ABSTRACT

A marked increase in the amount of cisternal-like cytoplasmic membranes was observed after ice encasement of winter wheat (Triticum aestivum L.) seedlings. Linear sucrose gradients were employed to separate the various membrane components of the microsomal membrane fraction. NADH- and NADPH-cytochrome c reductase, two specific enzyme markers for plant endoplasmic reticulum (ER) were used to locate the ER in the linear gradients. The identity of the ER fraction was confirmed by determining the effect of EDTA and Mg2+ in the preparative media on the distribution of NADH- and NADPH-cytochrome c reductase activity within the gradient. In the presence of EDTA which dissociates ribosomes from ER, peaks of activity for the two enzymes were observed at a density corresponding to that for “smooth” ER. When the media also contained an appropriate concentration of Mg2+ to maintain the attachment of ribosomes to the ER, the peaks of activity for the enzymes shifted to a density corresponding to that for “rough” ER. NADH-cytochrome c reductase activity was similar for 24 C-grown and 2C-grown iced seedlings, but significantly lower for 2 C noniced seedlings. No preferential increase in uptake of radioactive leucine or choline in the ER was observed during ice encasement. The accumulation of electron microscopically visible membrane arrays was not inhibited by the presence of protein synthesis inhibitors at concentrations which severely inhibited incorporation of [1-14C]leucine into membrane protein, but did not affect survival and growth of the seedlings. These observations indicate that the apparent proliferation of ER during ice encasement does not result from net membrane synthesis, but rather from reorganization of existing membrane elements within the cell.

Ice encasement of winter wheat seedlings, either under controlled environment conditions in the laboratory (14) or under artificially modified field conditions (13), results in damage to the seedlings. The shoot apex cells of these seedlings show proliferation of the cytoplasmic membranes but this is not necessarily related to plant injury (14). Ultrastructural and metabolic studies (2, 3, 14) have revealed the rapid formation of parallel arrays and concentric whorls of membranes and the accumulation of several anaerobic metabolites during icing at nonlethal temperatures. The proliferated membranes disintegrate rapidly when the stress of ice encasement is removed from the seedlings by thawing. Augmentation of cellular membranes also has been induced in winter wheat by exposure of seedlings at −1 C to combinations of metabolites at concentrations similar to those generated within the seedlings during ice encasement (3).

Electron microscopic studies on many plant species (1, 11, 12, 16) have shown the presence of membrane configurations similar to those observed after ice encasement in the cells of plants exposed to many types of stress, including mechanical effects, irradiation, exposure to CO, CO2, or CN, dehydration, anaerobiosis, and chilling. The membranes formed in response to these stresses have not been identified biochemically, but ultrastructural evidence indicates that they are ER. How these membranes proliferate during stress is not known, although it has been suggested that they arise through reorganization of existing membrane elements, not through net membrane synthesis (1, 14, 16).

The present study was undertaken to confirm the identity of the cytoplasmic membranes which accumulate during ice encasement. This paper describes selected enzymic properties of membrane fractions isolated from noniced and ice-encased winter wheat seedlings, and provides evidence to support the hypothesis that the proliferated membranes originate from existing membrane elements in the cell.

MATERIALS AND METHODS

Plant Material. Cold-acclimated and nonacclimated seedlings of Kharkov 22 MC winter wheat (Triticum aestivum L.), grown either in light or dark, were used throughout this investigation. Dark-grown seedlings were produced by sowing sterilized seed on moist filter paper in trays, and growing seedlings in dark at 24 C for 64 h or for 40 h at 24 C and then at 2 C day (16 h)/−2 C night (8 h) for 3 weeks. Cold-hardened seedlings were encased in ice by covering with water at 0 C and transferring the trays to a dark freezing cabinet at −1 C to −2 C (2 C iced seedlings). After 1 week, the seedlings were thawed under running tap water and immediately harvested for membrane isolation. For cold-hardened controls, trays of seedlings were transferred directly to −1 C for 1 week (2 C seedlings).

