Plant Physiol. (1980) 66, 349–352
0032-0889/80/66/0349/04/$00.50/0

Spin-label Studies of Membranes in Rye Protoplasts during Extracellular Freezing[1, 2]

Received for publication August 23, 1979 and in revised form March 12, 1980

JAS SINGH AND RICHARD W. MILLER
Chemistry and Biology Research Institute, Research Branch, Agriculture Canada, Ottawa, Ontario K1A OC6 Canada

ABSTRACT

Protoplasts isolated from epicotyls of nonhardened winter rye seedlings were spin-labeled with the *N*-oxyl-4-4-dimethyloxazolidine derivatives of 5-ketostearic (5NS) and 16-ketostearic (16NS) acids. Spectra of the membrane-bound labels showed motional broadening with a rotational correlation time of 1.5×10^{-8} second for 5NS and 1.5×10^{-10} second for 16NS at 0 C. A procedure was developed to follow membrane changes in these protoplasts during extracellular freezing. With freezing, molecular motion of 5NS, but not of 16NS, spin probes was restricted. The increase in molecular order near the hydrated end of the membrane did not result from lowered temperatures inasmuch as no such change was observed in supercooled samples. These changes are probably due to dehydration of protoplast membranes during extracellular freezing. Similar results were obtained with multilayered egg yolk lecithin and are consistent with previous observations of changes in lecithin multibilayers during dehydration. Such alterations in membrane order might lead to irreversible membrane damage during extracellular freezing of plant cells.

Primary sites of extracellular freezing injury in plant cells are cellular membranes, especially the plasma membrane, and cellular dehydration is an important factor in this injury (12, 14, 15). Loss of resilience of the plasma membrane has been suggested as the cause of lysis after plant cells have been subjected to dehydration from extracellular freezing (25). Damage to the plasma membrane after freezing also impairs semipermeability and ion-transport mechanisms (13) and causes irreversible breakdown of membrane ultrastructure (20). Other cellular membranes, such as thylakoid and mitochondrial membranes, can also be damaged by extracellular freezing (6, 9), although in some cases, different levels of sensitivity to freezing exist between the plasmalemma and the other membranous organelles (16, 23). Ultrastructural evidence indicates that both extracellular freezing and plasmolysis result in irreversible membrane damage as shown by characteristic reorganization of membrane bilamellar structure during freezing (20). Cold-hardened plant cells can withstand lower temperatures before these changes occur (20). Unsaturation of the phospholipid fatty acids in the membranes might be important in increased membrane tolerance to freezing, but recent evidence indicates that this is probably not the case (3, 5, 18, 22, 24).

Little is known about molecular changes in membranes when cells are subjected to extracellular freezing. One approach to the study of the molecular response of plant cell membranes to freezing is to incorporate membrane probes into the membranes and follow changes that occur when the cell is frozen. The ease with which large numbers of winter rye protoplasts can be isolated (4), the fact that they undergo dehydration upon extracellular (slow) freezing similar to that observed in the intact plant cell (19, 21), and the ease with which fatty acid labels can be incorporated into the membranes in the absence of the cell wall make this a good system to use for such studies. We report the successful fatty acid spin labeling of isolated protoplasts from the epicotyls of winter rye seedlings and the detection of structural changes in these membranes after extracellular freezing.

MATERIALS AND METHODS

Seeds of *Secale cereale* L. cv. Puma were germinated in the dark on moist filter paper at 24 C for 60 h. Epicotyls were dissected from the seedlings and protoplasts were isolated by a modification (21) of the method of de la Roche *et al.* (4). Seven to 10 million protoplasts were suspended in either 1 ml medium containing 0.3 M concentrations of mannitol and sucrose or in 1 ml sugar-free saline medium (21). In both bases, 1 mM potassium ferricyanide was included to prevent reduction of the label. Ten μl 5NS[3] or 16NS from 10 mg/ml ethanol stock solutions was added. The suspensions were allowed to stand overnight at 4 C. Labeled protoplasts then were washed twice with 5 ml suspension medium by centrifugation at 150*g* for 5 min. Supernatants were removed and the pelleted protoplasts were aspirated into 100-μl pipettes. The pipettes were sealed at the bottom and placed into a quartz sample holder which was 0.5 cm shorter than the pipette. ESR spectra were recorded with a Varian E-3 spectrometer fitted with a temperature-controlled quartz Dewar assembly (Varian) within the microwave cavity. The field was set at 3250 gauss, the microwave power was set at 2 mw, and the modulation amplitude was set at 1.0 gauss.

