Section 1

Fundamental principles

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Physico-chemical principles in low temperature biology

M.J. Taylor
MRC Medical Cryobiology Group
Cambridge

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Introduction

Much of our current understanding of the effects of reduced temperatures on biological systems has evolved from studies on a large variety of biological samples from both the animal and plant kingdoms. Furthermore, many evaluations of the influence of low temperatures have been based solely upon some measurement of survival. This approach has provided a considerable degree of understanding of the factors and variables which govern whether a biological system either withstands decrease in temperature successfully or succumbs to injury. Nevertheless, the exact mechanism of cryoprotection or cryoinjury at the molecular and macromolecular level is ill-understood. A knowledge of the physico-chemical aspects of low temperatures is

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fundamental to a complete understanding of the nature of resistance to low temperature or cryoinjury and cell death.

The major effect of reduced temperatures on any system is the reduction of molecular motion which, for biological systems, has important consequences. The inhibitory effects of low temperatures on chemical and physical processes, including the biochemical reactions of living tissue, provide the basic means for achieving long-term preservation of cells, tissues and organs. In the extreme, temperature reduction to absolute zero ($-273.16°C$) brings to a halt all molecular motion and causes all reactions to cease. In practice, however, no changes of biological importance occur at temperatures below $-150°C$ (Mazur, 1964) and it is only under these conditions that a state of true 'suspended animation' can exist. However, cooling below $0°C$ brings about dramatic changes in biological systems, especially if the water in the intra- and extracellular solutions freezes and separates as ice, giving rise to physico-chemical changes which may be lethal to cells. In nature some organisms have adapted to low temperature environments to avoid injury and in the laboratory, by careful manipulation of cooling and rewarming conditions and application of certain protective compounds, injury may also be avoided to allow the long-term preservation of a wide variety of biological cells and some tissues.

Methods of inhibiting cellular activity by temperature reduction are determined by principles of thermodynamics and heat transfer. These physical methods are complemented by the use of added cryoprotectant compounds whose mode of action is based on physiological and pharmacological principles. The purpose of this chapter is to summarize the physical aspects of reduced temperature and its effects on water and aqueous solutions as a basis for discussion of the effects of low temperatures on biological systems. The treatment of many of the concepts in this review will, of necessity, be brief and in some cases simplified, so the reader requiring greater detail will be directed to more comprehensive articles in the literature.

Heat energy and temperature

Heat is a particular form of energy, and the science of thermodynamics, which arose from interest in processes involving work, heat and material transformations, can be defined as the study of changes in energy of bulk matter and radiation. However, since it is conjectured that the total energy of the universe as a whole is constant (First Law of Thermodynamics), it is necessary to limit consideration to a special isolated part of the universe, a 'system'. The term 'surroundings' thus denotes the rest of the universe. An 'equilibrium' condition is said to apply when the macroscopic properties (volume, temperature, pressure, concentration, energy, etc.) of a system are constant with time. A 'state' is defined as an equilibrium condition for a system. Thermodynamics thus enables a number of useful predictions of what must happen in going from one state to another, as in a chemical reaction, a phase change, translocation of solvent and/or ions across a semi-permeable membrane, flow of electric current, or change in temperature.

As with all forms of energy, heat is a product of an intensity factor (temperature) and a capacity factor (the heat capacity). When heat is removed from a substance, its temperature will fall. Just how much the temperature will fall depends on:

(1) the amount of heat removed;
(2) the amount of substance present;
(3) the chemical nature and physical state of the substance;
(4) the conditions under which heat is removed.

In general, the temperature change (ΔT) for a given amount of substance is directly proportional to the heat (q) removed or added:

$$\Delta T \propto q \qquad\qquad\qquad \textbf{1}$$
$$\Delta T = Cq \qquad\qquad\qquad \textbf{2}$$

where C, the proportionality constant, is the heat capacity. Heat capacities vary greatly from substance to substance. Liquid water has a large heat capacity (75.3 J $(mol\ K)^{-1}$), so that it requires more heat to raise the temperature of 1 mole of water by 1 degree than it would for 1 mole of copper (24.47 J $(mol\ K)^{-1}$) under similar conditions. Many different conditions under which heat is added or removed from a system can be realized in practice, but the two important cases are constant volume (Cv) and constant pressure (Cp). For liquids and solids, Cv and Cp are very nearly the same because the volume change with temperature is quite small. For gases, however, Cp is larger than Cv by the amount of heat necessary to expand the gas. Heat capacities are, in general, temperature-dependent and should be defined precisely only in terms of a differential heat flow (dq) and temperature change (dT) thus:

$$C = \frac{dq}{dT} \qquad\qquad\qquad \textbf{3}$$

The experimental values are usually summarized in the form of a polynomial in temperature; three items suffice to represent data over a considerable range of temperature:

$$C = a + bT + cT^2 \qquad\qquad\qquad \textbf{4}$$

Heat can be defined as that form of energy which passes from one body to another solely as a result of a difference of temperature and manifests itself as disordered motion of molecules. This motion can be resolved into:

