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Thermal study of simple amino-alcohol solutions[☆]

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Abstract

Widely regarded as the most promising approach to long-term cryopreservation of organs for transplantation, vitrification is a process where liquid is transformed into a disordered solid state free from crystals, known as the amorphous state. The vitreous state is obtained by rapid cooling to cryogenic temperatures in the presence of antifreeze substances called cryoprotectants, such as polyalcohols, which are known to be very good vitrification agents. This work reports on the thermal properties of a new class of compounds, the amino-alcohols, studied for its similarity to the structure of the equivalent polyalcohols. We studied by differential scanning calorimetry the glass-forming tendency and stability of the amorphous state for de-ionized water solutions containing 2-amino-1-ethanol and 3-amino-1-propanol at the concentrations of 35%, 40%, 43%, and 45% (w/w). A comparison is made with previous results obtained by Mehl [Cryobiology 27 (1990) 687–688] on the same compounds under different experimental conditions. The results are also compared with those obtained by Boutron [Cryobiology 30 (1993) 87–97] for the corresponding dialcohols. A further comparison is made with a few results obtained for the 1-amino-2-propanol and the 2-amino-1-propanol tested under the same conditions. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Amino-alcohols; Water; Differential scanning calorimetry; Vitrification; Devitrification; Polyalcohols

The current transplant window for a given organ is far too short to reliably screen the donor tissue, trace compatible recipients, and carry out surgery. Hence, one of the major challenges facing cryobiologists is extending this window, with the

ultimate goal of creating a sustainable organ bank in the future. This would require the use of very low storage temperatures to arrest the chemical reactions, as well as the development of a technique to avoid ice crystallization. Complete vitrification of biological tissues offers at the moment the best potential to meet these criteria [17]. This method prevents the formation of ice crystals during cooling to cryogenic temperatures by using very fast cooling rates, necessary to fix the liquids in a disordered solid state free from ice crystals. To vitrify a solution using a cooling rate that is

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technically achievable, we have to use cryoprotectants that disturb ice nucleation and inhibit the growth of ice crystals.

By studying the methods that nature employs to defend against extreme cold [14] one can gain some insights into which molecules are effective cryoprotectants. In general, one observes that cryoprotectants differ from one another by their penetrative properties, their biological toxicity, and their ability to reduce the melting point of ice, T_m , as well as the homogeneous nucleation temperature, T_h [22] and to slow down ice crystal growth. Cryoprotectants all have a strong affinity with water. Most of them contain hydroxyl or amino groups that enable them to create hydrogen bonds with water molecules. Thus, the structure of a molecule is an important parameter in determining the effectiveness of a cryoprotectant [16,21].

It is now well recognized that polyalcohols are very useful cryoprotectants in cryopreservation [28,30]. In our study, we chose to test some compounds of the amino-alcohol family, due to its similarities to the polyalcohol family. As illustrated in Fig. 1, the chemical structure of amino-alcohols is very similar to that of the polyalcohols, with just one amino group replacing a hydroxyl group. Simple amino-alcohols are less basic than the corresponding amines (e.g., pK_b (21AE) = 4.5; pK_b (ethylamine) = 3.3), due to the withdrawing effect of the OH group, and, for the range of concentrations used in our studies, the concentration ratio of the cation of the amino-alcohols to their neutral forms is, in all cases, less than 0.25%. We made the supposition that the presence of two different electronegative groups in the same molecule would make the vitrification of an aqueous solution more efficient and that is what we will tend to demonstrate.

The typical pH of aqueous solutions of these materials is in the region 11–13. In most cryobiological applications, where pH is close to neutral, the solutes would be used in the presence of a buffer and thus the amino-alcohols would be present substantially in their cationic form. As a

first step in understanding the properties of these complex neutral solutions we have studied in this paper the more simple case of solutions of the solutes at their natural pH. This paper will present the findings of our study on 2-amino-1-ethanol (21AE) and 3-amino-1-propanol (31AP), 43% 1-amino-2-propanol (12AP), and 43% 2-amino-1-propanol (21AP), and make a number of comparisons with previous works.

