J. exp. Biol. (1977), 69, 13–21
With 2 figures
Printed in Great Britain

A CALCIUM-DEPENDENT MECHANISM RESPONSIBLE FOR INCREASING THE FREEZING TOLERANCE OF THE BIVALVE MOLLUSC *MODIOLUS DEMISSUS*

BY DENNIS J. MURPHY*

Department of Zoology, University of Maryland, College Park, Maryland 20742

(*Received 27 July 1977*)

SUMMARY

1. A time course of the changes in blood Ca^{2+} and freezing tolerance of *Modiolus demissus* (Dillwyn) demonstrated that increases in freezing tolerance parallel increases in blood Ca^{2+}. The increases in freezing tolerance occurred rapidly, suggesting that Ca^{2+} affects freezing tolerance directly by its presence in the blood.

2. The presence of La^{3+} reduced the freezing tolerance of isolated foot muscle. Thus, Ca^{2+} appears to increase freezing tolerance directly by binding to cell membranes.

3. The loss of the contractile response of freeze–thawed foot muscle to Ach, KCl and caffeine and the continued response to $CaCl_2$ suggested that cell membranes are the primary sites of freezing injury.

4. The increase in blood Ca^{2+} following low-temperature acclimation accounted for only 40% of the total change in freezing tolerance. Therefore, other mechanisms responsible for increasing the freezing tolerance of *M. demissus* following low temperature acclimation also exist.

INTRODUCTION

The primary site of extracellular freezing injury in many cell types is the plasma membrane (Lovelock, 1957; Farrant, Walter & Armstrong, 1967; Menz, 1971; Pribor, 1971). The mechanism of injury involves the disruption of the cell membrane caused either mechanically as cells shrink beyond a minimum cell volume (Meryman, 1971), or chemically as specific salts attain toxic concentrations in the frozen state leading to protein or lipoprotein denaturation (Lovelock, 1957; Pribor, 1971). In fact, solutes such as glycerol and various amino acids and sugars reduce injury to cells resulting from extracellular ice formation by interacting directly with membranes (Heber, Tyankova & Santarius, 1971; Santarius, 1973; Mazur, Miller & Leibo, 1974). Ca^{2+} binds to cell membranes (e.g. Shaltz & Marinetti, 1972) and also increases the freezing tolerance of algal cells (Terumoto, 1967) and the bivalve mollusc *M. demissus* (Murphy, 1977) when present in the extracellular medium. However, the mechanism of Ca^{2+} protection during freezing remains unknown.

* Present address: Department of Zoology, Connecticut College, New London, Connecticut 06320.

In order to determine the relative importance of Ca^{2+} as a solute for increasing freezing tolerance and to define the mechanism of Ca^{2+} protection during extracellular ice formation, I have examined the Ca^{2+}-dependent mechanism responsible for increasing the freezing tolerance of the intertidal, marsh-dwelling mussel *Modiolus demissus* following low-temperature acclimation. The results of these experiments indicate that the site of Ca^{2+} action appears to be at the cell membrane and that the cell membrane is the primary site of freezing injury. Furthermore, although Ca^{2+} is the only blood solute associated with the increased freezing tolerance of *M. demissus*, the increase in blood Ca^{2+} resulting from low-temperature acclimation can account for only 40% of the total increase in freezing tolerance resulting from low temperature acclimation. Therefore, the total freezing tolerance mechanism involves other components.

MATERIALS AND METHODS

Freezing injury determinations

Modiolus demissus were collected and stored as previously described (Murphy, 1977). The procedures for freezing intact *M. demissus* and for determining freezing mortality followed the methods of Murphy & Pierce (1975). Isolated strips of foot muscle from *M. demissus* were frozen and changes in freezing injury expressed as changes in the minimum voltage stimulus necessary to elicit a twitch contraction (Murphy, 1977).

