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PROTEIN AND GLYCOPROTEIN ANTIFREEZES IN THE INTESTINAL FLUID OF POLAR FISHES

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SUMMARY

Measurements of ion concentrations, freezing points and melting points of intestinal fluid were made for several Antarctic fishes and two North Atlantic species. These measurements indicated that plasma and intestinal fluid are nearly isosmotic. Freezing points of intestinal fluid were approximately 0·9 °C below the melting points, suggesting the presence of glycoprotein antifreeze within the intestinal fluid of the Antarctic fishes. Polyacrylamide gel electrophoresis and specific immunoprecipitation with glycoprotein antifreeze antibody confirmed the presence of appreciable quantities of antifreeze and showed that the major antifreeze fractions present in the intestinal fluid are low molecular weight glycopeptides.

INTRODUCTION

Polar fishes, particularly Antarctic species, live at extreme and constant low temperatures of −2 °C. These fishes avoid freezing injury in at least two ways. First, it has been shown that the blood contains peptide or glycopeptide antifreezes that lower the freezing point in a non-colligative manner and thus have little effect on the osmotic pressure of the blood (Raymond & DeVries, 1977). Secondly, plasma Na^+ levels are considerably elevated compared with most temperate marine teleosts (250 *v.* 160 mM-Na^+), which accounts for approximately 30–40% of the total freezing-point depression (−2·5 °C) observed in the plasma of Antarctic fishes (Duman & DeVries, 1974; O'Grady & DeVries, 1981). The elevated plasma electrolyte levels have also been suggested to represent an energy conserving adaptation to extreme cold (Prosser, Mackay & Kato, 1970; Hazel & Prosser, 1974).

Nearly all Antarctic fishes from the McMurdo Sound region synthesize glycoprotein antifreezes (DeVries, 1980). Eight glycoproteins showing antifreeze activity have been isolated from the serum of these fish (DeVries, Komatsu & Feeney, 1970). The glycoproteins range in molecular weight between 2600 and 25000 daltons. The peptide portion of the molecule consists of repeating units of ala-ala-thr. The smaller glycoproteins, designated 6, 7 and 8, contain proline which is not found in the larger glycoproteins 1 through 5. The disaccharides (Galactosyl-*N*-acetylgalactosamine) are

attached to the peptide via glycosidic linkage to threonine residues (DeVries & Lin, 1977). Antifreeze glycoproteins make up approximately 3% (w/v) of serum protein in Antarctic fishes, yet make a negligible contribution to the total osmotic concentration of the fishes' body fluids (DeVries & Wohlschlag, 1969).

The freezing-melting behaviour of specific body fluids such as blood, urine and coelomic fluid has been investigated in Antarctic fishes. Analysis of freezing and melting point differences as well as analysis of the crystal habit of the ice formed in urine indicate that antifreeze is not present in bladder urine. Renal conservation of glycopeptide antifreeze is due to aglomerular nephrons in the kidney, where urine formation is the result of secretion rather than filtration and reabsorption (Dobbs & DeVries, 1975; Eastman *et al.* 1979). These observations, along with a slow turnover of antifreeze (*ca.* 3 wk), would suggest that glycopeptide antifreezes are conserved within the circulation. However, nothing is known about the susceptibility of the intestinal fluid to freezing in Antarctic fishes. This is an important consideration since ingestion of ice may occur as a result of feeding or drinking. Propagation of ice from the environment into the rectum could also occur because the anal sphincter is not tightly closed. The present work is, therefore, directed towards investigating the composition and freezing behaviour of intestinal fluid of Antarctic fishes. Initially, data are presented establishing the presence of substantial quantities of glycoprotein and protein antifreeze in this fluid and determining its molecular size in a variety of Antarctic and Arctic species. Further data include analysis of the ionic composition of this fluid which can be used to estimate fluid absorption based on Skadhauge's approach using luminal Mg^{2+} concentrations as an indirect index of fluid absorption (Skadhauge, 1980). Ideally, studies of drinking rate and branchial epithelium fluxes are necessary to complete the picture for water and salt balance in these fishes. Although confined to intestinal studies, the present results allow some conclusions regarding salt and water transport in Antarctic fishes to be made, and also provide evidence of a biological role for the small glycoprotein antifreezes.

