# CALORIMETRIC STUDIES OF THE STATE OF WATER IN DEEPLY FROZEN HUMAN MONOCYTES

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ABSTRACT Intra- and extracellular phase transitions in human peripheral blood monocyte suspensions with and without the cryoprotectant 1 M dimethylsulfoxide were measured using differential scanning calorimetry. Using an fluorescence diacetate/ethdium bromide assay for membrane integrity and a phagocytosis assay for cell function, it was found that mortality was correlated with several phase transitions under a variety of cooling and warming regimens. As a result of these studies we concluded that: (*a*) intracellular freezing is lethal, but avoidance of freezing during fast cooling is not sufficient to provide complete protection; (*b*) a subtle freezing injury in the cryoprotected monocytes can be correlated with a measurable increase in devitrification on warming; and (*c*) the cell contents form more stable glasses than the Hanks' balanced salt solution with fetal calf serum used as the extracellular medium.

## INTRODUCTION

It has become common to preserve various types of cells and tissues at −80 or at −196°C in liquid nitrogen using cryoprotectants. The cryoprotectants are classified according to two categories: (*a*) membrane penetrating cryoprotectants, such as glycerol, dimethyl sulfoxide ($Me_2SO$), ethylene glycol, etc., and (*b*) nonpenetrating cryoprotectants, such as polyvinylpyrrolidone, dextran, hydroxyethyl starch, etc. There is uncertainty as to the protective mechanism of the latter cryoprotectants, but the former are believed to suppress the increase of intra- and extracellular electrolyte concentration during freezing (1, 2). Protection by penetrating cryoprotectants is observed when cells are cooled at optimal cooling rates (usually relatively slowly, ~1 to 10°C/min); if cells are cooled more rapidly, they are injured by the formation of intracellular ice. Thus the cooling rate is a critical factor in protection against freezing injury when penetrating cryoprotectants are used (3). Still, it is not well understood why and how cells loaded with penetrating cryoprotectant and cooled at their optimal cooling rates can survive at very low temperatures when several physical changes of state occur in the solutions during both cooling and warming at these optimal rates. It has been shown by several workers (4, 5, 6, 7) that penetrating cryoprotective solutions, even without cells present, exhibit freezing (heterogeneous and homogeneous nucleation) and glass formation during cooling and phase changes such as glass melting, devitrification, recrystallization, and melting, upon warming. The characteristics of such phase changes in the cytoplasm of cryoprotected cells and the correlation of such changes with viability have not been studied extensively.

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Human peripheral blood monocytes migrate into tissues and play an essential role in many different types of immune and inflammatory reactions. A highly reliable method to cryopreserve them has been established (8, 9, 10). Deviation from the optimal procedure greatly reduces survival. Here we report on studies in which we froze cryoprotective solutions with and without concentrated monocytes, and noncryoprotective (no $ME_2SO$) solutions with and without concentrated monocytes by various freezing protocols in the differential scanning calorimeter (DSC). We obtained both freezing and thawing records, checked the viability of the samples, and compared the mortality of cells with the phase changes detected by DSC. Correlation of monocyte mortality to thermal analysis by DSC shows several critical events in cells frozen at low temperatures.

## MATERIALS AND METHODS

Whole human blood was collected in full units drawn with citrate phosphate dextrose anticoagulant by the Washington Regional Blood Services (Washington, DC). Platelet-rich plasma was removed within 2 h of collection by centrifugation at 2,600 *g* for 3 min, a buffy coat (40 ml of the top layer of packed cells) was collected. The buffy coat was layered on a Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), centrifuged at 400 *g* for 30 min at room temperature, then mononuclear cells were collected. Monocytes were isolated from the mononuclear cells using a J2-21 centrifuge (Beckman Instruments, Palo Alto, CA) equipped with a JE-6 elutriator rotor and two Sanderson's separation chambers, following a procedure modified from Lionetti et al. (11). The purity of the monocytes was 95% as determined by both morphology (stained with May-Grünwald-Giemsa) and nonspecific esterase staining (Technicon Instruments, Tarrytown, NY).

Purified monocytes were suspended in Hanks' balanced salt solution without calcium and magnesium (HBSS) (Gibco Laboratories, Grand Island, NY), HBSS containing 5% (vol/vol) fetal calf serum (FCS) (M.A. Bioproducts, Walkersville, MD) or HBSS containing 5% FCS and 7.5% (vol/vol) $Me_2SO$ (Fisher Scientific Corp., Fair Lawn, NJ). These

