normal to the field lines and zero on the parallel areas. The average value of U_m can be estimated by dividing U_p by the number of cell membranes crossed by electric field lines in the pellet. Because every cell is crossed by the field lines twice, U_m can be expressed through the number of cell layers L in the pellet:

$$U_m = U_p/2L \quad (3)$$

The problem, however, is that L is not a well defined value. It depends on the cell packing in the pellet. Reorientation of nonspherical cells may change L and, therefore, change the pellet thickness and U_m considerably. These uncertainties make quantitative studies on electroporation and related phenomena in cell

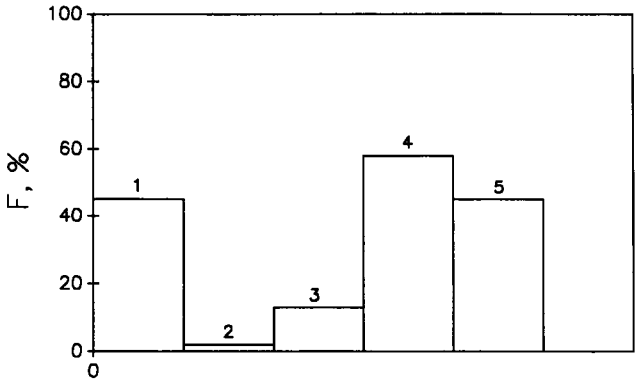

FIGURE 8 Effect of different manipulations with supernatant on electrofusion in pellets formed by centrifugation of 2×10^7 erythrocytes at $900 \times g$ for 20 min. in isotonic PBS (columns 1–4) or 300 mM sucrose (+5 mM NaPi) solution (column 5). All pellets were treated with 1 kV, 30-μs pulses. (Column 1 (control)) Pellets were subjected to pulse treatment immediately after formation. (Column 2) After cell pelleting, 40% PEG (8 kDa) was added to the supernatant; 5 min later, pellets were treated with electric pulses then diluting PEG to count FY. (Column 3) The same as in column 2, except that 40% dextran (8.8 kDa) was used instead of PEG. (Column 4) Conditions were the same as for column 3; however, before pulse treatment, the dextran-containing supernatant was replaced with PBS. (Column 5) Pellets were formed in sucrose solution; after pulse treatment, the supernatant was replaced with PBS.

pellets more difficult. On the other hand, with the same applied electric fields, the transmembrane potentials in cell pellets are much higher compared with that in cell suspensions. This makes the pellet method experimentally advantageous, because much lower voltages are required by the pellet to achieve the same effect in cell suspension.

What happens in cell pellets during electrical breakdown induced by a rectangular high-voltage pulse? In relatively strong fields, the current passing through a cell pellet system rises to a maximum very rapidly (Fig. 3 *a*). Fast (in μs-scale) reversible electrical breakdown is well known for cell suspensions; thus, at the first sight, in strong electric fields, cells in pellets and in suspensions behave similarly. However, there is an essential difference: during electrical breakdown, suspending cells experience the same electric field, whereas in pellets the electric field drastically decreases. The latter is obvious because the pellet resistance R_p decreases as cell membranes become more conductive. The pellet voltage U_p (and, therefore, the membrane voltage U_m) first drops very fast and then becomes almost independent of the applied voltage U_o. Such behavior of U_p (calculated using Eq. 2 and Fig. 3 *b*) at increasing U_o indicates that during electrical breakdown the pellet resistance is determined by membranes rather than by the intercellular space of the porous pellet structure, such that the transmembrane potential U_m remains constant (~0.8 V), and the membrane resistance decreases proportionally to the passing current. A similar effect was also observed earlier with mouse L-cell pellets (Abidor et al., 1993).

The fast increase of the pellet conductivity in strong electric fields is believed to be caused by the appearance of a large number of pores in cell membranes. Because during short electric pulses used for cell electroporation the pellet structure hardly changed, cell pellets seem to be a convenient system to study the pore formation process. However, caution should be used with the pore resealing process in this system. Generally, after electrical breakdown the pellet resistance restores within seconds, and in a few minutes even exceeds the initial value by many times (Fig. 4 *a*). Because this resistance restoration process depends on the suspension buffer, some osmotic effects must be involved. Specific osmotic properties of cell pellets are discussed below.

