SOME PHYSICAL FACTORS THAT INFLUENCE THE SURVIVAL OF BRUCELLA ABORTUS DURING FREEZE-DRYING[1]

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Since Shackell (1909) described the preservation of biological material by freeze-drying, or drying by sublimation of ice, other workers have improved the process until, at the present time, it is used to preserve a variety of biological products. Flosdorf (1949) has reviewed the course of this development. A few of the workers in the field have published the results of attempts to use this technique for the preservation of maximum numbers of viable, vegetative bacteria. Rogers (1914) made comparisons between numbers of viable cells present in bacterial suspensions before and after preservation. Naylor and Smith (1946) and Stamp (1947) showed that the chemical composition of the fluid used to suspend the cells that were preserved influenced the percentage of cells that remained viable after drying.

Insofar as the authors are aware, no one has yet studied the effect of some factors such as time, rate, and extent of drying on the survival of bacteria during freeze-drying. Elser, Thomas, and Steffen (1935), Greaves and Adair (1939); Flosdorf, Hull, and Mudd (1945), Greaves (1946), and others found that such factors as these had a marked influence on the physical properties of preserved biological materials. Consequently, in the work done for this paper the influence of the nature of the drying process on the percentage of viable vegetative bacteria recoverable after drying was determined. The factors studied were the influence of the temperature of subliming ice, the rate of sublimation, and the moisture content of the dried product. The test organism was *Brucella abortus* (BAI strain 19).

Cells to be dried were suspended in fluid similar to that recommended by Naylor and Smith (1946) in their studies with *Serratia marcescens*. The results of experiments are described in terms of the percentage of cells, viable in the suspension originally, which remained viable after drying.

MATERIALS AND METHODS

Cultures of *B. abortus* were produced in 250-ml quantities of 2 per cent Difco tryptose broth contained in 2-liter Erlenmeyer flasks. Sterilization was for 20 minutes in steam at 20 psi. Cultures used experimentally were freshly prepared 24-hour-old cultures that had been incubated at 34 C. During incubation all

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cultures were shaken on a machine that operated at a rate of 100 oscillations per minute with a stroke of 3 inches. Cultures for experimental use were inoculated with 2 per cent by volume of another similar culture whose inoculum was one agar slant of the stock culture.

After incubation, cells were centrifuged at 3,000 rpm for 30 minutes, the culture supernatant was discarded, and the cells from all flasks were pooled. These cells were then resuspended in a volume of fluid equal to one-tenth the volume of the original culture. The composition of the resuspending fluid was 0.1 per cent of Difco tryptose, 0.25 per cent of NaCl, 2.0 per cent of dextrin, and 0.5 per cent each of ascorbic acid (pH adjusted to 6.5), ammonium chloride, and thiourea. This is essentially the fluid that Naylor and Smith (1946) used to suspend cells of *S. marcescens* for freeze-drying and that was found, in preliminary experiments, to be effective also for freeze-drying of *B. abortus*. The suspension to be dried contained from 200 to 400 $\times$ 10^8 viable cells per ml.

Numbers of viable cells present in either the original suspension or in reconstituted, dried samples were determined by the usual technique of diluting and plating in Difco tryptose agar. Dried samples were restored in a solution containing 0.2 per cent of Difco tryptose. This solution was also used for diluting samples prior to assay. Colonies were counted after 5 days' incubation at 34 C. The time lapse during plating, the temperature of the diluting fluid, the extent and vigor of shaking dilution bottles, and the temperature of agar for pouring were standardized.

When the conventional manifold type "lyophile" apparatus was used, it was difficult to measure and control temperatures, to measure drying rates, or even to standardize treatment of individual samples. Hence, in order to accomplish more precise control of all physical factors and to enable uniform handling of replicate samples, a special freeze-drying apparatus was constructed. The device consists of two chambers, one of which is illustrated by the drawing in figure 1. Except for the fact that both chambers are evacuated by a single pump, either half of the unit can be operated independently of the other. Sublimed water is collected on dry-ice-cooled condensers located at the top of each chamber. The bottom of each chamber is a brass plate 1 inch thick, recessed to a depth of $\frac{3}{4}$ of an inch, to hold nine 1-ml samples, each contained in a 15-ml vaccine bottle. Either plate can be adjusted to any desired temperature between -40 C and $+50$ C by regulation of the flow of cold or hot alcohol, from reservoirs through coils soldered to the bottom side of the recessed plate. The entire base of each chamber is insulated to facilitate control of the temperature.

