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The Mechanics of Injury to Isolated Protoplasts following Osmotic Contraction and Expansion[1]

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MICHAEL F. DOWGERT[2], JOE WOLFE, AND PETER L. STEPONKUS*
Department of Agronomy, Cornell University, Ithaca, New York 14853-0144 (M.F.D., P.L.S.); *and School of Physics, University of New South Wales, Kensington New South Wales 2033 Australia* (J.W.)

ABSTRACT

Micro-osmotic manipulation was used to determine the influence of osmotic contraction on the expansion potential of individual protoplasts isolated from rye (*Secale cereale* L. cv Puma) leaves. For protoplasts isolated from leaves of nonacclimated plants (NA protoplasts), osmotic contraction in sufficiently hypertonic solutions (>1.53 osmolal) predisposed the protoplasts to lysis during osmotic expansion when they were returned to isotonic conditions (0.53 osmolal). In contrast, for protoplasts isolated from leaves of cold acclimated plants (ACC protoplasts), osmotic contraction in either 2.6 or 4.0 osmolal solutions was readily reversible. Following osmotic contraction, the resting tension (γ_r) of NA protoplasts was similar to that determined for protoplasts in isotonic solutions (*i.e.* 110 ± 22 micronewtons per meter). In contrast, γ_r of ACC protoplasts decreased from 164 ± 27 micronewtons per meter in isotonic solutions to values close to zero in hypertonic solutions. Following expansion in hypotonic solutions, γ_r's of both NA and ACC protoplasts were similar for area expansions over the range of 1.3 to 1.6. Following osmotic contraction and reexpansion of NA protoplasts, hysteresis was observed in the relationship between γ_r and surface area—with higher values of γ_r at a given surface area. In contrast, no hysteresis was observed in this relationship for ACC protoplasts. Direct measurements of plasma membrane tension (γ) during osmotic expansion of NA protoplasts from hypertonic solutions (1.53 osmolal) revealed that γ increased rapidly after small increments in surface area, and lysis occurred over a range of 1.2 to 8 millinewtons per meter. During osmotic expansion of ACC protoplasts from hypertonic solutions (2.6 osmolal), there was little increase in γ until after the isotonic surface area was exceeded. These results are discussed in relation to the differences in the behavior of the plasma membrane of NA and ACC protoplasts during osmotic contraction (*i.e.* endocytotic vesiculation *versus* exocytotic extrusion) and provide a mechanistic interpretation to account for the differential sensitivity of NA and ACC protoplasts to osmotic expansion from hypertonic solutions.

One form of freeze/thaw injury to isolated protoplasts is termed expansion-induced lysis (5). During slow cooling (<3°C/min) over the range of 0 to −5°C, protoplasts contract osmotically in response to the decreased chemical potential in the partially frozen external solution. Subsequently, during warming and thawing of the extracellular solution, the protoplasts expand osmotically in response to the decreasing osmolality. However, for protoplasts isolated from nonacclimated rye leaves (nonacclimated protoplasts), and cooled below −3°C, more than 50% of the protoplasts lyse before the original volume and surface area are regained (1). In contrast, in protoplasts isolated from acclimated rye leaves (acclimated protoplasts), expansion-induced lysis is rarely observed (<10%) in protoplasts cooled to any temperature over the range of 0 to −40°C (1).

Studies of the stress-strain relation of the plasma membrane of nonacclimated protoplasts using micropipette aspiration indicate that the large surface area changes incurred during osmotic contraction and expansion cannot be a consequence of intrinsic expansion or contraction of the membrane (9, 11). Intrinsic expansion or contraction obeys an elastic law, $\Delta\gamma = k_A\,\Delta A/A$ where k_A is the area elastic modulus (≈200 mN m^{-1}). Tensions greater than 2 to 3% of k_A cause lysis of the membrane so the maximum extent of intrinsic 'elastic' expansion is about 3%. Therefore, it was suggested that (a) sufficiently large osmotic excursions involve the exchange of material between the plane of the membrane and an extrinsic reservoir of membrane material and (b) the transfer of material depends on the bifacial surface tension, γ, of the membrane. The plasma membrane of isolated protoplasts equilibrated in isotonic solutions is under a small tension, referred to as the resting tension (γ_r) (9). If the membrane tension is increased, by micropipette aspiration or osmotic expansion, to a value greater than γ_r, material is incorporated into the membrane at a rate which increases strongly with γ. If γ is lowered below γ_r, γ_r is reestablished. For area contractions and expansions of <0.15 in nonacclimated protoplasts, γ_r is independent of the area change so the stress-strain relation of the membrane is said to approach a surface energy law over long time scales (10).

The existence of the proposed reservoir into which material is transferred during osmotic contraction has since been demonstrated by both light (1) and EM (2). Freeze-induced osmotic contraction of nonacclimated protoplasts results in endocytotic vesiculation of the plasma membrane, and numerous clusters of vesicles (0.1–1.5 μm in diameter) are observed in the cytoplasm. Freeze fracture studies reveal that the intramembrane particle density on both the PF_p[3] and EF_p of the plasma membrane remains unchanged following osmotic contraction (2). This suggests that endocytotic vesiculation involves a unit membrane deletion. Following osmotic reexpansion, the cytoplasmic vesicles are not observed to be reincorporated into the plasma membrane.

