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Low temperature crystallization of glasses in the H_2O–NaCl–dimethyl sulphoxide ternary system

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The determination and application of ternary H_2O–NaCl–cryoprotective agent phase diagram information appears to offer materials scientists and biologists a unique opportunity for co-operative research. Dimethyl sulphoxide (DMSO) is a widely used cryoprotective agent and in this investigation the H_2O–NaCl–DMSO system appears to develop a glassy phase even at relatively low cooling rates (25° C min^{-1}) for those water-rich compositions where the ratio of DMSO to NaCl varies from 2 to $\frac{1}{2}$. This glassy phase is relatively unstable and the kinetics of the glass-to-crystalline phase transition have been investigated and shown to be first order. Activation energies (Q) and the times required for the completion of this first order transformation have been evaluated by differential thermal analysis. Such information may be useful in interpreting the effects of long term storage on rapidly frozen biological materials perfused with DMSO.

1. Introduction

The salt content of physiological materials is usually taken as approximately 0.85 wt % NaCl [1]. All cells and tissues also contain, of course, a substantial fraction of H_2O. During freezing and thawing, these tissues may also contain dimethyl sulphoxide (DMSO), which is widely used as a cryoprotective agent because, for many biological materials, it provides protection against damage during such freezing and thawing (2-5). Hence, knowledge of the ternary system H_2O–NaCl–DMSO is very relevant to cryobiology.

Two major mechanisms of freezing and thawing damage to biological materials are thought to exist: (1) osmotic dehydration of cells due to increasing concentration of solute in the extracellular liquid as ice precipitates [6, 7], and (2) cell destruction through the formation of intracellular ice [8, 9]. Prevention of the latter can be achieved through the optimization of freezing rate. The minimization of damage due to the former mechanism, however, requires a knowledge of the segregation of physiological salt during freezing and the concomitant influence of cryoprotective agents on such segregation. It is for the evaluation and prediction of such segregation effects that low temperature phase diagram information is especially needed.

In addition to composition segregation effects, physiological substances perfused with cryoprotective agents and subsequently frozen may also show the development of glassy phases due to the increased viscosity brought about by the addition of the cryoprotective agent. At room temperature, for example, the viscosities of the two most widely used cryoprotective agents, dimethylsulphoxide (DMSO) and glycerol, are 1.98 cP and 954 cP, respectively [10, 11] compared to 1.0 for water. For solutions containing such materials, glassy phases may be formed even at relatively low cooling rates. In the H_2O–NaCl–DMSO ternary system investigated here, the development of glassy phases has been investigated as a function of system composition for an essentially constant cooling rate of 25° C min^{-1}. The heating of such vitreous solid phases can result in massive devitrification (crystallization) resulting either from the nucleation of crystals or the growth of pre-existing crystals or a combination of both [12-14].

Such effects would, of course, be important for physiological samples frozen under similar conditions, for which one might expect: (1) concentration of solute as the temperature

decreases, (2) formation of a vitreous solid, and (3) crystallization of the glassy phase either upon thawing or during long-term storage.

The criteria for substantial glass formation are a low driving force for the liquid to crystalline phase transition and a high activation energy for the decomposition of the glassy phase [13]. The long term stability of this glass will then depend on the time and temperature conditions to which the glass phase is exposed.

Composition effects have three primary influences on the crystallization process. The starting composition will determine the liquidus temperature, the path of crystallization and the resulting liquid phase composition as the temperature is lowered. The system's viscosity at any given temperature will in turn depend on this solution composition [14]. In this investigation we have aimed principally at the determination of the liquidus temperatures over a range of ternary compositions and the determination of the appearance or non-appearance, as well as the devitrification, of glassy phases as a function of initial composition. In particular, quantitative kinetic data have been developed for the recrystallization of the observed glassy phase. These data are then applied to the question of the low temperature long-term stability of the glassy phase.

2. Experimental

Phase diagram and glass-to-crystalline data have been obtained for the system H_2O–Na–Cl DMSO by means of low temperature differential thermal analysis (DTA). These experiments were made using a DuPont Model 900 thermal analyser together with a low temperature Dewar cell which allows rapid (25°C min^{-1}) cooling. The specimens used consisted of 0.02 ml of solution dispersed over approximately 0.02 g of thermally inert glass beads in a 4 mm × 28 mm glass tube. The reference specimen was, in every case, silicone oil similarly dispersed over glass beads. Heating rates varied from 1 to 30°C min^{-1}.

