MANIFESTATIONS OF INJURY IN YEAST CELLS EXPOSED TO SUBZERO TEMPERATURES

II. Changes in Specific Gravity and in the Concentration and Quantity of Cell Solids

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ABSTRACT

Mazur, Peter (Oak Ridge National Laboratory, Oak Ridge, Tenn.). Manifestations of injury in yeast cells exposed to subzero temperatures. II. Changes in specific gravity and in the concentration and quantity of cell solids. J. Bacteriol. **82**:673-684. 1961.—It has previously been established that subjecting cells of *Saccharomyces cerevisiae* to rapid cooling to −30 C results in cell death and in certain morphological alterations. The alterations consisted of the loss of the central vacuole and a 50% decrease in volume. The present experiments were concerned with determining whether the volume decrease was the result of the loss of water alone or of water plus cellular solutes. The density of the "frozen-thawed" cells was found to increase from 1.14 to 1.25 g/cm^3 on the basis of measurements of the sedimentation rate of the cells. Interferometric and refractometric measurements indicated, furthermore, that the concentration of cell solids increased from 20 to 28%, whereas the total mass of cell solids decreased from 25 to 17 $\mu\mu$g/cell. The decrease in cell volume was thus shown to be the result of loss of solution from the cells, a solution containing 11 to 16% solids. Measurements of the rate of dialysis suggested that most or all of these solids had a molecular weight below 600. The findings are consistent with the view that low-temperature exposure destroyed the vacuolar membrane and sufficiently damaged the permeability barriers of the cell to permit escape of low molecular weight compounds. The damage was present a few seconds after thawing, and may, therefore, have been a direct result of intracellular ice crystals which, on the basis of previous studies, are believed to be responsible for death from low-temperature exposure.

[1] Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

More than 99.99% of cells of the yeast *Saccharomyces cerevisiae* are killed by rapid cooling to −30 C or below and subsequent slow warming. On the other hand, only 50% are killed if cooling is slow, and none are killed if the temperature remains above −10 C.

These and other findings have been interpreted as indicating that death of these cells results from the formation of intracellular ice crystals (Mazur, 1961*a*). The dead cells remain visible as structurally intact entities, but differ from untreated living cells in two morphological respects: They have lost the large central vacuole that is present in the living cell, and their volume has decreased by one-half (Mazur, 1961*b*).

Since a decrease in volume signifies the loss of material from the cells, the nature of this material should be a reflection of the injury that has occurred. The material could be water, water plus solutes, or cytoplasm per se. Two approaches were taken to determine its nature. One was to estimate the concentration and size of solids in the water surrounding "frozen-thawed" cells by refractometry and dialysis. The other approach was to measure the changes in the concentration and quantity of solids that were produced within the cells by lethal exposure to low temperatures. These changes were detected first by estimating the specific gravity of the cells and then, more definitively, by interference microscopy and immersion refractometry. The term "frozen-thawed" is used for convenience to describe cells subjected to subzero temperatures and rewarmed to room temperature. Quotation marks are used only when no inference is drawn as to whether the cellular water was, in fact, frozen.

MATERIALS AND METHODS

Preparation of cell suspensions and low-temperature exposure. The cells used were obtained from a liquid culture of a diploid strain of *S. cerevisiae* (NRRL-Y-2235), shaken 24 hr at 29 to 30 C, washed three times in deionized distilled water, and concentrated approximately 10-fold to yield 2×10^8 cell/ml of suspension. Samples (0.1 ml) of the suspension were cooled to -10 or -30 C. Suspensions at -10 C usually supercooled and were made to freeze by seeding. Suspensions were cooled to -30 C either rapidly at 50 C/min or slowly at 1 C/min. All were held at -10 or -30 C for 10 to 15 min and then warmed slowly at 1 C/min. Details of these procedures have been given previously (Mazur, 1961*a*).

Determination of cell density. Densities were calculated from Stokes' Law by measuring the rate of fall of cells through water. The sedimentation chamber was a groove 60 mm long, 1 mm wide, and 1 mm deep, cut in one face of a Lucite slide (4.5 by 25 by 75 mm). A no. 1 (24 by 50 mm) cover slip was cemented over this groove with Permount (Fisher Scientific Company). The Lucite slide, in turn, was cemented over a standard glass microslide (25 by 75 mm) to form an inverted "T", the Lucite slide being the stem of the "T". The groove was filled with a 1,000-fold dilution of a cell suspension, and the openings formed where the grooves passed beyond the cover slip were sealed with paraffin. The "⊥" arrangement of slides was then placed on the mechanical stage of a microscope and the stage tilted so that the groove in the Lucite slide was vertical.

