Preservation by Freeze-Drying and the Stability of Virulence of *Salmonella typhimurium*

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Received for publication 27 March 1963

ABSTRACT

SIMON, ELLEN M. (University of Wisconsin, Madison), K. L. STAHL, AND J. B. WILSON. Preservation by freeze-drying and the stability of virulence of *Salmonella typhimurium*. Appl. Microbiol. **11**:371–376. 1963.—Differences in the ability to withstand freeze-drying were demonstrated among strains of *Salmonella typhimurium*. On the average, the number of viable cells in freeze-dried cultures stored at 5 C for 12 to 18 months was approximately one half as large as that found 24 hr after freeze-drying. The viability in samples stored at higher temperatures declined rapidly and was correlated with the dryness of the sample. The virulence for mice of three strains of *S. typhimurium* did not change appreciably when samples were kept for 1 or 2 years as freeze-dried samples stored at 5 C, or as agar cultures stored at 5 C or at room temperature.

Decreases in virulence of cultures of the salmonellae stored under laboratory conditions have plagued many investigators. The objectives of this study were (i) to obtain quantitative data on the survival of *Salmonella typhimurium* when freeze-dried and (ii) to compare the stability of colonial morphology and virulence of this species when stored under different conditions.

MATERIALS AND METHODS

Apparatus. The apparatus used for freeze-drying bacteria was essentially a "lyophilization" device as described by Flosdorf and Mudd (1935) and was similar to that of Thom and Raper (1945). It consisted of a glass manifold with 12 outlets, each permitting the attachment of glass connections with 3 suboutlets to which 8-mm Pyrex tubes could be attached. For freezing, the 36 tubes were immersed in a partially insulated stainless-steel tank containing a mixture of solid carbon dioxide and methyl Cellosolve. Vacuum was obtained with a Welch Duo-Seal Pump (model 1405-6) and measured with a swivel-type McLeod gauge. The vapor trap contained chips of solid carbon dioxide and a small volume of methyl Cellosolve.

Standard procedure for freeze-drying. An 18-hr culture grown on a slant of brucella agar (Albimi) was harvested in a 0.1% solution of peptone (Difco). Of this suspension, 1 ml was transferred to a Roux bottle, containing nutrient agar, which was incubated at 37 C for 18 hr. The cells were harvested in 6 ml of nutrient broth (Difco) plus 7.5% glucose, unless otherwise indicated. This suspending fluid was chosen because a medium of similar composition was the best of several used by Fry and Greaves (1951). Samples (0.1 ml) of this suspension were transferred to freeze-drying tubes into which cotton plugs were then inserted approximately 5 mm below the top of the tube. After the tubes were attached to the manifold, the samples were placed in a bath at −40 C for 15 min before the vacuum pump was started. In early trials, when a vacuum of 50 μ had been obtained, the temperature of the bath was allowed to rise gradually during the next 3 hr to −5 C. Foaming, however, occurred occasionally with different suspending fluids or dilute suspensions of cells. This could be eliminated by keeping the temperature of the bath at −30 C until the samples appeared to be dry. In later trials, the temperature was kept below −30 C routinely, because the organisms dried in this way survived better during storage. A vacuum of 10 μ was obtained consistently during the drying cycle. The tubes were removed from the bath, dried for another hour at room temperature, and sealed under vacuum with a Hoke-Jewel no. 61 "crossfire-torch attachment."

Plate counts of the original suspensions in nutrient broth plus 7.5% glucose were done in duplicate or triplicate. Freeze-dried samples were reconstituted by introducing a small volume of a 0.1% solution of peptone from a 10-ml blank into the tube by means of a pipette with a drawn-out tip, thus permitting recovery of practically the entire sample. The tube was rinsed again to recover the remaining cells, and plate counts of 10^{-8} and 10^{-9} or 10^{-7} and 10^{-8} dilutions were made. The peptone solution was used as diluent in all freeze-drying experiments.

