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Physiological Response of *Neurospora* Conidia to Freezing in the Dehydrated, Hydrated, or Germinated State

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This study concerned the response to freezing of *Neurospora crassa* conidia in four different states: air-dry, hydrated in water, hydrated in Vogel medium lacking only sucrose, or hydrated in complete Vogel medium. All hydrated conidia were incubated in one of the above media for various times before freezing and were then washed and frozen in distilled water. Viability was estimated by three techniques, and the agreement among them was good. Hydration of air-dry conidia was found to be very rapid and, once hydrated, the conidia were much more sensitive to rapid freezing than they were before hydration. Rapidly cooled conidia survived freezing to a much higher extent when the warming rate was rapid than when it was slow; slowly cooled conidia showed little or no dependence on the warming rate. This sensitivity to rapid cooling and slow warming was attributed to the effects of intracellular ice. The sensitivity to freezing could be reversed by dehydrating the conidia in vacuo before freezing; thus, it was concluded that the presence or absence of water is the determining factor in the initial sensitivity due to freezing. In water, the sensitivity remained constant from 2 min to 15 days after hydration. Although conidia hydrated in growth medium lacking sucrose remained metabolically inactive, their sensitivity to rapid freezing decreased as a function of time in the medium before freezing. The reason for this decreased sensitivity is not understood. Conidia hydrated in complete growth medium (i.e., containing sucrose) became metabolically active and, after the initial sensitivity associated with hydration, became increasingly more sensitive to freezing as a function of their time in the medium. Drying itself was deleterious to metabolically active conidia, and those that survived dehydration did not exhibit a large absolute increase in resistance to subsequent freezing. The increase in sensitivity to freezing and to drying seems associated with the presence of metabolic activity; however, the precise cause of the sensitization remains obscure.

Although there have been numerous studies on the effects of freezing on cells of animals, higher plants, and bacteria (see reference 26 for a review), there have been relatively few studies on fungi other than yeast (1, 4, 5, 7, 10-13, 18, 22, 32, 34). Most of the cryobiological studies of fungi have been concerned with spores and, in general, it has been concluded that the survival of spores is higher when cooling to subzero temperatures is slow rather than rapid, but that it is high and usually independent of the cooling rate when warming is rapid.

The dependence of the survival of various cells on the cooling and warming rates has been demonstrated in many studies (16, 19, 23-27). There have also been studies on freezing sensitivity as a function of the stages of the growth cycle in bacteria (26), yeast (19-21), and mammalian cells in synchrony (15, 17). In this last case, Koch et al. (15), for example, observed different sensitivities to freezing depending on the stage of the cell cycle the population was in at the time of freezing.

The sporulating fungi, however, offer an especially attractive opportunity for examining the relation between a broad range of physiological states of a cell and its response to subzero temperatures. Although there is ample evidence that conidia collected from aerial mycelia (air-dry, i.e., in equilibrium with the relative humidity) are more resistant to freezing than they are after being hydrated (5, 10, 22), there exists no systematic study comparing the response of populations of metabolically active conidia to inactive ones after freezing. The present study was de-

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signed to make this comparison and to examine further the role of cell water in freezing damage.

The comparison was facilitated by the fact that the conversion from air-dry spore to actively growing mycelium involves at least two sequential and separable steps. The first, which requires only water, is hydration of the air-dry spore. The second is germination and subsequent growth, a step that requires the presence of a carbon source, at least in *Neurospora*, and is directly associated with the onset of metabolic activity. Accordingly, the chief approach used in the experiments was to compare survival after freezing of air-dry conidia with the survival of conidia after they had been suspended in water or in a medium with a carbon source (Vogel medium with sucrose). The three states (air-dry, hydrated, and metabolically active) were found to exhibit major differences in freezing sensitivity. As a control, we also examined the freezing response of conidia preincubated in Vogel medium without sucrose. The response was still different from the other three cases.

The nature of the medium in which cells are suspended during freezing can exert major effects on the response of cells apart from its effects on the physiological state. Since we were concerned with the latter and not the former, the conidia were transferred from the preincubation medium into distilled water just before actual freezing and thawing. The two cryobiological factors studied were cooling rate (1 to 500°C/min) and warming rate (1 to 1,000°C/min). The survival of the conidia was assessed in terms of their ability either to form colonies on an agar medium or to germinate.

MATERIALS AND METHODS

N. crassa 74A (wild type) was used for these studies. Cultures were grown at 25°C on agar slants made with 2% Difco agar in Vogel medium (VMM) (33). Conidia were harvested from 4- to 5-day-old slants by aseptically transferring the mycelial mat to a tube containing sterile glass-distilled water. The tube was vigorously agitated with a Vortex mixer for approximately 15 s to disperse the conidia, and the contents were filtered through two layers of sterile gauze to remove mycelial fragments. The conidia were suspended in either VMM, Vogel medium lacking sucrose but containing the other 14 constituents of the medium ($V^{w/o}$), potassium phosphate buffer (PO_4) at pH 6.0, or distilled water. Just before freezing, the conidial suspensions in VMM, $V^{w/o}$, or PO_4 were diluted 20-fold with water and centrifuged for 15 min at 200 × *g*; the supernatant was then decanted, and the conidia were resuspended in glass-distilled water to a concentration of 10^7/ml, as determined by duplicate hemacytometer counts. Conidial suspensions (0.1 ml) were delivered to Pyrex freezing tubes (90 mm long; ID, 4 mm; OD, 6.8 to 7.1 mm) with a Hamilton microliter syringe.

Gram quantities of air-dry conidia, essentially free from other components of the mycelial mat, were obtained as follows. Approximately 10^6 conidia suspended in 0.1 ml of VMM were inoculated onto VMM containing 2% agar in petri dishes (100 by 15 mm) and incubated at 30°C for 3 to 5 days. Air-dry conidia were harvested by replacing the tops of the petri dishes with sterile, dry tops, inverting the dishes, and striking them against a padded surface 5 to 10 times. The free conidia dislodged by this procedure fell onto the dry top and were then transferred to sterile test tubes.

