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Critical Cooling Rates to avoid Ice Crystallization in Solutions of Cryoprotective Agents

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Emulsion isothermal calorimetry has been used to construct time–temperature–transformation (TTT) curves and hence continuous cooling (CT) curves for aqueous solutions of a number of established and potential cryoprotective agents, namely glycerol, dimethyl sulphoxide, (*2R,3R*)-(−)-butane-2,3-diol and mixtures of the *meso* isomer and racemate of butane-2,3-diol. The cooling rates required to avoid crystallization in these solutions are calculated from the TTT curves. The presence of the *meso* isomer of butane-2,3-diol at concentrations below 7% is shown not to affect the critical cooling rate for the racemic mixture. For vitrification at a cooling rate of 100 K min^{-1} the concentration of (*2R,3R*)-(−)-butane-2,3-diol required is 31.7% whilst for dimethyl sulphoxide the concentration is 42.1% and for glycerol 48.5%. (*2R,3R*)-(−)-butane-2,3-diol and mixtures of (±)-butane-2,3-diol with up to 7% of the *meso* isomer are thus shown to vitrify at cooling rates much lower than those for other cryoprotective agents.

Ice formation is one of the major problems that must be overcome before long-term storage of organs and tissues at sub-zero temperatures becomes a reality.[1] One possible avenue currently being vigorously investigated is storage in the vitreous state,[2] *i.e.* perfusion of the biological material with a suitable cryoprotectant followed by cooling at such a rate that ice crystallization is avoided and the vitreous state is achieved. Despite the current interest in vitrification little is known about the cooling rates required to reach the glassy state for a large number of commonly used cryoprotective agents. This cooling rate, also known as the critical cooling rate, is of fundamental importance in the design of perfusates and solutions for vitrification and also reflects the glass-forming ability of the solution. To date, the only direct determination of this parameter has been for propane-1,2-diol.[3] Boutron has published an extensive number of observations on the glass-forming abilities of a large number of aqueous solutions, particularly dihydric alcohols, from which the critical cooling rate may be inferred.[4] However, these experiments do not provide a direct measurement of the critical cooling rate as they depend on the determination of the amount of ice crystallising from the solution as a function of cooling rate in a differential scanning calorimeter, and as such are imprecise. This paper extends the previous study on propane-1,2-diol to the other commonly used cryoprotective agents glycerol and dimethyl sulphoxide (DMSO) as well as butane-2,3-diol. Butane-2,3-diol has recently been identified as a material of potentially great utility for vitrification.[5] The concentrations of butane-2,3-diol required to achieve the vitreous state are significantly lower than the corresponding concentrations of other cryoprotectants, and the glasses so formed are also more stable than those formed by other cryoprotective agents. The *meso* isomer of butane-2,3-diol is known to crystallize a hydrate very easily[6] and this hydrate has been reported[7] to haemolyse erythrocytes. The (2*R*,3*R*) and (2*S*,3*S*) isomers will crystallise a hydrate only with difficulty.[5] Hence, it is the (*2R,3R*), and (*2S,3S*) isomers and the racemic mixture of butane-2,3-diol that are of interest to the cryobiologist. However, the high cost of the individual enantiomers precludes their use in any practical vitrification solution. The racemic form is available only with varying amounts of the *meso* isomer, the proportions varying from batch to batch. A further aim of this work was to assess the effects of small (up to 11% w/w) concentrations of the *meso* isomer on the critical cooling rates, and thus the glass-forming ability of the racemic form of butane-2,3-diol.

