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Mechanical Study of the Deformation and Rupture of the Plasma Membranes of Protoplasts during Osmotic Expansions

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Summary. The stress and strain (surface tension and fractional change in area) in the plasma membrane of protoplasts isolated from rye leaves (*Secale cereale* L. cv Puma) were measured during osmotic expansions from isotonic into a range of more dilute solutions. The membrane surface tension increases rapidly to a maximum and then decreases slowly with some protoplasts lysing in all phases of the expansion. The maximum surface tension is greater for rapid expansions, and protoplasts lyse earlier during rapid expansion. Over the range of expansion rates investigated, the area at which lysis occurs is not strongly dependent on expansion rate. The value of the maximum tension is determined by the expansion rate and the rate at which new material is incorporated into the membrane. During osmotic expansion, protoplasts isolated from cold-acclimated plants incorporate material faster than do those from nonacclimated plants and thus incur lower membrane tensions.

Key Words membrane mechanics · plant protoplasts · osmotic expansion · cold acclimation

Introduction

The extent to which isolated protoplasts may be expanded osmotically by resuspension in a more dilute solution is limited by the expansion in area of the plasma membrane. The area increase at which 50% of a population lyse is called the Tolerable Surface Area Increment (TSAI), and it is largely independent of the time course of the osmotic treatment (Wiest & Steponkus, 1978). Plant protoplasts isolated from leaf mesophyll cells are spherical over a range of osmotic pressures, and their plasma membranes appear smooth and continuous in both light and electron micrographs. (Dowgert & Steponkus, 1984; Gordon-Kamm & Steponkus, 1984). Typically, protoplasts isolated from nonacclimated plants can survive surface area increases of 25% without lysis. Those isolated from cold-acclimated plants can be expanded in area by typically 60% without lysis. In either case, some individuals will expand rather more than the mean value without lysis (Wiest & Steponkus, 1978).

Over sufficiently short periods (less than about 1 sec) the plasma membrane behaves elastically, and changes in the membrane surface tension γ are related to changes in the area A by the equation

$$\Delta\gamma = k_A \, \Delta A/A \qquad (1)$$

where the area elastic modulus k_A is about 200 mN · m^{-1} (Wolfe & Steponkus, 1981; Wolfe, Dowgert & Steponkus, 1985). The membrane lyses under surface tensions in excess of about 4 mN · m^{-1} and so the maximum intrinsic, elastic stretching[1] of the membrane is around 2%. Larger changes in membrane area do not conserve the amount of material in the membrane. Wolfe and Steponkus (1981, 1983) proposed that the plasma membrane has a resting or equilibrium tension, γ_r (about 0.1 mN · m^{-1}) and that this resting tension is achieved at different degrees of expansion by the exchange of material between the plasma membrane and a reservoir of membrane material. When $\gamma > \gamma_r$ (during, for example, an osmotic expansion) material is transferred into the membrane from a reservoir and relatively large area increases are possible. If $\gamma = 0$ (immediately following an osmotic contraction, for example), the flaccid membrane undergoes endocytotic vesiculation until spherical shape and a resting tension are regained.

[1] The membrane whose mechanical properties are most studied is that of the erythrocyte (Evans & Skalak, 1979). The elastic modulus to the protoplast plasma membrane is about half that of the erythrocyte, and both lyse under intensive area expansion of 2 to 3%. Unlike the protoplast plasma membrane, the erythrocyte membrane is apparently incapable of extensive changes in area.

The dependence of the rate of incorporation of new material on the tension in the plasma membrane was measured by Wolfe et al. (1985) using micropipette aspiration (Mitchison & Swann, 1954). This dependence yielded a differential equation, which was solved to predict the time variation in γ in a membrane during an area expansion with a given time course $A(t)$. The form of $A(t)$ used in that study was an analytical function whose parameters were related to the osmotic pressures and an effective hydraulic conductivity for the membrane (assumed constant during the expansion). Hitherto, studies of the osmotic behavior of protoplasts (Wiest & Steponkus, 1978) had been made using equilibrium measurements of the diameters of protoplasts in large populations before and after abrupt changes in the osmotic pressure of the suspending media. This allowed comparison of population means before and after different osmotic treatments. The time course of osmotic expansion has not previously been studied in detail for individual protoplasts. Wolfe et al. (1985) measured the dependence of the frequency of membrane lysis as a function of tension, but did not use these measurements to calculate the tolerable surface area increment before lysis (the TSAI) because of a lack of experimental information about $A(t)$ and $\gamma(t)$ during expansions.

In this study one set of experiments was designed to determine: (i) $A(t)$ for individual protoplasts during an osmotic expansion produced by abrupt dilution of the suspending medium, and (ii) the TSAI and its possible dependence on variation in the expansion time course $A(t)$, and on variation of the tension in the membrane.

These time-dependent strain studies and the published dynamic stress-strain relations allow one to infer the time-dependent stresses in the membrane during an osmotic expansion. To obtain more direct information, a series of experiments was conducted in which the stress and strain in the plasma membrane were simultaneously monitored during an osmotic expansion. In these experiments a protoplast was attached to a micropipette by suction, which was used to measure the tension in its membrane. Both were located in a chamber through which flowed solution whose osmotic pressure decreased with time.

Cold acclimation of rye seedlings increases the TSAI of protoplasts isolated therefrom: such protoplasts can withstand larger osmotic expansions without lysis than can protoplasts isolated from nonacclimated leaves (Dowgert & Steponkus, 1984). One of the aims of this study is to relate this difference directly to the different dynamic properties of the plasma membranes of protoplasts of each class. For brevity, protoplasts isolated from nonacclimated seedlings are referred to as nonacclimated protoplasts and those from acclimated seedlings are termed acclimated protoplasts.

Materials and Methods

Plant Material and Protoplast Isolation

Seeds of *Secale cereale* L. cv Puma were sown in vermiculite and germinated in a controlled environment at 20°C (day) and 15°C (night) with a 16-hr photoperiod. Nonacclimated plants (LT_{50} −5°C) remained in this environment for 2 weeks. Acclimation was achieved by exposing 1-week old plants to 13°C (day) and 7°C (night) with an 11.5-hr photoperiod for one week and then to a 2°C controlled environment (10-hr photoperiod) for 4 weeks. The LT_{50} for plants thus acclimated is −25°C.

Protoplasts were enzymically isolated from the leaves as previously described (Dowgert & Steponkus, 1984). After digestion, the protoplasts were resuspended in isotonic sorbitol, 0.53 and 1.03 osm for nonacclimated and acclimated protoplasts, respectively. The higher isotonic osmolality for acclimated protoplasts was required to maintain the estimated in situ volume due to increases in internal solute concentration during cold acclimation. Acclimated and nonacclimated protoplasts have similar sizes when suspended in their respective isotonic solutions.

