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Possible Role of Transient Electric Fields in Freezing-Induced Membrane Destabilization

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Summary. Evidence is presented to support the hypothesis that electrical potentials generated during the freezing of aqueous solutions (the Workman-Reynolds effect) may contribute to the destabilization of the plasma membrane and cryoinjury of isolated protoplasts. Specifically, (1) electric potential differences of sufficient magnitude to cause lysis of the plasma membrane occur during the rapid freezing of isolated protoplasts suspended in sorbitol; (2) survival of protoplasts is inversely correlated with the magnitude of the potential difference and (3) cold acclimation increases the stability of the plasma membrane to applied electric fields. A discussion is given of the different physical phenomena thought to be involved in the Workman-Reynolds effect. The basis equations for these phenomena are outlined.

Key Words isolated protoplasts · plasma membrane · freezing injury · freeze-induced electrical transients · Workman-Reynolds effect

Introduction

The plasma membrane plays a central role in the behavior of a cell during a freeze-thaw cycle (Steponkus, 1984). First, because the plasma membrane is semipermeable, the cell behaves as an osmometer in response to changes in the solute concentration of the partially frozen extracellular solution. Second, the intact plasma membrane is an effective barrier to extracellular ice and precludes seeding of the intracellular solution.

Disruption of the semipermeability or lysis of the plasma membrane is a primary cause of freezing injury (Steponkus, 1984). Cryomicroscopic observations of isolated protoplasts reveal that destabilization of the plasma membrane can occur at various times during a freeze-thaw cycle and may cause any one of several distinctly different symptoms (Steponkus et al., 1983; Dowgert & Steponkus, 1984). These include expansion-induced lysis during warming and thawing of the suspending medium when the decreasing osmolality results in osmotic expansion of the protoplast; loss of osmotic responsiveness following cooling so that the protoplast is osmotically inactive during warming; or altered osmometric behavior during warming suggesting a transient loss of intracellular solutes or "leakiness" of the plasma membrane. In addition, cryomicroscopic observations suggest that mechanical failure of the plasma membrane is a cause rather than a consequence of intracellular ice formation.

While expansion-induced lysis is the result of mechanical stresses (surface tensions in the range of 4 to 6 $mN \cdot m^{-1}$), the other forms of injury occur when the membrane is flaccid and isotropic tension, γ, is zero and curvature energies are negligible (Wolfe & Steponkus, 1981; 1983*a*,*b*). In this case, lysis or loss of semipermeable characteristics are effected by other stresses. Most often, the chemical stresses of electrolyte concentration and dehydration are considered to be responsible for injury. This view, however, is disputed (*see* Steponkus, 1984). Heretofore, membrane destabilization due to electrical perturbations has not been considered in relation to cryoinjury. Electric fields applied across biological and artificial membranes result in electrical breakdown, increases in permeability to solutes, membrane fusion, and mechanical breakdown (*see* Zimmermann et al., 1981; Zimmermann, 1982, for comprehensive reviews). This spectrum of membrane disruption (fusion excepted) is quite similar to that observed for freezing injury and prompts us to inquire whether electrical perturbations contribute to cryoinjury—especially mechanical breakdown that is associated with intracellular ice formation.

In 1950, Workman and Reynolds reported measurements of large steady and transient potential differences across ice-water interfaces generated during the freezing of dilute aqueous solutions. Subsequently, many groups have reported potentials as high as tens (or even hundreds) of volts during the

freezing of dilute solutions of electrolytes (Lodge et al., 1956; Levi & Milman, 1966; Gross, 1971; Carnati & Illingworth, 1980). Although the Workman-Reynolds effect has been studied extensively by scientists concerned with atmospheric electrical phenomena, its possible significance in the area of cryobiology has apparently not been previously considered.

In this report, we consider the magnitude of the Workman-Reynolds effect during the freezing of isolated protoplast suspensions, its correlation with freeze-thaw injury, and whether cold acclimation alters the stability of isolated protoplasts in applied electric fields.