monocytes were packed by centrifugation at 150 *g* for 5 min at room temperature. Packed monocytes were placed in the sealable aluminum sample pans of a DSC (Perkin-Elmer DSC-4, Norwalk, CT), weighed, and cooled and/or warmed following the protocols of freezing experiments. Seeding of biological samples was accomplished by cooling the sample pan in the DSC-4 until a freezing event was observed on the microcomputer's videoscreen. As soon as this event was observed, the DSC-4 was warmed at ≥100°C/min to a preset subfreezing temperature, at which bulk samples are not injured when the extracellular solution is seeded. This temperature would be in the range −2 to −5°C for HBSS and FCS-HBSS, and −7 to −10°C for $Me_2SO$-FCS-HBSS. After annealing at that temperature for at least 5 min, cooling of the sample was resumed. The lowest temperature to which samples were cooled in these experiments was −160°C, the lower limit of accurate measurement by the DSC. After freezing and thawing, cells were resuspended in the same solution. The cells suspended in $Me_2SO$-FCS-HBSS were washed by slow dropwise addition of FCS-HBSS so as to dilute $Me_2SO$.

Monocyte viability (membrane integrity) was checked by the contrast fluorescence test as described by Martel et al. (12). The final concentrations of fluorescein diacetate (FDA) (Sigma Chemical Co., St. Louis, MO) and ethidium bromide (EB) (Sigma Chemical Co.) were 2 μg and 4 μg/ml cell suspension, respectively. In this FDA/EB assay, viable cells show green fluorescent cytoplasm and dead cells show red fluorescence of the nuclei under epifluorescence illumination using a fluorescent microscope (BH-RFL fluorescence illuminator mounted on system microscope model BHA; Olympus Corporation of America, New Hyde Park, NY). Phagocytosis was detected by a modification of the fluorochrome acridine orange staining method described by Pantazis and Kniker (13). In this assay, monocytes were incubated for 30 min with stationary phase *Escherichia coli* which had been opsonized by pooled serum, then stained by acridine orange (Sigma Chemical Co.). Monocytes that phagocytized more than five bacteria were regarded as cells that retained their phagocytic function.

## RESULTS

### Warming Curves of Monocyte-free Solutions

Monocyte-free solutions, HBSS, FCS-HBSS, and $Me_2SO$-FCS-HBSS were cooled at 1.3°C/min to −20°C, cooled to −160°C at 100°C/min, then warmed to −20°C at 80°C/min. The warming curves of HBSS (solid curve), FCS-HBSS (dashed curve), and $Me_2SO$-FCS-HBSS (dot-dashed curve) are summarized in Fig. 1. The glass transition of aqueous salt (labeled *Tg* aqueous salt) was seen in every curve. In $Me_2SO$-FCS-HBSS solution (dot-dashed curve), the aqueous salt glass transition was observed ~5°C higher than in $Me_2SO$-free solutions (solid and dashed curves). In the dot-dashed curve, the glass transition of aqueous $Me_2SO$ (labeled *Tg* $Me_2SO$) was seen at −115°C. The major devitrification (labeled *Td*) was observed in both $Me_2SO$-free solutions at approximately −60°C. In $Me_2SO$-FCS-HBSS, devitrification at −60°C was suppressed. There was ~5°C difference in the devitrification temperature between HBSS and FCS-HBSS solutions.

### Comparison of Warming Curves of $Me_2SO$ Solution and Cells in $Me_2SO$ Solution

The $Me_2SO$-FCS-HBSS solution, and cells equilibrated with $Me_2SO$-FCS-HBSS and then packed were both cooled to −80°C at 1.3°C/min, cooled to −160°C at

