J. Membrane Biol. 189, 45–54 (2002)
DOI: 10.1007/s00232-002-1003-y

The Journal of
Membrane
Biology

Intracellular Delivery of Trehalose into Mammalian Cells by Electropermeabilization

R. Shirakashi*, C.M. Köstner, K.J. Müller, M. Kürschner, U. Zimmermann, V.L. Sukhorukov
Lehrstuhl für Biotechnologie, Biozentrum der Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany

Received: 28 February 2002/Revised: 10 May 2002

Abstract. The disaccharide trehalose is increasingly being used as a very efficient stabilizer of cells, membranes and macromolecules during cryo- and lyoconservation. Although extracellular trehalose can reduce cryo- and lyodamage to mammalian cells, the sugar is required on both sides of the plasma membrane for maximum protection efficiency. In the present study, mouse myeloma cells were loaded with the disaccharide by means of reversible electropermeabilization in isotonic trehalose-substituted medium, which contained 290 mM trehalose as the major solute. By using the membrane-impermeable fluorescent dye propidium iodide as the reporter molecule, optimum electropulsing conditions were found, at which most permeabilized cells survived and recovered (i.e., resealed) their original membrane integrity within a few minutes after electric treatment. Microscopic examination during the resealing phase revealed that electropulsed cells shrank gradually to about 60% of their original volume. The kinetics of the dye uptake and the volumetric response of cells to electropulsing were analyzed using a theoretical model that relates the observed cell volume changes to the solute transport across the transiently permeabilized cell membrane. From the best fit of the model to the experimental data, the intracellular trehalose concentration in electropulsed cells was estimated to be about 100 mM. This loading efficiency compares favorably to other methods currently used for intracellular trehalose delivery. The results presented here point toward application of the electropermeabilization technique for loading cells with membrane-impermeable bioprotectants, with far-reaching implications for cryo- and lyopreservation of rare and valuable mammalian cells and tissues.

Key words: Electroporation — Disaccharide — Membrane permeability — Electric breakdown — Electroinjection — Volumetry

Introduction

The disaccharide trehalose is found at high concentrations in organisms that are capable of withstanding various environmental stress conditions. Many yeasts, plants and some animals naturally synthesize trehalose, which protects the structural integrity of cells during extreme dehydration and cold (Drennan et al., 1993; de-Araujo, 1996; Crowe & Crowe, 2000). Due to its extraordinary properties, trehalose is increasingly being exploited in biomedicine and biotechnology as a very efficient stabilizer of frozen and dry macromolecules (e.g., antibodies, enzymes, etc.), artificial and natural membranes (Rossi et al., 1997; March & Clark, 2000; Puhlev et al., 2001;). Medical applications of trehalose include lyo- and cryopreservation of blood, sperm, tissues and whole organs for storage, transport and surgical transplantation (Beattie et al., 1997; Woelders, Matthijs & Engel, 1997; Eroglu et al., 2000; Guo et al., 2000; Wolkers et al., 2001).

Several molecular mechanisms have been suggested by which trehalose protects cells during freezing and drying. These involve stabilizing effects on both cellular proteins and membranes by formation of hydrogen bonds and/or by water replacement, inhibition of intracellular ice formation, increase of the surface energy between the cell membrane and bulk solvent, etc. (de-Araujo, 1996; Oliver, Crowe & Crowe, 1998; Wolfe & Bryant, 1999; Tsvetkova et al., 1998; Takahashi, 1999, Crowe & Crowe, 2000; Lambruschini et al., 2000). Although extracellular trehalose can reduce cryo- and lyodamage to cells, the

**Permanent address:* Institute of Industrial Science, The University of Tokyo, 4-6-1 Komaba Meguro-ku, Tokyo 153-8505, Japan

Correspondence to: U. Zimmermann; email: zimmermann@biozentrum.uni-wuerzburg.de

disaccharide is usually required on both sides of the cell membrane for maximum protection efficiency (Paiva & Panek, 1996; Eroglu, Toth & Toner, 2001; Wolkers et al., 2001). Depending on the cell type, 150–200 mM intracellular trehalose is necessary for improved survival of frozen mammalian cells after thawing (Eroglu et al., 2000, 2001). In contrast to *freeze-thaw* protocols, much less intracellular trehalose (20–25 mM) is required for efficient preservation of mammalian cells by *freeze-drying* (Wolkers et al., 2001).

Various experimental approaches have been developed for the introduction of trehalose into mammalian cells, which can neither synthesize nor actively accumulate this disaccharide. Some of these in vitro techniques make use of the leakiness of cell membranes during the phase transition (Beattie et al., 1997), the fluid-phase endocytosis (Wolkers et al., 2001) or a pore-forming protein inserted into the plasma membrane to introduce exogenous trehalose (Eroglu et al., 2000). In a different approach, the genes for trehalose synthesis have been transferred into the target mammalian cells using viral vectors (Guo et al., 2000). Irrespective of the technique, the combination of intra- and extracellular trehalose was found to enhance greatly the survival of mammalian cells in the frozen or dry state.

An alternative approach that might allow to overcome the natural impermeability of cell membranes to trehalose is electropermeabilization (for review *see* Zimmermann & Neil, 1996). This technique (also known as electroporation or -injection) provides a well-proven tool for the introduction of various membrane-impermeable xenomolecules (such as drugs, hormones, proteins, plasmids, etc.) into living cells as well as the controlled release of intracellular substances. Electropermeabilization is based on the temporary increase of the membrane permeability due to reversible electric breakdown of the plasma membrane upon application of external high-intensity field pulses of very short duration (Zimmermann, Pilwat & Riemann, 1974). This field-pulse technique has gained common acceptance because it is more controllable, reproducible, and efficient than other methods for intracellular delivery of foreign molecules (Friedrich et al., 1998, Spiller et al., 1998; Delteil, Teissié & Rols, 2000).

In the present study, the introduction of trehalose into mouse myeloma cells (Sp2 line) was performed by means of electropermeabilization. Cells were suspended in isotonic trehalose medium (ITM, containing 290 mM trehalose as the major solute) and subjected to mild electropulsing conditions that produced no detectable loss of cell viability. The permeabilized cells recovered (i.e., resealed) within few minutes their original membrane impermeability to trehalose and other small solutes. The resealing time constant was about 4 min at room temperature, as assessed from the kinetics of the uptake of the fluorescent dye propidium iodide (PI). The volumetric response of cells to electropulsing was analyzed using a theoretical model that allowed the quantitative evaluation of the intracellular trehalose delivery. Using mild field-pulse conditions, about 100 mM trehalose could be introduced into the cytosol of living cells. The loading efficiency obtained here with electroporation compares favorably to those reported for other techniques currently used for intracellular trehalose delivery.