In this study the technique of emulsion isothermal calorimetry[8] is used to produce time–temperature–transformation (or TTT) curves from which the critical cooling rate may be calculated. TTT curves represent the time required for a given volume fraction of material to crystallise from the liquid phase as a function of temperature.[9] In the calorimetric determination of TTT curves the time recorded is that for the first detectable appearance of ice.[10]

Data for constructing a TTT curve are obtained by quenching an emulsified sample of the solution to a preselected temperature in a DSC and then recording the time for crystallization to occur, as indicated by an exotherm, at this temperature.[10] The sample is emulsified in order to inhibit heterogeneous nucleation.[11] In a finely divided sample, such as an emulsion containing a large number of very small droplets of aqueous solution, any contaminant particles capable of causing nucleation by a heterogenous mechanism will be confined to a statistically relatively small number of droplets, the crystallization of which will not affect the remainder of the droplets. Thus, heterogeneous nucleation will be avoided in most of the sample and the crystallization times measured will result from nucleation *via* a homogeneous mechanism. The locus of crystallization times plotted against isothermal hold temperature is then the TTT curve. In general, the time to crystallization initially decreases with falling temperature to a minimum value and then increases (see Fig. 1). The minimum in the TTT curve, usually referred to as the 'nose' of the curve, results from two competitive processes, namely the tendency of the solution to crystallise, which increases as the temperature is decreased, and the molecular mobility within the liquid, which decreases with decreasing temperature.[9] The critical cooling rate may be estimated from the nose of the TTT curve,[3] but this tends to overestimate the result as this approach assumes that the amount of crystallization is the same at all temperatures.[12] A more accurate method is to calculate a continuous cooling time–temperature–transformation (or CT) curve from the experimentally determined TTT curve. This represents the time for ice to crystallize when a sample is cooled to a given temperature rather than isothermally. The end point of the CT curve represents the critical cooling rate.

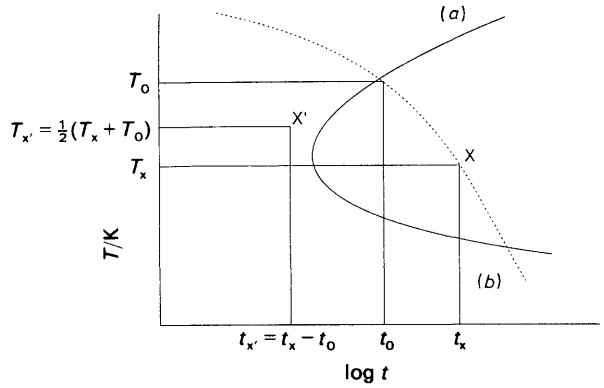

Fig. 1 Method of calculation of a continuous cooling curve from an experimentally determined TTT curve following the method of Grange and Keifer.[17] (*a*) TTT curve, (*b*) linear cooling curve. Reproduced from ref. 1 with permission

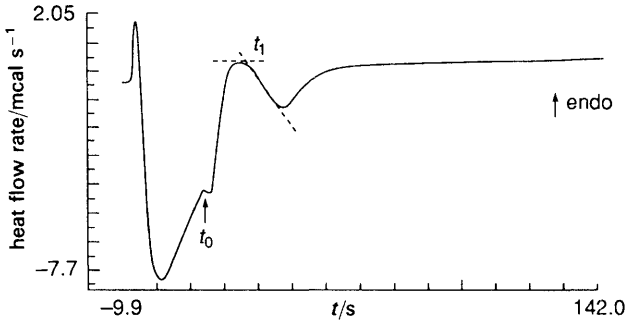

Fig. 2 DSC thermogram for 32% (w/w) (*2R,3R*)-(−)-butane-2,3-diol illustrating the measurement of crystallization times. t_0 is the point when the calorimeter reaches thermal equilibrium and t_1 is the time at which crystallization occurs. The measured crystallization time is thus $t_1 - t_0$

Experimental

(*2R,3R*)-(−)-Butane-2,3-diol and racemic butane-2,3-diol containing 3% and 11% *meso* isomer were obtained from Aldrich. A sample containing 7% *meso* isomer was obtained by mixing the 3% and 11% mixtures together. All other materials used in this study were from BDH. Emulsions were made with a 1 : 1 mixture of the aqueous solution and a 4% (w/w) solution of sorbitan tristearate (Span 65) in either a silicone oil (Dow Corning 200/1) (11) or a 1 : 1 mixture of methylcyclohexane and methylcyclopentane.[8] Silicone oil cannot be used below 170 K as it crystallizes at this temperature.[13] It was found that above 170 K the results were identical whichever carrier was used. Emulsions were made using a tissue homogenizer rotating at 15000 rpm for periods in excess of 2 min. The diameters of the drops produced using this technique were found to be in the range 5–50 μm, with the majority tending to be between 10 and 25 μm. The size distribution of the emulsion droplets was found not to change significantly over the time period of a complete TTT curve determination (*ca.* 3 h).

