Lyophilization of *Pasteurella pestis*[1]

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The general subject of lyophilization, with its specific applications, has been reviewed recently by Harris (1954). Although lyophilization has become a generally accepted method for long term preservation of stock cultures of viruses and bacteria it has not always been satisfactory for the preservation of *Pasteurella pestis*. This study was undertaken to determine some of the factors that affect viability of *P. pestis* during and after lyophilization.

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Materials and Methods

Cultures. Several strains of *P. pestis* were studied but strain A-1122 (avirulent) and strain 139 L (virulent) were used for most of the experiments. Cultures in 2.5 per cent Difco heart infusion broth (HIB) were incubated on a shaker for 48 hr at 32 C.

Containers. Most of the cultures were lyophilized in 60 ml Army style vaccine bottles, 5 ml per bottle. A few were lyophilized in 6 ml spherical Pyrex ampules, 0.5 ml per bottle. The bottles were stoppered with exhaust tube stoppers[4] fitted with brass ring clamps to facilitate sealing.

Lyophilization. The apparatus shown in figure 1 was designed to accommodate two complete manifolds, each with 24 outlets. Each manifold was housed in an

[4] Obtained from F. J. Stokes Machine Co., Philadelphia, Pennsylvania.

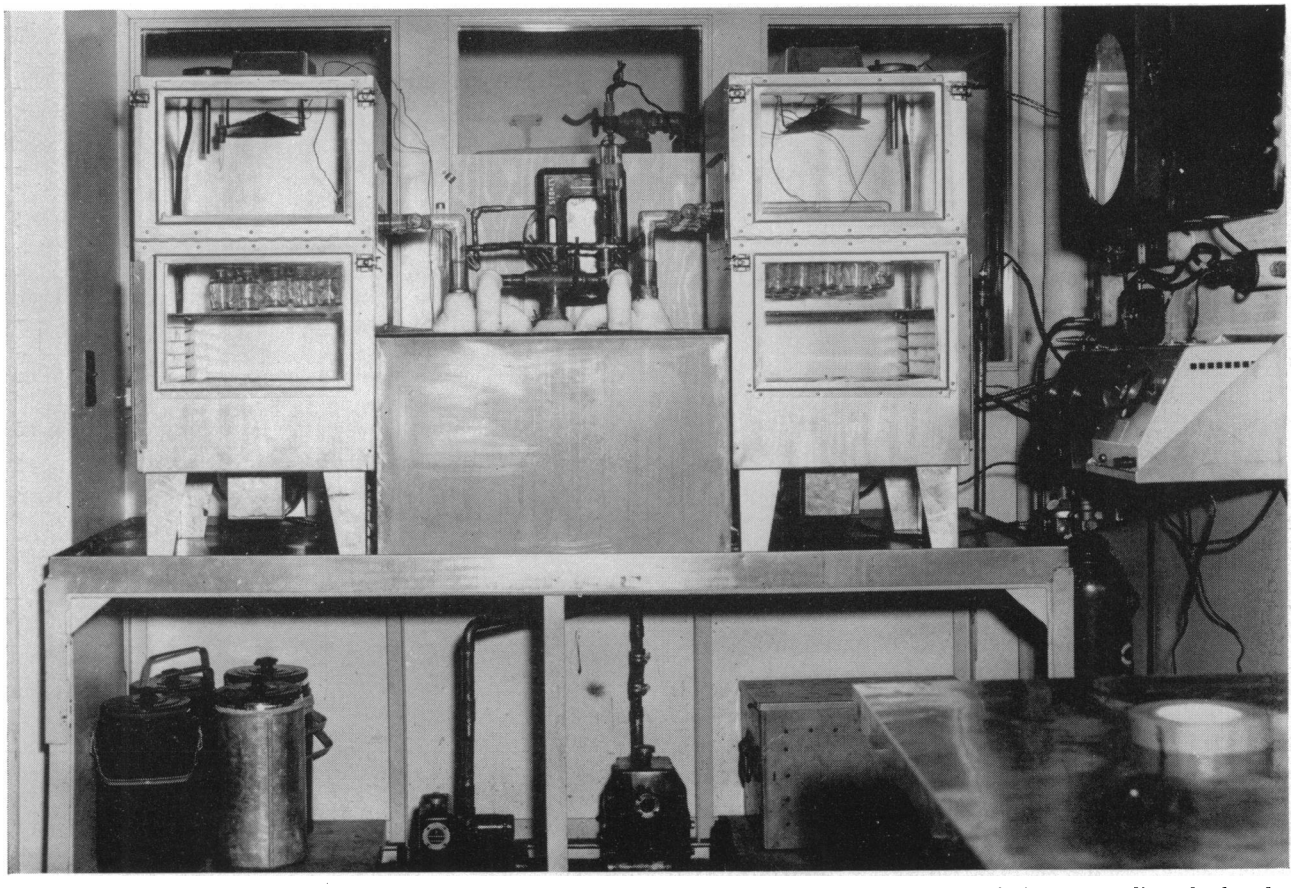

Figure 1. Manifold-type lyophilization apparatus with incubators for controlling temperature of air surrounding the bottles

incubator equipped to circulate air of constant temperature past the bottles.

Temperatures were determined at various locations within the drying system by the use of iron-constantan thermocouples. The temperature of the cultures was −15 to −18 C at the beginning of the drying cycle, and increased slowly until it approached that of the surrounding air, generally within 3 hr. After the first hr of each drying cycle, the air in the incubator was maintained at 32 ± 3 C.

The cultures were not cooled or frozen before attachment to the manifold unless otherwise indicated, but were degassed and "snap-frozen" in situ by slowly evacuating the system.

At the end of the drying period the bottles were sealed at the existing vacuum by compressing the brass ring on the exhaust tube stopper. The glass ampules were sealed by fusing the neck.