The cooling rates used were approximately 1, 10, 100, and 500°C/min, and the final temperature was −196°C. The warming rates were 1, 25, and 1,000°C/min (16). All preparations were equilibrated for 5 min at 0°C before freezing except for those frozen after 2 and 4 min of hydration. Preparations cooled at rates of 1, 10, and 100°C/min were further cooled to −2°C and then seeded with a crystal of ice to initiate freezing. Those cooled at 500°C/min were transferred directly from the 0°C bath to liquid nitrogen (−196°C). After warming, all preparations were held at 0°C until initiation of the viability assays (ca. 1 to 2 h).

The viability of frozen-thawed conidia was determined by one or both of two types of assays. The first viability assay (percent germination) was based on morphological changes leading to germination and was measured by phase-contrast microscopy at a magnification of ×400. For each conidial suspension to be evaluated, 0.1 to 0.3 ml of the contents of a freezing tube previously diluted 1:10 with appropriate medium was placed in 2 ml of VMM in sterile test tubes (18 by 150 mm). The rack of tubes was set into a New Brunswick gyratory (100 rpm) water bath at 30°C. Samples were removed with sterile Pasteur pipettes at hourly intervals; 200 to 400 conidia in randomly selected microscope fields were scored for the presence or absence of germ tubes. To determine the percentage of germination beyond 8 h, the procedure was modified because extensive growth of germ tubes made it difficult to identify the conidium from which the germ tube originated; immediately after thawing and subsequent inoculation into VMM, hemacytometer counts were made to determine the number of ungerminated conidia per ml. Then counts were made after 12 and/or 24 h of incubation, and the number of ungerminated conidia per milliliter was again determined. In this way one could obtain an indirect estimate of the percentage of germination by the disappearance of ungerminated conidia.

The second viability estimate was based on the ability of the conidia to form colonies on an agar medium developed by Brockman and de Serres (6) and Davis and de Serres (9). The conidial suspensions were diluted to contain 1,000 conidia per ml, and 0.1 ml was plated on disposable petri dishes (100 by 15 mm) containing 20 ml of the above medium. The cultures were allowed to grow for 4 to 5 days at 35°C to hasten colony growth, and the colonies were then counted under a standard bacterial colony counter.

Oxygen consumption of 1-ml samples containing approximately 10^7 cells was measured in a Gilson differential respirometer at 25°C. The suspending me-

dium was either water, PO_4 buffer at pH 6.0, VMM, or $V^{w/o}$.

To determine the water content of air-dry conidia, approximately 0.25 to 0.5 g of such conidia was transferred to tared weighing bottles, and the weight of the conidia was determined. The samples were then dried at 60°C in vacuo. Samples were dried for 6, 8, 12, or 24 h and were then capped, removed, cooled, and weighed to estimate the fractional water loss.

In some experiments, hydrated conidia were dried to various extents before freezing. The procedure was as follows. Conidia were equilibrated with water for 1 h at room temperature. The suspensions were then concentrated to a dense slurry by centrifugation at 200 × *g* for 15 min, the supernatant was removed, 0.1 ml of the cell suspension was transferred to tared, sterile Beem capsules, and the capsules were weighed. In a given experiment, one capsule was retained as undried, unfrozen control. Two preparations were undried but frozen at 500°C/min. The remaining capsules (six to eight per experiment) were placed in vacuo at room temperature and, after various times, duplicate capsules were removed, capped, and weighed. They were then cooled at 500°C/min and warmed at either 25 or 1°C/min. The dried conidia were then hydrated and plated to estimate viability.

Two techniques were used to measure the degree and rate of hydration of air-dry conidia when placed in aqueous media. Large quantities of these conidia were harvested as previously described and transferred to hematocrit tubes with an ID of 3 mm, an OD of 7 mm, and a length of 45 mm. They were centrifuged at 15,000 × *g* in a hematocrit centrifuge, and the column length of the packed cells was measured; it was generally between 25 and 30 mm. Water was then added with a Pasteur pipette and flushed back and forth until all conidia were suspended. After various times, the hematocrit tubes were again centrifuged, and the column length of packed cells was again measured. Four different cultures were grown from which four separate collections of conidia were harvested. The above determination was done in triplicate on samples from each collection of air-dry conidia.

A second estimate of hydration was obtained by measuring the diameters of conidia with a calibrated filar micrometer under a phase-contrast microscope at a magnification of ×400. First, 15 to 20 randomly selected conidia were measured in the air-dry state; then the slides were flooded from the side by a Pasteur pipette, and the diameters of an approximately equal number of conidia were measured again; this process was repeated 10 times. Calculations of cell volumes were made with the assumption that the conidia are spherical.

RESULTS

Requirements for respiration and germination. The results (Fig. 1 and 2) indicate that sucrose was required for germination and respiration of *N. crassa* conidia. Approximately 90% of the conidia in VMM germinated within 6 h (Fig. 1). Conidia in media other than VMM did not germinate even after 48 h; however, they remained viable (Fig. 1), since, within 24 h after the addition of sucrose to conidia in $V^{w/o}$ or after the addition of concentrated VMM to conidia in phosphate buffer or water, essentially all of the conidia germinated. The results for the respiration experiments were comparable (Fig. 2).

Survival after freezing and thawing. Table 1 shows the percentage of survival based on colony counts of frozen-thawed conidia as a function of the incubation medium, the length of time of incubation, and the cooling and warming rates.

Representative data have been plotted three ways (Fig. 3 to 5) to clarify relationships not immediately discernible from Table 1: (i) percentage of survival versus the time of preincu-

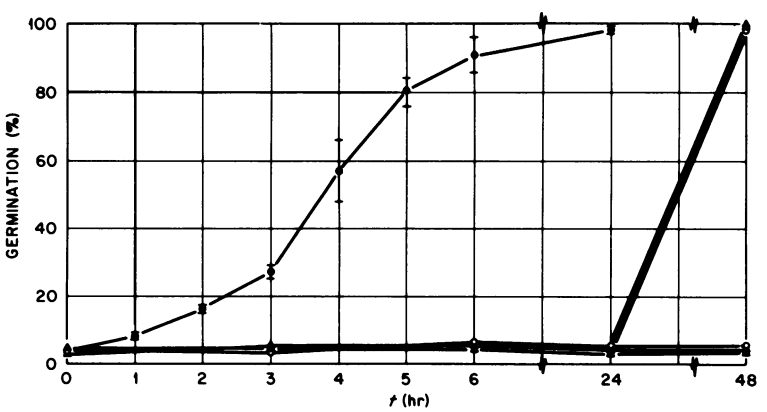

FIG. 1. *Percent germination of N. crassa as a function of time in various media: VMM (●), $V^{w/o}$ (△), PO_4 buffer (○), water (▲). After incubation for 24 h, VMM was added to half the samples in the last three media, and incubation was continued for another 24 h. The results are shown by three newly overlapping lines at the right side of the graph.*

