STUDIES ON THE EFFECTS OF SUBZERO TEMPERATURES ON THE VIABILITY OF SPORES OF ASPERGILLUS FLAVUS

I. THE EFFECT OF RATE OF WARMING*

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Living cells vary widely in their sensitivity to subzero temperatures, since some are destroyed when cooled to 0°C. or slightly below, whereas others tolerate even those temperatures approaching absolute zero (Becquerel, 1950). This extreme range of susceptibility raises questions not only as to the factors that determine whether a cell lives or dies, but also as to the causes of death of those cells which fail to survive. Answers to both these questions could be of considerable interest to the cell physiologist, for the effects of subzero temperatures are undoubtedly influenced by fundamental cell properties and processes such as the amount and nature of intracellular water, the permeability and osmotic behavior of cell membranes, and the nature and action of protoplasmic colloids and solutes.

Information on the fundamental effects of subzero temperatures could also aid in solving the problems involved in prolonging cell viability by either low temperatures alone or by freeze-drying. The use of freeze-drying to conserve the viability of fungous spores was first reported by Raper and Alexander (1945). They found that dehydrated suspensions contained enough viable spores to produce vigorous cultures several years later. But more recent investigations by Weston, Buell, and Mazur (unpublished data) have shown that as many as 95 to 99 per cent of certain spores, such as those of *Aspergillus flavus*, are killed during the actual treatment. Other studies (Mazur, 1953; Mazur and Weston, 1956) indicated that the death of freeze-dried *A. flavus* spores was not a result of the drying. The present investigation was originally begun, therefore, to see whether the lethal effects of freeze-drying could have been due to the presence of subzero temperatures.

* A preliminary report of the investigation was presented before the Mycological Society and the Microbiological Section of the Botanical Society of America at the annual meeting held at Cornell University, September 8, 1952.
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Spores (conidia) of A. flavus were excellent experimental material. Perhaps the most important reason for this excellence was the fact that they were not injured by being suspended in distilled water; hence, by using distilled water as a suspending vehicle, the spores could be cooled to low temperatures under conditions eliminating the complications of extracellular solutes. It is hoped that the advantages of this simplification will become apparent.

To simplify experimental conditions further, the studies were restricted in the main to determining the effects of the chief physical variables involved in subjecting cells to low temperatures; namely, the rate of cooling, the minimum cooling temperature, and the rate of subsequent warming.

In order to determine the viability of spores cooled to low temperatures, it is necessary, of course, to warm and thaw them. The rate of that warming has been shown to affect the viability of several types of cells in the comparatively few instances in which it has been studied (Smith, 1954; also, see Discussion). It was conceivable that it might affect the survival of A. flavus spores as well. Accordingly, the present study began with an analysis of the effects of various rates of warming. This first paper reports the results of that investigation which showed not only that rate of warming is a factor affecting viability, but also that under certain conditions it is a most important factor. Subsequent papers will describe the effects of the rate of cooling and the temperatures to which the spores are cooled.

The words “cooling,” “warming,” “freezing,” and “thawing” can be ambiguous unless defined. In these papers, freezing, and thawing (or melting) will refer specifically to changes of state from water to ice and from ice to water. Cooling and warming, on the other hand, will be used in a more general sense to indicate changes in temperature regardless of whether or not they promote changes of state. Thus, cooling will refer to a lowering of the temperature; warming, to a raising of the temperature. In accordance with the above definitions, the rate of cooling or warming will refer to the average change in temperature with respect to time, and the rate of thawing or melting will refer to the time required for the actual melting of a given amount of ice after reaching the melting point.

Methods and Materials

Obtaining Spore Suspensions.—The original culture of Aspergillus flavus was isolated from a pupa of Platysamia cecropia, as described elsewhere (Mazur and Weston, 1956). It was preserved under mineral oil by the technique of Buell and Weston (1947) and secondary cultures derived from it. To obtain spores for experimental purposes, inocula from these secondary cultures were subcultured on culture tube slants of standard 2 per cent potato-dextrose agar. The slants were held in an incubator at 24 ± 1.5°C. for 12 to 13 days. The spores from one slant were used for one experiment only; hence, the ages of spores were about the same in each experiment.

The spores were subjected to subzero temperatures while suspended in a liquid
vehicle, usually distilled water. Two methods were used to prepare the suspensions. (a) In the first technique the spores were tapped onto the dry glass surface of the culture tube that faced the agar slant, and poured as a dry powder into a sterile Hopkins centrifuge tube previously filled with 5 ml. of distilled water. The spores were then wetted and evenly suspended by thorough mixing with a sterile camel's-hair brush. This technique had the advantage of producing a suspension the spores of which had not been in contact with any solute; but it had the disadvantage of requiring laborious mixing to produce an even suspension, for minute echinulations on the spore wall tended to trap air and prevent wetting. (b) To eliminate this last difficulty, a second technique was used in later experiments. With this second method, 5 ml. of a sterile 0.1 per cent solution of the wetting agent naccanol-NR (1 gm. naccanol in 100 ml. distilled water) were poured directly into the culture tube which was shaken until a heavy even spore suspension had been produced. The resulting suspension was then poured into a sterile empty Hopkins tube. The spores were not at all harmed by exposure to the naccanol.

