Freezing Resistance in Some Antarctic Fishes

Abstract. *Measurements of serum freezing points in three Antarctic marine fishes indicated that they do not freeze in the −1.87°C seawater because their blood is isosmotic to seawater. Concentrations of sodium chloride, urea, and free amino acids in the serum accounted for only half of the freezing-point depression of the serum. A protein containing carbohydrate was isolated which accounted for 30 percent of the freezing-point depression of the serum.*

Water temperatures in McMurdo Sound (77°51′S,166°38′E), Antarctica, average −1.87°C throughout the year (*1*). During the winter months (July through December) water temperatures below freezing result in the formation of ice platelets which aggregate as a layer beneath the solid sea ice and as masses of anchor ice on the ocean floor. The platelet layer, being a loose matrix of ice platelets, has many tunnels and holes into which the semipelagic fish *Trematomus borchgrevinki* often swims. Both the benthic fishes *Trematomus bernacchii* and *Trematomus hansoni* often rest on masses of anchor ice, which forms only to depths of 30 m (Fig. 1) (*2*). Adaptational mechanisms for survival under these freezing conditions are ecologically and physiologically significant. During the winter of 1965 we studied freezing resistance in these three fishes.

The *T. borchgrevinki* were caught by hook and line through holes in the ice; *T. bernacchii* and *T. hansoni* were caught with baited wire traps set on the bottom. Immediately after the fish were caught, blood from the heart was collected by hypodermic syringe and allowed to clot at 0°C for 6 hours. Freezing points of serum were measured with a Fiske osmometer. Concentration of chloride in the serum was determined according to the method of Keys (*3*). Filtrates prepared by the addition of one volume of serum to 20 volumes of 10 percent trichloroacetic acid (TCA) were analyzed for nonprotein nitrogen by the micro-Kjeldahl method (*4*), free amino acids by the ninhydrin method (*4*), urea by the ammonia diffusion method (*4*), protein soluble in TCA (*5*), and total carbohydrate by the anthrone method (*6*).

A test for the significance of a difference between two means (*7*) indicates that the mean freezing point of serum of *T. borchgrevinki* is different from those of both *T. bernacchii* and *T. hansoni* at $P = 0.01$ (Table 1). The mean freezing points of serum from the shallow-water and deepwater populations of *T. bernacchii* are different, as are those for the two populations of *T. hansoni*. There is no apparent explanation for the higher freezing points in the deepwater *T. bernacchii* and *T. hansoni*, although, if the effect of pressure at 300 m is considered, the serum freezing points of the deepwater populations at such a depth would be nearly the same as those of the shallow-water populations at 20 m.

The blood of the shallow-water fishes is hyperosmotic to seawater (Table 1). In freshwater fishes, hyperosmotic fluids lead to an influx of water. The fact that large kidneys and urinary bladders occur in Antarctic fishes suggests that a similar phenomenon occurs with these organisms, though perhaps to a lesser extent than with freshwater fishes. In *T. borchgrevinki* the kidneys are approximately three times larger than those of *Embiotoca jacksoni*, a temperate marine black perch of comparable weight. The bladders of *T. borchgrevinki* contained 1 to 3 ml of urine, which has a mean freezing point of −1.11°C ± 0.01° (seven specimens).

In most temperate marine fishes sodium chloride accounts for 80 to 90 percent of the freezing-point depression of serum (*8*); however, in Antarctic fishes less than half the freezing-point depression of serum could be attributed to sodium chloride. Similarly, Gordon *et al.* (*9*) found that sodium chloride accounts for only slightly more than half of the freezing-point depression of serum in the shallow-water sculpins inhabiting the near-freezing water of the fjords of northern Labrador.

In an attempt to find what other serum constituents were responsible for the freezing-point depression of serum, we investigated the fraction of serum soluble in TCA. A protein containing carbohydrate (glycoprotein) was isolated which was responsible for 30 percent of the freezing-point depression of serum in *T. borchgrevinki*. This compound was isolated from the supernatant of serum from which most of the proteins were removed by heat precipitation (5 minutes at 95°C) and centrifugation. Freezing points of such supernatants were always within 0.05°C of the freezing point of whole serum. The glycoprotein was separated from other compounds present in the supernatant by chromatography on a Sephadex G-200 column (2.5 by 80 cm) equilibrated with 0.05*M* sodium phosphate (*p*H 7.0) in 0.1*M* sodium chloride. The freezing-point-depressant activity of the glycoprotein appeared in the first of two carbohydrate-protein peaks; when the first peak was again chromatographed on Sephadex, the freezing-point-depressant