MATERIALS AND METHODS

Animals

Pagothenia borchgrevinki (Boulanger), *Trematomus bernacchii* Boulanger, *Trematomus centronotus* Regan, *Trematomus hansoni* Boulanger and *Dissostichus mawsoni* Norman were collected by hook and line at McMurdo Sound, Antarctica (77° 54′ to 166° 40′ E). These animals were kept in large fibreglass holding tanks in a circulating sea water system. Animals were fed chopped pieces of fish once a week. Tank temperatures were held at $-1{\cdot}5 \pm 1$ °C and salinity was constant at 35‰. *Myoxocephalus scorpius* (L.) and *Pseudopleuronectes americanus* Walbaum were obtained from shallow water near the Mount Desert Island Biological Laboratory and held in sea water aquaria at the University of Illinois. *Notothenia rossii* were obtained by courtesy of Martin White, British Antarctic Survey, Cambridge. The fishes were collected from South Georgia. Water temperatures were maintained at 2 °C in artificial sea water at Cambridge.

Blood and intestinal fluid sampling

Blood samples (0·4 ml) were taken from the dorsal vein using a 30 gauge needle. Samples were allowed to clot at room temperature for one hour before centrifugation. After centrifugation at 5000 *g*, the serum was removed and frozen for later analysis. Intestinal fluid samples from *P. borchgrevinki*, *T. bernacchii*, *T. centronotus*, *T. hansoni* and *N. rossii*, were taken, following opening of the body cavity, by clamping the intestine at the pyloric junction and anus, then removing the intestine from the body cavity and allowing the contents to drain into 1·5 ml microfuge tubes. Intestinal fluid samples from *D. mawsoni*, *M. scorpius* and *P. americanus* were taken by insertion of a short length of tubing (P.E. 200) into the rectum and drawing the fluid out with a syringe. No evidence of blood contamination was observed in either sampling method. Each sample was centrifuged at 5000 *g* to remove particulate matter and the supernatant removed and frozen for later analysis.

Freezing and melting point analysis

Freezing and melting points were determined using a cryoscope following the method of Duman & DeVries (1974). Samples of intestinal fluid were placed in 10 μl capillary tubes and sealed with mineral oil. A small seed crystal was formed in the sample using a spray refrigerant (Cryokwik) and the sample immersed in a temperature-controlled viewing chamber ($\pm$ 0·01 °C) containing isopropanol. Melting points were determined by raising the temperature 0·01 °C every 2 min until the seed crystal melted. This temperature was taken as the melting point of the sample. Freezing points were determined by lowering the temperature 0·01 °C every 5 min until growth of the seed crystal was observed. This temperature was taken as the freezing point of the sample. Solutions containing antifreeze proteins show a substantial difference between melting and freezing points by this method (DeVries, 1980).

Antibody preparation

An antibody against glycoprotein antifreeze from Antarctic fish was developed in BABL/c mice and partially purified following the procedure of Tung *et al.* (1976): after three months, pooled ascites fluid was partially purified using sodium dextran sulphate followed by ammonium sulphate precipitation. The precipitate was redissolved in 0·01 M-Tris and dialysed against 0·01 M-Tris + 0·15 M-NaCl for 24 h to remove excess ammonium sulphate. The dialysed extract was divided into aliquots of 1·0 ml and stored at −20 °C until use.

Immunoprecipitation

Preparation of samples for immunoprecipitation involved treatment of intestinal fluid samples from *P. borchgrevinki*, *T. bernacchii*, *T. centronotus*, *T. hansoni* and *D. mawsoni* with 2 vols. of cold 10% trichloroacetic acid (TCA) for 2 h to precipitate any proteolytic enzymes that might degrade the antibody (glycoprotein antifreeze is soluble in 10% TCA, VanVoorhies, Raymond & DeVries, 1978). Each sample was centrifuged at 10000 *g* for 5 min. The supernatant was dialysed in Spectrapor-3

dialysis tubing against distilled water at 4 °C for 24 h, then lyophilized. The lyophilized material was resuspended in distilled water at a concentration of 20 mg/ml.