Osmotic Manipulation of Individual Protoplasts

Pipettes of 40 μm diameter were used to capture, to hold, and to transfer individual protoplasts from isotonic solutions to hypotonic solutions. The protoplasts were then ejected, and the increase in area as a function of time was determined. Protoplast suspensions were loaded by capillary suction into a 0.2-mm path length microslide (Vitro Dynamics, Rockwaway, N.J.). The pipette was inserted in the microslide, and a single protoplast was drawn into it by negative pressure applied to the pipette. The pipette was then withdrawn from the microslide and inserted in one containing the desired final osmolality. The protoplast, along with a small "plug" of the original solution, was ejected into the new solution by a short application of a very small positive pressure to the pipette. The pipette was immediately withdrawn to prevent further mixing of the two solutions. To ensure that the protoplast was not exposed to dilute solution before ejection, it was held 100 μm or more from the end of the pipette during transfer. The volume of the plug transferred with the protoplast (of order 100 pl) was not necessarily constant for each transfer. Following ejection, protoplast diameters were measured as a function of time. Protoplast volume and surface area were calculated assuming spherical geometry. A video camera mounted on a microscope produced images that were digitized in real time and stored on a Winchester fixed disc. After the experiment, the digitized images were displayed on a video monitor and the operator measured diameters by manipulating cursors on the screen using an image processor (*see* Steponkus, Dowgert, Ferguson & Levin, 1984).

Measurement of Membrane Tension during Osmotic Expansion

The chamber used to conduct these experiments is shown in Fig. 1. A microslide positioned under a microscope was connected by a flexible tube at one end to a solution of the initial osmolality and filled with this solution. A flexible tube at the other end was connected to a reservoir of the desired final osmolality. The height of this reservoir could be changed to cause flow of this solution through the microslide. A small slit was ground in the side of the microslide; the meniscus at this aperture was sufficiently curved to prevent the escape of solution. Protoplasts were introduced to the microslide through this slit.

A micropipette mounted on a micromanipulator was introduced through the slit. (Micropipettes were pulled on a commercial pipette puller and snapped off to form a neat planar annulus normal to the pipette axis. The inner diameter of the pipettes was generally between 9 and 13 μm.) A flexible tube connected the micropipette to a manometer and pressure transducer. A protoplast was attached to the micropipette by applying a small suction with the manometer.

The reservoir of more dilute solution was raised, causing dilute solution to flow past the protoplast on the end of the micropipette. As the protoplast expanded, the manometer was lowered (and thus the pressure in the micropipette made more negative) to maintain a constant, approximately hemispherical, intrusion into the micropipette. The surface tension in the membrane was obtained from the pressure in the pipette (measured to ± 0.1 Pa with the transducer) and the curvatures of the membrane (measured on a video screen using cursors of a video image processor) using the Laplace-Young equation. The area of the membrane was determined from the diameter of the protoplast, the internal diameter of the pipette, and the length of the intrusion therein. (Further details of the method and its accuracy are given by Wolfe & Steponkus, 1983.) During the transfer of protoplasts, we suppose that evaporation at the solution-air interface causes a very small local increase in the osmotic pressure, and therefore the membrane may be slightly nonspherical at the start of the experiment. As a consequence, a very small volume expansion would be possible without an increase in area and therefore without stretching of the membrane.

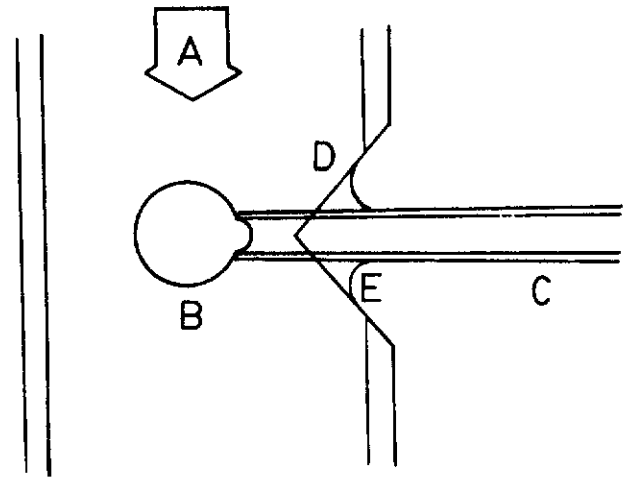

Fig. 1. A section through the glass microslide in the apparatus used for the measurement of $\gamma(t)$ and $A(t)$ during an osmotic expansion. (*A*) Flow of solution; (*B*) protoplast; (*C*) micropipette; (*D*) hole filed in microslide; (*E*) meniscus

Results

Expansion on Transfer to Dilute Solutions

Individual protoplasts were transferred from isotonic solution to hypotonic solution to determine the time dependence of the membrane area [$A(t)$], including the maximum expansion before lysis (the Tolerable Surface Area Increment or TSAI). Figure 2 plots the fractional increase in area [$\Delta a = A(t)/A_I - 1$ where A_I is the initial area] for nonacclimated protoplasts transferred into solutions with a range of hypotonic osmolalities. It should be noted that the final osmolality is, in each experiment, slightly greater than that of the solution into which the protoplasts were transferred due to the transfer with the protoplasts of a small "plug" of isotonic solution whose volume varied but was typically 100 pl or about 0.5% of the volume of the microslide into which they were transferred. A filled circle at the end of a curve indicates lysis at that Δa and t. Curves without such circles indicate protoplasts which reached a steady final size ($da/dt = 0$) without lysis. Time after transfer was measured to $<\pm 1$ sec, but accurate calculation of area was limited by the optical resolution of edges used to measure diameters, D. For a sphere $A = \pi D^2$ so $dA = 2\pi DdD$ and so $dA/A = 2dD/D$. For a typical diameter measurement of 30.0 ± 0.5 μm, the proportional error in A is therefore $\pm 3\%$.

In all cases, expansion was initially rapid, but the rate of expansion decreased with time if and when the protoplasts survived long enough to approach osmotic equilibrium. For the smallest dilution (0.53 to 0.34 osm) 7 out of 10 protoplasts survived the expansion, whereas for the two largest dilutions (0.53 to 0.14 and 0.00 osm) all protoplasts lysed during expansion. For all treatments, however, lysis among the population occurs over a range of increases in area. It cannot be argued that lysis occurs in any particular phase of the expansion. In the intermediate dilutions (0.53 to 0.29 or 0.24 osm) some protoplasts lyse soon after transfer, during the rapid phase of the expansion, and others lyse much later as they approach osmotic equilibrium. For small dilutions most survive the expansion. In the population that was transferred to distilled water there is not much change in the rate of expansion with time, but lysis occurs over a range

