THE FREEZING RESISTANCE OF ANTARCTIC FISH: II. THE FREEZING POINTS OF BODY FLUIDS

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ABSTRACT. Methods for measuring freezing points are critically reviewed with particular emphasis on their application to biological fluids. A modified version of the Ramsay and Brown (1955) freezing-point method is described by which the freezing points of small samples (4×10^{-2} mm.[3]) may be measured to $\pm$ 0·02° C. The freezing points of serum, aqueous humour, urine, cerebrospinal fluid, bile and tissue fluid of nine Antarctic teleosts were found to lie between −0·65° and −1·34° C. No marked differences ($\not>$32 per cent) were found in the freezing points of different body fluids in the same fish. Measured and calculated values of serum freezing-points were in reasonable agreement. Aqueous humour and urine were, on average, hypo-osmotic to serum in all cases, whereas bile (of all species except *Notothenia neglecta*), cerebrospinal fluid and tissue fluid were hyper-osmotic to serum. Diuresis occurred in fish that were used for freezing experiments and may have been the result of handling the fish. No obvious correlation was found between the amounts of urine produced and the urine freezing-points. The freezing-point measurements confirmed the results of chemical analysis and the principal conclusion was that the fish are supercooled at low environmental temperatures.

THE body fluid freezing points of polar fish (in conjunction with body temperatures) show whether or not the fish are supercooled when they encounter low sea temperatures. In addition, freezing-point measurements, together with the results of chemical analysis, reveal the response of the fish to change of temperature, that is whether changes in salt concentration occur or whether specific "antifreeze" substances are released into the body fluids.

The body fluid freezing points of some Antarctic teleosts are presented in this paper. The body fluids were found to be hypo-osmotic to sea-water in all cases, and so the fish are likely to be supercooled at low environmental temperatures. Some of the data have been published in a preliminary account of this work (Smith, 1970), and the chemical composition of the serum has been ascertained (Smith, 1972).

MEASUREMENT OF FREEZING-POINT DEPRESSION—REVIEW OF METHODS

The freezing point of a solution is defined as the temperature at which the vapour pressure of the solution and the vapour pressure of pure frozen solvent are equal. In very dilute solutions the freezing-point depression is proportional to the mole fraction of solute (or solute ions) and can be calculated. In more concentrated solutions, deviation from ideal behaviour occurs and only approximate freezing-point depressions can be calculated from solute concentrations. In the case of animal body fluids, discrepancies between measured and calculated freezing-point depressions are small if the effects of all the osmotically important constituents are included in the calculations. Thus, in addition to being an important physical parameter, the freezing point of a solution can indicate omissions and errors in the chemical analysis of the solution.

The freezing point of pure water does not vary with the amount of ice present and is thus relatively easy to measure. The freezing point of a solution, however, is less easy to measure, since it changes with the concentration changes that occur as freezing progresses. Methods for measuring the freezing points of solutions must either take these concentration changes into account or avoid them. In the former category, analysis of solutions in equilibrium with ice over a range of temperatures can relate composition to freezing-point depression (Adams, 1915; Scatchard and others, 1932) but this method is not suitable for biological fluids. In the latter category, methods which avoid complications due to concentration changes on freezing are eminently suitable for biological fluids and are of three main types. These are: (i) methods in which the melting point rather than the freezing point of the solution is measured; (ii) methods in which the temperature changes that occur on freezing a supercooled solution are measured; and (iii) methods in which the vapour pressures of solutions and standards are compared and related to freezing-point depressions. These three types of method will now be discussed in more detail.

Measurement of melting points

Melting points are generally measured by freezing a sample of solution, warming it slowly, and measuring the temperature at which the last minute ice crystal just melts or is in equilibrium with the solution. This temperature is, by definition, the freezing point (to within the limits of experimental error) so the method does not require calibration with an external standard and, in addition, it avoids the possibility of errors due to supercooling. To obtain accurate results, an efficient controlled-temperature bath is required and the rate of temperature rise must be low enough to maintain thermal equilibrium between the bath liquid, the sample and the thermometer. The samples are usually contained in capillary tubes and, since the conductivities of ice and glass are low, small samples and thin-walled capillary tubes are essential if thermal equilibrium between bath and sample is to be maintained at any but extremely low heating rates. In addition, small samples are necessary so that concentration gradients that form on thawing the frozen sample are quickly eliminated by diffusion. The requirements for accuracy are met, with varying degrees of success, by the experimental procedures that have been devised.

