FREEZING AVOIDANCE AND THE DISTRIBUTION OF ANTIFREEZE GLYCOPEPTIDES IN BODY FLUIDS AND TISSUES OF ANTARCTIC FISH

By JEFFREY A. AHLGREN*, CHI-HING C. CHENG, JOSEPH D. SCHRAG† AND ARTHUR L. DEVRIES
University of Illinois, 524 Burrill Hall, 407 South Goodwin Avenue, Urbana, IL 61801, USA

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Summary
The distribution of antifreeze glycopeptides (AFGPs) in the body fluids and tissues of antarctic notothenioid fish was determined. In Dissostichus mawsoni (Norman), the peritoneal, pericardial and extradural fluid, like the blood, contained all eight AFGPs and in concentrations sufficient to depress freezing points below that of sea water (—1.9°C). Secreted fluids including urine, endolymph and aqueous and vitreous humour either lack all AFGPs or have very low concentrations of only the low molecular weight forms and have freezing points of about —1.0°C, and are therefore undercooled with respect to environmental temperature.

Fluids with high concentrations of AFGPs also contain high levels of proteins similar to plasma proteins. Systemic administration of tritiated AFGPs in the closely related species Trematomus bernacchii (Boulenger) yielded a distribution pattern similar to that of the native AFGPs in D. mawsoni. This suggests passive distribution of AFGPs into the various fluid compartments following secretion from the liver; a pattern typical of secreted blood proteins.

Tissue distribution of AFGPs was determined by comparison with that of the extracellular space marker [14C]polyethylene glycol. AFGPs were found in the interstitial fluid of all body tissues examined except brain tissue. No tissue showed any intracellular accumulation of tritiated AFGPs from the blood.

Introduction
The ability of antarctic notothenioid fishes to survive in the perennially freezing sea water (—1.9°C) of the Antarctic ocean depends on the presence of a series of

* Present address: Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, USA.
† Present address: Department of Biochemistry, University of Arizona, Tucson, AZ 85721, USA.

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antifreeze glycopeptides (AFGPs) synthesized by their livers (O'Grady, Clarke & DeVries, 1982), which effectively lower the freezing point of their blood by a non-colligative mechanism to \(-2.2^\circ\text{C}\) to \(-2.7^\circ\text{C}\), depending on the species (for reviews, see DeVries, 1984, 1988). Antifreeze glycopeptides are present at high levels in the blood of these fishes (Schneppenheim & Theede, 1982; Ahlgren & DeVries, 1984). They have also been shown to be present in the intestinal fluid and bile (O'Grady, Ellory & DeVries, 1982, 1983), and at very low levels in ocular fluids (Turner, Schrag & DeVries, 1985). However, the only known site of intracellular AFGPs is the hepatocytes, the liver being the synthetic organ for AFGPs (O'Grady et al. 1982). Little is known about the amounts or sizes of AFGPs in the other body fluids, or the distribution of AFGPs in the body tissues of these fishes.

In this study, we analysed and quantified the AFGP contents of the major body fluids of the large antarctic cod, \textit{D. mawsoni}, and determined their distribution pattern in relation to that of non-AFGP proteins. In a small related fish, \textit{Trematomus bernacchii}, tritium-labelled AFGPs and the extracellular marker \([^{14}\text{C}]\text{polyethylene glycol (PEG)}\) were injected into the blood and the radioactivities of the two labels in the body fluids and tissues were subsequently assayed, allowing us to follow the movement of AFGPs from the blood to the body fluids and tissues. These experiments enabled us to describe the AFGP contents of various body compartments and to determine whether AFGP distribution is passive like that of other secreted proteins, and solely extracellular, or if there is also intracellular uptake. The results led to a better understanding of the role of AFGPs in freezing avoidance in the fish body fluids and tissues.

**Materials and methods**

**Animals and sampling procedure**

Specimens of \textit{Dissostichus mawsoni} weighing 40–60 kg were captured by cable and hook in 400–500 m of water at \(-1.9^\circ\text{C}\) in McMurdo Sound, Antarctica. Fish were taken from the cable and killed, and the following fluids were sampled immediately: blood, peritoneal fluid, pericardial fluid, extradural fluid, bile, urine, aqueous and vitreous humour, and endolymph. Blood was allowed to clot at \(4^\circ\text{C}\), centrifuged at 5000 g for 10 min, the serum removed and stored at \(-20^\circ\text{C}\) along with the other fluids until analysis.

