Viability and Estimation of Shelf-Life of Bacterial Populations[1]

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ABSTRACT

DEARMON, IRA A., JR. (U.S. Army Chemical Corps, Fort Detrick, Frederick, Md.), MICHAEL D. ORLANDO, ALBERT J. ROSENWALD, FREDERICK KLEIN, ALBERT L. FERNELIUS, RALPH E. LINCOLN, AND PAUL R. MIDDAUGH. Viability and estimation of shelf-life of bacterial populations. Appl. Microbiol. **10**:422–427. 1962—Mathematical concepts associated with the exponential and probit models are developed, and the similarity of the two methods is discussed. Because of its greater flexibility in design, the probit method was used to estimate the shelf-life for four bacterial populations, wet and dry spores of *Bacillus anthracis* and wet and dry cells of *Pasteurella tularensis*. On the basis of data gained by storing these organisms at high temperature, the probit method was used to predict the time at which 50% viability would occur for cells stored at 3 C. A plane passing through a three-space showing change in percentage viability of bacteria was formulated by a multiple regression method. With this functional technique, the percentage viability, expressed as a probit, was linearly related to a logarithm of storage time and storage temperature. The use of this method to study the effect of controlled variables on the viability of cells is demonstrated by comparing the effect of viability associated with three additives used prior to drying. The results of the test gave shelf-life estimates which were too low for all additives; however, the order of stability was ranked properly as confirmed by long-term tests.

When an essential characteristic of a product is viability, the length of time the product remains useful will bear some relationship with the percentage of cells remaining viable. The time of useful life or shelf-life may, in fact, be defined as the time at which a given per cent of the original population remains viable after storage under designated conditions. Viability of cells is of greatest interest in stock-culture repositories and in breweries, creameries, and other fermentative industries interested in maintaining viable stock cultures. With live vaccines again coming into wide medical use, viability will determine how frequently the vaccine needs to be replaced; therefore, the shelf-life will determine the amount of product that should be produced during any period of operation. Occasionally, information on changes in the viability of a culture for use in experiments requiring sampling through time may be needed. It is, therefore, desirable to know for these products, as for any viable material requiring surveillance, the length of time the cells will remain viable and how viability will be influenced by environment. This knowledge can be acquired directly by observation or indirectly by prediction.

Most stock-culture work has been conducted on a qualitative basis, and, on this basis, stock of many species of microorganisms are known to remain viable through 20 or more years. Among the literature reports giving quantitative data on stability of bacterial preparations during storage are those of Riley and Solowey (1958) for survival of *Serratia marcescens* at 5 C; Mead et al. (1960) for stability of *Pasteurella pestis* at 5 and −23 C; and Heckley, Anderson, and Rockenmacher (1958) and Heckley, Faunce, and Elberg (1960), who reported increased survival of freeze-dried *P. pestis* and *Brucella melitensis* when carbohydrates were added to cultures prior to drying.

Employing empirical techniques, thermal-inactivation levels of *Bacillus anthracis* and *B. subtilis* var *niger* spores were interpolated and extrapolated from the experimental range of 50 to 90 C by Fernelius et al. (1958). The same empirical approach used by Fernelius et al. (1958) can readily be expanded into a device for estimating the shelf-life of bacterial populations. The shelf-life end point selected for study in this paper is 50% viability, but other end points may be appropriate for other work. For example, in stock culture depositories an end point of less than 1% viability might be adequate.

In this paper, we consider three variable factors (storage time, storage temperature, and per cent survival), the interrelation of which will provide information for predicting shelf-life in advance of the time at which the desired end point is reached. The study of these interrelationships had a threefold purpose: (i) verification of the mathematical concepts associated with the probit method (Fernelius et al., 1958); (ii) investigation of techniques designed to accelerate the effects of storage on various bacterial populations, thereby providing rapid information on which shelf-life predictions could be based; and (iii)

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development of experimental designs for use in the study of shelf-life end points.

