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The equilibrium and non-equilibrium thermal behaviour of aqueous ternary solutions based on complex physiological support media, containing NaCl, and dimethyl sulphoxide or glycerol

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The effects of substituting a physiological support medium for water on phase diagram relationships in ternary systems composed of an aqueous component base (water or Eagle's minimum essential medium), NaCl, and a cryoprotective agent (glycerol or dimethyl sulphoxide) have been studied by differential thermal analysis. Primary solidification and eutectic transformations were observed, and both the transition temperatures and devitrification behaviour of non-equilibrium glassy phases were recorded. For most solutions it was found that substitution of the complex support medium for water had very little effect on equilibrium phase relationships. However, the aqueous component substitution resulted in small but measurable changes in the thermal behaviour of non-equilibrium glassy phases. In general, glass stability is enhanced. It is believed that the formation of stable glass phases plays a significant role in cryoprotection.

1. Introduction

Phase diagrams have been widely applied in the physical sciences. On the other hand, determination and application of phase relationships in the biological sciences is relatively recent. Within the past few years, for example, the low temperature behaviour of ternary systems consisting of water, salt, and a cryoprotective agent have been experimentally determined. Two such systems which have been studied extensively are H_2O–NaCl–dimethyl sulphoxide (DMSO) [1] and H_2O–NaCl–glycerol [2]. The application of this phase diagram information to the freezing and thawing of biological systems has been discussed [1–3].

In addition to approximately 99 wt % water and 0.85 wt % NaCl, physiological materials contain small amounts of many biological materials. In order to survive *in vitro,* such materials require a minimum number of supportive metabolites. A medium containing these metabolites was developed by Eagle [4] in 1959. This medium, which is now widely used in many tissue culture laboratories, is normally modified with Hanks' salts which are added to improve the supportive capacity of the medium. The composition of Eagle's minimum essential medium as modified with Hanks' salts (hereafter jointly referred to as MEM) is given in Table I. Eagle also maintained that such a support medium, supplemented with serum proteins, would permit large scale cultivation of a wide variety of cell lines. These serum proteins are frequently provided in the medium by addition of heat inactivated foetal calf serum (FCS). Thus, a typical support medium, and the one used in these studies, consists of MEM supplemented with 20 vol % FCS, hereafter designated (MEM + FCS). Of course, such a system contains, in the phase diagrams sense, a very large number of components. As we shall see, however, in terms of the liquidus

TABLE I Composition of Eagle's minimum essential medium with Hanks' salts (MEM). Grand Island Biological Company (1974) after Eagle (1959)

Compound	Concentration (mg/ litre^{-1}
L-amino acids	
arginine.HCl	126.00
cystine.2HCl	31.29
glutamine	292.00
histidine HCl.H_2O	42.00
isoleucine	52.50
leucine	52.40
lysine. HCl	72.50
methionine	15.00
phenylalanine	32.00
threonine	48.00
tryptophan	10.00
tyrosine	52.10
valine	46.00
Vitamins	
D-CaPantothenate	1.00
choline chloride	1.00
folic acid	2.00
i-inositol	1.00
nicotinamide	1.00
pyridoxal.HCl	1.00
riboflavin	0.10
thiamine.HCl	1.00
Salts	
$CaCl_2$ (anhydrous)	140.00
KCl	400.00
KH_2PO_4	60.00
$MgSO_4$ (anhydrous)	97.72
NaCl	8000.00
$Na_2HPO_4.2H_2O$	60.00
Other components	
glucose	1000.00
phenol red	10.00

surface at which ice first forms, systems containing both MEM and FCS may behave as essentially water–salt–cryoprotective agent ternary systems. It is primarily with regard to glass phases that MEM and FCS have their greatest effect.

Because of the potentially significant influence of the biological constituents of MEM and FCS on the behaviour of ternary solutions during freezing and thawing, the H_2O-based ternary phase diagrams have been redetermined substituting MEM and (MEM + FCS) in place of water. From the effect of these additions on viscosity, it is to be expected that the metastable glassy phases formed in the water-based systems [5, 6] would be particularly affected. The formation of such glasses in biological systems exposed to cryogenic temperatures has been proposed as a possible mechanism of cryoprotection [7–10]. Such amorphous phases might preclude the formation of lethal intracellular ice crystals and minimize damaging osmotic concentration effects.