\textit{Trematomus bernacchii} weighing 100–300 g were captured in traps set on the bottom at about 200 m in McMurdo Sound. Specimens were held alive in flow-through seawater aquaria (mean temperature \(-1.5 \pm 0.2^\circ\text{C}\)) and fed chopped fish weekly until they were used for experimentation.

\textit{Notothenia angustata} (Hutton), a related nototheniid fish but without AFGPs, were captured by trap or set-net at a depth of 3–5 m in the coastal waters of Otago harbour, New Zealand. They weighed 0.5–3.0 kg and were held in an aerated seawater aquarium at 6°C and fed pieces of fish several times weekly.
Freezing point and melting point determinations

Freezing points of the fluids were determined by slowly lowering the temperature of a small volume of the fluid in a 10 µl capillary tube in the presence of a small polycrystalline seed ice crystal (approx. 50 µm in diameter) as previously described (DeVries, 1986). We define the freezing point to be the temperature at which ice propagates from the polycrystalline seed crystal. The melting point is defined as the temperature at which the frozen fluid sample melts as it is slowly rewarmed, and it is the same as the melting temperature of the polycrystalline seed crystal in the fluid. The osmolality of the various fluids was measured with a Wescor model 5100B vapour pressure osmometer. Calculated melting points were then obtained by multiplying the osmolality values by $-0.001858°C \text{mosmol}^{-1} \text{kg}^{-1}$ (Pauling, 1953). The calculated and experimentally determined melting points were found to be identical.

Protein analysis and polyacrylamide gel electrophoresis

Protein concentrations of the fluids were determined by the method of Bradford (1976; Bio-Rad Laboratories protein assay) using bovine serum albumin as a standard. The non-antifreeze proteins were characterized qualitatively by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) with 10% acrylamide gels and staining with Coomassie brilliant blue. Antifreeze glycopeptide contents of the fluids were analysed by resolving the fluorescently labelled (with Fluorescamine; Pierce Chemical Co.) AFGPs on 10% non-denaturing acrylamide gels. The gels of the fluorescently labelled AFGPs were photographed under ultraviolet illumination with Kodak 2145 technical pan film through a 25 Å red filter.

$[^3]H\text{AFGP}$ preparation

Tritium-labelled AFGP was prepared from purified native AFGP as previously described (Lin & DeVries, 1974). To assess if there was autoradiolysis, samples of $[^3]H\text{AFGP}1-5$ and $[^3]H\text{AFGP}8$ of known radioactivity (in disints min$^{-1}$) and native AFGPs were fluorescently labelled and electrophoresed in adjacent lanes on a 10% non-denaturing polyacrylamide gel polymerized with the disulphide crosslinker BAC ($N,N'$-bis-acrylylcystamine; Bio-Rad). Gel slices of 0.5 cm down each radioactive lane were cut out, depolymerized in 0.14 mol l$^{-1}$ dithiothreitol, and the radioactivity (in disints min$^{-1}$) of each slice was assayed by liquid scintillation counting. The mobilities of the tritium-labelled and the native AFGPs were found to be the same and usually 90–95 % of the radioactivity was found to reside in the fluorescent radioactive AFGP bands. Fluorescamine, the fluorescent label, imparts an extra negative charge to the AFGPs and, in the case of AFGP8, which is a small molecule, causes it to migrate slightly faster on the gel than AFGP8 that fails to become labelled during sample preparation. This results in radioactivity showing up in two vertically adjacent regions. Without fluorescamine treatment, the majority of radioactivity of $[^3]H\text{AFGP}8$ appeared in one single location, thus the radiochemical purity of $[^3]H\text{AFGP}8$ was ensured. Stocks of
$[^3]H$AFGPs with small radiolytic products, if any, as determined by the radioactivity in the gel slice at the dye front, were repurified using DEAE ion-exchange chromatography. The specific activities of $[^3]H$AFGPl-5 and $[^3]H$AFGP8 were 195 and 12 $\mu$Ci mg$^{-1}$, respectively.

**Cannulation**

Specimens of *T. bernacchii* and *N. angustata* were anaesthetized with MS-222 (Sigma Chemical Co.) at 1 g/81 of sea water. A cannula of PE 50 tubing 25 cm long was implanted in the caudal artery or vein using the method of Watters & Smith (1973). The cannula was filled with buffered heparinized saline (280 mmol l$^{-1}$ NaCl, 2 mmol l$^{-1}$ KCl, 10 mmol l$^{-1}$ Hepes buffer, pH 7.8, 100 i. u. sodium heparin/ml) and the free end closed by inserting a pin.


