VISUALIZATION OF FREEZING DAMAGE

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ABSTRACT

Freeze-cleaving can be used as a direct probe to examine the ultrastructural alterations of biological material due to freezing. We examined the thesis that at least two factors, which are oppositely dependent upon cooling velocity, determine the survival of cells subjected to freezing. According to this thesis, when cells are cooled at rates exceeding a critical velocity, a decrease in viability is caused by the presence of intracellular ice; but cells cooled at rates less than this critical velocity do not contain appreciable amounts of intracellular ice and are killed by prolonged exposure to a solution that is altered by the presence of ice. As a test of this hypothesis, we examined freeze-fractured replicas of the yeast *Saccharomyces cerevisiae* after suspensions had been cooled at rates ranging from 1.8 to 75,000°C/min. Some of the frozen samples were cleaved and replicated immediately in order to minimize artifacts due to sample handling. Other samples were deeply etched or were rewarmed to -20°C and recooled before replication. Yeast cells cooled at or above the rate necessary to preserve maximal viability (∼7°C/min) contained intracellular ice, whereas cells cooled below this rate showed no evidence of intracellular ice.

INTRODUCTION

Freezing has become an increasingly important method of stabilizing biological structure and is an inherent aspect of such techniques as freeze-substitution, ultracryotomy, and freeze-cleaving. Although these techniques have been used primarily in studying ultrastructure, they can also be used as a direct probe to examine the ultrastructural alterations that result from freezing. Previous reports have shown that after freezing and thawing there is little correlation between structural alterations in the thawed cells and cell survival. For example, freezing and thawing cells at different rates can result in nearly identical cell survival but often cause distinctly different types of structural alterations. Interpretation of the causes of freezing injury in such studies is confounded by changes that occur during thawing. Such ambiguities can be eliminated by studying the cell in the frozen state.

The thesis has been advanced that at least two independent events determine the survival of frozen cells. According to this thesis, cells cooled at rates below a critical velocity are killed by prolonged exposure to a solution that is altered by the presence of ice. At higher cooling rates the cells are exposed to these altered solutions for shorter intervals and there is a commensurate in-
crease in cell survival, until a second factor limiting cell survival becomes significant. Cells cooled at rates at or above the critical value have insufficient time to equilibrate osmotically with the frozen external media before freezing internally, so the amount of water lost by the cells during cooling decreases and the probability and extent of intracellular ice formation increases. This results in a decrease in cell survival.

We have examined the ultrastructural alterations in bakers' yeast that result from freezing, as one test of the hypothesis that the loss in viability at optimal and supraoptimal rates of cooling is correlated with the presence of intracellular ice. These cells have been well characterized morphologically by a variety of conventional and low temperature preservation techniques (5, 8, 14, 22, 23, 27), and extensive data is available on their survival after freezing (19). Our experiments consisted of suspending Saccharomyces cerevisiae in dilute sodium chloride and then freezing samples at five rates, ranging from 1.8° to 75,000°C/min. The specific rates were chosen to be equal to, above, or below the cooling rate needed to yield the optimum survival values. After freezing, the samples were treated in three ways. Those in the first group were cleaved and replicated immediately, to ensure that any structural changes seen were due only to differences in the rate of cooling and to minimize artifacts due to sample handling. Those in group two were deeply etched to determine whether the crystals were present but not discernible immediately after cleaving. The third group was warmed to -20°C and recooled to -196°C to determine whether ice can form in cells that show no direct evidence of crystalline ice after freezing. Under these conditions if any intracellular ice is present, large intracellular ice crystals should be formed by migratory recrystallization (9, 12).

MATERIALS AND METHODS

Yeast Cells

A diploid strain of the yeast S. cerevisiae (NRRL Y-2235) was grown on fresh nutrient agar slants at 30°C for 48 h. The cultures were then suspended in 5.0 ml of sterile 0.15 M phosphate buffer, and 0.4 ml of the resulting suspension was added to 100 ml of Nickerson's liquid media contained in a 250 ml culture flask and incubated at 30°C in a rotary shaker (15). After 24 h the cells had reached early stationary phase (~2 × 10^6 cells/ml), and 40 ml of the suspension was decanted and sedimented at 500 g for 10 min. The pellet was washed three times in 5 mM NaCl and decanted until 0.1 ml remained over the pellet. The cells were resuspended in dilute NaCl because it was difficult to obtain good preparations of cells in distilled water. The available evidence indicates that the presence of small amounts of electrolyte in the suspending medium has little effect on the survival of cells subjected to freezing and thawing (15). Then a small portion of this dense cell slurry was drawn into a fine capillary tube (0.4 mm ID), and ~0.5 µl was loaded into each half of a hinged specimen holder (obtained from Denton Vacuum Inc., Cherry Hill, N. J.). The hinge was then closed to form a thin layer of cells at the interface.