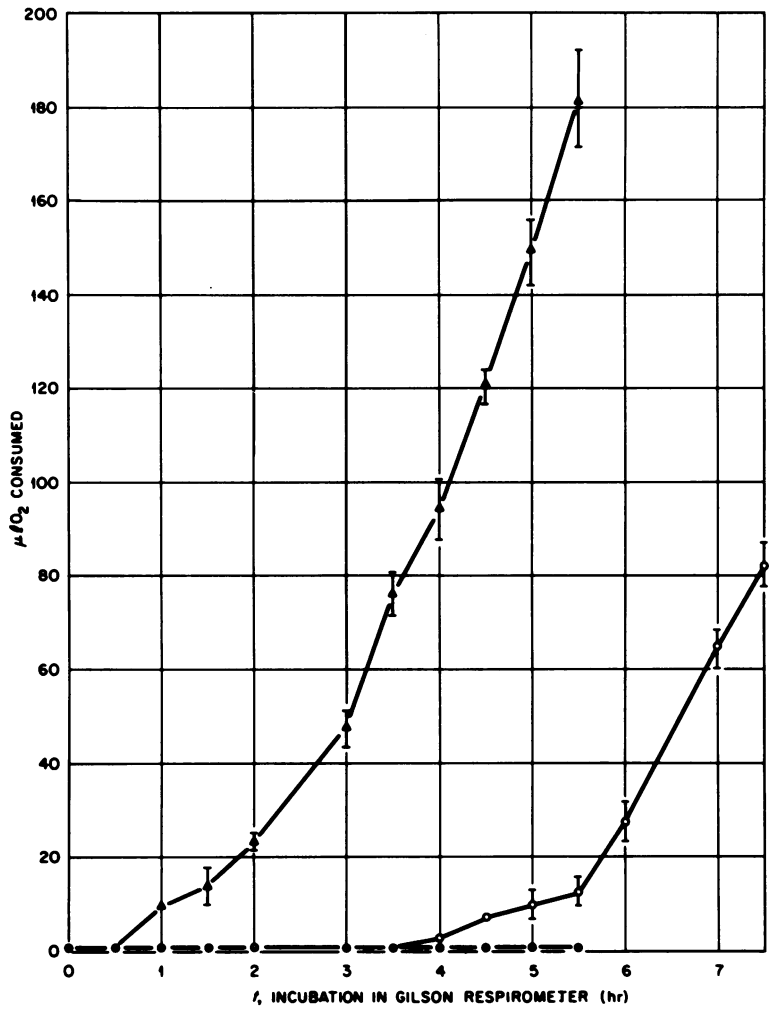

FIG. 2. *Oxygen consumption of N. crassa conidia as a function of time in various media; 10^7 conidia in VMM (▲), in $V^{w/o}$ (●), and in $V^{w/o}$ to which sucrose was added after 3.5 h* (○).

bation in various media before freezing, (ii) percentage of survival versus the warming rate; and (iii) percentage of survival versus the cooling rate.

Survival versus hydration and preincubation. The arrow at 90% on the ordinate of Fig. 3 is the mean survival of frozen-thawed air-dry conidia after different samples were subjected to all of the combinations of cooling and warming rates used in this study. The results show that hydration alone (0 h on abscissa) caused increased sensitivity of conidia to freezing. Figure 3 also shows that the sensitivity of conidia to cooling at 500°C/min further increased with time of preincubation in VMM before freezing; Table 1 shows a similar response for the other cooling rates. Conidia in $V^{w/o}$ behaved differently. At high cooling rates (500°C/min), they showed a decrease in sensitivity to freezing with increasing preincubation time (Fig. 3); at moderate cooling rates (10 and 100°C/min), they showed no effect of incubation time; and at slow cooling rates (1°C/min), they showed increased sensitivity to freezing with increased preincubation time.

Survival versus warming rate. When the results in Table 1 are plotted as a percentage of survival versus warming rate (Fig. 4), it becomes evident that rapidly cooled conidia (500 or 100°C/min) were more sensitive to the warming rate than were slowly cooled conidia (10 or 1°C/min). The slower the warming of rapidly cooled conidia, the lower was the survival. In contrast, the survival of slowly cooled conidia

TABLE 1. *Percent survival of conidia versus cooling rate, warming rate, and time of incubation in various media before freezing*

Cooling rate (°C/min)	Warming rate (°C/min)	Survival (%) after freezing for conidia suspended initially[a] in:						
		Water at 0 h[b]	$V^{w/o}$ for:			VMM for:		
			1 h	3 h	5 h	1 h	3 h	5 h
1	1	—[c]	70	73	56.2	80.2 ± 12	65	12.0
	25	93.0 ± 9.1[d]	97.0 ± 2.8	87.0 ± 14	44.3	98.5 ± 0.7	69.0 ± 2.6	20.8
	1,000	99.8 ± 17.2	96.0 ± 4.2	86.0 ± 4.6	70	81.0 ± 2.8	51.3 ± 9.8	25
10	1	—	56.2 ± 18	68.1	52.0	80.0 ± 20	66.2	4.5
	25	—	75 ± 19	78.2	75.0	96.1 ± 18	71	21.2
	1,000	—	93 ± 12	77.1	85.1	104.4 ± 20.3	50.0	9.1
100	1	—	4.3 ± 2.1	—	5.4	0.68 ± 4.5	—	0
	25	—	21.5 ± 8.3	—	29.7	3.03 ± 12	—	0
	1,000	—	77.2 ± 7.3	—	70.2	58.8 ± 19	—	0
500	1	—	4.3[e]		10.7	4.3	—	0
	25	39.7 ± 16.8	34.9 ± 18.5	46.9 ± 18	79.6 ± 21	3.1 ± 1.2	0.3 ± 0.5	0
	1,000	67.9 ± 12.8	62.5 ± 7.8	111.2 ± 8.4	121.2 ± 8.8	53.1 ± 16	6.6 ± 4.7	0

[a] After preincubation in VMM or $V^{w/o}$ for the indicated period of time, the conidia were washed and resuspended in distilled water just before freezing.