*T. bernacchii* were injected via the cannula with either $[^3]H$AFGP1–5 or $[^3]H$AFGP8, along with the inert extracellular space marker $[^14]C$polyethylene glycol (PEG) ($M_r$ 4000, specific activity 0-91 mCi g$^{-1}$; New England Nuclear). *T. bernacchii* was used in place of *D. mawsoni* because much less radiolabel is needed for its small size, and the two fishes are closely related. The single injection was delivered in a volume of 33 $\mu$l 100 g$^{-1}$ fish, and at a dose of 3 $\mu$Ci $[^3]H$AFGP and 0.6 $\mu$Ci $[^14]C$PEG per 100 g of fish. *N. angustata*, received $[^14]C$PEG only, as a control for the use of the label as an extracellular space marker. The cannula was then flushed with a minimal volume of buffered saline. Twelve hours after the injection the fishes were anaesthetized with MS-222 and killed, and samples of blood, peritoneal fluid, pericardial fluid, extradural fluid, urine, bile, and aqueous and vitreous humour were obtained with needle and syringe. All samples were centrifuged for 3 min in a microcentrifuge. Appropriate duplicate samples of serum and supernatants of the other fluids were diluted with distilled water to a total volume of 1 ml, and 9 ml of Aquasol II scintillation fluid (New England Nuclear) was added. Triplicate samples of the following tissues were immediately taken after the fish had been killed and the body fluids sampled: red muscle (pectoral), white muscle (dorsal epaxial), heart muscle, gill filaments, brain, stomach, intestine, head and caudal kidney, and spleen. Tissue samples were placed in tared scintillation vials, and their masses (usually about 50 mg) measured with a Mettler analytical balance. The tissue samples were liquefied with 0.5 ml of 0.2 mol l$^{-1}$ NaOH at 60°C, neutralized with 0.5 ml of 0.2 mol l$^{-1}$ HCl, and then 9 ml of Aquasol II scintillation fluid was added. All samples were assayed for radioactivity (in disintegrations min$^{-1}$) with a Beckman LS6800 liquid scintillation counter (Beckman Instruments, Inc.) programmed for dual-label counting and quench correction.

**Extracellular space volume analysis**

Estimation of extracellular space volumes for the various tissues is based on $[^14]C$PEG levels (disintegrations min$^{-1}$) in the plasma and in the tissue sample. The volumes are expressed as a percentage of tissue wet mass (i.e. $\mu$l 100 mg$^{-1}$ or ml 100 g$^{-1}$),
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and calculated as (disints min⁻¹ of [¹⁴C]PEG 100 mg⁻¹ tissue)/(disints min⁻¹ of [¹⁴C]PEG µl⁻¹ plasma).

*Tissue [³H]AFGPs distribution analysis*

Tissue radioactivity measurements were expressed as the ratio [³H]/[¹⁴C] and were compared with the same ratio in the plasma:

\[ \frac{T/P}{\text{ plasma [³H]}} = \frac{\text{ tissue [³H]} / [¹⁴C]}{\text{ plasma [³H]} / [¹⁴C]} \]

Assuming complete equilibration of the radiolabels within the 12-h period, and no uptake of [³H]AFGPs intracellularly, the tissue ratio should equal the plasma ratio, and thus the T/P value should equal 1. The tissue [³H]AFGP level in this case reflects that in the interstitial fluid of the tissue alone. If [³H]AFGPs were accumulated intracellularly in any tissue, the T/P value would be greater than unity. Student’s t-test was used to determine the statistical significance of the difference of T/P values from unity at the 95 % confidence level.

**Results**

*Polyacrylamide gel electrophoresis*

The electrophoretogram of the fluorescent-labelled fluid samples (Fig. 1) shows that the fluids can be divided into two groups. The first group includes peritoneal, pericardial and extradural fluids, which contain all the different sizes of AFGPs and in comparable amounts of those in the blood, based on the intensity of the fluorescent bands. The second group includes endolymph, urine, and aqueous and vitreous humour, which either lack all AFGPs or contain only small quantities of the low molecular weight AFGPs. The bile shows moderate amounts of only AFGP 7 and AFGP 8.

The denaturing polyacrylamide gels stained with Coomassie blue revealed non-AFGP proteins in these fluids (Fig. 2). AFGPs do not stain with Coomassie blue and therefore do not appear on the gel. The first group of fluids contained proteins that appeared identical in electrophoretic mobilities and staining intensities to those in the blood, suggesting that they are the same proteins. The second group of fluids, as well as bile, all appeared nearly free of non-AFGP proteins, even though five times as much sample was applied.

