



# NON EQUILIBRIUM ICE CRYSTALLIZATION IN AQUEOUS SOLUTIONS: COMPARISON WITH THEORY, CASE OF SOLUTIONS OF POLYALCOHOLS WITH FOUR CARBONS, ABILITY TO FORM GLASSES, COMPOUNDS FAVORING CUBIC ICE

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**NON EQUILIBRIUM ICE CRYSTALLIZATION IN AQUEOUS SOLUTIONS : COMPARISON WITH THEORY, CASE OF SOLUTIONS OF POLYALCOHOLS WITH FOUR CARBONS, ABILITY TO FORM GLASSES, COMPOUNDS FAVORING CUBIC ICE**

P. BOUTRON<sup>\*,\*\*</sup> and P. MEHL<sup>\*</sup>

<sup>\*</sup>Laboratoire d'Hématologie, Département de Recherche Fondamentale, INSERM U-217, CEN.G, 85 X, F-38041 Grenoble Cedex, France

<sup>\*\*</sup>Laboratoire Louis Néel, CNRS, 166 X, F-38042 Grenoble Cedex, France

**Résumé :** La tendance à former un verre au refroidissement depuis l'ambiante jusqu'à très basse température et la stabilité de l'état totalement amorphe au réchauffement ont été mesurées sur des solutions aqueuses de polyalcools à 4 carbones. Pour de mêmes concentrations en solutés, le 1,3-butanediol stabilise le plus l'état vitreux, presque aussi bien que le 1,2-propanediol, le soluté le plus efficace connu jusqu'ici. Ceci pourrait permettre d'étendre la gamme des produits disponibles pour la cryopréservation de cellules par vitrification totale. La cinétique de cristallisation de la glace a été comparée avec un modèle théorique à la fois au refroidissement et au réchauffement, pour ces solutions et des solutions étudiées antérieurement. L'accord est très satisfaisant. Le 1,2- et le 1,4-butanediol favorisent de façon surprenante la formation de glace cubique.

**Abstract :** The glass-forming tendency on cooling from room temperature to very low temperatures and the stability of the wholly amorphous state on rewarming have been measured on aqueous solutions of 4 carbons polyalcohols. For same solutes concentrations, 1,3-butanediol most favours the vitreous state, almost as well as 1,2-propanediol, the most efficient known till now. New solutions will be available for cryopreservation of cells by complete vitrification. The kinetics of ice crystallization have been compared with a theoretical model both on cooling and on rewarming, for the present solutions as well as for previously studied solutions. There is a very good agreement. 1,2- and 1,4-Butanediol surprisingly favour cubic ice crystallization.

## **I - INTRODUCTION**

The present study is the continuation of a systematic investigation of low toxicity solutes which most favour the complete vitreous state at low temperature in their aqueous solutions, even at low concentration (1-9). The aim of the study is to find the most suitable solutes for cryopreservation of cells, tissues or organs or living beings by complete vitrification in liquid nitrogen (10). In the presence of additives called cryoprotectants, there are two ways of preserving cells without damage. In the first case, they are surrounded with ice. On cooling pure ice first crystallizes outside the cells. Water is then attracted outside by osmotic pressure. At an optimum cooling rate, cells may survive : the shrinkage is sufficient to avoid intracellular ice crystallization but is not yet damaging in itself (11-13). A great variety of isolated cells, including red blood cells, lymphocytes, fibroblasts, spermatozoa, etc... have been successfully frozen at the optimum cooling rate (14). However, it has not yet been possible till now to freeze the major organs such as

the heart, the kidneys, etc... of man and large mammals by this method. The problem is that the interior of the organ is cooled more slowly than the surface, and that an organ may be constituted of several kinds of cells which require different optimum cooling rates. The second way to preserve cells or organs is to avoid any ice crystallization even outside the cells and obtain a completely vitreous state. Unfortunately with the commonly used cryoprotectants such as glycerol or dimethylsulfoxide, this requires too large solutes concentrations which are toxic, or too fast cooling rates, which cannot be reached for large samples.

