J. exp. Biol. **118**, 121–131 (1985)
Printed in Great Britain © *The Company of Biologists Limited 1985*

OCULAR FREEZING AVOIDANCE IN ANTARCTIC FISH

BY JEFFREY D. TURNER*, JOSEPH D. SCHRAG† AND ARTHUR L. DEVRIES

AND *Department of Physiology and Biophysics, University of Illinois, 524 Burrill Hall, 407 S. Goodwin Avenue, Urbana, Illinois 61801, U.S.A.*

Accepted 4 March 1985

SUMMARY

A series of experiments was undertaken to determine the underlying mechanism of ocular freezing avoidance in a variety of antarctic fish. The three possible mechanisms that were examined include the depression of the freezing point through (1) colligative or (2) non-colligative means or (3) the supercooling of the ocular fluids. The ocular fluids of antarctic fish, like those of most vertebrates, are slightly hypotonic with respect to plasma and contain very low levels of serum proteins. The chief means of ocular freezing avoidance appears to involve supercooling of the ocular fluids. The presence of suitable barriers to prevent ice propagation into the eye has been demonstrated both *in vitro* and *in vivo*.

INTRODUCTION

Fish living in the ice-laden Antarctic oceans avoid freezing through the accumulation of macromolecular antifreezes which depress the freezing point of the blood in a non-colligative fashion (DeVries & Wohlschlag, 1969; DeVries, Vandenheede & Feeney, 1971; Duman & DeVries, 1975). The majority of these molecules are glycopeptides composed of a repeating tripeptide (Ala-Ala-Thr) with the threonine-linked dissaccharide *N*-acetyl-galactosamine and galactose (Shier, Lin & DeVries, 1975). There are at least eight different sizes of glycopeptides, denoted as 1–8 with respect to their relative mobility during polyacrylamide gel electrophoresis (PAGE), and with sizes ranging from 2600 to 33 000 Da (DeVries *et al.* 1971). In the smaller two glycopeptide antifreezes (GPAFs), 7 and 8, there is an occasional substitution of proline for alanine (Lin, Duman & DeVries, 1972). Glycopeptides are synthesized in the liver and then secreted into the circulatory system (Hudson, DeVries & Haschemeyer, 1979; O'Grady, Clarke

*Present address: Department of Animal Science, University of Illinois, 205 MSL, 1503 S. Maryland Dr., Urbana, IL 61801, U.S.A.

†Present address: Department of Biochemistry, Biosciences West, University of Arizona, Tucson, AZ 85721, U.S.A.

Key words: Antarctic teleost, ocular freezing.

& DeVries, 1982). The mechanism by which these blood-borne GPAFs are translocated into and confer freezing avoidance to certain fluid compartments has not been elucidated.

One such important compartment which would appear to require protection because of its proximity to ice-laden sea water is ocular fluid. The importance of maintaining the functional integrity of an animal's eyes cannot be overemphasized. Antarctic fish are no exception, particularly the deep water forms which possess large eyes with a variety of adaptations for increased visual capacity in dim environments resulting from thick ice, water depth and the darkness of the austral winter (Meyer-Rochow & Klyne, 1982).

There are at least three possible strategies that antarctic fish could employ to avoid ocular freezing: depression of the freezing point of the ocular fluids below that of sea water, $-1{\cdot}9\,^{\circ}C$, using either (1) colligative or (2) non-colligative means, or (3) ensuring that these fluids are maintained in a supercooled state.

Increasing the ocular fluid salt content offers the means to depress the freezing point in a colligative manner. This involves making the humoural fluids hypertonic to serum by at least two-fold; such a situation is not known to occur in fish where ocular fluids are slightly hypotonic or in some cases isotonic with respect to plasma (Davson & Grant, 1960; Hoffert & Fromm, 1966; Zadunaisky, 1972, 1973).

Some theoretical problems arise when considering the second option, the non-colligative freezing point depression by a glycopeptide antifreeze. Firstly, the quantities of GPAF required in the ocular fluids to lower the freezing point below that of sea water would increase protein levels 60-fold over that typically found in vertebrate eyes (Cole, 1974). Such protein levels could compromise humoural transparency thereby affecting visual acuity. Further, the selective transport of large molecular weight GPAFs across the blood-aqueous humour barrier would be a unique process.