Both exothermic and endothermic peaks may be observed upon heating of an initially rapidly cooled specimen. A typical DTA thermogram is shown in Fig. 1 for the solution 78% H_2O–7.3% NaCl–14.7% DMSO. As shown, these peaks can be identified as glass transitions, G, exothermic glassy phase recrystallization, C, and endothermic melting peaks associated with a two-fold saturation line, T'', and finally, the liquidus surface, T'. Related DTA effects have been observed for binary solutions of DMSO–H_2O [2, 3], polyvinylpyrrolidone–H_2O binary solutions [15], hydrazine–H_2O binary solutions [16], glycerine–H_2O binary solutions [17], and also for the H_2O–NaCl–DMSO ternary system [18].

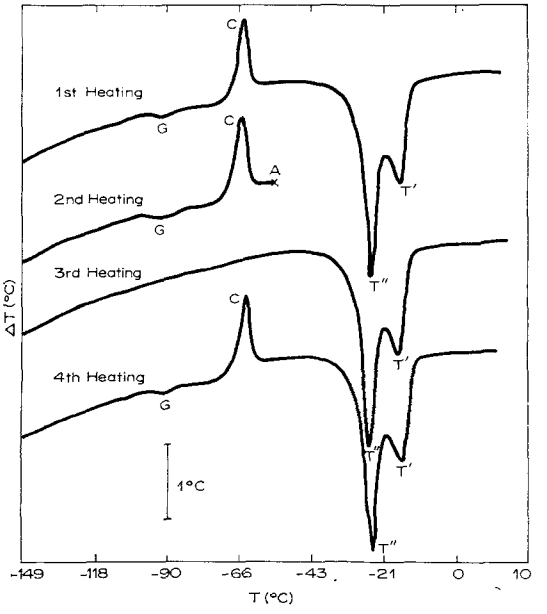

Figure 1 A series of DTA thermograms for the solution 78 wt % H_2O-14.7 wt % NaCl-7.3 wt % DMSO ($R' = \frac{1}{2}$; 78 wt % H_2O). Note the absence of both the glass transition endotherm (G) and the recrystallization endotherm (C) following quenching (A) at −54°C (curve 3). These features return after complete melting followed by rapid refreezing (curve 4).

In particular, the interpretation of the peaks labelled G and C may be confirmed, as shown in Fig. 1, by means of an interrupted heating experiment. In this experiment, the frozen sample is slowly heated (1°C min^{-1}) to a temperature, A, where $T'' > A > C$ after an initial rapid cooling to −196°C. At A, this sample is then quenched again to −196°C. Such an interrupted heating experiment demonstrates the metastable nature of the glassy solid phases present in the sample because the exothermic peak is not present in the second heating (see Fig. 1). In addition to the disappearance of the exothermic crystallization peak, the glass transition (base line slope change) at −93°C also did not appear during reheating after quenching at A. Both

observations indicate that the glassy phase is no longer present in significant quantity.

The range of solution compositions investigated is shown in Fig. 2. The solutions chosen all lie in one or more lines of constant composition ratio, R', where R' equals the ratio of the wt % DMSO to the wt % NaCl. Since ice, the first solid phase which forms, contains effectively no DMSO or NaCl, the residual solution composition will remain on the R' line defined by the initial solution composition. Thus, the solution used in Fig. 1, 78% H_2O–7.3% NaCl–14.7% DMSO may be described as the solution $R' = \frac{1}{2}$ (+ 78 wt % H_2).

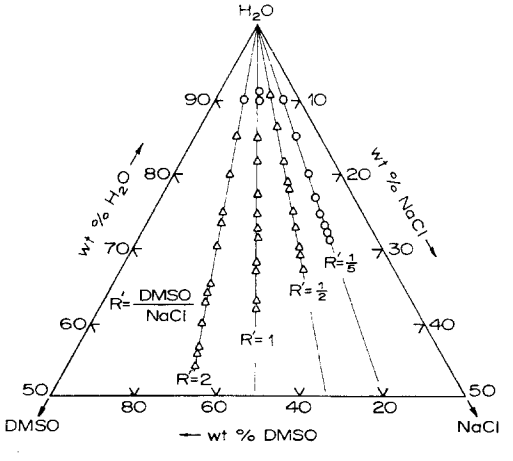

Figure 2 The points marked show the initial ternary system compositions. △ indicates solutions that formed a glassy phase on rapid cooling. O indicates solutions which did not appear to form such a phase.

