THE EFFECT OF THE INITIAL CELL CONCENTRATION UPON SURVIVAL OF BACTERIA AT −22 C

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That there are conflicting views as to the effect of cold upon bacteria is readily apparent from only a casual perusal of the literature, and no effort will be made here to review the voluminous work on the subject except as it pertains directly to our own research. For a review of the literature dealing with the effect of cold on organisms in general, one may refer to the monograph by Luyet and Gehenio (1940), and for reviews dealing specifically with bacteria, to the dissertations of Major (1953) and McDougal (1954).

We believe that crucial experiments can be devised to attack the main problem—the actual cause of death—only after understanding the effect of the variables inherent in the process of freezing and thawing. Two obvious variables are the species of bacteria and the initial cell concentration. As far as we know, no critical study has been made on the effect of the initial cell concentration upon survival. Rivers (1927), however, did note that the percentage survival of 2 dilutions of *Escherichia coli* in Locke's solution, frozen (−185 C) and thawed (16 to 18 C) 4 successive times, was greater in the less dilute suspension than in the more dilute suspension. No such relationship was observed to occur with broth suspensions.

MATERIALS AND METHODS

The test organism was cultivated in 100 ml of broth in 250 ml Erlenmeyer flasks. The flask cultures were pooled, centrifuged, the spent broth decanted, and the packed cells resuspended in a minimal amount of fresh broth. From this concentrate, using broth as diluent, suspensions of the test organism at a number of different cell concentrations were prepared. Five ml aliquots of the cell suspension were dispensed into sterile, cotton-plugged test tubes (16 mm by 150 mm). The suspensions were frozen by immersion of the tubes in an alcohol bath at −22 C. (The contents of the majority of the tubes had solidified within 5 to 10 minutes; those which were not frozen at this time were seeded with an ice crystal which brought about immediate solidification.) All tubes were then removed from the alcohol bath but kept within the cold chest at −22 C.

The number of viable cells initially present was determined by diluting and plating a 1 ml aliquot from each of 2 tubes of each suspension prior to freezing in the −22 C alcohol bath. After storage intervals of 3 days, 1 week, 2 weeks, 4 weeks, and 6 weeks, 2 tubes of each suspension were removed from the cold chest, thawed in a water bath at 34 C (this required about 3 minutes), and a 1 ml aliquot from each tube was diluted and plated. All platings were made in duplicate. (In some experiments additional counts were made after storage for 1 day, 8 weeks, and 10 weeks.)

The following bacteria were employed as test organisms: *Lactobacillus acidophilus*, strain 18L-2, *Lactobacillus fermenti*, strain 69L-3, *Micrococcus pyogenes* var. *aureus*, strain 72L-1, *Escherichia coli*, strain 69L-15, *Pseudomonas aeruginosa*, strain 60L-4, *Microbacterium flavum*, strain OJ 10, *Chromobacterium orangium*, strain ATCC 7319, *Serratia marcescens*, strain ATCC 274, *Bacillus coagulans*, strain NRS 784, *Bacillus pumilus*, strain NRS 236, and a strain of *Salmonella gallinarum*. The first 5 cultures have been characterized in detail by Harrison (1952) and Harrison and Hansen (1954); *M. flavum* was obtained from the collection of Orla-Jensen, *C. orangium* and *S. marcescens* from the American Type Culture Collection, the 2 bacilli from the collection of Nathan R. Smith at the U. S. Department of Agriculture, and the strain of *S. gallinarum* from the Vanderbilt Medical School.

A broth of the following percentage composition was used for the cultivation of *E. coli*, *S. gallinarum*, *M. pyogenes*, *M. flavum*, *C. orangium*, and the 2 bacilli: yeast extract (B.B.L., dehydrated), 1.0; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.01; and "tween 80" (Atlas Pow-

der Co.), 0.1. In the case of *P. aeruginosa* and *S. marcescens*, respectively, 1.3 per cent peptone (Difco) and 1.0 per cent trypticase (B.B.L., dehydrated) were employed *in lieu* of the yeast extract. The 2 lactobacilli were cultivated in broth of the following percentage composition: yeast extract, 1.0; trypticase, 1.0; glucose (anhydrous), 2.0; $CaCO_3$, 1.0; K_2HPO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.01; and tween 80, 0.1.