(1) translation of molecules from one position to another;
(2) rotation of molecules and parts of molecules;
(3) vibration of parts of molecules in relation to each other.

Each type of molecular motion contributes to the heat capacity of a body. The energy content of a system is therefore a function of its temperature. However, if ice absorbs heat at 0°C or if two blocks of ice are rubbed together, melting, but not necessarily any change in temperature, occurs. Thus there is a difference in the energy content of ice and water. This is called a latent energy or latent heat. The text of Adamson (1979) is recommended as

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further reading for detailed accounts of the concepts of heat, energy and
related thermodynamic topics.

Heat transfer

During cooling or freezing of a biological system the cold environment acts as
a heat sink which absorbs the thermal energy given off by the biomaterial
until that material is in equilibrium with the surroundings. The rate of heat
transfer has important consequences during the cooling and warming of a
biological system and is a major determinant of cell survival during cryo-
preservation (Mazur *et al*., 1970; Taylor, 1984 and elsewhere in this volume).
Within a solution, the number of ice nuclei that form after freezing has been
initiated, the rate at which they grow and the size they attain are all dependent
upon the rate at which heat is removed from the immediate environment.
During warming, the rate at which ice crystals melt and the opportunity for
crystal growth and recrystallization will depend on the rate at which heat is
pumped into the system. These rates are influenced not only by temperature
gradients outside the solution but also by gradients within it, as determined
by the *thermal conductivity* and *specific heat* (heat capacity per unit mass of
substance) of the solution both in the liquid and frozen state, by its latent heat
of fusion and by the overall geometry of the system.

Heat transfer is usually categorized into three modes of transmission:
conduction, convection and radiation.

Conduction is a process in which heat is transferred through a material
(solid, liquid or gas), as a result of a temperature differential, or between
different materials in direct physical contact. It is a convenient way of cooling
and warming a material and involves the transmittance of energy by direct
molecular communication without appreciable displacement of the
molecules involved.

Convection, on the other hand, is a process of energy transfer which
involves the actual movement of bulk material, as either liquid or gas, and it
is the most important mechanism of heat transfer between a solid surface and
a surrounding liquid or gas. This process of energy transfer has been
described as combining the action of heat conduction, energy storage and
mixing motion in a number of steps (Burdette, 1981). Initially, heat flows by
conduction from the solid surface to adjacent molecules of fluid and this
transferred energy causes an increase in the temperature and internal energy
of adjacent fluid molecules. Next, these molecules move to a region of lower
temperature in the liquid where mixing and energy transfer to other fluid
molecules occurs. Thus, energy is stored in the fluid molecules and is carried
as a result of their mass motion. Convection is the primary means of heat
transfer during cooling and freezing of biological systems.

Heat transfer by radiation involves heat flow by emission of electro-
magnetic energy from a material at a high temperature to a material at a lower
temperature, when the materials are separated in space (even by a vacuum).
This mode of heat transfer is important at relatively high temperature differ-
entials, such as that between liquid nitrogen used in cryogenic storage
($-196°C$) and room temperature.

The influence of temperature

The removal of heat from a system slows down both physical and chemical processes in proportion to the loss of heat and therefore to the fall in temperature. Physical phenomena such as osmotic pressure depend solely on the rate of molecular motion so that the decrease in the rate of the process is proportional to the fractional change in absolute temperature. Many chemical reactions, however, depend upon an energy of activation, which is the minimum energy required for molecules to react, and this results in a special relationship between the rate of reaction and temperature (Arrhenius, 1889). Arrhenius found that the log of the reaction rate was proportional to the reciprocal of the absolute temperature. This relationship can be expressed as:

$$\ln K = a \text{ constant} - E/RT \qquad \qquad \textbf{5}$$

From this it follows that a plot of log K against 1/T should be a straight line (the so-called Arrhenius plot) with a slope of $-E/2.3R$, where E is the activation energy and R is the gas constant. It is generally true that rate of reaction decreases with decreasing temperature and a plot of log K against 1/T yields a linear relation over a significant range in accordance with the Arrhenius equation. Such behaviour is not, however, always observed, for an Arrhenius plot can show curvature (and sometimes distinct breaks) probably reflecting changes in reaction mechanism. For example, when applied to the cooling of certain enzyme systems Arrhenius plots have a discontinuity of slope and approximate to two straight lines meeting at an angle, possibly indicating that there is a change in the value of activation energy at the transition temperature (Douzou, 1977).

Furthermore, the phenomenon of cooling retarding metabolism by reducing the number of activated molecules available to take part in physiological processes does not mean that all reaction rates are affected to the same extent or even in the same way by a reduction of temperature. For example, a disparate effect of temperature on the permeability of dog erythrocytes to cations has been reported (Elford and Solomon, 1974) where sodium flux was shown to increase during cooling from 37°C to 20°C and then decrease during subsequent cooling. Also, potassium flux demonstrated a minimum at 12°C and then increased during further cooling, whereas water transport decreased in accordance with a typical Arrhenius relationship. These observations suggest that sodium, potassium and water are transported across the dog red cell membrane by independent mechanisms that are affected differently by temperature. It is clear that the effect of cooling on integrated metabolizing systems is complex, and often unpredictable, such that reaction pathways may become uncoupled producing harmful consequences. Pegg (1981) has recently summarized, in general terms, the cellular mechanisms essential for cell survival and outlined some specific aspects of cooling which have practical implications for cell survival *in vitro*. These include the reduction in demand for metabolites, the effect on active ion transport and cell volume regulation, the consequences of these effects for the design of storage media for tissues to be held at low temperatures, and the phenomenon of thermal shock.

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Water – structure and physical properties as liquid and as ice

Some physico-chemical properties of water and its role in life processes

The universal importance of water as a biological solvent for life processes is obvious, and all such processes are sensitively attuned to the physical properties of water (Franks, 1977). Many of the important chemical properties of macromolecules in biological systems, including conformational stability and biochemical specificity, depend on the interaction of their constituent groups with the surrounding solvent medium. These interactions are, in turn, markedly influenced by the structural features of liquid water and by structural changes in water caused by solutes and changes in temperature.

Cooling alters these interactions and freezing may be influenced by the presence of solutes. Although little is known specifically about solute interactions during the freezing of water, an understanding of this process undoubtedly requires an appraisal of the properties of aqueous solutions. With regard to our current understanding of the structure and properties of water, particularly at sub-zero temperatures, reference must be made to volume seven of the major reference exposition entitled *Water: A Comprehensive Treatise* (Franks, 1982a). The reviews on the properties of super-cooled water (Angell, 1982) and on the properties of aqueous solutions at sub-zero temperatures (Franks, 1982a) are particularly useful.

Water has often been described as an 'unusual' solvent and there are many distinctive properties of water which allow it to play its important role in biological processes. Firstly, the *dielectric constant* or *permittivity* (ϵ), of water is one of the highest known (80 at 20°C). Since the force of attraction between ions varies inversely with ϵ, the attraction between ions is diminished when they are dissolved in water and, as a result, ionic compounds are very water-soluble. Secondly, water has an unusually high heat capacity, a major factor in temperature control in homeothermic animals, and is important in protecting plants from adverse effects of fluctuating temperature. Thirdly, the high heat of vaporization of water enables man and certain animals to effect surface cooling by evaporation of water from the skin, or from the surface of the tongue by panting. These, and other important properties including high surface tension, low viscosity, high melting and boiling points and higher density of the liquid state compared with the solid state (ice) are all ascribed to features of the unique structure of water and to the presence of hydrogen-bonding as described below.