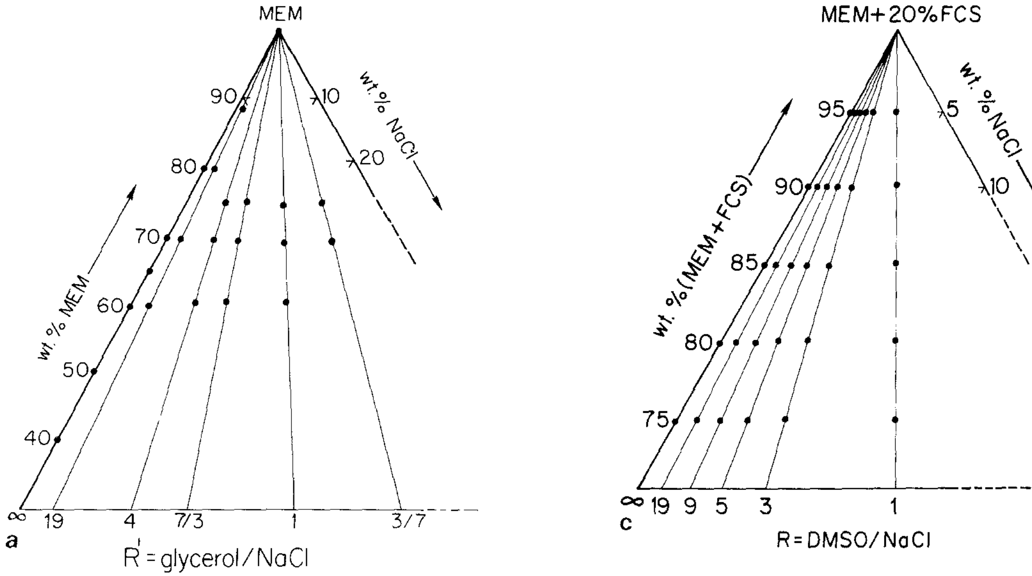
2. Experimental

Both equilibrium and non-equilibrium thermal behaviour were determined for ternary solutions composed of either MEM or (MEM + FCS) together with NaCl and a cryoprotective compound (DMSO or glycerol). Primary crystallization temperatures and temperatures indicating the onset of pseudo-binary eutectic solidification were recorded by standard differential thermal analysis techniques. Similarly, the transition temperatures and devitrification characteristics of glasses formed in these solutions were recorded. The specific systems studied were MEM–NaCl–glycerol, MEM–NaCl–DMSO, and (MEM + FCS)–NaCl–DMSO. The thermal data obtained were then compared to similar data for corresponding H_2O-based systems that were determined previously [1–3].

The compositions of all solutions studied were limited to the aqueous-rich regions of the phas diagrams. Solutions rich in the aqueous component are characteristic of physiological fluids and are of greatest interest in cryobiology since they will be, at least initially, isotonic to cells suspended in them. Thus, cellular damage due to osmotic effects will be reduced in such solutions.

At the onset of freezing, pure ice crystallizes from all solutions. Ice continues to precipitate until at least one of the components in the solute-enriched residual liquid, or a compound based on these components, reaches its solubility limit and begins to co-precipitate with pure ice as the temperature of the system is further lowered. During cooling, these solutions, whose initial compositions lie within the primary ice phase field, thus follow crystallization paths consisting of a tie-line which originates at a composition of 100 wt % H_2O and passes through the initial solution composition. This tie line extends into the ternary phase diagram along a line of constant ratio of the other two components, cryoprotective compound and NaCl, until a line of two-fold saturation is reached.