Materials and methods

To determine the thermal properties of the compounds, we conducted a series of experiments using a differential scanning calorimeter (DSC). Dynamic measurements were made to evaluate the critical cooling rate above which a given solution vitrifies entirely, and the critical warming rate necessary to avoid devitrification on warming, which results from the instability of the amorphous state produced by rapid cooling. 2-Amino-1-ethanol and 3-amino-1-propanol (from Aldrich, purity 99+%) and de-ionized water were used. Aqueous solutions with 35%, 40%, 43%, 45% (w/w) 21AE or 31AP and 43% 21AP or 12AP were prepared in a glove box under nitrogen atmosphere just before the DSC experiments because those compounds are hygroscopic. Thermograms on cooling and warming were obtained with a Perkin–Elmer DSC₇ apparatus controlled with Pyris Software (version 3.7), on samples of a few milligrams (3.89 ± 0.97 mg). Initially, we conducted the experiments as in previous work [2,29] using standard aluminium pans (Perkin–Elmer, 0219-0062) designed for volatile samples. However, chemical description of these compounds indicates that they are corrosive to Al [19]. Consequently, we covered by sputtering the inside of the pans with one micrometer of gold over a 100 Å layer of tungsten/titanium alloy, which was needed to prevent the gold from peeling off. This thin layer of gold prevented any chemical reactions.

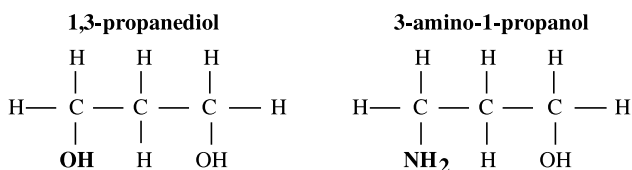


Fig. 1. Comparison of the chemical structure of a simple linear amino-alcohol: 3-amino-1-propanol, with its corresponding dialcohol: 1,3-propanediol.

The calibration of the DSC₇ for the temperatures and for the heat flow was made from the melting of ice from de-ionized water ($T_m = 0^\circ\text{C}$ and $\Delta H = 333.8\text{ J/g}$) and the crystallographic transition of cyclohexane in its solid state ($T = -87.1^\circ\text{C}$). All cryoprotective solutions were studied during cooling and warming at a constant rate which varied from one experiment to another from 1 to 500 K/min, between 120 K (-153°C , which is below the vitreous transition of the solutions) and 285 K (12°C , above the end of ice melting). Heats of solidification were measured at selected programmed cooling rates of 1, 2.5, 5, 10, 20, 40, 80, 160, and 320 K/min. The actual cooling rates are not determinable on this DSC₇ but according to our previous studies on a DSC₂, we consider that they must be at least identical to the indicated rates up to 80 K/min [3]. The temperatures of the transitions on warming were determined at warming rates of 1, 2.5, 5, 10, 20, 40, and 80 K/min, after quenching (programmed cooling rate within the range 320–500 K/min) and once after the slowest cooling rate for all the samples. Although other values for cooling and warming rate are available on the DSC₇, we chose the values enabling comparisons with results obtained in the past on other cryoprotectants with less up to date DSC. For most of the solutions, measurements were done on two or more samples.

Results

Heats of solidification on cooling and glass-forming tendency

To estimate the critical cooling rates, we used the semi-empirical model developed from the classical theory of crystallization by Boutron [4]. Its basic hypothesis states that the ice crystals are spherical and have equal size. The rate of growth is a function of temperature and the equation includes a term that takes into consideration reduction in growth rate due to crystal impingement [7]. By measuring the area of the exothermic peak of crystallization, we can determine the heat of ice solidification q for a given cooling rate. After having plotted these values as a function of the cooling rate, the aforementioned theory is used to fit a curve to the experimental points and estimate the critical cooling rate, which is deemed to be the rate above which less than 0.2% of the solution will crystallize.

The theoretical curves correspond as usual to the fourth model given by Boutron [4]:

$$A_1(x) = -\ln(1 - x^{1/3}) + \frac{1}{2} \ln(1 + x^{1/3} + x^{2/3}) + \sqrt{3} \text{Arc tangent} \left[\frac{\sqrt{3}x^{1/3}}{2 + x^{1/3}} \right] = \frac{k_4}{|v|}, \quad (1)$$

where x is the ratio of the total quantity of ice crystallized on cooling to the maximum crystallizable ice ($0 \leq x \leq 1$), v is the cooling rate, and k_4 is a constant. The theoretical value of q , considered as before [1,2,4,5,11,29] to be proportional to x , is chosen such that its maximum value q_{\max} fits the experimental values. Since $x = 1$ corresponds to $q = q_{\max}$, $x = q/q_{\max}$. After cooling at the lowest rate, no devitrification peak occurred on subsequent warming for the two compounds and all concentrations tested (meaning that $q = q_{\max}$ at this cooling rate), except at 45% (w/w) 21AE. For this last solution, a rough estimation of q_{\max} has been calculated from the area of the melting peak of ice at 2.5 K/min, neglecting the variation of the latent heat of melting with the temperature due to the difference between the specific heats of water and ice. This method gives an underevaluation of q_{\max} corresponding to a relative error smaller than 24% in our measurements. It was not possible to use the warming recorded at 1 K/min because of the irregular shape of the baseline.