Statistical evaluation of the freeze-drying procedure. The techniques and conditions that would contribute to variation in quantitative results were analyzed statistically. In each of four trials conducted on different days, two suspensions each of two strains (84 and TM2) of *S. typhimurium* were tested. Plate counts on each of the original suspensions were performed independently by two technicians. In reconstituting samples after storage at 5 C for 24 hr and 1, 2, and 3 months, each technician handled one of the duplicate samples from each group. The results were subjected to an analysis of variance.

Bacterial strains. The sources of the strains of *S. typhimurium* were as follows.

Strain 84, obtained from C. V. Seastone, was isolated

during an epizootic in a mouse colony at the University of Wisconsin in 1952.

Strain LT2 (Zinder and Lederberg, 1952) was obtained from N. D. Zinder.

Strain TM2, originally LT2 (Stocker, Zinder, and Lederberg, 1953), was obtained from D. T. Berman.

Strain SR11, originally BA_2 (Pike and MacKenzie, 1940; Schneider and Zinder, 1956), was obtained from H. A. Schneider. The parent strain will be referred to as SR11-Iln. SR11-S2[(4)]] is a smoother colonial variant isolated from a broth culture of SR11-Iln.

The two strains of *S. typhimurium* with which the major portion of the freeze-drying experiments was done were chosen because of a characteristic difference in colonial morphology when examined with a dissecting microscope and obliquely transmitted white light. Strain 84 produced extremely smooth, convex, pink colonies. Strain TM2 produced larger, more granular, translucent, somewhat flatter, greenish colonies. The in vitro studies with these two strains were well started when mice became available for titrations of virulence. Unfortunately, both strains were avirulent, so that subsequent in vitro work and the in vivo experiments were done with the following strains of *S. typhimurium*: SR11-Iln and SR11-S2[(4)]], both of which are highly virulent for the white Swiss mice used and LT2, a strain of intermediate virulence for these mice.

In vivo experiments. Young, randomly bred white Swiss or inbred C57Bl/6 mice (usually 4 to 6 weeks of age), from the *Salmonella*-free colonies maintained in our laboratory, were used in all in vivo experiments. Unless otherwise stated, the freeze-dried cultures were reconstituted and grown overnight on nutrient agar or brucella agar (Albimi). Inocula were prepared by suspending and diluting the growth in physiological saline. Cultures stored on agar slants were similarly transferred once before preparing inocula.

Intraperitoneal titrations were performed by inoculating groups of from 6 to 20 mice with 0.1 or 0.2 ml of several dilutions chosen to encompass the LD_{50} of the strain as determined in previous experiments. Inocula of highly virulent strains usually included 7 or 8 twofold dilutions calculated so that some animals received 1 cell or less. Inocula of less virulent strains included 4 to 8 tenfold dilutions.

In oral titrations, mice were fed 0.05- or 0.1-ml volumes from a blunt 27-gauge needle after having been deprived of drinking water for 24 hr (white Swiss) or 48 hr (C57Bl/6). Three to five 10- or 100-fold dilutions were employed.

The percentage of mice which died in 21 days was calculated per dose by cumulative totals (Reed and Muench, 1938) and plotted on logarithmic probability paper (Codex Book Co., Norwood, Mass.); the LD_{50} values, 95% confidence limits, and chi-squared values were determined by the method of Litchfield and Wilcoxon (1949).

RESULTS

Statistical analysis of the procedure. The F ratios obtained in the analyses of variance of the freeze-drying procedure in repeated trials with strains 84 and TM2 (Table 1) led to the following conclusions. (i) There is a highly significant difference between the two strains of *S. typhimurium* tested. (ii) Significant differences were not found after freeze-drying of individual strains in trials conducted at different times. (iii) There was no consistent evidence of interaction between strains and trials. (iv) A difference in the number of cells in the original suspensions of one strain which was still apparent 24 hr after treatment disappeared after longer periods of storage, probably because of increased variation within individual samples. (v) A significant difference did not exist between the results obtained by the two technicians in diluting and plating of samples, but the differences between duplicate samples reconstituted after storage at 5 C for 1 month or longer were highly significant.

