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Dynamics of Membrane Exchange of the Plasma Membrane and the Lysis of Isolated Protoplasts during Rapid Expansions in Area

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Summary. The plasma membrane of protoplasts isolated from rye leaves (*Secale cereale* L. cv. Puma) can withstand a maximum elastic stretching of about 2%. Larger area expansions involve the incorporation of new material into the membrane. The dynamics of this process during expansion from isotonic solutions and the probable frequency of lysis have been measured as a function of membrane tension in populations of protoplasts isolated from both cold-acclimated and nonacclimated plants. To a first approximation, both increase exponentially with tension. An analytical solution is reported for the membrane tension as a function of time during an arbitrary expansion in area.

Key Words membrane mechanics · plant protoplasts · osmotic expansion

Introduction

Recent investigations of the mechanical properties of the plasma membrane of isolated protoplasts[1] (Wolfe & Steponkus, 1980, 1981, 1983*a*) have shown that these membranes rupture when subjected to tensions above about 4 mN · m^{-1}. Because their area elastic modulus is about 200 mN · m^{-1}, the maximum possible elastic or intrinsic stretching of such membranes is about 2%. These studies have also shown that larger area variations are possible via the exchange of material between the plane of the membrane and a reservoir of membrane material. The net direction of such exchange is determined by whether the membrane tension is above or below an equilibrium or resting value. This resting tension is very small (typically 0.1 mN · m^{-1}) and so, when tensions of 1 mN · m^{-1} or more are applied, material is incorporated into the membrane until the tension is relaxed. When the tension is zero and the membrane is flaccid, material is deleted and transferred to the reservoir until the membrane regains its resting tension. This resting tension is, at most, weakly dependent on the degree of previous expansion of the membrane.

The aims of this study are three: (i) a quantitative account of the dynamics of the membrane, including the incorporation of new material; (ii) a calculation of the tensions generated in the plasma membrane (and hence the probability of lysis) during a rapid expansion of its area; and (iii) a comparison of the dynamical membrane properties of protoplasts isolated from nonacclimated plants, and from cold-acclimated plants (i.e., plants exposed previously to cold but not lethal temperatures). The second and third aims are motivated by the thesis that a major cause of damage to nonacclimated protoplasts caused by a freeze-thaw cycle is the lysis of the plasma membrane during the osmotic expansion which accompanies thawing of the suspending medium (Steponkus & Wiest, 1979).

When protoplasts from nonacclimated plants (hereinafter called nonacclimated protoplasts) are contracted osmotically, they become spherical. Electron microscopy reveals that the plasma membrane undergoes a large decrease in area, rather than, say, bending or folding (Gordon-Kamm & Steponkus, 1984). On return to an isotonic medium, the plasma membrane lyses if the associated expansion is sufficiently large or fast (Wolfe & Steponkus, 1983*a*). Protoplasts from acclimated plants (hereinafter called acclimated protoplasts) survive return to isotonic from any degree of contraction (Dowgert & Steponkus, 1984).

A complete study of the lysis caused by osmotic manipulation would involve consideration of the past history of the protoplasts: the dynamics of ex-

[1] This membrane behaves very differently from that of the erythrocyte, which is the most studied system (Evans & Skalak, 1979). The capacity for extensive area change and the possession of an equilibrium resting tension are properties shared by the protoplast plasma membrane and bilayer lipid membranes in equilibrium with a torus of lipid solution. The erythrocyte membrane can only stretch intensively, and its tension under normal conditions is zero.

pansion are likely to depend on whether or not the protoplast has previously been contracted or expanded. In the current study we confine ourselves to rapid expansions from suspension in an isotonic solution, that is, a solution whose osmotic pressure gives the protoplast a volume equal to that inferred for the state *in vivo*.

We aim to give a quantitative, analytical explanation of the lysis of isolated protoplasts subjected to a given area expansion in terms of the hydraulic conductivity and mechanical properties of the membrane. The steps involved are: (i) to understand the membrane elastic properties (how stretching increases tension); (ii) to measure the dynamics of incorporation of membrane material as a function of tension (since this process relaxes tension induced by stretching); (iii) to solve a differential equation for the tension produced by a given expansion; (iv) to measure the probable frequency of lysis as a function of tension; (v) to determine the area expansion of a protoplast with a plasma membrane of given hydraulic conductivity following a decrease in the osmolality of the suspending medium; and (vi) to account for variation of and correlation between parameters of cells comprising the population.

Two populations of protoplasts are investigated: one isolated from the leaves of plants which have been cold-acclimated and one from nonacclimated plants. Dowgert and Steponkus (1984) have reported that greater probabilities of lysis during a given osmotic expansion are observed for nonacclimated protoplasts than for acclimated protoplasts. We relate this behavior to the continuum mechanical properties of their plasma membranes.

Materials and Methods

Seeds of *Secale cereale* L. cv. Puma were germinated and grown for 7 days in vermiculite under a controlled environment (16-hr light period at 20°C and 8-hr dark period at 15°C). Nonacclimated plants (LT_{50} −3 to −5°C) were grown an additional 7 days. Acclimated plants were transferred to 13°C/7°C (11.5-hr photoperiod) for one week and then to 2°C (10-hr photoperiod) for four weeks, after which they were fully acclimated (LT_{50} −25 to −30°C). Protoplasts were enzymically isolated from excised leaves in a solution of 1.5% (wt/vol) Cellulysin (Calbiochem, LaJolla, Calif.) 0.5% macerase (Calbiochem), and 0.3% potassium dextran sulfate as described previously (Wiest & Steponkus, 1978) with the following modifications. The leaves were brushed with carborundum (329 grit) prior to digestion. Protoplasts from nonacclimated and acclimated leaves were isolated and suspended in 0.5 M sorbitol (0.53 Osm) and 0.9 M sorbitol (1.03 Osm), respectively. The different osmotic pressures were required to maintain the *in vivo* volume of the protoplasts because of increases in the internal solute concentration during cold acclimation. All solution osmolalities were determined using a freezing point osmometer.