The critical cooling rate v_{ccr} above which the amount of crystallization on cooling can be considered as negligible [5] was calculated from the theoretical curve. It is given by

$$v_{\text{ccr}} = \frac{k_4}{3 \left(\frac{0.2}{q_{\max}} \right)^{1/3}} \quad (2)$$

and can therefore be deduced from the values of k_4 and q_{\max} . For 45% (w/w) 2-amino-1-ethanol, there was no crystallization even on cooling at 1 K/min (the solution remained wholly amorphous). In this case, v_{ccr} is lower than 1 K/min.

Fig. 2 represents the experimental values of q versus the cooling rate for 35%, 40%, 43%, and 45% (w/w) 21AE or 31AP in water, together with the theoretical curves. For 35% (w/w) the theoretical curve is of limited significance due to the uncertainties in the zone of decrease of q (see Discussion). For the other concentrations, the experimental points fit well with the theoretical curves. The parameters of the theoretical curves (q_{\max} and k_4) are given in Table 1 for all four compounds. The values of v_{ccr} are given in Table 2.

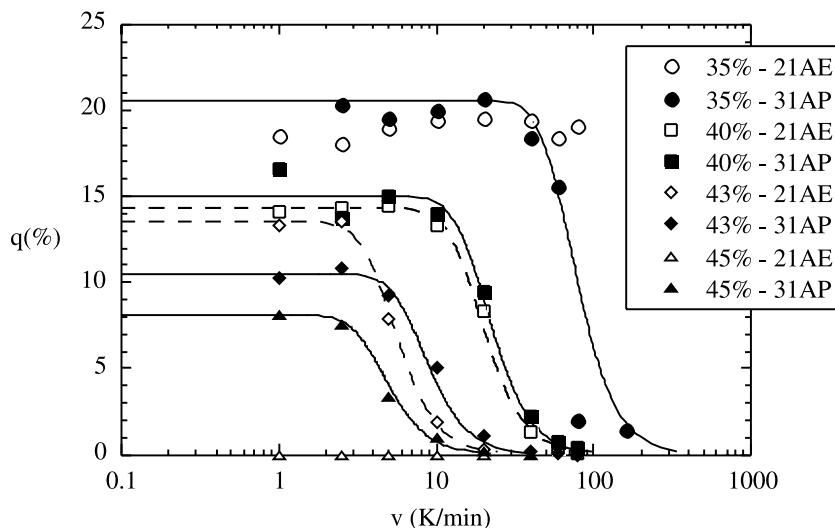


Fig. 2. Variation with concentration and cooling rate of the heat of ice crystallization q in solutions of amino-alcohols in water. The percentages of 2-amino-1-ethanol and 3-amino-1-propanol are in weight by weight. Isolated points are the experimental points; solid lines are the theoretical curves according to the fourth model [4]. The heat of ice solidification is represented as usual by the number q of grams of ice, the solidification of which at 0°C would liberate the same amount of heat as that of 100 g of solution on crossing the corresponding peak. These units are chosen because with these units the heat of solidification is close to the quantity of ice crystallized in percentage (w/w) of solution when only ice forms [29]. The heat in calories per 100 g of solution is obtained by multiplying q by 79.78.

Table 1
Values of the parameters necessary to evaluate the critical cooling rate

Concentration (w/w) (%)	21AE		31AP		21AP		12AP	
	q_{\max} (%)	k_4 (K/min)	q_{\max} (%)	k_4 (K/min)	q_{\max} (%)	k_4 (K/min)	q_{\max} (%)	k_4 (K/min)
35	19.6	–	20.6	220 ^a				
40	14.3	57	15	63				
43	13.5	16	10.5	25	10.7	4	6.4	5
45	7.1 ^b	–	8.1	14				

^a Value corresponding to the rather arbitrary theoretical curve of Fig. 2.

^b Estimation of q_{\max} from the thermogram on warming at 2.5 K/min.

Table 2
Values of the critical cooling rate v_{cer} (K/min)

Concentration (w/w) (%)	21AE	31AP	21AP	12AP	EG ^a	13PD ^a
35	>500	344				
40	79	89				
43	22	31 ^b	5 ^b	5		
45	<1	16			260	1100

^a Previous work [5].