Freezing Procedures

Most samples cooled at 1.8° or 7°C/min were seeded before cooling to -196°C. The procedure was as follows. Sample hinges were inserted into a multiple sample holder, and this assembly was screwed onto a threaded support post. The temperature of the hinges was lowered to -4°C by placing the lower portion of the post in a cold ethanol bath. The cell suspension to be seeded was placed on one half of the hinge, and a few small ice crystals were placed on the other half. Closing the hinge caused the media containing the cells to nucleate. The samples were held at -4°C for 3-5 min and then transferred to the appropriate cooling vessel as its temperature reached -3°C. In the case of nonseeded samples, the specimen hinges containing the cell suspensions were placed in plastic BEEM capsules and were then cooled at rates of 1.8°, 7°, 55°, 1,500°, and 75,000°C/min, the rates being measured between -5° and -65°C with a 40-gauge copper-constantan thermocouple soldered to a blank specimen hinge.

For ultrarapid cooling (75,000°C/min) the specimen hinges were dropped into liquid propane at its freezing point (~180°C). For lower rates of cooling, plastic BEEM capsules were attached to Lucite supports, and a specimen hinge containing the cell suspension was gently placed in the bottom of the capsule. The capsules were then transferred to cooling baths and cooled as follows: a cooling rate of 1,500°C/min was obtained when the capsules were immersed in liquid nitrogen; 55°C/min was obtained by placing the capsules on a cork support platform in the bottom of a glass test tube (50 × 320 mm), which was then immersed in a large Dewar flask containing liquid nitrogen; and 7°/min was obtained by placing the capsules on a cork support platform in the bottom of a glass test tube (50 × 320 mm), which was then immersed in a large Dewar flask containing liquid nitrogen; when the temperature of the sample cooled at 55°C/min or slower reached -100°C, the capsules were transferred directly into liquid nitrogen (-196°C); 6°C/min was obtained by placing the capsules (unseeded samples) or multiple holder (seeded sample) in an unevacuated Dewar flask.

Vacuum Inc., Cherry Hill, N. J.). The hinge was then closed to form a thin layer of cells at the interface.
blank (85 X 280 mm) which was immersed in a Dewar flask containing liquid nitrogen; and the slowest rate, 1.8°C/min, was obtained by placing 500 ml of absolute ethanol in an unevacuated Dewar flask blank (85 X 280 mm) and immersing this flask in a large Dewar flask containing liquid nitrogen. When the temperature of the constantly stirred ethanol reached −3°C, the capsule or the multiple sample holder was transferred to the cooling bath.

Recrystallization

Samples to be tested for the presence of intracellular ice were transferred from liquid nitrogen to an ethanol bath at −20°C ± 0.5°C. The samples were held at this temperature for 5 min, after which they were rapidly recooled to −196°C. (Due to the large mass of the multiple sample holder, the temperature of seeded samples approached the bath temperature asymptotically; hence, the 5 min timing of the holding period was initiated when the sample reached −22°C.) Other samples were exposed to lower temperatures, longer holding times, or both.