Freezing of protoplasts in the pipette was usually initiated at −4 C by dropping in a minute grain of dry ice while the pipette was in the quartz sample holder in the microwave cavity. The spectrometer microwave cavity detuned when freezing was initiated. When this process ceased, freezing was completed at the set temperature and the spectrometer was retuned to record the spectra. The temperature then was lowered approximately 2 C every 30 min. Spectra were recorded after equilibration at each temperature. Apparent rotational correlation times of 5NS were calculated by the method of Goldman *et al.* (7) for rotational motion at "intermediate" rates. Rotational correlational times for 16NS were calculated by the methods of Canon *et al.* (1) and

[1] This work is contribution No. 1121 of the Chemistry and Biology Research Institute, Agriculture Canada.

[2] Part of this work was reported at the Annual Meeting of the American Society of Plant Physiologists, 1978, Blacksburg, Va.

[3] Abbreviations: 5NS and 16NS: *N*-oxyl-4,4-dimethyloxazolidine derivatives of 5- and 16-ketostearic acids, respectively; ESR: electron spin resonance; EYL: egg yolk lecithin.

Kivelson (11).

Multilayered liposomes were prepared from purified EYL (Supelco Co. Ltd.). One hundred mg of the lipid dissolved in 10 ml chloroform was dried by a stream of N_2 in a clinical centrifuge tube. One ml distilled H_2O and a small glass bead were introduced and the centrifuge-tube was agitated on a Vortex mixer. The resultant liposome suspension was spin-labeled with 5NS and 16NS as described above except that ferricyanide was excluded. Freezing of the liposomes was carried out as described for protoplasts.

RESULTS

ESR spectra at 0 C with rye protoplasts and EYL liposomes labeled with 5NS are shown in Figure 1. The spectrum of rye protoplasts have a rotational rate of motion intermediate between the rapid isotropic and rigid limits. Compared to protoplasts labeled with 5NS, the spectrum for EYL indicated more rapid anisotropic motion, as shown by the resolution of the *g* resonances and a diminished hyperfine splitting ($2A_{11}$) of the g_{11} resonances (Fig. 1). The rotational correlation time for 5NS in EYL was 0.7 $\times 10^{-8}$ s compared to 1.5 $\times 10^{-8}$ s in the protoplasts.

Motion of the 5NS spin label in protoplasts and EYL liposomes before and after freezing is compared in Figure 2. Half the separation of the high-field and low-field peaks A_{11}, a motion-dependent parameter, was plotted against the temperature before and after freezing. Before freezing, motional broadening was linear with the lowering of temperature below 0 C (Fig. 2B). If

