ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Cryobiology 46 (2003) 88–98

CRYOBIOLOGY

www.elsevier.com/locate/ycryo

Ice nucleation in nature: supercooling point (SCP) measurements and the role of heterogeneous nucleation☆

P.W. Wilson,[a,*] A.F. Heneghan,[b] and A.D.J. Haymet[c]

[a] *Physiology Department, Medical School, University of Otago, Dunedin, New Zealand*
[b] *Department of Chemistry, University of Houston, Houston, TX 77204-5003, USA*
[c] *CSIRO Marine Research, GPO Box 1538, Hobart, Tasmania 7001, Australia*

Received 27 June 2002; accepted 27 November 2002

Abstract

In biological systems, nucleation of ice from a supercooled aqueous solution is a stochastic process and always heterogeneous. The average time any solution may remain supercooled is determined only by the degree of supercooling and heterogeneous nucleation sites it encounters. Here we summarize the many and varied definitions of the so-called "supercooling point," also called the "temperature of crystallization" and the "nucleation temperature," and exhibit the natural, inherent width associated with this quantity. We describe a new method for accurate determination of the supercooling point, which takes into account the inherent statistical fluctuations of the value. We show further that many measurements on a single unchanging sample are required to make a statistically valid measure of the supercooling point. This raises an interesting difference in circumstances where such repeat measurements are inconvenient, or impossible, for example for live organism experiments. We also discuss the effect of solutes on this temperature of nucleation. Existing data appear to show that various solute species decrease the nucleation temperature somewhat more than the equivalent melting point depression. For non-ionic solutes the species appears not to be a significant factor whereas for ions the species does affect the level of decrease of the nucleation temperature.

Keywords: Heterogeneous; Homogeneous; Nucleation; Supercooling point; Ice; Solutes

The temperature at which a solution spontaneously freezes when cooled below its equilibrium freezing temperature, T_f, is denoted variously as the "kinetic freezing point" [26], the "temperature of crystallization" [36], and the "nucleation temperature" [21]. For biological solutions, or even for whole organisms, this temperature of spontaneous freezing is also often called the "supercooling point" (SCP) [19,40], the notation we shall adopt in this paper.

The stochastic nature of the SCP [1,16] is not always realized or defined. Often the SCP is

☆ This work was funded by the Welch foundation (Grant E-1429).

* Corresponding author. Fax: +64-3-4797323.
E-mail address: peter.wilson@stonebow.otago.ac.nz (P.W. Wilson).

0011-2240/03/$ - see front matter
doi:10.1016/S0011-2240(02)00182-7

measured by sealing the solution into a small capillary, and decreasing the temperature of the capillary linearly as a function of time at some preset rate until the solution freezes. This heating/cooling process is then often repeated a few times employing different samples from the stock solution in each successive run. We show here that this misses one of the most important aspects of this phenomenon, namely the inherent width of the distribution of SCP. In the case of whole animals, the procedure is essentially the same, but great care is taken to ensure that the animal is not seeded with ice, which would prematurely induce freezing in supercooled fluids [29]. In this method, the lowest temperature reached prior to the sample freezing is defined as the supercooling point of the solution. Freeze-tolerant animals may survive the experience, and be used several times to investigate the distribution of this supercooling point temperature. However, even with freeze-tolerant species, there may be accumulating damage which makes comparison of successive SCP measurements less meaningful.

Here we attempt to clarify some of the accumulated misconceptions about the nucleation of supercooled solutions, particularly within biological systems. We argue that all nucleation in these biological systems is heterogeneous rather than homogeneous. Unfortunately, experiments performed in the 1950s by Bigg [2], which at first appeared to show "homogeneous" nucleation, were shown to be irreproducible by Langham and Mason (from the same laboratory) [22] a few years later, but only the original Bigg reference has been propagated through extremely important and influential biological literature [41,42]. We also study how the addition of solutes may affect the heterogeneous nucleation temperature of aqueous solutions. We exhibit a new method for determining both the statistically significant SCP and its correct, natural, inherent distributions of freezing temperatures, defined here as the "inherent width." To examine the proposed definitions of the supercooling point (SCP) and the inherent width, we describe an automated lag-time apparatus (ALTA), which repeatedly supercools the same sample linearly as a function of time many hundreds of times to gather the statistics of the nucleation process. Note this method does not solve the issue of reproducibility for freeze avoiding insects where damage may occur after even a single thermal cycle.

Definitions

Homogeneous nucleation

The homogeneous nucleation temperature of liquid water is generally accepted to be, and often quoted to be, approximately −39 °C [9]. Below this temperature even finely dispersed water droplets will freeze instantly. It has also been proposed that this temperature is somewhat volume dependent and this concept, together with the reported anomalous density data of supercooled water, is discussed by Hare and Sorensen [11]. An excellent review of the homogeneous nucleation of liquids can be found in the volume by Debenedetti [5, Section 3.1].

