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PROTOPLASMA

The Influence of Cold Acclimation on the Behavior of the Plasma Membrane Following Osmotic Contraction of Isolated Protoplasts[1]

W. J. GORDON-KAMM and P. L. STEPONKUS *

Department of Agronomy, Bradfield Hall, Cornell University, Ithaca, New York

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Summary

Osmotic contraction of protoplasts isolated from cold acclimated leaves of *Secale cereale* L. cv. Puma results in the formation of exocytotic extrusions of the plasma membrane. Numerous knobs or polyps were observed on the surface of the protoplasts with scanning electron microscopy. In thin sections, the extrusions were bounded by the plasma membrane with a densely osmiophilic interior. Cross-fracturing of the extrusions revealed aparticulate bodies within, a further indication that the interior of the extrusions was predominantly lipid material. Freeze-fracture of the plasma membrane suggests a possible source of this lipid material. Following osmotic contraction, the particle density on the plasma membrane protoplasmic face (PFp) increased, being reflected in both a substantial increase in paracrystalline arrays and an increase in the particle density in non-crystalline regions. This increase in particle density indicates that lipid material is preferentially lost from the plasma membrane during contraction. The density on the exoplasmic face (EFp) did not change. Together, these findings suggest that during hypertonic contraction of acclimated protoplasts, lipid material is preferentially subducted from the plasma membrane and sequestered into lipid bodies (the osmiophilic regions). The formation of lipid bodies and extrusions was readily reversible. Following osmotic expansion of acclimated protoplasts, the extrusions were retracted back into the plane of the plasma membrane.

Keywords: Cold acclimation; Exocytotic extrusions; Freeze-fracture; Isolated rye protoplasts; Lipid bodies; Osmotic contraction; Plasma membrane; Ultrastructure.

1. Introduction

During a freeze-thaw cycle, volumetric contraction and subsequent expansion must be successfully contended with if cells are to survive. With protoplasts isolated from non-acclimated rye leaves (*Secale cereale* L. cv. Puma) sufficiently large volumetric contractions are irreversible (WIEST and STEPONKUS 1978, STEPONKUS and WIEST 1978, 1979). From studies of the stress-strain relationship of non-acclimated protoplasts, it has been proposed that membrane material is deleted from the plasma membrane during osmotic contraction (WOLFE and STEPONKUS 1981, 1983, STEPONKUS *et al.* 1981). Contraction-induced deletion of plasma membrane material into cytoplasmic vesicles has been confirmed (GORDON-KAMM and STEPONKUS 1984). Sufficiently large contractions and deletion of the plasma membrane into vesicles are irreversible; and upon expansion, non-acclimated protoplasts lyse before regaining their original size.

Cold acclimation effectively ameliorates this form of injury, termed expansion-induced lysis (DOWGERT and STEPONKUS 1984). Although expansion-induced lysis quantitatively accounts for injury to non-acclimated protoplasts frozen to the LT_{50} of -3 to $-5\,°C$, this form of injury occurs at a very low frequency in acclimated protoplasts and does not account for the injury incurred at the LT_{50} of -25 to $-30\,°C$ (STEPONKUS *et al.* 1982). Cryomicroscopy demonstrates that major differences in the behavior of the plasma membrane during freeze-induced osmotic contraction are associated with the decreased sensitivity of acclimated protoplasts to osmotic contraction/expansion (DOWGERT and STEPONKUS 1984). The cytoplasmic vesicles observed in non-acclimated protoplasts

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* Correspondence and Reprints: Department of Agronomy, Bradfield Hall, Cornell University, Ithaca, NY 14853-0144, U.S.A.

following extracellular ice formation (and concomitant contraction) are not observed in acclimated protoplasts. Instead, extrusions appear on the surface of the protoplasts during contraction. Upon subsequent expansion, the exocytotic extrusions retract. Lysis does not occur and the acclimated protoplasts regain their original size. In the present study, we report on the behavior and ultrastructure of the plasma membrane of isolated acclimated protoplasts subjected to osmotic contraction and expansion.

2. Materials and Methods

2.1. Protoplast Isolation and Osmotic Manipulation

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-hour photoperiod). Acclimation was achieved by exposing 1 week old plants to 13 °C (day) and 7 °C (night) temperatures (11.5-hour photoperiod) for one week, and then to a 2 °C controlled environment (10-hour photoperiod) for 4 weeks. Protoplasts were enzymatically isolated from the leaves (see WIEST and STEPONKUS 1978) and resuspended in isotonic sorbitol (1.0 osm).

2.2. Osmotic Manipulation

Contraction and expansion of acclimated protoplasts was accomplished by manipulating the osmolality of the suspending medium. Protoplasts were contracted in hypertonic sorbitol (2.54 osm) and then partially re-expanded by dilution of the suspending medium (1.28 osm). These osmolalities resulted in an average (for a population of protoplasts) volumetric reduction during contraction and increase during expansion of 11.4 and 7.7 pl, respectively, with an initial isotonic volume of 17.5 pl.

2.3. Electron Microscopy

For each osmotic manipulation, protoplasts were fixed in glutaraldehyde followed by postfixation in OsO_4, and then prepared for either transmission or scanning electron microscopy. For details of fixation and EM techniques, see GORDON-KAMM and STEPONKUS (1984).

The diameter of the extrusions was measured using an eyepiece micrometer on the micrographs. Likewise, the width and length of the tethers was measured on extrusions around the periphery of the protoplasts.

2.4. Freeze-Fracture

Following osmotic contraction in hypertonic sorbitol, samples were quenched in liquid propane, fractured on a Balzers-360, platinum coated and observed under TEM. For further details concerning preparation and particle counting, see GORDON-KAMM and STEPONKUS (1984).

3. Results

The morphology of acclimated protoplasts changes dramatically following osmotic contraction. In isotonic sorbitol (1.0 osm) the surface of acclimated protoplasts appeared smooth (Figs. 1 *a* and *b*), although very slight bumps were often seen on the surface. However, when exposed to hypertonic sorbitol solutions (2.54 osm), extrusions were observed on the surface of the protoplasts (Figs. 1 *c* and *d*). Exocytotic extrusions were observed in nearly all acclimated protoplasts exposed to hypertonic solutions, although the extent to which the extrusions occurred varied between protoplasts (Fig. 1 *c*). The formation of extrusions was readily reversible during subsequent osmotic expansion. When contracted protoplasts were subsequently equilibrated in 1.20 osm sorbitol, the extrusions were reabsorbed into the plane of the plasma membrane (Figs. 1 *e* and *f*).

