Nucleation Kinetics of Ice in Undercooled Yeast Cells: Long-term Stability against Freezing

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Undercooling and ice nucleation in yeast cells exposed to increasing hypertonic polyvinylpyrrolidone (PVP) concentrations were measured. Ice nucleation rates were analysed in terms of classical nucleation theory. Contrary to earlier reports, nucleation was found to be of the catalysed type, by active catalytic sites within the cell. With increasing PVP concentration, the nucleation temperature tends to a limiting value (approximately 236 K), whereas the homogeneous nucleation temperature of the extracellular PVP solution continues to decrease with increasing PVP concentration. The calculated parameters in the nucleation equation indicate that the nucleation mechanism within the cell is unaffected by hypertonic stress. The experimentally determined freezing kinetics of undercooled water (in oil emulsions) were found to parallel closely previously reported death rates of yeast cells as a function of temperature. The observed kinetics are compatible with a slow crystallization or precipitation of emulsifying agent at the oil/water interface, to yield catalytic sites capable of promoting ice nucleation. Experiments with water-(or yeast)-in-oil dispersions containing no emulsifying agents led to long-term freezing resistance and high recoveries of viable cells.

INTRODUCTION

The short- and long-term responses of cells to low temperatures and to freezing form important fields of study from several points of view. Since cold is the most widespread stress condition facing many forms of life, an understanding of seasonal acclimation mechanisms may provide methods for the laboratory preservation of valuable germ-plasm, improving on conventionally used techniques, such as the application of chemical cryoprotectants (glycerol, dimethyl sulphoxide), coupled with storage at liquid nitrogen temperatures. A better understanding of the factors which determine survival at chill and freezing temperatures may also make possible the development, by breeding or genetic manipulation, of cold-tolerant varieties.

Much of the early pioneering work on freezing injury/survival was done on erythrocytes and yeast (Saccharomyces cerevisiae) (Mazur, 1961, 1966); the effects of cryoprotectant type and concentration, cooling and warming rates and storage temperature on the degree of survival and viability have been studied in great detail. One significant principle that has been established is that for a cell to survive low subzero temperatures, freezing of the cell fluids must be avoided. Conventionally, this is achieved by the osmotic freeze dehydration of the cell and the concomitant replacement of cell water by cryoprotectant as the temperature is lowered and the extracellular fluid becomes progressively more freeze-concentrated.

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Abbreviations: DSC, differential scanning calorimeter (calorimetry); DTA, differential thermal analysis; PVP, polyvinylpyrrolidone.

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Following Mazur (1961), who noted that yeast cells in bulk suspensions can be undercooled to 263 K without deleterious effects on their subsequent viability, at least after short exposures to undercooling, we have chosen to examine how the cell responds to subzero temperatures per se, under isotonic conditions, where the probability of ice nucleation in the extracellular liquid phase has been minimized. The technique of dispersing cells contained in microdroplets of medium in a continuous oil phase (Rasmussen et al., 1975), permits such conditions to be achieved. We have applied this method to study the effects of low temperatures on a variety of cell types and found that all cells examined are able to undercool, to varying degrees, but always to well below the equilibrium freezing point of the aqueous phase (Franks et al., 1983).

A related question is whether, during long-term storage in the undercooled state, slow changes in and around the cell can occur which eventually cause the extracellular medium and/or the cell fluids to freeze. Since undercooled water is thermodynamically metastable with respect to ice, the undercooled state must be kinetically safeguarded against inadvertent ice nucleation. Rasmussen et al. (1975) determined the survival rates of undercooled yeast cultures as a function of temperature and found that the number of cells surviving for a given period dropped rapidly with decreasing temperature. They proposed that the kinetics of cell death were consistent with a heterogeneous (catalysed) nucleation process, and they speculated on the generation of catalyst particles inside the cells as a result of exposure to low temperatures (see below).

Broto & Clausse (1976) did similar experiments, except that they studied the freezing of undercooled water droplets in oil emulsions as a function of time and degree of undercooling. They also found that the rate of freezing was proportional to the degree of undercooling. Thus, the time taken for 50% of the drops to freeze at 252, 246.5 and 240 K were 360, 74 and 34 h respectively. However, the fact that all emulsions could be initially undercooled to 234 K indicates that some process occurs during storage which renders the water droplets susceptible to freezing.