Materials and Methods

Wet B. anthracis and B. subtilis var. niger spores. The methods for growing and enumerating these test organisms and exposing them to heat have been described previously (Fernelius et al., 1958).

Dry B. anthracis spores. Growth conditions were the same as those for the two preceding organisms. Spore suspensions were concentrated by centrifugation, resuspended in distilled water, and 1-ml volumes were freeze-dried in 17-ml vaccine bottles by a procedure described by Monk et al. (1956). Dry spore preparations were exposed to heat by placing the vaccine bottles in ethylene glycol baths held at 80, 90, or 100 C. To maintain dryness, the samples were not removed from the bottles prior to heat treatment. At predetermined time intervals, samples were removed, 10 ml of gelatin-phosphate diluent added to the dry spores, and the resulting suspensions shaken for 20 min on a Kahn-type shaker. Viable spores were enumerated by the method described for wet *B. anthracis* spores.

Wet P. tularensis cells. Ten separate cultures of *P. tularensis* were grown in a casein acid digest medium as described by Hodge and Metcalfe (1958). Samples from each culture were packaged in 4-ml amounts in polyethylene ampules and stored at constant temperatures of 37, 26, 15, 4, 3, and 0 C for time periods varying between 30 min and 111 days. The 37-C storage facilities consisted of water baths, and lower temperature equipment consisted of air incubators or refrigerators. Viable-cell determinations were made by a plate-count method.

Dry P. tularensis cells. These cells were grown in the same manner as described for wet *P. tularensis* cells, then concentrated in a Servall SS-1 centrifuge at 23,500 $\times$ g for 7 min. Cells were suspended in: additive A, a mixture of skim milk and sucrose; additive B, a mixture of peptone, ammonium chloride, and thiourea; or additive C, a modification of Naylor and Smith's (1946) additives. Samples (1 ml) of cells plus additive were freeze-dried by the method used to freeze-dry *B. anthracis* spores. All dry materials were stored under vacuum in desiccator jars until sampled for viability. The storage temperatures investigated were 37, 32, 27, 15, 4, 3, and -18 C, and sampling times varied from 1 day to 1 year. Dry *P. tularensis* samples were reconstituted with 10 ml of gelatin-saline diluent, shaken on a Kahn-type shaker for 10 min, serially diluted, plated, and counted after 72 hr of incubation.

Mathematical Concepts

Exponential model. The model of the simple exponential death curve for bacteria is well known, and a derivation was given by Clark (1933). The model can be expressed as

$$N = N_0 10^{-kt} \tag{1}$$

where N is the number of survivors at time t, N_0 is the initial number, and k is a constant, namely, the rate of decrease in log number with time, and is related to a specific experimental condition. When the experimental conditions are held constant, except for controlled changes in storage temperature, then k is a function of storage temperature and

$$k = A.10^{bT} \tag{2}$$

where A is an arbitrary constant and b is the rate of change that relates k and T, the storage temperature.

Expression 2 is the form used by Rahn (1932) to derive the temperature coefficient expression by the relation of temperature and k from which the Q_{10} was evolved.

Experimental model of storage time and storage temperature. By substitution for k in equation 1, an expression in N, t, and T gives the form of an experimental model relating storage time to storage temperature for any degree of viability:

$$N/N_0 = 10^{-t(A\ 10^{bT})} \tag{3}$$

Whenever N/N_0, the proportion of cells remaining viable, is a specific value of interest for any given experimental condition, the equation (3) can be expressed as

$$t = -\log (N/N_0)/A\ 10^{bT}$$

where, if

$$F = \frac{-\log N/N_0}{A}$$

$$\log t = \log F - bT \tag{4}$$

The very important constant b can be estimated experimentally by obtaining a least-squares fit from data in the functional form; mathematically, this rate of change can be written as

$$b = \frac{\log t_2 - \log t_1}{T_2 - T_1} \tag{5}$$

where the subscripts 1 and 2 represent corresponding points of log t and T from the fitted curve. (One must be cognizant of the signs of the rate of changes when going from the mathematical model to data fitting.) The data of Bigelow and Esty (1920), in terms of t and T, fitted this model, as did the data of Fernelius et al. (1958), when $0.95 > N/No > 0.05$.