At higher magnifications (Figs. 2 *a–c*), the extrusions were more clearly visualized as tethered knobs or polyps on the surface of the protoplast. The diameter of the knobs was $0.48 \pm 0.13\,\mu m$ ($n = 574$) while the length and diameter of the tethers was $0.3 \pm 0.24\,\mu m$ and $0.32 \pm 0.04\,\mu m$, respectively ($n = 522$).

In thin sections (Fig. 3), the interior of the extrusions appeared densely osmiophilic, suggesting a high lipid content. The extrusions were bounded by the plasma membrane and the neck of the extrusions contained cytoplasm (Figs. 3 *d* and *e*). Thus, they were not merely globules adhering to the surface of the protoplast. The amorphous, aparticulate texture seen in cross-fractured extrusions also indicates that the material within the extrusions was lipid (Fig. 4). Concentric circles were often seen around these aparticulate regions, suggesting

Fig. 1. Scanning electron micrographs of protoplasts isolated from cold acclimated *Secale cereale*. Scale markers for Figs. 1 *a*, *c*, and *e* represent 10 μm, while scale markers for Figs. 1 *b*, *d*, and *f* represent 5 μm. *a* and *b* Acclimated protoplasts suspended in isotonic sorbitol (1.0 osm). The surface of the protoplasts is generally smooth (Fig. 1 *a*) although at higher magnifications, slight bumps protrude from the surface (Fig. 1 *b*). *c* and *d* When acclimated protoplasts are transferred to hypertonic (2.54 osm) sorbitol, numerous exocytotic extrusions appear on the surface of the protoplasts (*E*). This is a common response to dehydrative stress in acclimated protoplasts (Fig. 1 *c*) and is not restricted to selected individuals. At higher magnifications (Fig. 1 *d*) the extrusions appear as tethered spheres (arrow). *e* and *f* After exposure to hypertonic sorbitol (2.54 osm) and subsequent transfer into a more hypotonic solution (1.28 osm) which results in partial re-expansion of the protoplasts, the exocytotic extrusions have partially retracted back into the protoplast

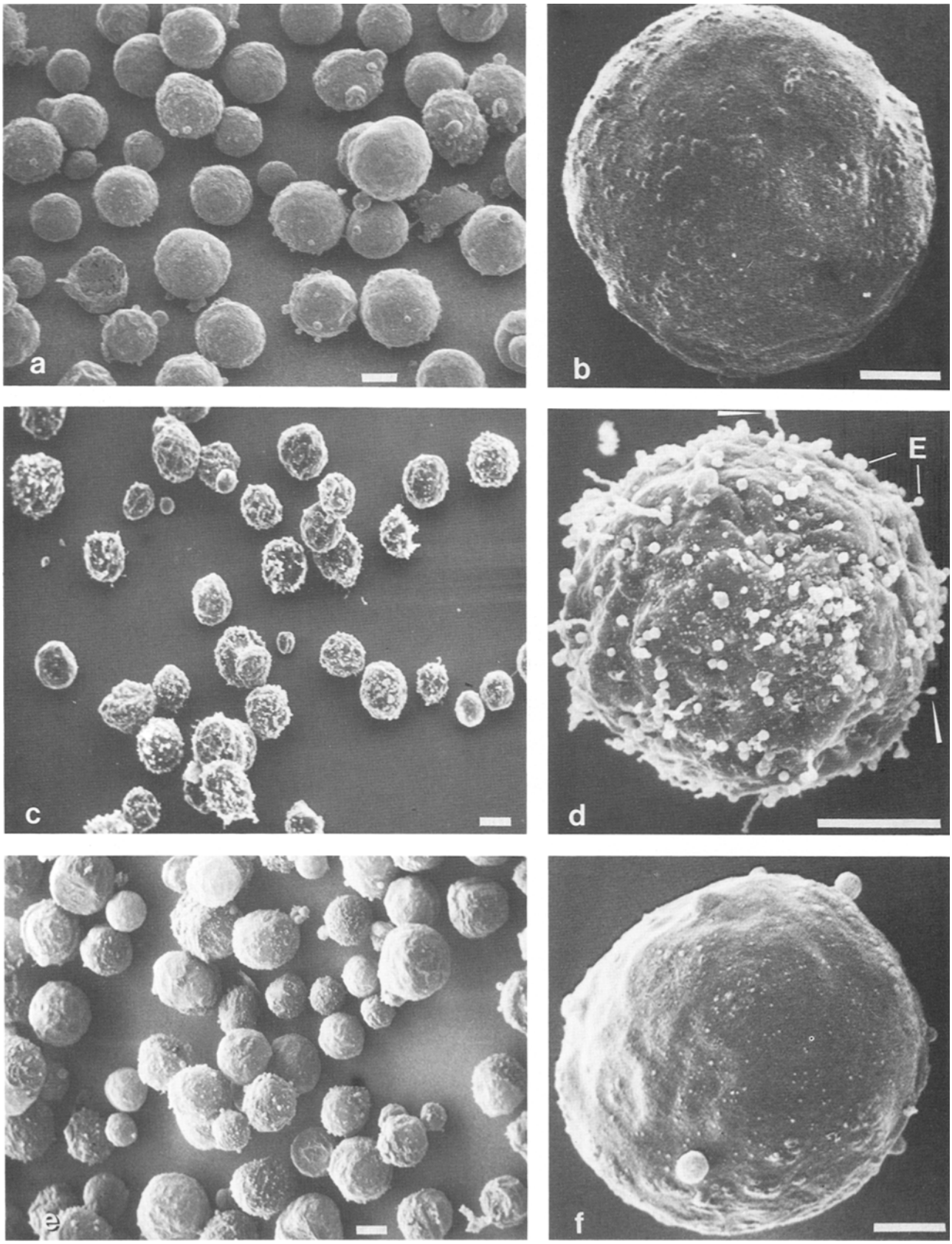
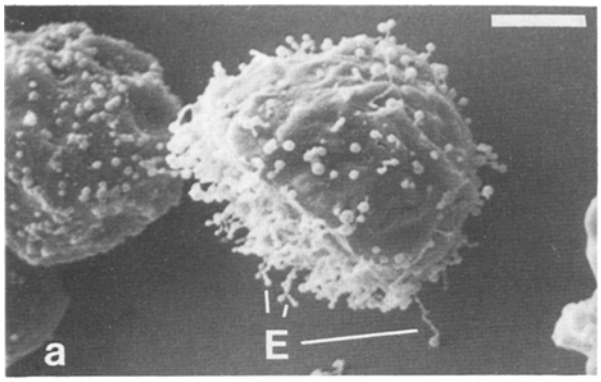
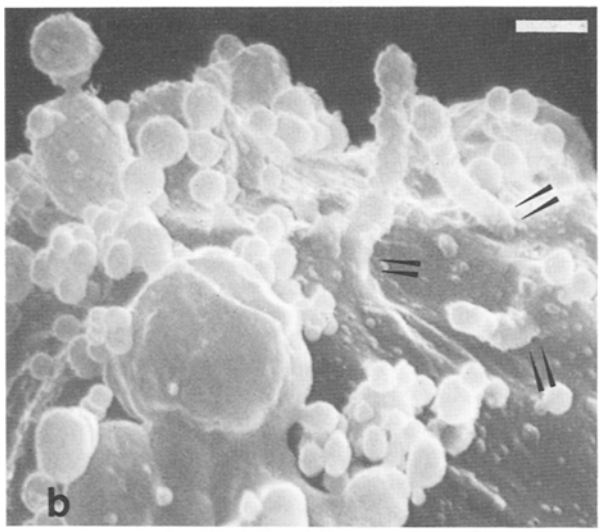
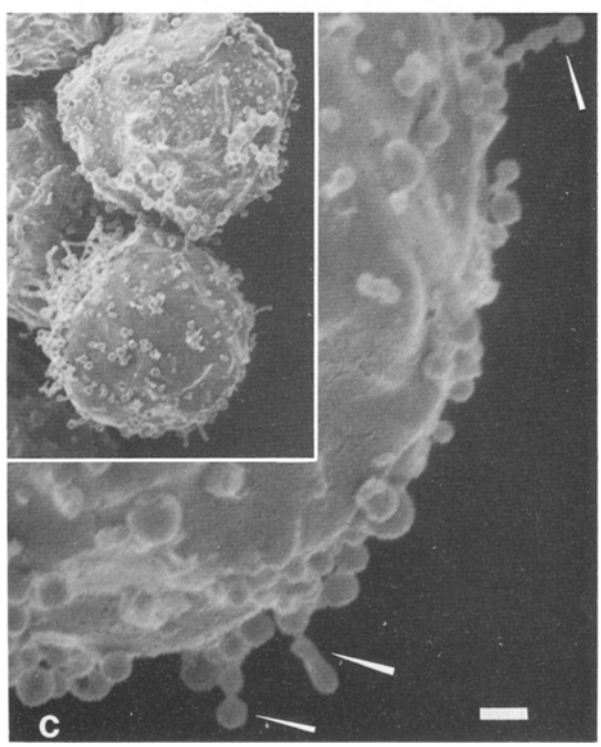