Effect of bacterial strain on survival of freeze-dried cultures. Further trials with strains 84 and TM2 confirmed the difference in their abilities to survive freeze-drying and subsequent storage. The results of 20 trials in which samples of the two strains were treated simultaneously are shown in Fig. 1. For strain 84, the per cent survival 24 hr after freeze-drying varied from 60 to 140 with a median of 91, and 12 months later the per cent survival ranged from 28 to 91 with a median of 74. For strain TM2, the per cent survival 24 hr after freeze-drying varied from 27 to 67 with a median of 40, and, after 12 months, from 11 to 38 with a median of 19. In some groups of samples reconstituted after storage for 9 to 12 months, we failed to obtain counts from 1, 2, or 3 samples, and these have been ignored in calculating the averages shown in Fig. 1. However, the points have been identified which would have been affected had low enough dilutions been plated to determine the actual number of viable cells. The via-

TABLE 1. *F ratios from analyses of variance of freeze-drying procedures*

Procedure	Degrees of freedom	F ratios				
		Original suspension	After storage			
			24 hr	1 month	2 months	3 months
Strains	1	89.72*	90.29*	62.16*	29.31†	223.34*
Trials	3	54.59*	5.40	1.49	0.78	9.04
Strains × trials	3	0.19	1.29	17.01*	39.9*	1.53
Suspensions in strains in trials	8	2.71†	3.70†	0.26	0.06	0.98
Samples in suspensions in strains in trials	1‡	1.81	0.075	3.24	0.01	0.036
	15	2.6†	1.65	4.27*	19.18*	3.73*

* Highly significant.

† Significant.

‡ One degree of freedom for technicians.

bility of samples stored at 5 C for 18 months was equal to or better than the viability after 12 months.

A number of other strains of *S. typhimurium* and colonial variants derived from stock cultures or from aging broth cultures were freeze-dried, and replicate samples were reconstituted after 1 day and after storage for 6 or 12 months, or both, at 5 C. The data from these experiments have been pooled in Table 2. Here again, some strains were able to withstand freeze-drying and storage better than others. No consistent correlation could be detected between survival and colonial type or parent strain from which the variants were derived. Furthermore, the cultures which survived the freeze-drying process best did not necessarily retain this position after storage.

Results with several strains of *S. abortivoequina* treated in the same manner were essentially the same as those with *S. typhimurium*.

Effect of medium on which cells were grown on survival of freeze-dried cultures. Cells grown on nutrient agar withstood freeze-drying and storage for 6 months better than did cells grown on richer media [brucella agar (Albimi), blood agar base, and Penassay agar]; i.e., 1 day after freeze-drying, 51% of 43 × 10^9 cells grown on nutrient agar were viable, as compared with 19 to 25% of 109 to 145 × 10^9 cells grown on the three richer media. Had the actual number of cells remaining viable been the criterion for judging these trials, rather than the per cent survival, the results would have been different because the suspensions of cells grown on the richer media were more concentrated than were those of cells grown on nutrient agar.

Effect of temperature on survival of freeze-dried cultures. (i) During the drying cycle in the early experiments, most of the killing occurred as the temperature of the bath rose from −5 to + 5 C; i.e., the per cent survival of strain TM2 after freezing at −40 C for 15 min was 94; after reaching −5 C, 80; after reaching +5 C, 22. (ii) The temperature of the peptone solution (5, 29, or 37 C) used for reconstituting and diluting samples did not affect the recovery of viable cells. (iii) In limited experiments, the temperature at which samples were stored affected their survival. Little difference was found between samples stored below 0 C and at 5 C, but at 20 to 25 C and at 37 C viability declined very rapidly. In one experiment in which the original suspension contained 5.2 × 10^{10} cells and 30% remained viable 24 hr after freeze-drying, dilutions from 10^{-3} to 10^{-8} of eight samples which had been stored for 1