Probit model. The probit model is of special interest, not only for obtaining shelf-life estimates but also for its value in experimental design, since the probit model permits simultaneous interpretation in more dimensions than the previously described model.

Plots of data have indicated that the probit per cent recovery is linearly related to the logarithm of time for a fixed temperature and is also linearly related to temperature for a fixed time. Thus, the joint effect of time and temperature might be represented by

$$Y = B_0 + B_1 \log t + B_2 T \tag{6}$$

where Y is probit ($100\ N/N_0$) or probit per cent viability; t is storage time; T is storage temperature (C); and B_0, B_1, and B_2 are the unweighted partial regression coefficients and constants estimable from experimental data in the given transformation. Finney (1952), in studying toxicity, used a similar representation for time and concentration of the toxic agent. This representation is consistent with the theory of log normal distribution of survival times as given by Withell (1942) and defended by Irwin (1942). It can be shown mathematically that, when ($100\ N/N_0$) is taken as the shelf-life proportion of live microorganisms required to be viable at time t for any controlled temperature, then equation 6 becomes

$$\log t = \frac{Y - B_0}{B_1} - \frac{B_2}{B_1} T$$

or

$$t = D\ 10^{-bT} \tag{7}$$

where D is a composite constant equal to $10^{\frac{[Y-B_0]}{B_1}}$ and $b = B_2/B_1$, the rate of change relating $\log t$ and T. It can be shown from equation 7 that

$$b = \frac{d \log t}{dT}$$

hence, for a special case

$$b = \frac{\log t_2 - \log t_1}{T_2 - T_1} \tag{8}$$

Equation 5 is equivalent to equation 8, and the constant b can be estimated from the partial regression coefficients B_1 and B_2 of equation 6; thus, the Q_{10} also can be determined from the partial regression coefficients. This model, relating the viability of microorganisms as a simultaneous function of time and temperature of storage, was fitted very well by the data observed by Fernelius et al. (1958). When an estimate of b and D are available, shelf-life estimations (t) can be made directly.

Regarding experimental designs, the model represented by equation 6 is useful, since, with equation 6 plus an appropriate interaction term, not only can independence of time and storage temperature be tested, but weights, B_1 and B_2, can be estimated for each respective component. Thus, a test for the interaction between time and temperature can be made directly in controlled experimental designs by using the probit model.

Equation 6 plus an appropriate interaction term leads immediately into factorial experimental designs, storage time and storage temperature being two factors studied primarily for their independence, and the mean being a third factor studied for, say, its estimate of stabilization.

RESULTS

The results of the present work are first given in the form of a number of prediction equations obtained from the probit mathematical model and the observed data on the viable recovery of microorganisms. Prediction equations for shelf-life estimations were obtained for wet and dry *B. anthracis* spores and wet and dry *P. tularensis* cells. Secondly, data are presented to illustrate the use of an accelerated storage technique and the usefulness of data obtained by the method to extrapolate to the storage properties of viable microorganisms.

The t given in the prediction equation refers to a storage time which is associated with the so-called shelf-life of the microorganism, which has been defined as the time at which 50% viability occurs. The symbol T refers to the storage temperature in degrees centigrade.

