*Biochimica et Biophysica Acta*, 730 (1983) 111–118
Elsevier Biomedical Press



BBA 71611

# POLY(ETHYLENE GLYCOL)-INDUCED FUSION OF PLANT PROTOPLASTS

## A SPIN-LABEL STUDY

W.F. BOSS

*Department of Botany, North Carolina State University, Raleigh, NC 27650 (U.S.A.)*

(Received September 21st, 1982)
(Revised manuscript received January 12th, 1983)

*Key words: Poly(ethylene glycol); Protoplast fusion; ESR; Glutaraldehyde; Membrane fluidity*

**Carrot protoplasts were used to study the mechanism of poly(ethylene glycol)-induced fusion in vivo. In contrast to human erythrocytes, plant protoplasts can be induced to undergo massive fusions by adding recrystallized $PEG_{6000}$. ESR spectra of 5-doxylstearic acid (5DS)-labeled protoplasts indicate that $PEG_{6000}$ (> 20% w/v) causes a decrease in membrane fluidity and the appearance of a fluid signal indicating isotropic motion of the fatty acid spin label. The same ESR spectra are obtained if spin-labeled protoplasts are pretreated with glutaraldehyde. The 3-carboxyl-2,2,5,5-tetramethylpyrroline-1-oxyl derivative of poly(ethylene glycol) does not appear to intercalate into the hydrophobic region of the lipid bilayer; however, the apparent extraction of the 5DS-label from the protoplast membrane indicates that poly(ethylene glycol) can interact with membrane lipids.**

## Introduction

Poly(ethylene glycol) (PEG) is a commonly used fusogen which will induce interspecies as well as interkingdom fusion of plant protoplasts and animal cells [1–4]. The precise mechanism by which poly(ethylene glycol) induces fusion appears to be quite complex. Data now indicate that contaminating antioxidants and polymerization catalysts or terminators present in poly(ethylene glycol) are essential for fusion of human erythrocytes [5]. We have found this not to be so with plant protoplasts. Recrystallized poly(ethylene glycol) will fuse protoplasts with the same efficiency as non-recrystallized poly(ethylene glycol). Thus plant protoplasts afford a good system for studying the mechanism of poly(ethylene glycol)-induced fusion in vivo.

Ahkong et al. [6] hypothesized that chemical fusogens alter membrane structure to allow aggregation of intramembranous proteins and subsequent mixing of lipid rich regions of adhering bilayers. Knutton [7] found that in erythrocytes, poly(ethylene glycol) enhanced formation of protein denuded regions of the membrane. He observed discrete cytoplasmic continuities between cells which he proposed arose from fusion of lipid regions of the bilayer. The spherical fusion product formed as the cytoplasmic continuity expanded. The response of a membrane to a given fusogen should depend on membrane structure. That is, how readily do membrane proteins aggregate and is further stimulus necessary to cause mixing of lipid rich bilayers?

Studies of lipid fluidity measured by 8-anilino-1-naphthalene sulfonic acid fluorescence indicate that while poly(ethylene glycol) decreases fluidity

Abbreviations: PEG, poly(ethylene glycol); 5DS, 5-doxylstearic acid, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

in liposomes it causes an initial increase and then decrease in human erythrocytes [8]. Although poly(ethylene glycol) was initially used to fuse plant protoplasts [1], most reports of protoplast fusion are concerned with optimizing fusion conditions rather than studying the mechanism of fusion itself. We have used electron spin resonance to study the effects of commercial and recrystallized poly(ethylene glycol) on protoplast membrane fluidity. Fatty acid spin labels have been used to monitor membrane structure of plant protoplasts during freezing [9] and in response to divalent cations [10]. We initially found no change in fluidity with up to 32% (w/v) $PEG_{1540}$ [10]. Our studies were limited by low fusion percentages and protoplast breakage. We report here a more extensive study using $PEG_{6000}$ and $PEG_{8000}$. The higher molecular weight poly(ethylene glycol) induced massive fusions with minimal protoplast breakage. We have also measured poly(ethylene glycol)-induced fluidity changes after glutaraldehyde treatment which prevents protoplast lysis.