Partially purified ascites fluid containing glycoprotein antifreeze antibody was placed in the centre well of an Ouchterlony plate with TCA-treated intestinal fluid samples in three of the four outer wells. The fourth well contained distilled water. The plate was incubated at room temperature (23 °C). Precipitin lines appeared within 24 h.

Polyacrylamide gel electrophoresis

Intestinal fluid samples were prepared in the same manner as those used for immunoprecipitation, except that the lyophilized material was resuspended in borate buffer (0·18 M-NaOH, 0·9 M-H_3BO_3, pH 8·6). Five μl of fluorescamine (Fluram, Roche Diagnostics) (4 mg/ml in acetone) was added to 20 μl samples of resuspended material from each species. Purified glycoprotein antifreeze from the serum of *D. mawsoni* (in borate buffer at 30 mg/ml) was used as a standard. Fluorescently labelled samples (10 μl) were loaded on to a 10% polyacrylamide gel and run at 10 V/cm for 2·5 h.

Intestinal fluid osmolalities and ion concentrations

Determinations of ion concentrations and osmolalities were made for intestinal fluid samples from six Antarctic fishes. Osmolalities were measured using a Wescor vapour-pressure osmometer. Sodium and potassium concentrations were determined by flame photometry using a Corning 455 model flame photometer with internal lithium standardization. Chloride concentrations were measured with a Buchler–Cotlove chloridometer. Magnesium concentrations were determined by atomic absorption spectrophotometry (Perkin-Elmer model 305A).

RESULTS

Cryoscopy

Freezing and melting point analyses of serum and intestinal fluid are presented in Table 1. Intestinal fluid samples show a substantial difference between melting and freezing points as well as a freezing point much lower than predicted from the osmotic concentration. Serum samples from these species have similar characteristics with slightly larger differences between freezing and melting points. Warm acclimated *P. americanus* (8 °C) showed no significant differences between freezing and melting points in either serum or intestinal fluid and served as a control in these measurements.

Biochemical analysis

Fig. 1 shows the results of an Ouchterlony analysis of TCA-treated intestinal fluid from *P. borchgrevinki*, *T. centronotus* and *D. mawsoni*. The precipitation indicates that glycoprotein antifreeze is present within the intestinal fluid of these species.

Polyacrylamide gel electrophoresis was used to determine which glycoprotein antifreezes were present in the intestinal fluid samples from Antarctic fish. Fig. 2 shows a comparison of intestinal fluid samples with glycoprotein antifreeze standard

