A mathematical model for the freezing process in biological tissue

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A mathematical model has been developed to study the process of freezing in biological organs. The model consists of a repetitive unit structure comprising a cylinder of tissue with an axial blood vessel (Krogh cylinder) and it is analysed by the methods of irreversible thermodynamics. The mathematical simulation of the freezing process in liver tissue compares remarkably well with experimental data on the structure of tissue frozen under controlled thermal conditions and the response of liver cells to changes in cooling rate. The study also supports the proposal that the damage mechanism responsible for the lack of success in attempts to preserve tissue in a frozen state, under conditions in which cells in suspension survive freezing, is direct mechanical damage caused by the formation of ice in the vascular system.

INTRODUCTION

Freezing of biological tissue has important applications in medicine, both for controlled destruction of tissue (cryosurgery) and, with the addition of appropriate protective agents, for preservation of viability in the frozen state (cryopreservation). Cryosurgery now has an established place in the surgeon’s armamentarium (Gage 1982), including its use in the treatment of malignant disease. Recent studies, in which the extent of the frozen region during treatment of non-resectable tumours in the liver was continuously monitored by ultrasound, have shown that the extent of the destroyed tissue corresponds to that of the frozen region, within the limits of resolution of the method (Gilbert et al. 1985; Onik et al. 1986; Rubinsky 1986). The mode of tissue freezing in the absence of cryoprotective agents is therefore of considerable interest.

On the other hand, in the presence of cryoprotective agents, many biological materials can be enabled to survive freezing, which permits the reduced temperature to be used to retard the process of deterioration ex vivo and ultimately to secure essentially infinite preservation of viability. The conditions necessary to achieve this objective include not only the addition of a cryoprotective solute,
which reduces the amount of ice formed at any given subzero temperature and ameliorates the effect of rising electrolyte concentration (Lovelock 1954), but also optimization of rates of change of temperature to avoid intracellular ice (Mazur et al. 1970). These phenomena, as they apply to isolated cell systems, are now reasonably well understood and techniques for the cryopreservation of many different cell types, such as red blood cells (Smith 1950), lymphocytes (Ashwood-Smith 1964), spermatozoa (Polge et al. 1949) and embryos (Whittingham et al. 1972) are in everyday use. However, although cells from organs such as the kidney (Chagnon & Pavilanis 1966), the heart (Alink et al. 1977) and the liver (Fuller et al. 1980) can, to varying extents, survive freezing in cellular suspensions, attempts to preserve whole organs in a frozen state have proved unsuccessful (Jacobsen & Pegg 1984). Although whole organs will not survive freezing, individual cells within those organs may survive (Alink et al. 1977; Pegg et al. 1984). Thus there appear to be additional mechanisms causing damage to organs and tissues that do not apply to isolated cells (Pegg et al. 1979; Pegg & Jacobsen 1983).

We have mentioned that the survival of cells in suspension is dependent on the cooling rate during freezing, that is the temperature change in unit time. Experimental evidence indicates that the relation between the cooling rate and the percentage of cells remaining viable after cryopreservation can generally be plotted as an inverse U-shaped curve, with maximal survival at a certain optimal cooling rate and with survival decreasing for cooling rates both above and below the optimal rate. The generally accepted explanation for the effect of cooling rate was proposed by Mazur (1963, 1970), who also developed a mathematical model for the process. Mazur suggested that during the freezing of a cellular suspension the probability that a critical ice nucleus forms in the extracellular space is much higher than in the intracellular space; ice will therefore form first in the extracellular space. Mazur also assumed that ice will not propagate through the cell membrane and consequently that ice will form independently in the intracellular and the extracellular spaces. Ice formation in the cell will depend on the probability of a critical ice-nucleus forming in the cell, which is an inverse function of the absolute temperature and a direct function of the dimensions of the cell. For freezing with sufficiently low cooling rates, where temperature is relatively high for long periods of time, the intracellular water can remain unfrozen while the extracellular solution freezes; when ice forms in the extracellular space, solute is rejected and the remaining solution becomes hypertonic. To equilibrate the difference in chemical potential between the intracellular and extracellular solutions, water must then leave the cell through the cell membrane, which is permeable to water but essentially impermeable to most of the solutes that are present. Consequently the cell dehydrates and shrinks, and the intracellular solute concentration increases. It was proposed by Lovelock (1953) and developed by Mazur (1977a) that high intracellular electrolyte concentrations have a damaging effect, which increases with the time of exposure. Because water transport is a rate-dependent process, freezing at higher cooling rates decreases the time for which a given cell is exposed to damaging conditions, which explains the increase in cell viability as cooling rate is increased towards the optimum. However, when
cells are frozen at supra-optimal cooling rates, the probability that intracellular ice will form increases (Mazur 1970). Experimental evidence shows that intracellular ice is usually damaging to cells (Mazur 1977a), and this is assumed to be responsible for the decrease in cell viability at cooling rates above the optimal.