The plating media were of the same composition as the respective broths, except that agar (1.5 per cent) was an additional ingredient, and in the case of the lactobacilli the calcium carbonate was omitted.

For resuspending the bacteria after centrifugation, broths of the same composition as the culture broths were employed, except in the case of the lactobacilli where the storage broth lacked calcium carbonate.

S. marcescens was incubated at approximately 26 C; the *Chromobacterium*, *Microbacterium*, and 2 bacilli at 32 C; whereas the other 6 species were incubated at 37 C. All bacteria were incubated for about 24 hours, which ensured that growth was well within the maximum stationary phase.

Only a small fraction of the cells of *B. coagulans* forms spores, and by means of parallel spore counts (obtained by heating an aliquot of the suspensions to kill the vegetative cells) corrections for spores were made since in this work we have been interested only in the survival of vegetative cells. Strain 236 of *B. pumilus* under our experimental conditions is asporogenous.

RESULTS AND DISCUSSION

In figure 1 are constructed the survival curves obtained upon storing *L. fermenti* and *E. coli* at a number of different cell concentrations. Before discussing the effect of the initial cell concentration upon survival, we would like to call attention to the general shape of these curves.

Observe that in all the curves the negative slope is initially steep, representing rapid destruction of the cells, but with the passing of each increment of time the slope decreases, representing slower and slower rates of death; after a time (2 to 3 weeks in the case of *E. coli*, but only 2 days with *L. fermenti*), the curves approximate horizontal lines, indicating that the frozen suspensions have become relatively "static". (That the curvature of the survival curves is smooth and continuous to the initial point was verified in several short-term experiments with *E. coli* and *L. fermenti* in which platings were made after storage intervals of 2, 6, 10, 24, 48, and 72 hours.)

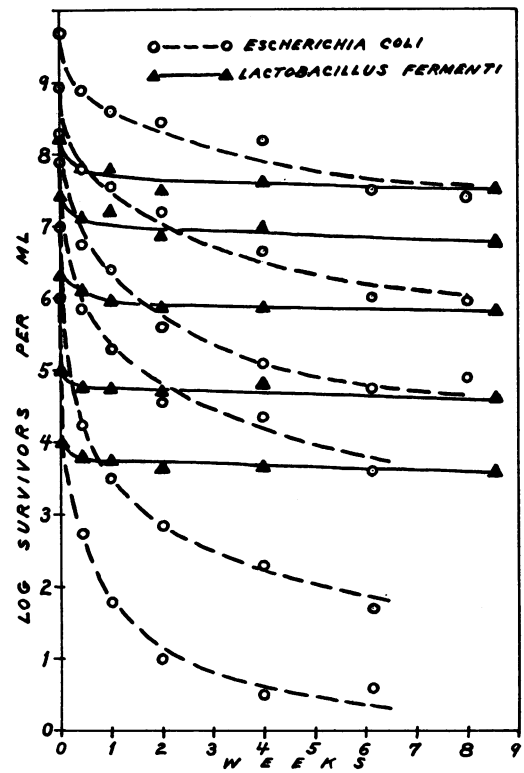

Figure 1. Survival of *Escherichia coli* and *Lactobacillus fermenti* when suspended in broth at various cell concentrations and stored at −22 C.

In order that survival curves represent the true picture of death versus time, the test organism must exist in the suspensions as single, discrete cells—not in clumps or chains. For this reason, not all bacteria are suitable as test organisms. The survival curves obtained from bacterial suspensions containing many aggregates of cells would be expected to vary from the type seen in figure 1 since plate counts would not represent cell counts. Furthermore, a longer time would be required to destroy an aggregate than a single cell; thus, on the curve, death would appear to be delayed. Moreover, the process of freezing and thawing might break up some aggregates, thereby concealing further the destruction of individual cells. Such survival curves, therefore, could be expected to show first a period of no death or even a slight rise in count, then would begin to slope downward, and,

finally, would assume the shape of the normal survival curve. The curves obtained with our strain of *M. pyogenes* are of this form. The period of apparent no death lasts several days; then for about 2 weeks the death rate appears to increase, but after 2 weeks the curves assume the usual form with an ever decreasing negative slope, and by 4 weeks the curves have become practically horizontal lines. The other 10 test organisms give undistorted curves of the type in figure 1.