Centrifugation of the initial suspensions was the next step in both procedures and served four purposes: (1) It permitted the removal of the naccanol used in the second technique (b). (2) It was a method of washing the spores. (3) It permitted the preparation of a final suspension of any desired solute concentration. And (4) it yielded a suspension with a constant concentration of spores. Centrifugation was effected at 750 g in an International clinical centrifuge (model CL). When naccanol had been used to harvest the spores (b, above), the suspension was centrifuged for 2 minutes and the naccanol decanted. Five ml. of distilled water were added, the spores again evenly dispersed, and the resulting suspension centrifuged an additional 10 minutes. When naccanol had not been used (procedure a, above), the initial suspension of spores in water was merely centrifuged for 10 minutes. All ensuing procedures were the same irrespective of which method (a or b) had been used to prepare the original suspension.

The supernatant distilled water was decanted, and sufficient sterile distilled water re-added to make the final volume of water equal to twenty times the volume of spores packed in the graduated capillary stem of the Hopkins tube. The volume of packed spores varied from 0.01 to 0.05 ml. The spores were once again evenly dispersed, and 0.01 ml. of the resulting suspension transferred to a small test tube (15 × 75 mm.) containing 1 ml. of the vehicle which was to surround the spores during treatment. The vehicles used in the present experiments were glass-double-distilled water, normal horse serum, 0.16 molal sodium chloride, and 0.29 molal sucrose. Counts with a hemocytometer slide showed that these final suspensions contained about 2 × 10⁶ spores per ml.

To expose the spores to low temperatures, 0.1 ml. volumes of the final suspension were carefully pipetted into small sterile Pyrex “cooling” tubes, 6.8 to 7.2 mm. in outside diameter and 85 mm. long. The volume of suspension was kept small both to minimize variation in the rates at which different portions of the suspension cooled or warmed, and also to permit rapid warming rates to be obtained. The open ends of the cooling tubes were plugged with cotton, and inserted into 6 inch lengths of rubber tubing which facilitated handling.

Low Temperature Treatment.—Suspensions were cooled to −70 to −75°C. by quickly immersing the bottom 2 inches of the cooling tubes into a Dewar flask or
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insulated beaker containing a mixture of methyl cellosolve and dry ice. The average rate of cooling from 0 to -60°C. was about 250°C. per minute. This rate was determined by inserting a 30 gauge copper-constantan thermocouple into a cooling tube containing 0.1 ml. of distilled water. The tube was placed in the cellosolve bath, and the change in temperature measured with a Leeds-Northrup K-2 potentiometer. Time was measured with a stop-watch.

The suspensions were held at -70 to -75°C. for 5 to 15 minutes. They were then warmed and thawed. Three standard rates of warming were used in the majority of the experiments. They will be referred to as slow, medium, and rapid. These rates and the methods of achieving them were as follows:

(a) Slow warming was achieved by placing the tubes in a liter beaker containing 300 ml. of methyl cellosolve (or in a 600 ml. beaker with 210 ml. of cellosolve) previously cooled to -65 to -70°C. The cellosolve was then allowed to warm by contact with room temperature air (23 to 28°C.). The change in temperature of the cellosolve was measured with a calibrated thermometer or a thermocouple. Tests showed that between -70 and 0°C. the temperature of the spore suspensions and the cellosolve bath agreed within 1°C. at any given time. The average rate of warming from -70 to 0°C. was 0.7 to 0.9°C. per minute. In most experiments, the tubes were allowed to remain in the cellosolve bath until the frozen suspensions had completely thawed. The thawing (which should be distinguished from the subzero warming from -70 to 0°C.) required about 15 minutes.

(b) Medium warming was achieved by removing the cooling tubes from the dry ice-methyl cellosolve bath and suspending them in direct contact with room temperature air. The resulting rate of subzero warming from -70 to 0°C. was 25 to 30°C. per minute. Subsequent thawing required about 3.5 minutes.

(c) Rapid warming was effected by removing the tubes from the cellosolve bath and plunging them into a liter beaker containing water at 35°C. The average rate of subzero warming from -70 to 0°C. was 700°C. per minute. The subsequent thawing of the ice required about 30 seconds. The rate was measured by noting the time interval between the instant the tubes were placed in the water bath and the instant that melting of the frozen suspensions became first apparent. In most cases, the spores were suspended in distilled water; hence, melting began at 0°C. The first signs of melting were clear cut.

Rates of warming other than the above three standards were used in two experiments. These non-standard rates will be described later.

Controls.—In all experiments at least one control was maintained. It consisted of the final spore suspension from which the cooling tubes had been filled. This control suspension was not cooled but was held at room temperature throughout the experiment. Other types of controls were sometimes present; they will be described under the pertinent experiment.

Determining Spore Viability.—The percentage of viable spores was determined immediately after the suspensions had been warmed and thawed. A spore was considered viable if it sent forth a germ tube during incubation for 11 to 17 hours on an appropriate agar surface. A non-germinating spore was considered dead.

As a first step in assaying for viability, the spore suspension in each cooling tube or in the control tube was mixed thoroughly and 0.03 ml. of the suspension pipetted
upon the surface of a 22 mm. diameter agar disk mounted on a standard microscope slide. These disks were prepared from Difco corn meal agar with sufficient Difco Bacto agar added to produce a medium containing 2 per cent agar. The same lots of agar were used in all the experiments reported in the papers constituting the present series. The slides supporting the disks were then placed in a moist chamber consisting of a Petri dish 6 inches in diameter containing wet sand. Germination of the spores was induced by placing the moist chambers in a 24°C. incubator for 11 to 17 hours. Longer incubation did not produce a higher percentage germination.

