

Selective osmotic effect on diffusion of plasma membrane lipids in maize protoplasts

(sterols/phospholipids/domains/fluorescence photobleaching recovery/trehalose)

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ABSTRACT Osmotic levels in the range typically used during plant protoplast isolation and incubation were investigated with regard to effects on the lateral diffusion of lipid probes in the plasma membrane. The lateral diffusion coefficient of a fluorescent sterol probe in the plasma membrane of maize (*Zea mays* L.) root protoplasts in a medium containing 0.45 M mannitol was 4 times faster than when the medium contained 0.9 M mannitol. The lateral diffusion coefficient of a fluorescent phospholipid probe, however, did not change over this range of mannitol concentrations. Similar diffusion characteristics were observed when the medium contained trehalose instead of mannitol. Slower lateral diffusion of the sterol probe at higher osmolality was also observed when KCl/CaCl₂-based osmotic media were used with protoplasts isolated by a mechanical, rather than by an enzymic, method. Extraction and quantitation of total lipids from protoplasts showed that both the phospholipid and sterol contents per protoplast decreased with increasing osmolality, while the sterol/phospholipid ratio increased. These results demonstrate that osmotic stress induces selective changes in both the composition and biophysical properties of plant membranes.

Evidence for the existence of lipid domains in plant and other biological membranes has come from a variety of experiments (1). One source of such evidence has been studies of lipid dynamics involving long-range motions—i.e., motions over distances that are large compared to molecular dimensions. Use of fluorescence techniques to measure such lipid motions in the plasma membrane of plant protoplasts was first reported by Metcalf *et al.* (2). In that work, fluorescence redistribution after photobleaching was used to measure the lateral diffusion of various fluorescent lipid probes in protoplasts from suspension-cultured soybean cells. Two diffusion coefficients, one in the $2\text{--}5 \times 10^{-10}$ cm²/sec range and another in the $1\text{--}6 \times 10^{-9}$ cm²/sec range, were simultaneously observed under certain conditions of temperature and probe concentration. The mobile fraction, summed for the two diffusing species, was in the 0.56–0.76 range—i.e., a significant portion of the lipid probe moved so slowly as to appear immobile on the time scale of the experiment. These heterogeneous diffusion parameters were interpreted as evidence for the existence of lipid domains in the plasma membrane of soybean protoplasts (1).

Fluorescence photobleaching recovery experiments carried out in our laboratory (3, 4) have yielded results that are comparable to those reported by Metcalf *et al.* (2). Fluorescent lipid probes (5) were used that enabled the consistent resolution of two diffusion coefficients over a range of conditions. The lateral diffusion coefficient of a fluorescent sterol probe in the plasma membrane of root cortical protoplasts from two different maize lines was $3\text{--}7 \times 10^{-10}$ cm²/sec

for temperatures in the 3.5°C–37°C range (3). Over the same temperature range, the diffusion coefficient of a fluorescent phospholipid probe was $2\text{--}4 \times 10^{-9}$ cm²/sec. The mobile fractions measured for both probes were 0.4–0.7. Similar results were observed in a study of lateral diffusion of plasma membrane components in protoplasts from suspension-cultured rose cells (4).

Although heterogeneous diffusion characteristics seem to indicate the existence of lipid domains in the plasma membranes of soybean, maize, and rose protoplasts, uncertainty remains with regard to whether or not these lipid properties arise during removal of the cell wall and are thus peculiar to protoplasts. Evidence from various experiments has shown that the plasma membrane is altered or damaged by preparations of cell wall-degrading enzymes (ref. 3 and references therein). Other reports (6–13) have shown that osmotic stress alters the lipid composition of plant cells. While this problem might be best addressed by comparing lateral diffusion characteristics in the membranes of protoplasts and whole cells, the cell wall interferes with selective fluorescence labeling of the plasma membrane and thwarts meaningful measurements on whole cells. In the present work, the effect of osmotic stress on protoplasts is addressed by measuring diffusion characteristics over a range of osmotic levels. The effect of cell wall-degrading enzymes on protoplasts is addressed by measuring diffusion characteristics for both enzymically and mechanically isolated protoplasts.