[b] Survival of conidia held in water for ~60 min before freezing. As will be seen (Fig. 8), survival after freezing was unaffected by times in water ranging from 2 min to 15 days.

[c] —, Not measured.

[d] Standard deviation.

[e] Single experiments done, mean of triplicate samples. Standard deviation not calculated.

was essentially unaffected by the warming rate (Fig. 4).

Survival versus cooling rate. Figure 5 shows the percentage of survival versus the cooling rate. There was a marked difference in the percentage of survival when the cooling rate of 10°C/min was compared with cooling rates of 100 or 500°C/min. The two higher cooling rates were damaging in all cases, especially when warming was slow. Furthermore, the two highest cooling rates were damaging for conidia preincubated both in VMM and in $V^{w/o}$.

Germination of frozen-thawed conidia. Table 2 summarizes the survival of frozen-thawed conidia based on the percentage of germination. In general, the effects on conidia of the preincubation medium, the length of incubation, and the cooling and warming rates, as assessed by this method, were similar to the effects as assessed by colony counts (Table 3). For example, conidia preincubated in $V^{w/o}$ before freezing again showed a higher percentage of germination than did those preincubated in VMM. Also, conidia cooled and warmed rapidly attained a higher percentage of germination than did those cooled rapidly but warmed slowly.

Figure 6 compares the kinetics of germination of air-dry conidia that were cooled at 500°C/min and warmed at either 25 (slow) or 1,000°C/min (rapid) with that of unfrozen conidia; there was little or no effect of freezing. Figure 7 shows the kinetics of germination for conidia that were frozen and thawed after preincubation for 1 h in either VMM or $V^{w/o}$. Conidia that were rapidly cooled and rapidly warmed reached TG_{50} (time to 50% of the maximum germination) much faster than did those warmed slowly. However, after 3 or 5 h of preincubation in VMM before freezing, most conidia were killed by the freeze-thaw event and, therefore, no further change in percentage of germination was noted (Table 2).

Freezing sensitivity versus hydration. Air-dry conidia were found to hydrate extremely rapidly. These conidia had an extremely irregular cell border and a mean cell volume of approximately 119 μm^3 as determined from micrometer readings. After contact with water, the cell border became regular very rapidly (less than a second), and the mean volume increased to approximately 196 μm^3, an increase in volume of 65%. Similar changes in the volume were found by hematocrit measurements; the mean column length of the packed cells increased by a factor of 1.86 after hydration. The increase in cell volume appeared to occur at the moment water came in contact with a given conidium. However, diameter and volume measurements could not be made in less than 1 min after the addition of water to the sample.

Once the conidia were suspended in water, their resistance to rapid freezing immediately decreased from the 90% survival for air-dry co-

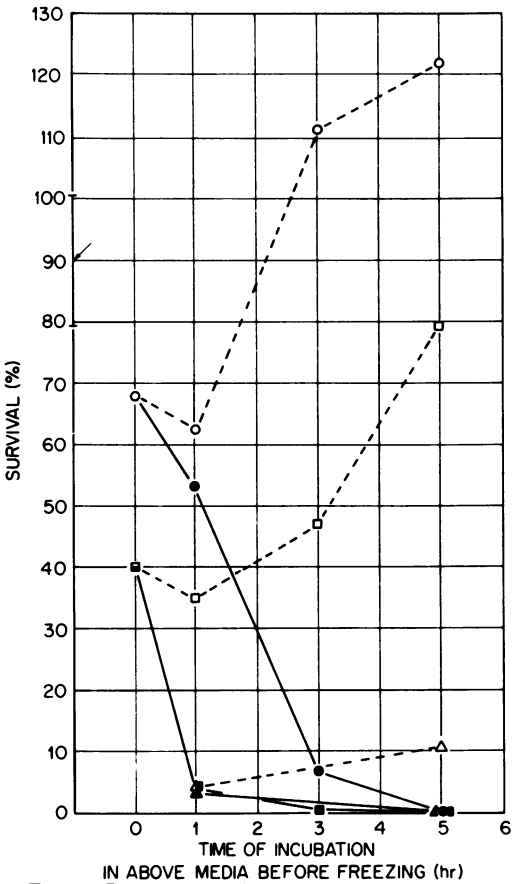

FIG. 3. *Percentage of survival based on colony counts of frozen-thawed conidia as a function of the length of incubation in VMM* (——) *or* $V^{w/o}$ (-----) *before freezing. The survival of frozen air-dry conidia is 90% (arrow on ordinate). In the experiment, air-dry conidia were suspended in water for 60 min. Zero hour refers to the survival of air-dry conidia suspended in water for 60 min before freezing. After the initial 60 min of equilibration in water, the other conidial suspensions were transferred to VMM or* $V^{w/o}$ *for 1, 3, or 5 h, frozen at 500°C/min, and warmed at 1,000°C/min* (●, ○), *25°C/min* (■, □), *or 1°C/min* (▲, △).

nidia to about 40 and 65% with slow and rapid warming, respectively. However, the survival of slowly cooled hydrated conidia remained above 90% (Table 1). For a given set of cooling and warming rates, the resistance to rapid freezing of hydrated conidia remained about the same between 2 min and 15 days (Fig. 8).

Since the hydration of conidia produces a decrease in their resistance to rapid freezing, it was of interest to determine whether that resistance would be restored by subjecting the hydrated conidia to dehydration before freezing. Drying alone was found to be somewhat detrimental to conidia that had been hydrated in water for 1 h; survivals decreased to approximately 75 and 52% after drying in vacuo for 2 and 5 h, respectively. However, the conidia that survived drying became much more resistant to subsequent rapid freezing than they were when they had been fully hydrated (Fig. 9).