Viability and virulence. The lyophilized cultures were reconstituted to the original volume with sterile distilled water at room temperature. Viability was determined by a modification of the drop counting technique of Miles *et al.* (1938) using 1 per cent peptone as the diluent and Difco blood agar base medium. Reported values represent the average of triplicate plates on each of three samples.

Virulence was determined by inoculating 0.2 ml of serially diluted culture intraperitoneally into 8- to 12-week old Namru mice. The LD_{50} was determined by the Linearized Mortality Grid Method of Goldberg *et al.* (1954).

Moisture determinations. Pressure in the bottles and moisture content of the lyophilized cultures were determined manometrically after various periods of storage utilizing the apparatus described by Heckly (1955).

Results

Effect of suspending medium and freezing methods. In preliminary experiments, the addition of albumin, methocel, starch, cysteine, and ascorbic acid to broth cultures of *P. pestis* had little or no protective effect during lyophilization and subsequent storage. Since Rogers (1914) had shown the value of skim milk in increasing the survival of lyophilized organisms, its individual components were assayed for their protective effect on *P. pestis* during lyophilization.

The results in table 1 show that the protective effect of lactose alone or with phosphate buffer was comparable to that of whole skim milk, while casein and dialyzed skim milk had little effect. It is of interest that precooling to −5 C in the presence of milk or lactose with either casein or buffer was not superior to snap-freezing, but precooling markedly increased survival when the culture was mixed with an equal volume of distilled water. Since survival in snap-frozen cultures containing lactose was comparable to that in precooled cultures without lactose the snap-freezing technique was used subsequently because of the greater ease of operation. The conventional method of shell-freezing at −78 C prior to lyophilization resulted in significantly lower survival than either of the other two methods (table 1).

Effect of pH and phosphate. The results in table 2 indicate that the cultures should be buffered since survival was higher at pH 7.6 than at 7.2 or 7.0. In other experiments, it was found that varying the final phosphate concentration between 1/30 M and 1/15 M had no effect on survival but the use of 1/10 M phosphate did result in slightly lowered survival.