Materials and Methods

Plant Culture and Protoplast Isolation

Seedlings of *Secale cereale* L. cv Puma were grown for 7 days in vermiculite in a controlled environment (16-hr light period at 20°C and 8-hr dark period at 15°C). Nonacclimated plants were maintained in this environment for an additional 7 days prior to isolation of protoplasts. Plants to be acclimated were transferred to a 13°C light period (11.5 hr)/7°C dark period (12.5 hr) regime for one week and then transferred to a 2°C (10-hr light period) regime for an additional 4 weeks.

Protoplasts were isolated from leaves in a solution of 1.5% (wt/vol) cellulysin (Calbiochem), 0.5% macerase (Calbiochem), and 0.3% potassium dextran sulfate as previously described (Dowgert & Steponkus, 1983). For the isolation of protoplasts from nonacclimated leaves, 500 mol · m^{-3} sorbitol was used as an osmoticum. Isolation of protoplasts from acclimated leaves required 900 mol · m^{-3} sorbitol as an osmoticum due to the increase in the internal solute concentration during cold acclimation. Protoplasts were washed 3 times in sorbitol and resuspended in a sorbitol plus KCl (0 to 3.0 mol · m^{-3}) solution at a final titer of 1.50×10^5 protoplasts/ml. The LT_{50} (temperature at which 50% of the protoplasts survived) was −3 to −5°C for nonacclimated and −25 to −30°C for acclimated protoplasts frozen and thawed in sorbitol solutions. The typical value of the radius of both the acclimated and nonacclimated protoplasts was 15 μm.

Measurement of the Workman-Reynolds Effect

The apparatus used for the measurement of the Workman-Reynolds effect consisted of a Plexiglas® trough (2 cm long, 1 cm wide and 1 cm deep), the ends of which were sealed to mylar-clad blocks. A temperature differential was established by varying the temperature of the two copper blocks via circulating alcohol baths. Initially, the temperature differential was such that the entire suspension was unfrozen. Ice growth proceeded in a unidirectional manner when the temperature of the warm block was lowered rapidly.

Electrical potentials were measured with platinum electrodes, one of which became encased in ice immediately. The other electrode was 1 cm away in the unfrozen solution. Potentials were measured with a high impedance electrometer (Keithley Model 610C) with the output recorded on a strip chart recorder.

Protoplast Stability in Applied Electric Fields

The electrical stability of isolated protoplasts was studied using three different methods: (1) microscopic determination of lysis in an external electric field between closely spaced platinum electrodes; (2) determination by conductivity measurements of the release of electrolytes into the suspending medium in response to external electric field pulses; and (3) monitoring of the protoplast resistance following the application of an internal pulsed electric potential via a microelectrode. All studies were conducted at room temperature ($T \approx 26$°C).

For the microscopic determinations of lysis, a chamber was constructed on a microscope slide by securing two parallel platinum electrodes 200 μm apart. A 2-msec pulse of varied amplitudes (1 to 30 volts) was applied. Lysis was determined visually from digitized video images taken at 6-sec intervals following the pulse. For the conductivity measurements, the conductivity of the protoplast suspension was determined prior to the pulse, immediately after the pulse, and then after three supra-critical (30 volts) pulses.

For the intracellular electrode studies, individual protoplasts were gently held with a suction pipette and then impaled with a microelectrode. A sequence of ± voltage pulses of 35-msec length of increasing magnitude was applied through the microelectrode and at the same time the current flow was monitored. From these measurements we derived the membrane resistance R_m as a function of the intracellular potential V_m.

Theory

The potential differences between the liquid and solid phases in the Workman-Reynolds effect are large compared with the typical values encountered in electrochemistry. These large potentials are caused by a space charge that is maintained against the electric field by the large energy flux in a system which is far from equilibrium. Some early theoretical models of the Workman-Reynolds effect have been discussed (Gross, 1954; LeFebre, 1967), but these involve highly specialized assumptions which were made so as to give simple analytical solutions.