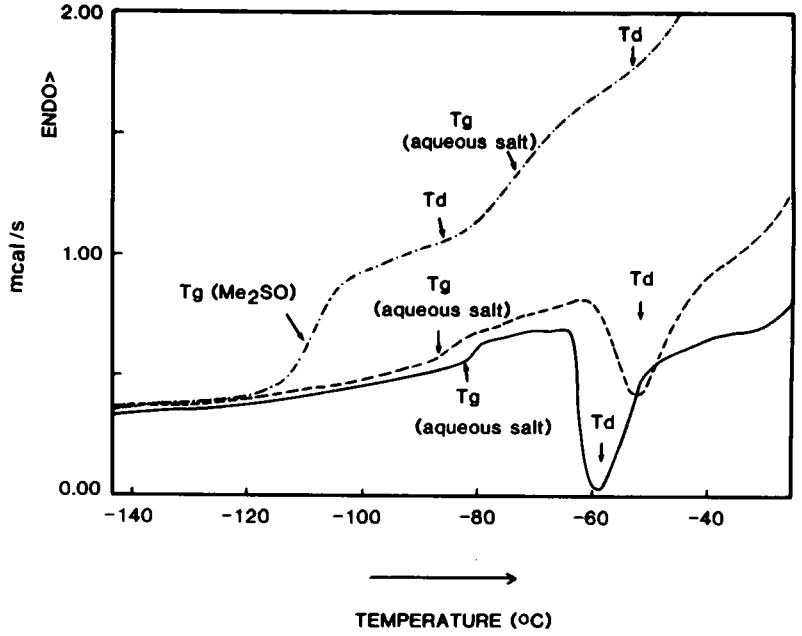


FIGURE 1 Warming curves of various solutions. *Abscissa:* an arrow on the temperature scale shows the direction of DSC run (i.e., warming → or cooling ←). *Ordinate:* heat diffusion rate (mcal/s). Up indicates an endothermic event and down indicates an exothermic event in the sample. *Tg* $Me_2SO$, *Tg* aqueous salt: glass transition temperatures of aqueous $Me_2SO$ and aqueous salt, respectively. *Td*: devitrification temperature of same. Solid curve: HBSS cooled to −20°C at 1.3°C/min, cooled to −160°C at 100°C/min, then warmed to −20°C at 80°C/min. Dashed curve: FCS-HBSS cooled and warmed in the same way as solid curve. Dot-dashed curve: $Me_2SO$-FCS-HBSS cooled and warmed the same way as solid and dashed curves.

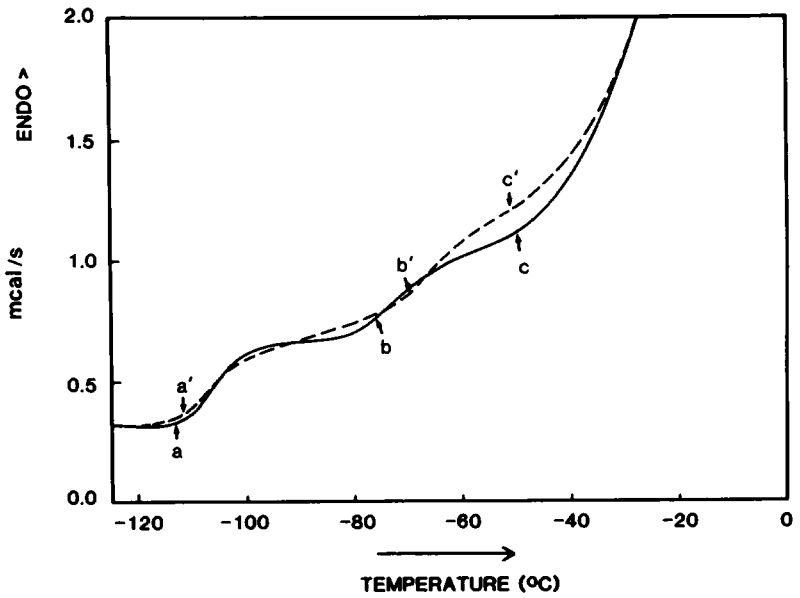


FIGURE 2 $Me_2SO$-FCS-HBSS vs. monocytes in $Me_2SO$-FCS-HBSS. Solid curve: $Me_2SO$-FCS-HBSS cooled to −80°C at 1.3°C/min, cooled to −160°C at 100°C/min, warmed to −20°C at 80°C/min. Dashed curve: same as solid curve, but solution contained densely packed suspension of monocytes. *a* and *a'* show *Tg* $Me_2SO$, *b* and *b'* show *Tg* salt, and *c* and *c'* show *Td*.

100°C/min, then warmed to −20°C at 80°C/min. An aqueous-$Me_2SO$ glass transition was seen at −115°C (labeled *a* and *a'*) in both cell-free (Fig. 2, solid curve) packed cell samples (dashed curve). In the curve representing a cell-free solution, an aqueous salt glass transition was seen at −80°C (*b*), but in the curve representing packed cells, it was seen at −70°C (*b'*). Little devitrification was seen in either solution (*c* and *c'*), but it was slightly greater in the cell-free suspension. Monocytes frozen in this condition and thawed completely retained complete membrane integrity (FDA/EB 98.0 ± 0.8%, mean ± SD, $n = 4$) and phagocytic ability (94.5 ± 1.3%) compared with nonfrozen cells (FDA/EB: 98.5 ± 1.0%, phagocytosis: 95.3 ± 0.8%).

