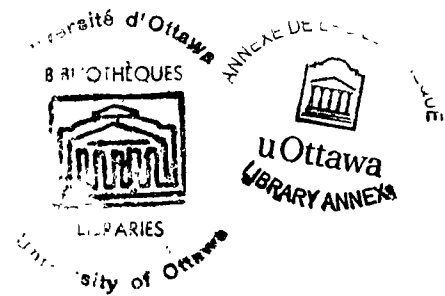


**PHYSIOLOGICAL RESPONSES OF WINTER RYE**  
*(Secale cereale L. cvs PUMA AND ANIMO)*  
**TO NON-LETHAL FREEZING TEMPERATURES**

by  
**Robert Langis**



A thesis

presented to the University of Ottawa  
in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy in Biology

Faculty of Science, Department of Biology  
University of Ottawa  
Ottawa, Ontario, Canada



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A MA BIEN AIMEE CAROLE

A CAMILLE

A MES PARENTS OMER ET MARGUERITE

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## ABSTRACT

The response of cold-acclimated winter rye (*Secale cereale* L. cvs Puma and Animo) during prolonged exposure to non-lethal freezing temperatures was investigated. The primary leaf tissues and their isolated protoplasts were maintained in the frozen state over the temperature range of  $-4$  to  $-16^{\circ}\text{C}$  for periods of time up to 240 hours.

In a first study, the leakiness of tissue sections after thawing was measured by the release of electrolytes. At non-lethal temperatures (e.g.,  $-10^{\circ}\text{C}$ ), the amount of electrolyte released by the tissues was proportional to the duration of the freezing treatment. Leakiness of the tissues was related to survival as estimated by expansion of the tissue sections in water and by the synthesis of chlorophyll under light. Leakiness was associated with a lag phase in chlorophyll synthesis, which was restored in the tissues only when the release of electrolytes reached a plateau. In Puma, freezing to  $-10^{\circ}\text{C}$  for 6 days led to the release of  $5.6 \times 10^{-5}$  mole of electrolytes $\cdot\text{g}^{-1}$  fr wt (equivalent  $\text{K}^{+}$ ) or 32% of the total electrolyte content of the tissues during the first four hours following thawing. Incubation in the presence of 1 mM calcium reduced this amount by almost 25%. In spite of this leakage, the tissue sections expanded fully in water and developed chlorophyll uniformly with no sign of local injury after 48 hours. However, in samples frozen to  $-10^{\circ}\text{C}$  for 10 days, the release of electrolytes reached 42% of the total in the tissue samples. In this case, the temperature of incubation influenced the post-thawing release of electrolytes. The amount of electrolytes released after thawing was significantly less at  $5^{\circ}\text{C}$  than at  $25^{\circ}\text{C}$ . This temperature effect on electrolyte leakage influenced survival: after 48 hours, only 86% of the tissue sections incubated at  $25^{\circ}\text{C}$  fully expanded and developed chlorophyll compared to 93% at  $5^{\circ}\text{C}$ . The other sections remained unexpanded and yellow-white after 48 hours. It was

concluded that the lack of stability of the cellular membranes and particularly the plasma membrane soon after thawing is manifested by leakiness. This leakiness determines the development of lethal injury after thawing. Low temperature during the post-thaw incubation period reduced the extent of electrolyte release by the tissues probably via a stabilizing effect on the injured membranes. Such a stabilizing effect reduces the leakiness of the membranes and consequently the probability of attainment of irreversible damage in critically stressed cells.

In a second series of experiments, mesophyll protoplasts were extracted from primary leaf tissue sections and were frozen to similar temperatures ( $-8^{\circ}\text{C}$ ,  $-10^{\circ}\text{C}$ ) for durations varying from 16 to 66 hours to investigate the response at the cellular level with a direct access to the plasma membrane and to the intracellular organization (compartmentation). The release of electrolytes from frozen-thawed protoplasts in the absence of physical disruption of the plasma membrane was evidenced by the reduction in the osmotic volume of the surviving protoplasts (spherical) as compared to the unfrozen controls in both cultivars (mean volume of 7900 vs. 9600  $\mu\text{m}^3$ ). The physical integrity of the plasma membrane of the surviving, but leaky, protoplasts was evidenced by the retention of sphericity and normal osmotic responsiveness of the protoplasts. The reduction in osmotic volume was not the result of an alteration in passive permeability since fluorescein was retained to the same extent in frozen-thawed, surviving protoplasts and since the non-penetrating fluorescent dye DAPI was excluded in both unfrozen controls and frozen-thawed protoplasts. In addition, there was no major difference in the fluorescence polarization signal of the probe TMA-DPH (at  $10^{\circ}\text{C}$ ) in frozen-thawed and unfrozen protoplasts indicating a similar average fluidity of the plasma membrane.

Vacuolar compartmentation of neutral red and acridine orange (sequestration of

the dye molecules in the vacuole) was observed in the majority of the unfrozen protoplasts (more than 90%). In the surviving protoplasts of frozen-thawed suspensions, there was proportionally more of the apparently intact frozen-thawed protoplasts showing decompartmentation (accumulation of the dyes in the cytoplasm). These protoplasts were unable to hydrolyse FDA and to accumulate fluorescein probably because of cytoplasmic acidification. In many cases, cytoplasmic acidification was accompanied by polyvacuolization of the protoplasts and the lack of accumulation of the dye in the vacuole. As the freezing temperature decreased, the relative frequency of the decompartmented response increased indicating that the accumulation of chemical damage with time in the frozen state depends on the extent of dehydration. It was concluded that in severely damaged protoplasts vacuolar decompartmentation is associated with cytoplasmic acidosis after thawing.

In suspensions frozen to  $-9^{\circ}\text{C}$  for 16 hours, vacuolar compartmentation was observed in the majority of the surviving protoplasts, with only about a 10% increase in the decompartmented protoplasts as compared to the controls. Nevertheless, a reduced osmotic volume was a general manifestation in the surviving population. In this case, the decrease in osmotic volume was accompanied by uptake of acridine orange in the vacuole, indicating vacuolar acidification in the absence of decompartmentation. Recovery for 8 hours at  $5^{\circ}\text{C}$  was associated with the recovery of the normal vacuolar pH in surviving, compartmented protoplasts.

It was concluded that the vacuolar acidification observed in apparently physically intact, frozen-thawed protoplasts is a consequence of prolonged exposure to non-lethal freezing temperatures. Recovery and long term survival depend on the maintenance of semipermeable characteristics of the tonoplast, and the development of lethal damage after freezing may be the result of the altered

capacity of the vacuole to accumulate and retain protons, leading to cytoplasmic acidosis and death. The results emphasize the primary importance of the proton pumps at the tonoplast for the regulation of cytoplasmic pH and the maintenance of homeostasis and vitality of the plant cell under freezing stress conditions.

## RESUME

La réponse de deux cultivars de seigle d'hiver (*Secale cereale* vars Puma et Animo) à des conditions non-létales de gel prolongé est étudiée. Des sections de tissus provenant de la jeune feuille primaire de plantules étiolées endurcis au froid, ainsi que les protoplastes des cellules mésophylles provenant de ces mêmes tissus, sont gelés à des températures variant de -8 à -10°C pour une durée allant jusqu'à 240 heures (10 jours).

Dans une première série d'expériences, la conductivité du milieu d'incubation des sections de tissus gelés est mesurée pendant la période de recouvrement afin d'évaluer les pertes en électrolytes des tissus. A une température de gel non-létale (-10°C), la quantité d'électrolytes perdus par les tissus après le dégel est proportionnelle à la durée de l'exposition au gel. Après un gel à -10°C durant 6 jours, on mesure, pendant les quatre premières heures suivant le dégel, des pertes en électrolytes équivalente à  $5.6 \times 10^{-5}$  mole d'électrolytes-g<sup>-1</sup> fr wt (équivalent K<sup>+</sup>) ou 32% du contenu total des tissus en électrolytes. En dépit de ces pertes, la totalité des sections de tissus prennent une expansion équivalente à celle des tissus témoins dans l'eau et produisent de la chlorophylle uniformément, ne présentant aucun indice de dommage localisé après 48 heures. Cependant, la production de chlorophylle par les cellules mesophylles est retardée dans les tissus gelés, et ne se rétablit que lorsque les efflux d'électrolytes cessent à environ 4 heures suivant le dégel.

Dans les échantillons maintenus à -10°C pendant 10 jours, les pertes en électrolytes des sections de tissus atteignent 42% du contenu total diffusible. Dans ce cas, la température initiale d'incubation a une influence sur les pertes en électrolytes, les pertes étant moindres à 5°C qu'à 25°C. Ces pertes en électrolytes plus importantes déterminent apparemment le développement d'un dommage

irréversible dans les tissus puisque, après 48 heures d'incubation, seulement 86% des sections de tissus incubées à 25°C prennent de l'expansion dans l'eau et produisent de la chlorophylle comparé à 93% dans les échantillons incubés à 5°C. On en déduit que les pertes en électrolytes des tissus dépendent d'un manque de stabilité et d'intégrité des membranes cellulaires après le dégel. Une incubation à basse température diminue l'importance de ces pertes probablement via un effet stabilisateur sur les membranes endommagées et réduit ainsi la probabilité du développement d'un dommage irréversible dans les cellules.

Dans une seconde série d'expériences, les protoplastes des cellules mésophylles sont isolées des jeunes feuilles primaires et gelés pour des durées allant de 16 à 66 heures. Ici, la réponse à un gel modéré prolongé est étudiée au niveau cellulaire avec un accès direct à la membrane plasmique et à l'organisation intracellulaire (compartimentation). Après le dégel, les pertes en électrolytes des protoplastes sont associées à une diminution du volume osmotique des protoplastes. En dépit de ce changement apparent de perméabilité, l'intégrité physique de la membrane plasmique des protoplastes gelés est préservée comme le démontre la sphéricité des protoplastes en suspension. Des mesures de rétention de la fluorescein et de l'exclusion du marqueur fluorescent DAPI permettent de rejeter la possibilité d'une altération dans les propriétés de perméabilité passives de la membrane plasmique.

Dans les suspensions non-gelées, une compartimentation vacuolaire des marqueurs rouges neutre et acridine orange (séquestration du marqueur dans la vacuole), est observée dans la majorité des protoplastes (plus de 90%). Dans les protoplastes gelés mais apparemment intacts à la suite du dégel, une proportion plus importante des protoplastes présentent une décompartimentation du marqueur (accumulation du marqueur dans le cytoplasme). Ces protoplastes décompartimentés sont incapables d'hydrolyser la diacétate de fluorescein et d'accumuler la fluorescein

probablement à cause d'une acidification du cytoplasme. Dans plusieurs cas, l'acidification du cytoplasme est accompagnée d'une polyvacuolisation ainsi que par l'incapacité d'accumuler le marqueur acridine orange dans la vacuole. A mesure que la température de gel est amenée à des valeurs plus négatives, la fréquence des protoplastes présentant une décompartmentation du marqueur est augmentée, ce qui indique que le développement de cette forme de dommage dépend de la déhydratation cellulaire. On peut conclure de ces résultats que dans les cellules endommagées, une décompartmentation de la vacuole est associée à une acidification du cytoplasme à la suite du dégel.

Dans les suspensions gelées à  $-9^{\circ}\text{C}$  durant 16 heures, les protoplastes qui récupèrent présentent une compartimentation vacuolaire du marqueur. Ce résultat indique que le recouvrement après le dégel dépend du maintien des propriétés de semipermeabilité du tonoplaste. Néanmoins, une diminution du volume osmotique est mesurée dans l'ensemble de la population. Dans ce cas, la diminution du volume osmotique s'accompagne d'une accumulation du marqueur acridine orange dans la vacuole (acidification de la vacuole). Cette acidification de la vacuole est réversible si les protoplastes sont incubés à  $5^{\circ}\text{C}$  pendant 6 heures après le dégel avant l'addition du marqueur. Puisque le recouvrement et la survie à long terme dépendent du maintien des propriétés de semipermeabilité du tonoplaste, le développement d'un dommage irréversible après le dégel peut être le résultat d'une incapacité de la vacuole à accumuler et retenir des protons en excès dans le cytoplasme. Une telle situation correspond à une acidification du cytoplasme et à la mort de la cellule. Ces résultats soulignent le rôle du tonoplaste dans la régulation du pH cytoplasmique et le maintien de l'homéostasie cellulaire et de la vitalité.

**Table of Abbreviations**

$A_i$	Intracellular amount of acridine orange dye
$A_o$	Extracellular amount of acridine orange dye
ACTCC	Apparent conductivity temperature compensation coefficient of bathing solution containing tissue sections (% increase in conductivity for an increase of 1°C in the temperature of incubation of the samples)
AH	Cold-acclimated Animo winter rye
AHF	Frozen-thawed, cold-acclimated Animo winter rye
AHNF	Unfrozen, cold-acclimated Animo winter rye
ANH	Nonacclimated Animo winter rye
AO	Non-protonated acridine orange molecule
$AOH^+$	Protonated acridine orange molecule
BSA	Bovine serum albumine
CTCC	Conductivity temperature compensation coefficient. % increase in conductivity of an electrolytic solution for an increase of 1°C in temperature ( $\% \cdot ^\circ C^{-1}$ )
DAPI	4-6 diamidino-2 phenyl indole
DPH	Diphenyl-hexatriene
DTT	Dithiothreitol
$E_m$	Emission wavelength (fluorescence wavelength)
ETOH	Pure ethanol
$E_x$	Excitation wavelength
FC	Fusicoccin
FDA	Fluorescein diacetate
GDW	Glass distilled water
IMP	Intramembranous particle

LT <sub>50</sub>	Lethal temperature 50: the freezing temperature where 50% of the individuals in a population or a sample are killed
PH	Cold acclimated Puma winter rye
PHF	Frozen-thawed, cold-acclimated Puma winter rye
PHNF	Unfrozen, cold-acclimated Puma winter rye
PNH	Non-acclimated Puma winter rye
PMF	Proton electromotive force
pH	$-\log_{10} [\text{H}^+]$ of a solution; $[\text{H}^+]$ expressed in $\text{ions}\cdot\text{g}\cdot\text{L}^{-1}$
pH <sub>o</sub>	Extracellular pH
pH <sub>i</sub>	Intracellular pH
<sup>31</sup> P NMR	<sup>31</sup> P phosphorus nuclear magnetic resonance
R 5/25	Temperature regime for recovery: 24 h at 5°C followed by 24 h at 25°C
R 25/5	Temperature regime for recovery: 24 h at 25°C followed by 24 h at 5°C
rpm	Rotation per minute
SD	Standard deviation
SEM	Standard error of the mean
TDCR	Temperature dependent conductivity ratio (conductivity value at 25°C divided by conductivity value at 5°C)
TMA-DPH	Trimethylamino-diphenylhenatriene
V <sub>i</sub>	Intracellular volume
V <sub>o</sub>	Extracellular volume

**Time Units**

s	second
ms	millisecond
min	minute
h	hour
d	day

**Length and Volume Units**

cm	centimeter ( $10^2$ m)
mm	millimeter ( $10^{-3}$ m)
$\mu\text{m}$	micrometer ( $10^{-6}$ m)
nm	nanometer ( $10^{-9}$ m)
L	liter
ml	( $10^{-3}$ L)
$\mu\text{l}$	microliter ( $10^{-6}$ L)

**Weight and Concentration Units**

g	gram
mg	milligram ( $10^{-3}$ g)
$\mu\text{g}$	microgram ( $10^{-6}$ g)
$\text{g}\cdot\text{ml}^{-1}$	grams per milliliter
$\mu\text{g}\cdot\text{ml}^{-1}$	micrograms per milliliter
$\mu\text{g}\cdot\text{g}^{-1}$ fr $\text{wt}\cdot\text{h}^{-1}$	microgram per gram fresh weight per hour
$\mu\text{C}$	microcurie
$\text{mC}\cdot\text{mg}^{-1}$	millicurie per milligram
% (w/v)	percent weight per volume (gram per 100 milliliter)

% (v/v)	percent volume per volume (milliliter per 100 milliliter)
M	molar
mM	millimolar
$\mu$ M	micromolar
prot $\cdot$ ml <sup>-1</sup>	protoplast per milliliter

**Conductivity Unit**

$\mu$ MHO(s) micro MHO

**Temperature Unit**

$^{\circ}$ C degree Celsius

**Centrifugation unit**

*g* Gravitational constant equal to  $6.6732 \times 10^{-11} \text{ N}\cdot\text{m}^2\text{kg}^{-2}$

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## CHAPTER I

### GENERAL INTRODUCTION

#### A. Overview

##### 1. Physiology of Crop Plants Under Non-Lethal Conditions of Environmental Stress.

The primary motivation for the study of the physiology of crop plants under environmental stress resides in the need for improving crop production in unfavorable environments and to reduce the risk of loss due to unpredictable climatic variations. An ever-increasing world population implies that food productivity must increase in a similar way. Actually, the average yield of most crops is far below the potential yield, and this is largely dependent on environmental factors such as drought and low temperature stresses (Weiser, 1982). As pointed out by Weiser (1982), important gains in productivity have been and can still be achieved by selecting crops for increased environmental adaptation rather than yield potential. Therefore, attention must be directed to the identification and transfer of genetically controlled characteristics of tolerance of plants adapted to unfavorable environments either via traditional breeding technology or via the application of gene transfer technology coupled to plant cell and tissue culture technology.

The transfer of stress tolerance characteristics to sensitive crop plants primarily depends on the identification of the characteristics at the molecular level and on the understanding of the physiology associated with their expression. In low temperature stress physiology, there are many studies of the biochemical changes that occur during cold acclimation, but the significance of the measured changes is

often unknown. As stressed by Steponkus (1984), in the absence of an understanding of the nature of freezing injury, it is difficult to envisage how the numerous biochemical changes occurring during cold-acclimation can be accurately assessed. Until now, the study of freezing injury of crop plants has concentrated largely on the understanding of the mechanism(s) of lethal injury at the cellular level (Steponkus, 1981; Gordon-Kamm and Steponkus, 1984; Mazur, 1969; Singh and Johnson-Flanagan, 1986; Singh and Miller, 1982; Willemot, 1983; Yoshida and Sakai, 1974; Dexter, 1932), but little attention has been given to the non-lethal effects of freezing stresses on plants.

Non-lethal effects can be associated with a certain degree of injury after thawing. Palta and his colleagues (Palta *et al.*, 1977a,b) clearly demonstrated that onion bulb cells either degenerate or recover following thawing. Their objective was to characterize the mechanism of development of lethal injury after thawing. In spite of a significant contribution, this mechanism is still poorly understood and many questions still remain to be answered. In addition, an important aspect of the non-lethal response of plants refers to the capability of the plant cells to express active recovery or repair mechanisms following thawing. Almost nothing has been done to understand the mechanisms by which frozen cells can recover. There is a need for the identification of cellular activity that may contribute to the avoidance of lethal injury after prolonged exposure to non-lethal freezing temperatures. Therefore, since cold acclimated plants are naturally able to undergo freezing and to recover from it, the study of their behavior under non-lethal conditions constitutes an important avenue for the identification of potentially transferrable characters of tolerance.

## 2. Extracellular Freezing and Freeze-Induced Cellular Dehydration.

Generally, plants adapted to frost do not avoid freezing; rather they accommodate ice within their tissues (Levitt, 1980). However, intracellular ice formation constitutes a lethal stress for all plant cells, whether or not they are adapted to freezing (Levitt, 1980). In most cases, intracellular ice does not form because heterogeneous, extracellular ice nucleation takes place first. As a result, the negative vapor pressure that develops outside the cells induces the efflux of water from the cells to the extracellular ice, and the cells achieve vapor pressure equilibrium by dehydration (Mazur, 1969). The extent of dehydration depends on the chemical potential of the ice, which is a direct function of the subzero temperature (Mazur, 1969), and on the intracellular solute concentration (Meryman and Williams, 1985).

Under natural freezing conditions (more or less prolonged frost characterized by slow variations in temperature), freeze-induced dehydration is the most critical stress experienced by plants and results in a variety of consequences for the cells. Cellular membranes, especially the plasma membrane and tonoplast, are subjected to mechanical stresses during osmotic excursions (Steponkus, 1984). Solution effects are associated with decreased water activity and increased concentration of cell solutes, notably electrolytes, and the resultant chemical stresses will induce chemical damages which are cumulative with time (Levitt, 1980).

## 3. Mechanical and Chemical Effects of Freeze-Induced Cellular Dehydration

During freezing, cells contract osmotically because of the gradient in vapor pressure between the intracellular solution and the extracellular ice (Mazur, 1969). In Steponkus' view (1981), the rapidity with which lethal injury is manifested during freezing suggests that injury is not due to metabolic dysfunction but rather

to a major, irreversible physical alteration of the plasma membrane. Here survival is essentially determined by the capability of the plasma membrane to resist the physical and chemical stresses associated with extreme freeze-induced dehydration. According to this view, one facet of cold acclimation includes alterations that allow avoidance or tolerance of excessive cell volume reduction and water loss, which may irreversibly destabilize cellular membranes (Meryman and Williams, 1985). Therefore, a very important adaptive aspect of the tolerance to freezing is the stability of cellular structures and constituents to severe dehydration at extreme temperatures. Destabilization and physical alterations of the plasma membrane have been associated with the following lethal responses (Steponkus, 1985):

1. Expansion-inducing lysis during thawing. Osmotic contraction results in the deletion of plasma membrane material in the form of endocytotic vesicles (Steponkus, 1984; Johnson-Flanagan and Singh, 1986). To avoid development of a critical tension in the plasma membrane on thawing (deplasmolysis), re-incorporation of membrane material is required (Wolfe and Steponkus, 1983). Expansion-inducing lysis is related to the limited capacity of the plasma membrane to reincorporate membrane material during osmotic expansion.
2. Loss of osmotic responsiveness such that the protoplast does not expand during thawing and rehydration and which may be the consequence of bilayer-to-hexagonal<sub>II</sub> phase transitions during osmotic dehydration.

The increase in the intracellular solute concentration (particularly sugars and amino acids) during cold acclimation reduces the extent of dehydration at a given subzero temperature (Meryman and Williams, 1985) and consequently reduces the probability of occurrence of these lethal responses. In addition, sugars can interact with membranes by means of hydrogen bonding providing stability to the membranes at low water activities (Crowe *et al.*, 1984). Finally, the increase in

concentration of non-toxic cryoprotectants such as sugars and some amino acids proportionally reduces the concentration of salts and other potentially toxic compounds (colligative cryoprotection) (Heber *et al.*, 1971).

Nevertheless, under prolonged moderate freezing conditions, there is the potential for damage as a result of degradative reactions that cannot be compensated by metabolic activity because of the low water activity and the temperature at which freezing occurs. In thermodynamic terms, natural freezing temperatures are not sufficiently low to prevent chemical reactions. This situation creates the potential for physiological dysfunction or non-lethal damage after thawing.

#### 4. Non-Lethal Damage and Recovery

Alteration of the permeability properties of the plasma membrane after thawing can be observed in the absence of physical disruption or alteration of the passive permeability properties of the membrane (Palta *et al.*, 1977a; Palta and Li, 1980). Pomeroy *et al.* (1983) reported that uptake of  $^{86}\text{Rb}^+$  by isolated winter wheat cells declined over prolonged exposure to icing stress at  $-1^\circ\text{C}$ , a response that was associated with amino acid efflux from the cells. In spite of these changes, electron spin resonance spectroscopy studies revealed no significant change in molecular ordering within the cell membranes. These results were taken as evidence that damage to the ion transport system is the earliest manifestation of injury to cellular membranes over prolonged freezing. Recovery, when possible, would be associated with repair and reactivation of damaged membrane systems.

Levitt (1980) has suggested that membrane proteins may be dislodged in the membrane and form aggregates in the frozen state because of (a) weakness of the hydrophobic bonds, (b) lipid displacement or (c) phase changes in the membranes.

This view is supported by the freeze-etch study of Pearce and Willison (1985) of the effects of extracellular freezing on cellular membranes of wheat. This study demonstrated that although the membranes generally retained their lamellar structures, as indicated by the abundance of typical membrane fracture faces in all treatments, the usual distribution of intramembranous particles (IMP) was lost. Instead, an increase in the occurrence of IMP-free areas in the membranes of frozen tissues was observed. The frequency of IMP-free areas increased with decreasing freezing temperature and was associated with increasing leakiness of the tissues.

According to Levitt (1980), protein dislodgement may be reversed on thawing as the lipids spontaneously return to the normal state. According to Levitt (1980), recovery may be much slower for the proteins than for lipids. This concept attempts to explain how freezing could depress active transport mechanisms without damaging the passive permeability of the plasma membrane or other membranes such as the tonoplast. One extreme situation might be that recovery is limited by strong protein aggregation and denaturation *in situ* via low-temperature-induced unfolding followed by intramolecular S-S bonding (Levitt, 1980). This may lead to irreversible physiological damage without immediate lysis of the plasma membrane or other membranes (*i.e.*, "physiological death"). Another extreme case might involve the coagulation or precipitation of cytoplasmic or soluble proteins (Levitt, 1980). However, this is not likely to constitute a critical factor, at least for adapted plants, since these plants accumulate large amounts of sugars in their protoplasm. Although the exact nature of the sugar-protein interaction is still a matter of controversy, sugars can protect the proteins against salt stress (Levitt 1980).

Although direct damage to the proteins can explain the physiological alteration, it is also essential to consider the possibility of progressive lipid degradation over

prolonged freezing. This is because of the importance of lipid-protein interactions in the regulation of membrane protein activity (Sandermann, 1978). Enzymatic degradation of phospholipids and other lipids in the membrane may take place after freezing at non-lethal freezing temperatures and may contribute to a progressive physical and physiological alteration of the membrane systems. However, most studies on freeze-induced phospholipid degradation report that degradation takes place after freezing to temperatures below the lethal temperature (Yoshida, 1978; Willemot, 1983). Thus, it appears that enzymatic lipid degradation is a consequence, rather than a cause, of lethal freezing injury.

Another possible cause of progressive membrane degradation is the involvement of free radicals in the oxidation of membrane lipids during freeze-induced dehydration and the accumulation of free fatty acids in the membranes (Senaratna *et al.*, 1985). This mechanism has been studied under lethal conditions (Kendall and McKersie, 1985), and the involvement of free radicals in mediating cumulative freezing injury to the cellular membranes in response to a prolonged exposure to a non-lethal freezing temperature remains to be established.

Although the fact that lethal damage can develop after freezing is generally accepted, the mechanism by which damage occurs is still highly controversial and the key physiological parameter(s) involved remain poorly understood. Similarly, the mechanism by which plant cells recover and repair physiological damage is also unknown.

## B. Objective, Strategy and Hypothesis

The overall objective of this thesis was to characterize the response of winter rye (*Secale cereale* L.) to prolonged exposures to non-lethal freezing temperatures.

The experimental strategy consists of a study of the behavior of two winter rye cultivars (Puma and Animo) that differ in their freezing tolerance. The lethal freezing or critical temperature (LT<sub>50</sub>) is -25°C for Animo and -40°C for Puma (Cloutier and Imbeault, 1988). Cold-acclimated samples (young primary leaf tissues and their isolated cells and protoplasts) were submitted to prolonged exposure to freezing temperatures (-8°C, -10°C), which are well above the critical temperature (LT<sub>50</sub>) for each cultivar. The responses of the tissues, cells and protoplasts were evaluated immediately following thawing until complete recovery or death. The responses were compared to those of unfrozen or lethally frozen controls or were used to define the nature, extent, and progress of the damage and to characterize the recovery process.

Since cellular membranes appear to be a primary site of freezing damage, special attention was given to the response of the plasma membrane and other membranes, such as the tonoplast, to prolonged freezing stresses. The first hypothesis considered was as follows:

Hypothesis 1: Alteration of the permeability properties of the plasma membrane (and tonoplast?) via leakiness primarily determines the development of lethal damage following thawing.

To test the first hypothesis, electrolyte leakage from primary leaf tissue sections was measured. Frozen and unfrozen primary leaf tissue sections were incubated in water after thawing at 5°C and 25°C, and the conductivity of the bathing solution was measured over time. The extent of electrolyte leakage was

related to the duration of the exposure to a non-lethal freezing temperature and was taken as a measure of the damage to cellular membranes (especially the plasma membrane and tonoplast). The effect of temperature of incubation on electrolyte leakage by the tissue during the first four hours following thawing was related to survival after 48 hours.

The plasma membrane not only delimits the cellular environment from the external environment but also regulates the exchange of materials between the cytosol and the external environment. The cytosol (consisting of all the material outside the cellular organelles) is particularly sensitive to the plasma membrane functions. In turn, the physiological integrity of the cellular organelles is, in part, determined by efficient biochemical interactions with the cytosol which make the link between the different compartments. The normal physiological activity of the whole cell depends on the integration of the different metabolic activities in the different compartments and membranes (Alberts *et al.*, 1983). This integration depends, in part, on the regulation of metabolite translocation from one compartment to another and on the ionic (and other solutes) environment of the different membranes. Regulation of metabolite translocation is a fundamental role of cellular membranes. This can be accomplished either by fusion of vesicles or by direct transport across the membranes. Therefore, physiological dysfunction or metabolic damage in response to freezing stress should be understood in terms of an effect on cellular compartmentation. Because the vacuole is acidic, cytoplasmic acidosis would be a major consequence of the decompartmentation via a damage to the semipermeable characteristics of the tonoplast. From this rationale, the second hypothesis was formulated:

Hypothesis 2: Development of lethal damage in the absence of physical disruption of cellular membranes after thawing is primarily determined by an alteration in the semipermeable characteristics of the tonoplast; which results in cytoplasmic acidosis.

The experimental approach to the second hypothesis involved the use of *in vivo* computerized microspectrophoto- and microspectrofluorometry of acridine orange in stressed and unstressed isolated mesophyll protoplasts. This technique permits studies of the intracellular proton compartmentation especially compartmentation of the acidic vacuole and regulation of intracellular pH in response to prolonged exposure to non-lethal freezing temperatures.

## CHAPTER II

### GENERAL MATERIALS AND METHODS

#### A. Plant Material

Two cultivars of winter rye (*Secale cereale* L.) were used: Puma a hardy cultivar (LT<sub>50</sub> = -40°C) and Animo a moderately hardy cultivar (LT<sub>50</sub> = -25°C). Puma seeds were obtained from the harvest of 1985 at the Central Experimental Farm, Agriculture Canada, Ottawa. Animo seeds were generously supplied by Dr H.G. Nass, Charlottetown, P.E.I. All seeds used in these experiments were surface-sterilized using 5% (v/v) sodium hypochlorite solution (15-min wash). After surface sterilization, the seeds were rinsed with tap water. The seeds were germinated on a moist filter paper in large plates (45 cm x 24 cm) in the dark. A 1 cm space was kept around each seed on the filter paper. Cold-acclimated seedlings of both cultivars were produced by maintaining the seedlings at 5 ± 1°C for four weeks. Non-acclimated seedlings were grown at 17.5°C for four d. These treatments produced seedlings with yellow primary leaves 3 to 5 cm in length.

#### 1. Preparation of leaf samples

The primary leaf tissue samples were prepared under green light in a cold room at 5°C. The primary leaf and coleoptile were removed from the seedlings by cutting with a razor blade above the crown. The coleoptile was removed from the primary leaf with fine forceps, and all the primary leaves were pooled. The pooled primary leaves were then cut transversely into sections 3 mm in length using a razor blade. The sections were not washed. All sections were pooled before being split into individual samples. From the pool, approximately 70

sections (0.1 g fresh wt) were randomly selected and used as individual samples. Each sample was placed on a wet, Whatman #1 filter paper in a covered petri dish 3.5 cm in diameter.

## 2. Isolation of leaf mesophyll cells

Mesophyll cells were isolated from the young, primary leaf of cold-acclimated and non-acclimated seedlings using the method of Singh (1981). The primary leaves, free of their coleoptile, were pooled as described and sliced into 0.5 to 1 mm cross-sectional pieces with a razor blade. 1 g fr wt of these pieces was vacuum infiltrated with 10 ml of isolation medium for 5 min in a 20-ml erlenmeyer flask. The composition of the isolation medium was the following:

- 0.1 M mannitol
- 5 mM KCl
- 5 mM MgCl<sub>2</sub>
- 1.5 mM CaCl<sub>2</sub>
- 1.5 mM BSA
- 1.5% glycerol (w/v)
- 0.3  $\mu$ M L-arginine
- 3 mM Tris-Mes, pH 6
- 1% (w/v) dextran sulfate (Pharmacia, MTL, Quebec)

After vacuum infiltration, the supernatant was discarded and replaced by 7 ml of fresh isolation medium containing 3 mM DTT and 0.0005% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Chiloa-ken, Japan).

The resultant suspension was incubated at room temperature in a 40 ml beaker with a disk-type magnet on a magnetic stirrer. To prevent heating of the suspension, the beaker was placed on a pad. The entire preparation was covered by

foil. The suspension was sampled regularly to observe the release of free cells. After approximately 2 h incubation, the suspension was filtered through Nitex (#44). The filtrate was distributed into 15 ml centrifuge tubes and centrifuged at 380g for 5 min. The pellet was resuspended in 1 ml of isolation medium, and the supernatant was recombined with the undigested tissues and further incubated. After 1 h, the suspension was ground very gently with a mortar and pestle for 1 min before being filtered and centrifuged again. If necessary, another 1-h incubation was given to the tissues after recombining with the supernatant.

Once the incubation period was completed and the final filtrate centrifuged, all of the resuspended pellets were pooled together, centrifuged and resuspended in isolation medium minus dextran sulfate. This final suspension was dialyzed overnight against 1 L of tap water at 5°C in the dark.

The next morning, the suspension was purified by centrifugation on Percoll (Sigma Chem. Co. St-Louis, Mo). The procedure was the following. The dialysed cells were resuspended in 10 mM CaCl<sub>2</sub> and transferred into 15 ml Corex centrifuge tubes. The suspension was centrifuged at 380g for 5 min and the supernatant was discarded. The pellet was resuspended in 1.03 density Percoll and the suspension was centrifuged at 740g for 5 min. After this centrifugation, the dead cells formed a layer on the top of the liquid with a thin layer on top of the pellet. The layer on top of the liquid and the supernatant were removed with a pasteur pipette within 0.5 ml from <sup>the</sup> top of pellet. The layer on the top of the pellet was removed by gently bubbling air with the pipet to stir up the dead cells. All the liquid was then discarded. The pellet of living cells was then resuspended in 10 mM CaCl<sub>2</sub> and washed twice by centrifugation at 380g for 5 min. The cells were finally resuspended in 10 mM CaCl<sub>2</sub> and used for experimentation.

### 3. Isolation of leaf mesophyll protoplasts.

Mesophyll protoplasts were isolated using a modified version of the method of de la Roche (1976). Two g of primary leaf tissue transverse sections (0.5 mm) were prepared from either non-acclimated or cold-acclimated seedlings. The tissue sections were transferred into a 50 ml erlenmeyer flask and 20 ml of isolation medium minus enzyme was added. The isolation medium consisted of:

0.6 M (non-acclimated) or 0.85 M mannitol (cold acclimated)

1 mM  $\text{CaCl}_2$

1 mM  $\text{KH}_2\text{PO}_4$ , pH 5.7

1 mM L-arginine

The suspension was vacuum infiltrated for 5 min and the liquid was discarded and replaced by isolation medium containing:

2% (w/v) cellulase R-10 Onozuka

1% (w/v) macerozyme R-10

(from Kanematsu Goshu, Canada Ltd., Montreal, Que.)

The suspension was incubated at room temperature in the dark for 3 to 4 h. The protoplasts were separated from the undigested tissues and debris by passage through 88- $\mu\text{m}$ -mesh nylon screens. The partially digested tissues in the screen were washed with isolation medium minus enzyme and the filtrate was centrifuged at 100g for 5 min. The pellet containing the intact protoplasts was further purified by two washes with suspension or freezing medium and finally resuspended and used for experimentation.

## B. Freezing Procedures

### 1. Leaf samples

#### *a. Prolonged freezing:*

Each set of petri dishes containing sample sections was placed onto a large aluminum plate and covered with clear plastic film and aluminum foil before being transferred into the freezing cabinet. Two types of freezing cabinet were used during the course of these experiments, a Tenney Benchmaster Digitenn system and a Wood freezer #221 controlled by a Conviron CMP2244 system. The freezing cabinet was equilibrated to 2°C before the beginning of the cooling process and was programmed to cool at a rate of 1°C·h<sup>-1</sup> down to -8°C or -10°C according to the experiment. It was maintained at the freezing temperature for periods varying from 0 to 10 d. After freezing was completed, the temperature was raised to 2°C within 2 h. The samples were kept at this temperature in the dark to allow a complete thaw. The progress of the cooling and warming processes was monitored by temperature probes placed in the freezing cabinet (ambient temperature) and into some of the petri dishes (tissue temperature). Readings were recorded automatically every 30 min on a Digistrip II (Kaye Instruments) during the freezing process.

#### *b. Vacuum-Infiltrated Controls:*

Sample sections (0.1 g) were prepared in the same way as the frozen ones except that they were maintained at 5°C. To achieve a water infiltration state equivalent to frozen-thawed samples, control tissues were repeatedly vacuum-infiltrated with glass distilled water before starting the incubation under light.

*c. Lethal Freezing Control:*

Tissue sections (0.1 g) were taken from unfrozen samples (5°C) and placed in dry ice (-78°C) for 15 min to kill the tissues.

2. Cell and protoplast suspensions

*a. Prolonged freezing*

i) Cells

Cell suspensions containing between  $5 \times 10^4$  to  $3 \times 10^5$  cells in 2 ml of a 10 mM CaCl<sub>2</sub> solution (cells) were cooled to -8°C at a rate of 1°C·h<sup>-1</sup> and then maintained at this temperature for 16 h using a programmed freezing cabinet (Trop-arctic, Guardite Co, Wheeling, ILL., USA). Following freezing, duplicate samples were placed on ice for slow thawing.

ii) Protoplasts

The protoplasts were frozen in either:

- The Meyer and Abel R06 saline medium (Meyer and Abel 1975) or:

- a sorbitol-phosphate medium (≈0.85 osmolar) containing:

0.1 M phosphate buffer pH 7 (≈0.2 osmolar)

0.65M sorbitol (≈0.65 osmolar)

1 mM L-arginine

1 mM CaCl<sub>2</sub>

The use of the sorbitol-phosphate medium reduces potential salt stress since, in this case, the major osmoticum is sorbitol.

Test tubes containing approximately  $6 \times 10^5$  protoplasts suspended in 2 ml of freezing medium (Meyer and Abel R06 or sorbitol-phosphate depending on the experiment) were equilibrated at 5°C in the dark for 30 min before being transferred into the freezing cabinet at 2°C in the dark. The protoplasts were

cooled to -8, -9, -10, -13 or -16°C, depending on the experiment at a rate of 1°C·hour and then maintained at the freezing temperature for at least 16 h in the dark. A Wood freezer cabinet #221 controlled by a Conviron CMP 2244 system was used during the course of these experiments. Ice crystallisation was initiated at -4°C using dry ice crystals.

*b. Unfrozen controls*

Unfrozen, control cells and protoplasts were maintained in the freezing medium in the dark at 5°C for the duration of the freezing process.

