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Recovery of Biological Indicator Organisms after Sublethal Sterilization Treatment

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ABSTRACT: An important factor affecting the recovery of biological indicators (BI's) is the time elapsed between exposure to a sterilant and growth testing. The length of time and the temperature at which the BI's are stored after sterilization were found to significantly affect growth. BI's stored at 20–25 °C for 48 hr prior to testing showed a 90% reduction in growth when compared to those that had been refrigerated at 2–8 °C for the same time period. This was consistent for B. subtilis *and* B. stearothermophilus *spores and independent of the type of sterilization process studied.*

Introduction

A biological indicator is a calibrated preparation of microorganisms used to monitor or validate the effectiveness of a sterilization process. The growth response of a biological indicator is routinely used by manufacturers of sterile products to demonstrate the efficacy of a sterilization process prior to product release (1–3).

Factors affecting the growth or recovery of biological indicators after a sterilization process should be evaluated and, when necessary, controlled. For example, Pflug et al. (4) showed that using different lots of soybean casein digest medium can cause the number of *B. stearothermophilus* organisms recovered after heating to increase or decrease. Obviously, with a decreased recovery count, the resistance of the organism would be underestimated and the efficacy of the sterilization process overestimated.

The objective of this study was to determine the effect of storage time and temperature on the recovery of biological indicators after a sublethal exposure to a sterilizing agent. The elapsed time between exposure of the biological indicator to the sterilization cycle and growth testing is defined as the post-sterilization hold time.

Materials and Methods

Biological Indicators

Bacillus subtilis var. niger (globigii) spores on paper carriers prepared at Travenol Laboratories, Morton Grove, IL, and from a commercial vendor were used as test samples in this study. The *Bacillus stearothermophilus* spores in glass ampules were purchased from a commercial vendor.

D-Value

A *D*-value analysis was performed on each organism under each test condition using previously published methods (5–7).

Sublethal Sterilization (SST)

All biological indicators used in this study received a sublethal dose of one specific sterilizing treatment. By using the *D*-value and the log of the initial population of spores (N_0) per biological indicator, the amount of time necessary to reduce the N_0 to approximately 1–3 survivors per carrier was calculated. This level was chosen to ensure approximately 95% positive growth units per total units tested in

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TABLE I. Effect of Incubation Temperature on Recovery of *B. subtilis* (globigii)

Optimum Incubation Period

Days	32°C	37°C	Days	32°C	37°C
1	2.5%[a] 18[b]	9.3% 47	8	99.7% 708	99.0% 501
2	83.7% 594	86.4% 437	9	99.7% 708	99.2% 502
3	94.2% 669	93.6% 474	10	99.8% 709	99.4% 503
4	96.2% 683	96.2% 487	11	99.8% 709	99.6% 504
5	97.7% 694	97.4% 493	12	99.8% 709	99.8% 505
6	98.4% 699	98.0% 496	13	99.8% 709	100% 506
7	99.3% 705	98.6% 499	14	100% 710	100% 506

[a] Percent positive growth relative to 14-day incubation period

[b] Number of positive growth tests from 1000 tested

a fraction negative recovery procedure. It was assumed that the majority of surviving spores would be stressed but viable organisms.

Sterilizing Parameters

The sterilizing parameters used in this study were:

1. Ethylene oxide 600 mg/liter ± 20 with 60% ± 10 relative humidity at 54 °C ± 1
2. Saturated steam at 250 °F ± 1

Research vessels at Travenol Laboratories were used to deliver these parameters.

Storage Conditions

After the SST treatment duplicate size samples of each biological indicator were stored at 2–8 °C and 20–25 °C for varying post-sterilization hold times prior to recovery.

Incubation and Recovery

Fraction negative (growth/no growth response) recovery procedures were used in all experiments. All *B. stearothermophilus* indicators were incubated under the conditions recommended by the manufacturer. *B. subtilis* (globigii) biological indicators were incubated for 14 days at 32 °C. This temperature was chosen because according to the results of previous unpublished experiments, recovery percentage is improved at 32 °C over that achieved at 37 °C, the temperature normally used. Data substantiating this finding are summarized in Table I.