Counts of the proportions of germinated and non-germinated spores were made with a pair of Veeder counters while observing the cells at a magnification of 600. Spores found in clumps were not counted unless each individual spore could be clearly resolved from its neighbor. Between 400 and 1700 spores were counted per agar disk. Counts were made along a randomly selected diameter of the disk from edge to edge. If a single diameter did not contain a sufficient number of spores, additional counts were made along a diameter at right angles to the first.

Percentage germination was simply \[ \frac{\text{No. germinated spores}}{\text{Total no. counted}} \times 100 \]

It is conceivable that the percentage of germination could be a false measure of viability if the treatment actually disintegrated spores, but two observations indicated that disintegration did not occur. In the first place, few if any fragments of spores were ever observed. In the second place, since the number of spores in the suspensions was held fairly constant by the techniques described above, any marked destruction should have resulted in many fewer being visible on the agar surface; however, the spore density was always about the same whether the spores had been subjected to the harshest conditions of cooling and warming, or as in the controls, had not been cooled at all.

RESULTS

Over-All Effects of Rate of Warming.—Two experiments were performed to obtain preliminary information on the effect of the rate of warming of frozen suspensions. In each, duplicate sets of suspensions of spores in distilled water were cooled to \(-75^\circ\text{C.}\), held for 5 minutes, and then warmed at the standard slow, medium, or rapid rate until the ice surrounding the spores had thawed. The resulting percentage recoveries are given in Table I. An examination of these data shows that the lower the rate of warming, the lower the percentage germination.

It should be noted, however, that slow subzero warming from \(-75\) to \(0^\circ\text{C.}\) also produced slow melting of the ice in which the spores were suspended. Similarly, medium and rapid subzero warming produced medium and rapid melting. Because the rate of melting of the vehicle was correlated with the rate of subzero warming, it was impossible to tell which of the two factors affected spore viability.

Fortunately, the use of distilled water as a vehicle permitted an experiment
to separate the effects of one factor from those of the other. Since the melting point of distilled water is 0°C., it was possible to warm frozen spore suspensions slowly to within a few degrees of zero without melting the vehicle. The vehicle in turn could then be thawed at a slow, medium, or rapid rate. In two such experiments, suspensions of spores in double-distilled water were cooled to and held at −73°C. for 5 minutes. They were then allowed to warm slowly in a methyl cellosolve bath at 0.8 to 0.9°C. per minute to −4.3 and −3.3°C. in the first and second experiments, respectively.

At this point, the ice surrounding the spores was thawed at a rapid, medium, or slow rate. Rapid thawing required 20 to 30 seconds and was achieved by plunging duplicate tubes of spore suspensions into water at 35°C. Thawing at a medium rate was effected by removing duplicate tubes from the cellosolve bath and holding the tubes in air (24°C.). The ice melted in 3.5 to 4.2 minutes. Finally, the ice in a third set of duplicate tubes was allowed to melt slowly, in 10 to 12 minutes, by leaving the tubes in the slowly warming methyl cellosolve bath.

From the resulting percentages of germination shown in Table II, it is evident that slow subzero warming to −4°C. produced low recovery irrespective of the rate at which the vehicle was thawed. Even when the vehicle was thawed rapidly, only 17 per cent of the spores were viable. In contrast, when both subzero warming and thawing are rapid, recovery is much higher (68 per cent, Table I).

Although all three rates of thawing produced low recovery, different rates did exert an apparent slight effect, for viability was slightly but significantly higher with rapid thawing of the vehicle (16.8 per cent) than with slow (9.3 per

<table>
<thead>
<tr>
<th>Rate of warming of frozen suspension</th>
<th>Percentage of germination*</th>
<th>Mean germination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
</tr>
<tr>
<td>Slow</td>
<td>7.2</td>
<td>9.3</td>
</tr>
<tr>
<td>Medium</td>
<td>25.6</td>
<td>26.6</td>
</tr>
<tr>
<td>Rapid</td>
<td>63.7</td>
<td>66.5</td>
</tr>
<tr>
<td>Control: Suspensions held unfrozen at room temperature</td>
<td>97.3</td>
<td>96.6</td>
</tr>
</tbody>
</table>

* Each percentage based on a sample from one cooling tube. Between 450 and 1500 spores counted per sample.
cent). In the former case, however, not only was thawing itself rapid, but sub-
zero warming from −4 to 0°C. was rapid as well. On the other hand, in the
latter case both thawing and subzero warming from −4 to 0°C. were slow.
It may be, therefore, that the apparent effect of rate of thawing of the sur-
rounding ice was, in fact, due to the effect of rate of subzero warming between
−4 and 0°C.

The range between −4 and 0°C. was investigated by performing an exper-
iment in which frozen suspensions of spores in distilled water were warmed
slowly (a) to −2.3°C., (b) to −0.8°C., (c) until about one-half the vehicle had
thawed, and (d) until the vehicle had completely thawed. The resulting per-

TABLE II
Effect of the Rate of Thawing of the Suspending Vehicle on the Percentage Germination
of A. flavus Spores

Spores suspended in distilled water without using naccanol (see Methods section), cooled
to −73°C. for 5 minutes, and warmed slowly to −4°C. before thawing vehicle at indicated
rate.