That is why a systematic investigation of other possible cryoprotectants has been done. Polyalcohols have been first studied because they are generally of low toxicity and strongly disturb the organization of water molecules by their hydrogen bonds. Therefore, first, aqueous solutions containing polyalcohols with 2 or 3 carbons or one of these polyalcohols and one monoalcohol have been studied (1-9). It was found that among the binary systems water-polyalcohol, for same water contents, the glass-forming tendency on cooling and the stability of the wholly amorphous state on rewarming was the highest with 1,2-propanediol (1,5) and that the stability of the amorphous state was still higher in the ternary system water-1,2-propanediol-1-propanol with 15 % 1-propanol/(1,2-propanediol+1-propanol) (1,8).

More recently, the aqueous solutions of the linear polyalcohols with 4 carbons have been investigated (9). The numerous experimental data concerning these solutions and the previously studied ones have been compared with a semi-empirical theory of the kinetics of ice crystallization on cooling or on rewarming (15). The main results of these studies are presented here. For details, justification of the nature of the phases observed and of equations, see ref (9) and (15).

## II - MEASUREMENTS

As usually, the phase transitions were observed on cooling or on rewarming generally between  $-153^{\circ}\text{C}$  and a temperature above the end of melting using a Perkin-Elmer DSC-2 calorimeter at programmed rates varying from 2.5 to about  $300^{\circ}\text{C}/\text{min}$ . The states between the transitions were observed by X-ray diffraction.

## III - NATURE OF THE PHASES OBSERVED WITH 4 CARBONS POLYALCOHOLS AQUEOUS SOLUTIONS

### A. Systems with or without hydrate

With 1,3-butanediol, 1,2,3- or 1,2,4-butanetriol, only ice could be observed on cooling or on rewarming. With 45 % (w/w) of any of these solutes, the solutions are wholly amorphous after cooling at the fastest rates (Fig. 4) ; the solution is mostly amorphous with 35 % (w/w) 1,3-butanediol. On rewarming these solutions after quenching, that is after cooling at the fastest rate available of about  $300^{\circ}\text{C}/\text{min}$  on the DSC-2, only the glass transition, the devitrification of ice (crystallization on rewarming) and the ice melting are observed (Fig. 1). With 35 % (w/w) 1,2,3- or 1,2,4-butanetriol, after cooling at any rate, or with the other above solutions after cooling slowly enough to allow ice crystallization on cooling, only the glass transition of an amorphous residue and the ice melting peak are observed on rewarming. It was confirmed by X-ray diffraction that ice is first cubic (1b) then hexagonal as 1,3-butanediol solutions are rewarmed from the wholly vitreous state, as for previously studied solutions (1-9).

Hydrates have been observed with 1,2-, 1,4- and 2,3-butanediol (9) (Fig. 1). With 35 or 45 % 1,2- or 1,4-butanediol, only ice crystallizes on cooling. Hydrate crystallizes only on rewarming (Fig. 1). With 35 or 45 % 1,2-butanediol, only ephemeral hydrate crystallization is observed : hydrate melts as soon as it has formed (Fig. 1). The incongruent melting of the hydrate of 1,4-butanediol occurs at  $-32^{\circ}\text{C}$ . At warming rates higher than  $20^{\circ}\text{C}/\text{min}$ , the hydrate has not enough time to crystallize. With 45 % of this solute, on rewarming after fast cooling, where the solution remained wholly amorphous (Fig. 4), ice also crystallizes on rewarming, at a sensibly lower temperature than the hydrate (Fig. 1). With 35 or 45 % 2,3-butanediol, ice and hydrate crystallize simultaneously either on cooling (Fig. 3 and 4) or, if cooling was too fast, on rewarming (Fig. 1). The hydrate melts at about  $12-13^{\circ}\text{C}$  for 35 or 45 % solute.

Supplemented phase diagrams showing the phases observed on rewarming after quenching are given for 1,3- and 2,3-butanediol solutions in Ref. (9).