Freeze-Cleaving

In the case of the unseeded samples, the individual sample hinges were inserted into a multiple holder under the surface of liquid nitrogen (the seeded samples had already been loaded on the multiple holder before cooling). The specimen support stage of a Denton DFE freeze-etching unit (29) was precooled to −175°C, and the multiple sample holder was then transferred to the support stage and a vacuum was rapidly obtained. To minimize specimen contamination, the roughing pump was isolated from the vacuum chamber by a molecular sieve trap. When the pressure was reduced to less than 10⁻⁴ torr with a Varian 140 liter/min ion pump (Varian Associates, Palo Alto, Calif.) and a titanium sublimation pump. (The partial pressure in the vicinity of the specimen would be lower due to the pumping action of the cryogenic chamber.) The temperature of the frozen samples, as measured by a copper-constantan thermocouple embedded in the specimen support post, did not exceed −125°C until replication was completed. Rotating the specimen support post within this inner chamber resulted in the simultaneous opening of the specimen hinges. The fractured samples were immediately replicated with platinum and carbon, and this replica was further stabilized with an additional layer of carbon. Samples to be etched were cleaved at −125°C, warmed rapidly to −100°C, held at this temperature for 5 min, and then recooled to −125°C before replication. After replication the vacuum system was depressurized with dry nitrogen. The specimens were allowed to warm to 5°C and were then removed from the freeze-cleaving unit, and the individual replicas were floated on the surface of the suspending media. Adhering organic material was digested by the addition of 7 M sodium hydroxide containing 1% sodium hypochlorite. The replicas were washed and further digested in 16 M sulfuric acid, rewashed, and if necessary further cleaned with 3% sodium hypochlorite. The replicas were then transferred to a small Petri dish containing distilled water. The replicas were recovered on 75-mesh Formvar-coated, carbon-stabilized grids and examined in a Hitachi HU-11C electron microscope operated at 75 kV.²

Size Measurements

Direct measurements of cell size were made by placing a ruler on the fluorescent screen of the electron microscope. While we cannot be certain that all measurements were made on cells that had been cleaved through the exact center, all cells that showed any evidence of tangential fracture were excluded. At least 50 cells were measured for each cooling rate. Measurements of the diameter of cells cooled at 1.8° and 7°C/min were made along several axes of the same cell, and the measurements from each cell were pooled and averaged to provide an estimate of the true mean diameter. This averaging was done to compensate for the asymmetrical shapes found in the majority of cells cooled at these low rates.

Survival

The survival values cited in the Results and Discussion sections are from Mazur and Schmidt (19) or Mazur (18) and correspond to the percentage of yeast cells in distilled water surviving freezing followed by rapid warming. (Cell survival was operationally defined in these studies as the ability to produce colonies.)

² The currently applied yardsticks for the quality of freeze-fractured preparations are most applicable for glycerinated specimens. As one departs from the "ideal conditions" of high glycerol concentrations and ultrarapid freezing the technical quality of the resulting replica progressively decreases. Nevertheless we believe that much useful information can be derived from such preparations. In addition, the susceptibility of ice to etching changes as the rate of cooling decreases. Evidently ice formed during slow cooling is more stable hence more resistant to etching than ice formed during rapid cooling.
RESULTS

Glycerol-Impregnated Yeast Cooled at 75,000°C/min

We find that the ultrastructure of yeast cells equilibrated with 20% glycerol (vol/vol), frozen at 75,000°C/min, cleaved, and etched for 1 min at -100°C (Fig. 1), is similar to that reported by other workers (5, 14, 22). Since the literature for "normal" yeast morphology is extensive, only those aspects that relate to freezing damage are discussed in the Results and Discussion sections. The structure of these cells serves as a control for comparison with the structure of yeast frozen in dilute sodium chloride.

Yeast Suspended in 5 mM NaCl

COOLING AT 75,000°C/MIN: Yeast suspended in 5 mM NaCl, frozen at 75,000°C/min (an ultrarapid rate), and freeze-cleaved show structural details comparable to cells frozen in glycerol (Fig. 2). They are usually round and average 5.1 μm in diameter. Even without etching, discrete organelles—including the nucleus, vacuoles, and mitochondria—can be unambiguously identified in most cross-fractured cells. A small percentage of the cells in this preparation show distinct evidence of intracellular freezing, as characterized by the presence of angular crystal facets in the vacuole (Fig. 3). The cytoplasm of these cells is covered with large numbers of particles, and few cytoplasmic organelles can be seen.

COOLING AT 1,500°C/MIN: Cells frozen at 1,500°C/min and freeze-cleaved are smaller than those frozen at 75,000°C/min. They average 4.1 μm in diameter, are rounded, and have a relatively smooth contour (Fig. 4). The vacuole seen in cross-section is rounded and well defined; distinct cleavage planes are usually present, often extending across the vacuolar membrane and con-

![Figure 1](image-url)

**Figure 1** Yeast cells in 20% glycerol, frozen at 75,000°C/min, and freeze-etched. CW, cell wall; P, plasmalemma; V, vacuole; M, mitochondrion; N, nucleus; NP, nuclear pore. Arrow indicates direction of shadowing. Bar represents 1 μm. × 29,500.
Figure 2  Yeast cell in distilled water, frozen at 75,000°C/min, and freeze-fractured. FR, fracture ridge. X 18,500.