The microscope, fitted with a 20× objective and 10× ocular with cross hairs, was focused on the groove and, at a preselected time, the cell nearest the horizontal cross hair was chosen for observation, provided it was clearly single and not budding. The cell was kept in the field of view for 15 or 20 min by moving the mechanical stage, and the distance it had fallen was recorded to the nearest 0.1 mm every 5 min. About 20 times in the 15- to 20-min observation period, the stage was held stationary and the time required for the cell to fall across the horizontal cross hair was measured with a stop watch calibrated in tenths of a second. This time interval and the velocity of fall were used to calculate the diameter of the cell. The temperature near the groove was recorded to within ± 0.5 C with a 40-gauge copper constantan thermocouple, and the corresponding values for the viscosity and density of water were obtained from standard tables.

Two slides were prepared. Slide A was observed for 1 hr and slide B kept horizontal. Then slide B was observed for 1 hr, and slide A held inverted. Finally, slide A was observed.

Interference microscopy and immersion refractometry. Phase retardation measurements through cells were made with an AO-Baker interference microscope with a 100× water-immersion shearing objective and matching condenser. The light source was a 110-v tungsten filament microscope lamp provided with a Bausch and Lomb 550 mμ interference filter. Diameters of cells and vacuoles were measured with a 12.5× filar micrometer.

Prior to observation, the control and treated cell suspensions were diluted 20 times with either deionized distilled water or solutions of bovine plasma albumin (BPA) in distilled water (Armour Pharmaceutical Company, lot no. V68802). The concentrations of protein in the solutions were determined both before and after addition of the cell suspension from measurements of the refractive index of the solution with an Abbe refractometer. The relation between protein concentration (in g/100 ml) and refractive index is

$$C = \frac{n_1 - n}{\alpha} \tag{1}$$

where n_1 and n are the refractive indices of the solution and water, respectively, and α is the specific refractive increment for protein (the change in refractive index per gram of protein in 100 ml H_2O) and was taken to be 0.0018 (Barer, 1956; Hale, 1958).

Solids in supernatant. Measured volumes of cell suspensions in precision-bore capillaries were cooled to -10 or -30 C and thawed either while being centrifuged at 22,000 × g or 30 or 60 min prior to such centrifugation. The procedures have been detailed previously (Mazur, 1961*b*). The refractive index of the cell-free supernatant was determined with an Abbe refractometer held at 20 ± 0.2 C, and used to calculate the concentration of solids.

Measurement of escape rates. A concentrated suspension of cells in deionized distilled water (1 volume of cells per 3 to 4 parts water) was

prepared; 1-ml portions of the suspension were transferred to each of five 1-ml capacity bulb-type lyophil tubes, which were cooled rapidly to −75 C (cooling rate, 87 C/min from −5 to −60 C) in an ethanol dry-ice bath, held for 10 min, and then warmed slowly to 0 C at 0.9 C/min and thawed. One hour after thawing, the suspensions were pooled and centrifuged for 20 min at 25,000 × *g*. The supernatant liquid was collected and its refractive index determined with an Abbe refractometer; 2 ml were then placed in a bag of Visking 8/32 dialysis tubing partially immersed in a stirred bath of water at 25 C. Every 15 min, 0.05 ml of the supernatant was withdrawn and its refractive index determined. Readings were continued until the refractive index showed no further decrease. Similar procedures were followed in measuring the rate of dialysis of 2% solutions of glucose, sucrose, raffinose, and BPA in deionized distilled water.

Statistical analyses. Differences among the values obtained for the various treatments in a given experiment were tested for statistical significance by analysis of variance or other appropriate procedures. Statements of significance are based on the 0.05 level of probability.

RESULTS

Densities of cells. Cells cooled rapidly to −30 C and warmed slowly had a higher density than untreated cells (1.25 vs. 1.14 g/cm³, Table 1).

These values were calculated from sedimentation rates as follows:

$$D = \frac{9R\eta}{2gr^2} + D_{H_2O} \qquad (2)$$

where R = the rate of fall of the cell (cm/sec), η = the viscosity of the extracellular water (poises), g = gravitational acceleration (980 cm/sec²), r = cell radius (cm), and D_{H_2O} = density of the extracellular water = 0.996 g/cm³.

Corrections for the finite diameter and length of the sedimentation chamber (McGoury and Mark, 1949) would have affected the density values by less than 0.1% and, therefore, were not made.

Equation 2 applies to spheres falling in a fluid, and measurements reported elsewhere show that the yeast cells were nearly spherical (Mazur, 1961*b*). More serious sources of error were the estimations of the cell radius (which enters Stokes' Law as the square) and temperature control. The former was calculated by measuring the time required for a cell to fall a distance equal to its diameter. This time was 1 to 3 sec and the timing error was a minimum of ±0.1 sec. Nevertheless, the diameters so calculated (Table 1, column 4) agree well with the more precise values of 4.82 and 5.94 μ for "frozen" and unfrozen cells obtained previously by micrometry (Mazur, 1961*b*). Temperature was measured to ±0.5 C so that the viscosity values used could be in error by some ±1.5%. Although measured to ±0.5 C, the temperature varied slowly over the several-hour duration of an experiment to the extent of 2 to 3 C. This variation and local heating could have produced convection currents in the water. But if such currents were present, they apparently were too small to affect the results appreciably, for the distance a cell settled usually was the same in each of the three or four successive 5-min observation periods.