## Materials and Methods

*Carrot cell cultures*

Two cell culture lines were used for these studies. One line was maintained on a modified Murashige-Skoog medium with 1 mg/l naphthalene acetic acid and 0.2 mg/l kinetin as described previously [11]. These cells gave large vacuolated protoplasts (average diameter 30 $\mu$m). The second cell line was maintained on a modified Whites medium with 0.1 mg/l 2,4-dichlorophenoxyacetic acid [12]. The protoplasts from these cells were smaller (average diameter 15 $\mu$m) and thus gave better resolution for the election spin resonance (ESR) experiments. Both suspension cultures were transferred weekly and were used during log phase of growth (3–4 days after transfer). ESR and fusion data described below were similar with both cell cultures.

*Protoplast isolation*

Protoplasts were isolated in 2% Driselase and 0.4 molal sorbitol, 1 mmolal Mes (pH 5.8) as described previously [11]. The protoplasts were washed three times in 0.4 molal sorbitol, 1 mmolal Mes (pH 6.0) prior to use.

*Spin labeling*

Washed protoplasts were spin labeled with 5-doxylstearic acid (5DS) as described previously [10]. Although label incorporation was best at pH 6.0, ESR signal stability was enhanced by increasing the pH; thus label protoplasts were washed immediately in 0.4 molal sorbitol containing 10 mmolal potassium phosphate pH 7.2. The higher pH did not alter the maximum hyperfine splitting ($2A_{max}$). Labeled protoplasts were drawn up into a quartz capillary tube and the spectrum recorded on a Varian 109 B ESR spectrometer at room temperature (14 to 16 dB microwave power, 9.52 GHz microwave frequency, 100 G scan range 4 G modulation, 0.25 s time constant, 4 to 8 min scan time and a variable gain of approx. of $1.6 \cdot 10^4$.

*PEG-induced fusion*

For ESR studies of poly(ethylene glycol)-induced fusion two methods were employed. (1) Spin-labeled protoplasts were mixed gently with an equal volume of 40% (w/v) poly(ethylene glycol) in 0.4 molal sorbitol, centrifuged, and the spectrum run in quartz capillary tubes as described above. (2) Labeled protoplasts were taken up into disposable capillary tubes containing either 20% or 40% (w/v) poly(ethylene glycol) solutions; one end of the capillary was sealed; and the capillary was centrifuged at $40 \times g$. The capillary containing the pelleted protoplasts was placed in a quartz tube and the ESR spectrum run. The latter method enhanced protoplast packing and was especially useful with the larger diameter protoplasts.

*Synthesis of spin-labeled poly(ethylene glycol)*

The 3-carboxyl-2,2,5,5-tetramethylpyrroline-1-oxyl derivative of $PEG_{6000}$ was synthesized by a modification of the procedure of Boss et al. [13] and purified on a Sephadex G25 column. The infrared spectrum of the spin-labeled product showed a carbonyl ester stretching at 1745 $cm^{-1}$ versus 1730 $cm^{-1}$ for the acid starting material; the melting point was 55°C (lit. 55.5°C, [14]), and the ESR spectrum had 3 sharp lines with $A_N$ = 32.75 G in $H_2O$ versus 32 G for the starting material.

*Other chemicals*

The 5DS-spin label was obtained from Syva

Chemical Co. (U.S.A.). $PEG_{6000}$ (lot No. 190-0183) and $PEG_{1000}$ (lot No. 108C-0150) were obtained from Sigma Chemical Co. (U.S.A.) and were used without further purification or were recrystallized in $CHCl_3$/ether as described by Honda et al. [5]. The latter is denoted as recrystalized poly(ethylene glycol). $PEG_{8000}$ was a gift from Dr. John Wojcieszyn, UNC, Chapel Hill and was manufactured by BASF Wyandotle Corp. The $PEG_{8000}$ does not contain antioxidants and was shown by Dr. Wojcieszyn to be inefficient in causing fusion of erythrocytes with human fibroblasts (personal communication).

