Reprinted from
BULLETIN of the PARENTERAL DRUG ASSOCIATION
MAY–JUNE, 1976 – Vol. 30, No. 3

Thermal Resistance of Bacillus Stearothermophilus Spores Suspended in Parenteral Solutions*

IRVING J. PFLUG, GERALDINE M. SMITH, MIRA SCHEYER, and PAUL A. CHAPMAN
University of Minnesota
Minneapolis, Minnesota

ABSTRACT: The wet heat destruction characteristics of Bacillus stearothermophilus spores suspended in four parenteral solutions and Sorensen's M/15 phosphate buffer were evaluated by both end-point and survivor curve methods.

The spores exhibited the largest D(121°C)-values in Water for Injection and Sorensen's buffer. The D-values were about one minute less for 5% Dextrose in Water and 5% Dextrose in Lactated Ringer's solution. The lowest D-values were in 5% Dextrose in Saline (.9%).

The effect of initial numbers on destruction characteristics of Bacillus stearothermophilus spores suspended in Water for Injection and heated at 121°C was determined. The initial inoculum was of the order of 10^5, 3 x 10^6 and 3 x 10^7 spores per replicate unit. The results suggest that the initial number of spores per replicate unit does not appear to affect the destruction rate.

Bacillus stearothermophilus spores have many attributes as biological indicators; they have high heat resistance are generally nonpathogenic and are relatively easy to culture. However, they are sensitive to many environmental factors. Brown (1) found that a lower percentage of *Bacillus stearothermophilus* spores germinated when they were heated in M/10 phosphate buffer than when they were heated in distilled water. Buhlmann, Gay, and Schiller (2) found that *Bacillus stearothermophilus* spores suspended in physiological saline had an appreciably higher heat resistance than when they were suspended in distilled water.

One method of monitoring the heat sterilization process received by bottles of parenteral solution is to inoculate directly the parenteral solution. At least two questions quickly arise: 1) what is the effect of the parenteral solution on the survival characteristics of the spores, and 2) is there an effect of spore concentration? This study was carried out to develop data that would help answer these questions.

In this study the wet heat resistance of *Bacillus stearothermophilus* spores suspended in four parenteral solutions and M/15 Sorensen's phosphate buffer (pH 7.0) was determined. Both fraction negative and survivor curve spore

*Presented at the Annual Meeting of the Parenteral Drug Association in New York City on November 1, 1974. These studies were supported in part by DHEW/FDA Contract No. 72-306. The work is represented in Scientific Journal Series Paper No. 9054, Minnesota Agricultural Experiment Station, St. Paul, Minnesota 55108.

survival tests were carried out in addition to preliminary work to determine the volume of recovery medium to be used in fraction negative tests and the effect of the initial number of spores per replicate unit on spore survival characteristics.

MATERIALS AND METHODS

Spores

Spores of *Bacillus stearothermophilus* coded PCIF were used in all tests. The PCIF spores were grown from a culture of NCA 1518 spores originally supplied to our laboratory by Dr. C. F. Schmidt of Continental Can Co., Chicago, Illinois.

The spores were grown on nutrient agar supplemented with 5 ppm manganese. Incubation was at 55°C for 48 hours. The spore crop was cleaned by repeated washings with distilled water and centrifugation. The working spore suspension was in distilled water and storage was at 4°C.

Preparation of Commercial Parenteral Solution

The manufacturer's specifications for the commercial solutions are presented in Table I.

Screw cap test tubes, 18 x 150 mm, whose cap liners had been removed and replaced with neoprene gaskets were sterilized by heating in an autoclave at 121°C for 30 minutes and then dried in an oven at 125°C for 30 minutes. The sterile tubes were transferred to a Class 100 clean room for filling.

The bottle of parenteral solution was aseptically opened and 2 or 5 ml of solution was added to each sterile tube using a Cornwall 10 ml repetitive pipetting syringe. Enough tubes were prepared to complete one heating test plus two tubes to be used for determining the pH of the solution. The pH of each solution was measured using a Beckman Zeromatic pH meter before heating and after the longest heating time.

In the event that sufficient parenteral solution remained in the bottle to run a second test, the rubber stopper was aseptically replaced in the bottle, the top of the bottle covered with sterile aluminum foil and the bottle stored in the clean room. The filled tubes were stored in the refrigerator until used.