The combined errors are probably sufficient to restrict to three the number of significant figures in the density values. But even with this limitation, the difference in density between

TABLE 1. *Sedimentation rates and densities of untreated yeast cells and of cells subjected to rapid cooling to −30C*[a]

Treatment	Mean rate of fall	Mean time fall 1 diameter	Mean cell diameter[b]	Mean cell density ± $s_{\bar{x}}$[c]
	cm/sec × 10⁻⁴	*sec*	*cm × 10⁻⁴*	*g/cm³*
Unfrozen cells	3.0	2.03	5.9	1.14 ± 0.01
Exposed to −30 C	3.5	1.45	4.7	1.25 ± 0.02

[a] Twenty-four untreated and 21 "frozen-thawed" cells were observed.

[b] The diameter of each cell was calculated separately as the product of columns 2 and 3 for each cell. The mean volumes of unfrozen and "frozen-thawed" cells were 114 and 59 μ³, respectively. Volumes were calculated separately for each cell as $V = \pi d^3/6$.

[c] The density of each cell was calculated separately according to equation 2 in text. The temperatures ranged from 25.5 to 30 C, and the viscosity of the extracellular water ranged from 88 to 80 × 10⁻⁴ poises, respectively.

untreated and "frozen-thawed" cells is highly significant statistically.

Concentration of solids and total solids in yeast cells. The concentration and quantity of solids in untreated cells and in "frozen-thawed" cells were estimated by interference microscopy and immersion refractometry. The theory and procedures for these techniques have been extensively reviewed (Barer, 1956; Hale, 1958). Briefly, the interference microscope permits the measurement of the phase retardation of light passing through a cell. Knowing the phase retardation, the refractive index of the suspending medium, and the thickness of the cell, one can calculate the refractive index of the cell. Or alternatively, the refractive index of the cell can be calculated without knowledge of cell thickness if phase retardation measurements are obtained for cells suspended in two or more media of different refractive indices. The refractive index of the cell, in turn, is directly proportional to the concentration of solid materials. The proportionality constant (α) is known as the specific refractive increment, and for the solids in cells is close to 0.0018 (Barer, 1956; Hale, 1958), the value used in our calculations.

Interference microscopy of cells suspended in water. Phase retardation measurements were made on untreated cells and on cells cooled rapidly to −30 C and warmed slowly, water being the suspending medium in both instances. The presence of a large central vacuole in untreated cells necessitated different measurements and calculations than for "frozen-thawed" cells.

1) Untreated cells:—The concentration of solids in g/100 ml of solution in the nonvacuolar portion of these cells is

$$C_1 = \frac{\phi_1}{\alpha t_1} \qquad (3)$$

where ϕ_1 is the phase retardation in microns and equals $\frac{\Delta_1 \lambda}{360°}$ when Δ_1 is the measured phase retardation in degrees and λ the wavelength of the illuminating light (550 mμ); α is the specific refractive increment (0.0018); and t_1 the thickness of the region of the cell through which the phase retardation was measured. [The subscripts in this and in succeeding equations (4 through 9) refer to the correspondingly numbered columns in Table 2.]

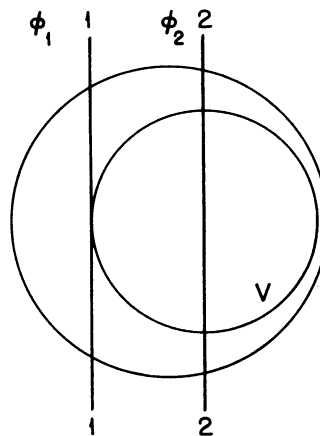

FIG. 1. *Schematic of a normal unfrozen cell of Saccharomyces cerevisiae (1 cm = 1 μ). V is the vacuole. Lines 1–1 and 2–2 show the regions through which phase retardations ϕ_1 and ϕ_2 were determined (see text).*

The geometric relations between a cell and its vacuole are depicted schematically to scale in Fig. 1. Line "1–1" indicates the region in which ϕ_1 was measured. This thickness was 5.2 μ, compared to the full cell diameter of 5.9 μ. Accordingly, one would expect the value of t_1 to be 5.2 μ. But Ross (1957) has observed that phase retardations through bacteria of circular cross-section were the same at the mid-line and at the thinner-edge portions. Moreover, it is possible that the vacuole could bend light by refraction and reflection into the path "1–1." In view of these uncertainties, calculations were made both with t_1 equal to 5.2 μ and with it equal to 5.9 μ, the full diameter.

The concentration of solids in the vacuole (C_3) was calculated by the following equation, derived by substituting our equation 1 in equation 25 published by Barer and Dick (1957):

$$C_3 = \frac{\phi_2}{\alpha t_3} - C_1 \frac{t_2 - t_3}{t_3} \qquad (4)$$

ϕ_2 is the optical retardation through the central portion of the vacuole and cell in microns (i.e., line "2–2" in Fig. 1); t_2 is the thickness of the whole cell in the region at which ϕ_2 was measured (i.e., line "2–2" of Fig. 1) and was taken as equal to the full diameter of the cell; and t_3 is the thickness of the vacuole in microns as estimated by measurement of the horizontal vacuole diameter.