Individual ternary solutions were made up for constant ratios of the cryoprotective compound, either glycerol or DMSO, to NaCl, with varying amounts of the aqueous component, MEM or (MEM + FCS). Hereafter, solutions containing glycerol as the cryoprotective agent will be desig-



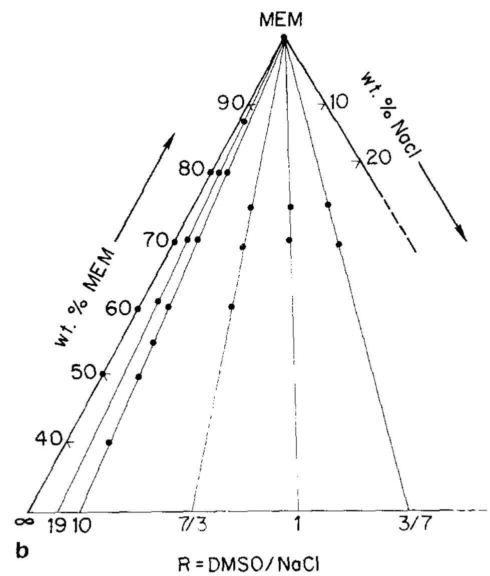


Figure 1 Composition of solutions studied by DTA
(a) MEM–NaCl–glycerol system.
(b) MEM–NaCl–DMSO sytem.
(c) (MEM + FCS)–NaCl–DMSO system.

nated by R', the weight ratio of glycerol to NaCl. Similarly, the weight ratio of DMSO to NaCl will be denoted by R. The thermal behaviour of these ternary solutions was investigated between -196 and 0° C using a DuPont model 900 thermal analyser with a rapid cool attachment. Details of these experiments have been described previously [1–3]. Samples were quenched to -196° C and heating thermograms were obtained using one of three constant heating rates; 2.5, or 10° C/min^{-1}. The thermal characteristics observed include endothermic primary solidification and eutectic transformation temperatures, glass transition temperatures, and exothermic devitrification temperatures. These thermal characteristics have been superimposed on several isoplethal sections taken from H_2O-based DTA data [1–2]. The compositions of the solutions studied are shown in the appropriate ternary diagrams in Fig. 1.

3. Results and discussion

Substitution of MEM for H_2O in solutions containing glycerol resulted only in some small variations in the liquidus surface of the primary ice phase field. Fig. 2 is an isoplethal section taken along the ratio line $R' = 7/3$ shown in Fig. 1a. Examination of this figure shows that high concentrations of glycerol and NaCl are less effective in depressing the freezing point of MEM than pure water. For solutions containing a constant amount of aqueous component, MEM (Fig. 3), this deviation becomes more pronounced at lower ratios, R', of glycerol to NaCl. However, eutectic solidification temperatures, indicated by points on the horizontal line in Fig. 2 and on the sloping, dashed line in Fig. 3, are essentially the same in both H_2O- and MEM-based systems. The dashed line in Fig. 3 represents a projection of the line of two-fold saturation (pseudo-binary eutectic trough) onto the 75 wt% H_2O isoplethal section of the H_2O–NaCl–glycerol system [2].

Examination of the isoplethal sections in Figs. 2 and 3 reveals variations in non-equilibrium be-

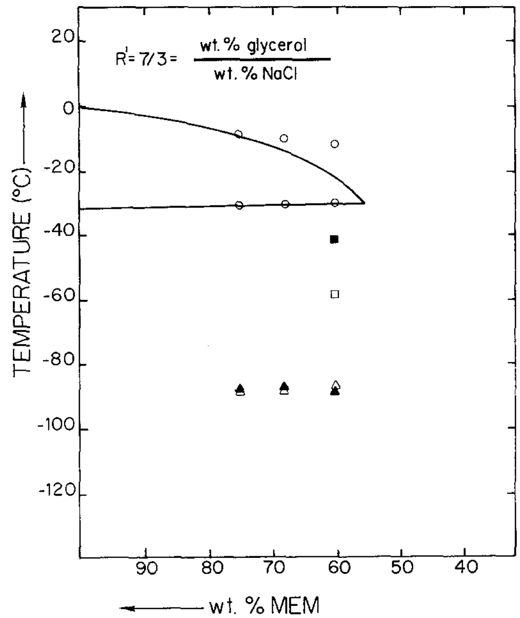


Figure 2 MEM $- R' = 7/3$ isoplethal section in MEM–NaCl–glycerol system. Solid lines and symbols for H_2O-based solutions, open symbols for MEM-based solutions. ○ = equilibrium transformations; △ = glass transitions; □ = glass devitrification.