The suspension of cells to be dried was pipetted into sterile, cotton-stoppered, 15-ml vaccine bottles. The individual samples were then shell-frozen and held in a tray containing an alcohol-dry-ice slurry until time for transfer to the previously cooled driers. In early experiments, the bottles were removed from the dry-ice slurry and allowed to drain free of alcohol before transfer. Then all bottles were placed in the drier at as near the same time as possible. As noted in the experimental results below, this technique was not satisfactory and was changed. Instead of being allowed to drain off, alcohol was wiped off the sample

bottles, and each individual bottle was transferred from the dry-ice-alcohol slurry to the cooled drier as quickly as possible. After transfer the cotton stoppers in the bottles were removed and discarded. In standard operation, the base of each chamber was cooled to at least −30 C before samples were transferred to them,

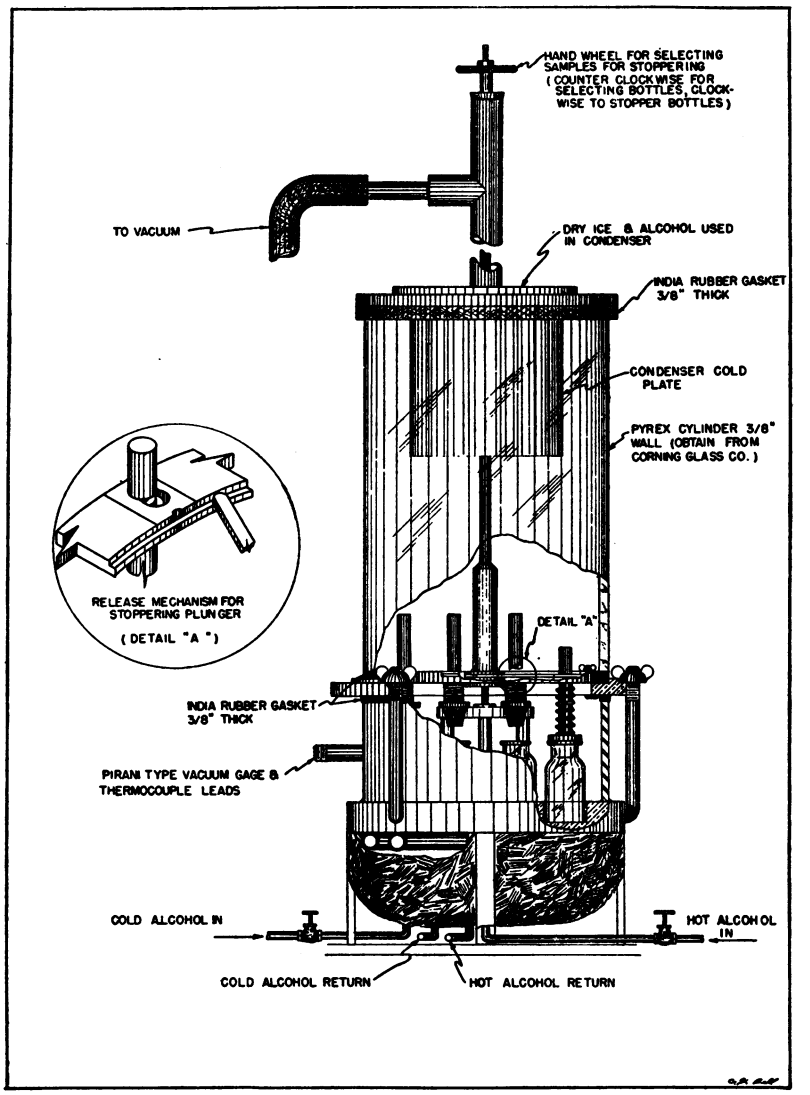

Figure 1. Diagram illustrating the construction of the special freeze-drying apparatus.

and the temperature of the base was not changed until the desired vacuum was obtained.

It may be noted that each chamber of the apparatus is equipped with a device consisting of an assembly of 9 spring-activated plungers, each of which

holds a stopper in position above each sample bottle. These plungers are controlled by means of a lever, which is located outside the chamber. By using this lever, any bottle can be stoppered at any desired time during the drying process and each sample can be stoppered while the chamber is still evacuated. Also, when pathogens are being handled, risk of accidental infection can be minimized by spraying a disinfectant into the chamber before it is opened. Five per cent phenol was used and found to be satisfactory.

