

A NEW METHOD OF FREEZING-POINT DETERMINATION FOR SMALL QUANTITIES

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(With Two Text-figures)

I. INTRODUCTION

To a very large extent progress in the field of invertebrate physiology is a measure of progress in the technique of manipulating small structures and analysing small samples. Within this field the study of osmotic regulation is no exception. Krogh (1938) has summarized the known methods of measuring osmotic pressure, applicable to quantities of fluid of the order of 0.1–1.0 cu.mm. The freezing-point method now to be described is capable of dealing with quantities one thousand times smaller.

II. PRINCIPLE

The main difficulty of the orthodox freezing-point methods, when applied to small quantities, is the difficulty of avoiding undercooling. Drucker & Schreiner (1913) showed that this could be overcome by first freezing the sample and then warming slowly so as to observe (with a hand lens) the disappearance of the last crystal of ice; the temperature at which this happens is the freezing-point, provided that certain precautions have been taken. This method was used by Fritsche (1916), who determined the freezing-points of 1–3 cu.mm. samples of *Daphnia* blood to the nearest 0.01°C. As described by Fritsche the method is difficult, requiring care and practice—'die ganze Methode ziemlich individuell ist'—and does not seem to have been used by others. It is apparent, however, that the main difficulty of the method lay in the control of temperature, and that the apparatus was not such as to make temperature control at all easy. It was therefore decided to examine the possibility of developing the method along the lines of better temperature control while retaining the principle of observing the disappearance rather than the formation of ice. In addition, it was proposed to observe the ice-crystals with a microscope, so as to make it possible to work with smaller quantities, and to keep the sample under liquid paraffin, so as to avoid loss of water by evaporation.

III. APPARATUS

The apparatus is illustrated in Figs. 1 and 2. 30% alcohol at -5°C . from a refrigerator unit is circulated through the outer jacket which is separated by an air-space from the inner vessel, also containing 30% alcohol, in which the sample and the thermometer are mounted. This inner vessel is stirred by compressed air which is first passed through a cooling coil in the outer jacket; since moisture in

the compressed air tends to freeze in the cooling coil and block it, it is necessary to bubble the air through 95% alcohol before it enters the apparatus. The alcohol in the inner vessel can be warmed by passing a current through a loop of nichrome wire. This current is adjusted to give the desired rate of heating by means of a variable resistor, and when rapid heating is required the variable resistor is shorted out by a key. When the current is switched off the temperature of the alcohol in the inner vessel falls slowly to -5°C . Rapid cooling is brought about by introducing cold alcohol from the outer circulation through a pipe reaching to the bottom of the inner vessel, excess alcohol being drawn off from the top by the suction of a filter pump.

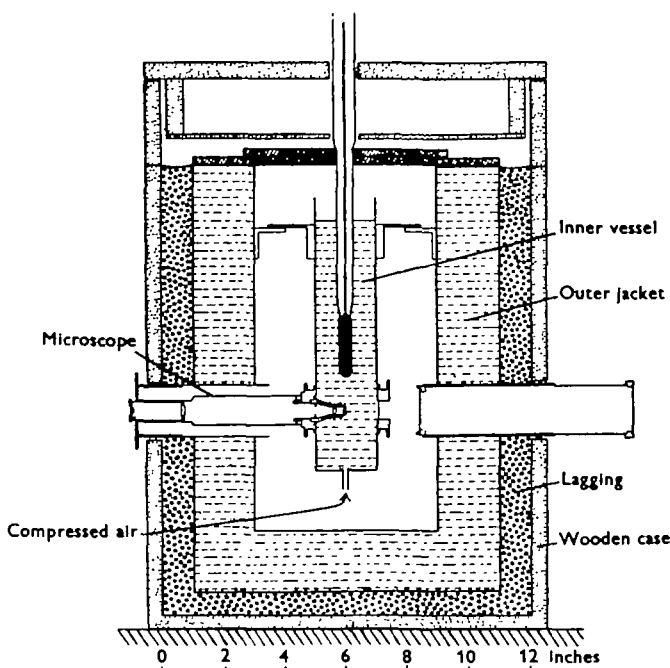


Fig. 1. To show the optical system and cooling system. The following features have been omitted for the sake of clarity: the coil on the compressed air supply, the pipes taking alcohol into and out of the inner vessel, the heating loop and the mounting carrying the sample.