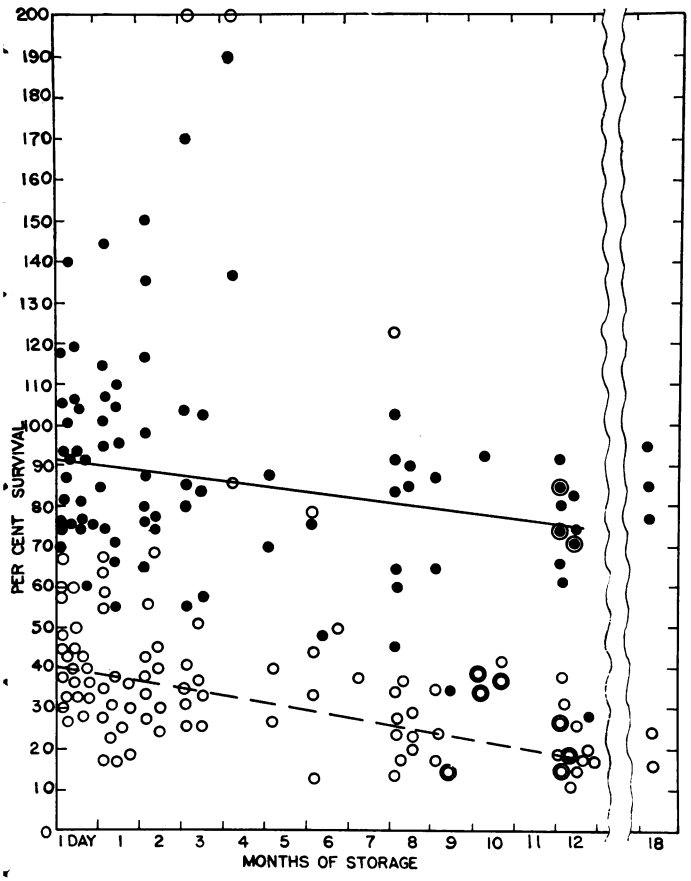

FIG. 1. *Survival of Salmonella typhimurium strains 84 (solid dots) and TM2 (open circles) after freeze-drying and storage at 5 C. Each point represents the average of the samples from a trial reconstituted at the same time. During the first 8 months, only two samples were routinely reconstituted; from 9 to 18 months, the averages represent four to eight samples with very few exceptions. The lines are drawn from the median per cent survival at 1 day to the median per cent survival at 12 months. The solid dots with circles around them and the concentric circles indicate reconstitutions in which the viability in one, two, or three samples had decreased so much that counts were not obtained. These samples were disregarded in calculating the averages.*

TABLE 2. *Survival of Salmonella typhimurium after freeze-drying and storage at 5 C*[a]

Survival	Group I (1 strain, 7 colonial types)[b]		Group II[c] (13 strains, 26 colonial types)[b]		Group III[d] (12 strains, 28 colonial types)[b]	
	Range (%)	Median %	Range (%)	Median %	Range (%)	Median %
At 1 day.....	1.4[e]–43	32	20–93	53	12–130	67
At 6 months..	—	—	8–95	27	8–84	45
At 12 months.	?[e]–27	18	—	—	6–86	29

[a] Number of viable cells in suspensions before freeze-drying varied from 2 × 10^{10} to 10 × 10^{10} per ml. The average number of replicate samples reconstituted for each culture was two at 1 day, usually four to eight at 6 months, and four to eight at 12 months. Percentages reported are means of samples of each culture reconstituted at one time, not of individual samples.

[b] Total number of colonial types tested, including the original type of each strain and variants derived from most of them.

[c] Variation in counts of replicate samples ranged from < two-fold to tenfold at 6 months.