The survival curves obtained by Harrison *et al.* (1952) with a number of different species stored at −25 C and also at −60 C as broth suspensions, as concentrates in physiological sodium chloride, and as packed cells are, in the vast majority of cases, the same type as those in figure 1.

Contrariwise, Tanner and Williamson (1928) have stated unequivocally that "the rate of death follows the curve of a monomolecular reaction, the death rate being proportional to the number of living cells". Hence, plotting logarithms of survivors versus time should result in a curve which is a straight line. Four species of bacteria were stored at −13 to −15 C in broth and also in physiological sodium chloride. When one plots their data, it is apparent that 2 of the resulting curves (*E. coli* in sodium chloride and *S. marcescens* in broth) are of the same form as our survival curves (figure 1). Two other curves constructed from their data (*S. marcescens* in sodium chloride and *B. subtilis* in broth) are of the same form as we obtained with *M. pyogenes*. Obviously, none of these curves represents the curve of a first order reaction. Admittedly, the survival curves of *B. subtilis* in sodium chloride, *Bacillus mesentericus* in broth and in sodium chloride, and *E. coli* in broth are straight lines although the points in the latter instance are scattered widely. However, the degree of sporulation by the bacilli was not mentioned by Tanner and Williamson, and it may be that the manner of dying of spores and vegetative cells is quite different. At any rate, it appears to us that the conclusion stated by these authors is not in accord with their data.

Haines (1938) stored *E. coli*, *P. aeruginosa*, and *M. pyogenes* at a number of different subfreezing temperatures. The survival curves obtained with the first 2 organisms at −1 to −5 C were of the same shape as we always obtain (figure 1). However, at storage temperatures of −10 C and −20 C Haines obtained curves which approximate straight lines. On the other hand, his strain of *M. pyogenes* gave survival curves like those in figure 1 at all subfreezing temperatures—from −1 to −20 C. It should be pointed out that his experimental technique differs somewhat from ours. He used a different suspending medium (water), stored the suspensions in smaller volumes (0.1 ml), and froze them at a lower temperature (−78 C) prior to storage. Although we have varied our technique in numerous ways, we have always obtained curves of the type in figure 1. We are investigating this matter further.

In reference to the effect of the initial cell concentration upon survival, it may be observed in figure 1 that in the case of *L. fermenti* the relative drop in viable cell count is much the same regardless of the initial count. On the other hand, with *E. coli* the drop in count is inversely proportional to the initial count, or, stated another way, the fraction of the cells initially present which survive a given storage interval is proportional to the initial cell concentration. This relationship may be expressed as follows:

$$\frac{[B]}{[B_0]} \propto [B_0] \quad \text{or} \quad [B] = k[B_0]^2$$

where [B] is the number of viable cells per ml after a given storage interval, $[B_0]$ is the number of viable cells per ml initially present, and k is a proportionality constant. That k is indeed constant over a wide range of initial cell concentrations is apparent in table 1 where the values for k have been calculated for a 4 week storage interval in one experiment with *E. coli*. These k values show fairly good agreement, especially when the limitations of the plate count technique are kept in mind. In all calculations we have arbitrarily selected 4 weeks as a common point of reference; actually, any other time interval on the relatively flat portion of the curves would probably serve as well. In replicate experiments with *E. coli* the average value of k has varied as much as 5-fold, indicating that there are variables affecting the relationship equated above which, in spite of our efforts towards a uniformity in technique, are not held constant from one experiment to another; however, in any single experiment the values for k show good agreement.