Endocytotic vesiculation of the plasma membrane is not observed in acclimated protoplasts. Instead, freeze-induced osmotic

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[2] Present address: Glie Farms, 1818 Bathgate Ave., Bronx, NY 10457.

[3] Abbreviations: PF_p, protoplasmic face; EF_p, exoplasmic face; N, Newton; TEM, transmission electron microscopy; TSAI, tolerable surface area increment; LT_{50}, minimum temperature for 50% survival; osm, osmolal; NA, nonacclimated; ACC, cold acclimated.

contraction results in the formation of exocytotic extrusions over the surface of the protoplasts (1). Ultrastructural studies show that the extrusions have a densely osmiophilic interior, and cross-fracturing of the extrusions reveals aparticulate lamellae (3). These observations suggest that the interior of the extrusions is predominantly lipid. Freeze-fracture studies and determination of the intramembrane particle density of the plasma membrane fracture faces are consistent with the interpretation that the osmiophilic material in the extrusions is lipidic material resulting from the preferential deletion of lipids from the plasma membrane. Following osmotic contraction, there was a substantial increase in the frequency of paracrystalline arrays and an increase in the particle density in the noncrystalline regions of the PF_p. As volumetric contraction of acclimated protoplasts is readily reversible, so too is the formation of the exocytotic extrusions. During osmotic expansion, the extrusions are drawn back into the plasma membrane (1). Apparently, the lipid material that was in the interior of the extrusions is also reincorporated into the plasma membrane because there is no evidence of its presence in electron micrographs of expanded protoplasts.

The difference between the ultrastructural behavior of the plasma membrane of nonacclimated protoplasts and that of acclimated protoplasts during osmotic contraction (endocytotic vesiculation *versus* exocytotic extrusion) has been proposed to explain (a) the irreversibility of osmotic contraction in nonacclimated protoplasts and (b) the ability of acclimated protoplasts to survive large surface area contractions and expansions (1). The objective of this study was to determine the influence of endocytotic vesiculation and exocytotic extrusion formation on the stresses and strains imposed on the plasma membrane by osmotic manipulation.

Previous studies of the influence of large osmotic excursions on protoplast survival have involved determination of survival of the population following changes in tonicity of the suspending medium (1, 6, 8). Plots of percent survival as a function of the increase in the surface area, calculated using the Boyle-van't Hoff relation, showed that lysis of nonacclimated protoplasts occurred if the area increase exceeded a given value, the Tolerable Surface Area Increment. The TSAI determined for nonacclimated protoplasts was independent of previous osmotic contraction. The TSAI so determined is a population parameter. The object of the present study was (a) to characterize the TSAI of individual protoplasts by osmotic manipulation of single protoplasts while under microscopic observation, (b) to provide a mechanistic interpretation for the ability of acclimated protoplasts to survive large osmotic contractions and expansions by measuring tension in the membrane following equilibration for 30 min in solutions of varied tonicities, and measuring membrane tension during area expansion from hypertonic solutions.

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) temperatures (16-h photoperiod). Nonacclimated plants (LT_{50} of −5°C) remained in this environment for 2 weeks. Cold acclimation was achieved by exposing 1-week-old plants to 13°C (day) and 7°C (night) temperature (11.5-h photoperiod) for 1 week and then to a 2°C constant day and night temperature (10-h photoperiod) for 4 weeks, after which the plants were 'fully acclimated' (LT_{50} of −25°C).

Protoplasts were enzymically isolated from leaf tissue as previously described (1). Following digestion, the protoplasts were resuspended in isotonic sorbitol: 0.53 and 1.03 osm for nonacclimated and acclimated protoplasts, respectively. Because the internal solute concentration increases during cold acclimation, a higher isotonic osmolality was required to maintain the *in situ* volume of acclimated protoplasts. Protoplasts are referred to as either acclimated or nonacclimated to denote the state of the tissue from which they were isolated.

Osmotic Manipulation of Individual Protoplasts. Osmotic manipulation of individual isolated protoplasts was accomplished by capturing individual protoplasts in pipettes of 40 μm diameter and transferring the protoplasts from hypertonic solutions to isotonic or hypotonic solutions. The protoplasts were then ejected and the increase in area as a function of time was determined. Protoplast suspensions were loaded by capillarity into a 0.2 mm path length microslide (Vitro Dynamics, Rockaway, NJ). The micropipette was maneuvered in the microslide and the protoplast captured by applying a negative pressure to the pipette. The pipette was withdrawn from the microslide and transferred to one containing the desired final osmolality. The pipette was inserted into the new microslide and the protoplast ejected by applying a positive pressure. Following ejection of the protoplast, the pipette was immediately removed to minimize mixing of the two solutions. Some of the isotonic solution was ejected along with the protoplast. The volume of solution ejected was typically <1% of that of the solution volume in the microchamber. Following ejection, protoplast diameter was measured over time. Volume and surface area changes were calculated assuming sphericity. Protoplast diameters were measured on digitized video images by using an interactive mensuration procedure (7).