TABLE I

Manufacturer's Specifications for Parenteral Solutions Used In Heat Resistance Studies

Solution	Approx. pH	Cal per liter	Milliequivalents per 1000 ml				
			Na^+	K^+	Ca^{++}	Cl^-	HCO_3^- Precursor
Sterile Water	5.7						
Dextrose, 5% In water	5.0	170					
Dextrose, 5% In Lact. Ringer's	5.1	179	130	4	3	109	Lact. 28
Dextrose, 5% In Saline (0.9%)	4.7	170	154			154	

Preparation of M/15 Sorensen's Phosphate Buffer

The Sorensen's phosphate buffer (pH 7.0) was prepared by mixing 61.1 ml of a stock solution of M/15 disodium phosphate (Na_2HPO_4) with 38.9 ml of a stock solution of M/15 potassium acid phosphate (KH_2PO_4). To minimize variability between the buffer and parenteral solutions, stock solutions were prepared using Water for Injection. Two ml or 5 ml were pipetted into each tube and then the filled tubes were autoclaved for 15 minutes at 121°C. The tubes were stored at 4°C until used.

The Effect of the Volume of Trypticase Soy Broth on the Outgrowth of Heated Bacillus stearothermophilus Spores

The procedure we proposed to use in performing fraction negative tests was to suspend *Bacillus stearothermophilus* spores in 5 ml of parenteral solution. After heating, a measured amount of single strength BBL Trypticase Soy broth (TSB) plus .04% bromcresol purple indicator would be aseptically added to each tube and the tubes incubated for two weeks at 55°C.

Tests were carried out to determine the optimum amount of Trypticase Soy broth to be added to give the lowest number of negative tubes after heating.

About 10^5 spores were added to 5 ml of M/15 Sorensen's phosphate buffer (pH 7.0) in 150 x 18 mm screw-cap test tubes. The tubes were heated in a miniature retort at 121°C. The heating time was chosen to give both positive and negative tubes. In some of the tests two different volumes of broth were added to the tubes while in other tests three different volumes of broth were evaluated. When two broth volumes were evaluated, 16 replicate tubes were used making a total of 32 tubes per test. When three broth volumes were evaluated, 15 tubes of each volume were tested for a total of 45 tubes. Since all of the tubes could not be heated at the same time in one retort, the tubes heated in any one retort were divided equally among the media volumes to be evaluated. In this way, the tubes in any one test all received exactly the same heat treatment regardless of the volume of media that was being added.

After heating, the measured amount of Trypticase Soy broth was aseptically added to each tube of spore suspension. In the test program 5, 10, 15 and 20 ml amounts of broth were added to 5 ml of spore-buffer solution. The tubes were incubated at 55°C for two weeks and scored as either being positive (growth) or being negative (no growth).

Procedure for the Study to Determine the Effect of Spore Concentration

The spores were suspended in 5 ml of Water for Injection in screw cap test tubes. The initial spore concentrations in each experiment were about 1.0×10^5, 3×10^6, and 3×10^7 spores per tube. The inoculum volume was .05 ml. Three replicate tubes were used at each test condition. Tubes from all three spore concentrations were heated at the same time in the same retort whenever possible. Duplicate experiments were carried out; each experiment consisted of three survivor curves.

The tubes were heated in the miniature retort at 121°C. After heating, the tubes were cooled in an ice bath for 1.5 minutes, and then taken to the clean room for processing.

A 1.0 ml aliquot from each tube of the heated solution was appropriately diluted in Butterfield's phosphate buffer (pH 7.2) and duplicate 0.1, 1.0, and 10 ml aliquots of the dilution were pipetted into 100 mm diameter plastic petri plates. BBL Trypticase Soy agar (TSA) medium was used; 30 ml of single strength was added to plates inoculated with 0.1 and 1.0 ml aliquots and 25 ml of 1.5 strength TSA was added to plates inoculated with 10 ml aliquots.

Tests were carried out to enumerate the number of surviving organisms as this number approached one per tube. This was done so we would have plate counts near the fraction-negative portion of the survivor curve. Our procedure was to plate the entire 5 ml of solution. Four 1.0 ml aliquots were pipetted into four petri plates and 30 ml of TSA added. Thirty ml of TSA were then added to the parenteral solution remaining in the tube and the contents poured into a fifth plate. The counts from all five plates were added together for the total plate count of surviving organisms in the 5 ml of heated solution. Plates were incubated at 55°C for 48 hours and the colonies counted with the aid of a Bactronic colony counter.