The average or integrated value for the concentration of solids in the cell as a whole (C_4) is given by

$$C_4 = \frac{V_3}{V_4}(C_3) + \left(1 - \frac{V_3}{V_4}\right)(C_1) \quad (5)$$

where V_3 and V_4 are the volumes of the vacuole and the whole cell, respectively.

And finally, the total solids in the cell in $\mu\mu g$ (10^{-12} g) is

$$T_4 = V_4C_4 \quad (6)$$

2) "Frozen-thawed" cells:—Since these cells lacked a central vacuole, the concentration of cell solids is simply

$$C_5 = \frac{\phi_5}{\alpha t_5} \quad (7)$$

and the total mass of solids is

$$T_5 = V_5C_5 \quad (8)$$

Finally, one can calculate the concentration of solids in the material lost from the "frozen-thawed" cells (C_E) as

$$C_E = \frac{T_4 - T_5}{V_4 - V_5} \times 100, \text{ g/100 ml of solution} \quad (9)$$

The observed and calculated values for these several quantities are given in Tables 2 and 3. The calculated concentrations of solids and total solids were somewhat affected by which of the two values of thickness (t_1) was used, but not enough to blur the distinctions between untreated and "frozen-thawed" cells. The unfrozen control cells consisted of a nonvacuolar portion containing 23 to 27% solids and a vacuole with about 14% solids, the whole cell having an average solid concentration of 20 to 22%. After the cells were cooled to −30 C and rewarmed, the *concentration* of solids in the cells increased to 28%, but the *total* quantity of solids decreased from about 25 to 17 $\mu\mu g$ per cell. The fact that concentration increased while total solids decreased is explained by the 50% decrease in the volume of "frozen-thawed" cells. The calculations indicate that the solution lost from the cells during this volume decrease contained about 13 to 16% solids.

Immersion refractometry. Interference microscope measurements of the phase retardations by cells suspended in various concentrations of BPA provided a second estimate of the concentration of solids in the yeast cells, an estimate requiring no knowledge of cell thickness.

The phase retardations of unfrozen control cells in solutions of BPA ranging from 22 to 38% are shown by the center line of Fig. 2. The retardation measurements were made on the non-

TABLE 2. *Phase retardations, thickness, and volumes of untreated yeast cells and of cells cooled rapidly to −30 C*

Measured parameter	Expt	Untreated control cells					"Frozen-thawed" cells	
		Column (1)		(2)	(3)	(4)	(5)	
		No. observed	Nonvacuole portion	Nonvacuole + vacuole	Vacuole only	Whole cell	No. observed	Whole cell
Mean phase retardation (Δ in degrees)	K	15	163.6 ± 5.9	95.3 ± 7.1			14	147.7 ± 6.4
	O	175	163.3 ± 1.7	122.5 ± 1.8			300	156.3 ± 2.2
Thickness (t in microns)	K[a]	15	—[b]	5.90 ± 0.21	4.32 ± 0.27	5.90 ± 0.21	14	4.13 ± 0.23
	O	298	—[b]	5.93 ± 0.05[c]	4.29[d]	5.93 ± 0.05[c]	198	4.80 ± 0.06[c]
Volume (V in cubic microns)	K[a]	15			48.6 ± 7.3	113.0 ± 11.2	14	41.5 ± 7.1
	O	298			41.4[d]	116.5 ± 3.0[c]	198	62.8 ± 2.2[c]

[a] Values measured separately for each cell observed.

[b] Two values (5.93 and 5.20 μ) used in calculations (*see* text).

[c] Values are weighted averages of data from Table 1 and from Mazur (1961*b*, Table 5).

[d] Vacuole volume calculated by multiplying volume of whole cell (116.5 μ^3) by 0.355, the vacuole/cell volume ratio obtained previously (Mazur, 1961*b*). Vacuole thickness was computed as $t = \sqrt{6V/\pi}$

TABLE 3. *Interferometric estimates of solids in untreated yeast cells and in cells subjected to rapid cooling to −30 C*

Parameter	Untreated control cells		"Frozen-thawed" cells
	When $t_1 = 5.20\mu$[a]	When $t_1 = 5.93\mu$[a]	
Concn of solids in nonvacuolar portion (g/100 ml)	$C_1 = 26.7$[b]	$C_1 = 23.4$	—
Concn of solids in vacuole (g/100 ml)	$C_3 = 13.6$	$C_3 = 14.9$	—
Concn of solids in whole cell (g/100 ml)	$C_4 = 22.0$	$C_4 = 20.4$	$C_5 = 27.8$
Total solids in whole cell ($\mu\mu$g)	$T_4 = 25.6$	$T_4 = 23.6$	$T_5 = 17.1$
Concn of solids in solution lost from "frozen" cells (g/100 ml)	—	—	$C_E = 15.9$ (when $t_1 = 5.20\ \mu$)
	—	—	$C_E = 13.2$ (when $t_1 = 5.93\ \mu$)

[a] The two values of t_1 refer to different assumed thicknesses for the region of the cell through which nonvacuolar phase retardations were measured. See text.