Figure 3  Yeast cell in distilled water, frozen at 75,000°C/min, and freeze-fractured. X 17,500.
continuing through the cytoplasm. (Other organelles are present in the cytoplasm, but the lack of structural detail makes it difficult to differentiate between the various types of organelles.) Cells cooled in an identical manner, cleaved, and then deeply etched (Fig. 5) provide additional information on the sites of ice crystal formation. Two well-defined phases exist within the vacuole and the cytoplasm. During etching, the ice phase recedes, revealing the vacuolar solutes as irregularly shaped islands and sites of small cytoplasmic ice crystals.

When cells are cooled at this same rate, warmed to $-20^\circ C$ for 5 min, and recooled before freeze-etching (Fig. 6), they show extensive recrystallization (Fig. 11). These cells differ in appearance from cells cooled more rapidly, mainly in the large size of the ice crystals within the vacuole.

COOLING AT $6^\circ C$/MIN: In these experiments, the cells were frozen by seeding at $-4^\circ C$, held at that temperature several minutes, and subsequently cooled at $6^\circ C$/min. This procedure permits a linear rate of cooling, since a major portion of the heat of crystallization is evolved before the cooling procedure. A cooling rate of $\sim 6^\circ C$/min is optimal for the survival of this strain of yeast, yet the structure of these cells after freeze-cleaving differs greatly from the well-organized structure seen at the ultrarapid rates of cooling. Cells cooled at this rate average 3.4 $\mu m$ in diameter and are contracted and compacted (Fig. 11). The irregular but well-defined vacuole is relatively smooth but occasionally has one or more fracture ridges transversing it; such ridges indicate the presence of ice. The cytoplasm appears compacted, with numerous membrane-like folds closely appressed to one another. The cell wall of slowly cooled cells is seen in freeze-cleaved preparations, whereas in cells that are cooled at higher rates it is visible only after etching. The appearance of the cells is
**Figure 6** Yeast cell frozen at 1,500°C/min, warmed to -20°C for 5 min, and freeze-cleaved. I, ice. × 21,500.

**Figure 7** Yeast cell frozen at 1,500°C/min, warmed to -20°C for 5 min, and freeze-etched. × 19,500.
essentially unchanged, even after they are warmed to $-20^\circ C$ for 5 min, recooled, and freeze-etched (Fig. 12), indicating that little intracellular ice is present or that the ice is relatively stable.

When cells are cooled at $\sim 6^\circ C$/min without being seeded they supercool before freezing. Since heat is liberated during freezing, such a sample warms to temperatures close to the freezing point.
Figure 11  Yeast cell seeded at $-4^\circ$C, frozen at $6^\circ$C/min, and freeze-cleaved. $\times$ 18,000.

Figure 12  Yeast cell seeded at $-4^\circ$C, frozen at $6^\circ$C/min, warmed to $-20^\circ$C for 5 min, recooled, and freeze-etched. $\times$ 30,500.

Figure 13  Yeast cell cooled at $6^\circ$C/min and freeze-cleaved. $\times$ 12,500.

Figure 14  Yeast cell seeded at $-4^\circ$C, frozen at $1.8^\circ$C/min, and freeze-cleaved. $\times$ 27,000.

Figure 15  Yeast cell seeded at $-4^\circ$C, cooled at $1.8^\circ$C/min, and freeze-etched. $\times$ 18,500.

Figure 16  Yeast cell seeded at $-4^\circ$C, cooled at $1.8^\circ$C/min, warmed to $-20^\circ$C for 5 min, recooled, and freeze-etched. $\times$ 14,500.
of the solution and subsequently cools at a high rate until its temperature approached that of the cooling bath. (In 0.2 ml samples of water in glass tubes, the actual cooling rate of unseeded samples cooled at 6°C/min was found to be \( \sim 15°C/min \) over the temperature range of \(-2°C\) to \(-20°C\).) Unlike the seeded cells cooled at the same rate, the unseeded cells have fracture ridges present in almost every vacuole and they closely resemble cells cooled at 55°C/min.