The present study was done to determine (1) the mechanistic and kinetic details of intracellular ice nucleation and freezing of *S. cerevisiae* under conditions of isotonicity and when subjected to gradually increasing hypertonic water stress, and (2) the effects of longer-term storage on undercooled water droplets in emulsified form and on cell viability, with the particular aim of establishing whether a relationship exists between freezing kinetics and cell survival rates.

Differential scanning calorimetry (DSC) was chosen as the most suitable method to monitor undercooling and ice nucleation, because the cooling and warming thermograms permit a very detailed analysis of the various thermal processes which take place in the cells and the suspending medium. The latent heats of crystallization and fusion of water are large, so that small samples can be used and the capability of the instrument exploited to the full.

**METHODS**

*Materials and preparation of dispersions.* Cells of *S. cerevisiae* (NCYC 914) were incubated in 2% (w/v) aqueous sucrose solution for 30 min at 300 K; they were then washed and resuspended in distilled water. The moisture content of the ‘dry’ cells (11–5%, w/w) was obtained by weighing samples which had been dried at 403 K for 3 d.

Silicone fluid (Dow-Corning, 1 cP), containing 5% (w/w) sorbitan tristearate (Fluka SPAN 65) was homogenized with the aid of a Polytron coaxial cylinder homogenizer at about 10000 r.p.m., while the cell suspension was added dropwise from a Pasteur pipette. The final oil : aqueous phase volume ratio was usually 6:5 which was close to 1:1 by weight. Precise weight ratios were determined by weighing each phase before homogenization.

An aqueous stock solution containing 60% (w/w) of polyvinylpyrrolidone (PVP) (Sigma PVP-40T, average molecular mass 40 kDa), which had been previously dialysed against distilled water and freeze-dried, was diluted with water or the aqueous yeast suspension to yield aqueous phases containing respectively 10, 20, 30 or 40% (w/w) PVP.

*Undercooling and nucleation studies.* A Perkin-Elmer DSC-2, fitted with autoscanning zero and subambient temperature accessories was used (Michelmore & Franks, 1982). Dispersion samples, of the order of 2 mg, always containing <1 mg water, were weighed to ±10 μg in sealed aluminum sample pans. An empty sealed pan was used as a reference sample. Cooling rates of 2.5 K min⁻¹ were used in most cases, although some samples were cooled at 5 K min⁻¹. For the kinetic analysis of the freezing exotherms, the power–time curves were divided into
equal time intervals and each area was estimated by applying the trapezium method to the digitized data from the experimental output.

**Medium- and long-term undercooled storage**. Samples of water-in-oil emulsions, prepared as described above, were rapidly cooled in the DSC (40 K min\(^{-1}\)) to the chosen storage temperature. Storage periods ranging from 10 min to 8 h were used at the following temperatures: 260, 250, 245 and 240 K. After storage, the sample was warmed and the ice melt endotherm recorded. The sample was then completely frozen by cooling it slowly to 230 K and rethawed, the melt endotherm being recorded again. The ratio of the two endotherm areas gave a measure of the percentage of droplets which had frozen during undercooled storage.

In order to ascertain whether the dispersions were freeze-thaw stable, a water-in-oil emulsion sample was subjected to three cool/thaw cycles between 278 and 240 K and held each time for 1 h at the lower temperature. After each cycle the area of the melting endotherm was recorded.

Long-term storage trials were done on yeast dispersions in mineral oil. Samples were stored at 253 K for periods of up to 4 months, after which the cells were recovered, diluted and plated, as described by Mathias et al. (1985).

**RESULTS**

**Kinetics of ice nucleation in yeast-containing systems**

The general features of the bimodal power–time curves recorded during cooling and heating an aqueous yeast/oil dispersion (Fig. 1) resemble those previously reported for dispersions of yeasts, erythrocytes and cultured plant cells (Franks et al., 1983). The larger exotherm corresponds to the heat liberated due to the freezing of water in the cells and in the aqueous phase surrounding the cells, while the smaller exotherm at the lower temperature reflects the freezing of aqueous droplets which did not contain cells. This is borne out by the freezing temperature, 234.5 K, which is taken to be the homogeneous nucleation temperature (\(T_n\)) of ice in undercooled water. The fact that the cell fluid freezes at a temperature substantially higher than \(T_n\) indicates that catalysed (heterogeneous) ice nucleation triggers intracellular freezing (\(T_c\)).