TABLE 1

The effect of various substances and methods of freezing on survival of Pasteurella pestis strain A-1122 during lyophilization

Solution Added to the Culture*	Method of Freezing					
	Precooled†		Snap-frozen		Shell-frozen at −78 C	
	No. trials	Per cent survival‡	No. trials	Per cent survival‡	No. trials	Per cent survival‡
Skim milk	3	57 (38–76)	8	60 (46–74)	3	21 (7–35)
Casein 3% in lactose 6%	3	75 (51–99)	6	72 (65–79)	—	—
Casein 3%	—	—	6	15 (12–18)	—	—
Lactose 6%	3	72 (66–78)	6	49 (41–57)	3	12 (9–15)
Lactose 6% in 2/15 M phosphate buffer	—	—	6	67 (63–70)	—	—
Dialized skim milk	—	—	3	16 (12–20)	—	—
Distilled water (control)	9	37 (30–44)	12	9 (7–11)	9	1.7 (1.2–2.3)

* Equal volumes of the solutions were added to heart infusion broth cultures which had an original viable cell count of about 1.1 × 10^9 per ml.

† Preparations were slowly cooled to −5 C and held at this temperature for 24 hr before lyophilization.

‡ Mean survival immediately after lyophilization. 95 per cent confidence interval in parentheses.

TABLE 2

The effect of pH on the survival of Pasteurella pestis strain 139L lyophilization

pH*	No. of Trials	Per Cent Survival†
7.0	6	15 (11–19)
7.2	3	18 (9–27)
7.6	5	50 (36–64)

* The pH was adjusted with 10 per cent NaOH after adding to the culture an equal volume of 6 per cent lactose, buffered at pH 7 with 2/15 M phosphate. Cultures contained about 10^9 viable cells per ml.

† Mean survival immediately after lyophilization with 95 per cent confidence intervals in parentheses.

Comparison of sugars. A comparison of glucose, sucrose and lactose as individual additives (table 3) showed that the three sugars were about equally effective on a weight basis in maintaining viability. Increasing the sugar concentration apparently increased survival. The claim of Fry and Greaves (1951) that the addition of 5 to 10 per cent glucose would improve almost every drying medium for bacteria was substantiated in this study as far as initial drying and limited storage were concerned (table 3 and figure 2*A*). However, as shown in figure 2*A*, viability of *P. pestis* cultures to which glucose had been added decreased rapidly after 1 month's storage at room temperature. The addition of either lactose or sucrose was more effective than glucose in preserving viability.

Effect of moisture and storage. After satisfactory viability during lyophilization was achieved, attention was directed toward minimizing the loss in viability during prolonged storage. Fry and Greaves (1951) had reported that viability was decreased by excessive drying of the culture and suggested that the optimal residual moisture content was not the same for all organisms. In an attempt to obtain conditions of extreme drying, cultures were dried 4 hr at elevated temperature after the usual lyophilization. The results presented in figure 3, seem to confirm those of Fry and Greaves in that the cultures dried at the higher temperatures showed the greatest loss during lyophilization. However as shown in figure 3 organisms dried at the higher temperatures survived storage for 12 weeks as well or better than those dried at 25 C. It is possible that the moisture content of the cultures immediately after drying was below the optimum for storage and diffusion of moisture through the stopper increased the mositure content to the optimum for at least a portion

TABLE 3

Comparative effect of three sugars on the survival of Pasteurella pestis strain A-1122 lyophilization

Sugar	Mean Percentage Survival				
	Final sugar concentrations*				
	None	0.5%	1.5%	3.0%	6.0%
Glucose	—	36	40	58	—
Sucrose	—	23	34	65	—
Lactose	—	50	78	83	93
None (control)	9	—	—	—	—

* Equal volumes of double strength sugar solutions were added to cultures before lyophilization.