Samples that were to be stored were placed in vessels that were evacuated by continuous pumping to pressures of less than 200 microns of mercury.

EXPERIMENTAL RESULTS

The killing effect of certain ice film temperatures. In some experiments that were done to test for uniformity of results, samples were dried for 24 hours at pressures not exceeding 200 microns of mercury. During drying the temperature of the base of the apparatus was allowed to come to equilibrium with the temperature of the room and to remain there for 20 hours. Then the temperature of the base was raised to 40 C over a 2-hour period and, finally, was held at 40 C for another 2 hours. At the end of this time samples were stoppered, pressure in the chamber was raised to atmospheric, and the samples were removed from the drier.

The numbers of viable cells recovered from individual samples dried in these first experiments were not always uniform. Sometimes recovery was uniform and high, sometimes uniform and low, but usually there was significant variation between supposedly identical samples within a given experiment. In these first experiments shell-frozen samples had been allowed to drain free of alcohol before transfer to the drier. When samples were wiped off and transferred immediately and not allowed to stand to drain, it was found that the results were uniform and reproducible. Later it was found that shell-frozen samples of *B. abortus* (strain 19) suspensions are quite susceptible to changes in temperature between −30 C and −10 C, and it was demonstrated that the previous irregularity of results might have been caused by samples' being subjected to unfavorable temperatures during the time that they were transferred to the drier. For example, it was found that if the temperature of a film of frozen *B. abortus* suspension was never allowed to rise above −30 C before drying that the dried material remained white and fluffy and 50.8 ± 7.6 per cent of the viable cells originally present could be recovered. On the other hand, if the temperature of the film was permitted to warm to −10 C, the dried material was not noticeably changed in appearance but only 1.4 ± 0.9 per cent of the viable cells that were present originally could be recovered. The data contained in table 1 are those that resulted when experiments were performed in which frozen suspensions were allowed to warm to several temperatures between −10 C and −25 C. These data show not only that the elevation of temperatures of ice in samples to as high as −10 C causes a severalfold reduction in the number of viable cells recoverable after drying, but also that moderate warming, to temperatures between −20 C and −15 C, causes the recoveries after drying to be quite irregular.

It was noted that if frozen suspensions of cells were substantially free of salt, elevation of ice film temperatures did not cause a reduction in the numbers of viable cells present. The data presented in table 2 allow comparison of recoveries obtained when the concentration of salt was varied and when frozen suspensions were subjected to an elevated temperature. In the course of these experiments

TABLE 1

The influence of warming ice films on the recovery of viable cells of B. abortus (strain 19) after freeze-drying

TEMPERATURE TO WHICH ICE FILM WAS WARMED BEFORE FREEZE-DRYING STARTED	REPLICATION	PER CENT RECOVERY OF VIABLE CELLS PRESENT ORIGINALLY
−10 C	1	1.1
	2	1.3
	3	13.0
	4	10.0
−15 C	1	6.1
	2	7.9
	3	51.0
	4	57.0
−20 C	1	39.0
	2	55.0
	3	86.0
	4	59.0
−25 C	1	44.0
	2	55.0
	3	59.0
	4	62.0

TABLE 2

The influence of the presence of dissolved sodium chloride on the freezing and thawing of suspensions of B. abortus (strain 19)

SODIUM CHLORIDE PRESENT IN THE SUSPENSION	PER CENT RECOVERY OF VIABLE CELLS FROZEN AND THAWED* AS INDICATED	
	Held frozen until thawed	Temperature of frozen suspension alternated between −80 C and −15 C five times, then thawed
None	87.0 ± 6.3	79.0 ± 12.8
0.5 per cent	97.0 ± 2.6	8.0 ± 2.5

* Suspensions tested were frozen in a dry-ice alcohol bath. Frozen suspensions were thawed by the addition of 0.1 per cent sterile Difco tryptose broth at 80 C. The volume of broth added was that which was required to bring the contents of the bottle to equilibrium at approximately room temperature.

it was found that the recovery of viable cells after freeze-drying was extremely low unless salt was present in the suspension to be preserved. Consequently, the ice films that were tested were not dried but rather were exposed to controlled temperatures above −80 C and were then thawed rapidly. For this reason, the data in table 2 do not contribute directly to an explanation for the effect of

variation of temperatures of ice films on the recovery of viable cells after freeze-drying. Nevertheless, there is an indication that the presence of dissolved salts is in some way related to the destruction of cells that occurs when the frozen suspensions of bacteria are subjected to unfavorable temperatures.

