Lyophilization of *Brucella melitensis*

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The preservation of *Brucella melitensis* was of interest because Elberg and Faunce (1957) had shown that a nondependent mutant derived from a streptomycin-independent strain of *B. melitensis* was immunogenic when administered as a living vaccine. Hutton et al. (1951) and, more recently, Alexander and van Drimmelen (1956), showed that lyophilization was practical for preserving the viability of the *Brucella abortus* (strain 19) vaccine. Although lyophilization is a generally accepted method for maintaining viability of bacterial cultures (Harris, 1954), the conditions for the optimal preservation of bacteria by lyophilization are not identical for all species or strains of given species. This report describes the results of limited studies on the lyophilization and storage of a live vaccine strain of *B. melitensis*.

**Materials and Methods**

**Culture.** The nondependent mutant clone (Rev 1-S) was grown on Albimi brucella agar and collected as a thick suspension in 0.85 per cent NaCl solution. The solid medium was employed to minimize reversion to the rough (nonsmooth) colonial form. The cell suspension was diluted with an equal volume of a 6 per cent sugar solution just prior to lyophilization.

**Containers.** Most of the cultures were lyophilized in 60 ml Army style vaccine bottles with exhaust tube stoppers, which were fitted with brass ring clamps to facilitate sealing.

**Lyophilization.** The apparatus and general procedure for lyophilization were essentially those described by Heckly et al. (1958). Cultures were dried for a total of about 22 hr. The temperature of the air surrounding the bottles was about 20°C for the first 4 to 6 hr and then was raised to 32°C overnight. The rate of drying, and hence the ice temperature, was governed primarily by the size of the connection between the culture and the condenser manifold. Generally, the cultures were not cooled or frozen before attachment to the manifold, but were degassed and “snap frozen” in situ by slowly evacuating the system.

**Viability.** The lyophilized cultures were reconstituted with an 0.85 per cent NaCl solution, and the numbers of viable organisms were estimated by surface plating of appropriate dilutions on Albimi brucella agar. All reported values are the average obtained from at least three bottles or ampules with five plates counted per dilution.

**Results**

**Effect of suspending medium.** Lactose, sucrose, and glucose protected the organisms about equally well during freezing and drying. In all instances, about 50

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per cent of the organisms were viable when tested immediately after lyophilization. As shown in figure 1, the viability of the culture with glucose was markedly less than the others when tested after storage for 125 days at room temperature. Because viability of the organisms dried in lactose and sucrose was comparable, and since sucrose can be sterilized by autoclaving without decomposition, the latter was used in all subsequent experiments.

Effect of freezing and drying conditions. The effects of plug and snap freezing and of two drying temperatures (−18 C and −28 C) were compared. Plug freezing at −78 C was effected by immersing the bottles in a bath of Dry Ice and ethanol after they were attached to the manifold, but before the system was evacuated. The bath was removed only after the full vacuum, about 20 \( \mu \) Hg, had been attained. Neither the method of freezing nor the drying temperature had a measurable effect on viability when the samples were reconstituted and tested immediately after lyophilization. However, as shown in figure 2, both the method of freezing and the ice temperature during drying affected the stability of the cells on storage. Viability, when tested 120 days after lyophilization, was best maintained in cultures which had been snap frozen and dried at about −18 C. The greatest loss of viability occurred in plug frozen cultures which had been dried at −28 C. Apparently, the lower temperature rendered the organisms less resistant to the adverse storage conditions. A temperature above −18 C might, therefore, result in an even greater stability. This point was not investigated.

Effect of container. Viabilities of lyophilized cultures stored in glass sealed ampules and rubber stoppered bottles are compared graphically in figure 3. Survival apparently depended more on temperature during storage than on the type of closure. In glass sealed ampules there was no appreciable loss of viability at 0 C, whereas there was approximately a 3 log loss in 110 days at room temperature, and a 6 log loss at 37 C. At 20 C the influence of the glass sealed ampule and rubber stoppered bottles was not significantly different until after a period of 110 days, as shown in figure 3.

The same percentage survivals were obtained when the bottles were either filled with dry nitrogen or sealed under the original vacuum.

Effect on colonial types. All plates were carefully examined to ascertain the colonial morphology. The proportion of smooth to nonsmooth colonies on plates from the reconstituted cultures did not differ significantly from the proportion present in the original culture before lyophilization. The observation that the

![Figure 2](http://aem.asm.org/)

**Figure 2.** Effect of two methods of freezing and of two drying temperatures on the viability of *Brucella melitensis* strain Rev 1-S lyophilized and stored at room temperature in rubber stoppered bottles. Equal volumes of a 6 per cent sucrose solution and culture were mixed immediately before lyophilization.

![Figure 3](http://aem.asm.org/)

**Figure 3.** Effect of storage conditions on the viability of lyophilized *Brucella melitensis* strain Rev 1-S.
colonial properties of the culture were maintained, even
when only 0.001 per cent of the original culture re-
ained viable, indicated that there was no difference
in the resistance of the cell types to lyophilization.

**Discussion**

The results of the experiments on *B. melitensis* de-
scribed above are in general agreement with those ob-
tained on *B. abortus* by Hutton et al. (1951), even
though the methods differed considerably. Hutton's
procedures were not followed because it was desired to
keep the number of operations at a minimum, and
because it had been shown (Heckly et al., 1958) that
simple addition of either a sucrose or lactose solution
to a liquid culture adequately protected *Pasteurella
pestis* during lyophilization and storage. It is significant
that both *B. melitensis* and *P. pestis* survived better
during storage in either sucrose or lactose than in glu-
cose, although in all instances no significant differences
were observed when the cells were tested immediately
after lyophilization.

Alexander and van Drimmelen (1956) reported that
*B. abortus* survived for 1 month when lyophilized in a
medium containing lactose, presumably with refrigeration,
although the storage temperature was not indi-
cated in these experiments. As shown in figure 1,
viability of *B. melitensis* after prolonged storage was
satisfactory only at a reduced temperature, 0°C.

Since many laboratories preserve their stock cultures
by simply freezing them, one may question the value of,
or need for, lyophilization if refrigeration is required.
Lyophilized cultures can be stored at room temperature
for a short period, but the principal advantage over
frozen cultures for long term storage is that little or no
cellular damage would be anticipated if the refrigeration
system failed. It is well known that repeated freez-
ing and thawing is rather detrimental to most organisms
and, specifically, van Drimmelen (1956) showed that
freezing and thawing markedly reduced the viability of
*B. abortus*.

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**Summary**

Survival of cultures of a vaccine strain of *Brucella
melitensis* (Rev 1-S) to which equal volumes of 6 per
cent solutions of lactose, sucrose, or glucose had been
added was comparable when tested immediately after
lyophilization. However, after 125 days of storage at
room temperature, the viability of the culture with
sucrose was markedly less than that of cultures con-
taining either lactose or sucrose.

At 0°C there was no loss of viability of cultures
lyophilized with sucrose after storage for 225 days, but
at room temperature only 0.1 per cent survived, and at
37°C less than 10⁻⁴ per cent of the cells remained viable.
In no instance was there a significant change in the
ratio of smooth to nonsmooth colonial types.

Although neither the freezing nor the drying method
affected viability when tested immediately after ly-
ophilization, viability was best maintained in cultures
which were snap frozen and dried at about −18°C.

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