Crystallization times were measured using a Perkin-Elmer DSC-2 equipped with a liquid-nitrogen sub-ambient accessory. Data acquisition and manipulation was performed using an IBM PS/2 microcomputer running DARES, a dedicated DSC software package.[14] The calorimeter was calibrated for temperature at the melting point of 99.99% indium (429.8 K) and the $S_{II} \rightarrow S_I$ crystal transition temperature of cyclohexane (186.2 K) using the method of Franks.[15] As the calibration range was rather large and some of the isothermal hold temperatures are below the crystal transition temperature of cyclohexane, the calibration of the instrument was checked at the melting point of cyclohexane (279.7 K) and the crystal transition temperature of cyclopentane (138.1 K) and was found to be accurate and reproducible to within ±0.5 K. Small samples (*ca.* 5 mg) of the emulsion were sealed in aluminium pans and crash-cooled at the maximum rate possible (nominally 320 K min^{-1}, but in practice only around 140 K min^{-1} [16]) to a preselected temperature. Crystallization times were measured from the point at which the calorimeter reached thermal equilibrium at the preselected temperature to the point when a crystallization exotherm was first detected. This is illustrated in Fig. 2. The reference cell contained a sample of the Span 65–organic phase mixture such that the reference and sample pans were of the same weight.

Calculation of CT Curves

Continuous cooling curves were calculated from TTT curves using the method of Grange and Keifer[17,18] (Fig. 1). It is necessary to assume that during quenching negligible crystallization occurs and that in cooling from a temperature T_0 to some other temperature T_x the amount of crystallization is the same as is obtained in the isothermal case at the mean temperature $(T_x + T_0)/2$ after a time interval $t_x - t_0$.

Crystallization times were fitted to an equation of the form.

$$\log t = a_0 + a_1 T + a_2 T^2 + \cdots \quad (1)$$

For a given cooling rate the point of intersection of the TTT curve and the cooling curve was calculated *i.e.* (t_0, T_0). The amount of crystallization at increasingly lower temperatures on the same cooling curve was then determined. Considering Fig. 1, for point X, corresponding to temperature T_x and time t_x, the amount of crystallization in the isothermal experiment is given by the point X', *i.e.* $[t_x - t_0, (T_x + T_0)/2]$. Since X' lies to the left of the TTT curve no detectable crystallization has yet occurred. The calculation was then repeated until a temperature was found such that the point X' lay on the TTT curve. The values of T'_x and t'_x, which define point X', are thus the coordinates on the continuous cooling curve for the chosen cooling rate. These calculations were then repeated for increasing values of cooling rate until a value was found such that no crystallization ever occurred. This cooling rate is thus the critical cooling rate.

Results and Discussion

The range of solution concentrations that may be studied using emulsion isothermal calorimetry is restricted by the cooling rates available on the calorimeter. In the case of the butane-2,3-diol isomers used in this study, the maximum cooling rate available was insufficient to prevent crystallization occurring during the quenching phase in samples where the solution concentration was below 29% (w/w). The upper concentration limit is generally determined by the glass-forming properties of the solution. For (*2R,3R*)-(−)-butane-2,3-diol and the racemic mixtures containing up to 7% *meso*-isomer the maximum concentration that could be studied was *ca.* 33% (w/w). Evolution of enthalpy of crystallization is most rapid when the isothermal hold temperature is near the nose temperature. Crystallization becomes slower and the resulting exotherms more spread out at hold temperatures removed from the nose. The crystallization process also becomes increasingly sluggish as the solution concentration is increased. For solutions of butane-2,3-diol containing up to 7% *meso* isomer where the total solute concentration was greater than 33% crystallization was very slow so making the uncertainties in the crystallization times very large whilst at concentrations above 35% the solutions vitrified. This was found not to be the case

for the mixture containing 11% *meso*-butane-2,3-diol and will be discussed later.