MATERIALS AND METHODS

Materials. The fluorescent lipid probes dilithium 4-amino-*N*-{[β -(carbo(5-cholesten-3 β -yl)oxy)hydrazinocarbonyl]amino}-1,8-naphthalimide-3,6-disulfonate (LY-Chol) and dilithium 4-amino-*N*-{3-[β -(dilauroyl-*sn*-glycero-3-phosphoethanolamino)ethylsulfonyl]phenyl}-1,8-naphthalimide-3,6-disulfonate (LY-DC_{12:0}PE) were synthesized as described by Nothnagel (5). Sources of maize seeds, cell-wall degrading enzymes, and other materials were as described by Dugas *et al.* (3).

Plant Material and Protoplast Isolation. The methods for germination and growth of maize seedlings and for isolation of the primary root cortex have been described (3, 14). Both mechanical and enzymic methods were used for the isolation of protoplasts from the root cortex.

The mechanical method for isolation of cortical protoplasts will be described in detail elsewhere. Briefly, the root cor-

Abbreviations: LY-Chol, dilithium 4-amino-*N*-{[β -(carbo(5-cholesten-3 β -yl)oxy)hydrazinocarbonyl]amino}-1,8-naphthalimide-3,6-disulfonate; LY-DC_{12:0}PE, dilithium 4-amino-*N*-{3-[β -(dilauroyl-*sn*-glycero-3-phosphoethanolamino)ethylsulfonyl]phenyl}-1,8-naphthalimide-3,6-disulfonate.

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tices were plasmolyzed for 30 min in a medium of 0.25 M KCl/0.03 M CaCl₂/0.01 M Mes-KOH/0.5 mM dithiothreitol/0.05% (wt/vol) bovine serum albumin, pH 6.0. In some experiments, higher KCl and CaCl₂ concentrations (0.40 and 0.05 M, respectively) were used. At the end of the 30-min plasmolysis, the cortices were fed with a stream of medium into a cutter composed of a stainless steel block that housed a brass shaft, which was turned at \approx 1900 rpm by an electric drill. On the end of the shaft was mounted a double-edged razor blade that sliced the cortices into cross-sections as thin as 100 μ m. Protoplasts escaping from the cut ends of cells were separated from the debris by filtration and centrifugation steps similar to those described by Gronwald and Leonard (14) for enzymic isolation of protoplasts. The KCl/CaCl₂ medium described above, minus dithiothreitol and bovine serum albumin, was used in these purification steps and in the final suspensions of protoplasts.

The method for enzymic isolation of cortical protoplasts with Cellulysin and Pectolyase Y-23 was a modification of the method of Gronwald and Leonard (14). Instead of using a razor blade to manually cut the cortices into 2-mm-thick cross-sections (14), the electrically driven cutter described above was used to cut the sections. The resulting thinner size of the sections allowed the incubation time in the enzyme mixture to be shortened from 3.5 to 2 hr. Other details of the procedure were as described (14), except 0.45 or 0.90 M mannitol was used in some experiments instead of the usual 0.70 M concentration in the medium. The final suspensions of protoplasts were in medium containing mannitol at one of these three concentrations plus 2 mM Tris Mes (pH 6.5). In some experiments, trehalose was substituted for mannitol in all steps, including cell wall digestion.

Lateral Diffusion Measurements. Protoplasts were routinely labeled with either 0.1 mg (115 μ M) of LY-Chol per ml or 0.3 mg (265 μ M) of LY-DC_{12:0}PE per ml for 20 min at 25°C and then washed as described by Dugas *et al.* (3). In preliminary experiments, labeling of mechanically isolated protoplasts was done in KCl/CaCl₂-based medium and resulted in relatively low fluorescence levels. Greater fluorescence intensities resulted when the protoplasts were transferred into mannitol-based medium of equivalent osmolality before labeling and were then transferred back into the KCl/CaCl₂-based medium after washing to remove excess label. Since the two labeling procedures yielded similar diffusion parameters in preliminary experiments, the temporary transfer into mannitol-based medium was routinely used when labeling mechanically isolated protoplasts.