Measurement of the mechanical properties of the plasma membrane of isolated protoplasts was accomplished using micropipette aspiration. Protoplasts in suspension were loaded into a microslide (Vitro Dynamics, Brockway, N.J.), of 0.2 mm path length. A micropipette filled with osmoticum and attached to a manometer was abutted to a protoplast in the microslide. A negative pressure was applied and a portion of the plasma membrane delimited by the pipette was distorted to produce a curvature larger than that in the remainder. From the diameter of the pipette, the radius of the protoplast outside the pipette, the length of the membrane intrusion into the pipette along with the negative pressure applied, the tension (γ) in the plane of the membrane and the change in the area (ΔA) can be determined. Tension in the plane of the membrane was obtained from the pressure applied and the curvature of the plasma membrane using

$$\gamma = -P/(2/r - 2/R)$$

where P is the negative pressure applied, R is the radius of the protoplasts outside the pipette and r is the radius of the deformed section. From geometry

$$r = \begin{cases} (D^2 + a^2)/2D & D \leq a \\ a & D > a \end{cases}$$

where a is the radius of the pipette and D is the length of the intrusion into the pipette. The cell volume is unchanged by the deformation and the total increase in membrane area ΔA is given by

$$\Delta A = \begin{cases} \pi[D_o^2 + D^2 - D^3/3R - 2DD_o] & D \leq a \\ \pi[a(2D - a + 4D_o/3) + D_o^2 - 4DD_o] & D > a \end{cases}$$

where $D_o = a^2/2R$. The derivation of these equations and the assumptions used in their derivation are given and discussed by Wolfe and Steponkus (1983*a*). Micropipette aspiration was conducted under observation with a Nikon Biophot microscope fitted with differential interference contrast optics. Individual experiments along with time and pressure were recorded on a video cassette recorder and projected on a 48-cm color monitor. Measurement of pipette diameter and the length of intrusion of the membrane into the pipette was accomplished during replay of the video cassettes using the freeze-frame on the video cassette recorder.

In general, pipettes of between 8 and 12 μm diameter were used in all experiments. The resting tension γ_r for individual protoplasts was estimated as the slope of a line through $(2/R,0)$ on a plot of P *vs.* curvature of the membrane deformation in the pipette. The experimental procedure consisted of rapidly (~0.1 sec) applying a small negative pressure (8 to 30 Pa) to the protoplast's plasma membrane and measuring the resulting curvature. From the slope of the line through this point and $(2/R,0)$, γ_r was determined. When low pressures were used, a slow application of pressure did not always result in a 'seal' between the membrane and the pipette lip. A rapid application of pressure alleviated this problem in most cases.

The area modulus of elasticity (k_A) was calculated from $k_A = A\delta\gamma/\delta A$ where δ_γ and δA are the change in tension and resultant change in membrane area, respectively. Protoplasts were expanded in area by the application of a tension of between 200 and 2000 $\mu N \cdot m^{-1}$ for approximately 2 min. After this time the tension was rapidly reduced to ~100 $\mu N \cdot m^{-1}$ and δ_γ, δA and A determined. The determination of k_A required the application of large tensions and this resulted in the exchange of material be-

tween the reservoir and the plane of the membrane. Determination of k_A during contraction minimized this contamination, providing a more accurate estimate of k_A.

The probable frequency of lysis and the rate of proportional increase in membrane area were measured in two different experimental modes. In one mode, the membranes of protoplasts were subjected to the tension generated by a constant pressure in the pipette (this produces a very nearly constant tension) and the area was measured regularly until the membranes lysed. This was repeated for different tensions. In another mode, a linearly decreasing pressure was applied to the pipette (producing a linear increase in membrane tension) and the area was measured regularly until the membranes lysed. This was repeated for different rates of increase in tension. The probable frequency of lysis and the rate of proportional increase in membrane area may be deduced directly from the results of experiments of the first type, or from a simple mathematical analysis of the results of experiments of the second type. These analyses are described in the next section.

When constant negative pressure was applied to the pipette, the tension produced in the membrane decreased very slightly with time because, as the protoplast intruded into the pipette, the radius of the external portion decreased. This decrease was less than 5% in all experiments reported here; hereinafter such experiments are called constant tension experiments. Similarly, linearly decreasing pressures produced tensions which increased nearly linearly with time. The rate of increase in tension varied by less than 5% in any one of such experiments; hereinafter such experiments are called linear tension experiments.

Analysis

From the definition of the area elastic modulus one may write for the area A of the membrane:

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

where A_o is the area that would be occupied by the material currently comprising the membrane if the tension were zero. Thus changes in A may be the result of changes in γ (elastic stretching), changes in A_o (i.e., changes in the amount of material in the membrane) or, more usually, a combination of both. In the experiments reported here, the area varies with both tension and time so we may write

$$\frac{dA}{dt} = \left(\frac{\partial A}{\partial t}\right)_\gamma + \left(\frac{\partial A}{\partial \gamma}\right)_t \frac{d\gamma}{dt}.$$

Using Eq. (1) this yields

$$\frac{1}{A_o}\frac{dA}{dt} = \frac{1}{A_o}\frac{dA_o}{dt} + \frac{1}{k_A}\frac{d\gamma}{dt} \tag{2}$$

where the two terms on the right signify respectively incorporation and stretching. We define a dynamic variable, *the rate of proportional incorporation of area* by

$$Z = \frac{1}{A} \cdot \frac{dA_o}{dt}. \tag{3}$$

Since membranes lyse at tensions above about 4 $mN \cdot m^{-1}$, $\gamma/k_A \leq 2\%$ and so, from Eq. (1), $\Delta A \ll A_o$. Using Eqs. (2) and (3) and this approximation, Z can be expressed in terms of measurable quantities:

$$Z = \frac{1}{A}\frac{dA}{dt} - \frac{1}{k_A}\frac{d\gamma}{dt}. \tag{4}$$

In the introduction we argued that Z was possibly a function of both the tension and the amount of material in the reservoir, and thus that $Z = Z(\gamma, A_o)$. In previous work (Wolfe & Steponkus, 1983*a*) and in this study, we found that for cells subjected to a constant tension at isotonic, Z was not measurably dependent on A_o for expansions of less than about 10–15%. (Expansions larger than this are possible using micropipette aspiration, but for such expansions only a limited range of tensions is available.) In this paper we shall treat Z as a function of γ alone, and note that strict application of the results obtained must therefore be limited to expansions of less than about 10–15% for cells at isotonic.