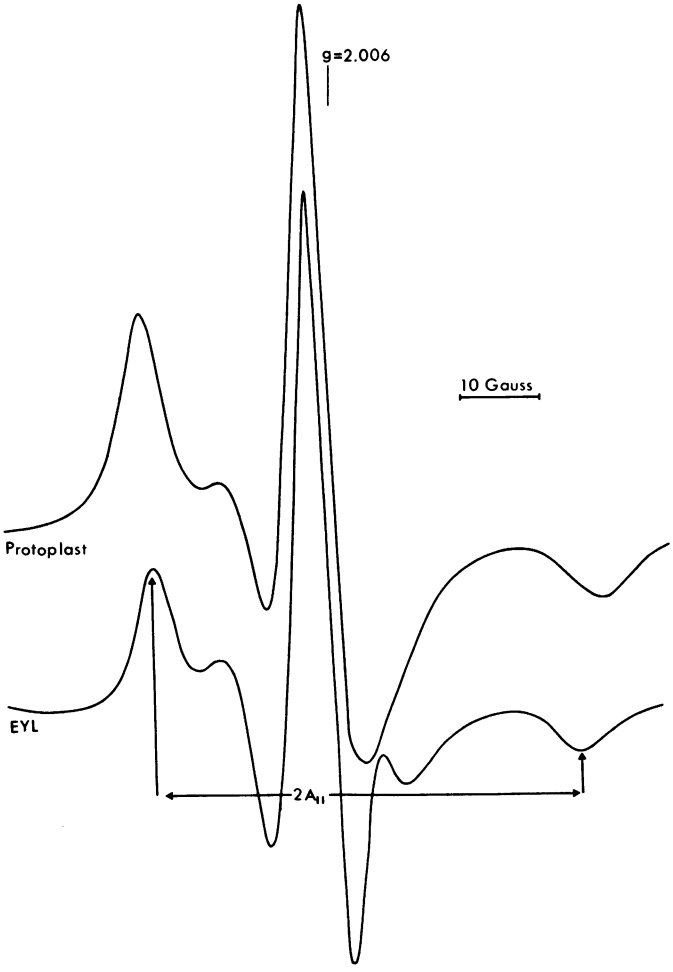

FIG. 1. ESR spectra at 0 C of 5NS label in rye protoplasts and in EYL liposomes.

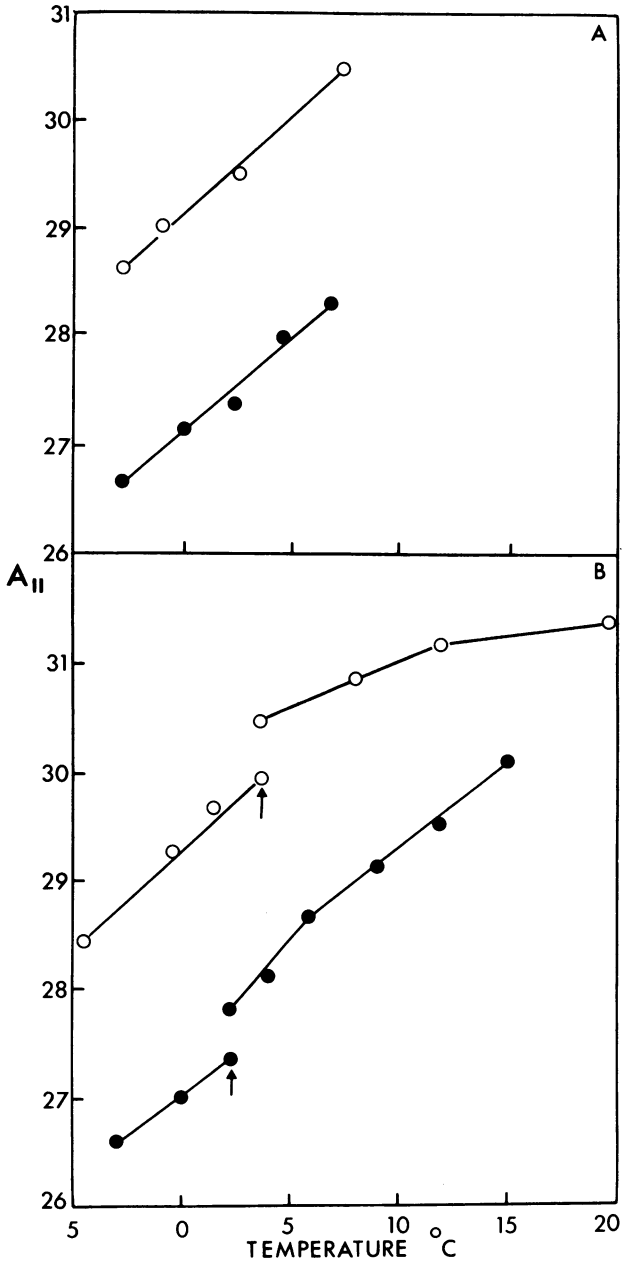

FIG. 2. Plots of hyperfine splitting A_{11} *versus* temperature in rye protoplasts and EYL spin-labeled with 5NS. ○, ●, spin-labeled nonhardened protoplasts and liposomes, respectively. In A, the samples were supercooled (no freezing) and, in B, freezing in the samples was initiated as described under "Materials and Methods." Arrow indicates initiation of freezing.