The procedure, in its original form, lacked efficient temperature control, though results accurate to $\pm 0 \cdot 01^\circ$ C were possible if sufficient care was taken (Drucker and Schreiner, 1913; Fritsche, 1917; Mosebach, 1940). Ramsay (1949) described an improved technique with which results accurate to $\pm 0 \cdot 006^\circ$ C could be obtained using 10^{-3}–10^{-4} mm.^{3} samples. A modified version of Ramsay's apparatus was described by Kinne (1954). Hargitay and others (1951) devised a similar type of apparatus, later modified by Zwicky (1954), capable of measuring freezing points to within $0 \cdot 002^\circ$ C. A novel feature of this apparatus was the use of a polarizing microscope to simplify the observation of ice crystals due to their birefringence. Jones (1941) and Gross (1954) employed a different approach to the measurement of freezing points. In this method, frozen samples and standards were warmed at a constant rate and the times at which they melted were recorded. Freezing points were obtained from a calibration curve of freezing point against melting time and an accuracy of $\pm 0 \cdot 02^\circ$ C using 0·01 ml. samples was claimed (Gross, 1954). This method does not require a thermometer which is, however, a questionable advantage.

Scholander and others (1957) devised a method based on that of Pounder and Masson (1934) in which extremely small samples are not necessary since thermal and concentration gradients in the thawing samples are prevented by stirring. In this method, Scholander and others measured the incipient freezing temperature (the temperature at which the last small ice crystal starts to grow) as well as the melting point (the equilibrium temperature of the last small ice crystal in the solution), and gave the average of these two results as the freezing point. It is, however, unnecessary to include the incipient freezing temperature in the measurement since by definition the melting point is equivalent to the freezing point. In an ideal solution, the incipient freezing temperature would equal the melting or freezing point, but in practice it is lower because of the effects of solutes on the kinetics of ice propagation (Lusena, 1955). The results given by Scholander and others are thus slightly lower than the true freezing points.

A frequently used method of measuring melting points of solutions is that described by Ramsay and Brown (1955). This method combines the better features of previous methods while avoiding most of their drawbacks. Accurate temperature control is combined with ease of operation and the standard deviation of the results is $\pm 0 \cdot 003^\circ$ C when sample volumes of 10^{-3}–10^{-4} mm.^{3} are used. The lower limit of sample volume is about 10^{-5} mm.^{3}; with smaller volumes the effect of slight contamination of the samples by the capillary tubes becomes obvious. The minute sample volumes required by this method and its high accuracy are obvious advantages in biological studies.

Freezing of supercooled solutions

Measurement of the temperature rise that occurs on freezing a supercooled solution provides a means of determining freezing points. If no heat loss occurs to the surroundings, the latent heat released on freezing raises the temperature of the solution to its freezing point, which can then be measured directly. This situation is attained in practice if minimal supercooling is employed and if the cooling-bath temperature is only slightly lower than the expected freezing

point. If these conditions are not complied with, the final temperature of the frozen solution is lower than its true freezing point and it is necessary to calibrate the apparatus with solutions of known freezing point.

The Beckmann apparatus (Glasstone, 1960) enabled the first accurate freezing-point determinations to be made and has been used to determine the freezing points of blood and tissue (Atkins, 1909; Collip, 1920*a*, *b*). A refined form of Beckmann apparatus, the Hortvet cryoscope, is now used to measure the freezing point of milk to $\pm 0 \cdot 002°$ C (Sato and others, 1957; Shipe, 1960). Johlin (1931) devised a modified version of the Beckmann apparatus capable of measuring the freezing point of 1 ml. of solution to $\pm 0 \cdot 005°$ C of the true value. Errors are minimized in Johlin's method by using a small sample volume, limiting super-cooling to a few hundredths of a degree, keeping the cooling-bath temperature not more than 0·4° C below the expected freezing point, and freezing the supercooled sample with a metal ring cooled in dry ice and frosted in moist air. Many replicate measurements on the same sample are possible since the ice on the frosted metal ring causes very little dilution. A similar but less precise method was described by Olmstead and Roth (1957). Stadie and Sunderman (1931) used a somewhat different approach. In their method the solution is pre-cooled to its approximate freezing point and transferred to a chamber containing ice. Several consecutive transfers of aliquots of the sample eliminate dilution errors and a final temperature stable to 0·001° C is taken as the end point. This method has the disadvantage of requiring 10 ml. or more of solution and a single determination takes about 45 min.