Light-grown seedlings were obtained by sowing seed in soil in fiber flats (10 × 20 cm) for 7 days at 20 C light 15 C dark with a 16-h day of 35,000 lux (about 525 μE m−2 s−1) or for 5 days under the same conditions and then transferred to 2 C light 0 C dark with a 16-h day of 10,000 lux (about 145 μE m−2 s−1) for 6 weeks. Seedlings were prepared for ice encasement and other treatments by washing the roots free of soil. For icing treatments seedlings were placed in plastic saucers, immersed in cold water, and frozen for 1 week at −1 C in dark.

Membrane Isolation and Enzyme Assays. Shoots (8–10 g fresh weight) from dark-grown seedlings were harvested, ground with a mortar and pestle for 60 to 90 s at 2 to 4 C in a grinding medium (GM) containing 3 μm sucrose, 5 mm 2-mercaptoethanol, 25 mm Tris-Mes (pH 7.5), and 3 mm EDTA. Membrane pellets were obtained by differential centrifugation, as previously described by Nagahashi and Bevers (10). For experiments to determine the effect of EDTA and Mg2+ on the distribution of membranes in sucrose gradients, shoots (18 g fresh weight) were ground in GM

1 Contribution 1045 of Chemistry and Biology Research Institute, Agriculture Canada.

2 Abbreviation: GM: grinding medium.
minus EDTA. The crude homogenates were divided into three equal fractions and the final 80,000g pellets were washed, either in a medium containing 0.25 m sucrose, 1 mM 2-mercaptoethanol, and 1 mM Tris-Mes (pH 7.2), or in the same medium plus 3 mM EDTA, or plus 3 mM EDTA and 9 mM MgCl₂. Washed pellets were suspended in 2 ml of their respective wash media and overlaid on 25 ml linear sucrose gradients.

Density gradients of 15 to 50% (w/w) sucrose in 1 mM Tris-Mes (pH 7.2) plus 1 mM 2-mercaptoethanol, or in this medium plus 3 mM EDTA or plus 3 mM EDTA and 9 mM MgCl₂ were prepared over a 3-ml cushion of 60% (w/w) sucrose using a Buchler Instruments density gradient maker. Membrane pellets were layered over the appropriate gradients and centrifuged at 80,000g for 15 h in a Beckman SW 25.1 rotor, and fractionated with an ISCO model 180 density gradient fractionator into 25 fractions (1 ml/ fraction). Sucrose concentration in each fraction was determined with a Bausch & Lomb refractometer. Protein in all preparations was determined by the method of Lowry et al. (9).

NADH- and NADPH-Cyt c reductase activities were assayed spectrophotometrically (6) ± 1 μM antimycin A by following the reduction of Cyt c at 550 nm. Cyt c oxidase activity was determined by following the oxidation of Cyt c at 550 nm (6).

**Incorporation Experiments.** Sterilized seeds were germinated in a minimal volume of water for 24 h at 24 C in dark in small trays without moist filter paper. Small aliquots (3-4 ml) of aqueous solutions containing 50 μCi of DL-[1-14C]leucine or [1,2-14C]choline chloride (Sigma Chemical Co.) were added to the seedlings and they were returned to 24 C for an additional 24 h. The seedlings were then cold-hardened, in the dark for 4 weeks, encased in ice for 1 week, and membranes isolated on linear sucrose gradients from 4 to 5 fresh wt of shoots, as described above. Aliquots of 0.2 ml from each fraction were mixed with 10 ml New England Nuclear Aquasol and counted in a Beckman LD 8000 scintillation counter with 90% counting efficiency.

**Protein Synthesis Inhibition Experiments.** Light-grown, cold-hardened seedlings were washed free of soil and placed in 50-ml beakers (30 plants per beaker) containing 30 ml of aqueous solutions of cycloheximide (1-10 mg/l) or chloramphenicol (50- 600 mg/l). The seedlings were returned to 2 C for 3 days after which the roots and shoots of half of the seedlings were returned to 2 C and the seedlings transplanted into damp Vermiculite. The roots and shoots of the remaining seedlings were returned to 2 C and they were then encased in ice for 1 week prior to transplanting. Survival and regrowth of seedlings were recorded after 2 weeks at 20 C day (16 h; 35,000 lux-525 μM m⁻² s⁻¹) 15 C night.