Survivor Curve Procedures for Study of the Five Solutions

A distilled water suspension of spores (0.05 ml) was added to each tube containing 5 ml of parenteral solution 5 minutes before heating was started. Three replicate tubes were heated at each time interval and three unheated tubes were processed to obtain the N_0 count. In the first series of survivor curve tests performed, 10^5 spores were added per tube. This number of spores proved to be too low for solutions where the spores had low heat resistance; therefore, in the second series of survivor curve tests, 10^6 spores were added per tube.

The heating, cooling, and recovery of the spores were the same as described above.

Fraction Negative Procedures for the Study of the Five Solutions

For the first series of fraction negative tests performed, the spores were suspended in 5 ml of parenteral solution. The results of these tests showed a definite difference in the heat resistance of the spores suspended in the various solutions. The possibility existed that these differences were caused by the effect of the solution present in the incubated tube on the outgrowth of the spores rather than by the effect of the solution on the spores during heating. A tube containing 5 ml of solution plus 20 ml of Trypticase Soy broth has a parenteral solution concentration of 20%. A study was carried out to determine if the presence of parenteral solution in the culture medium affected the outgrowth of the heated spores.

The results of the solution concentration studies indicated that in some cases a concentration of 20% of parenteral solution in the culture medium could have an effect on the out-

growth of spores. Therefore the second series of fraction negative tests were performed using 2 ml of solution per tube. This resulted in a parenteral solution concentration of 9% in the incubated tube.

A distilled water suspension of spores (0.05 ml) was added to each tube of solution five minutes before heating was started. Ten replicate tubes were heated at each time interval. Heating was done in a miniature retort at 121°C. After heating the tubes were cooled in an ice bath for 1.5 minutes and 20 ml of TSB was added to each tube. The tubes were incubated at 55°C for two weeks. The initial population of spores per tube (N_0) was determined by plating 1.0 ml of inoculated solution from three replicate unheated tubes.

RESULTS

The results of the several experiments to determine the effect of TSB media volume are summarized in Table II. The results suggest that larger volumes of broth added to the 5 ml of spore-buffer solution show no significant tendency to increase the outgrowth of the surviving spores. The dilution factor therefore appears to be without effect. The use of 20 ml of broth to 5 ml of spore-buffer solution or 2 ml as used in later experiments should produce results that are not influenced by relative volume of broth and buffer.

The results of the survivor curve tests to evaluate the effect of initial spore concentration on the heat resistance of *Bacillus stearothermophilus* spores are summarized in Tables III and IV. The results are presented graphically in Figures 1 and 2.

When we compare the results of the six tests on the basis of D-value and intercept ratio (Table III), the data indicate that the initial number of spores suspended in water has little

TABLE II

Results as Numbers of Negative Tubes of the Tests to Determine the Effect of Media Volume on the Outgrowth of Spores Heated at 121°C

Test No.	Heat. time (min)	No. of tubes heated	No. of negative tubes			
			ml of TSB added			
			5	10	15	20
T04010	18	16	10			1
MS4015	18	16	2			2
MS4038	18	16		12		5
MS4039	18	16		10		3
MS4043	18	15		8	2	2
MS4045	18	15		7	2	0
	Total fraction negative		12/32	37/62	4/30	13/94
	Ratio		0.38	0.60	0.13	0.14

TABLE III

The Effect of Initial Spore Concentration on the Heat Resistance of Bacillus stearothermophilus Spores Suspended in Water and Heated at 121°C

Test No.	N_o per tube	D-value[a] (min)	95% C.I.	IR[b]
MS4101A	1.1×10^5	3.5	3.2 - 3.8	.92
MS4101B	2.9×10^6	3.2	3.0 - 3.4	.92
MS4101C	3.3×10^7	3.2	3.1 - 3.4	.92
MS4112A	2.9×10^4	3.5	3.0 - 4.1	.86
MS4112B	3.2×10^6	3.4	3.1 - 3.7	.91
MS4112C	3.3×10^7	3.4	3.2 - 3.6	.93

[a] Times have been corrected for lag in heating and cooling.

[b] Intercept ratio (IR) = $\log y_o / \log N_o$

effect on the heat resistance characteristics of the spores heated at 121°C. The D-values ranged from 3.2 to 3.5 minutes and the intercept ratios from 0.86 to 0.93.

The semilogarithmic survivor curves in Figures 1 and 2 are not straight lines but curve slightly. The survivor curves are concave upward; therefore the rate of destruction is higher (smaller D-value) at the beginning of heating than at later times. The intercept ratio reflects this in that the intercept ratio in all tests is less than 1.0. We have calculated a D-value for the line connecting adjacent data points in Figures 1 and 2.