Suppose that the position of a planar ice front is given by $X = Rt$ where R, its velocity, is assumed constant. The ionic solution occupies the region $x > Rt$, and, at $t = 0$, it is neutral and homogeneous with $Z_+c_+ = Z_-c_- = c_o$, where $z_\pm$ and $c_\pm$ are the valence and concentration of the ions. The electrochemical potential $\tilde{\mu}$ of all species is a continuous function of x, and, therefore, the ratios of the concentrations of ions in the layers on either side of the interface may be given to a first approximation by the partition coefficients $K_\pm$ of each species between ice and water:

$$\frac{c_{\pm}(X_i)}{c_{\pm}(X_w)} = K_{\pm} = \exp\left(\frac{\mu^{\circ}_{\pm w} - \mu^{\circ}_{\pm i}}{kT}\right) \tag{1}$$

where $\mu^{\circ}_{\pm}$ are standard chemical potentials in ice (i) and water (w), k is Boltzmann's constant and T the thermodynamic temperature.

Initially, $Z_+c_+(X_w) = Z_-c_-(X_w)$ so that

$$\frac{Z_+c_+(X_i)}{Z_-c_-(X_i)} = \frac{K_+}{K_-} = r, \tag{2}$$

where X_w denotes the value of the x coordinate in the liquid phase just outside of the ice front, and X_i the x coordinate just inside the ice. The $K_{\pm}$ are individually large and differ greatly for different ions, so r can be either a very large or very small number (Gross, McKee & Wu, 1975; Gross, Wu et al., 1975). Initially, therefore, one ionic species is deposited in the ice at a much larger concentration than the other. Though these concentrations are themselves small, they give large charge densities. (A concentration difference of 1 mmol $\cdot$ m^{-3} of ions produces a charge density of 96 Coul $\cdot$ m^{-3}.) We shall neglect for the moment the difference between concentration and activity, while noting that this approximation may be improved by using the Debye-Huckel theory in which the activities $a_{\pm}$ are given by

$$a_{\pm} = c_{\pm} \exp[-Ac_{\pm}^{1/2}/(1 + Ba_ic_{\pm}^{1/2})] \tag{3}$$

where a_i, A and B are constants for each species (MacInnes, 1961).

The deposition of a space charge in the ice is diminished at $t > 0$ by two effects: First, both the selective deposition of ions and the electric field it sets up in the solution produce a concentration excess of the counter-ion in the region of the interface. As $Z_+c_+(X_w)/[Z_-c_-(X_w)] \to 1/r$, the rate of charge deposition approaches zero, $R[Z_+c_+(X_i) - Z_-c_-(X_i)] \to 0$. Second, the electric field set up in the ice sets up an ion flux in the ice which acts to reduce that field. Hydrogen ions have the largest mobility in ice. Therefore, for simplicity we consider the case where the moving interface is accompanied by a flux of protons which gradually neutralizes the space charge in the ice.

The real steady state of this system is achieved only when the concentrations of all species in the ice near the interface equal their bulk values, that is, when $Z_{\pm}c_{\pm}(X_i) = c_{\alpha}$. It seems likely that the one-dimensional approximation breaks down (due, for example, to convection in the solution and irregularities in the shape of the interface) before this steady state is achieved. A quasi-steady state (QSS) in which the electric field in the ice is no longer increasing rapidly is approached by the system as $Z_+c_+(X_w)/[Z_-c_-(X_w)] \to 1/r$.

Electric fields that occur in the liquid phase can be ascribed to two effects. First, as the system approaches the QSS, the charge density in the Stern layer (a layer with a thickness of approximately one ion diameter in the solution adjoining the interface) is very large. In a system at equilibrium such a surface charge sets up an ionic double layer described by the Poisson-Boltzmann equation. In this system, too, the field set up by this charge decays rapidly due to screening by the counter-ions within a few Debye lengths of the interface. A second contribution to the electric field in the liquid is made by the different mobilities of the ion species.

Neglecting diffusion in ice by all ions except hydrogen, the conservation of the ions implies

$$\frac{\partial}{\partial t} c_{\pm} = \begin{cases} -\dfrac{\partial}{\partial x} J_{\pm}, & x \geq Rt \geq 0, \\ 0, & 0 \leq x \leq Rt, \end{cases} \tag{4a}$$

where

$$J_{\pm} = -c_{\pm}D_{\pm}\left[\frac{\partial}{\partial x} \ln c_{\pm} + \frac{q_{\pm}}{kT}\frac{\partial}{\partial x}\Phi\right],$$

and where $D_{\pm}$ are the diffusion coefficients, $q_{\pm}$ the ion charges, and Φ the electrostatic potential.