Power–time curves were deconvoluted and the freezing exotherms processed as previously described. The nucleation rate \(J\) (nuclei m\(^{-3}\) s\(^{-1}\)) is given by

\[
J = -\frac{1}{v} \ln \left[ \frac{A(t_1 + \Delta t)}{A(t_1)} \right]
\]

where \(A(t_1)\) is the total area under the peak minus the area after time \(t_1\), and \(v\) is the mean drop volume averaged over all the aqueous droplets. \(A(t_1)\) is thus a measure of the fraction of the droplets which remains unfrozen at time \(t_1\). The applicability of eqn (1) is based on the
assumption that compared to \( J \), crystallization is rapid, so that nucleation is the rate-determining process under the pertaining experimental conditions. This assumption was validated by Michelmore & Franks (1982).

When yeast cells are suspended in hyperosmotic PVP solutions, water will be drawn from the cells until osmotic equilibrium is re-established. The osmotic activities of concentrated PVP solutions were reported by Franks (1982), and they enable the change in the water content of the extracellular solution to be calculated on the assumption that the cell acts as a perfect osmometer. In principle, a more detailed analysis of the power–time curves than that described above would also permit the estimation of the solvent composition around the cells and the water content of the cells under osmotic stress, from which the heats of crystallization of ice outside and inside the cells can be obtained. In other words, the high temperature exotherm contains a contribution from the freezing of the extracellular solution. At the present stage, however, instrumental factors render such calculations of limited value. In particular, the curvature of the DSC instrumental base line can become a significant source of uncertainty in the calculation of the latent heat of freezing, particularly at high PVP concentrations and high instrumental sensitivity settings. Attempts will be made to overcome these problems and to provide a complete analysis of the power–time curves.

According to classical theory, active crystal nuclei (i.e. those capable of spontaneous growth) are formed by the stepwise condensation of water molecules onto clusters which aggregate and decay through random density fluctuations in the body of the liquid (Dufour & Defay, 1963). The steady-state rate of nucleation of ice in an undercooled aqueous mother phase at temperature \( T \) is given by

\[
J(T) = L(aT)^{1/2} \phi^3 \exp \left[ -\Delta G^\ddagger/RT \right] \exp \left[ -Qa^3/(\Delta T)^2 T^3 \right]
\]  

where \( L \) is a function of the molar volumes of ice and undercooled water, \( \phi \) is the volume fraction of water and \( Q \) contains the equilibrium melting temperature \( T_m \) and the latent heat of crystallization of ice at the temperature of nucleation \( T \); \( \Delta G^\ddagger \) is the free energy of activation of diffusion of water, \( \sigma \) is the interfacial free energy between the nucleus (assumed to be ice-like) and the undercooled liquid, and \( \Delta T \) is the degree of undercooling (\( = T_m - T \)). For present purposes, eqn (2) can be recast into a simpler form in terms of reduced temperatures

\[
J(\tau) = \exp (B\tau)
\]  

where \( \tau = [\Delta\theta^{-1} \theta^{-3}] \), \( \theta = T/T_m \) being the reduced temperature and \( \Delta\theta = (T_m - T)/T_m \), the reduced degree of undercooling. The conversion of actual temperatures to reduced temperatures makes possible the direct comparison of \( J(\tau) \) for systems of different melting temperatures.

Although it is not clear how the classical model of nucleation is to be applied to an extensively hydrogen-bonded liquid like water, it has been established experimentally that eqn (3) is able to account for the shapes of the exotherms, at least over several orders of magnitude in \( J \). This is illustrated in Fig. 2 for yeast cells suspended in water and in aqueous solutions of PVP. Table 1 summarizes the experimental nucleation and melting temperatures and the kinetic constant \( B \) for PVP solutions in the absence of yeast and for droplets which contained yeast cells. Fig. 3 shows the trends in \( T_m \), \( T_h \) and \( T_c \) as functions of PVP concentration. They were taken to be the temperatures at which \( J(T) \) becomes rapid and were read off the power–time curves by extrapolation of the linear portions back to the base line, as indicated in Fig. 1.

**Freeze resistance of undercooled emulsions**

The kinetics of droplet freezing in moderately undercooled water-in-oil emulsions were qualitatively similar to those reported by Broto & Clausse (1976) and Clausse et al. (1983). Fig. 4 represents a summary of the results. It is seen that the freezing process is complex. After an initial induction period, the length of which depends on the storage temperature, freezing assumes first-order kinetics.

Replicate experiments on different samples taken from the same emulsion and from different emulsion preparations gave rise to considerable scatter in the results, suggesting that catalysed nucleation was involved. The freeze-thaw stability of individual emulsion samples which had
Ice nucleation by undercooled yeast cells

Fig. 2. Plots of ln (Jv) (where J is the nucleation rate and v the mean drop volume) against the temperature function τ0, according to eqn (3), for yeast in distilled water (○), yeast in 10% (w/w) PVP (Θ) and yeast in 30% PVP (●).