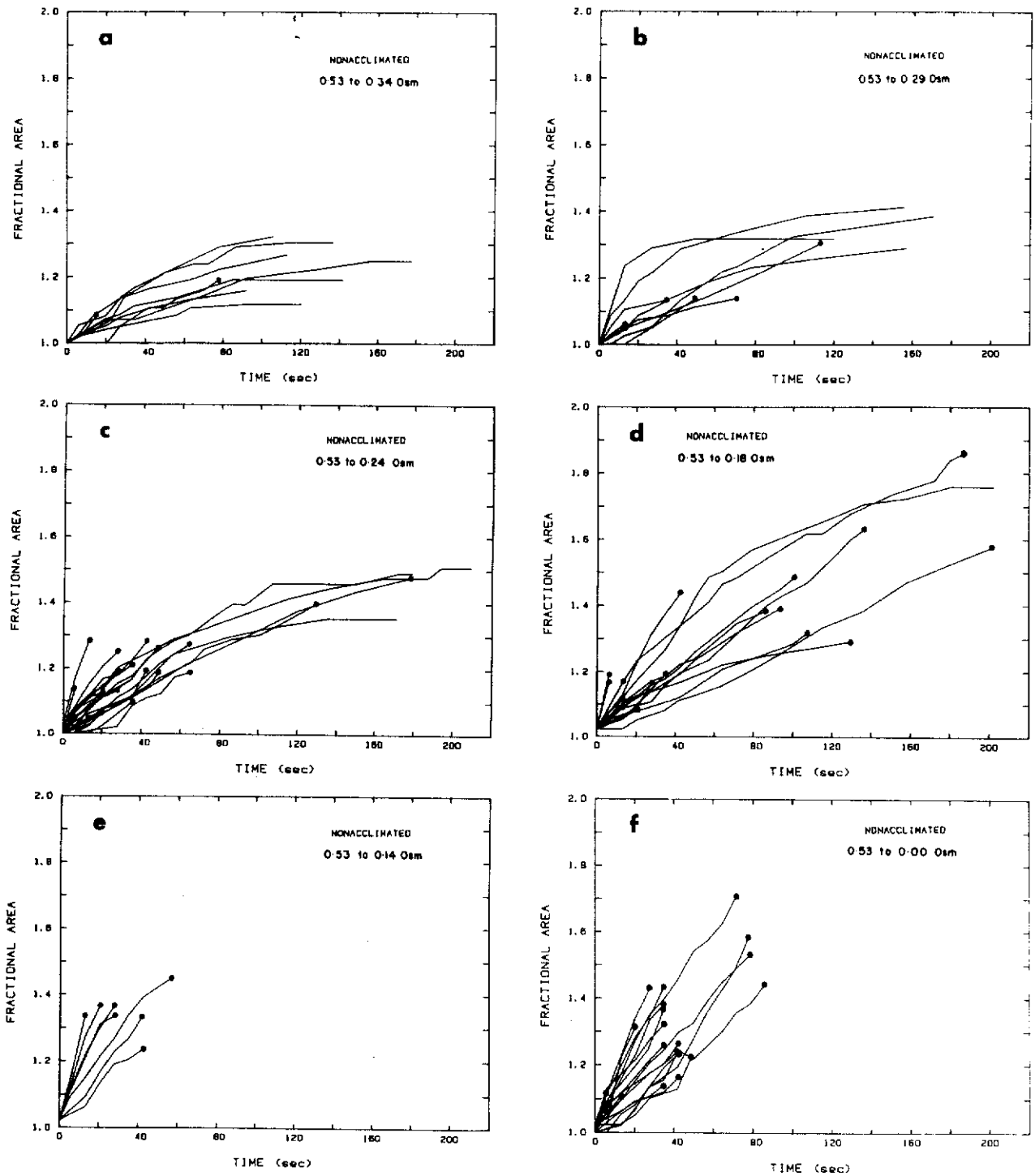

Fig. 2. The surface area as a function of time is plotted for nonacclimated protoplasts transferred to solutions whose osmolalities were (*a*) 0.34, (*b*) 0.29, (*c*) 0.24, (*d*) 0.18, (*e*) 0.14 and (*f*) 0.00 osm from isotonic solution (0.53 osm). In this figure and in Fig. 4 lysis is represented as a filled circle terminating the curve

of areas. The range of areas over which lysis occurs is similar in all treatments among nonacclimated protoplasts.

Figure 3 shows the results of a similar series of experiments conducted on acclimated protoplasts. The results are qualitatively similar: most protoplasts survive small dilutions and none survive transfer to distilled water; expansion is initially rapid but slows if protoplasts survive to approach osmotic equilibrium; among the population, lysis

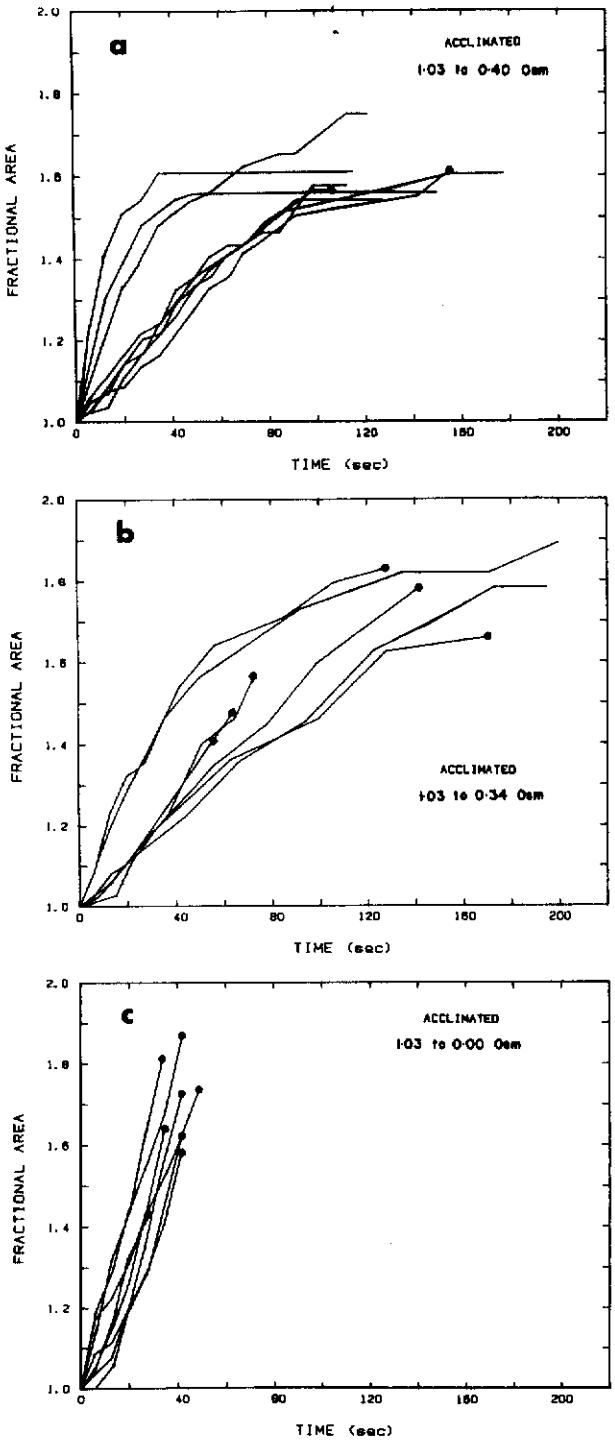

Fig. 3. The surface area as a function of time is plotted for acclimated protoplasts transferred to solutions whose osmolalities were (*a*) 0.40, (*b*) 0.34 and (*c*) 0.00 osm from isotonic solution (1.03 osm)

occurs over a range of areas during all phases of the expansion. For each different dilution, lysis occurs over a similar range of increases in area. Acclimated protoplasts lyse after larger expansions in area, however, than do nonacclimated protoplasts.