In practice, the phenomenon of homogeneous nucleation is actually very difficult to achieve, especially under laboratory conditions. In order to approach the limit of homogeneous nucleation, much care must be taken in the preparation of the sample to avoid impurities, which may lead to heterogeneous nucleation. One attempted method to achieve homogeneous nucleation takes advantage of extremely small volumes of ultra-pure water, which are immersed within oil emulsions [4]. As the sample volume becomes smaller, there is a greater probability that impurities are absent from the liquid droplet. The oil acts to prevent extra "surfaces" or "sites" on which the sample may nucleate. Hence in the limit of extremely small droplets of water, it may be possible to approach the limit of homogeneous nucleation, depending on the influence of the surrounding curved surfaces. A second method to achieve homogeneous nucleation involves levitating a small volume of aqueous solution by using an electromagnetic field. Although this elegant method eliminates the use of a container, which again may introduce unwanted "sites" for nucleation, it does not ensure a sample free of impurities. For example, there may be small dust particles, or impurities, in some samples but not others, which act as heterogeneous nucleation

sites. Hence, most, if not all, nucleation encountered in the laboratory and in practical experience is heterogeneous. Even nucleation of clouds in the atmosphere is now believed to occur entirely or mostly on a seed particle such as ammonium sulfate [43]. We believe this assertion is especially true when dealing with solutions of biological origin where undoubtedly impurities such as surfaces, membranes, and large molecules are present.

Since experiments in cryobiology are not actually conducted within environments of ultra pure water, the effect of solutes on the SCP must be addressed. Solute-induced decrease of the homogeneous nucleation temperature is difficult to measure. However, Rasmussen and McKenzie [30] did measure the homogeneous nucleation temperature for a variety of aqueous solutions of increasing concentration, and their data suggested that the homogeneous nucleation temperature decreased by about twice the equivalent melting point depression, for a given volume and measurement technique. McKenzie [26] re-plotted this data some years later, and added further data for sucrose. He also pointed out this twofold ratio but offered no real explanation for it.

Recently, Koop et al. [20] have analyzed the reported homogeneous nucleation temperatures of 18 different solutes as a function of solute molality. In essence, they found that the ratio is in fact unity for typical physiological strengths, of less than approximately 500 mOsm, and that the identity of solute species is unimportant. More recently, Miyata et al. [27] argue that there exists a strong correlation between the homogeneous nucleation temperature T_H and ionic radius of alkali ions and/or halide ions. They have shown that some of the results of Koop et al. [20] for ionic solutions are at best an approximation.

In a recent review in this journal, Zachariassen and Kristiansen [42] discuss homogeneous nucleation, and they argue that homogeneous nucleation may be the mechanism of freezing in some biological systems. They contend that nucleation in some insects is not triggered by ice nucleation agents of any sort and that they instead undergo homogeneous nucleation. We find their argument flawed due to its reliance on the interpretation of the results of Bigg [2]. To our knowledge the results of Bigg have never been reproduced. The high T_H values reported by Bigg are possibly due to some heterogeneous process, perhaps involving a reaction at the water–oil–surfactant interface. This possibility has already been discussed by McFarlane et al. [25].

Zachariassen [41] some time ago also suggested the term "semi-homogeneous nucleation" to describe situations where the SCP is as low as −30 °C, resulting from nucleation sites of low efficiency, rather than homogeneous nucleation. Since homogeneous nucleation is so difficult to achieve, even with the methods described previously, we believe that homogeneous nucleation is impossible to achieve in biological samples, and that there is no such thing as semi-homogeneous nucleation. There are so many surfaces and particles about which nucleation might be seeded within an organism, extra-cellular fluid, or cell, that nucleation within biological solutions will undoubtedly be heterogeneous.

The claims of Zachariassen and Kristiansen [42] seem to be based on their observations that adding solutes to certain insect haemolymph samples does tend to decrease the nucleation temperature by about twice the equivalent melting point depression, for a given volume and container. They also found that adding solutes to other insect haemolymph samples containing potent ice nucleating proteins (INPs) only lowered the SCP by the equivalent melting point depression (i.e., a onefold ratio). In the latter case, since nucleation was clearly heterogeneous (due to the INPs), they reasoned that if the twofold rule was present in any given haemolymph sample then that sample must be undergoing homogeneous nucleation. They do note however that "the basis for any strong solute-induced SCP depression in insects is not understood."

We examine the "twofold phenomenon" below and show that no conclusions about the nature of homogeneous or heterogeneous nucleation can be drawn from the value of the ratio of the SCP decrease to the melting point depression.

Heterogeneous nucleation

Apart from the example of ultra pure water sequestered in emulsions to reduce the contact

with surfaces discussed above, all other aqueous solutions will undergo heterogeneous nucleation. The liquid sample must be housed in a container of some form and even so-called "pure" water will, in general, have some impurities about which nucleation might proceed. From a free energy point of view, it is more favorable to grow an ice embryo on a two-dimensional surface than in a three-dimensional surface-free volume of water. This point is easily demonstrated by looking at the minimum work required to create the critical nucleus leading to the new phase [28]:

$$W_{\min} = \frac{16\pi\sigma^3}{3\rho^2\Delta\mu^3}.$$

This is the work required for the formation of a critical nucleus in a free volume. When nucleation proceeds about a surface, this equation is multiplied by the so-called catalytic potency factor defined as the following:

$$f(\theta) = \frac{(1+\cos\theta)^2(2-\cos\theta)}{4},$$

where theta is a function ranging between 0 and 1.