^b Good agreement between the theoretical critical cooling rate and the experimental measurement of the amount of ice crystallization at this rate: $q(v) < 0.2\%$.

We tested experimentally the critical cooling rate estimated for 43% (w/w) 31AP and obtained a very good agreement between the theoretical prediction and v_{ccr} obtained by direct experimental evaluation of the minimum cooling rate which leads to $q < 0.2\%$.

Stability of the wholly amorphous state on warming

On warming, the thermograms of 21AE or 31AP are not as simple for most polyalcohols [2]. When the solution is initially wholly amorphous (no crystallization peak observed on previous cooling) and when warming is not fast enough to avoid ice formation, one observes first the glass transition, then the devitrification peak corresponding to ice formation on warming, and before the nonisothermal peak corresponding to ice melting, a small exothermic peak (examples are given for 21AE in Fig. 3 and the 31AP in Fig. 4). The vitreous transition temperature T_g is determined as usual [1] at the inflexion point between the limits at which the slope of the curve changes through the glass transition. The top of the devitrification peak is called T_d as usual [1,2,5,11,29].

The temperature T_m of the end of ice melting can be defined, as for most of the solutions studied [1,2,5,11,29], as the top of the temperature of the ice melting peak on warming at the smallest warming rate (here 1 K/min). The corrections using the leading edge method [5,9,23] are negligible at this warming rate for the concentrations of amino-alcohols presently studied. The top temperature of the small feature between T_d and T_m is T_r .

When a cryoprotective solution is warmed from a completely amorphous state, the temperature corresponding to the peak of vitrification, T_d , rises as the warming rate increases. The critical warming rate is reached when T_d approaches the melting temperature, T_m , and ice crystallization is avoided. Boutron and other authors have observed that T_d is inversely proportional to the logarithm of the warming rate [1,2,10,29]. Hence, to estimate the critical warming rate, T_m is divided by T_d and the experimental values of T_m/T_d (where T_m and T_d are in K) are plotted versus the warming rate. As usual [1,2,5,7], the stability of the amorphous state is defined by the critical warming rate v_{cwr} above which there is not enough

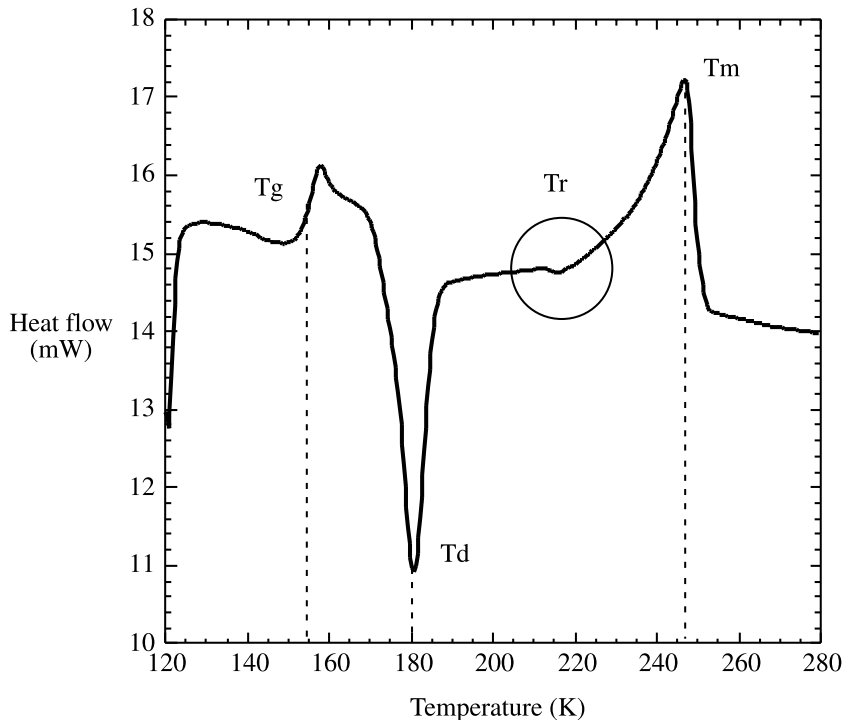


Fig. 3. Thermogram on warming at 20 K/min for 2.76 mg of 40% (w/w) 2-amino-1-ethanol in water after quenching at the programmed cooling rate of 500 K/min. The small feature at T_r is indicated by a circle.