Influence of Ca^{2+} on freezing tolerance

A time course of the changes in both blood Ca^{2+} and freezing tolerance of *M. demissus* was determined. A group of 200 mussels was acclimated for 2 weeks in 33·7‰ artificial sea water (ASW) (Wilkins, 1972) with a 3·0 mM-Ca^{2+} concentration at 15 °C. Subsequently, these mussels were transferred to ASW (33·7‰) containing 9·6 mM-Ca^{2+}. At various times following the transfer, ten mussels were removed and either blood Ca^{2+} concentrations were measured using a murexide assay (as previously described by Murphy (1977)) or the mussels were frozen to −10 °C for 12 h to determine percent mortality. The salinity of all sea-water samples was determined with a freezing-point depression osmometer (see Murphy, 1977).

The influence of Ca^{2+} on freezing tolerance was also determined by specifically displacing Ca^{2+} from cell membranes with lanthanum ions (La^{3+}) (Weiss, 1974). Isolated strips of foot muscle were first incubated in either ASW for 6 h, or ASW for 5 h followed by incubation in ASW containing 2·0 mM-La^{3+} for 1 h. Next, the tissue was frozen for 12 h at −8·0 °C, thawed for 1 h in ASW, and the responses (threshold voltages^{-1}) compared. The salinity (31·9‰) and temperature (25 °C) of the ASW solutions were constant throughout. Furthermore, in order to dissolve $LaCl_3$, the pH of the ASW was lowered from 7·8 to 6·5 using 0·1 N-HCl. Control studies showed that a pH as low as 4·1 had no significant effect on freezing tolerance ($P > 0·5$).

Response of freeze-thawed tissue to various chemical stimuli

Foot tissue was isolated from *M. demissus*, cut into strips, and immediately frozen for 12 h to various temperatures between −6·0 °C and −13·1 °C. The degree of freezing injury was then determined by measuring the contractility of the muscle following a 1 h thawing period according to the following procedure. Each tissue strip was suspended vertically in air with a silk suture between a fixed metal hook and a force-displacement transducer (Grass Instrument Co., Model FT. 03 C). The suspended tissue was then immersed in a series of 31·8‰ ASW solutions containing progressively increasing concentrations of a specific chemical stimulus. The degree of contractility was expressed as the concentration of the stimulus needed to elicit a sustained contraction for 1·5 min as displayed on an oscillograph (Grass Instrument Co., Model 79 C).

The response of the freeze-thawed tissue to KCl was determined by thawing the tissue in K^+-free ASW and then exposing the tissue to KCl concentrations between 5×10^{-2} M and 1·0 M. Similarly, the response to $CaCl_2$ was measured after thawing the tissue in Ca^{2+}-free ASW and then exposing the tissue to $CaCl_2$ concentrations between 1×10^{-4} and 5×10^{-3} M. The responses to acetylcholine (Ach) and caffeine were determined over concentration ranges of 1×10^{-4} to 1×10^{-2} M and 5×10^{-4} to 5×10^{-2} M respectively. The temperature of the thawing media was always 25 °C. Changes in the response of the freeze–thawed tissue to the chemical stimuli were expressed as the difference between the minimum concentration stimuli needed to elicit a response in the freeze-thawed tissue and that of a non-frozen control tissue incubated in the same thawing media. Response values were calculated according to the formula:

$$\text{response relative to control} = \log(\text{chemical concentration})_{\text{test}} - \log(\text{chemical concentration})_{\text{control}}.$$

Influence of the blood solute changes following low-temperature acclimation on freezing tolerance

The total change in freezing tolerance resulting from temperature acclimation was obtained by acclimating groups of mussels in 31·8‰ Instant Ocean at either 0 or 23 °C for 5 weeks. Strips of foot tissue were obtained from both groups of mussels and incubated for 6 h (25 °C) in 20 ml of blood from mussels acclimated to the same conditions. Blood osmotic concentrations were 945 m-osmol/kg H_2O and 941 m-osmol/kg H_2O for the 0 and 23 °C acclimated groups respectively. Following incubation, the total change in freezing tolerance was determined by freezing both groups of tissue to −10 °C for 12 h, thawing the tissue in ASW (940 m-osmol/kg H_2O) at 25 °C for 1 h, and then measuring the difference in the threshold voltages. To determine the effect of changes in total blood solutes resulting from temperature acclimation on freezing tolerance, a similar experiment was performed following incubation of foot tissue from 23 °C-acclimated mussels in blood from either 0 or 23 °C acclimated mussels. Finally, the influence of the changes in blood Ca^{2+} resulting from temperature acclimation on freezing tolerance was determined. Strips f foot muscle from 23 °C-acclimated mussels were incubated in ASW containing