Osmotic effects in cell pellets

Although far from equilibrium, erythrocytes in suspension behave like perfect osmometers (Kinosita and Tsong, 1977). Because cell pellets are also in thermodynamic equilibrium with the supernatant, they should adjust to changes in supernatant compositions. This is indeed the case (Fig. 1, *curves* 2, 4, and 6′), although this process can take several minutes. However, when the supernatant is replaced with a more diluted solution, R_p first rises then drops to a level lower than that in the original solution (compare *curve* 3 with 1′ and 5′ in Fig. 1). Reasons for such complicated behavior of the pellet resistance in the second case are not yet clear. Probably, in more diluted solutions the pellet structure changes so fast that the system does not come to equilibrium.

Adding macromolecules like PEG or dextran in supernatant leads to a drastic increase of the pellet resistance (Fig. 2). The supernatant effect can be explained with a simple kinetic model described as follows (Fig. 9): (a) cell membranes are permeable to water molecules and impermeable to all other molecules and ions; (b) in the intercellular space of pellets, diffusion of water, ion and other small molecules are similar to those in bulk solutions; and (c) diffusion of macromolecules into the intercellular space of pellets is much slower than that in the bulk solutions. One can estimate the diffusion characteristics times τ_1, τ_2, and τ_3 for water to move (1) through cell membranes thickness of 5 nm (diffusion coefficient $D = 10^{-11}$ cm^2/s; Sachs et al., 1975); (2)through the distance of 2 μm from the plasma membrane to the cell center ($D = 10^{-5}$ cm^2/s), and (3) through the intercellular space of half a pellet thickness of 240 μm. For a pellet containing 30×10^7 erythrocytes, the respective times are $\tau_1 \approx \tau_2 \approx 1$ ms and $\tau_3 \approx 10$ s, i.e., $\tau_1 \approx \tau_2 \ll \tau_3$. Thus, if the pellet is not too thick, cells and supernatant attain osmotic equilibrium in tens of seconds to several minutes (as compared with milliseconds in cell suspensions). Because macromolecules cannot enter the intercellular space of the pellet, increasing supernatant tonicity leads to water being removed from the pellet. As a result, cells are shrunk. The intercellular space is also compressed until its osmotic pressure and repulsive forces between cell membranes equalize the osmotic pressure in supernatant and cells (Fig. 2, *curves* 2–4). However, if certain low-weight macromolecules in the supernatant are able to penetrate into the intercellular space, with much longer characteristic time then those for