Hence, the work of formation of the new phase is actually lowered when nucleation proceeds about a surface as manifest in the equation below, and the minimum work of formation may be written in the following form:

$$W_{\min} = \frac{16\pi\sigma^3}{3\rho^2\Delta\mu^3}\cdot f(\theta).$$

Since any growing ice embryo will form about some surface, be that on the side of the glass container, on a piece of dirt or on a cell wall, all nucleation known to date in supercooled solutions is heterogeneous. This is especially true of biological systems, such as a small beetle, where there is a large surface area containing the aqueous solution.

A look at some heterogeneous nucleation data

When the volumes of water used in experiment are larger than the micron-sized droplets found in emulsions, and they are supercooled, the heterogeneous nucleation temperature varies markedly due to varying containers and varying purity of the aqueous sample used. There has been a great deal of experimental work on the freezing of supercooled water under a variety of experimental conditions and a variety of experimental methods. Below is a brief outline detailing only some of the work starting from the extremely small volumes extending to much larger samples. For water we find that:

1. Nanoliter samples of water which are surrounded by oil and used for the calibration of the nL osmometer (Otago Osmometers, Dunedin, New Zealand) typically freeze at temperatures between −20 and −30 °C (D. Wharton, personal communication). These temperatures are far from −40 °C; hence, impurities are most likely responsible for heterogeneous nucleation.
2. Hosler and Hosler [17] used a variety of sizes of capillary tubes and found that, even when the capillaries had an extremely small diameter of only 0.2 mm, the lowest temperature they could reach with water samples was −33 °C, at which point nucleation occurred.
3. Most workers who use differential scanning calorimeters (DSC) use water as a control, at one time or another. These experiments usually find that the typical sample volume comprised of only 5 μL of "pure" water will invariably freeze in the DSC pan at temperatures ranging between about −21 °C and −25 °C [39], which is again far from the homogeneous nucleation limit.
4. With slightly larger volumes, studies have shown that 200 μL of clean, reagent grade distilled water sealed in a glass NMR tube typically freezes at temperatures around −14 °C, and that this temperature is somewhat container dependent when there are no strong (efficient) nucleators present in the aqueous sample [14]. The SCP is independent on the rate of cooling for low enough cooling rates, and this effect has also been studied, see for example [14].
5. Using even larger volumes tends to make it more difficult to achieve low nucleation temperatures, simply because it enhances the probability that an efficient nucleator is present as the

total volume of the solution increases. In 1948, Dorsey [6] published a very thorough study of the effects of cleanliness, the effects of glass type, and the effects of water conductivity on the heterogeneous nucleation temperature of water. In his study, sample sizes of approximately 4 mL were used. At no time was he able to cool the water below about −19 °C before heterogeneous nucleation occurred. Recently Inada et al. [18] have managed to supercool several hundred milliliters of water down to temperatures around −12 °C, a major achievement for such a large volume of water.

Effect of solutes on SCP

We now examine the effect of various solutes on the SCP. In the context of recent literature [42], the important question is whether, for a given solution and container, added solutes actually decrease the level of supercooling ability of that solution in that container by an amount which is the same as the MP, or twice as much, or three times? In fact, it would seem that the actual measurement of the level of supercooling is far too stochastic and difficult to achieve to be able to even give any sensible value to this ratio, a factor which we address below.

Zachariassen [42] places great importance in the early results of Bigg [2] and McKenzie [26]. The latter claim that the effect of solutes on (so-called) homogeneous nucleation yields a twofold increase. To examine closely the "twofold" claim, we analyze the data from Rasmussen and McKenzie [30] for the decrease of T_H as a function of solute concentration for a variety of solutes. Their data has been digitized and re-plotted here as Fig. 1, as solute concentration versus the ratio of the decrease of T_H to the melting point depression. By definition, this ratio is unity at zero concentration, and at first glance it appears that all the species they tested plateau at a ratio of about two. Although McKenzie [26] claimed that the data were "almost all grouped into one band with a gradient of 2.0 ± 0.2," we do not agree. In both his plot and ours, the ratio varies from 1.3 to about 2.5 (excluding PVP and PEG which have even higher ratios).

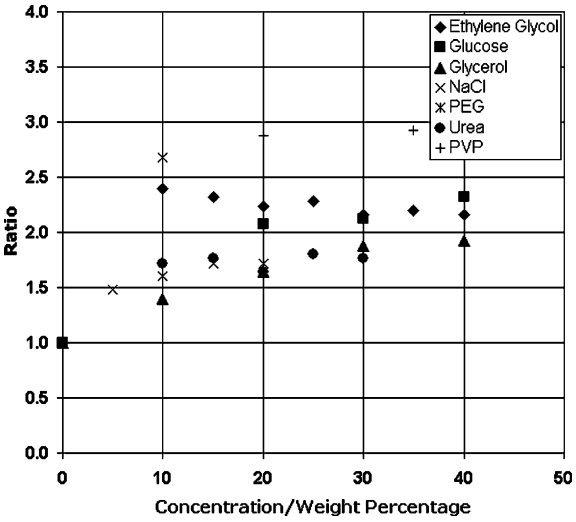

Fig. 1. Data of McKenzie [26] replotted with solute concentration as a function of the ratio of the decrease of the homogeneous nucleation to the depression of the melting point.