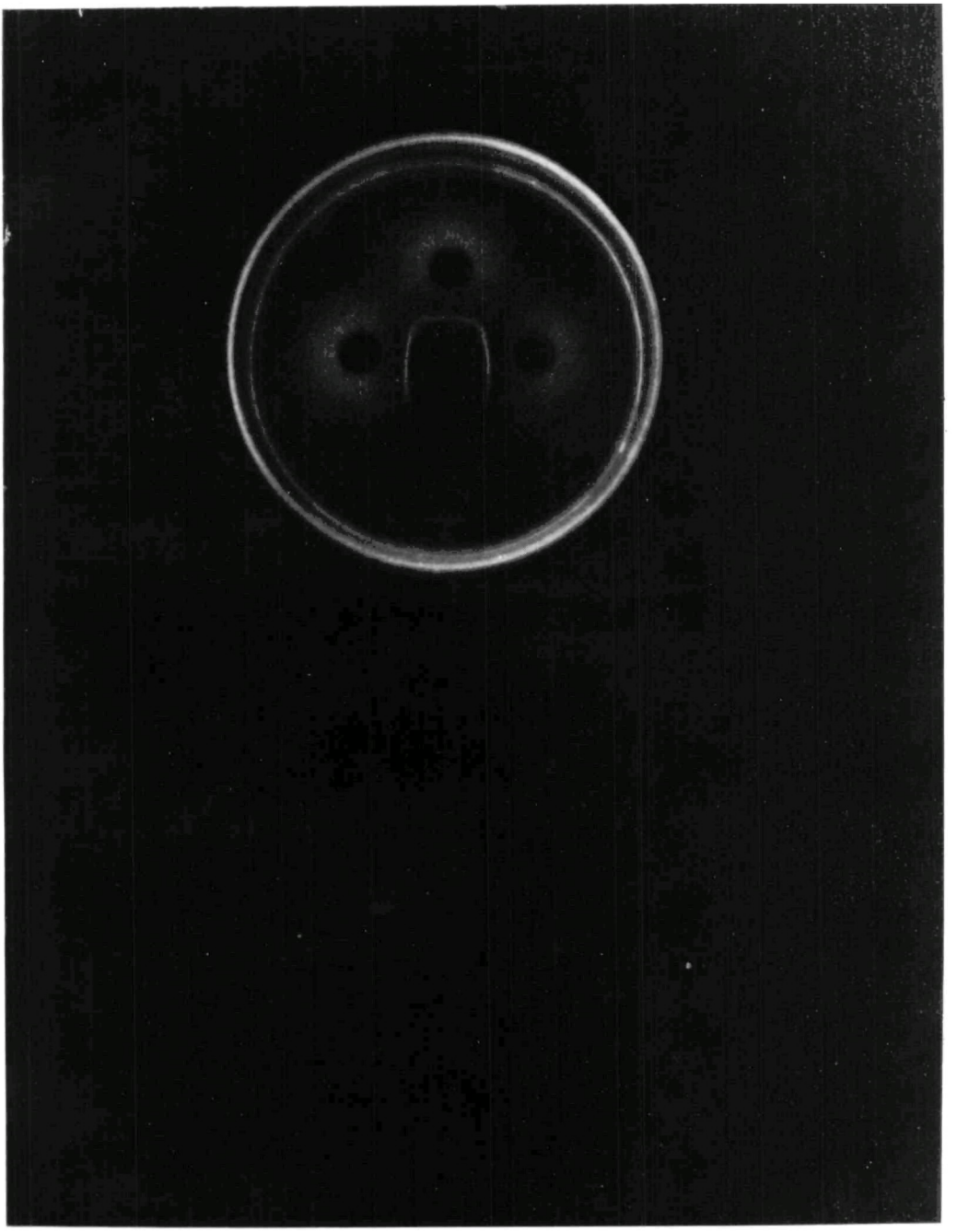

Fig. 1. Ouchterlony analysis of intestinal fluid samples from *P. borchgrevinki* (on the right); *D. mawsoni* (top) and *T. bernacchii* (on the left). The bottom well contained distilled water. Precipitation occurred within 24 h.