**COOLING AT 1.8°C/MIN:** There are extensive alterations in cells after seeding at \(-4°C\) and subsequent cooling at 1.8°C/min (Fig. 14). We observed few structural differences between seeded and nonseeded cells cooled at the same rate. No structural changes were seen that indicated the presence of intracellular ice. These cells average 5 μm in diameter in freeze-cleaved preparations, compared to \( \sim 5 \mu m \) for rapidly cooled cells. The only intracellular organelle recognizable is a non-granular vacuole, but the vacuole wall is poorly demarcated from the surrounding cytoplasm. The cell wall can be seen, but the remainder of the cell is a jumble of convoluted elements that presumably represent the remnants of the cellular organelles. Much additional detail is seen in deeply etched cells cooled at the same rate (Fig. 15), but due to the gross alterations in structure (presumably caused by dehydration) little additional structural information, other than the location of membranes, can be obtained. The structure of these cells is essentially unchanged by warming to \(-20°C\) for 5 min before recooling and freeze-etching (Fig. 16).

**DISCUSSION**

Approximately 60% of yeast cells cooled at 7°C/min are viable after thawing (19). Raising or lowering the rate of cooling results in a decrease in viability (Table I, Fig. 17). Since survival decreases both below and above the optimal rate, two or more rate-dependent factors must be responsible for the loss in viability. At suboptimal cooling velocities, the loss in viability apparently is caused by the so-called “solution effects” (17, 19). These solution effects are a result of the progressive conversion of extracellular water to ice and include cellular dehydration, pH changes, and concentration and precipitation of solutes (10, 11, 16, 20). (Finally, when the temperature is sufficiently low, the system solidifies without ever forming intracellular ice.)

| COOLING VELOCITY | VIABILITY (%) after warming at 1°C/min | VIABILITY (%) after warming at 1,500°C/min |
|------------------|--------------------------------------|------------------------------------------|
| 1.2°C/min        | 5 (0.5)†                            | 43 (12)†                                 |
| 7.2°C/min        | 2                                   | 55 (10)†                                 |
| 45°C/min         | 3 \( \times 10^{-3} \)               | 10                                        |
| 1,400°C/min      | 1.7 \( \times 10^{-5} \)            | 0.24                                      |
| 75,000°C/min     | —                                   | —                                         |

† Values in parentheses refer to unseeded samples.

* From Mazur and Schmidt (19). The original paper gives data for a number of other cooling and warming rates.

**TABLE I**

Effect of Cooling Rate on Viability

Injury at supraoptimal cooling velocities is believed due to the formation of intracellular ice and the recrystallization of that ice during warming. A thermodynamic analysis indicates that at rates of 10°C/min and greater there is not sufficient time during the cooling of yeast cells for the vapor pressures of the external medium and the “free” intracellular water to reach equilibrium. As a result, the cell water becomes increasingly supercooled and eventually freezes internally (16). The drop in survival of cells cooled at supraoptimal rates apparently is due to the formation of such intracellular ice during cooling and to the recrystallization of that ice during warming. Several lines of evidence support this view. For example, with increasing cooling velocity the survival of cells becomes increasingly dependent on the rate of warming (Table I). Presumably, the abrupt drop in the survival of rapidly cooled cells with decreasing warming velocity reflects the
recrystallization of intracellular ice. Secondly, measurements on rapidly cooled freeze-substituted cells have shown that their volume is larger than it would be if they lost all free water; and calorimetric measurements have shown that all free water, in fact, freezes at −20°C and below (17). Such evidence, however, is indirect. Freeze-cleaving offers a direct test of the hypothesis that the drop in survival at supraoptimal rates is associated with the formation of intracellular ice. The process can be illustrated in the yeast cells cooled at 1,500°C/min, a rate that is far above the optimum. Cells cooled at this rate, cleaved, and immediately replicated show numerous small fracture ridges both in the vacuole and in the bulk cytoplasm (Fig. 4). Such fracture ridges can be caused by a deviation in the plane of cleavage at the boundary between large, randomly oriented crystals of ice (7, 28).