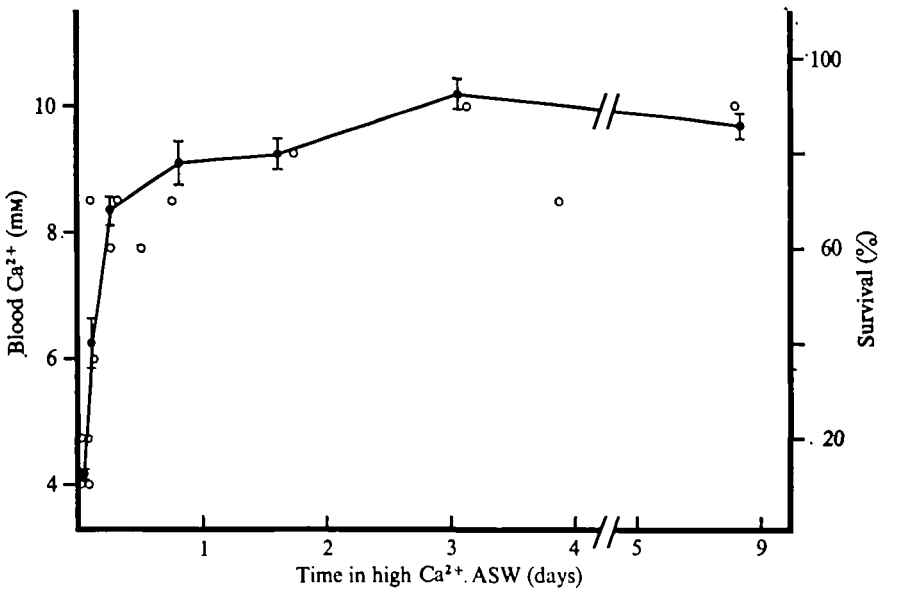

Fig. 1. Relationship between the rates of change in the blood Ca^{2+} and in the freezing tolerance of *M. demissus*. Mussels were acclimated in 33·7‰ ASW (15 °C) with 3·0 mM-Ca^{2+} for 2 weeks and transferred to isosmotic ASW with 9·6 mM-Ca^{2+}. ●—●, Represents blood Ca^{2+} concentrations. Each open circle represents a percentage survival based on ten mussels. Error bars indicate ± 1 × standard error of the mean, $n = 10$.

either 8·0 or 12·0 mM-Ca^{2+} for 6 h at 25 °C. Following incubation, both groups were frozen, thawed, and the difference in the threshold voltages determined.

RESULTS

Influence of Ca^{2+} on freezing tolerance

A time course of the changes in blood Ca^{2+} and freezing tolerance showed that blood Ca^{2+} concentrations of *M. demissus* acclimated in 33·7‰ low Ca^{2+}-ASW (3·0 mM) and transferred to isosmotic, high Ca^{2+}-ASW (9·6 mM) increased from 4·2 to 6·3 mM within 2·5 h. Simultaneously, the percentage survival of *M. demissus* frozen at −10 °C increased from 10–20% to 40–70% following the 2·5 h exposure to high Ca^{2+}-ASW (Fig. 1). Six hours after the transfer to high Ca^{2+}-ASW, the blood Ca^{2+} concentration increased further to 8·5 mM and the percentage survival ranged between 60 and 70%. By 19 h, blood Ca^{2+} concentrations had stabilized between 9·0 and 10·3 mM and did not increase any further for the duration of the experiment (8·25 days). Percentage survival values also levelled off after 19 h and ranged between 70 and 90% thereafter (Fig. 1).