C. Post-Thaw Recovery

1. Leaf samples: illumination and temperature regime for recovery.

Once the thawing process was completed (at 2°C in the dark), samples (controls and frozen-thawed) were equilibrated at 5°C for 15 min in the dark. Tissues were either left in the petri dish or transferred to 20 ml beakers. Incubation was started by adding 2 or 4 ml of GDW at 5°C to the containers. Triplicate samples were then placed under light (wide spectrum fluorescent Agro light, 65  $\mu$ Einsteins<sup>1</sup>) at 5°C and 25°C and were maintained under continuous illumination with moderate shaking (50 rpm) using a gyrotary shaker (New Brunswick Co., Model G2). The experimental arrangement for incubation for recovery is illustrated in Figures 2.1 A and B.

After 24 h of incubation, the samples at 5°C were transferred to 25°C (R5/25) and those at 25°C were transferred to 5°C (R25/5) for another 24 h of incubation

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<sup>1</sup>The Einstein corresponds to a unit of photochemical energy and is defined as  $E = NH\nu$  where E stands for Einstein, N is the Avogadro constant, h the Planck constant and  $\nu$  is the light velocity.

**Figure 2.1 Post-thaw recovery (tissue samples)**

**A- Experimental arrangement for incubation for recovery under light**

**B- Close up of A showing samples**

**C- Survival estimates following incubation**

**1- Frozen-thawed, 100% survival, R05**

**2- Frozen-thawed, 100% survival, R25**

**3- Lethal freezing control, 0% survival, R05**

**4- Lethal freezing control, 0% survival, R25**

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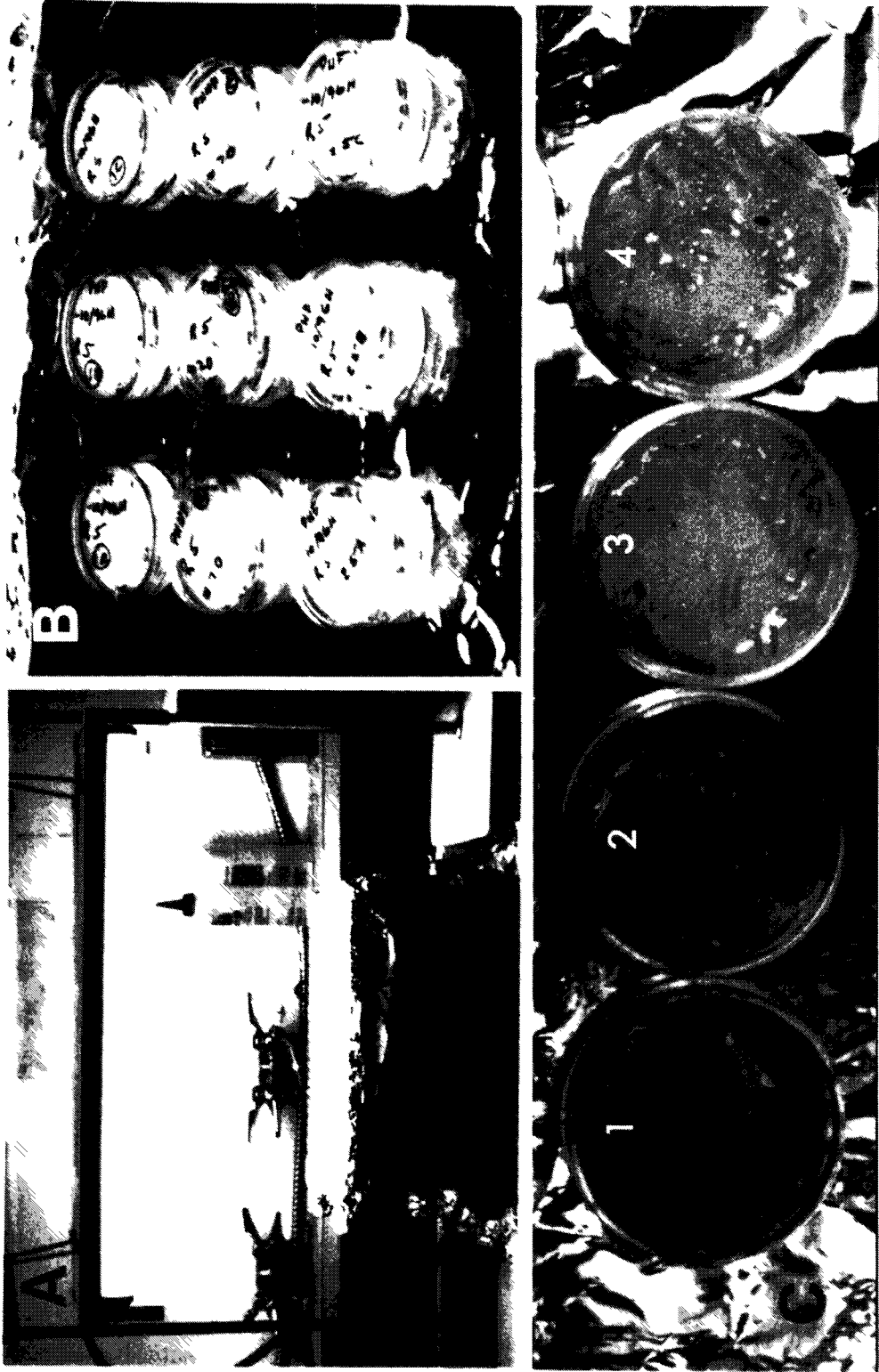
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QUALITY OF THE THESIS SUBMITTED  
FOR MICROFILMING.

UNFORTUNATELY THE COLOURED  
ILLUSTRATIONS OF THIS THESIS  
CAN ONLY YIELD DIFFERENT TONES  
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ILLUSTRATIONS EN COULEURS DE CETTE  
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to achieve equivalent growth (equivalent degree-h exposure of the samples) before estimating survival at 48 h.

## 2. Cell and protoplast suspensions

### a) Cells

The tubes containing cells were placed on ice in the dark for thawing and maintained in 10mM CaCl<sub>2</sub>.

### b) Protoplasts

Once the freezing time was completed, the tubes were removed from the freezing cabinet and placed on ice for thawing in the dark. Immediately after completion of thawing, the freezing medium was decanted and replaced by 12 ml of fresh incubation medium. The incubation medium consisted of either:

(i) a sorbitol-phosphate medium: *i.e.*, the freezing medium (the pH of which ranged from 5.5 to 8.6) or

(ii) a mannitol-tris-maleate medium containing:

0.56 M mannitol ( $\approx 0.56$  osmolar)

50 mM tris-maleate ( $\approx 0.1$  osmolar)

7 mM to 87 mM KOH ( $\approx 0.014$  to 0.174 osmolar)

87 mM to 7 mM KCl ( $\approx 0.174$  to 0.014 osmolar)

The different concentrations of KOH were used to create a pH gradient ranging from 5.1 to 8.6. The different concentrations of KCl were used to counter-balance the ionic effect of KOH.

Before centrifugation, the protoplasts were resuspended with a Pasteur pipette. The suspensions were then centrifuged at 120g for 5 min to pellet the protoplasts. Following centrifugation, the supernatant was discarded and the protoplasts were resuspended in fresh incubation medium. The suspensions were centrifuged once

more and the protoplast population was then split into different subsamples. All glassware used in these experiments was treated with Sigmacote (Sigma Chem Co. St-Louis, Mo.) to minimize surface charges.

The unfrozen suspensions were submitted to the same wash procedures as the frozen suspensions following thawing and split into the same number of subsamples. The subsamples were split into paired groups. A first pair (frozen and control) was used to compare the state of the non-lethally frozen-thawed protoplasts immediately after thawing with the state of unfrozen control protoplasts. All observations on this pair were made between 60 and 180 min following addition of the dye. A second pair was maintained in the dark at 5°C for 6 h before being examined to estimate the reversibility of the measured changes in the first pair. The observations and measurements on these samples were also made between 60 and 180 min following addition of the dye.

#### D. Survival Determinations

##### 1. Leaf samples

###### *a. Visual observations:*

Survival was visually estimated after 48 h of incubation by counting the number of green and expanded tissues sections in each sample (Cloutier and Imbeault, 1988). Survival, green and expanded tissue sections in water under light, was expressed as a percentage of the total number of sections incubated. Dead tissue sections remained yellow and did not expand (Fig 2.1 C). Photographs were taken during and after incubation using Kodak Ektachrome EL-135 400 ASA film.

###### *b. Measurement of fresh and dry weight*

The fresh weight of individual samples was determined after removing excess

water from the tissue sections. First, the incubating water was removed with a pasteur pipette. Then, the tissue sections plus their paper substrate (Whatman #1, 3.5 cm in diameter) were placed onto dry filter paper (Whatman #1, 5.5 cm in diameter) to remove the residual excess water by capillarity. Finally, the tissue sections were lifted from the paper and weighed in a plastic weighing boat. The samples were then dried in an oven at 50°C for determination of the dry weight.

## 2. Isolated cells

### *a. Hydrolysis of fluorescein diacetate and accumulation of fluorescein.*

Survival of isolated cells was determined using the vital stain fluorescein diacetate (FDA) purchased from Sigma Chem. Co., St Louis, MO. Cells showing fluorescence after 20 min of incubation at  $5 \pm 1^\circ\text{C}$  in a suspension medium containing  $10^{-5}$  M FDA were counted as surviving. Survival in the frozen sample was normalized against survival in the unfrozen control suspension. Fluorescein fluorescence was observed using a Zeiss epi-illumination fluorescence microscope (excitation wavelength, 470 nm; emission wavelength, 540 nm).

### *b. Osmotic responsiveness*

Unfrozen and frozen-thawed cells were centrifuged, and resuspended in 1 M (non-acclimated cells) or 1.2 M (acclimated cells) mannitol containing  $10^{-5}$  M FDA for 5 min and deplasmolysed by adding suspension medium. Successful plasmolysis/deplasmolysis, indicating osmotic responsiveness, was observed in individual cells on the fluorescence microscope after FDA staining.

### 3. Isolated protoplasts

#### *a. Protoplast sphericity*

Unless a different criterion of viability is specified for a given experiment (*e.g.*, vital staining), spherical protoplasts were assumed to be alive and survival was estimated on this basis. Survival was expressed as a percentage of the number of spherical protoplasts present in the unfrozen, control population.

## CHAPTER III

### CHLOROPHYLL SYNTHESIS IN ETIOLATED TISSUES AS A PARAMETER OF RECOVERY AND ELECTROLYTE RELEASE BY TISSUES AS A MEASURE OF INJURY

#### A. Introduction

##### 1. Chlorophyll Synthesis in Etiolated Tissues as a Parameter of Recovery

Parameters such as vitality, injury, recovery and death are difficult to define with precision. Nevertheless, they are essential concepts of stress physiology. In the absence of precise quantitative methods, the estimation of these parameters remains essentially subjective and does not allow for the development of models with predictive value. For example, observations of loss of turgor and water infiltration following freezing of plant tissues have little practical use unless one can find a way to quantify them in such a way that a specified degree of freezing injury can be indicated.

Chlorophyll synthesis in etiolated tissues ("greening") following freezing stress has been taken as an expression of survival in screening tests for freezing tolerance in winter cereals (Cloutier and Imbeault, 1988). The greening process is known to be affected by water stress in plants; and, in general, water stressed plants return to original turgidity much faster than metabolic activities (Misra and Misra, 1987). Because freeze dehydration may potentially affect all cellular membranes and not only the plasma membrane (Steponkus, 1984) and because chlorophyll synthesis is a membrane associated process (Goodwin and Mercer, 1983), the greening response was used as a measure of membrane physiological recovery following prolonged freezing stress.

## 2. Electrolyte Release by Frozen-Thawed Tissues as a Measure of Injury

The release of electrolytes from injured tissues has been frequently used as an indicator of viability and to estimate the degree of injury to tissues. The principle of increased leakiness of injured tissue has been generally accepted and has been applied to freezing stress (Dexter *et al.*, 1932; Wilner, 1960; Palta *et al.*, 1977a), dehydration or desiccation stress (Senaratna and Mckersie 1983), and chemical stress (Mckersie *et al.*, 1982).

An important method of measuring release of electrolytes from tissues relies on the measurement of the electrical conductivity of an effusate from plant tissue immersed in water (Heald, 1902). Release of solutes from cells depends on a change in permeability (or resistance) of the plasma membrane and possibly the tonoplast. If the plant is injured and the semipermeable characteristics of the plasma membrane and tonoplast are altered, the release of electrolytic solutes gives a measure of the amount of injury (Osterhout, 1922). In the case of a disrupted membrane, the leakage reaches diffusional equilibrium.

Other techniques can be used to monitor a change in permeability of the plasma membrane. Examples include the release of amino acids (Siminovitch, 1962) or sugars (Dubois *et al.*, 1951) and the measurement of radioactive rubidium influx and efflux (Pomeroy *et al.*, 1985). In addition, there are indirect ways to monitor solute loss, such as measurement of the change in the extracellular solute concentration required for plasmolysis (Osterhout, 1922). Finally, an alteration of the permeability of the plasma membrane can be evidenced by volumetric measurements of the rate of penetration of different solutes with low permeability coefficients (Overton, 1895). Nevertheless, because of its simplicity and accuracy, the release of electrolytes as measured by the conductivity of the bathing solution

has been the most commonly used method of measuring freezing injury (Dexter, 1932; Palta *et al.*, 1977a).

When the measurement of injury is coupled to estimates of recovery and survival, it can lead to the determination of a critical level of injury. In his pioneer works on the effect of increasing salt stress, Osterhout (1922) measured both the extent of injury and the % recovery relative to a normal control. In these experiments Osterhout (1922) measured the electrical resistance or conductance of tissues using electrodes. The electrodes were either directly applied to the tissues or to a saline solution, which created a bridge between the electrodes and the tissues. Although in such a system, the cell sap (and the cell wall of the plant cell) contribute to the electrical conductance, it is widely recognized that the rise and fall of conductivity of the whole tissue depends essentially on an alteration of the permeability of the plasma membrane (and tonoplast) to electrolytes (Osterhout, 1922). Osterhout (1922) found that the resistivity of the tissues was reduced with increasing salt stress. As a general rule, the greater the injury (% decrease in resistivity), the less complete was the recovery (return to initial resistivity with time). Osterhout (1922) found that if injury reached 5%, the tissues of the marine alga Laminaria recovered its normal resistance when replaced in sea water. If injury reached 25%, recovery was incomplete, reaching only 90% of the normal control. When injury reached 90%, there was no recovery. From these results, an alteration of the resistance of the plasma membrane (and tonoplast) in the absence of physical disruption was recognized for salt stress (Osterhout, 1922) and the existence of a critical degree of injury was proposed.

In freezing stress, both the conductivity method using direct application of electrodes (also called resistivity method) and the measurement of the release of electrolytes from tissues were generally assumed to measure the percent of killed

cells and therefore the extent of plasma membrane disruption in tissues submitted to freezing stresses (Dexter *et al.*, 1932; Wilner, 1960; Wilner *et al.*, 1960). Palta *et al.* (1977a, b) clearly demonstrated that this is not necessarily the case, and following prolonged exposure to a non-lethal freezing temperature, the permeability of the plasma membrane can be altered in the absence of physical disruption or lysis. Palta *et al.*, (1977a) reported that as much as 76% of the total electrolyte content of onion scale tissues could leach out following thawing without any sign of mortality - as indicated by the apparent intactness of the cells, normal osmotic responsiveness, and maintenance of protoplasmic streaming immediately after thawing. A major result of the study made by Palta and his colleagues (1977b) was that the leaking cells, which apparently are alive following thawing, can either recover or degenerate with time after thawing. Consequently, death can be the result of the development of lethal damage following thawing. From these results, Palta and Li (1978) proposed a model for the development of lethal injury after thawing. This model was based on the premise of physiological damage to the plasma membrane.

Although alteration of the semipermeable characteristics of the plasma membrane (and tonoplast) after thawing in absence of physical disruption certainly constitutes a measure of the degree of injury, there is no clear evidence in the current literature that the progressive development of lethal damage after thawing is determined by the extent of injury to the plasma membrane (and tonoplast) *via* leakiness. Here the phenomenon of expansion-induced lysis (Steponkus, 1984), during rehydration is excluded and the progress of damage is assumed to take place in fully rehydrated and reexpanded cells. In addition, in spite of the important contributions from Osterhout (1922) and Palta *et al.*, (1977ab), a critical level of injury still remains to be determined.

Therefore, in addition to assessing the greening parameter for recovery, the objectives of the present study were to measure the release of electrolytes from injured tissues to determine:

- a. if the alteration of the permeability properties of the plasma membrane and possibly the tonoplast (leakiness) in the absence of physical disruption after thawing primarily determines the progressive development of irreversible damage following thawing and
- b. the critical amount of injury (leakiness) associated with the development of lethal damage following a prolonged exposure to a non-lethal freezing temperature.

## B. Materials and Methods

### 1. General

Primary leaf tissue sections of cold-acclimated winter rye seedlings (*Secale cereale* L. cvs Puma and Animo) were frozen to  $-8^{\circ}\text{C}$  for 5 d or to  $-10^{\circ}\text{C}$  for 1 h, 4 d, 6 d or 10 d and then slowly thawed. The chlorophyll content of the tissues was measured and survival was assessed on the basis of greening. The extent of injury was determined after thawing by measuring the electrolyte release at different times during recovery in the light and under different temperature regimes (R5/25 and R25/5). The different temperature regimes were used to evaluate the effect of temperature during recovery on the extent of electrolytes released by the tissues. The two temperature regimes consisted of (i) R5/25= 24 h at  $5^{\circ}\text{C}$  followed by 24 h at  $25^{\circ}\text{C}$  and (ii) R25/5= 24 h at  $25^{\circ}\text{C}$  followed by 24 h at  $5^{\circ}\text{C}$ . Survival was estimated after 48 h. The response of the tissues to prolonged freezing stress was compared to the response of unfrozen or lethally frozen controls. The release of electrolytes by the different treatments was related to survival after 48 h of

incubation. The extent of reabsorption of the released electrolytes was also evaluated at 48 h. The details of the experimental protocol are presented in Chapter II, except for the measurement of conductivity which is described below.

## 2. Quantitation of chlorophyll content during greening:

Total chlorophyll content of the primary leaf sections was extracted at intervals during recovery under light (up to 24 h) using 20 ml of 80% (v/v) acetone. The quantitation of chlorophyll in  $\text{mg}\cdot\text{L}^{-1}$  was obtained for the more concentrated samples following exposure to illumination for 24 h using the Arnon method (Holden, 1965). This method relies on the measurement of absorbance of the solution at 663 nm and 645 nm in a 1 cm path using the specific coefficients included in the following equation:

$$\text{EQUATION 2.1- Total chlorophyll (mg}\cdot\text{L}^{-1}) = 20.2A_{645} + 8.02A_{663}$$

It was then possible to express the total amount of chlorophyll in  $\mu\text{g}\cdot\text{g}^{-1}$  fr wt of tissues knowing the exact fresh weight of the samples and the volume of the solvent used.

Since most of the extracts contained very small amounts of chlorophyll, their quantitation was done by fluorescence measurements. The fluorescence intensity of the chlorophyll (Ex 405, 436 nm; Em 670 nm) was measured using a fluorescence spectrophotometer (Hitachi Perkin Elmer MPF-2A) equipped with a xenon lamp. For the transformation of fluorescence intensity into concentration ( $\mu\text{g}\cdot\text{g}^{-1}$  fr wt), a dilution series of the concentrated sample was measured and the fluorescence to concentration ratio was obtained. From this ratio, a dilution value within the range of concentration where chlorophyll fluorescence increased linearly with concentration was selected. Then a diluted sample of every extract was made to determine the relative concentration of all extracts from their relative fluorescence

intensities. The chlorophyll content of the total extracts at 0, 1, 2, 4, and 8 h of illumination was then expressed in  $\mu\text{g}\cdot\text{g}^{-1}$  fr wt on the basis of relative fluorescence intensity compared to the diluted, 24-h incubation reference sample.

### 3. Measurement of Electrolyte Release

Four ml of GDW at 5°C were added to each sample at 5°C. Immediately, one set of replicate samples was placed under light on a gyrotory shaker (moderate speed) at 5°C and a second set of replicate samples was transferred to 25°C to be incubated under identical illumination and shaking conditions (see incubation for recovery Chapter II). After a 15-min equilibration period, the conductivity of the water containing frozen-thawed or unfrozen tissues at 5°C (R5/25) and 25°C (R25/5) was monitored at intervals for 48 h using a conductivity bridge (YSI model 31). The electrode was used in reverse mode to allow for the measurement of small volumes (1.5 ml). The electrode was always rinsed with GDW before each measurement and the incubation water was returned to the petri dish after each measurement. The measurements were made at 5°C for the samples incubated at 5°C and at 25°C for the samples incubated at 25°C. Water evaporation was compensated for by adding water regularly during the incubation. The volume (weight) of water was then kept constant. The conductivity of the GDW was routinely checked and was always less than 1  $\mu\text{MHO}$ . In one experiment, 1 mM  $\text{CaCl}_2$  or  $\text{MgCl}_2$  was used instead of GDW to monitor the effect of calcium on the release of electrolytes by the tissues.

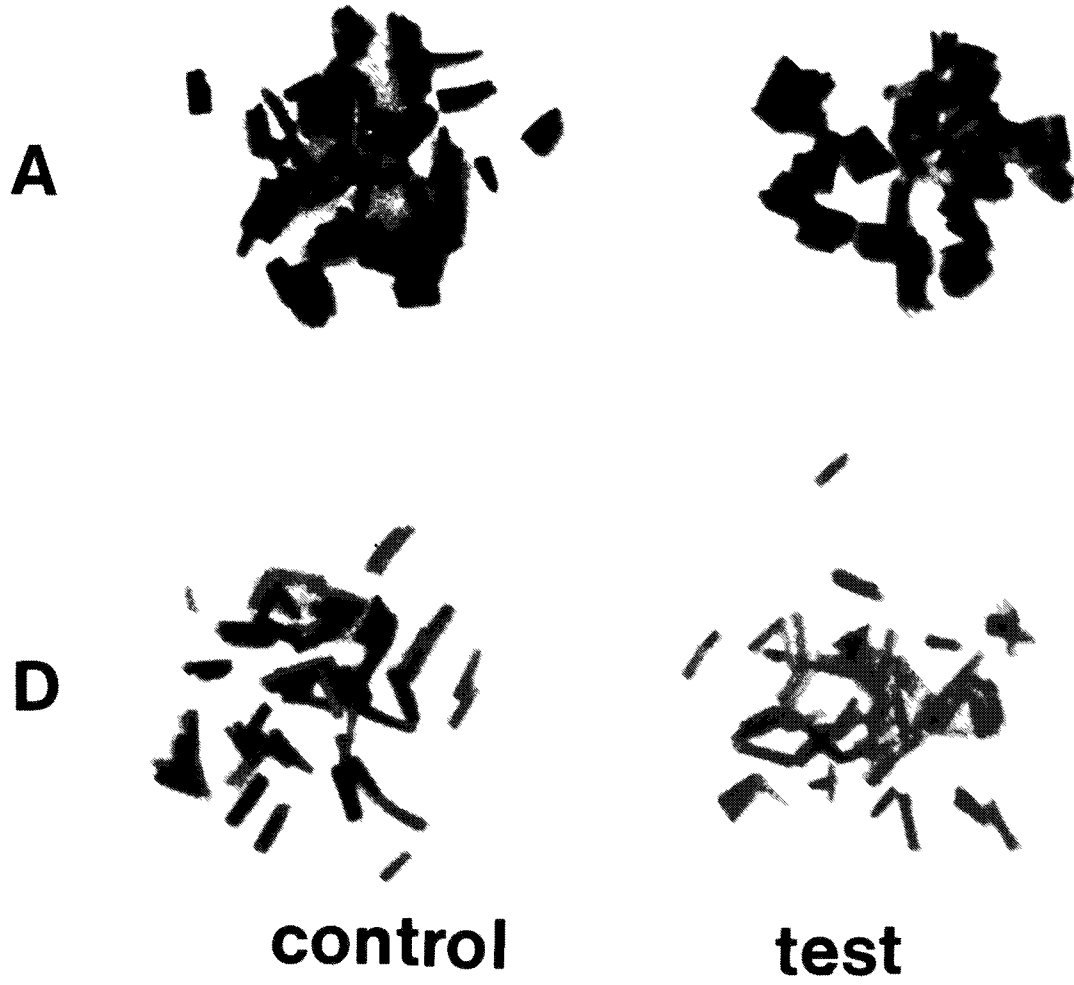
### C. Results and Discussion

#### 1. Chlorophyll Synthesis (Greening) and Survival Following Prolonged Exposures to Non-Lethal Freezing Temperatures.

In both cultivars, freezing to  $-8^{\circ}\text{C}$  for 5 d did not kill any of the tissue sections of the two cultivars when these were incubated at  $25^{\circ}\text{C}$  (R25/5) in water under light. All the tissue sections of both the unfrozen, control samples and non-lethally, frozen-thawed samples expanded fully in water and developed chlorophyll uniformly under light with no sign of local injury in any of the tissue sections after 48 h (Fig 3.1 A). However, frozen tissues were slower to green than the unfrozen controls during the first 4 h of incubation in water under light (Fig 3.1 D).

Analysis of the chlorophyll content verifies this visual impression (Table 3.1). In the case of Puma, the stressed tissues produced  $120 \mu\text{g}\cdot\text{g}^{-1}$  fr wt compared to the  $212 \mu\text{g}\cdot\text{g}^{-1}$  fr wt of the unstressed controls after 4 h of incubation. In percentage this is 56.8% of the control value. In Animo, the difference was greater; the stressed tissues produced  $84 \mu\text{g}\cdot\text{g}^{-1}$  fr wt compared to  $215 \mu\text{g}\cdot\text{g}^{-1}$  fr wt of the control value (39.2%) at 4 h. Importantly, during the first h of incubation, the stressed tissues of both cultivars produced an amount of chlorophyll approaching the value of the controls. This may indicate that the damage is affecting an early step in the series of reactions leading to chlorophyll synthesis. This is consistent with the results of Virgin (1965) who demonstrated that diminished chlorophyll formation in water-stressed plants is due to slower formation of precursors for protochlorophyllide. During the subsequent 2 to 4 h of incubation, the frozen samples fell behind. Between 4 and 8 h after the beginning of the recovery period, Puma tissues that had been frozen produced chlorophyll at a rate equivalent to that

**Figure 3.1.** Non-lethally, frozen-thawed (TEST:-10°C for 6 d) and unfrozen (CONTROL) primary leaf tissue sections of cold-acclimated Puma rye seedlings (dark grown) at 2 h (D) and 48 h (A) after incubation in continuous light.



**Table 3.1.** Chlorophyll content of unfrozen and frozen-thawed ( $-8^{\circ}\text{C}/5$  d) Puma and Animo winter rye leaf tissues over time of incubation under light. Survival was 100% in all treatments after 48 h of incubation.

Cultivar	Time	Total Chlorophyll		Rate	
		Control	Frozen	Control	Frozen
	(h)	--- ( $\mu\text{g}\cdot\text{g}^{-1}$ fr wt) ---		- ( $\mu\text{g}\cdot\text{g}^{-1}$ fr-wt $\cdot\text{h}^{-1}$ )-	
Puma	0	< 7	< 7	--	--
	1	59 $\pm$ 5	50 $\pm$ 9	59	50
	2	109	65	50	15
	4	212 $\pm$ 3	120 $\pm$ 20	52	28
	8	417	291	51	43
	24	1137	809	45	33
Animo	0	< 7	< 7	--	--
	1	50 $\pm$ 9	34 $\pm$ 2	50	34
	2	93	43	43	9
	4	215 $\pm$ 38	84 $\pm$ 18	61	21
	8	300	225	21	35
	24	632	740	21	32

Means of triplicates  $\pm$  S.D. Data without statistics = one sample.

Chlorophyll content was quantified fluorometrically using the method described in

Materials and Methods

of the controls ( $43 \mu\text{g}\cdot\text{g}^{-1} \text{ fr wt}\cdot\text{h}^{-1}$  by 8 h as compared to the controls =  $51 \mu\text{g}\cdot\text{g}^{-1} \text{ fr wt}\cdot\text{h}^{-1}$ ). In Puma, after 24 h of incubation under light, the stressed tissues contained 70% of the amount present in the control. In Animo, the control tissues contained 85% of the amount of chlorophyll present in the non-lethally stressed tissues. Therefore, on the basis of chlorophyll synthesis, metabolic recovery was observed by 4 h of the beginning of the incubation in both cultivars. Therefore, in addition to tissue expansion in water, the greening parameter was selected to monitor recovery and survival in experiments using etiolated tissues.

## 2. Effect of Prolonged Freezing on the Release of Electrolytes.

In preliminary experiments freezing injury in winter rye primary leaf tissue sections was evaluated by electrolyte release i.e., by measurement of the conductivity of the bathing solution. A series of experiments were then conducted over a period of two months to relate the manifestation of injury in the tissues with the expression of recovery and survival. The results are summarized in Table 3.2. All six experiments (A to F) were done with cold-acclimated tissues: A-D Puma, E-F Animo winter rye. Each experiment dealt with two triplicate sets of vacuum-infiltrated control samples and two triplicate sets of frozen-thawed samples. One set of each (control and frozen samples) was incubated for recovery at  $5^{\circ}\text{C}$  (R5/25) and one set at  $25^{\circ}\text{C}$  (R25/5). The samples were either frozen to  $-78^{\circ}\text{C}$  (lethal; A and E) or frozen to  $-10^{\circ}\text{C}$  (sublethal) for 4 d (B), 6 d (C and F) or 10 d (D). Survival was estimated to be 100% in all unfrozen controls and 0% in samples frozen to  $-78^{\circ}\text{C}$  for 15 min. This was true both for Puma and Animo. This means that all the tissue sections (100%) expanded and uniformly developed chlorophyll in unfrozen samples and that all the tissue sections did not expand in water and did not develop chlorophyll in samples frozen at  $-78^{\circ}\text{C}$  for 15 min.

**Table 3.2.** Survival of cold-acclimated winter rye primary leaf tissue sections and conductivity of the bathing solution after 4 and 48 h of incubation in GDW following prolonged freezing stresses. GDW at 5°C was added after complete thawing. The incubation was done under light and under two different temperature regimes:

1. R5/25 = 24 h at 5°C followed by 24 h at 25°C.
2. R25/5 = 24 h at 25°C followed by 24 h at 5°C.

**TABLE 3.2**

Experiment	Cultivar	Treatment	Conductivity of the bathing solution in $\mu\text{MHOS}^2$					
			Survival <sup>1</sup>		After 4 h		After 48 h	
			R 5/25	R 25/5	R5/25	R 25/5	R 5/25	R 25/5
A	Puma	Unfrozen	--	100 $\pm$ 0	--	36.0 $\pm$ 1.7	--	11.4 $\pm$ 0.4
		-78 ° C/1 h	--	0 $\pm$ 0	--	185.0	--	222.0 $\pm$ 7.0
B	Puma	Unfrozen	100 $\pm$ 0	100 $\pm$ 0	30.0 $\pm$ 1.7	43.0 $\pm$ 3.2	16.5 $\pm$ 0.7	17.0 $\pm$ 1.2
		-10 ° C/4 d	100 $\pm$ 0	100 $\pm$ 0	45.7 $\pm$ 2.3	66.0 $\pm$ 0.6	20.3 $\pm$ 1.2	16.0 $\pm$ 0.6
C	Puma	Unfrozen	100 $\pm$ 0	100 $\pm$ 0	26.3 $\pm$ 0.3	36.0 $\pm$ 1.2	11.3 $\pm$ 0.9	11.4 $\pm$ 0.4
		-10 ° C/6 d	100 $\pm$ 0	100 $\pm$ 0	52.3 $\pm$ 2.2	71.7 $\pm$ 2.3	11.3 $\pm$ 1.2	10.3 $\pm$ 0.9
D	Puma	Unfrozen	100 $\pm$ 0	100 $\pm$ 0	28.2 $\pm$ 1.1	39.5 $\pm$ 2.2	17.0 $\pm$ 1.2	15.9 $\pm$ 1.3
		-10 ° C/10 d	93 $\pm$ 2	86 $\pm$ 4	54.0 $\pm$ 7.0	94.0 $\pm$ 2.2	26.0 $\pm$ 2.3	25.4 $\pm$ 3.8
E	Animo	Unfrozen	100 $\pm$ 0	100 $\pm$ 0	28.7 $\pm$ 1.3	40.7 $\pm$ 1.6	22.5 $\pm$ 1.5	20.5 $\pm$ 1.5
		-78 ° C/1 h	0 $\pm$ 0	0 $\pm$ 0	150.0	205.0	240.0 $\pm$ 15	230.0 $\pm$ 12
F	Animo	Unfrozen	100 $\pm$ 0	100 $\pm$ 0	28.7 $\pm$ 1.3	40.7 $\pm$ 1.6	22.5 $\pm$ 1.5	20.5 $\pm$ 1.5
		-10 ° C/6 d	86 $\pm$ 5	82 $\pm$ 1	55.3 $\pm$ 7.5	90.0 $\pm$ 7.4	33.0 $\pm$ 1.5	29.5 $\pm$ 0.5

Data without statistics are means of duplicates.

<sup>1</sup> survival after 48 h expressed as a % greening and expanding tissue sections (approximately 70 sections incubated per samples). Dead tissue sections remained yellow and did not expand in water.

<sup>2</sup> The conductivity values are not compensated for temperature after 4 h. All conductivity readings at 25 ° C after 48 h.

In Puma, freezing to  $-10^{\circ}\text{C}$  for 4 or 6 d was apparently non-lethal since all the tissue sections expanded fully in water and developed chlorophyll uniformly under light with no sign of local injury (Table 3.2, B and C). However, in the case of Puma samples frozen to  $-10^{\circ}\text{C}$  for 10 d (Table 3.2 D), only 93% of the sections expanded and developed chlorophyll at  $5^{\circ}\text{C}$  (R5/25) and 86% at  $25^{\circ}\text{C}$  (R25/5). An interesting observation was that partial survival of individual tissue sections (3 mm x 1 mm) was not observed i.e., the sections were either all uniform in expansion and greening with no sign of local injury or they were totally killed with no sign of any surviving cells in them. This may indicate that cell-cell interactions in the organized tissue sections are important in the modulation of the lethal response. The same phenomenon was observed in Animo frozen to  $-10^{\circ}\text{C}$  for 6 d (Table 3.2 F) where 86% of the tissue sections in the samples expanded and uniformly greened at  $5^{\circ}\text{C}$  (R5/25) and 82% at  $25^{\circ}\text{C}$  (R25/5). Although it is not statistically significant at the 95% probability level, survival was always higher at  $5^{\circ}\text{C}$  (R5/25) than at  $25^{\circ}\text{C}$  (R25/5).

The conductivity of the bathing solution after 4 h of incubation was always higher in frozen samples than in their unfrozen controls. This was the case for both an incubation at  $5^{\circ}\text{C}$  (R5/25) and at  $25^{\circ}\text{C}$  (R25/5). This increase in conductivity was taken as a measure of the electrolyte release by the tissue sections during the first 4 h following thawing and as a measure of injury in frozen-thawed tissues. At 4 h of incubation, the conductivity was always higher at  $25^{\circ}\text{C}$  than at  $5^{\circ}\text{C}$ ; but since the measurements were made at  $5^{\circ}\text{C}$  and  $25^{\circ}\text{C}$  respectively, the difference depends on the effect of temperature on the mobility of electrolytes in solution. Nevertheless, by considering a conductivity temperature compensation coefficient, an analysis of the effect of incubation temperature on the

release of electrolytes from the frozen tissues during the first four h after thawing was made. This analysis is presented in a next section of this chapter.

The conductivity of the bathing solution of the frozen-thawed samples showing 100% survival (Puma 4 and 6 d of freezing; Table 3.2 B,C; both R5/25 and R25/5) returned to the control level within 48 h indicating an apparent reabsorption of the electrolytes by the cells. The conductivity was only partially reversed with a residual difference of almost 10  $\mu$ MHOs in the case of the samples showing reduced survival (Puma 10 d of freezing and Animo 6 d of freezing; Table 3.2 D,F). By contrast, the conductivity increased in the cases of the samples frozen to  $-78^{\circ}\text{C}$  (Puma and Animo lethal; Table 3.2 A, E). In this case, the differential permeability of the membranes was totally and irreversibly lost (Steponkus, personal communication), and reabsorption was not possible. These samples frozen to  $-78^{\circ}\text{C}$  (lethal freezing) were used to calculate the total amount of electrolytes in the cells available for leaching by comparing the conductivity of the leachate with the conductivity of KCl solutions of known concentration. It was estimated to be  $1.6 \times 10^{-5}$  mole of electrolytes ( $\text{K}^{+}$  equivalents). Taking the molecular weight of  $\text{K}^{+}$ , this corresponds to  $6.24 \times 10^{-4}$  g or 2.6% of the dry weight of the tissue samples which was measured ( $24 \pm 1$  mg). Since most plant tissue contains approximately 1% of  $\text{K}^{+}$  on a dry weight basis and since  $\text{K}^{+}$  constitutes the major electrolyte in plant cells, this estimate appears to be reasonable. Because the conductivity to concentration relation was found to be linear in the range of concentration reached in the bathing solution of all treatments, the % leakage<sup>was</sup> estimated directly from conductivity measurements.

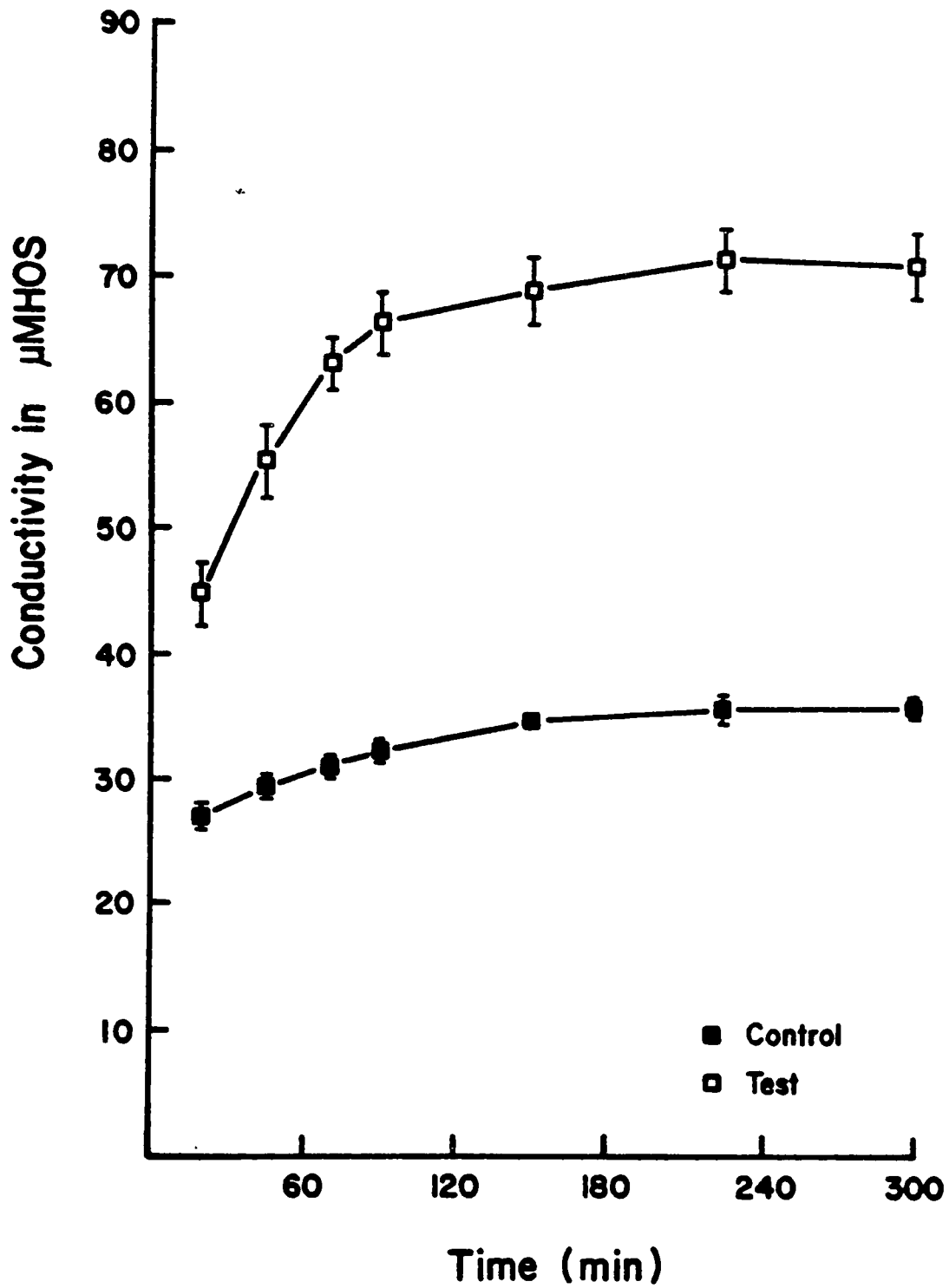
Figure 3.2 shows the time course of electrolyte release for Puma tissue sections during incubation at  $25^{\circ}\text{C}$  following freezing at  $-10^{\circ}\text{C}$  for 6 d. After a 15-min equilibration, the control sample gave a reading of 25  $\mu$ MHOs, presumably

resulting from electrolytes released by the debris associated with the cut surfaces and to apoplastic electrolytes. What is of interest though, is the further slight increase in conductivity of the bathing solution to approximately 33  $\mu$ MHOs after 4 h (240 min). This slight increase may indicate that the membrane of unfrozen control cells is not perfectly impermeable to electrolyte diffusion since some leakage was measured from these normal tissues. The increase in conductivity was much greater for tissues frozen to  $-10^{\circ}\text{C}$  for 6 d (test) reaching a value of  $71.7 \pm 2.3$   $\mu$ MHOs after less than 4 h (240 min). However, there was no apparent membrane disruption since survival was still 100% after 48 h of incubation as shown in Table 3.2 C.