freezing was initiated at −4 C and the temperature was maintained at −4 C, an increase in motional broadening, compared to that before freezing, was observed (Fig. 2B). The rotational correlation time changed from 2 to 3 $\times 10^{-8}$ s. Since both spectra were observed at −4 C, the cause can be attributed to decreased probe motion, resulting from extracellular freezing, and not to temperature effects. Lowering the temperature after freezing produced further motional broadening until, at around −15 C, a rigid limit spectrum was obtained (Fig. 2B). In the absence of nucleation, no changes were observed with lowering of temperature (Fig. 2A). It was not possible to obtain spectra of 5NS in supercooled protoplasts lower than −8 C since nucleation occurred spontaneously below that temperature. On freezing of the EYL liposomes at

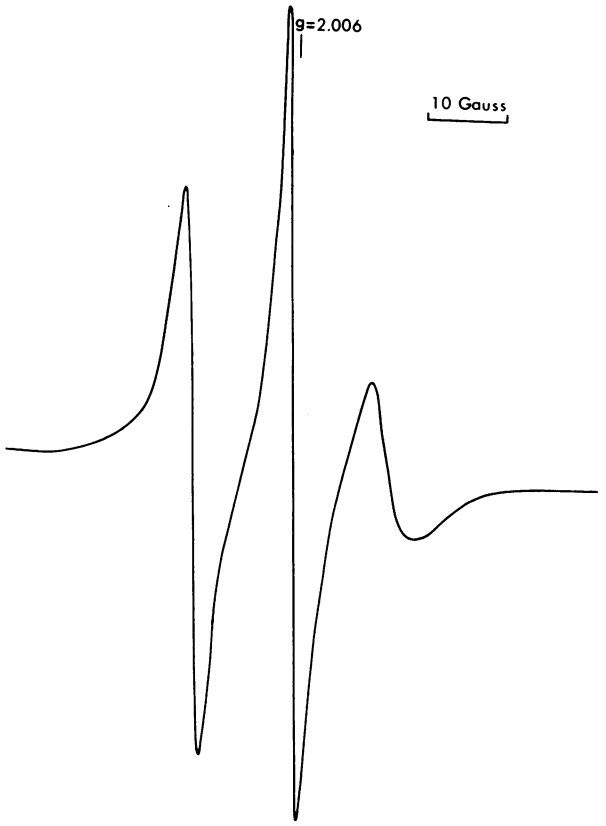

FIG. 3. ESR spectra of 0 C of 16NS label in protoplasts isolated from winter rye.

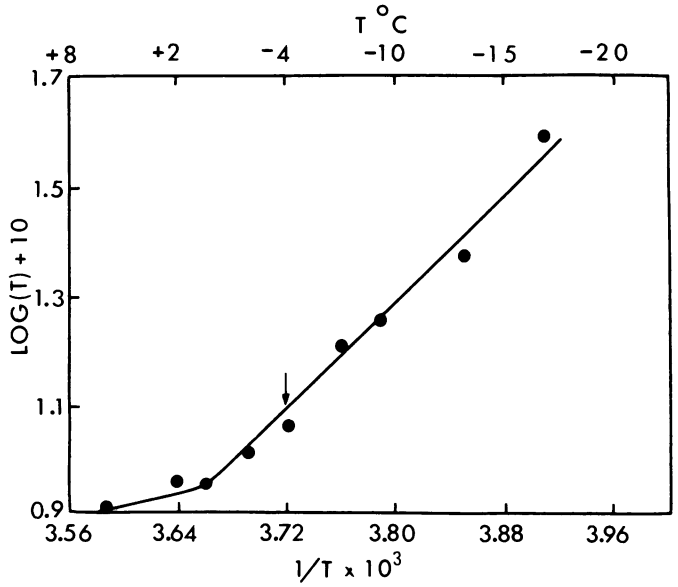

FIG. 4. Arrhenius plot of rotational correlation time (T) of 16NS in rye protoplasts. Arrow indicates initiation of freezing.