Other compositions in addition to $R' = \frac{1}{2}$ (+ 78% H_2O) also displayed a glassy phase when rapidly cooled. Fig. 2, in addition to initial system compositions, also indicates those compositions that showed an exothermic crystallization peak. Also, in some low water content and DMSO rich (high R') solutions, a low temperature thermal effect was observed even though an exothermic peak was not. Such effects may indicate the existence of a glass phase which did not recrystallize or they may indicate a low temperature ternary eutectic reaction.

3. Results and discussion

Our principal aim has been the investigation of the kinetic stability of the glassy phase and the determination of kinetic data for the prediction of the long term stability of this phase at low temperatures. Such information may be of particular value to cryobiologists because recrystallization may affect the long term viability of biological materials prepared under conditions which lead to the generation of such a glassy phase with DMSO.

Many methods for the extraction of kinetic data from DTA curves have been proposed [19-23]. Most of these methods, however, were developed for stirred solutions and therefore might not be applicable to what are effectively solid solutions. Reduction of sample size by a factor of four resulted in no observable deviation of calculated values of Q. Thus, the initial sample size used was sufficiently small, Thompson [24], to introduce only small errors when applying the method of Kissinger. In applying this method to the system H_2O–NaCl–DMSO, what is measured is the shift in the temperature position of the exothermic crystallization peak maximum as the heating rate is varied. For recrystallization or other rate equations of order n, one may write

$$\frac{df}{dt} = k(1 - f)^n \qquad (1)$$

where f is the fraction of reacted material and k is the temperature-dependent rate constant. In this method, Q is determined by plotting $\ln(\lambda/T_p^2)$ versus $1/T_p$ where λ is the heating rate (K min^{-1}) and T_p is the temperature position of the exothermic crystallization peak (c) and $k = Q\lambda/RT_p^2$. The temperature dependence of k is given by

$$k = k_0 \exp(-Q/RT) \qquad (2)$$

where k_0 (the frequency factor) and Q (the activation energy) are constants and R is the gas constant.

Table I shows the results of the Q and k_0 calculations for two ratios of DMSO to NaCl, R', as a function of water content of the solutions.

With this information, the time, t, for a fraction of the reaction to go to completion can be predicted if the reaction order is known. This reaction order, n, can be evaluated from the actual observed shape of the exothermic DTA peak [20]. This evaluation is accomplished by means of the relationship

$$n = 1.26S^{1/2}$$

where S is a shape factor defined by

$$S = a/b\,.$$

TABLE I Activation energies as a function of composition on lines of constant ratios of DMSO to NaCl.

Composition ($R' = \frac{1}{2}$ + wt % H_2O)	Activation energy, Q (kcal mol^{-1})	Standard of deviation in Q (kcal mol^{-1})	Log frequency factor, k_0 (min)
67.0	17.3	5.1	18.804
69.0	12.4	4.5	13.562
70.0	15.8	4.7	16.865
73.0	12.3	4.4	13.384
75.0	15.3	4.5	16.579
78.0	8.6	3.1	9.429
78.8	8.4	3.1	8.896
81.3	11.7	4.2	12.637
86.4	11.6	3.4	12.546
90.6	9.0	3.2	9.797

Composition $R' = 1$ + wt % H_2O	Activation energy, Q (kcal mol^{-1})	Standard of deviation in Q (kcal mol^{-1})	Log frequency factor, k_0 (min)
62.0	12.3	3.1	12.308
62.9	7.2	1.5	6.917
67.0	11.4	3.3	11.306
68.0	12.8	4.8	12.740
71.5	15.8	15.6	15.824
73.0	9.7	2.8	9.433
75.0	18.9	6.7	18.752
77.6	17.9	4.5	17.605
81.5	12.8	6.4	12.301
90.6	—	—	—

where b is the distance between the tangent to the initial point of inflection (of the DTA peak) and the intersection of this tangent with the third point of inflection tangent. In this case a is the difference between b and the separation distance between the first and third points of inflection. Table II gives the reaction order as calculated by this method for all of our solutions. Fig. 3 illustrates the effect of water composition on reaction order for solutions with $R' = \frac{1}{2}$ and also shows the definition of a and b.