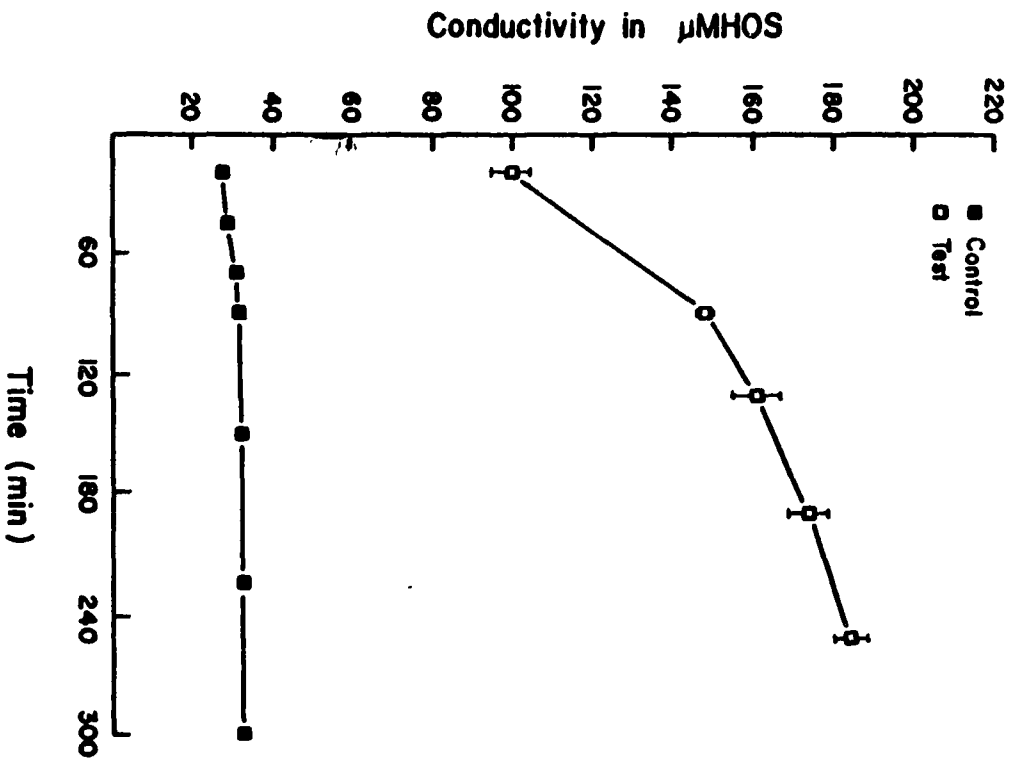
Figure 3.3 shows the post-thawing time course of conductivity for tissue sections frozen to  $-78^{\circ}\text{C}$  (lethal freezing). The conductivity increased to 185  $\mu$ MHOs by 4 h (240 min). The comparison of the extent of electrolyte release by non-lethally frozen-thawed tissues (Fig. 3.2) with the extent of electrolyte release by lethally frozen-thawed tissues (Fig. 3.3) indicates that the tissues can lose an important fraction of their total electrolyte content ( $\sim 35\%$ ) and still remain viable. Since mesophyll cells are dominant in the tissues, it was assumed that they significantly contribute to the response. In addition, because the vacuole of the plant cell constitutes an important reservoir of electrolytes, a significant proportion of the electrolytes released by the frozen tissue sections must come from the vacuole. This indicates that the tonoplast is also leaky.

Figure 3.4 is derived from the conductivity data obtained for 4 and 6 d of freezing by a  $e^{x/10}$  transformation. It shows that the increase in conductivity of the incubating medium, presumably resulting from electrolyte diffusion, was a single exponential function of time following thawing. This further indicates that the

**Figure 3.2.** Change in conductivity of the incubating medium of unfrozen (CONTROL) and non-lethally frozen-thawed (TEST -10°C for 6 d) Puma rye primary leaf tissue sections over time of recovery at 25°C. Conductivity of the water was <1  $\mu$ MHO. Survival was 100% in both cases after 48 h of incubation. All values are the mean of triplicates, bars indicate SEM, same tissues as in table 3.2.



**Figure 3.3.** Change in conductivity of the incubating medium of unfrozen (CONTROL) and lethally frozen-thawed (TEST -78 °C) Puma rye primary leaf tissue sections (Table 3.2 A) over time of incubation for recovery at 25°C. Conductivity of water was <1 uMHO. Survival was 100% in control and 0% in lethally frozen (no recovery) after 48 h of incubation. All values are the mean of triplicates, bars indicate SEM.



diffusion of electrolytes out of the tissue sections was limited by a single factor.

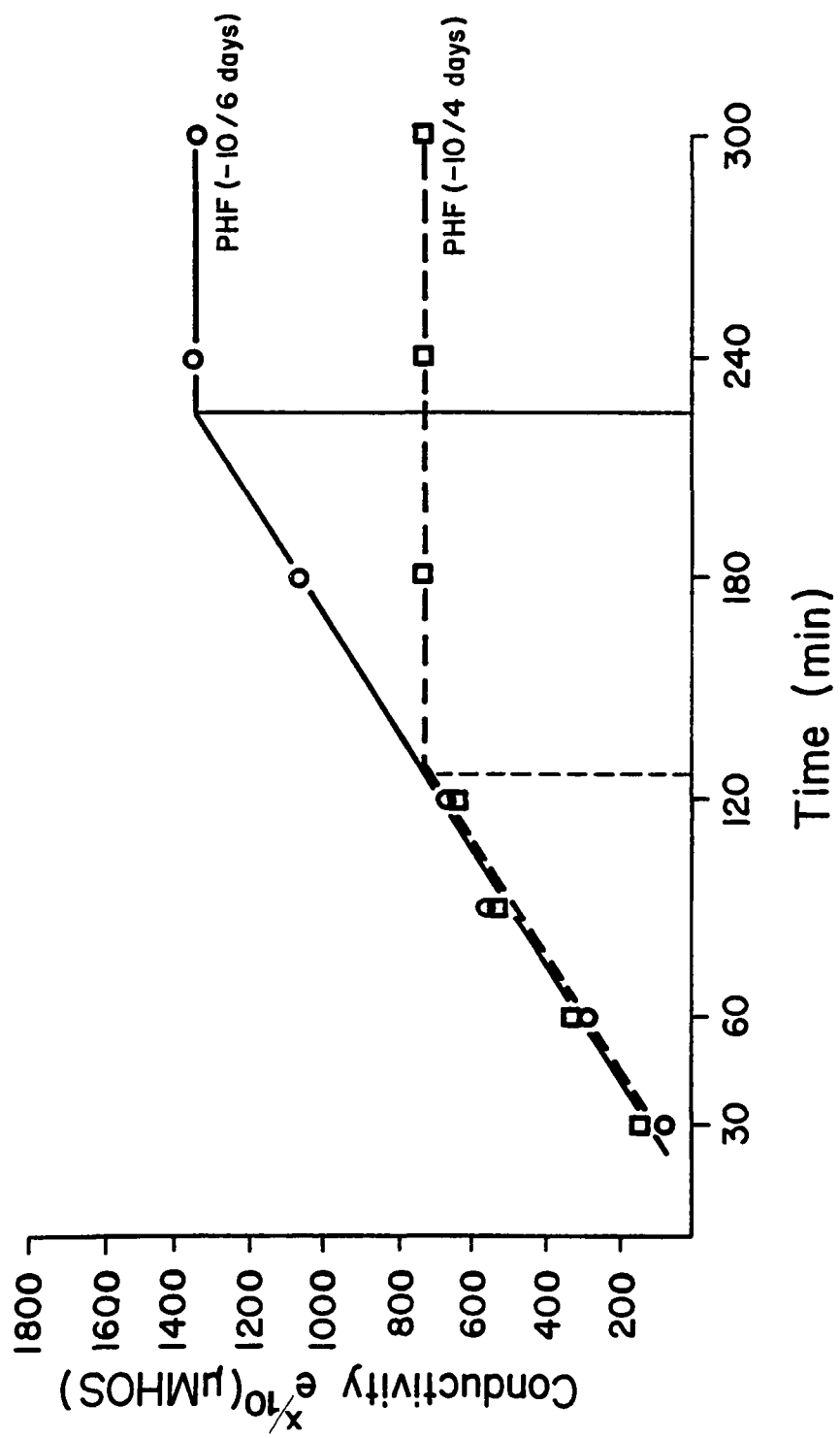
Limitations to the diffusion of the electrolytes from the cells include:

- a. symplastic limitations: plasma membrane, tonoplast, cell walls.
- b. apoplastic limitations: tissue organization, conducting elements (size, number), infiltration state (influence of extracellular freezing), cuticle, tissue size.

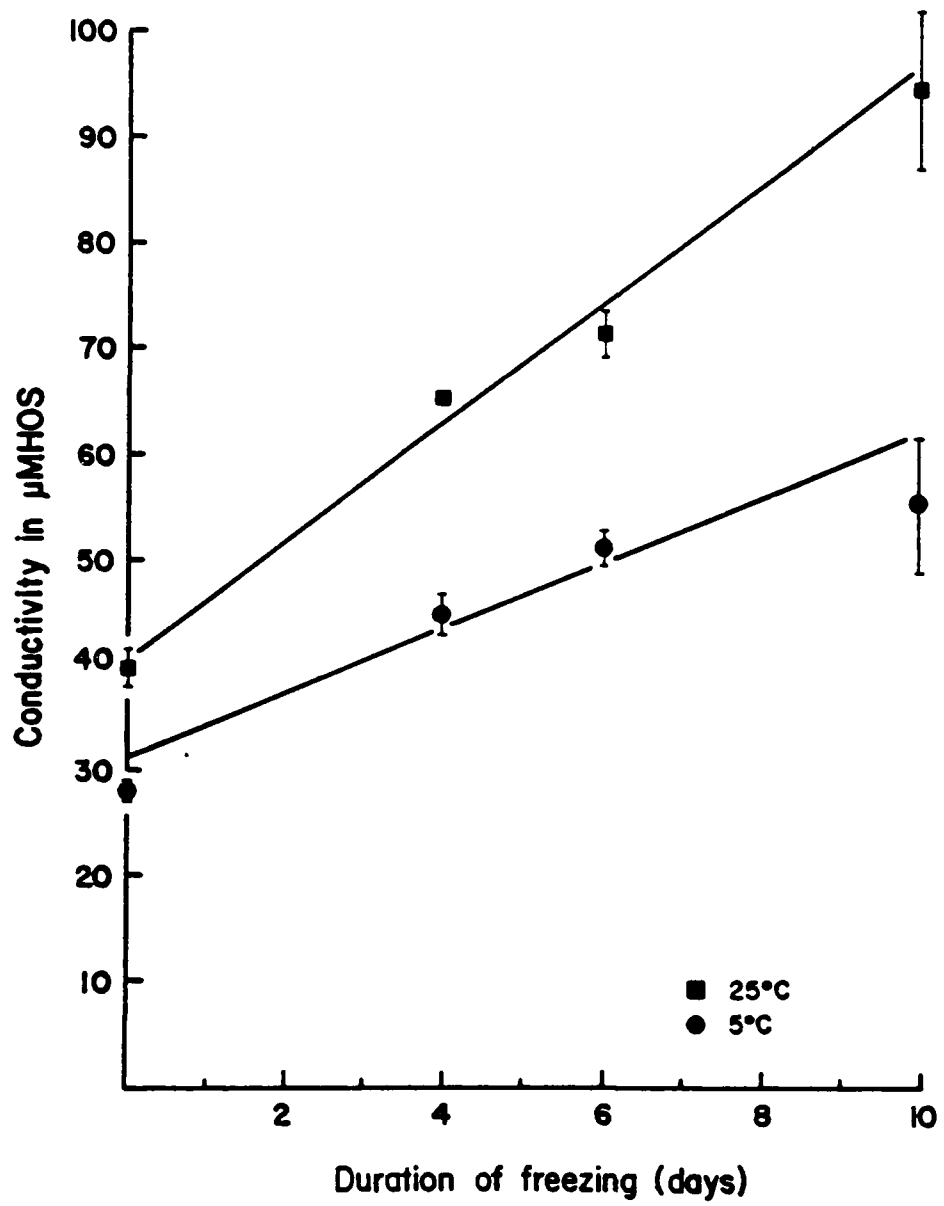
Therefore, the kinetics of release of electrolytes may be primarily determined by one of these limiting factors which is not necessarily plasma membrane resistance. The exponential relation holds for approximately 2 h in the case of a freezing of 4 d and for 4 h in the case of a freezing of 6 d duration. In these cases, an increase in the duration of post-thawing leakage may account for the higher loss of electrolytes from the frozen tissues (Table 3.2 B, C). In addition, this time of electrolyte leakage more or less corresponds to the time during which chlorophyll synthesis is inhibited as indicated in Table 3.1 for a freezing at  $-8^{\circ}\text{C}$  for 5 d.

Figure 3.5 gives the conductivity of the incubating medium after 4 h of incubation at  $5^{\circ}\text{C}$  or  $25^{\circ}\text{C}$  following thawing plotted against duration of the freezing stress at  $-10^{\circ}\text{C}$ . It is evident from this figure that conductivity of the bathing solution increases with the duration of freezing. This means that the extent of post-thawing leakage is directly related to the duration of the freezing treatment. Assuming that the plasma membrane constitutes the limiting barrier for the release of electrolytes from the cells (not necessarily the factor determining the kinetics of diffusion out of the tissue sections), it is reasonable to consider that the increase in conductivity is a consequence of a loss of this control and thus a measure of damage to the plasma membrane. In Puma frozen for 4 and 6 d, the damage is non-lethal and the cells recover. However after 10 d of freezing, the maximum tolerable degree of injury is reached in a certain proportion of cells and

**Figure 3.4.** Plot of exponential values ( $\log e^{x/10}$ ) of the conductivity of the incubating medium of non-lethally frozen ( $-10^{\circ}\text{C}$  for 4 and 6 d) Puma rye primary leaf tissue sections over time of incubation at  $25^{\circ}\text{C}$ . Data for 6 d shown in Fig 3.3. Survival is 100% in both cases after 48 h of incubation.



**Figure 3.5.** Conductivity of the incubating medium of frozen-thawed Puma primary leaf tissue sections after 4 h of incubation (y axis) plotted against duration of the freezing treatment (x axis). Incubation at 5°C and 25°C. All values are the mean of triplicates, bars indicate SEM (Data from experiment presented in table 3.2).



survival is reduced. At this point, it is difficult to establish if death depends on physical disruption of the unstable plasma membrane or if it depends on a critical amount of solute lost by the injured cells.

The extent of freezing injury as measured by the release of electrolyte must be corrected for the unfrozen controls. In Table 3.3, this is presented as the mean conductivity difference (frozen - control, at 4 and 48 h). Assuming that the semipermeable characteristics of the plasma membrane are unchanged in unfrozen tissues, the release of electrolyte by the unfrozen controls depends only on apoplastic contributions and very little on symplastic contributions. Therefore, the mean conductivity difference between frozen tissues and unfrozen controls is taken as a measure of the intracellular contribution to the release of electrolytes by the frozen tissues. The mean conductivity difference after 4 h increases with increasing duration of freezing to  $-10^{\circ}\text{C}$  from 23.0  $\mu\text{MHOs}$  at 4 d to 54.5  $\mu\text{MHOs}$  at 10 d for Puma (Table 3.3). The mean conductivity difference at 4 h was taken as a measure of freezing injury or the stress-dependent change in plasma membrane resistance to electrolyte diffusion. The difference between unfrozen control samples and samples frozen to  $-78^{\circ}\text{C}$  (lethal) represents the total electrolyte available for diffusion from the cells or 100% leaching. Since diffusional equilibrium was not reached by 4 h in the tissues frozen to  $-78^{\circ}\text{C}$  (lethal), 100% leaching (186  $\mu\text{MHOs}$ ) was estimated by subtracting the control value at 4 h (36  $\mu\text{MHOs}$ ) from the conductivity of the lethal treatment at 48 h (222  $\mu\text{MHOs}$ ) (data from Table 3.2 A). The % leakage for the different non-lethal freezing treatments ( $-10^{\circ}\text{C}$  for 4, 6 or 10 d) was then estimated by dividing the mean conductivity difference at 4 h for each treatment (Table 3.3) by 186  $\mu\text{MHOs}$ . In Puma, the % leakage estimated by this method is 12.4% for freezing to  $-10^{\circ}\text{C}$  for 4 d, 19.2% for 6 d and 29.3% for 10 d. Comparing the % leakage with estimated % survival, one obtains:

**Table 3.3** Difference in conductivity between frozen and control samples (mean conductivity difference) after 4 and 48 h of incubation at 25°C following a prolonged freezing stress (data from Table 3.2; conductivity of the bathing solution after 4 h; R25/5).

Cultivar	Freezing Treatment (d)	Mean Conductivity Difference [frozen - control] ( $\mu$ MHOS)		Survival 48 hrs After Thawing
		4 h	48 h	
Puma	4 days @ -10°C	23.0	-1.0	100
	6 days @ -10°C	35.7	-1.1	100
	10 days @ -10°C	54.5	9.5	86
	15 min at -78°C (Lethal)	149.0	210.6	0
Animo	6 days @ -10°C	49.3	10.5	82
	15 min at -78°C (Lethal)	164.3	209.5	0

100% survival when leakage reaches 12.4%

100% survival when leakage reaches 19.2%

86% survival when leakage reaches 29.3%

These results indicate that prolonged exposure to non-lethal freezing temperatures can lead to a loss of at least 20% of the total cellular electrolytes without lethal damage. A loss of about 30% is associated with lethal damage in 14% of the tissue sections incubated.

At 48 h in Puma, the mean conductivity difference (Table 3.3) is  $-1.0 \mu\text{MHO}$  at 4 d,  $-1.1 \mu\text{MHO}$  at 6 d,  $9.5 \mu\text{MHO}$ s at 10 d and  $210.6 \mu\text{MHO}$ s in tissue sections frozen at  $-78^\circ\text{C}$  (lethal). A negative value indicates that the conductivity of the bathing solution of the frozen samples is lower than of the unfrozen control. The mean conductivity difference at 48 h was used as an indication of the extent of reabsorption of the electrolytes by the cells between 4 and 48 h. In Puma frozen to  $-10$  for 4 and 6 d, the small negative values indicate complete reabsorption of the leaked solutes. This apparent complete recovery is consistent with the estimates of 100% survival based on both expansion of the tissue sections in water and uniform development of chlorophyll by the etiolated tissues exposed to light after thawing. The reabsorption of electrolytes was only partial in the case of frozen samples with reduced survival. In Puma frozen to  $-10^\circ\text{C}$  for 10 d, the mean conductivity difference was  $9.5 \mu\text{MHO}$ s after 48 h. In Animo frozen to  $-10^\circ\text{C}$  for 6 d, the mean conductivity difference was  $10.5 \mu\text{MHO}$ s. Finally, no reabsorption was observed in the lethal treatment, which is consistent with the observed 0% survival.

The values for electrolyte leakage after prolonged exposures to  $-10^\circ\text{C}$  for 4, 6 or 10 d are lower than those reported for onion bulb cells by Palta *et al.* (1977 a,b;1978). These authors reported that up to 76% of the electrolyte content of

**Table 3.4.** Effect of prolonged freezing stress on electrolyte release, initial viability and long term survival of:

A - onion bulb scale parenchyma cells (from Palta *et al.*, 1977 a,b) (Shaking time 1 hour).

Treatment	% of leakage		Survival After			
	ABS <sup>1</sup>	CC <sup>2</sup>	1 h	2 d	7d	
-11°C for 6 d	76%	64%	(exp 1)	100%	ND	40%
			(exp 2)	100%	ND	90%
- 4°C for 6 d	42%	12%	100%	ND	100%	
+ 3°C (control)	34%	0%	100%	ND	100%	

B - Puma rye primary leaf tissues (repeat from table 3.3)

-10°C for 10 d	42%	29%	ND	89.5%	ND
-10°C for 6 d	32%	19%	ND	100%	ND
-10°C for 4 d	29%	12%	ND	100%	ND

<sup>1</sup>ABS = Calculation of % leakage based on absolute values of conductivity of the bathing solutions (100% leakage = total electrolyte released by killed tissues).

<sup>2</sup>CC = Calculation of % leakage based on conductivity values corrected for unfrozen controls (100% leakage = conductivity difference between killed tissues and unfrozen controls).

the cells was lost and yet the cells remained alive. Apart from the fact that a different system was used, two factors may contribute to this difference:

- a. Palta *et al.*, estimated survival immediately after thawing and not after 48 h.
- b. Palta *et al.*, estimated % leakage on absolute values of conductivity; uncorrected for controls.

Table 3.4 compares the data for onion scale and rye leaves tissues. When one takes into account long-term survival (7 d) and expresses the results of Palta *et al.*, as the difference between the stressed and the controls, the disparity between the results is reduced.

### 3. Effect of Temperature during Post-Thaw Recovery on Electrolyte Release

It is well-known that temperature affects the conductivity of a salt solution by increasing or decreasing the viscosity of the milieu and by affecting the dissociation coefficient of the salt (Besson, 1962). Over small temperature intervals, the temperature dependence is almost linear (Besson, 1962). This effect can be referred to as the Conductivity Temperature Compensation Coefficient (CTCC) of an electrolytic solution and is expressed as % increase per °C ( $\% \cdot ^\circ\text{C}^{-1}$ ) over certain ranges of temperatures (International Critical Table, McGraw Hill, 1986). Temperature also affects the permeability of membranes to electrolytes. Therefore, analysis of the data in Table 3.2 (conductivity measurements at 4 h, uncorrected for temperature) was carried out in an attempt to separate these two possible effects of temperature. First, the conductivity values of samples incubated at 25°C (R25/5) were divided by that of samples incubated at 5°C (R5/25) after 4 h of incubation for recovery to give a Temperature Dependent Conductivity Ratio (TDCR) for each treatment (control and frozen tissues). The results of this simple calculation on

the data of Table 3.2 are given in Table 3.5. In a calculation similar to the CTCC (conductivity temperature compensation coefficient) of solutions, the % increase represented by the TDCR (e.g., for TDCR = 1.37, the % increase = 37%) was divided by the temperature difference (20°C) to give a value called here Apparent Conductivity Temperature Compensation Coefficient (ACTCC) (Table 3.5).

In the samples of Animo frozen to -78°C (lethal freezing), the TDCR at 4 h was 1.37, and the ACTCC was 1.85 (Table 3.5). The mean TDCR of the unfrozen control samples of Animo was also 1.37 giving the same ACTCC as the samples frozen to -78°C (1.85). The Animo samples frozen at -10°C for 6 d showed a significantly higher TDCR than did the unfrozen control (1.65 vs. 1.37). The ACTCC was thus also higher (3.25 vs. 1.85). The mean TDCR of Puma samples frozen at -10°C for 4 d was 1.44 and for 6 d was 1.37. These values are not significantly different from the values obtained for their respective unfrozen controls. The ACTCC obtained for these samples was 2.20 and 1.85 respectively, as compared to 2.15 and 1.85 for the unfrozen controls. In Puma samples frozen at -10°C for 10 d, the TDCR was 1.76, significantly higher (95% probability level) than that obtained for the controls (1.40). The ACTCC calculated was almost twice that of the unfrozen control (3.80 vs 2.00).

The TDCR (1.37) obtained for lethally frozen samples containing dead tissues is independent of the potential effect of the incubation temperature on the plasma membrane since the plasma membrane was disrupted. All of the cells are killed by the extreme freezing (-78°C), and they do not retain their electrolytes which are free for diffusion up to diffusional equilibrium. The similar TDCRs obtained for unfrozen control samples in Animo indicate the absence of a specific effect of incubation temperature on the permeability of the plasma membrane of normal unstressed cells. The actual difference in conductivity of the bathing solution

**Table 3.5.** Temperature Dependent Conductivity Ratio (TDCR)<sup>1</sup> and Apparent Conductivity Temperature Compensation Coefficient (ACTCC)<sup>2</sup> of the effusates from winter rye primary leaf tissues after 4 h of incubation following prolonged freezing stresses. (Adapted from data in Table 3.2, 4 h incubation time).

Cultivar	Treatment	TDCR	ACTCC (%·°C <sup>-1</sup> )
Animo	unfrozen control	1.37 ± .03	1.85
	-78°C for 15 min	1.37	1.85
Animo	control	1.37 ± .03 s	1.85
	-10°C for 6 d	1.65 ± .11	3.25
Puma	control	1.43 ± .04 ns	2.15
	-10°C for 4 d	1.44 ± .06	2.20
Puma	control	1.37 ± .04 ns	1.85
	-10°C for 6 d	1.37 ± .03	1.85
Puma	control	1.40 ± .03 s	2.00
	-10°C for 10 d	1.76 ± .07	3.80

<sup>1</sup> TDCR = Conductivity value at 25°C divided by conductivity value at 5°C. Adapted from Table 3.2; conductivity values after 4 h incubation time.

<sup>2</sup> ACTCC = % increase in conductivity of the bathing solution containing tissue sections for an increase of 1°C in the temperature of incubation.

ns= not-significantly different at the 95% probability level.

s= significantly different at the 95% probability level

measured at 5°C (R5/25) and at 25°C (R25/5) for unstressed control tissue samples and for dead tissues must be understood in terms of a change in the mobility of the electrolytes in solution. The ACTCCs obtained for killed tissues and those of unfrozen controls are close to those reported in the literature for solutions of NaCl, KCl and KNO<sub>3</sub> of similar concentrations and for the same temperatures (Table 3.6). For these solutions, the CTCC is virtually the same and is relatively independent of concentration over the range 1 to 100 mM and is also independent of the nature of the electrolyte. Therefore, similar or dissimilar values of ACTCCs for control and frozen tissues are not significantly affected by these physico-chemical parameters, but reflect the response of the plasma membrane.

The TDCR of the frozen samples showing 100% survival and complete reabsorption of the leaked electrolytes after 48 h (Puma frozen at -10°C for 4 and 6 d Table 3.5) is the same as that of the respective unfrozen controls. Here again, the TDCRs can also be understood in terms of an effect of temperature on the mobility of the electrolytes rather than by a specific effect of temperature on the permeability of the plasma membrane soon after thawing. On the other hand, the elevated TDCR of the frozen samples showing partial recovery after 48 h (Puma frozen to -10°C for 10 d and Animo frozen to -10°C for 6 d) cannot be entirely explained by the physical phenomenon of mobility of electrolytes in solution. It must rather be understood in terms of a very specific effect of temperature on the permeability of the plasma membrane of these stressed cells in the early stage of the post-thawing period. This effect must be taken in terms of the sensitivity of a critically damaged plasma membrane in a significant proportion of the cells. Under such critical conditions, the plasma membrane is sufficiently unstable to be affected by a difference of 20°C in the incubation temperature in water after thawing. An incubation at 5°C instead of 25°C may stabilize the plasma membrane and thus

**Table 3.6.** Effect of concentration on the conductivity temperature compensation coefficient (CTCC)<sup>1</sup> over the 5 to 25°C interval for different salt solutions.

Electrolytes	Concentration (mM)	CTCC (%·°C <sup>-1</sup> )
NaCl	100 mM	1.88
	10 mM	1.92
	1 mM	1.93
KNO <sub>3</sub>	100 mM	1.79
	10 mM	1.80
KCl	100 mM	1.80
	10 mM	1.84
	1 mM	1.88

<sup>1</sup> CTCC = % increase in conductivity of an electrolytic solution for an increase of 1°C in the temperature of incubation.

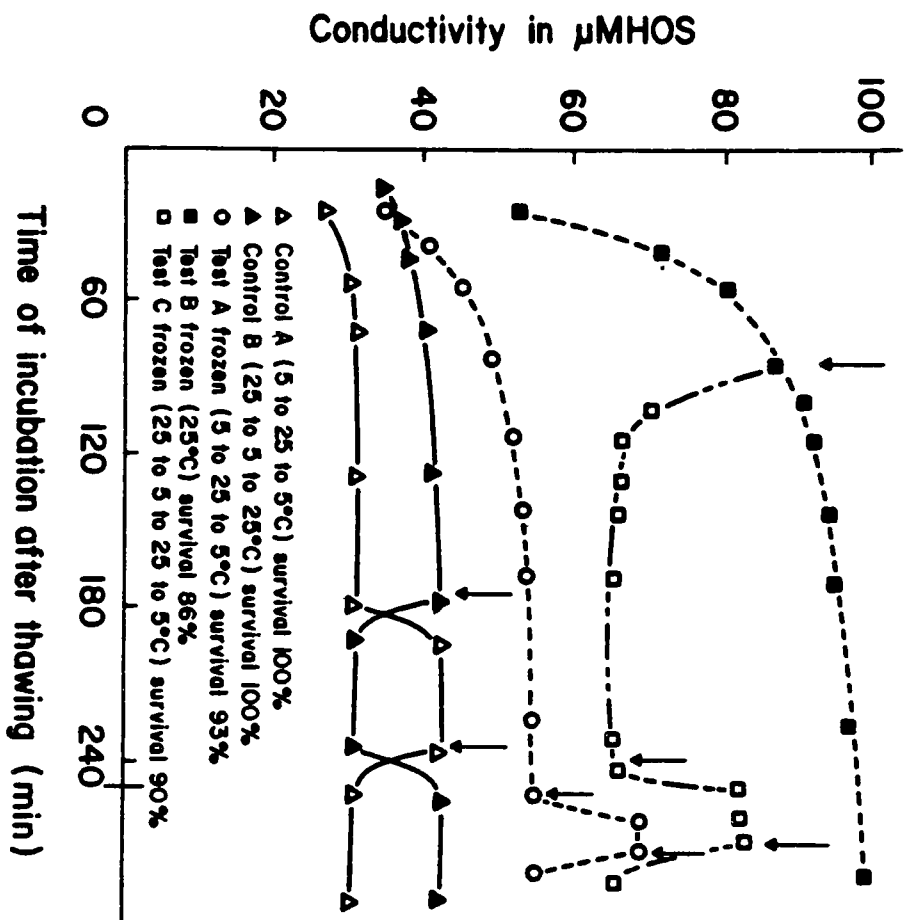
Values obtained from the International Critical Tables Vol VI McGraw Hill, N.Y. pp. 229-253 (1986).

influences the occurrence of an irreversible lesion. In the case of an irreversible lesion depending on enzymatic degradation, it is reasonable to expect that incubation at low temperatures would reduce such activity and consequently favor recovery.

#### 4. Effect of Transfer from 25°C to 5°C (or *vice versa*) at Various Times during Post-Thaw Recovery.

Figure 3.6 shows that if the transfer of the samples from 25°C to 5°C was made after 90 min from the beginning of the incubation, the conductivity dropped by almost 30% (Test C). This immediate readjustment was taken in terms of an effect of temperature on the mobility of electrolytes already present in the bathing solution. Subsequently, the conductivity remained stable as long as the samples was maintained at 5°C and was in contrast to the continuing increase in sample maintained at 25°C (test B). This indicates a stabilizing effect of low temperature on the plasma membrane. Importantly, the conductivity did not go as low as the conductivity of the samples maintained at 5°C from the beginning of the incubation (Test A). Since (i) the release of electrolyte was terminated at about the same time (about 240 min) for both incubation at 5°C and incubation at 25°C and (ii) transfer from 5°C to 25°C of both Test A and Test C at that time did not restore conductivity to the value of the frozen-thawed samples maintained at 25°C throughout (Test B), it was concluded that a certain proportion of the damage that developed during the first 90 min of incubation at 25°C was irreversible. The effect of temperature on electrolyte release in the early stages of the post-thaw period in Puma frozen to -10°C for 10 d may emphasize the primary role of the plasma membrane, and possibly the tonoplast, in the transition from reversibly damaged cells to irreversibly damaged cells. There is a net permanent effect of the

**Figure 3.6.** Change in conductivity of the incubating medium of unfrozen (CONTROL solid lines) and non-lethally, frozen-thawed (-10°C for 10 d, TEST dashed lines) Puma rye primary leaf tissue sections over time of incubation at 5°C (A) or 25°C (B). Arrows indicate time of transfer of samples from 5°C to 25°C or from 25°C to 5°C. All values are mean of triplicates, bars indicate SEM.

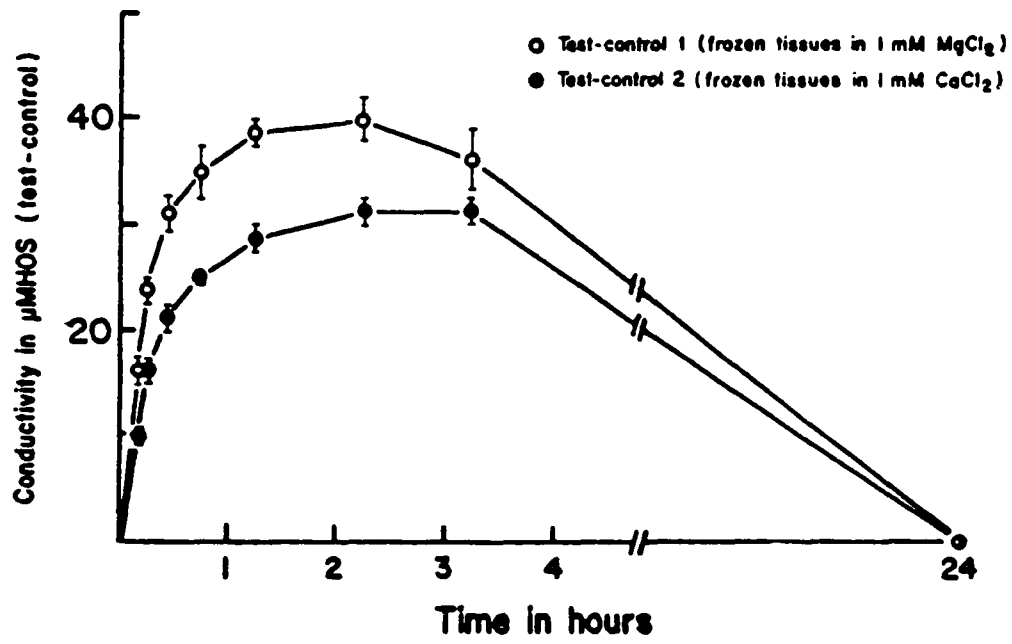


initial temperature of incubation on the extent of post-thaw cellular leakage and this may contribute to the attainment of an irreversible level of damage. It is reasonable to think that the differences in survival between samples at 5°C (93% survival), those transferred from 25 to 5°C at 90 min (90% survival) and those at 25°C (86% survival) are consequences of the early effect of temperature on the stressed membranes after thawing. This may be due to the stabilizing effect of low temperature or due to an increase in the rate of degradative processes at high temperatures. Unless we actually observe disruption of the plasma membrane or cytoplasmic acidosis after thawing, it is difficult to establish if mortality was determined by physical disruption of the plasma membrane of the damaged cells or if it was dependent on an alteration of the semipermeable properties of the tonoplast leading to cytoplasmic acidosis.

##### 5. Effect of Calcium on Electrolyte Leakage

Incubation in presence of 1 mM  $\text{CaCl}_2$  following freezing of Puma (-10°C for 6 d) reduced the post-thaw increase in conductivity as compared to samples incubated in 1 mM  $\text{MgCl}_2$  (Fig 3.7).  $\text{Ca}^{++}$  ions are thought to interact with membranes and to contribute to the maintenance of the membrane integrity (Van Dijck *et al.*, 1975). The reduction of the post-thaw electrolyte leakage in  $\text{Ca}^{++}$ -treated samples is indicative of the stabilizing effect of this agent on the plasma membrane. These results are consistent with those of Arora and Palta (1986) who observed that post-thaw injury can be prevented by  $\text{Ca}^{++}$  and further emphasizes the importance of the destabilisation of the plasma membrane in the progress of injury following thawing.

**Figure 3.7.** Change in conductivity of the incubating medium (expressed as the difference in conductivity between the stressed and unstressed samples) of frozen-thawed ( $-10^{\circ}\text{C}\cdot 6\text{ d}$ ) Puma rye primary leaf tissue sections over time of incubation. Both frozen and control tissue sections were incubated in 1 mM  $\text{CaCl}_2$  (TEST-CONTROL 2) or in 1mM  $\text{MgCl}_2$  (TEST-CONTROL 1). All values are the mean of triplicates, bars indicate SEM. Survival = 100% in both cases after 48 h of incubation.



#### D. Conclusions

Although the kinetics of electrolyte diffusion out of the tissues sections may be determined by apoplastic limitations (structural), the extent of electrolyte release by the tissues in response to prolonged exposure to non-lethal freezing temperatures depends on an alteration of the semipermeable properties of the plasma membrane and possibly the tonoplast of stressed cells. Because they are largely dominant in the primary leaf tissue sections, mesophyll cells must contribute significantly to the amount of electrolytes released by the tissues. Apparently, leakiness is a measure of injury and is associated with an inhibition of the synthesis of chlorophyll in the thylakoid membrane of the mesophyll cells. In the non-lethal treatments, recovery of chlorophyll synthesis in the mesophyll cells is associated with the arrest of electrolyte leakage by the tissues. Under these conditions, the frozen-thawed tissues incubated in water can lose up to 32% of their total electrolyte available for leakage (% based on absolute value of conductivity) or 20% of their cellular electrolytes (% based on conductivity corrected for unfrozen controls) and still totally recover.