Residual Ethylene Oxide

The amount of residual ethylene oxide on paper carriers was determined using an acid hydrolysis/GC procedure.

Results

Bacillus subtilis (*globigii*) *and Ethylene Oxide*

Effect of Post-Sterilization Hold Time—Six hundred biological indicators each of sample type A and B composed of paper carriers inoculated with 10^6 *B. subtilis* (globigii) spores were exposed to SST with ethylene oxide gas as described above. One hundred biological indicators of each type were tested for growth immediately after the SST treatment, while the remaining biological indicators were stored at 20–25 °C. Equal sample sets (100 of each type) were tested for growth daily on five consecutive days.

The data as shown in Figure 1 indicate that, as the post-sterilization hold time at 20–25 °C was increased, the number of biological indicators positive for growth decreased to less than 10%. This was true for both types of biological indicators tested.

Effect of Post-Sterilization Hold Temperature—Eleven hundred sample type A

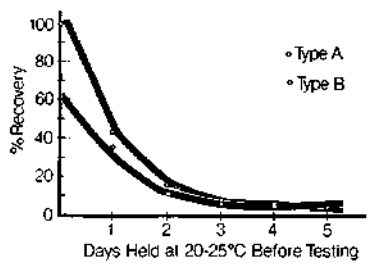

Figure 1—Graph of the effect of post-sterilization hold time at 20–25 °C using two types of B. subtilis (*globigii*) *biological indicators.*

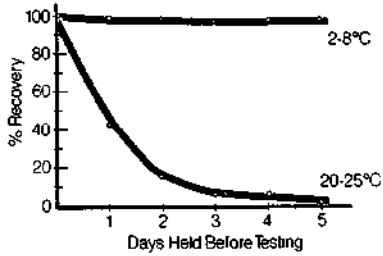

Figure 2—Graph of the effect of post-sterilization hold time at temperatures of 2–8 °C and 20–25 °C on B. subtilis (*globigii*) *biological indicator type A.*

biological indicators were exposed to the SST conditions described above. Immediately after the exposure, 100 biological indicators were tested for viability. The remaining 1000 biological indicators were divided into two groups. One group was placed in storage at 2–8 °C and the other at 20–25 °C. On five consecutive days, thereafter, 100 biological indicators stored at 2–8 °C and an equal number stored at 20–25 °C were tested for growth.

The data in Figure 2 show that biological indicators stored under refrigeration for up to 5 days prior to testing, had a 97% positive growth rate, while biological indicators held at 20–25 °C for 5 days produced only 2% positives.

The growth/no growth data obtained from these two studies were used to calculate *D*-values for the biological indicators. The *D*-values are summarized in Table II. There was no change in the *D*-values calculated for biological indicators stored up to 5 days at 2–8 °C. Biological indicators stored at 20–25 °C exhibited a decrease in *D*-value. For example, biological indicator type A had a *D*-value of 2.3 min when tested 5 days after exposure, as compared to a *D*-value of 3.0 min when tested 24 hr after exposure. Under these conditions, indicator sample type A would no longer meet the necessary resistance requirements listed in USP XIX, in which a minimum *D*-value of 2.5 min is suggested for the cycle parameters utilized in this study.

TABLE II. Effect of Post-Sterilization Hold Temperature on the *D*-Value in Minutes of *B. subtilis* (globigii) Biological Indicator, Type A

STORAGE TEMP.	DAYS OF STORAGE 1	2	3	4	5
2-8°C	3.4	3.5	3.4	3.4	3.4
20-25°C	3.0	2.8	2.6	2.6	2.3

TABLE III. Concentration of Residual Ethylene Oxide (Micrograms per Unit) after Storage at 2–8 °C and 20–25 °C

		2-8°C		20-25°C	
Days→	0	1	2	1	2
Type A	39	48	39	33	27
Type B	12	8.3	0.7	5.4	2.7

Effect of Residual Ethylene Oxide—An analysis of ethylene oxide residuals was performed on each type of biological indicator after exposure to determine whether residual EtO had an effect on recovery. As can be seen in Table III, the amount of residual EtO present at any time or temperature tested was minimal. The low EtO levels could not have affected lethality during the post-sterilization hold time. This suggests that different organisms subjected to an SST treatment using other modes of sterilization could have a similar response to post-sterilization storage.