Previously (Wolfe & Steponkus, 1983*a*), approximate estimates of $Z(\gamma)$ were obtained using Eq. (4) in two ways. If the tension were varied so as to maintain constant area, then the first term is zero and $Z(\gamma)$ is calculated from $(d\gamma/dt)$. The limitations of optical resolution made it difficult to extract accurate values of $Z(\gamma)$ using this method. In the second method, the tension was held constant and the area was measured regularly. Here the last term in Eq. (4) is zero, and so $Z(\gamma)$ is obtained directly. This method was used for some of the results reported in this paper. Despite its directness, this method has several shortcomings: (i) at very low applied tensions the experiment is very slow, and some of the assumptions used in obtaining γ and ΔA (*see* Wolfe & Steponkus, 1983*a*) are violated; (ii) at very high applied tensions the experiment is over so quickly that it is difficult to obtain accurate measurements; and (iii) variations of Z with γ must be compared among a population of protoplasts—i.e., it is impossible to derive detailed information about $Z(\gamma)$ from a single cell. All the shortcomings of both these methods are overcome by measuring A regularly during a linear increase in tension. Putting $\gamma = \beta t$, we have

$$Z(\gamma) = \frac{1}{A}\frac{dA}{dt} - \frac{\beta}{k_A} \tag{5}$$

from which $Z(\gamma)$ is readily calculated.

This method also provides an alternative method of calculating k_A to that described in Materials and Methods. If γ_r be the resting or equilibrium tension, then by definition $Z(\gamma_r) = 0$. Providing $Z(\gamma)$ is continuous,

$$\frac{1}{A}\cdot\frac{dA}{dt} \to \frac{\beta}{k_A} \text{ as } \gamma \to \gamma_r.$$

Thus k_A may be calculated from the slope of a plot of $A(t)$ at $t = 0$ during a linear increase in tension.

We define the *probable frequency of lysis*, $\omega(\gamma)$ as the probability per unit time that the membrane of a given protoplast which is subject to a tension γ will lyse in a small interval of time. Let $p(t)$ be the probability that lysis does not occur before time t, i.e., the probability that the protoplast survives until or after t. The probability that the membrane does not lyse before $(t + dt)$ is therefore

$$p(t + dt) = p(t)[1 - \omega(\gamma)dt]$$

so

$$dp = p(t + dt) - p(t) = -p \cdot \omega(\gamma) \cdot dt$$

whence

$$\omega(\gamma) = -\frac{d}{dt}\ln p. \tag{6}$$

If all protoplasts were identical, then ω would be a constant of the population under given conditions. In such a population, ω could be simply derived as the slope of a plot of the log of the fraction surviving at time t. Our sample is assumed to be derived predominantly from mesophyll cells of entire leaves, and the protoplasts vary in size and age. Our results suggest that ω varies in the population, so the derivation of ω is not so straightforward.

At a given tension γ, if the probable frequency of lysis of a particular protoplast be $\omega(\gamma)$, then the probability that that protoplast lyses at time t is $p(t) = \exp(-\omega t)$. Out of a population of N_o protoplasts, let the distribution function of ω be $F(\omega)$, i.e., the number of protoplasts dN with a probable frequency of lysis between ω and $\omega + d\omega$ is

$$dN = N_o F(\omega)d\omega.$$

Because $\omega \geq 0$, the expected number of cells $\bar{N}(t)$ surviving at time t is

$$\bar{N}(t) = N_o \int_0^\infty F(\omega)e^{-\omega t}d\omega$$

or

$$P(t) = \frac{\bar{N}(t)}{N_o} = \int_0^\infty F(\omega)e^{-\omega t}d\omega. \tag{7}$$

Thus $P(t)$ is the Laplace transform of $F(\omega)$. $F(\omega)$ cannot, in this instance, be recovered by numerical inversion of the transform because of the sensitivity of the procedure to noise in the data (Davis & Rabinowitz, 1967).

If one approximates (ω) with a normal distribution with mean $\bar{\omega}$ and standard deviation σ_ω, then it follows that

$$P(t) \approx \tfrac{1}{2}\exp(\bar{\omega}t + \sigma_\omega^2 t^2/2)\,\text{expfc}(\sigma_\omega t/\sqrt{2}). \tag{8}$$

Third and higher moments of the distribution may be recovered by expanding Eq. (7) about $t = 0$ to yield

$$P(t) = 1 - t\bar{\omega} + \frac{t^2}{2}\overline{\omega^2} + \frac{t^3}{\sigma}\overline{\omega^3} + \ldots \tag{9}$$

[In this case, $F(\omega)$ is positively skewed but the third moment cannot reliably be estimated from the stochastic data.]

As with $Z(\gamma)$, it is impracticable to obtain ω for a large range of γ using a series of experiments in each of which γ is held constant. Equation (6), however, suggests that an estimate, ω', of the probable frequency of lysis for the whole population may be gained from

$$\omega' = \frac{d}{dt}\ln\left(\frac{N(t)}{N_o}\right).$$

For the experiments in which the tension was increased linearly with $\gamma = \beta t$, we obtain

$$\omega' = \beta\frac{d}{d\gamma}\ln\left(\frac{N(\gamma)}{N_o}\right). \tag{10}$$

This equation is strictly only applicable to a homogeneous population, and, as we shall report, the dynamic variables in the population studied have a substantial variation. We are obliged to accept this approximation, however, as the best available to us.

Results and Discussion

Resting Tension

The plasma membrane of both nonacclimated and acclimated protoplasts in isotonic solution maintained a small but finite resting tension (γ_r). It is this resting tension that gives isolated protoplasts their characteristic spherical shape. The median resting tension for nonacclimated protoplasts was 120 $\mu N \cdot m^{-1}$ with measured values ranging from 40 to 290 $\mu N \cdot m^{-1}$. The median resting tension for acclimated protoplasts was 170 $\mu N \cdot m^{-1}$ with a range from 60 to 490 $\mu N \cdot m^{-1}$. The median was used as a measure of γ_r since the values were not normally distributed. While the median γ_r was higher in acclimated than in nonacclimated protoplasts there was significant overlap in the observed values. The accuracy of the linear measurements was ± 0.25 μm and the pressure was determined with ± 1 Pa giving an error in γ_r of typically 50 $\mu N \cdot m^{-1}$. We conclude that the range of values measured for γ_r reflects a large proportional variation in the γ_r of individual protoplasts, rather than measurement errors.

Compared with the area elastic modulus, or even with the tension necessary for lysis, γ_r is small and the difference in this parameter between the acclimated and nonacclimated populations is small.

In previous papers (Wolfe & Steponkus, 1981, 1983*a*), we have argued that this equilibrium or resting tension is a surface energy, i.e., that it is numerically equal to the free energy per unit area of membrane for the transfer of material between a reservoir and the membrane. For a typical membrane lipid, the measured γ_r corresponds to a free energy difference of about 3×10^{-25} *J* or 0.006 times the thermal energy per lipid molecule. Chemical potential increases with concentration, and so γ_r is expected to decrease with increasing concentration of reservoir material (Gruen & Wolfe, 1982). We would expect, then, that the value of γ_r at equilibrium would be higher if the reservoir were substantially depleted by expansion.