Under critical freezing conditions (partial recovery and reduced survival), the ability of the plasma membrane (and tonoplast) to recover after thawing is temperature dependent. This temperature dependence can determine the development of lethal injury after thawing as indicated by the permanent effect of the initial temperature of incubation on the extent of electrolyte leakage and survival. These results were taken as evidence for the primary importance of damage to cellular membranes in the post-thawing transition from reversible to irreversible damage. Finally, under equivalent freezing conditions ( $-10^{\circ}\text{C}$  for 10 d in Puma, 90% survival and  $-10^{\circ}\text{C}$  for 6 d in Animo, 85% survival), the behavior of the two cultivars

appeared to be very similar both in terms of % leakage and extent of reabsorption. This may indicate that the lethal response depends on the attainment of a critical level of injury in both cultivars.

## CHAPTER IV

### MEASUREMENT OF RADIOACTIVE RUBIDIUM UPTAKE BY PRIMARY LEAF TISSUES AS INFLUENCED BY PROLONGED FREEZING AND FUSICOCCIN

#### A. Introduction

Reversible damage to the semipermeable characteristics of the plasma membrane in response to prolonged exposures to non-lethal freezing temperatures has been demonstrated in onion bulb cells (Palta *et al.*, 1977a,b) and in winter rye primary leaf tissues (Chapter III). Palta *et al.* (1977a,b) reported that as much as 76% of the total electrolyte content of onion scale tissue can leach from the cells following thawing without any sign of mortality, as based on the apparent intactness of the cells, normal osmotic responsiveness and the maintenance of protoplasmic streaming immediately after thawing. Sugars constituted almost 80% of the solute content of the leachate.  $K^+$  (plus its assumed counterion) was found to be the most abundant electrolyte accounting for almost 100% of the conductivity of the leachate. Because the plasma membrane apparently remained physically unaltered, these authors described the leakage in terms of an alteration in active transport systems rather than as a change in passive permeability of the plasma membrane (Palta *et al.*, 1977a; Palta and Li, 1978). Although leakage was observed for substances which are known to be specifically associated with active transport systems of the plasma membrane (sugars,  $K^+$ ), their conclusion was drawn in the absence of any direct evidence of an inhibition of active transport. Nevertheless, Palta and Li (1978) suggested that proteins in the plasma membrane could be affected during the early stage of freezing injury.

Proton ( $H^+$ ) pumping at the plasma membrane and tonoplast constitute major ATP-consuming activities and primary transport processes of the plant cell (Poole, 1978; Spanswick, 1981; Lin, 1985). The general finding is that  $H^+$  efflux at the plasma membrane is energy dependent, while influx is passive at extracellular pH values below 9 (Smith and Raven, 1979). Active  $H^+$  extrusion contributes to the hyperpolarization of the plasma membrane (negative potential) (Spanswick, 1981). A variety of transport processes such as  $K^+$  uptake are facilitated by  $H^+$  extrusion because of the resultant proton electromotive force (PMF) (Poole, 1978; Lin, 1985). The high intracellular concentration of  $K^+$  is dependent on this primary transport process. Therefore, inhibition of  $H^+$  pumping at the plasma membrane could be responsible for a net efflux of  $K^+$  from the stressed cells. Importantly, Palta *et al.* (1977a) reported a decrease in the  $H^+$  concentration of the incubating medium as the  $K^+$  content increased, which suggested the possibility of an exchange between  $H^+$  and  $K^+$ . Such an exchange is in the opposite direction to the normal mechanism of active  $H^+$  extrusion coupled with uptake of  $K^+$  and other solutes at the plasma membrane (Smith and Raven, 1979). In addition, cold treatment and other injurious actions and agents increased  $^{45}Ca^{++}$  influx into corn root tissues, a response which was also found to depend on depolarization of the plasma membrane (Rincon and Hanson, 1986). Since hyperpolarization is primarily a result of electrogenic  $H^+$  pumping (Spanswick, 1981), it is reasonable to assume that inhibition of  $H^+$  pumping was responsible for this response.

Fusicoccin (FC) has been used to demonstrate the coupling of  $H^+$  extrusion to uptake of  $K^+$  and other ions at the plasma membrane of the plant cell (Marre *et al.*, 1974). FC specifically binds to a protein present in a plasma membrane-enriched preparation (Dohrmann *et al.*, 1977) and influences active  $K^+$  (or  $Rb^+$ ) uptake at the plasma membrane of plant cells, probably via the stimulation of electrogenic  $H^+$

extrusion (Marre, 1979). The stimulation of ion uptake by FC can be used as evidence for the activation of functional proton pumps and hyperpolarization of the plasma membrane of the plant cell (Marre, 1979).

Radioactive rubidium ( $^{86}\text{Rb}^+$ ) has been used as an analog of  $\text{K}^+$  to probe the permeability properties of the plasma membrane (Pomeroy *et al.*, 1983; 1985). The uptake of  $^{86}\text{Rb}^+$  by root tissues of winter wheat declined following exposure to  $-6^\circ\text{C}$  for several weeks (Pomeroy *et al.*, 1985). A similar response was obtained for ice encasement ( $-1^\circ\text{C}$ ) of winter wheat isolated cells where  $^{86}\text{Rb}^+$  uptake declined more rapidly than viability, which was based on osmotic responsiveness of the cells and uptake of neutral red (Pomeroy *et al.*, 1983).

The objective of this Chapter was to study the effect of prolonged freezing ( $-10^\circ\text{C}$  for 6d) on the uptake of  $^{86}\text{Rb}^+$  by winter rye primary leaf tissues after thawing and to evaluate the effect of temperature of incubation and the FC sensitivity of the response.

## B. Materials and Methods

### 1. General

Samples of Animo and Puma rye tissue sections were frozen to  $-10^\circ\text{C}$  for 6 d and incubated for recovery as previously described (Chapter II). After freezing, the tissue sections (including controls) were processed in the same way as described in Chapter II (incubation for recovery) except that 2 ml of water was added instead of 4 ml. In the experiment with FC, a freezing-infiltration control was used in addition to the vacuum-infiltration control.

### 2. Freezing-Infiltration Controls

Tissues were frozen to  $-4^\circ\text{C}$  for 1 h to achieve an infiltration state of the tissue sections similar to that of those maintained under prolonged freezing. The

samples were placed into the freezing cabinet and removed shortly after crystallization had been completed (at approximately  $-4^{\circ}\text{C}$  according to the development of an exotherm on the Digistrip recording). These samples were then placed on ice for slow thawing in the dark and used as freezing-infiltrated controls.

### 3. Measurement of Rubidium Uptake and Fusicochin Treatment

Two  $\mu\text{C}$  of radiolabelled  $^{86}\text{RbCl}$  (specific activity =  $1.69 \text{ mC}\cdot\text{mg}^{-1}$ ) were added to the beakers containing the tissue sections, and the incubation was allowed to proceed at  $5^{\circ}\text{C}$  or  $25^{\circ}\text{C}$  for 1, 15, 60, 180 min or 21 h. These times were selected to correlate  $^{86}\text{Rb}^{+}$  uptake with the electrolyte leakage experiments presented in Chapter III (Fig. 3.2). One min is taken as the zero time; 15 min corresponds to the time at which the first conductivity measurements were made; 60 min corresponds to the time of maximum rate of electrolyte leakage (increase in conductivity); 180 min corresponds to the time at which electrolyte leakage reached a plateau; 24 h (data not shown) corresponds to the time when the reabsorption of electrolytes was almost complete at  $25^{\circ}\text{C}$ , but only partial at  $5^{\circ}\text{C}$ . Note that 24 h of incubation also corresponds to the time when samples at  $25^{\circ}\text{C}$  were transferred at  $5^{\circ}\text{C}$  (R25/5) and those at  $5^{\circ}\text{C}$  were transferred to  $25^{\circ}\text{C}$  (R5/25) for a further 24 h for the estimate of survival at 48 h (see Chapter II). The incubation was stopped by decanting the incubation medium and followed immediately by repeated washing as follows:

1. Add 2 ml of GDW and immediately decant the wash.
2. Add 2 ml of GDW, shake for 1 min at 100 rpm on a gyrotory shaker and then discard the wash.
3. Add 5 ml of 5 mM KCl, shake for 15 min at 100 rpm. Discard each wash

into an individual identified flask to measure the efficiency and reproducibility of this wash in removing readily exchangeable cations.

4. Add 5 ml of GDW to tissues, shake 1 min at 100 rpm and then discard the wash.

After washing, the tissue sections were placed in scintillation vials containing 10 ml of GDW and the radioactivity was measured using a Beckman scintillation counter, Model LS-8000. Net uptake was expressed as % of incorporation of radiolabel into tissue sections after washing compared to a total activity blank value of  $2\mu\text{C}$  RbCl. The blank was used throughout the experiment to monitor the normal decay of the radiolabel. All experiments were repeated three times.

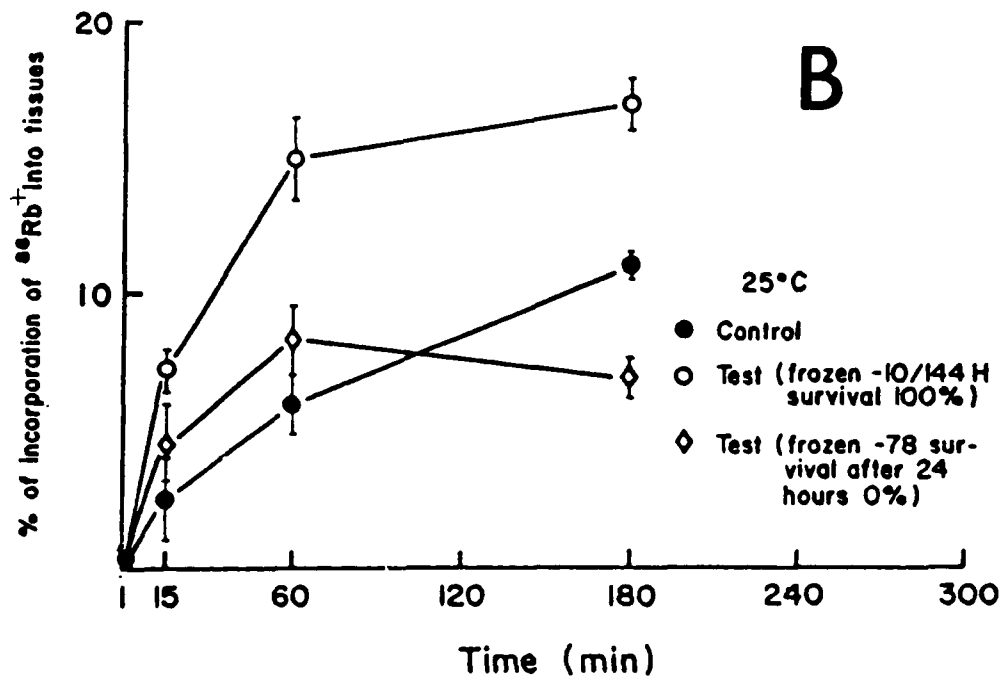
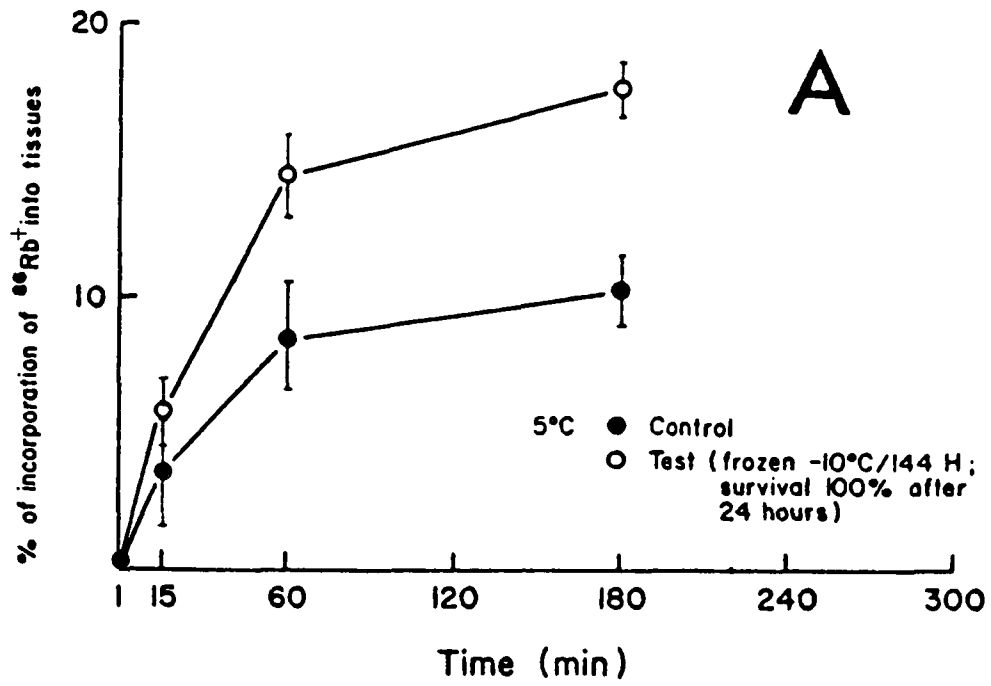
In addition to the influence of temperature, the uptake process was examined for its FC sensitivity. Samples were incubated with  $2.5 \times 10^{-5}$  M FC after addition of 10  $\mu\text{l}$  of a stock solution ( $5 \times 10^{-3}$  M FC in ETOH) to 2 ml of suspending medium. Controls (-FC) were prepared by addition of 10  $\mu\text{l}$  of pure ETOH added to the 2 ml of the suspending medium (final concentration of 0.5% v/v ETOH)

### C. Results and Discussion

#### 1. Effect of Prolonged Freezing ( $-10^{\circ}\text{C}$ for 6 d)

The first observation in Figure 4.1 is that all curves show a rapid initial increase during the first 60 min followed by a clear deceleration in the rate after 60 min. At  $5^{\circ}\text{C}$  (Fig 4.1A) the initial uptake of  $^{86}\text{Rb}^{+}$  is greater in the frozen tissues ( $-10^{\circ}\text{C}$  for 6 d) than in the unfrozen controls such that the frozen tissues contain almost twice the amount of radioactivity than did the unfrozen controls after 180 min. At  $25^{\circ}\text{C}$  (Fig. 4.1B), the same phenomenon was observed, the tissues frozen to  $-10^{\circ}\text{C}$  for 6 d (open circles) accumulated more  $^{86}\text{Rb}^{+}$  than the unfrozen

FIGURE 4.1:  $^{86}\text{Rb}^+$  incorporation into unfrozen (CONTROL) and frozen-thawed (TEST) Puma rye primary leaf tissue sections from dark-grown seedlings during recovery for 3 h under light in water containing  $2\mu\text{C}$  of  $^{86}\text{Rb}^+$  radiolabel. Incubation at  $5^\circ\text{C}$  (A) and  $25^\circ\text{C}$  (B). Survival after 48 h of incubation is 100% in all cases. All values are the mean of triplicates; bars indicate SEM. B includes the response of lethally frozen tissues (lethal control). Fig. 4.1 A gives the kinetics for unfrozen vacuum infiltrated control at  $5^\circ\text{C}$  ( $\bullet$ ) and for tissue sections frozen at  $-10^\circ\text{C}$  for 6 d ( $\circ$ ). Fig. 4.1 B gives the kinetics for unfrozen vacuum infiltrated control at  $25^\circ\text{C}$  ( $\bullet$ ), for tissue sections frozen at  $-10^\circ\text{C}$  for 6 d ( $\circ$ ), and for tissues frozen at  $-78^\circ\text{C}$  (lethal).



controls (filled circles). At 25°C, the tissues frozen to -78°C (open diamond) accumulated more  $^{86}\text{Rb}^+$  than the unfrozen controls during the first 60 min, however between 60 and 180 min, the radioactivity decreased. Such a decrease was observed only in the tissues frozen to -78°C (lethal).

Comparison of the kinetics of  $^{86}\text{Rb}^+$  uptake at 5°C (Fig 4.1 A) and at 25°C (Fig 4.1 B) shows that the behavior of the nonlethally frozen tissues (○) was independent of the temperature. The initial behavior of the unfrozen control (●) was also relatively independent of temperature at least for the first 60 min. Nevertheless, in the unfrozen controls, the increase between 60 and 180 min was greater at 25°C than at 5°C suggesting that in those unstressed tissues a temperature dependent component of  $^{86}\text{Rb}^+$  uptake was active. Most of the difference in  $^{86}\text{Rb}^+$  uptake between non-lethally frozen and the unfrozen controls developed during the first h of incubation. When compared with the kinetics of electrolyte leakage under identical freezing conditions (Fig. 3.2), it appears that the stress dependent increase in  $^{86}\text{Rb}^+$  uptake takes place during the phase of maximum rate of electrolyte leakage. Finally, at 25°C the initial uptake of  $^{86}\text{Rb}^+$  by tissues frozen to -78°C (lethal) suggests that some physiological activity was still present in those tissues, at least for 60 min.

The experiments presented in Table 4.1 were conducted to permit the analysis of the measurement of  $^{86}\text{Rb}^+$  uptake by the tissues in relation to recovery and survival of both Puma and Animo. In Puma, prolonged freezing (-10°C for 6 d) had no deleterious effects, i.e. 100% survival after 48 h of incubation for both an incubation started at 5°C (R5/25) and for an incubation started at 25°C (R25/5) (Table 4.1 A). The prolonged freezing was partially lethal for Animo. Only 89% of the Animo tissue sections survived an incubation started at 5°C and 87% for an incubation started at 25°C (Table 4.1 B). Nevertheless, as discussed below, the

**Table 4.1.** Effect of a prolonged exposure to a non-lethal freezing temperature ( $-10^{\circ}\text{C}$  for 6 d) on survival and  $^{86}\text{Rb}^{+}$  incorporation into unfrozen and frozen-thawed Puma (A) and Animo (B) primary leaf tissue sections (3 mm x 1 mm) after 3 h and 21 h of incubation under two different temperature regimes (R5/25 and R25/5). Means of 3 experiments  $\pm$  SEM.

Cultivar	Treatment	Temperature Regime for Recovery	Survival	% of $^{86}\text{Rb}^{+}$ Incorporation	
			48 h After Thawing (%)	Into Tissues	
				Time After Thawing	
				3 h	21 h
Puma	Control	R 5/25	100 $\pm$ 0	9.9 $\pm$ 1.2	23.6 $\pm$ 2.6
	$-10^{\circ}\text{C}$ for 6 d	R 5/25	100 $\pm$ 0	18.0 $\pm$ 0.6	30.3 $\pm$ 1.6
	Control	R 25/5	100 $\pm$ 0	10.9 $\pm$ 0.4	45.2 $\pm$ 3.6
	$-10^{\circ}\text{C}$ for 6 d	R 25/5	100 $\pm$ 0	17.0 $\pm$ 0.5	45.2 $\pm$ 1.2
	$-78^{\circ}\text{C}$	R 25/5	0	7.0 $\pm$ 0.8	2.0
	Animo	Control	R 5/25	100 $\pm$ 0	9.1 $\pm$ 1.8
$-10^{\circ}\text{C}$ for 6 d		R 5/25	89	20.9	30.7
Control		R 25/5	100 $\pm$ 0	11.6 $\pm$ 1.2	46.5 $\pm$ 1.2
$-10^{\circ}\text{C}$ for 6 d		R 25/5	87	17.5	44.8
$-78^{\circ}\text{C}$		R 25/5	0	4.2	2.7

Data without statistic are means of two experiments.

response of the two cultivars was essentially the same with regard to  $^{86}\text{Rb}^+$  uptake. Exposure to  $-78^\circ\text{C}$  was lethal for both cultivars (Table 4.1 A,B).

As previously shown, prolonged non-lethal freezing ( $-10^\circ\text{C}$  for 6 d) resulted in a greater amount of  $^{86}\text{Rb}^+$  uptake by the tissues over that of the unfrozen controls during the first 3 h of recovery following thawing (Table 4.1). In Puma at  $5^\circ\text{C}$  (R5/25), unfrozen control tissue sections accumulated 9.9% of the total counts during the first 3 h of incubation compared to 18.0% by the non-lethally frozen tissues. Similar values were obtained at  $25^\circ\text{C}$  where unfrozen controls took up 10.9% of total counts present in the medium at the beginning of the incubation compared to 17.0% for non-lethally frozen tissues. This shows that the incubation temperature did not influence the differential uptake since the response was of a similar amplitude at both temperatures.

In Animo, the same phenomenon was observed and was of similar amplitude in spite of 11 to 13% mortality in the non-lethally frozen samples after 48 h compared to 100% survival in Puma. These values correspond to very small amounts of  $^{86}\text{RbCl}$  taken up on a molar basis with 18% of incorporation corresponding to  $\approx 2.33 \times 10^{-9}$  mole  $^{86}\text{RbCl}$ . This corresponds to only 0.004% of the amount of electrolytes lost by the same tissue sections in  $\text{K}^+$  equivalents (Chapter III).

One possible explanation for this enhanced  $^{86}\text{Rb}^+$  uptake by non-lethally frozen tissues is the following. Freezing enhances  $\text{K}^+$  leakage, which would depolarize the membrane and activate  $\text{H}^+$  pumps. The  $\text{H}^+$  pumping activity would hyperpolarize the membrane, a situation that could enhance  $\text{K}^+$  uptake by the cells. This is attractive, but the absence of influence of incubation temperature on the initial  $^{86}\text{Rb}^+$  uptake during the first 3 h by the non-lethally frozen tissues indicates that a temperature-sensitive component of uptake did not contribute to this early response. Pumping should be faster at  $25^\circ\text{C}$  than  $5^\circ\text{C}$ . The samples frozen to  $-78^\circ\text{C}$  (lethal)

accumulated a significant amount of radioactivity during the initial 3 h of incubation, an amount approximately equal to the difference between that of frozen and control tissues (Table 4.1). This suggests that the radioactivity present in dead tissues may explain, at least partially, the difference between the unfrozen controls and the non-lethally frozen samples.

In Puma, after a further 18 h of incubation, radioactivity in the control tissues increased from 9.9 to 24% for an incubation at 5°C and from 10.9 to 45.2% for an incubation at 25°C (Table 4.1 A). In tissues frozen to -10°C for 6 d, it increased from 18.0 to 30.3% for an incubation at 5°C and from 17.0 to 45.2% for an incubation at 25°C. The tissues frozen to -78°C on the other hand, lost most of their radioactivity by 21 h at 25°C. Similar behavior was observed for Animo (Table 4.1 B). Taking account of the differences between non-lethally frozen and control samples after 3 and 21 h of incubation, the results indicate that the differential uptake was no longer observable after 21 h of incubation at 25°C (~45% both for controls and samples frozen to -10°C for 6 d), but was still detectable, to a large extent, after 21 h of incubation at 5°C (~24% for the controls and ~30% for samples frozen to -10°C). Assuming that the infiltration of the tissues by extracellular ice during freezing (freezing infiltration) produces adsorption sites for  $^{86}\text{Rb}^+$  on thawing, the disappearance of the difference at 25°C could depend on the release of  $^{86}\text{Rb}^+$  from the adsorption sites during recovery. Recovery of the tissue may be faster at 25°C than at 5°C and this could explain why the difference is still observable after 21 h at 5°C. However, the fact that killed tissues lost most of their radioactivity may argue against such a conclusion. Therefore, it is likely that the differential uptake corresponds, at least partially, to a greater intracellular accumulation of  $^{86}\text{Rb}^+$  in the frozen tissues and that the lack of any difference at 25°C may depend on the masking effect of the total incorporation at 25°C.

## 2. Effect of Fusicochin on Radioactive Rubidium Uptake

In the absence of FC, the samples frozen to  $-10^{\circ}\text{C}$  for 6 d accumulated more  $^{86}\text{Rb}^{+}$  than the unfrozen, vacuum-infiltrated controls (Table 4.2; 23.2% vs 10.8%). This result is consistent with the results obtained in the previous experiments during an incubation at  $25^{\circ}\text{C}$  (Table 4.1). In addition, the very short and mild freezing given to the frozen-infiltrated control ( $-4^{\circ}\text{C}$  for 1 h) led to a slightly greater incorporation of the radiolabel as compared to the unfrozen control (13.6% as compared to 10.8%; Table 4.2). These differences in  $^{86}\text{Rb}^{+}$  uptake in the absence of FC (-FC) may indicate that, as infiltration of the extracellular space by ice increases during freezing, because of increased cellular dehydration with decreasing temperature, there were more sites for  $^{86}\text{Rb}^{+}$  adsorption upon thawing.

FC at  $25\ \mu\text{M}$  effected an increase in the initial uptake (first 3 h of incubation) from 10.8 to 21.1% total added radioactivity in the Puma control tissues (vacuum infiltrated) (Table 4.2). The extent of the stimulation was greater for the sample frozen to  $-4^{\circ}\text{C}$  for 1 h where the initial uptake went from 13.6 to 28.8% of the total radioactivity. The extent of the FC stimulation was much less in Puma tissues subjected to a long-duration freezing treatment at  $-10^{\circ}\text{C}$  for 6 d (23.2 to 28.2%) i.e., an increase of only 5% compared to the increases of 10.3% for the vacuum-infiltrated controls and of 15.2% in the frozen-infiltrated controls.

FC binds directly to a membrane protein to accomplish its stimulatory effect (Dohrmann *et al.*, 1977), and ultimately FC-stimulated uptake activity depends on coupling with a FC-stimulated  $\text{H}^{+}$  extrusion that hyperpolarizes the plasma membrane (Marre, 1979; Spanswick, 1981). The greater FC-stimulation of the uptake in the frozen-infiltrated control ( $-4^{\circ}\text{C}$  for 1 h) as compared to the vacuum-infiltrated

Tabel 4.2.  $^{86}\text{Rb}^+$  incorporation into unfrozen and frozen-thawed ( $-10^\circ\text{C}$  for 6 d) Puma primary leaf tissues after incubation for 3 h at  $25^\circ\text{C}$  in the presence or absence of  $25\ \mu\text{M}$  of fusicoccin ( $\pm$  FC). Means of triplicates  $\pm$  SEM. Survival is 100% in all treatments after 48 h.

Treatment	% of $^{86}\text{Rb}^+$ Incorporation Into Tissues after 3 h of Incubation Following Thawing
Control A (vacuum-infiltrated control)	
-FC	$10.8 \pm 2.0$
+FC	$21.1 \pm 1.9$
Control B (frozen-infiltrated control); $-4^\circ\text{C}/30$ min	
-FC	$13.6 \pm 2.0$
+FC	$28.8 \pm 1.7$
Frozen A ( $-10^\circ\text{C}$ for 6 d)	
-FC	$23.2 \pm 4.0$
+FC	$28.2 \pm 1.6$

control further indicates that the access of radioactive ions to the plasma membrane was effectively favored by the freezing infiltration. This indicates that the reduced FC-stimulation in the tissues frozen at  $-10^{\circ}\text{C}$  for 6 d cannot be explained by a reduced infiltration of the tissues after thawing. In addition, this reduced stimulation cannot be explained by disruption of the plasma membrane since survival was still 100% after 48 h in all treatments.

Two possible interpretations of the results can be proposed. First, FC is unable to stimulate the pump further because it is already operating at maximum rate. Here again, the absence of any temperature effect on the enhanced  $^{86}\text{Rb}^{+}$  uptake in frozen-thawed tissues in the absence of FC may argue against this interpretation. For the  $^{86}\text{Rb}^{+}$  uptake to operate at maximum rate in the absence of FC stimulation both at 5 and  $25^{\circ}\text{C}$  would imply that temperature does not affect the system. But, active  $^{86}\text{Rb}^{+}$  uptake is an ATP-dependent enzymatic process that is sensitive to temperature. This active mechanism should be slower at  $5^{\circ}\text{C}$  than at  $25^{\circ}\text{C}$ . The second interpretation is that the reduced FC-stimulation may indicate an inhibition of the FC-stimulated  $\text{H}^{+}$  extrusion system associated with active  $^{86}\text{Rb}^{+}$  uptake at the plasma membrane. However, to be valid, this interpretation requires that the ATP level in the cell is not a limiting factor to the FC-stimulation and that no antagonists to FC are present in the frozen cells. Such an antagonist could compete with FC for the binding site on the membrane.

#### D. Conclusions

Although  $^{86}\text{Rb}^{+}$  uptake by rye primary leaf tissue sections was increased by a prolonged freezing treatment, the stimulation was temperature insensitive and was associated with an inhibition of the FC-stimulated active  $^{86}\text{Rb}^{+}$  uptake. The greater accumulation of radioactivity in the non-lethally frozen tissues was

explained by an effect of the infiltration of the tissues by extracellular ice during freezing. On thawing there would be more sites for passive uptake and for  $^{86}\text{Rb}^+$  adsorption in the tissues. The reduced FC-stimulated active  $^{86}\text{Rb}^+$  uptake was taken to indicate damage to the  $\text{H}^+$  pumps at the plasma membrane. Therefore, in the absence of plasma membrane disruption in the non-lethally frozen tissues, the release of a large amount of electrolytes after thawing may be the result of a dissipation of the proton electromotive force (PMF) at the plasma membrane.

## CHAPTER V

### EVALUATION OF THE PHYSICAL INTEGRITY OF THE PLASMA MEMBRANE OF WINTER RYE PRIMARY LEAF CELLS AND PROTOPLASTS SUBJECTED TO PROLONGED FREEZING

#### A. Introduction

In this chapter the post-thaw response of the plasma membrane of mesophyll cells and protoplasts isolated from the primary leaf of non-acclimated and cold-acclimated winter rye seedlings was studied. The objective was to examine the physical integrity and stability of apparently intact mesophyll protoplasts after a prolonged exposure to freezing. The hypothesis that leakiness may be associated with increased fluidity or reduced molecular ordering of the plasma membrane after thawing was also tested. In all experiments, only intact, spherical protoplasts were considered.

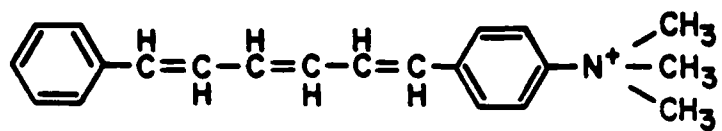
All biological membranes, including the plasma membrane, are dynamic structures with a common overall structure provided by lipid molecules that spontaneously form a bilayer in the presence of water. The lipid bilayer constitutes an effective barrier to the flow of water-soluble molecules and many of the essential transport properties of the biological membranes are accomplished by proteins inserted in the lipid bilayer. The stability and integrity of a lipid bilayer depends largely on hydration of the polar head groups in aqueous environments. This is why freeze-induced dehydration is thought to affect the stability and physical integrity of the lipid bilayer. Leakiness of a stressed cell could then be the result of an alteration in (i) the physical integrity of the lipid bilayer, (ii) the

functional properties of membrane transport proteins, or (iii) lipid-protein interactions.

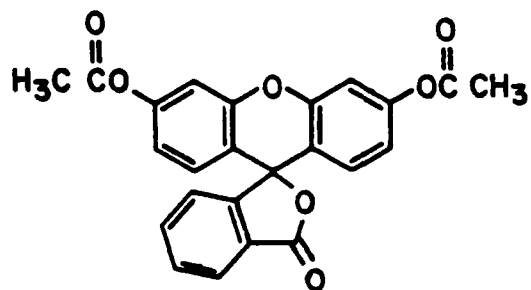
Different observations were made during the estimate of survival after thawing to evaluate the physical integrity of the plasma membrane. The osmotic responsiveness of the frozen-thawed cells was monitored following plasmolysis/deplasmolysis cycles (Palta and Li, 1978). The passive permeability of the plasma membrane of isolated protoplasts was studied by retention of fluorescein after incubation with fluorescein diacetate (FDA). FDA is a fluorogenic ester (Fig 5.1), which permeates biological membranes and which can be hydrolysed to a positively charged fluorescein molecule in the cytoplasm of living cells. The charged fluorescein molecules are trapped in the cytoplasm of the cells and their retention can be used as an indicator of normal permeability of the plasma membrane (Rotman and Papermaster, 1966). The permeability of the plasma membrane was also studied by exclusion of a non-penetrating fluorescein dye, 4-6 diamidino-2 phenylindole (DAPI). The chemical structure of DAPI is given in Figure 5.1. The fluorescent dye is known to have a high affinity for nucleic acids and has been used as a chromosome staining agent (Schnedl *et al.*, 1981). At pH 6, the dye molecule is positively charged and staining of living plant protoplasts requires that the plasma membrane be made temporarily permeable by giving a heat shock or detergent treatment (Meadows and Potrykus, 1981). Therefore, incubating frozen-thawed cells with DAPI makes it possible to compare the effect of non-lethal prolonged freezing on the plasma membrane with the effect of heat shock or detergents.

According to the fluid-mosaic model, all the constituents of the membrane, including lipids and proteins, are held together in a coherent way by non-covalent interactions (Singer and Nicholson, 1972). Both lipids and proteins can move

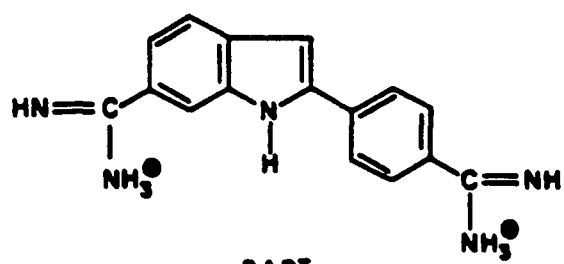
**Figure 5.1** Chemical structures of TMA-DPH (trimethylamino-diphenylhexatriene), FDA (fluorescein diacetate) and DAPI (diamidino-phenylindole)



TMA-DPH



FDA



DAPI

laterally in the plane of the membrane within a monolayer (Poo and Cone, 1974). In addition to lateral diffusion, the individual lipids present rotational motions about their long axes. This indicates that the lipids are flexible (Alberts *et al.*, 1983). This "flexibility" depends on the saturation of the acyl chains, and the greatest degree of flexion occurs near the center of the bilayer (Kates <sup>and Kuksis</sup>, 1980). This flexibility is used to define membrane fluidity. Membrane fluidity depends greatly on the lipid composition and temperature and is an important effector of membrane protein activity (Riordan, 1980).

Because of the possibility of an accumulation of chemical damage to the lipids over prolonged freezing and because such a damage could alter the lipid composition after thawing, it is possible that the average fluidity of the plasma membrane might be changed early after thawing. For this reason, an evaluation of the fluidity of the plasma membrane of non-lethally stressed protoplasts was made.

Different techniques can be used to directly measure membrane fluidity and molecular ordering. One is electron spin resonance (Singh and Miller, 1982). In this case the probe, which can be a modified lipid or a free fatty acid, carries a spin label of which the motion and orientation in the bilayer can be measured. In the present study, the fluidity of the plasma membrane of protoplasts from cold-acclimated and non-acclimated winter rye seedlings was studied by steady state fluorescence polarization spectroscopy (Cossins, 1981). In this technique, a fluorescent molecule, or fluorophor, is intercalated into the membrane and the rotation that occurs during its fluorescence lifetime is followed by measuring the polarization of fluorescent light given a polarized excitation.

Although the behavior of lipid molecules in synthetic or reconstituted bilayers is generally thought to be the same as in cell membranes (Alberts *et al.*, 1983), the development and use of membrane probes that can specifically label a defined

membrane *in situ* and *in vivo* or a defined locus in a membrane present important advantages. The probe trimethylamino-diphenylhexatriene (TMA-DPH) permits studies of the fluidity adjacent to the polar heads in the plasma membrane of intact plant protoplasts and can be used as an indicator of the overall molecular ordering at that level (Kuhry *et al.*, 1983). TMA-DPH is a derivative of the well-known membrane probe DPH (diphenylhexatriene) with a cationic moiety (trimethylamino phenyl) affixed to the para position of one of the phenyl rings (Kuhry *et al.*, 1980). The chemical structure of TMA-DPH is given in Figure 5.1. Because of its trimethylamino group, TMA-DPH labels the external surface of the plasma membrane and measures the fluidity of the upper region of the acyl chains proximate to the polar heads (Kuhry *et al.*, 1980). Fluorescence polarization of TMA-DPH is influenced by the fluidity (order and dynamics) of the membrane lipids and at the polar head level by lipid compaction. An ordered membrane ultrastructure greatly limits the dynamics of the probe and leads to a high fluorescence polarization.

## B. Materials and Methods

### 1. General

The protoplasts and cells used in the present study were isolated from the primary leaves of non-acclimated and cold-acclimated winter rye seedling following the methods defined in Chapter II (General Materials and Methods). The cells and protoplasts were frozen at  $-8^{\circ}\text{C}$  for 16 hours. The cells were frozen in 1 mM  $\text{CaCl}_2$  and the protoplasts in Meyer and Abel R0.6 saline medium. In one experiment, (Table 5.3;  $1.5 \times 10^5$  prot. $\cdot\text{ml}^{-1}$ ), a sorbitol-phosphate buffer was used as the freezing medium. Post-thaw recovery was done in the mannitol-phosphate buffer system. The full procedures for freezing and recovery are described in Chapter II. Survival was estimated on the basis of hydrolysis of FDA and

accumulation of fluorescein in cells and spherical protoplasts. Volumetric measurements were made using an eyepiece micrometer (10X) calibrated against a hemocytometer micrometer (graduated volumetric slide, Neubauer, Fein-Optic, Blankenburg, GDR). Retention of fluorescein and exclusion of DAPI were estimated microspectrofluorometrically as described below.

## 2. Retention of fluorescein

Following 20 min of incubation at  $20 \pm 1^\circ\text{C}$  with  $10^{-5}$  M FDA, unfrozen and frozen-thawed protoplasts of the two cultivars were centrifuged at 130g for 5 min and transferred to FDA-free buffer for 4 h at  $5 \pm 1^\circ\text{C}$ . Retention of fluorescein was estimated on the basis of visual observation of fluorescence photomicrographs (Kodak EL-135 400 ASA film) taken before and after 4 h incubation without FDA and on the basis of fluorescence intensity measurements using a Zeiss UMSP80 microspectrofluorimeter (Carl Zeiss LTD., Oberkochen, W. Germany; Ex 470 nm, Em 540 nm). Fluorescence intensities were expressed relative to a standard uranyl stained glass.

## 3. Exclusion of DAPI

Immediately following thawing, frozen-thawed and non-frozen protoplast suspensions ( $1.5 \times 10^5$  prot·ml<sup>-1</sup>, pH 6) from Puma and Animo were incubated with DAPI ( $50 \mu\text{g}\cdot\text{ml}^{-1}$ ) at  $5 \pm 1^\circ\text{C}$  for 60 min. DAPI was observed by fluorescence microscopy under epi-illumination using a filter combination (Ex 365 nm, Em 420 nm). Fluorescence photomicrographs were taken.

## 4. Steady State Fluorescence Polarization Spectroscopy of TMA-DPH

The fluorescence polarization of the probe localized in the plasma membrane was determined using a conventional Perkin-Elmer fluorescence spectrophotometer (model MPF 44A) equipped with polaroid filters, a thermostated bath and a magnetic stirrer. The excitation and emission wavelengths selected for fluorescence polarization measurement of TMA-DPH were 350 nm and 430 nm, respectively. Two ml of protoplast suspensions of concentration  $2.5 \times 10^4$  to  $3 \times 10^5$  prot·ml<sup>-1</sup> were incubated at  $5 \pm 1^\circ\text{C}$  for 30 min with TMA-DPH at a final concentration of  $10^{-6}$  M. The final concentration was obtained by adding a volume of a stock solution ( $2 \times 10^{-4}$  M TMA-DPH in dimethylformamide or ethanol) to the suspension medium. Suspensions of protoplasts were centrifuged at 130g for 5 min and resuspended in fresh medium just before measurements to remove cellular debris and dead protoplasts from the frozen-thawed suspension.