The displacement of Ca^{2+} from cell membranes by La^{3+} influenced the freezing tolerance of isolated foot muscle. The contractile responses of the freeze–thawed tissue incubated in 2·0 mM-$LaCl_3$ relative to non-frozen control tissue were significantly lower than the relative contractile responses of freeze–thawed tissue incubated without $LaCl_3$ present ($P < 0{\cdot}001$) (Table 1).

Table 1. *The freezing tolerance of isolated foot muscle of* M. demissus *incubated and frozen in the indicated media and thawed in ASW for* 1 *h at* 25 °C*

Group	Prefrozen incubation conditions	Threshold voltage non-frozen control tissue log (voltage^{-1}),† n = 10	Threshold voltage freeze-thawed tissue‡ log (voltage^{-1}), n = 10	Change from control Δ log (voltage^{-1})
I	ASW§	−0·341 ± 0·036‖	−0·705 ± 0·041‖	+0·364 ± 0·027‖ ¶
II	ASW + 2 mM-La^{3+}	−0·373 ± 0·032	−1·402 ± 0·096	+1·029 ± 0·051

* Animals used in this experiment were collected during the summer from Little Sippewissett Marsh, Massachusetts, and were held on sea tables in continuously running sea water (32‰) at 20 °C for 1–2 weeks prior to use.

† Threshold voltages are expressed as voltage^{-1} so that increasing values represent increases in freezing tolerance.

‡ Tissue was frozen for 12 h at −8·0 °C.

§ Artificial sea-water mixture (ASW) contained 10 mM calcium with a total osmotic pressure of 930 m-osmol/kg H_2O. For details of complete ionic composition see Materials and Methods section.

‖ Error values are standard errors.

¶ Change in group I was significantly less than the change in group II ($P < 0{\cdot}001$) (a t-test was used to determine the statistical difference).

Response of freeze–thawed tissue to chemical stimuli

The relative response of freeze-thawed foot muscle from *M. demissus* to $CaCl_2$ remained constant following exposure to temperatures between −8·0 and −13·1 °C ($P > 0{\cdot}5$) (Fig. 2). In contrast, although the relative responses of freeze–thawed tissue to Ach and KCl followed the same pattern as the relative $CaCl_2$ responses

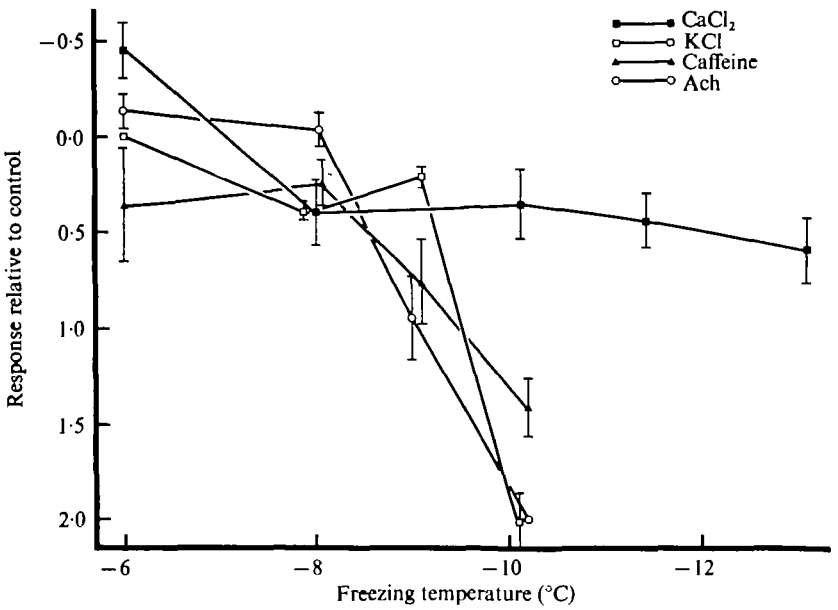

Fig. 2. Contractile response of freeze–thawed foot muscle of *M. demissus* to the indicated chemical stimuli relative to non-frozen controls. Increasing values represent increases in the relative concentrations of stimuli necessary to elicit contraction, or increases in freezing injury. Error bars indicate ± 1 × standard error of the mean, n = 20 (for further details see Materials and Methods section).