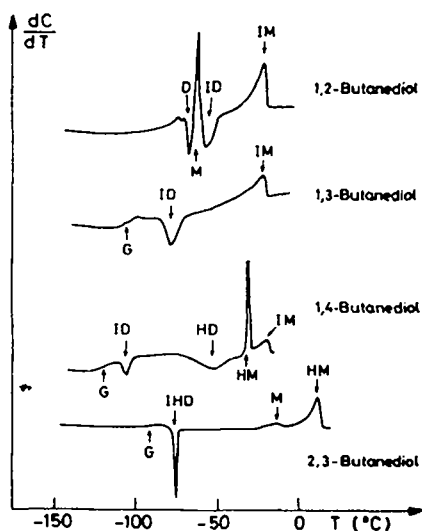


Fig. 1 : Warming thermograms at low warming rates after quenching for aqueous solutions with 45 % (w/w) butanediol. Peaks ID and IM correspond to ice devitrification and melting, HD and HM to hydrate devitrification and melting and IHD to devitrification of both ice and hydrate. G is a glass transition. Peaks D and M may correspond to hydrate devitrification and melting for the 1,2-butanediol solution ; for that with 2,3-butanediol, the ice disappears as the peak M is crossed. The warming rate is 2.5°C/min except for the solution with 45 % 2,3-butanediol where it is 10°C/min.  $dC/dt$  is the derivative of the specific heat vs the time ; its scale is not represented.

#### B. 1,2- and 1,4-butanediol favour cubic ice-gigantic glass transition with 1,2-butanediol

When a 35 % 1,2-butanediol solution is cooled ice crystallized is cubic at a cooling rate of more than 40°C/min, cubic and hexagonal between 10 and 40°C/min, hexagonal below 10°C/min (9). This is the first time that we have observed cubic ice crystallization on cooling. In the other solutions with polyalcohols or dimethylsulfoxide, cubic ice was always observed only on rewarming from the wholly amorphous state. With 45 % 1,2-butanediol, the solution remains wholly amorphous on cooling at 20°C/min or more and cubic ice crystallizes on rewarming ; hexagonal ice crystallizes on cooling at 2.5°C/min. In the previously studied solutions, it was observed (10) that the kinetics of the transition from cubic into hexagonal ice was very similar to that in pure water (9) (Fig. 2). It is a little slower on rewarming a 35 % 1,2-butanediol solution (Fig. 2).

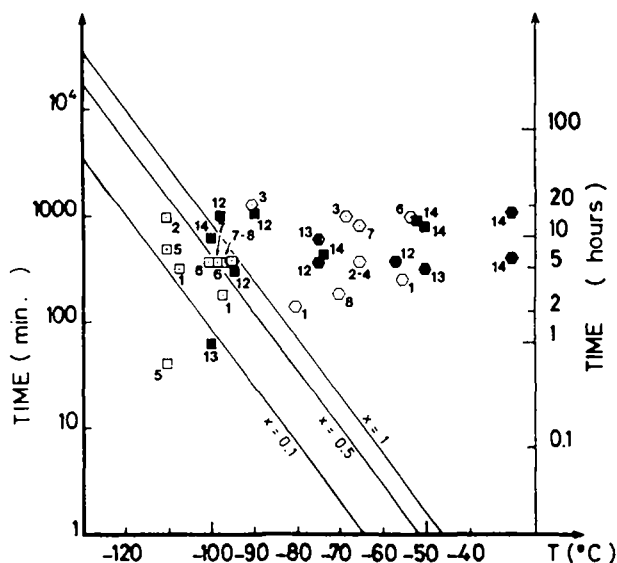


Fig. 2 : Structure observed by X-ray diffraction of the ice formed on rewarming wholly amorphous aqueous solutions ; comparison with the case of pure water (16) : hexagonal ice ( $\odot$ ), cubic or cubic and a few hexagonal ice ( $\square$ ), cubic and hexagonal ice ( $\oplus$ ). Abscissa : approximative temperature during the photos ; ordinate : duration of the photos. Lines  $x=0.1$ ,  $x=0.5$  and  $x=1$  correspond to the conversion of 10, 50 and 100% of cubic into hexagonal ice in pure water (10). The numbers from 1 to 8 correspond to solutions with alcohols with 2 or 3 carbons or dimethylsulfoxide (see ref. 10 for the detailed enumeration of these solutions). Numbers 12, 13 and 14 correspond respectively to aqueous solutions with 35 % (w/w) 1,2-butanediol, 40 % (w/w) 1,3-butanediol and 45 % (w/w) 1,4-butanediol. Filled cubes and hexagons are used for these three solutions for a better visualization.

Hexagonal ice crystallizes on cooling a 35 % 1,4-butanediol solution. With 45 % of this solute, when ice crystallizes on cooling, it is cubic even at a cooling rate as low as 10°C/min. On rewarming, after more than 15 hours at - 50°C, ice is still mainly cubic, while the transition occurs in about 2 min with the other solutes at - 50°C ! (Fig.2).