Initially, the efficiency of the probe in monitoring changes in fluidity or order in the plasma membrane was estimated by the variation of fluorescence polarization of the probe in the plasma membrane at temperatures ranging from 5 to 40°C. This was done, using suspensions of  $1.5 \times 10^5$  prot·ml<sup>-1</sup> of non-acclimated and acclimated protoplasts of both Puma and Animo cultivars and led to an evaluation of the effect of the cold acclimation process on the average fluidity of the plasma membrane. Subsequently, the effect of protoplast concentration on fluorescence polarization was determined by examining a dilution series of the suspensions, from approximately  $3 \times 10^5$  prot·ml<sup>-1</sup> to  $3.75 \times 10^4$  prot·ml<sup>-1</sup> (1:8 dilution).

Finally, the state of the plasma membrane in frozen-thawed ( $-8^\circ\text{C}/0^\circ\text{C}/10^\circ\text{C}$ ) protoplasts immediately after thawing was compared with the state of the plasma membrane in unfrozen control protoplasts ( $5^\circ\text{C}/10^\circ\text{C}$ ) by comparing the fluorescence polarization of the probe within the plasma membrane of the protoplasts at  $10 \pm 1^\circ\text{C}$ . Each polarization value was the mean of 4 to 11 measurements, with each

measurement being the average value of 60 readings over a 30-s measurement interval (2 readings·s<sup>-1</sup>). The concentration of the control suspension was reduced before measurement in order to compensate for mortality in the frozen-thawed sample. The measurements were done both on low density (2.5 x 10<sup>4</sup> prot·ml<sup>-1</sup>) suspensions and high density (1.5 x 10<sup>5</sup> prot·ml<sup>-1</sup>) suspensions.

### 5. Fluorescence Microscopy:

Unfrozen (5°C) and frozen-thawed (-8°C/0°C/5°C) protoplasts were labeled with TMA-DPH (10<sup>-5</sup> M) for 30 min and observed under epiillumination using a Zeiss filter combination (Ex 365 nm, Em 420 nm) during the first 4 h following the thaw. Photomicrographs were taken.

## **C. Results and Discussion**

### 1. Survival of Cells and Protoplasts After Freezing

Preparations of non-acclimated cells and protoplasts of both Puma and Animo showed approximately 40% survival after exposure to -8°C for 16 h (Table 5.1). Survival of cold-acclimated cells (~80 to 90% survival) was higher than survival of the cold-acclimated protoplasts (~65% survival) for the two cultivars. Acclimated cells of hardy Puma rye were slightly more tolerant (~92% survival) to freezing stress than were those of Animo rye (~77% survival); however, the protoplasts (63-65% survival) showed no cultivar differences.

The freezing protocol used in these experiments was lethal for a certain proportion of cells and protoplasts in the different populations tested. The greater survival of Puma acclimated cells over that of Animo is expected from the greater resistance to freezing of Puma primary leaf tissues after cold acclimation (Cloutier and Imbeault, 1988). The greater survival of acclimated cells over that of

**TABLE 5.1:** Survival of Puma and Animo protoplast and cell suspensions after a freezing treatment. Cells in 10mM CaCl<sub>2</sub> and protoplasts in isotonic saline solution were frozen to -8°C for 16 h. Survival of cells and protoplasts was monitored after FDA staining. Mean of four replicates ± SEM.

Sample/Treatment	Survival (%) ± SEM		
	Cells		Protoplasts
Puma, non-acclimated	40 ± 7	ns	40 ± 15
	s		ns
Puma, cold-acclimated	92 ± 7	ns	65 ± 12
Animo, non-acclimated	40 ± 7	ns	50 ± 2
	s		ns
Animo, cold-acclimated	77 ± 7	ns	63 ± 2

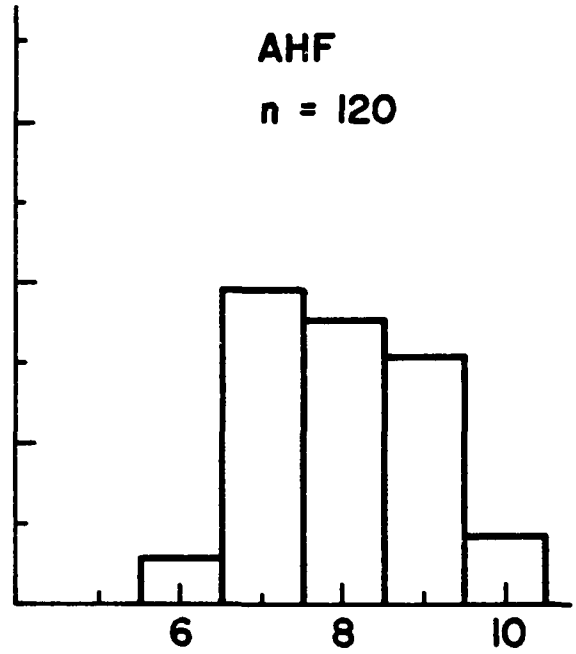
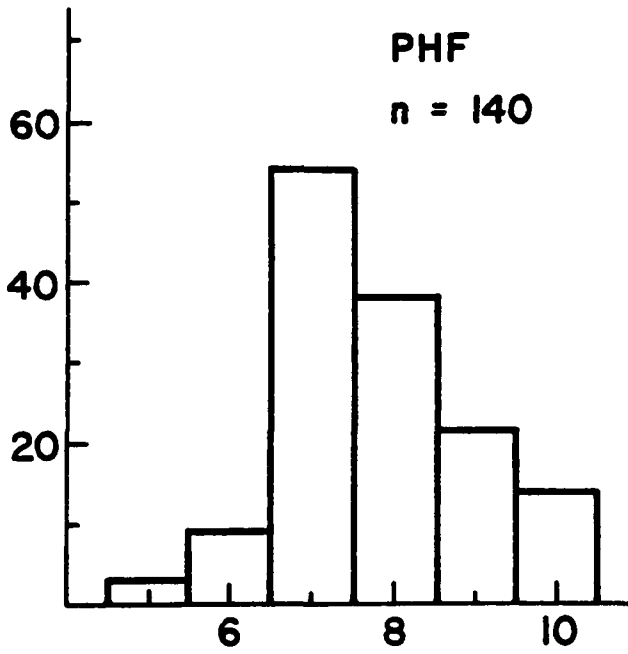
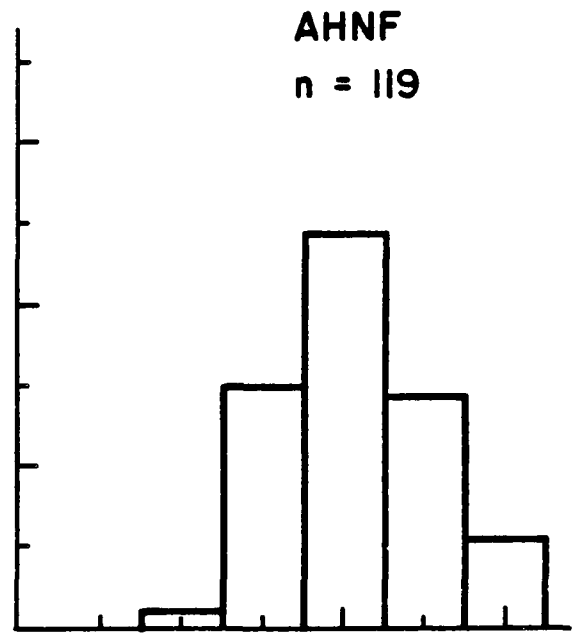
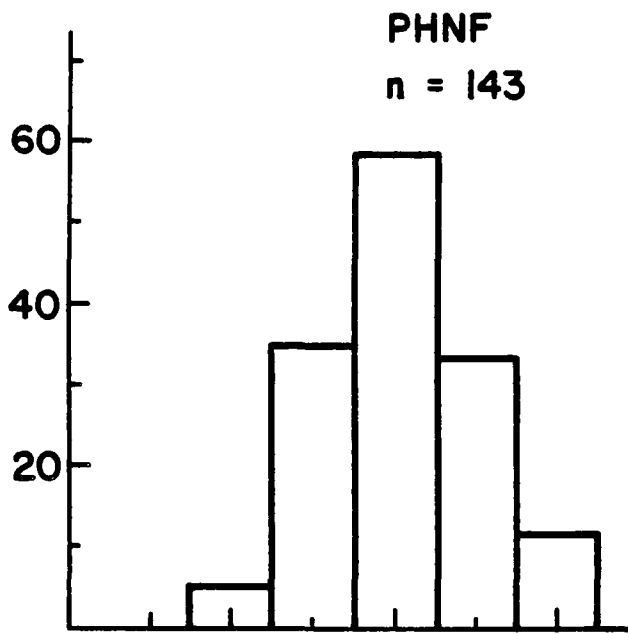
s, ns - significant or non significant difference at the 95% probability level.

acclimated protoplasts, particularly those of Puma, further emphasizes the fact that the freezing stress experienced by protoplasts suspended in a saline osmoticum is not equivalent to the freezing stress experienced by cells in aqueous suspensions (Singh, 1981). It is known that some media for protoplasts show a cryoprotective effect during freezing (Singh, 1979). The Meyer and Abel saline medium R 0.6 was used in the present study to avoid the protective effect of media containing sugar. However, the Meyer and Abel saline medium may constitute a factor of increased stress as it concentrates over the freezing process. Therefore, in these experiments with protoplasts, the resultant strain was dependent on the combined effects of low temperature, freezing and high salt stresses. Nevertheless, it is possible that some structure(s) associated with intact cells but not with protoplasts may be important for the complete expression of freezing tolerance. This structure may well be the cell wall, although membrane protein digestion by contaminating proteases in the cellulase treatment of the protoplasts may also be a cause of reduced freezing tolerance of protoplasts (Fitzsimons and Weyers, 1985). Nevertheless, in different species it has been demonstrated that cells can be more tolerant than their protoplasts (Bartolo *et al.*, 1987). These findings are in contrast to those of Tao *et al.*, (1983) who showed that isolated cells of cultured potato were less tolerant than their protoplasts. However, this contrast presumably reflects undefined differences between the systems. Finally, the fact that cold acclimation in various tissues is accompanied by a reduced digestibility of the cell wall may also indicate that changes in the cell wall may be associated with the increase in freezing tolerance (Bartolo *et al.*, 1987).

**Figure. 5.2.** Distribution of rye primary leaf mesophyll protoplasts into size class (1 unit of diameter =  $3.3\mu\text{m}$ ; n = 140).

AHF<sup>1</sup> Amino cold-acclimated, frozen protoplasts  
AHNF Amino cold-acclimated, unfrozen protoplasts  
PHF<sup>1</sup> Puma cold-acclimated, frozen protoplasts  
PHNF Puma cold-acclimated, unfrozen protoplasts

<sup>1</sup> protoplast diameter measured 2 h after thawing



Protoplast diameter

## 2. Leakiness of the Frozen-Thawed Protoplasts

A decrease in protoplast diameter was measured in the protoplasts recovering from freezing as compared to the unfrozen protoplasts (Fig. 5.2) indicating a reduction in osmotic volume of the spherical protoplasts in suspension. This reduction in osmotic volume from about  $9600 \mu\text{m}^3$  for unfrozen to about  $7900 \mu\text{m}^3$  for frozen-thawed spherical protoplasts was taken as evidence of solute loss by the apparently physically intact frozen-thawed protoplasts. This reduction in protoplast volume was not due to the release of solute by dead protoplasts since the freezing medium was replaced by fresh incubating medium before the measurements.

## 3. Properties of the Plasma Membrane of Frozen-Thawed Cells and Protoplasts

### *a. Retention of Fluorescein as Measured Microspectrofluorometrically*

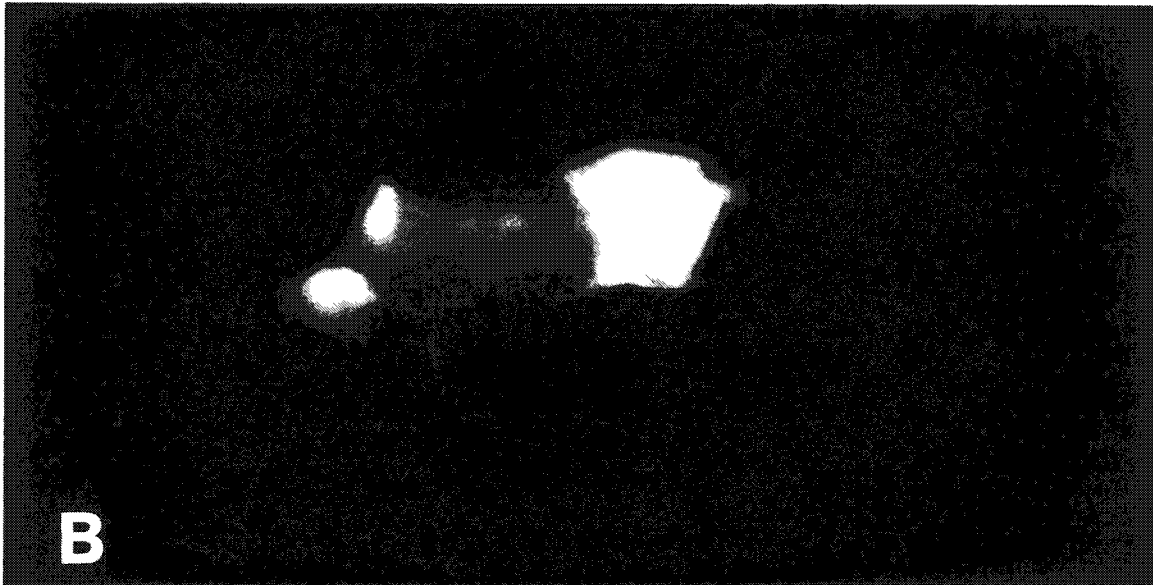
In these experiments (Table 5.2) only intact spherical protoplasts were considered, independent of the survival in the entire populations. That is, in cold-acclimated Puma, the comparison was made between the fluorescein fluorescence of individual, unfrozen protoplasts that were spherical and that of intact, frozen-thawed protoplasts. Percent survival was not considered since the focus was on the measurement of potential damage to apparently, normal (intact and spherical) protoplasts. Two identical experiments with cold-acclimated Puma protoplasts are presented in Table 5.2. The differences in fluorescence intensities in Experiment 1 and 2 were the result of a difference in the standardization of the fluorescence intensity at the beginning of the experiment. In Experiment 1 the mean fluorescence intensity at 540 nm of individual unfrozen protoplasts after 20 min of incubation with  $10^{-5}$  M FDA was  $45.9 \pm 5.5$  vs  $50.9 \pm 5.5$  for intact, frozen-thawed protoplasts. However, this difference between unfrozen and frozen-thawed

**Fig 5.3** Photomicrographs of frozen-thawed (-8°C for 16 h) non-acclimated, Puma rye cells and protoplasts.

A protoplasts incubated with DAPI (50  $\mu\text{g}\cdot\text{ml}^{-1}$ , pH 6.0). The fluorescent molecule penetrates the cytoplasm and nuclei only in the case of dead protoplasts (bright white, lower protoplast). The surviving protoplasts did not take the dye (upper, osmotically active protoplast). Magnification 1200X.

B and C Frozen-thawed, non-acclimated Puma winter rye plasmolyzed cells showing normal osmotic responsiveness in mannitol. (B= FDA positive cell, C= FDA negative cell). Magnification 1200X

n



**B**



**C**

protoplasts was not significantly different. After a further 4 h incubation in absence of FDA, the mean fluorescence intensity in the unfrozen protoplasts was reduced to  $33.9 \pm 5.0$  and to  $33.4 \pm 5.1$  in the frozen-thawed protoplasts. Again, these values are not significantly different. In the second experiment, the mean fluorescence intensity of unfrozen protoplasts was also not significantly different from the mean fluorescence intensity of the frozen-thawed protoplasts, both after the 20 min incubation with  $10^{-5}$  M FDA ( $27.4 \pm 1.7$  vs.  $24.6 \pm 2.1$ ) and after a further 4 h incubation without FDA ( $18.5 \pm 1.7$  vs.  $16.9 \pm 1.9$ ). The mean loss of fluorescence during the 4 h incubation in absence of FDA, as measured by the difference between intensity at 20 min and intensity at 4 h (-12, -18, -9, -8) was taken as a measure of leakage of fluorescein from the intact protoplasts. This loss was almost the same for unfrozen and frozen-thawed protoplasts corresponding to approximately 30% of the fluorescence intensity at 20 min. These results indicate that the plasma membrane of the stressed but apparently intact protoplasts conserved its normal semipermeability to fluorescein.

*b. Exclusion of DAPI from Protoplasts*

In all experiments with non-lethally, frozen-thawed protoplasts as well as with unfrozen protoplasts, DAPI was excluded from intact and spherical protoplasts. Only dead protoplasts took up the stain and became brightly fluorescent while intact and spherical protoplasts, even those which had been frozen-thawed, never accumulated DAPI (Fig 5.3 A). The exclusion of DAPI from all frozen-thawed protoplasts observed (acclimated and non-acclimated) in both cultivars indicates that the physical integrity of the plasma membrane is preserved in surviving protoplasts. Therefore, the non-lethal cellular leakage from intact protoplasts during the post-thaw period cannot be accounted for by a change in passive semipermeable

**TABLE 5.2:** Fluorescence intensity at 540nm (Ex 470nm) of fluorescein accumulated and retained in the cytoplasm of unfrozen and frozen-thawed (-8°C for 16 h) cold acclimated Puma rye protoplasts. Protoplasts were incubated for 20 min at  $20 \pm 1^\circ\text{C}$  with  $10^{-5}$  M FDA.

The protoplast suspension was then centrifuged at 130g for 5 min and transferred to FDA-free buffer for 4 h. Retention of fluorescein was estimated on the basis of fluorescence intensity of individual protoplasts (relative to a standard uranyl stained glass) measured before and after the 4 h incubation without FDA using the microspectrofluorimeter.

Treatment	Relative Fluorescence Intensity		
	20 min	4 h	mean loss (20 min-4 h)
<b>EXPERIMENT 1</b>			
Unfrozen	45.9 $\pm$ 4.6 n.s. (n=20)	33.9 $\pm$ 5.0 n.s. (n=20)	-12
Frozen-Thawed	50.9 $\pm$ 5.5	33.4 $\pm$ 5.1	-18
<b>EXPERIMENT 2</b>			
Unfrozen	27.4 $\pm$ 1.7 n.s. (n=40)	18.5 $\pm$ 1.7 n.s. (n=24)	-9
Frozen-Thawed	24.6 $\pm$ 2.1	16.9 $\pm$ 1.9	-8

(n.s. non significant differences at the 95% probability level)

characteristics of the plasma membrane or a transient membrane rupture/resealing event.

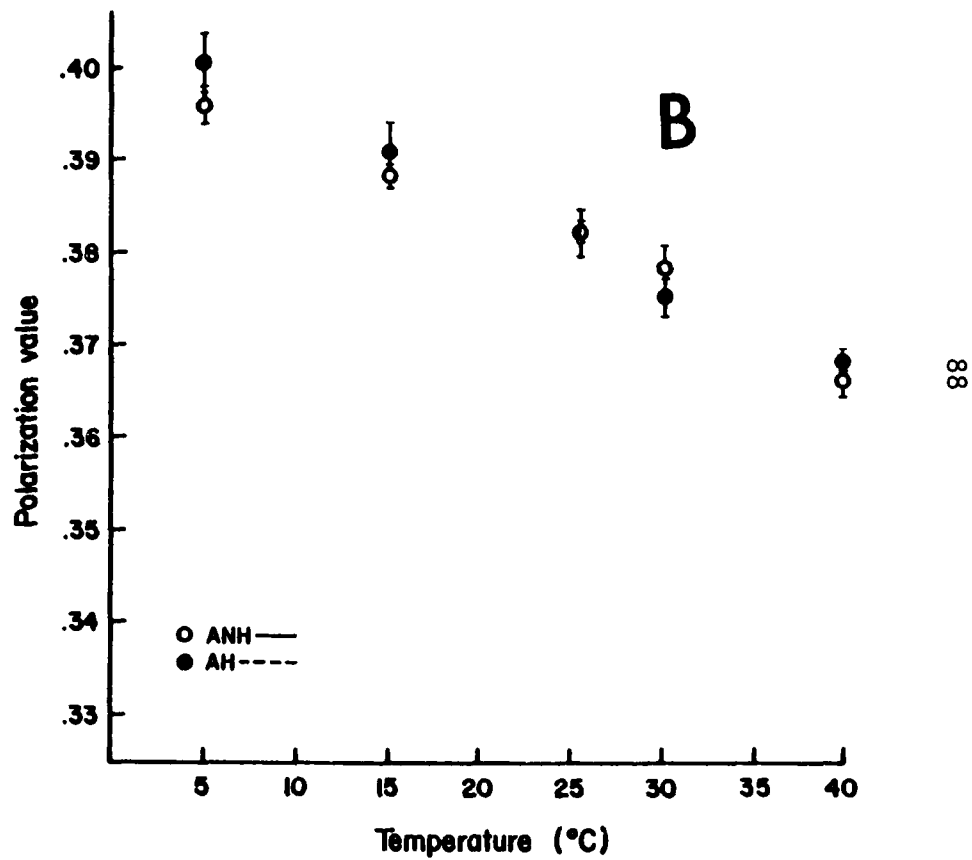
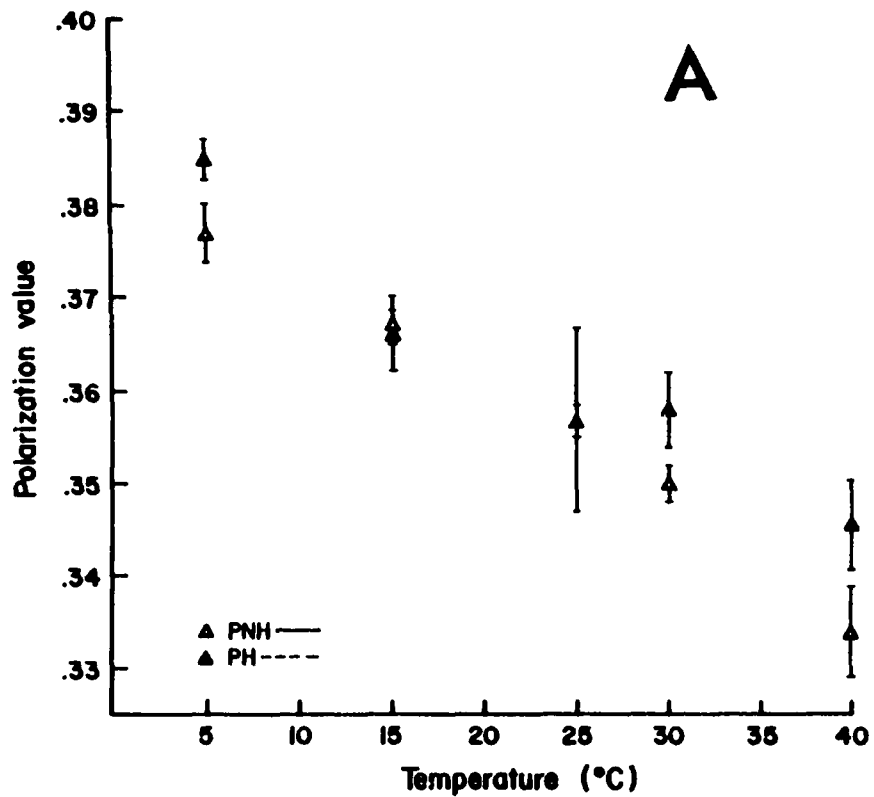
*c. Osmotic Responsiveness of Cells*

All unfrozen (5°C) and frozen-thawed (-8°C/0°C/5°C) cells that were where stained with FDA were also able to undergo a plasmolysis/deplasmolysis cycle (Fig 5.3 B). In all cases, plasmolysis was reversible after 5 min. In some cases, normal osmotic responsiveness was also observed in cells that were unable to accumulate fluorescein (Fig 5.3 C). The normal osmotic responsiveness of the frozen-thawed cells of both cultivars is another indication of the physical integrity of the plasma membrane.

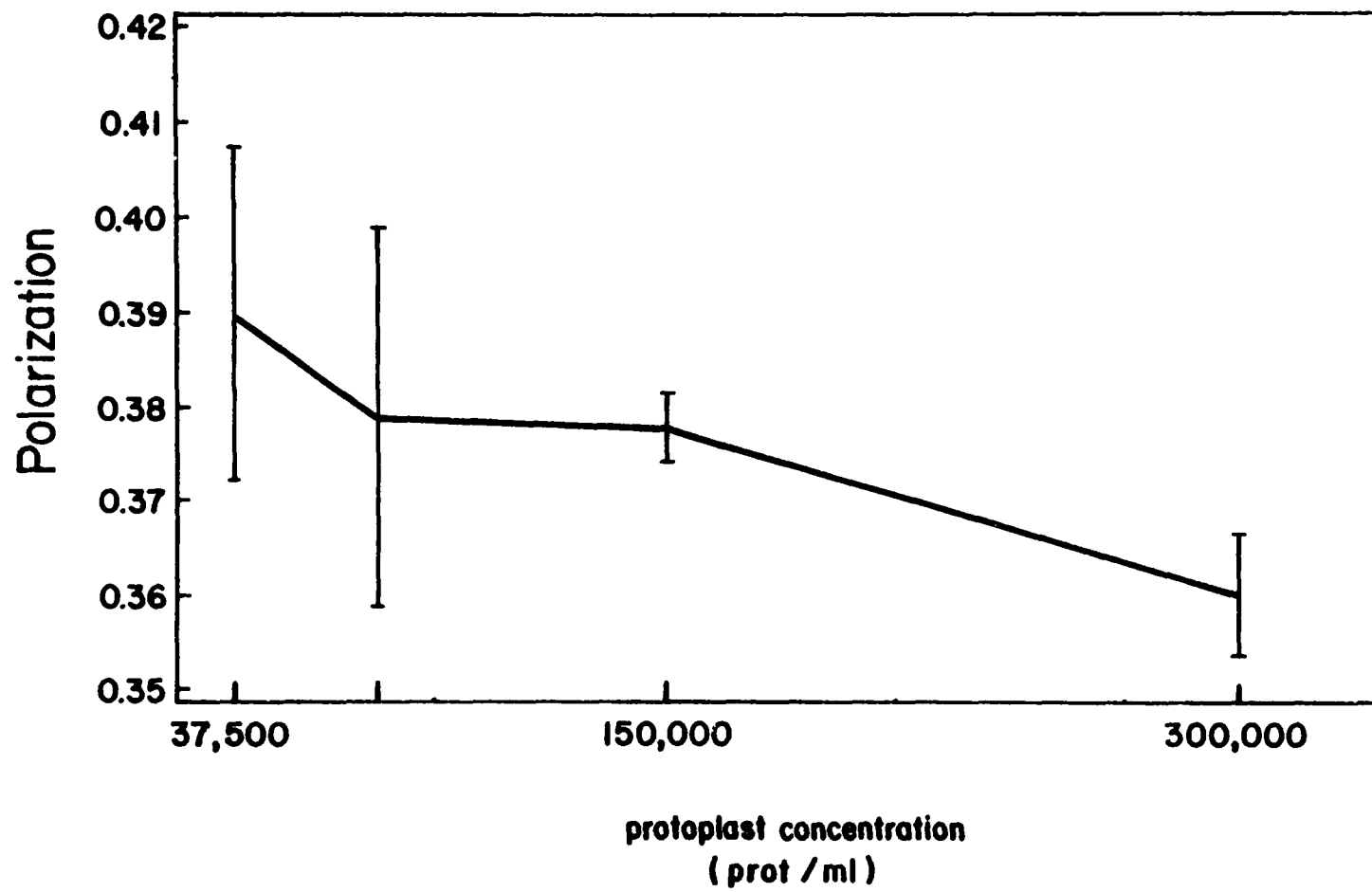
*d. Fluidity of the Plasma Membrane of Protoplasts*

Fluorescence polarization of TMA-DPH was related to temperature changes (Fig 5.4). Increasing temperature decreased the fluorescence polarization. The relations, plotted as polarization vs. T(°C) were almost the same for cold-acclimated protoplasts and non-acclimated protoplasts for both cultivars although the cold-acclimated protoplasts may present a slightly higher polarization value than the non-acclimated protoplasts (Fig 5.4). Increasing temperature increases molecular motion in the bilayers, including motion of TMA-DPH resulting in fluorescence depolarization. The similar relationship for non-acclimated and cold-acclimated protoplasts shows that TMA-DPH is insensitive to the very specific and complex changes in lipid composition observed by Lynch and Steponkus (1987) during cold acclimation of winter rye seedlings. As pointed out by Lynch and Steponkus (1987), the role of lipid compositional changes in the cold acclimation process should be considered from a perspective of freeze-induced alterations in lipid mesomorphism rather than solely from a perspective of average membrane fluidity.

**Figure 5.4** Fluorescence polarization vs temperature relation (over the range of 5 to 40°C) of TMA-DPH embedded in the plasma membrane of Puma (A) and Animo (B) non-acclimated (PNH, ANH) and cold-acclimated (PH, AH) protoplasts. Triplicates  $\pm$  SEM. Protoplast concentration was  $1.5 \times 10^5$  prot $\cdot$ ml $^{-1}$ .



**Figure 5.5** Fluorescence polarization in relation to protoplast concentration.  
Mean of 6 measurements  $\pm$  1 SD. Puma cold acclimated protoplasts  
were used (maximum concentration  $3 \times 10^5$  prot $\cdot$ ml $^{-1}$ )



**TABLE 5.3:** Fluorescence polarization as measured by steady state fluorescence spectroscopy of TMA-DPH embedded in the plasma membrane of cold acclimated Puma rye protoplasts and non-acclimated Puma and Animo protoplasts before and after a non-lethal freezing stress. All measurements done at  $10 \pm 1^\circ\text{C}$ . Mean values of 5 to 11 measurements made on duplicate samples  $\pm$  SEM.

Cultivar	Treatment	Polarization Value ( $P_0=0.5$ )	
		(25,000 prot/ml)	(150,000 prot/ml)
Puma (cold acclimated)	Control	$0.459 \pm 0.014$ n.s.(n=5)	$0.359 \pm 0.002$ s.(n=11)
	Frozen-thawed	$0.444 \pm 0.020$	$0.350 \pm 0.002$
Puma (non-acclimated)	Control	$0.426 \pm 0.019$ n.s.(n=7)	
	Frozen-thawed	$0.422 \pm 0.014$	
Animo (non-acclimated)	Control	$0.442 \pm 0.008$ n.s.(n=6)	
	Frozen-thawed	$0.428 \pm 0.010$	

(s.,n.s.= significant or non-significant difference at the 95% probability level)

Fluorescence polarization was also inversely related to protoplast density or concentration of the suspension (Fig 5.5). Increasing protoplast density led to a fluorescence depolarization and also reduced the spread of the measurements as indicated by the reduction of the standard deviation. The smaller variability in the measurements at high protoplast concentrations probably results from a more uniform distribution of protoplasts in the stirred suspension. The effect of protoplast density on polarization values may be due to scattering (Teale, 1969). As the density of the material increases, the scattering increases and depolarizes the excitation source.

The mean fluorescence polarization of unfrozen control protoplasts was always slightly greater than that of frozen/thawed protoplasts regardless of conditioning or variety (Table 5.4). The small difference was not statistically significant when the measurement was made on suspensions of low protoplast density. However, when made on suspensions of high protoplast density, the difference was statistically significant at the 95% probability level (Table 5.4). This small, but apparent reduction in molecular dynamics at the polar head of the plasma membrane of frozen-thawed protoplasts may be related to a reorganization of the lipids during rehydration and recovery. Although this difference in polarization may be sufficient to be indicative of changes that can affect lipid-protein interactions (Kates and Kuksis, 1980) it certainly does not indicate a dramatic change in the fluidity of the lipids.

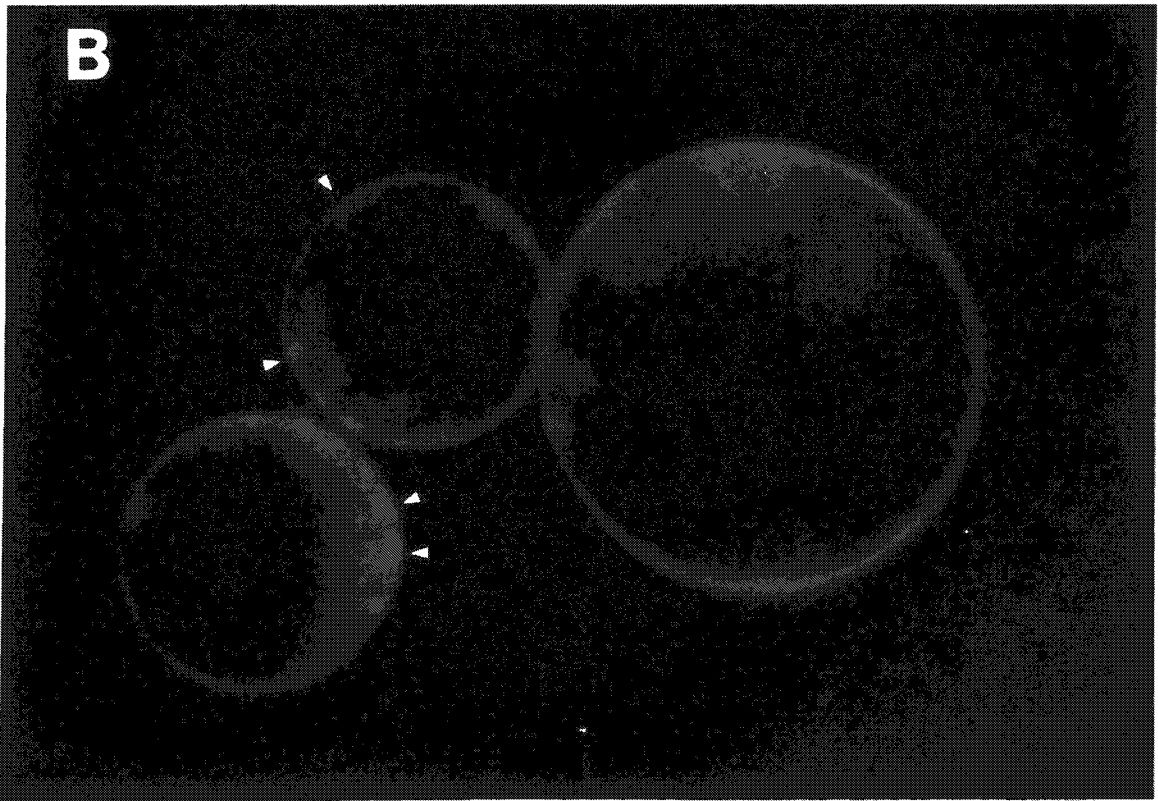
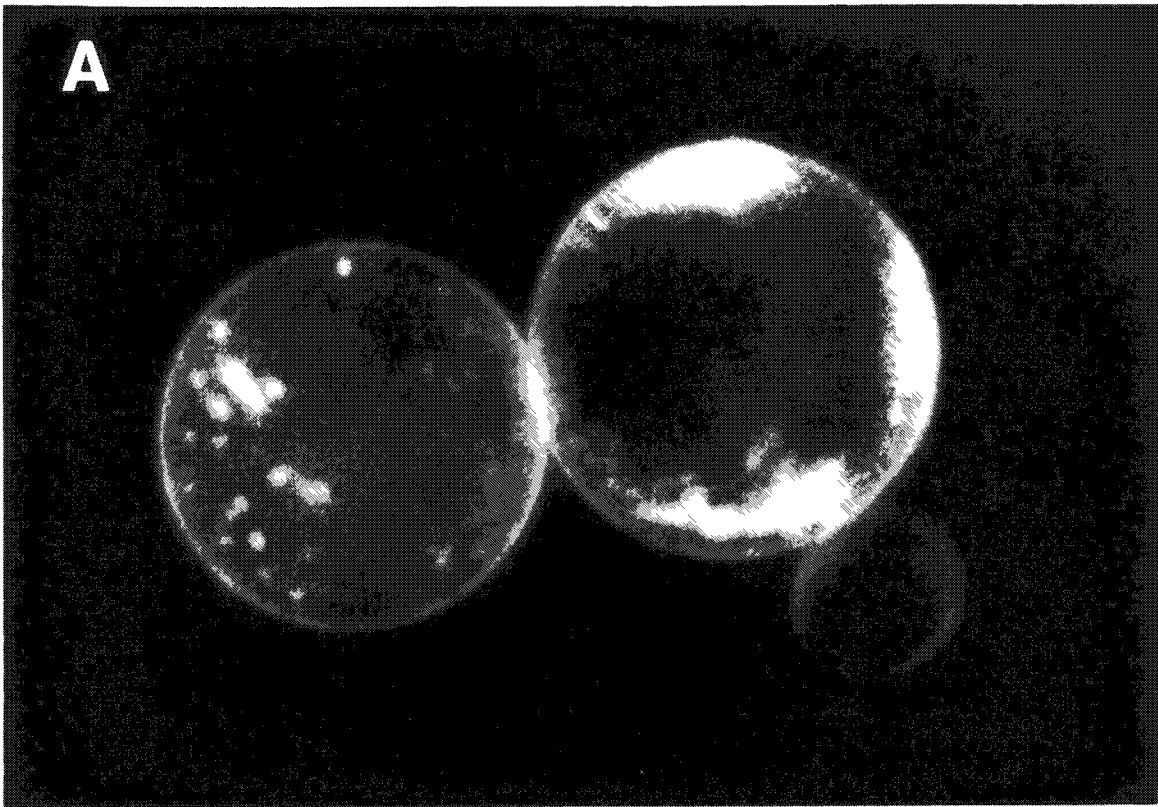
*e. Fluorescence Microscopy of the Plasma Membrane of Protoplasts During Recovery*

The location of TMA-DPH at the plasma membrane was clearly evidenced by microscopic observations (Fig 5.6 A and B). TMA-DPH apparently shows no specificity or preference for any specific domains of the plasma membrane, but is

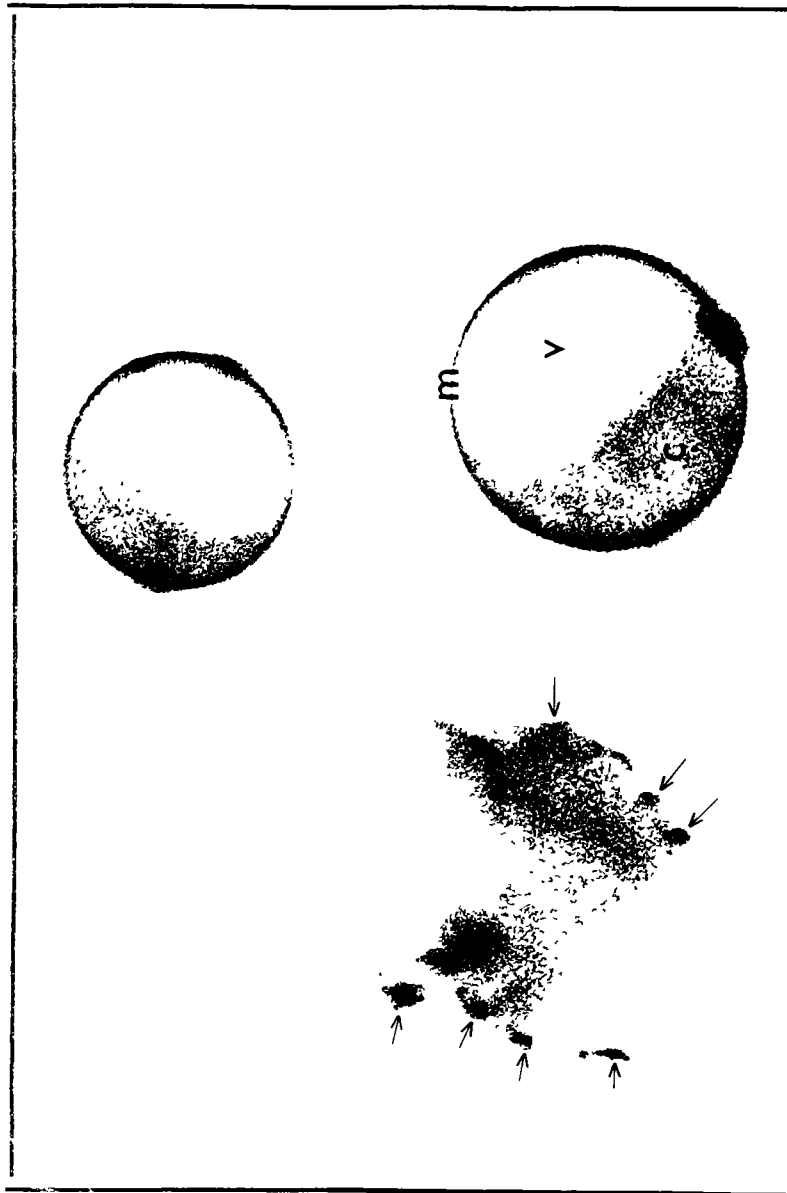
**Figure 5.6.** TMA-DPH (blue fluorescence) in the plasma membrane of unfrozen, cold-acclimated Puma rye protoplasts.