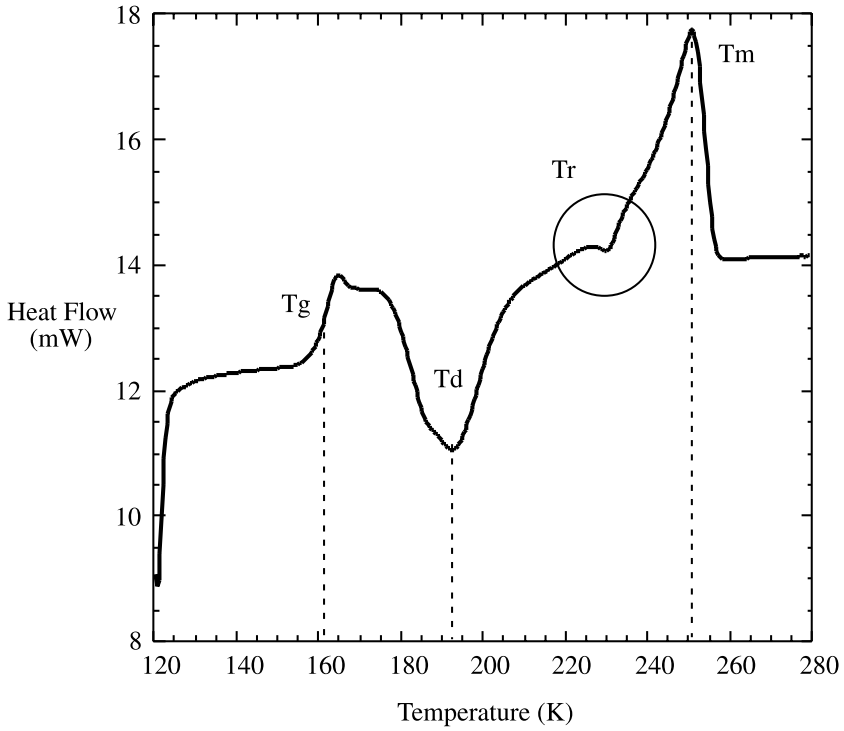


Fig. 4. Thermogram on warming at 20 K/min for 3.37 mg of 40% (w/w) 3-amino-1-propanol in water after quenching at the programmed cooling rate of 320 K/min. The small feature at T_r is indicated by a circle.

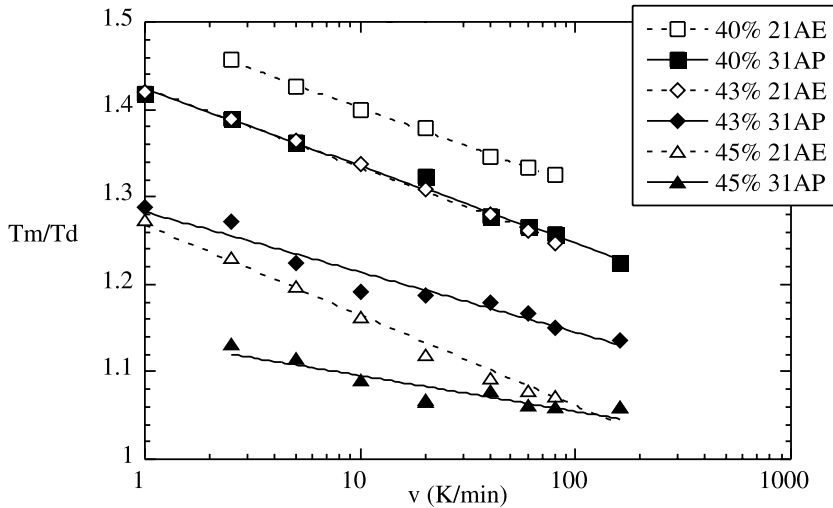


Fig. 5. Variation of the experimental values of T_m/T_d with warming rate for 40%, 43%, and 45% (w/w) of 2-amino-1-ethanol and 3-amino-1-propanol in water. Isolated points are the experimental points; solid straight lines result from interpolation by the least-squares method.

time for crystallization on warming the wholly amorphous solution; v_{cwr} is obtained by the extrapolation of the experimentally observed linear

variation of $1/T_d$ with $\log(v)$ to $T_d/T_m = 0.95$, which normally corresponds to 0.5% crystallization [1,2,5,10,29].

Table 3
Values of the critical warming rate v_{cwr} (K/min)

Concentration (w/w) (%)	21AE	31AP	21AP	12AP	EG ^a	13PD ^a
35	–	$1.8 \times 10^{9\text{b}}$				
40	115,373	17,807				
43	12,368	1984	50 ^c	121		
45	>80 and <160 ^c	70 ^c			3.3×10^5	1×10^9

^a Previous work [5].