*Osmalalities, freezing points, and AFGP and protein concentrations of body fluids*

The osmolalities of the body fluids of *D. mawsoni* were only about 60 % of that of sea water (Table 1). Based on the osmolality values, the calculated melting points (MPs) of these fluids were about −1.1 to −1.2°C, substantially higher than that of sea water (−1.9°C). The freezing points (FPs), based on the temperature at which a polycrystalline seed ice crystal grows, fell into two groups. The first group of fluids – serum, pericardial and extradural fluids – had FPs lower than −2.0°C,
Fig. 1. Non-denaturing polyacrylamide gel electrophoresis of fluorescently labelled antifreeze glycopeptides in the body fluids of *Dissostichus mawsoni*. Lane 1, serum; lane 2, peritoneal; lane 3, pericardial; lane 4, extradural; lane 5, bile; lane 6, urine; lane 7, endolymph; lane 8, aqueous humour; lane 9, vitreous humour. The relative molecular masses (×10^3) for the AFGPs are indicated.

i.e. below that of sea water. The second group of fluids – the ocular fluids, endolymph and urine – had FPs of about −1.2°C, i.e. above that of sea water, and were very close to the calculated MPs. From a standard curve of MP − FP versus known concentrations of pure AFGPs, the concentration of AFGPs in each of these fluids was determined. The first group of fluids had high levels, and the second group had very low or negligible levels of AFGPs (Table 1). This is in agreement with the fluorescence intensities of AFGP bands of these fluids in the non-denaturing gel (Fig. 1). The direct correlation between the levels of AFGPs and non-AFGP proteins in these fluids was confirmed with the protein concentrations obtained by the quantitative Bradford protein assay, which excludes the non-Coomassie-staining AFGPs. The fluids with high levels of AFGPs also had high levels of non-AFGP proteins, and those with low levels of AFGPs also had
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Fig. 2. SDS-PAGE of body fluid proteins from Dissostichus mawsoni. Lanes 1, 6 and 12, molecular weight marker proteins (A, bovine serum albumin, $M_r 66000$; B, hen egg albumin, $M_r 45000$; C, lysozyme, $M_r 14400$); lane 2, serum; lane 3, peritoneal; lane 4, pericardial; lane 5, extradural; lane 7, bile; lane 8, urine; lane 9, aqueous humour; lane 10, vitreous humour; lane 11, endolymph. Lanes 2-5, 1 μl of fluid in sample buffer; lanes 7-11, 5 μl of fluid in sample buffer.

low levels of non-AFGP proteins (Table 1). Again, the non-AFGP protein concentrations of these fluids are in agreement with the staining intensities of the protein bands in the denaturing gel. Bile had an intermediate MP – FP difference as well as AFGP and non-AFGP protein levels.

Distribution of $[^3]H$AFGP and $[^{14}C]$PEG in body fluids

Serial blood sampling after injection of the radiolabels showed that the level of radioactivity declined steeply during the first hour due to blood mixing, and declined gradually in the next 11 h due to transport into the various body
Table 1. Osmolality, calculated melting points, measured freezing points, melting point—freezing point differences, antifreeze glycopeptide concentrations and protein concentrations of body fluids of Dissostichus mawsoni.