To determine the effect of protein synthesis inhibitors on incorporation of amino acids into membrane protein in the whole plant system, groups of cold-hardened seedlings with endosperm removed were incubated for 3 days at 2 C in 30 ml of water containing 25 μCi DL-[1-14C]leucine in the presence or absence of cycloheximide or chloramphenicol. One-cm segments of crowns from 25 to 30 seedlings (1-2 g fresh weight) were then harvested, ground with a mortar and pestle, and the homogenate centrifuged at 13,000g for 15 min (6). The total microsomal membrane fraction was obtained by centrifuging the 13,000g supernatant at 80,000g for 30 min. The membrane pellet was resuspended, centrifuged again at 80,000g for 30 min, resuspended in 1 ml water, and radioactivity determined. Aliquots of 10 μCi of [1-14C]choline chloride (data not shown) were added to the 13,000g pellets and the activity determined.

**Electron Microscopy.** Shoot apices dissected from light-grown, cold-hardened seedlings prior to and after exposure to protein synthesis inhibitors and ice encasement were processed for electron microscopy as previously described (14). KMnO₄ was used as fixative because our earlier studies indicated that better preservation of membranes was obtained using it, instead of first fixing in glutaraldehyde followed by postfixation in osmic acid (14).

**RESULTS**

**Effect of EDTA and Mg²⁺ on Gradient Fractionation.** In the absence of either EDTA or MgCl₂ in the grinding or washing media, a large peak of NADH-Cyt c reductase activity, and smaller peaks of NADPH-Cyt c reductase and Cyt c oxidase activities all occurred between 1.17 and 1.19 g/ml in linear sucrose density gradients (Fig. 1A). A minor peak of NADPH-Cyt c reductase also was observed at 1.06 g/ml. Major and minor peaks of total protein were found at densities of 1.18 and 1.06 g/ml, respectively. When EDTA was included in the wash media and in the sucrose gradient, the peaks of activity for NADH- and NADPH-Cyt c reductase were found at approximately 1.11 g/ml, whereas the peak for Cyt c oxidase remained at 1.17 g/ml (Fig. 1B). The small peak of NADPH-Cyt c reductase activity observed in the absence of EDTA was not present. The protein peak had broadened somewhat, and the maximum concentration of protein was found in a fraction between the major enzyme peaks. When both EDTA and MgCl₂ were included in the wash media and in the gradient, the pattern of enzyme distribution (Fig. 1C) was similar to that observed in the absence of these compounds. The protein peak was sharper than in the presence of only EDTA and the maximum concentration of protein was at 1.19 g/ml.

The inclusion of 1 μM antimycin A in the assay system did not significantly change the activities of either NADH- or NADPH-Cyt c reductase in the 13,000 to 80,000g membrane fraction. In contrast, Cyt c oxidase activity was reduced by approximately 40% under the same assay conditions. When the mitochondrial fraction (1,000-13,000g pellet) was assayed in the presence of antimycin A, NADH-Cyt c reductase activity decreased about 50%. These observations demonstrated that the 13,000 to 80,000g membrane fractions were not contaminated significantly by mitochondria.

**Enzyme Distribution in Membranes from Noniced and Iced Seedlings.** The sucrose gradient distribution of antimycin A-insensitive NADH- and NADPH-Cyt c reductases, and Cyt c oxidase activity in the 13,000 to 80,000g membrane fractions from 24 C, 2 C, and 2 C ice-encased seedlings was similar (Fig. 2). The inclusion of EDTA in the media during preparation of the membranes resulted in peaks of activity for NADH- and NADPH-Cyt c reductase at a density of 1.10 to 1.11 g/ml. The peaks of activity for Cyt c oxidase were observed at 1.16 to 1.18 g/ml for membranes of seedlings from the three treatments. The distribution of protein within the gradients was similar for the three environmental treatments (Fig. 2), with the highest concentrations present in fractions between those showing greatest reductase and oxidase activities.

Total activity of NADH-Cyt c reductase was more than 10-fold greater than that of NADPH-Cyt c reductase in membranes from all three treatment conditions (Table 1). The level of NADH-Cyt c activity was the same for 24 C and 2 C iced seedlings, but was markedly reduced in 2 C noniced seedlings. NADPH-Cyt c reductase activity was significantly higher in 24 C than in either 2 C or 2 C iced seedlings. Cyt c oxidase activity was similar for the three treatment conditions, but the maximum observed level of activity was always less than 10% of that observed in mitochondrial fractions (data not shown). Total protein in the microsomal fraction of 24 C, 2 C, and 2 C iced seedlings was not significantly different (Table 1).