Materials and Methods

Cell Culture

The murine myeloma cell line Sp2/0-Ag14 was cultured in RPMI 1640 complete growth medium (CGM), supplemented with 10% (v/v) fetal calf serum (FCS; PAA, Linz, Austria), at 37°C under 5% CO_2. The cells were kept in the exponential growth phase by subculturing two or three times a week.

Pulsing Medium

Isotonic trehalose medium (ITM) contained 290 mM trehalose (Sigma, Deisenhofen, Germany) and 5 mM KCl as the major osmoticum and electrolyte, respectively. Osmolality and conductivity were determined to be 300 mOsm and 0.9–1.1 mS/cm (at 22°C) by means of a cryoscope (Osmomat 030, Gonotec, Berlin, Germany) and a conductometer (Knick, Berlin, Germany), respectively. pH of the medium was about 7.3. The reasons for using low-conductivity medium are discussed in detail elsewhere (Mussauer et al., 1999; Mussauer, Sukhorukov & Zimmermann, 2001).

Electroporation

Electropermeabilization of Sp2 cells suspended in ITM was performed by means of the Multiporator distributed by Eppendorf (Hamburg, Germany). This instrument generates exponentially decaying field pulses with peak intensities of up to 1.2 kV and decay times between 15 and 500 μsec (Friedrich et al., 1998). Most experiments were performed with the commercial cuvettes purchased from Eppendorf (Hamburg, Germany). These cuvettes, consisting of two planar aluminum electrodes (2 cm^2 area) spaced by $d = 2$ mm, were filled with 400 μl of cell suspension. Alternatively, a chamber consisting of two parallel stainless steel wire electrodes with a diameter of 200 μm was used for microscopic observations of cells during and after electropulsing. The electrodes were mounted on a glass substrate at a distance d of 560 μm. Typically, 10–20 μl of cell suspension was pipetted between the wire electrodes and covered with a glass coverslip. For both electroporation chambers, the initial applied field strength E_0 was calculated from the supplied pulse voltage V_0 as $E_0 = V_0/d$. All electropermeabilization experiments were performed at room temperature (20–22°C).

Microscopy

Observations were made with a microscope (BX 51 Olympus, Hamburg, Germany) using transmitted light to examine the volumetric cell response to electropulsing (*see* Results and Appendix 2). Microphotographs were taken before and every one minute after

electropulsing, using a high-resolution digital camera (ColorView 2, Soft Imaging System, Münster, Germany) attached to the microscope.

Flow Cytometry

In a series of experiments, the pulse medium (i.e., ITM) was supplemented with 20 μg/ml (≈30 μM) propidium iodide (PI, Sigma, Deisenhofen, Germany) as the fluorescent reporter molecule. This membrane-impermeable cationic dye reveals strong red fluorescence after binding to nucleic acids. PI served two purposes: 1) as a vital stain, it distinguished living cells from dead ones, and 2) as an indicator of the transient and reversible electropermeabilization, it allowed the determination of the resealing time constant of the plasma membrane (*see* Appendix 1). PI-staining of cells was analyzed at various time intervals after field-pulse application in a flow cytometer Epics XL system (Beckman Coulter, Fullerton, CA) equipped with a 15-mW 488-nm argon laser using a band-pass filter of 675/15 nm. Cellular red fluorescence (RF) signals from samples containing about 5000 cells were presented as one-dimensional frequency histograms. The short-term viability in electropulsed cell samples was evaluated 10–15 min after electropulsing. Because of a large difference in their RF intensity, the populations of viable and dead cells could easily be distinguished in the RF histograms (for detail, *see* Mussauer et al., 2001). The viability was defined as the ratio of the count of weakly fluorescent living cells to the total cell number.

Results

Viability of Cells Electropermeabilized in Isotonic Trehalose Medium

In order to optimize the electropulsing conditions for intracellular trehalose delivery, we first examined the electrosensitivity of Sp2 cells by studying the dependence of their short-term viability on both field strength E_0 and duration of the applied field pulses. The viability was quantified by flow-cytometry using the membrane-impermeable fluorescent dye PI as the reporter molecule (Sukhorukov et al., 1995; Mussauer et al., 2001).

In the absence of PI, viable unpulsed cells (Fig. 1*A*, curve *1*) with an intact membrane showed a very weak autofluorescence centered at about 5 arbitrary units (a.u.). After the addition of 30 μM PI to ITM, this peak shifted slightly to about 8 a.u. (Fig. 1*B*, curve 0), which was apparently due to the dye adsorption to the outer membrane surface. Treatment of cells with saponin resulted in 100% permeabilization and gave rise to a very high red fluorescence (RF) intensity, with the G_0/G_1-peak centered at 1000 a.u. (*data not shown*). This high RF level corresponds to near saturation of the cellular binding sites for PI (Sukhorukov et al., 1995).

Curve *2* in Fig. 1*A* shows a typical PI content distribution in a cell sample measured 15 min after application of a single electric pulse of 5 kV/cm strength and 40 μsec duration, in the presence of PI. Two readily distinguished cell populations appeared

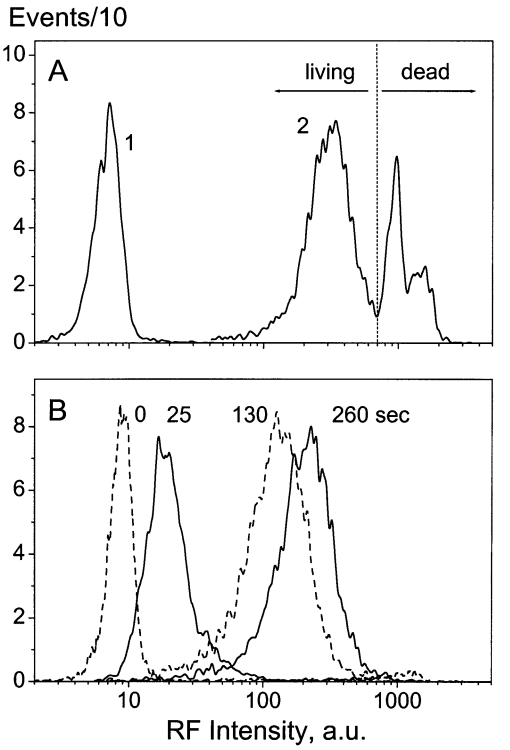

Fig. 1. Flow cytometric assessment of electropermeabilization of Sp2 cells using PI as the reporter molecule. In ***A***, curve *1* represents the autofluorescence of control cells suspended in media without PI. Curve *2* shows cells treated with a supracritical field pulse (5 kV/cm, 40 μsec). In ***B***, the RF histograms were measured before (0), 25, 130, and 260 sec after mild electropulsing (2 kV/cm, 20 μsec) in the presence of 30 μM PI.