The maintenance of ocular fluids in a supercooled state has been proposed as a possible mechanism for ocular freezing avoidance in antarctic fish (DeVries, 1974). Indeed, whole body supercooling is frequently observed as an adaptation of northern fishes (Scholander *et al.* 1957; Leivestad, 1965). However in these northern fish, supercooling carries with it an obligate avoidance of environmental ice. Antarctic fish frequently contact ice, so if supercooling is important for avoiding ocular freezing, certain conditions must exist that prevent nucleation of the supercooled state. Firstly, suitable barriers must exist to prevent ice propagation into the eye from the environment. This is consistent with the thickness and permeability characteristics of piscine cornea (Edelhauser, 1968; Edelhauser & Siegesmond, 1968). Secondly, spontaneous nucleation of the ocular fluids must not occur. The probability of nucleation with $1\,^{\circ}C$ supercooling would be expected to be small, unless nucleating surfaces are present within the eye (Dorsey, 1948). The urine from antarctic fish does appear to exist in a supercooled state. The maintenance of this metastable state is possible because the bladder contains no nucleation sites and is surrounded by tissues fortified with GPAF (DeVries, 1984).

This communication considers the mechanism(s) used by antarctic fish to prevent ocular freezing. Experiments have focused on the ionic and protein compositions of the ocular fluids and the ability of the cornea to prevent ice propagation into the eye.

MATERIALS AND METHODS

Animals and sampling procedure

Specimens of *Pagothenia borchgrevinki* (Boulenger), *Trematomus hansoni* (Boulenger), *T. bernacchii* (Boulenger), *T. centronotus* (Regan), ranging in weight from 100 to 300 g, were caught in baited traps and *Dissostichus mawsoni* (Norman), ranging in weight from 40 to 60 kg, were caught on baited hooks in McMurdo Sound, Antarctica (77°54′S, 166°40′E). Fish were held in a flow-through sea water system at $-1{\cdot}7 \pm 0{\cdot}2$ °C for a minimum of 7 days prior to experimentation. Fish remained disease-free and ate chopped fish *ad libitum*.

Under MS222 anaesthesia (1:15 000, Sigma), 2 ml of blood was sampled by haemal-arch penetration with a cold syringe, permitted to clot for 60 min at -1 °C, then centrifuged at 7500 ***g*** for 2 min and the serum removed and frozen. Immediately following blood sampling, animals were killed by concussion and both eyes were enucleated. Aqueous humour was sampled from the anterior chamber with a 100-μl Hamilton syringe and frozen. Vitreous humour was aspirated from an incision made in the sclera, triturated five times to reduce viscosity then frozen. Samples contaminated with blood were discarded.

Glycopeptide antifreeze characterization and quantitation

Freezing-melting point analysis

The presence of glycopeptide antifreeze (GPAF) in the serum, aqueous and vitreous humour was determined by measuring thermal hysteresis – the difference between the freezing and melting points. Freezing points were determined visually in a 10-μl capillary tube sealed with mineral oil, as described by Duman & DeVries (1975). Melting point was calculated from total osmolarity values determined with a Wescor vapour pressure osmometer.

PAGE with fluorescently-labelled GPAF

Samples of serum, aqueous humour, vitreous humour and pure GPAF were added to 20 μl of 0·3 mol l^{-1} borate buffer, pH > 8·0 and 10 μl of 30 mg ml^{-1} fluorescamine (Roche Diagnostics) in acetone (Udenfriend *et al.* 1972). Glycopeptides were separated using a 10 % non-denaturing polyacrylamide gel polymerized by the soluble disulphide linker BAC (*N*,*N*′-bis-acrylylcystamine, Bio-Rad) after the method of Hansen, Pheiffer & Boehnert (1980) and Hansen (1981). The gels were run at a constant 25 mA for a period of 2 h. Gel slices containing individual protein bands were dissolved for 10 min in a Tris-borate buffer at pH 8·3 containing 0·012 g dithiothreitol g^{-1} of gel. Upon appropriate

dilution, the fluorescence was measured using fluorometry (Turner fluometer, Model III, excitation <365 nm, emission >455 nm). GPAF concentration was estimated from a standard curve of GPAF 8, which was linear from 1 to 100 nmol. Dissolved BAC-polyacrylamide gel has little intrinsic fluorescence. Photographs of the gels were made with high contrast technical film (Kodak 2415) with ultraviolet lighting.

High performance liquid chromatography (HPLC) quantitation

Twenty-μl samples of plasma or aqueous humour were chromatographed on a 10-μm, reverse phase column (Partisil ODS-3, Whatman) with a 5% acetonitrile in water isocratic separation at a flow rate of 1 ml min^{-1}. Absorbance at 230 nm was detected by a Spectroflow 773 (Kratos) variable wavelength detector and peak areas were integrated with a Spectrophysics SP4270. The sensitivity of the serum measurements was 0·050 absorbance units full scale (AUFS) while that of aqueous humour was 0·0050 AUFS. Glycopeptide antifreeze concentration was proportional to the integrated area when pure GPAF 7 and 8 were analysed.