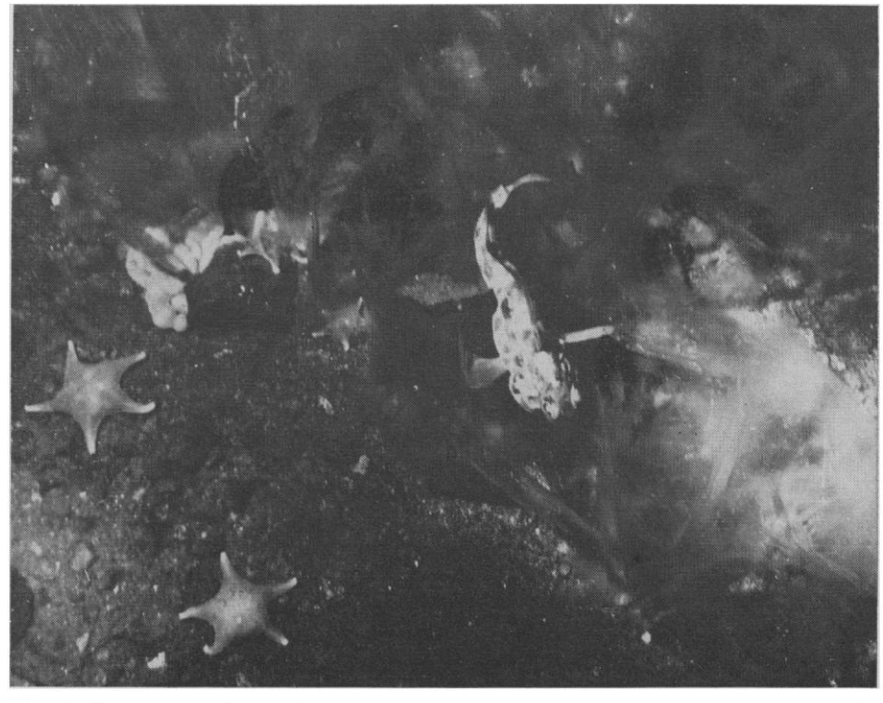


Fig. 1. *Trematomus bernacchii* resting on mass of anchor ice in 20 m of water in McMurdo Sound, Antarctica. [Photo by Paul Dayton]

Table 1. Data on blood serum of three Antarctic fishes expressed as mean ± standard error. Numbers of samples are shown in parentheses.

Fish	Serum freezing point (°C)	Serum chloride (mmole/liter)	Percent freezing-point depression due to NaCl*	Concentration (mg/100 ml)†				
				Nonprotein nitrogen	Urea nitrogen	α-Amino nitrogen	Carbohydrate	Protein
				Shallow water (20 m)‡				
T. borchgrevinki	−2.07 ± 0.014(28)	235 ± 1.6(26)	39	504 ± 8.5(26)	61 ± 1.3(6)	20 ± 4.0(5)	831 ± 35.0(10)	1890 ± 67.8(10)
T. hansoni	−2.01 ± 0.019(24)	259 ± 4.3(24)	44	480 ± 12.0(14)		12 ± 1.2(3)	838 ± 41.5(7)	1830 ± 93.0(8)
T. bernacchii	−1.98 ± 0.007(25)	254 ± 1.9(25)	44	481 ± 10.3(25)	52 ± 2.0(22)		880 ± 30.6(8)	1900 ± 83.4(8)
				Deep water (300 m)				
T. hansoni	−1.92 ± 0.015(13)	258 ± 3.3(13)	46	375 ± 17.9(13)			594 ± 43.1(5)	1620 ± 65.8(7)
T. bernacchii	−1.87 ± 0.008(14)	254 ± 4.4(14)	46	343 ± 24.7(14)			587 ± 24.5(6)	1120 ± 91.5(9)

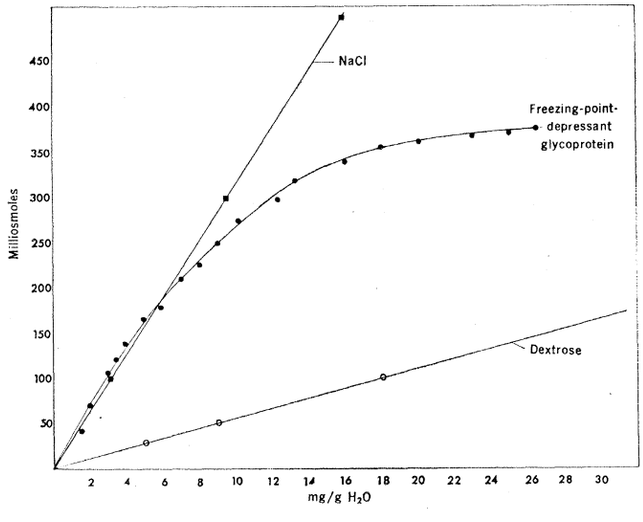
* Sodium chloride estimated on basis of chloride measurements. † Determinations made on 10 percent TCA filtrates. ‡ Mean freezing point of shallow water was −1.90°C.