The equation governing the proton flux in the ice is:

$$\frac{\partial}{\partial t} c_p = -\frac{\partial}{\partial x} J_p \qquad 0 < x < Rt \tag{4b}$$

where

$$J_p = -c_pD_p\left[\frac{\partial}{\partial x} \ln c_p - \frac{e}{kT}\frac{\partial}{\partial x}\Phi\right],$$

and where c_p is the proton concentration, and D_p is the proton diffusion coefficient. Notice that the rate of dissociation of H_2O has been neglected in comparison with fluxes in Eq. (4*b*).

Poisson's equation relates potential and charge density, i.e.

$$\frac{\partial}{\partial x}\left(\epsilon_{\alpha}\frac{\partial}{\partial x}\Phi\right) = q_+c_+ + q_-c_- + ec_p, \tag{5}$$

where ϵ_{α} is the dielectric constant of the solid or liquid phase, and e is the magnitude of the electron charge.

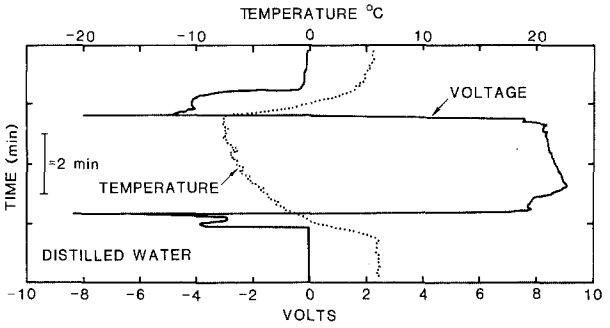

Fig. 1. Electrical transients during the freezing of distilled water. The electrostatic potential is that measured between an electrode imbedded in the ice and an electrode in the liquid

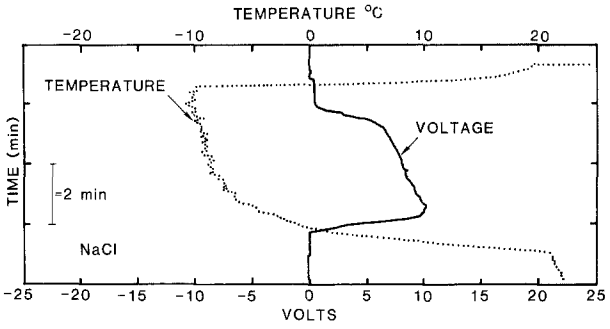

Fig. 2. Electrical potential during the freezing of a 1-mM NaCl solution

For the general case involving transients, the solution of Eqs. (4) and (5) appears to require numerical methods, which are currently being initiated. Nevertheless, some general observations may be made: Two characteristic lengths appear in Eqs. (4) and (5) in the region of the liquid phase. One is the Debye length: $\lambda_{\pm} = [\epsilon kT/(2c_{\pm}q_{\pm}^2)]^{1/2}$ which is of the order of a few nanometers. The other is the diffusion length:

$$L_{\pm} = u_{\pm}kT/q_{\pm}R = D_{\pm}/R, \tag{6}$$

where $u_{\pm}$ are the ionic mobilities, and R is the speed at which the ice front advances. For typical values for electrolytes and for $R \sim 10^{-5}$ m · sec^{-1}, we find $L_{\pm} \sim 200$ μm. The large electric fields set up by the charge in the Stern layer are expected to diminish rapidly over a few Debye lengths. However, without detailed solutions of Eqs. (4) and (5), we cannot rule out the possibility that significant transient electric fields extend into the diffusion layer.

The total potential difference produced between the bulk ice and the bulk solution phases is thus the sum of potentials produced across three different regions: 1) a relatively small field over a large distance in the ice between the interface and the end of the space charge; 2) a very large field over a few Debye lengths in the ionic double layer; and 3) a relatively small field possibly extending across the diffusion layer in the fluid phase. The sizes of these three components is not yet known, but they are the subject of continuing experimental and theoretical investigation.