The use of thermocouples or thermistors in greatly modified types of Beckmann apparatus enables freezing points of small samples to be determined quickly and easily (Bishop, 1923; Zeffert and Hormats, 1949; Crawford and Nicosia, 1952; Wesson, 1952; Conway and McCormack, 1953; Bowman and others, 1954; Hervey, 1955; Lucarain, 1962; Levine and Musallam, 1964), and has resulted in the development of various commercial thermistor cryoscopes or "osmometers". Such osmometers consist of a cooling bath in which a small sample of solution (0·2–2 ml.) is supercooled and then frozen by vigorous stirring or a sudden shock. The resulting temperature rise is measured by a thermistor located in the frozen sample. Some of the latent heat released on freezing is lost to the surroundings and so the temperature does not rise to the true freezing point of the sample. It is therefore necessary to calibrate the instrument with solutions of known freezing point. It is essential that the kinetics and thermodynamics of the operating cycle are the same for both samples and standards, otherwise errors are liable to occur. The rate of ice formation on freezing the supercooled sample is of particular importance in this respect. In the case of sodium chloride solutions (which are commonly used as standards), ice propagation rapidly leads to the formation of a layer of ice crystals round the thermistor. The thermal insulation of this layer retains latent heat within the frozen sample, and the temperature at the centre of the sample where the thermistor is located reaches a maximum which remains constant for up to several minutes. This maximum temperature is measured by the thermistor. If the sample contains solutes that retard the propagation velocity of ice more effectively than sodium chloride (Lusena, 1955) or if ice formation does not reach completion, more latent heat will be lost to the surroundings than in the case of a standard sodium chloride solution and an anomalously low freezing point will be recorded. This effect will be more marked if crystallization is initiated at only one point in the solution rather than at many points throughout the solution. A "seeding" method such as vigorous stirring that produces many crystallization nuclei is therefore to be preferred. If differences are found in the temperature–time curves of the operating cycle for samples and standards, this is an indication that the kinetics and thermodynamics are not the same in each case. A slow temperature rise indicates slow ice propagation or insufficient seeding, and a poorly defined maximum in the temperature–time curve may result from these causes or from incomplete freezing. Most biological fluids, however, are sufficiently similar to sodium chloride solutions in their properties for the method to be applied to them; in general, the speed and accuracy of freezing-point measurements on small samples recommend the method for routine biological or clinical applications.

Vapour-pressure measurements

The vapour pressures and freezing-point depressions of solutions are related and the

relationship may be used to determine freezing points. Such determinations are most easily done by isopiestic methods, i.e. measurement of the changes that occur on equilibration of the sample and a standard in a closed system. Equilibrium under these conditions is reached when enough solvent to equalize the vapour pressures of the two solutions has distilled from the solution of higher vapour pressure and condensed in the solution of lower vapour pressure. Changes in the volumes and temperatures of the solutions occur as a result, and determination of either of these changes gives a measure of the vapour-pressure differential between sample and standard, which can then be related to the difference in their freezing points. In practice, the changes are generally less than those that are theoretically possible, so calibration with standards of known freezing point is necessary. Volume changes are easily determined by measuring changes in length of columns of solutions contained in uniform bore capillary tubes. This is the principle of Barger's (1904) method which was used originally for molecular-weight determination. In this method, alternating drops of sample and standard solutions are put in capillary tubes with air spaces between the drops. The more concentrated drops increase, and the less concentrated ones decrease in length. The changes can then be related to the particular colligative property that is being measured. This method has been modified by various workers and applied to biological fluids (White, 1928; Ursprung and Blum, 1930; Keosian, 1938) but great care is essential if accurate results are required and a single determination may take 24 hr. or more.