The process of freezing in cellular suspensions has been studied extensively by light microscopy (Diller 1982), with special temperature-controlled microscope stages, in which the temperature of the specimen is uniform spatially and can be varied in time. These experiments have indeed shown that low cooling rates permit the cells to shrink, whereas high cooling rates cause intracellular ice to form. Thus the freezing process in cellular suspensions has been studied extensively both through analytical models (Mazur 1963; Diller & Lynch 1983) and experimentally (Mazur 1977b; Leibo 1977; Diller 1982). The process of freezing in tissues and organs has also been studied experimentally using isothermal freeze-fixation and freeze-substitution techniques with transmission light or electron microscopy (Taylor & Pegg 1983; Jacobsen et al. 1984; Hunt 1984; Pegg & Diaper 1982; Pegg 1987), and these studies have suggested that extracellular, and particularly intravascular, ice is extremely damaging in such systems, although it is innocuous to cell suspensions.

Recently a new experimental technique has been developed which allows controlled directional freezing in tissue and permits us to study the thermophysical phenomena which occur during freezing (Rubinsky & Ikeda 1985; Rubinsky et al. 1987). The experimental studies, which were performed with slices of liver tissue, will be discussed in more detail later; they are entirely compatible with the whole organ or tissue studies referred to above. However, before this work, no mathematical model for the freezing process in tissue has been described. The model, which is based upon the experimental observation of freezing within the capillaries, accurately accounts for the observed experimental results. The results from this model will be compared with a mathematical model for the freezing process in cellular suspensions that is similar to that developed first by Mazur (1963), and an explanation will be presented for the specific mechanism responsible for damage in vascularized tissue, which can cause an organ to be destroyed under conditions that permit the cells to survive.

**Experimental results**

An experimental technique using directional solidification of tissue samples and low-temperature scanning electron microscopy (LTSEM) was developed to study the process of freezing in tissue, and to provide the information needed for the development of the mathematical model. Livers were excised from adult female Sprague–Dawley rats under ether anaesthesia. Tissue slices (8 mm × 3 mm × 2 mm) were cut by hand, and frozen in a longitudinal direction on the directional-solidification stage previously described (Rubinsky & Ikeda 1985; Rubinsky et al. 1987); the stage has two separate bases separated by a 3 mm air gap and maintained at different constant temperatures, one above and the other below the phase transition temperature. A substrate, on which the liver sample rests, is placed across the bases, and the system achieves a linear temperature distribution.
in the substrate and the sample between the bases. In typical experiments the substrate is moved with a constant velocity across the bases from the high to the low temperature, causing the sample to freeze in the longitudinal direction, with a constant cooling rate between prescribed temperatures. Liver slices were frozen at several cooling rates from 2 to 220 °C min\(^{-1}\), between +35 and −40 °C. After freezing, the samples were immersed in liquid nitrogen, fractured, gold-coated and studied in the fully hydrated state by LTSEM.