after electropulsing: 1) irreversibly permeabilized dead cells with very bright RF (1000 a.u.), which was similar to that of saponin-lyzed cells (*not shown*); 2) cells with much lower PI staining (i.e., far from equilibrium with the medium), whose peak RF is centered at about 400 a.u. This weakly fluorescent population apparently consisted of viable cells, which had been transiently permeabilized and which then recovered (i.e., resealed) their original membrane impermeability to PI. The PI histograms, such as shown in Fig. 1, were used to evaluate short-term cell viability, which was defined as the percentage of weakly fluorescent cells referred to the total cell number. Qualitatively similar PI-content distributions (*data not shown*) were obtained for cell samples subjected to single field pulses of 20 or 40 μsec duration and a field strength ranging from 1 to 5 kV/cm.

Figure 2 summarizes the viability data obtained by flow cytometry. The portion of living cells in unpulsed control samples usually varied between 85 and 93% (Fig. 2, $E_0 = 0$). For 20- and 40-μsec pulses (Fig. 2, *circles* and *squares*, respectively), the percentage of viable cells was not affected by electropulsing with field strengths smaller than or equal to

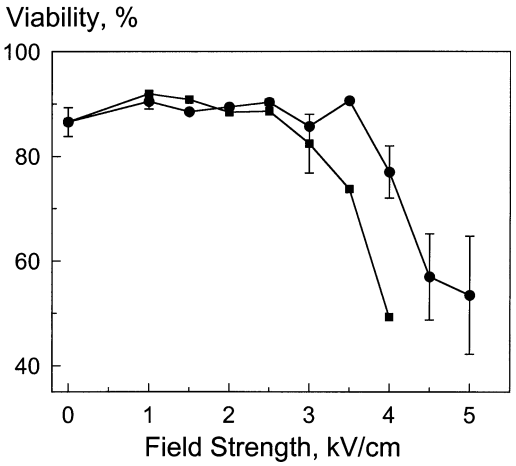

Fig. 2. Field strength dependence of viability of Sp2 cells exposed to a single electric pulse in isotonic trehalose medium at 21–23°C. Circles and squares denote pulses of 20 and 40 μsec duration, respectively. The data (mean ± SE) were obtained from 2–3 flow cytometric measurements, such as shown in Fig. 1. The percentage of dead cells before pulsing showed typical day-to-day fluctuations of 7–15%.

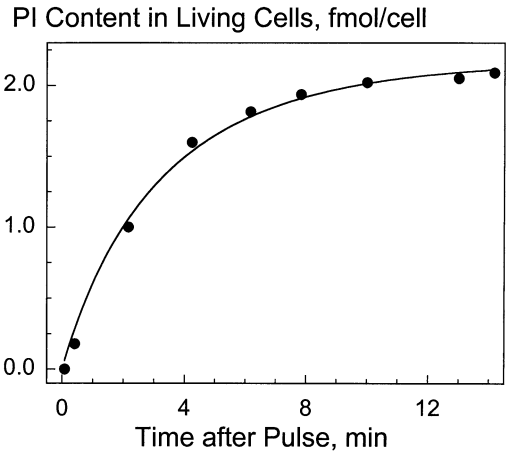

Fig. 3. Time course of PI uptake in reversibly permeabilized (living) Sp2 cells after application of one pulse of 2 kV/cm intensity and 20 μsec duration. Pulsing and resealing were performed in the presence of 25 μg/ml PI (for further experimental details, *see* legends to Figs. 1 and 2). The continuous curve is the best least-squares fit of Eq. A6 to the data. The two parameters obtained by fitting are: the resealing time constant $\tau_R = 4.4 \pm 0.4$ min, and the initial effective permeability of the cell membrane to the dye, $P_{PI} = (5.5 \pm 0.3) \times 10^{-10}$ m/sec (±SE of the fit).

3.5 and 3 kV/cm, respectively. At higher field strengths, the percentage of living cells decreased sharply. The data in Fig. 2 also allowed rough estimation of the field-strength values LE_{50}, at which 50% of initially living cells were irreversibly permeabilized (dead). As expected, LE_{50} decreased from about 5 to 4 kV/cm when the pulse duration was increased from 20 to 40 μsec, respectively. Based on the viability results presented in Fig. 2, mild or moderate pulsing conditions (i.e., $E_0 = 2$–2.5 kV/cm for both pulse durations), at which no detectable loss of cell viability occurred, were chosen for the following experiments.

Kinetics of the Electrically Driven Dye Uptake by Viable Cells

In addition to the viability measurements (Fig. 2), the use of PI also allowed the determination of the resealing time constant τ_R of the plasma membrane from the kinetics of the fluorescent cell staining after electropulsing. Figure 1*B* illustrates that upon application of an electric pulse (2 kV/cm, 20 μsec), the intracellular PI content in the viable cell population grew gradually with time, as suggested by the progressive increase of the cellular red fluorescence (RF) intensity. The RF histograms allowed the quantification of the intracellular PI content in electropulsed living cells. Because of the relatively broad RF-distributions, the mean RF signal was used as a measure for the dye uptake by living cells. The strongly fluorescent lyzed cells, whose RF signal corresponds to ~10 fmol PI per mean Sp2 cell, were used as the internal standard of the intracellular dye content (for details, *see* Sukhorukov et al., 1995).

The mean PI content estimated from the RF histograms (Fig. 1*B*) was plotted as function of time as illustrated by the symbols in Fig. 3. The PI content increased asymptotically to the maximum value of about 2 fmol/cell. This low PI content (i.e., far from equilibrium with the external medium) as well as the kinetics of the dye uptake indicate that the cells underwent transient and reversible permeabilization upon the moderate electropulsing used in these experiments. The fact that the dye influx terminated at about 10 min after field exposure (Fig. 3) suggests that within this time the cell membrane restored its original impermeability to PI.

Volumetric Cell Response to Electropulsing

The intracellular trehalose concentration C_{tr}^{i} was estimated from the kinetics of the cell volume change induced by electropermeabilization of Sp2 cells in isotonic trehalose medium. These experiments were performed in a chamber consisting of two parallel cylindrical electrodes (*see* Material and Methods) that enabled microscopic observation of the electropulsed cells. From the microphotographs taken before and at various time intervals after electropulsing (upper row in Fig. 4), the volume of single cells in the course of time was determined from the projected cell area, assuming spherical geometry.