On cooling at 20°C/min or more a 45 % 1,2-butanediol solution, it remains wholly amorphous. A gigantic glass transition is observed in calorimetry on cooling, or on rewarming at 40°C/min or more (9) (at lower warming rates, the crystallization and melting of the hydrate is superimposed to that transition). It has a peak like shape and its size is comparable to that of the ice melting peak ! (9) . It is still sensibly higher than the already large glass transition observed with 45 % 1,2-propanediol (5).

#### IV - QUANTITY OF ICE CRYSTALLIZED ON COOLING POLYALCOHOL SOLUTIONS, GLASS FORMING TENDENCY, COMPARISON WITH THEORY

##### A. Experimental results .

Experimental heats of solidification versus cooling rate  $v$  are given in Fig. 3 for aqueous solutions with 35 % of various 3 or 4 carbons polyalcohols and Fig. 4 for aqueous solutions with 45 % solutes according to data given in ref. (1-9, 17). 1,2- butanediol is not represented on Fig. 4. With 45 % of this solute  $q = 0$  for  $v = 20$  or more °C/min,  $q = 20.6$  for  $v = 2.5$  °C/min and could not be determined for  $v = 5$  and 10 °C/min since the crystallization peak overlap with the glass transition.

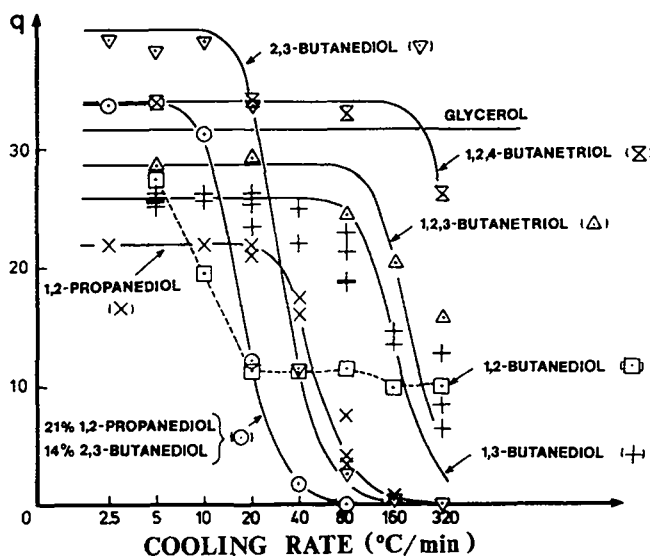
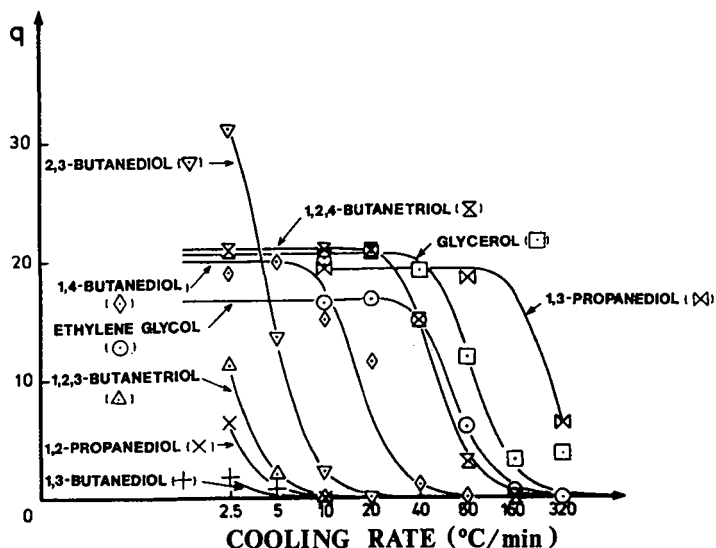


Fig. 3 : Variation with cooling rate of the heat of crystallization  $q$  for aqueous solutions with 35 % (w/w) alcohol. It is hexagonal ice which crystallizes except for the 1,2-butanediol solution where cubic or hexagonal ice or both crystallize depending on cooling rate (see text) and the two solutions with 2,3 butanediol where ice and hydrate crystallize simultaneously. The heats of solidification are represented by the numbers  $q$  of grams of ice whose solidification at 0°C would liberate the same amount of heat as that from 100 g of solution on crossing the corresponding peaks (Ref. 8, p 554) ;  $q$  is close to

the real quantity of ice crystallized (17) in % (w/w) of the solution when it is ice which crystallizes. Isolated points : experimental points, continuous lines : theoretical curves, --- : experimental curve obtained by interpolation.