Fluorescence photobleaching recovery experiments were performed as described (3–5), except for replacement of the argon-ion laser by a helium-cadmium laser operating at 441.6 nm in the present work. Appropriate bandpass filters and a dichroic beamsplitter for use with this laser line were designed and supplied by Omega Optical (Brattleboro, VT). When focused on the protoplast surface, the laser beam had a radius of 0.66 μ m at e^{-2} of the peak intensity. All diffusion measurements were performed at 25°C.

Lipid Analyses. The solvent system of Folch *et al.* (15) was used for the extraction of total lipids from protoplasts. Phospholipid content in the extracts was determined through measurement of lipid phosphorus (16). Total sterol content was determined by the assay of Kates (17) with β -sitosterol as the standard. The same assays were used to quantitate phospholipids and sterols in extracts prepared from whole cortices by either the Bligh and Dyer (18) or Folch *et al.* (15) method.

The amount of LY-DC_{12:0}PE incorporated into protoplasts during labeling was quantitated by the method described for quantitating incorporation of the dipalmitoyl analog of this probe (3).

Other Measurements. Numbers of protoplasts per volume of suspension were determined through use of a hemacytometer slide and a microscope. Protoplast diameters were measured with a microscope having an eyepiece with a calibrated reticle. The diameters reported are means \pm SD from measurements on at least 50 protoplasts. Osmolalities of protoplast media were measured with a vapor-pressure osmometer (model 5100C; Wescor, Logan, UT). Micrographs of fluorescence-labeled protoplasts were made through use of violet (435.8 nm) excitation light and Kodak Tri-X film with 9-min development in Kodak D-19 developer.

RESULTS

Use of LY-DC_{12:0}PE to label live cells has not been previously reported, although similar probes having longer fatty acyl chains have been used with both plant and animal cells (3, 5). Protoplasts labeled with LY-DC_{12:0}PE showed a peripheral ring of fluorescence that indicated localization primarily at the plasma membrane (Fig. 1 A and B). The intensity of autofluorescence from the protoplasts (Fig. 1 C and D) was appreciable compared to fluorescence from the LY-DC_{12:0}PE label and was subtracted from the fluorescence photobleaching recovery curves. Fig. 2 shows that incorporation increased nearly linearly with the applied concentration of LY-DC_{12:0}PE. For equivalent applied concentrations, the incorporation of LY-DC_{12:0}PE into protoplasts was less than the incorporation of its dipalmitoyl analog (Fig. 2; cf. figure 2 of ref. 3), yet the fluorescence intensity with LY-DC_{12:0}PE was more than twice as great. Judging from the relative fluorescence increases observed when labeled protoplasts were dissolved in a buffer containing SDS detergent, fluorescence quenching of the dipalmitoyl analog of LY-DC_{12:0}PE in intact protoplasts was much greater than the quenching of either LY-DC_{12:0}PE itself or LY-Chol (results not shown).

Diffusion characteristics of lipid probes in the plasma membrane of enzymically isolated protoplasts from the root cortex of A634 maize seedlings are summarized in Table 1. The lateral diffusion coefficient of LY-Chol in protoplasts

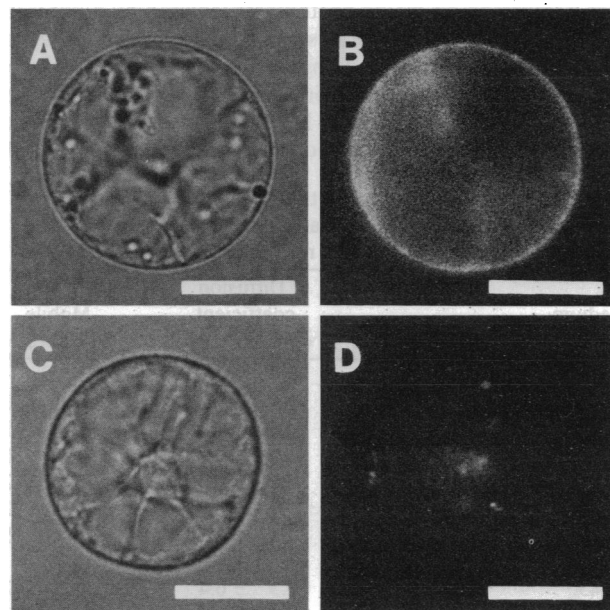


FIG. 1. Localization of fluorescence in enzymically isolated A634 maize protoplasts in medium containing 0.7 M mannitol. (A and B) Bright-field and fluorescence micrograph pair show labeling with LY-DC_{12:0}PE (0.3 mg/ml). (C and D) Autofluorescence. Micrographs of maize protoplasts labeled with LY-Chol have been published elsewhere (5). (Bars = 25 μ m.)