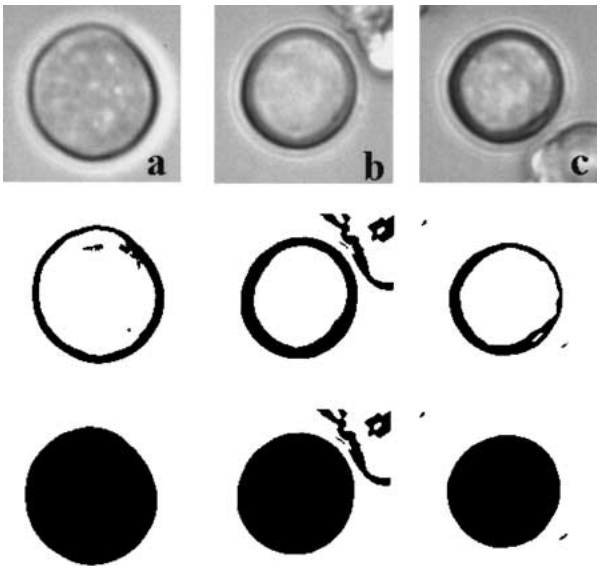

Fig. 4. Selected frames from a video sequence demonstrating cell shrinkage induced by a single electric field pulse of 2 kV/cm and 20 μsec duration. Frames *a*, *b* and *c* show the same cell shortly before, 4 and 8 min after electropulsing, respectively. The middle and bottom rows illustrate the principle of cell sizing by evaluating the projected cell area.

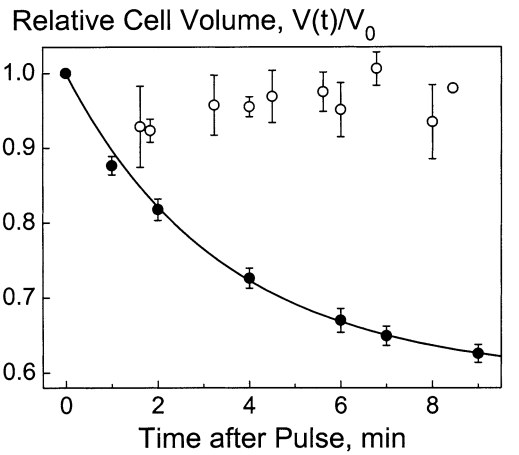

Fig. 5. Kinetics of cell volume change after electropulsing (filled circles). The continuous curve shows the best least-squares fit of Eq. B9 to the data. The final intracellular concentration of trehalose obtained from the fit is about 100 mM. Open circles denote unpulsed control cells. Each symbol represents the mean ± SE from 12 cells. The data were obtained by image analysis as illustrated in Fig. 4.

The processed images in the middle and bottom rows of Fig. 4, highlighting the cell boundary and projected cross-area, respectively, illustrate the principle of cell size measurement by means of quantitative image analysis.

Figure 5 (filled symbols) summarizes the volumetric behavior of Sp2 cells permeabilized by a single electric pulse of 2 kV/cm strength and 20 μsec duration. Within 10 minutes upon electropulsing in ITM, the cells shrank to about 60% of their initial isotonic volume. In contrast, unpulsed control cells did not exhibit any significant volume change within this time interval (Fig. 5, open circles).

Discussion

Trehalose-Mediated Electroprotection of Cells

Comparison of the field-strength dependence of cell viability in isotonic trehalose medium (Fig. 2) with the data of Sp2 cells electropulsed in inositol medium of the same osmolality and ionic composition (5 mM KCl, *see* Djuzenova et al., 1996) reveals that trehalose is able to protect this mammalian cell line against excessive electropermeabilization. Thus, the substitution of inositol by trehalose produced a twofold increase of the LE_{50} value (i.e., the field strength at which 50% of cells are irreversibly electropermeabilized), from about 2 kV/cm in inositol to about 4 kV/cm in ITM, for electric pulses of the same 40-μsec duration (*compare* Fig. 4*C* in Djuzenova et al., 1996 and Fig. 2 in the present study). The trehalose-mediated electroprotection of Sp2 cells is in accordance with the earlier observation that this disaccharide improves both short- and long-term survival of electrotransfected human lymphocytes and other mammalian cells (Mussauer et al., 2001).

An interesting parallel has to be noted between the effects of trehalose and EDTA-like chelators on the electroporated plasma membrane of mammalian cells. Similarly to trehalose, EDTA and its derivatives protect cells against excessive electroporation (Schneeweiss, Zimmermann & Saleemuddin, 1977; Nanda & Mishra, 1994). It has been recognized that the chelator-mediated increase of the membrane rigidity is the main reason for the improved stability and survival of electropulsed mammalian cells (Mussauer et al., 1999). The ability of trehalose to increase the rigidity of the phospholipid head group region via hydrogen bond formation has been demonstrated in experiments with model phosphatidylcholine liposomes (Bárdos-Nagy, Galántai & Fidy, 2001). Therefore, trehalose-induced alterations of cell membrane rigidity might be responsible for the observed electroprotective effect of this disaccharide on electroporated mammalian cells reported here and elsewhere (Mussauer et al., 2001). It is also possible that both membrane stabilization and cell survival have benefited from the electroinjected intracellular trehalose at the secondary stages following membrane breakdown.

Determination of the Resealing Time Constant of the Plasma Membrane

The rate of the resealing process (i.e., restoration of the plasma membrane integrity) is one of the key parameters influencing not only the cell survival but also the amount of solutes taken up by electropermeabilized cells. In order to determine the resealing time constant τ_R, we analyzed the PI uptake kinetics measured by flow cytometry (Fig. 1*B*) with the theoretical model presented in Appendix 1 (Eqs. A1–A6). The continuous curve in Fig. 3 shows the best least-square fit of Eq. A6 to the mean PI content data (symbols in Fig. 3). For these calculations, we used the value $K_a \approx 2 \times 10^5\ M^{-1}$ for the equilibrium constant of PI binding to mammalian cell DNA reported in the literature (Bertuzzi et al., 1991; Djuzenova & Flentje, 2001). The mean number of PI binding sites in Sp2 cells, $N_0 \approx 10$ fmol/cell, has been determined earlier by fluorimetric titration (Sukhorukov et al., 1995). The mean cell radius $a = 7$ μm, measured with a calibrated microscope, was used to calculate the cell surface area S_0 and volume V_0. The only two unknown parameters τ_R and P_{pi0} (i.e., the initial permeability of the electroporated membrane to the dye) were determined by curve fitting using the nonlinear least-squares method.