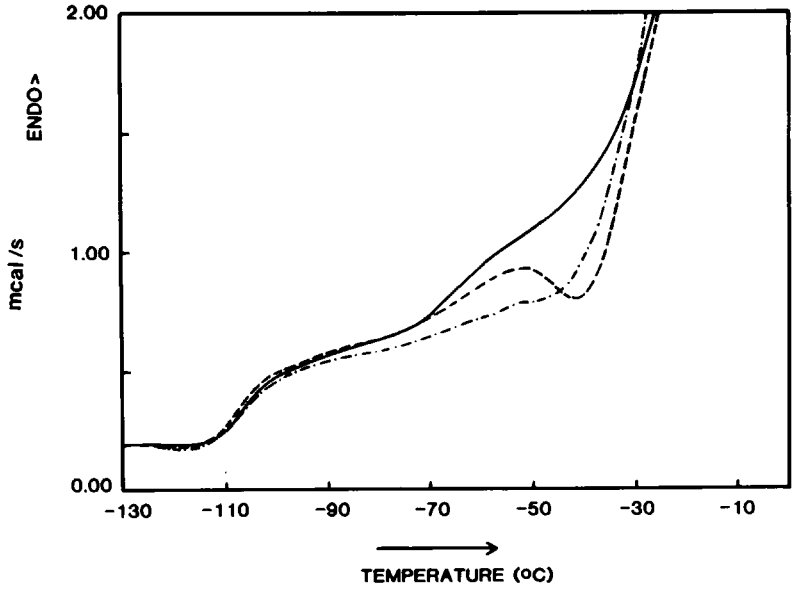


FIGURE 3 Comparison of first, second, and third warming curves. Solid curve: monocytes in $Me_2SO$-FCS-HBSS cooled to −80°C at 1.3°C/min, cooled to −160°C at 100°C/min, warmed to −20°C at 80°C/min (first warm, same as dashed curve in Fig. 2). Dashed curve: the above sample was held at −20°C for 3 min, recooled to −160°C at 100°C/min, then rewarmed to −20°C at 80°C/min (second warm). Dot-dashed curve: the above (dashed curve) was again held at −20°C for 15 min, then recooled to −160°C at 100°C/min, then rewarmed to −20°C at 80°C/min (third warm).

## Comparison of First, Second, and Third Warming

Monocytes in $Me_2SO$-FCS-HBSS were cooled to −80°C at 1.3°C/min, cooled to −160°C at 100°C/min, warmed to −20°C at 80°C/min (first warming, Fig. 3, solid curve), held for 3 min at −20°C, recooled to −160°C at 100°C/min, then warmed to −20°C at 80°C/min (second warming, dashed curve). Rapid recooling from −20°C and rewarming yields a large devitrification (beginning at −60°C) even in the presence of $Me_2SO$ (dashed curve), which was not seen in the first warming curve. These cells were once again cooled from −20 to −160°C at 100°C/min, then warmed (third warming, dot-dashed curve). When the once devitrified cells (dashed curve) were recooled and rewarmed again (dot-dashed curve), the devitrification began at a much lower temperature and was at least as extensive as that during the second warming. This is clear because not only is the dot-dashed curve significantly exothermic with respect to the dashed curve from −95 to −50°C, but at higher temperatures it is uniformly endothermic in the comparison, indicating more ice is melting during the third warming than during the second.

## Comparison of Cooling Curves of Monocytes Rapidly Cooled from −20° and −35°C

Monocytes in $Me_2SO$-FCS-HBSS were seeded at −7°C, cooled to −20°C or to −35°C at 1.3°C/min, and then cooled to −160° at 100°C/min. Cells in $Me_2SO$-FCS-HBSS cooled quickly from −35°C (Fig. 4, dashed curve) showed no very low temperature event upon cooling. Cells cooled quickly from −20°C showed a substantial freezing event upon cooling in the range of −90 to −110°C (Fig. 4, solid curve). Cells cooled rapidly from −20°C were seriously injured (FDA/EB: 63.3 ± 3.1%, phagocytosis: 0.0 ± 0.0%). In cells cooled rapidly from −35°C, ~40% lost phagocytic ability although most retained their membrane integrity (FDA/EB: 92.0 ± 1.3%, phagocytosis: 61.3 ± 1.3%).

## Comparison of Warming Curves of Cells Fast Cooled from −35 and from −50°C

Monocytes in $Me_2SO$-FCS-HBSS were cooled to −35 or to −50°C at 1.3°C/min, cooled to −160°C at 100°C/min, then warmed to −20°C at 80°C/min. In the warming curves of these cells (Fig. 5), there were small devitrifications at −75°C (labeled $x_1$) and at −45°C ($y_1$) if cells were cooled quickly from −35°C (solid curve). The low temperature devitrification ($x_1$) observed upon warming in the cells cooled quickly from −35°C became much smaller ($x_2$) when cells were warmed after being cooled quickly from −50°C (dashed curve). A high temperature devitrification ($y_1$) in the cells cooled from −35°C also became smaller ($y_2$) and both devitrifications occurred at higher temperatures in the cells cooled from −50°C. Injury in cells cooled slowly to −50 and the rapidly cooled to −160°C was hard to detect (FDA/EB: 98.0 ± 1.0%, phagocytosis: 92.5 ± 1.0%), but quite noticeable by phagocytosis assay in cells cooled slowly to −35°C as mentioned.