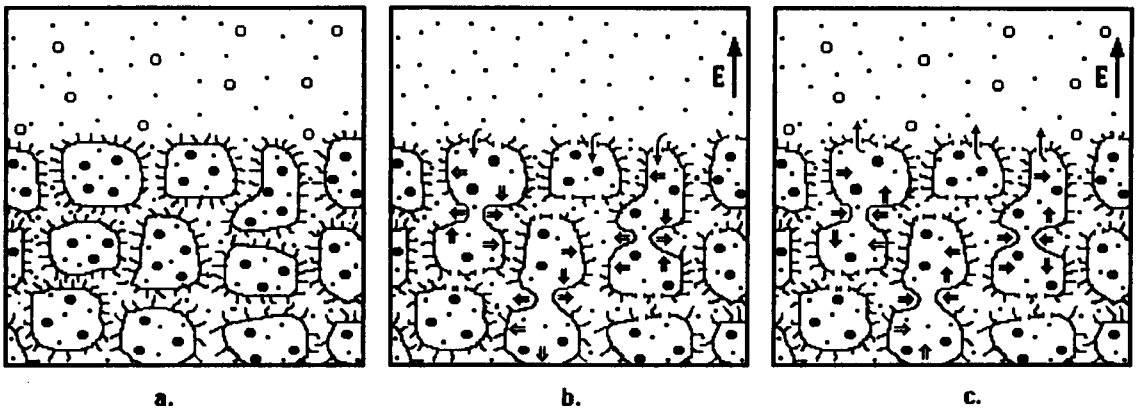

FIGURE 9 Colloid and colloid-osmotic effects in erythrocyte pellets. (*a*) Colloid compression of cell pellets by adding macromolecules in supernatant. Although intermembrane gaps are bigger than macromolecules in supernatant, these macromolecules cannot move (at least not very fast) in the gaps because of overlapping glycocalyx. Because in gaps and in cells, water is in osmotic equilibrium with the supernatant, osmotic pressure in supernatant may be much higher than in the pellet. For this reason, the pellet is effectively compressed and the intermembrane distance drops up to 50–60 Å. (*b*) Colloid-osmotic effect in isotonic PBS. Ion concentration in the gaps between cell membranes is higher then inside cells. Application of a high voltage electric pulse leads to formation of minute pores, mostly in membranes normal to the field direction. Water and ions move together inside cells (as shown by *thin arrows*), increasing osmotic pressure there. This results in osmotic compression of the pellet (intermembrane distance effectively decreases). The same effect is evidently observed when cells first are exposed to electric pulses and then pelleted. Increasing osmotic pressure (shown by *double-line arrows*), on one hand, prevents cell lysis because swelling may become slower than pore healing. On the other hand, if some cells start to fuse, forming intermembrane bridges, it promotes fusion expanding these bridges. (*c*) Reverse colloid-osmotic effect in presence of macromolecules in supernatant. If an erythrocyte pellet subjected to colloid compression by supernatant (as in *a*) is exposed to a high-voltage electric pulse, ion concentration in the gaps between cells becomes higher than inside cells. By this reason, ions and water move out of cells and pressure is oriented in the opposite direction compared with the previous case (*b*). It should not affect electrohemolysis significantly, because the pellet stays under high osmotic compression; however, it may suppress electrofusion, destroying primary fusion bridges formed during pulse delivery.

water and ions, a temporary compression of the intercellular space, as represented by an increase in R_p, is seen in Fig. 2 (*curve* 1 for 8 kDa dextran).

It is important to note that, when one supernatant is replaced with another, the behavior of cell pellets depends not only on the supernatant tonicities and the presence of macromolecules in the second supernatant, but also on the ratio of ion concentrations in both supernatants. As seen from Fig. 2 (*curve* 3), the pellet resistance increases even when isotonic PBS is replaced with isotonic 20% dextran (500 kDa) solution. To explain this effect, one should take into account that the initial ion concentration in the intercellular solution is higher than that in the second supernatant. Ion movement along the concentration gradient apparently results in decreasing its tonicity and, finally, compresses the pellet. This, so-called colloid-osmotic effect, is especially important when cell pellets are treated with high-voltage pulses.

Cell membrane interactions

High molecular weight PEG and Dextran cannot penetrate the pellet, yet at equilibrium, the osmotic pressure in the intracellular space in the pellet equals that of the supernatant. As seen from Fig. 2, replacement of PBS supernatant with PEG or dextran solutions leads to a significant increase of R_p, reflecting a decrease of the pellet porosity from ~3 to 0.5%, and the equivalent intermembrane distance from 250 to 50 Å, as the result of this osmotic pressure. As shown in electron micrographs (see preceding paper), contacts between erythrocyte membranes in a pellet extended like flat-parallel plates, with openings in cell junctions. The only way to decrease the pellet porosity is to bring cell membranes closer. As the distance between membranes d decreases, repulsive forces between membranes become significant. These forces include the electrostatic and steric repulsion due to cell surface glycocalyx; the electrostatic forces between interacting diffuse double layers; and the steric forces and hydration entropic forces (Israelashvili and Wennestrom, 1991) of the lipid bilayer. These forces are balanced by osmotic pressure and Van der Waals attraction. Provided that the relation between the pellet resistance R_p and the equivalent intercellular distance d (derived from pellet porosity ϵ) is valid, as given in the Appendix of the preceding paper, the relation between the supernatant osmotic pressure and the equivalent intercellular distance can be determined.