Wet B. anthracis spores. The results of exposure to high temperatures were given by Fernelius et al. (1958). The constants B_0, B_1, and B_2 for calculating shelf-life, using the probit model, are given in Table 1. Using these constants, the prediction equation for the shelf-life is

$$t = 2030 \cdot 10^{-0.0951\,T} \tag{9}$$

where t is storage time in years and T is storage temperature in degrees C. For temperatures of 3 C, the shelf-life is predicted to be 620 years. This estimate is an extrapolation of data observed at high temperature, and it is doubtful if the prediction will be confirmed. We have had samples of wet *B. anthracis* spores in the laboratory for approximately 5 years at 27 C and have observed no

TABLE 1. *Observed constants of the probit model associated with four bacterial populations and predicted and actual shelf-life at 3 C*

Bacterial populations	Estimates of partial regression coefficients[a]			Range of storage conditions		Shelf-life at 3 C	
				Time	Temperature[b]	Predicted	Observed
	B_0	B_1	B_2	*days*	*C*		*days*
Wet *Bacillus anthracis*[c]	26.02	−2.39	−0.228	0–43	*50, 60, 70, 80, 90*	620 years[d]	[e]
Dry *B. anthracis*	24.41	−2.41	−0.149	0–4	*80, 90, 100*	146 years[d]	[e]
Wet *Pasteurella tularensis*	17.66	−2.59	−0.121	0–111	0, 4, 15, *26, 37*	33 days	38
Dry *P. tularensis*	15.69	−1.84	−0.122	0–365	3, 15, 18, *27, 37*	254 days	279

[a] Based on storage time expressed as minutes.
[b] Italicized temperatures were used in prediction of shelf-life.
[c] Data taken from paper of Fernelius et al. (1958).
[d] Subject to experimental error of at least ±45%.
[e] Shelf-life not observable.

decrease in viability of the spores. To determine how well the predicted shelf-life, based on viability when stored at high temperature, corresponds to actual shelf-life, at the desired temperatures, sets of comparable value are included in Table 1.

Dry B. anthracis spores. The mean percentages of original concentration remaining viable after storage at three temperatures for a number of time periods are shown in Table 2. These data were used to estimate the constants, B_0, B_1, and B_2 (see Table 1), in the probit model by multiple regression analysis. By use of these constants and equation 6, the predicted shelf-life for storage at any temperature can be calculated from the following:

$$t = 208 \cdot 10^{-0.0617 T}, \quad (10)$$

where t is storage time in years and T is storage temperature in degrees C. Solving the equation for T at 3 C, the shelf-life is estimated to be about 165 years and the Q_{10} estimated to be 4.1.

Wet P. tularensis cells. In determining the constants for the probit model for wet *P. tularensis*, ten cultures were utilized. Eight were cultures with viable-cell concentrations ranging from 26×10^9 to 36×10^9 cells/ml, and two were concentrated by centrifugation to about 67×10^9 cells/ml. All ten samples were stored at six different temperatures, and each sample was observed in a time sequence depending on the storage temperature. The mean percentage viability of all samples at each temperature for each observed time is shown in Table 3. However, for each sample, constants for the probit model were computed and, because these independent constants were homogeneous, a mean B_0, B_1, and B_2 was obtained (Table 1).

By use of the established constants, B_1 and B_2 (based on all the data), the shelf-life of each sample of material was predicted for storage at 3 C based on observations made at 37 and 26 C. This predicted shelf-life was compared with the observed shelf-life as shown in Table 4. Tests of significance indicate the mean predicted shelf-life of all ten materials is not significantly different from the observed mean shelf-life at 3 C.

The prediction equation for estimating the shelf-life of wet *P. tularensis* was determined to be

$$t = 53.7 \cdot 10^{-0.046 T} \quad (11)$$

where t is storage time in days, and T is storage temperature in degrees C. Thus, for a storage temperature of 3 C, the predicted shelf-life is approximately 39 days and the Q_{10} is estimated to be 2.9.