[b] Symbols refer to those used in equations in text. Values were calculated separately for experiments K and O (Table 2) and a weighted average was taken.

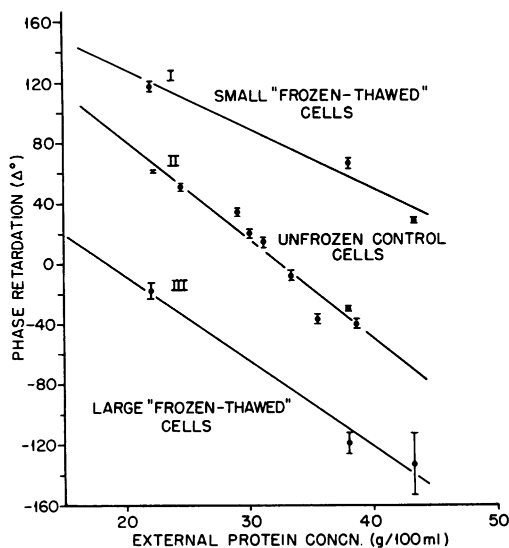

FIG. 2. *Phase retardations of untreated yeast cells and of cells subjected to rapid cooling to −30 C as a function of the concentration of bovine plasma albumin in the external suspending medium. Lines fitted by method of least squares. The slopes of lines I, II, and III are −3.96 ± 0.18, −6.55 ± 0.17, and −5.65 ± 0.90, respectively.*

vacuolar portion of the cells (line 1-1 in Fig. 1). The phase retardation was 0° when the external protein concentration was 32%; hence, the concentration of solids in the nonvacuolar portion of the cell would be 32% provided three assumptions were valid: One assumption is that the cells were impermeable to the plasma albumin. Impermeability of yeast to substances of this size has been demonstrated by the studies of Conway and Downey (1950*a*). Second, it is assumed that α, the specific refractive increment, is the same for the cell solids as for BPA and is equal to 0.0018. The studies by Barer (1956) and Hale (1958) indicate that this assumption introduces insignificant error. The third assumption is that the volume of the yeast cell was constant and independent of the concentration of protein in the external solution. Micrometry measurements of the diameters of cells in water and in 32% BPA were made. They indicated that this third assumption was not valid (Table 4). The volume of cells in the protein solution was 81% of that in water, a significant decrease. Since the shrinkage was almost certainly a result of the loss of water by osmosis, the concentration of solids within the cells in protein solution was higher than it would have been in the absence of shrinkage. If the observed concentration of 32% is corrected for the volume decrease by multiplying 32% by 0.81, the concentration of solids in the nonvacuolar portion of the cells becomes 26%, a value agreeing well with those of 23 and 27% obtained from the phase retardation measurements of cells in water.

Knowing the relation between the concentration of external protein and the phase retardation, one can calculate an independent estimate

TABLE 4. *Measured diameters and calculated volumes of unfrozen yeast cells in water and in a 32% solution of bovine plasma albumin (BPA)*

Suspending fluid	Cell diameter[a]	Cell volume[a]	Cell vol in protein / Cell vol in H_2O
	μ	μ^3	
Deionized water	5.74	107.5	0.81
32% (w/v) BPA	5.35	87.2	

[a] One hundred cells were measured in each suspending fluid. The standard errors of the mean diameters and volumes were 0.1 μ and 5 μ^3, respectively. Volumes were calculated separately for each cell as $V = \pi d^3/6$.

of cell thickness by the following equation:

$$t = \frac{\phi_1 - \phi_2}{\alpha(C_2 - C_1)} = \frac{\lambda}{360°\alpha}\left(\frac{\Delta_1 - \Delta_2}{C_2 - C_1}\right) = -0.849b \qquad (10)$$

when ϕ_1, ϕ_2, Δ_1, and Δ_2 are the phase retardations (in microns and in degrees, respectively) of cells suspended in external protein concentrations C_1 and C_2, respectively, and b is the slope of the plot of phase retardation vs. concentration.

This calculation yields a value of 5.56 ± 0.14 μ for the thickness of the unfrozen cells in protein solution, a figure not statistically different from the value of 5.35 μ obtained by micrometry (Table 4).

Measurements were also made of the phase retardations of cells that had been rapidly cooled to −30 C, rewarmed, and suspended in three concentrations of BPA. In protein solutions, these "frozen-thawed" cells fell into two sharply delineated groups. One group, comprising about two-thirds of the population, consisted of small cells with an extremely high concentration of solids. The upper curve of Fig. 2 shows the phase retardation of these cells as a function of the concentration of BPA in the external medium. Extrapolation of the curve to 0° phase retardation indicates a concentration of solids of more than 50%. The cell thickness calculated by equation 10 is 3.36 ± 0.15 μ. The volume occupied by a sphere of this diameter would be 20 μ^3, which is less than one-fifth the volume of untreated cells (116 μ^3) and less than one-third the volume of "frozen-thawed" cells suspended in water (63 μ^3) (Table 2).