Fig. 1







that these bodies might be multilamellar or associated with lamellae lying under the plasma membrane. However, this feature was not preserved by the chemical fixation used for the TEM portion of this study. These underlying lipid bodies were responsible for the "knobbed" appearance of the extrusions (as seen in SEM).

Changes in freeze-fracture morphology following hypertonic contraction of acclimated protoplasts suggest that the lipid bodies occur as a result of deletion of lipid material from the plasma membrane. Following exposure to hypertonic conditions, the particle density on the plasma membrane protoplasmic face (PFp) increased significantly (Fig. 5, Tab.1; Students T-test, $P = 0.01$). In addition, a substantial increase in the percentage of the PFp surface area in paracrystalline arrays was also observed (Tab. 1, see Figs. 5 *b* and 6 for examples). In isotonic medium, no paracrystalline arrays were seen on the PFp of acclimated protoplasts. The particle density on the exoplasmic face (EFp) remained constant following hypertonic exposure (Fig. 5, Tab. 1). Particle size did not change significantly ($P = 0.05$) on either face following hypertonic exposure. The density and size of particles on the PF or EF of the plasma membrane extending over the extrusions did not appear to differ from the regions of plasma membrane that did not bound the extrusions (Tab. 1, Fig. 7).

4. Discussion

Of the sequential stresses encountered by plant cells during a freeze-thaw cycle, volumetric contraction/expansion is one of the foremost that must be overcome (STEPONKUS 1984). Cryomicroscopy and osmotic manipulation studies have shown that protoplasts isolated from non-acclimated rye leaves are unable to tolerate substantial contraction and reexpansion, and that irreversible volumetric contraction/expansion is associated with the behavior of the plasma membrane during freeze-induced osmotic contraction of non-acclimated protoplasts (DOWGERT and

Figs. 2 *a–c*. Higher magnification scanning electron micrographs of acclimated protoplasts suspended in hypertonic sorbitol (2.54 osm). Numerous exocytotic extrusions appear on the surface of the protoplasts, often extending on tethers (*E*). Some tethered extrusions appear to contain tandem spheres (arrows). In Fig. 2 *b* the continuity of the tethers with the plasma membrane (double arrow) can be seen. Scale markers for (2 *a–c*) represent 5,1 and 1 μm, respectively

STEPONKUS 1984). Following hypertonic contraction of non-acclimated protoplasts, electron microscopy reveals that the plasma membrane is smooth and numerous vesicles are present in the cytoplasm (GORDON-KAMM and STEPONKUS 1984). Fluorescence labeling of the plasma membrane prior to osmotic contraction shows that these vesicles are derived from the plasma membrane. The internalization of fluorescence indicates that endocytotic vesiculation occurs when non-acclimated protoplasts are hypertonically contracted. Freeze-fracture morphology of the plasma membrane is consistent with contraction-induced endocytosis. Even though a substantial surface area reduction occurs during contraction, the intramembrane particle (IMP) density remains constant. Thus, membrane lipid together with the integral proteins are being pinched off into vesicles (*i.e.*, a unit-membrane deletion occurs). In contrast to the vesiculation observed in non-acclimated protoplasts, no vesicles were seen when cold acclimated protoplasts were osmotically contracted. Instead. exocytotic extrusions of the plasma membrane were observed with densely osmiophilic regions inside.