Area Elastic Modulus

The area elastic moduli determined for nonacclimated and acclimated protoplasts were not significantly different. The mean k_A calculated from 38 nonacclimated protoplasts was 180 ± 130 mN $\cdot$ m^{-1}, while from 70 acclimated protoplasts the mean k_A was 190 ± 140 mN $\cdot$ m^{-1}. The variability in the values determined for k_A are admittedly large. However, considering the k_A values determined, a large applied tension (2.0 mN $\cdot$ m^{-1}) would result in an area change of ~1%. Thus the variability in the determination of k_A can be largely attributed to the optical resolution of small changes in area rather than to a large variation in the k_A's for a population of protoplasts. In addition, determination of k_A is complicated by the exchange of material between the plane of the membrane and the proposed reservoir. This was minimized by determining k_A during contraction when extrinsic exchange is much slower (Wolfe & Steponkus, 1981, 1983*a*). The effect of this possible artifact is an underestimation of k_A. Previous calculations of k_A were slightly larger than reported here; however, the difference is less than the error expected due to optical resolution, which is 50 mN $\cdot$ m^{-1}.

It has been argued elsewhere (e.g., Bates & Wolfe, 1980; Evans & Kwok, 1982) that the most important factor determining the area elastic modulus is the possible existence of local regions of solid phase lipids in the membrane. In the liquid phase, this modulus is not expected to depend strongly on the lipid composition, though its dependence on protein concentration is harder to predict. Since the measurements reported here were conducted at room temperature, it is assumed that there are no regions of solid phase lipid in the membranes of either population. Thus the rather large and similar values of k_A are not unexpected.

Proportional Rate of Area Incorporation

Figure 1*a* and *b* shows the increase in area as a function of time for protoplasts subjected to a linearly increasing membrane tension with different rates, β. The tension at any time is indicated on the upper scale. At very low tension ($\gamma \approx \gamma_r$) the rate of area increase is very small, approximately equal to the increase expected from purely elastic stretching, i.e., $\gamma A/k_A$. As the tension increases from 2 to 5 mN $\cdot$ m^{-1}, however, the rate of increase in area (the slope of the plot) increases markedly. This large increase in area is interpreted as the tension-dependent incorporation of new material into the membrane (*see* Wolfe & Steponkus, 1981, 1983*a*, and Analysis). In expansion at low γ, the total area increase before lysis is greater in nonacclimated cells because, even though the *rate* of area increase is greater in acclimated protoplasts, their membranes lyse at lower tensions.

Using Eq. (5), the proportional rate of area incorporation $\left[Z(\gamma) = \frac{1}{A}\frac{dA}{dt}\right]$ was calculated and

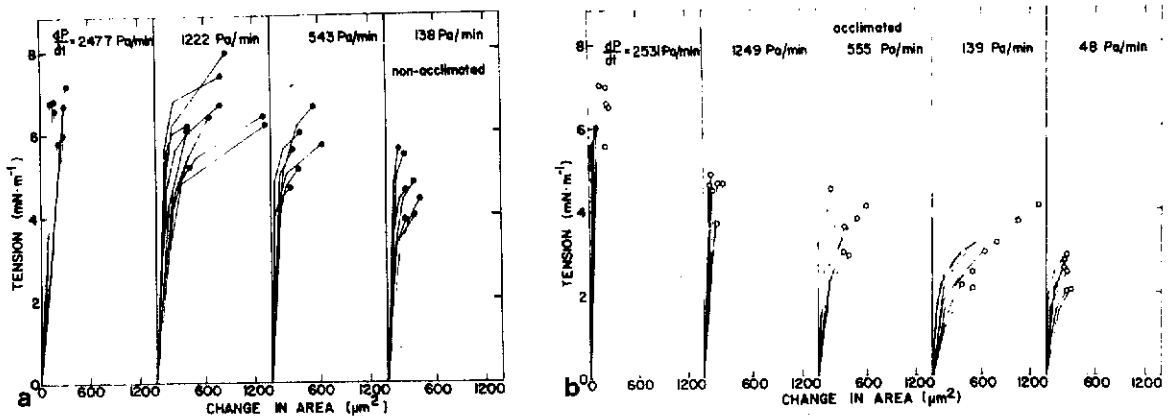

Fig. 1. The pressure in the micropipette was decreased at the constant rates indicated on each plot. This produced a tension (plotted on the ordinate) which was almost exactly proportional to time. The change in area at each time and tension is plotted on the abscissa. Each circle represents lysis of the plasma membrane. (*a*) The behavior of nonacclimated protoplasts and (*b*) that of acclimated protoplasts is shown

values are presented in a semi-log plot in Fig. 2. Average Z and γ were calculated for cells within a given range of Z, and typical standard error bars are shown on a point in each group.[2] For both populations, Z increases strongly with γ over the investigated range (Z varies from 0.05 to about 2% per sec). The error bars show that there is a large variation within each population—for some cells the tension required to produce a given Z is 2 mN · m^{-1} greater than that for others in the same population. For this range of β (3–100 μN · m^{-1} sec^{-1}) there is no systematic dependence of Z on β, the rate at which the tension was increased in a particular experiment.

In comparing the acclimated and nonacclimated samples, it is noted that at any given tension, the incorporation rate is much larger in acclimated cells than in nonacclimated cells. If a nonacclimated protoplast and an acclimated protoplast are to incorporate area at the same rate, then, on average, the membrane of the nonacclimated protoplast must be exposed to a tension about 1.7 mN · m^{-1} larger than that to which is exposed the membrane of the acclimated protoplast. For example, using linear regressions of ln Z on γ, an incorporation rate of 1% per sec requires a tension of 6.0 mN · m^{-1} in nonacclimated and 4.2 mN · m^{-1} in acclimated protoplasts.

Plots of area increase versus time for membranes under constant tension give similar though less detailed results (*see* Analysis). With a couple of puzzling exceptions the area increases more or less linearly with time, and the rate is disproportionately greater at high tensions. Again, there is a large population variation in $Z(\gamma)$.

Acclimated protoplasts incorporate area more rapidly than do nonacclimated protoplasts; however, their membranes rupture at lower tensions. As a consequence, the total area increase before lysis is larger for nonacclimated cells in experiments with the same tension or rate of increase in tension. Superficially, this seems contrary to the observation (Dowgert & Steponkus, 1984) that acclimated protoplasts can withstand larger osmotic expansions. The two experiments are not simply comparable, however. In these experiments the tension is the independent variable, and all membranes experience the same tension. In osmotic expansions, the area is (effectively) the independent variable and the tensions are determined in each protoplast by the relationship $Z(\gamma)$. As we shall show in the final section, acclimated cells incorporate material sufficiently rapidly that their membranes are never exposed to high tensions during osmotic expansions.