In contrast, drying alone was more detrimental to conidia that had been preincubated in VMM for 5 h. Survival decreased to approximately 22% after only 2 h of drying, and the remaining viable cells showed very little absolute increase in resistance to freezing (Fig. 9). However, on a relative basis, the increase was significant. Leef and Gaertner (Cryobiology 12:584, 1975; Gaertner, personal communication, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.), using a much more concentrated inoculum than that used here, found the survival of conidia held in VMM for 5 h before rapid freezing to be about 0.0001%. Therefore, the 3% survival observed in Fig. 9 for conidia dehydrated after preincubation for 5 h in VMM before freezing represents an increase in survival of more than four orders of magnitude over those frozen in the hydrated state.

In another series of experiments, air-dry conidia were dried in vacuo at 60°C to determine their water content only; it was found to be 53.1 ± 4.3% by weight.

DISCUSSION

This study was concerned with the response to freezing of *N. crassa* conidia in four states: (i) air-dry, (ii) in water, (iii) in $V^{w/o}$, and (iv) in VMM.

Air-dry conidia are known to be metabolically inactive, as measured by morphological change, respiration, and protein synthesis, and to remain inactive after hydration. Schmit and Brody (31) have confirmed this; yet they have shown that the act of hydration of *N. crassa* conidia is accompanied by changes in amino acid pools and that polysomes are rapidly formed. Their findings substantiated earlier observations by Mirkes (28), who found that air-dry conidia of *N. crassa* contained 3% of their ribosomes as polysomes and that after hydration, in the absence of a carbon source such as sucrose, 30% of the ribosomes were in the form of polysomes. When conidia were suspended in VMM for 15 to 30 min, the percentage of ribosomes that sedimented as polysomes increased even further, to approximately 75%. The mechanisms of these changes are not known (28).

Our results also support the view that conidia

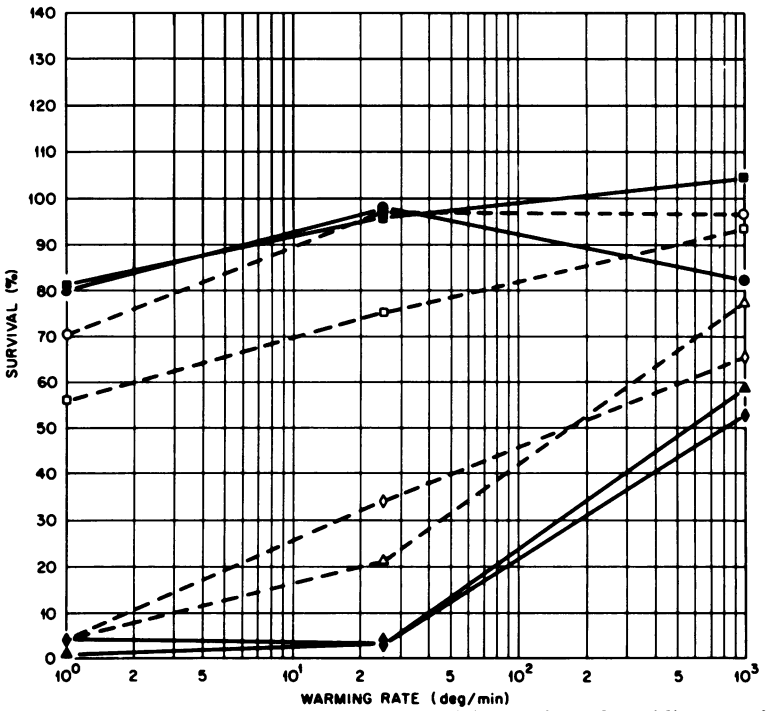

FIG. 4. *Percentage of survival based on colony counts of frozen-thawed conidia as a function of the warming rate of conidia incubated 1 h in VMM* (——) *or* $V^{w/o}$ (-----) *before freezing. The cooling rates were 1°C/min* (● *or* ○), *10°C/min* (■ *or* □), *100°C/min* (▲ *or* △), *and 500°C/min* (◆ *or* ◇).

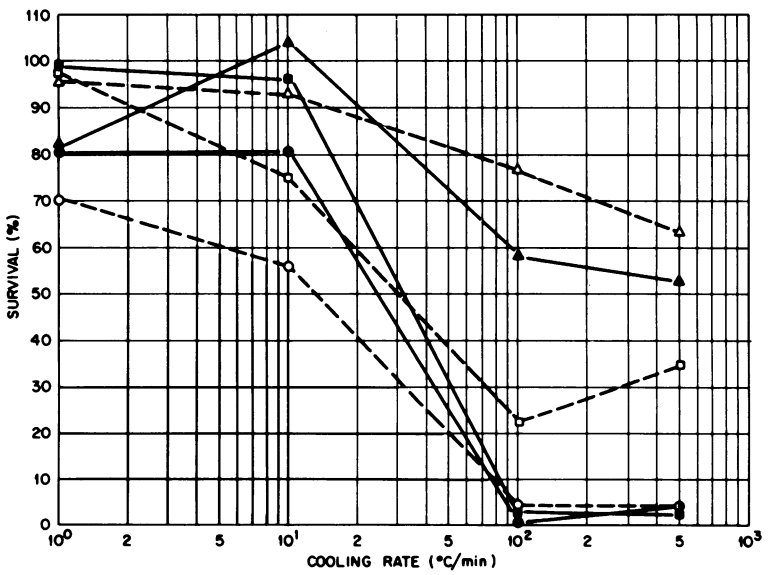

FIG. 5. *Percentage of survival based on colony counts of frozen-thawed conidia as a function of the cooling rate of conidia incubated for 1 h in VMM* (——) *or* $V^{w/o}$ (-----) *before freezing. The warming rates used were 1°C/min* (● *or* ○), *25°C/min* (■ *or* □), *and 1,000°C/min* (▲ *or* △).

incubated in the absence of a carbon source remain metabolically inactive; they neither germinate nor consume measurable quantities of oxygen during the lengths of time examined (Fig. 1 and 2). In contrast, conidia in VMM both germinate and consume measurable quantities of oxygen during identical lengths of time. The lack of metabolic activity caused by the omission