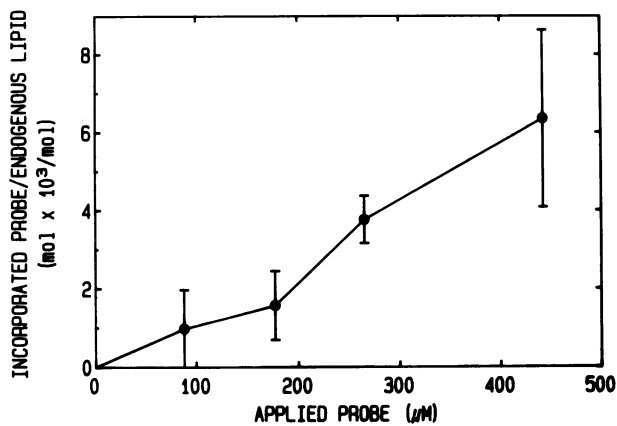


FIG. 2. Levels of incorporation of LY-DC_{12:0}PE into enzymically isolated A634 maize protoplasts in medium containing 0.7 M mannitol. Protoplasts were incubated in various concentrations of LY-DC_{12:0}PE for 20 min at 25°C and were then washed by centrifugation before the amounts of probe and total endogenous phospholipids were measured. Results are expressed as mean \pm SD from three determinations. Data on the incorporation of LY-Chol into maize protoplasts have been published elsewhere (3).

isolated in medium containing 0.45 M mannitol was nearly 4 times faster than in protoplasts isolated in 0.90 M mannitol. The lateral diffusion coefficient of LY-DC_{12:0}PE, in contrast, was nearly constant over this concentration range. Although the mobile fractions of the two probes seemed to vary with mannitol concentration, the changes were generally not statistically significant.

When trehalose was substituted for mannitol in the medium, the diffusion coefficient of LY-Chol, but not LY-DC_{12:0}PE, was again observed to decrease at a higher sugar concentration (Table 1). The osmolality ranges over which the LY-Chol diffusion coefficient changed were roughly similar for trehalose and mannitol.

To further test the generality of the osmotic effect on the diffusion coefficient of LY-Chol, additional measurements were performed on maize protoplasts isolated by a different method. Table 2 summarizes diffusion characteristics of LY-Chol in the plasma membrane of mechanically isolated protoplasts from the root cortex of WF9 \times Mo17 maize seedlings. In this system, the lateral diffusion coefficient of LY-Chol in protoplasts isolated in medium containing 0.25 M

Table 1. Effects of mannitol or trehalose level on lateral diffusion of lipid probes in the plasma membrane of enzymically isolated A634 maize root protoplasts

Medium sugar, M	Probe	Diffusion coefficient, $\text{cm}^2 \times 10^9/\text{sec}$	Mobile fraction
Mannitol			
0.45	LY-Chol	1.3 ± 0.3	0.36 ± 0.03
0.70	LY-Chol	0.79 ± 0.45	0.35 ± 0.07
0.90	LY-Chol	0.34 ± 0.12	0.50 ± 0.16
0.45	LY-DC _{12:0} PE	2.2 ± 0.9	0.52 ± 0.12
0.70	LY-DC _{12:0} PE	1.7 ± 0.4	0.36 ± 0.07
0.90	LY-DC _{12:0} PE	2.3 ± 1.2	0.46 ± 0.05
Trehalose			
0.45	LY-Chol	1.3 ± 0.4	0.30 ± 0.07
0.70	LY-Chol	0.19 ± 0.12	0.36 ± 0.10
0.45	LY-DC _{12:0} PE	2.5 ± 1.7	0.47 ± 0.02
0.70	LY-DC _{12:0} PE	1.8 ± 0.8	0.40 ± 0.13

Results are expressed as means \pm SD. Osmolalities of the 0.45 and 0.70 M trehalose media were 512 and 912 mmol/kg, respectively. Osmolalities of the mannitol medium are shown in Table 3. Medium contained 2 mM Tris Mes (pH 6.5) in addition to the indicated sugar.