## Results

*Effects of poly(ethylene glycol) on spin-labeled protoplasts*

The following criteria were used to substantiate the presence of 5DS spin label in the plasma membrane of protoplasts: (1) the signal was enhanced by $10^{-5}$ M to $3 \cdot 10^{-2}$ M $K_3Fe(CN)_6$, a non-permeant ion, which stimulates metabolic reoxidation of spin labels associated with the outermost membrane [15]; (2) ascorbic acid (10 mM), which penetrates membranes, caused a reduction in the 5DS signal ($t_{1/2} = 4$ min); (3) $NiCl_2$ (3 mM), which will exchange broaden these spin labels in solution and which does not readily penetrate protoplasts, did not alter the ESR signal [16]; (4) lanthanum, a non-permeant cation, which has been purported to be a specific label for protoplast plasma membrane [17] perturbed the 5DS probe resulting in a 6 G increase in $2A_{max}$ and exchange broadening similar to that seen with calcium.

Protoplast fusion was routinely carried out by adding poly(ethylene glycol)/sorbitol solutions to protoplasts in a sorbitol/Mes osmoticum pH 6.0 (Fig. 1). The protoplasts fused equally well with $PEG_{6000}$, recrystalized $PEG_{6000}$, and $PEG_{8000}$. A completely ionic osmoticum of 0.25 M KCl, 0.01 M $MgCl_2$ and 0.05 M Tris, pH 7.2, could be used in place of sorbitol, and poly(ethylene glycol)-induced fusion was not affected. The fusion products, however, were less stable in the ionic osmoticum and tended to burst over the 20 min period necessary to complete the ESR studies. Thus, the sorbitol/Mes osmoticum was used for these studies. None of the osmoticums tested would, by itself, induce fusion of the carrot protoplasts. When 5DS-labeled protoplasts were treated with $PEG_{6000}$ at a final concentration of $\geq 20\%$ (w/v) the ESR spectrum indicated decreased membrane fluidity in the region of the 5DS label (Table I, Fig. 2A).

A unique feature of the ESR spectrum was the 'free spin' signal which was consistently found (denoted by the arrows) at these poly(ethylene glycol) concentrations. This signal is characteristic of a spin label undergoing isotropic motion such as that found when 5DS is in an organic solvent (for example MeOH), a solution of 20% poly(ethylene glycol), or a very fluid environment in the membrane. The 'free spin' signal was not tightly associated with the protoplasts (it could be recovered in the supernatant after one wash) and was exchange broadened by 3 mM $NiCl_2$. These data suggested that poly(ethylene glycol) had extracted some of the fatty acid spin label from the membrane bilayer. Thus poly(ethylene glycol) not only altered membrane fluidity but also appeared to solubilize the membrane associated fatty acid spin label. $PEG_{8000}$ and recrystallized $PEG_{6000}$ gave the same results.

When poly(ethylene glycol)-treated protoplasts were washed in a simple sorbitol osmoticum the original ESR signal was obtained (Table I, Fig. 2A); however, there was always some loss of signal intensity due to breakage. To test the validity of our data in a system in which the ESR signal was more stable, we used two approaches. One, we repeated these studies using isolated membranes, and two, we pretreated the protoplasts with glutaraldehyde thus preventing breakage.

*Effects of poly(ethylene glycol) on spin-labeled membranes*

A microsomal membrane fraction was isolated by differential centrifugation as previously described [11]. The membrane pellet was washed once in 0.4 molal sorbitol 1 mM Mes, pH 6.0, prior to spin labeling. The microsomal fraction afforded a membrane system which did not readily reduce the spin-label signal and which gave us a higher concentration of membranes per unit volume of sample. Thus the signal to noise was enhanced over that of the protoplast system. The