The smaller the cooling rates when the quantity of ice decreases then reaches zero, the higher is the glass-forming tendency of the solution. For a given solute, the glass-forming tendency increases rapidly with the concentration. Fig. 3 and 4 allow to classify the solutes according to their glass-forming tendency. Among the solutions where only ice crystallizes, with 35 % solute the 1,2-propanediol solution is the most efficient followed by that with 1,3-butanediol, and 1,2-butanediol which has a particular behaviour, while with 45 % solute, 1,3-butanediol is the first followed by 1,2-propanediol. The wholly amorphous state is particularly easy to obtain with 35 % 2,3-butanediol, but large quantities of ice and hydrate crystallize on slow cooling or on rewarming . This disadvantage can be

Fig. 4 : Variation with cooling rate of the heat of crystallization  $q$  for aqueous solutions with 45 % (w/w) alcohol. It is hexagonal ice which crystallizes except with 1,4-butanediol where it is cubic ice and with 2,3-butanediol where ice and hydrate crystallize simultaneously. The same comments hold as for Fig. 3



partially suppressed by using ternary systems with both 1,2-propanediol and 2,3-butanediol with still higher glass-forming tendencies (Fig.3).

#### B. Comparison with theory

Four semi-empirical models have been compared with experiment (15). Only one of them, called the fourth model or "spherical crystals with an  $1-x$  term" model fits the experiments (15). In this model, the ratio  $x$  of the total quantity of ice crystallized on cooling to the maximum crystallizable ice ( $0 < x < 1$ ) is related to the cooling rate  $v$  by (15) :

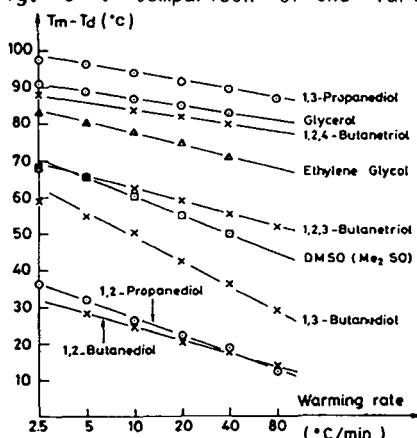
$$-\text{Log}_e (1-x^{1/3}) + \frac{1}{2} \text{Log}_e (1+x^{1/3}+x^{2/3}) + \sqrt{3} \text{Arctg} \left( \frac{\sqrt{3} x^{1/3}}{2 + x^{1/3}} \right) = \frac{k_4}{|v|} \quad [1]$$

where  $k_4$  is a constant.

The theoretical curves on Figs 3 and 4 have been calculated in the fourth model using Eq. [1] assuming, in a first approximation, that  $x = q/q_{\max}$ , where  $q$  is the heat of solidification and  $q_{\max}$  its maximum value. The only adjustable parameter  $k_4$  has been chosen to fit to one of the experimental points. The agreement between theory and experiment is very good not only for the solutions where hexagonal ice crystallizes, but also for those of 2,3-butanediol where both ice and hydrate crystallize. Experiment is in disagreement with theory for the 1,2-butanediol solution. This is normal since cubic or hexagonal ice crystallizes according to cooling rate. The agreement is poor for the 35 % 1,3-butanediol solution, where also the dispersion of the experimental points is large. Indeed the experiments have been repeated several times also for all the other solutions but the dispersions are very small. One notes that agreement between experiment and theory is less good at "160" and "320"°C/min programmed cooling rates. This is not surprising since the real cooling rates are smaller.

Theoretical crystallization peaks on cooling have been calculated in the fourth model for a 35 % 1,2-propanediol and for a 50 % glycerol (15) aqueous solution. The theoretical peaks are very similar to the experimental ones (15).  $k_4$  can be considered as characteristic of the glass-forming tendency: the smaller is  $k_4$ , the larger is the glass-forming tendency. Values of  $k_4$  and of other constants  $k_i$  are given in Refs (7), (9) and (15).