The osmiophilic nature of these regions in thin section (observed without uranyl acetate or lead citrate staining; data not shown) and the characteristic amorphous, aparticulate texture of these areas when cross-fractured indicate a high lipid content. These inclusions of closely packed lipid material appeared to be readily exchanged with the plasma membrane during contraction and subsequent expansion. This proposal is based on our observations that 1. these osmiophilic regions are usually juxtaposed to the plasma membrane in acclimated protoplasts and increase following hypertonic contraction and 2. after the protoplasts have regained sphericity upon returning to isotonic conditions, few osmiophilic regions are observed.

Freeze-fracture evidence suggests that the plasma membrane is the source of this lipid material. For a given species and cell type, the fracture faces for the various cellular membranes have characteristic particle densities (BRANTON 1971, VERKLEIJ and VERVERGAERT 1978, MARTY 1982). It is generally accepted that intramembrane particles (IMP) represent integral membrane proteins (VERKEIJ and VERVEGAERT 1978, LANG and BROWN 1981). Thus the particle density represents the ratio of protein particles per membrane surface area. Because the matrix of the membrane is composed of a lipid bilayer, this density can be thought of as reflecting the relative amount of protein and lipid in the membrane. Changes in the IMP density for a particular membrane would indicate an altered IMP/lipid-surface-area ratio. Following contraction of acclimated protoplasts, a rise in PFp particle density was reflected in both an increase in the particle density in non-crystalline regions and in the incidence of paracrystalline arrays. This could be explained through either 1. an increase in membrane protein during hypertonic contraction, 2. vertical displacement of membrane proteins effectively pushing proteins into the fracture plane (as proposed by ARMOND and STAEHELIN 1979, NIEDERMEYER *et al.* 1976), or 3. a preferential loss of lipid material. It is unwarranted to assume that protein would be synthesized and/or inserted into the plasma membrane in response to a relatively rapid hypertonic contraction (*i.e.*, a few minutes). The vertical displacement of integral proteins is unlikely because it is thermodynamically unfavorable (HOUSLEY and STANLEY 1982). Because the increase in IMP density accompanies the loss of plasma membrane surface area during contraction, it appears that the surface area loss is due to the preferential subduction of lipid material. Because this coincides with an increase in lipid bodies next to the plasma membrane, we propose that lipid is subducted from the membrane into these lipid regions. In contrast to the PFp, the particle density on the EFp does not change following hypertonic contraction. This is an excellent example of the autonomy of the two membrane faces, and is consistent with the growing consensus that the two faces are compositionally and functionally distinct (HOUSLEY and STANLEY 1982). However, although the subduction of lipid material into lipid bodies is reflected in an increased particle density on the PFp, at present it is not clear how or even if an equivalent amount of EFp material is reversible exchanged. This aspect of the deletion process still requires further investigation.

The appearance of lipid bodies or lipid regions following freeze-induced dehydration is not a phenomenon unique to isolated protoplasts. Lipid bodies are observed next to the plasma membrane of cells from wheat seedlings following both the addition of cryoprotectants and extracellular freezing (GAZEAU 1980). Lipid inclusions are also observed in thin section following extracellular freezing of tall fescue leaf tissue (PEARCE 1982). In cells from tall fescue leaf tissue that survived the freezing (dehydrative) protocol, osmiophilic regions were apparent following contraction due to extracellular freezing. In addition, PEARCE finds that in the cells that survive, the osmiophilic regions are no