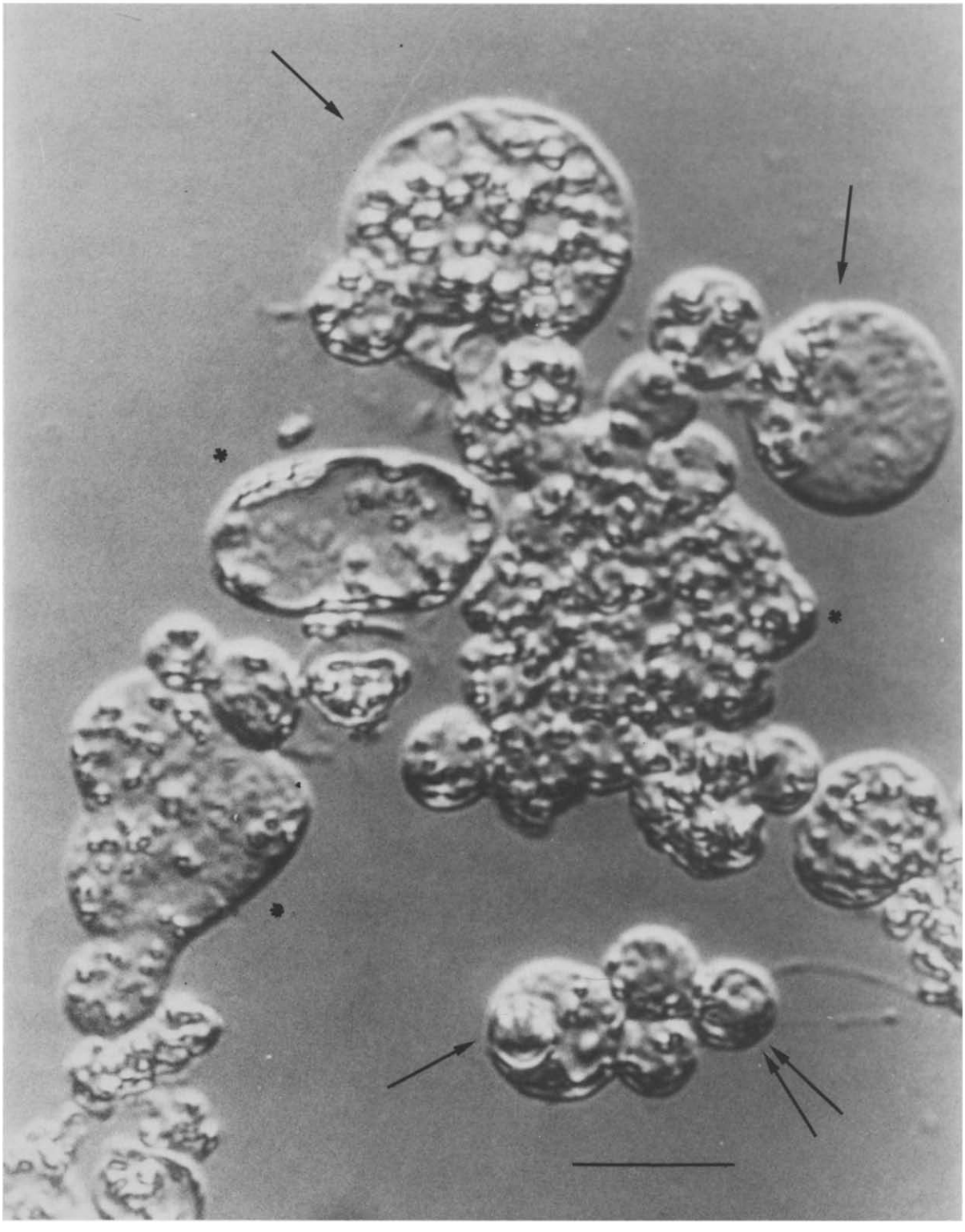

Fig. 1. Poly(ethylene glycol) fusion of carrot protoplasts. The carrot protoplasts prior to fusion are on the average 15 $\mu$m in diameter (double arrow). One drop of 40% (w/v) $PEG_{6000}$ was added to two drops of carrot protoplasts in sorbitol. Fusion occurred immediately at the PEG/sorbitol interface. Note some fusion products have rounded out (arrow) while others have not yet formed a complete sphere (*). (Bar = 30 $\mu$m; Nomarski differential interference contrast optics.)