A wide tube passing through the outer jacket allows a microscope to be mounted so as to observe the sample in the inner vessel. The objective, $\frac{3}{8}$ in., is screwed into an adaptor in the wall of the inner vessel which is then lowered into position inside the outer jacket, and the tube of the microscope is then passed through and screwed into the other end of the adaptor. On the opposite side the inner vessel carries a window and light is admitted by another wide tube passing through the outer jacket.

The fact that the objective is rigidly fixed to the wall of the inner vessel makes it necessary to provide means of adjusting the sample to lie in the field of view and in focus. The arrangement for doing this is shown in Fig. 2 and is described in the

legend to that figure. A fine adjustment to the focus is afforded by moving the microscope eye-piece in and out of the tube.

The thermometer used is a Beckmann, graduated in 0.01°C .

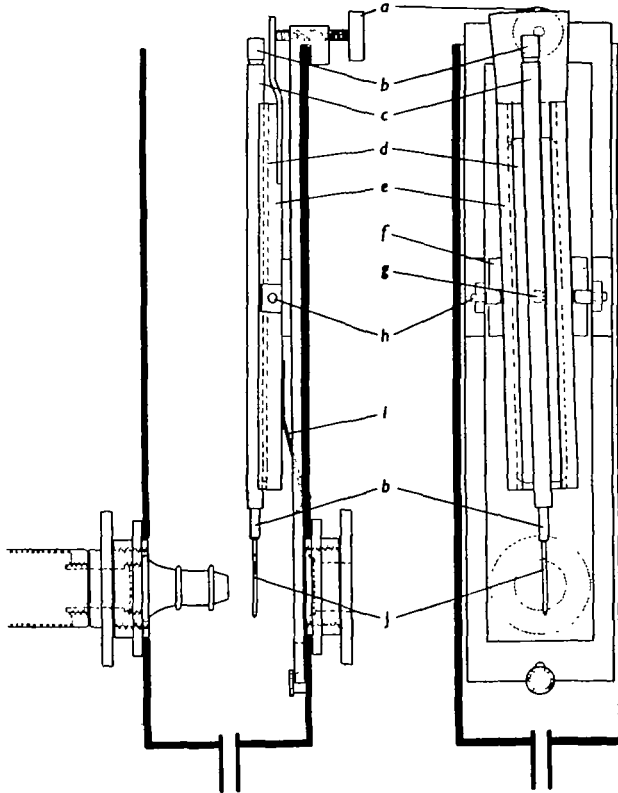


Fig. 2. To show the mounting by which the sample is adjusted in the field of view of the microscope. The sample is enclosed in the glass tube, *j*, which is fixed with sealing-wax to the brass rod, *b*. The brass rod, *b*, fits into the tube, *c*, which is fixed to the slide, *d*; this gives the up-and-down movement. The slide-guide, *e*, is mounted on the plate, *f*, and is free to move about the bearing, *g*; this gives the side-to-side movement. The plate, *f*, is carried on the bearings, *h*, and moved by the focusing screw, *a*, against the spring, *i*. The up-and-down and the side-to-side movements are friction-fitted.

IV. PROCEDURE

In order to present a concise and connected account of the procedure under the new method the reasons for certain of the steps have been omitted and are given at the end of this section under the 'Notes on Procedure', with numbers referring to the preceding text.

The pipettes are drawn from thin-walled 'Vitreosil' silica (see n. 1, p. 61) tubing of about 1 mm. external diameter. The collecting pipettes may be adapted in shape and dimensions to the problem in hand, but it is generally desirable that a collecting pipette should be drawn with a steep taper so that it is sufficiently rigid to be thrust into the tissues; the opening at the tip may be about 10μ in diameter. The pipette

is then fixed to a wider glass tube with sealing-wax and is connected through pressure-tubing to a mercury reservoir and screw-plunger. Mercury is driven to the tip and then withdrawn under liquid paraffin which is allowed to fill the tapering portion. The pipette is now ready for use. When a sample has been collected a further small quantity of liquid paraffin is drawn into the tip to prevent exposure of the sample to the air.