In Table IV are shown these two-

TABLE IV

Point to Point D-values for Bacillus stearothermophilus Spores Suspended in Water and Heated at 121°C

Heating time Interval (min)	D-value[a] (min)					
	MS4101A $N_o=1.1\times10^5$	MS4112A $N_o=2.9\times10^4$	MS4101B $N_o=2.9\times10^6$	MS4112B $N_o=3.2\times10^6$	MS4101C $N_o=3.3\times10^7$	MS4112C $N_o=3.3\times10^7$
0 - 1	1.88	1.79	1.87	1.72	1.68	2.16
1 - 4	2.57	2.21	2.31	2.38	2.17	2.40
4 - 7	3.84	2.51	2.76	2.76	2.93	2.56
7 - 10	3.68	7.60	3.56	3.73	3.00	3.55
10 - 13	3.96	4.98	4.02	5.15	4.25	3.56
13 - 16			3.72	3.75	4.17	5.62
16 - 19					2.96	3.64

[a] Times have been corrected for the lag in heating and cooling

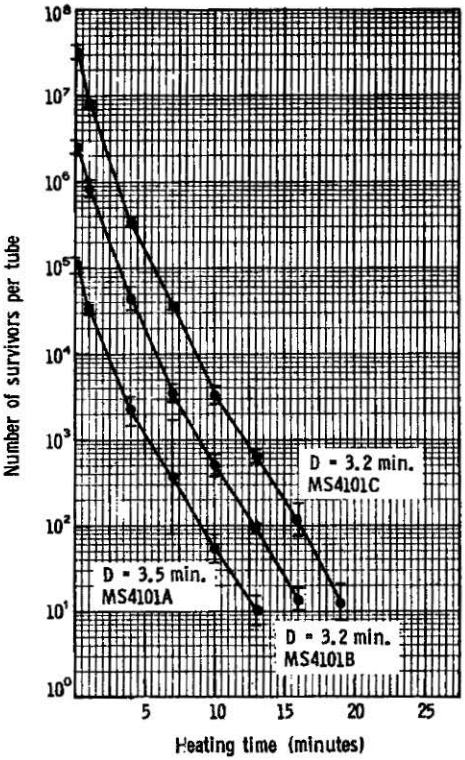

FIGURE 1: Survivor curves for *Bacillus stearothermophilus* (PCIF) spores suspended in 5 ml of Water for Injection, heated at 121°C. The 95% confidence band is shown around each plotted mean survivor data point.

point D-values for the results from tests MS4101A, B and C and MS 4112A, B and C. The trend in all tests is for the D-value to be smaller during the initial heating periods and larger at the later heating periods. The two-point D-values for the same time interval data for the six tests are in close agreement.

pH of Parenteral Solutions

The pH of the parenteral solutions before and after heating for the longest time are summarized in Table V. The pH of Water for Injection was higher than the manufacturer's specified approximate pH in all cases. After heating it remained the same or changed by ± 0.2 of a pH unit. The 5% dextrose in water solution was very close to the specified pH before heating. In three of the five tests, the pH increased by about .2 but in two tests it decreased by .35 and .75.

The pH of 5% Dextrose in Lactated Ringer's solution and 5% Dextrose in saline was very stable and changed very little after heating. The ions present in these solutions probably act as a buffering agent. Dextrose in Lactated Ringer's solution had a pH very close to that specified, but the dextrose in saline solution was consistently lower by 0.7 of a pH unit.

Survivor Curve Tests

The results of the survivor curve tests are presented in Table VI. The heat resistance of *Bacillus stearothermophilus* spores suspended in M/15 Sorensen's phosphate buffer (pH 7.0) was higher than for spores suspended in Water for Injection. The mean D-values were 3.8 minutes and 3.6 minutes respectively.

The D-values at 121°C for both the 5% Dextrose in Water and 5% Dextrose in Lactated Ringer's were about one minute less than for the Water for Injection and Sorensen's buffer; mean D-values were 2.6 and 2.5 minutes respectively. The spores had the least resistance in 5% Dextrose in Saline (.9%); the mean D-value was 1.5 minutes.