Table 2. Effects of KCl/CaCl₂ level on lateral diffusion of the LY-Chol sterol probe in the plasma membrane of mechanically isolated WF9 \times Mo17 maize root protoplasts

Medium KCl/CaCl ₂ , M/M	Osmolality, mmol/kg	Diffusion coefficient, $\text{cm}^2 \times 10^9/\text{sec}$	Mobile fraction
0.25/0.03	535	2.1 ± 1.0	0.39 ± 0.08
0.40/0.05	847	0.38 ± 0.15	0.45 ± 0.11

Results are expressed as means \pm SD. Medium contained 10 mM Mes-KOH (pH 6.0) in addition to KCl and CaCl₂.

KCl and 0.03 M CaCl₂ was 5 times faster than in protoplasts isolated in 0.40 M KCl and 0.05 M CaCl₂. The mobile fraction did not differ between these concentrations of KCl and CaCl₂.

The mechanically isolated protoplasts were relatively small, with the mean diameter being $16 \pm 5 \mu\text{m}$ for protoplasts isolated in medium containing 0.25 M KCl and 0.03 M CaCl₂ and $15 \pm 5 \mu\text{m}$ for protoplasts isolated in 0.40 M KCl and 0.05 M CaCl₂. Staining with Hoechst 33258 dye showed that only $\approx 50\%$ of these protoplasts contained a nucleus. Thus, the preparation of mechanically isolated protoplasts was a mixture of protoplasts and subprotoplasts. The diffusion measurements reported in Table 2 were performed on the larger protoplasts in the preparation, since these were considered most likely to be whole protoplasts.

The effects of mannitol concentration on several characteristics of the enzymically isolated protoplasts are summarized in Table 3. Protoplast yield per g of cortex was not affected by mannitol concentration in the range of 0.45–0.90 M, although protoplasts isolated in 0.70 M mannitol had a smaller diameter than did protoplasts isolated in 0.45 M mannitol. The phospholipid content per protoplast decreased steadily with increasing mannitol concentration. Although total sterol content per protoplast also decreased with increasing mannitol concentration, the relative decrease was not as great as for the phospholipid content. Thus, the molar ratio of sterols to phospholipids increased from 0.63 at 0.45 M mannitol to 0.79 at 0.90 M mannitol. By comparison, the molar ratio of sterols to phospholipids in whole cortices was 0.47 ± 0.04 .

DISCUSSION

The central observation of the present work is the significant reduction of the lateral diffusion coefficient of a sterol probe in the plasma membrane of protoplasts at elevated osmotic levels (Table 1). Comparison of different protoplast preparations (Tables 1 and 2) shows that the effect is neither limited to a single maize genotype nor caused by exogenous enzyme preparations used to degrade the cell wall. Likewise, the effect is not particularly dependent on the type of solute, since mannitol, trehalose, and KCl/CaCl₂ produce similar diffusion effects (Tables 1 and 2) at roughly similar osmolalities (Tables 1–3). The similarity of results with trehalose and the other solutes has added interest because trehalose has been shown to preserve membrane fluidity under dehydrating conditions (19).

Lateral diffusion of the phospholipid probe, in contrast, is not altered by elevation of the osmotic level (Table 1). The phospholipid probe used in this work, LY-DC_{12:0}PE, has shorter fatty acyl chains and labels the protoplast surface brighter (Fig. 1) than does its dipalmitoyl analog, yet these two phospholipid probes exhibit comparable diffusion characteristics (Table 1 and ref. 3).