TABLE I

MAXIMUM HYPERFINE SPLITTING OF 5DS-LABELED PROTOPLASTS AND MEMBRANES

Each figure is the mean of four values, S.D. $\pm$ 0.5 gauss.

| | $A_{max}$ (gauss) | | | |
|---|---|---|---|---|
| | Control | $Ca^{2+}$ (10 mM) | $\geq$ 20% $PEG_{6000}$ | PEG washed out |
| Protoplasts | 51.5 | 56 [a] | 54 [b] | 51.5 |
| Protoplasts in 0.25% (or 2.5%) glutaraldehyde | 51.5 | 56 [a] | 54 [b] | 54 |
| Microsomal membrane fraction from cells | 53 | 55.5 [a] | 55.5 [b] | 53 |

[a] Exchange broadened.

[b] Free spin evident.

5DS-labeled membranes responded to poly(ethylene glycol) in the same manner as the protoplasts. At concentrations $\geq$ 20% (w/v) $PEG_{6000}$ membrane fluidity decreased, the 'free spin' signal appeared, and when washed in sorbitol without poly(ethylene glycol) the original ESR signal was observed (Fig. 2B, Table I). $PEG_{6000}$, recrystallized $PEG_{6000}$, and $PEG_{8000}$ gave the same results.

*Effects of poly(ethylene glycol) on glutaraldehyde-treated protoplasts*

Studies have indicated that although glutaraldehyde crosslinks proteins, it does not alter the fluidity of the lipid bilayer of erythrocytes and phospholipid vesicles [18,19]. We have found this also to be true with protoplasts. After spin labeling, protoplasts were washed in 0.4 molal sorbitol and 10 mmolal sodium phosphate, pH 7.2, containing either 0.25% (v/v) or 2.5% (v/v) glutaraldehyde. Glutaraldehyde-treated spin-labeled protoplasts showed a typical response to 10 mM calcium (Table I). This indicated that the spin label was indeed in a very fluid environment in the membrane which, although it was not responsive to perturbations of membrane proteins resulting from glutaraldehyde treatment, could still respond to changes in lipid structure. Glutaraldehyde treatment stabilized the 5DS signal and increased protoplast packing such that the signal to noise ratio was greatly enhanced. When poly(ethylene glycol) was added to glutaraldehyde-treated protoplasts, the 5DS signal indicated decreased fluidity (Fig. 2C) and solubilization of the spin label (note arrow) similar to that seen in non-glutaraldehyde-treated protoplasts. In the presence of poly(ethylene glycol) the glutaraldehyde- treated protoplasts plasmolyzed and agglutinated. If calcium was present some of the protoplasts appeared to fuse at the points of adhesion. Since membrane proteins were crosslinked, the 'fusion' products could not round out. Thus we could not ascertain by light microscopy whether the protoplasts had, in fact, fused. (Electron microscopy studies are in progress to answer this question.) The glutaraldehyde-treated protoplasts retained their plasmolyzed state and corresponding ESR signal even after several washings in poly(ethylene glycol)-free osmoticum (Table I, Fig. 2C). As with non-glutaraldehyde treated protoplasts and with the microsomal membranes, $PEG_{6000}$, recrystallized $PEG_{6000}$, and $PEG_{8000}$ gave the same results.

In earlier studies where fusion rates were 10% or less, we observed no fluidity change or free spin for up to 30% $PEG_{1540}$ [10]. Rapid signal loss confounded these studies and was most likely due to protoplast breakage. We repeated the experiments using $PEG_{1000}$ (Sigma Chem. Co.) and glutaraldehyde-pretreated protoplasts and still found no change in fluidity at up to 30% poly(ethylene glycol) (data not shown). Even at 40% $PEG_{1000}$, where aggregation and plasmolysis of the protoplasts was extreme, we could only detect slightly decreased fluidity (less than 1 gauss) and no free spin evident with glutaraldehyde-pretreated protoplasts. Although agglutination was extensive under these conditions, fusion was only on the order of 10% if protoplasts were not treated with glutaraldehyde. The low fusion percentage corre-