The influence of rate of drying on viability immediately after drying. In the first experiments that were done to determine the effect of the rate of drying, the actual procedure for drying was the same as that described in the preceding section. The rate of flow of water vapor from samples to condenser was regulated mechanically. In some instances the opening leading from the samples to the condenser was partly closed, and in others a light cotton stopper was left in each sample bottle during drying. In these experiments it was found that a decreased rate of flow of water vapor caused a slight but consistent improvement in the percentage of viable cells recoverable after drying.

Experiments of this kind could not be considered conclusive because it had been noted that any restriction of the flow of water vapor that was great enough to influence the number of cells recoverable after drying also caused an increase in the temperature of the ice in the samples; thus it could not be fully determined whether the reduced rate of sublimation or the changed temperature was the factor that influenced the recovery of viable cells. The influence of the rate of drying was finally determined in another way. Advantage was taken of the fact that there is a relationship between total pressure in the drying chamber and the temperature of ice during drying, which is nearly independent of the temperatures of the metal surfaces surrounding the sample bottles. Consequently it was possible to hold constant the temperature of ice during drying by controlling the pressure in the drying chamber and to adjust the rate of sublimation by variation of the temperature of the recessed plate that contained the samples. Thus the rate of drying was expressed in terms of the difference between the temperature of the film of ice and the temperature of the surrounding surfaces. Data that permit comparison of the percentage of recovery of viable cells from samples of *B. abortus*, which were dried at various rates, are presented graphically in figure 2. Groups of samples were dried at different pressures and, consequently, at different temperatures of ice film. All the samples in this experiment were dried for 24 hours.

One will note that the recovery of viable cells is poor when the difference between the temperature of the ice film and the surfaces surrounding the sample is small, that recovery is increased to a maximum as this temperature differential is increased, and, finally, that recovery is decreased markedly as the temperature differential (rate of sublimation) continues to increase. Data from other similar experiments in which samples were dried until further drying no longer changed the percentage of recovery of viable cells are presented in figure 3. Here it can be noted that extension of the time of drying has eliminated the low recoveries that were obtained when the difference between the temperature of the ice in the sample and the temperature of the surrounding surface is small. When figures 2 and 3 are compared, it becomes evident that, when the drying process is continued long enough for samples to reach the point at which further drying has no effect, the slowest rate of drying produces the highest percentage of viable

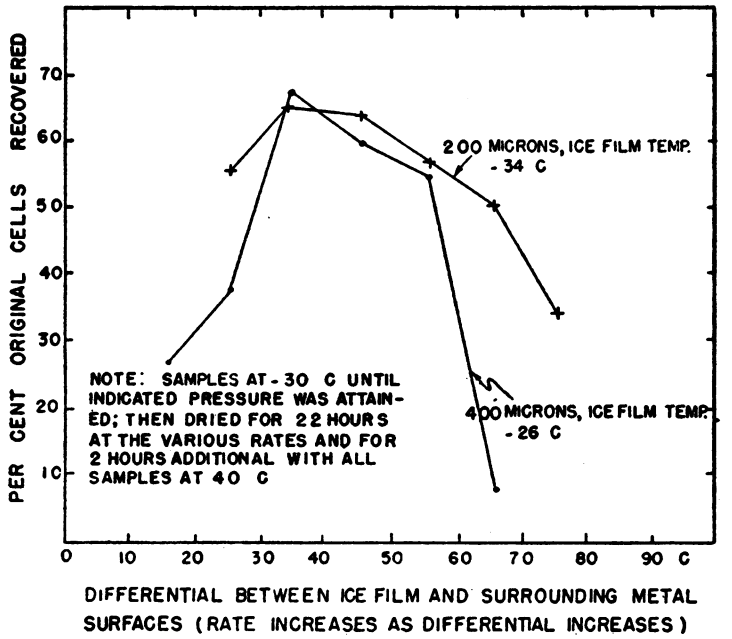

Figure 2. The influence of the rate of freeze-drying on the recovery of viable cells of *B. abortus* (strain 19).