A- Unfrozen protoplasts showing homogeneous staining of their plasma membrane. Magnification 1500X.

B- Frozen protoplasts showing fluorescence aggregations at the plasma membrane (indicated by arrows). Magnification 1500X.



**Figure 5.7.** Negative print of a black and white slide of frozen-thawed ( $-8^{\circ}\text{C}$  for 16 h) Puma winter rye protoplasts. The protoplasts were photographed under epi-illumination using the Zeiss universal microscope. The plasma membrane (m) was labelled with TMA-DPH ( $10^{-5}$  M). One protoplast in the process of bursting shows clear fluorescent aggregations (arrows). It is surrounded by two intact protoplasts. The shaded area corresponds to the cytoplasm (c). The clear area corresponds to the vacuole (v). Magnification 1200X.



apparently distributed uniformly in the membrane. Because of this, the fluorescent polarization signal was taken as an indicator of the average fluidity of the plasma membrane.

Disruption of the plasma membrane of a few protoplasts was observed during the post-thaw period in both cultivars. The disruption of the plasma membrane was immediately preceded by a rapid appearance of large, discrete fluorescent aggregates at or near the plasma membrane (Fig 5.6 B and Fig 5.7). In many cases, appearance of the aggregates was associated with a loss of sphericity. These large discrete TMA-DPH fluorescent aggregates may be indicative of a physical alteration of the lipid bilayer before disruption. The rapidity with which this response develops suggests that a major perturbation in the lipid matrix is a very late event in the process of membrane damage, taking place rapidly and irreversibly at a critical time.

#### D. Conclusions

The physical integrity of the plasma membrane is associated with protoplast shape integrity and maintenance of the normal resilience in the bilayer structure (Wolfe and Steponkus, 1983). The effect of a prolonged freezing upon the physical integrity of the plasma membrane is apparently an all or nothing event associated with the loss of the bilayer structure during freezing or thawing or rapid recovery of the membrane after rehydration. This is evidenced by the fact that, in spite of a reduced osmotic volume of the protoplasts (indicating leakiness), the physical properties of the plasma membrane of surviving protoplasts are totally preserved. Only a very small increase in fluidity was measured. Therefore, alterations of the physical integrity of the plasma membrane appear to be a consequence rather than a cause of the development of lethal injury after thawing and are manifested as

lysis. If the development of lethal damage after thawing is related to an alteration of the permeability properties of the plasma membrane (leakiness) after thawing, the cause of the alteration in the semipermeable characteristics apparently is not associated with a change in passive permeability.

Although the experiments of this chapter do not provide an explanation for the mechanism of development of lethal damage after thawing, they provide useful information with regard to the stability of the observed protoplasts (recovering and surviving). In the next chapter, the degree of vacuolar  $H^+$  compartmentation in these protoplasts is presented. The objective was to demonstrate that the development of lethal injury after thawing depends on an alteration of the semipermeable characteristics of the tonoplast leading to cytoplasmic acidification of apparently, physically stable protoplasts (irreversible physiological damage).

## CHAPTER VI

### UTILIZATION OF NEUTRAL RED AND ACRIDINE ORANGE IN THE EVALUATION OF CELLULAR PROTON COMPARTMENTATION AND INTRACELLULAR pH

#### A. Introduction

The normal physiological activity of the whole cell depends on the integration of the different metabolic activities in the different compartments and membranes (Alberts *et al.*, 1983). This integration depends, in part, on the regulation of translocation of metabolites from one compartment to another and on the ionic (and non-ionic) environment of the different membranes. Regulation of metabolite translocation is a fundamental role of cellular membranes and a fundamental aspect of cellular compartmentation. Metabolite translocation can be achieved either by fusion of vesicles or by direct transport across the membranes (Alberts *et al.*, 1983).

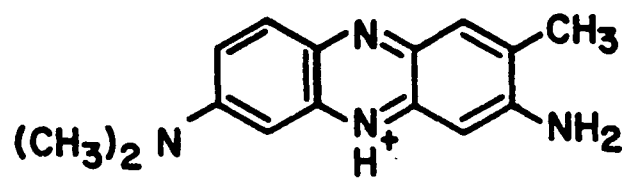
One of the most distinguishing features of plant cell compartmentation is the presence of a large acidic central vacuole delimited by the tonoplast. Consequently, an important indicator of the integrity of cellular compartmentation is the maintenance of vacuolar compartmentation. At the tonoplast, the proton motive force (PMF) is associated with the maintenance of a steady state pH gradient (Poole, 1978; Lin, 1985). The proton ( $H^+$ ) gradient is related to the energetics of the tonoplast because of the involvement of the  $H^+$  electrochemical potential in cell vacuolar compartmentation regulation (Poole, 1978; Boller and Wiemken, 1986).

Inhibition of the tonoplast  $H^+$ -pumping activity can lead to vacuolar leakage and to cytoplasmic acidosis. An important aspect of plant cell pH regulation is related to  $H^+$  transport and compartmentation, *i.e.*, the biophysical pH stat model (Smith and Raven, 1979). Thus from this rationale, the following hypothesis was formulated: Post-thawing development of lethal damage is determined primarily by an alteration in the semipermeable properties of the tonoplast inducing cytoplasmic acidosis. In an attempt to test this hypothesis, attention was given to the effect of prolonged freezing on vacuolar  $H^+$  compartmentation and intracellular pH of physically intact protoplasts before and after prolonged freezing.

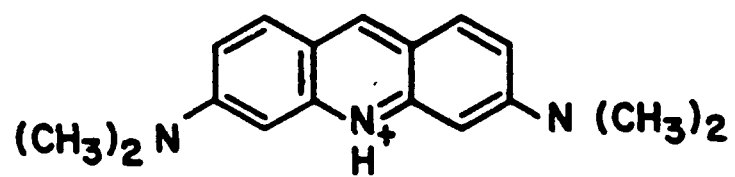
There are several techniques for determination of intracellular pH. The cell sap can be extracted and its pH determined with a glass electrode (Kurkdjian and Guern, 1981). A direct measurement of the intracellular pH can be done using pH sensitive microelectrodes of the Thomas type (Shen and Steinhardt, 1978).  $^{31}P$  NMR can also be used (Roberts *et al.*, 1980). The technique used in the present study relies on the differential distribution at equilibrium of weak bases across the cell membranes (Kurkdjian and Guern, 1981; Manigeault *et al.*, 1983). A variant of this technique uses fluorescent amines such as acridine orange to study intracellular pH of both animal and plant cells. It has been used successfully in sea urchin gametes in relation to the initiation of sperm motility, in porcine gastric microsomes from acid-secreting cells and in various reconstituted liposomes (Lee *et al.*, 1982). In plants, it has been used to study vacuolar pH of isolated cells, protoplasts and isolated vacuoles (Kurkdjian *et al.*, 1984) and to estimate the  $H^+$  concentration difference across the tonoplast of isolated vacuoles (Weigel and Weis, 1984).

The chemical structures of neutral red and acridine orange are given in Figure 6.1. Neutral red (5,3-amino-7-dimethylamino-2-methyl phenazine) contains primary and secondary amines. Acridine orange is a 3,6-bis (dimethylamino) derivative of

**Figure 6.1.** Chemical structure of neutral red (5,3-amino-7-dimethylamino-2-methyl phenazine) and acridine orange (3,6-bis dimethylaminoacridine).



**Neutral Red**



**Acridine Orange**

the acridine molecule and also contains primary and secondary amines. The primary and secondary amine residues give basic properties which are similar for neutral red and acridine orange.

Because of their basic character, acridine orange and neutral red can be in a protonated or non-protonated form. That is, at any given pH, the charged and uncharged forms are in equilibrium with the  $H^+$  concentration (Lee *et al.*, 1982). Since the  $pK_a$  of these molecules is approximately 10, most of the molecules of acridine orange and neutral red will be in the protonated form at physiological pHs. The non-protonated dye molecules are those that can diffuse into the protoplasts and cells, assuming that biological membranes are freely permeable to only this form of the molecules (Lee *et al.*, 1982). The inward diffusion, which depends on the presence of a gradient of the non-protonated permeable form across the membrane, continues until intracellular equilibrium is reached (Lee *et al.*, 1982). The mechanism for the equilibrium process for an acidic vesicle delimited by a single lipidic membrane is illustrated in Figure 6.2. Once into the lumen of the vesicle, the non-protonated molecules of acridine orange (AO) will interact with protons and become protonated or not depending on the intravesicular pH. The protonated molecules ( $AOH^+$ ) will be trapped and accumulate there because they cannot permeate the membranes. The accumulation of the protonated molecules continues until no more protonation can take place. At that point, the non-protonated form begins to accumulate and the gradient of the permeable form will rapidly disappear. If movement of the permeable molecules across the membrane is still possible when equilibrium is reached, it will have no effect on the total intravesicular dye concentration. If the intravesicular pH is more acidic than the extracellular pH, there will be a net accumulation of the protonated dye molecules inside the vesicle

at equilibrium, with most of these intravesicular dye molecules being in the protonated form.

Acridine orange also possesses interesting and usable fluorescence properties and constitutes a probe that exhibits a concentration-dependent shift in fluorescence spectra with a concentration dependent fluorescence quenching (Lee *et al.*, 1982). The accumulation of acridine orange in response to the existence of a pH gradient across a membrane is logarithmically related to the magnitude of the  $H^+$  gradient (Lee *et al.*, 1982). When excited with intense blue light, the monomers emit a green fluorescence (540nm). At a critical concentration, polymerization takes place and multimeric dye aggregates are formed. The formation of polymers may be related to the formation of ionic bridges between the acridine orange monomers or to Van der Waals interactions. When multimeric dye aggregates are formed, a new fluorescent emission peak appears at approximately 625 nm (Lee *et al.*, 1982) and is correlated with the disappearance of the emission peak of the monomer at 540 nm. Thus, in addition to visible light absorption, quenching of the emission peak at 540 nm and the development of the orange fluorescence at 625 nm are direct measures of dye uptake (Lee *et al.*, 1982).

The model illustrated in Figure 6.2 is a simplified version compared to the complexity of a compartmented living cell. However,  $H^+$  compartmentation of intact cells can be visualized with acridine orange (Zeitz, 1980). Since the central acidic vacuole can occupy up to 90% of the protoplasmic volume, the vacuolar proton compartmentation of an intact plant cell can also be easily visualized. By adjusting the dye concentration and the pH of the incubation medium, a normal plant cell will produce at equilibrium:

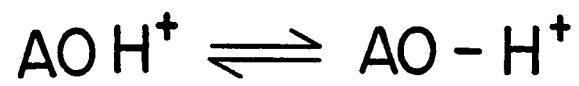
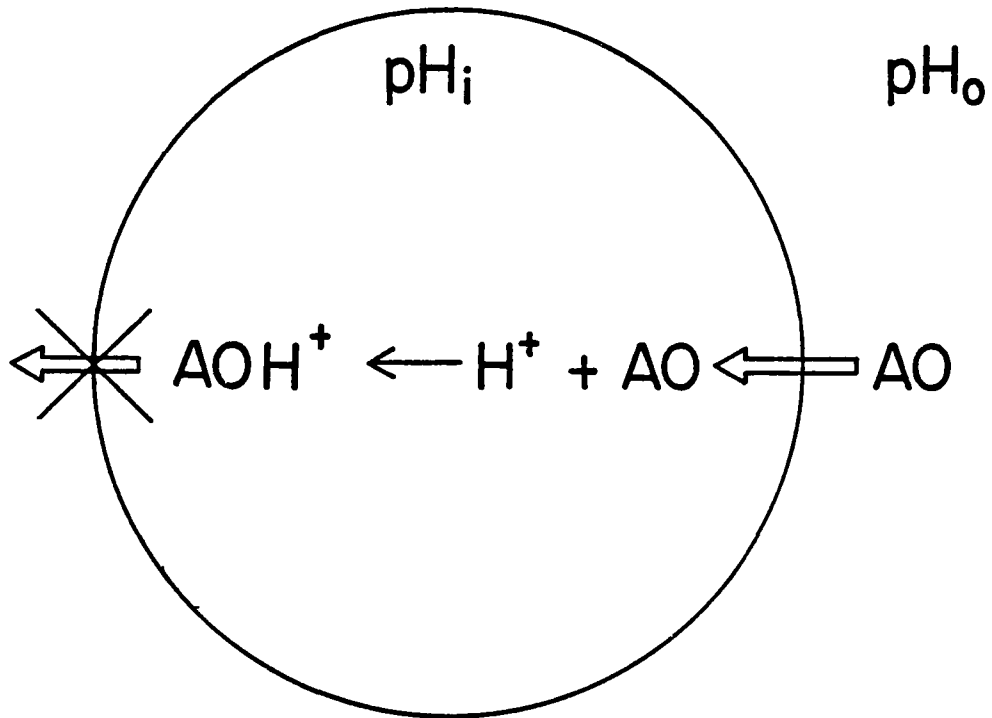
**Figure 6.2.** Illustration of the mechanism of accumulation of acridine orange in an acidic vesicle delimited by a single lipidic membrane.

$\text{pH}_o$  = extravesicular pH

$\text{pH}_i$  = intravesicular pH

AO = non-protonated acridine orange molecule

$\text{AOH}^+$  = protonated acridine orange molecule



$$\text{pK}_{\text{A}} \text{AO} = 10$$

$$\text{pH}_i < \text{pH}_o$$

- i) a green fluorescence characteristic of the monomer in the cytoplasm (pH value close to neutral=low accumulation if pH of the medium is equal or lower than the cytoplasmic pH), and
- ii) an orange fluorescence characteristic of the polymers in the acidic vacuole

Since protonation of dye molecules as shown in Figure 6.2 is reversible (Lee *et al.*, 1982), any change in compartmentation of pH in the cell resulting from prolonged freezing should affect the compartmentation of the dye molecules at equilibrium. In addition, the total acridine orange uptake of the plant cell is largely determined by the large acidic vacuole where protonation is favored (Kirkdjian *et al.*, 1984). Measurement of intracellular and extracellular dye concentrations at equilibrium permits determination of vacuolar pH when the pH of the incubation medium is known (Lee *et al.*, 1982). The distribution of the dye should conform with the following equation:

$$\text{Equation 6.1} \quad \text{pH}_i = \text{pH}_o - \log(A_i/A_o) - \log(V_o/V_i)$$

where  $A_i$  and  $A_o$  correspond to internal and external amounts of dye as measured by fluorescence or absorption and  $V_i$  and  $V_o$  refer to the corresponding internal and external volumes.

In the present chapter, microspectrophotometry and microspectrofluorometry of acridine orange were used to compare the distribution of vacuolar pH within populations of protoplasts before and after a prolonged freezing and under different conditions for recovery. The vacuolar compartmentation of the protoplasts was evaluated by brightfield transmission microscopy of neutral red and by fluorescence microscopy of acridine orange.

## **B. Materials and Methods**

### **1. General**

The protoplasts used in this study were isolated from primary leaves of cold-acclimated Puma winter rye seedlings and processed following the protocol described in Chapter II (General Materials and Methods). They were frozen to temperatures varying from  $-9^{\circ}$  to  $-16^{\circ}\text{C}$  and maintained in the frozen state for a minimum of 16 h depending on the experiment. The freezing medium used was the Meyer and Abel R0.6 saline for the neutral red experiments and the sorbitol-phosphate buffer system for the acridine orange experiments. Post-thaw incubation was in either the mannitol-tris-maleate medium or the mannitol-phosphate medium as defined in Chapter II (General Materials and Methods). Survival was assessed according to sphericity and expressed in either absolute numbers (n) or as a percent of the unfrozen controls following the procedure described in Chapter II (General Materials and Methods).

### **2. Staining with Neutral Red**

Neutral red was obtained from Fisher Scientific Company as a Fisher certified biological stain (# N-129, purity 70% total dye content). It was used at the concentration of  $25\text{ g}\cdot\text{ml}^{-1}$  at pH 7. Intracellular neutral red compartmentation was estimated visually using a bright-field transmission microscope. The spherical protoplasts observed were distributed into different groups according to the pattern of dye compartmentation at equilibrium (vacuolar, cytoplasmic, or vacuolar and cytoplasmic).

### 3. Staining with Acridine Orange

Acridine Orange Base was purchased from Aldrich Chemical (# 23547-4, purity 75% total dye content) and was used at concentrations varying from  $4 \times 10^5$  M (0.001% w/v) to  $1.2 \times 10^4$  M (0.003% w/v) in media of pH varying from 5.1 to 8.6. The incubation temperature was 20°C. Incubation was initiated by adding stock solution (100 to 600  $\mu$ l of AO) to the protoplast suspension to get 1 or 2 ml total volume with the desired concentration of the dye (0.01% in incubating medium). The suspensions were then incubated for 60 min to reach equilibrium before observation and measurement. Fluorescence microscopy was used to estimate intracellular acridine orange compartmentation in individual protoplasts (n = 130 for each treatment). Acridine orange fluorescence was observed under epi-illumination using a Zeiss filter combination (Ex 470 nm, Em >510 nm). Photomicrographs were taken using Kodak EL 135 400ASA film.

### 4. Measurement of Intra- and Extracellular Concentration of Acridine Orange

A Zeiss UMSP 80 microspectrofluorometer was set up for epifluorescence and bright field transmission. Three different sources of illumination were used, a 75W xenon lamp, a 100W mercury lamp for the fluorescence, and an halogen lamp for transmission. Protoplasts were placed in a hemocytometer (graduated volumetric slide, Neubauer, Fein-Optic, Blankenburg, GDR) of 0.1 mm in depth and observed through a Zeiss 10X Neofluor objective (0.30 N.A.). The irradiated part of the slide was restricted by a circular diaphragm adjusted to allow illumination of a surface slightly larger than the diameter of the largest protoplasts to be measured.

First, the positions of 10, 20, 25, 30, 40, 50, 60 or 100 individual protoplasts under bright field illumination were registered automatically by the computer controlled electronic microscope stage. At the same time, individual protoplast

diameters were measured using the eyepiece micrometer (10X) calibrated against the hemocytometer micrometer. Then, individual protoplasts were measured sequentially and automatically both for fluorescence emission at 540 and 625 nm (ex 470nm) and for absorption at 500 nm using the cytological fluorescence analysis program (Cyflan). These wavelengths for absorption and emission measurements were selected on the basis of protoplast absorption, excitation and emission spectra obtained using a spectral scanning program (Lamda-Scan). The fluorescence was expressed relative to a standard uranyl glass (GG17) emission at 540 nm. For automatic individual protoplast measurements, the use of automatic shutters together with ms excitation pulses was used to prevent the introduction of variability resulting from fading of the fluorochrome under prolonged irradiation.

#### 5. Measurement of Intracellular pH

For the precise estimation of intracellular pH values using equation 6.1 (p. 102), the external pH was set to 7.1 and the quantitation of  $A_i$  and  $A_o$  was related to light absorption by the following measurements made on the cylindrical measuring field of the hemocytometer containing or not one protoplast in suspension (Figure 6.3).

$$A_o = M_{tr} - AOM_{tr}$$

$$A_i = PR_{tr} - PRAO_{tr} - [(1 - V_i/V_o)(M_{tr} - AOM_{tr})]$$

where:

$A_o$  = Amount of dye in the medium at equilibrium (per unit volume of the measured field).

$M_{tr}$  = Light transmittance of the incubation medium (per unit volume of the measured field).

$AOM_{tr}$  = Light transmittance of the incubation medium containing acridine orange at equilibrium (per unit volume of the measured field).

$A_i$  = Amount of dye accumulated in the protoplast at equilibrium.

$PR_{tr}$  = Light transmittance of one protoplast suspended in incubation medium.

$PRAO_{tr}$  = Light transmittance of one protoplast suspended in incubation medium containing acridine orange at equilibrium.

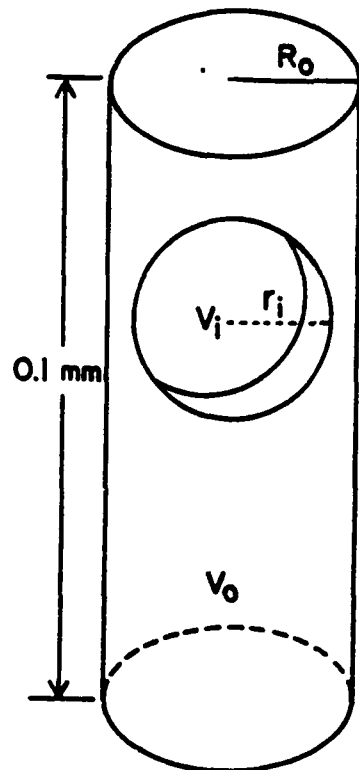
$V_i$  = intracellular volume

$V_o$  = extracellular volume

The external reference absorbance ( $A_o$ ) was set to 1% using an acridine orange concentration of 0.003% in the incubation medium ( $A_o = M_{TR} - AOM_{TR} = 1$ ). Therefore,  $\log (A_i/A_o) = \log A_i$ . The acridine orange absorbance of individual protoplasts ( $A_i$ ) was estimated by subtracting the mean transmittance of stained protoplasts ( $PRAO_{TR}$ ) from the mean transmittance of unstained protoplasts ( $PR_{TR}$ ) of the same size ( $A_i = PR_{TR} - PRAO_{TR}$ ). The value  $[(1 - V_i/V_o)(M_{TR} - AOM_{TR})]$  was subtracted from  $A_i$  to correct for the contribution of the suspending medium in the measurement of the absorption of an individual protoplast.  $(1 - V_i/V_o)$  corresponds to the fractional volume occupied by the suspending medium in the cylindrical measuring field when one protoplast is observed (see Fig 6.3).

$V_o$  and  $V_i$  were determined by applying the appropriate volumetric equations to the cylindrical measuring field of the hemocytometer containing one protoplast in suspension (Fig 6.3). The depth of the measuring field was 0.1 mm.

**Figure 6.3.** Schematic representation of the cylindrical measuring field of the hemocytometer containing one protoplast in suspension. The depth of the measuring field is 0.1 mm and the radius of the surface disk ( $R_0$ ) is  $1.65 \times 10^{-2}$  mm. The radius of individual protoplasts ( $r_i$ ) in suspension varies from  $9.9 \times 10^{-3}$  to  $1.65 \times 10^{-2}$  mm. From these values it is possible to determine the respective external volume ( $V_0$ ) and intracellular volume ( $V_i$ ) using the appropriate volumetric equations.



$$V_i = \frac{4}{3} \pi r_i^3$$

$$V_o = 0.1 \pi R_o^2$$

$$D_o = 0.033 \text{ mm}$$

$$d_i = 0.0198 \text{ mm to } 0.033 \text{ mm}$$

$$R_o = 0.0165$$

$$r_i = 0.0099 \text{ mm to } 0.0165 \text{ mm}$$

$$V_o = 85.5 \times 10^{-6} \text{ mm}^3$$

$$V_i = 4.0 \times 10^{-6} \text{ mm}^3 \text{ to } 18.8 \times 10^{-6} \text{ mm}^3$$

Measurements obtained experimentally were introduced into equation 6.1 to permit determination of the vacuolar pH of unfrozen control and non-lethally frozen-thawed protoplasts.

## C. Results and Discussion

### 1. Intracellular Compartmentation of Neutral Red

Three types of neutral red compartmentation were identified microscopically on the basis of intracellular distribution of the dye in osmotically active, spherical protoplasts. These three distributions were referred to as i) vacuolar compartmentation (V), ii) vacuolar and cytoplasmic compartmentation (V+C), and iii) cytoplasmic compartmentation (C). The populations of protoplasts (frozen and unfrozen) were characterized by the distribution of individual spherical protoplasts in these three categories giving histograms such as those presented in Figure 6.4. Each column of the histograms gives the absolute number of protoplasts per unit suspension volume (frequency) in each category (V, V+C, C). Each different experiment is represented by two histograms (A-B, B-E, C-F) representing the unfrozen control populations (A, B, C) and the frozen populations (B, E, F). In unfrozen control populations (Fig 6.4 A,B,C), the majority (70 to 90%) of the individual protoplasts showed vacuolar compartmentation (V). However, there was always a certain number of protoplasts (frequency) that also accumulated the dye in the cytoplasm (V+C) and in a few cases, the dye accumulated only in the cytoplasm (C).

The freezing temperature was  $-10^{\circ}\text{C}$  in experiments A-D,  $-13^{\circ}\text{C}$  in experiments B-E, and  $-16^{\circ}\text{C}$  in experiments C-F. The duration of the freezing temperature was 66 h in experiments A-D, 24 h in experiments B-E and 24 h in experiments C-F. In the histograms representing the stressed populations (D, E, F), a fourth category

**Figure 6.4.** Histograms showing the relative distribution of the three steady state neutral red compartmentation responses V, V+C, and C at pH<sub>o</sub> = 7.0 in unstressed (PHNF) (A, B, and C) and frozen-thawed (PHF) (D, E, and F) Puma protoplasts following prolonged freezing at -10 (D), -13 (E) and -16°C (F) in Mayer and Abel saline medium R0.6 (Meyer and Abel, 1975).

V = vacuolar compartmentation only

V+C = vacuolar + cytoplasmic compartmentation

C = cytoplasmic compartmentation

M = missing or dead protoplasts: unfrozen samples were taken as 100% viable.

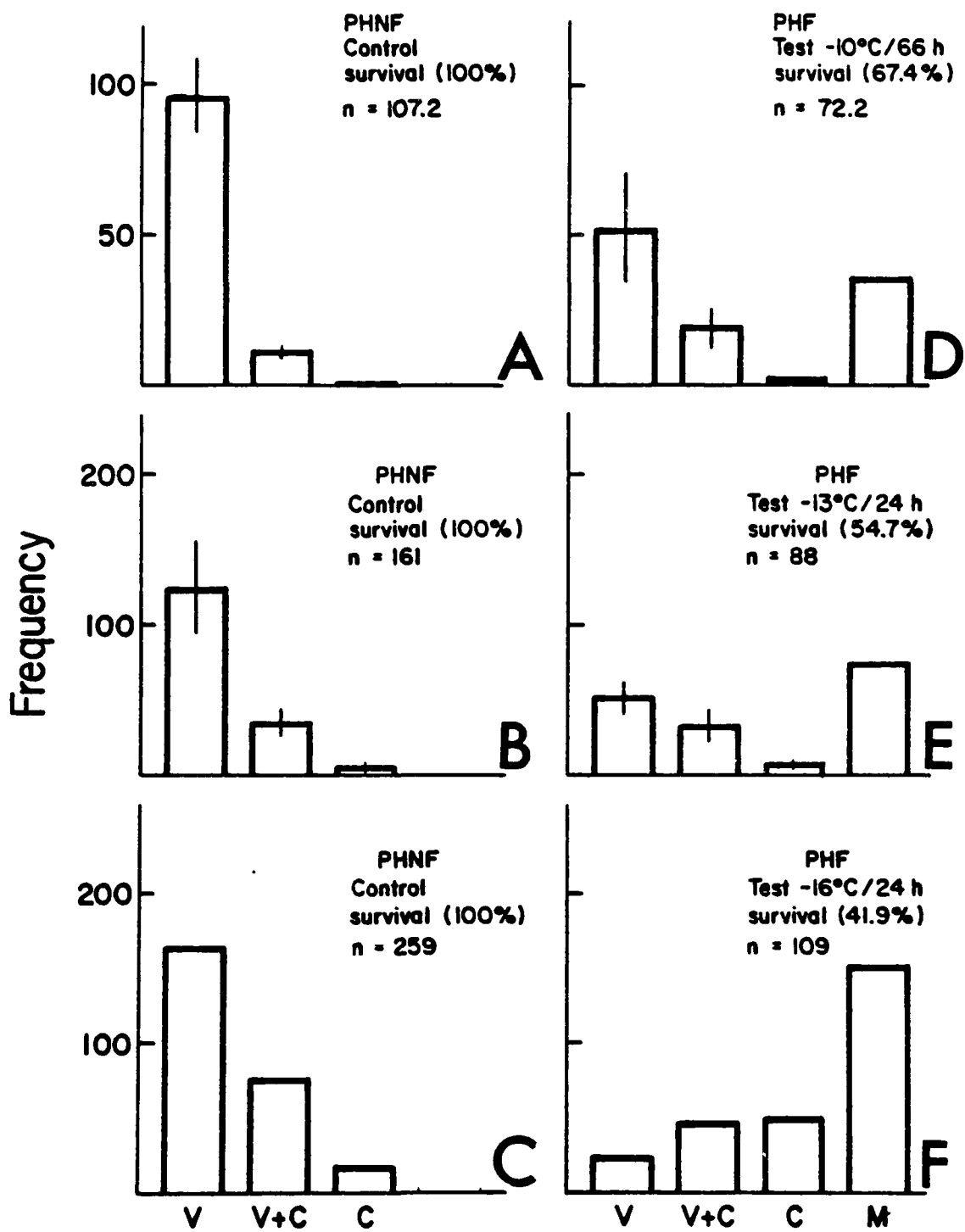
Frequency = number of protoplasts counted in each category

A-D = mean of 3 experiments

B-E = mean of 3 experiments

C-F = 1 experiment

Vertical bars ± SEM



was added (The "M" column) which gives the number of missing or non-spherical (dead) protoplasts calculated as the difference in the cumulative frequency of the three compartmentation categories ( sum of V, V+C and C) between unfrozen controls and frozen populations. The cumulative frequency of the three compartmentation categories for each histogram is represented by "n" in the legend of each histogram. "n" represents the total number of spherical protoplasts observed for compartmentation per unit volume. It is therefore an estimate of abundance and can be used to estimate survival if the "n" value of the unfrozen control is used as a reference for 100% survival. The "M" column represents the difference in "n" value between control and frozen and gives mortality. As the "M" column increases, survival decreases.

Survival decreased from 67.4% at  $-10^{\circ}\text{C}$  to 54.7% at  $-13^{\circ}\text{C}$  and to 41.9% at  $-16^{\circ}\text{C}$ . This is shown by the increased height of the "M" column (Figures 6.4 D, E, F). In these stressed populations, the number of protoplasts showing vacuolar compartmentation of the dye (v) per unit volume of suspension (frequency) was much less than in controls. This decrease in absolute number of protoplasts showing vacuolar compartmentation of the dye (V) is consistent with an increase in the "M" column (mortality). The same relation with the "M" column does not exist for the two other dye compartmentation categories ( V+C and C). The absolute number of protoplasts in these categories remains stable or even increases in spite of the increasing mortality. In other words, these two categories have become proportionally greater in the stressed population as compared to unfrozen control. These two categories correspond to the decompartmented responses as opposed to the vacuolar compartmentation (V) which is taken as the normal dye distribution in intact and viable protoplasts.

The decompartmented protoplasts were also unable to hydrolyse FDA and did not accumulate fluorescein probably because of cytoplasmic acidification. This indicates that these protoplasts are non-viable. Therefore, these protoplasts may be more susceptible to progressive degeneration following thawing and will eventually die. The results indicate that an alteration of the semipermeable properties of the tonoplast can determine the development of lethal injury following thawing via cytoplasmic acidification.

The lowest temperature of freezing resulted in the greatest incidence (80% of all surviving protoplasts) of the two decompartmented responses ( V+C and C) (Fig 6.4 F). This may indicate that the extent of freeze-induced dehydration, which increases with lower freezing temperatures (Mazur, 1969), is an important factor for the incidence of the damage under prolonged freezing.

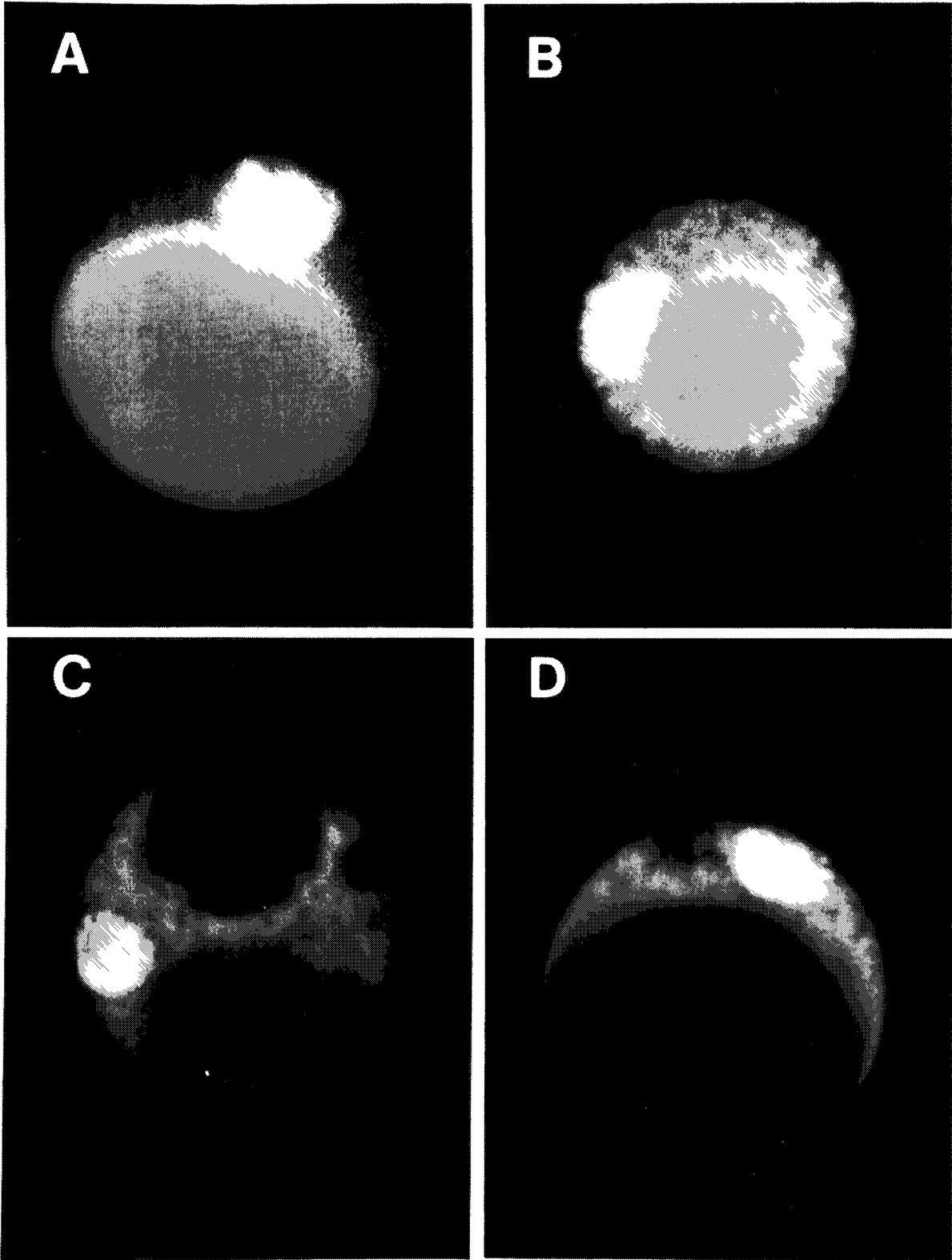
## 2. Survival and Intracellular Compartmentation of Acridine Orange

For normal protoplasts maintained in a nearly neutral incubation medium, most of the dye is accumulated in the acidic vacuole as evidenced by the orange-red fluorescence (Fig 6.5 A). The green fluorescence of the cytoplasm corresponds to the fluorescence of dye monomer and is indicative of a low dye concentration in this compartment. In addition, the nucleus took up the stain and showed a yellow fluorescence. The protoplasts that did not exhibit vacuolar compartmentation of the dye developed different staining patterns (Fig 6.5 B,C,D). In these protoplasts, the dye tended to accumulate in larger quantities in the cytoplasm as evidenced by the development of the orange fluorescence. These protoplasts can show polyvacuolization (Fig 6.5 C); and, in many cases, the vacuoles did not accumulate the dye as shown by their black color (Fig 6.5 C,D). This condition was called vacuolar decompartmentation. Therefore, it appears that vacuolar

**Figure 6.5. Acridine orange protoplast staining at equilibrium:**

A - Fluorescence micrograph of a Puma rye primary leaf protoplast showing the normal vacuolar dye compartmentation pattern with the orange-red fluorescence of dye multimers in the acidic vacuole, the green fluorescence of the dye monomers in the nearly neutral cytoplasm and a yellow fluorescence in the nucleus.

B, C, D - Fluorescence micrograph of Puma rye primary leaf protoplast showing the decompartmented responses where more of the dye molecules accumulate in the cytoplasm and the orange fluorescence of the dye multimers can be observed (B). This cytoplasmic compartmentation can be associated with a vacuolar decompartmentation of the dye (C and D black empty vacuoles) and a polyvacuolization of the protoplast (C).  $\text{pH}_0=7.1$  Magnification 1500X



decompartmentation is effectively associated with cytoplasmic acidosis although the osmotic responsiveness of the tonoplast is apparently preserved. As for neutral red, these decompartmented responses do not constitute viable states as determined by the incapacity of these protoplasts to hydrolyse FDA and to accumulate fluorescein (data not shown).