By reason of its steep taper the collecting pipette is not suitable for use in the determination of freezing-point (see n. 2). It is therefore necessary to transfer the sample to a freezing pipette which has a gentle taper from about $20\ \mu$ at the opening to about $70\ \mu$ at a distance of 1 cm. from the opening. The freezing pipette is mounted with sealing-wax upon a wider tube, connected to a mercury reservoir and screw-plunger in the same way as for the collecting pipette, and liquid paraffin is drawn into the tapering portion. Both pipettes are brought over the stage of a microscope, end to end and dipping into liquid paraffin. The tip of the collecting pipette is inserted into the tip of the freezing pipette and by manipulation of the screw-plungers the sample, or part of it, is transferred to the freezing pipette. A sample of volume 0.1×10^{-3} cu.mm. will fill the freezing pipette for a length of about 0.3 mm. It is then drawn up, followed by liquid paraffin, until in the wider part of the pipette its length is approximately double its diameter (see n. 3). The tip of the pipette is then removed from the liquid paraffin and is brought up against a drop of paraffin wax held in a loop of platinum wire. The wax is melted by passing current through the wire and a small amount of molten wax is drawn up into the tip of the pipette and serves to seal it (see n. 4). The pipette can now be removed from the wider tube on which it was mounted.

A piece of thin-walled glass tubing (see n. 5), about 1 mm. in diameter and about 4 cm. long, is sealed at one end and half-filled (see n. 6) with liquid paraffin. The freezing pipette is inserted into the open end of this tube and is pushed in as far as the taper will allow; it is then snapped off with forceps at this point and the end-portion is pushed right in until the part containing the sample passes beneath the surface of the liquid paraffin. The tube is then fixed with sealing-wax to the end of a brass rod (*b*, Fig. 2).

The brass rod is placed in the apparatus and adjusted so that the sample lies in the field of view. It is then removed and dipped for a few moments into 95% alcohol which has been cooled with carbon dioxide snow. Meanwhile, the temperature of the alcohol in the inner vessel is adjusted so as to be below the expected freezing-point of the sample. The sample, now frozen, is quickly transferred to the apparatus, the lid is replaced and the thermometer is inserted.

If the temperature of the alcohol is well below the freezing-point the sample appears more or less homogeneous. As the temperature is raised rapidly—at about 0.1°C./min. —by use of the shorting key, a sudden transformation is observed and the sample is seen to be made up of numerous round or oval ice-crystals with a small amount of liquid water. The temperature is allowed to rise rapidly until the volume of ice remaining is about one-fifth of the volume of the sample; the rate of heating must then be reduced. In the final stages a rate of heating of approximately 0.01°C.

in 2 min. has been found satisfactory (see n. 7). When the crystals become smaller than the diameter of the tube they float upwards and eventually the last crystal becomes hidden behind the curved surface of the meniscus (see n. 8). At this point the temperature is read to the nearest 0.005°C .

NOTES ON PROCEDURE

(1) Even with hard glasses such as pyrex it appears that substances from the glass dissolve into the sample and can appreciably lower the freezing-point.

(2) It is necessary to withdraw the sample far enough from the tip of the pipette to avoid any risk of its being boiled in the process of sealing with paraffin wax; in the steeply-tapered collecting pipette the sample forms a short column which breaks down and adheres to the wall after being withdrawn a short distance.

(3) If the sample formed a long narrow column the ice-crystals, floating to the top, would tend to cause dilution at the top as they melted, and in a long column diffusion might not be adequate to ensure uniform concentration throughout. The column is therefore made as short as is possible without risk of its breaking down.

(4) If sealing is omitted the sample is likely to be lost as a result of the sudden changes of pressure involved on removing the pipette from its mounting.

(5) Besides protecting the fine pipette containing the sample, this tube and the liquid paraffin contained in it fulfil important functions by virtue of their heat capacity. Notwithstanding the very rapid stirring which is maintained by the compressed air stream, mixing is never perfect. A small eddy, slightly warmer than the bulk of the alcohol, may strike the sample, and if this not protected by the wide tube its heat-capacity is so low that the last ice-crystal may be melted. The thermometer, measuring the bulk-temperature and unaffected by the eddy, records a temperature which is lower than that of the eddy which melted the ice and a false freezing-point is obtained.