Probable Frequency of Lysis

In Fig. 1, a circle indicates the lysis of a cell at that time and tension.

It is evident from Fig. 1 that either a large tension for a short time or a smaller tension for a longer time causes lysis and that the time for lysis under any condition varies among protoplasts. (This is consistent with a tension-dependent, stochastic lysis; *see* Analysis.) The large tensioning rates reach high tensions quickly, so lysis occurs early at a

[2] We have averaged over γ, which is the independent variable in these experiments, because we apply these data to osmotic expansions in which γ is largely determined by Z rather than the reverse.

high tension and conversely for low tensioning rates. Thus high tensioning rates give information about $Z(\gamma)$ and $\omega(\gamma)$ at high γ, and low rates give information on these parameters at low γ. This is evident in Fig. 2.

There is a dependence on the rate of increase in tension of the total area change before lysis (ΔA_{max}). For the nonacclimated population, ΔA_{max} is largest when $\beta = 76\ \mu N \cdot m^{-1}\ sec^{-1}$ and smaller for both smaller and larger β, although the effect is small. For nonacclimated protoplasts, there is a strong dependence, and ΔA_{max} is largest for $\beta = 10.2\ \mu N \cdot m^{-1}\ sec^{-1}$.

Equation (10) in the Analysis section shows how an estimate, ω', of the probable frequency of lysis may be obtained from the number $N(t)$ of cells surviving at time t out of an initial population N_o at $t = 0$. Using the data presented in Fig. 2 ω' is calculated by numerical differentiation using Eq. (10). A semi-log plot of ω' is presented against γ in Fig. 3*a* and *b*.

As with Z, ω' shows a strong dependence on tension. There is a large scatter, which we attribute largely to the inevitable noise in stochastic data, in this case amplified by the process of numerical differentiation.

Comparing the nonacclimated and acclimated populations in Fig. 3*a* and *b* shows a result we did not expect: at the same tension, the membrane of an acclimated protoplast is more likely to lyse than is the membrane of a nonacclimated protoplast. Figure 3 suggests that, on average, a similar probability of lysis is produced in the populations if the membranes of nonacclimated cells are exposed to a tension about $10\ mN \cdot m^{-1}$ higher than that in the membranes of acclimated cells. Note, however, that this difference is less than the difference (about $1.7\ mN \cdot m^{-1}$) between the tensions required to produce equal rates of incorporation in the membranes of the two populations.

Thus the greater capacity of acclimated cells to incorporate new membrane area (and thus relax the membrane tension imposed by an expansion in area) more than compensates for their greater tendency to lyse at a given tension. We deal with this point in more detail in the following sections.

Variation within the Population

In one series of experiments, a constant tension of $\gamma = 5.0\ mN \cdot m^{-1}$ was applied to the membrane of a population of N_o cells, the time of lysis of each, and thus the number $N(t)$ surviving at time t, were noted. The value of Z for each cell was also recorded. Figure 4 plots $\ln[N(t)/N_o]$ versus t for acclimated and nonacclimated cells. Since these plots are nonlinear, it follows that ω is not a constant within the population. Both plots are concave-up; that is they exhibit a positive second derivative. Qualitatively, one may explain this by noting that the slope is steepest at small t while those cells with a large ω—the more fragile cells—are lysed. Only the least fragile cells—those with low ω—survive till large t, where the slope is smaller.

More formally, this behavior is explained by Eqs. (7)–(9): $N(t)/N_o$ is simply the Laplace transform of the distribution function $F(\omega)$ in the population. Equation (8) yields $\bar{\omega} = 0.023\ sec^{-1}$, $\sigma_\omega = 0.012\ sec^{-1}$ for nonacclimated cells and $\bar{\omega} = 0.056\ sec^{-1}$, $\sigma_\omega = 0.024\ sec^{-1}$ for acclimated cells at this particular value of the tension. It is reasonable to suppose that this variation in ω in each population may be the result of differences in size and development of protoplasts in the population. In particular, the probability of lysis is expected to be proportional to the number of sites (or "defects") which could nucleate lysis, and the number of such sites might be larger for a larger area of membrane.

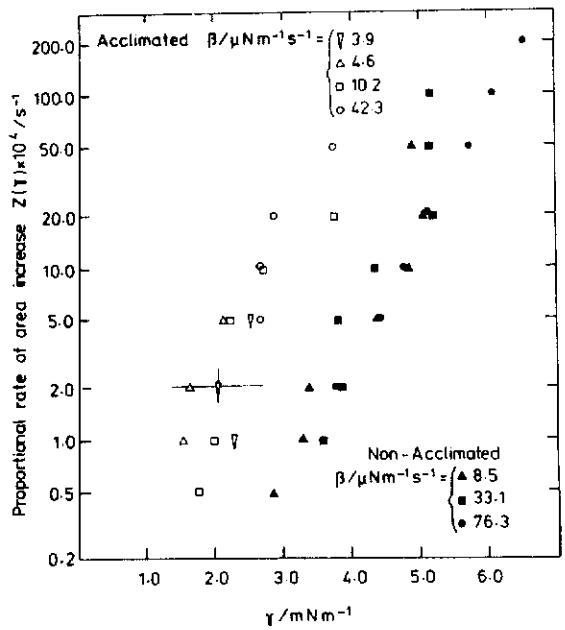

Fig. 2. Z, the proportional rate of area incorporation, is plotted on a semi-log plot against the tension. Filled and open symbols denote nonacclimated and acclimated protoplasts, respectively. The different symbols indicate different rates of increase in the tension, which were chosen to investigate the different ranges of Z as described in the Analysis section. The bars shown on one point represent the standard deviation in the population. These bars are typical of all data points; the others have been omitted for clarity