Cross-sectional and longitudinal views of sinusoids and surrounding hepatocytes were studied and compared with published scanning electron micrographs of the normal liver by using perfusion fixation (Motta et al. 1978; Macchiarelli & Motta 1986). On the electron micrographs, ice crystals appear as smooth surfaces whereas the tissue surface appears rough; single ice crystals were observed along the sinusoids at all cooling rates but for lower cooling rates, of the order of 1 °C min\(^{-1}\), the sinusoids were expanded relative to the normal liver, and the adjacent hepatocytes were dehydrated without intracellular ice. This can be observed in figure 1, plate 1, which shows SEM images of normal liver tissue (figure 1a) and tissue frozen at about 4 °C min\(^{-1}\) (figure 1b, c). Comparison of the micrographs of normal liver (a) with the frozen liver (b, c) shows the sinusoids to be expanded and the hepatocytes dehydrated, but the distance between the centres of adjacent sinusoids is similar to that in the normal tissue.

At higher cooling rates, intracellular ice was seen; figure 2, plate 2, was obtained by freezing liver tissue slices at 220 °C min\(^{-1}\). Here, single ice crystals with dimensions between 3 and 7 μm can be seen in addition to the ice in the sinusoids. These smaller ice crystals are located between sinusoids and surrounded by a layer of cellular material; the distance between the centres of adjacent small ice crystals is much smaller than that between adjacent sinusoids. For these reasons we believe that the small ice crystals are intracellular.

Both in cryosurgery and in attempted cryopreservation organs are frozen from the outer surface towards the interior and the freezing interface propagates in the direction of the temperature gradient. We may assume, with Mazur (1963, 1970), that ice does not propagate through the cell membrane (Chaw & Rubinsky 1985). Ice forming in the vascular system will propagate along the blood vessels where there is no barrier to the crystal growth process; such ice will propagate in the general direction of the temperature gradient but in the particular direction of the blood vessel, which would be consistent with the experimental observation that single ice crystals are seen within the blood vessels. Again, with Mazur, we may assume that water in the cells surrounding the frozen blood vessel, being compartmentalized in small volumes, will at first remain supercooled. As the intravascular ice forms, water is removed from the solution in the vascular system, which renders that solution hypertonic, and will cause a flow of water from the cells to equilibrate the chemical potentials. Consequently the cells will dehydrate, and the water that leaves the cell will freeze in the blood vessels, which will therefore expand. This would explain the observed expansion of the sinusoids during freezing at low cooling rates. Water transport into the vascular system is a rate-governed process, which means that the cells in the tissue that is frozen at high cooling rates will supercool sufficiently for intracellular ice to form; this again is consistent with our observations.
Figures 1. SEM images of liver sinusoids (S), also showing adjacent hepatocytes (H). The structure of the normal liver (a) is compared with that of tissue frozen at a cooling rate of 4°C min⁻¹ longitudinally in (b) and transversely in (c) (scale bar 10 μm). (a) is reprinted with permission from Macchiarelli & Matta (1985).

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Figure 2. Transverse section through sinusoids (S), adjacent hepatocytes (H), and intracellular ice (I) in liver frozen at a cooling rate of 220 °C min⁻¹ (scale bar 10 μm).
Analysis

Our purpose is to establish a basic mathematical model which can be used to predict and analyse the physical processes that occur during the freezing of organs. From our experimental results it is evident that the freezing process, and the ultimate structure of the frozen tissue, depend on the mass transport of water from cells into the vascular system. Several analytical models have been developed for studying this process, and an extensive review of the literature in this area can be found in several texts (Crane & Larsen 1970; Middleman 1972; House 1974). A basic assumption in all models is that the mass transport across the vascular system occurs mostly in the smaller blood vessels (capillaries in general, sinusoids in the liver), where the ratio of surface area to tissue volume is much greater than that for the larger vessels. In most of the studies, the geometrical regularity of the small blood vessels is used to justify the concept of a repetitive tissue–blood-vessel unit such as that shown in figure 3. The mass transfer process in an organ can then be modelled by a large number of identical units, and by considering the mass process in such a unit to be typical and representative of the processes in the whole organ. The mass transfer process in such a ‘unit structure’ was first analysed by the Danish physiologist Krogh (1919), and this unit is usually referred to as a Krogh cylinder. A model was therefore developed to study the freezing process in an organ using a repetitive, tissue–blood-vessel unit structure.