Metcalf *et al.* (2) have shown that a slow-diffusing component appears when some lipid probes are incorporated at higher levels into protoplasts. The slow diffusion of LY-Chol observed at elevated osmotic levels in the present work is not

Table 3. Effects of mannitol level on yield, size, and total lipid composition of enzymically isolated A634 maize root protoplasts

Medium mannitol, M	Osmolality, mmol/kg	Protoplast yield, $\times 10^{-5}$ per g of cortex	Protoplast diameter, μm	Phospholipid, fmol per protoplast	Sterol/phospholipid, mol/mol
0.45	498	2.4 ± 0.1	33 ± 11	117 ± 2	0.63 ± 0.07
0.70	798	2.7 ± 0.3	26 ± 9	83 ± 18	0.64 ± 0.04
0.90	1032	2.5 ± 0.5	27 ± 9	54 ± 7	0.79 ± 0.05

Results are expressed as means \pm SD from four determinations. Medium contained 2 mM Tris Mes (pH 6.5) in addition to mannitol.

likely an effect of incorporation level. Application of LY-Chol at 0.1 mg/ml to protoplasts in 0.7 M mannitol results in 2.6 mmol of probe incorporated per mol of endogenous sterol (3), and application of LY-DC_{12:0}PE at 0.3 mg/ml results in 3.8 mmol of probe incorporated per mol of endogenous phospholipid (Fig. 2). Both of these incorporation levels are lower than those needed to observe a slow-diffusing component in the work of Metcalf *et al.* (2). As judged from prebleach fluorescence intensities in fluorescence photobleaching recovery experiments, the incorporation of LY-Chol did not vary over the mannitol range of 0.45–0.90 M. Furthermore, the diffusion characteristics of LY-Chol in protoplasts at 0.90 M mannitol were not significantly altered when the labeling concentration of the probe was lowered from 0.1 to 0.025 mg/ml (results not shown).

Steponkus *et al.* (20) have shown that the plasma membrane can form exocytotic extrusions up to several micrometers long in some types of protoplasts exposed to high osmotic stress. While no such extrusions were detected by fluorescence microscopy of labeled protoplasts in the present work, extrusions smaller than the resolution limit of the microscope could have gone undetected. The potential presence of extrusions is a concern, since fluorescence photobleaching recovery underestimates the diffusion coefficient when undulations such as microvilli are present in the membrane. Theoretical analysis of this effect has shown that the apparent diffusion coefficient is not less than half of the real one (21), however, and experimental evidence indicates that the effect is even less pronounced (22). Thus, it seems that membrane extrusions, if present, could not be principally responsible for the observed 4- to 6-fold smaller diffusion coefficient of LY-Chol at elevated osmotic levels (Tables 1 and 2). Furthermore, since the diffusion of LY-DC_{12:0}PE did not vary with osmotic level (Table 1), any assumed effect of extrusions on the apparent diffusion of LY-Chol would necessitate an assumption that LY-DC_{12:0}PE was excluded from the extrusions.

The data presented in Table 3 provide some clues to understanding the cause of slow sterol diffusion at elevated osmotic levels. Since the yield of protoplasts does not vary over the mannitol range of 0.45–0.90 M, it is unlikely that protoplasts isolated at different osmotic levels come from different populations of cortical cells. Thus, the smaller mean size of protoplasts isolated at higher osmotic levels probably reflects osmotic contraction (20). Accompanying this contraction and reduced membrane area are reduced contents of both phospholipid and sterol on a per protoplast basis, with a net increase in the sterol/phospholipid ratio (Table 3).

The mechanism of disappearance of lipids at elevated osmotic levels is not addressed by the present experiments. Previous studies have shown, however, that osmotic or water-deficit stress (6, 9) and protoplast isolation (7, 8, 10, 13) both lead to loss of phospholipids, particularly those containing unsaturated fatty acyl chains. Concomitant with this loss of polar lipids is a relative increase in neutral lipids, which are thought to be mostly triacylglycerols in lipid bodies (6–8, 13). Interconversions of free sterols and sterol derivatives have been reported in connection with osmotic or

water-deficit stress (6, 9, 12) and protoplast isolation (11). While net loss of total sterols occurs during these stresses (9, 11), the loss is not as great as the loss of total fatty acids (9). The results presented in Table 3 are consistent with these previous studies.