Table 6.1 presents the results obtained with one experiment that is representative of results obtained with up to 5 repetitions of each condition. The protoplast concentration of the unfrozen control suspension (Control 2, Table 6.1) was estimated at  $1.8 \times 10^5$  prot/ml and was used as a reference for 100% survival. In this unfrozen suspension, 122 of 130 (94%) individual protoplasts showed a clear vacuolar compartmentation of the dye. Survival in the frozen-thawed suspension 2 h after thawing (Frozen recovered 2, Table 6.1) was only  $60 \pm 2\%$ . Of 130 protoplasts observed for compartmentation, 85% showed vacuolar compartmentation of the dye. Incubation of the frozen-thawed protoplasts in the mannitol phosphate buffer medium for 6 hours at  $5^\circ\text{C}$  in the dark before the addition of the dye (Frozen 6+2, Table 6.1) gave a slightly higher survival (65%) and also a greater proportion of vacuolar compartmentation (93%). This indicates that the protoplasts are stable and effectively recover from the freezing stress in the absence of the dye. In addition, the high degree of normal dye compartmentation in these protoplast populations clearly indicates that maintenance of vacuolar compartmentation is an essential factor for the expression of long term survival following freezing. Incubation for recovery for 8 h following thawing in the presence of the dye (Frozen 8, Table 6.1) reduced survival to 38% but the proportion of protoplasts showing vacuolar compartmentation remained very high (96%). This result indicates that a significant proportion of the population is very sensitive to the extra stress resulting from the accumulation of the dye at high

**TABLE 6.1** Survival (%) and acridine orange vacuolar compartmentation in unfrozen (Control 2; unfrozen protoplasts incubated for 2 h at 20°C with acridine orange, pH<sub>0</sub> 7.1) and frozen-thawed protoplasts of Puma rye primary leaves under the following conditions of incubation for recovery.

Frozen 2 = protoplasts (frozen at -9°C for 16 h) incubated for 2 h at 20°C with acridine orange immediately after thawing.

Frozen 6+2 = protoplasts (frozen at -9°C for 16 h) incubated for 6 h at 5°C after thawing in the dark before addition of acridine orange for 2 h at 20°C.

Frozen 8 = protoplasts (frozen at -9°C for 16 h) incubated for 8 h at 20°C with acridine orange immediately after thawing.

TREATMENT	SURVIVAL		COMPARTMENTATION	
	# prot/ml	(%)	# protoplasts. observed for compartmentation	% of vacuolar compartmentation
Control 2	179,166	100	130	94
Frozen 2	108,333	60 ± 2	130	85
Frozen 6+2	116,666	65 ± 5	130	93
Frozen 8	68,750	38 ± 2	130	96

concentrations in the protoplasts (presence of the dye in the nucleus and phototoxicity).

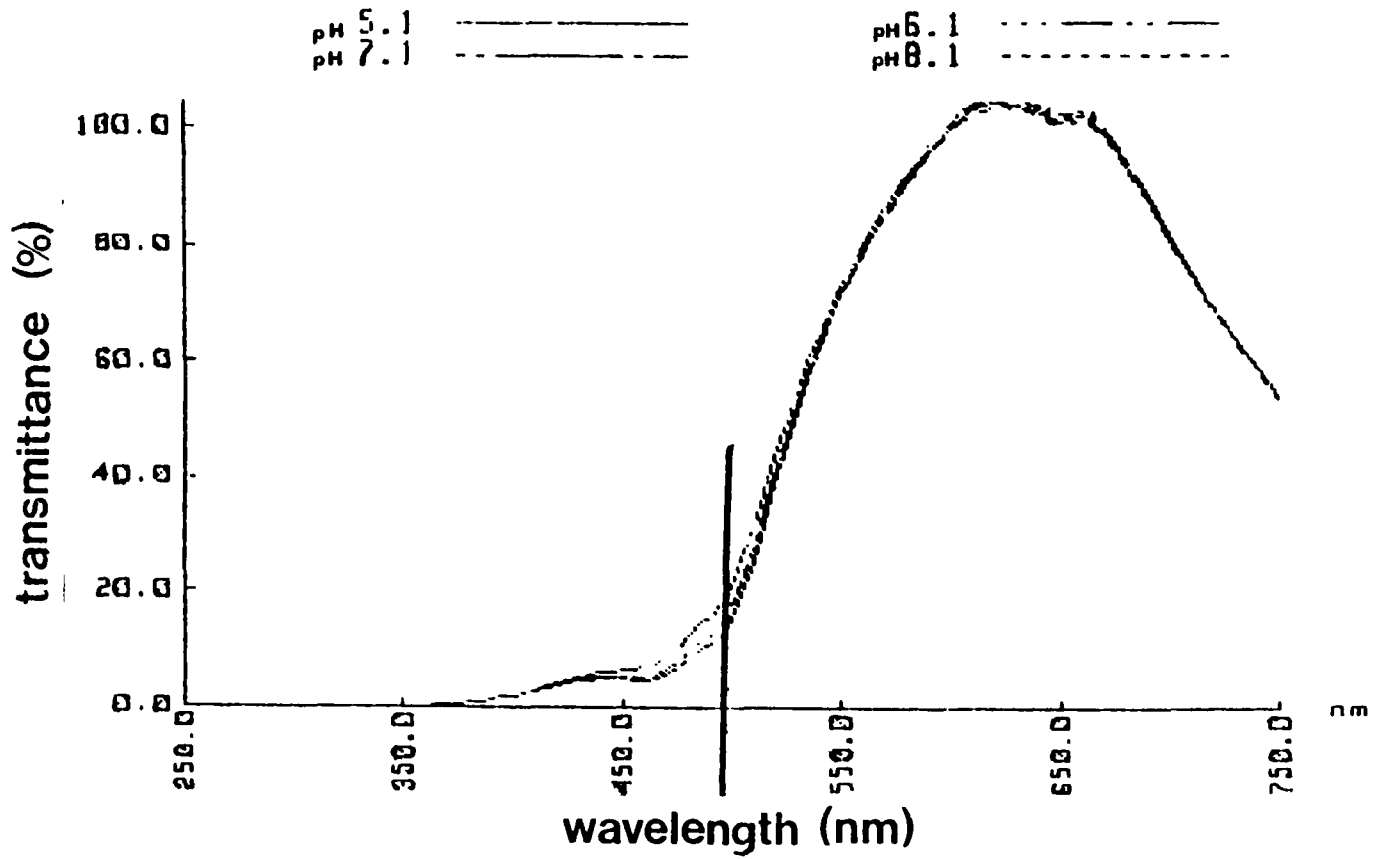
### 3. Microspectrophotometric Properties of Acridine Orange

The transmittance spectrum of an acridine orange solution (0.01% w/v) is given in Figure 6.6. The spectrum is the same at pH 5.1, 6.1, 7.1 and 8.1. The transmittance spectrum of individual protoplasts stained with acridine orange (0.001% w/v) is given in Figure 6.7. Relative transmittance of the individual protoplasts was reduced as the pH of the incubating medium increased from 5.1 to 8.1. From these spectra, 500 nm was selected for the quantitation of intracellular acridine orange by absorbance because at this wavelength there is no interference from chlorophyll.

### 4. Fluorescence Spectrum of a Normal Protoplast Stained with Acridine Orange

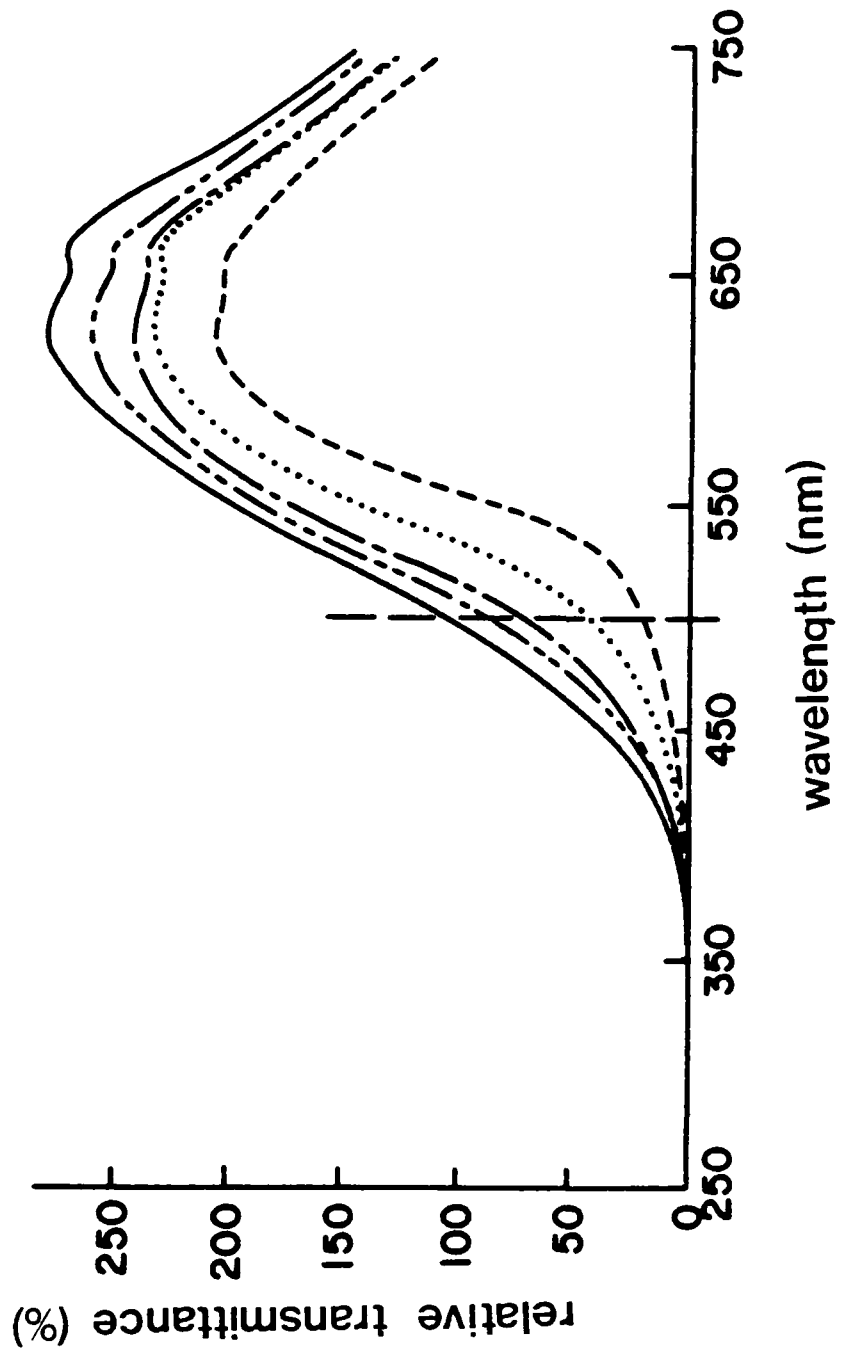
The fluorescence spectrum of a normal, compartmented protoplast incubated in acridine orange is shown in Figure 6.8. Despite the fact that the vacuole, which effectively emits in the orange, occupies up to 90% of the protoplast volume and contains most of the accumulated dye, the orange-red fluorescence (shoulder at 625 nm) is only 50% of the intensity of the green fluorescence (peak at 540 nm) produced by the cytoplasm. This clearly shows that the quantum yield of the monomer form (green fluorescence at 540 nm in the cytoplasm) is much higher than the quantum yield of the polymer form (orange-red fluorescence at 625 nm in the vacuole).

**Figure 6.6.** Transmittance spectrum from 250 to 750 nm of an acridine orange solution (0.01% w/v) in mannitol-tris-maleate buffer,  $\approx 0.85$  osmolar, pH 5.1, 6.1, 7.1, 8.1



**Figure 6.7** pH dependent transmittance spectra from 250 to 750 nm of individual protoplasts stained with 0.001% acridine orange in mannitol-tris-maleate buffer, 0.85 osmolar, pH 5.1, 6.1, 7.1, 8.1 relative to the transmittance of unstained protoplasts (continuous line). The standard 100% corresponds to the transmittance of the blank at 500 nm.

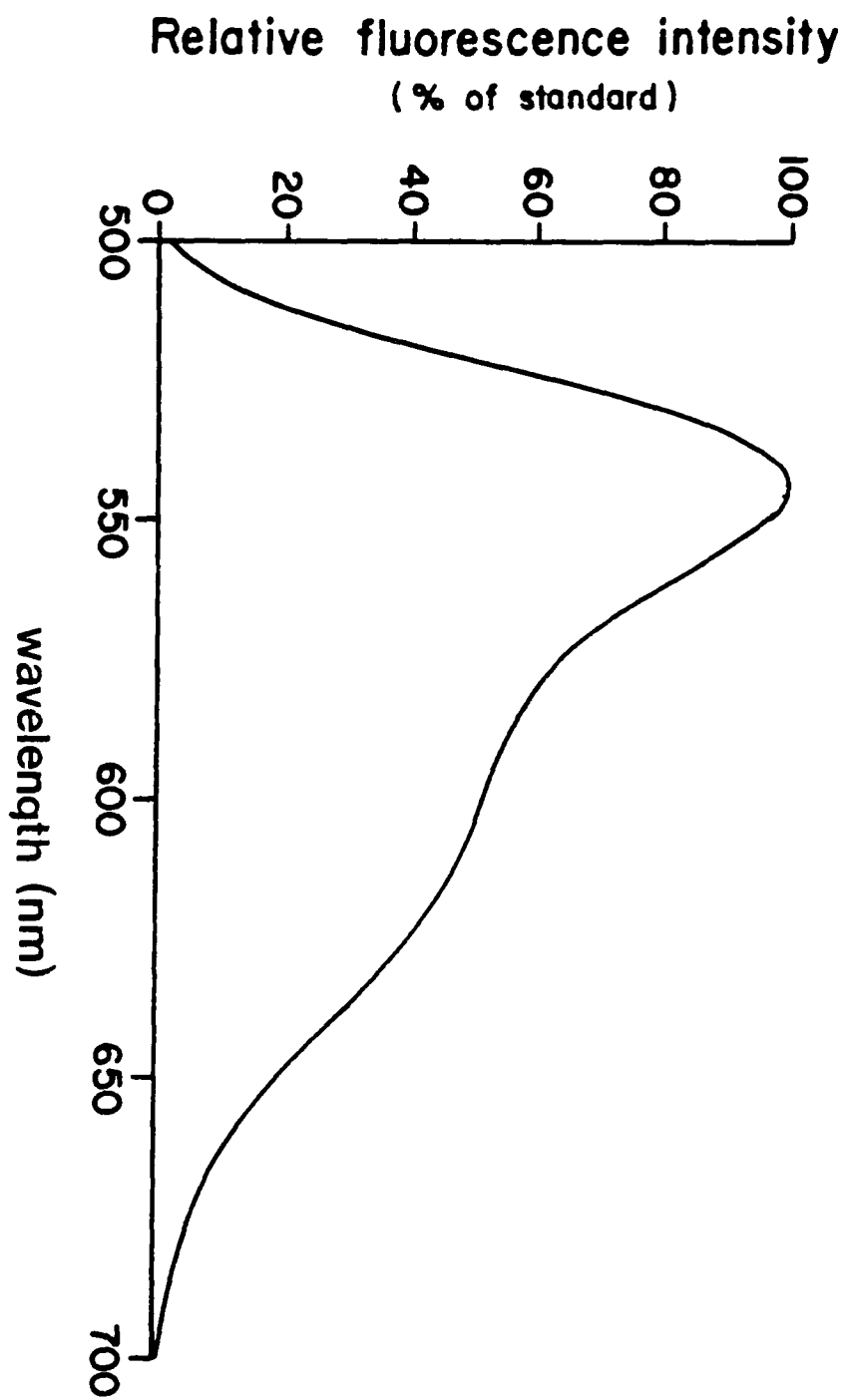
pH 5.1 --- - - ---  
pH 6.1 ---- - ----  
pH 7.1 .....  
pH 8.1 - - - - -



**Figure 6.8.** Fluorescence spectrum (Em 500 to 700 nm Ex 470 nm) of a protoplast stained with acridine orange (0.001% pH<sub>0</sub>=7.1). This corresponds to the fluorescence spectrum of a protoplast showing the normal vacuolar compartmentation of the dye (cf., Fig 3.5 A).

X axis = Emission wavelength for a 470 nm excitation

Y axis = Fluorescence intensity relative to a uranyl glass standard



## 5. Effect of Continuous Illumination on the Stability of Absorbance and Fluorescence of Protoplasts Stained with Acridine Orange

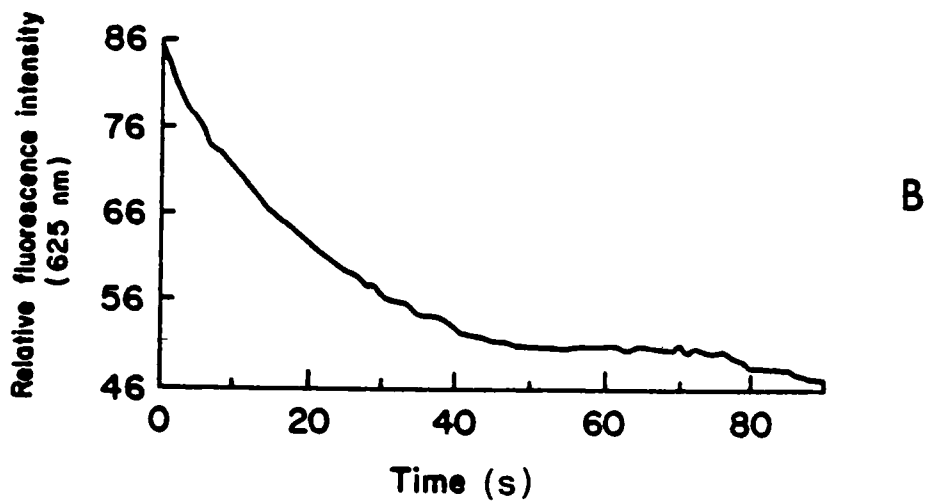
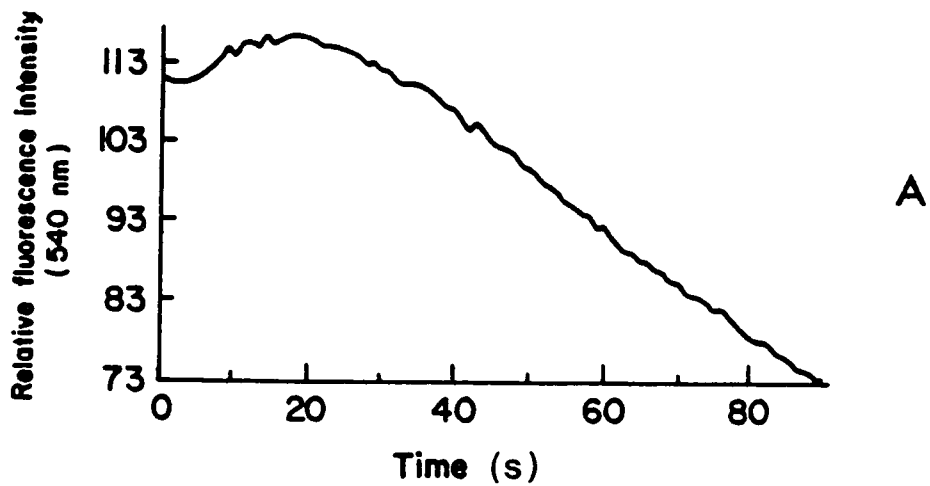
While the transmittance of a stained protoplast was stable over the time of illumination with the halogen lamp, the fluorescence emission rapidly faded over time of excitation at 470 nm (Fig 6.9). The green fluorescence initially increased slightly and subsequently gradually faded (Fig 6.9 A). The 625 nm orange fluorescence faded rapidly to 50% relative fluorescence intensity by 40 seconds (Fig 6.9 B). Consequently, the use of an automatic shutter for flash illumination was required to eliminate variability resulting from fading, especially for the fluorescence at 625 nm. Using ms flash illuminations, the staining of individual protoplasts over time of incubation in the dark with the dye was found to be stable for at least 3 h as illustrated in Figure 6.10.

## 6. Effect of the Nucleus on Total Protoplast Fluorescence

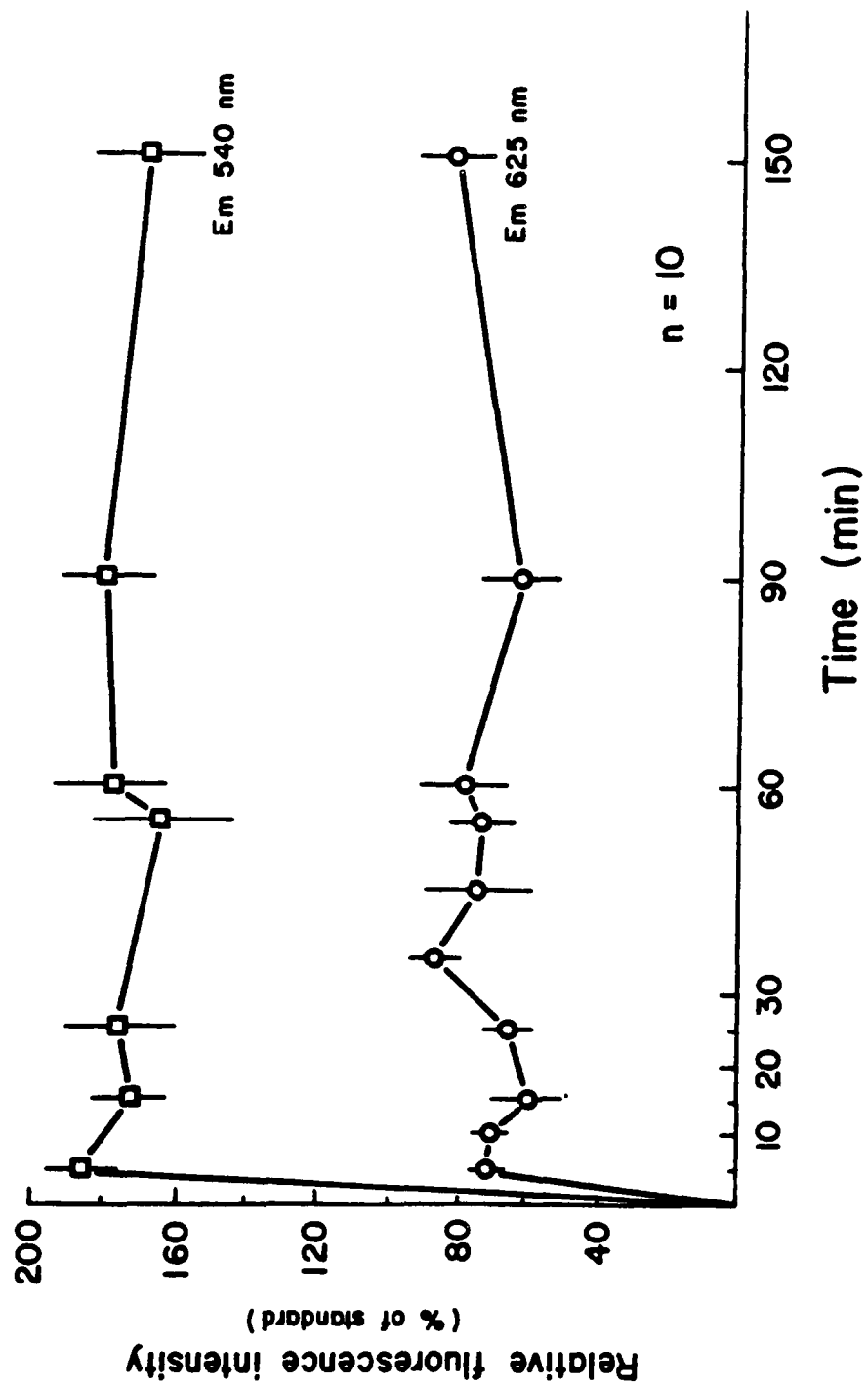
Because acridine orange also stained the nucleus of the protoplasts (Fig 6.5), the contribution of the nuclear fluorescence was evaluated by adjusting the measuring diaphragm to the size of the nucleus of individual protoplasts. The contribution of the nucleus to the green fluorescence of individual protoplasts varied from 13 to 44% with a mean of 23% of the total fluorescence of the protoplast at 540 nm (Table 6.2). Thus the cytoplasmic contribution primarily determines the fluorescence emission at 540 nm.

**Figure 6.9.** Time-dependent fading in relative fluorescence intensity (uranyl glass standard) at 540 nm (A) and 625 nm (B) of an individual, cold-acclimated unfrozen Puma protoplast stained with acridine orange ( $\text{pH}_0=7.1$ ) over a steady state illumination of 90 s.

TIME (s) corresponds to time of illumination in s.



**Figure 6.10.** Steady state staining of individual protoplasts over time of incubation with acridine orange. Mean fluorescence intensity at 540 and 625 nm of protoplasts incubated with 0.001% acridine orange at 20°C in the dark for up to 150 min ( $\text{pH}_0=7.1$ ). Each value is the mean of 10 individual protoplast measurements made under short-flash illumination using automatic shutters. This was to eliminate the effect of fading over time of illumination as illustrated in Figure 6.9.



**TABLE 6.2** Contribution of the nucleus to the acridine orange fluorescence of individual protoplasts at 540 nm.

PROTOPLAST #	FLUORESCENCE INTENSITY			NUCLEAR CONTRIBUTION (%)
	TOTAL (A)	NUCLEAR (B)	DIFFERENCE (A-B)	
1	43.2	13.4	29.8	31.0
2	128.1	25.3	102.8	19.8
3	36.1	4.8	31.3	13.3
4	50.8	14.2	36.6	27.9
5	46.9	7.3	39.6	15.6
6	83.4	11.4	72.0	13.7
7	58.0	12.4	45.6	21.4
8	37.1	10.0	27.1	27.0
9	43.9	13.1	30.8	29.8
10	63.5	18.3	45.2	28.8
11	90.5	22.3	68.2	24.6
12	117.7	21.6	96.1	18.4
13	35.9	10.1	25.8	28.2
14	22.6	9.9	12.7	43.8
15	79.3	11.7	67.6	14.8
16	76.4	19.5	56.9	25.5
17	79.9	16.6	63.3	20.8
18	141.6	30.0	111.6	21.2
19	74.5	18.7	55.8	25.1
20	102.1	14.8	87.3	14.5
Sum	1411.5	305.4	1106.1	
Mean	70.6	15.3	55.3	23.3
St. Dev.	33.1	6.3	28.0	

- (A) = Total protoplast fluorescence obtained by adjusting the measuring field in the hemocytometer to the size of the individual protoplasts  
 (B) = Nucleus fluorescence obtained by adjusting the measuring field to the size of the nucleus of the individual protoplasts

### 7. Quantitation of Intracellular Acridine Orange

Both fluorescence intensity (Em 540 and 625 nm) and absorbance were used as measures of dye uptake (Figs 6.11 and 6.12). Dye uptake continued as external pH ( $\text{pH}_0$ ) increased from 5.1 to 8.5 as shown by the increase in absorbance at 500 nm (Fig 6.11 B). The deviation from an ideal logarithmic relation around  $\text{pH}_0$  7.1 may result from polymer formation. Dye uptake was also shown by relative fluorescence intensities; but, as a result of polymerisation of monomers and intramolecular interactions in the polymers, quenching occurred (Fig 6.11 A and Fig 6.12). The increase in relative fluorescence intensity at 625 nm (Fig 6.11 A) was indicative of the accumulation of the polymer form. Again, this was not an ideal curve and is a phenomenon that may reflect the complexity of a living cell. The drop observed above  $\text{pH}_0$  8.0 probably resulted from intramolecular interactions (*i.e.*, quenching of fluorescence). Dye uptake, as shown by relative fluorescence intensity at 540 nm, was a logarithmic function only to  $\text{pH}_0$  6.5 (Fig 6.12). Quenching of the fluorescence of the monomer was observed for higher  $\text{pH}_0$  values and occurred as the polymeric form increased (Fig 6.12 vs Fig 6.11 A). The  $\text{pH}_0$  at which the quenching of fluorescence occurs can be used as a parameter to assess intracellular vacuolar pH ( $\text{pH}_i$ ).

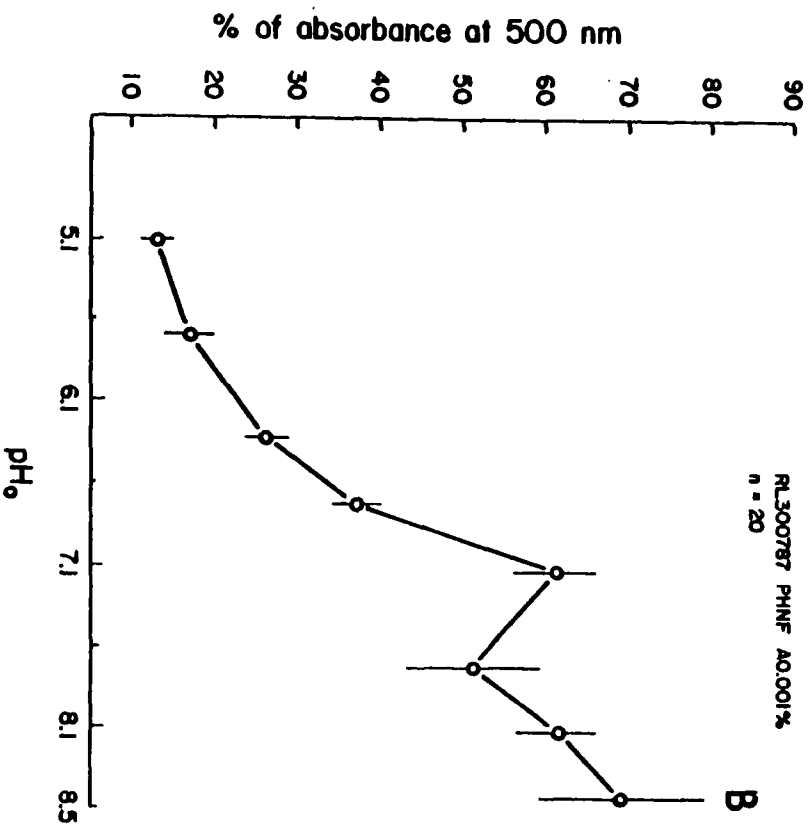
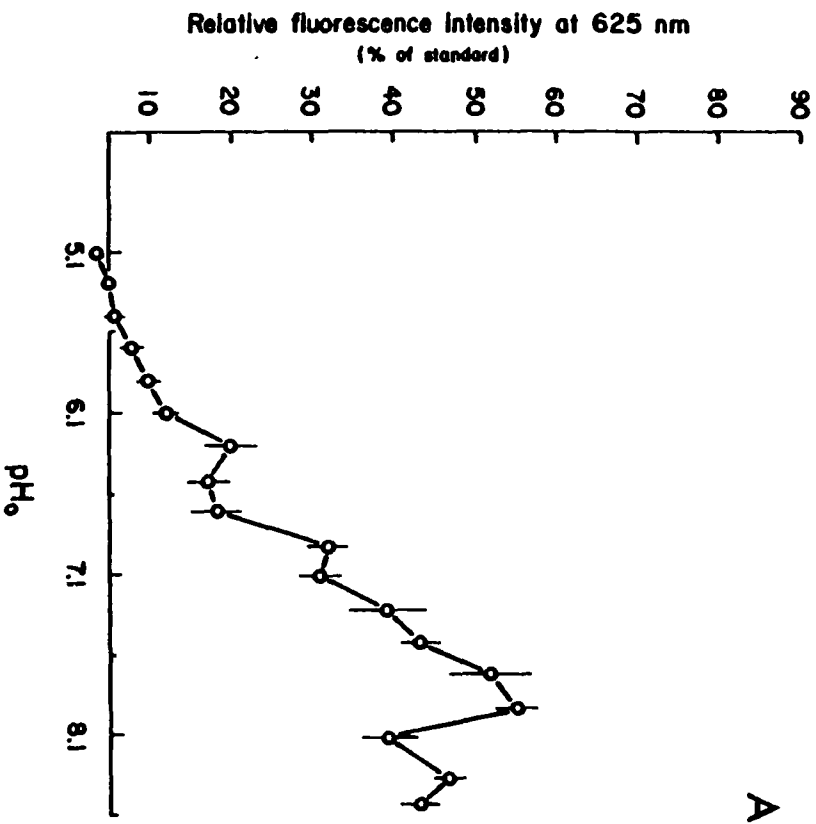
### 8. Effect of Freezing on Acridine Orange Uptake and Vacuolar pH

Between 1 and 3 h incubation time following thawing, the relative fluorescence intensity at 540 nm was higher in frozen-thawed ( $-9^\circ\text{C}$  for 16 h) than in unfrozen, control protoplasts for external pH 5.1 to 6.5 (Fig 6.13). This indicates that the frozen protoplasts accumulated more acridine orange monomer than the unfrozen controls within that range of  $\text{pH}_0$ . The occurrence of fluorescence quenching at a lower  $\text{pH}_0$  value for the frozen samples also indicates a greater

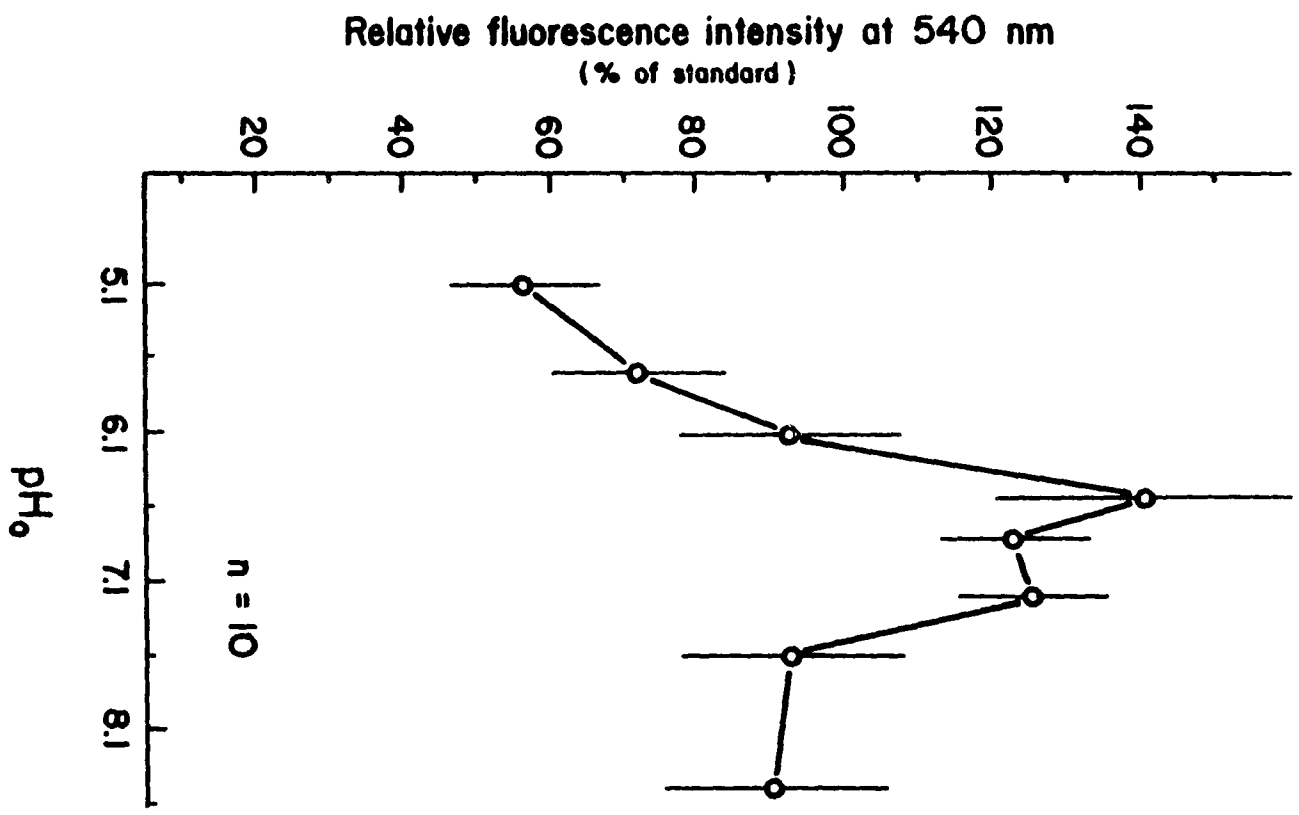
**Figure 6.11.** Accumulation of acridine orange in protoplasts in relation to an artificially imposed pH gradient:

A - Relative fluorescence intensity of intracellular acridine orange at 625 nm (Ex 470 nm) as a function of increasing pH of the incubation medium ( $\text{pH}_0$ ).

B - Intracellular acridine orange absorbance at 500 nm as a function of increasing pH of the incubation medium ( $\text{pH}_0$ ). Each point corresponds to the mean fluorescence intensity or absorbance taken individually on 20 protoplasts over the range  $\text{pH}_0 = 5.1$  to 8.5. (bars = SEM).



**Figure 6.12.** Relative fluorescence intensity of intracellular acridine orange at 540 nm (Ex 470 nm) as a function of increasing pH of the incubation medium ( $\text{pH}_0$ ). Each point corresponds to the mean of 10 individual protoplast measurements over the range  $\text{pH}_0 = 5.1$  to 8.5 (bars=SEM).



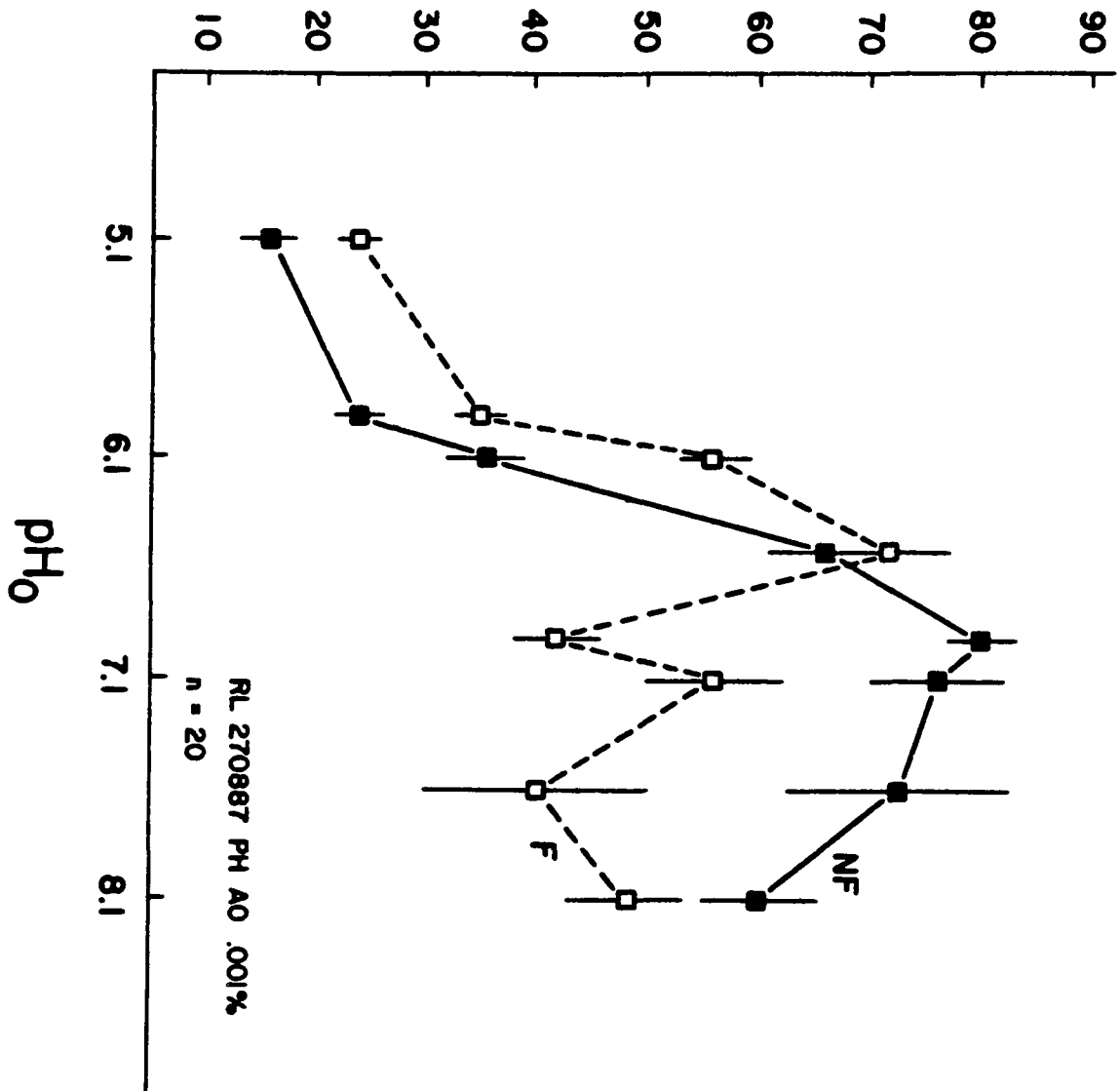
uptake and suggests that the intracellular pH ( $\text{pH}_i$ ) of the recovering protoplasts was lower than that of the unfrozen controls. Since the vacuolar compartmentation of the dye molecules was generally observed and no sign of accumulation of the dye in the cytoplasm was observed in frozen protoplasts (Table 6.1), the accumulation was assumed to take place in the vacuole of frozen protoplasts and was indicative of vacuolar acidification.