Fig. 1 does show that the type of solute appears not to be a significant factor determining how much extra decrease in the SCP the solute molecules afford the solution. Koop et al. [20] recently also claim that the identity of the solute species has no effect whatsoever on the ratio. As mentioned above, the claim for some ions is different, and the correlation between ionic radius and the SCP ratio has been studied [27].

It is also clear that the level of SCP enhancement varies from a ratio unity to as much as 2.5, and that generalizing to a value of 2 is misleading, especially at physiological concentrations of solutes, which ranges between 0 and 10 or even 20 wt%. Leyendekkers and Hunter [24] have offered an explanation for the linear relationship found by Rasmussen and McKenzie [30] and show that T_H decrease should in fact be approximately linear in concentration.

Fig. 1 also shows that sodium chloride, urea, glycerol, and glucose all appear to decrease the SCP by *about* twice the equivalent melting point depression at high concentrations. Although these reanalyzed data are claimed to be for homogeneous nucleation, we have no reason to suspect that the effects of solutes will be any different on the water molecules when the solution freezes at a

warmer temperature and undergoes heterogeneous nucleation.

Other workers who have made similar measurements have found a great variety of results. Block and Young [3] have reported that added glycerol decreases the SCP of some particular solutions by more than three times the equivalent melting point depression.

Wang and Haymet [37] have shown recently that even within the simple sugars, the amount of supercooling enhancement for a given volume differs from one isomer to another. They found that trehalose and sucrose decreased the nucleation temperature in a modulated DSC further than glucose and fructose.

This ratio has also been examined closely by Duman et al. [7]. After surveying the literature, their conclusions included:

1. It has been argued theoretically that the ratio should be unity, even for homogeneous nucleation [8]. The ratio has been found to vary from unity to about two for heterogeneous nucleation [3,31]. In studies on whole animal (invertebrate) species lacking ice nucleating agents, the ratio appears to be closer to three. See for example Somme [35] (but note that we believe this observation may be due to too few sample repetitions, as discussed below).
2. In general, all other studies have found that, when potent nucleators are present, the ratio tends to have a value which is very close to unity [23].
3. Zachariassen [40] found that adding either saline or glycerol to water at concentrations up to 2.5 Osm increases the SCP of that solution (same volume, same container) by a factor between 1.4 and 1.5.

Zachariassen and Kristiansen [42] contend that the polyol accumulation in freeze-tolerant insects generally only decreases the SCP by a ratio of unity. Freeze-tolerant insects have potent ice nucleators, and nucleation is then unambiguously heterogeneous. They note that McKenzie [26] quotes (incorrectly, we now observe) a ratio of two for homogeneous nucleation, and from this they conclude that when a ratio of two is found in nature, the nucleation is therefore homogeneous. We argue that when INPs are not present, nucleation will occur on the container wall or a piece of dirt or some other site, and nucleation will still be heterogeneous. The ratio appears to vary from unity to more than three for both types of nucleation, and no conclusions can be drawn from that value. It is also clear that to measure accurately the nucleation temperature, many more measurements are needed than have typically been made on the experiments published to date. Also, the solute dilution (or concentration) series must be carried out in the same container and under the same conditions, such as rate of cooling, and even then the ratio may not be a reasonable quantity to measure. We demonstrate a reproducible method for making this measurement below.

There have also been reports of extraordinarily low supercooling points, some as low as −54 °C [32] (but with only three repetitions and 0 SD). However these reports tend to be in samples where there is as little as 30% water available. The level of desiccation of a plant or invertebrate sample will greatly influence the measured SCP, and great variation of SCPs in field samples is observed regularly, mainly as a result of the varying levels of sample water content. The very high solute concentrations may mean that, instead of simple heterogeneous ice nucleation, there is involved a complicating glassy state (which may not be detected by the thermocouple). McFarlane et al. [25] found that for ionic solutions of compositions richer in LiCl than only 12%, the glassy state was obtained easily.

Reproducible measurements of the SCP

We now address the stochastic nature of any SCP measurements. It has been relatively common in cryobiology literature to report SCPs accurate to ±0.1 °C, for example, in [3,10,38]. Quite often, SCP determinations have been made from only a handful of measurements on each sample, or on each stock solution or on each group of animals, and the resulting values quoted with standard deviations calculated in the usual way. We realize that in most cases each such measurement results in the sacrifice of an animal and it is not possible to run this experiments many times on the same sample. However, if so few data points are determined then the likelihood of measuring the most

probable nucleation temperature is small. In this case, caution is needed in reporting results, and especially "error bars" or "confidence limits." In fact, we have shown that up to 200–300 measurements are needed on a single sample to determine accurately the nucleation temperature [15,16].