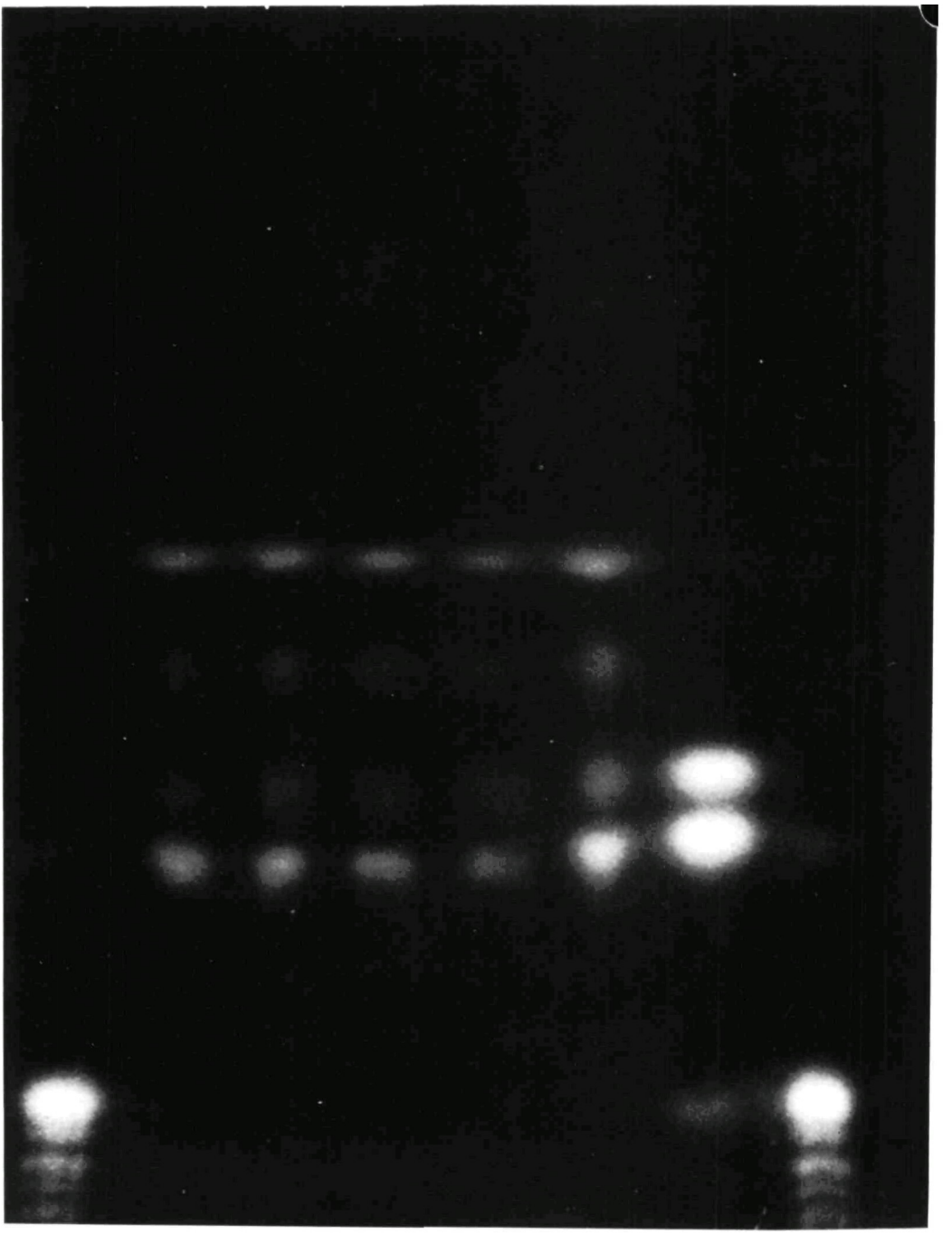

Fig. 2. Polyacrylamide gel comparing intestinal fluid samples from five Antarctic fishes to glycoprotein antifreeze standards isolated from the serum of *D. mawsoni*. From left to right: (1) glycoprotein antifreeze STD (1–5), (2) glycoprotein antifreeze STD (7–8), (3) intestinal fluid from *P. borchgrevinki*, (4) intestinal fluid from *T. bernacchii*, (5) intestinal fluid from *T. centronotus*, (6) intestinal fluid from *T. hansoni*, (7) intestinal fluid from *D. mawsoni*, (8) glycoprotein antifreeze STD (1–5).