<table>
<thead>
<tr>
<th>Rate of thawing of the vehicle</th>
<th>Percentage of germination*</th>
<th>Mean germination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
</tr>
<tr>
<td>Slow</td>
<td>9.2</td>
<td>5.9</td>
</tr>
<tr>
<td>Medium</td>
<td>10.3</td>
<td>9.8</td>
</tr>
<tr>
<td>Rapid</td>
<td>12.4</td>
<td>16.2</td>
</tr>
<tr>
<td>Control: Suspensions held unfrozen at room temperature</td>
<td>83.6</td>
<td>91.6</td>
</tr>
</tbody>
</table>

* Each percentage based on a sample from one cooling tube. At least 450 spores counted per sample.

percentages of germination are shown in Table III. The overwhelming proportion
of the spores must have been killed by the slow subzero warming, for by the
time the temperature had risen to −0.8°C., only 1.6 per cent were viable.
Subsequent slow thawing of the ice surrounding the spores produced only a
slight further reduction to 0.6 per cent.

Hence, it is evident that the lethal effects of slow warming occurred below
0°C., and, therefore, were not due to the slow melting of the ice surrounding
the spores, for that ice was essentially pure, and should not have started to
melt until the temperature reached zero. The purity of the ice is attested to by
the fact that the spores were not only surrounded by double-distilled water
during treatment, but were initially suspended, centrifuged, and resuspended
in distilled water as well (no naccanol was used in the experiments under
discussion).

Since the rate of thawing of the vehicle had practically no effect, no further
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A. flavus Spores When Frozen Suspensions Were Slowly Warmed and Thawed to Indicated Extent and Any Residual Thawing Completed Rapidly

<table>
<thead>
<tr>
<th>Extent of slow warming and thawing</th>
<th>Percentage germination</th>
<th>Mean germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>To $-2.3^\circ\text{C}$</td>
<td>3.45</td>
<td>2.78</td>
</tr>
<tr>
<td>To $-0.5^\circ\text{C}$</td>
<td>1.87</td>
<td>1.62</td>
</tr>
<tr>
<td>Until suspension half-thawed</td>
<td>1.10</td>
<td>0.88</td>
</tr>
<tr>
<td>Until suspension fully thawed</td>
<td>0.53</td>
<td>0.73</td>
</tr>
<tr>
<td>Control: Suspensions held unfrozen at room temperature</td>
<td>95.5</td>
<td>94.6</td>
</tr>
</tbody>
</table>

* Temperature higher than $-70^\circ\text{C}$. through error; however, experiments have shown that temperatures between $-60$ and $-70^\circ\text{C}$. exert the same effect (Mazur, 1953).

† Each percentage based on a sample from one cooling tube. More than 900 spores counted per sample.

The resulting percentages of germination from the two experiments have been plotted in Fig. 1, a graph in which both coordinates are logarithmic. Data from Table I have also been included in this figure. Clearly, survival was a function of the rate of subzero warming since the percentage of germination increased with increasing rapidity of warming. Moreover, the fact that straight lines provide an excellent fit for the plotted points indicates that the logarithm of the percentage germination, \( G \), was a linear function of the logarithm of the rate of subzero warming, \( R \); or \( G = a - R^b \), in which \( a \) is the Y-axis intercept and \( b \) the slope of the line. The linearity of the relationship is supported statistically by regression analysis. While the two upper curves have almost
identical slopes and Y-axis intercepts, the lowest line differs in both respects. These differences may be associated with the fact that the percentage germination of the untreated control spores was lower in that experiment (84 per cent) than in the two experiments represented by the upper two lines (95 per cent). The differences, however, do not negate the fact that there is a fundamental relationship between the percentage germination and the rate of subzero warming.

TABLE IV

<table>
<thead>
<tr>
<th>Technique of warming</th>
<th>Average rate of warming from -70°C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Tubes warmed in indicated amount of methyl cellulose:</td>
<td>°C/min.</td>
</tr>
<tr>
<td>650 ml. in 2 liter beaker*</td>
<td>0.12</td>
</tr>
<tr>
<td>210 ml. in 600 ml. beaker (standard slow warm)</td>
<td>0.90</td>
</tr>
<tr>
<td>120 ml. in 250 ml. beaker</td>
<td>1.3</td>
</tr>
<tr>
<td>3 ml. in 16 X 150 mm. test tube</td>
<td>5.4</td>
</tr>
<tr>
<td>B. Tubes warmed by contact with air at 25°C.:</td>
<td></td>
</tr>
<tr>
<td>Suspended in air (standard medium warm)</td>
<td>27</td>
</tr>
<tr>
<td>Placed in holes drilled in wood block</td>
<td>60</td>
</tr>
<tr>
<td>C. Tubes immersed in water bath at:</td>
<td></td>
</tr>
<tr>
<td>2.5°C</td>
<td>140</td>
</tr>
<tr>
<td>19°C</td>
<td>530</td>
</tr>
<tr>
<td>35°C. (standard rapid warm)</td>
<td>700</td>
</tr>
<tr>
<td>73°C.‡</td>
<td>1000</td>
</tr>
</tbody>
</table>

* Beaker insulated with glass wool.
‡ The tubes containing the spore suspensions were held in the bath only long enough to melt the suspensions. They were then placed in a 35°C. water bath for a few seconds.