Dry P. tularensis. Dry cells of *P. tularensis*, suspended in additive A (skim milk and sucrose), were stored at five temperatures and for various time periods within each temperature. The percentages of cells remaining viable at the several storage periods are given in Table 5. From these data, we first estimated values of B_1 and B_2 as 1.83 and 0.12, respectively, then, using viable responses for storage at 37 and 27 C, we derived a tentative B_0:

$$B_0 = \bar{Y} + 1.83 \log \bar{t} + 0.12 \bar{T} \quad (12)$$

where $\bar{Y}$ is mean probit per cent viability, $\log \bar{t}$ is the mean

TABLE 2. *Per cent viability of dry Bacillus anthracis spores after storage at indicated temperatures*

Storage temp	Storage time	Number of samples	Mean viability of spores[a]
C	*min*		%
100	10	3	84.7
	20	3	94.8
	30	6	82.6
	60	6	74.2
	120	6	23.3
	180	3	20.1
	300	3	4.7
90	60	6	94.4
	120	6	89.1
	180	3	87.7
	240	3	76.4
	300	3	85.1
	420	3	48.0
	480	3	45.6
	1,440	2	2.0
80	120	2	92.7
	255	2	89.1
	435	5	91.5
	1,440	3	34.8
	1,890	3	45.6
	2,880	3	14.0
	4,320	3	8.1

[a] Based on mean probit of individual observations.

TABLE 3. *Observed mean viability of ten Pasteurella tularensis cultures stored at indicated temperatures*

Storage temp	Storage time	Mean viability[a]
C	*days*	%
37	⅓	96.7
	1	51.2
26	1	93.2
	2	85.3
	4	19.5
	8	1.2
15	4	83.9
	8	59.5
	16	47.4
	34	14.5
3	16	74.9
	34	68.1
	67	28.4
	93	23.8
0	34	75.8
	67	25.0
	93	18.5
	111	8.6

[a] Mean is based on mean probit of individual samples.

log of storage time, and $\bar{T}$ is the mean storage temperature. By using equation 6 with B_1, B_2, and tentative B_0, an extrapolation was made for shelf-life at 3 C, which was predicted to be 254 days. Actually, the observed shelf-life at storage of 3 C was 279 days; thus, the predicted and observed shelf-life correspond favorably. The general relation of shelf-life of dry *P. tularensis* and storage temperature was estimated to be:

$$t = 434 \cdot 10^{-0.067T} \quad (13)$$

where t is storage time in days, and T is storage temperature in degrees centigrade. The Q_{10} for the dry *P. tularensis* cells was estimated to be 4.6.

It was tentatively concluded that data collected on cells of dry *P. tularensis* stored at relatively high temperatures can be used to predict shelf-life for material stored at a lower temperature. On the extrapolation to −18 C, the shelf-life would be predicted to be about 12 years, provided linearity of the function was unaffected by passage through the freezing point of the material. A single observation made after 1 year of storage at −18 C (Table 5) indicated that dry *P. tularensis* had a viability of 70%. By using equation 6 and the constants B_0, B_1, and B_2, it can be predicted that the viable recovery expected at −18 C for 1 year of storage is about 98%. This observation indicates that extrapolation too far outside the experimental data may be of great risk; therefore, extensive extrapolation might be reserved for planning of experimental conditions to be used in collecting data.

Accelerated-storage technique in experimental work. An example of the use of the accelerated-storage technique illustrates its usefulness in experimental work. We used this concept to determine whether any of three additives, introduced prior to drying, would change the storage life of *P. tularensis* cells (Table 6).

Based on the accelerated-storage technique, a decision was made at the end of a 4-day experiment in favor of additive B. Storage at 3 C through 1 year confirmed the decision in favor of additive B as well as the order of superiority of additives C and A. Based on data observed at 37 and 32 C, shelf-life of the *P. tularensis* cells with the various additives was predicted as follows: B, 584 days; C, 243 days; and A, 148 days. Although the predictions were lower than the values actually observed, they were in the proper order, and, because they were obtained after only 4 days of observation, tentative decisions could be made that allowed experimental work to proceed.