**Incorporation of Labeled Membrane Precursors during Ice Encasement.** The linear sucrose gradient distribution of radioactivity in the 13,000 to 80,000g membrane fraction from seedlings incubated with [1,2-14C]choline chloride (data not shown) was similar for 2 C and 2 C iced seedlings. With EDTA in the preparation media, the highest levels of radioactivity were observed in fractions 10 and 11, corresponding to a density of 1.10 to 1.12 g/ml. The peak of total protein was relatively broad, with maximum levels in fractions 10 through 15. When both MgCl₂ and EDTA were included in the preparative media, the peaks of radioactivity for both noniced and iced seedlings shifted to a density of approximately 1.16 to 1.18 g/ml, with a concomitant shift in the peak for total protein (Fig. 3B). The total amount of radioactive choline recovered from all gra-
dient fractions was considerably greater in iced than noniced seedlings (Table II). However, no significant increase, either in uptake of radioactive leucine or in total membrane protein (Table II) was observed after ice encasement.

Effect of Protein Synthesis Inhibitors. The effect of the protein synthesis inhibitors cycloheximide and chloramphenicol on incorporation of radioactive leucine in the noniced whole seedling system was determined. Incorporation of [1-\textsuperscript{14}C]leucine into membranes of the total microsomal fraction during incubation of cold-hardened seedlings for 3 days at 2°C was inhibited about 60% at a concentration of cycloheximide (1 mg/l) which did not reduce survival or regrowth. At higher cycloheximide concentrations (2.5–5 mg/l), uptake of the label was further reduced, but survival and regrowth also decreased slightly. In contrast with the results obtained with cycloheximide, exposure of seedlings even to high levels of chloramphenicol (500 mg/l) did not result in a significant decline in survival, and uptake of label actually increased 10 to 20%.

Regrowth of seedlings decreased at the higher levels of chloramphenicol.

The effect of exposure of seedlings to protein synthesis inhibitors on survival and regrowth after ice encasement is shown in Table III. In the presence of relatively low concentrations of cycloheximide (1 mg/l) or chloramphenicol (500 mg/l), survival and regrowth after icing were reduced by approximately 50%, the same level of inhibition observed after icing in the absence of inhibitor. When cycloheximide concentration was increased to 5 mg/l, survival and regrowth of noniced seedlings were reduced only slightly, but both parameters were reduced markedly in iced seedlings. In contrast, higher levels of chloramphenicol (500 mg/l) did not further reduce survival of seedlings after ice encasement, although additional inhibition of growth was observed.

Electron microscopic studies were conducted to examine the effect of protein synthesis inhibitors on the proliferation of cellular membranes during ice encasement. Ultrastructural features of shoot apex cells from noniced (Fig. 4) and ice-encased (Fig. 5) cold-hardened seedlings, not exposed to inhibitors, were similar except for a marked accumulation of parallel arrays and concentric whorls of membranes in the ice-encased treatment. Exposure of seedlings to a cycloheximide concentration (5 mg/l) sufficient to reduce survival and regrowth (Table III), and inhibit the incorporation of labeled leucine into membrane protein by approximately 75% had no major effect on the ultrastructure of noniced seedlings (Fig. 6). Similarly, the highest level of chloramphenicol (500 mg/l) also did not alter ultrastructural features of noniced cells.

When seedlings were encased in ice after exposure to various levels of cycloheximide, the lowest concentration (1 mg/l) did not inhibit the proliferation of membranes (Fig. 7) that normally occurs during ice encasement (Fig. 5), although at this level of inhibitor, 60% decrease in incorporation of [1-\textsuperscript{14}C]leucine was observed. Cycloheximide at 5 mg/l completely arrested the accumulation of membranes (Fig. 8), and general ultrastructural features of these cells were similar to those of noniced cells (Figs. 4 and 6). In contrast to the results obtained with cycloheximide, incubation of seedlings, even in very high levels of chloramphenicol (500 mg/l) prior to icing did not inhibit the formation of parallel arrays and concentric whorls of membranes during ice encasement (Fig. 9).