Relationship between the Harmful Effects of Slow Warming and Temperature.---Since more than 60 per cent of the spores were viable after being cooled to -70°C. and warmed rapidly, at least these 60 per cent must have been alive just prior to warming. Yet when warming was slow, 10 per cent or fewer were viable by the time the temperature reached 0°C. These findings raise interesting questions as to whether the lethality of slow warming was exerted equally at all temperatures between -70 and 0°C. or was exerted only within a narrow span of temperatures.

These questions were studied in two experiments in which frozen spore suspensions were warmed slowly to various subzero temperatures and then warmed and thawed rapidly. In the first experiment, suspensions of spores in distilled water were cooled to -75°C. and held at that temperature for 15
minutes. One duplicate set of suspensions underwent no slow warming, but was warmed rapidly by the usual immersion in water at 35°C. The other sets were allowed to begin warming slowly at 0.6 to 0.8°C. per minute in a methyl cellosolve bath. As the temperature of the bath reached --54, --43, --36, --27, --15, and --5°C., duplicate tubes of suspensions were removed from the cellosolve and warmed and thawed rapidly by immersion in water at 35°C. The suspensions in a final duplicate set of tubes remained in the slowly warming cellosolve bath until the suspensions had thawed and warmed to +5°C. The

![Graph](https://via.placeholder.com/150)

**Fig. 1.** The percentage of germination of *A. flavus* spores as a function of the rate of subzero warming from -70 to 0°C. (Both coordinates are logarithmic.) The dotted line represents a plot of the data from Table I. Each point on the other two lines represents the mean of three samples, with 450 to 1000 spores being counted per sample.

second experiment was similar, differing only in the initial cooling temperature (-70°C.) and the temperatures at which slow warming was terminated (-50, -40, -30, -20, -10, -0.1°C.).

The rates of subzero warming to the various temperatures were all similar (0.6 to 0.8°C. per minute); hence, any differences in survival among the treatments could not be attributed to variations in the rate of slow warming, but should be ascribable to differences in the temperature at which slow warming was terminated. The relationship between the percentage of germination and the temperature to which slow warming was carried is shown in Fig. 2.

An examination of the two curves shows that in both cases slow warming was more harmful at higher temperatures than at lower ones. In Experiment 1, slow warming produced little injury until the temperature reached -27°C.; but
between −27 and 5°C., viability dropped more than sixfold from 63 per cent to 10.3 per cent, a decline of 1.6 per cent per degree. In Experiment 2, viability decreased slowly between −70 and −20°C., dropping 1.6-fold or 0.7 per cent per degree; but, in contrast, between −20 and −0.1°C., recovery dropped fifteenfold from 54 per cent to 3.5 per cent, or 2.5 per cent per degree. Although there were differences between the results in the two experiments, it is clear, nevertheless, that slow warming was not equally harmful at all temperatures. To the contrary, the lethal effects were especially pronounced at temperatures between about −27 or −20 and 0°C.

![Diagram](image)

**Fig. 2.** The viability of *A. fumus* spores following slow warming from −70°C. to indicated temperatures with any residual warming and thawing carried out rapidly. Each point represents the mean of two samples. From 350 to 1200 spores were counted per sample.

**Effect of Length of Exposure to Subzero Temperatures.**—Warming involves two factors: (1) a change in temperature, and (2) the time required for the change. This time obviously would increase as the rate of warming decreased; hence, slowly warmed spores not only underwent a slow change in temperature, they also were exposed to subzero temperatures for a relatively long length of time. Conceivably, either the slow change or the long exposure could have been responsible for the lethal effects.

An experiment was performed to study the effect of long exposure by itself. The technique consisted of warming frozen suspensions of spores as rapidly as possible to various temperatures between −70 and 0°C., maintaining them at these temperatures for 1 hour, and then again warming them as rapidly as possible until they were thawed. By carrying out all warming rapidly, the
effects of slow changes in temperature would be minimized, and any decreases in viability might then reflect the effects of the 1 hour exposure.

In the experiment, suspensions of spores in distilled water were initially cooled to \(-72^\circ\text{C}\) and held for 5 minutes. Duplicate tubes of suspensions were thereupon transferred to beakers containing methyl cellosolve at \(-48\), \(-32\), \(-22^\circ\), or to ice-salt baths at \(-8^\circ\) and \(-4.5^\circ\text{C}\). A final duplicate set of tubes remained at \(-72^\circ\text{C}\). The baths were held within \(\pm 1^\circ\text{C}\) of these temperatures for 1 hour, at the end of which time, the suspensions were warmed rapidly by immersing the tubes in water at \(35^\circ\text{C}\). The percentages of germination were determined and are plotted in Fig. 3.

![Graph](attachment:image)

**Fig. 3.** The viability of *A. flavus* spores after a 1 hour exposure at indicated temperatures. Suspensions of spores in water were warmed rapidly from \(-72^\circ\text{C}\) to each exposure temperature and subsequently warmed and thawed rapidly. Each point represents the mean of two samples with 300 to 750 spores being counted per sample.