From the best fit (solid curve in Fig. 3), the values $\tau_R = 4.4 \pm 0.4$ min and $P_{pi0} = (5.5 \pm 0.3) \times 10^{-10}$ m/sec (means ± SE of the fit) were found for the plasma membrane of Sp2 cells suspended in ITM. The quantity P_{pi0} represents the effective membrane permeability averaged over the whole cell surface. In practice, both electropermeabilization and PI uptake occur in the membrane areas facing the electrodes (Zimmermann & Neil, 1996). It is interesting to note that the τ_R value of 4.4 min obtained in the present study for Sp2 cells suspended in ITM is significantly larger than the resealing time constant of about 2 min measured for the same cell line in isotonic inositol medium (Sukhorukov et al., 1995; Djuzenova et al., 1996). This means that besides its electroprotective effect discussed above, trehalose also reduced markedly the rate of the resealing process, which can similarly be explained by the trehalose-mediated alteration of the mechanical properties of the cell membrane.

Although the trehalose content in electropulsed cells could not be determined from the experiments with PI shown in Figs. 1 and 3, the substantial electro-uptake of the propidium cation (MW = 414) by Sp2 cells is a strong indication that electropulsing also rendered cell membranes permeable to the smaller (MW = 342) and flexible molecule of trehalose. Therefore, the electropermeabilization technique might also be useful for the intracellular delivery of trehalose. It is unlikely that electropermeabilized cell membranes are selectively permeable to the cationic propidium, because neutral solutes (e.g., sucrose) cross electropermeabilized cell membranes as fast as ionic compounds (Rutter & Denton, 1992).

Estimation of the Electrically Mediated Uptake of Trehalose

Cell volumetry is widely used for studying transport phenomena across cell membranes (Gimsa et al., 1994; McGrath, 1997). This in situ technique is also useful for measuring the uptake of membrane-permeable cryoprotective agents (Shirakashi & Tanasawa, 1998) and particularly for the determination of the intracellular sucrose concentration taken up through a genetically engineered protein pore in the plasma membrane (Russo, Bayley & Toner, 1997). For modeling the volumetric behavior of cells, the permeability coefficients for water and solutes are usually treated as *time-invariable* quantities. This traditional assumption, however, is not valid in the case of the *transient* permeabilization of cell membranes subjected to high-intensity, short-duration electric field pulses.

In the present study, the theoretical model relating the cell volume changes to the fluxes of water and solutes across the cell membrane was extended in order to account (1) for the rapid permeabilization of the plasma membrane via the electric breakdown mechanism and (2) for the subsequent gradual decrease of the membrane permeability to osmolytes during the resealing phase. The model and equations used here for the analysis of the volumetric cell response to electropulsing are outlined in Appendix 2. The model also allowed the quantitative determination of the concentration of intracellular trehalose taken up by electropermeabilized cells during the resealing phase.

For the evaluation of the intracellular trehalose concentration, Eq. B9 was fitted to the volumetric cell response data using the least-squares method. In general, this equation contains three unknown parameters: (1) the resealing time constant τ_R, (2 and 3) the initial membrane permeabilities to trehalose P_{tr0} and electrolyte P_{el0}. All other quantities appearing in Eq. B9 are known: the extracellular trehalose concentration was adjusted to $C_{tr}^{o} = 290$ mM, the initial cell volume V_0 and the cell surface S_0 (assumed invariable) were determined microscopically as described above. The time-dependent cell volume V was the fitting variable. When all three unknown parameters (i.e., τ_R, P_{tr0} and P_{el0}) were varied in the fitting procedure, the fit function converged at $\tau_R = 4.2$ min, $P_{tr0} = 2.7 \times 10^{-9}$ m/sec and $P_{el0} = 7.4 \times 10^{-9}$ m/sec. However, the fitted values had large standard errors.

In order to reduce the number of unknown parameters, we assumed the value τ_R = 4.4 min obtained from the PI uptake kinetics (Fig. 3). In this case, curve fitting gave the following estimates for the two remaining unknown parameters: P_{tr0} = (3.1 ± 0.4) × 10^{-9} m/sec and P_{el0} = (7.8 ± 0.5) × 10^{-9} m/sec (mean ± SE of the fit). In Fig. 5, the fitted result is shown as a smooth curve together with the experimental volumetric data. The correlation coefficient close to unity (r = 0.9994) implies that the theoretical model provides good approximation to the data. Moreover, the fact that the τ_R value of 4.4 min based on the *independent* PI uptake measurements matches well the volumetric data is further justification of the model (Appendix 2) used here for the evaluation of trehalose taken up after electropermeabilization. Using the fitted parameters, the intracellular concentration of trehalose C^i_{tr}∼100 mM after pulsing and resealing can be calculated by applying the following expression $C^i_{tr} = A(V_0/V-1)$ (A is defined in Appendix 2).

Electropermeabilization *vs.* Other Membrane Permeabilization Techniques

The theory of electropermeabilization and the biomedical applications of this field-pulse technique are reviewed in detail elsewhere (Zimmermann & Neil, 1996; Zimmermann et al., 2000). As estimated from the volumetric cell response after electropulsing (Fig. 5), mild electropermeabilization conditions enabled the introduction of about 100 mM trehalose into the mouse myeloma cells without detectable loss of their viability (Fig. 2). This intracellular concentration of trehalose (obtained for cells shrunk to about 60% of their original isotonic volume) corresponds to the loading efficiency of 21–34%, which is defined as the ratio of the intracellular disaccharide concentration to its external concentration (290 mM). By using the permeability and loading efficiency values obtained here, the reversible electropermeabilization can be compared quantitatively to other techniques currently used for the loading of mammalian cells with membrane-impermeable cryoprotectants. Thus, immediately upon electropulsing, the membrane permeability of Sp2 cells to trehalose was found to be P_{tr0} = 3.1×10^{-9} m/sec. This is close to the value of 2.85×10^{-9} m/sec for the sucrose permeability measured in mouse fibroblasts whose plasma membrane contained engineered porins developed by site-directed mutagenesis of *staphylococcus* α-toxin (Russo et al., 1997).

Small-sized cells, such as human platelets, can be loaded with trehalose by long-term incubation at 37°C in trehalose-rich medium (Wolkers et al., 2001). Although high loading efficiency of 50–60% can be achieved by this method, the uptake of trehalose, involving the fluid-phase endocytosis mechanism, requires very long incubation times of up to 4 hr. In contrast, only 10–15 min of post-pulse incubation are necessary for the intracellular delivery of comparable amounts of trehalose by means of electropermeabilization.