If the value of k remains the same at very

TABLE 1

Survival of bacteria in broth suspensions at various initial cell concentrations after 4 weeks' storage at −22 C

Test Organism	$[B_0]$ (Initial Viable Cell Count per Ml)	$[B_4]$* (Viable Cell Count per Ml after 4 Weeks)	$\frac{[B_4]}{[B_0]}$	$k = \frac{[B_4]}{[B_0]^2}$
Escherichia coli	4.8×10^9	8.0×10^7	1.7×10^{-2}	3.5×10^{-12}
	9.1×10^8	3.2×10^6	3.5×10^{-3}	3.8×10^{-12}
	1.9×10^8	1.3×10^5	6.8×10^{-4}	3.6×10^{-12}
	8.3×10^7	1.6×10^4	1.9×10^{-4}	2.3×10^{-12}
	9.8×10^6	2.1×10^2	2.1×10^{-5}	2.1×10^{-12}
	1.0×10^6	4.0×10^0	4.0×10^{-6}	4.0×10^{-12}
Salmonella gallinarum	5.7×10^9	2.5×10^7	4.4×10^{-3}	7.7×10^{-13}
	5.7×10^8	3.1×10^5	5.4×10^{-4}	9.5×10^{-13}
	5.7×10^7	3.2×10^3	5.6×10^{-5}	9.8×10^{-13}
Serratia marcescens	3.7×10^8	2.2×10^7	5.9×10^{-2}	1.6×10^{-10}
	3.6×10^7	1.7×10^5	4.7×10^{-3}	1.3×10^{-10}
Micrococcus pyogenes var. *aureus*	3.6×10^9	1.8×10^9	5.0×10^{-1}	—
	3.6×10^8	1.1×10^8	3.1×10^{-1}	—
	3.6×10^7	8.3×10^6	2.3×10^{-1}	—
	3.6×10^6	5.6×10^5	1.6×10^{-1}	—
Bacillus pumilus	1.0×10^9	9.5×10^7	9.5×10^{-2}	—
	9.2×10^6	1.9×10^5	2.1×10^{-2}	—
	1.1×10^5	1.3×10^3	1.2×10^{-2}	—
Lactobacillus fermenti	1.1×10^8	4.3×10^7	3.9×10^{-1}	—
	2.2×10^7	8.7×10^6	4.0×10^{-1}	—
	2.2×10^6	7.3×10^5	3.3×10^{-1}	—
	1.1×10^5	4.7×10^4	4.3×10^{-1}	—
	1.1×10^4	4.5×10^3	4.1×10^{-1}	—
Lactobacillus acidophilus	7.9×10^9	2.5×10^9	3.2×10^{-1}	—
	4.2×10^8	1.3×10^8	3.1×10^{-1}	—
	6.9×10^7	1.9×10^7	3.6×10^{-1}	—
	7.8×10^6	1.8×10^6	4.3×10^{-1}	—
Microbacterium flavum	5.2×10^8	1.0×10^8	1.9×10^{-1}	—
	5.2×10^6	1.0×10^6	1.9×10^{-1}	—
	5.5×10^4	1.0×10^4	2.0×10^{-1}	—
Chromobacterium orangium	3.4×10^8	1.4×10^8	4.1×10^{-1}	—
	3.5×10^6	1.7×10^6	4.9×10^{-1}	—
	3.4×10^4	2.0×10^4	5.9×10^{-1}	—
Bacillus coagulans	2.0×10^8	2.0×10^7	1.0×10^{-1}	—
	1.8×10^6	2.5×10^5	1.4×10^{-1}	—
	1.8×10^4	2.5×10^3	1.4×10^{-1}	—

* In those cases where the actual count falls above or below the smooth curve, $[B_4]$ has been estimated from the curve.

high initial cell concentrations, one might expect to be able to obtain 100 per cent survival by setting $[B_0]$ equal to the reciprocal of k, since then [B] would equal $[B_0]$. We have not tried this due to the technical difficulties in acquiring a sufficient volume of viable cells at a concentration of 3×10^{11} per ml—the concentration which would be required with *E. coli*. In this connection,

though, Harrison *et al.* (1952), upon centrifuging broth suspensions of *E. coli*, decanting the clear supernatant, and storing the cell "paste" at −25 C, obtained after 4 weeks survivals close to 100 per cent.