Table 1. *Comparison of freezing and melting points of intestinal fluid and serum from five Antarctic fishes and two northern species*

		Intestinal fluid			Serum		
Species	*N*	MP ± S.L.	FP ± S.E.	(MP − FP)	MP ± S.E.	FP ± S.E.	(MP − FP)
Antarctic fishes							
Pagothenia borchgrevinki	5	−1·15 ± 0·05	−2·10 ± 0·09	0·95	−1·02 ± 0·04	−2·74 ± 0·03	1·72
Trematomus bernacchii	5	−1·01 ± 0·10	−1·95 ± 0·08	0·94	−1·04 ± 0·03	−2·55 ± 0·03	1·51
T. centronotus	4	−1·10 ± 0·08	−1·99 ± 0·04	0·89	−1·08 ± 0·05	−2·54 ± 0·04	1·46
T. hansoni	4	−1·08 ± 0·07	−1·94 ± 0·06	0·86	−1·05 ± 0·04	−2·61 ± 0·04	1·56
Dissostichus mawsoni	5	−1·35 ± 0·06	−2·32 ± 0·05	0·97	−1·07 ± 0·06	−2·68 ± 0·05	1·61
Northern fishes							
*Myoxocephalus scorpius**	1	−0·88	−1·26	0·38	−0·69	−1·23	0·54
	2	−0·84	−1·19	0·35	−0·72	−1·38	0·66
Pseudopleuronectes americanus	4	−0·73 ± 0·05	−0·75 ± 0·03	0·02	−0·67 ± 0·30	−0·68 ± 0·04	0·01

* Values from individual animals reported.

from the serum of *D. mawsoni*. The gel shows that two low molecular weight glycoproteins corresponding to the smallest glycoprotein antifreeze within the standard are present in the intestinal fluid. The third band, just behind the solvent front, is sometimes observed in serum samples from Antarctic fish. Whether it is a fragment from one of the larger glycoproteins or an independently synthesized glycoprotein antifreeze is unknown. Although the gel shows only the low molecular weight glycoproteins within the intestinal fluid, the freezing characteristics (i.e. fine spicular crystals, little intermittent growth and large freezing-point depression) indicate that small amounts of large glycoproteins may also be present.

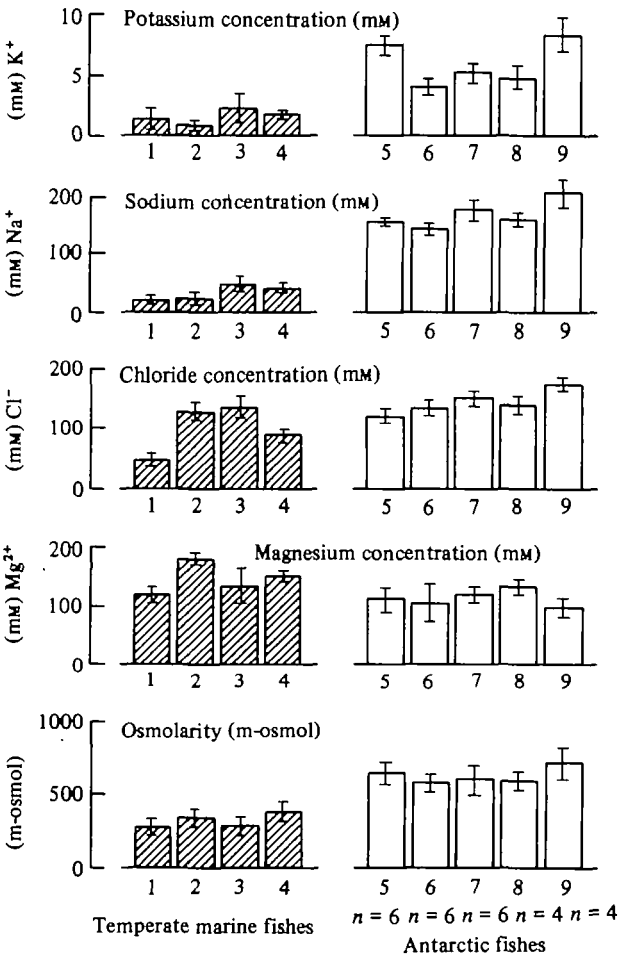

Fig. 3. A comparison of intestinal fluid electrolyte concentration and osmolalities from five Antarctic fishes and four selected temperate marine species. All values are ± standard error. (1) SW rainbow trout (*Salmo gairdneri*), (2) southern flounder (*Paralichthys lethostigma*), (3) cod (*Gadus callarias*), (4) toadfish (*Opsanus tau*), (5) *P. borchgrevinki*, (6) *T. bernacchii*, (7) *T. centronotus*, (8) *T. hansoni*, (9) *D. mawsoni*.