Fig. 3. Melting points (●, ○), heterogeneous nucleation temperatures (▲) of yeast cells and homogeneous nucleation temperatures (○, ★) of aqueous solutions as functions of PVP concentration. ○, ○, Emulsions not containing yeast cells; ●, ▲, ★, emulsions containing yeast cells.

been subjected to several freeze-thaw cycles was, however, found to be high, indicating that repeated freezing does not adversely affect the homodispersity of the emulsion.

Storage results of yeast dispersions in mineral oil in the absence of emulsifying agent were
Table 1. Melting points, nucleation temperatures and kinetic parameter B in eqn (3) for emulsions with and without yeast, as functions of PVP concentration

<table>
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<tr>
<th>PVP concn (%w/w)</th>
<th>Without yeast</th>
<th>With yeast</th>
<th>Without yeast</th>
<th>With yeast</th>
<th>Without yeast</th>
<th>With yeast</th>
<th>Without yeast</th>
<th>With yeast</th>
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reported by Mathias et al. (1985). Survival (and freezing) patterns were completely different from those shown in Fig. 4, in that storage at 253 K did not lead to freezing during periods of several months, nor was there a significant reduction in the number of viable cells.

DISCUSSION

Nucleation kinetics of ice in yeast dispersions

The only data with which our experimental results can be compared are those of Rasmussen et al. (1975) who used differential thermal analysis (DTA) to monitor the freezing of yeast cells in water and hypertonic solutions of polyethylene glycol (PEG). In the present investigations it was decided to use PVP in preference to PEG, because the latter is able to crystallize from aqueous solution during warming; the additional crystallization and eutectic melting features in the power–time curve further complicate the analysis of the thermal data, and they also impose an additional osmotic stress on the dispersed cells.
Even allowing for the fact that the sensitivity and stability of the Perkin-Elmer DSC-2 is greatly superior to earlier DTA systems, it is nevertheless surprising that none of the earlier thermograms show a bimodal freezing behaviour (Rasmussen et al., 1975), and that all the reported nucleation temperatures are substantially lower (<238 K) than those reported here. However, it was noted that emulsions which did not contain cells undercooled more than those that did, and also that 'yeast cell contents did not supercool as much as the PEG solutions exterior to the cells where the PEG solution was hypertonic to the yeast' (Rasmussen et al., 1975). Even so, they found that 'where yeast was suspended in PEG, the yeast undercooled to a greater extent than did distilled water', a finding which directly contradicts our results. We are unable to reconcile the two sets of results but emphasize that our yeast results are compatible with our previous DSC studies (Franks et al., 1983; Mathias et al., 1984). It should also be noted that the yeast results here presented are in line with all the recent reports on so-called bacterial 'ice nucleators' (Lindow, 1983). The possibility exists that \( T_c \) could be affected by the age of the cells and/or any acclimating pretreatment, as reported for bacterial 'ice nucleators' (Anderson et al., 1982).

The \( T_h \) values measured in the ternary systems PVP/yeast/water (Fig. 3, Table 1) were consistently lower than those measured in aqueous solutions of PVP (in the absence of yeast), because the weight ratio PVP:water is higher in the ternary mixtures (see Methods). The value of \( T_h \) was determined only by this ratio and was not affected by the yeast content of the mixture. If the PVP concentrations of the ternary mixtures in Fig. 3 are adjusted for the weight of dry yeast and the \( T_h \) data replotted, then all \( T_h \) values for the binary and ternary mixtures fall on the same curve (○).

The dependence of \( T_c \) on PVP concentration does not parallel that displayed by \( T_h \). It appears that \( T_c \) tends to a limiting value, while \( T_h \) decreases with PVP concentration, in the manner to be expected for an aqueous solution (Franks, 1982). Although DSC is not a particularly sensitive method for the measurement of \( T_m \), the constant \( T_c/T_m \) ratio for yeast-free droplets is in line with earlier observations of Rasmussen & MacKenzie (1971). The claim that constancy for the ratio \( T_c/T_m \) is evidence that the yeast cells freeze by a homogeneous nucleation mechanism (Rasmussen et al., 1975) is, however, not supported by our results. The \( T_c \) behaviour (Fig. 3 and Table 1) clearly demonstrated that freezing was catalysed by the cells and ice was nucleated under conditions which were only indirectly and moderately affected by the increase in hypertonicity. We therefore infer that freezing was initiated within the cells; the plasma membrane will increasingly resist the osmotic pressure as cell shrinkage takes place. This supports the findings of Morris et al. (1986) that Boyle–van't Hoff plots for yeast cells were highly non-linear and that an osmolality increase (NaCl) from 1 to 4 osm only resulted in a 10% reduction in cell volume. By comparison, a 40% PVP solution (molecular mass 40 kDa) has an osmolality of 780 mosm (Franks, 1982).