TABLE 2. *Percent germination of frozen-thawed conidia*

Medium before freezing	Incubation time before freezing[a]	Cooling rate[b]	Warming rate[c]	Germination (%) after indicated times of incubation in VMM after freezing in water at:							
				0 h	4 h	5 h	7 h	8 h	12 h	24 h	Δ%G[d]
VMM	1	RC	RW	8.0 ± 1.5[e]	67 .1			85.0	80.1		72.1
		RC	SW	8.0 ± 1.5	14.7			14.0	12.5	23.6	15.6
	3	RC	RW	27.1 ± 1.9	21.5 ± 6.4			25.0 ± 0.1	17.0 ± 9.9	13.0 ± 5.0	0
		RC	SW	27.1 ± 1.9	26.5 ± 3.5			18.0 ± 1.4	17.0 ± 4.2	19.0	0
		SC	RW	27.1 ± 1.9	50.5 ± 10.6			63.0 ± 2.8	73.0		45.9
		SC	SW	27.1 ± 1.9	66.0 ± 1.4			85.5 ± 3.5			58.4
	5	RC	RW	80.3 ± 3.7					80.0		0
$V^{w/o}$	1	RC	RW	3.7 ± 1.5	54.0			85.0	87.1		83.4
		RC	SW	3.7 ± 1.5	12.2			18.0	37.0	38.0 ± 4.0	34.3
	3	RC	RW	3.7 ± 1.5	55.5 ± 3.5	65.2		70.0 ± 4.2	80.0 ± 22	77 ± 21	73.3
		RC	SW	3.7 ± 1.5	11.5 ± 3.5	32.4		37.5 ± 20	57.5	91.3	87.6
		SC	RW	3.7 ± 1.5	53.0 ± 2.8			78.0 ± 7.1			74.3
		SC	SW	3.7 ± 1.5	48.0 ± 2.8			83.0 ± 1.4			79.3
	5	RC	RW	3.7 ± 1.5		64.2	77.2			67.0	93.3
		RC	SW	3.7 ± 1.5		48.1	56.0			98.0	94.3

[a] Time conidia were incubated in VMM or $V^{w/o}$ before centrifugation and resuspension in distilled water for freezing. The conidia had been hydrated in water 1 h before suspension in above media.

[b] RC, Rapid cool (500°C/min); SC, slow cool (1°C/min).

[c] RW, Rapid warm (1,000°C/min); SW, slow warm (25°C/min).

[d] Δ%G, Change in percent of germination after freezing; the value is equal to the percentage of germination recorded at either 8, 12, or 24 h (highest value) minus the percentage of germination at 0 h of incubation after freezing. The 0-h values are the percentages of germination reached in VMM or $V^{w/0}$ before freezing. Therefore, the 0-h values for given time points in VMM are identical, and, since essentially no germination occurred with time in $V^{w/o}$, all 0-h values are identical.

[e] Standard deviation.

TABLE 3. *Comparison between percentage of survival based on plate counts and the percentage of germination of frozen-thawed conidia*

Medium before freezing	Incubation time before freezing (h)	Cooling rate[a]	Warming rate[b]	Survival plate counts (%)	Germination at 12 h (%)	TG_{50} (h)[c]
VMM	—[d]	Control	—	100	98[e]	3.9
	1	RC	RW	53.1	80.1	2.2
		RC	SW	3.1	12.5	NM[f]
	3	RC	RW	6.6	17.0	NM
		RC	SW	0.3	17.0	NM
		SC	RW	51.3	73.0	1.5
		SC	SW	69.0	85.5[g]	1.6
	5	RC	RW	0	80	NM
		RC	SW	0	80	NM
$V^{w/o}$	1	RC	RW	62.5	87.1	3.2
		RC	SW	34.9	37.0	7.8
	3	RC	RW	111.2	80.0	2.8
		RC	SW	46.9	57.5	4.9
		SC	RW	86.0	78.0[g]	1.1
		SC	SW	87.0	83.0[g]	2.8
	5	RC	RW	121.2	97[e]	2.8
		RC	SW	79.6	98[e]	3.6
Water	1	RC	RW	67.9	89	4.0
		RC	SW	39.7	65.0	6.1

[a] RC, Rapid cool; SC, slow cool; 500 and 1°C, respectively.

[b] RW, Rapid warm; SW, slow warm; 1,000 and 25°C, respectively.

[c] TG_{50}, Time to reach 50% "ultimate" percentage of germination.

[d] —, Control was not frozen.

[e] Percentage of germination at 24 h.

[f] NM, Not measurable. When incubation produced no change in the percentage of germination after freezing, the TG_{50} was reported as not measurable.

[g] Percentage of germination at 8 h.

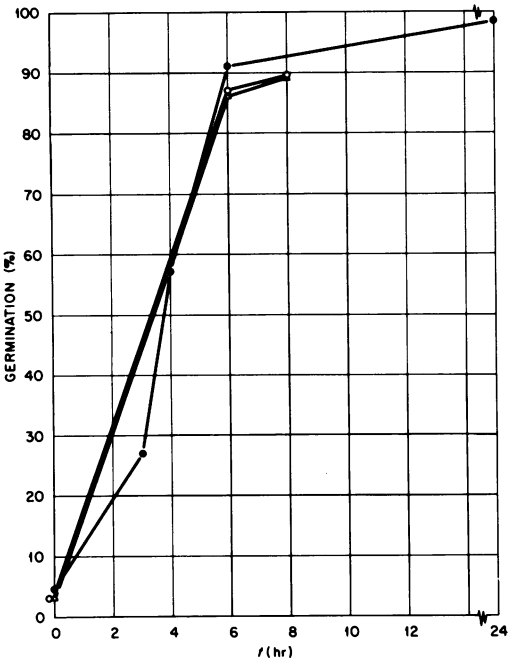

FIG. 6. *Effects of freezing and thawing on the kinetics of germination of air-dry conidia. Symbols:* ●, *control;* △, *rapid cool-rapid warm; and* ○, *rapid cool-slow warm.*