Temperature changes that occur on equilibrating dissimilar solutions are utilized more easily than volume changes in freezing-point determination. The original vapour-pressure osmometer based on this principle was devised by Hill (1930). In Hill's method, the sample and standard solutions are applied to filter papers on the opposite sides of a thermopile and allowed to equilibrate at constant temperature in a chamber which is lined with filter paper moistened with standard solution. The temperature changes that occur in the sample and standard activate the thermopile and the resultant e.m.f. is registered by a galvanometer. When equilibrium is reached, the galvanometer deflection is noted and the process is repeated with the positions of the sample and standard reversed. The freezing-point depression (or any other colligative property) is calculated from the average of the two deflections by reference to the deflections produced by standard solutions used to calibrate the instrument. The method is accurate, reasonably fast and requires only small sample volumes. In addition, measurements may be made at any required temperature. Modifications of the apparatus were described by Margaria (1930), Grollman (1931) and Culbert (1935).

A considerable improvement was effected by Baldes (1934), who replaced the thermopile by a single thermocouple, thus enabling smaller volumes to be used and increasing the speed and accuracy of the method. A later modification was the use of several thermocouples situated in the same equilibrium chamber (Johnson and Baldes, 1938; Baldes and Johnson, 1939). Roepke and Baldes (1938) made a critical study of the errors and accuracy of the method and Baldes (1939) has discussed its theory. Further modifications were introduced by Kinsey (1950) and Goyan and Reck (1955).

Brady and others (1951) used thermistors instead of thermocouples in their version of the apparatus and Müller and Stolten (1953) made a detailed study of the use of thermistors in the vapour-pressure osmometer. Instruments employing thermistors were described by Iyengar (1954) and Neumayer (1959). Higuchi and others (1959) introduced various refinements and devised an instrument for use in thermodynamic studies. Such refinements, however, are not necessary for freezing-point measurements. The use of thermistors eventually led to the production of a commercial vapour-pressure osmometer, and a useful account of the theory and use of such an instrument has been published (Ehrmantraut, 1966). Freezing points of biological fluids may be measured quickly and easily by vapour-pressure osmometry on samples as small as 15 μl. (Ehrmantraut, 1966), but care must be taken to ensure that factors such as thermal decomposition, chemical reaction or volatilization of solutes do not invalidate the results (Wolf and Prentiss, 1968).

Freezing-point measurement—conclusions

Many of the older methods of determining the freezing points of biological fluids have been superseded by thermistor cryoscopy or vapour-pressure osmometry using commercially

available instruments. The melting-point method is still used, however, particularly in cases where only very small sample volumes are available and a commercial version of the apparatus has recently been marketed. Melting-point determination is the most basic of the three methods since it alone requires no calibration with standard solutions. The errors that may be encountered in thermistor cryoscopy or vapour-pressure osmometry have been discussed. If doubtful results are obtained by either of these methods, they may be checked by melting-point determination. In conclusion, it may be said that, providing their limitations are understood and allowed for, all three methods may be usefully employed in biological studies.

Apparatus and Methods

Collection of samples

Fish were caught at Signy Island in the South Orkney Islands, at South Georgia and at Montevideo. Details of the various species and localities have been given previously and the collection of serum has been described (Smith, 1972). Aqueous humour, urine, cerebrospinal fluid, bile and tissue samples as well as serum were collected from some fish. Aqueous humour was removed by syringe from the anterior chamber of each eye. Urine and bile were extracted by syringe from the urinary bladder and gall bladder after opening the fish. Cerebrospinal fluid was obtained by cutting off the top of the skull and removing the fluid by syringe. Tissue samples consisted of skinned fillets which were quickly rinsed in fresh water, blotted dry and sealed in polythene bags. Body fluid and tissue samples were stored deep-frozen. On standing for a short while at room temperature, a small crystalline precipitate formed in most urine samples, showing that supersaturation of the urine occurred *in vivo*. The precipitates were not analysed but probably consisted of $MgPO_4.3H_2O$ (Grafflin and Ennis, 1934; Pitts, 1934). The urine freezing-point depressions reported here were not corrected to allow for the precipitates, so they are slightly lower than the true values. Fluid was obtained from tissue samples by compressing small pieces of tissue in a 10 ml. syringe. A cotton wool pad in the syringe served to filter the extracted fluid. The freezing points of tissue fluids were measured immediately after extraction.