An assumption of uniform first order reaction kinetics would seem justified because of the small departure of n from unity and the small effect of fractional changes in the exponent n when integrating Equation 1 with respect to time, thus Equation 1 reduces to

$$\frac{df}{dt} = k_0(1 - f) . \qquad (3)$$

Using the kinetic data from Table I for values

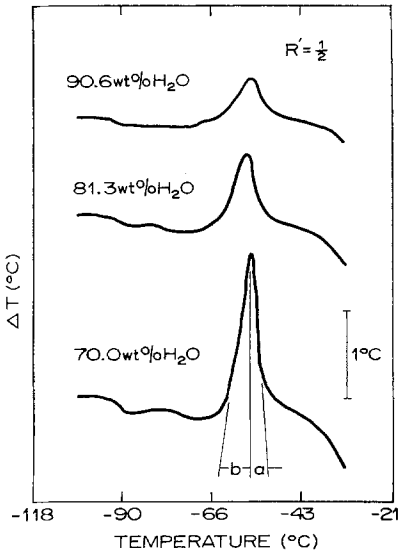

Figure 3 Typical DTA exotherms for the crystallization of the glassy phase which occurs in $R' = \frac{1}{2}$ solutions as a function of water content. Note the decrease in peak size with increasing water content.

of $R' = \frac{1}{2}$ and $R' = 1$ in conjunction with Equations 2 and 3, the time for any fraction of the transformation to be completed may be calculated by integrating Equation 3.

These times are shown in Fig. 4 as a function of temperatures that might be used for storing biological specimens. Fig. 4 indicates that if the crystallization of glass contributes to biological damage, storage temperatures will have to be of the order of $-130°C$ for prolonged storage. This temperature is also thought to be a minimum for both biological activity [25] and significant crystal growth rate of ice [26]. The variation in these times which result if the actual fractional orders, shown in Table II, are negligible.

It might be expected that both the kinetic behaviour and the relative amount of glassy material formed during initial rapid cooling should be a function of the initial solution composition. In the case of kinetic behaviour, this is not found to be the case. As may be seen from Table I, neither the activation energy nor the frequency factor is found to vary substantially with either initial water or initial DMSO concentration. This result leads to the conclusion that the composition of the glassy phase remains effectively constant and only the relative amounts of glassy phase changes as the initial ternary solution composition changes. This

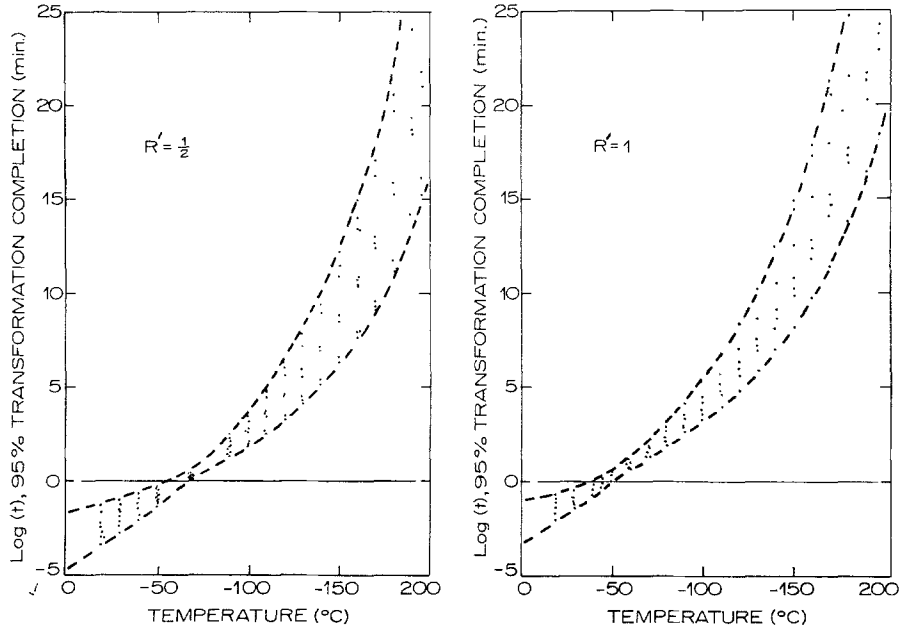

Figure 4 Log time required for 95% completion of crystallization transformation for DMSO/NaCl ratios—$R' = \frac{1}{2}$ and $R' = 1$. Points used for all compositions given in Table I.