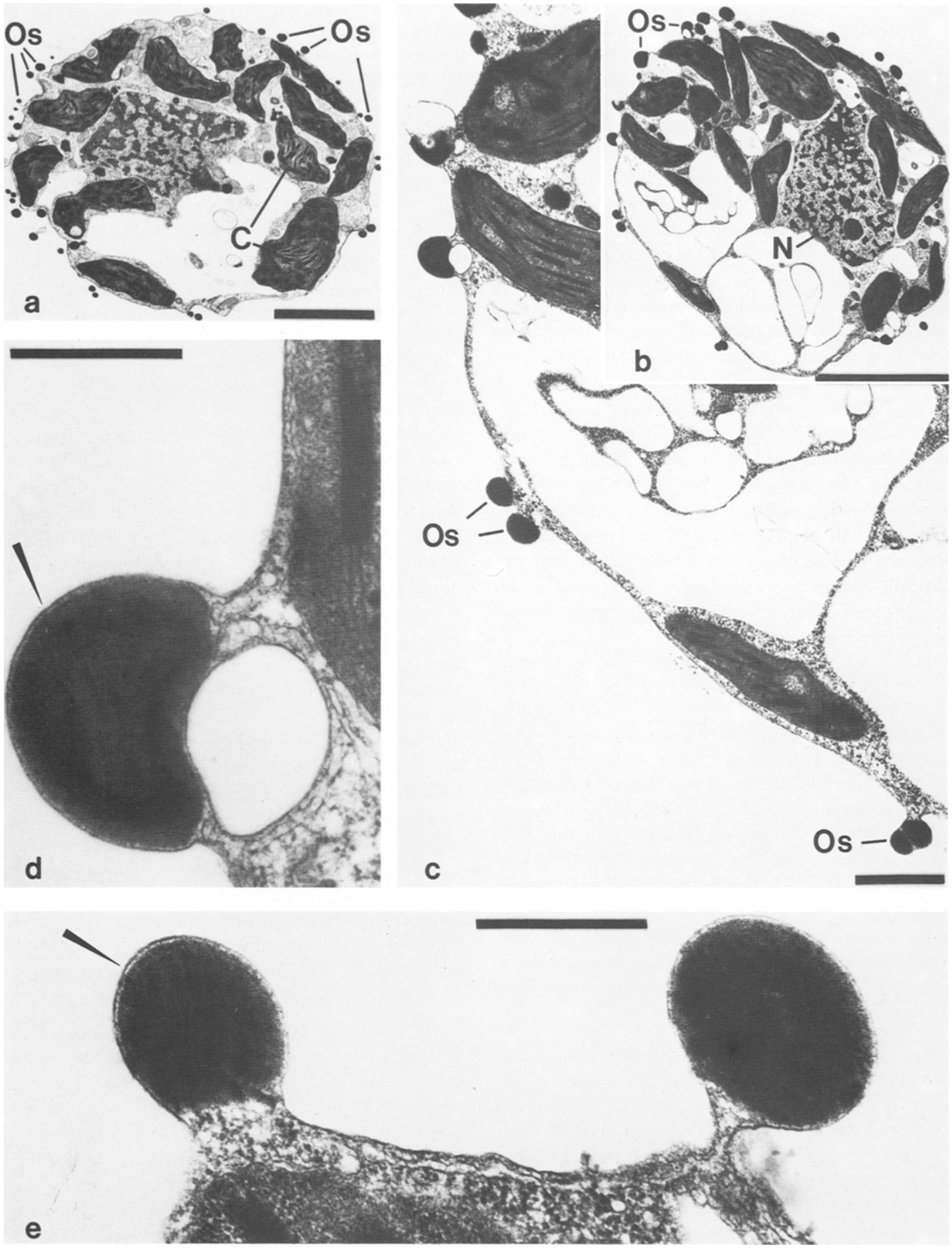


Fig. 3. Transmission electron micrographs of acclimated protoplasts suspended in hypertonic (2.54 osm) sorbitol. *a* and *b* At low magnifications, numerous osmiophilic regions (*Os*) appear around the periphery of the protoplasts. *C* chloroplast, *N* nucleus. Scale marker represent 5 μm. *c–e* Higher magnifications (Figs. 3 *c* and *d*) of a portion of Fig. 3 *b*, showing more details of the osmiophilic regions. As seen in both Figs. 3 *d* and *e*, these osmiophilic regions are bounded by the plasma membrane (arrows) and the neck of the extrusion contains cytoplasm. The osmiophilic regions seen in thin section represent the material inside the exocytotic extrusions seen under the scanning electron microscope. The osmiophilic nature suggests that this material is lipid. Scale markers represent 1 μm in Fig. 3 *c*, while in Figs. 3 *d* and *e* the scale markers represent 0.5 μm

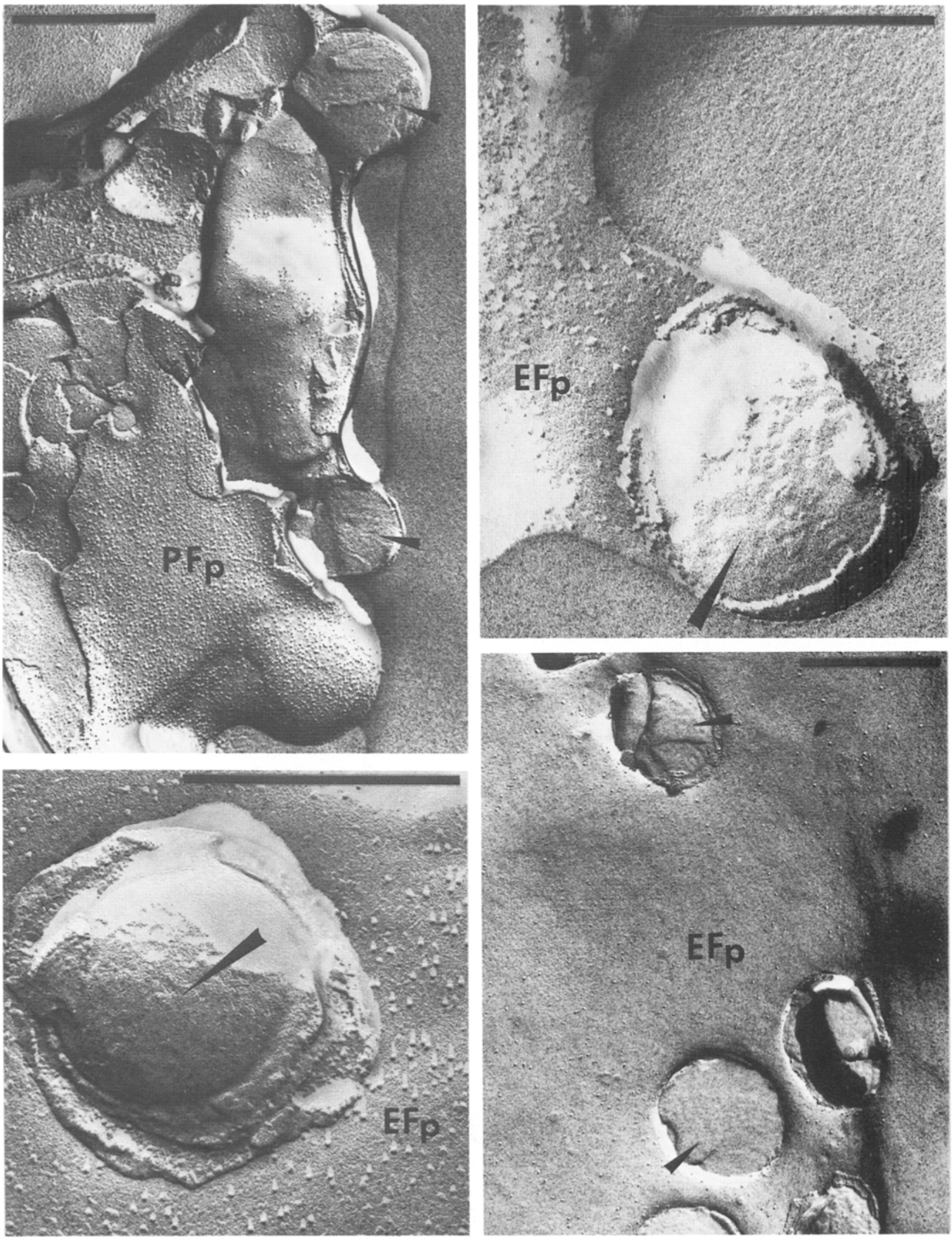


Fig. 4. When the knobs or extrusions are cross-fractured, the interior appears aparticulate (arrows) suggesting that this material may be lipid. PFp protoplasmic face of plasma membrane. EFp exoplasmic face. Scale markers represent 0.5 μm