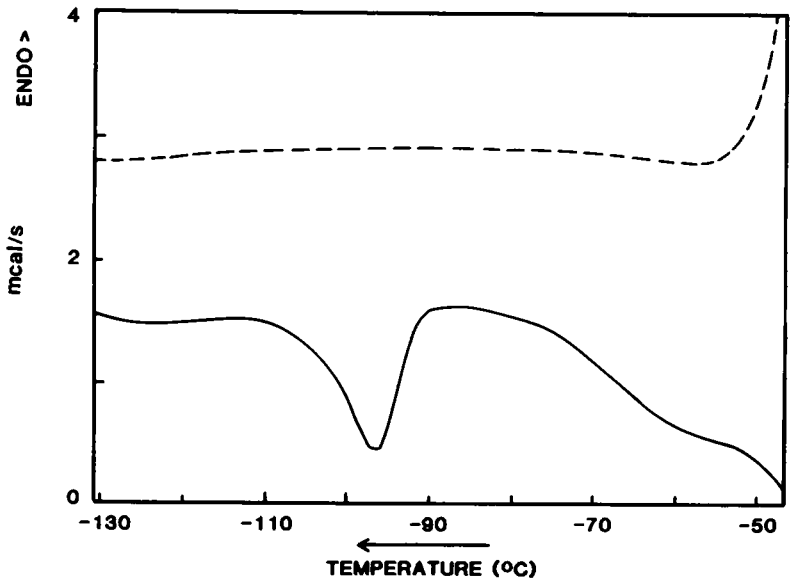


FIGURE 4 Cooling curves of monocytes rapidly cooled from −20°C vs. monocytes rapidly cooled from −35°C. Solid curve: monocytes in $ME_2SO$-FCS-HBSS cooled to −20°C at 1.3°C/min, then to −160°C at 100°C/min. Dashed curve: same as solid curve, but monocytes cooled to −35°C at 1.3°C/min, and then cooled to −160°C at 100°C/min.

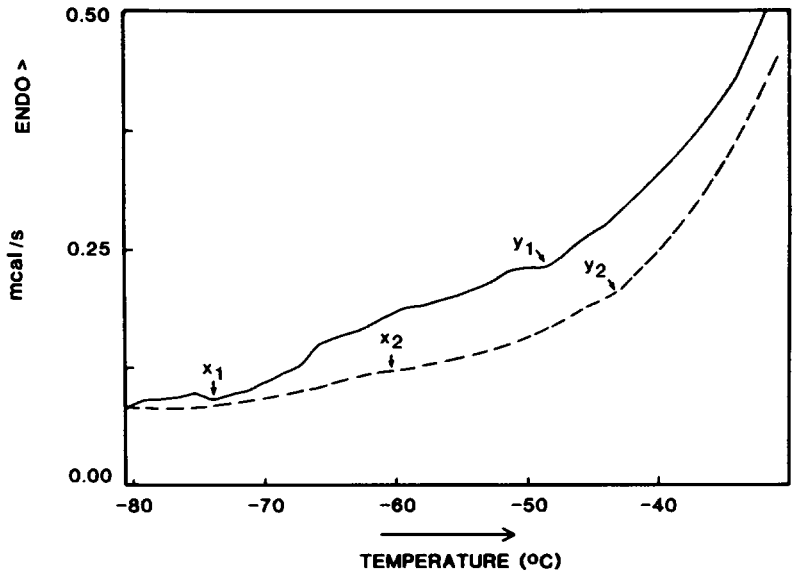


FIGURE 5 Warming curves of fast cool from −50°C vs. fast cool from −35°C. Solid curve: monocytes in $Me_2SO$-FCS-HBSS cooled to −35°C at 1.3°C/min, to −160°C at 100°C/min, warmed to −20°C at 80°C/min. Dashed curve: monocytes in $Me_2SO$-FCS-HBSS cooled to −50°C at 1.3°C/min, to −160°C at 100°C/min, warmed to −20°C at 80°C/min. Explanation of labeled points in text.

## Cells vs. No Cells in $Me_2SO$-free Solution

Monocytes suspended in FCS-HBSS, but not protected with $Me_2SO$ were cooled to −80°C at 1.3°C/min, cooled to −160°C at 100°C/min, then warmed to −20°C at 80°C/min (first thaw, Fig. 6, solid curve). These cells were recooled immediately to −160°C at 100°C/min, rewarmed to −20°C at 80°C/min (second thaw, not shown), held at −20°C for 15 min, recooled to −160°C at 100°C/min, then warmed to −2°C at 80°C/min (third thaw, dashed curve). Alternatively, the same FCS-HBSS, but without cells, was cooled at −20°C at 1.3°C/min, then cooled to −160°C at 100°C/min, then warmed to −2°C at 80°C/min (dot-dashed curve). Cells slowly cooled to a very low temperature without $Me_2SO$ (solid curve) showed greatly reduced devitrification upon thawing in compari-