Transmembrane ice propagation

The ability of both the cornea and corneal epithelium (the clear extension of skin over the cornea) to act as a barrier to ice propagation was determined using an Ussing chamber modified after Helman & Miller (1971). The corneal epithelium from either *P. borchgrevinki* or *D. mawsoni* or the cornea of the *D. mawsoni* were mounted in the chamber using high vacuum grease (Dow-Corning) and placed in a flow-through sea water system at −1·7 °C. Chamber fluid was 550 mosmol l^{-1} NaCl. After thermal equilibrium, one chamber was nucleated with a small ice crystal and the propagation of ice through the membrane was noted by the freezing of fluid in the adjacent chamber. As a control, thin latex rubber could be shown to prevent ice propagation while a 0·4-μm polycarbonate filter (Nucleopore) did allow propagation.

The physiological analogy was performed by removing the corneal epithelium from the eyes of eight anaesthetized *P. borchgrevinki*. After 24 h of recovery, fish were placed in ice-laden sea water (−1·9 °C) for 45 min and observed for ocular freezing.

Freezing behaviour of ocular fluids

The temperature of heterogenous nucleation was determined using *T. centronotus* serum and aqueous humour. One hundred μl of fluid was placed under *n*-hexane in an aluminium crucible and the temperature slowly reduced using a Peltier effect cold plate (Thermoelectric Unlimited Inc.) Temperature was monitored with a telethermometer (Yellow Springs Instr.) and nucleation noted visually.

Analytical techniques and statistical analysis

Soluble protein concentration in plasma and aqueous humour were assayed by the method of Bradford (1976) with bovine serum albumin (Sigma) as a standard. Antifreeze glycopeptides do not react with this Coomassie based assay.

Sodium and potassium concentrations were determined by flame photometry (Corning, Model 455) with internal lithium standardization.

Statistical analysis was done using the unpaired Student's two-tailed *t*-test, with significance limits set to $P \leqslant 0{\cdot}05$. All values are reported as the mean $\pm$ 1 S.E.M. (N).

RESULTS

Colligative freezing point depression

In all the fish studied the ocular fluids were found to be significantly hypo-osmotic (15–25 mosmol l^{-1}) to the serum (Table 1). The single exception was *T. bernacchii*, where vitreous humour values were not significantly lower. Decreased sodium concentration appeared to be responsible for the decreased osmolarity. The vitreous humour possessed significantly less sodium than the aqueous humour. Potassium levels in the serum, aqueous and vitreous humours were not significantly different from each other in any of the fish studied (Table 1).

Non-colligative freezing point depression

Non-colligative freezing point depression does not appear to be of sufficient magnitude to prevent ocular freezing in the species examined (Table 2). The freezing point of ocular fluids is difficult to determine accurately as the initial ice growth is not well defined and may be complicated by the increased viscosity of

Table 1. *Sodium and potassium concentration and total osmolarity of serum, aqueous humour (AH) and vitreous humour (VH) of four species of antarctic fish*

Species		Na^+ (mequiv l^{-1})	K^+ (mequiv l^{-1})	Total osmolarity (mosmol l^{-1})
Pagothenia borchgrevinki	Serum	253 ± 2	2·54 ± 0·29	590 ± 4
	AH	234 ± 2*	2·67 ± 0·09	576 ± 3*
	VH	236 ± 3*	3·08 ± 0·15	573 ± 5*
Trematomus bernacchii	Serum	288 ± 11	3·31 ± 0·24	558 ± 5
	AH	250 ± 4*	2·49 ± 0·18	539 ± 5*
	VH	235 ± 7†*	4·14 ± 0·61	542 ± 5
Trematomus hansoni	Serum	276 ± 5	2·52 ± 0·23	560 ± 8
	AH	251 ± 7*	2·51 ± 0·19	536 ± 7*
	VH	227 ± 4†*	2·93 ± 0·13	538 ± 9*
Trematomus centronotus	Serum	276 ± 3	3·11 ± 0·36	579 ± 5
	AH	257 ± 10*	2·33 ± 0·17	549 ± 5*
	VH	227 ± 2†*	2·87 ± 0·41	549 ± 5*

* Indicates a significant difference ($P \leqslant 0{\cdot}05$) from serum.
† Indicates a significant difference ($P \leqslant 0{\cdot}05$) from aqueous humour.
$\bar{X} \pm 1$ S.E.M., $N = 8$.

the ocular fluids. Nevertheless, in the presence of a seed crystal, ice grows in aqueous humour at temperatures 0·4–0·6 °C warmer than the freezing point of sea water. Serum gives a characteristic large hysteresis with the mean freezing points always lower than −2·30 °C. The crystal growth consisting of very fine spicules is typically observed in the presence of larger molecular weight GPAF.