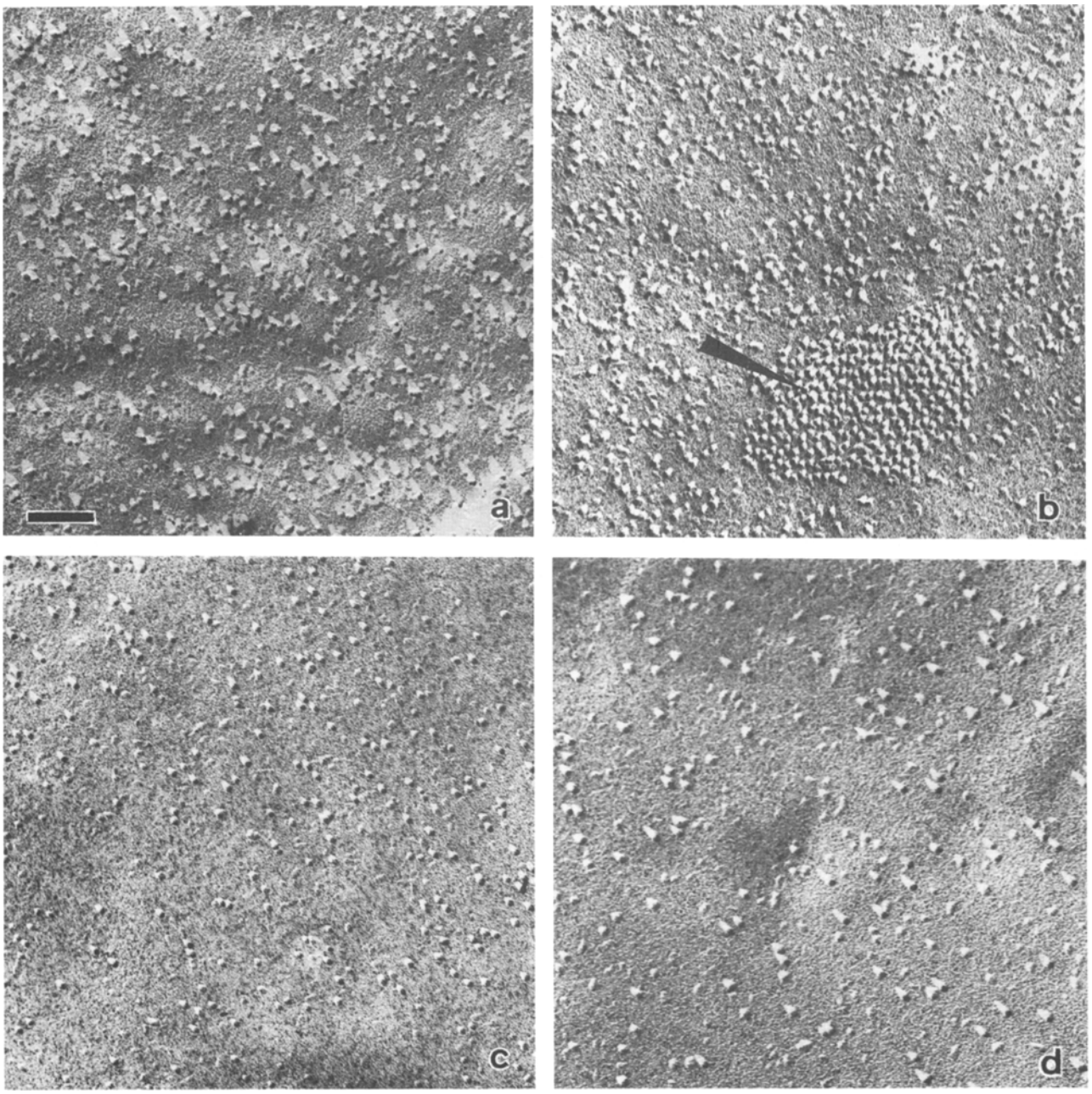


Fig. 5. When acclimated protoplasts were taken from isotonic (*a*) to hypertonic (*b*) conditions, the particle density on the PFp increased. The increase in particles was reflected in the appearance of paracrystalline arrays (arrow) which covered 7.1% of the PFp following contraction and in an increase in particle density in the non-crystalline areas. No change was observed in EFp particle density when protoplasts were taken from isotonic (*c*) to hypertonic sorbitol (*d*). Scale marker represents 100 nm

longer present following warming (*i.e.*, re-expansion). These observations suggest that the preferential subduction of lipid from the plasma membrane into lipid bodies may also occur in wheat and tall fescue, resulting in freeze-induced dehydration being reversible. While the results of both GAZEAU (1980) and PEARCE (1982) are consistent with our observations in acclimated protoplasts, other studies of acclimated cells are not.

Exocytotic extrusions are not observed by SINGH (1979, 1982) in acclimated rye cells following either hypertonic contraction or extracellular freezing. Instead, he reports that when acclimated cells are contracted, infolding and invagination of the plasma membrane occurs

Table 1. *Analysis of intramembrane particles on plasma membrane faces of acclimated protoplasts exposed to either isotonic (1.0 osm) or hypertonic (2.54 osm) conditions*

	Particle density (particles/μm²)		*Mean particle diameter* (nm)		% paracrystalline array (PFp)
	PFp	EFp	PFp	EFp	
Isotonic	735 ± 84 (16)	339 ± 119 (19)	11.1 ± 2.1	11.3 ± 1.8	0
Hypertonic	1,191 ± 276 (18)	337 ± 80 (21)	11.4 ± 2.2	11.8 ± 2.5	7.1 ± 2.2
Hypotonic (on extrusion) [1]	1,251 ± 244 (22)	358 ± 77 (17)	11.3 ± 1.7	11.5 ± 2.1	7.0 ± 5.0

[1] Measurements taken from membrane fracture faces on the extrusions, particle densities are corrected for curvature.

which conserves surface area. Thus, the irreversible plasma membrane "roll-up" and fusion that he observes in non-acclimated cells is precluded (SINGH 1982). Although the conservation of plasma membrane surface area reported by SINGH is consistent with our observations in acclimated protoplasts, the manner in which the surface area is conserved differs. Furthermore, SINGH does not address the reversible loss of membrane material. His mechanistic interpretations are based on ultrastructural changes observed at —10 °C and —20 °C (or the equivalent in osmotic manipulations) for non-acclimated and acclimated cells, respectively. At these temperatures he relates injury to loss of plasma membrane surface area. However, almost all (~ 95%) of the surface area reduction that occurs in rye cells has already occurred by —4 °C for non-acclimated and —8 °C for acclimated cells. Therefore, the ultrastructural changes observed by SINGH are more likely to be manifestations of stresses (*i.e.*, the loss of osmotic responsiveness, STEPONKUS *et al.* 1983, STEPONKUS 1984) that occur under more severe cellular dehydration.