TABLE II Reaction order as a function of water content on lines constant ratio of DMSO to NaCl

$R' = \frac{1}{2}$		$R' = 1$	
Composition (wt % H_2O)	Reaction order n	Composition (wt % H_2O)	Reaction order n
67.0	0.72	62.0	1.09
69.0	0.00	62.9	0.97
70.0	0.75	67.0	1.13
73.0	0.51	68.0	1.14
75.0	0.75	71.5	1.21
78.0	0.82	73.0	1.20
78.9	0.95	75.0	0.73
81.3	0.95	77.6	0.89
86.4	1.00	81.5	1.00
90.6	1.12	90.6	1.03

latter conclusion is verified by the observed relative integrated areas under the exothermic crystallization peak. That is, for nearly constant sample size, the area under the DTA curve can be expected to be proportional to the total heat released during crystallization. With the assumption that the energy release per gram of glassy phase crystallized is a constant, this integrated area will be proportional to the amount of glassy phase initially present. The three curves of Fig. 3 show this effect clearly; the higher water content samples clearly show a decreased fraction of glassy phase. This behaviour is precisely that which would be expected if the glassy phase were formed from the residual liquid present at the intersection of the appropriate R' ratio line with a line of two-fold saturation.

4. Conclusions

This investigation has shown that rapidly solidified biological material containing the cryoprotective agent dimethyl sulphoxide may not be entirely stable unless stored at very low temperatures. This is so due to the formation of a metastable glassy phase during initial rapid solidification. Such a phase is shown to form in the ternary H_2O–NaCl–DMSO system even for solutions very dilute in DMSO due to the rejection of DMSO into the residual liquid by the advancing ice phase. Quantitative kinetic data for the decomposition of this glassy phase is given and used to predict the stability of rapidly solidified H_2O–NaCl–DMSO solutions.

The development of additional phase diagram information relative to this and other H_2O–NaCl–cryoprotective agents appears to be a particularly appropriate area for the co-operation of materials scientists and biologists.

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References

1. A. U. SMITH, "Biological Effects of Freezing and Super-cooling" (Edward Arnold, London, 1961).
2. J. H. KITE, JUN and G. F. DOEBBLER, *Fedn. Proc.* **205** (1961) 149.
3. J. E. LOVELOCK and M. W. H. BISHOP, *Nature (London)* **183** (1959) 1394.
4. C. E. HUGGINS, *Science* **139** (1963) 504.
5. K. K. NAG and H. E. STREET, *Nature (London)* **245** (1973) 270.
6. J. E. LOVELOCK, *Biochim. Biophys. Acta* **16** (1953) 414.
7. *Idem, ibid* **11** (1953) 28.
8. P. MAZUR, *Science* **168** (1970) 939.
9. K. E. DILLER, E. G. CRAVALHO and C. E. HUGGINS, *Cryobiology* **9** (1972) 429.
10. "Dimethyl Sulfoxide Technical Bulletin", Crown Zellerbach Corporation, Camas, Washington (1973).
11. R. C. WEAST, ed., "Handbook for Chemical and Physics", 46th Edn. (The Chemical Rubber Company, Cleveland, 1965).
12. B. J. LUYET, *Biodynamica* **10** (1969) 277.
13. W. D. KINGERY, "Introduction to Ceramics", (Wiley, New York, 1960).
14. G. O. JONES, "Glass', (Wiley, New York, 1956).
15. B. LUYET and D. RASMUSSEN, *Biodynamica* **10** (1967) 137.
16. J. A. MCMILLAN, *J. Chem. Phys.* **42** (1965) 3497.
17. R. L. BOHON and W. T. CONWAY, *Thermochim. Acta* **4** (1972) 321.
18. W. E. BROWER and F. H. COCKS, *Cryobiology* (1974) to be published.
19. H. E. KISSINGER, *J. Reas. N.B.S.* **57** (1956) 217.
20. H. E. KISSINGER, *Anal. Chem.* **29** (1957) 1702.
21. H. J. BORCHARDT and F. DANIELS, *J. Amer. Chem. Soc.* **79** (1957) 41.
22. G. O. PILOYAN, I. D. RYABCHIKOV and O. S. NOVIKOVA, *Nature (London)* **215** (1966) 1229.
23. E. S. FREEMAN and B. CARROLL, *J. Phys. Chem.* **62** (1958) 394.
24. D. S. THOMPSON, in "Thermal Analysis", Vol. 2 (edited by R. F. Schwenker and P. D. Garn) (Academic Press, New York, 1966) p. 1147.
25. P. MAZUR, "Basis of Freezing Injury", in "Cryobiology", (edited by H. T. Meryman) (Academic Press, New York, 1966).
26. H. T. MERYMAN and E. KAFIG, *J. Appl. Phys.* **24** (1953) 1416.

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