^b Critical warming rate evaluated for a partially amorphous state.

^c Estimation of the experimental critical warming rate by measuring the area of the melting peak of ice to deduce when $q(v) < 0.5\%$.

Fig. 5 represents the variations of the experimental values of T_m/T_d with warming rate for 40%, 43%, and 45% (w/w) 21AE or 31AP. They are approximately in agreement with theory [10] between 1 and 160 K/min. The straight lines were drawn using the least-squares methods. Critical warming rates values are given for all four compounds in Table 3. As one can see, the critical warming rates tend, as for previously studied compounds, to be particularly high, and are often well beyond the capabilities of the DSC₇. As a result, the determination of the warming rate is somewhat less reliable than that of the critical cooling rate. Moreover, when the compounds have a good stability of the amorphous state (the smaller v_{cwr} , the greater the stability of the amorphous state), this method may give a wrong estimation of v_{cwr} when no extrapolation is required. The critical warming rate is defined as being equal to the experimental warming rate for which the amount of ice melted becomes less than 0.5% [10]. It is the case of 45% (w/w) of 21AE and 45% (w/w) of 31AP in water. The heats of melting have therefore been evaluated at the highest warming rates for these compounds. For 31AP, $q(60\text{K/min}) = 0.64\%$ and $q(80\text{K/min}) = 0.44\%$, giving v_{cwr} around 70 K/min whereas v_{cwr} estimated by extrapolation is 113 K/min. For 21AE, $q(80\text{K/min}) = 1.29\%$ and $q(160\text{K/min}) = 0.24\%$ giving $80 < v_{\text{cwr}} < 160\text{K/min}$, whereas the extrapolated value of v_{cwr} is 121 K/min.

Discussion

Fig. 2 illustrates the results we obtained for 21AE and 31AP during cooling. As already said, the values for the 35% (w/w) concentration do not fit well the theoretical curve. This is due to a large supercooling effect that makes the determination of the crystallization peak area very difficult. The

position of the baseline is hard to find because the peak at this concentration is very close to the vitreous transition. Moreover, a deviation may exist at the highest cooling rate 320 K/min, due to the fact that the real cooling rate is smaller. However, it is clear that q_{max} decreases as the compound concentration increases and it is apparent that the critical cooling rate decreases with increasing amino-alcohol concentration, as it is used to be for other cryoprotectants [1–3,29]. When comparing the glass-forming tendency of the two compounds, we can see from 40% to 45% (w/w) that 21AE is more effective than 31AP. This observation is backed up by quantum chemical calculations and matrix-isolation infrared experiments conducted on these compounds [12,31], which showed that the intramolecular hydrogen bonding of 31AP is stronger than in 21AE. A significant amount of intramolecularly bond monomers of 31AP was found for this compound in the pure liquid [12], clearly indicating that for this molecule intramolecular hydrogen bonding is of similar strength as intermolecular interactions. As a result, 31AP is less able to create the hydrogen bonds with the water molecules required to disturb and inhibit ice nucleation than 21AE, where no evidence of the presence of intramolecularly bond monomers species was found in the liquid phase [31].

In Fig. 5, we did not plot the results obtained at 35% (w/w) as the solutions do not form a completely amorphous state during cooling. All the other concentrations follow the same trend in warming as they do in cooling: the stability of the amorphous state increases with the compound concentration. This result is similar to what is generally obtained with cryoprotectants [1–3,29]. However, if we compare the stability of the amorphous state of the two compounds, we can see from 40% to 45% (w/w) that 21AE is less effective than 31AP. This might be due to the higher

basicity of 31AP. Even if the number of hydrogen bonds that 31AP can establish is smaller (because of the existence of monomers with intramolecular hydrogen bonds), they are stronger than in the case of 21AE and the amorphous state is more stable.

The origin of the small peak at T_r , observed between T_m and T_d , is uncertain. A possible explanation would assume that recrystallization of the smallest ice crystals previously formed at T_d occurs. This has already been observed for several systems, including various cryoprotective solutions [20] and is due to the lack of stability of the small ice crystals when the temperature increases because their surface-to-volume ratio becomes too high [20]. At a given temperature, these small crystals melt and their molecules are transferred to the large crystals, which grow bigger. The quasi-simultaneous occurrence of the melting of the small crystals and recrystallization on big crystals would give rise to the feature observed at T_r , which in fact shall contain overlapped contributions due to these two events.