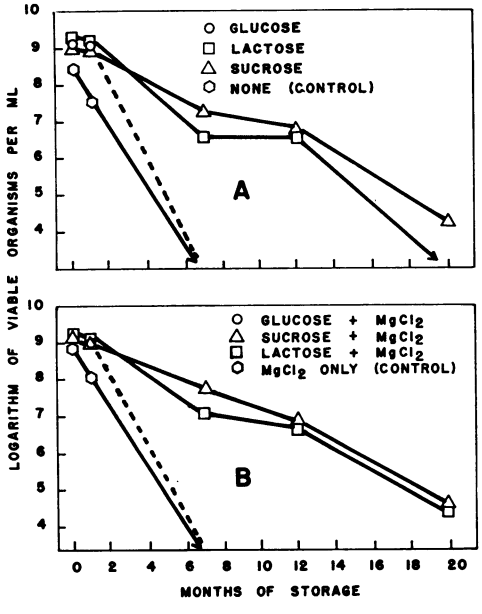

Figure 2. The effect of three different sugars alone (*A*) and combined with magnesium chloride (*B*) on viability of *Pasteurella pestis* strain A-1122 lyophilized and stored in rubber stoppered bottles.

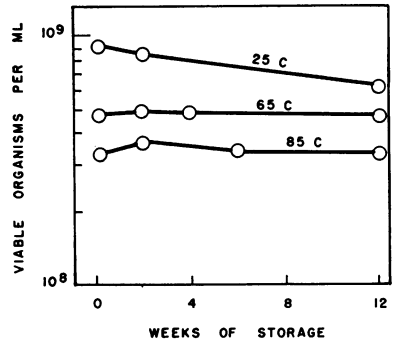

Figure 3. The effect of drying temperature on survival of *Pasteurella pestis* in rubber stoppered bottles. Cultures were mixed with an equal volume of 6 per cent lactose containing 2/15 M phosphate buffer at pH 7.6. All preparations were dried 18 hr at 25 C and the temperature was raised to 50 C for 2 hr before the final drying at 65 and 85 C. Pressure in the manifold ranged from 10 to 30 μ Hg.

TABLE 4

Air pressure and moisture content of lyophilized cultures in various menstrua after 7 months' storage at room temperature in rubber stoppered bottles

Sugar Added*	Final Salt Concentration					
	0.5 per cent $CaCl_2$		0.25 per cent $MgCl_2$		None	
	Per cent water† by wt	Pressure‡	Per cent water by wt	Pressure (mm Hg)	Per cent water by wt	Pressure (mm Hg)
Glucose	9.9	133	9.0	126	8.0	136
Lactose	7.0	160	8.0	172	8.0	144
Sucrose	7.0	129	9.0	121	9.6	135
None	7.5	123	10.0	125	8.0	138

* Equal volumes of culture and 6 per cent sugar solutions containing either one per cent $CaCl_2$ or 0.5 per cent $MgCl_2$ were mixed.

† Water removed by distillation at 50 μ pressure for 10 min at 100 C. Immediately after lyophilization all materials contained about 0.2 per cent water.

‡ Pressure within bottle at time of sampling. All bottles were sealed at 0.02 mm Hg pressure.

of the storage period. The permeability of the rubber stoppers to moisture and air is indicated in table 4. After 7 months' storage, the moisture had increased from 0.2 per cent to approximately 8 per cent. On the basis of determinations made earlier, the moisture had approached 8 per cent after as little as 12 weeks' storage. Since moisture was determined on individual samples in table 4, differences may not be as significant as they appear to be. The stoppers were more permeable to water than air since the pressure within all bottles had increased from 0.02 mm to only 150 mm Hg pressure, whereas, the moisture approached equilibrium with that in the atmosphere. This difference in permeability to air and water is probably correlated with molecular size.

Effect of calcium or magnesium chloride. In an attempt to minimize the detrimental effect of moisture, calcium or magnesium chloride (as drying agents) was added to the culture before lyophilization. As shown in figure 2*B* the addition of magnesium chloride alone did maintain viability on storage and comparison of figure 2*B* with figure 2*A* shows that survival of cultures containing sugars was not improved significantly by the addition of magnesium chloride. Comparable results were found with calcium chloride.

Effect of volume per bottle. The volume of culture lyophilized per bottle was not critical in the presence of 3 per cent lactose. Approximately 50 per cent of the original cells survived in bottles containing between 0.5 and 10 ml of culture.