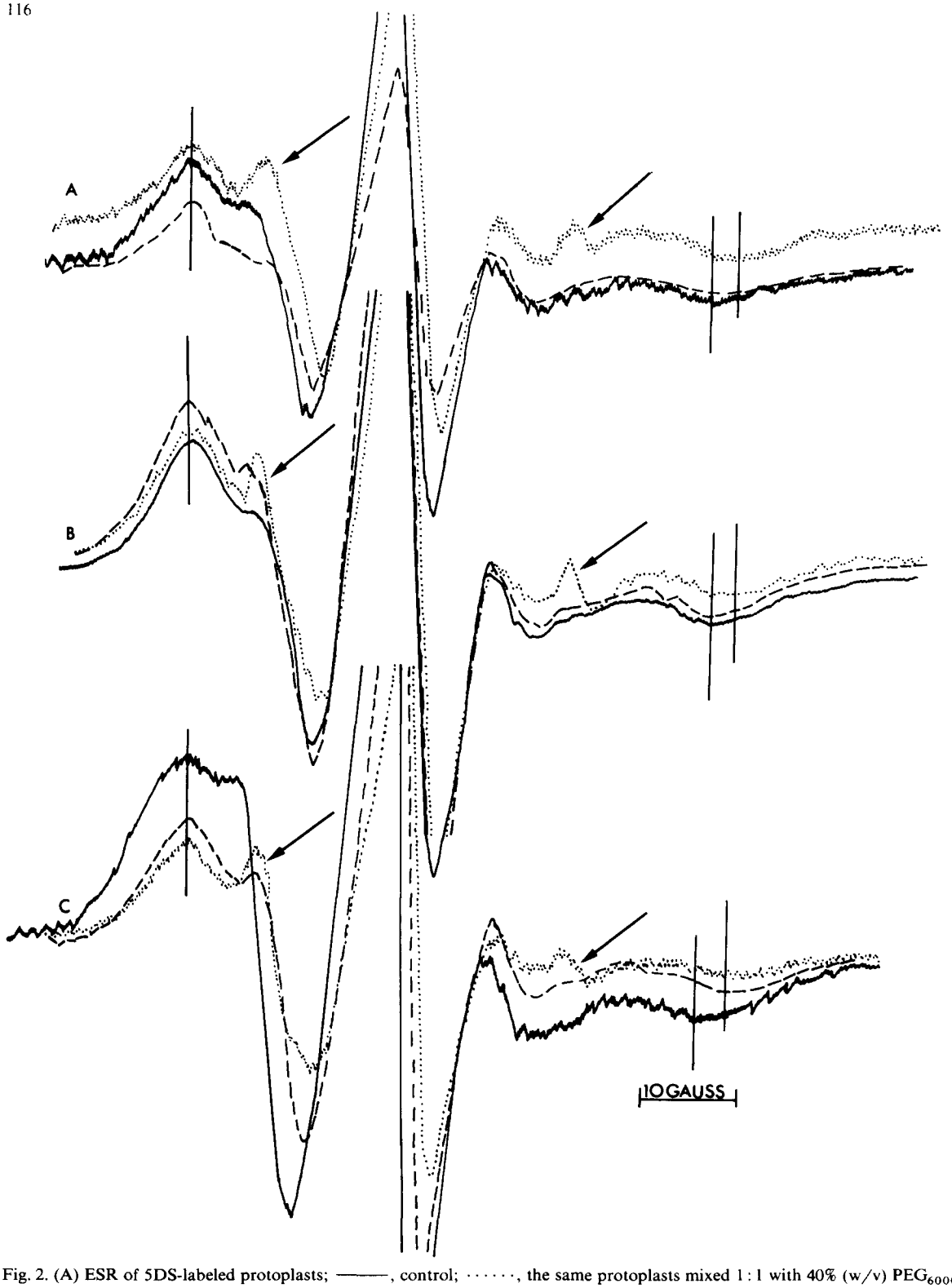


Fig. 2. (A) ESR of 5DS-labeled protoplasts; ———, control; · · · · · ·, the same protoplasts mixed 1 : 1 with 40% (w/v) $PEG_{6000}$; — — —, the same protoplasts washed in 5 ml sorbitol osmoticum and rescanned. (The dashed line represents an average of the noise and is run under the same conditions as the dotted line spectrum.) (B) ESR treatments are as described for (A): ———, control; · · · · · ·, plus PEG; — — —, PEG-treated protoplasts after one wash in sorbitol. (C) ESR of 5DS-labeled protoplasts pretreated with 0.25% glutaraldehyde and then treated as described for (A): ———, control; · · · · · ·, plus $PEG_{6000}$; — — —, PEG-treated protoplasts after several washes in sorbitol osmoticum.

lates with the lack of detectable free-spin signal and the marginal change in membrane fluidity.