Figure 3. The Krogh cylinder. $r$, radius of blood vessel; $K$, outer radius of unit; $l$, length of unit.

The typical structure of a group of adjacent sinusoids can be seen in figure 1. At a local level, the sinusoids appear as a group of parallel cylinders surrounded by cellular material. Adjacent sinusoids share between them one or two hepatocytes, in a variety of geometrical arrangements imposed by the shape of the hepatocytes. For this study, typical values for the diameter of sinusoids and for the tissue material around the sinusoids were determined by averaging measurements from
scanning electron micrographs of the normal liver. In the classical application of the Krogh cylinder to studies of mass transfer with bulk fluid flow, the length of the cylinder enters into the calculations, but this is not so in our study, which deals only with local thermodynamic phenomena. Nevertheless we shall define the concept of the model more fully by assuming that the length of the Krogh cylinder is that of a single hepatocyte, about 20 μm. This will then justify the assumptions that the diameter of the sinusoid is uniform along the length of the tissue unit and that the tissue unit is isothermal during freezing. In the experiment analysed here, the maximum possible temperature difference across such a tissue unit was of the order of 0.2 °C.

In the analysis of the water transport process it will be assumed that the transport occurs only across the boundary between the blood vessel lumen and the tissue and not between adjacent tissue units or by diffusion along the vascular system, which is justified if the temperature difference between adjacent units is not large. It is also assumed that the tissue can be represented by one compartment containing a solution of one solute in water, initially in osmotic equilibrium with the solution in the blood vessel, and that the concentration in the tissue and in the blood vessel can be represented by one lumped value for each region. In reality there are two pathways for the flow of water from hepatocytes into the blood vessel, through the fenestrations in the basement membrane of the blood vessel and through the endothelial cells, but we assume that the permeability to water of the interface between the blood vessel and the tissue has a set of constant lumped properties and is dominated by the permeability of the hepatocytes. The membrane is assumed to be impermeable to the solutes present in the cell and the vascular system. These simplifying assumptions are not specific to this analysis, but are commonly made in other studies of mass transport across the vascular system (Bassingthwaite 1979; Pegg et al. 1986; Hempling 1988). Although more complex models could be used, they are quite controversial, and it was felt that this first model of the freezing process in biological tissue did not justify greater complication; moreover, it should be sufficiently basic for its actual application not to be prevented by lack of experimental data. Finally, we also assume that the change in density of water upon freezing can be neglected.

The equations developed by Kedem and Katchalsky using the methods of irreversible thermodynamics (Katzir-Katchalsky 1976) will be used to describe the volumetric flow of water, \( J \), into the blood vessels during freezing.

\[
J = SL[(P_t - P_i) - \sigma RT(C_{st} - C_{sf})],
\]

where \( P_t - P_i \) is the difference in hydrostatic pressure between the tissue and the fluid in the blood vessel, and \( C_{st} - C_{sf} \) is the difference in molar concentration between the solute in the tissue and in the blood vessel. Here, \( S \) is the surface area between the blood vessel and the lumen, and \( RT \) is the product universal gas constant multiplied by absolute temperature. The equation contains two permeability parameters: the reflection coefficient, \( \sigma \), which in this work is taken to be unity, and the permeability, \( L \), of the interface between the blood vessel and the tissue, which will be taken to be that of the hepatocyte. In this study we will
assume that the membrane permeability, \( L \), to water is temperature-dependent and given by Diller & Lynch (1983),

\[
L = L_0 \exp \left[ -\frac{E}{R} \left( \frac{1}{T_0} - \frac{1}{T} \right) \right],
\]

where \( E \) is an activation energy. Under normal physiological conditions the maximal difference in hydrostatic pressure between the tissue and the fluid in the vascular system is equivalent to a difference in osmolality of less than 5 mosmol kg\(^{-1}\), and the contribution of the pressure difference will therefore be neglected.