[d] Variation in counts of replicate samples ranged from < 2-fold to 68-fold at 6 months, and upward from < 2-fold (counts on a few samples were not obtained at the dilutions plated) at 12 months.

[e] A nonsmooth strain with which plate counts were highly inaccurate. The per cent survival of the other six variants in this group was 32 or better at 1 day and 8 or better after 12 months.

month at 37 C were plated. No colonies appeared, indicating that the number of viable cells had decreased by a factor of more than 10,000,000. The survival of samples stored at these temperatures was improved by keeping them at −30 C during the freeze-drying cycle until they appeared to be completely dry. In samples treated in this manner, 3% of the cells were recovered after storage for 4 months at 37 C as compared with 47% of those stored at 5 C.

Stability of virulence and colonial morphology during storage. In one experiment designed to study the effect of the actual freeze-drying process on virulence, a suspension of *S. typhimurium* strain SR11-Iln was prepared as usual and freeze-dried. A portion of the same suspension was diluted appropriately, and five groups of 10 white Swiss mice received intraperitoneal inocula ranging from 1 to 24 cells. On the following day, a freeze-dried sample was resuspended and diluted, and mice were inoculated intraperitoneally with doses ranging from 1 to 10^5 cells. An agar slant inoculated with some of the resuspended cells was incubated at 37 C for 18 hr. Groups of mice were again inoculated with doses ranging from 1 to 10^5 cells. The LD_{50} values were as follows: 6 cells for the original suspension with 95% confidence limits of 4 to 10 cells; 12 cells for the freeze-dried culture with confidence limits of 7 to 20 cells; and 70 cells for freeze-dried cells subsequently grown on agar with confidence limits of 10 to 497 cells. As the confidence limits of the three titrations overlapped, it was not possible to show any effect of freeze-drying on virulence, although the culture subsequently grown on agar had a higher LD_{50} and a more variable dose response.

In a series of experiments, the stability of the virulence of three strains of *S. typhimurium* (SR11-Iln, SR11-S2(4), and LT2) was tested during storage under different conditions for periods of 1 or 2 years. One suspension of each strain was prepared and used for the following: freeze-drying 36 samples; titration in mice; and inoculation of agar slant and stab cultures which were stored at room temperature or at 5 C for future comparisons with the freeze-dried organisms. These cultures were titrated periodically by intraperitoneal inoculation in white Swiss mice, and by intraperitoneal and oral inoculation in C57Bl/6 mice when sufficient numbers of this inbred strain were available.

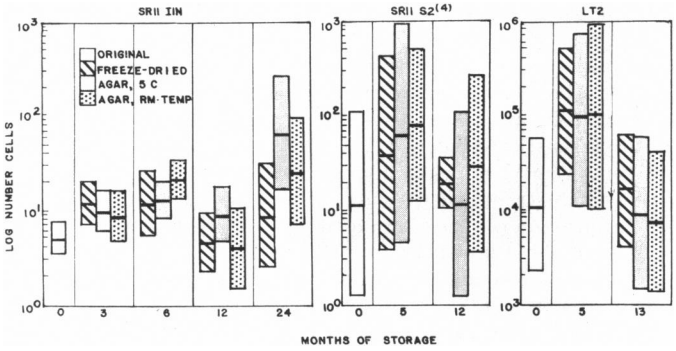

FIG. 2. *Effect of storage of three strains of Salmonella typhimurium on virulence for white Swiss mice which had been inoculated intraperitoneally. The 95% confidence limits are represented by bars and the LD_{50} by a center line in the bar.*

The data in Fig. 2 indicated that the virulence of the three strains, as assessed by intraperitoneal inoculation of mice, was extremely stable whether the strains were freeze-dried or stored as agar cultures. The LD_{50} values of strain SR11-Iln for white Swiss mice ranged from 4 to 66 cells; those of strain SR11-S2(4) from 12 to 80 cells; and those of LT2 from 10^4 to 10^5 cells. The LD_{50} levels of all three strains for C57Bl/6 mice were under 10 cells.