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Osmolality (mosmol kg⁻¹)</th>
<th>Calculated MP (°C)</th>
<th>Observed FP (°C)</th>
<th>MP—FP (°C)</th>
<th>AFGP (mg/ml)</th>
<th>Protein (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>647 ± 9</td>
<td>-1.24</td>
<td>-2.46 ± 0.1</td>
<td>1.22</td>
<td>32.0</td>
<td>47.9 ± 3.9</td>
</tr>
<tr>
<td>Peritoneal</td>
<td>614 ± 5</td>
<td>-1.17</td>
<td>-2.22 ± 0.1</td>
<td>1.05</td>
<td>24.0</td>
<td>31.5 ± 6.0</td>
</tr>
<tr>
<td>Extradural</td>
<td>598 ± 5</td>
<td>-1.16</td>
<td>-1.74 ± 0.1</td>
<td>0.98</td>
<td>20.0</td>
<td>23.3 ± 1.8</td>
</tr>
<tr>
<td>Bile</td>
<td>605 ± 10</td>
<td>-1.11</td>
<td>-1.26 ± 0.2</td>
<td>0.88</td>
<td>7.5</td>
<td>5.1 ± 1.7</td>
</tr>
<tr>
<td>Aqueous humour</td>
<td>583 ± 11</td>
<td>-1.10</td>
<td>-1.20 ± 0.1</td>
<td>0.15</td>
<td>2.0</td>
<td>0.35 ± 0.12</td>
</tr>
<tr>
<td>Vitreous humour</td>
<td>578 ± 8</td>
<td>-1.18</td>
<td>-1.23 ± 0.1</td>
<td>0.13</td>
<td>1.0</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>Urine</td>
<td>616 ± 11</td>
<td>-1.13</td>
<td>-1.16 ± 0.1</td>
<td>0.05</td>
<td>0.35 ± 0.12</td>
<td>0.18 ± 0.11</td>
</tr>
<tr>
<td>Endolymph</td>
<td>591 ± 10</td>
<td>-1.13</td>
<td>-1.16 ± 0.1</td>
<td>0.03</td>
<td>0.22 ± 0.02</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Sea water</td>
<td>995</td>
<td>-1.90</td>
<td>-1.90</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± S.E.M., N = 5. Melting points (MP) were calculated from osmolality value: MP = osmolality x (-0.001858°C mosmol⁻¹ kg⁻¹). Freezing points (FP) were observed in the presence of a polycrystalline seed ice crystal. AFGP concentration was determined from a standard curve of MP—FP versus known concentrations of pure antifreeze.
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compartments. Twelve hours after injection, whole-body extracellular space was calculated by the method of Holmes & Donaldson (1969), i.e. the quotient of the amount of $[^{14}\text{C}]$PEG (disint min$^{-1}$) injected and the extrapolated zero-time equilibrium concentration of $[^{14}\text{C}]$PEG (disint min$^{-1}$ ml$^{-1}$) in the blood. The extracellular volume was estimated to be 15.5% of body mass. This value is within the range of reported values of 12.5–16.6% of body mass for other marine teleosts when measured with sucrose (Thorson, 1961). This indicates that 12 h is probably sufficient for equilibration of the radiolabel and for distribution analysis. For distribution analysis, the level of radioactivity of $[^{3}\text{H}]$AFGP 1–5, $[^{14}\text{C}]$PEG and $[^{3}\text{H}]$AFGP 8 in each fluid is expressed as a percentage of its respective level in the plasma. Since $[^{14}\text{C}]$PEG distribution is independent of that of a second radiolabel, its percentage plasma values for each fluid from fish injected with $[^{3}\text{H}]$AFGP 1–5 and from those with $[^{3}\text{H}]$AFGP 8 were pooled and averaged (Fig. 3). There were various degrees of movements of AFGPs and PEG into the peritoneal, pericardial and extradural fluids within the 12-h period after injection, as indicated by the different but significant levels of $[^{3}\text{H}]$ and $[^{14}\text{C}]$ radioactivity. The remaining fluids showed very low levels of either label at 12 h. $[^{3}\text{H}]$ and $[^{14}\text{C}]$ levels in the urine, aqueous and vitreous humour were less than 3% of the plasma levels, indicating

Fig. 3. Radioactivity in the body fluids of *Trematomus bernacchii* relative to plasma value (100%) 12 h after injection. Mean values + s.e.m., $N = 5$ for $[^{3}\text{H}]$AFGP 1–5 (○); $N = 13$ for $[^{14}\text{C}]$PEG (●); $N = 8$ for $[^{3}\text{H}]$AFGP 8 (●).
Table 2. Extracellular volume (percentage of wet mass) of tissues determined by \[^{14}\text{C}]\text{PEG} distribution

<table>
<thead>
<tr>
<th>Tissue</th>
<th>\text{T. bernacchii}</th>
<th>\text{N. angustata}</th>
<th>\text{S. gairdneri}</th>
</tr>
</thead>
<tbody>
<tr>
<td>White muscle</td>
<td>4-2 ± 0-5</td>
<td>3-8 ± 1-5</td>
<td>3-8 ± 0-5</td>
</tr>
<tr>
<td>Red muscle</td>
<td>11-5 ± 1-0</td>
<td>8-0 ± 2-2</td>
<td></td>
</tr>
<tr>
<td>Heart muscle</td>
<td>18-6 ± 2-0</td>
<td>20-0 ± 2-5</td>
<td>19-3 ± 1-7</td>
</tr>
<tr>
<td>Gill</td>
<td>18-0 ± 1-2</td>
<td>21-0 ± 6-3</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>11-3 ± 0-6</td>
<td>12-4 ± 2-1</td>
<td>23-4 ± 1-6</td>
</tr>
<tr>
<td>Brain</td>
<td>1-7 ± 0-3</td>
<td>3-1 ± 1-8</td>
<td>6-3 ± 0-7</td>
</tr>
<tr>
<td>Intestine</td>
<td>22-0 ± 1-0</td>
<td>23-2 ± 1-2</td>
<td>42-0 ± 1-2</td>
</tr>
<tr>
<td>Stomach</td>
<td>42-8 ± 2-3</td>
<td>33-0 ± 1-8</td>
<td>42-0 ± 1-2</td>
</tr>
<tr>
<td>Caudal kidney</td>
<td>18-8 ± 1-7</td>
<td>24-0 ± 1-9</td>
<td></td>
</tr>
<tr>
<td>Head kidney</td>
<td>23-8 ± 1-4</td>
<td>28-6 ± 3-0</td>
<td></td>
</tr>
</tbody>
</table>