activity was eluted as a single peak. The purity of this material was checked by electrophoresis on acrylamide gel slabs at *p*H 8.6 (*10*). The gel was stained for carbohydrate with alpha-naphthol and concentrated sulfuric acid. Three closely grouped bands that migrated toward the anode were observed. No bands were observed when the gel was stained for protein with nigrosine or aniline blue black. More gentle methods of isolation from whole serum by means of filtration on Sephadex and ion-exchange chromatography on carboxymethylcellulose and diethylaminoethylcellulose also yielded glycoprotein which migrated as three bands when subjected to electrophoresis. The freezing-point-depressant activity of the material in each of the three bands was nearly the same.

Freezing points of aqueous solutions of the glycoprotein, sodium chloride, and dextrose were measured at several concentrations (Fig. 2). At low concentrations the glycoprotein is as efficient as sodium chloride in depressing the freezing point of water. At higher concentrations (above 6 mg per gram of H_2O) the glycoprotein is less efficient than sodium chloride.

The molecular weight of the glycoprotein (30 g/mole) was calculated from the freezing point data (Fig. 2) at a concentration of 6 mg per gram of H_2O. This finding is inconsistent with estimates of molecular weight obtained by other methods. The glycoprotein did not pass through 8/32-inch dialysis tubing (Visking) in 24 hours. Ultrafiltration experiments indicated that the glycoprotein slowly passes through an Amnicon UM 50 membrane (molecular weight cutoff at 50,000 *g* but does not pass through a UM 2 membrane (molecular weight cutoff at 10,000 *g*). Determination of molecular weight by means of an analytical Sephadex G-200 column (2.5 by 50 cm) gave a value of 78,000 *g*; this estimate may be high in view of the anomalous behavior of proteins containing carbohydrate on Sephadex (*11*). A molecular weight of 26,000 *g* was calculated from measurements of osmotic pressure at concentrations of 0.05, 0.075, 0.1, and 0.25 percent (by weight) made with a recording membrane osmometer (Melabs). The sedimentation of the glycoprotein was measured in an analytical ultracentrifuge (Beckman model E) at 59,780 rev/min at a concentration of 0.4 percent (by weight) in 0.1*M* sodium chloride. An observed sedimentation coefficient of 1.4*S* was calculated by the method of Schachman (*12*); no molecular weight was calculated because the diffusion coefficient and partial specific volume were not known. The physical data suggest that the glycoprotein is a relatively large molecule, and that its depression of the freezing point of water (Fig. 2) is much greater than expected for the number of particles in solution.

In order to determine how the freezing-point-depressant activity of the glycoprotein is related to its size and structure, we digested it with Pronase;



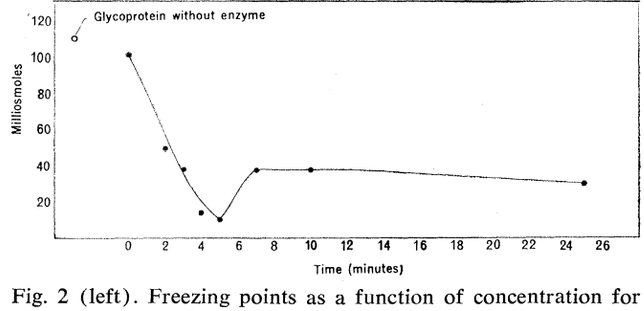


Fig. 2 (left). Freezing points as a function of concentration for aqueous solutions of the freezing-point-depressant glycoprotein, sodium chloride, and dextrose. The freezing point of a 100-milliosmolar solution is −0.186°C. Fig. 3 (above). Pronase digestion of the freezing-point-depressant glycoprotein from serum of *T. borchgrevinki* (8.1 mg of the glycoprotein in 2.2 ml of 0.05*M* phosphate buffer containing 0.25 mg of Pronase, incubated at 39°C). Enzyme was added at 0 minutes. The contribution of phosphate buffer to the freezing point of the glycoprotein digest was subtracted.

the freezing point was measured as digestion proceeded (Fig. 3). Apparent cleavage of peptide bonds resulted in nearly complete inactivation of the glycoprotein, which was accompanied by the appearance of dialyzable protein containing carbohydrate. Digestion with subtilisin (strain BPN′) (Nagarse) also inactivated the glycoprotein. Thus the freezing-point-depressant activity is an intrinsic property of the glycoprotein molecule and is not due to the presence of small amounts of substances of low molecular weight which could not be separated from the glycoprotein by dialysis. If the freezing-point-depressant activity were due to the presence of retained ions, no loss in activity would be observed upon degradation of the glycoprotein molecule.