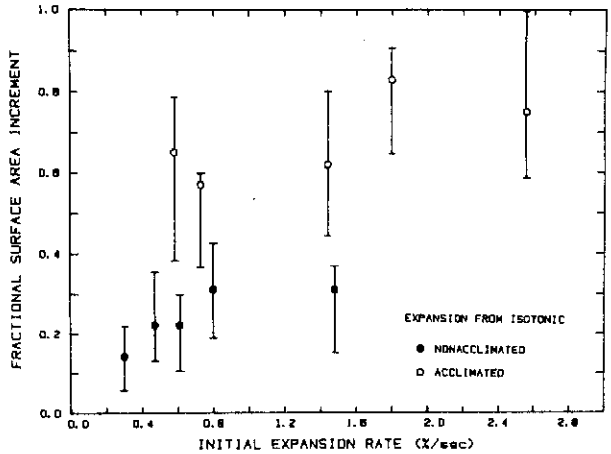

Fig. 4. The fractional area increase at which lysis occurred (the TSAI) as a function of the initial area expansion rate. Open circles represent acclimated and filled circles represent nonacclimated protoplasts. Larger maximum expansion rates are possible for acclimated protoplasts because their interior osmotic pressure at isotonic is greater

The time course of expansion shown in Figs. 2 and 3 varies substantially both among and within treatments; in particular the initial expansion rate varies among protoplasts as well as among treatments. In order to examine the possible dependence of TSAI on expansion rate, all the data displayed in Fig. 2 were divided into pentiles according to the initial rate of area expansion. The data displayed in Fig. 3 were likewise divided. (The initial expansion rate in each case was calculated from the derivative at $t = 0$ of a curve with the form of Eq. (A4), which was fitted to the data (t, A) by the method of least squares. See Appendix I for details.) The results are shown in Fig. 4. Within each population (acclimated and nonacclimated) there is a small increase in TSAI with initial rate of area expansion over the range of expansion rates produced by the dilutions used in this study.

Figures 5 and 6 show the results of experiments in which both area and membrane tension γ were monitored as a function of time during an osmotic expansion achieved by passing a range of dilute solutions past protoplasts attached to a micropipette inside a microslide (*see* Fig. 1). Three different final concentrations were used for each population. In the dilutions into finite osmolality (Figs. 5*a* and 6*a*), some of the expansion curves show a decrease in rate as osmotic equilibrium is approached; in the other instances, lysis occurs before there is any reduction in expansion rate. In the dilutions into distilled water (Figs. 5*c* and 6*c*), the rate of expansion either remains steady or increases with time.

The plots of tension as a function of time all show a region of steep increase. For acclimated

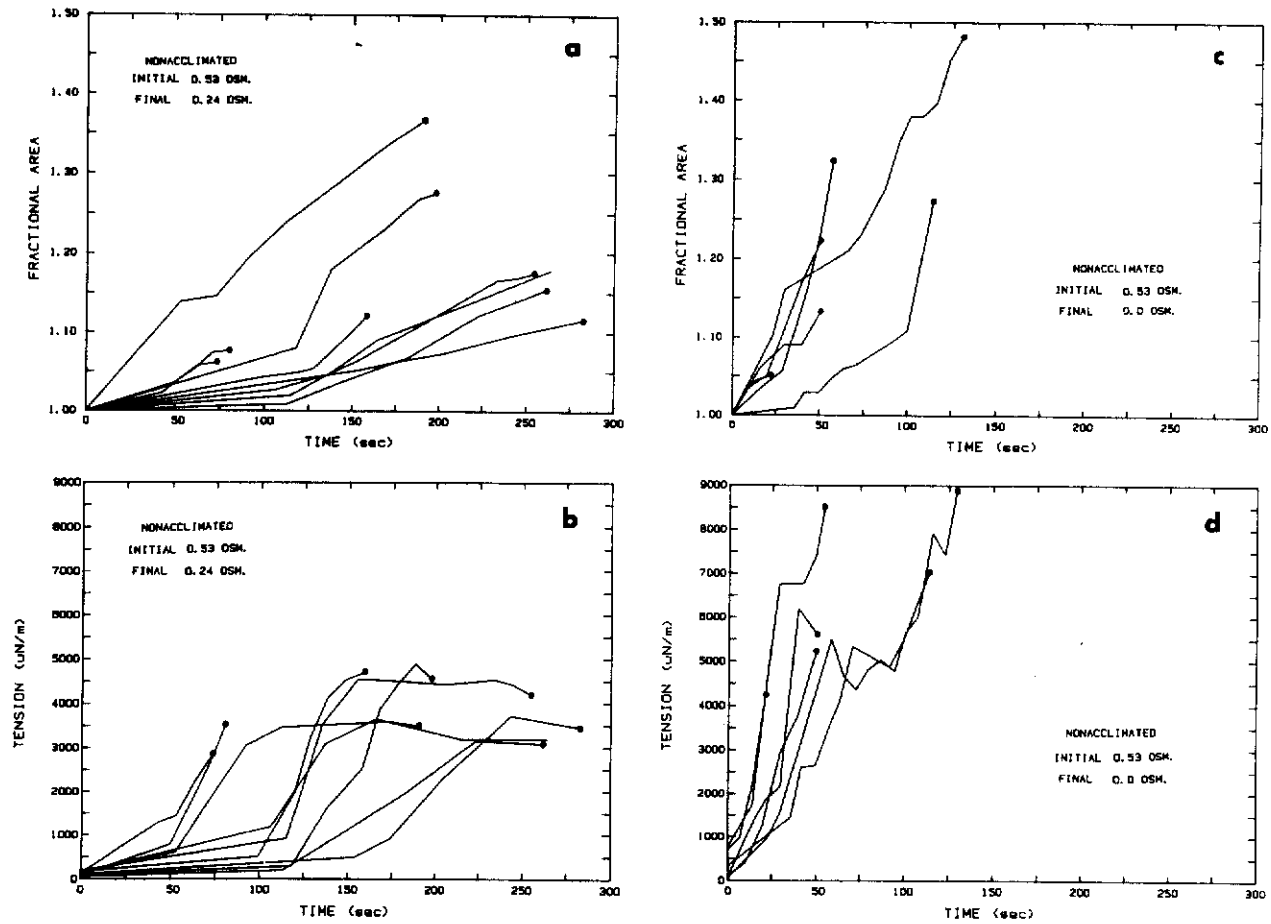

Fig. 5. *a* shows $A(t)$ for nonacclimated protoplasts which were osmotically expanded in the solution chamber (Fig. 1) in a solution whose osmolality fell from 0.53 to 0.24 osm. *b* shows $\gamma(t)$ for the same protoplasts, monitored during the expansion. *c* and *d* show $A(t)$ and $\gamma(t)$ for nonacclimated protoplasts expanded in a solution whose osmolality fell from 0.53 to 0.00 osm. In this figure and in Fig. 6 lysis is represented by a filled circle terminating a curve

protoplasts this is often followed by a region in which tension does not increase, and then decreases with time. For nonacclimated protoplasts, the tension stops increasing and begins to decrease in several of the expansions in finite dilution, but in the dilution into distilled water the tension increases until lysis. In all treatments there are some protoplasts which lyse during the rapid increase in tension. In some cases, the area expansion begins very slowly and this is accompanied by an initially slow increase in tension.

Discussion

It is our aim in this study to answer the question: what determines the extent of expansion that a plasma membrane can support before lysing? The mechanical failure of most materials is discussed in terms of the stress to which they are exposed and the time over which the stress is applied. The protoplast plasma membrane approximates a two-dimensional fluid so the isotropic surface tension γ is the appropriate stress. We shall discuss first the experimental study of $\gamma(t)$ during an expansion. We note that the osmotic expansion used in the experiments designed for the measurement of $\gamma(t)$ differs somewhat from an abrupt dilution with good mixing. This is due to the experimental constraints imposed by the simultaneous measurement of γ and A. In contrast, the experiments in which only $A(t)$ was measured allowed more abrupt dilution of the supporting medium, and these experiments yielded a range of total expansion and expanion rates. The area increase at membrane lysis is of particular interest since it determines whether a protoplast of given size can support a given dilution.