**DISCUSSION**

The identity of the ER fraction on linear sucrose gradients was
confirmed through the use of specific enzyme markers and by determining the effect of EDTA and Mg²⁺ on gradient fractionation. Antimycin A-insensitive NADH-Cyt c reductase and NADPH-Cyt c reductase are known enzyme markers for plant ER (4, 6, 8, 10). Peaks of activity for these enzymes were observed at densities corresponding to those previously reported for other plant systems (8, 10). Furthermore, the location of the ER in the gradient shifted in the presence and absence of EDTA and Mg²⁺. The controlled use of EDTA results in dissociation of the ribosomes from the RER, converting it to a lighter "smooth" ER, while appropriate concentrations of Mg²⁺ maintain the attachment of ribosomes, even in the presence of the EDTA (8). In our experiments, the peaks of activity for the two enzyme markers were observed at a density (1.11 g/ml) corresponding to that for smooth ER in the presence of EDTA, and at a density (1.17–1.19 g/ml) corresponding to that for RER in the presence of EDTA plus Mg²⁺. These observations confirmed the identity and location of the ER fraction in our preparations.

The proliferation of cytoplasmic membranes similar to that observed during ice encasement of winter wheat has been reported previously from ultrastructural studies of several plant species exposed to a wide variety of external stresses (1, 11, 12, 16). However, the manner in which these membranes arise has remained open to speculation. Several lines of evidence support the conclusion that the formation of linear arrays and concentric whorls of ER during ice encasement is not the result of net membrane synthesis. No significant differences were observed, either in total membrane protein or in the gradient distribution of protein, from the three treatment conditions. The level of NADH-Cyt c reductase activity was the same for ER from 24 C seedlings as from 2 C icced seedlings, although the amount of electron microscopically visible cisternal ER was much greater in the icced seedlings. The lower level of activity of this enzyme in the 2 C noniced seedlings may result from repression of the enzyme during growth at low temperature. The increase in activity observed after icing is probably associated with ice encasement-induced release

Table 1. Total Activities of Antimycin A-insensitive NADH- and NADPH-Cyt c Reductase, and Cyt c Oxidase and Total Protein in the 13,000 to 80,000g Membrane Fractions from Noniced and Ice-encased Winter Wheat Seedlings

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NADH-Cyt c Reductase</th>
<th>NADPH-Cyt c Reductase</th>
<th>Cyt c Oxidase</th>
<th>Protein mg/g fresh weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 C Noniced</td>
<td>0.29 ± 0.02</td>
<td>0.024 ± 0.004</td>
<td>0.046 ± 0.012</td>
<td>1.47 ± 0.08</td>
</tr>
<tr>
<td>2 C Noniced</td>
<td>0.17 ± 0.01</td>
<td>0.011 ± 0.001</td>
<td>0.041 ± 0.010</td>
<td>1.42 ± 0.09</td>
</tr>
<tr>
<td>2 C Iced</td>
<td>0.31 ± 0.01</td>
<td>0.007 ± 0.001</td>
<td>0.038 ± 0.001</td>
<td>1.45 ± 0.12</td>
</tr>
</tbody>
</table>

Each value represents an average ± the standard error of at least three determinations.

Fig. 2. Effect of three different environmental treatments on the linear sucrose gradient distribution of antimycin A-insensitive NADH- and NADPH-Cyt c reductase, Cyt c oxidase, and total protein in the 13,000 to 80,000g membrane fraction. In all treatments, the preparative media contained 3 mM EDTA and no MgCl₂: A: 24 C seedlings; B: 2 C seedlings; C: 2 C icced seedlings.
Fig. 3. Linear sucrose gradient distribution of radioactive label and total protein in the 13,000 to 80,000 g membrane fractions isolated from 2 C (○—○) and 2 C iced (□—□) seedlings grown in the presence of [1,2-3C]choline chloride. A: preparative media contained 3 mM EDTA; B: preparative media contained 3 mM EDTA + 9 mM MgCl₂.