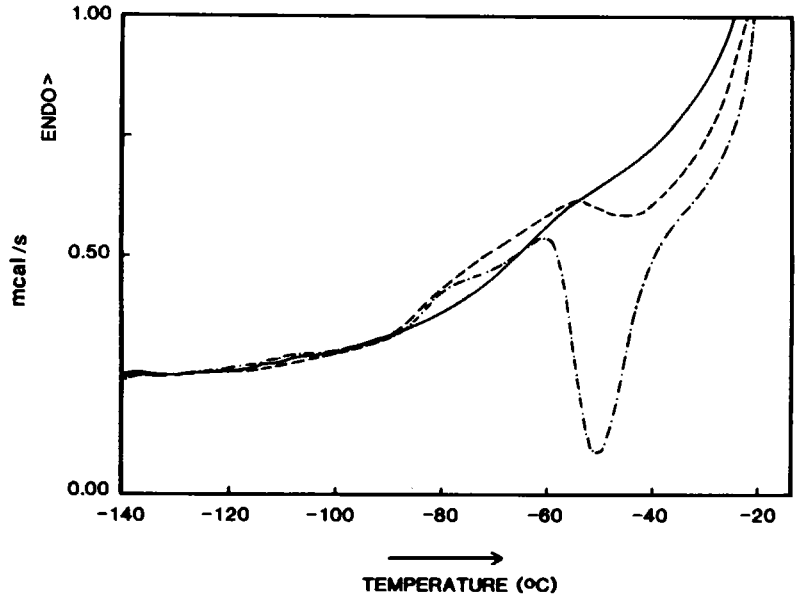


FIGURE 6 Cells vs. no cells in $Me_2SO$ free solution. Solid curve: monocytes suspended in FCS-HBSS were cooled to −80°C at 1.3°C/min, cooled to −160°C at 100°C/min, then warmed to −20°C at 80°C/min (first warm). Dashed curve: the above sample was immediately cooled from −20°C to −160°C at 100°C/min, rewarmed to −20°C at 80°C/min, held at −20°C for 15 min, recooled to −160°C at 100°C/min, in this record warmed again to −2°C at 80°C/min (third warm). Dot-dashed curve: the same solution as in solid and dashed curves, but no cells, cooled to −20°C at 1.3°C/min, then cooled to −160°C at 100°C/min, here warmed to −20°C at 80°C/min.

son to FCS-HBSS without cells. The temperature of the glass transition was also much higher than in the cell-free solution. But cells frozen once without $Me_2SO$ were completely injured (FDA/EB: 0.0 ± 0.0%, phagocytosis: 0.0 ± 0.0%). If cells were recooled quickly from −20°C and then rewarmed (an additional cycle of freeze-thaw) as observed in the third warming curve (dashed curve), the glass transition occurred at about the same temperature as that of the cell free solution, but devitrification was still suppressed.

## DISCUSSION

The data in Fig. 1 illustrate the extent to which thermal events observed in monocyte suspensions are characteristic of the cell-free suspension media. The endothermic event (glass transition) at approximately −115°C in the dot-dashed curve is about where one observes the glass transition in aqueous $Me_2SO$ solutions (5, 6, 7). The glass transitions at about −85°C in the solid curve (HBSS) and at about −90°C in the dashed curve (FCS-HBSS) indicate that the major glass transition in these solutions is of water associated with salt (aqueous salt), not water associated with protein (FCS). Thus the major effect of the FCS appears to be to raise the general viscosity of the solution and thereby raise the devitrification temperature ~5°C and reduce the amount of devitrification. If $Me_2SO$ is also added, as in the dot-dashed curve, the glass transition temperature of the aqueous salt is almost the same, but devitrification of the aqueous salt is severely depressed (the *Td* points on the dot-dash curve are virtually undetectable). Thus both FCS and $Me_2SO$ appear to effect devitrification (*Td*) more profoundly than they affect the glass transition temperature (*Tg*). Since $Me_2SO$ is a powerful colligative agent (5), suppression of devitrification in an aqueous $Me_2SO$ solution was not surprising. An unexpected result was that FCS, which has a low osmotic coefficient, even at relatively high concentrations, should affect devitrification so much more than it affects the aqueous salt glass transition temperature. This is despite its undoubted ability to increase viscosity greatly at high concentration and low temperature. It argues that the water associated with aqueous salt is literally in a separate microdomain, with its own viscosity, largely independent of protein associated water (14).