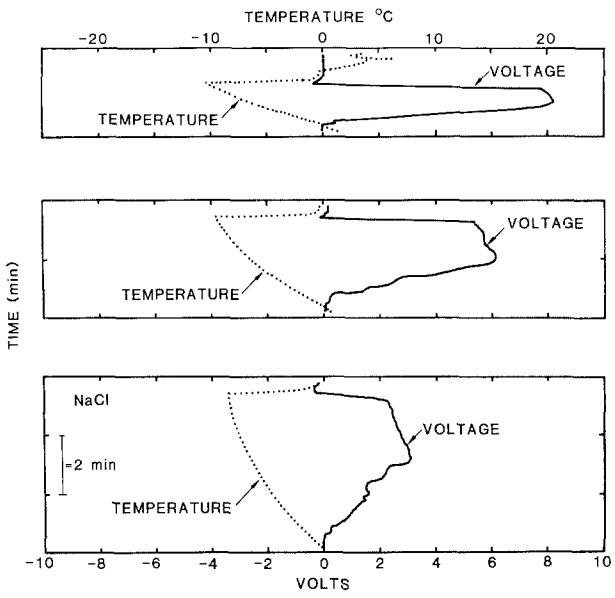

Fig. 3. Cooling rate dependence of the magnitude of the electrostatic potential during the freezing of a 1-mM NaCl solution

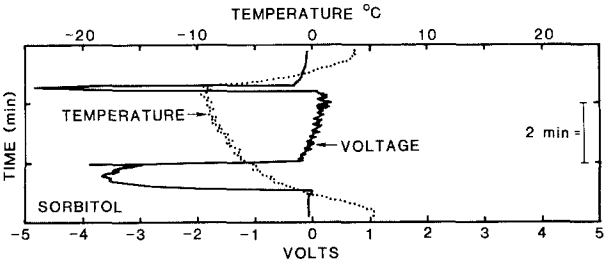

Fig. 4. Electrical transients during the freezing of a 0.5-M sorbitol solution

Results

Workman-Reynolds Effect

During the freezing of distilled water (10 kΩ m resistivity), a transient potential of about −8 V developed initially (the unfrozen water being negative with respect to ice) (Fig. 1). As freezing progressed, the potential reversed polarity to +9 V and was

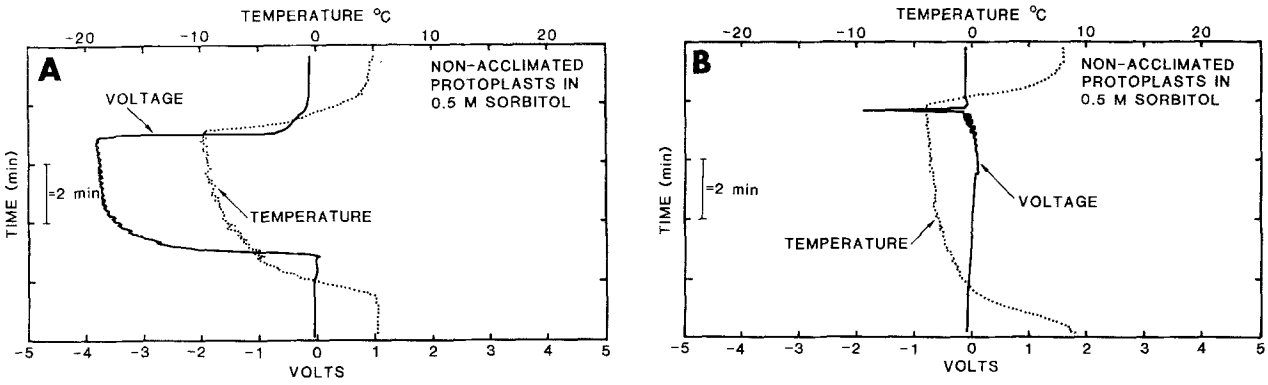

Fig. 5. Electrical potential during the freezing of isolated protoplasts suspended in 0.5 M sorbitol. *A*) cooled rapidly to −10°C; *B*) cooled slowly to −4°C

maintained until freezing was stopped. At that time, a transient negative potential occurred but rapidly diminished.

With a 1 mM NaCl solution, a potential of +10 V was developed during ice formation (Fig. 2). This potential gradually declined as the rate of ice formation diminished and abruptly returned to zero when the second electrode became encased in ice. The magnitude of the potential increased at faster cooling rates (Fig. 3). Although not measured here, this is a result of the faster rate of ice formation (Levi & Milman, 1966).