We summarize here an automatic lag time apparatus (ALTA), which we use regularly to study the statistics of liquid-to-crystal nucleation [1,12,15,16]. The machine, which we refer to as ALTA4 to distinguish it from earlier versions of the apparatus [1], repeatedly cools, nucleates, and thaws a single, unchanging sample of solution. For biological studies, it operates in a linear cooling mode, in which the temperature of a single sample of liquid is decreased by a constant value in time until the sample freezes. The set up and operation of the apparatus for inorganic aqueous solutions has been described elsewhere [14], but we will summarize the main components of operation as used in the biological context here.

A single aqueous sample, in volume typically 200 μL (although much less volume could be used) is placed in a shortened NMR tube (5 mm OD, length 100 mm) which resides snugly in a hollowed-out 10 mm thick aluminum block of a cross sectional area of 25 cm^2. A K-type thermocouple rests outside of the sample NMR tube, but within 2 mm of the actual sample, to measure temperature accurately, yet prevent unwanted nucleation sites. The aluminum block sample-holder is sandwiched between two 65 W thermoelectric (Peltier) modules which are used to heat and cool the sample as desired by computer control. Excess heat is removed from the outsides of these modules by brass heat sinks, which are cooled at a constant temperature by a flowing isopropanol cooling bath. A schematic outlining the major components of the ALTA4 device is shown in Fig. 2. The freezing of the sample is monitored by the (interrupted) transmission of a low power (<1 mW) diode laser ("laser pointer"), which illuminates a photodiode. When a sample freezes, the light is scattered and results in a decrease of intensity at the photodiode detector. This decrease in the intensity signals the computer to activate a relay to switch direction of the current applied to the Peltiers. The Peltiers then heat the sample up to 283 K

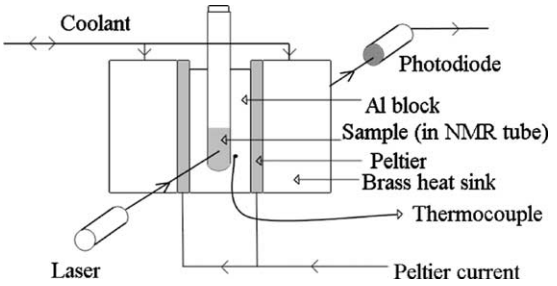

Fig. 2. A schematic of ALTA4, showing the cooling system and the optical sensing of solidification of the sample. Note that the thermocouple is close to, but outside the sample.

for 4 min to ensure melting of all residual ice crystals prior to commencing another run. This cycle is repeated as many times as necessary to generate the unambiguous statistics of nucleation. The rate of cooling is controlled experimentally at the start of a series of runs, and we use a value of 1.08 K min^{-1} in this work.

The data presented here has been chosen simply to illustrate the typical results generated by ALTA4 and to help clarify our description of measuring and defining the SCP. The primary data collected in this experiment are the time, t_i, and the temperature, T_i, at which nucleation occurs, as a function of run number i. Fig. 3 shows these data

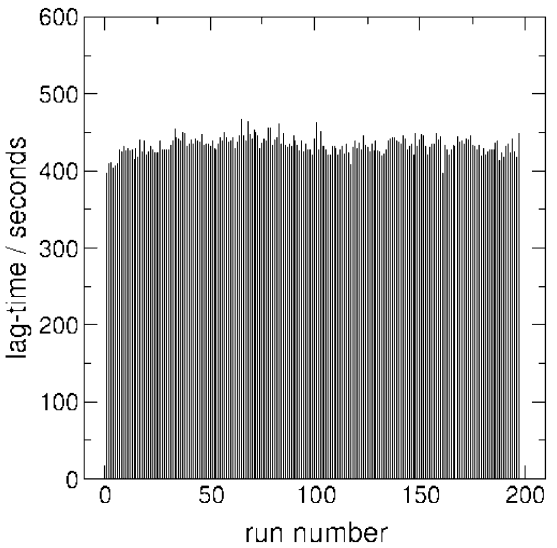

Fig. 3. The lag-time as a function of run number for a single aqueous sample of the notothenoid, *N. angustata*.

collected from 198 consecutive heating/cooling cycles on a single sample which was cooled at a rate $\alpha = 0.018\,\mathrm{K\,s^{-1}}$ or equivalently $1.08\,\mathrm{K\,min^{-1}}$. We call this type of plot a "Manhattan" [1] and it neatly demonstrates the stochastic nature of the nucleation process. Although we are using the exact same sample from run to run, the sample does not freeze at the same temperature on each run. Instead, there exists a distribution of temperatures which we have defined as the inherent width, spanning approximately 2 K in this case, over which nucleation occurs. This range of temperatures is an inherent, reproducible, and important quantity which must be considered in SCP measurements. It must be considered when determining the SCP of any solution or organism. Thus, making only a few measurements of the SCP introduces a source of error into reporting a value for the SCP.