Data on R_p of rabbit erythrocyte pellets at different compressions by means of centrifugation, electroporation (colloid-osmotic effect), or by adding macromolecules in supernatant (pure osmotic effect) are summarized in Fig. 10. Because the value of d in Fig. 10 is derived from R_p, based on the assumption that cell volume remained constant at all pressures, a certain error is implied. As seen, the function $\log\Delta P(d)$ is divided in two parts: at $\Delta P > 10^4$–10^5 Pa, it decreases linearly, whereas at lower ΔP it changes more slowly. At $d \approx 50$–60 Å, intermembrane repulsion changes in character and becomes much stronger. The high ΔP part of the function follows approximately an exponential relation $P_h = P_0 \cdot \exp(-d/\lambda)$ where $P_0 = 10^{10}$ N/m^2 and $\lambda = 2$ Å as depicted by Parsegian and Rand (1991). The low ΔP part of

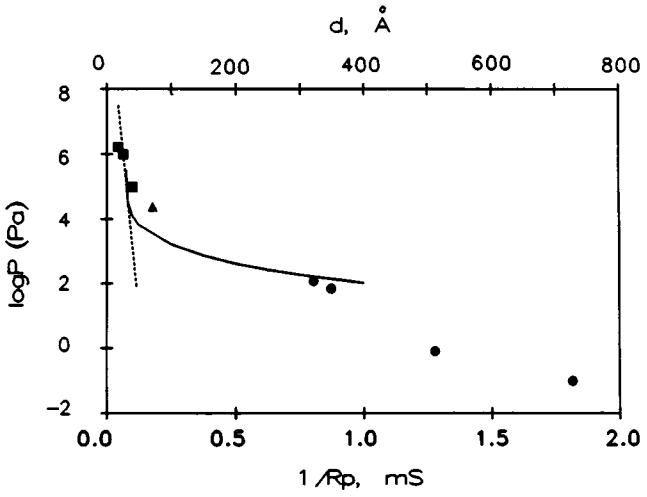

FIGURE 10 Intermembrane forces (in log-scale) as functions of the pellet resistance R_p and the derived intermembrane distance d. Experimental points were obtained from data on mechanical (centrifugation) (*circles*) colloid-osmotic (*triangle*) and osmotic (*squares*) compression. The dashed line shows a dependence of hydration forces P_h on d, calculated according to Parsegian and Rand (1991). The solid line is a similar dependence for electrostatic repulsion P_{el} estimated for a simple model with moderate charge density (5 mC/cm^3) homogeneously distributed in the intermembrane gap.

the function is approximately linear, therefore 10-fold increase of ΔP causes ~120 Å decrease of d. Similar semilogarithmic characteristic have been observed for lipid bilayers in the range of electrostatic repulsion (Parsegian and Rand, 1991). It seems reasonable that, in this range, membrane repulsion is also caused by interaction of electric double layers. However, future studies are necessary to be more conclusive. Nevertheless, further studies in this direction might open unique opportunities for direct measurements of intermembrane interactions between intact cells. Such measurement is not yet possible.

Osmotic effects associated with electroporation in cell pellets

Electropermeabilization is commonly considered as a result of the formation of numerous minute pores in the membrane lipid matrix, hence the membrane becomes permeable to both water and ions. Suppose that suspended red cells are in osmotic equilibrium with isotonic PBS. The total molar concentrations of all particles (ions and hemoglobin) inside and outside of cells are roughly equal; the ion concentration in cells, therefore, is lower than that in PBS. When cell membranes become electropermeabilized, ions move in along their concentration gradient, while hemoglobin molecules cannot pass out through pores. In result, the total concentration of particles in cells becomes higher than in PBS. To balance tonicity, water moves in and cells swell. Should cell membranes reseal rapidly, further increase of the cell volume could not exceed 5–6%. Cell membrane resealing proceeds rather slowly at room temperature, but colloid-osmotic swelling of cells takes only minutes. Continuous swelling leads to hemolysis (Abidor et al., 1994).

A restricted volume and a rather high diffusional or hydrodynamic resistance of intercellular space affect the colloid-osmotic process in cell pellets. After electropermeation, ions together with water start to move into cells from intercellular solution, but their replenishment from the supernatant is very slow. Simple calculations show that even if each cell in a 30-million-cell pellet has only one pore of 3-Å radius, the diffusional flux in cells from the intercellular solution equals that for a pellet with $\epsilon = 3\%$. Electrical breakdown, of course, creates many pores per cell (Stenger and Hui, 1986); therefore, replenishment of ions and water from the supernatant to the intercellular space in the pellet is negligible when compared to the influx into cells. Removing ions and especially water from intercellular space leads to a further decrease of the pellet porosity and a further increase in the pellet resistance. All of these changes might be temporary. After membrane resealing, osmotic equilibrium among cell cytoplasm, intercellular solution, and supernatant is restored, and the pellet is decompressed. This process is reflected in a slow decrease of the pellet resistance after reaching its maximum (Fig. 4 *a*, *curve* 2). Reducing the pellet diffusion rate by covering electroporated pellets with untreated cells impedes this R_p decrease, (Fig. 5 *b*, *curve* 4), whereas adding macromolecules in supernatant intensifies pellet compression.