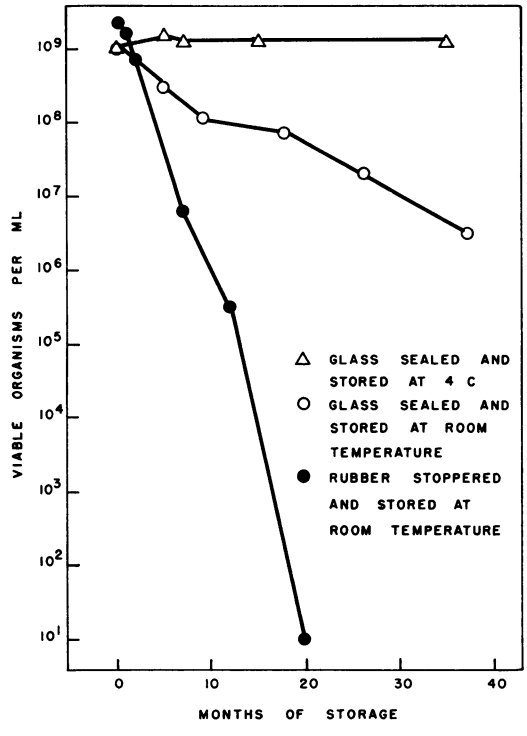

Figure 4. Survival of *Pasteurella pestis* strain A-1122 under various storage conditions. Equal volumes of 6 per cent lactose and culture were mixed immediately before lyophilization. All containers were sealed under vacuum after lyophilization.

Effect of type of container. The viability of lyophilized cultures stored in sealed ampules and rubber stoppered bottles is compared graphically in figure 4. Viability apparently depended on temperature during storage as well as on the closure. In glass sealed vials there was no appreciable loss in viability at 4 C while there was approximately a log loss in 15 months at room temperature. At 6 and 12 months, approximately 100 times more organisms remained viable in sealed glass ampules than in the rubber stoppered bottles; the difference was, presumably, due to the diffusion of moisture through the stopper.

Table 5 summarizes the results of tests during storage for 450 days with 10 different strains of *P. pestis.* Although the average survival on lyophilization was only 18 per cent, there was no significant additional loss on storage of any of the strains.

Virulence of lyophilized cultures. Table 6 presents some results, showing the difference in virulence between cultures stored in glass ampules and in rubber stoppered bottles. Immediately after reconstitution of cultures stored 9 months in rubber stoppered bottles, 7700 organisms constituted an LD_{50} while the LD_{50} of cultures stored 15 months in glass ampules was only 450 organisms.

Of special interest is the observation that, as the interval between reconstitution of the lyophilized cultures and testing was increased (up to 32 hr), the virulence increased, approaching the original virulence of about 15 organisms per LD_{50}.

That this unexpected effect was not peculiar to one strain is shown in table 7. Furthermore, the number of organisms per LD_{50} for guinea pigs as well as for mice

TABLE 5

Survival of various strains of lyophilized Pasteurella pestis during prolonged storage at 4 C

Strain	Viable Cells before Lyophilization* × 10^8 per ml	Percentage Survival after Storage			
		0 day	135 days	195 days	450 days
A-1122	11	14	29	24	22
Java	10	25	30	20	23
K-120	13	16	17	19	22
TRU	4.5	33	42	21	50
Tijwidej	2.3	12	19	8.4	13
53H-1	9.1	8	13	16	7.5
Bombay	22	10	13	7	10
EV 76	6.2	9.0	10	11	8.5
Soemedang	8.8	30	36	15	15
#14	8.7	21	19	13	9.7
Avg	9.7	18	23	16	18

* Six per cent lactose in $\frac{2}{15}$ M phosphate buffer, pH 7.6, was added to an equal volume of each culture immediately before lyophilization in 6 ml Pyrex ampules, 0.5 ml per ampule, and stored at 4 C.