In the past, some authors have used histograms to bin the data. However, based on the size of the bin width, results will vary. A simpler and hence better way to evaluate these data is to calculate a "survival curve." We denote by N_0 the total number of repetitions on the same sample. Simple statistical analysis shows that to determine unambiguously the inherent width and location of the SCP, N_0 should be of order 200–300. The survival curve is simply the fraction unfrozen of samples as a function of time or scaled time, and we denote this $F(t)$,

$$F(t) = N(t)/N_0, \tag{1}$$

where $F(t)$ equals unity at the time zero, when the sample cools below its equilibrium melting point, at which point all of the samples are unfrozen, and evolves to the value 0 at some finite time later, when all of the samples have frozen. Since the supercooled temperature decreases linearly with time, $\Delta T = \alpha t$, we may convert the time axis to a scaled time by multiplying by the cooling rate, α. We have made this measurement on a wide variety of inorganic and biological samples, including pure water, water seeded with an AgI crystal, other biological samples, and samples containing bubbles. These measurements are reported elsewhere [13]. Here, we show how this measured "survival curve" leads naturally to a reproducible definition of the SCP, and in fact is the key quantity which any SCP measurement must determine.

The sample used for measurement and the example of the supercooling point is a 200 μL sample of the notothenoid, *Notothenia angustata*. Fig. 4 shows the "survival curve" for 198 continuous heating/cooling cycles. We have found that after about 200 runs, the survival curve does not change magnitude or shape as a result of more repetitions. Hence, we suggest measuring the SCP with heating/cooling cycles as many times as necessary until the survival curve does not change. In the limit that this curve does not change, enough data is collected to analyze the statistical spread of this process, and this curve becomes the focal point for this new, proposed definition of the SCP.

After measuring the survival curve for any aqueous solution, the definition of the SCP becomes obvious. The natural definition of the SCP is the temperature at which the survival curve crosses the 50% unfrozen mark, namely the temperature at which on average half of the samples are frozen and half of the samples are unfrozen. Fig. 4 shows this survival curve for this solution.

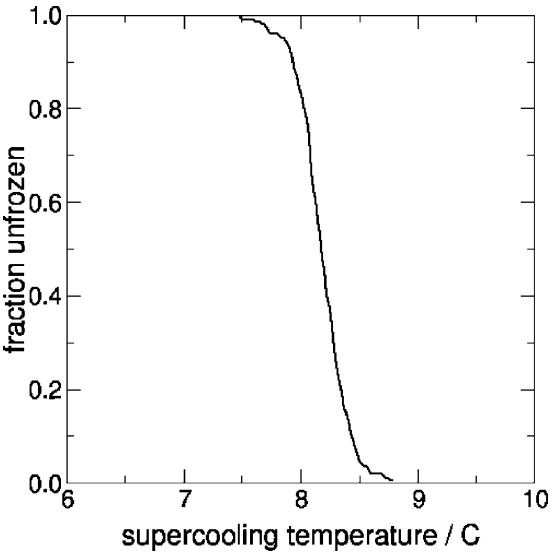

Fig. 4. The survival curve for 198 runs on the same sample of *N. angustata*. The cooling rate $\alpha \times$ time is equivalent to the amount of supercooling, in Celsius. We argue that the SCP should be taken as the temperature where 50% of the samples survive, which in this case is about 8.17 °C. Also, the spread of values should be the 10–90 width, which in this case is 0.61 °C.

For these data, the proposed supercooling point is 8.17 K. However, this survival curve also provides a natural definition for the inherent width and hence error bars for the supercooling point. By measuring the 10–90 width (the range of temperature where the sample is 90% unfrozen to the temperature where the sample is 10% unfrozen), upper and lower bounds emerge naturally from this analysis. Here the 10–90 width is 0.61 K. This spread in the temperature of nucleation for the *exact same sample* demonstrates further the point that many repetitions are needed.

Again, this method does not solve the problem of measuring SCP for whole animals or samples where few repetitions are possible, but it does, however, define unavoidable limits on the accuracy and reproducibility of such measurements.

Conclusions

1. All nucleation of supercooled biological solutions or whole animals is heterogeneous, and the term homogeneous should be avoided and only used for situations where great care is taken by employing, for example, ultra-pure water samples sequestered in oil emulsions or levitation.
2. Many repetitions on the same or provably identical samples are required to measure accurately the SCP, and its partner quantity, its inherent width.
3. Although solutes appear to decrease the SCP of solutions, ratios of SCP decrease to melting point depression have been reported ranging from 1 to at least 3. The effect is solute species independent, except for ions as mentioned in the text above. Possibly the data available for other solutes cannot be used sensibly since they are made up of insufficient measurements and do not adequately represent the true SCP. It is likely that, if all previously reported measurements were to be repeated and made many more times each, in order to measure the inherent statistical nature of nucleation and the inherent width of the SCP as described above, this ratio would be found to be much closer to unity, as shown by Koop et al. [20].

Acknowledgments

This research was supported by the Welch Foundation (Grant E-1429) to whom grateful acknowledgement is made. We thank Professor A.L. DeVries, University of Illinois, for many interesting discussions and the kind donation of the fish serum used in the measurement of the supercooling point.