It should be noted that, unlike cell suspensions, cell pellets cannot change their volume freely, and the membrane surface tension stays much lower than its critical value. Therefore, there is no reason to expect massive hemolysis in erythrocyte pellets. As seen from Fig. 6, cells in pellets are much more resistive to hemolysis compared with those in suspension. For the same reason, pelleting cells right after pulse treatment, before significant colloidal-osmotic swelling, efficiently prevents cells from hemolysis (Fig. 6). R_p of pellets formed from cells just treated with a high-voltage electric pulse in suspensions behave almost the same as pulse-treated pellets (Fig. 5 *a*).

Electrofusion in cell pellets

Electrofusion mechanism have been studied extensively by us and others (Abidor and Sowers, 1992a; Hui and Stenger, 1992; Kuzmin et al., 1988). As in dielectrophoretic chain, fusion yield of cells in pellets depends strongly on the pulse amplitude. It was true for erythrocytes in this study (Fig. 7 *a*) as well as for different nucleated cells earlier (Abidor et al., 1993). Despite some uncertainties in measuring transmembrane potentials U_m induced by electric fields in cell pellets, U_m required for electroporation of different cells (~1 V, from Fig. 4 *b*) is only half that required to induce notable electrofusion (~2 V, from Fig. 7 *a*). Therefore, in electrofusion, electric fields might play a more involved role than simply inducing pores in cell membranes (Stenger et al., 1991).

Valuable information on the mechanism of cell electrofusion can be provided by kinetics studies based on measurements of the fusion yield, F, as a function of pulse duration. This question was not studied in an earlier work

(Abidor et al., 1993) on cell electrofusion in pellets. In dielectrophoretic chains, the fusion thresholds for erythrocytes, as for many other cells, decrease with increasing pulse duration (Stenger et al., 1991). Although for rabbit erythrocyte ghosts in dielectrophoretic chains, the dependence of F on pulse duration was found to be exponential, i.e., in this system electrofusion kinetics can be described as a first-order process (Abidor et al., 1992a, b); for erythrocyte pellets, F does not depend on the pulse duration, as seen in Fig. 7 *b*.

The difference in electrofusion kinetics for pellets and dielectrophoretic chains reflects different behaviors of these two systems in strong electric fields. In dielectrophoretic systems, the electric field distribution does not change during the fusion process, because cell chains do not affect the electrical parameters of the chamber. On the contrary, the resistance of cell pellets is relatively high, and a significant part of the voltage applied to the chamber drops on the pellet. In strong electric fields used for electrofusion, the pellet resistance drastically falls in the first few microseconds in the beginning of the pulse application, and for the remaining of the pulse the pellet experiences a much lower field. If electrofusion is induced only during the first microseconds, the rest of the pulse is actually useless. It is not surprisingly, therefore, that at constant amplitude, pulses longer than 10 μs give the same fusion yields.

Another interesting question concerns the so-called reverse colloid osmotic effect. Usually, one expects that the tighter the membrane contacts the higher the fusion yield. Generally, this is true. However, following Fig. 8, adding PEG or dextran before the application of fusogenic pulses effectively suppresses electrofusion, although according to electrical measurements, the intermembrane distance becomes very small. The explanation of these results is that, after electropores are formed, the osmotic pressures in cells and in intercellular space are still lower than that in the supernatant, because PEG or dextran cannot enter the pellet (Fig. 2). The cells are still shrunken, as opposed to swollen in control cells. Cell shrinkage is detrimental to fusion.

This work was supported by grant GM30969 from the National Institutes of Health. Lin-Hong Li was supported in part by Training Fellowship of the World Health Organization.

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