Comparison with literature

We were surprised to find a distinct lack of papers studying these compounds. We believe that this is probably due to the awkwardness of studying this particular family of compounds, as they are irritant, corrosive, and hygroscopic, and have to be stored under nitrogen according to the manufacturer. It appears that only Patrick Mehl has studied them [24,25]. Table 4 reports the critical cooling and warming rates obtained by this author. Upon comparing the present values with those previously reported, it is clear that they are different. However, whilst our measurements were conducted on a DSC₇ with gold pans, Mehl used a DSC₄ and standard aluminum pans. He agreed that there was significant chemical reactivity between the compounds and his pans (private communication), and this probably affected

his results. Furthermore, it should also be noted that we calculated the critical warming rates using a different criteria than Mehl, whose method was more constrained [26]. His critical warming rate is defined as the rate above which the size of the crystals is not damaging cells in organized tissues (radius of ~60 nm). It involves the knowledge of the nucleation kinetics from data on isothermal experiments and the corresponding crystallized fraction is lower than the one considered by the method used in this work [26]. This could also explain why his critical warming rates are systematically greater than ours.

Additionally, we compared our results with those previously obtained by Boutron [5] on the corresponding diols; namely ethylene glycol [8] for 21AE and 1,3-propanediol [6] for 31AP. These earlier experiments were conducted with aluminium pans, since the polyalcohols do not react chemically with them. As can be seen from Tables 2 and 3, showing the results for solutions of 45% (w/w) with water, the critical cooling and warming rates for the amino-alcohols are consistently lower than those of the corresponding diols. This shows that by substituting a hydroxyl group by an amino group, we can improve the properties of the compounds in regard to their glass-forming tendencies and the stability of their amorphous state.

Comparison of the four amino-alcohols

To study the effect of the position of the substituents on the relevant physicochemical properties, we made a short study of two additional amino-alcohols with 3 carbon atoms: 1-amino-2-propanol (12AP) and 2-amino-1-propanol (21AP). Fig. 6 shows the experimental and theoretical variations of q with cooling rate v for solutions with 43% (w/w) amino-alcohol in water. At lower and higher concentrations no data are given because thermograms of both 12AP and 21AP are too difficult to interpret due to their complicate shape perturbed by the presence of hydrates.

Table 4
Comparison of our critical rates for 40% (w/w) solute with literature data

40% (w/w)	Cooling (K/min)		Warming (K/min)	
	This work ^a	P. Mehl ^b	This work ^a	P. Mehl ^b
21AE	79	80	1.2×10^5	2.6×10^6
31AP	89	10	1.8×10^4	1.4×10^5

^a Measurements conducted with gold covered aluminium pans, on a DSC₇.

^b Measurements conducted with pure aluminium pans, on a DSC₄ [24,25].

Table 1 presents the values of k_4 and q_{\max} and Table 2 the critical cooling rates of aqueous solutions of 12AP and 21AP together with those of aqueous solutions of 21AE, 31AP, and the corresponding diols. Fig. 7 presents the variation of T_m/T_d with warming rate v at the concentration of 43% (w/w) and Table 3 gives the critical warming rates v_{crw} of aqueous solutions of simple amino-

alcohols studied in this work together with those of the corresponding diols.

It is noticeable that the critical rates for 21AP and 12AP are lower than those obtained for 31AP, for both cooling and warming, illustrating the significance of the position of the OH and NH_2 in the amino-alcohol molecule. The lower critical rates observed for 12AP and 21AP can be