Polyacrylamide gel electrophoresis of fluorescently-treated serum and ocular fluid proteins can be seen in Fig. 1. In all fish species studied, ocular fluids contained predominantly GPAF 8 and traces of GPAF 7. None of the larger molecular weight GPAFs were detected even when the amount of ocular fluid load on the gel was increased five-fold. Serum contained the typical complement of large and small molecular weight GPAFs. Fluorescence quantitation of GPAF 7 and 8 concentrations with fluids from *T. centronotus* indicated that serum contained some 10–20 times more of these low molecular weight antifreezes than did ocular fluids (Fig. 2). Quantitation by this method was time consuming and tedious therefore further determinations were made using HPLC techniques.

The values obtained for GPAF 7 and 8 concentrations using either fluorescence or HPLC were not significantly different (Fig. 2). The HPLC data confirmed that aqueous humour concentrations of GPAF 7 and 8 were consistently below 1 mg ml^{-1} for all species tested, while serum concentrations for GPAFs 7 and 8 always exceeded 12 mg ml^{-1}. Clearly, a disequilibrium in antifreeze concentration exists between the serum and aqueous humour.

Non-antifreeze protein levels show a similar gradient, with ocular fluids having 1 mg ml^{-1} and serum having levels above 20 mg ml^{-1} (Fig. 2). This suggests that both the antifreeze and non-antifreeze protein are being handled in a similar fashion.

Supercooling and barriers to ice propagation

The degree to which aqueous humour and serum could supercool was evaluated using *T. centronotus* samples. Cooled under *n*-hexane to prevent nucleation from the air, aqueous humour could be supercooled to −13·50±0·16 °C (6) and serum

Table 2. *The freezing point (fp), melting point (mp) and thermal hysteresis (Δ) of serum and aqueous humour of five antarctic fish*

	Serum			Aqueous humour		
Species	fp (°C)	mp (°C)	Δ (°C)	fp (°C)	mp (°C)	Δ (°C)
Pagothenia borchgrevinki	−2·73±0·06	−1·10±0·01	1·63±0·06	−1·40±0·05	−1·07±0·01	0·33±0·05
Trematomus bernacchii	−2·62±0·04	−1·04±0·01	1·58±0·03	−1·29±0·06	−1·00±0·01	0·29±0·06
Trematomus hansoni	−2·66±0·06	−1·04±0·02	1·62±0·05	−1·31±0·01	−1·00±0·01	0·31±0·02
Trematomus centronotus	−2·35±0·04	−1·08±0·01	1·27±0·04	−1·49±0·05	−1·02±0·01	0·47±0·04
Dissostichus mawsoni (N=5)	−2·50±0·04	−1·20±0·02	1·29±0·04	−1·31±0·03	−1·09±0·02	0·21±0·03
McMurdo sea water	−1·91	−1·91				

$\bar{X}$±1 S.E.M., N=8.