TABLE 6

The effect of storage on viability and virulence of lyophilized cultures of Pasteurella pestis, strain 139L

	Interval between Reconstitution and Titration*							
	0 hr		2 hr		4 hr		32 hr	
	Organisms per ml × 10^7	Organisms per LD_{50}	Organisms per ml × 10^7	Organisms per LD_{50}	Organisms per ml × 10^7	Organisms per LD_{50}	Organisms per ml × 10^7	Organisms per LD_{50}
Before lyophilization	44.0	16	—	—	—	—	—	—
Immediately after lyophilization	4.4	60	5.0	4.5	—	—	—	—
After 3 months in glass ampules†	1.6	370	—	—	1.4	170	—	—
After 6 months in glass ampules†	2.1	220	—	—	2.2	35	—	—
After 15 months in glass ampules†	1.2	450	1.3	325	1.2	200	1.2	43
After 7 months in bottles‡	3.1	1175	2.1	950	1.8	913	2.1	22
After 9 months in bottles‡	1.8	7700	0.6	4000	0.7	1500	1.0	45

* Reconstituted with distilled water and held at about 20 C.

† Lyophilized in 6 ml all-glass ampules, 0.5 ml per ampule and stored at 4 C.

‡ Lyophilized in 60 ml Army style vaccine bottles, 5.0 ml per bottle, and stored at room temperature with rubber closure.

TABLE 7

*Virulence in mice and guinea pigs of lyophilized Pasteurella pestis after 6 months' storage**

Interval between Reconstitution and Titration	Strain								
	139L			499559			Substrain 122 of 139L (streptomycin resistant)		
	Organisms per ml × 10^7	Organisms per LD_{50}		Organisms per ml × 10^7	Organisms per LD_{50}		Organisms per ml × 10^7	Organisms per LD_{50}	
		Mice	Guinea pigs		Mice	Guinea pigs		Mice	Guinea pigs
0 hr	2.1	220	600	1.8	1000	7000	8.4	90	2800
4 hr	2.2	35	590	1.9	170	2000	7.2	250	1000
24 hr subculture	140	6	40	120	4.5	130	220	70	550
Before lyophilization	44	12	32	82	12	—	120	110	—

* The cultures were diluted by adding an equal volume of 6 per cent lactose in 2/15 M phosphate buffer, lyophilized in 6 ml glass ampules, 0.5 ml per ampule, and stored at 4 C.

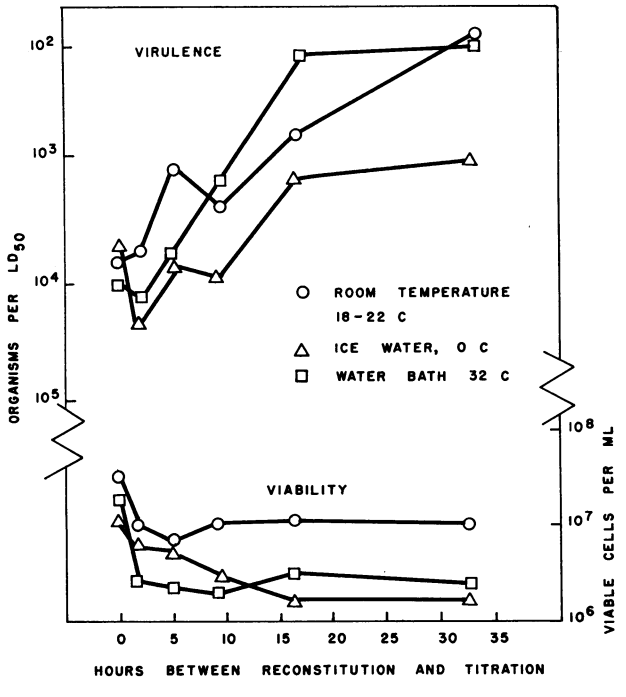

Figure 5. The effect of temperature on virulence and viability of lyophilized cultures of *Pasteurella pestis* strain 139 L at intervals after reconstitution with distilled water. The lyophilized cultures had been stored 9 months at room temperature in rubber stoppered bottles.

usually decreased as the interval between reconstitution and testing was increased. In all instances the virulence of the lyophilized culture was restored to its original value by a single 24-hr subculture in HIB.