An experimental design to study the effect of additives on cell stability is illustrated by the arrangements of

TABLE 4. *Predicted and observed shelf-life of Pasteurella tularensis cultures and concentrates*

Culture number	Predicted shelf-life for 3 C[a]	Observed shelf-life at 3 C
	days	*days*
1	36	53
2	26	36
3	35	44
4	24	44
5	37	34
6	28	56
7	39	44
8	26	31
9	35	29
10	47	25
Mean (geometric)	33	38

[a] Based on data observed at 37 and 26 C.

TABLE 5. *Observed viability of dry Pasteurella tularensis cells suspended in additive A (skim milk and sucrose) and stored at various temperatures*

Storage temp	Storage time	Mean viability
C	*hr*	%
37	24	64.1
	48	50.0
	72	31.9
	144	13.4
	204	4.9
27	48	79.7
	72	70.6
	144	55.6
	204	35.2
	504	15.4
15	144	94.8
	204	91.0
	504	84.2
	984	65.0
	1,400	43.7
3	984	93.2
	1,400	80.0
	3,840	64.1
	4,800	59.0
	8,760	45.0
−18	8,760	70.0

TABLE 6. *Observed viability of dry Pasteurella tularensis cells dried with three additives and stored at high and low temperatures*[a]

Storage temp	Storage time	Mean viability associated with indicated additive		
		A	B	C
C	*days*	%	%	%
37	1	43.7	69.2	52.4
	2	26.4	62.2	28.8
	4	11.0	56.0	23.6
Mean		25.0	62.6	34.1
32	1	64.8	92.1	78.8
	2	46.4	79.4	65.2
	4	30.2	70.9	48.4
Mean		46.8	82.3	64.9
3	365	45.3	58.7	52.0

[a] All values are based on the mean probit value from two independent trials.

temperatures and time sequence in Table 6. Decisions relative to additives made after only 4 days of testing were confirmed by follow-up studies. Our observations support use of the probit model when studying behavior of cells under stress. A test for the homogeneity of B_1 and B_2 is an integral part of this model. When B_1 and B_2 are found to be either nonhomogeneous or to be in interaction with each other, decisions related to various stresses are not directly interpretable. Second- and third-order interactions may be very informative, and an analysis of variance should be made for this information.

Discussion

Two models, the so-called exponential and probit, have been discussed mathematically. Both seem to be similar and both have practical value in the study of viability of microorganisms with respect to storage time and storage temperature. The probit model has been especially useful, having characteristics preferable to the exponential model, because weights in the form of partial regression coefficients can be attached independently to the two major sources of stress (time and temperature). Furthermore, the ratio of the two regression coefficients is associated with the temperature coefficient of the product under study and from which the Q_{10} can be estimated. In addition, the probit model is well adapted to experimental designs of the factorial type in which a number of combinations of variables and their effect on viability could be investigated simultaneously.

The relationship between stability, storage time, and storage temperature in our studies differed for each of the four bacterial populations studied. This relationship is characterized by the partial regression coefficients, B_0 for stability, B_1 for log of storage time, and B_2 for storage temperature.

It appears that the predicted values of shelf-life correspond quite well with those actually observed in those cases for which observations are available. Therefore, one concludes that this general method of approach (i.e., use of high temperatures and short observation times) may be generally useful in the estimation or prediction of shelf-life at lower temperatures.

One application of this technique was demonstrated in screening the effect of additives on the stability of dry *P. tularensis* cells. Based on a 4-day testing procedure, decisions were made relative to the order of superiority of three additives and these decisions were substantiated by actual observations on *P. tularensis* stored at refrigerator conditions. By use of this accelerated-storage procedure, in which the storage temperature differed by only 5 C, the predicted shelf-life itself underestimated the actual shelf-life observed. It is believed that this technique has wide adaptability in experimental work; however, more experience is needed to determine the method's usefulness with regard to any specific problem.

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