\[^{a,b,c,d}^{ \text{Houston} \& \text{Mearow (1979): a subdorsal epaxial white muscle; b estimated from Figs 1, 2; c values from text; d tissue listed as gut.}}\]

highly restricted movements, if any, of AFGPs and PEG into these fluid compartments.

**Extracellular space analysis for body tissues**

The extracellular space volumes for various tissues expressed as a percentage of wet mass (v/w) from \text{T. bernacchii} and \text{N. angustata}, a related notothenioid fish but without AFGPs, were compared with values from the freshwater teleost \text{S. gairdneri} obtained in a similar way using the same radiolabel, \[^{14}\text{C}]\text{PEG} (M, 4000) (\text{Houston} \& \text{Mearow, 1979}) (Table 2). The extracellular space values are very similar for \text{T. bernacchii} and \text{N. angustata}, and in most cases in good agreement with those of \text{S. gairdneri}, indicating that the 12-h PEG equilibration period is sufficient for the calculation of extracellular space. This is important since the determination of any intracellular accumulation of AFGPs utilizes the level of extracellular \[^{14}\text{C}]\text{PEG} radioactivity as a reference, as described in the following section. It should be noted that the extracellular space volumes for brain tissues really reflect the vascular volumes, since the blood-brain barrier excludes the passage of macromolecules into the interstitium of the brain tissue. The measured PEG space of the brain was 1-7\% of brain wet mass, which corresponds to the brain vascular space of other marine teleosts (Cserr, Fenstermacher \& Rall, 1978).

[^{3}\text{H}]\text{AFGP} and \[^{14}\text{C}]\text{PEG} distribution in the body tissues

Table 3 lists the T/P values for the tissues. Except for the brain, all the tissues examined in the \[^{3}\text{H}]\text{AFGP} 1–5 distribution experiments had a T/P value significantly lower than 1. The fact that the T/P values were not greater than 1 indicates that \[^{3}\text{H}]\text{AFGP} 1–5 did not enter into the intracellular space, and was therefore distributed only extracellularly. The fact that the T/P values were lower than 1 suggests that \[^{3}\text{H}]\text{AFGP} 1–5 did not partition equally between the plasma and the interstitial space. This uneven distribution can be explained if there is
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Table 3. T/P values – ratio of tissue $[^3H]/[^14C]$ radioactivity to plasma $[^3H]/[^14C]$ radioactivity 12 h after injection of $[^3H]$AFGP and $[^14C]$PEG into the blood of Trematomus bernacchii

<table>
<thead>
<tr>
<th>Tissue</th>
<th>T/P (AFGP 1–5)</th>
<th>T/P (AFGP 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$(M_r 34,000–10,500)$</td>
<td>$(M_r 2,600)$</td>
</tr>
<tr>
<td>White muscle</td>
<td>0.63 ± 0.05*</td>
<td>1.41 ± 0.18</td>
</tr>
<tr>
<td>Red muscle</td>
<td>0.54 ± 0.02*</td>
<td>1.24 ± 0.21</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.71 ± 0.08*</td>
<td>0.89 ± 0.08</td>
</tr>
<tr>
<td>Heart</td>
<td>0.67 ± 0.05*</td>
<td>1.13 ± 0.10</td>
</tr>
<tr>
<td>Gill</td>
<td>0.81 ± 0.03*</td>
<td>1.01 ± 0.06</td>
</tr>
<tr>
<td>Brain</td>
<td>0.96 ± 0.07</td>
<td>1.00 ± 0.09</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.59 ± 0.02*</td>
<td>1.01 ± 0.11</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.83 ± 0.04*</td>
<td>0.96 ± 0.05</td>
</tr>
<tr>
<td>Bladder</td>
<td>0.62 ± 0.03*</td>
<td>0.83 ± 0.09</td>
</tr>
<tr>
<td>Head kidney</td>
<td>0.80 ± 0.06*</td>
<td>1.09 ± 0.07</td>
</tr>
<tr>
<td>Caudal kidney</td>
<td>0.89 ± 0.03*</td>
<td>0.95 ± 0.07</td>
</tr>
<tr>
<td>Skin</td>
<td>0.53 ± 0.03*</td>
<td>0.95 ± 0.12</td>
</tr>
</tbody>
</table>

* Significantly different from 1:00, $P < 0.05$.