Figure 5 presents graphically the effect on viability and virulence of the temperature at which the reconstituted cultures were held. The virulence of the reconstituted cultures held at 0 C increased more slowly than that of those held at higher temperature. However, both 0 and 32 C were inferior to room temperature (18 to 22 C) for maintaining viability of reconstituted cultures.

Discussion

Optimal methods of lyophilizing cultures of *P. pestis* as described in this report may or may not apply to other organisms. For instance, many investigators have found rapid freezing to be suitable for other organisms, but contrariwise, as shown above, slow freezing increased survival of *P. pestis*. The protective effect of sugars likewise differs. Various theories have been proposed to explain the role of sugars and rate of freezing. Fry and Greaves (1951) suggested that the added sugar increased survival by retaining moisture and preventing over-drying of the cultures; however, the studies with *P. pestis* indicated that maintaining

osmotic equilibrium may be an alternative explanation. Osmotic pressure of protein solutions is generally affected by changes in temperature and pH. Since the relative rate of change in osmotic pressure of the bacterial suspension would be decreased by adding sugar it would be expected, and has been demonstrated above, the protective effect of slow cooling is more pronounced in the absence of sugar.

Meryman (1956), in his discussion of the process of freezing and its effect on living matter, indicated that slow freezing in the absence of colloids resulted in a concentration of electrolytes sufficient to injure living cells, whereas, the presence of sugar or glycerin prevented the concentration of these electrolytes. It is important to recognize that what has been referred to in this report as fast freezing is really slow freezing as defined by Meryman. It may be more realistic to consider 3 speeds of freezing: fast, slow, and very slow. Fast freezing would not benefit by the presence of sugar since the rate of freezing is so rapid that only minute crystals form and electrolytes are not concentrated. The very slow freezing would, also, not benefit by the addition of a protective colloid if the rate of freezing were sufficiently slow to permit the cells to adjust to changes in electrolyte concentration. Only the slow (intermediate) rate would be benefited by the addition of sugar as suggested by Meryman (1956).

Throughout this work two separate problems were considered: losses during drying and losses during subsequent storage. Often these losses seem to be correlated, but, as shown in figure 2, optimal conditions for drying were not necessarily optimal for prolonged storage. It was shown that glucose or sucrose could be substituted for lactose without decreasing survival during lyophilization, but significantly more cells survived prolonged storage in preparations containing sucrose or lactose than in those containing glucose. This would indicate that the protection of *P. pestis* during storage by sugars was not entirely dependent on the presence of colloid or moisture.

Wasserman and Hopkins (1957) showed that under certain conditions the number of viable cells recovered from lyophilized *Serratia marcescens* was greater when reconstituted with 0.05 M malate than with water. Therefore it is possible that recovery of viable *P. pestis* might have been generally higher if conditions of rehydration had been optimal. The optimal conditions should define the rate of rehydration, diluent, composition of the plating medium and temperature of incubation as well as temperature and composition of the reconstituting fluid.

The apparent loss of virulence of lyophilized cultures after prolonged storage may not be peculiar to *P. pestis*. Reconstituted cultures are seldom used directly; transplants to growth medium from lyophilized stocks are usually used instead. A possible explanation of our observations may be that lyophilization and storage in the dried state adversely affect the cells without destroying their viability *in vitro*. Such cells require some time to rehydrate or repair sublethal damage and when injected into an animal the majority of such damaged cells are destroyed by the host before they become fully reactivated and functional as infectious units. Hence the lower apparent virulence.

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SUMMARY

Survival of *Pasteurella pestis* during lyophilization was increased comparably by precooling the culture or by adding an equal volume of buffered lactose before snap-freezing. Shell-freezing with or without additives at −78 C resulted in significantly lower survival. Survival was the highest at about pH 7.6 but the concentration of phosphate buffer within limits was not critical. The volume of culture lyophilized per bottle was also not critical; about 50 per cent of the cells survived in 60 ml bottles containing between 0.5 and 10 ml of culture.

Survival of lyophilized *P. pestis* during prolonged storage was affected by moisture and perhaps air, and to a lesser extent by storage temperature. There was no appreciable loss in viability in glass-sealed vials during 3 years' storage at 4 C while there was a log loss per year at room temperature. Viability in rubber stoppered bottles at room temperature decreased by approximately 3 log per year.