Measurement of freezing point

Serum freezing points were measured at Signy Island using a modified Ramsay and Brown (1955) apparatus. Other body fluids and the tissue samples were deep-frozen and transported to the United Kingdom where their freezing points were measured by thermistor cryoscopy using an Advanced Instruments Inc. osmometer (Model 67–31LAS).

The modified Ramsay and Brown apparatus resembled the original version of the apparatus in the basic design of the controlled-temperature bath but differed from it in the following details. Heating or cooling of the bath was effected by the addition of warm (room temperature) or cold (-15° C) aqueous alcohol (50 per cent). The capillary tubes containing the frozen samples were not mounted in protecting tubes but were clipped to an adjustable holder and aligned vertically alongside the thermometer with the samples on a level with the top of the mercury column. The thermometer was graduated in $0\cdot1^\circ$ C intervals and its zero was checked *in situ*. Observation windows were cut in the insulation at the front and rear of the bath; the sample tubes and thermometer were illuminated through the rear window and observed through the front window with a $\times10$ binocular microscope. Condensation of moisture on the windows was eliminated by fitting perspex covers to the outside of the insulation and putting some silica gel in the cavities thus formed. Observation of ice crystals in the samples was enhanced by viewing them through crossed polaroids, troublesome reflections were avoided by painting the inside of the bath black and an eyepiece graticule in the binocular microscope enabled the thermometer to be read to $0\cdot01^\circ$ C. Serum samples (approximately 4×10^{-2} mm.^{3}) were mounted between columns of liquid paraffin in capillary tubes (outer diameter $0\cdot3$–$0\cdot4$ mm.). Two or three samples of the same serum were put in the same tube, the ends of which were sealed with paraffin wax. A novel feature of the apparatus was that several capillary tubes could be clipped to the holder at once, allowing simultaneous observation of up to a dozen separate samples and reducing the average time for a single determination to a few minutes. The melting point of a sample was measured by allowing the bath to warm

slowly until most of the ice in the sample had melted, and then controlling the temperature so that the last-minute ice crystal remained in equilibrium with the thawed sample for 1–2 min. This temperature was taken as the melting point. When dealing with a number of samples at the same time, the determinations were carried out in a step-wise manner beginning with the sample with the lowest freezing point.

The accuracy of the modified Ramsay and Brown apparatus was checked by carrying out 12 determinations each on de-ionized water and six standard sodium chloride solutions, covering a freezing-point range of 0·00° to −1·00° C. The means of the measured values agreed with the true values to within 0·01° C and the standard deviations varied between 0·01° and 0·02° C. The results obtained with serum are probably slightly less accurate, i.e. within 0·02° C of the true values, than those obtained with the standards since fewer replicate determinations were made on each sample. In addition, the ice crystals in frozen serum did not glow so brightly under polarized light as those in the standards, making it less easy to observe the end point in the case of serum. The accuracy obtained in practice is in reasonable agreement with that predicted by theory, since it was calculated that enough heat to melt a typical frozen sample would pass through the wall of the capillary tube in 1–2 min. with a temperature differential of 0·01° C between sample and bath.

The Advanced Instruments osmometer was used to measure the freezing points of aqueous humour, urine, cerebrospinal fluid, bile, tissue fluid and some serum samples. With this instrument, results reproducible to 0·002° C were obtained with sample volumes of 0·2 ml.

Degradation of Deep-frozen Samples during Storage and its Effect on Freezing-point Determination

Deep-frozen serum and cerebrospinal fluid samples were found to be unstable on storage, particularly if they were thawed and re-frozen at intervals. Over a period of months, cloudy precipitates and, in some cases, semi-solid aggregates formed in the samples. Aqueous humour, urine and bile samples were unaffected. It is not known whether tissue samples underwent degradation during storage, since fluid was not extracted from them on collection but only after storage.