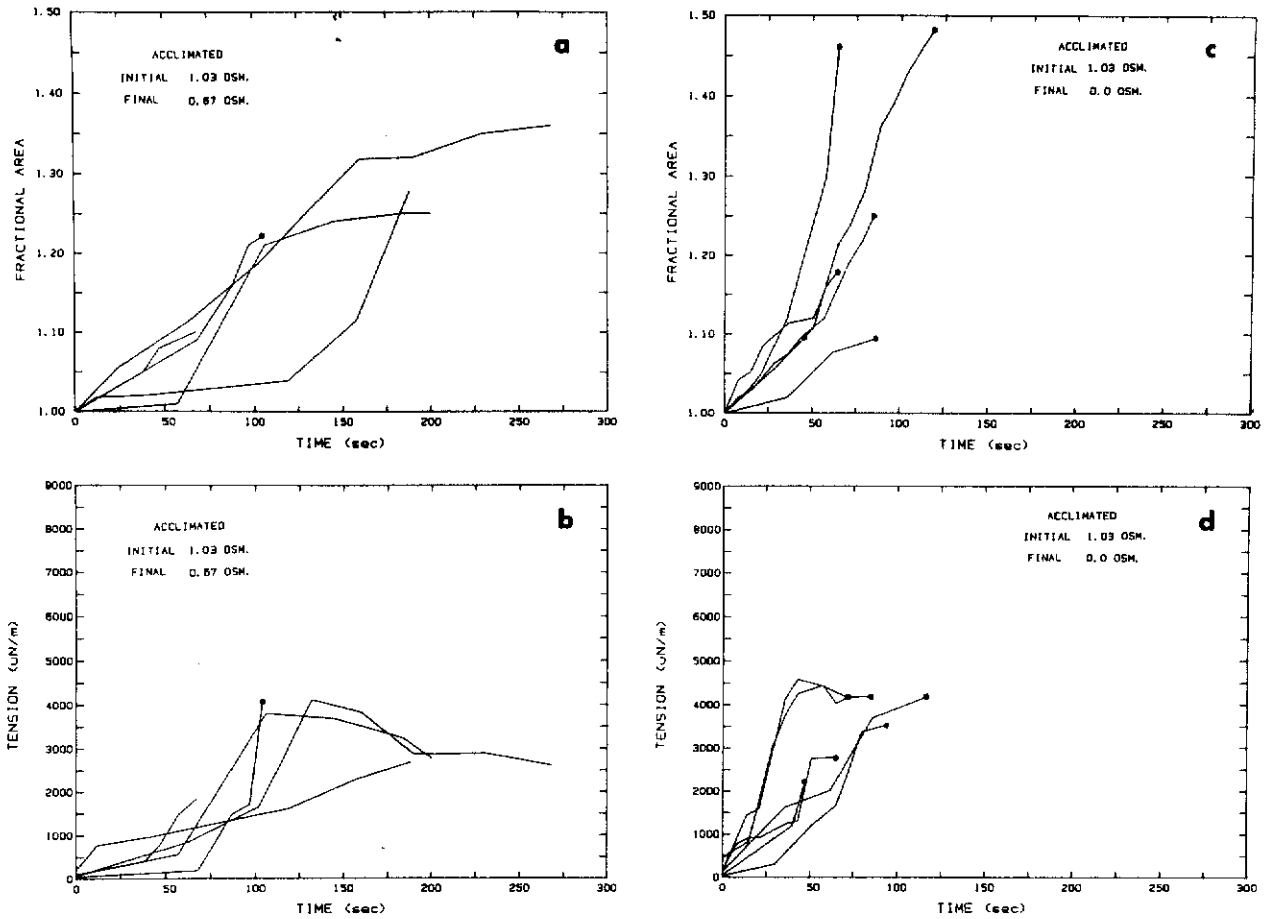

Fig. 6. *a* and *b* show, respectively, $A(t)$ and $\gamma(t)$ for the acclimated protoplasts expanded in a solution whose osmotic pressure fell from 1.03 to 0.67 osm. *c* and *d* show $A(t)$ and $\gamma(t)$ for acclimated protoplasts expanded in a solution whose osmotic pressure fell from 1.03 to 0.00 osm

Mechanical Analysis

The tension in the membrane during an expansion is jointly determined by the intrinsic elasticity of the membrane (which increases the tension during expansion) and by the rate at which membrane material enters the membrane (and thus relaxes the tension). The tensions necessary to overcome membrane viscosity are negligible over timescales of seconds and greater. An analysis of the tension so generated was given by Wolfe et al. (1985), and we summarize the results of that analysis below.

(i) The AREA ELASTIC MODULUS k_A is defined by

$$\gamma = k_A(A - A_o)/A_o \tag{1}$$

where the membrane has area A when subjected to an isotropic surface tension γ, and where A_o is the area occupied by the membrane (comprising the same amount of material) when $\gamma = 0$. k_A is measured to be about 200 mN · m^{-1}, whereas tensions of several mN · m^{-1} cause lysis. It is therefore assumed that $\gamma \ll k_A$, and it follows that the amount of intrinsic stretching is limited to a few percent.

(ii) The rate of proportional increase in area is defined by

$$Z = \frac{1}{A}\left(\frac{\partial A_o}{\partial t}\right)_\gamma. \tag{2}$$

From Eqs. (1) and (2) and for $\gamma \ll k_A$ it follows that for any function $A(t)$, $\gamma(t)$ satisfies the differential equation

$$\frac{d\gamma}{dt} = k_A\left(\frac{1}{A}\frac{dA}{dt} - Z(\gamma)\right) \tag{3}$$

where the first term on the right represents the expansion rate and the second the rate of incorporation of new membrane material.

(iii) In the simplest model, Z is assumed to be a function of γ only, and experiments show that Z is a strong function of γ. The empirical relation

$$Z = M(\exp \gamma/\Gamma_i - \exp \gamma_r/\Gamma_i), \qquad \gamma \geq \gamma_r \qquad (4)$$

is used, where M and $1/\Gamma_i$ are the coefficients of a linear regression of ln Z on γ. The last term in Eq. (4) is immeasurably small (its value is typically 10^{-6} sec^{-1}) and is inserted to satisfy the formal requirement that $Z = 0$ when γ takes its resting value γ_r. Γ_i shows fairly small variation among protoplasts, but the largest and smallest values of M differ by a factor of nearly 100.

(iv) Equations (3) and (4) can be solved analytically for any monotonic increasing function $A(t)$. The solution is a lengthy expression. An exact solution for $A(t)$ is possible for a spherical ideal osmometer transferred abruptly to a more dilute solution, assuming that ideal mixing occurs (this solution is given in Appendix I). In such an expansion, the greatest expansion rate ($r = [1/A][dA/dt]$) is achieved immediately and r decreases gradually to zero as the protoplast comes to osmotic equilibrium at the lower osmotic pressure. As a consequence, γ increases rapidly till it reaches a maximum then gradually decreases to γ_r—providing, of course, that the protoplast survives the expansion.