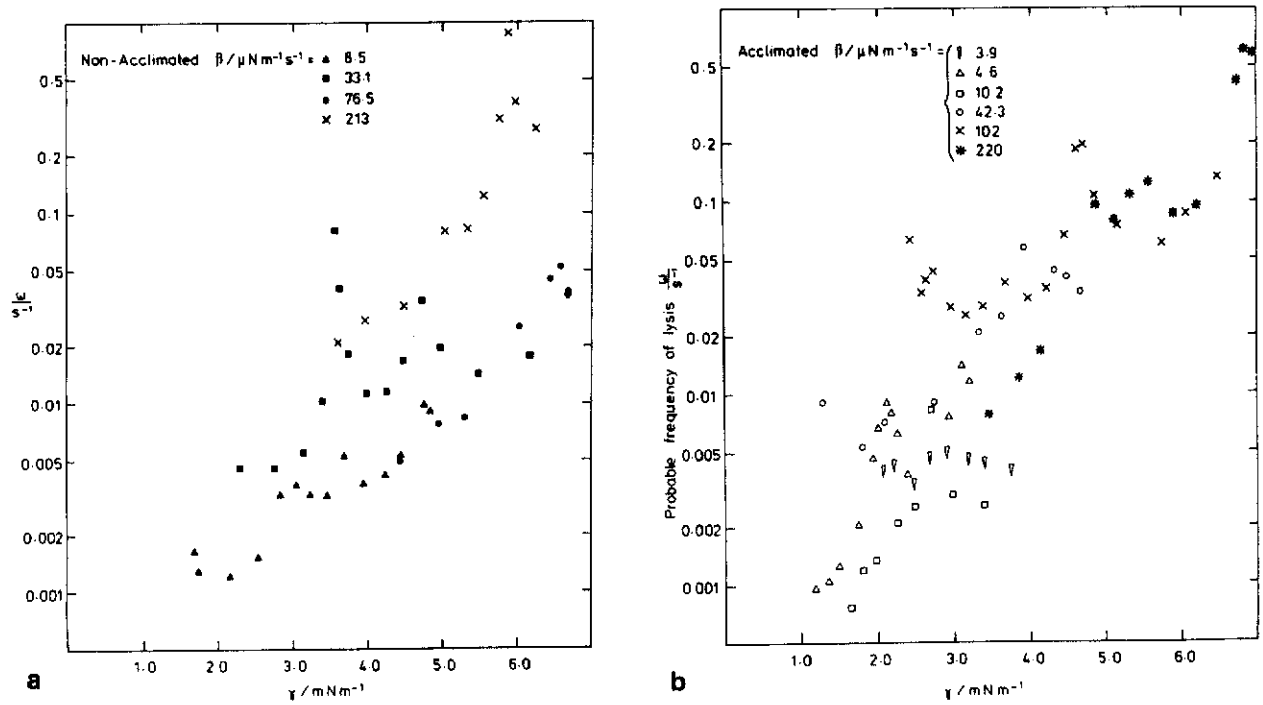

Fig. 3. The probable frequency of lysis, ω, is plotted as a function of γ on a semi-log plot. (*a* and *b*) Nonacclimated and acclimated protoplasts, respectively. The different symbols represent different rates of increase in tension. Each point derives from the lysis of a single protoplast, so considerable scatter is evident

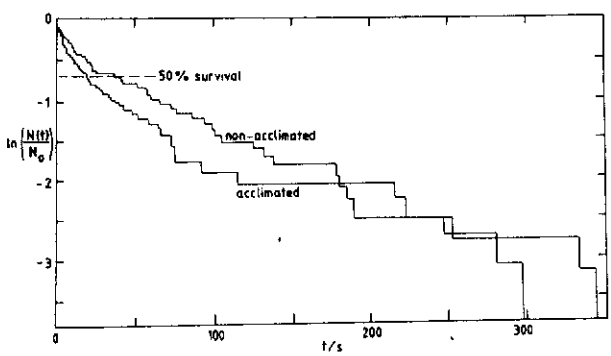

Fig. 4. For protoplasts whose plasma membranes were maintained under a constant tension of 5.0 mN · m^{-1}, the log of the surviving fraction at time t is plotted against t

Correlation between ω and Z

Correlations between the variations in a population of $Z(\gamma)$ and $\omega(\gamma)$ cannot be investigated in detail because ω is a probability value and because lysis can only be measured destructively. If, however, lysis at a given tension were a purely stochastic process (like, e.g., radioactive decay), then ω is the reciprocal of the expected lifetime, $\bar{T}$.

Using Z and T calculated for cells whose membranes were exposed to a constant tension of 5.0 mN · m^{-1}, reciprocal mean lifetimes were plotted against Z (Fig. 5). In both populations of cells, a strong correlation is evident. Most importantly, we note that this plot shows that, *at the same rate of area incorporation, nonacclimated cells have a smaller average lifetime than do acclimated cells.*

The strong correlation between incorporation and lysis may be more than coincidental. One likely identification of the primary membrane reservoir is a population of very small vesicles (or other aggregates of membrane material) in the cytoplasm. If this be the case, then large expansions in the plasma membrane must involve the fusion of that membrane with very many vesicles. The topology of this fusion must require the formation of a hole in each of the membranes which then reseal to produce a continuous membrane (though it is possible that bilayer regions of the membrane might fuse by forming holes and sealing only two half-bilayers at one time, as suggested by Fisher and Parker, 1984). If the initial formation of a hole were the rate-determining step in incorporation and also in lysis, then a strong correlation between the rates of the two processes would result.

A conceptually simple model of membrane lysis was proposed by Derjaguin and Gutop (1962) and developed by Taupin Dvolaitzky and Sauterey (1975), Abidor et al. (1979) and Petrov, Mitov and

Derzhanski (1980): this model is the two-dimensional analogue of three-dimensional cavitation of a fluid. In this model, defects or nucleation sites exist in the membrane and may be thermally activated to produce tiny holes (imagined, for simplicity, to be circles with radius r) in the membrane. The expansion of these holes is abetted by an isotropic tension which contributes a term $-\pi r^2\gamma$ to the energy of formation of the hole. The energy associated with their perimeter tends to close the holes and contributes an energy term which is, in the simplest model, proportional to r. Above a critical radius the negative quadratic term dominates and the hole expands indefinitely, lysing the membrane. The critical radius decreases, and so lysis becomes more probable, at large tensions.

The edges of holes through the membrane must be rather hydrophilic (a hydrophobic surface is energetically prohibited). One possible picture of the defects which nucleate membrane lysis is that of the inner part of a torus in the plane of the membrane. Such a defect would comprise molecules with a geometry like that of lysolipids, which would aggregate as two-dimensional micelles in the fluid membrane (Wolfe, 1978). It is plausible, therefore, that both ω and Z would depend on the number of such defects in the plasma membrane.