The opacity of stored deep-frozen serum and cerebrospinal fluid samples precluded measurement of their freezing points by the modified Ramsay and Brown method. Degraded samples gave anomalous results with the Advanced Instruments osmometer. The effect of degradation on the temperature–time cycle of the instrument is shown diagrammatically in Fig. 1 and is probably due to loss of latent heat resulting from slow or incomplete crystallization of the degraded samples on freezing. No such problem was encountered with aqueous humour, urine or bile samples. With tissue fluids, the plateau portions of the temperature–time curves were shorter than with standard solutions (Fig. 1), so it is possible that the measured freezing points were slightly lower than their true values.

A comparison of serum freezing points measured by the modified Ramsay and Brown method and by thermistor cryoscopy could not be obtained for all of the species of fish studied, since, by the time the Advanced Instruments osmometer became available, all the samples had been deep-frozen for several months and many were badly degraded. *Chaenocephalus aceratus* serum, however, suffered little obvious degradation and some South Georgia *Notothenia rossii* sera were only slightly degraded. The freezing points of these sera were therefore measured with the Advanced Instruments osmometer and compared with the freezing points previously obtained by the modified Ramsay and Brown method. The results are included in Table I. Good agreement was obtained with the *C. aceratus* sera, showing that the two methods gave equally valid results. The agreement was less good with the *N. rossii* sera, but this was probably due to the degradation of the samples. It is reasonable to conclude that the two methods of freezing-point determination are essentially comparable.

Results

Serum freezing points, both measured and calculated, are given in Table I. The calculated values were derived from the mean concentrations of sodium, potassium, chloride, reducing sugar and protein in the sera (Smith, 1972). The contributions of sodium, potassium, chloride

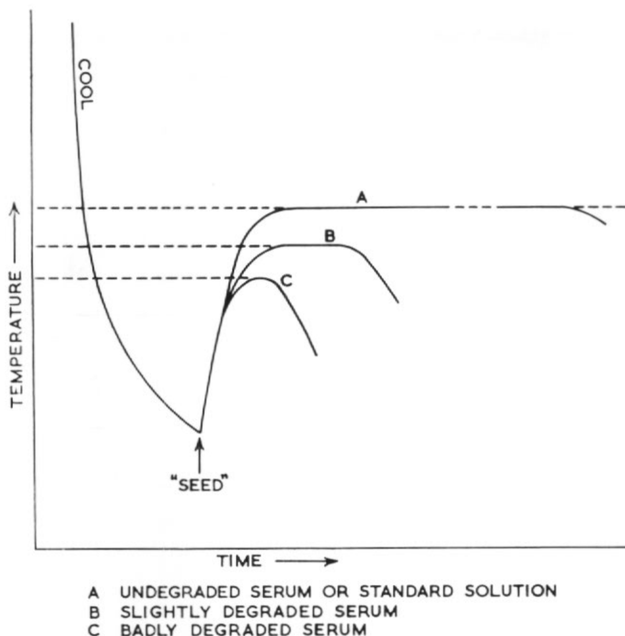

Fig. 1. Effect of serum degradation on temperature–time cycle of thermistor cryoscope.

and reducing sugar (as glucose) towards the freezing-point depressions were calculated from values recorded in the literature. The small contributions of protein towards the freezing-point depressions were estimated from a calibration curve prepared with bovine albumin solutions whose freezing points were measured by thermistor cryoscopy. In cases where the chloride concentrations were not measured, they were assumed to be equal to the sodium concentrations for the purposes of the calculation, and in the single case (*Parachaenichthys charcoti*) where the reducing sugar concentration was not measured it was assumed to be 5 mg./ml.

The freezing points of body fluids other than serum are given in Table II.

The amounts of urine (as percentages of body weight) found in some species of fish are given in Table III. "Frozen" fish are fish that were experimentally supercooled and then seeded with ice, as a result of which they died.

Discussion

In general, the results show that the body fluids of the Antarctic fish studied at Signy Island and South Georgia are hypo-osmotic to sea-water and so the fish are liable to be supercooled and possibly frozen in winter. This is conclusively shown for *Notothenia neglecta* and *N. rossii* which were studied in winter as well as in summer, and for *Trematomus newnesi* which was studied only in winter. It is assumed that the conclusion applies equally to the species that were studied only in summer.