*Studies with spin-labeled poly(ethylene glycol)*

If poly(ethylene glycol) intercalates into the hydrophobic regions of membranes the $2A_{max}$ of spin-labeled poly(ethylene glycol) could reflect such a change in environment; thus, spin-labeled poly(ethylene glycol) was added to the protoplasts in the presence of 0, 20, and 40% unlabeled poly(ethylene glycol). Although the maximum hyperfine splitting decreased slightly (0.5 G) with increasing poly(ethylene glycol) concentration, in all cases the presence of protoplasts did not alter the splitting. The sharp, three-lined spectrum could be exchange broadened with 3 mM $NiCl_2$ and was readily washed free from the protoplasts.

## Discussion

$PEG_{6000}$ was used for these studies because it caused massive fusions (nearly 100% fusion when 40% poly(ethylene glycol) was mixed 1 : 1 with protoplasts) with minimal protoplast damage. With plant protoplasts, poly(ethylene glycol)-induced fusion occurs when the poly(ethylene glycol) is added and does not require removal of most of poly(ethylene glycol) as reported for erythrocytes [5,7]. At these concentrations of $PEG_{6000}$, 5DS spin-labeled protoplasts showed decreased fluidity of membrane-associated probe and the appearance of free spin or spin label undergoing isotropic motion. Similar results were obtained with isolated membranes and with glutaraldehyde-pretreated protoplasts, suggesting that the ESR signal was due to changes in structure of the lipid bilayer of the protoplast membrane and that these changes could take place even when membrane proteins were immobilized.

These data represent an average of the protoplast population present; therefore, it is not surprising that we did not observe a significant change in the ESR signal under conditions where the fusion percentage was low ($< 20\%$ $PEG_{6000}$ or $\leq 40\%$ $PEG_{1000}$).

Spin-labeled $PEG_{6000}$ did not show significant change in $2A_{max}$ in the presence of protoplasts. However, the fact that free spin is evident in 5DS-labeled protoplasts at poly(ethylene glycol) concentrations which cause massive fusion suggests that poly(ethylene glycol) can interact with membrane lipids. Our data showing that poly(ethylene glycol) can solubilize membrane-associated fatty acid spin label and electron microscopy studies showing lipid droplets associated with poly(ethylene glycol)-treated erythrocytes [7] indicate that when studying effects of poly(ethylene glycol) with membrane probes one should consider the potential for solubilization of the probe as well as the membrane by the amphaphite, poly(ethylene glycol).

Spin-label studies of plant protoplasts are difficult to do for two major reasons. First, the relatively large size (15 to 30 $\mu$m diameter) means less signal per volume. Secondly, the signal that is present is rapidly reduced with time. By labeling the protoplasts at pH 6.0 and washing at pH 7.2 we were able to increase signal stability. Similarly, 0.25% glutaraldehyde treatment enhanced signal stability while also maintaining membrane integrity. At 2.5% glutaraldehyde there was no detectable signal loss over the time course of these studies. Thus glutaraldehyde treatment may prove to be useful for long term ESR studies of the responses of protoplast membrane lipids.

There are at least two major differences in poly(ethylene glycol)-induced fusion of plant protoplasts and that of animal cells. One, fusion of plant protoplasts takes place almost immediately upon the addition of poly(ethylene glycol) and without further dilution. Two, antioxidants or free radical chain terminating contaminants necessary for erythrocyte fusion [5] are not essential for protoplast fusion. Although these differences may reflect inherent differences in the animal and plant cell membrane, it is reasonable to suspect that a more direct effect of the 1 to 2 h treatment in Driselase is the cause for the different fusion responses. Driselase, like most cellulase preparations, contains some protease activity. Akhong et al. [6] have proposed that chemical induced fusion depends on how effectively a fusogen can displace intramembrane proteins, thus exposing lipid rich regions. These lipid rich regions in adjacent membranes are then amenable to the action of the chemical fusogen or may fuse spontaneously. If this hypothesis is valid, then the proteins of plant protoplasts either respond more readily to the