The remaining one-third of the cells exposed to −30 C and suspended in BPA formed a strikingly different group. The plot of their phase retardation as a function of the BPA concentration (bottom curve of Fig. 2) shows 0° phase retardation with an external BPA concentration of 18.5%; hence, the concentration of solids in the cells is assumed to be 18.5%, much lower than that in the cells of the first group. Visually, these cells appeared much larger than those of the first group, an impression confirmed by a calculation of cell thickness by equation 10, which yielded a value for thickness of 4.8 μ with, however, a rather large standard error of 0.8 μ.

The existence of two groups of "frozen-thawed" cells was also evident, although less distinct, in the previous interference measurements on cells suspended in distilled water (Table 2). There, in addition to a measurement of phase retardation, each cell of Experiment O was qualitatively classified as "large" or "small." Those classified as "large" constituted 22% of the sample and had a mean phase retardation of 127°. Their diameters were not actually measured, but can be estimated from micrometry measurements made on similarly treated cells. The frequency distribution of the diameters of 163 cells after exposure to −30 C is shown in Fig. 3, and the mean diameter of the largest 22% was 5.92 μ. (The values for the upper quartile and quintile were 5.87 and 5.95 μ, respectively). Substitution of this value and the observed phase retardation of 127° into equation 7 yields a value of 18.3% for the concentration of solids in the "large" cells. This value is almost identical with the concentration of 18.5% found for the unambiguous group of "large" cells observed in

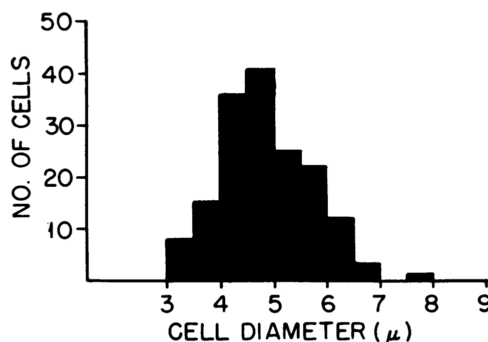

FIG. 3. *Frequency-distribution histogram of the diameter of yeast cells after rapid cooling to −30 C and subsequent slow warming.*

the BPA solutions. It is also close to the average concentration of solids in unfrozen control cells (20 to 22%). Control cells showed no evidence of falling into distinct groups separable on the basis of concentration of solids. Their relative homogeneity is shown by the small standard errors of the datum points for the middle curve of Fig. 2.

Concentration of solids in the solution lost from "frozen-thawed" cells. The interference microscope measurements provided an estimate of the concentration of solids in the solution lost from cells after exposure to −30 C. Further support of this estimate, and hence support for the various assumptions and calculations used in interference microscopy, was obtained by direct measurement of the refractive index of the extracellular water. In the preceding paper (Mazur, 1961*b*), experiments were described in which packed-cell volume was determined by permitting cell suspensions frozen in precision-bore capillaries to thaw during high speed centrifugation. This procedure quickly separated the cells from the surrounding water and also provided a quantitative measurement of the volume of the supernatant liquid. The supernatant not only represented the water surrounding the cells before treatment, but also contained whatever solution was withdrawn from the cells as a consequence of the low temperature exposure. The concentration of solids in the whole supernatant was determined from measurements of the refractive index as

$$C_s = \frac{\Delta_n}{\alpha} \tag{11}$$

where Δ_n is the difference between the refractive indices of the supernatants from frozen and unfrozen suspensions, and α, the specific refractive increment, is 0.0018, as before.

The fraction of the supernatant ascribable to the solution lost from the cells is

$$V_E = \frac{V_f - V_u}{V_f} \tag{12}$$

where V_f and V_u are the lengths of the capillaries occupied by the supernatants from frozen and unfrozen suspensions, respectively, and are corrected for any differences in the quantity of cell suspension initially drawn into the capillaries. These lengths are equivalent to volumes, since the diameter of the capillary bore was constant throughout. Knowing the fraction V_E, one can calculate the concentration of solids in that portion of the supernatant representing the solution lost from the cells because of low temperature exposure (C_E) as being

$$C_E = \frac{C_s}{V_E} \tag{13}$$

TABLE 5. *Refractometric estimates of the concentration of solids in the solution lost from cells exposed to subzero temperatures and thawed during centrifugation or prior to centrifugation*

Treatment	Expt	No. of samples	Time between thawing and centrifuging	Δ_n[a]	C_s[b]	V_E[c]	C_E[d]
			min	$\times 10^{-5}$	*g/100 ml*		*g/100 ml*
Cooled to and seeded at −10 C	r	5	0	0	—[e]	—	—
	t	8	0	4	—	—	—
Slow cooling to −30 C	p	9	0	120	0.67	0.123	5.4
Rapid cooling to −30 C	n	3	0	188	1.04	0.134	7.8
	u	4	0	165	0.92	0.132	7.0
		4	60	258	1.43	0.136	10.5
		4	120	274	1.52	0.138	11.0

[a] Refractive index of frozen supernatant minus that of unfrozen control. The standard errors ranged from 2 to 14 $\times 10^{-5}$.