The serum freezing points of the Antarctic fish lay mainly within the freezing-point range of non-Antarctic teleost serum (Parry, 1966). The agreement between the measured and calculated values indicated that no major osmotically active serum constituent had been omitted from the analytical study (Smith, 1972). The inorganic serum constituents (sodium, potassium and chloride) accounted for much of the observed freezing-point depression in all cases, their calculated contributions ranging from 72 to 95 per cent of the total. In temperate marine fish the situation is similar, the inorganic (sodium chloride) contribution to the freezing-point depression being 80–90 per cent (Dill, 1964).

The measured serum freezing points of *Notothenia neglecta* and *N. rossii* were slightly lower in winter than in summer, and differences of approximately the same magnitude were found

Table I. Comparison of the measured and calculated values of teleost serum freezing points

(Measured freezing points are given as means. Figures in parentheses are the standard deviation followed by the number of specimens in the sample. Calculated freezing points were obtained from the mean results of chemical analysis. Freezing points given to two decimal places were measured by the modified Ramsay and Brown method, those given to three places were measured with the Advanced Instruments osmometer.)

Species	*Location*	*Season*	*Freezing points* (°C) *Measured*	*Calculated*	*Difference* (°C)	*Percentage of measured freezing-point depression due to* Na, K *and* Cl
Notothenia neglecta	Signy Island	Summer	−0·93 (0·04, 30)	−0·84	0·09	81
		Winter	−1·07 (0·04, 27)	−0·97	0·10	78
	South Georgia	Summer	−0·86 (0·07, 5)	−0·77	0·09	80
N. rossii	Signy Island	Summer	−0·88 (0·03, 4)	−0·73	0·15	77
		Winter	−1·07 (−1·06, −1·07)*	−0·88	0·19	74
	South Georgia	Summer	−0·77 (0·03, 30) −0·982 (0·047, 7)	−0·74	0·03	91
N. gibberifrons	Signy Island	Summer	−1·01 (0·06, 9)	−0·95	0·06	82
Trematomus bernacchii	Signy Island	Summer	−1·34 (—, 1)	−1·07	0·27	74
T. newnesi	Signy Island	Winter	−1·01 (0·05, 14)	−0·80	0·21	72
Chaenocephalus aceratus	Signy Island	Summer	−1·10 (0·12, 13) −1·115 (0·151, 13)	−0·97	0·13	84
	South Georgia	Summer	−0·81 (−0·79, −0·83)* −0·816 (−0·822, −0·809)*	−0·72	0·09	87
Parachaenichthys georgianus	South Georgia	Summer	−0·93 (—, 1)	−0·79	0·14	78
Muraenolepis microps	South Georgia	Summer	−0·65 (—, 1)	−0·65	0·00	95

* Individual results for two fish given in parentheses.

TABLE II. SUMMER VALUES OF BODY-FLUID FREEZING POINTS OF SIGNY ISLAND FISH

(Results are given as means; figures in parentheses are the standard deviation followed by the number of specimens in the sample.)

Species	Freezing points (°C)				
	Aqueous humour	*Urine*	*Cerebrospinal fluid*	*Bile*	*Tissue fluid*
Notothenia neglecta	−0·886 (0·050, 47)	−0·855 (0·071, 38)	—	−1·012 (0·039, 4)	−1·032 (0·058, 15)
N. rossii	−0·739 (0·497, 22)	−0·768 (0·087, 16)	−0·932 (1·056, 17)	−0·772 (0·082, 22)	−0·975 (0·074, 10)
N. gibberifrons	−0·845 (0·059, 27)	−0·885 (0·079, 12)	−1·085 (0·022, 3)	−0·977 (0·191, 3)	−1·034 (0·105, 10)
Chaenocephalus aceratus	−1·002 (0·106, 9)	−0·970 (0·117, 8)	—	−1·038 (0·103, 6)	−1·149 (0·102, 10)
Parachaenichthys charcoti	−0·934 (—, 1)	−0·887 (—, 1)	—	—	—

TABLE III. URINE CONTENT OF SIGNY ISLAND FISH IN SUMMER

(Results are given as percentage urine (urine volume, ml. × 100/body weight, g.). "Frozen" fish are fish that were supercooled and killed by contact with ice.)