Table II. Total Radioactivity and Protein Recovered in the 13,000 to 80,000 g Membrane Fractions from Noniced and Iced Winter Wheat Seedlings

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>³⁵S]Leucine</td>
<td>³⁵S]Choline</td>
</tr>
<tr>
<td></td>
<td>dpm·10⁻⁶/g fresh weight</td>
<td>mg/g fresh weight</td>
</tr>
<tr>
<td>2 C Noniced</td>
<td>35 ± 2</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>2 C Iced</td>
<td>36 ± 5</td>
<td>103 ± 10</td>
</tr>
</tbody>
</table>

of this repressed activity.

In experiments where seedlings were exposed to two different radioactive membrane precursors (leucine, and choline) prior to ice encasement, preferential accumulation of label did not occur specifically in the ER fraction from ice-encased seedlings. If new membrane material was being rapidly synthesized during this period, it would be expected that the levels of the two labeled precursors would increase preferentially in these membranes, due to accelerated incorporation of the radioactive compounds accumulated prior to icing. However, no increase in the uptake of leucine, and only a uniform increase in uptake of choline throughout the gradients were observed. These observations, and the absence of a significant increase in protein level during icing, suggest that ice encasement induced a more rapid turnover of choline in all membranes of the microsomal fraction, but not synthesis of new membrane components.

The results obtained from experiments in which seedlings were exposed to inhibitors of protein synthesis prior to ice encasement also support the view that protein synthesis, and hence net membrane synthesis, are not prerequisites for the proliferation of membranes during icing. In accord with a previous report (15), our results showed that low levels of cycloheximide (1 mg/l) severely inhibited protein synthesis without observable effect on survival and growth of the plant. Under these conditions, no inhibition of membrane accumulation was observed electron microscopically in ice-encased plants, indicating that net protein synthesis is not required for membrane proliferation.

The results obtained when seedlings were exposed to chloramphenicol differ somewhat from those previously reported. Trunova and Zvereva (15) showed that chloramphenicol at comparable levels partially inhibited protein synthesis in wheat, while Hooper et al. (7) reported a 30 to 50% reduction in [³⁴C]leucine uptake in Chlamydomonas cells. In contrast, we found a slight increase in uptake of leucine, greater inhibition of growth than previously
Figs. 4 and 5. General ultrastructural features of shoot apical cells of cold-hardened Kharkov winter wheat prior to (Fig. 4, x 5,400) and after ice encasement for 1 week at -1 C (Fig. 5, x 6,700). Fixation in KMnO₄, for all figures. (cw): cell wall; (d): dictyosome; (er): endoplasmic reticulum; (l): lipid body; (m): mitochondrion; (n): nucleus; (p): plastid; (pm): plasmalemma.

Fig. 6. Ultrastructure of apical cells after exposure to 5 mg/l cycloheximide but not encased in ice (x 6,700). (d): dictyosome; (er): endoplasmic reticulum; (l): lipid body; (m): mitochondrion; (n): nucleus; (p): plastid.

Figs. 7, 8, and 9. Ultrastructure of apical cells after exposure to 1 mg/l cycloheximide (Fig. 7, x 10,000), 5 mg/l cycloheximide (Fig. 8, x 6,700), and 500 mg/l chloramphenicol (Fig. 9, x 10,000) followed by ice encasement for 1 week at -1 C. (cw): cell wall; (er): endoplasmic reticulum; (m): mitochondrion; (n): nucleus; (p): plastid.
reported (5, 15) but membrane accumulation was not inhibited. The absence of an effect of chloramphenicol on membrane accumulation was not unexpected since it inhibits protein synthesis on 70S ribosomes of chloroplasts (7), not in the cytoplasm. However, the lack of inhibition of membrane formation by cycloheximide which inhibits protein synthesis on cytoplasmic 80S ribosomes (7) supports the view that protein synthesis is not a prerequisite for proliferation of membranes during ice encasement.

The results obtained in this study have shown that the arrays of ER membranes seen in the electron microscope after ice encasement do not arise from net membrane synthesis. The actual mechanism by which these membranes develop in response to ice encasement stress has not been elucidated, but our evidence suggests that they arise through reorganization of existing membrane components, possibly vesicular ER not resolved in the electron micrographs. The function of this proliferated ER is unknown, but it may be involved in cell repair processes following stress-induced injury (1).

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