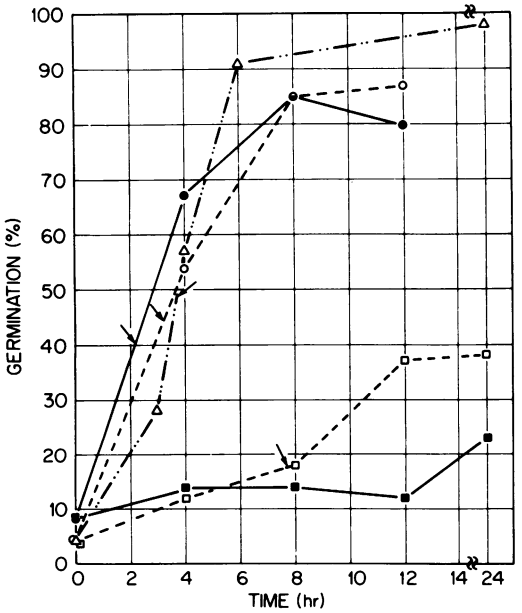

FIG. 7. *Comparison between the kinetics of germination of untreated conidia* (— — —) *and the kinetics of germination of conidia that were preincubated for 1 h in VMM* (——) *or* $V^{w/o}$ (·····), *cooled at 500°C/min, and warmed at 25°C/min* (■, □) *or 1,000°C/min* (●, ○). *The arrows indicate the* TG_{50}, *the time at which germination reached 50% of the maximum attained at 12 or 24 h.*

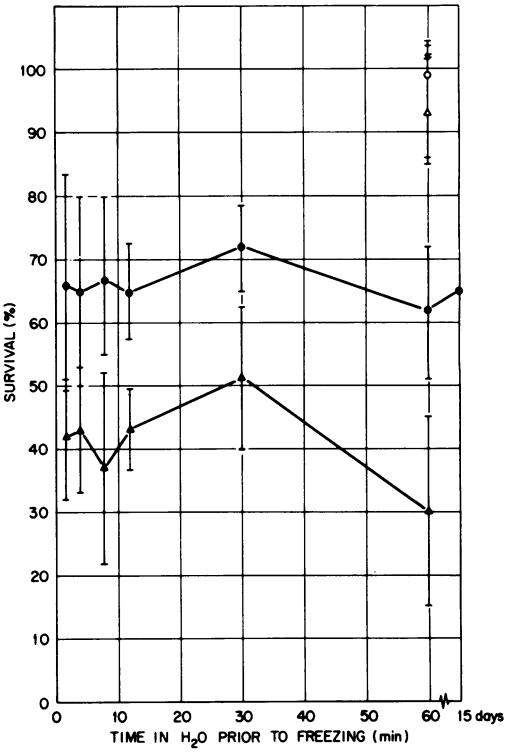

FIG. 8. *Effect of time in water before freezing on the ability of conidia to withstand rapid freezing. The warming rate used was either 1,000* (●) *or 25°C/min* (▲).

of sucrose from the medium has no effect on the viability of the cells. When sucrose was later added to conidia initially suspended in $V^{w/o}$, they responded metabolically in the same manner as those that had been initially suspended in VMM. Therefore, it was possible to compare the sensitivity of cells that apparently differed only in metabolic activity to various regimens of cooling and warming.

In spite of the fact that air-dry conidia, conidia in water, and conidia in $V^{w/o}$ were all metabolically inactive, they differed in their responses to freezing. Air-dry conidia were extremely resistant to freezing regardless of the cooling and warming rates used. When conidia were placed in water, they became hydrated in a fraction of a minute after they came in contact with water; they also became susceptible to freezing damage, with survival dependent on the cooling and warming rates used (Table 1; Fig. 8). However, their susceptibility to freezing was independent of the length of time (between 2 min and 15 days) they were held in water before freezing.

Conidia in $V^{w/o}$ are similar to conidia in water with respect to hydration and lack of metabolic

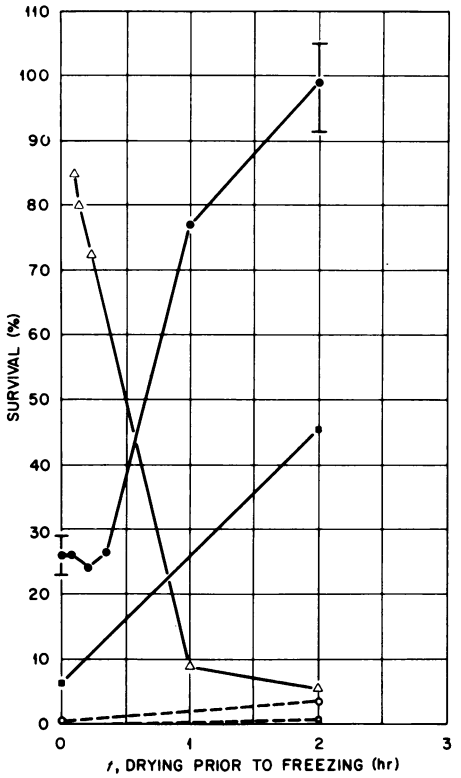

FIG. 9. *Effect of dehydration on the ability of previously hydrated conidia to withstand freezing and thawing. The conidia were preincubated in water* (——) *or VMM* (-----), *dried for the length of time shown on the abscissa, and then cooled at 500°C/min and warmed at either 25* (● *or* ○) *or 1°C/min* (■ *or* □). △, *Rate at which water was removed from the suspension during the drying phase.*

activity. Those cooled at 10 and 100°C/min also responded similarly to conidia in water in that survival after freezing was relatively unaffected by length of time in $V^{w/o}$ before freezing (Table 1). However, those cooled at lower or higher rates showed different responses. At the lower cooling rates, survivals decreased with time in $V^{w/o}$, but, at the higher cooling rates, survivals increased with time (Table 1). We are at present unable to explain these results.

The fourth physiological state investigated was that of conidia incubated in complete growth medium (VMM). In that medium, they became both hydrated and metabolically active. There was a marked decrease in the freezing resistance of the conidia with time in VMM before freezing and, after 5 h in VMM, most of the conidia were killed by freezing regardless of the cooling and warming rate used.

The finding that metabolically active, germinating conidia were more sensitive to freezing than inactive ones is not surprising. Germinating conidia are in the process of sending or have sent forth new cell wall material in the form of a hyphal tube. This tube could be a weak or altered structural site in the cell wall similar to the bud scar in yeast. The portion of the cell wall containing the bud scar has been shown to be structurally weaker than the rest of the wall (8); possibly the site of hyphal protrusion in *Neurospora* shares similar structural weaknesses.