The experiments in which both $\gamma(t)$ and $A(t)$ were measured (Figs. 5 and 6) show expansions that differ from those produced by abrupt dilution: they show an initial phase of increasing expansion rate. This is probably the result of mixing near the boundary between the isotonic solution and the dilute solution, which produces a region of gradually decreasing osmotic pressure in the chamber shown in Fig. 1. As this layer of solution moves past the protoplast under observation, the osmotic pressure difference across the membrane increases gradually, and this produces the increasing expansion rate. For protoplasts that attain or approach osmotic equilibrium the expansion rate must ultimately approach zero. The dilutions used here were chosen to produce lysis so that the TSAI could be measured. Thus, even in the dilution in solutions other than distilled water (Figs. 5*a* and 6*a*), most protoplasts lyse during the rapid expansion and only the longer-lived individuals show the slowing of expansion rate as they approach osmotic equilibrium.

The form of $\gamma(t)$ must be examined individually for each experiment, since the individual forms of $A(t)$ varied substantially due to differences in mixing between the chamber and the solution reservoir. In all cases, the beginning of the rapid increase in area is accompanied by a steep increase in the membrane tension. Over steady increases in area, the tension increases with time until either the protoplast lyses or the tension reaches a maximum value. From Eq. (3), the maximum γ is that value which satisfies $Z(\gamma_{max}) = [1/A][dA/dt]$. For the longer-lived protoplasts in which γ reaches a plateau, the slope of the $A(t)$ curve and the value of γ_{max} yield a datum (Z, γ). As we reported previously, there is a large scatter in Z in any population; nevertheless, Figs. 5*a,b* and 6*a,b* show a substantial difference between the two protoplast populations. The maximum rates of area expansion (r) for nonacclimated protoplasts in Fig. 5*a* are about 2×10^{-3} sec^{-1} or less, and the maximum tensions reached are in the range 3.5 to 4.8 mN · m^{-1}. Figure 6*a* shows acclimated protoplasts subjected to expansion rates greater than 2×10^{-3} sec^{-1} and maximum tensions less than 4.1 mN · m^{-1}. This is in agreement with the observation by Wolfe et al. (1985) that a given incorporation rate was produced by a lower tension in the membrane of acclimated protoplasts than in the membrane of nonacclimated protoplasts or, equivalently, that the same tension produced a greater incorporation rate in acclimated than in nonacclimated protoplasts. We argued previously that the greater Z in acclimated protoplasts conferred on them their greater ability to survive expansions by producing a lower maximum γ. (This greater ability is not due to a greater membrane strength: at any given tension the membranes of acclimated protoplasts are more likely to lyse than are those of nonacclimated protoplasts.)

In those cases in Figs. 5*a* and 6*a* in which the protoplasts survive long enough to show the decline in expansion rate, the tension is observed to fall very slowly from its maximum value, as predicted by the mechanical theory of Wolfe et al. (1985). In principle, a measured $A(t)$ such as in Figs. 5*a,c*, and 6*a,c* can be inserted in Eq. (3) and a solution $\gamma(t)$ obtained numerically for comparison with the measured $\gamma(t)$. This approach is unhelpful in practice because Eq. (3) requires the local slope of $A(t)$, which is very sensitive to errors in consecutive measurements of A. Optical resolution limits the accuracy of A, and the slope cannot be determined sufficiently accurately.

Lysis

Figures 5 and 6 show that lysis of the plasma membrane is not simply the result of the tension exceeding some critical value; rather, it appears that lysis depends on both the tension and the time of applica-

tion. In the slow expansions (Fig. 5*a*), the tension in the membranes of nonacclimated protoplasts has a maximum value in the range 3.5 to 4.8 mN · m^{-1}, and these tensions are supported for a range of periods of up to 130 sec before lysis. (Five of the protoplasts lyse when the membrane tension has declined from the maximum value.) In the fast expansion (Fig. 5*c*), the tension in the membranes of similar protoplasts quickly reaches values over 5.0 m^{-1}. These and larger values of γ are supported for a range of shorter periods before lysis. For acclimated protoplasts, the slow expansion (Fig. 6*a*) produces values of γ less than 4.1 mN · m^{-1}, which result in the lysis of only one protoplast during the expansion. The fast expansion (Fig. 6*c*) produces higher tensions, and the protoplasts are all lysed between 45 and 170 sec.

In a previous study (Wolfe et al., 1985), we argued that membrane lysis was stochastic and tension-dependent and we defined the probable frequency of lysis, ω, where ωdt is the probability that a protoplast lyses between t and $t + dt$. Measurements of the lifetimes of protoplasts whose membranes were subjected to a range of tensions indicated that, at least over the range studied, ω is a function only of γ; i.e., that the chance of lysis depended only on γ and the time of exposures to that γ. ω was a strongly increasing function of γ (the simplest analytical approximation is that γ is proportional to exp γ/Γ_1 where the constant Γ_1 is about 0.7 mN · m^{-1}). The results shown in Figs. 5 and 6 are qualitatively explained in terms of the proposed stochastic, tension-dependent lysis. Lifetimes vary widely over a given range of tensions, and higher tensions produce shorter lifetimes. The range of γ produced by the fast expansion in acclimated protoplasts (Fig. 6*d*) is similar to that produced by the slow expansion in nonacclimated protoplasts (Fig. 5*b*) because of the greater incorporation rate in acclimated protoplasts. Over this range of tensions, acclimated protoplasts have shorter lifetimes (and thus a greater ω) than do nonacclimated protoplasts. This observation agrees with our previous study (Wolfe et al., 1985) in which γ was the independent variable.

Area Increase Before Lysis

How does the range of $\gamma(t)$ affect TSAI, the area increase before lysis? A comparison of Fig. 5*a* and *c* shows that there is a larger variation in the area at which lysis occurs (from 7 to 37% for expansion into 0.25 osm, from 5 to 48% for dilution with pure water). The mean area at lysis is greater in the latter case, but there is considerable overlap in the range of areas. (Such a comparison cannot be made for acclimated protoplasts—Fig. 6*a* and *c*—since nearly all survived the expansion into 0.67 osm). The similarity in TSAI is due to the compensation between tension-dependent incorporation and tension-dependent lysis. A slow expansion requires a slow incorporation rate and thus a low γ, and at low γ the protoplasts have a long lifetime. A fast expansion requires a fast incorporation rate and a high γ, so the lifetime is short. The product of a small expansion rate and a long lifetime is not very different from the product of a large expansion rate and a short lifetime. We analyze this compensation formally in Appendix II.

The experiments in which $A(t)$ was measured for a range of dilutions allow a quantitative examination of the dependences of the TSAI on expansion rate. Each of the $A(t)$ curves was fitted to an equation of the form

$$A = A_I + \Delta A \exp(-t/\tau)$$

where ΔA and τ are constants which are related to parameters of the expansion in Appendix I. The initial expansion rate was calculated from this equation because of the difficulties with numerical differentiation, which were discussed above. The experiments were grouped in pentiles according to initial expansion rate and Fig. 4 plots TSAI against initial expansion rate.

In both populations, TSAI increases with increasing initial expansion rate. This may seem unexpected since viscous or plastic materials are more likely to fail during fast deformations than during slow deformations of the same magnitude. The stresses needed to overcome membrane viscosity, however, are negligible for deformations over seconds or longer (Wolfe & Steponkus, 1983) and so such effects are unimportant here. It should be stressed that the range of initial expansion rates is less than fivefold in each experiment, a limitation imposed by the maximum possible dilution and by the hydraulic conductivity of the membrane. The largest initial osmotic pressure difference that can be obtained is that which results from transfer to distilled water. At the other extreme, smaller dilutions than those used here produce small expansions which are survived by all the protoplasts and so the TSAI cannot be measured. It is possible, of course, that at sufficiently fast expansion rates the TSAI could decrease with expansion rate. Nevertheless, such large rates do not obtain during osmotic manipulation—even upon transfer to distilled water. Hence, for osmotically induced changes in area, the extent of expansion is not decreased by rapid rates of expansion.