The ice forming in the vascular system must be in thermodynamic equilibrium with the solution surrounding the ice. The relation between the change of phase temperature in aqueous solutions and molal concentration, \( m \), is given with reasonable accuracy (Mazur & Schneider 1986) by

\[
T = 273.15 - 1.86 \phi \nu m,
\]

where the temperature is given in kelvins, \( \phi \) is the osmotic coefficient and \( \nu \) is the number of species into which the solute dissociates. We assume that \( \phi = 1 \) and as the main solutes are sodium and potassium chloride, \( \nu = 2 \). To facilitate the mathematical solutions we will use molar concentrations in \( \text{(3)} \); in the range of concentrations encountered in this work, this should lead to errors not larger than 10% (Weast 1986).

As shown in figure 3, the volume of the tissue, \( V \), is given by

\[
V = l(2\sqrt{3}K^2 - \pi r^2),
\]

where \( l \) is the length of the unit, \( K \) is the outer radius of the unit and \( r \) is the radius of the blood vessel. Our experimental studies showed that water leaves the tissue to freeze in the vascular lumen, causing the radius of the blood vessel to expand, while the distance between the centres of the sinusoids remains unchanged. To satisfy continuity, the volumetric flow of water from the tissue, \( J \), where \( t \) is time, can be determined from \( \text{(5)} \):

\[
J = -\frac{dV}{dt} = 2\pi r l \frac{dr}{dt}.
\]

Also from considerations of mass conservation in the tissue, the molar concentration of solute in the tissue, \( C_{st} \), as a function of the isotonic concentration \( C_{st,0} \) and the transient radius of the capillary \( r_t \), is given by

\[
C_{st, t} = C_{st,0} \left( \frac{2\sqrt{3}K^2 - \pi r_t^2 - V_0}{2\sqrt{3}K^2 - \pi r_0^2 - V_0} \right),
\]

where \( r_0 \) is the radius of a sinusoid in the normal state and \( V_0 \) is the non-solvent volume of the tissue. We will assume that the non-solvent volume is equal to the non-osmotic volume derived from a Boyle van't Hoff plot; by extrapolation from the volume measurements on hepatocytes by Graut & Fuller (1982) this will be assumed to be 20% of the volume of the tissue.

\^ One osmole contains one mole of osmotically active particles.
When (1), (2), (3), (5) and (6) are combined, considering the surface area, $S$, between the blood vessel and the tissue to be $2\pi rl$, an expression can be obtained for the rate of change of the radius of the blood vessel as a function of time.

$$\frac{dr_e}{dt} = -LRT C_{st,0} \left( \frac{2 \sqrt{3K^2 - \pi r_0^2 - V_0}}{2 \sqrt{3K^2 - \pi r_0^2 - V_0}} \frac{273.15 - T}{1.860} \right),$$

where $T$ is the Kelvin temperature reached by the tissue at time $t$, after the onset of the freezing process. From knowledge of the thermal history at each location the radius of blood vessels during freezing in an organ can be estimated by solving (7). The concentration in the tissue can then be evaluated from (6). The constitutional supercooling, $\Delta T$, of the water in the tissue can be estimated from

$$\Delta T_t = 273.15 - T - 1.860C_{st}.$$

In most experiments in cryobiology, and therefore in this analysis, the tissue was frozen with a constant cooling rate, $H$.

$$\frac{dT_t}{dt} = H.$$

Equation (9) can be used to remove the time dependency in (7) and generate a direct dependency between all the variables in this problem and temperature,

$$\frac{dr_e}{dT} = -\frac{LRT C_{st,0}}{H} \left( \frac{2 \sqrt{3K^2 - \pi r_0^2 - V_0}}{2 \sqrt{3K^2 - \pi r_0^2 - V_0}} \frac{273.15 - T}{1.860} \right).$$