Determination of Membrane Tension following Osmotic Manipulation. Measurements of membrane tension using micropipette aspiration, described in detail by Wolfe and Steponkus (9, 10), were modified slightly in this study. Briefly, micropipettes with inside diameters of 7 to 12 μm were mounted on a micromanipulator and connected to a manometer via a flexible tube. Protoplast suspensions were loaded into a glass microslide of 0.2 mm path length (Vitro Dynamics, Rockaway, NJ) and the micropipette maneuvered in the microslide to abut a protoplast. A negative pressure was applied to the protoplast membrane by adjusting the height of the manometer and the protoplast membrane was deformed. Experiments were generally conducted at a distance of 2 to 4 mm from the end of the microslide. At this distance, the local solution osmolality was not measurably affected by evaporation from the end of the microslide for experiments lasting up to 10 min.

The tension of individual protoplasts was estimated as the slope of a line through $(0, 2/R)$, where R is the radius of the protoplast, on a plot of negative pressure *versus* curvature of the membrane in the pipette. The experimental procedure consisted of rapidly applying a small negative pressure (8–30 Pa) to the plasma membrane of the protoplast and measuring the resultant curvature. The slope of a line through this measured point and $(0, 2/R)$ provides an estimate of tension in the membrane. 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.

Measurement of Membrane Tension during Osmotic Expansion. A microslide positioned on a modified microscope stage was connected by a flexible tube to a reservoir 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 solution to flow through the microslide. A small slit was ground in the side of the microslide to allow entry of a micropipette used to measure membrane tension. The surface tension of the solution prevented solution from leaking from the slit. Protoplasts were placed in the microslide through this slit.

A 7 to 12 μm micropipette mounted on a micromanipulator was inserted through the slit in the microchamber and manipu-

lated to abut a protoplast. The reservoir of more dilute solution was raised, causing dilute solution to flow past a protoplast held on the tip of the measuring pipette by a small suction. As the protoplast expanded osmotically, the pressure in the pipette was made more negative by lowering the manometer and a nearly constant intrusion into the pipette was maintained. The surface tension of the membrane was obtained from the pressure in the pipette and the Laplace-Young equation (10). 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 into the pipette.

Volume and Surface Area Contraction of Isolated Protoplasts. Protoplasts behave as osmometers and exhibit characteristic Boyle-van't Hoff behavior. The volume of isolated protoplasts varies linearly with the reciprocal of the osmolality of the suspending medium. However, protoplast lysis is a function of the surface area. Following equilibration in hypotonic solutions, the plasma membrane remains smooth and the surface area is accurately estimated from diameter measurements. Following osmotic contraction the global geometry of both nonacclimated and acclimated protoplasts is spherical. However, only the surface of nonacclimated protoplasts remains smooth (2). In acclimated protoplasts, exocytotic extrusions are formed on the surface of the protoplast (3). The total amount of plasma membrane area which forms the extrusions and the total amount of material deleted into the putative lipid cores is unknown. Calculation of the surface area of nonacclimated protoplasts in all tonicities and the area of acclimated protoplasts in iso- or hypotonic media can be calculated from measurements of the diameter and the equation $A = 4\pi r^2$. However, for acclimated protoplasts in hypertonic solutions the same calculation underestimates the actual surface area. In this study we are interested in the effect of osmotic contraction on protoplast lysis. To facilitate discussion of lysis relative to surface area expansion the parameter routinely used is surface area of a smooth sphere of the same diameter. Where this is not equal to the membrane area (contracted acclimated protoplasts) this distinction is made explicitly.

RESULTS

Influence of Hypertonic Contraction on Protoplast Expansion. Nonacclimated protoplasts suspended in 0.98 osm solutions regain a spherical shape, with a fractional surface area of 0.70 predicted from the Boyle-van't Hoff relation. Following transfer to an isotonic solution (0.53 osm), over 90% of the protoplasts reached an equilibrium area without lysing (Fig. 1a). In some instances, the fractional area at equilibrium was less than 1.0. This occurred because when a protoplast was transferred into the isotonic solution, a small volume of the original hypertonic solution was also transferred. Some variation in the area measured at osmotic equilibrium is attributed to the variability in the volumes of hypertonic solution transferred along with the protoplasts. Following osmotic equilibration in 1.53 osm solutions, the fractional surface area predicted from the Boyle-van't Hoff relation was 0.55. Following transfer to an isotonic solution, 60% of the nonacclimated protoplasts lysed (Fig. 1b). Lysis occurred over a range of fractional areas from 0.62 to 0.79 with a median value of 0.71. The median value of the increase in area before lysis as a fraction of the isotonic surface area was 0.15.