WILLIAMS and HOPE (1981) observed refractile droplets in plasmolyzed cells of one acclimated wheat cultivar (Kharkov). The refractile droplets are interpreted to be vesicles and are postulated to act as lipid reservoirs which are readily reincorporated into the plasma membrane during expansion (WILLIAMS *et al.* 1981, MERYMAN and WILLIAMS 1982). We also see this behavior (*i.e.*, endocytotic vesiculation) during contraction—but only in *non-acclimated* protoplasts (GORDON-KAMM and STEPONKUS 1984). Furthermore, in non-acclimated protoplasts, the cytoplasmic vesicles remain in the cytoplasm following expansion and are not reincorporated into the plasma membrane. From these discrepancies one must question the hardiness of the Kharkov tissue in which the vesicles were seen.

Although cold acclimation dramatically alters the behavior of the plasma membrane during volumetric contraction of isolated protoplasts, how this occurs is not yet known. While it is likely that changes in membrane composition are responsible, compositional analysis of isolated plasma membrane fractions are required before the significance of any such changes can be accurately assessed. Nonetheless, it is well established that membrane changes occur, as evidenced by the striking morphological differences observed in a variety of plants following cold acclimation. Freeze-fracture results for isolated rye protoplasts indicate that a substantial decrease in plasma membrane particle density occurs on both the PF and EF during acclimation (*cf.*, Tab. 1 of this report with Tab. 1, GORDON-KAMM and STEPONKUS 1984). Decreases in particle densities following cold acclimation have been observed in many other plant cell types. Since this was first observed on the EF of spinach chloroplast thylakoids (GARBER and STEPONKUS 1976), decreases in IMP density following cold acclimation have been observed on the plasma membrane EF of Jerusalem artichoke tissue culture cells (SUGAWARA and SAKAI 1978) and on both the PFp and EFp of *Salix fragilis* shoot cambial cells (PARISH 1974) potato tissue culture cells (TOIVIO-KINNUCAN *et al.* 1981) and in meristematic cells of *Tilia europea* (CRAGG and WILLISON 1980). Another common morphological change that occurs is the proliferation of cellular membrane material during acclimation. This membrane augmentation (as termed by SIMINOVITCH *et al.* 1975) has been observed in ultrastructural studies on various species (POMEROY and SIMINOVITCH 1971,