$N = 8$ for AFGP 1–5; $N = 9$ for AFGP 8.

Restricted movement of these high molecular weight forms ($M_r 10,500–33,500$) relative to PEG ($M_r 4,000$) through the capillary walls into the interstitial fluids over the 12-h period.

The T/P values for the distribution of the low molecular weight form $[^3H]$AFGP 8 in all the tissues examined were not statistically different from 1. $[^3H]$AFGP 8 therefore distributes extracellularly and partitions evenly between the vascular and interstitial spaces.

The T/P value of 1 for both $[^3H]$AFGP 1–5 and $[^3H]$AFGP 8 in the special case of brain tissue, however, means that the distribution of the radiolabel is restricted to the vascular space because of the blood–brain barrier, rather than being equally partitioned between the vascular and interstitial space.

Discussion

The blood osmolality of antarctic notothenioid fishes (approx. 600 mosmol kg$^{-1}$) is higher than that of most marine fishes (approx. 350 mosmol kg$^{-1}$) (Prosser, 1973), but still significantly lower than the osmolality of sea water (approx. 1000 mosmol kg$^{-1}$). The freezing point of the blood of notothenioid fishes, calculated from blood osmolality, is approximately $-1.2^\circ C$, which is $0.7^\circ C$ higher than the freezing point of the sea water ($-1.9^\circ C$) they inhabit. The antifreeze glycopeptides depress the freezing point further to about $-2.2^\circ C$ to prevent freezing.

The results here in addition to a few others (Dobbs & DeVries, 1975; O'Grady et al. 1982, 1983; Turner et al. 1985) showed that the other body fluids in the
notothenioid fishes are also hypsomotic to sea water. Freezing of these fluids must
therefore be prevented by other factors in addition to the colligative lowering of
their freezing points. It has been shown that the intestinal fluid contains substantial
amounts, and bile moderate amounts, of the low molecular weight AFGPs
(O’Grady et al. 1982, 1983). Similar results were obtained for bile in this study.

The first group of fluids in this study, the peritoneal fluid, pericardial fluid and
extradural fluid, resemble blood in that they showed large melting point — freezing
point differences and contained all of the sizes of AFGPs and in quantities
sufficient to lower their freezing points to below that of sea water. Concomitantly
large were the quantities of non-AFGP proteins which are identical to plasma
proteins as judged by SDS-gel electrophoresis.

The second group of fluids, urine, endolymph, and aqueous and vitreous
humour, showed very small melting point — freezing point differences and
contained only minute amounts of the low molecular weight AFGPs, insufficient
to depress their freezing points significantly below their colligative melting points.
Their freezing points were therefore above that of sea water. The non-AFGP
protein contents of these fluids were also very low.

The reason for the lack of AFGPs and plasma proteins in the second group of
fluids is that they are formed by secretion across epithelia consisting of cells joined
to each other by tight junctions. Urine formation in these notothenioid fishes
involves a secretory process instead of filtration, because their kidneys are
composed entirely of agglomerular nephrons (Dobbs, Lin & DeVries, 1974;
Eastman & DeVries, 1986). An analogous situation occurs in the formation of the
ocular fluids, where a tight blood/ocular fluid barrier exists in vertebrates (Cole,
1974). These fluids are therefore essentially salt solutions.

The lack of AFGPs in these secreted fluids means that they are undercooled, by
about 0-7–0-8°C, and that this amount of undercooling must be stable over the
lifetime of these fishes since they do not freeze despite their ice-laden habitat. In
other words, ice propagation into these fluids to cause nucleation must in some
way be prevented. In the case of urine, ice entry through the only potential route,
the urethra, is prevented by a closed urinary sphincter lined with much mucus. In
the case of ocular fluids, both the clear head skin and the spectacle between it and
the underlying cornea have been shown to be effective barriers to ice propagation
(Turner et al. 1985). The transparent head skin lacks significant vascularization,
but the spectacle between the head skin and cornea contains fluid fortified with
antifreeze which will inhibit ice entry even if the head skin is disrupted. Entry of
ice into the endolymph is unlikely because of its deep-seated location and because
it is surrounded by tissues that are fortified with antifreeze.