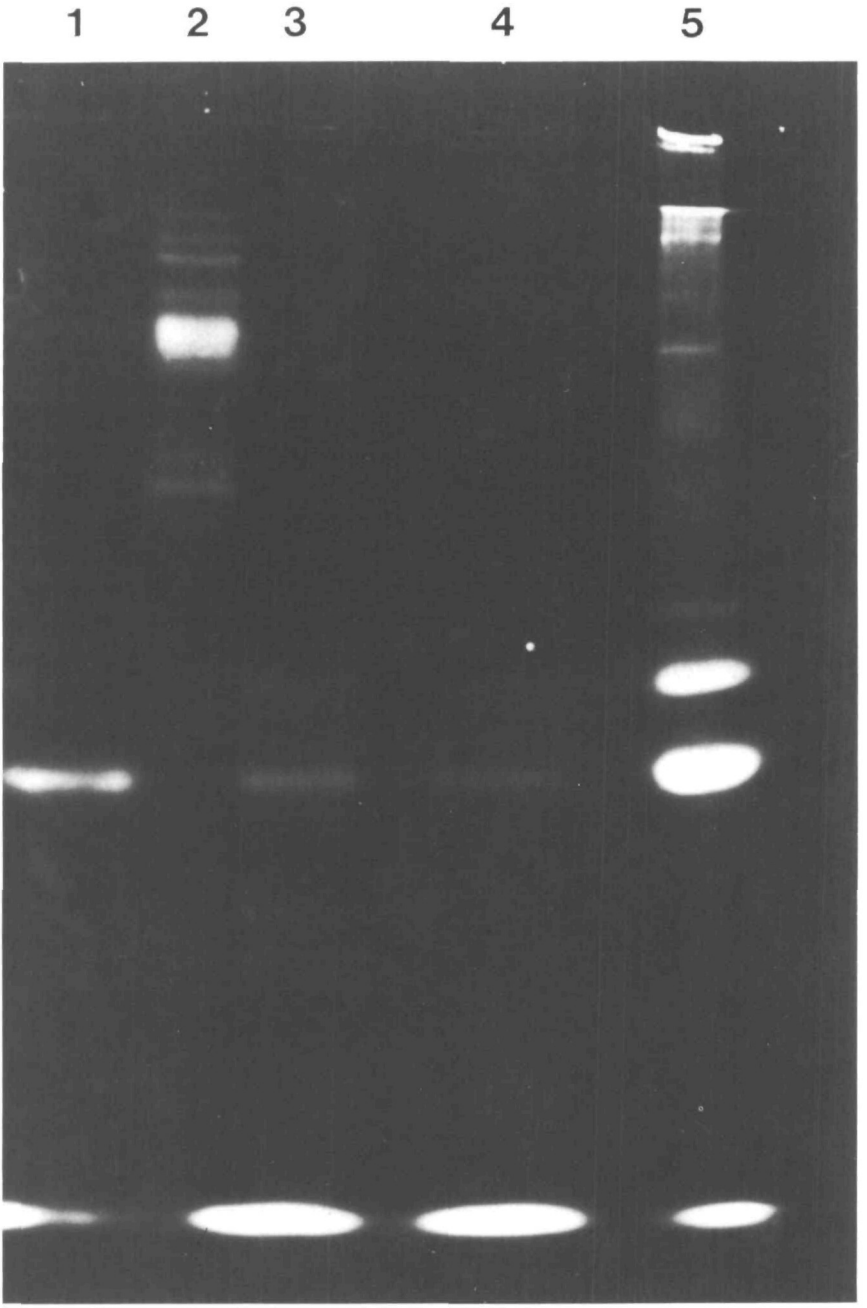

Fig. 1. Polyacrylamide gel electrophoresis of fluorescamine-treated samples from *Trematomus bernacchii*: lane (1) purified GPAF 8, (2) purified GPAF mixture 1–6, (3) aqueous humour, (4) vitreous humour and (5) serum. The dye front is indicated by large fluorescent bands near the bottom of the gel.