Intracellular Trehalose Concentration and Cell Preservation

Intracellular trehalose concentration required for efficient cell protection depends on the cell type and also on the preservation technique. Thus, at least 200 mM intracellular trehalose is necessary for high post-thaw survival (70–80%) of frozen human fibroblasts and keratinocytes (Eroglu et al., 2000). A somewhat lower concentration of intracellular trehalose (150 mM) is required for high recovery rates of frozen mouse and human oocytes (Eroglu et al. 2001). It is evident that the cytosolic trehalose concentration of 100 mM reported here is not sufficient for conservation of mammalian cells by freezing-thawing. Larger cytosolic concentrations of exogenous trehalose (and other solutes) can apparently be obtained by using higher field strengths and/or longer pulses (Friedrich et al., 1998; Mussauer et al., 2001). However, the introduction of larger amounts of trehalose into the cells via severe electropulsing will be achieved at the expense of cell viability (Fig. 2).

It has to be noted that successful preservation of mammalian cells by *freeze-drying* (lyophilization) requires much lower intracellular trehalose concentrations than those needed in *freezing-thawing* protocols. Thus, human platelets loaded with 20–25 mM trehalose show excellent recovery (85–88%) after lyophilization and subsequent rehydration from the vapor phase (Wolkers et al., 2001). Therefore, the intracellular trehalose concentration of 100 mM achieved in the present study by mild electropulsing in trehalose-substituted medium points toward application of the electropermeabilization technique for loading cells with trehalose, with far-reaching implications for preservation of rare and valuable mammalian cells and tissues by lyophilization. Finally, combination of the intracellular trehalose delivered by electropermeabilization with conventional membrane-permeable cryoprotectants, such as dimethylsulfoxide, glycerol, etc. (Beattie et al., 1997; Eroglu et al., 2000), also appears to be a promising approach for improving the survival of cryopreserved mammalian cells.

This work was supported by grants from BMBF (16SV1329) to U.Z. and V.L.S, from the Deutsche Forschungsgemeinschaft (Zi 99/12-1) to U.Z., and from the Ministry of Education of Japan to R.S.

Appendix 1

Theoretical Considerations: Electrically Induced PI-Uptake

In earlier studies (Sukhorukov et al., 1995; Djuzenova et al., 1996), a simplified model was used for the estimation of the resealing time constant τ_R of the plasma membrane from the cell-staining kinetics after electropulsing in the presence of PI. The theoretical analysis presented here was extended to account for the two steps involved in the cell staining with PI. These are: (1) the diffusion-driven uptake of PI through the electropermeabilized cell membrane (Eqs. A2 and A3); (2) PI binding to intracellular nucleic acids (Eqs. A4 and A5), which gives rise to a strongly fluorescent complex. The model is based on the assumption that during resealing the permeability of the plasma membrane to PI, $P_{pi}(t)$, decreases exponentially with time:

$$P_{pi}(t) = P_{pi0}\exp(-t/\tau_R), \tag{A1}$$

where P_{pi0} is the initial permeability immediately after pulse application; τ_R is the resealing time constant. The quantity $P_{pi}(t)$ represents the *effective* permeability of the electroporated plasma membrane to PI averaged over the whole cell surface.

The ruling equation for the flux of PI, J_{pi}, through the electroporated cell membrane during the resealing phase is:

$$J_{pi} = \frac{d(C^i_{pi}V_0)}{S_0 dt} = P_{pi0}\exp(-t/\tau_R)\left(C^o_{pi} - C^i_{pi}\right), \tag{A2}$$

where C^i_{pi} is the intracellular concentration of the free PI; $C^o_{pi} = 30$ μM is its external concentration; V_0 and S_0 are the cell volume and surface area, respectively.

Because the rate of resealing is not sensitive to the cell volume, the shrinkage of cells induced by electroporation (*see below*) was neglected in the analysis of the cell-staining with PI. Assuming both V_0 and S_0 to be invariable, the following expression for the total (i.e., both free and bound) intracellular PI concentration can be obtained by integrating Eq. A2 over time:

$$C^i_{pi} = C^o_{pi}\left[1 - \exp\left(\frac{S_0 P_{pi0}\tau_R}{V_0}(\exp(-t/\tau_R) - 1)\right)\right], \tag{A3}$$

Equilibrium is further assumed for the binding of the intracellular PI to the nucleic acids:

$$\mathrm{PI} + \mathrm{NA} \leftrightarrow \mathrm{PI}\cdot\mathrm{NA} \tag{A4a}$$

$$K_a = \frac{[\mathrm{PI}\cdot\mathrm{NA}]}{[PI][\mathrm{NA}]_{free}} = \frac{C^b_{pi}}{C^i_{pi}\left(N_0 - C^b_{pi}\right)} \tag{A4b}$$

where K_a is the binding constant; $C^b_{pi} = [\mathrm{PI}\cdot\mathrm{NA}]$ is the concentration of the bound (i.e., fluorescent) dye; N_0 is the total number of binding sites for PI; $(N_0 - C^b_{pi}) = [\mathrm{NA}]_{free}$ is the number of free binding sites. Equation A4b can be rearranged to obtain an expression for the bound intracellular PI:

$$C^b_{pi} = \frac{K_a N_0 C^i_{pi}}{1 + C^i_{pi}K_a} \tag{A5}$$

Substituting Eq. A3 into Eq. A5 gives the following result for the time-dependent concentration of the dye PI that (*i*) crossed the electropermeabilized cell membrane and (*ii*) subsequently intercalated into the cellular DNA, which in turn gave rise to a strong fluorescent cell staining:

$$C^b_{pi}(t) = \frac{N_0 C^o_{pi}K_a\left\{1 - \exp\left[\left(S_0 P_{pi0}\tau_R/V_0\right)(1 - \exp(-t/\tau_R))\right]\right\}}{C^o_{pi}K_a - \left(1 + C^o_{pi}K_a\right)\exp\left[\left(S_0 P_{pi0}\tau_R/V_0\right)(1 - \exp(-t/\tau_R))\right]} \tag{A6}$$

Fitting Eq. A6 to the PI uptake data gave the estimates for two unknown parameters: τ_R and P_{pi0}.