In table 1 the results of most of our experiments have been briefly summarized. It may be seen that *S. gallinarum* and *S. marcescens* manifest the same sort of survival as does *E. coli*. The survival of *P. aeruginosa* likewise improves with increasing initial cell concentrations, but we do not have sufficient data with this organism to make accurate calculations for the value of k and have not included it in the table.

L. acidophilus, *M. flavum*, *C. orangium*, and *B. coagulans* manifest the same sort of survival as does *L. fermenti*. With these bacteria, $[B_4]/[B_0]$ is constant and independent of the initial cell concentration. It is also apparent from the table that these organisms store much better than the first 3 species tabulated.

Unlike the other gram positive bacteria, *M. pyogenes* and *B. pumilus* show increasing survivals with increasing initial cell concentrations although the correlation is only slight. In the case of *M. pyogenes*, with each 10-fold increase in initial count, $[B_4]/[B_0]$ increases roughly 1½-fold. This relationship may be approximated by the following equation:

$$\frac{[B_4]}{[B_0]} = \frac{\log [B_0] - 5.5}{8.5}.$$

While we were still engaged in our work, a paper by Record and Taylor (1953) was published in which it was noted with 2 strains of *E. coli* that percentage survival to freeze-drying is proportional to the initial cell concentration; however, no such relationship was found to exist in the case of simple freezing-thawing. For suspending the cells they used phosphate buffer, and freezing was carried out at −78 C. The reason Record and Taylor did not observe the dependence of $[B]/[B_0]$ upon $[B_0]$ may be explained in our experiment summarized in table 2. In this experiment we suspended washed cells of *E. coli* in distilled water rather than in the customary broth. The nature of the suspending medium affects survival greatly, since with water the proportion of the cells surviving is much greater, and the effect of the initial cell concentration is less pronounced and slower in manifesting itself. The length of time Record and Taylor held their frozen suspensions prior to thawing was not indicated in their paper, and we have inferred that there was no appreciable storage period—that the suspensions were thawed immediately or shortly after freezing. In table 2 it is apparent that with water the effect of the initial cell concentration upon the proportion of the cells surviving is not detectable until after a lapse of time, and this may be the case also with phosphate buffer.

Even with broth as the suspending medium it is possible to greatly improve survival and to minimize the influence of the initial cell concentration, at least with *E. coli* and *S. marcescens*. This may be done by cultivating these bacteria under forced aeration or on a shaking apparatus. Percentage survivals then are of the same magnitude as obtained with the gram positive species.

SUMMARY

Eleven species of bacteria were stored in broth at various cell concentrations at −22 C. When the test organism occurred as single, discrete cells, plotting logarithms of survivors versus the storage interval always gave survival curves of the same general form. Initially, the negative slope of the curves is steep, representing rapid destruction of the cells, but with the passing of each increment of time the slope

TABLE 2

Survival of Escherichia coli when suspended in distilled water at 2 initial cell concentrations and stored at −22 C

$[B_0]$	Stored 10 Minutes		Stored 1 Week		Stored 4 Weeks	
	$[B]$	$\frac{[B]}{[B_0]}$	$[B]$	$\frac{[B]}{[B_0]}$	$[B]$	$\frac{[B]}{[B_0]}$
4.5×10^8	4.0×10^8	0.89	2.1×10^8	0.47	1.8×10^8	0.40
4.0×10^6	3.6×10^6	0.90	5.5×10^5	0.14	3.2×10^5	0.08

decreases, representing slower and slower rates of death until after a few days or weeks (depending upon the organism) the slope becomes negligible.

Escherichia coli, *Salmonella gallinarum*, *Serratia marcescens*, and *Pseudomonas aeruginosa* manifested a percentage survival which varies in proportion to the initial cell concentration. Likewise, *Micrococcus pyogenes* var. *aureus* and *Bacillus pumilus* also manifested a percentage survival which varies in proportion to the initial cell concentration, although in this instance the relationship is not as pronounced. On the other hand, *Lactobacillus fermenti*, *Lactobacillus acidophilus*, *Microbacterium flavum*, *Chromobacterium orangium*, and *Bacillus coagulans* manifested a percentage survival which is constant and independent of the initial cell concentration.

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