Species		*Percentage urine*	*Number of samples*	*Standard deviations*
Notothenia neglecta	Controls	0·09	12	0·09
	"Frozen"	0·19	13	0·24
N. rossii	Controls	0·13	11	0·18
	"Frozen"	0·20	13	0·22
N. gibberifrons	Controls	0·07	21	0·12
	"Frozen"	0·12	5	0·13
Chaenocephalus aceratus		0·09	11	0·09
Parachaenichthys charcoti		1·53	1	—

in the calculated winter and summer values. The inorganic serum constituents were responsible for over 50 per cent of the observed increases, although the total contribution of the inorganic constituents to the freezing-point depression was 3 per cent less in winter than in summer for both *N. neglecta* and *N. rossii*. A lowered serum freezing point at low environmental temperature combined with a reduced inorganic contribution to the freezing-point depression has been observed in other species of fish (Pearcy, 1961; Leivestad, 1965; Woodhead and Woodhead, 1965). In some Arctic fish the adaptation is taken to extremes and the serum freezing-point depression increases by about 75 per cent in winter, this increase being largely due to unidentified organic "antifreezes" (Scholander and others, 1957; Gordon and others, 1962). A glycoprotein thought to act as an "antifreeze" has been isolated from the serum of certain *Trematomus* species at McMurdo Sound, where the sea temperature is −1·87° C all year round (DeVries and Wohlschlag, 1969; DeVries, 1970). At low concentrations, the

glycoprotein was, despite its high molecular weight, comparable to sodium chloride as a freezing-point depressant when solutions were compared by thermistor cryoscopy using a Fiske osmometer. This apparent contravention of the colligative theory of solutions can, however, be explained by postulating that the glycoprotein markedly retards the propagation velocity of ice in solutions (Lusena, 1955). The effect of this on the results of thermistor cryoscopy has already been discussed and, in the case of the McMurdo Sound *Trematomus* species, it could account for the observed freezing-point depressions. This hypothesis could be tested by comparing the results obtained by thermistor cryoscopy with those obtained by melting-point measurement or vapour-pressure osmometry. In the case of Signy Island and South Georgia fish, the agreement between the modified Ramsay and Brown method and thermistor cryoscopy in the two cases where comparison was possible indicates that a glycoprotein "antifreeze" does not occur to any extent in the blood of these fish. A similar conclusion resulted from chemical analysis of the serum (Smith, 1972).

The body-fluid freezing points showed that the osmotic concentration was fairly constant throughout the bodies of the Signy Island fish, differences between maximum and minimum body-fluid freezing points in the same fish ranging from 19 to 32 per cent of the minimum freezing-point depression. Supercooling under winter conditions was therefore unlikely to be confined to any one part of the fish. While all the body fluids studied were hypo-osmotic to sea-water, aqueous humour and urine were, on average, hypo-osmotic to serum, whereas bile (of all species except *Notothenia neglecta*), cerebrospinal fluid and tissue fluid were hyper-osmotic to serum.

The amounts of urine in the Signy Island fish were considerably smaller than those reported for *Trematomus bernacchii* at McMurdo Sound in which the urine was found to be as much as 4 per cent of the body weight (Potts and Morris, 1968). The increased amounts of urine found in "frozen" Signy Island fish may have been a true response to the experimental conditions but were more probably the result of "laboratory diuresis" caused by handling the fish. When urine freezing points were plotted against the amounts of urine (either as volumes or as percentages of body weight), no obvious correlation was apparent nor were there any differences between the sexes.

The freezing-point measurements confirm and extend the results of chemical analysis (Smith, 1972) and the principal conclusion of this study is that, while small individual and seasonal differences occurred in body-fluid freezing points, the body fluids of all the Antarctic fish species studied were found to be hypo-osmotic to sea-water. Signy Island and South Georgia fish are therefore liable to be supercooled at low environmental temperatures.

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