The intercept ratios for the survivor

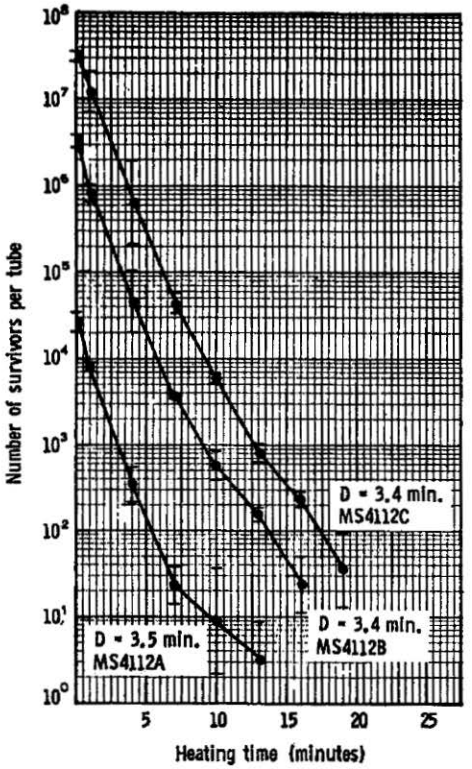

FIGURE 2: Survivor curves for *Bacillus stearothermophilus* (PCIF) spores suspended in 5 ml of Water for Injection, heated at 121°C. The 95% confidence band is shown around each plotted mean survivor data point.

curves for the five solutions were less than 1.0 indicating the survivor curves were not straight lines but were slightly concave upward.

Fraction-Negative Tests

The results of the fraction-negative tests are reported in Table VII. The spores had the highest heat resistance when suspended in Water for Injection with a mean D-value of 3.3 minutes. In M/15 Sorensen's phosphate buffer (pH 7.0) the mean D-value was 3.1 minutes, slightly lower than in Water for Injection. The spores in the 5% Dextrose in Water, 5% Dextrose in Lactated Ringer's, and 5% Dextrose in Saline have mean D-values of 2.4, 2.1 and 1.4 minutes respectively. The D-values of the spores decreased in the same order as the pH value of the solutions decreased.

Changing the volume of the solution in the tube from 5 ml to 2 ml lowered the concentration of the solution in the incubated tube from 20% to 9% and raised the concentration of spores per ml from about 4.0×10^4 to 1.0×10^5. Decreasing the volume of the so-

TABLE V

pH of Parenteral Solutions Measured Before and After Each Experiment

Solution	Mfg's Approx. pH	No. of tests	Actual pH			
			Before heating		After heating	
			Mean	Range	Mean	Range
Water for Injection	5.7	6	6.52	6.15 - 6.95	6.48	6.15 - 6.85
5% Dextrose in water	5.0	5	5.04	4.75 - 5.36	4.93	4.55 - 5.30
5% Dextrose in Lact. Ringer's	5.1	5	4.96	4.90 - 5.00	4.94	4.90 - 5.00
5% Dextrose in saline (.9%)	4.7	5	4.01	4.00 - 4.05	3.98	3.90 - 4.05

lution did not seem to affect the heat resistance of the spores suspended in the solution except for 5% dextrose in saline. For spores suspended in 5 ml of saline, the D-value was 1.2 minutes and in 2 ml of saline the D-value was 1.7 minutes.

DISCUSSION

The D-values for the survivor curve tests shown in Table VI are all larger than the comparable fraction negative tests (Table VII). This is an expected result since the intercept ratio of the survivor curves is less than 1.0 indicating that the semilogarithmic survivor curve is concave upward. With this shape of survivor curve the two-point method of calculating D-values required when FN methods are used

TABLE VI

Comparison of the Heat Resistance of Spores PCIF Heated at 121°C Suspended In Various Parenteral Solutions as Determined by Fraction-Negative Tests

Solution	Test No.	$N_o \times 10^{-5}$	D-value[a] (min)	95% C.I.
Water for Injection	MS4078A	2.76	3.4	3.3-3.6
	MS4136B[b]	2.47	3.2	3.0-3.3
	MS4143B[b]	2.03	3.4	3.2-3.7
Mean			3.3	
5% Dextrose in water	MS4066A	3.38	2.2	2.0-2.4
	MS4081B	2.38	2.7	2.6-2.9
	MS4162A[b]	1.36	2.3	2.1-2.5
Mean			2.4	
5% Dextrose in lact. Ringer's	MS4066B	2.70	2.1	2.0-2.3
	MS4085B	2.85	2.2	2.1-2.4
	MS4162B[b]	1.41	1.9	1.8-2.1
Mean			2.1	
5% Dextrose in saline (.9%)	MS4078B	2.90	1.2	1.1-1.4
	MS4085A	2.45	1.2	1.1-1.4
	MS4162C[b]	1.22	1.7	1.6-1.9
Mean			1.4	
M/15 Sorensen's buffer pH 7.0	MS4064A	1.24	3.2	3.1-3.3
	MS4081A	2.50	2.9	2.8-3.0
	MS4136A[b]	3.00	2.8	2.7-2.9
	MS4143A[b]	2.28	3.3	3.1-3.4
Mean			3.1	

[a] Times have been corrected for the lag in heating and cooling

[b] Tube contained 2 ml of parenteral solution

will give a smaller D-value than the slope of regression line through the points of the survivor curve determined at many heating times by plate count methods.