Table 2. *Influence of total blood solute and calcium changes following low-temperature acclimation on the freezing tolerance of isolated foot muscle of* M. demissus*

Group	Acclimation conditions†	Incubation medium‡	Average changes in contractile response (threshold voltage$^{-\frac{1}{2}}$)§, $n = 20$	Total change in freezing tolerance (%)
A	0 °C 5 weeks (¶ = 931)	Blood from 0 °C acclimated animals (¶ = 945)	(A − E) = 0·324^{a} ± 0·023‖	100
B	23 °C 5 weeks (¶ = 942)	Blood from 0 °C acclimated animals (¶ = 945)	(B − E) = 0·124^{b} ± 0·015	38
C	23 °C 6 weeks (¶ = 937)	ASW with 12 mM-Ca^{2+} (¶ = 937)	(C − D) = 0·134^{b} ± 0·016	41
D	23 °C 6 weeks (¶ = 931)	ASW with 8 mM-Ca^{2+} (¶ = 935)		
E	23 °C 5 weeks (¶ = 931)	Blood from 23 °C acclimated animals (¶ = 941)		

* Isolated foot muscle was frozen for 12 h following incubation and thawed for 1 h in ASW (940 m-osmol/kg H_2O) prior to testing.

† Animals were acclimated in artificial sea water (Instant Ocean); ¶ = osmolarity (m-osmol/kg H_2O).

‡ Incubation time was 6 h at 25 °C.

§ Square roots of the threshold voltages^{-1} were calculated to obtain homogeneity of variance.

^{ab} Means with identical superscripts are not significantly different ($P > 0{\cdot}05$) (The Student–Newman–Keuls (SNK) Test was used to determine statistical differences).

‖ Error values are standard errors.

when frozen to −8·1 °C ($P > 0{\cdot}2$), the responses to these stimuli dropped significantly below the $CaCl_2$ response when frozen to temperatures between −8·1 and −10·2 °C ($P < 0{\cdot}001$) (Fig. 2). Similarly, freeze–thawed foot tissue frozen to −8·1 °C showed the same relative response to caffeine as to $CaCl_2$, but exposure to temperatures between −8·1 and −10·2 °C significantly reduced the caffeine response below that of the response to $CaCl_2$ ($P < 0{\cdot}01$) (Fig. 2).

Influence of the blood solute changes following low-temperature acclimation on freezing tolerance

A comparison of the changes in the contractile responses of freeze–thawed foot muscle from *M. demissus* acclimated at 23 °C and incubated in blood from either 23 or 0 °C acclimated animals showed that an increased freezing tolerance could be induced in isolated foot muscle by incubation in blood from 0 °C acclimated animals (Table 2). This increase in freezing tolerance, however, accounted for only 38% of the total increase in freezing tolerance which occurred when foot tissue from mussels acclimated at 0 °C was incubated in blood from 0 °C acclimated animals. Similarly, the freezing tolerance of foot muscle from 23 °C acclimated animals incubated in ASW with 12·0 mM-Ca^{2+} was increased above that of foot muscle incubated in ASW with 8·0 mM-Ca^{2+}. This increase accounted for only 41% of the total increas

in freezing tolerance (Table 2). Thus, although the increases in blood Ca^{2+} which accompany low-temperature acclimation could account for the effect of blood solutes on freezing tolerance, changes in blood solutes alone do not account for the total change in freezing tolerance resulting from low-temperature acclimation.