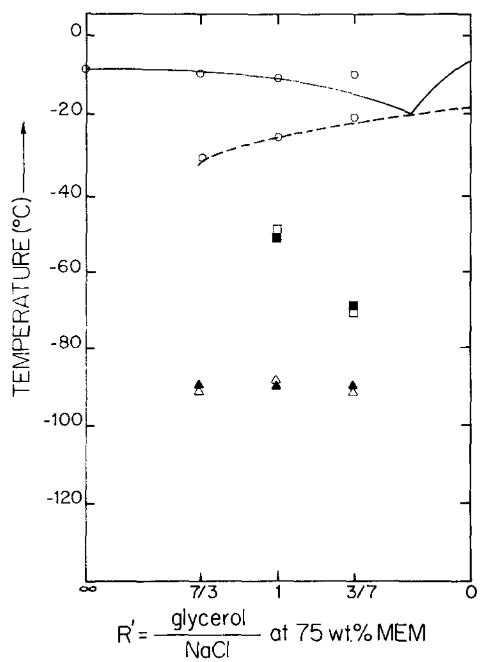


Figure 3 Isoplethal section at 75 wt % MEM in MEM–NaCl–glycerol system. Lines and symbols have same meaning as in Fig. 2.

haviour when MEM is substituted for H_2O. Glass transition temperatures in both MEM- and H_2O-based solutions occur at approximately $-90°$ C and are independent of both the aqueous component content (Fig. 2) and the ratio of glycerol to NaCl (Fig. 3). The stability of the glasses formed in these two systems was also found to be similar for either high H_2O or high MEM concentrations. Glass stability in both systems increased with increasing aqueous content (Fig. 2). This result derives from the observation that the glassy phases found at about 75 and 70 wt % MEM did not devitrify while those at about 60 wt % MEM did devitrify. However, the metastable glass formed in a solution with a ratio of glycerol to NaCl of 7/3 containing 60 wt% H_2 (Fig. 2) was more stable than the glass formed in the corresponding MEM-based solution. This conclusion is evidenced by the higher devitrification temperature for the H_2O-based glass implying that more thermal energy must be supplied, at a constant heating rate, to crystallize the H_2O-based glass than is necessary to devitrify the analagous MEM-based glass. Glass stability in both of these systems increased with increasing ratio of glycerol to NaCl (Fig. 3). This is in accordance with the greatly increasing viscosity of glycerol-rich solutions at higher ratios. The glasses formed at $-90°$ C in

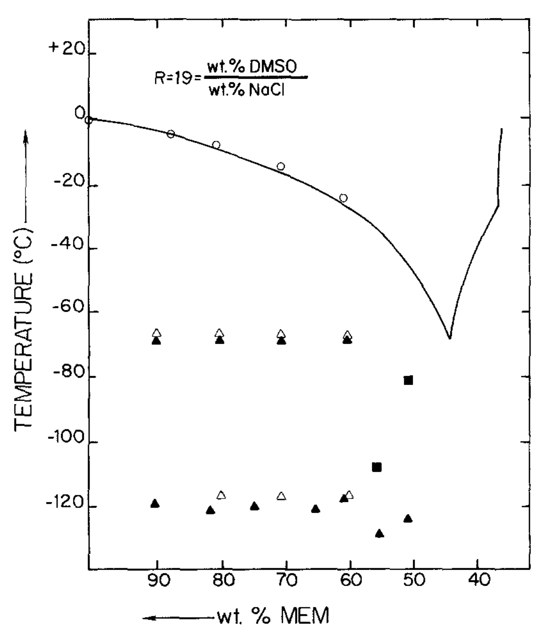


Figure 4 MEM $- R = 19$ isoplethal section in MEM–NaCl–DMSO system. Lines and symbols have same meaning as in Fig. 2.