## V - CRYSTALLIZATION ON REWARMING AND STABILITY OF THE WHOLLY AMORPHOUS STATE

Fig. 5 : Comparison of the variation of  $T_m - T_d$  with the warming rate after

quenching for different solutions without hydrate with 45 % (w/w) solute.  $\Delta$  : alcohol with two carbons ;  $\odot$  : alcohol with three carbons ;  $\times$  : alcohol with four carbons ;  $\square$  : dimethylsulfoxide.

For the aqueous solutions of 4 carbons polyalcohols (9) as well as for the previously studied solutions (1-8), the temperature  $T_d$  corresponding to the maximum of ice crystallization velocity during devitrification varies linearly with  $\log v$ , in agreement with theory (2) (except, as expected, when  $T_d$  is very close to the temperature  $T_m$  of the end of melting). On Fig. 5 is represented the

variation of the difference  $T_m - T_d$  for aqueous solutions with 45 % solutes where no hydrate is observed in our experiments and for the 45 % 1,2 butanediol solutions since hydrate crystallization has not enough time to occur on rewarming at 40 °C/min or more (1-9). Critical warming rates  $v_{cr}$  above which crystallization has no more enough time to occur on rewarming have been calculated from the variations of  $T_m - T_d$  with  $v$  (9). By convention, the smaller is  $v_{cr}$ , the larger is the stability of the amorphous state (1-9). By comparison with Fig. 3 and 4 one sees that the order of the solutes is almost the same on cooling or on rewarming. In all cases, 1,2-propanediol, 1,3- butanediol and 1,2-butanediol are the three most efficient solutes.

On rewarming also, the experimental crystallization peaks have been compared with theory (15). In the fourth model, the theoretical peaks are very similar to the experimental ones. Yet the individual variations from one solution to another are not the same. Values of  $k_i$  on rewarming are given Ref (7, 9, 15).

## VI - CONCLUSION

There are three conclusions, two of interest to physicists and one to biologists.

1,2- and 1,4- butanediol stabilize cubic ice in their aqueous solutions, while with most polyalcohol solutions the kinetics of the transition from cubic into hexagonal ice is the same as in pure water. From the behavior of these last solutions it was suggested (10) that hexagonal ice crystals would be formed by a reorganisation of the structure of preexisting cubic ice. Stabilization of cubic ice by 1,2- or 1,4- butanediol may be due for instance to change of interfacial energies. Takahashi (18) suggested that in pure water cubic ice crystals cannot exceed  $2.4 \times 10^{-6}$  cm. It is perhaps inexact with these solutions. When cubic ice was observed on cooling the X-ray diffraction lines were split into visible points, which was not the case in the other experiments. This suggests that the crystals were larger than usual. It would be therefore interesting to use such solutions to try to grow large cubic ice crystals.

The present theory of ice crystallization in solutions has been carried out assuming that the speed of advance  $U$  of a crystal edge is constant at constant temperature and varies in an Arrhenian manner with temperature. Though, in solutions, a diffusion controlled growth where  $U$  depends on time is generally

assumed (19). It would be interesting to understand why this simple model agrees so well with experiment and to observe crystallization kinetics in a cryomicroscope on rewarming at a constant rate. In any case, this model is very convenient to replace numerous experimental data by analytical expressions more suitable for theoretical applications both in physics and in cryobiology. To know the variations of the constants  $k_i$  with the solutes and their concentrations could have fundamental interest.

Together with 1,2-propanediol, 1,3-butanediol with an almost as high glass-forming tendency and stability of the amorphous state, 2,3-butanediol with its high glass-forming tendency and 1,2-butanediol which favours both cubic ice and glass have interesting physical properties for cryopreservation of cells by total vitrification.

### Acknowledgement

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hydrates with our photos of powder spectrums observed by X-ray diffraction. But we think that it would be very interesting if anyone could determine the structure of polyabutol hydrates doing more efficient X-ray experiments.

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W.F. KUHS

Do you have an idea what the (atomic scale) mechanism is, by which 1.2 (1.4) butanediol inhibits the growth of hexagonal ice crystals ?

Answer :

With the other alcohols, the kinetics of the transformation from cubic into hexagonal ice is the same than with pure water. It suggests that hexagonal ice crystals nucleate on cubic ice faces rather than in the solution. Then for the exceptions observed with 1.2 or 1.4 butanediol are very surprising. Perhaps a higher interfacial energy between the solution condition hexagonal ice nuclei would slow hexagonal ice crystallization.