Osmotic contraction of acclimated protoplasts in either 2.6 or 4.0 osm solutions was readily reversible (Fig. 2, a and b). All but one acclimated protoplast survived contraction in 2.6 osm and reexpansion in isotonic solutions (Fig. 2a). Similarly, only one acclimated protoplast lysed during reexpansion from 4.0 to 1.03 osm (Fig. 2b). In the figures illustrating the expansion of acclimated protoplasts from hypertonic solutions of 2.6 and 4.0 osm, the ordinate is the fractional area of a smooth sphere with volume equal to that of the protoplast, *i.e.* $(V/V_o)^{2/3}$. It is acknowledged

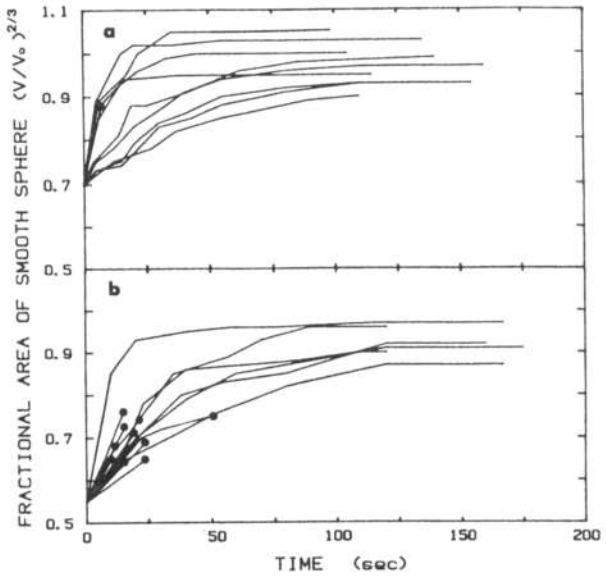

FIG. 1. Osmotic expansion of nonacclimated protoplasts following a step transfer from hypertonic to isotonic solutions. Osmotic expansion is expressed as the increase in fractional area, relative to the isotonic area, as a function of time. The protoplasts were suspended in either (a) 0.98 or (b) 1.53 osm and the diameter was measured. The fractional area at 0.98 and 1.53 osm was calculated to be 0.70 or 0.55, respectively, from the Boyle-van't Hoff relation. The protoplasts were then transferred to isotonic solutions and the increase in diameter measured over time. A closed circle at the end of a curve denotes lysis.

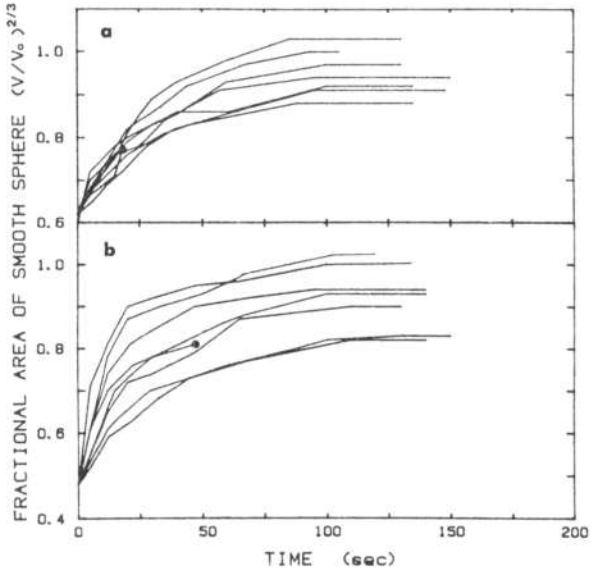

FIG. 2. Osmotic expansion of acclimated protoplasts following a step transfer from hypertonic to isotonic solutions. Procedure was the same as in Figure 1 except expansion was effected from (a) 2.6 osm or (b) 4.0 osm.

that the surface of acclimated protoplasts in hypertonic solutions is not smooth so this represents an underestimate of the membrane area in this case. This is not, however, a limitation on the quantitative use of the figures because acclimated protoplasts survive reexpansion to isotonic solutions and so the ordinate in all cases gives the membrane area at lysis.

Effect of Osmotic Manipulation on Membrane Resting Tension. The resting tension of nonacclimated protoplasts suspended in isotonic solutions was somewhat less ($110 \pm 22\ \mu N\ m^{-1}$) than

that of acclimated protoplasts (164 ± 27 μN m^{-1}) (Fig. 3). Following a 30- to 60-min equilibration period in hypotonic solutions, the tensions of both acclimated and nonacclimated protoplasts were greater than those measured in isotonic solutions. With the exception of the treatment resulting in a fractional area of 1.15, the resting tensions of nonacclimated and acclimated protoplasts were similar following equilibration in hypotonic solutions. It should be noted that a significant fraction of nonacclimated protoplasts lyse during osmotic expansion. As shown in Figure 1b, nonacclimated protoplasts lyse at an average fractional area expansion of 0.15 the original surface area. Thus, some of the difference in the resting tension of acclimated and nonacclimated protoplasts following an osmotic expansion may be the result of measuring tension in the surviving population of nonacclimated protoplasts. The resting tension of neither nonacclimated nor acclimated protoplasts returned to the isotonic values following area expansions greater than 0.15. A 30-min exposure to hypotonic solutions, resulting in a 0.30 area expansion, produced median resting tensions of 400 and 480 μN m^{-1} in nonacclimated and acclimated protoplasts, respectively. Following area expansion of 0.60, the median resting tension of both nonacclimated and acclimated protoplasts was greater than 800 μN m^{-1}. Thus, the resting tension of the protoplast membrane is either dependent upon the extent of area expansion or only slowly reverts to the isotonic value for resting tension following osmotic expansions of greater than 0.15.