It should be noted that the most harmful effects of the 1 hour exposure were found between \(-22\) and \(-4.5^\circ\text{C}\), a range of temperatures similar to the one over which slow warming is most harmful (Fig. 2). The similarity suggested that the lethality of slow warming might be partially due to long exposure to temperatures above \(-20^\circ\text{C}\). The results in Fig. 3 indicate, however, that such exposure will not account for more than a portion of the harmful effects of slow warming. In the first place, standard slow warming is more harmful (10 per cent recovery or less) than was the exposure of 1 hour at even the most injurious temperature of \(-8^\circ\text{C}\) (31 per cent recovery); moreover, it is more harmful in spite of the fact that slowly warmed spores are exposed to the lethal temperatures above \(-20^\circ\text{C}\) for only a half-hour. In the second place, slow warming becomes increasingly harmful as the temperature approaches...
0°C. (Fig. 2). In the present experiment, in contrast, an exposure of 1 hour at -4.5°C. appeared slightly less harmful than the hour exposure at the lower temperature, -8°C.

Although the concept of long exposure to subzero temperatures does not seem to explain the lethal effects of slow warming, it is possible that slowness of warming can account for the apparent harmful effects of the 1-hour exposures. As described above, the frozen suspensions were warmed to the various exposure temperatures by immersing them in baths held at those temperatures. Although this technique produced the most rapid rates of warming attainable, the rate of warming must have become slower and slower as the temperature of the suspensions approached that of the baths. In the case of the -8 and -4.5°C. baths, warming must have become markedly slower in the very temperature zone in which slow warming is especially harmful. On the other hand, in the case of the baths held at -22°C. or lower, the progressive slowing of the warming would have taken place primarily at temperatures below those at which slow warming is especially harmful.

If length of exposure to temperatures between -20 and 0°C. actually affects spore viability, one might expect that the longer the exposure time, the lower the viability. An experiment was performed to test this possibility. Suspensions of spores in distilled water were initially cooled to and held at -72°C. for 5 minutes. They were then transferred to a -15°C. cellosolve bath and held at -15°C. for 5, 30, 60, or 120 minutes before being warmed and thawed rapidly in a 35°C. water bath. The percentage recoveries are shown in Table V.

The time at -15°C. apparently affected viability slightly, for analysis of

### Table V

<table>
<thead>
<tr>
<th>Time at -15°C.</th>
<th>Percentage germination*</th>
<th>Mean germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>32.3</td>
<td>39.7</td>
</tr>
<tr>
<td>30</td>
<td>29.4</td>
<td>32.9</td>
</tr>
<tr>
<td>60</td>
<td>17.8</td>
<td>23.2</td>
</tr>
<tr>
<td>120</td>
<td>22.4</td>
<td>21.6</td>
</tr>
<tr>
<td>Control: Suspensions held unfrozen at room temperature</td>
<td>92.7</td>
<td>96.7</td>
</tr>
</tbody>
</table>

* Each percentage based on a sample from one cooling tube. Between 400 and 1100 spores counted per sample.
EFFECTS OF SUBZERO TEMPERATURES ON SPORES

variance showed that the decrease in recovery from 36 per cent after 5 minutes to 20 per cent after 2 hours was significant. Nevertheless, time appeared to be only a minor factor in survival, since most of the loss in viability occurred in the first 5 minutes. The 5 minute exposure period itself could have been responsible for this loss, but it is even more likely that the initial low recovery was the result of the unavoidable slow warming that must have occurred as the temperature of the suspensions approached −15°C. Irrespective, however, of which factor produced the initial loss, it should be noted that even after 2 hours at −15°C, recovery was still higher than that following standard slow warming from −70°C to 0°C.

TABLE VI

Percentage Germination of A. flavus Spores Suspended in Indicated Vehicle, Cooled to −75°C. for 15 Minutes, and Warmed at Indicated Rates

Spores were harvested without using naccanol (see Methods section)

<table>
<thead>
<tr>
<th>Rate of warming</th>
<th>Mean percentage* germination in</th>
<th>Horse serum</th>
<th>0.16 molal NaCl</th>
<th>0.29 molal sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>3.8 ± 0.4†</td>
<td>8.8 ± 0.6</td>
<td>12.8 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>69.5 ± 2.4</td>
<td>45.0 ± 2.9</td>
<td>29.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Rapid</td>
<td>88.6 ± 0.2</td>
<td>78.3 ± 0.5</td>
<td>71.4 ± 9.1</td>
<td></td>
</tr>
<tr>
<td>Control A: Spores in unfrozen vehicle at room temperature</td>
<td>90.1 ± 0.4</td>
<td>89.0 ± 2.8</td>
<td>90.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Control B: Spores in unfrozen water at room temperature</td>
<td>88.9 ± 0.8</td>
<td>92.5 ± 0.4</td>
<td>91.8 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

* Means based on counts of one sample from each of two cooling tubes. Between 500 and 1700 spores counted per sample.
† Error term is the standard error (standard deviation of the mean, s). In summary, then, the results of this and the immediately preceding experiment indicate that A. flavus spores were more harmed by being warmed slowly over a range of subzero temperatures than by being held at any of the constant temperatures studied. In other words, the lethal effects of slow warming seem to have been due more to a slow change in temperature than to the long exposure to subzero temperatures produced by slow warming.