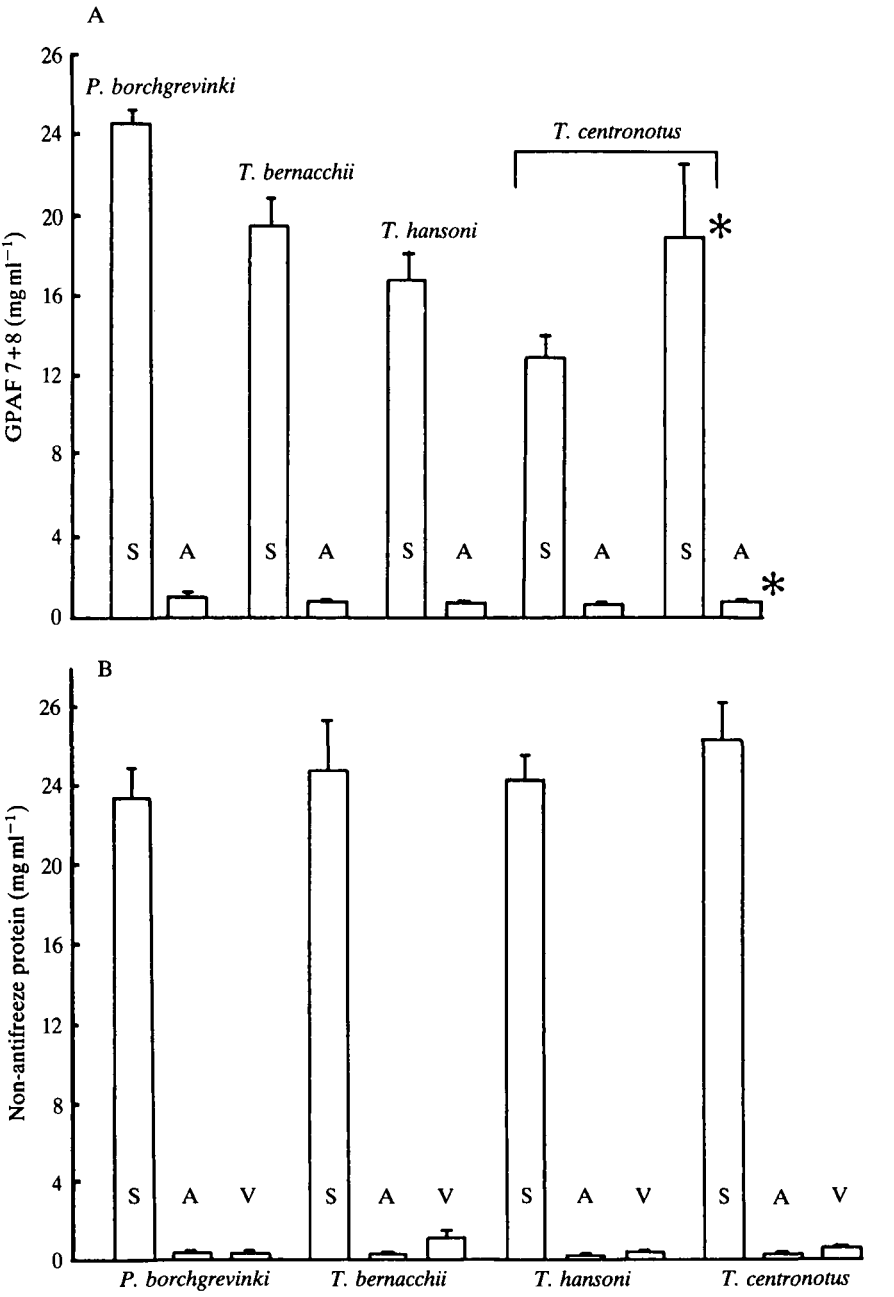

Fig. 2. (A) The concentration of glycopeptide antifreeze (GPAF) 7 and 8 in mg ml^{-1} ($\bar{X}\pm1$ S.E.M., $N=8$) in the serum (S) and aqueous humour (A) of *Pagothenia borchgrevinki*, *Trematomus bernacchii*, *Trematomus hansoni* and *Trematomus centronotus*. Fluorescence estimates of GPAF 7 and 8 for *T. centronotus* ($\bar{X}\pm1$ S.E.M., $N=3$) are shown for comparison (*). (B) The concentration of non-antifreeze protein (in mg ml^{-1}; $\bar{X}\pm1$ S.E.M., $N=8$) in the serum (S), aqueous humour (A) and vitreous humour (V) of four antarctic fish.

to $-12{\cdot}70 \pm 0{\cdot}36\,^{\circ}C$ (6) before spontaneous nucleation occurred. This suggests that no nucleation sites exist within aqueous humour that can initiate spontaneous freezing at sea water temperatures, $-1{\cdot}9\,^{\circ}C$.

A prerequisite for using supercooling as a mechanism for avoidance of ocular freezing is that a barrier to environmental ice must be present. The modified Ussing chamber proved useful as thin membranes could be held securely without edge damage for at least 3 h. When polycarbonate filters with a pore size of $0{\cdot}4\,\mu m$ were mounted in the chamber and the fluid on one side nucleated, ice propagated rapidly through the filter and nucleated the opposite bathing solution. Thin latex rubber prevented any ice propagation across the membrane for at least 60 min. Similarly, cornea or corneal epithelium acted as a complete barrier to ice propagation. This barrier remained intact for at least 60 min and prevented ice growth in either direction across the tissue.

In vivo studies involving surgical removal of the corneal epithelium did not result in visible damage to the cornea or alter behaviour. When the fish were placed in sea water at $-1{\cdot}9\,^{\circ}C$ in direct contact with crushed sea ice, no ocular freezing was observed.

DISCUSSION

It is quite clear from the total osmolarity measurements that ocular fluids are hypo-osmotic by some 10–30 $mosmol^{-1}$ with respect to serum. This relationship is consistent with findings from other marine teleosts and is thought to be characteristic of aqueous humour formation (Zadunaisky, 1972). Since ocular fluids are approximately half the osmolarity of sea water, colligative freezing point depression is insufficient as a mechanism of ocular freezing avoidance.

Three independent methods, thermal hysteresis, PAGE and HPLC, all indicate that very low levels of glycopeptide antifreezes are present in ocular fluids (Table 2; Figs. 1,2). Although the small amount of GPAF does account for most of the thermal hysteresis of these fluids, the measured freezing point is well below the freezing point of both the blood and environment. Clearly, the mechanism of freezing avoidance of the ocular fluids does not involve a significant non-colligative lowering of the ocular fluid freezing points by glycopeptide antifreezes.