In most experiments in which bacteria were administered *per os* to C57Bl/6 mice, it was not possible to calculate LD_{50} levels from the resulting data, usually because the range of doses chosen was too high. The only titrations in which the three methods of storage could be compared were those of SR11-S2(4) after storage for 5 months. The LD_{50} levels were as follows: 100 cells for the freeze-dried sample, 700 cells for the agar culture stored at 5 C, and 1,000 cells for the agar culture stored at room temperature. The difference in virulence suggested here may not be real, since the confidence limits were wide and overlapped slightly.

In the cultures examined in detail for dissociation (see Table 3), variants accounted for 1% or less of the total population, if they were detected at all. The only exception

TABLE 3. *Dissociation of Salmonella typhimurium after storage under different conditions*

Culture	Time of storage	Condition of storage*	Per cent dissociation	Remarks and colony types of variants
	months			
SR11-Iln	3	FD	<0.1	
	3	5C	<0.1	
	12	FD	<0.1	
	12	5C	0.25	S colonies
	12	RT	2.0	S and I2 colonies in same frequency
	24	FD	<0.1	
	24	5C	1.0	Very small colonies of I3 texture
	24	RT	10.0	Mostly I2, some I3, no S colonies
SR11-S(4)	12	FD	<0.1	
	12	5C	0.5	I colonies
	12	RT	<0.1	
LT2	13	FD	<0.1	
	13	5C	<0.1	50% of colonies were very small on initial streak but showed no difference in size upon restreaking
	13	RT	<0.1	

* FD, freeze-dried, stored at 5 C; 5C, agar slants or stabs stored at 5 C; RT, agar slants or stabs stored at room temperature.

to this was the SR11-Iln agar culture stored at room temperature, which at 12 months was found to contain 2% variants and at 24 months contained 10% variants.

Discussion

Freeze-drying. The statistical evaluation of the procedure used for freeze-drying and further work with *S. typhimurium* showed that different strains of the same species reacted differently to freeze-drying. Record and Taylor (1953) also reported wide variation in the survival of strains of one species (*Escherichia coli*). Fry and Greaves (1951) demonstrated differences in stability to freeze-drying of several bacterial genera. They obtained 100% survival with hemolytic streptococci even with suspending fluids which gave poor results with other organisms. With *S. typhimurium*, they obtained 30% survival 24 hr after treatment and 19% after storage for approximately 20 months. With *Neisseria gonorrheae* and *Vibrio cholerae* the survival was less than 10% 24 hr after treatment and less than 1% after storage.

The accuracy of much of the quantitative data in our work would have been increased had larger numbers of samples been reconstituted to determine the per cent survival at various times after storage. When two samples are counted by use of duplicate plates, as was done in the trials which were analyzed statistically and in much of the early work, the expected variation of the means is three to four times as great as it would be if six samples were counted by using the triplicate plates. The data on reconstitutions performed 1 day after freeze-drying would probably have been altered very little, since preliminary trials in which groups of eight samples were counted showed the deviation from sample to sample to be within the limits of normal variation. However, after storage for 1 month, this variation among individual samples was highly significant, and it continued to increase with time. The means for samples reconstituted between 1 and 8 months represented by the points in Fig. 1 show the effect of increasing variation, as most of them are the averages of only two samples. The means of the larger groups of samples reconstituted after storage for 12 to 18 months show less variation in spite of the greater differences in the counts of individual samples.

Possible explanations for the few points in Fig. 1 which seem unreasonably high are: (i) the chance selection of the one or two samples reconstituted from the tail of the normal distribution due to irreducible experimental error, (ii) a decrease in the number of viable cells in the original suspension before plate counts were completed, (iii) an increase in the number of colony-forming units in the freeze-dried samples by actual multiplication or separation of pairs or small aggregations of cells, and (iv) errors in preparing blanks of diluent, measuring samples, etc.