Tension Generated during Expansion

We have reported here experiments designed to measure the dynamic mechanical properties of the plasma membrane and its reservoir, rather than to investigate the biochemical or ultrastructural events and entities that give rise to these properties. The lack of a description of these processes at the molecular level does not, however, inhibit a mechanical analysis of expansion-induced lysis. In this section we make analytical approximations to the reported results and use these as empirical laws governing membrane mechanics (just as, for example, the empirical Hookes Law is used in classical mechanics without an analysis of intermolecular forces). The data presented in Figs. 1 to 5 show substantial scatter, which results variously from population variations, stochastic noise, and the measurement limitations imposed by optical resolution. Because of this scatter, simplicity and economy of parameters have determined the analytical approximations we make to the data presented.

It has been demonstrated that the maximum hydrostatic pressure that can be resisted by the plasma membrane per se (in the absence of the cell wall) (~300 Pa) is negligible in comparison to typical osmotic pressures in protoplasts and their suspending media (~10^6 Pa) (Wolfe & Steponkus, 1981, 1983*a*,*b*). It therefore follows that when spherical protoplasts are expanded osmotically, the areas of their plasma membranes (until lysis) are completely determined by the concentration difference across the membrane and the membrane's hydraulic conductivity. Neglecting for a moment the possibility[3] that hydraulic conductivity is dependent on tension, one may conclude that the time course of area expansion $A(t)$ imposed by a certain osmotic expansion is completely fixed. (Such an expansion is an independently complicated problem, which is discussed by Dowgert and Steponkus, 1984). Thus a given osmotic treatment may be considered to impose a certain $A(t)$ on a protoplast. The difference between this expansion rate and the induced rate of incorporation of new area determines the extent of intrinsic stretching, and thus γ.

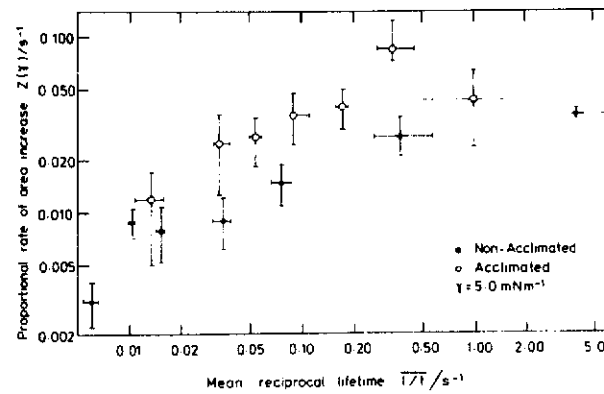

Fig. 5. Z is plotted against the mean reciprocal lifetime on a log-log plot. Filled and open circles represent nonacclimated and acclimated protoplasts, respectively. The plasma membrane tension was maintained constant at 5.0 mN · m^{-1}

The differential equation (4) relating membrane tension and time may be rewritten

$$\frac{d\gamma}{dt} = k_A\left(\frac{1}{A}\frac{dA}{dt} - Z(\gamma)\right). \tag{11}$$

To obtain a solution $\gamma(t)$, analytic forms are required for $Z(\gamma)$ and $A(t)$. [As we noted in the analysis, the approximation that $Z(\gamma,A) = Z(\gamma)$ is strictly valid for expansions in area of only about 10 to 15% from isotonic. Since the aim of this analysis is to determine the survival of the initial rapid phase of the osmotic expansion, the approximation is reasonable over this range.]

The plot of ln $Z(\gamma)$ vs. γ (Fig. 2) may be fitted, to a first approximation, by a straight line. We therefore write

[3] This neglect is prompted by an absence of data on this effect rather than a discounting of its possible importance.

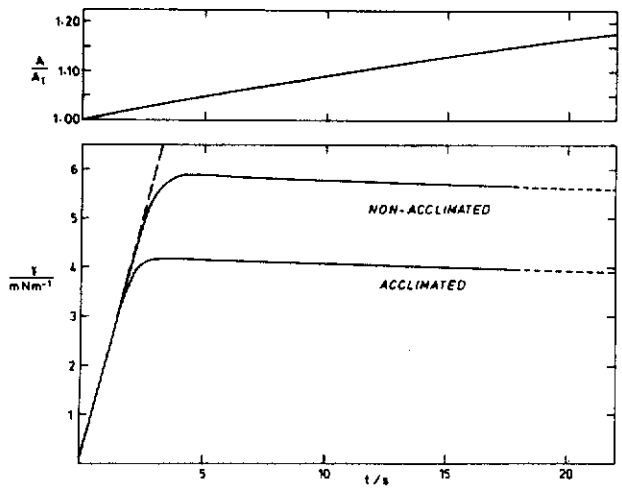

Fig. 6. The relative increase in area A/A_I given by Eq. (16) and $\gamma(t)$ given by Eqs. (14) and (15) are plotted against time for the initial phase of an osmotic expansion. The dashed line on the left indicates elastic behavior. $\gamma(t)$ curves are discontinued at large t because of the possible effects of reservoir depletion on Z, which would complicate Eq. (14)

$$Z(\gamma) = M(\exp \gamma/\Gamma_i - \exp \gamma_r/\Gamma_i) \tag{12}$$

where Γ_i is the slope of such a plot and ln M its intercept. The last term in Eq. (12) is insignificant except at tensions below the range investigated. We have added this term simply to satisfy the condition that $Z(\gamma_r) = 0$. [It is tempting to speculate that $Z(\gamma)$ represents the difference between a rate of incorporation and a rate of deletion, both of which might be tension dependent and which would be equal when $\gamma = \gamma_r$. Data to test such a speculation are lacking because of the difficulties in conducting experiments at very low tensions.]

Substituting Eqs. (12) and (11) gives

$$\frac{d\gamma}{dt} = k_A\left[\frac{1}{A}\frac{dA}{dt} - \frac{M}{k_A}e^{\gamma/\Gamma_i} + \frac{M}{k_A}e^{\gamma_r/\Gamma_i}\right] \tag{13}$$

which is the differential equation describing this system. It may be verified by substitution that the following is the solution to Eq. (13):

$$\gamma = -\Gamma_i \ln\left(\frac{Mk_A}{\Gamma_i}\right) + k_A \ln\left(\frac{A(t)}{A_I}\right) + Mk_A e^{\gamma_r/\Gamma_i t} - \Gamma_i \ln\left[\int_o^t \left(\frac{A(t')}{A_I}\right)^{k_A/\Gamma_i} \exp(Mk_A e^{\gamma_r/\Gamma_i t'}/\Gamma_i)dt' + \frac{\Gamma_i}{Mk_A}e^{-\gamma_I/\Gamma_i}\right] \tag{14}$$

where $\gamma = \gamma_I$ and $A(t) = A_I$ at $t = 0$. Equation (14) is an analytical solution to Eq. (13), which holds for all continuous, monotonically increasing functions $A(t)$. The features of Eq. (14) are not immediately evident upon inspection. Two limits are noted: When $t \to \infty$, $\gamma \to \gamma_r$ as required. When $t \to 0$ and $\frac{dA}{dt} \neq 0$, then $\gamma \to k_A(A - A_I)/A_I + \gamma_r$; that is, the membrane approaches simple elastic behavior. In order to plot Eq. (14), an explicit form for $A(t)$ is required.