The radius of the blood vessel will change during freezing as a function of the cooling rate $H$ and the permeability $L$. For freezing with infinitesimally slow cooling rates, the solute concentration in the tissue will be in equilibrium with that in the vessel. The dependence of the solute concentration on temperature can then be obtained directly from (3), and the radius of the blood vessel as a function of temperature can be derived from (6).

$$r_T = \sqrt{\frac{1}{\pi} \left[ 2 \sqrt{3K^2 - V_0} - C_{st,0} \left( 2 \sqrt{3K^2 - \pi r_0^2 - V_0} \right) \frac{1.860}{273.15 - T} \right]}.$$

To compare the freezing process in tissue with the freezing process in cells in suspension, a set of equations for the freezing process in hepatocytes in suspension will be derived. The analysis is similar to that first presented by Mazur (1963) but the equations have been modified to facilitate comparison with freezing in tissue. It is assumed that the hepatocyte can be modelled as a sphere, and that freezing occurs first in the extracellular space causing an increase in the extracellular solute concentration; water then leaves the cells to freeze in the extracellular space. The water transport from a cell can be modelled by an equation similar to (10), and the relation between temperature and the extracellular concentration is given by (3). The volume of the cell $V_c$ is approximated in this simplified analysis by a sphere,

$$V_c = \frac{4\pi r_{ce}^3}{3},$$

where $r_{ce}$ is the radius of the cell.

By using equations similar to (1), (2), (3), (5), (6) and (9), in addition to (12), the
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rate of change in the radius of the cell with temperature, the intracellular concentration $C_{sc}$, and the radius of the cell at equilibrium are given by (13), (14) and (15) respectively.

$$\frac{dr_{ce, T}}{dT} = -\frac{LRT C_{sc, 0}}{H} \left( \frac{4\pi r_0^3 - 3V_0}{4\pi r_T^3 - 3V_0} \frac{273.15 - T}{1.86\phi T} \right),$$

(13)

$$C_{sc} = C_{sc, 0} \left( \frac{4\pi r_0^3 - 3V_0}{4\pi r_T^3 - 3V_0} \right),$$

(14)

$$r_{ce, T} = \sqrt[3]{\left[ \frac{1}{4\pi} \left( \frac{4\pi C_{sc, 0} r_0^3 - 3V_0}{273.15 - T} \right) 1.86\phi T \right] + 3V_0}.$$  

(15)

Equations (9) and (13) are first-order, nonlinear differential equations. A numerical solution can be obtained to these equations using an Euler forward integration scheme, with a temperature step $\Delta T$, of the form,

$$\frac{dr_T}{dT} = \frac{r_{T+\Delta T} - r_T}{\Delta T}. $$

(16)

When (16) is introduced into (10) and (13) a general finite difference form, given by (17), is obtained.

$$r_{T+\Delta T} = r_T + \Delta T(r.h.s.)_T,$$

(17)

where r.h.s. stands for the right-hand side of (10) or (13), the subscript $T$ indicates terms evaluated at temperature $T$, and the subscript $T+\Delta T$ indicates terms evaluated at temperature $T+\Delta T$.

Results and Discussion

Simulations of the freezing process in liver tissue and in single hepatocytes were done for cooling rates of 0.1, 1, 10 and 100 °C min$^{-1}$. From the scanning electron micrographs of normal liver (Motta et al. 1978), it was determined that the average radius of a sinusoid is 4.6 μm, whereas the average outer radius of a tissue unit is 11 μm. According to Alpini et al. (1986), the permeability to water of hepatocytes is $0.74 \times 10^{-6}$ cm s$^{-1}$ atm$^{-1}$, and the average radius of a hepatocyte is 10 μm. The activation energy, $E$, was taken to be 20 kJ mol$^{-1}$ (Diller & Lynch 1983). Equations (10) and (13) were solved numerically to determine the radius of the blood vessel and of the hepatocytes as a function of temperature with a temperature step of 0.1 °C, which was determined through a halving procedure to produce accurate results. The volume of the tissue and of the hepatocyte, the concentration and the degree of supercooling were determined from (4), (12), (6), (14) and (8).