(6) If the broken-off portion of the freezing pipette, containing the sample, were wholly immersed in the liquid paraffin it would be free to alter its position under gravity according to the inclination of the tube. This would be a nuisance if it happened after the sample had been adjusted to lie in the field of the microscope. With only 2 cm. of the tube filled with liquid paraffin one end of the pipette remains above the surface and is held firmly against the side of the tube by surface tension.

(7) The rate of heating must of course be so slow that the lag of the thermometer does not introduce any appreciable error. Owing to the latent heat of fusion of ice and the relatively poor thermal conductivity of liquid paraffin, equilibrium between ice and solution in the sample is established with surprising slowness, and as the size of the sample and its enclosing tubes is increased this effect is very much more significant than the lag of the thermometer.

(8) From simple geometrical considerations it can be shown that the volume of an ice-crystal as it disappears behind the meniscus is about 0.25% of the volume of a sample whose length is twice its diameter. The disappearance of the last ice-crystal behind the meniscus may therefore be accepted as the complete disappearance of ice from the sample.

V. ACCURACY

The accuracy of the method has been tested only so far as was necessary to establish its suitability for the investigation of the urine of earthworms (Ramsay, 1949). Results of repeated determinations on solutions of NaCl are given in Table 1. Each figure for freezing-point in this table is for a separate sample; the freezing-point of any

given sample is normally reproducible to the nearest 0.005°C . From these results it appears that there is a reasonably linear relationship between Δ and % NaCl, with a value of $\Delta/\% \text{NaCl} = 0.60$. If this relationship is accepted, the standard deviation of the observations is 0.00625°C . and the probable error is therefore 0.00427°C . or 0.007% NaCl; but in view of the relatively small number of measurements, too small for proper statistical treatment, it would be better to put it that the probable error is less than 0.01% NaCl.

The data given by Landolt-Börnstein (*Physikalische-Chemische Tabellen*, p. 611) show that the relation between concentration and freezing-point depression of solutions of NaCl is non-linear, but that over the range of $0-2\%$ concentration the errors involved in assuming a linear relationship are little more than 0.005°C . An exact comparison cannot be made since the Landolt-Börnstein data are in terms of g. NaCl/100 g. H_2O or g. NaCl/100 g. solution, whereas the data in Table 1 are in terms of g. NaCl/100 c.c. solution. Making allowance for this it appears that a value

Table 1

Solution (g. NaCl/100 c.c.)	Δ ($^{\circ}\text{C}$.)	Volume (cu.mm. $\times 10^{-3}$)
0.50	0.295	Not recorded
	0.295	
	0.300	
	0.305	
1.00	0.595	0.20
	0.595	0.16
	0.605	0.06
	0.590	0.54
	0.605	0.04
2.00	1.200	0.54
	1.195	0.13
	1.205	0.14
	1.210	0.07
	1.205	0.29

for the ratio $\Delta/\% \text{NaCl}$ derived from the Landolt-Börnstein data would be somewhat lower, about 0.59 . In view of these uncertainties it seems better to treat the new method as a purely empirical one, calibrating the freezing-point depression with solutions of known concentration and accepting whatever relationship gives the greatest consistency.

Since the accuracies claimed for other methods are not defined in statistical terms a close comparison with the present method is not possible. Fritsche claims that his results are accurate to the nearest 0.01°C . but does not state whether this applies to repeated measurements on the same sample or to measurements on separate samples of the same solution. White (1928) states that his modification of the Barger method is capable of detecting the difference between 0.60 and 0.65% NaCl, i.e. a difference of about 8% of the total concentration. Keosian (1938), using the same method, claims that a difference of 2% is significant. For the Hill-Baldes vapour-pressure method Johnson & Baldes (1938) claim that the method is 'sensitive' to changes of 2 mg. NaCl in 100 g. water; Fox & Baldes (1935) state that it is

'accurate' to within 1% of the total concentration. If we assume that the error of any single determination should not exceed three times the probable error, the 'accuracy' of the present method is 0.03% NaCl in concentrations of up to 2% NaCl and so is approximately 1.5% of the total concentration; its accuracy is therefore of the same order as the accuracy of the other methods.