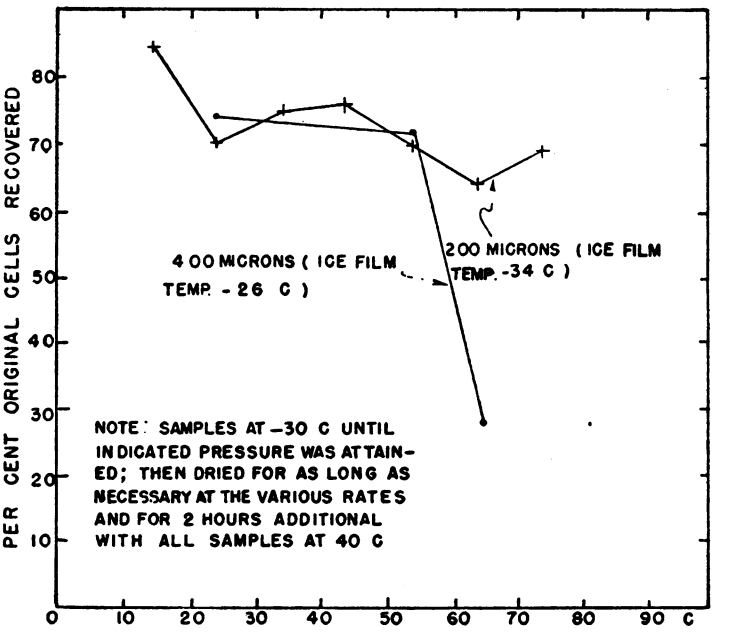

Figure 3. The influence of the rate of freeze-drying on the recovery of viable cells of *B. abortus* (strain 19).

cells recoverable after drying. Because drying at very slow rates requires such a greatly extended period of drying and because the improved recovery resulting from this is not highly significant, it is suggested that, for most purposes, an intermediate rate of drying is the most useful.

The effect of residual moisture in samples preserved by freeze-drying. An attempt was made, in the experiments described above, to standardize the moisture content of all samples, regardless of the rate or temperature of drying. This was done by heating the metal block that contained the samples to 40 C for the

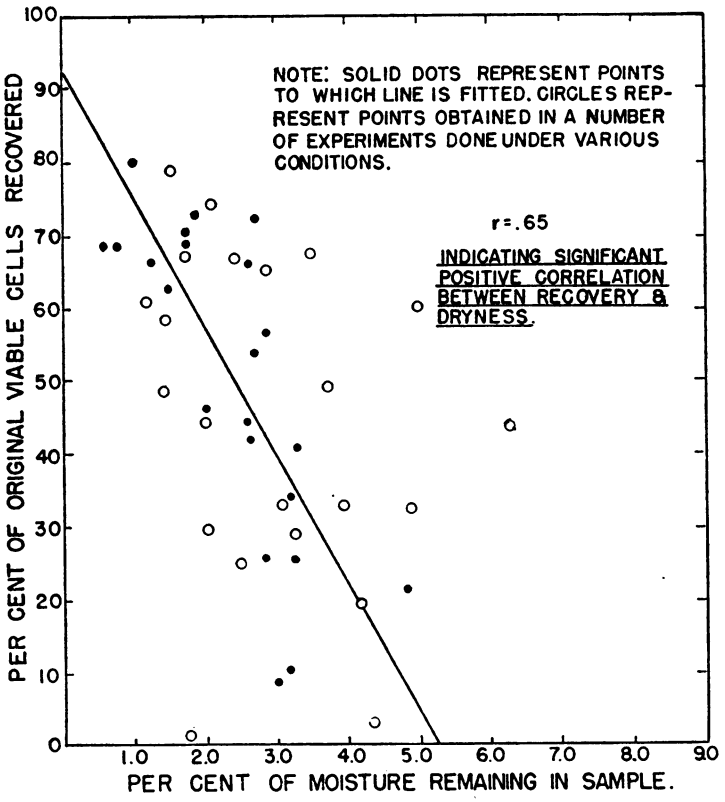

Figure 4. The influence of residual moisture on the recovery of viable cells of *B. abortus* (strain 19) preserved by freeze-drying.

last 2 hours of the drying period. The moisture content of all samples prepared in this way was below 2.0 per cent of the dry weight of the samples, as measured by the modified Karl Fischer technique described by Levy *et al.* (1945). In these samples it was doubtful that any correlation existed between the percentage of moisture of the preserved sample and the percentage of viable cells recoverable immediately after drying.