In previous studies (Lodge et al., 1956; Levi & Milman, 1966), the Workman-Reynolds effect was most pronounced during the freezing of dilute aqueous solutions of electrolytes and the potential went through a maximum as the concentration of the solution was increased. Therefore the occurrence of the Workman-Reynolds effect in highly concentrated solutions of neutral solutes such as sorbitol was of interest for application in the area of cryobiology. During the freezing of 0.5 M solutions of sorbitol (2.1 kΩ m resistivity), a transient potential of about −3.7 V initially occurred, but then diminished to zero (Fig. 4). A second transient of approximately −5 V occurred when freezing was halted.

For protoplasts suspended in 0.5 M sorbitol (1.6 kΩ m resistivity), a steady potential of −3.7 V occurred during freezing at a relatively rapid rate to −10°C (Fig. 5*A*). When freezing was halted, the potential returned to zero. Following thawing of the solution, only 12% of the protoplasts remained intact and were osmotically responsive. When the protoplast suspensions were cooled to −4°C at a slower rate, no potential developed during freezing and only a transient potential of less than −2 V occurred when freezing was halted (Fig. 5*B*). Following thawing of the suspension, 60% of the protoplasts were intact and osmotically responsive. [As this freeze-thaw protocol was to the LT_{50}, the 40% decrease in survival was attributed to expansion-induced lysis (*see* Dowgert & Steponkus, 1984).] Though only correlative, these observations corroborate the suggestion that the electric field generated during freezing may have resulted in lysis of the protoplasts. Further, the visual appearance of the lysed protoplasts was similar to that observed following thawing of protoplast suspensions in which intracellular ice formation has occurred (*see* Dowgert & Steponkus, 1983).

Protoplast Lysis in Applied Electric Fields

If electrical perturbations contribute to cryoinjury, it is reasonable to expect that cold acclimation might increase the stability of isolated protoplasts in applied electric fields. Microscopic observations of isolated protoplasts following an applied d-c pulse confirm this expectation. For field strengths (E) over the range of 25 to 150 kV · m^{-1} (2-msec pulse duration), the incidence of lysis was a function of E (Fig. 6). For nonacclimated protoplasts, 50% lysis occurred at E = 75 kV · m^{-1}; for acclimated protoplasts, 50% lysis occurred at E = 140 kV · m^{-1}. At E = 75 kV · m^{-1}, the V_m developed across the plasma membrane was calculated to be 850 mV. Since the pulse duration (2 msec) greatly exceeded the charging time (~10 μsec; Lovelace et al., 1984) of isolated protoplasts, the difference in the incidence of lysis between nonacclimated and acclimated protoplasts was not attributable to differences in charging time.

The stability of nonacclimated and acclimated protoplasts in an electric field was also contrasted by determining the release of intracellular electrolytes following a d-c pulse (Fig. 7). Again, a substantial difference in the stability of nonacclimated

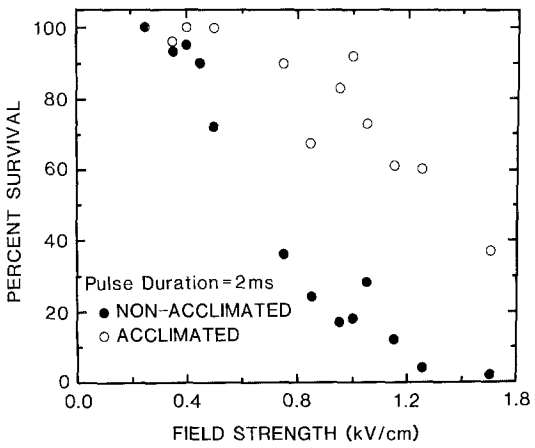

Fig. 6. Incidence of protoplast lysis as a function of electrical field strength

and acclimated protoplasts was observed. Approximately 50% of the intracellular electrolytes were released at approximately $E = 40$ and 90 kV · m^{-1} for nonacclimated and acclimated protoplasts, respectively. Thus, increased permeability of the plasma membrane occurred at values of E lower than those that resulted in lysis.