Cholesterol has been shown to increase ordering in fluid-phase membranes (1, 23). While cholesterol is not the dominant sterol in plant plasma membranes (24), Liljenberg and Kates (9) have suggested that an increase in the sterol/phospholipid ratio and a relative loss of unsaturated fatty acyl chains cause a reduction in membrane fluidity during water-deficit stress. In the same way, the increased sterol/phospholipid ratio observed for protoplasts at elevated osmotic levels (Table 3) might be responsible for the decreased diffusion rate of LY-Chol (Table 1). Since the data in Table 3 represent total protoplast lipids, however, caution must be exercised in using these data to draw conclusions about plasma membrane dynamics.

It is particularly noteworthy that elevated osmotic stress is selective in slowing the diffusion of LY-Chol but not LY-DC_{12:0}PE (Table 1). Alecio *et al.* (25) measured the lateral diffusion of two other sterol and phospholipid probes in cholesterol/dimyristoylphosphatidylcholine liposomes. At relatively low proportions of cholesterol, the diffusion coefficients of the probes were either equal or unequal, depending on the occurrence of lateral-phase separation at low temperature.

Hui (23) has recently summarized other evidence indicating the occurrence of domains in model membranes composed of cholesterol/phospholipid mixtures. When present at >33 mol %, cholesterol molecules are able to touch each other and are thought to form cholesterol-rich domains some tens of nanometers in size (23). Since sterols are more abundant in the plasma membrane than in endomembranes (24), it is likely that stressed protoplasts (Table 3) with an overall sterol/phospholipid ratio of 0.79 (44 mol % sterol if other lipids are ignored) have plasma membranes containing >33 mol % sterol. Indeed, even nonstressed plant cells typically have plasma membranes containing >33 mol % sterol. Highly purified plasma membranes isolated from rye leaves, for example, contain >50 mol % total sterol (24). Thus, plant plasma membranes may contain sterol-rich domains. The slower diffusion of LY-Chol at higher osmolality might represent diffusion within such domains, or even diffusion of small domains themselves. Hui (23) has emphasized, however, that the physicochemical description of model membranes composed of simple binary mixtures may not be applicable to biological membranes composed of extremely complex mixtures.

While physicochemical description may not yet be possible for biological membranes, considerable experimental evidence indicates that cholesterol is inhomogeneously distributed in animal cell membranes (1, 23). The present results suggest that sterols may also play a role in defining domains in plant cell membranes, at least under conditions of osmotic stress. This hypothesis may have implications for understanding membrane-related limitations in processes such as

the regeneration of dividing cells from protoplasts (3, 4) and the survival of cells under freezing conditions (20, 24).

The present observations also shed light on the heterogeneous diffusion characteristics that have been observed for lipids in the plasma membranes of various protoplasts (2–4). In all of these previous studies, protoplasts were studied in standard media that have osmolalities around 700 mmol/kg or higher. In each case, two lipid diffusion coefficients, one in the 10^{-10} cm²/sec range and another in the 10^{-9} cm²/sec range, were observed together with a substantial immobile fraction. The present study shows that the 10^{-10} cm²/sec component in maize can be brought into the 10^{-9} cm²/sec range by lowering the osmolality to \approx 500 mmol/kg. Using electron spin resonance, Boss and Grimes (26) have also observed evidence of decreased membrane fluidity at elevated osmotic levels.

Even with the 10^{-10} cm²/sec component merged into the 10^{-9} cm²/sec range, however, unexplained heterogeneity remains in the substantial lipid fraction that appears immobile (Tables 1 and 2). Wilkinson and Northcote (27) used freeze-fracture electron microscopy to show that up to 45% of the intramembrane particles form pseudocrystalline domains in the plasma membrane of some plant protoplasts. Whether or not some lipid molecules could be trapped and immobilized by such domains remains unclear. Also uncertain is whether or not a substantial fraction of immobile lipid is present in whole plant cells. Even at the lowest osmotic level tested in the present work, the sterol/phospholipid ratio of 0.63 is considerably greater than the 0.47 ratio observed for whole cortices. Thus, lipid dynamics in whole cells may be different from dynamics in protoplasts prepared even at low osmotic levels. Using electron spin resonance, however, Windle (28) has obtained evidence of immobilized lipid in both protoplasts and whole cells.

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