Addition of glucose, sucrose, or lactose increased survival during lyophilization. After storage for about 1 month at room temperature, viability of cultures to which glucose had been added decreased as rapidly as those with no additives. After storage for 7 months, less than 10^4 cells remained viable, whereas, 10^7 cells remained in similar cultures to which sucrose or lactose had been added.

The virulence of lyophilized cultures which had been stored in rubber stoppered bottles for 9 months was approximately 10^4 organisms per LD_{50} immediately after reconstitution but less than 10^2 organisms per LD_{50} 24 to 32 hr after reconstitution with distilled water.

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Combined Irradiation-Heat Processing of Canned Foods

II. Raw Ground Beef Inoculated with Spores of *Clostridium botulinum*

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If combined irradiation-heat processing is applied in the canned food processing industry, it could be desirable to preirradiate the products in the raw state. For example, it is conceivable that canned meat might be irradiated at one geographical location and heat processed in another. This idea prompted an investigation of the effectiveness of combined irradiation-heat processing of ground raw beef artificially contaminated with *Clostridium botulinum* 213B spores.

MATERIALS AND METHODS

Lean ground beef was purchased locally from the University of Michigan Food Stores. As soon as the meat was ground, it was spread in shallow enameled pans and placed in an evacuation chamber. Here the dissolved metabolic gases were removed by evacuation to about 25 in. of mercury after which the vacuum was released. This was repeated 3 times. Next the ground beef was packed into no. 1 picnic tin cans, being careful to avoid air pockets. Some of the cans were equipped with Ecklund (1949) thermocouples. The cans of meat were now placed in the evacuation chamber, where the degassing procedure was repeated 3 times. The meat was kept at about 40 F throughout this process. Following degassing, the meat was inoculated at the approximate geometrical center of each can with 1 ml of a spore suspension in distilled water. Finally the cans were sealed in a commercial type vacuum closing machine at a vacuum of about 26 in. of Hg. Experimental cans were then either irradiated or temporarily stored in a refrigerator; controls were immediately placed in an 85 F incubator. Following irradiation, the canned meat was heat processed to the desired F_0 value and then quickly cooled to the incubation temperature by immersion in cold water; after this, the processed experimental cans were also incubated at 85 F. During incubation, all cans were examined daily for gas development as evidenced by swelling. Since *C. botulinum* 213B spores were used as the inoculum, the development of toxin was also used as a criterion for *C. botulinum* growth in some of the cans showing gas production. Mouse inoculation was used for this purpose as previously described (Kempe *et al.*, 1954).

Irradiation was carried out in the "center-well" of the large cobalt^{60} gamma radiation source at the University of Michigan. For this work, eight cans were placed in the "center-well" along with sufficient Dry Ice to refrigerate the canned meat. The irradiation was allowed to proceed for the required time, based upon a gamma ray field intensity of approximately 130,000 rep[1] per hr in the center of the cans at the time these studies were made. Periodic verification of the dosage rate was made by ferrous-ferric sulfate dosimetry (Weiss, 1952) using ferrous sulfate solutions in glass vials placed in the center of the cans (Kempe *et al.*, 1954).

Seven cans at a time were processed in a steam heated autoclave as previously described (Kempe *et al.*, 1957). During heating, the cans were immersed in a pail of water located inside the autoclave. Temperatures at the center of three cans were measured by Ecklund thermocouples; the other four were the experimental cans whose temperature variations were assumed to parallel those found in the cans of meat containing thermocouples. F_0 values were calculated by a graphical modification of Ball's general method (Schultz and Olson, 1940). The Z value was assumed to be 18 for these calculations.

The *C. botulinum* 213B spores used in this study were

[1] One rep is a dosage of ionizing radiation capable of producing energy adsorption of 93 ergs per g of tissue. Ferrous-ferric sulfate dosimetry was based on the oxidation of 15.4 micromols of ferrous ions per L per 1000 rep.