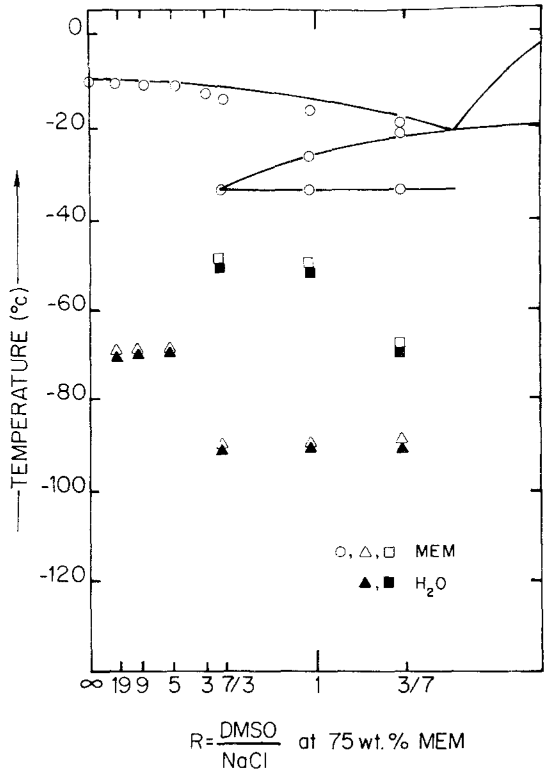


Figure 5 Isoplethal section at 75 wt % MEM in MEM–NaCl–DMSO system. Lines and symbols have same meaning as in Fig. 2.

solutions of $R' = 7/3$, containing either 75 wt % H_2O or MEM, did not exhibit devitrification peaks and were therefore assumed to be stable through the temperature range investigated.

Substitution of MEM or (MEM + FCS) for H_2O in aqueous based ternary solutions containing NaCl and DMSO resulted in only minor variations in both equilibrium and non-equilibrium behaviour. An isothermal section for a constant ration, $R = 19$, in the MEM–NaCl–DMSO system (Fig. 4) shows that the liquidus surface of this system is nearly identical to that of the corresponding H_2O-based ternary system. The similarity in equilibrium behaviour between these two systems is further illustrated on a constant 75 wt % aqueous component isopleth (Fig. 5). From this figure it can be seen that equilibrium transformation temperatures, including primary solidifications and eutectic reactions, are nearly identical for corresponding H_2O-based and MEM-based solutions.

The formation of non-equilibrium glassy phases in both H_2O-based and MEM-based solutions is also shown in Figs. 4 and 5. Examination of Fig. 4 shows glass transition temperatures in both systems at approximately $-120°$ C. As shown in this figure, the transition temperature of this glass is independent of the aqueous content of the solution for high ratios of DMSO/NaCl. In addition, what appears to be a second non-equilibrium reaction occurs in both MEM-based and H_2O-based solutions at approximately $-70°$ C. This transformation is also independent of the aqueous content of the solution at high ratios of DMSO/NaCl ($R = 19$). These relatively high-temperature non-equilibrium reactions exhibit thermal characteristics which are typical of those exhibited by glass transitions on DTA thermograms. Although this similarity exists, the possibility that these thermal characteristics may represent some non-equilibrium transformation other than glass formation has recently been suggested [11].

Similar non-equilibrium reactions were observed in aqueous-rich solutions between $R = 19$ and $R = 5$ for both MEM-based and H_2O-based solutions (Fig. 5). At 75 wt % aqueous component content, glasses form at about $-90°$ C in both systems, independent of the ratio of DMSO to NaCl between $R = 7/3$ and $3/7$. As in glycerol-containing solutions, the stability of these glasses increases as the ratio of cryprotective compound to NaCl is increase from 3/7 to 7/3.