The direct correlation of high levels of AFGPs and plasma proteins in the first
group of fluids suggests that AFGPs are passively distributed in a similar manner
to and along with many other plasma proteins by diffusion down a concentration
gradient from blood to these fluids. This is supported by the similar distribution
patterns of the native AFGPs and [3H]AFGPs after introduction of the radiolabe
into the circulatory system.
[14C]PEG is the extracellular marker of choice because studies have shown it to be the one most effectively confined to the extracellular space amongst the common markers used (Schmidt-Nielsen, Renfro & Benos, 1972; Hickman, Newcomb & Kinter, 1972; Beyenbach & Kirschner, 1976; Houston & Mearow, 1979). Its distribution in the extracellular space is passive and should reflect that of any extracellular macromolecule of a similar size. The presence of significant levels of [3H]AFGPs and [14C]PEG in the first group of fluids indicates that these fluid compartments are accessible to the antifreeze molecules and blood proteins. It appears that although 12 h may be sufficient for equilibration of [14C]PEG in most of the extracellular space, it may not be sufficient for complete equilibration in some fluid compartments, as indicated by the fact that the [14C]PEG in pericardial, peritoneal and extradural fluids did not reach plasma level within the 12-h period. The pericardial and peritoneal fluids are separated from the rest of the extracellular compartments by a continuous epithelium which conceivably may restrict the movement of the macromolecules and prolong equilibration time. Nevertheless, the results clearly indicate that blood-borne AFGPs and PEG do enter these fluid compartments, most probably in a passive way. The absence of significant levels of [3H]AFGP and [14C]PEG in urine, aqueous and vitreous humour is again due to the secretory nature of these fluids. Endolymph was not sampled for radioactivity because of its small volume, but its levels are not expected to be any different from those of the other secreted fluids.

Bile has been shown to contain low molecular weight AFGPs and is the source of antifreeze in the intestinal fluid through evacuation from the gall bladder into the intestine (O’Grady et al. 1983). Our recent preliminary studies show that AFGPs are translocated from blood to bile passively through the tight junctions to the bile canaliculi (A. L. DeVries, unpublished results). Some serum proteins in mammals are thought to enter bile in small amounts by diffusion through these tight junctions (Mullock & Hinton, 1981). Similar passive transport may account for the presence of moderate amounts of AFGPs and blood proteins in the bile. The presence of only low levels of [3H]AFGP and [14C]PEG in bile is probably due to the short equilibration time.

The blood-borne AFGPs also partition into the interstitial spaces of all the tissues examined except the brain, but do not accumulate intracellularly. The combined effect of the fluid and tissue distribution is that nearly the entire extracellular phase of the body of the fish is fortified with AFGPs. The body tissues therefore avoid freezing by virtue of AFGPs in their interstitium. The latter is particularly important in the case of the undercooled secreted fluids which lack AFGPs. One method to prevent ice propagation into these undercooled fluids is for the tissues surrounding them to be fortified with AFGPs. In the case of the brain, it is protected by the large amounts of AFGPs in the extradural fluid.

The distribution of AFGPs of antarctic fish appears similar to the distribution of the peptide antifreeze of the north Atlantic species Pseudopleuronectes americanus. Studies by Petzel (1982) on the distribution of [125I]-labelled peptide antifreeze (M, 3300) showed that the peptide partitioned into the extracellular...
space but not into the cytoplasm of the kidney, muscle, gut, bladder and liver tissues. Although all tissues examined, except the brain, showed distribution of AFGPs into their interstitial fluid, no intracellular uptake of AFGPs from the blood was detected. In both fishes, the possibility of intracellular synthesis in some body tissues is not precluded. However, it would appear energetically costly to have several major sites of synthesis other than the liver, especially when AFGPs secreted by the liver can be efficiently distributed to most body fluids and interstitial spaces of body tissues to confer protection from freezing.

An interesting problem is the purpose of this intricate distribution of AFGPs within the fish when there are physical barriers such as integument, head skin over eyes, and a tight urinary sphincter to prevent entry of ice from without. It has been observed that these fishes often sustain abrasions that expose their muscles to the sea water, which would constitute a route for inward ice propagation, and also that they drink sea water laden with ice crystals, which conceivably could become a source of internal ice growth. Consequently, there would be the need for body fluids and tissues to be protected by AFGPs.

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**References**


Antifreeze glycopeptides in antarctic fish