Following equilibration in hypertonic solutions, the resting tensions of nonacclimated protoplasts were reestablished to values that were similar to those in isotonic solutions (Fig. 3). This reestablishment of resting tension is interpreted as being a consequence of the deletion of membrane material (9, 10). In contrast, following osmotic contractions of $(V/V_o)^{2/3}$ to 0.85 and 0.7, the resting tensions of acclimated protoplasts were either zero or below the resolution of the current technique.

The resting tension in the membrane of nonacclimated protoplasts increased following osmotic expansion from the contracted state (Fig. 4a). For protoplasts contracted to a fractional area of 0.7 and reexpanded to a fractional area of 1.15 the median resting tension increased from 89 to 580 μN m^{-1}. Similarly, the median resting tension in the membrane of protoplasts contracted to a fractional area of 0.55 and reexpanded to a fractional area of 1.0 increased from 76 to 800 μN m^{-1}. Thus, hysteresis was observed in the resting tension of nonacclimated protoplasts contracted to fractional areas of 0.7 and 0.55 and reexpanded. The variability in the resting tension for any given extent of area expansion may be attributed, in part, to measuring tension in only those protoplasts that survived the expansion.

In contrast, the resting tension of acclimated protoplasts expanded from hypertonic solutions remained low until expanded beyond the isotonic surface area (Fig. 4b). The resting tensions of acclimated protoplasts osmotically contracted to volumes that correspond to smooth spheres of fractional areas of 0.7 and 0.55

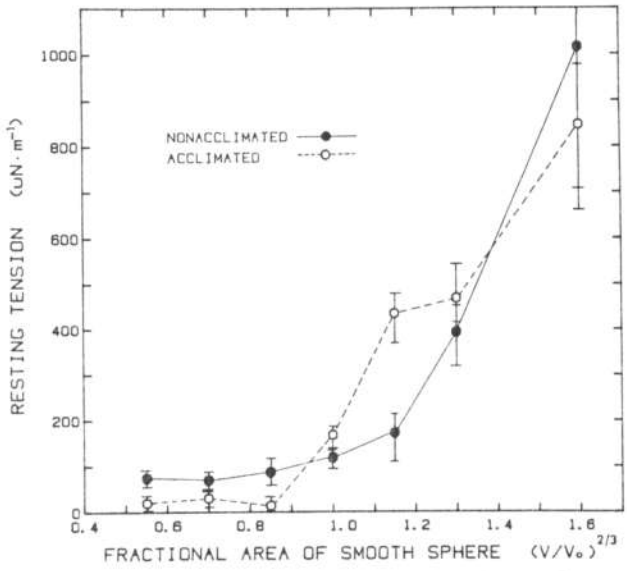

FIG. 3. The resting tension of the plasma membrane of nonacclimated (●——●) and acclimated (○——○) protoplasts equilibrated for a minimum of 30 min in solutions of varied tonicities. The tonicities of the suspending media were adjusted to result in a given protoplast volume predicted by Boyle-van't Hoff behavior. Area was calculated assuming a smooth sphere of a given volume. The median resting tensions and 95% confidence limits are presented in the figure.