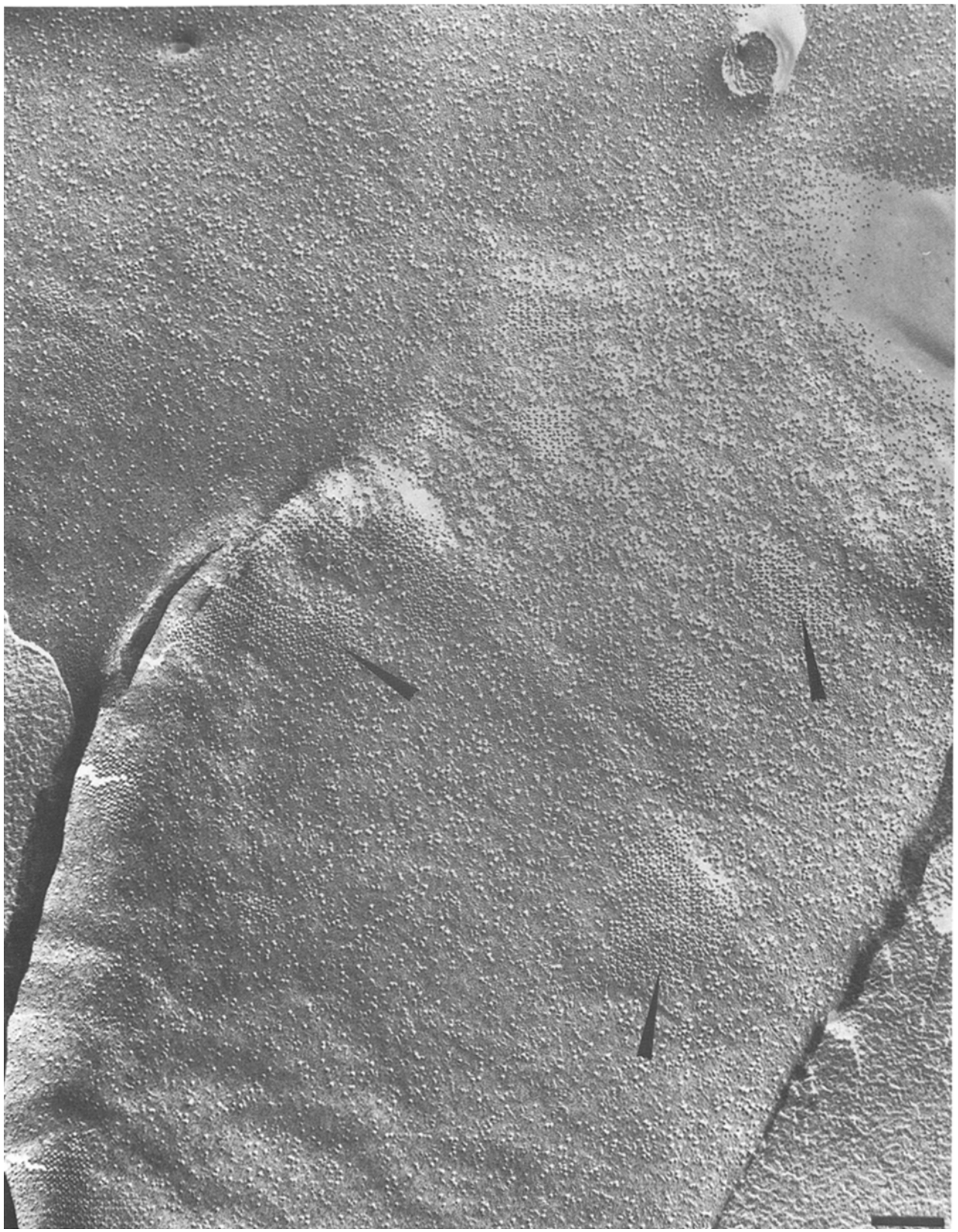


Fig. 6. The protoplasmic face (PFp) of an acclimated protoplast in hypertonic sorbitol (2.54 osm). Following hypertonic exposure, numerous paracrystalline arrays are observed (arrows). These arrays of intramembrane particles vary in size, but are typically under 0.1 μm^2 in surface area. Scale marker represents 0.2 μm

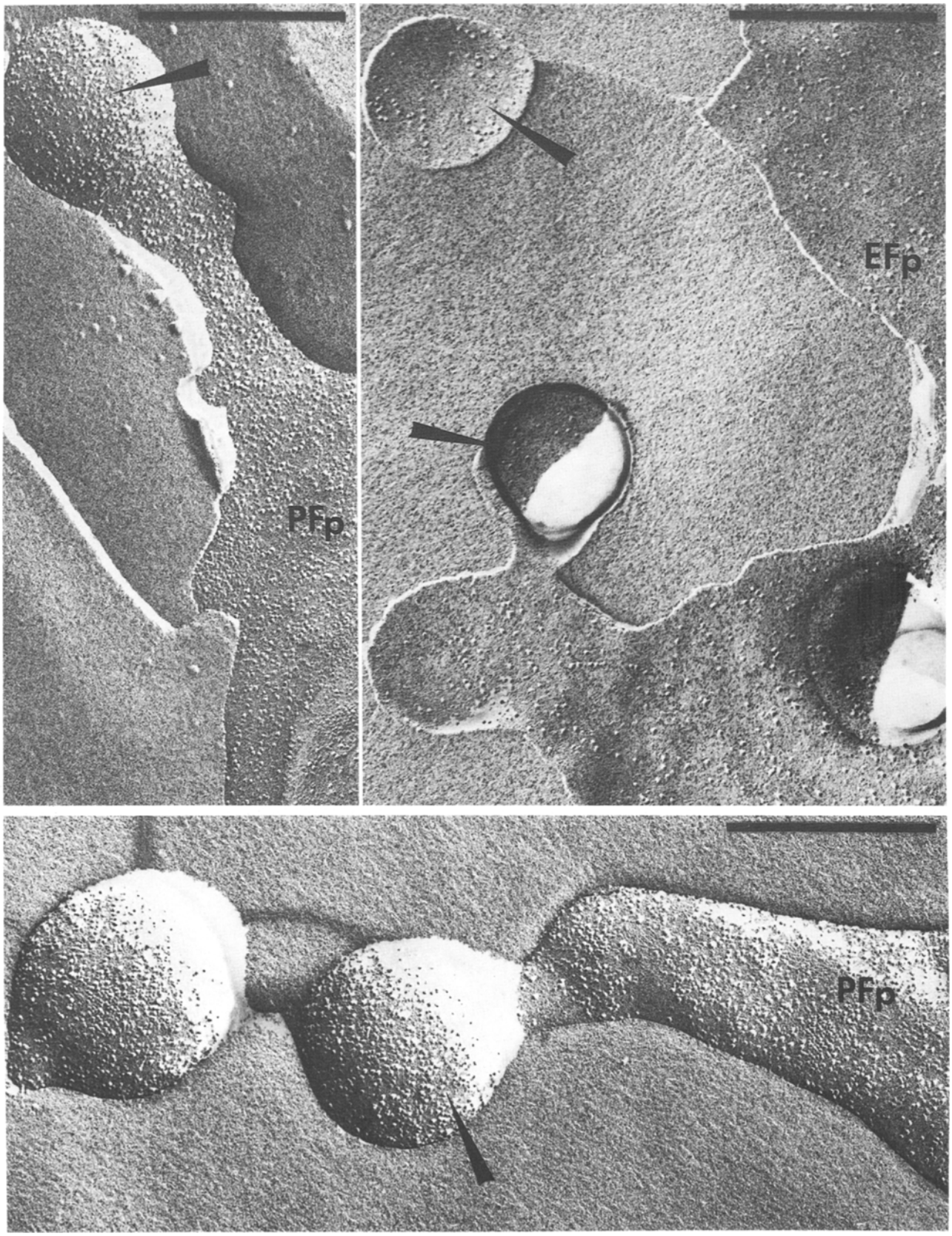


Fig. 7. The plasma membrane extending over the knobs does not appear to differ from the bulk plasma membrane. The particle density and distribution on the knobs (arrows) is similar to that of the rest of the plasma membrane for both the PF and EF. Scale markers represent 0.5 μm

ROBARDS and KIDWEI 1969, NIKI and SAKAI 1981, PIHAKASKI 1983). Likewise, changes in bulk lipid composition have been associated with the acclimation process (SIMINOVITCH *et al.* 1975, SINGH *et al.* 1975, HATANO *et al.* 1982). Although many changes are observed, the significance of either the morphological or biochemical alterations associated with cold acclimation have remained elusive.

Once the behavior of the plasma membrane during freezing and thawing is well understood, then these changes in plasma membrane morphology and composition which occur during cold acclimation can be put into perspective. In addressing one form of freeze-thaw injury (*i.e.*, expansion-induced lysis) by using osmotic simulation, we have characterized the behavior of the plasma membrane during hypertonic contraction of protoplasts. The distinct membrane responses observed during contraction of non-acclimated and acclimated protoplasts appear to represent functional differences in the plasma membrane, and suggest that changes in the intrinsic properties of the plasma membrane *per se* are likely to be responsible for the increased tolerance of acclimated protoplasts to osmotic contraction/expansion.

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