DISCUSSION

The results presented here indicate that the increase in the blood Ca^{2+} concentration of *M. demissus* following low-temperature acclimation increases freezing tolerance directly by binding to cell membranes. Transferring *M. demissus* from low Ca^{2+}-ASW to high Ca^{2+}-ASW showed that the resulting increases in blood Ca^{2+} with time were paralleled by similar increases in freezing tolerance. Furthermore, the effect of an increase in blood Ca^{2+} on freezing tolerance occurred rapidly (within 2·5 h). Taken together, these data indicate that Ca^{2+} affects freezing tolerance directly by its presence in the blood and not secondarily by altering some biochemical or physiological process. The reduced freezing tolerance of isolated foot muscle, incubated in ASW supplemented with La^{3+} prior to freezing, indicated that the Ca^{2+} effect on freezing tolerance is at the plasma membrane surface. La^{3+} does not penetrate the cell membrane and, in addition, binds specifically to Ca^{2+} binding sites at the cell membrane surface, displacing bound Ca^{2+} from such sites (Weiss, 1974). Mg^{2+} also competes for Ca^{2+} binding sites on plasma membranes (e.g. Hemminki, 1974). Therefore, since Mg^{2+} is only 20% as effective as Ca^{2+} for increasing the freezing tolerance of *M. demissus* (Murphy, 1977), the reduced blood Mg^{2+} concentrations observed following low-temperature acclimation (Murphy, 1977) may also serve to increase freezing tolerance by making available a greater proportion of Ca^{2+} for binding to membrane sites.

As stated previously, a mechanism for increasing the freezing tolerance of *M. demissus* appears to involve an increased binding of Ca^{2+} to cell membranes. This suggests that the primary site of extracellular freezing injury is the cell membrane. The cell membrane is, in fact, the primary site of freezing injury in a variety of cell types (Lovelock, 1957; Farrant *et al.* 1967; Menz, 1971; Pribor, 1971). Changes in the response of freeze–thawed foot muscle to chemical stimuli following exposure to a series of subfreezing temperatures suggests that the plasma membrane is also the primary site of injury in *M. demissus*. The threshold concentration of $CaCl_2$ needed to elicit a contraction of freeze–thawed foot muscle relative to non-frozen controls remained the same following exposure to temperatures as low as −13 °C. In contrast, exposure to temperatures between −8 and −10 °C resulted in an almost complete loss of response to Ach, KCl and caffeine. The loss of response of freeze–thawed muscle to both KCl or Ach which elicit muscle contraction by plasma membrane depolarization (for review see Prosser, 1973*a*) and caffeine which causes contraction by eliciting a Ca^{2+} release from either sarcoplasmic reticulum or plasma membranes (e.g. Huddart & Syson, 1975) implies that damage to the plasma membrane resulted from exposure to temperatures between −8 and −10 °C. The persistence of the response of freeze–thawed muscle to $CaCl_2$, however, is probably due to the direct interaction of Ca^{2+} with the intracellular contractile proteins (for review see Prosser, 1973*b*).

The increase in blood Ca^{2+} which occurs during low-temperature acclimation is the only blood solute change which is associated with the increase in the freezing tolerance of *M. demissus*. However, this increase in blood Ca^{2+} can account for only 40% of the total observed change in freezing tolerance resulting from low temperature acclimation. A comparison of the differences in the freezing tolerance of *M. demissus* acclimated either anaerobically (13 °C) or aerobically (0 °C) showed that although the blood Ca^{2+} concentration of the anaerobic mussels was 4-fold greater than that of the low-temperature acclimated mussels, the low-temperature acclimated mussels still had a higher freezing tolerance. Therefore, part of the total increase in freezing tolerance involves mechanisms other than the blood Ca^{2+} increase and, apparently, depends directly on exposure to low temperature.

In conclusion, the increased binding of Ca^{2+} to cell membranes may reduce membrane damage during freezing either by physically stabilizing the membrane against the mechanical disruption caused by cell shrinkage or, perhaps, by preventing the denaturation of membrane components as specific tissue solutes concentrate to toxic levels in the frozen state. The other possible mechanisms which exist may involve either changes in the concentrations of specific intracellular solutes such as amino acids, organic acids, or sugars; or changes in the structural components of membranes, specifically lipids. Such changes could stabilize membranes against the stresses imposed during freezing (Heber *et al.* 1971; Santarius, 1971, 1973; Siminovitch, Singh & De La Roche, 1975). These possible mechanisms are being investigated.

I wish to thank Dr. S. K. Pierce Jr. for his critical reading of the manuscript.

This work was supported by NSF Grant BMS 72-02465-A01 to Dr. S. K. Pierce Jr. and a Chesapeake Bay Funds Grant, University of Maryland.