Appendix A. Nucleation probabilities and the role of heterogeneous nucleation

In some alternate analyses of nucleation data [33,34], the first derivative of the survival probability plays a major role. This first derivative with respect to temperature yields an instantaneous nucleation probability as a function of temperature. Fig. 5 shows this quantity for the heterogeneous nucleation data presented in this paper, together with a "best guess" (hypothetical) data set for homogeneous nucleation of pure water. This plot shows the extremely low probability of homogeneous nucleation at temperatures warmer

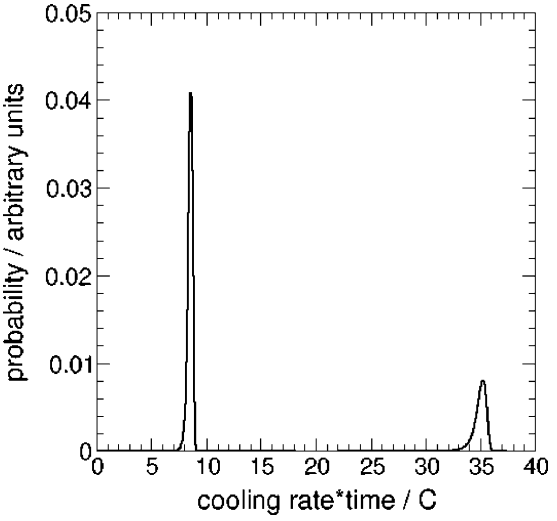

Fig. 5. The instantaneous nucleation rate (first derivative of the survival curve) for the data presented in this paper, together with a "best guess" synthetic curve for homogeneous nucleation. This figure demonstrates the vanishingly small probability of homogeneous nucleation at warmer temperatures.

than about −30 °C. Even though it is mathematically possible nucleation proceeds homogeneously at much warmer temperatures, Fig 5 demonstrates that the instantaneous homogeneous nucleation probability at these warmer temperatures is vanishingly small.

References

[1] T.W. Barlow, A.D.J. Haymet, ALTA: an automated lag-time apparatus for studying the nucleation of supercooled liquids, Rev. Sci. Instrum. 66 (1995) 2996–3007.

[2] E.K. Bigg, The supercooling of water, Proc. Phys. Soc. B 66 (1953) 688–694.

[3] W. Block, S.R. Young, Measurement of supercooling in small arthropods and water droplets, Cryo-Letters 1 (1979) 85–91.

[4] F. Broto, D. Clausse, A study of the freezing of supercooled water dispersed within emulsions by differential scanning calorimetry, J. Phys. C 9 (1976) 4251.

[5] P.G. Debenedetti, Metastable Liquids, Princeton University Press, Princeton, 1996.

[6] N.E. Dorsey, The freezing of supercooled water, Trans. Am. Philos. Soc. 38 (1948) 248.

[7] J. Duman, T. Olsen, K. Yeung, F. Jerva, The roles of ice nucleation in invertebrates, in: G.J.W.R.E. Lee, L.V. Gusta (Eds.), Biological Ice Nucleation and Its Applications, Amer Phytopathological Society, 1995, pp. 201–219.

[8] F. Franks, The nucleation of ice in undercooled aqueous solutions, Cryo-Letter 2 (1981) 27–31.

[9] F. Franks, J. Darlington, T. Schenz, S.F. Mathias, L. Slade, H. Levine, Antifreeze activity of antarctic fish glycoprotein and a synthetic polymer, Nature 325 (1987) 146–147.

[10] U. Gehrken, Supercooling and thermal hysteresis in the adult bark beetle *Ips acuminatus* Gyll., J. Insect Physiol. 35 (1989) 347–352.

[11] D.E. Hare, C.M. Sorensen, The density of supercooled water. 11. Bulk samples cooled to the homogeneous nucleation limit, J. Chem. Phys. 87 (1988) 4840–4845.

[12] A.D.J. Haymet, A.F. Heneghan, P.W. Wilson, K.E. Zachariassen, H. Ramlov, Nucleation in the presence of fish and insect ice-growth inhibition (“antifreeze”) molecules, in: B.N. Hale, M. Kulmala (Eds.), Atmospheric Aerosols and Nucleation, vol. 534, APS, New York, 2000, p. 410.

[13] A.F. Heneghan, A.D.J. Haymet, Liquid-to-crystal nucleation: effect of a bubble on nucleation, Chem. Phys. Lett. 368 (2002) 177–182.

[14] A.F. Heneghan, A.D.J. Haymet, Liquid-to-crystal nucleation: improved lag-time apparatus to study supercooled liquids, J. Chem. Phys. 117 (2002) 5319–5327.

[15] A.F. Heneghan, A.D.J. Haymet, Nucleation of Pure and AgI seeded supercooled water using an automated lag-time apparatus, in: B.N. Hale, M. Kulmala (Eds.), Atmospheric Aerosols and Nucleation, 534, APS, New York, 2000, p. 439.

[16] A.F. Heneghan, P.W. Wilson, G. Wang, A.D.J. Haymet, Liquid-to-crystal nucleation: automated lag-time apparatus to study AgI seeded supercooled water, J. Chem. Phys. 115 (2001) 7599.

[17] C.L. Hosler, C.R. Hosler, An investigation of the freezing of water in capillaries, Trans. Am. Geophys. Union 36 (1955) 126.