The weak dependence of TSAI on the time-

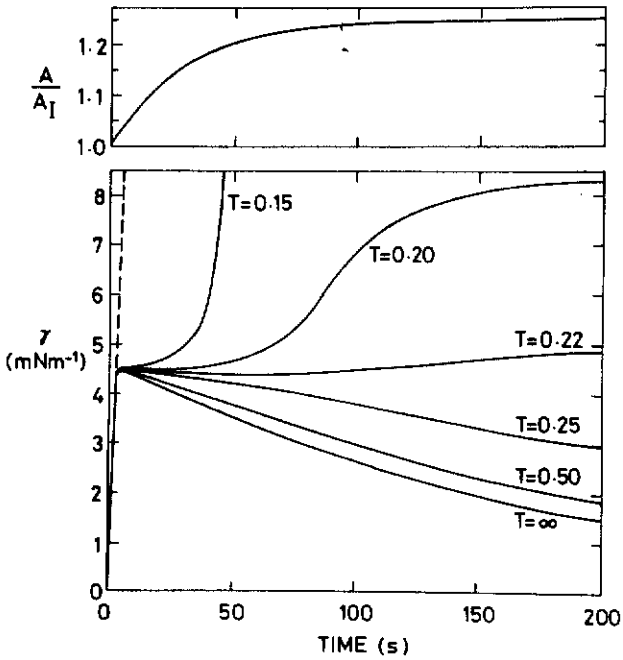

Fig. 7. *a* plots $A(t)$ for an expansion of 25% using Eq. (5). *b* plots solutions to Eq. (6) using different values of T, a parameter representing the area of the material available for incorporation as a fraction of the initial area. Because the incorporation rate at any given tension varies widely among protoplasts, the time over which the tension decreases to its resting value also varies widely—from several tens of seconds to tens of minutes

course of expansion is consistent with the results of the experiments of Wiest and Steponkus (1978) on the fractional survival of protoplast populations following osmotic manipulation, which indicated that the median TSAI was a constant of the population, independent of the time course of the expansion. One hypothesis we sought to test was that the extent of expansion at lysis was related to the extent of material in the reservoir available for incorporation of material. If, during an expansion, the reservoir was depleted so that the rate of incorporation fell to zero, then the membrane would expand only by intrinsic stretching. The tension would then increase at a rate of k_A times the fractional expansion rate. For an expansion rate of 0.005 sec^{-1} (a rather slow rate among those measured here) the tension would increase by 1 $mN \cdot m^{-1}$ in each second and the membrane would be ruptured in a few seconds.

Our previous mechanical analysis assumed that the incorporation rate depended only on γ. To include the possible effect of depletion of the reservoir we have introduced to our previous model one adjustable parameter[2] T, where TA_I represents the area of the material in the reservoir (available for expansion) when the protoplast is in isotonic suspension with initial area A_I. The area A_o occupied by the membrane at $\gamma = 0$ is $A/(1 + \gamma/kA)$, so, during an expansion, if the area is A then the area of material in the reservoir would be $TA_I - (A_o - A_I)$ or $A_I(1 + T) - A/(1 + \gamma/k_A)$. To model the putative mass action of the material in the reservoir, we made the proportional incorporation rate Z proportional to this quantity as well as being exponentially dependent on γ. Thus Z is zero when $A_o = A_I(1 + T)$, i.e., when the reservoir is depleted, and Z is a maximum when $A_o = A_I$. We then solved the differential equation[3] for $\gamma(t)$ for various values of T.

In Fig. 7 are shown the solutions for an expansion of 25% given by the analytic approximation in Eq. (5) and using parameters for Z and τ measured previously (Wolfe et al., 1985). When $T = \infty$, the result is the same as the analytical solution to Eq. (3) given in our previous study. For $T \geq 0.25$, γ rises to approximately the same maximum and then falls at a slower rate due to the greater proportional reduction in the amount available for incorporation, but γ finally returns to γ_r at large t. For $T < 0.25$, γ rises to approximately the same value and then changes only slowly for a while until, when the reservoir approaches depletion, it rises rapidly towards its final value given by $\gamma/k_A = 1.25/(1 + T) - 1$. Thus this mass-action model for incorporation predicts an abrupt rise in γ as the reservoir runs out of material, even if the expansion rate at that time is quite slow. Such behavior is not observed in Figs. 5 and 6, so we reject the hypothesis that the TSAI is simply determined by the amount of material available for incorporation. On the other hand, if the reservoir has enough or more than enough material to supply area for the expansion imposed on the membrane by osmotic constraints, then there is little qualitative difference in the form of $\gamma(t)$ which results. Thus if the reservoir capacity exceeds the expansion required by even a modest amount, the approximation that Z is independent of A or T is reasonable.

We therefore conclude that, for the range of area expansions from isotonic considered in this

[2] The model of Wolfe et al. (1985) has no parameters that are not independently measured.

[3] Because reservoir depletion would lead to large γ, we did not make the approximation $\gamma \ll k_A$ in this calculation. The differential equation for γ thus became

$$\frac{d\gamma}{dt} = k_A \left(\frac{1}{A_o} \frac{dA}{dt} - \frac{A}{A_o} Z(\gamma, A_o) \right) \quad (6)$$

for the solution of which we were reduced to the use of numerical methods.

study (typically 25% for nonacclimated and 60% for acclimated protoplasts), the rate of incorporation is a function of the tension in the membrane, and so the tension produced in the membrane during an expansion is determined by the rate of area expansion and the function $Z(\gamma)$. The weak dependence of the TSAI on the time course results from compensation between the $Z(\gamma)$ and the tension-dependent probability of lysis $\omega(\gamma)$. This compensation is analyzed in Appendix II; it is, however, qualitatively simple: a given increase in area can result from a short rapid expansion with a short exposure of the membrane to a large tension, or by a long slow expansion with a long exposure to a lower tension. Over the range of expansion rates generated by osmotic manipulation of these protoplasts (about 0.003 to 0.03 sec^{-1}) the chance of lysis due to small γ for a long time and that due to large γ for a short time are similar. Further, the greater capacity of acclimated protoplasts to expand from isotonic is not due to a greater ability to resist mechanical stress, but due to a greater capacity to relax the stress by incorporating new material.