In a different experiment where the external pH was set to 7.1, the mean absorbance of unfrozen control protoplasts was  $25.1 \pm 0.8$  (mean  $\pm$  SEM,  $n = 100$ ) compared to  $31.4 \pm 1.1$  for frozen-thawed ( $-9^\circ\text{C}$  for 16 h) protoplasts 2 h after thawing. These means were significantly different at the 95% probability level. In addition, the individual values in the frozen population were distributed less uniformly around the mean as can be seen in Figure 6.14 (B vs. A). The stressed population showed a greater standard deviation ( $\text{SD} = \pm 11.1$ ) as compared to the control ( $\text{SD} = \pm 8.4$ ). The comparison of the two distributions by a Chi-square analysis for goodness of fit, which takes the distribution of the control as the reference distribution (Zar, 1974), revealed that there was less than 0.1% probability that the distribution of frozen protoplasts would be obtained by sampling a population showing the distribution of the control. This major difference in the distribution curves may indicate that the response to the freezing treatment was general in the protoplast population.

When the frozen protoplasts were maintained at  $5^\circ\text{C}$  in the dark for 6 h before the addition of the dye there was a net tendency of the values to distribute more uniformly around the mean (Fig 6.14 C), and the mean ( $28.6 \pm 1.1$ ,  $n = 100$ ) was not statistically different from the mean of the control protoplasts at the 95% probability level. The standard deviation remained relatively large ( $\text{SD} = \pm 11.3$ ). The Chi-square analysis for the goodness of fit indicated that the two distributions

**Figure 6.13.** Relative fluorescence intensity of intracellular acridine orange at 540 nm (Ex 470 nm) as a function of increasing pH of the incubation medium ( $\text{pH}_0$ ) for unfrozen (NF) and frozen-thawed (  $-9^\circ\text{C}$  for 16 h) protoplasts of Puma winter rye primary leaf. Each point corresponds to the mean of 20 individual measurements over the range  $\text{pH}_0 = 5.1$  to 8.5 (bars = SEM) taken between one and three h following thawing.

Relative fluorescence intensity at 540 nm  
(% of standard)

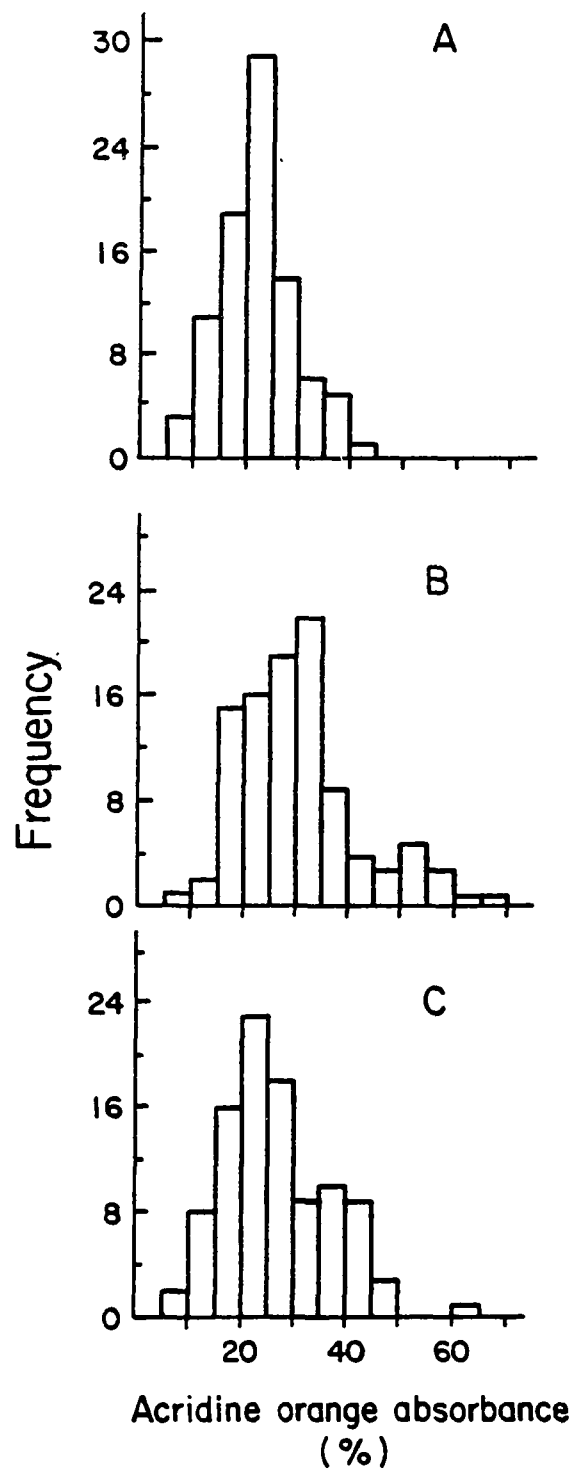


**Figure 6.14.** Histograms showing the distribution of unfrozen and frozen ( $-9^{\circ}\text{C}$  for 16 h) individual cold-acclimated Puma protoplasts within classes of absorbance at 500 nm after equilibrium staining with acridine orange ( $\text{pH}_0=7.1$ ). The distribution is expressed as the number of protoplasts (frequency) included in each class of absorbance (0-5%, 6-10%, 11-15%, etc) based on samples of 100 individual protoplasts measurements. The transmittance of a stained protoplast was subtracted from the transmittance of an unstained protoplast (blank) to get the specific acridine orange absorbance of the protoplast.

A - Control 2: unfrozen protoplasts after 2 h incubation with the dye, mean= $25.1 \pm 0.8\%$ , n = 100

B - Frozen 2: frozen-thawed ( $-9^{\circ}\text{C}$  for 16 h) protoplasts after 2 h incubation with the dye immediately after thawing, mean= $31.4 \pm 1.1\%$ ,  
n = 100

C- Frozen 6 + 2: frozen-thawed ( $-9^{\circ}\text{C}$  for 16 h) protoplasts after 6 h incubation for recovery at  $5^{\circ}\text{C}$  in the dark followed by 2 h incubation with the dye. mean= $28.6 \pm 1.1\%$ , n = 100



**Figure 6.15.** Distribution of unfrozen and frozen (-9°C for 16 h) individual cold-acclimated Puma protoplasts within classes of absorbance at 500 nm after equilibrium staining with acridine orange and subtraction of the blank (pH<sub>0</sub>=7.1). Samples of 30 individual protoplasts measurements (n=30).

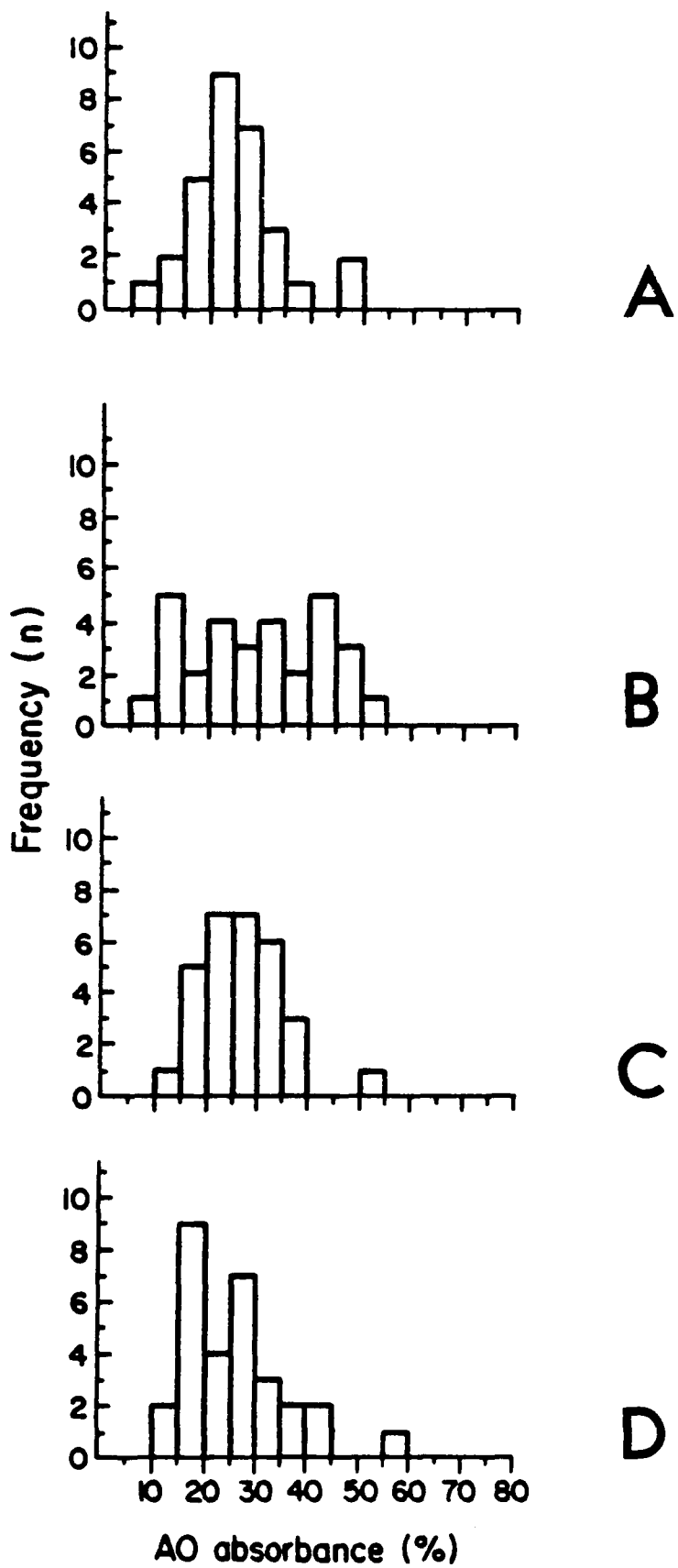
A - Control 2: unfrozen protoplasts after 2 h incubation with the dye, mean= 25.6 ± 1.6%

B - Frozen 2: frozen-thawed protoplasts after 2 h incubation with the dye immediately after thawing, mean= 31.4 ± 2.3%

C - Frozen 6+2: frozen-thawed protoplasts after 2 h incubation with the dye following 6 h incubation at 5°C in the dark, mean= 28.1 ± 1.4%

D - Frozen 8: frozen-thawed protoplasts after 8 h incubation with the dye immediately after thawing, mean = 26.9 ± 1.8%

\*\* see Table 6.1 for survival and compartmentation data.



were different, although the probability of obtaining the distribution of Figure 6.14 C from the reference distribution of Figure 6.14 A was greater than the probability of obtaining the distribution of Figure 6.14 B.

In a different set of measurements (Fig 6.15), a reduction in sample size to  $n = 30$  yielded similar distribution patterns as for Figure 6.14 where  $n = 100$ . A reduction in the size of the sample did not affect the means or the standard deviations, although the standard errors of the mean increased by a factor of two (the means and the SEM are given for each treatment in the figure headings). Figure 6.15 D gives the distribution obtained for stressed protoplasts allowed to recover for 8 h in the presence of the dye. The mean absorbance was  $26.9 \pm 1.8$ . As indicated by a distribution pattern and mean similar to control, there was an apparent recovery. However, this was associated with a decrease in survival to 38% (Table 6.1).

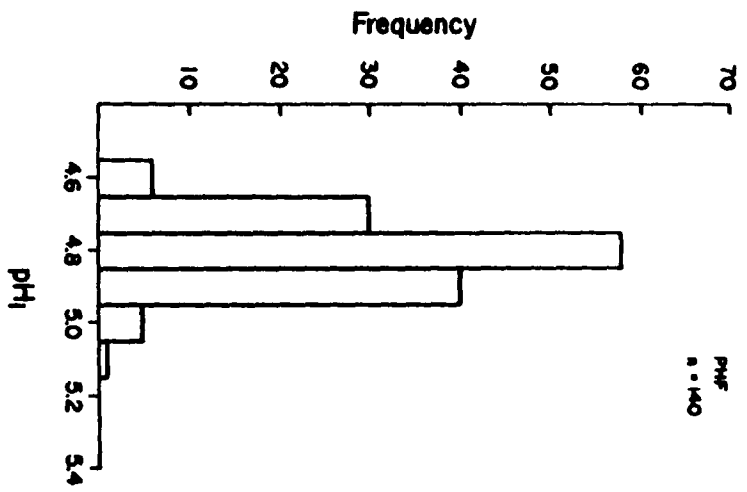
Calculation of vacuolar pH using absorbance permitted the distribution of individual protoplasts within classes of 0.1 pH units. The distribution differed between control (Fig 6.16 B) and frozen-thawed (Fig 6.16 A) protoplasts with the mean vacuolar pH value for the recovering protoplasts (4.8) 0.1 unit lower than for the unfrozen controls (4.9). This vacuolar acidification was general in the population and the difference was statistically significant at the 95% probability level.

A decrease in protoplast diameter was measured in the protoplasts recovering from freezing (Fig 6.17 A) as compared to the control (Fig 6.17 B) indicating that the vacuolar acidification was associated with a lower osmotic concentration of the protoplasts. Such a change in the osmotic volume of the frozen protoplasts would be expected if solute leakage occurred during the post-thaw period. The Chi-square analysis for goodness of fit revealed that the size classes distributions were

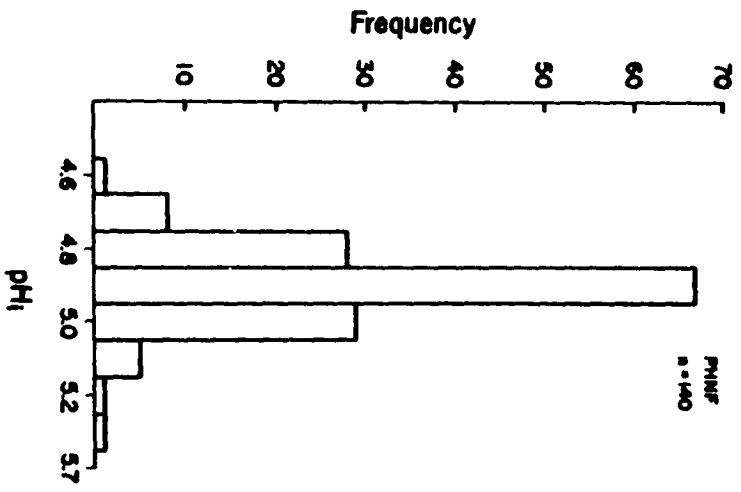
**Figure 6.16.** Histograms showing the distribution of intracellular (vacuolar) pH ( $\text{pH}_i$ ) of unfrozen and frozen ( $-9^\circ\text{C}$  for 16 h) individual Puma cold-acclimated protoplasts ( $n = 140$ ) calculated from equation 6.1 (page 106).

A - Frozen 2 (PHF): 2 h incubation after thawing, mean  $\text{pH}=4.9$ .

B - Control 2 (PHNF): 2 h incubation, mean  $\text{pH}=4.8$ .



A

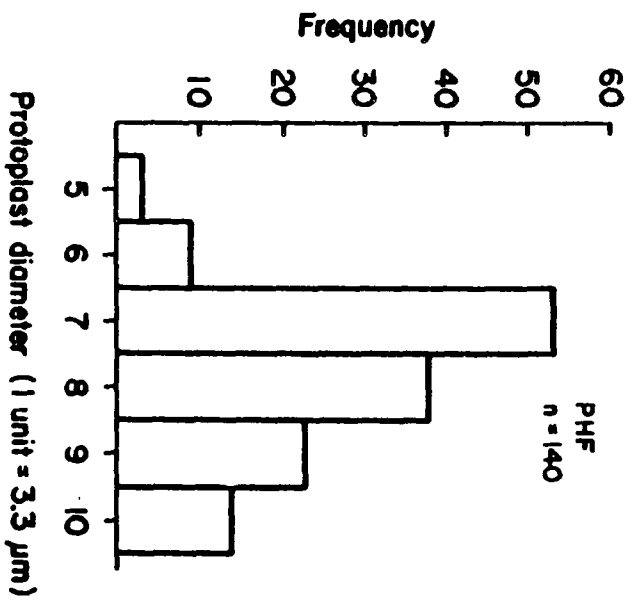


B

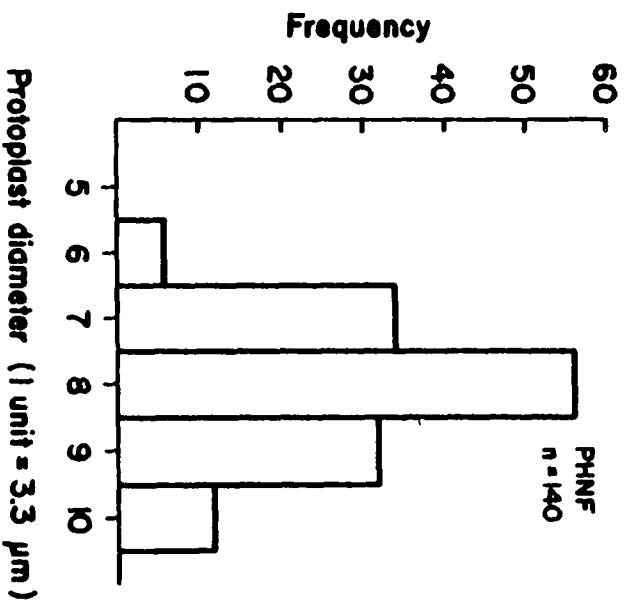
**Figure 6.17. Distribution of unfrozen and frozen (-9°C for 16 h) protoplast diameter  
(n=140)**

**A - frozen-thawed (PHF) 2 h after thawing**

**B - unfrozen control (PHNF)**



A



B

statistically different. This experiment was repeated many times always giving the same results.

#### **D. Conclusions**

The vacuolar acidification observed in apparently physically intact, frozen-thawed protoplasts is a consequence of prolonged exposure to non-lethal freezing temperatures. Recovery and long term survival depend on the maintenance of the semipermeable characteristics of the tonoplast, and the development of lethal injury after freezing may be the result of the altered capacity of the vacuole to accumulate and retain  $H^+$ , which leads to cytoplasmic acidosis and death.

## CHAPTER VII

### GENERAL CONCLUSIONS

The ability of plants to survive a freezing stress depends on (i) events that occur during cold-acclimation prior to the freezing event, (ii) events that occur during the freeze/thaw cycle, and (iii) events that occur following thawing. Traditionally, most attention has been directed to the first two with little attention given to the latter. The results presented in this thesis are consistent with the general view that cellular membranes, in particular the plasma membrane, are a primary site of injury following prolonged exposure to non-lethal freezing temperature (see Chapter III). In addition, an important contribution of this thesis is that it establishes that leakiness must be understood not only in terms of an alteration of the semipermeable characteristics of the plasma membrane but also in terms of an alteration of the semipermeable characteristics of the tonoplast (see Chapter VI). This is not surprising, considering that the vacuole is an important reservoir of solutes for the plant cell and that the tonoplast is as subjected to the same effects of freeze-induced dehydration as is the plasma membrane. The observation of vacuolar decompartmentation and cytoplasmic acidosis in protoplasts subjected to prolonged freezing indicates that the semipermeable characteristics of the cellular membranes are altered in the absence of physical disruption. On the other hand, recovery from prolonged freezing can occur in the absence of vacuolar decompartmentation and cytoplasmic acidosis. Thus, the development of lethal injury after thawing appears to be the result of alterations in the semipermeable characteristics of the tonoplast. An innovative aspect of the present thesis is its focus on events during the recovery period. The state of plant cells recovering

from a non-lethal prolonged freezing is definitely different from that of unfrozen cells. This state is initially associated with a certain degree of injury that is efficiently repaired during a post-thaw incubation period. The repair mechanism requires maintenance of vacuolar compartmentation and cytoplasmic pH regulation.

Plants such as winter rye must have evolved mechanisms of recovery as a specific component of their adaptation to survive a freezing stress. An increase in solute uptake via a feedback mechanism during the critical phase of solute loss may contribute to the avoidance of lethal injury after thawing (see Chapter IV). Similarly, as observed in the absence of vacuolar decompartmentation and cytoplasmic acidosis, reversible vacuolar acidification may contribute to the avoidance of the development of the lethal injury. There is evidence of a strong relationship between organic acid metabolism and alteration of  $H^+$  and other ion transport at the plasma membrane and tonoplast of plant cells i.e., the biochemical and biophysical pH stat model (Smith and Raven, 1979). An increased  $H^+$  concentration in the cytoplasm can be compensated for by an increased  $H^+$  transport into the vacuole. For example, there is evidence that anaerobic stress causes excess  $H^+$  production in the cytoplasm and that this change is associated with an acidification of the vacuole (Boller and Wiemken, 1986). Following prolonged freezing stress, an increased  $H^+$  influx into the cytoplasm may result from an exchange between intracellular  $K^+$  and extracellular  $H^+$  as indicated by the results of Palta (Palta *et al.*, 1977a) who measured an increase in extracellular pH during leakage of  $K^+$ . Such an exchange would contribute to the maintenance of electroneutrality but would increase the  $H^+$  concentration in the cytoplasm. Vacuolar acidification would be the result of the elimination of excess  $H^+$  in the cytoplasm to avoid cytoplasmic acidification. If such is the case, a measure of vacuolar acidification in the absence of cytoplasmic acidification in apparently

intact, frozen-thawed protoplasts isolated from cold-acclimated leaves would constitute a measure of the ability of the protoplast to recover after prolonged freezing stress. At this point, it would be important to verify if non-acclimated plant cells can express the same response under equivalent freezing conditions. Characterization of the response at the molecular level ( $H^+$  pumps at the tonoplast) could provide useful information for the identification of transferable characters of tolerance of acclimated plants. In addition, since non-lethal responses are reversible, time for recovery could be measured.

In conclusion, non-lethal responses to freezing play an important role in the adaptation of plants to subzero temperatures. Hopefully, the results of this thesis will stimulate further studies of this aspect of the plant's response to freezing.

## LITERATURE CITED

- Alberts B., B. Dennis, M. Raff, K. Roberts and J.D. Watson 1983. *Molecular Biology of the Cell*. Garland Publishing Inc., N.Y., 1146p.
- Arneau P. 1975. *Cours de Chimie Organique*. Bordas, Paris, 484p.
- Arora R. and J.P. Palta 1986. Protoplasmic swelling as a symptom of freezing injury in onion bulb cells: its stimulation in extracellular KCl and prevention by calcium. *Plant Physiol.* 82:625-629.
- Bartolo M.E., S.J. Wallner and R.E. Ketchum 1987. Comparison of freezing tolerance in cultured plant cells and their respective protoplasts. *Cryobiology* 24:53-57.
- Besson J. 1962 *L'électrochimie*. Presses universitaires de France, Paris, 125p.
- Boller T. and A. Wiemken 1986. Dynamics of vacuolar compartmentation. *Ann. Rev. Plant Physiol.* 37:137-164.
- Cloutier Y. and E. Imbeault 1988. A new test for freezing tolerance in winter cereals. *Phytoprotection* 69:3-7.
- Cossins A.R. 1981. Steady state and dynamic fluorescence studies of the adaptation of cellular membranes to temperature. in: *Fluorescent Probes*. Beddar G and M.A. West eds, Academic Press, N.Y., pp39-80.
- Crowe J.H., L.M. Crowe and D. Chapman 1984. Infrared spectroscopic studies on interactions of water and carbohydrates with a biological membrane. *Arch. Biochem. and Biophys.* 232:400-407.
- de la Roche A.I., W.A. Keller, J. Singh and D. Siminovitch 1976. Isolation of protoplasts from unhardened and hardened tissues of winter rye and wheat. *Can. J. Bot.* 55:1181-1185.
- Dexter S.T., W.E. Tottingham and L.F. Graber 1932. Investigation of the hardness of plants by measurement of electrical conductivity. *Plant Physiol.* 7:63-78.

- Dohrman U., R. Hertel, P. Pesci, S.M. Cocucci, E. Marre, G. Randazzo and G. Ballio 1977. Localization of in vitro binding of the fungal toxin fusicochin to plasma-membrane-rich fractions from corn coleoptile. *Plant Sci. Lett.* 9:291-299.
- Dubois M., K. Gille, J.K. Hamilton, P.A. Rebers and F. Smith 1951. A colorimetric method for determination of sugars. *Nature* 168: 167.
- Fitzsimons P.J. and J.D.B. Weyers 1985. Properties of some enzymes used for protoplast isolation. in: *The Physiological Properties of Plant Protoplasts*. PE Pilet ed., Springer-Verlag, Berlin, pp12-23.
- Goodwin T.W. and E.I. Mercer 1983. *Introduction to Plant Biochemistry*. Pergamon Press, N.Y., 677p.
- Gordon-Kamm W.J. and P.L. Steponkus 1984. Lamellar to hexagonal<sub>II</sub> phase transitions in plasma membrane of isolated protoplasts after freeze-induced dehydration. *Proc. Natl. Acad. Sci. U.S.A.* 81:6373-6377.
- Heald F. de F. 1902. The electrical conductivity of plant juices. *Bot. Gaz.* 34:81-92.
- Heber U., L. Tyankova and K.A. Santarius 1971. Stabilization and inactivation of biological membranes during freezing in the presence of amino acids. *Biochim. Biophys. Acta* 241:578-592.
- Holden M. 1965. Chlorophyll in: *Chemistry and Biochemistry of Plant Pigments*. Goodwin T.W. ed., Academic Press, London, pp 461-488.
- Johnson-Flanagan A.M. and J. Singh 1986. Membrane deletion during plasmolysis in hardened and non-hardened plant cells. *Plant Cells and Environment* 9:299-305.
- Kates M. and A. Kuksis 1980. *Membrane Fluidity: Biophysical Techniques and Cellular Regulation*. The Humana Press inc., Clifton, N.J., 445 p.

- Kendall E.J. and B.D. Mckersie 1985. Phase properties of membranes after freezing injury in winter wheat. *Can. J. Bot.* 63:2274-2277
- Kurkdjian A. and J. Guern 1981. Vacuolar pH measurement in higher plant cells: Evaluation of the methylamine method. *Plant Physiol.* 67:953-957.
- Kurkdjian A., H. Barbier-Brygoo, J. Manigault and P. Manigault 1984. Distribution of vacuolar pH values within populations of cells, protoplasts and vacuoles isolated from suspension cultures and plant tissues. *Physiol. veg.* 2:193-198.
- Kuhry J.G., P. Fonteneau, G. Duportail, C. Maechling and G. Laustriat 1983. TMA-DPH: A suitable fluorescence polarization probe for specific plasma membrane fluidity in intact living cells. *Cell Biophys.* 5:129-140.
- Lee H.C., J.G. Forte and D. Epel 1982. The use of fluorescent amines for the measurement of pH: Applications in liposomes, gastric microsomes and sea urchin gametes. in: *Intracellular pH: Its Measurement, Regulation and Utilisation in Cellular Functions.* Alan R. Liss ed., Alan R Liss Inc., N.Y., pp 135-160.
- Levitt J. 1980. *Responses of Plants to Environmental Stresses Vol 1 Chilling, Freezing and High Temperature Stresses.* Academic Press, N.Y., 497p.
- Lin W. 1985. Energetics of membrane transport in protoplasts. *Physiol. Plant.* 65: 102-108.
- Lynch D.V. and P.L. Steponkus 1987. Plasma membrane lipid alteration associated with cold-acclimation of winter rye seedlings (*Secale cereale* L. cv Puma) *Plant. Physiol.* 83:761-767.
- Lyons J.M., J.K. Raison and P.L. Steponkus 1979. The plant membrane in response to low temperature: an overview in: *Low Temperature Stress in Crop Plants.* Academic Press, N.Y., pp 1-24.

- Manigault P., J. Manigault and A. Kurkdjian 1983. A microfluorometric method for vacuolar pH measurement in plant cells using 9-amino acridine. *Physiol. veg.* 1:129-136.
- Marre E., P. Lado, F. Rasi Caldogno, R. Colombo and M.I. DeMichelis 1974. Evidence for the coupling of proton extrusion to K<sup>+</sup> uptake in pea internode segments treated with fusicoccin and auxin. *Plant. Sci. Lett.* 3:365-379.
- Marre E 1979. Fusicoccin: a tool in plant physiology. *Ann. Rev. Plant Physiol.* 30:273-288.
- Mazur P. 1969. Freezing injury in plants. *Ann. Rev. Plant Physiol.* 20:419-448.
- McKersie B.D., W.D. Beversdorf and P. Hucl 1982. Solute leakage from susceptible and tolerant cultivars of *Phaseolus vulgaris* L. following ozone exposure. *Can. J. Bot.* 60:73-78.
- Meadows M.G. and Potrykus 1981. Hoechst 33258 as a vital stain for plant cells protoplasts. *Plant Cell Reports* 1:77-79.
- Meryman H.T. and R.J. Williams 1985. Basic principles of freezing injury to plant cells: Natural tolerance and approaches to cryopreservation. in: *Cryopreservation of Plant Cells and Organs*. KK Kartha ed., CRC Press inc., Boca Raton, Fla. pp 13-47.
- Meyer Y. and W.O. Abel 1975. Budding and cleavage division of tobacco mesophyll protoplasts in relation to pseudo-wall and wall formation. *Planta (Berl)* 125:1-13.
- Misra A.N. and M. Misra 1987. Effect of age and rehydration on greening of wheat leaves. *Plant Cell Physiol.* 28(1):47-51.
- Osterhout W.J.V. 1922. *Injury, Recovery and Death, in Relation to Conductivity and Permeability*. JB Lippincott Co, Philadelphia, 259 p.

- Overton E. 1895. Ueber die osmotischen Eigenschaften der lebenden Pflanzen und Thierzelle. *Vierteljahrschr d. Naturfs. Ges. in Zurich* 40:159.
- Palta J.P., J. Levitt and E.J. Stadelman 1977a. Freezing injury in onion bulb cells: evaluation of the conductivity method and analysis of ion and sugar efflux from injured cells. *Plant Physiol.* 60:393-397.
- Palta J.P., J. Levitt and E.J. Stadelman 1977b. Freezing injury in onion bulb cells: Post-thawing injury or recovery. *Plant Physiol.* 60:398-401.
- Palta J.P. and P.H. Li 1978. Cell membrane properties in relation to freezing injury In: *Plant Cold Hardiness and Freezing Stress*. Academic Press, N.Y., pp 93-116.
- Palta J.P. and P.H. Li 1980. Alterations in transport properties by freezing injury in herbaceous plants: evidence against rupture theory. *Physiol. Plant.* 50:169-175.
- Pearce R.S. and J.H.M. Willison 1985. A freeze-etch study of the effects of extracellular freezing on cellular membranes of wheat. *Planta* 163:304-316.
- Pomeroy M.K., S.J. Pihakaski and C.J. Andrews 1983. Membrane properties of isolated winter wheat cells in relation to icing stress. *Plant Physiol.* 72:535-539.
- Pomeroy M.K., C.J. Andrews, K.P. Stanley and Ji Yin Gao 1985. Physiological and metabolic responses of winter wheat to prolonged freezing stress. *Plant Physiol.* 78:207-210.
- Poo M. and R.A. Cone 1974. Lateral diffusion of rhodopsin in the photoreceptor membrane. *Nature* 247:438-441.
- Poole R.J. 1978. Energy coupling for membrane transport. *Ann. Rev. Plant Physiol.* 29:437-460.

- Rincon M. and J.B. Hanson 1986. Controls on calcium ion fluxes in injured or shocked corn root cells: Importance of proton pumping and cell membrane potential. *Physiol. Plant.* 67:576-583.
- Riordan J.R. 1980 Plasma membrane  $Mg^{2+}$  ATPase activity is inversely related to lipid fluidity. in: *Membrane Fluidity; Biophysical Techniques and Cellular Regulation.* Kates M. and A. Kuksis eds, The Humana Press inc., Clifton, N.J., pp119-129.
- Roberts J.K.M., P.M. Ray, N. Wade-Jardetzky and O. Jardetzky 1980. Estimation of cytoplasmic and vacuolar pH in higher plant cells by  $^{31}P$  NMR. *Nature* 283:870-872.
- Rotman B. and B.W. Papermaster 1966. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. *Proc. Natl. Acad. Sci. U.S.A.* 55(1):134-141.
- Sandermann H. Jr 1978. Regulation of membrane enzymes by lipids. *Biochim. Biophys. Acta* 515:209-237.
- Schnedl W., R. Abraham, M. Forster and D. Schweizer 1981. Differential fluorescent staining of porcine heterochromatin by chromomycin A3, distamycin A, DAPI and D287/170. *Cytogenet. Cell Genet.* 31:249-253.
- Senaratna T., B.D. Mckersie and R.H. Stinson 1985. Simulation of dehydration injury to membranes from soybean axes by free radicals. *Plant Physiol.* 77:472-474.
- Shen S.S. and R.A. Steinhardt 1978. Direct measurement of intracellular pH during metabolic derepression of the sea urchin egg. *Nature* 272:253-254.

- Siminovitch D., H. Therrien, J. Wilner and F. Gfeller 1962. The release of amino acids and other ninhydrin-reacting substances from plant cells after injury by freezing: a sensitive criterion for the estimation of frost injury in plant tissues. *Can. J. Bot.* 40:1267-1269.
- Singer S.J. and G.L. Nicholson 1972. The fluid mosaic model of the structure of cell membranes. *Science* 175:720-731.
- Singh J. 1979. Freezing of protoplasts isolated from cold-hardened and non-hardened winter rye. *Plant Science Letters* 16:195-201.
- Singh J. 1981. Isolation and freezing tolerance of mesophyll cells from cold hardened and non-hardened winter rye. *Plant Physiol.* 67:906-909.
- Singh J., B. Iu and A.M. Johnson-Flanagan 1986. Membrane alterations in winter rye and *Brassica napus* L. cells during lethal freezing and plasmolysis. *Plant Cell and Environment* 10:163-168.
- Singh J. and R.W. Miller 1982. Spin-probe studies during freezing of cells isolated from cold hardened and non-hardened winter rye. *Plant Physiol.* 69:1423-1428.
- Smith F.A. and J.A. Raven 1979. Intracellular pH and its regulation. *Ann. Rev. Plant Physiol.* 30:289-311.
- Spanswick R.M. 1981. Electrogenic ion pumps. *Ann. Rev. Plant Physiol.* 32:267-289.
- Sperling F. 1976. Non-lethal parameters as indices of acute toxicity: Inadequacy of the acute LD<sub>50</sub> in: *New Concepts in Safety Evaluation*. Hemisphere Pub. Corp., John Wiley and sons, N.Y., pp 177-191.
- Steffen K.L., R. Arora and J.P. Palta 1985. Responses of photosynthesis and respiration to a freeze-thaw cycle in various potato species. *Plant Physiol.* 77:15 (supp).

- Steponkus P.L. 1981. Responses to extreme temperatures: cellular and subcellular bases. in: *Encyclopedia of Plant Physiology New Ser. Physiological Plant Ecology 1*. O.L. Lange, P.S. Nobel, C.B. Osmond and H. Zeigler eds, Springer Verlag, Berlin, pp 371-402.
- Steponkus P.L., M.F Dowgert and W.J. Gordon-Kamm 1983. Destabilization of the plasma membrane of isolated plant protoplasts during a freeze-thaw cycle: the influence of cold-acclimation. *Cryobiology* 20:448-465.
- Steponkus P.L. 1984. Role of the plasma membrane in freezing injury and cold acclimation. *Ann. Rev. Plant Physiol.* 24:543-584.
- Steponkus P.L. 1985. Fundamental aspects of cryoinjury as related to cryopreservation of plant cells and organ. in: *Biotechnology in Plant Science*. Academic Press, N.Y., pp145-159.
- Tao D., P.H. Li and J.V. Carter 1983. Role of the cell wall in freezing tolerance of cultured potato cells and their protoplasts. *Physiol. Plant.* 58: 527-532.
- Teale F.W.J. 1969. Fluorescence depolarization by light scattering in turbid solutions. *Photochem. Photobiol.* 10:363-374.
- Virgin H.I. 1965. Chlorophyll formation and water deficit. *Physiol. Plant.* 18:994-1000.
- Weigel H.J. and E. Weis 1984. Determination of the proton concentration difference across the tonoplast membrane of isolated vacuoles by means of -amino fluorescence. *Plant Science Letters* 33:163-175.
- Weiser C.J. 1982. Cold Hardiness and Stress Research: An Evolving Agricultural Science in: *Plant Cold Hardiness and Freezing Stress*, Vol. 2, Academic Press, N.Y., pp 1-3.

- Willemot C. 1983. Rapid degradation of polar lipids in frost damaged winter wheat crown and root tissues. *Phytochem.* 22:861-863.
- Wilner J., W. Kalbfleisch and W.J. Mason 1960. Note on two electrolytic methods for determining frost hardiness of fruit trees. *Can. J. Plant Sci.* 40:563-565.
- Wilner J. 1960. Relative and absolute electrolytic conductance tests for frost hardiness of apple varieties. *Can. J. Plant. Sci.* 40:630-637.
- Wolfe J. and P.L. Steponkus 1983. Mechanical properties of the plasma membrane of isolated plant protoplasts: Mechanism of hyperosmotic and extracellular freezing injury. *Plant Physiol.* 71:276-285.
- Yoshida S. and A. Sakai 1974. Phospholipid degradation in frozen plant cells associated with freezing injury. *Plant Physiol.* 53:509-511.
- Yoshida S. 1978. Phospholipid degradation and its control during freezing of plant cells. in: *Plant Cold Hardiness and Freezing Stress*. PH Li ed., Academic Press, N.Y., pp 117-135.
- Zar J.H. 1974 *Biostatistical Analysis*. Prentice-Hall inc., Englewood Cliffs, N.J., 620p.
- Zeitz M., K. Lange, K. Keller and H. Herken 1980 Distribution of acridine orange accumulating particles in neuroblastoma cells during differentiation and their characterization by subcellular fractionation and electron microscopy. *Cell. Mol. Biol.* 25:305-314