The factors which distinguish one microorganism from another when placed in an adverse environment, as for example freeze-drying and storage, remain to be determined. It might be assumed that the greater resistance of strain 84 of *S. typhimurium* could be related to the morphology of the cells, which are more nearly coccoid than are the cells of strain TM2, and might be subjected to less physical stress during the freezing and drying procedures. This hypothesis is untenable when other organisms are considered, since smooth strains of *S. typhimurium* did not consistently survive better than others, and Fry and Greaves (1951) obtained extremely poor results with *N. gonorrheae*. The fact that cells of the same strain grown in a relatively poor medium (nutrient agar) survived better than did cells grown on very rich media suggests that a consequent physiological difference may be responsible.

In the practical applications of a technique for preservation of microorganisms, the actual number of cells surviving after long periods of storage should be considered. Although the percentage of cells killed in extremely concentrated suspensions (10^{11} per ml) was greater than in less dense suspensions, the actual number of viable cells remaining in the former was equal to or greater than that in the latter. Data are not available on the survival of these very concentrated samples during storage for periods of longer than 6 months.

The temperature at which freeze-dried cultures were stored was of utmost importance, and the amount of residual moisture affected the keeping quality of freeze-dried cultures, especially at temperatures of 20 C or higher. The data of Fry and Greaves (1951) showed that some moisture was beneficial and suggested that glucose added to the suspending medium ensured the retention of a certain amount of water. Hutton, Hilmoe, and Roberts (1951) found that freeze-dried cultures of *Brucella* containing less than 1% moisture retained viability better than those with a higher moisture content, especially after storage at room temperature. The residual moisture in freeze-dried samples in our work was not determined quantitatively, but it was apparent that samples which appeared to be dry contained more viable cells after storage at room temperature or at 37 C than did other samples. Hutton (1948) and van der Scheer (1956) emphasized the necessity for keeping the temperature of the cultures below −20 C during the drying period, and the survival of our cultures stored at higher temperatures was greatly increased if they were held at −30 C until they were no longer visibly moist. These results, as well as those of Proom (1951), indicated the advisability of storing freeze-dried cultures at the lowest practicable temperature.

Stability of virulence. The in vivo experiments proved that virulence for mice was still a stable characteristic of strain SR11 of *S. typhimurium*, as it was when MacKenzie and his coworkers studied it more than 20 years ago (MacKenzie, Fitzgerald, and Pike, 1935; Pike and MacKenzie, 1940). These experiments showed that the freeze-drying procedure had little effect on the virulence of *S. typhimurium* (strain SR11), and the work of Lambin, German, and Sigrist (1958) with *S. typhi* and *S. paratyphi*

B showed that agglutination, antigenicity, and protective ability of antiserum were not markedly altered by this treatment. Furthermore, virulence of *S. typhimurium* was practically unaffected by storage for 2 years under different conditions. The results with strain LT2 emphasized the importance of the host in any determination of the virulence of a pathogenic organism. Relatively large numbers of cells were required to produce a fatal infection by intraperitoneal inoculation in our white Swiss mice, but extremely small inocula produced the same effect in C57Bl/6 mice. Furness and Rowley (1956) reported an LD_{50} of 17 cells for this strain for "Swiss white mice no. 1 injected intraperitoneally."

The methods of preservation tested apparently did not provide much of a selective advantage to the mutants with altered virulence or colonial morphology which arose in these cultures. If the agar cultures had been transplanted frequently or if the bacteria had been grown and stored in fluid media, the selective conditions would have been altered considerably.

ACKNOWLEDGMENTS

The authors are indebted to Lois M. Jones for calculation of the LD_{50} values and to Martha S. Dopp and Marcia Esposito for technical assistance. This work was supported by contract with U.S. Army Chemical Corps, Fort Detrick, Md.

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