The effects of supplementing MEM with 20 vol % FCS, and substituting this mixture for water in H_2O–NaCl–DMSO solutions, are shown in Figs. 6

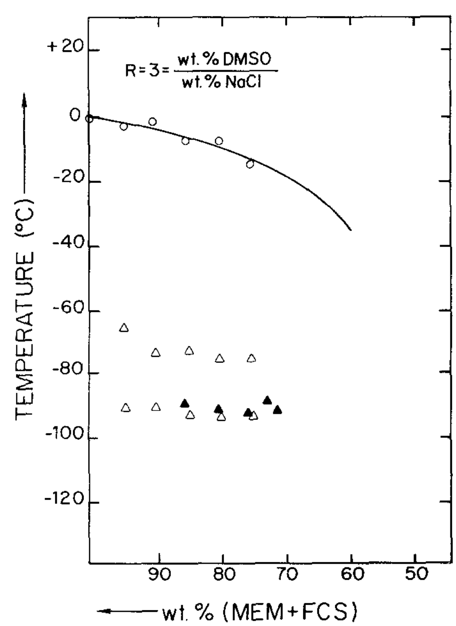


Figure 6 (MEM + FCS) – $R = 3$ isoplethal section in (MEM + FCS)–NaCl–DMSO system. Lines and symbols have same meaning as in Fig. 2.

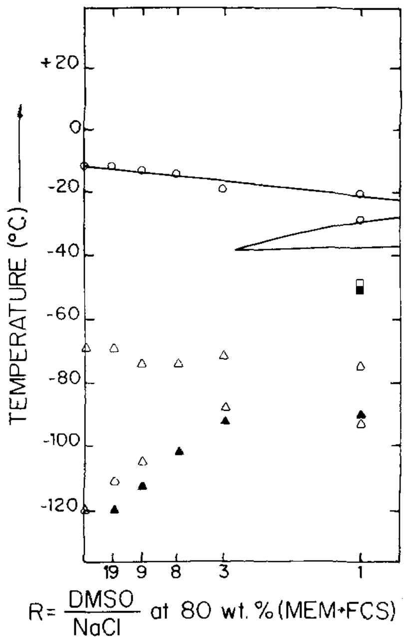


Figure 7 Isoplethal section at 80 wt % (MEM + FCS) in (MEM + FCS)–NaCl–DMSO system. Lines and symbols have same meaning as in Fig. 2.

and 7. The equilibrium liquidus points for all solutions based on (MEM + FCS) are within 1.5° C of the H_2O–NaCl–DMSO liquidus surface. However, the non-equilibrium behaviour of (MEM + FCS)-based solutions was found to be somewhat different from that of H_2O-based solutions. A typical comparison is shown in Fig. 6 for a ratio of $R = 3$. Stable glassy phases were formed at approximately −90° C in both systems. However, (MEM + FCS)-based solutions exhibited an additional glass transition, which ranged from −65 to −75° C depending on the aqueous content. Fig. 7, an isoplethal section for solutions containing 80 wt % (MEM + FCS), shows that the transition temperature of the low temperature glasses increased with decreasing ratios of DMSO/NaCl. This is to be expected since it is the presence of the cryoprotective compound, DMSO, which leads to the formation of this glass. The glass transition temperature of the high temperature glasses, on the other hand, was found to be independent of the ratio of DMSO to NaCl. This glass was found to exist at lower ratios of cryoprotective compound to NaCl in solutions containing foetal calf serum than in solutions without this additive. Thus, it appears that the presence of this highly viscous component promotes glass formation. Likewise, the presence of foetal calf serum tends to stabilize glassy phases. With the exception of the glass formed in the $R = 1 + 80$ wt % (MEM + FCS) solution, none of the glasses formed in solutions containing foetal calf serum devitrified over the temperature range −196 to 0° C.

4. Conclusions

The overall effect of both MEM and (MEM + FCS) on H_2O–NaCl–DMSO systems was found to be minor for both equilibrium and non-equilibrium behaviour. It has been shown that, with regard to initial equilibrium ice formation, the substitution of MEM or (MEM + FCS) for H_2O in H_2O–NaCl–CPA ternary solutions has little effect. Thus, available H_2O–NaCl–glycerol and H_2O–NaCl–DMSO ternary data may be used to predict solution compositions and the residual liquid fraction as functions of temperature, even in the case of MEM or FCS additions to these solutions. The primary effect of (MEM + FCS) is in the stability of the glassy phases which are formed during rapid cooling. In the case of MEM additions, the stability of some glassy phases formed at lower water content is decreased by such additions. The decrease in glassy phase stability may have a resultant influence on the viability of cells frozen in such media.

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