The volume of the hepatocyte-tissue portion of the tissue-blood-vessel unit, and of the hepatocyte, relative to their initial volumes, is shown as a function of temperature in figure 4. The results are presented for the different cooling rates. The behaviour of the systems can best be understood when compared with the equilibrium curve obtained for an infinitesimally low rate of freezing, which is the same for the tissue and the individual hepatocyte. The results show that for any

$\dagger$ 1 atm = 101325 Pa.
cooling rate the volume of the tissue and of the cell is initially larger than for the equilibrium case; however, at lower temperatures the volume decreases to the equilibrium-curve value. This is a consequence of the finite permeability of the membrane to water transport. The higher the cooling rate the larger will be the volume, which implies that for higher cooling rates less water will leave the tissue or the cell at any temperature simply because that temperature was reached more rapidly at higher cooling rates. For a cooling rate of 1 °C min⁻¹, the tissue dehydrates completely at about −6 °C; for cooling rates of 10 °C min⁻¹ the tissue is fully dehydrated at about −18 °C; for a cooling rate of 100 °C min⁻¹, the tissue is not completely dehydrated in the temperature range shown in the figure. The dehydration of a single hepatocyte is faster than that of the tissue unit because the surface-area:volume ratio is greater, but the behaviour is not so very different.

Figure 5 shows the intracellular solute concentration in the tissue and in a single hepatocyte as a function of temperature for the different cooling rates. The concentration during equilibrium freezing, which is given by (3), is also shown. The results show that, for higher cooling rates, the intracellular concentration is lower at all temperatures. These results are similar to those obtained by Mazur (1963, 1970) for cells in suspension. Freezing at higher cooling rates indeed results in a lower internal solute concentration at any temperature, which may be responsible for the general decrease in the extent of damage due to solute concentration (‘solution effects’) that is commonly observed in many cells (Mazur et al. 1970). The intracellular concentration starts to increase rapidly to the equilibrium value at a temperature of about −2 °C for a cooling rate of 0.1 °C min⁻¹; for freezing at 1 °C min⁻¹ the concentration starts to increase rapidly at −6 °C; for
freezing at 10 °C min⁻¹, the concentration increases only at about −17 °C. The figure shows that the concentration in tissue will always be lower than that in a suspended cell frozen under similar conditions, which implies that during the freezing of an organ, the damage due to 'solution effects' should be less than during the freezing of cells in a suspension. However, the difference is not great; again it is a manifestation of the difference in the ratio of surface area to volume.

Supercooling, in both the tissue and the hepatocyte, is shown as a function of temperature in figure 6. The figure shows that for cooling rates of the order of 1 °C min⁻¹, the maximum supercooling is about 4 °C, increasing to a supercooling of about 12 °C for cooling at 10 °C min⁻¹, and reaching very high values for cooling rates of the order of 100 °C min⁻¹. It is interesting to note that for all cooling rates the supercooling reaches a maximal value and then decreases rapidly. As the extent of supercooling indicates the probability of intracellular ice formation, the results in figures 6 and 4 imply that for freezing with a cooling rate of ca. 1 °C min⁻¹ the probability of intracellular freezing before complete dehydration is small in both the cell and tissue. This conclusion is consistent with the experimental results in figures 1 and 2 which show major dehydration of the tissue and expansion of the vascular system. For freezing at ca. 100 °C min⁻¹, the results in figure 6 indicate a high probability of intracellular freezing, which is also consistent with the experimental results in figure 2. Thus the mathematical model here predicts both qualitatively and quantitatively the behaviour of tissue during freezing. The results show that the probability of intracellular freezing, as expressed by

**Figure 5. Intracellular concentration of solutes (mol l⁻¹) in the tissue unit and a hepatocyte as a function of temperature during freezing with different cooling rates.** —, Tissue; ——, isolated cell. Cooling rates (degrees Celsius per minute) as indicated.
the degree of supercooling, is somewhat higher in tissue than in the suspended hepatocytes.