Interestingly, from a cryopreservation viewpoint, where solute toxicity is a major consideration, the concentrations of the more commonly used cryoprotectants glycerol and DMSO that could be studied were considerably higher than those for butane-2,3-diol. The minimum concentration of DMSO and glycerol that could be used were 41 and 46%, respectively.

Fig. 3 shows some representative TTT and CT curves. Previous[3,10] workers have observed that at temperatures *ca.* 20 K above the nose temperature crystallization times begin to decrease with increasing hold temperature. This phenomenon is thought to be due to a heterogeneous mechanism,[19] but is not fully understood.[10] Franks *et al.*[20] have shown that this phenomenon occurs only in systems containing emulsifier and that the kinetics indicate that the emulsifier slowly crystallizes at the organic/aqueous interface and thus acts as a catalyst for ice nucleation. This effect was also observed for all the solutes studied here but the crystallization times thus obtained are not plotted.

The TTT curves show in Fig. 3 represent solutions where the critical cooling rate is close to 100 K min^{-1}. The figure shows the temperature ranges over which the experiments were performed. For more concentrated solutions the noses of the TTT curves move out to longer times and down to lower temperatures, in accord with expectation.[10] The continuous cooling curves in Fig. 3 show the temperatures to which the solutions must be cooled at 100 K min^{-1} in order to avoid crystallization. Obviously this temperature must be the glass-transition temperature for the solution. It is gratifying to note that the end-points of the CT curves are in excellent agreement with the literature and experimentally determined values for the glass-transition temperatures. For example, in Fig. 3 the CT curve for 42.1% DMSO terminates at 144 K whilst Rasmussen and Mackenzie[21] report a value of 140 K for the glass transition. Similarly, the value of the glass transition in 31.7% (2*R*,3*R*)-(−)-butane-2,3-diol from Fig. 3 is 170 K whilst the experimentally determined value for this solution was 175 K (at a heating rate of 5 K min^{-1}).

Fig. 4 shows the calculated critical cooling rates for the butane-2,3-diol mixtures. It is apparent that there is no significant difference between the curves obtained for the pure (2*R*,3*R*) isomer and mixtures of the racemate with up to 7% *meso* isomer, the difference between the curves being within the experimental errors of the technique. The data here represent the crystallization of ice from the solutions. In most cases hydrate crystallization was extremely slow but could be observed in some cases, such as the higher concentrations of the 7% *meso* mixture. The upper curve of Fig. 4, representing the 11% *meso* mixture, is significantly different from the other curves. In this case the critical cooling rate goes through a minimum value and increases with concentration above 33%. Above 43% crystallization again becomes too rapid to be measured, *i.e.* crystallization occurs during the quenching phase of the procedure. The complete curve for the 11% *meso* mixture is shown in Fig. 5. The low-concentration limit of the curve represents ice crystallization whilst the higher concentration side corresponds to hydrate formation. This indicates that hydrate formation is more rapid than ice crystallization above 32%. This result is not too surprising in view of the equilibrium liquidus line for the *meso* hydrate,[6] which shows that the temperature of hydrate crystallization increases with concentration up to a concentration of 50% (w/w).

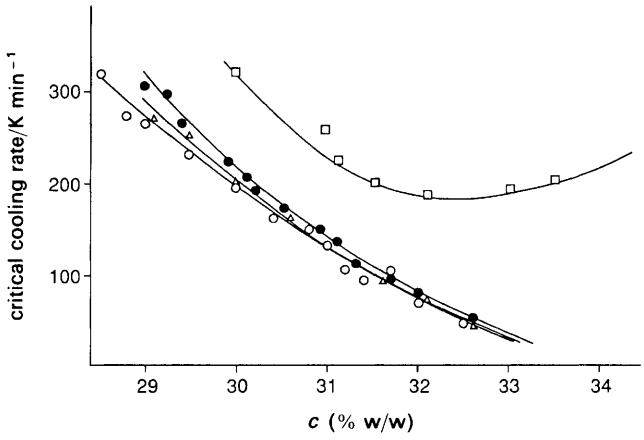

Fig. 4 Variation of critical cooling rate with concentration for the various butane-2,3-diol mixtures studied. ●,(2*R*,3*R*)-(−)-butane-2,3-diol; ○, racemate with 3% *meso* isomer; △, racemate with 7% *meso* isomer; □, racemate with 11% *meso* isomer. The error associated with each point is of the order of ± 10 K min^{-1}