Air-dry conidia were resistant to freezing regardless of the cooling and warming rates used (Fig. 3). This was not so for hydrated, metabolically inactive conidia (i.e., in $V^{w/o}$) or for hydrated, metabolically active conidia (i.e., in VMM). In these latter cases, a destructive event occurred at a cooling rate between 10 and 100°C/min which markedly reduced survival, especially when the higher rates of cooling were followed with slow warming (Fig. 5). This pattern of cellular response is consistent with the destructive event being the formation of intracellular ice. A similar pattern is observed with the yeast *Saccharomyces cerevisiae* (21): viability declines abruptly when the cooling rate is raised from 10 to 50°C/min, and freeze-cleaving studies (2, 3) provide direct evidence that intracellular freezing occurs in yeast over this range of cooling rates.

In general, when conidia were cooled slowly (1 to 10°C/min), there was less dependence on the warming rate than when they were cooled rapidly (100 or 500°C/min, Fig. 4). In fact, slowly cooled conidia remained essentially unaffected by any of the three warming rates used, whereas rapidly cooled conidia showed a profound dependence on the warming rate (Fig. 4). The same relationship between cooling rate and dependence or independence of survival on the warming rate was found regardless of the medium used for preincubation. The dependence of the survival of rapidly cooled cells on the warming rate has been observed with other cells and has been accounted for on the basis of the "two-factor hypothesis" of freezing damage (19, 22, 24; K. R. Diller and E. G. Cravalho, Cryobiology **10**:515, 1973; and M. Ushiyama, E. G. Cravalho, K. R. Diller, and C. E. Huggins, Cryobiology **10**:517, 1973). In yeast, the dependence on the warming rate stems from the fact that the recrystallization of intracellular ice is minimized if rapidly cooled cells are warmed rapidly, and it is maximized if they are warmed slowly. We believe that the results with *N. crassa* conidia are explicable on the same basis.

Survival based on colony counts reflects the ability of a conidium to both germinate and grow sufficiently to form a macroscopic colony. However, with a conidium, it is possible to base viability estimates on the ability of an individual

conidium to germinate. Essentially all conidia that were going to germinate had done so by 12 h. Determining the extent of germination after incubation for 24 h was technically difficult because of extensive hyphal growth; it therefore was generally not measured. However, when such measurements were made, they were similar to the values attained at the 12-h time points (Table 2). When the 12-h percentage of germination of frozen versus nonfrozen conidia was compared to the percentage of survival based on colony counts (Table 3), the results were in good agreement with one exception, i.e., the apparent discrepancy for conidia preincubated in VMM for 5 h before freezing, in which case the germination was 80% and the percentage of survival, based on colony counts, was zero (Table 3). The high germination is artifactual. It reflects the fact that 80% germination in the samples had occurred during the 5-h preincubation in VMM before freezing. The fact that there was no further change in the percentage of germination during incubation after freezing means that none of the ungerminated conidia survived the freezing.

In summary, then, a conidium that possessed the ability to germinate after freezing also had the ability to form a macroscopic colony.

It might be expected that the lower the percentage of germination after freezing, the longer would be the time for germination to reach 50% of the ultimate value (TG_{50}). That was indeed the case (Table 3). In nearly every case in which treatment was harsh (rapid cool-slow warm), there was a significant increase in the TG_{50} over comparatively gentle treatments (rapid cool-rapid warm, or slowly cooled, conidia) (Table 3). The mean TG_{50} for gently treated conidia was 2.62 ± 1.0 h (range, 1.1 to 4.0), whereas harshly treated conidia had a mean of 5.6 ± 1.8 h (range, 3.6 to 7.8). It is not clear whether the decreased rate of germination of conidia subjected to harsh freezing treatments represents time needed for cellular repair or the decreased efficiency of an injured population. A number of studies in bacteria show that at least a portion of freezing injury is reversible (and therefore perhaps repairable) if proper metabolites are furnished (14, 29, 30).

The TG_{50} determinations are also consistent with the finding already discussed: that conidia preincubated in $V^{w/o}$ before rapid cooling showed marked increases in survival with increases in the time of preincubation (Table 3). Since the conidia are somehow being afforded protection by incubation in $V^{w/o}$ before freezing, one might also expect the TG_{50} to decrease with increased time in $V^{w/o}$. The results (Table 3) show that this occurred. After 1 h in $V^{w/o}$ before freezing (rapid cool-rapid warm), the TG_{50} was 3.2 h, whereas after 5 h it had decreased to 2.8 h. Conidia cooled the same way but warmed slowly showed a decrease in the TG_{50} from 7.8 to 3.6 h over the same period of time (Table 3).

Since all three measures of viability (percentage of germination, percentage of conidia forming colonies, and reduction of TG_{50}) showed that the resistance of conidia to freezing increased with increasing time in $V^{w/o}$, we conclude that the phenomenon is a genuine one, although currently inexplicable.

The role played by water in sensitivity to freezing seems to be a direct effect of hydration in nonmetabolizing conidia. We have shown that conidia become hydrated very rapidly; J. E. Glasgow (Ph.D. thesis, Rice University, Houston, Tex., 1969) also reported it to be an "instantaneous" process, as have others (28, 31). Air-dry conidia remain at equilibrium with the ambient relative humidity, but, even though the water content of spores can be quite high (52 to 72% for *Erysiphe* sp.), nuclear magnetic resonance studies have shown that they contain no free water to freeze (22, 32). In the present study, the weight percent of water in air-dry *N. crassa* conidia varied from 40 to 60%. Once the conidia were suspended in aqueous media, they showed a marked decrease in resistance to freezing. Since the conidia were not metabolically active, and the only apparent difference between the hydrated and air-dry conidia was the presence of intracellular water, we concluded that the sensitivity to freezing was caused by the water alone.

To further substantiate our conclusion regarding the role of water in this system, hydrated conidia were dried in vacuo (Fig. 9) before freezing. After removal of water, the conidia that survived the dehydration were as resistant to rapid freezing as were air-dry spores. Thus, the role of water in the sensitivity to freezing of metabolically inactive cells was shown to be passive and reversible. However, once conidia become metabolically active, the role of water in their sensitivity to freezing is not as clear. They become extremely sensitive to dehydration per se (Fig. 3); furthermore, those that survive the dehydration exhibit only a slight absolute increase in resistance to subsequent freezing (Fig. 9).

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