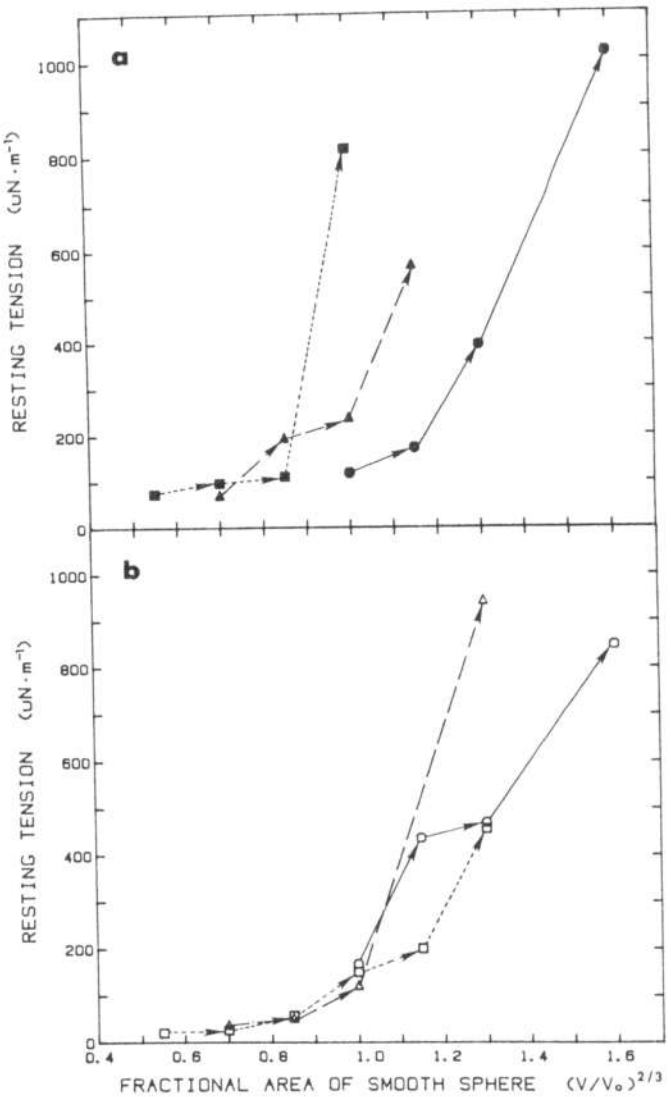

FIG. 4. a, Nonacclimated protoplasts were equilibrated in hypertonic solutions which resulted in protoplast volumes that correspond to surface areas of 0.7 and 0.55; b, acclimated protoplasts were equilibrated in hypertonic solutions which resulted in protoplast volumes that correspond to fractional surface areas of 0.7 and 0.55. As the surfaces of contracted acclimated protoplasts were not smooth, but covered with extrusions, the actual surface areas were greater than those calculated for a smooth sphere of the same volume. The resting tensions under these conditions were measured. The protoplasts were equilibrated in varied hypotonic solutions which result in volumes that corresond to the areas indicated and, following a 30-min equilibration, the tension measured again.

and reexpanded to the isotonic surface area were similar to the resting tensions of protoplasts that had not undergone the contraction/expansion sequence. The resting tension of acclimated protoplasts expanded beyond the isotonic surface area was independent of the previous extent of contraction and increased in a manner similar to that of protoplasts expanded from isotonic solutions.

Membrane Tension during Osmotic Expansion. The measurements of resting tension following osmotic contraction are consistent with the suggestion that membrane area of acclimated protoplasts is not irreversibly lost during contraction. In contrast, the hysteresis in resting tension as a function of area following contraction and reexpansion of nonacclimated protoplasts is consistent with the suggestion that membrane area is not conserved during contraction (1). These responses are directly contrasted by measuring membrane tension during osmotic expansion from hypertonic solutions. To accomplish this, nonacclimated and acclimated protoplasts were equilibrated in hypertonic solutions of 1.53 and 2.6 osm, respectively. The protoplast suspensions were loaded into a microchamber containing the same solution. A protoplast was selected and attached to a micropipette by applying a small suction. Subsequently, the tonicity of the solution in the chamber was decreased, and the surface area and the tension in the membrane during osmotic expansion were simultaneously measured as functions of time until lysis.

The increase in area of nonacclimated protoplasts exposed to a decreasing osmolality in the microchamber was not a linear function of time (Fig. 5a). The rate of area expansion was initially slow and increased over time for the majority of protoplasts. Lysis occurred over a range of fractional areas from 0.6 to 0.75. The tension in the membrane of nonacclimated protoplasts initially increased slowly with time, then increased rapidly. The protoplasts lysed over a range of tensions from 1.2 to 8.0 mN m^{-1} (Fig. 5b).

Acclimated protoplasts, transferred from 2.6 osm to distilled H_2O in the microchamber expanded at a wide range of rates (Fig. 5c). For the majority of protoplasts, the rate of expansion (expressed as the rate of expansion in area of a sphere with equal volume) was initially rapid and slowed over time. Except for one protoplast, lysis of acclimated protoplasts occurred only after the isotonic surface area was exceeded. The tension in the membrane of acclimated protoplasts was low during the initial area expansion then increased to a high value (Fig. 5d). Lysis of acclimated protoplasts occurred over a range of tensions from 1.5 to 4.2 mN m^{-1}.

The relation between osmotic contraction and the tension in the membrane is best illustrated when tension is considered as a function of the extent of expansion from hypertonic solutions (Fig. 6). During reexpansion of nonacclimated protoplasts from hypertonic solutions the tension increased after only a small increase in area, and the protoplasts lysed before they regained their isotonic area. In contrast, during osmotic expansion of acclimated protoplasts little tension was generated in the membrane until the isotonic surface area was achieved. Except for two protoplasts, a large increase in tension was not observed in acclimated protoplasts until after they expanded beyond the isotonic surface area.