Critical cooling rates plotted as a function of concentration for DMSO, glycerol and (2*R*,3*R*)-(−)-butane-2,3-diol are presented in Fig. 6. Data for propane-1,2-diol are given for comparison.[3] The curves for mixtures of the *meso* isomer and racemic mixture of butane-2,3-diol have not been drawn as they may simply be superimposed on the curve for the single

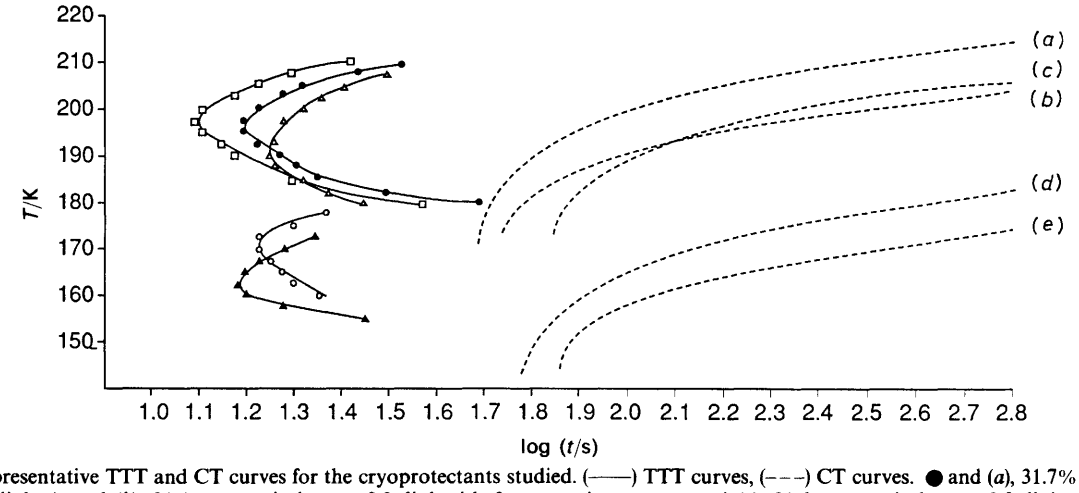

Fig. 3 Representative TTT and CT curves for the cryoprotectants studied. (——) TTT curves, (– – –) CT curves. ● and (*a*), 31.7% (2*R*,3*R*)-(−)-butane-2,3-diol; △ and (*b*), 31.4% racemic butane-2,3-diol with 3% *meso* isomer; □ and (*c*), 31.6% racemic butane-2,3-diol with 7% *meso* isomer; ○ and (*d*) 48.5% glycerol and ▲ and (*e*) 42.1% DMSO. All concentrations % w/w. All the concentrations here correspond to a critical cooling rate of *ca.* 100 K min^{-1}. Crystallization times measured in s

Table 1 The coefficients of eqn. (2) for the systems studied

cryoprotectant	concentration range (w/w %)	a_0	a_1	a_2	s.e.[a]
(2*R*,3*R*)-(−)-butane-2,3-diol	28.5–33	11 152	−643 (9)	9.3 (10)	4
DMSO	41–45	61 557	−2830 (15)	32.5 (15)	12
glycerol	45–50	12 682	−475 (76)	4.5 (86)	6
propane-1,2-diol[b]	33–37	741	−17 (5)	—	2

The numbers in brackets represent the percentage errors at 95% confidence for the coefficients. [a] Standard error of the fit. [b] Data from ref. 3.

isomer. It is immediately apparent that the critical cooling rates required to avoid crystallization and to reach the glassy state are significantly lower for butane-2,3-diol than for the other materials. The data in this figure may be represented by a simple polynomial expansion of the form

$$v_{cr} = a_0 + a_1 c + a_2 c^2 \quad (2)$$

where v_{cr} is the critical cooling rate in K min^{-1} and c represents the concentration in terms of a weight for weight percentage. The coefficients for this equation are given in Table 1.