The difference in the stability of the plasma membrane of nonacclimated and acclimated protoplasts was also measured directly with intracellular microelectrodes. Voltage pulses of 35-msec length were applied to the electrode so as to give intracellular potentials V_m increasing by increments of ±0.1 V between pulses to max $(V_m) = \pm 1.5$ V (limited by the available instruments). For the five nonacclimated protoplasts studied, the critical intracellular potential for lysis was found to be approximately 1 V $> (V_m)_{crit} \geq 750$ mV. The $(V_m)_{crit}$ values for lysis were clearly identifiable by the marked decrease of the membrane resistance from typical values of $R_m \sim 200$ to 500 MΩ to values of $R_m < 50$ MΩ. For the five acclimated protoplasts studied no lysis was observed for V_m's up to ±1.5 V.

Discussion

Destabilization of the plasma membrane resulting in either increased permeability to solutes ("leakiness" or loss of osmotic responsiveness) or mechanical breakdown (lysis) is a primary cause of freezing injury of isolated protoplasts. In addition, intracellular ice formation during rapid cooling of isolated protoplasts is a consequence of mechanical breakdown of the plasma membrane allowing for the supercooled intracellular solution to be seeded by the extracellular ice. The fact that altered semipermeable characteristics or mechanical breakdown of the plasma membrane of isolated protoplasts can also be effected by the application of electrical fields led us to query whether electrical perturbations contribute to freeze-thaw injury. The observations reported herein support this possibility.

First, large potentials are generated during the freezing of aqueous solutions of electrolytes (NaCl, Fig. 2) with the magnitude of the potential a function of the velocity of ice formation (Fig. 3). These potentials are a consequence of the Workman-Reynolds effect. Qualitatively, the Workman-Reynolds effect is easily understood. When a solution of electrolytes is frozen at a finite rate, ions of one sign tend to be included in the ice in a greater concentration than that of the other sign so that a charge separation occurs across the interface. The size of the electric potential thus generated depends on the freezing rate and the mobility and asymmetry of the electrolyte. Since the original report of Workman and Reynolds (1950), various authors have reported potentials as high as tens (or even hundreds) of volts in dilute solutions of electrolytes in which an ice interface moves at speeds of the order of 10^{-5} m · sec^{-1} (Levi & Milman, 1966; Gross, 1971; Carnati & Illingworth, 1980).

Second, the generation of electric potentials of sufficient magnitude to lyse the plasma membrane is observed during the freezing of protoplasts suspended in nonelectrolyte (sorbitol) solutions. Presumably, the low electrolyte concentrations (1.6 kΩ m resistivity) of the suspensions are due to the diffusion of electrolytes from the protoplasts. More important, however, was the fact that the survival of the protoplasts is correlated with the magnitude of the electric potential generated. When frozen at a rapid rate (> 3°C · min^{-1}) to −10°C, there is a high incidence of intracellular ice formation in nonacclimated protoplasts (Dowgert & Steponkus, 1983). Under similar freezing conditions, an electrical potential of nearly 4 V was measured (Fig. 5*a*). For nonacclimated protoplasts, a transmembrane potential of 0.75 V resulted in lysis (Fig. 6). When frozen at slow rates to −4°C, no electrical potential was observed and 60% of the protoplasts survived. Under this freeze-thaw protocol, approximately 50% survival is expected with injury being the result of expansion-induced lysis during thawing (Dowgert & Steponkus, 1984).

Third, if electrical perturbations contribute to cryoinjury one would expect cold acclimation to increase the stability of isolated protoplasts to electrical fields. This expectation was confirmed by the direct observation of protoplast lysis (Fig. 6) and measurement of the release of intracellular electrolytes (Fig. 7). The critical membrane voltage for

lysis was determined to be 0.85 V for nonacclimated protoplasts and ~2.00 V for acclimated protoplasts. Further, at voltages that did not result in lysis, significant increases in the conductivity of the suspending medium occurred—indicative of increased membrane conductance (increased solute permeability) at sublytic field strengths. The use of intracellular electrodes for characterizing the critical V_m yielded similar results.