The second test for the presence of intracellular ice involves warming the fractured surface to about −100°C to produce etching (Fig. 5). The fracture lines of etched cells are less evident. Since ice sublimes at a much higher rate than biological material, additional structural detail is exposed during etching and the etched regions must represent the locations of ice. The two phases seen within the vacuole represent an ice phase and a solute phase.

The presence of intracellular ice can be unequivocally confirmed by warming the frozen cells to a high subzero temperature (usually to −20°C) for several minutes and then recooling them before etching and/or replication. As a result of the increase in surface energy at the higher temperature, crystals grow larger either by fusing with other crystals or by sequestering water from adjacent regions (Figs. 6–8, 10). The size of the crystals is a function of initial crystal size, storage temperature, and time. This amplification of crystal size is useful in determining whether ice is present, but the precise location of that ice and its relationship to cellular membranes is altered by local migration of the crystals during their exposure to high subzero temperatures.

Controlled warming is an extremely sensitive method for showing the presence of intracellular ice. If intracellular ice was formed during cooling to −196°C, then subsequent warming to a relatively high subzero temperature should cause recrystallization of that ice into large, visible crystals. Thus when yeast cells that have been cooled at 1,500°C/min are warmed from very low temperatures to −20°C, recrystallization proceeds so rapidly that after 5 min the cell is scarcely recognizable (Figs. 6, 7). Large ice crystals often obscure much of the cell. Cross-fractures through such cells reveal portions of the cells showing angular facets that follow the crystallographic planes of large ice crystals. Rapidly cooled cells can undergo extensive recrystallization at temperatures far lower than −20°C. Although the rate of crystal growth is inversely proportional to temperature, with this technique appreciable recrystallization can be detected at temperatures as low as −45°C. This indicates that unless rapidly cooled cells are warmed extremely rapidly they will undergo extensive structural damage from migratory recrystallization and that such damage can account for the poor viability of rapidly cooled, slowly warmed cells (3).

On the basis of these morphological criteria for the presence of intracellular ice in yeast, we can examine other rates of cooling to determine whether or not the decrease in survival of cells cooled at supraoptimal rates is correlated with the formation of intracellular ice. A cooling rate of 55°C/min is above the optimum rate for maximum viability. When warming and thawing are rapid (1,400°C/min) the viability is 10%; when warming and thawing are slow (1°C/min) the viability is 0.003% (Table I). Cells that are freeze-etched after being cooled at this rate are smaller and show less structural detail than cells cooled at 1,500°C/min. As with the cells cooled at 1,500°C/min, the structure of cells cooled at 55°C/min is markedly altered after warming to −20°C for 5 min and recooling to −196°C before cleaving, etching, and replication. The vacuole shows the presence of massive crystals, whereas the bulk cytoplasm is disrupted by numerous small crystals. Such structural alterations indicate that these cells must have contained intracellular ice before warming and that the ice has the ability to recrystallize rapidly at high subzero temperatures and appreciable recrystallization occurs at temperatures as low as −45°C (Fig. 8).

A cooling rate of 1.8°C/min is suboptimal for yeast. Survivals are 43% for rapidly warmed cells and 5% for slowly warmed cells. After being cooled at this rate (Fig. 13), the cells are contracted, and there is no evidence of ice in freeze-cleaved or freeze-etched cells. The structure of these cells is
structures are relatively stable and that the cell essentially unchanged even after warming to -20°C (Fig. 16). The lack of structural alteration during the warming period indicates that the structures are relatively stable and that the cell cannot contain appreciable amounts of intracellular ice.

The optimal cooling rate for yeast viability is about 7°C/min. Survivals are 55% for rapidly warmed cells and 2% for slowly warmed cells. Most of the cells that have been freeze-cleaved after cooling at this rate resemble those cooled at 1.8°C/min, being contracted and showing few structural changes after etching or after warming to -20°C. On the other hand, a fraction of the cells (fewer than half) resemble those cooled at 55°C/min. They are relatively uncontracted, show fracture ridges, and are clearly frozen internally. When seeding is omitted, the ratio of the two types shifts, and the cells showing intracellular freezing predominate. As mentioned previously, the omission of seeding increases the cooling velocity (between 0° and 20°C) to about 15°C/min, and such an increase in rate has been shown to be sufficient to lower the survival from 50% to about 10% (Table I). The existence of two classes of cells at the optimum cooling rate is consistent with the survival data which indicate that the optimum occurs when the reduction in solution-effect injury produced by raising the cooling rate is counterbalanced by the appearance of intracellular ice in an increasing fraction of the cells.