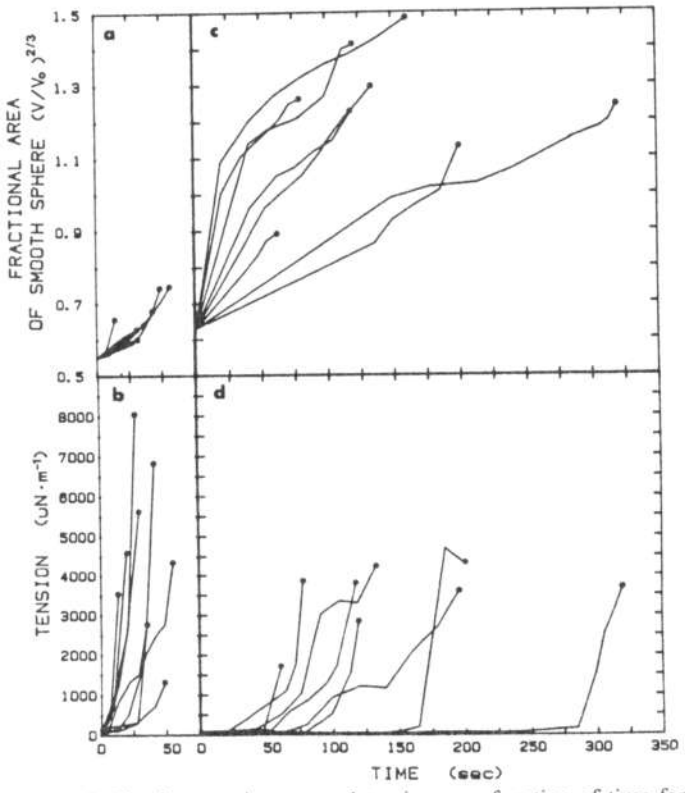

FIG. 5. The increase in area and tension as a function of time for nonacclimated (a, b) and acclimated (c, d) protoplasts expanded in a microchamber. Nonacclimated and acclimated protoplasts were initially contracted in 1.53 and 2.6 osm, respectively, loaded into the microchamber, and tension measured with a pipette connected to a manometer. Solution flow was effected in the chamber, and the tonicity of the solution lowered. Tension was measured during subsequent protoplast expansion. Closed circles terminating curves denote protoplast lysis.

DISCUSSION

We propose that the differential behavior of the plasma membrane (endocytotic vesiculation *versus* exocytotic extrusion formation) during osmotic contraction of nonacclimated and acclimated protoplasts is responsible for the difference in the sensitivity to an osmotic contraction/expansion excursion.

1. The tension generated in the plasma membrane during expansion of nonacclimated protoplasts from hypertonic solutions is similar to that generated by expansion from isotonic solutions (12). Large and lethal tensions are generated early in the expansion before the area has increased by a large fraction (Fig. 6). In contrast, the tension in the plasma membrane of acclimated protoplasts expanded from hypertonic solutions does not start to rise until after the isotonic volume is exceeded (Fig. 6).

2. The resting tension of the plasma membrane of nonacclimated protoplasts was reestablished to the same value after osmotic contraction (Fig. 3). In contrast, the resting tension of the membrane of acclimated protoplasts is reduced after contraction. Wolfe and Steponkus (10) suggest that γ_r is the free energy per unit area required to transfer material from a reservoir to the membrane, and that a larger or smaller γ_r would result from a smaller or larger concentration of material in that reser-

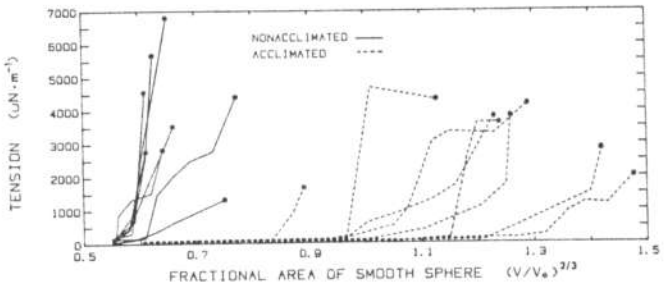

FIG. 6. The data shown in Figure 5 replotted to show tension as a function of fractional area for nonacclimated and acclimated protoplasts.

voir. If this is true, then the equilibrium is unchanged by contraction of nonacclimated protoplasts. The lower γ_r for acclimated protoplasts shows that the membrane may be expanded with application of very little work.

3. For nonacclimated protoplasts subjected to osmotic contraction followed by osmotic expansion (Fig. 4a), the increase in γ_r only depended on the area increase and not on the previous degree of contraction. This is consistent with the proposition that the reservoir available for expansion is little changed by previous contraction. For acclimated protoplasts the value of γ_r in isotonic solutions is regained after contraction/reexpansion. For expansions beyond isotonic, γ_r is not very different in populations previously contracted to different degrees (Fig. 4b).

Osmotic contraction of nonacclimated protoplasts results in endocytotic vesiculation of the plasma membrane (1, 2). The reestablishment of the nonzero resting tension over the range of fractional areas of 1.0 to 0.55 (Fig. 3) is the result of deletion of plasma membrane material (9, 10). The observation that the tension in the membrane of nonacclimated protoplasts is a function of the increase in area regardless of the extent of contraction (Fig. 4a), rather than a function of the absolute area, suggests that membrane material deleted during osmotic contraction is not readily reincorporated into the plasma membrane during subsequent reexpansion. For expansion from hypertonic solutions, the relationships between tension and area were similar to those determined for expansion from isotonic solutions (12). This suggests that increases in membrane area in nonacclimated protoplasts are derived from the same membrane reservoir (as yet unidentified) whether the expansion is from isotonic or hypertonic solutions. This interpretation is consistent with the observations at the light and electron microscopic levels that during osmotically induced volume reduction, vesicles form in the cytoplasm and these vesicles remain in the cytoplasm following reexpansion (1, 2). The deletion of membrane material into cytoplasmic vesicles that are not readily available for reincorporation into the membrane upon reexpansion is consistent with the constancy of TSAI.