[b] Concentration of solids in frozen supernatant calculated from Δ_n.

[c] Fractional increase in volume of supernatant resulting from low-temperature exposure.

[d] Concentration of solids in the solution lost from the cells as a result of low-temperature exposure.

[e] Not significantly different from zero.

Values of Δ_n, C_s, V_E, and C_E after several treatments are given in Table 5.

The solution withdrawn from cells subjected to rapid cooling to −30 C contained about 7% solids immediately after thawing, and about 11% 60 or 120 min after thawing. The value of 11% is reasonably close to that of 13 to 16% calculated for comparably treated cells from the interference microscope measurements (Table 3).

The concentration of solids in the extracellular water of cells cooled *slowly* to −30 C (C_s) was lower than that of rapidly cooled cells. It was lower primarily because slowly cooled cells undergo less volume decrease and hence less loss of cell solution than those cooled rapidly (Mazur, 1961b). The solution that escaped from slowly cooled cells also contained a somewhat lower concentration of solids, C_E (5.4 vs. 7.5%), but the difference may not be significant since slow and rapid cooling were not compared in the same experiment. No increase in solids was found in the water surrounding cells cooled to −10 C. This result was predictable, because previous experiments had shown that such cells undergo little volume decrease, and hence little loss of cell contents (Mazur, 1961*b*).

Size of solids in solution lost from "frozen-thawed" cells. A rough estimate of the molecular size of solids in the solution withdrawn from "frozen-thawed" cells was obtained by comparing the rate at which the solids escaped through a dialysis membrane with the escape rate of known compounds (Craig, King, and Stracher, 1957). This comparison is made in Fig. 4, the known compounds being BPA and raffinose (mol wt 595). The escape rates were determined by following the change in refractive index of the test solution as a function of the duration of dialysis. Half of the solids in the supernatant from "frozen-thawed" cells escaped in about 30 min, and no detectable solids remained after 130 min. This escape rate is higher than that of raffinose. BPA did not escape at all. Determinations were also made of the escape rates of glucose and sucrose; they were not distinguishable from that of the supernatant.

The determinations must be considered preliminary, for they were subject to considerable instrumental error. Initially, the difference in refractive index between the solutions and the external water was about 0.0030 (1.3360 vs. 1.3330). The error of the refractometer readings

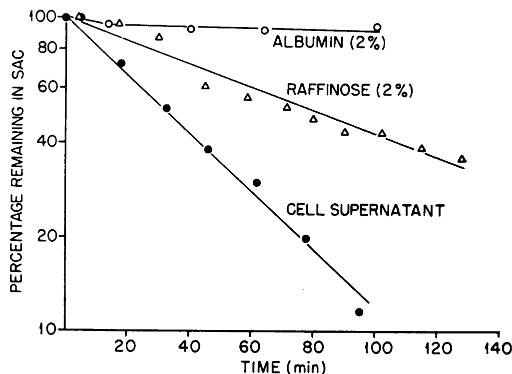

FIG. 4. *Escape rates of several solutes through 8/32 Visking dialysis tubing. "Cell supernatant" is the solution surrounding yeast cells after rapid cooling to −75 C and subsequent slow warming. Lines fitted by eye.*

was at least ±0.0001, so that the estimate of the concentration of solids had a minimal error of 3% at time zero, and an error of 33% after 90% of the material had escaped. Nevertheless, the method is sensitive enough to demonstrate that over 90% of the solids are considerably smaller than albumin and apparently smaller than raffinose. The possibility remains, however, that a small, undetectable portion of the solids was of high molecular weight.

DISCUSSION

The various alterations observed in cells killed by freezing and thawing indicate that the permeability barriers of such cells have been damaged by low-temperature exposure. The normal living cell consists of a ring of cytoplasm surrounding a central vacuole, which contains a lower concentration of solids (14%) than the cytoplasm (26%). Electron microscopy indicates that the vacuole is separated from the cytoplasm by a vacuolar membrane and that the cytoplasm, in turn, is surrounded by a plasma membrane (Agar and Douglas, 1957; Hashimoto, Conti, and Naylor, 1958, 1959). The existence of a plasma membrane is also supported by the physiological evidence for differential permeability and osmotic response (Conway and Downey, 1950*a*; Rothstein, 1959). Finally, the entire protoplast is enclosed in a cell wall. This wall apparently is elastic, for living yeast cells can decrease considerably in volume in hypertonic solutions without undergoing plasmolysis (Eddy,

1958; Drabble, Drabble, and Scott, 1907; Ebutt, 1961). The cell wall is permeable to low molecular weight compounds but not to large molecules such as inulin or peptone (Conway and Downey, 1950*a*).