Lysis during Osmotic Expansions

It is possible to derive an analytical solution for the area as a function of time of an ideal spherical osmometer bounded by an ideal semipermeable membrane with well-mixed solutions on either side which is subjected to a step decrease in external osmotic pressure (*unpublished*). The expansion of a protoplast with finite diffusion and mixing will only be approximated by this solution. The behavior of individual protoplasts during manipulations of the osmotic pressure of their supporting medium is the subject of continuing study; however, an excellent analytical approximation (average regression coefficient = 0.992) to the $A(t)$ produced by a step dilution is:

$$A(t) = A_F - (A_F - A_I)e^{-t/\tau} \tag{15}$$

where A_I and A_F are the initial and final areas of the membrane, and the time constant is a semi-empirical parameter which is determined by:

$$\tau = \left(\frac{A_F}{A_I} - 1\right)\left[\left(\frac{1}{A}\frac{dA}{dt}\right)_{t=0}\right]^{-1} = \left(\frac{A_F}{A_I} - 1\right)\frac{R_I}{2L_P(\pi_I - \pi_F)} \tag{16}$$

where L_p is the hydraulic conductivity and π_I and π_F are the initial and final osmotic pressures.

Using measured parameters from Figs. 2 and 3, we show in Fig. 6 the tension as a function of time which would be produced by an expansion of the form of Eq. (15).

Several observations may be made about this figure by referring to Eq. (11) to which it is a solution:

$$\frac{d\gamma}{dt} = k_A\left[\frac{1}{A}\frac{dA}{dt} - Z(\gamma)\right].$$

At $t = 0$, $\gamma = \gamma_r$, and so $z(\gamma) = 0$. Thus the asymptote at $t = 0$ is simple elastic behavior and the ten-

sion increases in proportion to the area (dashed line). Once the tension reaches a few mN · m^{-1}, material starts entering the membrane [$Z(\gamma) > 0$] so that the tension increases more slowly than the elastic law. After a couple of seconds the tension reaches its maximum value when [using Eqs. (11) and (12)]

$$0 = \frac{1}{A}\frac{dA}{dt} - Z(\gamma) \approx \frac{1}{A}\frac{dA}{dt} - Me^{\gamma/\Gamma_i}. \tag{17}$$

Because the initial rate of expansion in a step dilution is

$$\frac{1}{A}\frac{dA}{dt} = \frac{2}{R}\frac{dR}{dt} = \frac{2}{R}L_p(\pi_I - \pi_F) \tag{18}$$

it follows that the maximum tension achieved during an expansion is:

$$\gamma_{max} = \Gamma_i \ln\left[\frac{2L_p}{R_I M}(\pi_I - \pi_F)\right]. \tag{19}$$

[Further, the available evidence suggests that, in suspension at isotonic, there is little difference between the radii of acclimated and nonacclimated protoplasts and that there is not a very large difference in L_p (Dowgert & Steponkus, 1983). Thus the different tensions generated are primarily the result of different intrinsic dynamical properties of the membrane (M and Γ_i).] Because the incorporation rate is much higher for acclimated cells than for nonacclimated cells at all values of γ (i.e., M is larger for acclimated cells) the maximum tension to which the membranes of acclimated cells are exposed is lower than that which occurs in the membranes of nonacclimated cells exposed to the same expansion.

After the maximum tension is reached, it declines very slowly. During this phase, the slope of $\gamma(t)$ is seen in Fig. 6 to be very much smaller than $\frac{k_A}{A}\frac{dA}{dt}$, the slope at $t = 0$. For this phase $Z(\gamma) \approx \frac{1}{A}\frac{dA}{dt}$; i.e., the rate of incorporation is approximately equal to the expansion rate and so the tension varies only slowly. Since the expansion rate falls gradually to zero as the osmotic pressures come to equilibrium, so the incorporation rate falls gradually to zero. The behavior of γ at large t cannot be predicted with confidence (hence the dotted line) since in this region the approximation that $Z(\gamma,A_o) = Z(\gamma)$ is expected to break down. Indeed, if the reservoir becomes substantially depleted, the tension necessary to produce the required rate of incorporation may increase. (This aspect is the object of continuing research.)

A complete knowledge of $\gamma(t)$ and $\omega(\gamma)$ would give $\omega(t)$, which could, in principle, be integrated over time to give the probability of survival by a protoplast of a given dilution. In addition to the difficulty, mentioned above, in determining the behavior of $\gamma(t)$ at large t, we have also noted that $\omega(\gamma)$ cannot be measured for a single protoplast and that this quantity has a substantial variation in the population. At this stage, these difficulties prevent the *ab initio* calculation of population survival rates using only measured parameters. Thus the explicit equations (14) and (19) can be applied only to a hypothetical population of identical protoplasts and must be restricted to the initial, rapid phase of the expansion. Despite these limitations, these analytical expressions have the important advantage that they allow the higher survival rate which obtains among acclimated cells to be attributed to specific measurable properties of the membrane. A further possibility is the prediction of survival rates under different conditions of expansion such as the expansion that occurs during the thawing of frozen tissue. Finally, by indicating specific membrane properties which are altered by acclimation, we have indicated the area in which a molecular explanation of the process of acclimation may profitably be sought.

Conclusions

a) The resting tensions in the membranes of protoplasts from nonacclimated and acclimated plants are similar, and small.
b) The area elastic moduli of the membranes of nonacclimated and acclimated protoplasts are similar and are in the range expected for a fluid mosaic membrane.
c) The probable frequency of lysis of protoplast membranes increases approximately exponentially with tension and is substantially higher for acclimated protoplasts.
d) The rate of proportional increase in area increases approximately exponentially with tension at high tensions and is much larger in acclimated than in nonacclimated protoplasts.
e) An analytical expression is obtained for the tension generated in the membranes of protoplasts exposed to a given expansion in area, solely employing parameters measured in these experiments.

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