On the other hand, positive correlation was shown to exist between dryness and percentage of viability in samples preserved in other experiments in which the length of the drying period was varied and the period of final drying at 40 C was omitted.

The recoveries of viable cells obtained from samples of preserved material having varying moisture contents are shown in figure 4. The solid black dots represent the values obtained in experiments designed to determine the effect of residual moisture on viability. The circles define moisture contents of samples taken from experiments not specifically designed for establishing this regression. The r value of 0.65 for the regression shown in figure 4 indicates that the plotted points follow the line of best fit in a manner that suggests that a high degree of correlation exists between dryness and high recovery immediately after preservation by freeze-drying.

TABLE 3

Percentage of recovery of viable cells of B. abortus (strain 19) after storage of preserved samples having various moisture contents

PER CENT MOISTURE* OF REPRESENTATIVE SAMPLES	NUMBER OF DAYS SAMPLES WERE STORED AT ROOM TEMPERATURE				
	0	4	10	25	30
5.0	65	<1	<1	<1	<1
2.5	45	<1	<1	<1	<1
1.7	46	20	5	<1	<1
0.3	43	27	19	2	2
0.2	46	28	11	2	<1

* Levy *et al.* (1945) claim sensitivity of 25 to 100 micrograms of water when 1 to 25 mg of water are being determined. Note that determination of less than 0.5 per cent of moisture in these samples whose dry weight was about 35 mg would require the determination of 250 micrograms or less of water. Errors at this moisture level were so great that the results of determinations were meaningless.

TABLE 4

Percentage of viable cells of B. abortus (strain 19), preserved by freeze-drying to maximum dryness, surviving after exposure to various concentrations of water vapor

DAYS OF STORAGE AT ROOM TEMPERATURE BEFORE VIABLE CELLS WERE COUNTED	TWO-HOUR EXPOSURE TO WATER VAPOR PRESSURES (IN MICRONS OF MERCURY) AS INDICATED			
	1	50	300	1,250
None	78	69	60	58
7	47	50	36	14
14	38	47	42	16
30	43	60	24	2
90	<1	8.7	1.0	<1

The survival of viable cells after storage at room temperature was found to be influenced also by the moisture content of the preserved samples. The data are presented in table 3, which show that at high moisture contents the percentage of cells, viable after drying, which survived after very short periods of storage is very low and that, as dryness is increased, survival after storage is increased.

Because certain samples contain too little water to be measured by the titrimetric method, the water content of these samples was measured gravimetrically on composites of several individual samples. These samples were transferred,

for weighing, in a dry chamber, and all possible precautions were taken to make the determination as accurate as possible. Even so, the results were so variable that in all probability they are only approximations of the true water content of the samples.

When the difficulty with the determination of water in the dried samples was recognized, another method of determining the effect of residual moisture was tried. Following drying, groups of samples, all dried over an extended period and at a final temperature of 40 C, were exposed to various concentrations of water vapor. The data presented in table 4 show the effect on preserved samples of a 2-hour exposure to various water vapor pressures. These vapor pressures were obtained by adjustment of the temperatures of the condensers of the drying apparatus at the end of the period of drying. No significant difference between the moisture content of any of the samples in any of the groups could be determined by any method of moisture determination available; yet it can be noted that the storage properties of the samples in the various groups are different.

The data contained in table 4 may point to the existence of an optimum level of moisture content for the storage of samples preserved by freeze-drying. Further studies will be required to confirm this indication.

No data are supplied to show the effect of residual moisture on samples stored at refrigerator temperature because loss of viable cells in samples that were stored, after freeze-drying, at refrigerator temperatures for 90 days was negligible.

SUMMARY

An experimental freeze-drying apparatus was constructed and used. With this apparatus it was possible to maintain close control over such factors as the temperature of the ice film before and during drying and to vary rates of drying. Individual samples could be stoppered in the gaseous environment that existed at any time during the drying period. If desired, the interior of the apparatus and the exterior of the samples could be decontaminated before the apparatus was opened.

The existence of a critical temperature for the frozen samples above which recovery of viable cells was lessened was demonstrated, and this effect was associated with the salt content of the sample.

The existence of an optimum rate of drying was demonstrated. The effect of residual moisture in the samples on the viable cells recoverable from the samples was determined. Dryness was shown to be correlated with recovery immediately after drying, and storage at room temperature was shown to be influenced by the dryness of the sample. A technique for controlling moisture content at very low levels was used in these studies.

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