In Fig. 2, dashed curve, we compare the thermal characteristics of an initially dense suspension of cells (~90% of sample volume occupied by cells) cooled slowly to −80°C in $Me_2SO$-FCS-HBSS to $Me_2SO$-FCS-HBSS slowly cooled to −80°C without cells (solid curve). We note little change in the glass transition of the aqueous $Me_2SO$ (~115°C) (*a*, *a'*). The aqueous salt glass transition has risen by ~10°C in the cell suspension (from *b* to *b'*). Also, devitrification is further reduced in the cell suspension *c* and *c'*). A second freeze (homogeneous nucleation) is not observed upon slow cooling of these cells to −80°C, implying that the differences between the two curves of Fig. 2 reflect the differences between the intracellular solution with $Me_2SO$ and cell-free solution with $Me_2SO$. This conclusion is based on the assumption that if the initial cell concentration is high, such that most of the initial solution volume is intracellular, the high ratio of intra- to extracellular volume remains roughly constant even at extreme subzero temperatures. At least one condition could cause this assumption to be significantly in error. If the osmotic coefficient of the extracellular solution should deviate from ideality (>ideal) significantly more than the intracellular solution, then as the temperature is lowered most additional loss of liquid water to ice would be from the intracellular solutions, thus changing the ratio of the two compartments. On the other hand, if the osmotic coefficient of the two compartments are not very different, then the ratio of water in them will stay roughly constant. Thus the high temperature glass transition (*b'*) shown in Fig. 2 (dashed curve), can be presumed to represent an intracellular glass transition, whereas the congeneric transition (*b*) on the solid curve represents an aqueous salt glass transition in the $Me_2SO$-FCS-HBSS solution. Thus the record indicates that the cell interior becomes partially glassy ~10°C above the extracellular solution. Therefore, we can conclude: (*a*) the intracellular medium effects the aqueous $Me_2SO$ glass transition almost identically to the FCS-HBSS system, (*b*) depresses devitrification of the concentrated aqueous $Me_2SO$ glassy melt better than FCS-HBSS, (*c*) possesses a higher temperature aqueous salt transition than that of FCS-HBSS, and (*d*) suppresses devitrification of the aqueous salt and $Me_2SO$ melt better than the $Me_2SO$-FCS-HBSS system. Note that the efficiency of suppression of devitrification in this system is sufficient to allow complete survival of the cells.

In Fig. 3, the thermal record of the warming of packed cells in $Me_2SO$-FCS-HBSS after fast freezing from −80°C is compared with the warming of the same cells when fast freezing begins at −20°C. Thus the solid curve is the same as the dashed curve in Fig. 2 and represents the behavior of uninjured cells. The dashed curve illustrates what happens when the cells were warmed to −20°C (after slow cooling to −80°C and fast cooling to −160°C) recooled quickly to −160°C then rewarmed quickly (second warming). It implies that the intracellular water in equilibrium with ice at −20°C will form homogeneous nuclei upon quick cooling. This latter point is proved by Fig. 4, where the actual freezing event is demonstrated (solid curve). What is important to note here, however, is that during the second warming of the cells to −20°C, a large devitrification occurred above the glass transition of the aqueous salt. Thus a large intracellular ice formation upon warming was implied. This assessment is further strengthened by the dot-dashed curve, the results of a second fast freeze to −160°C from −20°C (third warming). Note that in the record of this third warming experiment, ice formation begins much sooner (compared

with the second warming), with the $Me_2SO$ associated water significantly less stable than during the second warming (in the range $-100$ to $-70$°C). This might indicate the dissociation of some of the cells, but no such structural damage is observed at temperatures $\leq 10$°C in the cryomicroscope. Consistent with the notion that even more ice is forming during the third warming is the smaller, higher temperature aqueous salts glass transition and the large melt (large endotherm) between $-30$ and $-50$°C.

Since Figs. 3 and 4 demonstrate that significant intracellular freezing occurs during both fast cooling from $-20$ to $-160$°C and subsequent fast warming from $-160$ to $-20$°C, how much of the cellular mortality is due to each event separately is not ascertainable from our data. However, the dashed curve of Fig. 4 represents cells slowly cooled to $-35$°C before fast cooling to $-160$°C and clearly shows no significant intracellular freezing during cooling. This suppression of homogeneous nucleation is not surprising. Aqueous solutes, which display nearly ideal behavior with respect to suppression of the equilibrium freezing point, suppress homogeneous nucleation according to the following approximate equation (15): $T_H = -40 - 1.8 \times \Delta T_m$; $\Delta T_m$ is the equilibrium suppression of melting in °C and $T_H$ is the homogeneous nucleation temperature in °C. Thus a nearly ideal solution with $\Delta T_m = 20$°C would have $T_H \approx -76$°C. A nonideal solution can be much more efficient. Thus if a nearly ideal solution has a $\Delta T_m$ of 35°C and $T_H \approx -101$°C, a nonideal solution with $\Delta T_m$ of 35°C and a coefficient of suppression of 2.6× would prevent all homogeneous nucleation by yielding $T_H = -133$°C. Even if $T_H$ is approximately $-100$°C, that is so low that if initial solution viscosity is very high, ice growth might be negligible at $-100$°C at a cooling rate of ~100°C/min, the rate used in the experiments represented in Fig. 4. Thus any mortality upon warming after cooling quickly from $-35$°C can be confidently ascribed to intracellular devitrification and possibly a subsequent recrystallization. That such events can be seen is illustrated by the points $x_1$ and $y_1$ on the solid curve of Fig. 5. These events are associated with ~40% loss of phagocytic ability and little loss of membrane integrity. We do not yet know if the damage occurs by the end of the event labeled $x_1$, or is dependent on passage through $y_1$ as well, or is partitioned between the two events. The dashed curve in Fig. 5 shows that after fast freezing from $-50$°C, residual devitrification continues to occur at $x_2$ ($\equiv x_1$) and $y_2$ ($\equiv y_1$) but by both phagocytic and FDA/EB assay all damage has disappeared. While it would be exciting to conclude that this shows a tolerable level of intracellular devitrification, it is not clear whether these residual events emanate from the small number (<10%) of damaged monocytes present in both experimental and control systems.