Influence of the Suspending Vehicle on the Effects of Rate of Warming.—Double-distilled water was the vehicle used in all the experiments described so far. As mentioned in the introduction, it was used to avoid the complicating effects of the presence of extracellular solutes. Other investigators in studying the effects of subzero temperatures, however, have used aqueous solutions as suspending vehicles. It seemed desirable, therefore, to see whether the effects of rate of warming observed when A. flavus spores were suspended in distilled water would be modified when solutes were present in the suspending fluid.
Three experiments were performed. They were identical to the first experiment described in the present paper (reported in Table I) except that spores were suspended in horse serum, 0.16 molal sodium chloride, or 0.29 molal sucrose instead of in distilled water. The molalities of sodium chloride and sucrose yielded solutions which were approximately isosmotic with horse serum. Tubes containing suspensions of spores in each of the three vehicles were cooled rapidly to \(-75^\circ\text{C.}\), held at \(-75^\circ\text{C.}\) for 15 minutes, and then warmed and thawed at a rapid, medium, or slow rate in the standard manner. The percentages of germination that resulted are shown in Table VI. Survival clearly was affected by the rate of warming, for irrespective of the vehicle, the slower the warming, the lower the recovery. Apparently, therefore, the harmful effects of slow warming occurred whether the vehicle was distilled water, horse serum, 0.16 molal sodium chloride, or 0.29 molal sucrose.

Although these three experiments indicated that the influence of warming rate was not appreciably modified by the three dilute aqueous solutions utilized, other experiments have shown that the nature and behavior of other vehicles can play an important role in determining the effects of subzero temperatures. This role will be the subject of a future report.

**DISCUSSION**

In the present study it has been shown that when *A. flavus* spores are cooled rapidly to \(-70^\circ\text{C.}\), the fate of most of the spores depends on the rate at which they are subsequently warmed. Thus, an average of 75 per cent survived when warming was rapid, but only an average of 7 per cent survived when warming was slow. These spores are not unique, however, in surviving rapid warming better than slow. Haskins (1955) has recently reported similar findings for spores of the fungi *Alternaria*, *Fusarium*, *Penicillium*, and *Pestalotia* as well as for *Aspergillus*. The injurious effects of slow warming were observed to occur both with spores suspended in water and those in blood serum.

Other cells which also have been found to be less injured by rapid warming include spirochetes of relapsing fever (Turner and Brayton, 1939), the yeast *Saccharomyces carlsbergensis* (Lund and Lundberg, 1949–50), one strain of leukemic cells of mice (Breedis, 1942), mammalian erythrocytes (Luyet, 1949, Lovelock, 1953), and frog spermatozoa (Luyet and Hodapp, 1938). Other instances demonstrating the superiority of the more rapid rates have been reported for epithelial cells and melanoblasts of rabbit earskin grafts (Billingham and Medawar, 1952), mouse fibroblasts and human epithelial cells (Scherer and Hoogasian, 1954), chick embryos (Luyet and Gehenio, 1954), and the moss *Mnium sp.* (Luyet and Gehenio, 1938).

Unfortunately, most of the above investigators made no attempt to separate the effects of subzero warming from those of the actual thawing of ice. Accordingly, the terms rapid and slow "warming" in the immediately preceding
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The effects of subzero temperatures on spores include both subzero warming and thawing. This lack of separation of the two factors makes it difficult to compare the measured rates of subzero warming and thawing used in the present paper with the non-separated rates of "warming" used by most others. Crude comparisons can be made, however, on the basis of the time required for the overall process of subzero warming and thawing. Moreover, data from the work of Lovelock and from Luyet and Gehenio (1954) can be compared on the more satisfactory basis of the average change in temperature with respect to time. Both types of comparisons are made in Table VII for those cases in which data are available.

One other study is of some interest. Strumia et al. (1941) reported that when human plasma that has been stored at −40°C. is warmed and thawed slowly in air, a heavy precipitate forms. It does not appear, however, when the frozen plasma is warmed more rapidly in a water bath. However, the authors gave no information as to the nature of the precipitate. If it were denatured protein, it would be interesting to speculate that a slow rise in temperature caused the denaturation. In this regard, Nikkilä and Linko (1954) report evidence indicating that the myosin of frozen fish may denature during defrosting.

It would be erroneous to leave the impression that rapid warming is always

<table>
<thead>
<tr>
<th>Investigators</th>
<th>Organism or Cell</th>
<th>Cooling temperature</th>
<th>Rapid warming</th>
<th>Slow warming</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yeast</td>
<td>−78</td>
<td>180−300</td>
<td>240</td>
</tr>
<tr>
<td>Lund &amp; Lundberg (1949-50)</td>
<td>−60 to −70</td>
<td>120</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Scherer and Hooasian (1954)</td>
<td>−70 to −75</td>
<td>30−40</td>
<td>11.7*</td>
<td>0.7−0.9*</td>
</tr>
<tr>
<td>Lovelock (1953)</td>
<td>Fungal spores</td>
<td>−70 to −75</td>
<td>30−40</td>
<td>5−100</td>
</tr>
<tr>
<td>Masur</td>
<td>Fibroblasts and epithelium</td>
<td>−70 to −75</td>
<td>30−40</td>
<td>5−100</td>
</tr>
<tr>
<td>Turner and Brayton (1939)</td>
<td>Spirochetes</td>
<td>−78</td>
<td>30−40</td>
<td>5−100</td>
</tr>
<tr>
<td>Bradis (1942)</td>
<td>Leukemic cells</td>
<td>−196</td>
<td>8−12</td>
<td>20−30</td>
</tr>
<tr>
<td>Luyet (1949)</td>
<td>Erythrocytes</td>
<td>−78 or −160</td>
<td>1.5−21</td>
<td>3−5</td>
</tr>
<tr>
<td></td>
<td>Embryo</td>
<td>[&lt;10]</td>
<td>Approximate ly 50%</td>
<td>60%</td>
</tr>
</tbody>
</table>