−2.3 C, a discontinuity similar to that in the protoplasts was observed, but this was absent in the supercooled sample. This change in motional restriction in the EYL membranes during freezing was of the same order of magnitude as observed for their protoplast counterparts.

When protoplasts were labeled with 16NS, where the nitroxyl group is in the hydrocarbon interior of the membranes and not as close to the polar head groups as 5NS, spectra as shown in Figure 3 were obtained. Rotational correlation times were in the order of 10^{-10} s at 0 C. Motion of this probe is less restricted than that of 5NS. This result is consistent with liposome studies which indicate increasing order in the bilayer when the paramagnetic probe is closer to the polar group regions in the phospholipid bilayer (8, 10). Arrhenius plots of rotational correlation times for 16NS *versus* temperature were linear below 0 C (Fig. 4) and, unlike 5NS, no major change in probe motion was observed after freezing at −4 C. This suggested that removal of water from the cell on extracellular freezing had a larger effect on the phospholipid bilayer close to the polar regions where water plays an important role in structuring and ordering polar head groups.

DISCUSSION

Decreases in rotational freedom near the hydrophilic regions of membrane phospholipids of 5NS spin-labeled rye protoplasts coincide with extracellular freezing. Protoplasts labeled with 16NS show less immobilization of the probe upon freezing than those labeled with 5NS. Once freezing was initiated, ordering of the phospholipid polar regions increased until a high degree of order was observed at −15 C. The initial decrease in molecular motion at freezing was not caused by temperature-induced changes in membrane fluidity since the changes occurred under isothermal conditions and not in supercooled samples. During extracellular freezing, rye protoplasts, as in intact plant cells, dehydrate and contract as a result of thermodynamic equilibration between vapor pressures of the cell fluids and that of the ice outside and also as a result of the concentration of solutes in the suspension medium (19). Thus, reductions in molecular motion near phospholipid head groups of protoplast membranes during extracellular freezing can result from dehydration induced by freezing or plasmolysis. The results presented here are consistent with observations which show that, when spin-labeled lecithin multibilayers are dehydrated by drying, membrane order increases and is more pronounced closer to the polar headgroups than at the hydrocarbon interior (8, 10).

Without cell walls, protoplasts require an osmoticum to prevent lysis. During extracellular freezing, many of the protoplasts reside in liquid channels of high solute concentration between the ice (19). It is possible that plasma membrane changes were caused by effects of high solute concentrations on the outer surface of the bilayer. However, EYL liposomes, spin-labeled with 5NS and prepared in distilled H_2O, behaved in a way similar to that of the protoplasts upon freezing. Such liposomes would not be exposed to high solute concentrations upon freezing but would be subjected to dehydration from ice formation (17).

It is unlikely that changes in the packing geometry of the phospholipids due to freezing at −4 C is directly related to the cause of lethal freezing injury since most protoplasts isolated from nonhardened rye survive freezing to this temperature. It is possible that further packing of the membranes at lower freezing temperatures (−10 to −15 C), indicated by the spectra approaching the rigid limit, may contribute indirectly to irreversible injury since unhardened protoplasts are killed at these temperatures (21). For instance, it has been proposed that "crystallization" of the lipid chains in membranes may lead to the formation of packing faults and subsequently to irreversible aggregation of membrane proteins (2). Furthermore, it has been shown ultrastructurally that when rye cells were lethally dehydrated by extracellular freezing or plasmolysis, parts of the normally bilamellar structure of the membrane were irreversibly converted to osmiophilic granules or regions (20). Such ultrastructural alterations suggest denaturation or aggregation of lipids or lipoproteins. Thus alterations of the phospholipid packing in protoplast and EYL liposome membranes shown by the spin probes here indicate not only that changes at the molecular level during extracellular freezing are similar to those observed during dehydration but also that such changes

may result indirectly in irreversible membrane damage. Spin probe studies of protoplasts isolated from hardened winter rye, which survives freezing to moderately low temperatures, are in progress to further elucidate the mechanism of freezing injury and tolerance in plant cells.

Acknowledgment—The authors wish to acknowledge the skillful technical assistance of Mrs. B. Iu.

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