Appendix 2

Theoretical Considerations: Cell Volume Changes after Electropulsing

Upon electropulsing in isotonic trehalose medium, myeloma cells shrank to about 60% of their original volume (Figs. 4 and 5). To analyze this effect, we used the following set of equations:

$$\left(C^o_{tr} - C^i_{tr}\right) + \left(C^o_{el} - C^i_{el}\right) = 0 \tag{B1}$$

$$\frac{d\left(C^i_{tr}V\right)}{S_0 dt} = P_{tr0}\exp(-t/\tau_R)\left(C^o_{tr} - C^i_{tr}\right) \tag{B2}$$

$$\frac{d\left(C^i_{el}V\right)}{S_0 dt} = P_{el0}\exp(-t/\tau_R)\left(C^o_{el} - C^i_{el}\right) \tag{B3}$$

where Eq. B1 describes the osmotic equilibrium between the cytosol and suspending medium. Equation B1 implies that the membrane permeability to water is much higher than its permeability to the osmolytes and that only two osmolytes, trehalose and KCl, maintain the osmotic equilibrium. Equation B2 relates the flux of trehalose (subscript "tr") across the membrane to the difference of its concentration $(C^o_{tr} - C^i_{tr})$ on the two sides of the membrane. Equation B3 is the corresponding flux relation for the electrolyte KCl (subscript "el"). The superscripts "o" "i" denote the outer and intracellular solutions, respectively. V and S_0 (S_0 = constant) are the volume and membrane surface area of the cell, respectively. For simplicity, the reflection coefficients equal to unity are assumed for both osmolytes.

As with the PI electro-uptake (treated in Appendix 1), the exponential terms $P_{tr0}\cdot\exp(-t/\tau_R)$ and $P_{el0}\cdot\exp(-t/\tau_R)$ represent, respectively, the effective permeabilities of the electroporated plasma membrane to trehalose and electrolyte averaged over the cell surface. Since the electroporated cell membrane resealed within few minutes at room temperature, both permeabilities are assumed to decrease exponentially from their initial values P_{tr0} and P_{el0}, with the same resealing time constant τ_R.

In order to solve the system of equations B1–B3, the following assumptions were applied. The initial intracellular concentrations of trehalose $C^i_{tr}(t \to 0) = 0$ and KCl $C^i_{el}(t \to 0) = 300$ mOsm. Because of the very low cell density used in this study, changes in osmolyte concentrations in the suspending medium were neglected: $C^o_{tr} = 290$ mM and $C^o_{el} = 10$ mOsm, corresponding to 5 mM KCl (i.e., both external concentrations are invariable). The system of equations B1–B3 was solved analytically as outlined below.

Combining Eqs. B1–B3 leads to the following equation:

$$\frac{1}{P_{tr0}}\frac{d\left(C^i_{tr}V\right)}{dt} + \frac{1}{P_{el0}}\frac{d\left(C^i_{el}V\right)}{dt} = 0 \tag{B4}$$

Substituting C^i_{el} in Eq. B4 by the expression $C^i_{el} = C^o_{tr} - C^i_{tr} + C^o_{el}$ (*see* Eq. B1) gives:

$$\frac{d(C_{tr}^{i}V)}{dt} = -\frac{P_{tr0}(C_{tr}^{o} + C_{el}^{o})}{P_{el0} - P_{tr0}}\frac{dV}{dt} \quad \text{(B5)}$$

Taking into account that $t = 0$ the intracellular trehalose concentration is zero, $C_{tr}^{i} = 0$, the integration of Eq. B5 over time t leads to:

$$C_{tr}^{i}V = -\frac{P_{tr0}(C_{tr}^{o} + C_{el}^{o})}{P_{el0} - P_{tr0}}(V - V_0), \quad \text{(B6)}$$

where V_0 is the initial cell volume $V_0 = 4\pi a^3/3 = 1.4$ pl. Equation B6 can be rewritten as

$$V = \frac{AV_0}{C_{tr}^{i} + A}; \text{ with } A \equiv P_{tr0}\frac{C_{tr}^{o} + C_{el}^{o}}{P_{el0} - P_{tr0}} \quad \text{(B7)}$$

Substitution of Eq. B7 into Eq. B3 and integration of the differential equation (Eq. B8) over time gives finally an algebraic expression (Eq. B9) for the time dependence of the volume of the transiently permeabilized cells during the resealing phase:

$$\frac{A^2V_0}{(C_{tr}^{i} + A)^2}\frac{dC_{tr}^{i}}{dt} = P_{tr0}S_0\exp(-t/\tau_R)(C_{tr}^{o} - C_{tr}^{i}) \quad \text{(B8)}$$

$$t = -\tau_R \ln\left[1 + \frac{A^2V_0}{P_{tr0}S_0\tau_R}\left[\frac{(V/V_0 - 1)}{A(C_{tr}^{o} + A)} + \frac{1}{(C_{tr}^{o} + A)^2}\ln\left|\frac{V}{V_0} + \frac{A}{C_{tr}^{o}}\left(\frac{V}{V_0} - 1\right)\right|\right]\right] \quad \text{(B9)}$$

Since no explicit expression for the cell volume V as function of time could be achieved, the inverse function given by Eq. B9 was fitted to the volumetric data, such as shown in Fig. 5, using the least-squares method (*see* Results).

References

Bárdos-Nagy, I., Galántai, R., Fidy, J. 2001. Effect of trehalose in low concentration on the binding and transport of porphyrins in liposome-human serum albumin system. *Biochim. Biophys. Acta*. **1512:**125–134

Beattie, G.M., Crowe, J.H., Lopez, A.D., Cirulli, V., Ricordi, C., Hayek, A. 1997. Trehalose: A cryoprotectant that enhances recovery and preserves function of human pancreatic islets after long-term storage. *Diabetes* **46:**519–523

Bertuzzi, A., D'Agnano, I., Gandolfi, A., Graziano, A., Starace, G., Ubezio P. 1991. Study of propidium iodide binding to DNA in intact cells by flow cytometry. *Cell Biophysics* **17:**257–267

Crowe, J.H., Crowe, L.M. 2000. Preservation of mammalian cells — learning nature's tricks. *Nature Biotechnol.* **18:**145–146

de-Araujo, P.S. 1996. The role of trehalose in cell stress. *Braz. J. Med. Biol. Res.* **29:**873–875

Delteil, C., Teissié, J., Rols, M.-P. 2000. Effect of serum on in vitro electrically mediated gene delivery and expression in mammalian cells. *Biochim. Biophys. Acta* **1467:**362–368

Djuzenova, C.S., Flentje, M. 2001. Light scatter and DNA accessibility to propidium iodide of *ataxia telangiectasia* and Fanconi anemia cells. *Biochem. Biophys. Res. Commun.* **286:**365–371