The low level of GPAFs, as well as low levels of other proteins in the ocular fluids relative to the blood of the antarctic fish, indicates that there are barriers between blood and the aqueous and vitreous humours. Recent experiments with tritiated GPAFs 1–5 and 8 also confirm the presence of these barriers, in that the movement of radio-labelled antifreeze derivatives from the blood into the ocular fluids is very slow and small in magnitude (J. A. Ahlgren & J. D. Turner, unpublished data). The low protein concentrations observed in these ocular fluids is in accord with very low protein concentrations found in the ocular fluids of most vertebrates (Cole, 1974).

The long-term supercooling of pure water is not unusual. Dorsey (1948) has shown that the spontaneous nucleation of pure water in glass ampoules usually

does not occur until temperatures are lowered below −6 °C and is independent of the length of time the sample has been in the supercooled state. Although fish ocular fluids contain salt and both soluble and structural protein, these solutes do not appear to affect the stability of the supercooled state. This is supported by the fact that aqueous humour can be routinely supercooled *in vitro* to −12 °C.

With extreme supercooling the probability of spontaneous nucleation increases dramatically. With slight supercooling, external factors such as the introduction of nucleators (ice) or cavitation may result in freezing. The apparent stability of slight supercooling in fishes has been documented. Supercooled fish taken from deep fjords in Labrador spend their entire lives at −1·7 °C even though the equilibrium freezing point of their body fluids is −0·9 °C; however, when brought to the surface and exposed to ice they freeze (Scholander *et al.* 1957). The site where ice propagates across the integument into the supercooled fluids of the fish is unclear, but the thin epithelia of the gills is a likely site.

Based on the high freezing points of the ocular fluids (−1·3 to −1·4 °C), it can be concluded that the ocular fluids of these antarctic fishes exist in a supercooled state and that the cornea plays a role in maintaining the stability of this state by prevention of the entry of ice from the sea. The cornea is an absolute barrier to entry of ice from the environment. In part, this view is supported by the fact that some of the large antarctic fishes are known to survive for decades, with much of their life spent in the ice-laden waters of McMurdo Sound.

In vitro measurements indicate that both cornea and corneal epithelium can prevent propagation of ice. Further, *in vivo* studies indicate that the cornea alone is a barrier to ice propagation into the ocular fluids. The fact that fish cornea are barriers to water and electrolyte movement in the marine environment (Edelhauser, 1968) may be related to their capability for preventing ice propagation. How it is related is not clear but it may be that the collagen in the cornea forms a strong, mechanical meshwork which would depress the freezing point of corneal cell water. Such mechanisms of freezing point depression have been documented in certain gel matrices with high tensile strength filaments (Bloch, Walters & Kuhn, 1963). Rabbit and bull corneal cell water remains stable in the supercooled state *in vitro* to at least −5 °C in the presence of extracellular ice (Smith & Smiles, 1953).

Maintenance of the ocular fluids in a stable supercooled state has adaptive significance. No energy is required for its maintenance, as would be the case if large osmotic gradients were in place between the serum and ocular fluid and a colligative mechanism was used to lower the freezing point below that of sea water. In addition, if antifreeze glycopeptides were used as a means of freezing avoidance, specific transport of proteins into the aqueous humour would be required.

In summary, antarctic fishes appear to avoid ocular freezing because of the presence of a corneal barrier which prevents the propagation of ice from the environment and allows the ocular fluids to remain slightly supercooled.

The authors wish to thank S. Munsell for his assistance concerning collection of the fish and J. A. Ahlgren for his critical evaluation of this manuscript. Thanks

also go to Dr C. Knight for valuable discussions concerning the nature of supercooled fluids and Dr J. T. Eastman for his help with ocular histology. This work was supported by NSF Grant DPP,81–16917 to ALD.

REFERENCES

BLOCH, R., WALTERS, D. H. & KUHN, W. (1963). Structurally caused freezing point depression of biological tissues. *J. gen. Physiol.* **46**, 605–615.

BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72**, 248–254.

COLE, D. F. (1974). Comparative aspects of intraocular fluids. In *The Eye: Comparative Physiology*, (eds H. Davson & L. T. Graham, Jr), pp. 71–147. New York: Academic Press.

DAVSON, H. & GRANT, C. T. (1960). Osmolarities of some body fluids in the elasmobranch and teleost. *Biol. Bull. mar. biol. Lab., Woods Hole* **119**, 293.

DEVRIES, A. L. (1974). Survival at freezing temperatures, biochemical and biophysical perspectives. In *Marine Biology*, Vol. 1, (eds D. C. & J. R. Sargent), pp. 289–330. London: Academic Press.