[18] T. Inada, X. Zhang, A. Yabe, Y. Kozawa, Active control of phase change from supercooled water to ice by ultrasonic vibration. 1. Control of freezing temperature, Int. J. Heat Mass Transfer 44 (2001) 4523–4531.

[19] S.L. Johnston, R.E. Lee, Regulation of supercooling and nucleation in a freeze intolerant beetle (*Tenebrio molitor*), Cryobiology 27 (1990) 562–568.

[20] T. Koop, B. Luo, A. Tsias, T. Peter, Water activity as the determinant for homogeneous ice nucleation in aqueous solutions, Nature (London) 406 (2000) 611–614.

[21] E. Kristiansen, S. Pedersen, H. Ramlov, K.E. Zachariassen, Antifreeze activity in the cerabycid beetle rhagium inquisitor, J. Comp. Physiol. B 169 (1999) 5–60.

[22] E.J. Langham, B.J. Mason, The heterogeneous and homogeneous nucleation of supercooled water, Proc. R. Soc. (London) Ser. A 247 (1958) 493.

[23] R.E. Lee Jr., K.E. Zachariassen, J.G. Baust, Effect of cryoprotectants on the activity of hemolymph nucleating agents in physical solutions, Cryobiology 18 (1981) 511–514.

[24] J.V. Leyendekkers, R.J. Hunter, Thermodynamical properties of water in the subcooled region, J. Chem. Phys. 82 (1985) 1440–1453.

[25] D.R. McFarlane, R.K. Kadiyal, C.A. Angell, Homogeneous nucleation and growth of ice from solutions: TTT curves, the nucleation rate, and the stable glass criterion, J. Chem. Phys. 79 (1983) 3921–3927.

[26] A.P. McKenzie, Non-equilibrium freezing behaviour of aqueous solutions, Philos. Trans. R. Soc. (London) B 278 (1977) 167–189.

[27] K. Miyata, H. Kanno, T. Niino, K. Tomizawa, Cationic and Anionic effects of the homogeneous nucleation of ice from aqueous alkali halide solutions, Chem. Phys. Lett. 354 (2002) 51–55.

[28] D.W. Oxtoby, Nucleation, in: D. Henderson (Ed.), Fundamentals of Inhomogeneous Fluids, Marcel Dekker, New York, 1992, pp. 407–442.

[29] H. Ramlov, P. Westh, Ice formation in the freeze tolerant alpine weta hemideina-maori hutton (Orthoptera, Stenopelmatidae), Cryo-Letters 14 (1993) 169–176.

[30] D.H. Rasmussen, A.F. McKenzie, Effect of solute on ice-solution interfacial free energy: calculation from measured homogeneous nucleation temperatures, in: Water Structure at the Water–Polymer Interface, vol. 161, Plenum Press, New York, 1972, pp. 126–145.

[31] D.S. Reid, A.T. Foin, C.A. Lem, The effect of solutes on the temperature of heterogeneous nucleation of ice from aqueous solution, Cryo-Letter 6 (1985) 189–198.

[32] R.A. Ring, Freezing-tolerant insects with low supercooling points, Comp. Biochem. Physiol. 73A (1982) 605–612.
[33] V.P. Skripov, Homogeneous nucleation in melts and amorphous films, Curr. Topics Mater. Res. 2 (1976) 327–378.
[34] V.P. Skripov, G.T. Butorin, Crystallization of supercooled water, Sov. Phys.—Crystallography 17 (1972) 322.
[35] L. Somme, The effect of temperature and anoxia on the hemolymph composition and supercooling in three overwintering insects, J. Insect Physiol. 13 (1967) 805–814.
[36] G. Vigier, R. Vassoille, Ice nucleation and crystallization in water–glycerol mixtures, Cryobiology 24 (1987) 345–354.
[37] G.M. Wang, A.D.J. Haymet, Trehalose and other sugar solutions at low temperature—modulated differential scanning calorimetry (Mdsc), J. Phys. Chem. B 102 (1998) 5341–5347.
[38] D.A. Wharton, M.R. Worland, Ice nucleation activity in the freezing-tolerant antarctic nematode *Panagrolaimus davidi*, Cryobiology 36 (1998) 279.
[39] P.W. Wilson, J.W. Arthur, A.D.J. Haymet, Ice premelting during differential scanning calorimetry, Biophys. J. 77 (1999) 2850–2855.
[40] K.E. Zachariassen, Ice nucleating agents in cold-hardy insects, in: Water and Life, Springer Verlag, Berlin, 1982.
[41] K.E. Zachariassen, Physiology of cold tolerance in insects, Physiol. Rev. 65 (1985) 799–832.
[42] K.E. Zachariassen, E. Kristiansen, Ice nucleation and antinucleation in nature, Cryobiology 41 (2000) 257–279.
[43] B. Zuberi, A.K. Bertram, T. Koop, L.T. Molina, M.J. Molina, Heterogeneous freezing of aqueous particles induced by crystallized $(NH_4)_2SO_4$, ice, and loetovicite, J. Phys. Chem. A 105 (2001) 6458–6464.