The coelomic fluid and urine of earthworms contain very little protein, but as a matter of interest tests were carried out to see whether the method would be upset by protein in greater concentration. For this purpose human serum was used. No difficulty whatever was experienced in observing the freezing-point. Sixteen determinations of vapour-pressure by the Hill-Baldes method and five determinations of freezing-point were made with the following results:

Hill-Baldes method: 0.93% NaCl
Freezing-point method: 0.944% NaCl

The time required to carry out a determination of freezing-point by the new method is about 45 min., of which about 25 min. is taken up in transferring the sample from the collecting pipette to the freezing pipette and mounting it on the brass rod. In the course of routine work it is found convenient to have several brass rods available and to mount each sample as soon as it has been collected. At the end of the day the freezing-points of all the samples are determined, the average time per sample being about 15 min.

VI. DISCUSSION

As stated in the previous section, the accuracy of the method has been tested only so far as was necessary for a specific problem, and for this it proved to be entirely adequate. One feature of the results which calls for comment is the slight discrepancy between the freezing-point depression of a salt solution as measured by orthodox methods and as measured by the method now under consideration; but the reason for this discrepancy is not at present understood. This and other points will have to be cleared up before the limitations of the new method are defined. One such limitation will obviously be the solubility of constituents of the sample in liquid paraffin. However, it appears that the method is likely to have a useful range of application.

The apparatus is simple to construct. Its main disadvantage will appear to be the requirement of a refrigerator unit, an expensive item of equipment. It would have been difficult to develop this method without a refrigerator unit and it is certainly a great convenience to have it available; but all that is basically necessary is to have a supply of alcohol at about -5°C ., and no doubt this could be provided by some system depending upon the conventional ice and salt mixtures.

The method works better with small quantities, e.g. 0.0001 cu.mm., than with larger quantities, e.g. 0.1 cu.mm. With large quantities the effect of the latent heat of fusion of ice in delaying equilibrium (see § IV, n. 7) is much more in evidence. A mass of small crystals of ice may appear to be in equilibrium when, in fact, the temperature is well above the freezing-point, the rate of melting being too slow to

be appreciated. As the mass becomes smaller the rate of melting of the remaining crystals is increased and the last few disappear very quickly. One is therefore almost certain to overshoot the freezing-point if the temperature is raised continuously, unless the rate of rise is so slow as to require hours for the determination. In order to get an accurate measurement in reasonable time it is necessary to be able to cool rapidly when only one small crystal remains and then to adjust the temperature until the crystal neither increases nor decreases in size.

What is perhaps more interesting is to consider the possibility of working with still smaller quantities. The smallest quantity dealt with so far is 0.04×10^{-3} cu.mm. To go below this size a higher power of the microscope would be required and some refinement of the apparatus would be necessary for convenient working under, say, a $\frac{1}{8}$ in. objective. On purely optical considerations quantities of the order of 10^{-6} cu.mm. are a possibility, the size of the crystal disappearing behind the meniscus being of the order of 1μ in diameter. A possible difficulty arises from the effect of surface tension at the ice-water interface, whereby crystals below a certain size become unstable. Dr A. E. Alexander, who kindly looked into this matter for me, finds that this effect will be negligible at crystal diameters greater than 0.1μ , even in pure water or salt solutions; in the presence of protein or other surface-active substances the critical size will be less. More probably the difficulties to be encountered in dealing with smaller quantities will be those of manipulation and of contamination with impurities coming from the liquid paraffin and the silica.

VII. SUMMARY

1. A method is described for determining the freezing-point of small quantities ($0.1 - 1.0 \times 10^{-3}$ cu.mm.) of fluid.
2. Tested on solutions of NaCl of concentration up to 2% the probable error of the method is less than 0.01% NaCl.
3. The potentialities of the method are discussed.

I am indebted to Lord Rothschild for the loan of a refrigerator unit, without which the development of this method would not have been undertaken.

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