Thus, although the evidence garnered to date is largely correlative, it consistently supports the hypothesis that electrical perturbations which arise during or following freezing of aqueous solutions contribute to freezing injury. To make a formal case, however, it is necessary to demonstrate that an electric potential sufficiently large to lyse the membrane is generated across it during the passage of the ice front. The spatial variation of the Workman-Reynolds potential is still unknown, so the intensity of the associated electric fields are unknown. The conductivity of the cytoplasm is very much greater than that of the plasma membrane, however, so most of any potential difference applied to the region containing the cell appears across the membrane. Two extreme cases illustrate this: The total potential is large, so (i) if the field is localized over a small region in space (say < 10 nm) then it must be very large ($> 10^9$ V · m^{-1}) and (ii) if the field is relatively small ($< 10^5$ V · m^{-1}) then it must extend over a large range ($> 10^{-4}$ m). We consider these possibilities in turn.

The cytoplasm is very much more conductive than the plasma membrane, so in an external electric field which does not vary rapidly with time, the electric potential is constant on the inner side of the plasma membrane (*see* Lovelace et al., 1984). If two different points on the exterior of the plasma membrane have potentials which differ by V, then this potential difference appears across the membrane.

Consider first a cell in an electric field which is localized over a distance d which is much smaller than the diameter of the protoplast. For example, d might be of the order of a few Debye lengths. The region of high field moves with the ice front and therefore the high field region passes the cell. The potential of the solution immediately outside the membrane changes from the potential of the bulk solution to that of bulk ice as the high field region passes by. By hypothesis, the field is highly localized, and so, at some time during the passage of the high field region two points outside the membrane separated by a distance greater than d have a potential difference which could be an appreciable fraction of the total freezing potential. For a freezing rate of $R \sim 10^{-5}$ m/sec and a Debye length of $\lambda \sim 1$ nm, the time-scale of the electric field "pulse" across the membrane is $\lambda/R \sim 10^{-4}$ sec, which is long compared with the RC charging time scale of the protoplast (Lovelace et al., 1984).

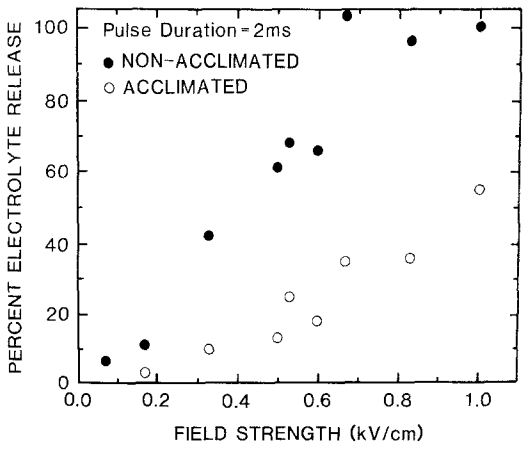

Fig. 7. Release of intracellular electrolytes from isolated protoplasts as a function of electrical field strength

At the other extreme, consider a cell in a relatively weak field which extends over a distance larger than a cellular diameter, $2r$. This can be approximated by a field (with strength E) which is constant far from the cell. Solving Poisson's equation with suitable boundary conditions shows that the transmembrane potential difference is $3Er/2$ at points on a cell axis parallel to the field. Suppose that a freezing potential of 10 V were spread over a distance of 200 μm, yielding a field of $\sim 5 \times 10^4$ V · m^{-1}. For a protoplast with $r = 15$ μm, a peak potential difference of ~1.1 V is generated across the membrane. Thus, whether the field extends over nanometers or microns, at some time during the passage of the high field region the membrane may be exposed to a potential which is a substantial fraction of the total Workman-Reynolds potential.

Cryomicroscopy reveals that the geometry of a cell being engulfed by ice is not adequately represented by a parallel field at a planar interface (Dowgert & Steponkus, 1983). Most often one or more channels of solution connect the pool of solution containing the engulfed protoplast to the receding interface. This channel of highly conducting solution would keep the potential near most of the plasma membrane near that of the bulk solution. In this state, the approach of ice towards the membrane at any point would produce a potential difference across the membrane at this point.

In summary, the experimental observations and theoretical analyses suggest that electrical transients that occur during the freezing of aqueous solutions may contribute to destabilization of the

plasma membrane and result in cryoinjury. Although direct measurements of membrane potential during a freeze-thaw cycle will present significant technical problems, such studies are warranted.

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