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Appendix I

The Time Course of Osmotic Expansion

Consider a protoplast bounded by a semipermeable membrane. The osmotic pressure of its suspending medium is rapidly reduced from Π_I to Π_F. The protoplast has radius R, internal osmotic pressure Π and its plasma membrane (or plasma membrane and tonoplast in series) has an effective hydraulic conductivity L_P. For reasons discussed by Wolfe and Steponkus (1983), the contribution to the chemical potential gradient due to hydrostatic pressure is neglected in comparison with that due to differences in osmotic pressure. Assuming spherical symmetry and that the solutions are well mixed (which is equivalent to assuming that diffusion is very much faster than permeation), the volume flux of water is given by

$$\frac{1}{A}\frac{dV}{dt} = \frac{dR}{dt} = L_P(\Pi - \Pi_F). \tag{A1}$$

Let the subscripts I and F represent initial and final values, and let the effective number of osmotically active particles within the membrane be n. Equation (A1) becomes

$$\frac{dR}{dt} = L_P\left(\frac{3nkT}{4\pi R^3} - \Pi_F\right) \equiv \alpha\left(\frac{1}{R^3} - \frac{1}{R_F^3}\right)R_F^4. \tag{A2}$$

The following equation may be shown to be a solution of Eq. (A2)

$$\alpha t = \frac{1}{s_I} - \frac{1}{s} + \frac{1}{6}\ln\left(\frac{1 - s^3}{1 - s_I^3}\right) - \frac{1}{2}\ln\left(\frac{1 - s}{1 - s_I}\right) - \frac{1}{\sqrt{3}}\tan^{-1}\left(\frac{\sqrt{3}\,(s - s_I)}{2 + s + s_I + 2ss_I}\right) \tag{A3}$$

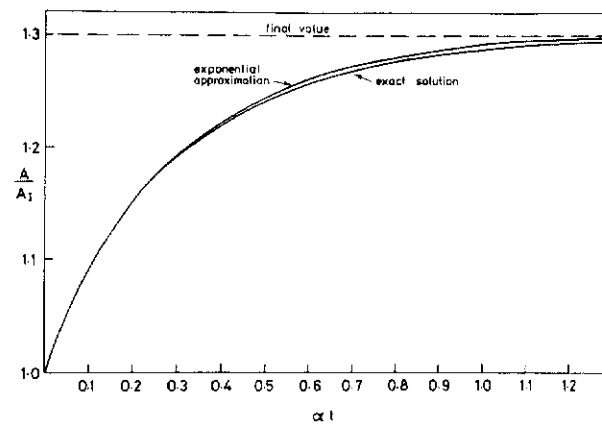

Fig. A1. This plot compares the analytical approximation for $A(t)$ given by Eq. (A4) with the exact solution Eq. (A3). The abscissa is nondimensional time where $\alpha = L_p\Pi_F/R_F$

where $s = R_F/R$, $s_I = R_F/R_I$ and $\alpha_i = L_P\Pi_F/R_F$. Equation (A3) is awkward to manipulate, and so we have approximated the function $A(t) = 4\pi R^2(t)$ obtained from Eq. (A3) with the simpler analytical expression

$$A(t) = A_F - (A_F - A_I)e^{-t/\tau} \tag{A4}$$

where $\tau = (A_F - A_I)/8\pi L_P R_I$. A solution of the form (A4) is obtained for the one-dimensional osmotic expansion, and so it is a better approximation to $A(t)$ derived from Eq. (A3) when the expansion is small. Nevertheless, Eq. (A3) is a very good approximation for most of the expansion. This is seen in Fig. A1 where the $A(t)$ calculated from both expressions are compared. It is noted that Eq. (A4) is a poor approximation for the asymptotic behavior; the asymptotic behavior, however, is difficult to observe due to the limitations of optical resolution.

Finally, we note that $A(t)$ produced by nonideal osmotic expansion (with finite unstirred layers and nonideal membranes) will only be approximated by either Eq. (A3) or Eq. (A4), and we have chosen to use the simpler expression. When the data (t, A) were fitted by least squares to Eq. (A4), the average value of r^2 was greater than 0.99.

Appendix II

An Analysis of Area Increase at Lysis

The data $\omega(\gamma)$ were gained by numerical differentiation of fraction surviving versus time (Wolfe et al., 1985) and were thus rather scattered due to the probabilistic lysis. The strong dependence of ω on γ is most simply fit by the empirical relation

$$\omega = \omega_o \exp \gamma/\Gamma_1 \tag{B1}$$

where ω_o and $1/\Gamma_1$ are the coefficients of a linear regression of ln ω on γ. Γ_1 is thus the tension increase which increases e-fold the probability of lysis. (Γ_i is the tension increase for which Z increases e-fold if $\gamma \gg \gamma_r$.)

Solutions to Eqs. (3) and (4) show that, except for the initial few seconds of elastic expansion, $d\gamma/dt$ is much smaller than the other terms in Eq. (3) and so

$$\frac{1}{A}\frac{dA}{dt} \approx Z(\gamma) \approx M \exp(\gamma/\Gamma_i)$$

so

$$\gamma \approx \Gamma_i \ln\left(\frac{1}{MA}\cdot\frac{dA}{dt}\right) = \Gamma_i \ln\left(\frac{1}{M}\frac{d\ln A}{dt}\right).$$

Substituting this result in Eq. (A1) gives

$$\omega = \omega_o \exp \gamma/\Gamma_1 \approx \omega_o \exp\left(\Gamma_i \ln\left(\frac{1}{M}\cdot\frac{d\ln A}{dt}\right)\Big/\Gamma_1\right)$$
$$= \omega_o\left(\frac{1}{M}\frac{d\ln A}{dt}\right)^{\Gamma_i/\Gamma_1}$$

whence

$$d(\ln A) \approx M\left(\frac{\omega}{\omega_o}\right)^{\Gamma_i/\Gamma_1} dt.$$

Integration from the initial area A_I to A gives

$$\ln\frac{A}{A_I} \approx \frac{M}{\omega_o}\int_{t'=0}^{t} \omega\left(\frac{\omega}{\omega_o}\right)^{\Gamma_i/\Gamma_1 - 1} dt' \tag{B2}$$

The value of this integral obviously depends on the relative magnitudes of Γ_i and Γ_1, i.e., whether incorporation is a stronger function of γ than is the probability of lysis ($\Gamma_i < \Gamma_1$) or the reverse. The results of Wolfe et al. (1985) indicated that $\Gamma_i \approx \Gamma_1$. Making this approximation, Eq. (B2) becomes

$$\ln\frac{A}{A_I} \approx \frac{M}{\omega_o}\int_{t'=0}^{t} \omega\, dt' = \frac{M}{\omega_o}(1 - P) \tag{B3}$$

where P is the probability of lysis at an area between A_I and A. Putting $A = A_I + \Delta A$ and rearranging,

$$\frac{\Delta A}{A_I} \approx \exp\left(\frac{M}{\omega_o}(1 - P)\right) - 1$$

and, in cases where second and higher order terms in $\Delta A/A_I$ may be neglected,

$$\frac{\Delta A}{A_I} \approx \frac{M}{\omega_o}(1 - P). \tag{B4}$$

Equation (B3) indicates that the fraction of survivors of any given proportional area increase is independent of the actual time course of the expansion. Equation (B4) indicates that, to this level of approximation, the percentage of survivors is a linearly decreasing function of $\Delta A/A_I$. The value of ΔA at which 50% lyse is just

$$\mathrm{TSAI}_{50} \approx A_I \exp\left(\frac{M}{2\omega_o}\right) - 1 \approx \frac{A_I M}{2\omega_o}. \tag{B5}$$

Thus the weak dependence of the TSAI on the time course of the expansion is a result of the approximate equality of Γ_i and Γ_1. The similar tension dependence of ω and Z could be related to the mechanisms of lysis and incorporation. It is possible that the membrane reservoir available for expansion is a collection of small cytoplasmic vesicles. Incorporation of these in the membrane requires the topological equivalent of lysis of the plasma membrane prior to or during their insertion, and thus it is less surprising that the two have similar dependence on tension. In our previous study we noted that, among the members of any population of protoplasts, Z and ω showed a strong correlation, which would also be expected if the two processes were limited by the same step.