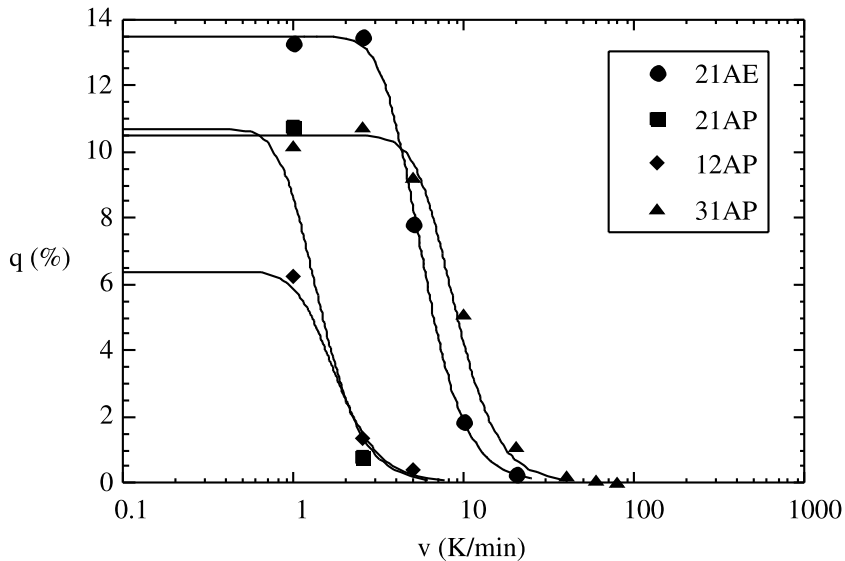


Fig. 6. Variation of the heat of ice crystallization q with cooling rate v for solutions with 43% (w/w) solute in water. The units of q are the same as in Fig. 2. Isolated points: experimental points; continuous lines: theoretical curves.

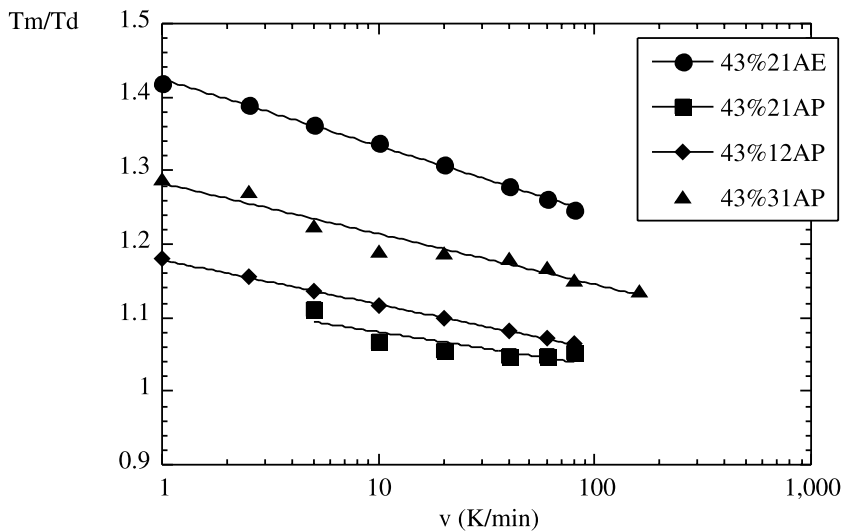


Fig. 7. Variation of T_m/T_d with warming rate for solutions with 43% (w/w) solute in water. T_m and T_d are in Kelvin. Isolated points: experimental points; straight lines: interpolation by the least-squares method.

explained by taking into consideration the fact that intramolecular hydrogen bonding in these molecules is weaker than in 31AP, since it involves the formation of a relatively less stable 5-membered ring, while in 31AP it involves the formation of an energetically more favourable 6-membered ring [12,13,18]. On the other hand, since in 12AP and 21AP the two electronegative groups occupy a relative position to each other that is similar to that found in 21AE, the difference in their respective performances must be due to other structural features: the length and asymmetry of the hydrophobic carbon chain, that are a consequence of the presence of the additional methyl group in both 12AP and 21AP. The longer and more asymmetrical this chain is, the lower the critical rates are, because these structural features make more difficult the establishment of the hydrogen bond network of the ice [16,27]. Furthermore, the methyl group in 12AP and 21AP also activates both OH and NH₂ substituents to participate in intermolecular hydrogen bonding, this being an additional factor contributing to the lower critical rates observed for these two molecules. It should however be pointed out that the analysis of the 12AP and 21AP thermograms is particularly difficult due to the presence of hydrates, whose occurrence is also related with the strong affinity of these compounds by water, but needs to be avoided in cryobiology.

Conclusion

Because the need to have available banks of biological tissues increases with the improvement of medical knowledge and transplantation procedures, we have studied simple linear amino-alcohols as antifreeze agents that could help to make vitrification and avoid crystallization at cooling and warming rates technically more achievable.

The 2-amino-1-ethanol and the 3-amino-1-propanol that we studied by differential scanning calorimetry have a glass-forming tendency, better than that of the corresponding di-alcohols, which is essentially due to their two different electronegative groups and the hydrogen bonds that they create with the water molecules. This study reinforced our belief that these compounds could be effective in vitrification, in association with other compounds to reduce their biological toxicity [15]. On the other hand, it must be noticed that the use of amino-alcohols as cryoprotectants will require

the regulation of pH by use of a suitable buffer (e.g., pH of a 30% solution of 21AE is ca. 12). Such requirement will then introduce an additional complexity that cannot be ignored in cryobiology applications.

Acknowledgments

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