Contribution number 60 from the Tallahassee, Sopchoppy and Gulf Coast Marine Biological Association.

REFERENCES

FARRANT, J., WALTER, C. A. & ARMSTRONG, J. A. (1967). Preservation of structure and function of an organized tissue after freezing and thawing. *Proc. R. Soc.* B **168**, 293–310.

HEBER, U., TYANKOVA, L. & SANTARIUS, K. A. (1971). Stabilization and inactivation of biological membranes during freezing in the presence of amino acids. *Biochim. biophys. Acta* **241**, 578–92.

HEMMINKI, K. (1974). Calcium binding to brain plasma membranes. *Biochim. biophys. Acta* **363**, 202–10.

HUDDART, H. & SYSON, A. J. (1975). The effect of caffeine on calcium efflux and calcium translocation in skeletal and visceral muscle. *J. exp. Biol.* **63**, 131–42.

LOVELOCK, J. E. (1957). The denaturation of lipid protein complexes as a cause of damage by freezing. *Proc. R. Soc. Lond.* B **147**, 427–33.

MAZUR, P., MILLER, R. H. & LEIBO, S. P. (1974). Survival of frozen-thawed bovine red cells as a function of the permeation of glycerol and sucrose. *J. Membrane Biol.* **15**, 137–58.

MENZ, L. J. (1971). Structural changes and impairment of function associated with freezing and thawing in muscle, nerve, and leucocytes. *Cryobiology* **8**, 1–13.

MERYMAN, H. T. (1971). Osmotic stress as a mechanism of freezing injury. *Cryobiology* **8**, 489–500.

MURPHY, D. J. (1977). Metabolic and tissue solute changes associated with changes in the freezing tolerance of the bivalve mollusc *Modiolus demissus*. *J. exp. Biol.* (in the Press).

MURPHY, D. J. & PIERCE, S. K. JR. (1975). The physiological basis for changes in the freezing tolerance of intertidal molluscs. I. Response to sub-freezing temperatures and the influence of salinity and temperature acclimation. *J. exp. Zool.* **193**, 313–322.

PRIBOR, D. B. (1971). Osmotic hemolysis contrasted with freeze–thaw hemolysis. *Cryobiology* **8**, 14–24.

PROSSER, C. L. (1973*a*). Excitable membranes. In *Comparative Animal Physiology* (ed. C. L. Prosser). pp. 457–504. Pennsylvania: W. B. Saunders.

PROSSER, C. L. (1973*b*). Muscles. In *Comparative Animal Physiology* (ed. C. L. Prosser), pp. 730–4. Pennsylvania: W. B. Saunders Co.

SANTARIUS, K. A. (1971). The effect of freezing on thylakoid membranes in the presence of organic acids. *Pl. Physiol., Lancaster* **48**, 156–62.

SANTARIUS, K. A. (1973). The protective effect of sugars on chloroplast membranes during temperature and water stress and its relationship to frost, dessication and heat resistance. *Planta* **113**, 105–14.

SHALTZ, L. & MARINETTI, G. V. (1972). Calcium binding to rat liver plasma membrane. *Biochim. biophys. Acta* **290**, 70–83.

SIMINOVITCH, D., SINGH, J. & DE LA ROCHE, J. A. (1975). Studies in membranes in plant cells resistant to extreme freezing. I. Augmentation of phospholipids and membrane substance without changes in unsaturation of fatty acids during hardening in black locust bark. *Cryobiology* **12**, 144–53.

TERUMOTO, I. (1967). Frost resistance in algae cells. In *Cellular Injury and Resistance in Freezing Organisms* (ed. E. Asahina), pp. 191–209. Hakkaido University Press, Japan.

Weiss, G. B. (1974). Cellular pharmacology of lanthanum. *A. Rev. Pharmac.* **14**, 343–54.

WILKINS, L. A. (1972). Electrophysiological studies in the heart of the bivalve mollusc, *Modiolus demissus*. I. Ionic basis of the membrane potential. *J. exp. Biol.* **56**, 273–91.