The thermal behavior of the monocytes at very low temperatures in HBSS or FCS-HBSS solutions without $Me_2SO$ is, unfortunately, not associated with loss of viability, all of which occurs about $-20$°C. In our previous experiments, we found that monocytes shrink under osmotic stress and freezing and lose as much as 70% of their original volume in HBSS, but cells lose phagocytic and chemotaxis functions when dehydrated to <60% of their original volume. One of the reasons for this loss of function is that cell membranes lose receptors for the Fc portion of immunoglobulin G and those of complement derivative C3b, therefore cells cannot ingest opsonized bacteria (16). Despite the lack of correlation between cellular injury and low temperature phase changes, studies of the cells in noncryoprotected solutions provide additional information on the role of $Me_2SO$ at low temperature. Such studies also elucidate the ability of cells to resist intracellular ice formation during repeated osmotic stress below the temperature of lethality. Fig. 6 compares the warming curves of cell-free FCS-HBSS solution, cells slowly frozen to lower temperature once, and those frozen and thawed three times. The cells slowly cooled to $-80$°C show (solid curve) little or no devitrification upon warming and display a glass transition ~20°C above those of cells repeatedly cooled from $-20$°C and those of cell-free medium. Thus the cells can form a stable intracellular glass by loss of water to extracellular ice at a temperature far below their killing point, which is about $-20$°C. In cells repeatedly frozen and thawed (dashed curve), despite the fact that the intracellular solution displays a glass transition of a similar magnitude and at about the same temperature (approximately $-80$°C) as the cell-free medium (dot-dashed curve), the devitrification was greatly suppressed. Since ice is present in the FCS-HBSS solution before fast cooling in both of the solutions cooled quickly from $-20$°C and since the annealing protocol for the cells insured the formation of intracellular ice as well (because the fast cooling began at $-20$°C), this is not a measure of inhibition of ice growth into the cell by the plasma membrane. This is a measure of the rate of ice growth in the intracellular vs. the extracellular solution. Thus the concentrated intracellular matrix is seen to be a better suppressor of ice growth (devitrification) than an equivalently concentrated solution of FCS-HBSS.

Note that the range of size among the transitions reported here is quite large. Since we wanted to display the largest transitions without attenuation, the minor transitions often appear quite small. However, expansion of the power scales shows that all of the minor transitions are >10× noise except on the dashed curve of Fig. 5. There the transitions are only clearly present because they correspond to larger transitions (as shown on the solid curve of that figure) at a lower temperature in glassy solutions of the same nonaqueous composition having a slightly higher water content. The broadness of many of the transitions is due to the fast cooling and warming rates and the poor thermal conductance of frozen aqueous solutions. Since significantly slower rates (<20°C/min), while increasing accuracy, would greatly increase the amount of ice formed

by homogeneous nucleation or devitrification and lead to massive cell damage, we were forced to examine the living systems under conditions as presented in this paper.

These data led us to the following conclusions.

(*a*) Freezing injury in the $Me_2SO$-FCS-HBSS treated monocyte can be correlated to a subtle but measurable increase in the devitrification events on warming.

(*b*) Avoidance of intracellular freezing upon fast cooling in solutions containing penetrating cryoprotectant is insufficient to provide complete protection if devitrification occurs upon warming.

(*c*) The intracellular solution of monocytes is a more stable glass former than FCS-HBSS solution without $Me_2SO$.

(*d*) Monocytes slowly frozen to a very low temperature without cryoprotectant show complete destruction even though there is far less devitrification than in cell-free solutions on fast warming. Thus avoidance of intracellular freezing in such cellular suspensions appears insufficient to protect the cells, osmotic stress having already proven totally lethal.

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