* Rate measured from cooling temperature to 0°C.
† Time and rate measured from −40 to −3°C.
§ The temperature range on which this rate was based was not stated by the investigators.
less harmful than slow, for there are instances reported in the literature in which either rate of warming had no effect, or else rapid warming was more harmful than slow. Stille (1950) has found the latter situation to apply to cells of *Saccharomyces cerevisiae*, *Pseudomonas fluorescens*, *P. pyocyanea*, *Bacterium prodigiosum*, and *B. rubidaeum*. Rapid warming, however, was only slightly more harmful than slow. On the other hand, Ulrich and Halvorson (1946–47) have found that the viability of similar microorganisms was about the same whether the cells were warmed slowly by contact with air or rapidly by immersion in a water bath at 35°C. The bacteria included *Escherichia coli*, *Proteus vulgaris*, *Lactobacillus casei*, *Staphylococcus aureus*, and *Alcaligenes fecalis*.

Bartetzko (1910) studied the effects of low temperatures on young hyphae of *Aspergillus niger*, *Botrytis cinerea*, *Penicillium glaucum*, and *Phycomyces nitens*, and found that they were no more harmed by slow than by rapid warming. This finding differs from that of the present paper, but then hyphae differ morphologically and physiologically from spores. Levitt (1941) has reviewed much of the literature on the effects of low temperatures on higher plants. He concludes that generally the survival of higher plant cells is not affected by rate of warming. When an effect has been found, however, it is rapid warming which is the more harmful.

Thus, different cells respond to warming in different ways. However, the very fact that the viability of some cells is affected by the rate of warming makes it quite evident that this factor should not be ignored when the effects of low temperatures are under study. Either the rate of warming should be carefully controlled or else its effects evaluated.

The observation of an effect inevitably raises questions as to the underlying causes. What then can be deduced as to the possible causes of the lethal effects of slow warming on *A. flavus* spores? In the majority of the experiments (except those reported in Table VI), the suspensions consisted of only two components: The spores and the water in which they were suspended. Hence, the warming of the frozen suspensions produced (1) warming of the spores, (2) warming of the surrounding ice, and (3) thawing of that ice. Death must have been associated with one of these three events. The thawing, however, could not have been the cause of death, for the lethality of slow warming occurred below 0°C, before the vehicle had even begun to melt. Moreover, the rate at which this extracellular ice melted had little or no effect on viability. It was the rate of subzero warming that determined survival.

Yet, it is difficult to see how the second event, mere slow subzero warming of the frozen vehicle, would be injurious to the spores. A more likely possibility is that slow warming of the spores themselves (1, above) produced some intracellular effect leading to death. An example of such an effect could be the slow melting of ice inside the spores, for the melting of intracellular ice would occur
below 0°C. owing to the fact that protoplasm is an aqueous solution. This example assumes, of course, that intracellular ice formed within the spores as a result of rapid cooling to −70°C. Since there is no experimental support for this assumption from the data reported in the present paper, the example is, for the time being, only one of several possible explanations of an intracellular effect. In succeeding papers, however, experimental results will be presented which suggest that the assumption is valid. These experiments deal with the effects of the rate of cooling, the temperatures to which the cells are cooled, and the nature and behavior of the vehicle in which the spores are suspended. It will become apparent that these factors influence the effects of warming rate with the result that the percentage of spores surviving low temperatures is a consequence of the combined effects of all the factors.

SUMMARY

1. The survival of spores of Aspergillus flavus suspended in distilled water and cooled rapidly to −70 to −75°C. was found to depend primarily on the rate of subsequent warming of the frozen suspension. Only 7 per cent of the spores germinated following slow warming at 0.9°C. per minute, whereas about 75 per cent germinated following rapid warming at 700°C. per minute.

2. Viability was dependent on the rate at which the suspensions warmed from −70 to 0°C. (subzero warming), but was not dependent on the rate of thawing of the frozen water in which the spores were suspended.

3. The logarithm of the percentage of germination appeared to be a linear function of the logarithm of the rate of subzero warming when spores were warmed at rates ranging from 0.12 to 1000°C. per minute.

4. The lethal effects of slow warming from −70 to 0°C. were more pronounced between about −20 and 0°C. than between −70 and −20°C. In the former range of temperatures, the percentage of germination decreased sharply as slow warming progressed towards 0°C.

5. Slow warming from −70 to 0°C. was more harmful to the spores than was a 1 or 2 hour exposure to constant temperatures between −70 and 0°C.

6. Slow warming was found to be more harmful than rapid warming when spores were suspended in horse serum, 0.16 molal sodium chloride, or 0.29 molal sucrose as well as in distilled water.

The author wishes to express his deep gratitude to Professor William H. Weston who provided not only the impetus for the studies reported in the papers constituting the present series, but the most expert guidance as well. Professor Carroll M. Williams offered many suggestions on various aspects of this work. His continued interest and aid are sincerely appreciated.
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