Ion analysis

A comparison of intestinal fluid electrolyte concentrations between Antarctic fishes and data for temperate marine fishes selected from the literature (Hickman, 1968; Shehadeh & Gordon, 1969; Fletcher, 1978; Howe & Gutknecht, 1978) is presented in Fig. 3. Sodium concentrations are, on the average, five times greater in Antarctic fishes (i.e. in the range of 140–205 mM compared with 20–25 mM for the Atlantic species). However, magnesium concentrations are significantly lower in Antarctic fishes ($P < 0.01$) than those for temperate marine fishes shown in Fig. 3. Potassium concentrations are also markedly higher but chloride concentrations were not significantly higher in Antarctic fishes compared to temperate fishes. Measurements of total osmolality by vapour pressure osmometry gave values in the range of 575–712 m-osmol for the intestinal contents of the Antarctic fishes. These values were in close agreement with the osmolalities calculated from melting point data.

Table 2 gives estimates of percent fluid absorption based on measurement of magnesium concentration in Antarctic and selected temperate fishes using the assumptions of Skadhauge (1980). On this basis, 65–80% of ingested sea water is absorbed in the intestines of temperate marine fishes while estimates of fluid absorption in Antarctic fishes range between 40 and 60%.

Table 3 shows a comparison of ion concentrations and freezing–melting point

Table 2. *Estimates of percentage fluid absorption based on Mg^{2+} concentration in samples from Antarctic fish and temperate marine species*

Species	Fluid absorption (%)
Temperate fishes	
S.W. rainbow trout (*Salmo gairdneri*)	80
Southern flounder (*Paralichthys lethostigma*)	76
Atlantic cod (*Gadus callarias*)	68
Toadfish (*Opsanus tau*)	72
Antarctic fishes	
Pagothenia borchgrevinki	55
Trematomus bernachii	48
T. centronotus	57
T. hansoni	60
Dissostichus mawsoni	43

Calculations: percentage fluid absorption $= \left(1 - \frac{\text{S.W.}[Mg^{2+}]}{\text{I.F.}\ [Mg^{2+}]}\right) \times 100.$

Table 3. *Comparison of ion concentrations and antifreeze activity between serum, bile and intestinal fluid from two specimens of* Notothenia rossii

		MP	FP	MP–FP	OSM	Na^+	K^+	Cl^-	Mg^{2+}
Serum	1	−0·77	−1·93	1·16	409	194	4·8	168	2·4
	2	−0·73	−1·24	0·51	396	182	3·6	140	2·7
Intestinal fluid	1	−0·78	−1·24	0·46	421	154	8·6	168	108
	2	−0·76	−1·14	0·38	413	138	9·2	156	98
Bile	1	−0·81	−1·23	0·42	452	130	3·4	54	—
	2	−0·83	−1·19	0·36	419	124	4·1	66	—

results between serum, bile and intestinal fluid from two specimens of *N. rossii*. The differences between melting and freezing points for bile and intestinal fluid are very similar, indicating that the antifreeze concentrations in these fluids are approximately the same.

DISCUSSION

Antarctic fishes represent an isolated group of fishes living in a constant thermal environment. Analysis of plasma ion concentrations from these fishes reveals some of the highest NaCl concentrations found in fishes (Dobbs & DeVries, 1975; O'Grady & DeVries, 1981). To avoid freezing, these fishes maintain a higher serum osmolality than temperate fishes and fortify their body fluids with glycoprotein antifreeze (DeVries & Lin, 1977).