Our results are essentially consistent with those reported by Moor (21), who used a different strain of yeast and a different freeze-etching procedure. Both his and our studies show that intracellular freezing occurs in cells cooled at about 60°C/min but does not occur in cells cooled at around 1°C/min. But there are differences. His starting material was pressed yeast cakes, whereas we used early stationary phase liquid cultures which had been derived from fresh agar slants. Perhaps these differences in the physiological state of the cells are responsible for the differences in our results. He reported two populations of cell types which differed in their tendency to form intracellular ice crystals. We found little evidence for two groups. He did not detect a decrease in viability at low rates of cooling whereas Mazur and Schmidt (19) have. Our study attempted to relate morphology to the factors that decrease viability on both sides of the survival optimum. Furthermore, he did not subject slowly cooled cells to brief warming to -20°C to test for the presence or absence of recrystallizable intracellular ice.

If our conclusions about the presence of intracellular ice in rapidly cooled cells are correct, cells cooled at a rate sufficiently high to cause the formation of intracellular ice should be less dehydrated and therefore larger than cells cooled at a rate at which no intracellular ice forms. In the present study, the mean cell size increased with increased with increasing cooling rate, from 3.0 µm for cells cooled at 1.8°C/min to 5.2 µm for cells cooled at 75,000°C/min (Table II). If we assume that the cells cooled at 1.8°C/min are fully dehydrated, we can use them as a basis for comparing the amount of water retained by cells cooled at a more rapid rate. The diameters of freeze-etched cells cooled at 7°, 55°, 1,500°, and 75,000°C/min are 1.1, 1.3, 1.4, and 1.7 times, respectively, the diameter of cells cooled at 1.8°C/min. This increase in cell size must represent a greater retention of cell water as cooling rate increases. Such excess water is converted to intracellular ice during cooling and must be responsible for the presence of recrystallizable ice in cells cooled at supraoptimal rates.

The growth of ice crystals during the warming of yeast cells cooled at 55°C/min or faster demonstrates that small intracellular ice crystals are capable of causing major structural damage (Fig. 10). The absence of recrystallizable ice in yeast cells cooled at 1.8°C/min (Fig. 16) indicates that the decrease in cell viability must be due to some factor other than intracellular ice, namely the so-called solution effects.

Ultrarapid cooling rates often provide remarkable preservation of structural integrity and have been reported to result in high survival of cells (2, 13, 21, 30). If both viability and structural integrity of yeast cells cooled at supraoptimal rates.

| Effect of Cooling Rate on Yeast Size |
|-------------------------------------|

| Cooling rate (°C/min) | Measured size (µm) | Size relative to fully dehydrated cell |
|-----------------------|--------------------|----------------------------------------|
| 1.8                   | 3.04 ± 0.06        | 1                                      |
| 7                     | 3.41 ± 0.08        | 1.1                                    |
| 55                    | 3.90 ± 0.07        | 1.3                                    |
| 1,500                 | 4.10 ± 0.07        | 1.4                                    |
| 75,000                | 5.15 ± 0.08        | 1.7                                    |

* Mean ± standard error for 50 measurements.
Integrity are improved as the size of intracellular ice becomes sufficiently small, one may say confidently that a "well-preserved" cell could survive if thawing were sufficiently rapid (1). Correlation of viability and structure for cells cooled at lower rates is somewhat more tenuous. Although cells cooled at 1.8° and 7°C/min show rather high survivals (~50%), their morphology is far from esthetically pleasing. Evidently the cell does not share the morphologists' conviction that a neatly organized structure is requisite for survival.

We are grateful to T. Makinodan for the generous use of his microscope facilities, E. H. Y. Chu for providing the cells, and J. L. Leef and S. P. Leibo for helpful discussions.

Preliminary reports on this material were presented at the 1972 meeting of the Biophysical Society.

This research was sponsored by the United States Atomic Energy Commission under contract with the Union Carbide Corporation. Dr. Bank was a National Institutes of Health Predoctoral Trainee supported by grant no. GM1974 from the National Institute of General Medical Sciences.

Received for publication 10 October 1972, and in revised form 8 February 1973.

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