The consequences of the lower critical cooling rates for butane-2,3-diol in terms of organ and tissue vitrification are the the concentrations of solute required to achieve the vitreous state at the cooling rates possible in large samples are significantly lower than for other solutes. The data indicate, for example, that for a cooling rate of 40 K min^{-1} the critical concentration for vitrification is 11% lower for butane-2,3-diol than DMSO and 15% lower than for glycerol. These concentration differences remain fairly constant with increasing cooling rate.

The reasons for the enhanced glass-forming ability of butane-2,3-diol over other cryoprotective agents, and indeed over butane-1,3-diol which shows no great propensity for glass formation,[22] are unclear. Recent work by Macfarlane[23] has shown that the basicity of the solute plays an important role and that those solutions which interact most strongly with water display the best glass-forming ability. However, more work is necessary before a complete understanding is reached.

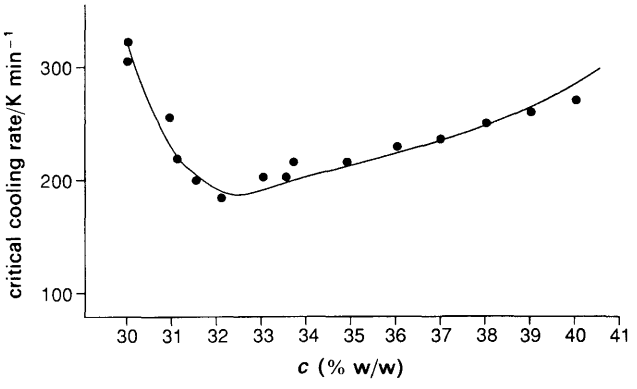

Fig. 5 Critical cooling rates for racemic butane-2,3-diol containing 11% (w/w) *meso* isomer

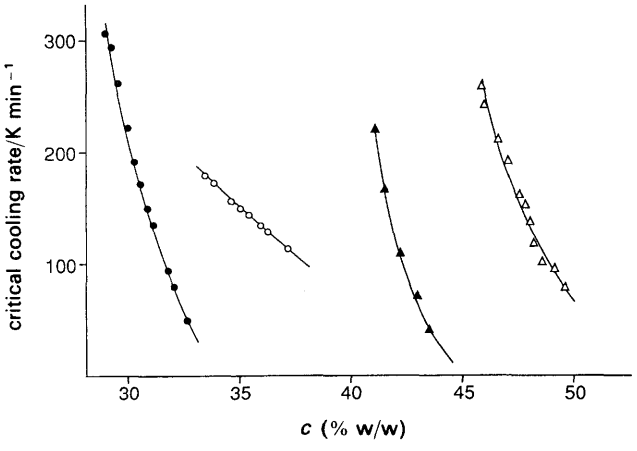

Fig. 6 Critical cooling rates for (2*R*,3*R*)-(−)-butane-2,3-diol ●, DMSO ▲ and glycerol △. Data for propane-1,2-diol (○) from ref. 1 included for comparison

Conclusion

This work has shown that the critical cooling rates required to avoid ice crystallization and thus promote the vitreous state are significantly lower for aqueous solutions of (2*R*,3*R*)-(−)-butane-2,3-diol than for DMSO, glycerol or propane-1,2-diol. This has important consequences for organ cryopreservation by vitrification where solute toxicity is an important consideration. Further, this work has demonstrated that the glass-forming properties of (2*R*,3*R*)-(−)-butane-2,3-diol are not affected by the presence of up to 7% (w/w) of the *meso* isomer, but that concentrations above this have a significant deleterious effect.

Note

After the acceptance of this paper a Ph.D. Thesis was brought to my attention (C. Roling, Rheinisch-Westfalischen Technischen Hochschule, Aachen, Germany) that contains TTT data for aqueous glycerol solutions. The results of this study are in good agreement with the results presented in this paper.

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