Our results show that intestinal fluid and serum osmolalities in Antarctic fishes are nearly isosmotic. Hence, without antifreeze the intestinal fluid would freeze since ion concentrations are not high enough to depress the freezing point sufficiently below their environmental temperature, and ingestion of ice precludes the possibility of supercooling. Freezing and melting point data, coupled with immunoprecipitation and polyacrylamide gel electrophoresis results on intestinal fluid indicate that glycoprotein antifreeze is present in high concentration within the intestinal fluid of six Antarctic fishes and one Arctic species.

One interesting question that arises from these results concerns the origin of glycoprotein antifreeze found in the intestine. Preliminary observations on the freezing–melting behaviour of bile (MP–FP = 0·52 °C) from *D. mawsoni* indicate that glycoprotein antifreeze is present. Measurements of freezing points and melting points of bile from *N. rossii* (Table 3) also indicate that antifreeze is present. Thus, the simplest way by which antifreeze could enter the intestine is via secretion from the liver into the bile, followed by biliary secretion into the small intestine. Other possibilities could be that the intestine synthesizes these glycoproteins and secretes them into the intestinal lumen; or that low molecular weight glycoproteins from the circulation are transported into the intestinal fluid. Whether or not reabsorption of antifreeze from the intestinal fluid occurs is unknown; this is an important question since excretion of antifreeze would require resynthesis at a cost of 2 ATP per peptide bond. In view of the fact that polar fishes may exhibit only partial compensation to low temperatures (Wohlschlag, 1964; Holeton, 1974) (hence less available energy for resynthesis of antifreeze) resynthesis of antifreeze may represent a considerable energetic demand. The fact that glycoprotein antifreezes are nearly 60% carbohydrate might explain their resistance to proteolytic digestion in the intestine. However, the peptide antifreeze of *M. scorpius*, also present in the intestinal fluid, is known to be trypsin-sensitive. Thus, some degradation of peptide antifreezes may occur.

The osmolality of gut contents from Antarctic fishes studied ranged between 575–712 m-osmol, being hypotonic to sea water, but approximately isotonic to serum. These values are higher than those normally obtained for temperate marine teleosts (Hickman, 1968; Fletcher, 1978; Howe & Gutknecht, 1978). Similar ion analyses typically revealed higher Na^+ and lower Mg^{2+} concentrations for Antarctic fish intestinal fluid than for that of other temperate species. Nevertheless fr

observations of the absolute Na^+ concentrations and the increased Mg^{2+} levels compared with sea water, it is likely that significant NaCl and water absorption occurs across the gut of these species. Using Mg^{2+} as an inert, non-absorbable marker (Skadhauge, 1980), it can be demonstrated that it is concentrated about 2-fold compared to sea water (i.e. 50% absorption of salt and water has occurred). This value is somewhat lower than that for other non-Antarctic species (see Table 2) but still indicates active intestinal fluid transport.

In the context of environmental adaptation, two previously proposed functions have been ascribed to the high plasma Na^+ concentrations in Antarctic fish. An obvious reason is avoidance of freezing (O'Grady & DeVries, 1981) where up to 40% of the total freezing point depression comes from plasma ion levels (0·9–1·0 °C). This also applies to the intestinal fluid in the present context. Another hypothesis proposes that elevated plasma ion concentrations represent a reduced energy requirement for osmoregulation in that the ionic gradient between plasma and the environment is reduced. This hypothesis, however, is difficult to verify, since quantitative estimates of the effect of temperature on ion permeability are not available for polar fishes. Without this information, it is difficult to postulate what adaptive significance a reduced ionic gradient has with respect to reducing the energetic cost of osmoregulation.

The Mg^{2+} analysis presented in this paper represents preliminary data on fluid absorption. Intestinal transport must also be directly determined via studies by Ussing chamber and everted sac techniques. Further, overall osmotic balance ideally requires measurement of gill function as well. Nevertheless, the intestine clearly demonstrated concentrative and hence absorptive capacity, like temperate species.

In conclusion, the present work demonstrates intestinal adaptation to the extreme cold of the Antarctic by two mechanisms: (1) the presence of significant levels of glycoprotein antifreeze, and (2) the maintenance of high ion concentrations in the intestinal fluid as compared to temperate marine teleosts.

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