poly(ethylene glycol), giving lipid-rich regions as described by Knutton [7] for erythrocytes, or discrete lipid-rich regions are already present in the bilayer. If the latter is true, then fusion is limited only by adhesion and lipid mixing and we should be able to see discrete cytoplasmic connections between glutaraldehyde-treated protoplasts after exposure to poly(ethylene glycol). Electron microscopy studies are in progress to substantiate this hypothesis. In comparing poly(ethylene glycol)-induced fusion of erythrocytes and liposomes, Ohno et al. [8] suggested that if a system existed where intramembrane proteins were already displaced such that lipid rich regions were present, fusion would proceed more like the liposome system. Perhaps plant protoplasts will prove to be such a system.

In conclusion, poly(ethylene glycol) concentrations (> 20% $PEG_{6000}$ or $PEG_{8000}$) which cause massive fusion of plant protoplasts decrease membrane fluidity in the region of the 5DS probe and at the same time mobilize some of the 5DS probe to a very fluid environment. Fusion occurs in the presence of poly(ethylene glycol) and does not require antioxidants. Washing the protoplasts free of poly(ethylene glycol) reestablishes the original ESR signal, and the freely mobile signal is lost. These data suggest that poly(ethylene glycol) both stabilizes (e.g. decreased fluidity) and destabilizes (e.g. mobilizes fatty acids) the membrane bilayer during protoplast fusion.

## Acknowledgement

This work was supported by the North Carolina Board of Science and Technology and by N.C. Agricultural Research Service. This is Paper No. 8512 of the Journal Series of the North Carolina Agricultural Research Service.

## References

1 Kao, K.N., Constabel, F., Michayluk, M.R. and Gamborg, O.L. (1974) Planta 120, 215–227
2 Wallin, A., Glimelius, K. and Eriksson, T. (1974) Z. Pflanzenphysiol. 74, 64–80
3 Dudits, D., Raski, I., Hadlaczky, C.Y. and Lima-de-Faria, A. (1976) Hereditas 82, 121–124
4 Jones, C.W., Mastranglo, I.A., Smith, Liu, H.Z. and Meck, R.A. (1976) Science 193, 401–403
5 Honda, K., Maeda, Y., Sasakawa, S., Ohno, H. and Tsuchida, E. (1981) Biochem. Biophys. Res. Commun. 101, 165–171
6 Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. (1975) Nature 253, 194–195
7 Knutton, S. (1979) J. Cell Sci. 36, 61–72
8 Ohno, H., Sakai, T., Tsuchida, E., Honda, K. and Sasakawa, S. (1981) Biochem. Biophys. Res. Commun. 102, 426–431
9 Singh, J. and Miller, R.W. (1980) Plant Physiol. 66, 349–352
10 Boss, W.F. and Mott, R.L. (1980) Plant Physiol. 66, 835–837
11 Boss, W.F. and Ruesink, A.W. (1979) Plant Physiol. 64, 1005–1011
12 Wetherell, D.F. and Dougal, D.K. (1976) Physiol. Plant. 37, 97–103
13 Boss, W.F., Kelley, C.F. and Landsberger, F.R. (1975) Anal. Biochem. 64, 289–292
14 Törmälä, P., Latillä, H. and Lindberg, J.L. (1973) Polymer 14, 481–487
15 Kaplan, J., Canonico, P.G. and Caspary, W.J. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 66–70
16 Hyde, J.S., Swartz, H.M. and Antholine, W.E. (1979) in Spin Labeling II, Theory and Applications (Berliner, L.J., ed.), pp. 71–113, Academic Press, New York
17 Taylor, A.R.D. and Hall, J.L. (1979) Plant Sci. Lett. 14, 139–144
18 Jost, P., Brooks, U.J. and Griffith, O.H. (1973) J. Mol. Biol. 76, 313–318
19 Komorowska, M., Koter, M. Bartosz, G. and Gomulkiewicz, J. (1982) Biochim. Biophys. Acta 686, 94–98