DEVRIES, A. L. (1984). Role of glycopeptides and peptides in inhibition of crystallization of water in polar fishes. *Phil. Trans. R. Soc. Ser. B* **304**, 575–588.

DEVRIES, A. L., VANDENHEEDE, J. & FEENEY, R. E. (1971). Primary structure of freezing point-depressing glycopeptides. *J. biol. Chem.* **246**, 305–308.

DEVRIES, A. L. & WOHLSCHLAG, D. E. (1969). Freezing resistance in some antarctic fishes. *Science, N.Y.* **163**, 1073–1075.

DORSEY, N. E. (1948). The freezing of supercooled water. *Trans. Am. phil. Soc.* **38**, 248–328.

DUMAN, J. G. & DEVRIES, A. L. (1975). The role of macromolecular antifreezes in cold water fishes. *Comp. Biochem. Physiol.* **52A**, 193–199.

EDELHAUSER, H. F. (1968). Sodium and water permeability of salt-water fish corneas. *Comp. Biochem. Physiol.* **24**, 665–667.

EDELHAUSER, H. F. & SIEGESMOND, K. (1968). Ultrastructure of trout cornea. *J. Fish. Res. Bd Can.* **25**, 863–866.

HANSEN, J. N. (1981). Use of solubilizable acrylamide disulfide gels for isolation of DNA fragments suitable for sequence analysis. *Analyt. Biochem.* **116**, 146–151.

HANSEN, J. N., PHEIFFER, B. H. & BOEHNERT, J. A. (1980). Chemical and electrophoretic properties of solubilizable disulfide gels. *Analyt. Biochem.* **105**, 192–201.

HELMAN, S. I. & MILLER, D. A. (1971). *In vitro* techniques for avoiding edge damage in studies of frog skin. *Science, N.Y.* **173**, 146–148.

HOFFERT, J. R. & FROMM, P. O. (1966). Effect of carbonic anhydrase inhibition on aqueous humour and blood bicarbonate ion in the teleost (*Salvelinus namaycush*). *Comp. Biochem. Physiol.* **18**, 333–340.

HUDSON, A. P., DEVRIES, A. L. & HASCHEMEYER, A. E. V. (1979). Antifreeze glycoprotein biosynthesis in antarctic fishes. *Comp. Biochem. Physiol.* **62B**, 179–183.

LEIVESTAD, H. (1965). Supercooling and osmoregulation in teleosts of the boreal and sub-arctic. *Spec. Publs int. Comm. N. W. Atlant. Fish.* **6**, 747–752.

LIN, Y., DUMAN, J. G. & DEVRIES, A. L. (1972). Studies on the structure and activity of low molecular weight glycoproteins from an antarctic fish. *Biochem. biophy. Res. Commun.* **46**, 87–92.

MEYER-ROCHOW, V. B. & KLYNE, M. A. (1982). Retinal organization of the eyes of three nototheniid fishes from the Ross sea (Antarctica). *Gegenbaurs morph. Jahrb., Leipzig* **128**, 762–777.

O'GRADY, S. M., CLARKE, A. & DEVRIES, A. L. (1982). Characterization of glycoprotein antifreeze biosynthesis in isolated hepatocytes from *Pagothenia borchgrevinki*. *J. exp. Zool.* **220**, 179–189.

SCHOLANDER, P. F., VANDAM, L., KANWISHER, J. W., HAMMEL, H. T. & GORDON, M. S. (1957). Supercooling and osmoregulation in Arctic fish. *J. cell. comp. Physiol.* **49**, 5–24.

SHIER, W. T., LIN, Y. & DEVRIES, A. L. (1975). Structure of the carbohydrate of antifreeze glycoproteins from an Antarctic fish. *FEBS Letts* **54**, 135–138.

SMITH, A. U. & SMILES, J. (1953). Microscopic studies of mammalian tissue during cooling to and rewarming from −79 °C. *Jl R. microscop. Soc.* **73**, 134–159.

UDENFRIEND, S., STEIN, S., BOHLEN, P., DAIRMAN, W., LEIMGRUBER, W. & WEIGELE, M. (1972). Fluorescamine: a reagent for assay of amino acids, peptides, proteins and primary amines in the picomole range, *Science, N.Y.* **178**, 871–872.

ZADUNAISKY, J. A. (1972). The electrolyte content, osmolarity and site of secretion of the aqueous humour in two teleost fishes (*Carassius auratus* and *Diplodus sargus*). *Expl Eye Res.* **14**, 99–110.

ZADUNAISKY, J. A. (1973). The hypotonic aqueous humor of teleost fishes, *Expl Eye Res.* **16**, 397–401.