Water structure in the solid and liquid state

The structure of water in the liquid phase is not nearly so clearly defined and understood as that of ice. It is therefore helpful to consider the structure of ice in the first instance.
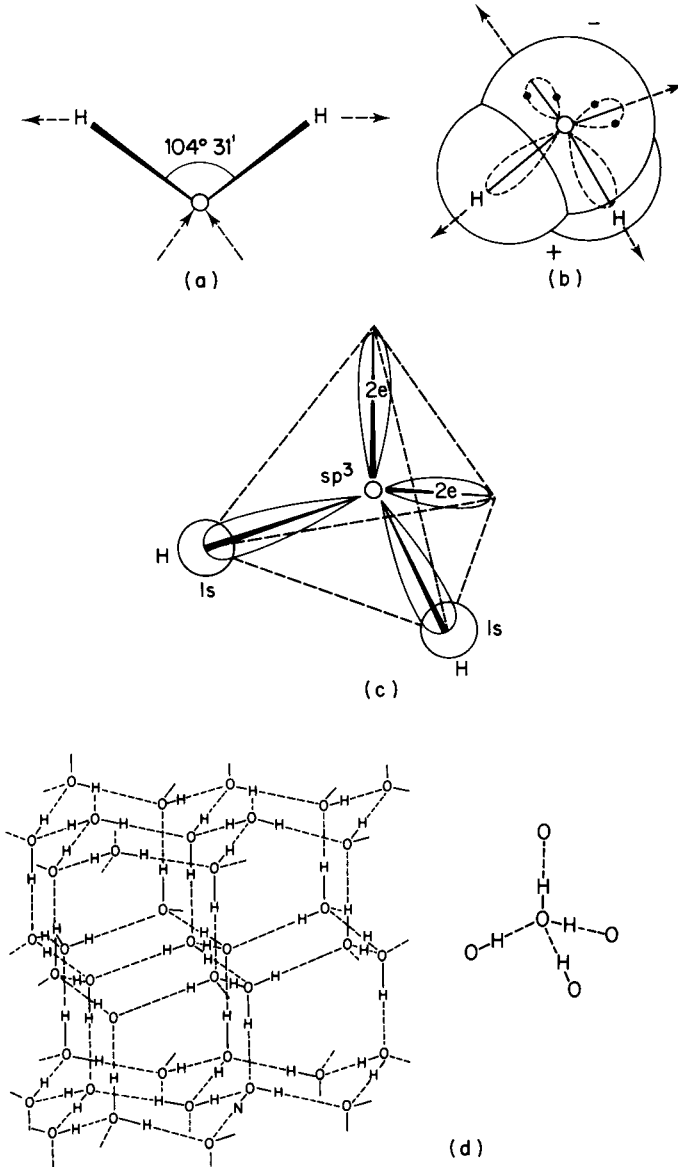
Structure of ice

As shown in Fig. 1.1a, a water molecule exists in the form of an isosceles triangle with an O–H bond length between 0.96 and 1.02Å and an H–O–H

Fig. 1.1   The water molecule and the hydrogen-bonding arrangement in the structure of *Ice I*. Details are given in the text. (a) The bond angle between oxygen and hydrogen in the water molecule. (b) The relative location of the unshared electron pairs. (c) The overall geometry of the molecule is a tetrahedron. (d) When formed into ice, water molecules are hydrogen-bonded to each other. The typical hexagonal ice form at low pressures (*Ice Ih*) is shown here. The tetrahedral hydrogen-bond arrangement for a single water molecule is shown separately for clarity.

The schematic representation of the three-dimensional network of molecules in the crystal structure of ice (d) is adapted from Némethy and Scheraga, 1962. Covalent bonds are represented by solid lines and hydrogen bonds as broken lines.

bond angle of 104°31′. There is a separation of charge within the molecule as a result of the attraction of the oxygen nucleus for electrons which are drawn away from the hydrogen atoms leaving the area around them with a net positive charge. This gives rise to a high molecular dipole moment of 1.87×10^{-18} electrostatic units. The dipole acts along the bisector of the H–O–H angle with the negative end towards the oxygen. The two unshared pairs of electrons of the oxygen atom are concentrated in orbitals which are directed away from the O–H bonds, as illustrated in Fig. 1.1b. The electronic structure of the water molecule is described by considering the O atom to be sp^3 hybridized (because the atomic orbitals arise from the mixing of one s orbital and three p orbitals). The geometry of this configuration is a distorted tetrahedron due to compression of the bond angle by the two lone pairs of electrons (Fig. 1.1c).

This highly polar structure gives rise to the formation of hydrogen bonds in which a positively charged region in one molecule orientates itself towards a negatively charged region of a neighbouring molecule. In water, the number of protons around each oxygen atom that is able to form the positive ends of hydrogen bonds is equal to the number of lone pairs of electrons on that oxygen atom that can form the negative ends; consequently, each molecule tends to have four nearest neighbours resulting in an extensive three-dimensional network in which each oxygen atom is tetrahedrally bonded to four hydrogen atoms in two covalent bonds and two hydrogen bonds (Fig. 1.1d).