Djuzenova, C.S., Zimmermann, U., Frank, H., Sukhorukov, V.L., Richter, E., Fuhr, G. 1996. Effect of medium conductivity and composition on the uptake of propidium iodide into electropermeabilized myeloma cells. *Biochim. Biophys. Acta* **1284:**143–152

Drennan, P.M., Smith, M.T., Goldsworthy, D., van Staden, J. 1993. The occurrence of trehalose in the leaves of the desiccation-tolerant angiosperm *Myrothamnus flabellifolius* Welw. *J. Plant. Physiol.* **142:**493–496

Eroglu, A., Russo, M.J., Bieganski, R., Fowler, A., Cheley, S., Bayley, H., Toner, M. 2000. Intracellular trehalose improves the survival of cryopreserved mammalian cells. *Nature Biotechnol.* **18:**163–167

Eroglu, A., Toth, T.L., Toner, M. 2001. Cryoprotection of mouse and human oocytes by intracellular trehalose. *Abstr. 38th Meeting Soc. Cryobiol.* p. 45

Friedrich, U., Stachowicz, N., Simm, A., Fuhr, G., Lukas, K., Zimmermann, U. 1998. High efficiency electrotransfection with aluminum electrodes using microsecond controlled pulses. *Bioelectrochem. Bioenerg.* **47:**103–111

Gimsa, J., Schnelle T., Zechel G., Glaser R. 1994. Dielectric spectroscopy of human erythrocytes: investigations under the influence of nystatin. *Biophys. J.* **66:**1244–1253

Guo, N., Puhlev, I., Brown, D.R., Mansbridge, J., Levine, F. 2000. Trehalose expression confers desiccation tolerance on human cells. *Nature Biotechnol.* **18:**168–171

Lambruschini, C., Relini, A., Ridi, A., Cordone, L., Gliozzi, A. 2000. Trehalose interacts with phospholipid polar heads in Langmuir monolayers. *Langmuir* **16:**5467–5470

March, J.B., Clark J. 2000. Enzymes by post-restriction enzyme stability. *Nature Biotechnol.* **18:**243

McGrath, J.J. 1997. Quantitative measurement of cell membrane transport: technology and applications. *Cryobiology* **34:**315–334

Mussauer, H., Sukhorukov, V.L., Haase, A., Zimmermann, U. 1999. Resistivity of red blood cells against high-intensity, short-duration electric field pulses induced by chelating agents. *J. Membrane Biol.* **170:**121–133

Mussauer, H., Sukhorukov, V.L., Zimmermann, U. 2001. Trehalose improves survival of electrotransfected mammalian cells. *Cytometry* **45:**161–169

Nanda, G.S., Mishra, K.P. 1994. Modification of electroporative response of erythrocytes by EDTA in isotonic solutions. *Bioelectrochem. Bioenerg.* **34:**189–193

Oliver, A.E., Crowe, L.M., Crowe, J.H. 1998. Methods for dehydration-tolerance: depression of the phase transition temperature in dry membranes and carbohydrate vitrification. *Seed Sci. Res.* **8:**211–221

Paiva, C.L.A., Panek, A.D. 1996. Biotechnological applications of the disaccharide trehalose. *Biotechnol. Annu. Rev.* **2:**293–314

Puhlev, I., Guo, N., Brown, D.R., Levine, F. 2001. Desiccation tolerance in human cells. *Cryobiology* **42:**207–217

Rossi, S., Buera, M.P., Moreno, S. Chirife, J. 1997. Stabilization of the restriction enzyme EcoRI dried with trehalose and other selected glass-forming solutes. *Biotechnol. Progress* **13:**609–616

Russo, M.J., Bayley, H., Toner, M. 1997. Reversible permeabilization of plasma membranes with an engineered switchable pore. *Nature Biotechnol.* **15:**278–282

Rutter, G.A., Denton, R.M. 1992. Effects of insulin and guanosine 5′-[γ-thio]triphosphate on fatty acid synthesis and lipolysis within electropermeabilized fat-cells. *Biochem. J.* **281:**431–435

Schneeweiss, F., Zimmermann, U., Saleemuddin, M. 1977. Preparation of uniform haemoglobin free human erythrocyte ghosts in isotonic solution. *Biochim. Biophys. Acta* **466:**373–378

Shirakashi, R., Tanasawa, I. 1998. Method of designing prefreezing protocol in cryopreservation of biological materials. *Ann. N.Y. Acad. Sci.* **858:**175–182

Spiller, D.G., Giles, R.V., Grzybowski, J., Tidd, D.M., Clark, R.E. 1998. Improving the intracellular delivery and molecular

efficacy of antisense oligonucleotides in chronic myeloid leukemia cells: a comparison of streptolysin-O permeabilization, electroporation, and lipophilic conjugation. *Blood* **91:**4738–4746

Sukhorukov, V.L., Djuzenova, C.S., Frank, H., Arnold, W.M., Zimmermann, U. 1995. Electropermeabilization and fluorescent tracer exchange: the role of whole-cell capacitance. *Cytometry* **21:**230–240

Takahashi, H. 1999. Interaction of disaccharide and sugar alcohol with phospholipids. *Membrane* **24:**207–214 (in Japanese)

Tsvetkova, N.M., Phillips, B.L., Crowe, L.M., Crowe, J.H., Risbud, S.H. 1998. Effect of sugars on headgroup mobility in freeze-dried dipalmitoylphosphatidylcholine bilayers: Solid-state ^{31}P NMR and FTIR studies. *Biophys. J.* **75:**2947–2955

Woelders, H., Matthijs, A., Engel, B. 1997. Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. *Cryobiology* **35:**93–105

Wolfe, J., Bryant, G. 1999. Freezing, drying and/or vitrification of membrane-solute-water systems. *Cryobiology* **39:**103–129

Wolkers, W.F., Waker, N.J., Tablin, F., Crowe, J.H. 2001. Human platelets loaded with trehalose survive freeze-drying. *Cryobiology* **42:**79–87

Zimmermann, U., Neil, G.A. 1996. Electromanipulation of Cells, CRC Press, Boca Raton, FL

Zimmermann, U., Friedrich, U., Mussauer, H., Gessner, P., Hämel, K., Sukhorukov, V.L. 2000. Electromanipulation of mammalian cells: Fundamentals and applications. *IEEE Trans. Plasma Sci.* **28:**72–82

Zimmermann, U., Pilwat, G., Riemann, F. 1974. Reversible dielectric breakdown of cell membranes by electrostatic fields. *Z. Naturforsch.* **29c:**304–305