The membrane properties that account for the irreversibility of vesicle formation in nonacclimated protoplasts are not known. However, one contributing factor may be the size of vesicles formed during osmotic contraction. Large vesicles have limited diffusional motion and, if formed, would have a low probability of encountering the plasma membrane in a manner that facilitates membrane fusion. In contrast, small vesicles, having greater diffusional motion, would have a higher probability of fusing with the plasma membrane. High resolution video microscopy has shown that during osmotic contraction the membrane becomes flaccid and a large number of vesicles are observed to form (1). Initially, the vesicles are quite large, up to 2 μm in diameter. As the protoplast regains sphericity, vesicle formation is less evident either because the vesicles formed are smaller or because of the lower frequency of vesicle formation at this stage of contraction. For a protoplast with a surface area of 2000 μm^2, a change in area of 0.05 is sufficient to raise the resting tension 0.1 mN m^{-1} because the resting tension of the protoplasts is extremely small compared to the elastic modulus (200 mN m^{-1}). Vesicles of this size or smaller, even if they underwent rapid exchange with the plasma membrane, would account for only a small amount of the deleted surface area.

Following osmotic contraction of acclimated protoplasts, exocytotic extrusions form on the protoplast surface (1). EM has revealed that the exocytotic extrusions consist of a densely osmiophilic core bounded by the plasma membrane (3). The formation of exocytotic extrusions occurs at either a zero or near zero value of resting tension in osmotically contracted acclimated protoplasts and does not result in a reestablishment of tension to the isotonic value. The observation that acclimated protoplasts appear to be globally spherical following osmotic contraction is consistent with a small nonzero tension in the membrane. (If the membrane surrounding the extrusions was on all sides attracted to the contents of the extrusion then this putative interaction could sustain a small, nonzero tension in the bulk of the membrane. Further, a local attraction or cohesion is consistent with the observation the extrusions tend to be stable, long-lived structures.) The contents of the extrusions are believed to be lipid material deleted from the plasma membrane because the particle density of the PF_p face increases following osmotic contraction and because freeze-fracture of the core of the extrusions reveals aparticulate lamellae (3). During osmotic expansion of contracted acclimated protoplasts, the irregularly shaped plasma membrane with membrane surface extrusions becomes smooth, and it appears that the material in the core is reincorporated into the membrane. This rearrangement occurs at tensions far below that necessary to lyse the membrane (<100 μN m^{-1} compared with 4000 μN m^{-1}). Thus, the ability of acclimated protoplasts to survive large volumetric contractions and expansions is the result of reversibility of extrusion formation at low tensions. The mechanics of the transformation of the plasma membrane of acclimated protoplasts from the irregular surface topography in the contracted protoplast to a smooth surface is not well understood because the tension at which these transformations occur is below the limit of the resolution of our techniques.

In the current study, osmotic contraction of acclimated protoplasts was reversible in the sense that protoplasts survived reexpansion to isotonic solutions. However, the surface area at lysis of acclimated protoplasts expanded from hypertonic solutions was less than the surface area at lysis of those expanded from isotonic solutions (12). Acclimated protoplasts osmotically expanded from isotonic solutions lysed at surface areas between 1.4 and 1.87 (12). In contrast, following osmotic contraction and reexpansion in the microchamber, lysis occurred at surface areas between 0.9 and 1.49 (Fig. 5c). In several cases, exocytotic extrusions were observed to become detached from the protoplast surface during transfer into the microchamber. This may have contributed to the lower surface area at lysis for reexpanded acclimated protoplasts, especially in the protoplasts where tension increased before the isotonic surface area was reached.

Previous studies have shown that the resting tension of nonacclimated protoplasts is reestablished to the value measured in isotonic solutions following equilibration in solutions that result in a change in area ≤ 0.15 (9, 10). The present study shows that following an increase in area >0.15 the resting tension is not reestablished to the resting value in either nonacclimated or acclimated protoplasts. In addition, following osmotic contraction of acclimated protoplasts the resting tension is not reestablished to the isotonic value. Wolfe and Steponkus (10) suggested that the resting tension may be influenced by the amount of material available in the reservoir. A resting tension determined by equilibrium between the molecules in a membrane and a reservoir is increased if the reservoir concentration, and thus the chemical potential of its components, is decreased (4). Thus, the increase in resting tension of nonacclimated and acclimated protoplasts following osmotic expansion suggests that depletion of the reservoir results in a higher resting tension. Contraction of nonacclimated protoplasts from isotonic solutions does not significantly change the resting tension, suggesting that the reservoir which gives rise to γ_r is little changed by osmotic contraction and endocytotic vesiculation. In contrast, the reduction in γ_r following contraction of acclimated protoplasts suggests that the chemical potential of the reservoir is reduced by contraction of acclimated protoplasts. Thus, contraction of acclimated protoplasts serves to concentrate and lower the chemical potential of the reservoir giving rise to a lower γ_r in acclimated protoplasts. However, the relation between extrusion formation and the

concentration of the reservoir is not known.

Finally, the results of the present study establish a causal relationship between exocytotic extrusion formation and the reversibility of osmotic contraction in acclimated protoplasts. The constancy of TSAI in nonacclimated protoplasts is, in part, the result of membrane material deleted into cytoplasmic vesicles not being available for incorporation into the membrane during reexpansion. The mechanics of area expansion of nonacclimated protoplasts is the same whether the protoplasts are expanded from isotonic or hypertonic solutions. A detailed study of the mechanics of area expansion from isotonic solutions is given by Wolfe *et al.* (12). In contrast, osmotic contraction of acclimated protoplasts alters the stress/strain relationship of the plasma membrane. The association among the plasma membrane, the extrusions and the reservoir present in isotonic conditions remains to be established.

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