Several forms of ice structure are known to exist depending upon pressure and temperature. The normal form of ice at low pressures is called *ice Ih* (hexagonal ice). This is a very open structure in which the individual water molecules, arranged tetrahedrally with a bond angle of 109°28′ as shown in Fig. 1.1d, retain a considerable degree of freedom and are capable of rotation in the crystal. This is reflected in the relatively high dielectric constant of ice. The hydrogen bonds responsible for this structure are straight and thus the structure is virtually strain-free. Interestingly, the density of ice at 0°C is 0.917, whereas that of liquid water at this temperature is 0.999 (if ice was a close-packed structure, its density would be about 1.7). This fact is of ecological significance because if it were not for this unique type of hydrogen bonding, ice, like most other solid substances would be heavier than the corresponding liquid and on freezing, would sink to the bottom of a pond causing all the water to freeze gradually. Most living organisms in the body of water would not survive. Fortunately, water reaches its maximum density at $+4°C$ so cooling below this temperature decreases the density, allowing it to rise to the surface where freezing occurs. The ice layer that forms on the pond surface does not sink and, more importantly, acts as a thermal insulator for the water below it.

At high pressures (>2000 atm) six different crystalline forms of ice can exist, each in a particular pressure and temperature range (Kamb, 1965; Eisenberg and Kauzmann, 1969; Fletcher, 1970; Franks, 1972) (see Fig. 1.3). Under these conditions the water molecules are fully hydrogen-bonded, but are arranged so that the space is more fully occupied, and often two inter-penetrating networks are formed (Kamb, 1965). In such structures the

hydrogen bonds are bent or the molecules compressed, however these forms of ice do not arise under the conditions of interest in low temperature biology.

At very low temperatures (below $-140°C$) a metastable form of ice I, cubic ice (*ice Ic*) can exist, but it can only form under special conditions, such as on condensation from the vapour (Bertie *et al.*, 1963). It cannot form by cooling ice Ih, although its hydrogen-bonding configuration is a modification of that of ordinary ice. It crystallizes in a cubical form instead of the hexagonal crystal structure and on warming it changes into ice Ih.

One other form of water crystal will be mentioned for completeness, although having restricted relevance in low temperature biology. *Clathrates* are inclusion compounds (or gas hydrates) that arise from the entrappment of a neutral 'guest' molecule such as Cl_2 or SO_2, which are accommodated in cavities of molecular size within the spatial network formed by the hydrogen-bonded water. No chemical bonds are formed between the two substances, although the crystals are stabilized by van de Waal's forces acting between the guest molecule and water molecules forming the cavity.

Among the various forms of ice, only ice I is produced during either slow or rapid freezing of water or of aqueous solutions at low pressures. The only other low pressure form, ice Ic, cannot form from the liquid.

Structure of liquid water

The structure of liquid water is not as well understood as that of solid ice; it is, however, generally agreed that the tetrahedral co-ordination of water molecules found in ice also exist in a more imperfect form in liquid water. (This configuration has been established by X-ray diffraction of ice, as well as by X-ray scattering of liquid water.) As we have seen, ice has a very open structure, and the increase in density on melting was at first explained as simply the result of the breakdown of the ice structure into individual water molecules, with perhaps some small hydrogen-bonded units present. However, this would not account for many of the properties of water, including its high critical temperature. Consequently, a number of models have been proposed to take account of the unique properties of this liquid. None of these models can account satisfactorily for all the observed properties of water, but progress has been made toward a detailed explanation of its structure and properties (e.g. Némethy and Scheraga, 1962; Némethy, 1968; Frank, 1970, 1972; Conway, 1981).

Bernal and Fowler (1933) suggested that the hydrogen-bonding found in ice also exists in water, although the regular three-dimensional network in the solid no longer exists – instead, some hydrogen bonds are broken and the coordination number increases above four. This explains why the volume decreases upon melting. The free monomeric water molecules, released as some of the hydrogen-bonds break, are able to occupy spaces in the remaining 'icelike' lattice; this explains why the density of water is greater than that of ice. As the temperature rises, so more hydrogen bonds are broken, but at the same time the kinetic energy of molecules increases. Higher temperature increases the density of water, while elevated kinetic