Experimental freezing of hepatocytes in suspension has been reported by Fuller et al. (1980) and these data are reproduced in figure 7. Biochemical function, measured by protein synthesis, was low for all cooling rates, but urea synthesis and structural integrity, as indicated by trypan blue exclusion, show the typical inverse U-shaped dependence on cooling rate. It is seen that the decreasing branch of the inverse U which, as we discussed earlier, can be attributed to the formation of intracellular ice, occurs at cooling rates higher than about $10^\circ C\, \text{min}^{-1}$. This would produce a maximum supercooling of $9^\circ C$ according to our analytical predictions (figure 6) which again is highly consistent.

It is hardly surprising that the freezing process in tissue as visualized by this analysis, and particularly the effect of cooling rate on cell dehydration and intracellular ice formation, is very similar to that in suspensions of single cells. However, there is one major difference: the extracellular space is very large (assumed to be infinite) in the case of the cell suspension, and extracellular ice formation is in any case assumed to be innocuous per se. In the tissue unit, however, the extracellular space (the vessel lumen) is smaller in volume than the tissue, and events within the vessels cannot be assumed to be irrelevant. Figure 8 compares the radius of the blood vessel with the radius of a hepatocyte as a function of temperature, for different cooling rates. The vascular distension will inevitably produce gross changes in the morphological structure of a frozen tissue; although a cell will shrink during freezing to only about 80% of its initial radius, the radius of the blood vessel will expand about 200% from its initial value. The ratio of the
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**Figure 7.** Viability of hepatocytes, frozen in suspension, as a function of cooling rate. ▲, Urea synthesis; ○, protein synthesis; ●, dye exclusion. Reproduced, with permission, from Fuller et al. (1980).

**Figure 8.** Normalized radius \( r/r_0 \) of the sinusoid within a Krogh unit, and of an isolated hepatocyte as a function of temperature for freezing with different cooling rates. ——, Sinusoid; ———, isolated cell. Cooling rates (degrees Celsius per minute) as indicated.

The volume of the vessel lumen during freezing to that of the cells in the tissue unit is given by

\[
\frac{\pi r_s^2}{2 \sqrt{3K^2 - r^2}},
\]

(18)

With the data from our study, this ratio is initially 0.19, but at the end of the freezing process it is 1.55, an eightfold increase. This result emphasizes the tremendous expansion of the vascular system during freezing, an expansion which must impair the structural integrity of the capillaries, and indeed such damage has been directly observed in frozen kidneys (Jacobsen et al. 1984). This result also
explains why individual cells can survive freezing, although an organ cannot survive under similar conditions. Also, we now have an explanation for the observation that, in the liver, cryosurgery is successful in destroying tissue up to the margin of the frozen region as visualized by ultrasound. Figure 8 shows that for freezing at the very low cooling rates that occur at the margin of the frozen region during cryosurgery, the radius of the blood vessels will expand very significantly, even for temperatures as high as $-1\,^\circ\text{C}$. Consequently, even if malignant cells should be spared destruction owing to the fact that the low cooling rate avoids intracellular freezing, the microcirculation will be destroyed in the frozen region, resulting in eventual ischaemic necrosis.

**Conclusions**

A mathematical model for the study of freezing in tissues has been established, by using the concept of a repetitive unit structure composed of a cylinder of tissue with an axial blood vessel; the use of the Krogh cylinder in this context is new. The model is able to account for both qualitative and quantitative experimental results obtained during the freezing of liver tissue. The results show that during freezing ice propagates along the vascular system; when the cooling rate is low, the cells around the vascular system become dehydrated and the blood vessel expands; with higher cooling rates, intracellular ice forms. The results show that the process of freezing has similar consequences for the cells in an organ and for cells in a cellular expansion but that the organ itself cannot survive primarily because of the structural damage to the vascular system and connective tissue elements.

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