Bacillus stearothermophilus *and Steam (250 °F)*

Effect of Post-Sterilization Hold Temperatures 20–25 °C and 2–8 °C—One hundred and sixty *B. stearothermophilus* spore biological indicators were subjected to SST with saturated steam at 250 °F as described previously. Forty units were tested for growth after SST while the remaining biological indicators were divided into two groups and stored at either 20–25 °C or 2–8 °C prior to growth testing at 1, 2, and 7 days after sterilization.

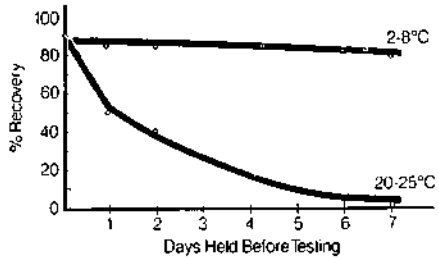

Figure 3—Graph of the effect of post-sterilization hold temperatures of 2–8 °C and 20–25 °C using ampules of B. stearothermophilus *biological indicators.*

As shown in Figure 3, the viability of the biological indicators, measured as percent of recovery, was unaffected if they were refrigerated. Without refrigeration, a significant decrease in viability was seen. This would effect a decrease in *D*-value similar to the one observed for *B. subtilis* (globigii). These data coupled with the above data for a different organism and SST treatment indicate that the effect of post-sterilization storage conditions on viability was independent of either the organism or the sterilant tested.

Conclusion

Three factors were shown to affect recovery of the biological indicators tested.

1. Growth testing incubation temperature
2. Post-sterilization hold time
3. Storage temperature after sterilization

Therefore, to ensure that the biological indicator response is a valid representation of the efficacy of the sterilization process, there should be adequate control over the biological indicator recovery system.

References

1. Bruch, C. W., "Guidelines for Sterilization of Intraocular Lenses by Manufacturer," Paper given at Conference on Effect of GMP on Industry, Madison, WI, Oct. 24–26, 1976.
2. Caputo, R. A., Odlaug, T. E., Wilkinson, R. L., and Mascoli, C. C., "Biological Validation of a Sterilization Process for a Parenteral Product Fractional Exposure Method," *J. Parenteral Drug Assoc.*, **33**, 214–221 (1979).
3. Frieben, W. R., Kreiger, R. A., Juberg, D. L, and Enzinger, R. M., "Validation of Steam Sterilization Cycles Used for Sterile Processing Equipment," *J. Parenteral Drug Assoc.*, **32**, 249–257 (1978).
4. Pflug, I. J., Smith, B. M., and Christensen, R., "Effect of the Lot of Soybean Casein Digest Medium on the Number of *Bacillus stearothermophilus* Spores Recovered," Paper given at British Spore Group Meeting, Cambridge University, England, Sept. 18–21, 1979.
5. Pflug, I. J., and Odlaug, T. E., *Syllabus for an Introductory Course in the Microbiology and Engineering of Sterilization Processes*, 3rd ed., Environmental Sterilization Services, St. Paul, MN, 1978.
6. Pflug, I. J., and Schmidt, C. F., *Thermal Destruction of Microorganisms in Disinfection, Sterilization and Preservation*, Lea & Febiger, Philadelphia, PA, 1976.
7. Stumbo, C. R., Murphy, J. R., and Cochran, J., "Nature of Thermal Death Time Curves for P.A. 3679 and *Clostridium botulinum*," *Food Technol.*, **4**, 321 (1950).

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