The kinetic parameter \( B \) in eqn (3) is sensitive to, and diagnostic of the mechanistic details of the nucleation process. The value of \(-0.08\) for yeast cells in water is very close to that \((-0.07\) reported for the nucleation of ice in cultured *Glycine max* cells (Franks et al., 1983). The relative insensitivity (within the limits of experimental error) of \( B \) for yeast cells with increasing concentration of PVP indicates that the nature of the catalytic site within the cell is unaffected by the hypertonic stress, as is the mechanism of nucleation. On the other hand, \( B \) for homogeneous nucleation becomes increasingly sensitive to temperature as the PVP concentration increases, a finding established for aqueous solutions of PEG by Michelmore & Franks (1982). In terms of classical nucleation theory, as described by eqn (1), this could be accounted for by the effect of PVP on the interfacial free energy \( \sigma \) and/or the heat capacity of undercooled water. We have previously shown that the partial heat capacity of PVP in water, which is positive at ordinary temperatures, changes sign and becomes increasingly negative at subzero temperatures (Wakabayashi & Franks, 1986; Franks & Wakabayashi, 1987).

It would be of interest to identify the nature of the molecular structures within a cell that are responsible for catalysing ice nucleation, but the limited scope of the DSC study does not permit this. However, the evidence for the existence of pro- and antifreeze proteins, coupled with the isolation of microbial 'ice-nucleating' genes (Green & Warren, 1983), suggests that a similar, although less potent, proteinaceous catalyst is involved.
Freezing resistance of undercooled dispersions

The similarity between the yeast death rates, as reported by Rasmussen et al. (1975), and the long-term droplet freezing results of Broto & Clausse (1976) is striking. Although there is a qualitative similarity of the results in Fig. 4 and those of Broto & Clausse, the lengths of the induction periods differ markedly. For instance, Broto & Clausse (1976) reported a period of >100 h before freezing could be detected at 252 K, compared with only 3 h in our studies. The only significant difference between the two experimental procedures lies in the choice of emulsifier: Broto & Clausse (1976) used a commercial lanolin preparation, whereas we used sorbitan tristearate. It seemed possible, therefore, that slow changes at the oil/water interface during prolonged undercooled storage were responsible for the observed freezing behaviour.

The above findings and similarities with the previous studies led us to develop techniques whereby aqueous media could be emulsified in oil to yield mechanically stable dispersions in the absence of emulsifying agents. Such preparations were not as homodisperse as those containing sorbitan tristearate and it proved impossible to achieve the required degree of stability with silicone fluids. However, the long-term resistance to freezing was vastly improved. For instance, simple water-in-oil emulsions are stable over periods of several months at temperatures as low as 240 K. Furthermore, yeast cells suspended in such emulsions do not exhibit the characteristic loss of viability reported by Rasmussen et al. (1975). Indeed, in the absence of freezing, survival rates should be enhanced with decreasing temperature, and our preliminary studies bear this out.

We therefore conclude that the results of Broto & Clausse (1976) and those of Rasmussen et al. (1975) are due to a slow phase change, perhaps a crystallization, of emulsifier at the water/oil interface, producing catalytic sites capable of raising the nucleation temperature of ice. Clausse et al. (1983) did further experiments in which emulsions were precooled to given temperatures and subsequently stored at different temperatures. The overall freezing kinetics were found to be consistent with two superimposed processes, one of which exhibited typical nucleation kinetics, and the other obeyed Arrhenius kinetics. The latter we would identify with the phase change in the interfacial layer which causes nucleation to become controlled by interfacial catalysis. In principle, and with very high precision data, this hypothesis might be tested, because the homogeneous nucleation rate is a function of the droplet volume, whereas the catalysed rate depends on the surface area of the droplet.

Finally, with regard to the effect of undercooled storage on the survival of yeast cells, the available evidence now suggests that it is the freezing of water droplets (through interfacial catalysis) that causes the cells to die.

REFERENCES