The density of untreated cells was 1.14 g/cm^3 according to our experiments. This value compares favorably with that of 1.12 reported by Silbereisen (1936). Haddad and Lindegren (1953), using procedures similar to those reported in this paper, obtained a density of 1.09 g/cm^3; however, they neglected to apply a correction to Stokes' Law for the finite dimensions of the sedimentation chamber, a hemocytometer slide (McGoury and Mark, 1949). Application of the required correction raises their value to 1.124 g/cm^3.

Yeast cells killed by exposure to subzero temperatures differed from the normal in a number of respects. There was a close correlation between the percentage killed and the percentage lacking the large central vacuole (Mazur, 1961*b*), a correlation indicating that lethal low-temperature treatment resulted in destruction of the vacuolar membrane.

Secondly, the dead cells exhibited conspicuous changes in volume, in specific gravity, and in the quantity and concentration of solids. Cell volume decreased by one-half (Mazur, 1961*b*) and was accompanied by an increase in specific gravity (from 1.14 to 1.25) and in the concentration of cells solids (from 21 to 28%). The increases in concentration and density occurred in spite of the fact that solids escaped out of the "frozen-thawed" cells into the surrounding water. They increased because the solution leaving the cells contained a lower concentration of solids (11 to 16%) than that originally present in the cell (20 to 22%). The escaped solids were all low molecular weight compounds as far as could be detected; high molecular weight compounds were retained within the cells.

The escape of the smaller molecules implies damage to the plasma membrane. The retention of the larger molecules indicates either than the plasma membrane continued to exist as a partial barrier or that the residual permeability barrier was the cell wall.

The loss of a large quantity of low molecular weight solutes must have produced a considerable decrease in the osmotic pressure within the cell. A decrease in osmotic pressure would decrease the tendency for water to enter the cell and, hence decrease the turgor pressure against the elastic cell wall. The lowered turgor pressure, in turn, would permit the cell wall to shrink and so produce a decrease in cell volume.

Up to now the dead "frozen-thawed" cells have been discussed as a homogeneous population. Actually, they fell into two groups. One group, comprising the great majority, behaved as described in the preceding paragraphs. But the second group of one-third to one-fifth of the cells behaved differently. They contained a concentration of solids (18.5%) close to that of normal living cells (20 to 22%). Moreover, their diameter and volume were about the same as the average of the living cells. These findings suggest that the plasma membrane of these cells remained sufficiently intact to prevent the loss of low molecular weight compounds. Therefore, the cells maintained their internal osmotic pressure and did not decrease in volume. Their vacuole did disappear, however, so that even in this group, low-temperature exposure apparently destroyed the vacuolar membrane.

Interference microscopy and refractometry yield no information on the nature of materials escaping from cells killed by freezing and thawing. Hansen and Nossal (1955), however, analyzed the washings from pressed commercial yeast frozen by intimate mixing with dry ice; they found that the washings contain coenzymes, organic acids, and particularly, amino acids, carbohydrates, and inorganic salts. They also found that 15% of the total solids in the washings was biuret-positive material (protein). In contrast, no nondialyzable material was detected by the present refractometric measurements. The latter method, however, was rather insensitive and might not detect protein concentrations of less than 5 to 10% of the solids. Sakagami (1959) has also found an increase in low molecular weight compounds extractable by water from yeast cells exposed to -30 or -190 C. The compounds included sugars, fatty acids, esters, and particularly, amino compounds. The concentration of extractable substances was higher with lower survivals (i.e., higher for cells exposed to -190 C than for cells exposed to -30 C).

An increase in the permeability of "frozen-thawed" yeast to external materials is well documented. It has been reported for citrate (Foulkes, 1954), hydrogen ions (Conway and

Downey, 1950*b*), and di- and tricarboxylic acids (Krebs, Gurin, and Eggleston, 1952). In those studies, however, no attempt was made to correlate the increased permeability with loss in survival, and there was no attempt to relate either effect to the physical factors introduced by subzero temperature exposure.

On the basis of earlier work, death of *S. cerevisiae* at subzero temperatures seems to be a consequence of the formation and growth of intracellular ice crystals (Mazur, 1960, 1961*a*, *b*). If this theory is correct, the destruction of the vacuolar membrane and the increased permeability of the plasma membrane must somehow be related to intracellular ice formation. The difficulty is to assign cause and effect. Does intracellular freezing produce membrane damage which leads to cell death, or does it produce cell death which leads to increased permeability? All one can say at present is that most of the loss in cell solids and the decrease in cell volume had already occurred within a few seconds after thawing of the suspensions (centrifugation experiments). Moreover, the changes in morphology and in the content of solids noted in those cells that subsequently failed to produce colonies on an agar medium were already evident several minutes after thawing and underwent little, if any, further change with time.

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