Chemical analysis by the method of Lowry *et al.* (*5*), in which bovine serum albumin was used as a standard, indicated that the glycoprotein is 50 percent protein (by weight). An estimation of the total carbohydrate content by the phenol-sulfuric acid method (*13*), in which dextrose was used as a standard, indicated that the glycoprotein is 34 percent carbohydrate (by weight). Amino acid analysis after hydrolysis for 24 hours in 6*N* HCl at 110°C indicated that alanine and threonine were the only amino acids present. Galactosamine accounted for approximately 20 percent of the hydrolyzed glycoprotein. The glycoprotein is soluble in most protein-precipitating agents, such as 10 percent TCA, zinc hydroxide, and 3 percent tungstic acid solution; however, it is insoluble in acetone and 95 percent ethanol.

The concentrations of nonprotein nitrogen, TCA-soluble protein, and carbohydrate in the serums of the Antarctic fishes are four to five times higher than those in most temperate marine fishes. The solubility in TCA and the chemical composition of the glycoprotein suggest that these high concentrations are largely due to the presence of the glycoprotein. In the deepwater populations of *T. bernacchii* and *T. hansoni* where the serum freezing points are 0.1°C higher than those of the shallow-water populations, the concentrations of nonprotein nitrogen, TCA-soluble protein, and carbohydrate are correspondingly lower. This most likely is an indication of lower amounts of the glycoprotein in the deepwater populations. A similar correlation between higher serum freezing points and lower serum concentrations of nonprotein nitrogen and TCA-soluble constituents was also found in another benthic Antarctic fish, *Notothenia larseni*, which has a serum freezing point of −1.51°C (*14*).

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15. Supported in part by NSF grants GA–45 and GA–661, administered by Drs. D. E. Wohlschlag and A. C. Giese, respectively. Part of this work was carried out in the Department of Food Science and Technology, College of Agricultural and Environmental Sciences, University of California, Davis, by A.L.D. with support of NSF grant GA–991 administered by Dr. R. E. Feeney. We thank A. C. Giese and R. E. Feeney for reading the manuscript, and members of the U.S. Navy Task Force 43 and the U.S. Antarctic Research Program for their support. A.L.D. was supported by an NIH postdoctoral fellowship [1-F2-AM-38,350-01 (h)] while in the terminal phases of this study.

23 August 1968; revised 4 November 1968

Do Trehalose and Trehalase Function in Renal Glucose Transport?

Abstract. *Maximum catalytic capacity for renal trehalase varied from 20 micromoles (of glucose produced per minute per gram of fresh tissue) for the armadillo down to zero for the rat, the cat, most turtles, fishes, and all birds and snakes investigated. Labeled trehalose could not be demonstrated in the kidney of the rabbit and the rat after injection with labeled glucose. These data do not support the hypothesis that trehalase and trehalose function in the transport of glucose through the kidney.*

After discovering trehalase in the blood serum of several mammals and turtles (*1*), we determined the trehalase activity in the kidneys of a representative number of mammals, birds, reptiles, amphibians, and fishes. Tissues were removed as quickly as possible and used fresh or after storage at −20°C. Absence of trehalase was not due to lability during storage. In rats, no activity was found, even when the assay was started within minutes after death. Human kidneys were removed 4 to 10 hours after death. Tissues were homogenized, diluted with water to a concentration of 100 mg/ml, and centrifuged at 1500 rev/min for 10 minutes. The supernatants contained 0.6 to 1.0 percent protein (60 to 100 mg per gram, fresh weight) and were assayed as described for serum (*1*); all reactions followed zero-order kinetics. By incubating homogenates with low activities up to 48 hours, we determined the sensitivity to be in excess of 0.005 μmole/min.

The rabbit kidney cortex contains all the enzymes for the synthesis of trehalose, and trehalase is localized within the cortical tubules of the human, the mouse, and the rabbit (*2*). Sacktor proposed that, in mammals, the reaction

2 Glucose → trehalose → 2 glucose

functions in a mechanism for tubular reabsorption of glucose; rapid synthesis of trehalose would provide a steep glucose concentration gradient between the glomerular filtrate and the tubular cell (*2*). A similar hypothesis has been used by Treherne to rationalize the rapid synthesis of blood trehalose from absorbed monosaccharides in insects (*3*).

The fasting normal human with a blood glucose concentration of 0.09 percent reabsorbs about 150 mg/min, which is 0.5 mg per gram of kidney or 0.8 mg per gram of cortex. The kidney would then have to produce from trehalose 2.8 μmole of glucose per minute per gram (4 μmole per gram of cortex); when the maximum tubular reabsorptive capacity is used, after a large sugar