The mean D-value for spores in Water for Injection by the fraction negative method is larger than the mean D-value for spores in M/15 Sorensen's phosphate buffer; however, in the survivor curve tests the reverse is true. The differences are small and the range of D-values overlap and therefore suggest that the D-value for spores in Water for Injection and for spores in M-15 Sorensen's phosphate buffer are not different.

The three formulated parenteral solutions show the same pattern of heat resistance in the survivor curve tests as in the fraction-negative tests; the lower the pH of the solution, the lower the heat resistance.

SUMMARY AND CONCLUSIONS

The effect of initial numbers on des-

TABLE VII

Comparison of the Heat Resistance of Spores PCIF, Heated at 121°C, Suspended in Various Parenteral Solutions as Determined by Survivor Curve Tests

Solution	Test No.	$N_o \times 10^{-5}$ per tube	D-value[a (min)	95% C.I.	IR[b
Water for Injection	MS4087A	1.53	4.1	3.4-5.0	.81
	MS4094B	2.03	3.2	2.9-3.6	.89
	MS4101A	1.15	3.5	3.2-3.8	.92
	MS4112A	.29	3.5	3.0-4.1	.86
Mean			3.6		.87
5% Dextrose in water	MS4098A	.42	2.4	2.1-2.8	.92
	MS4148A	29.10	2.8	2.6-2.9	.91
	MS4163A	33.70	2.6	2.5-2.8	.93
Mean			2.6		.92
5% Destrose in Lact. Ringer's	MS4098B	.51	2.5	2.0-3.4	.83
	MS4148B	29.80	2.5	2.3-2.7	.91
	MS4163B	35.10	2.5	2.3-2.7	.89
Mean			2.5		.88
5% Dextrose in Saline (.9%)	MS4105A	.54	1.6	1.5-1.8	.79
	MS4156A	30.60	1.4	1.3-1.4	.89
Mean			1.5		.84
M/15 Sorensen's Buffer pH 7.0	MS4091A	1.53	4.5	3.9-5.2	.90
	MS4094A	2.40	3.5	3.4-3.6	.92
	MS4156B	33.90	3.4	3.3-3.5	.97
Mean			3.8		.93

[a Times have been corrected for the lag in heating and cooling

[b Intercept ratio (IR) = $\log Y_o / \log N_o$

truction characteristics of *Bacillus stearothermophilus* spores suspended in Water for Injection and heated at 121°C was determined. The initial inoculum was of the order of 10^5, 3 x 10^6 and 3 x 10^7 spores per replicate unit. The initial number of spores per replicate unit did not appear to affect the destruction rate. D-values varied from 3.2 to 3.5 minutes; intercept ratios varied from 0.86 to 0.93. The semi-logarithmic survivor curves were not straight lines but curved slightly. All six survivor curves were similar in shape.

The destruction characteristics of *Bacillus stearothermophilus* spores in four parenteral solutions and Sorensen's M/15 phosphate buffer were determined by both fraction replicate unit negative (FN) and survivor curve methods. The spores had the largest D(121°C)-values in Water for Injection and Sorensen's buffer; the range of the means was 3.1 to 3.8 minutes. The range of mean D-values was 2.1 to 2.6 minutes for 5% Dextrose in Water and 5% Dextrose in Lactated Ringer's; this was about one minute less than in Water for Injection or Sorensen's buffer. The lowest mean D-values, range of 1.4 to 1.5 minutes, was for spores in 5% Dextrose in Saline (0.9%).

ACKNOWLEDGMENTS

Rebecca Gove, Mary Deziel, and Yvonne Heisserer assisted in carrying out these experiments. Richard Holcomb assisted in the statistical analysis.

REFERENCES

1. Brown, M. R. W. "Studies on the Heat Resistance of Bacterial Spores," A thesis for the degree of Ph.D., University of London (1962).
2. Buhlmann, X., Gay, M., and Schiller, J. "Test Objects Containing *Bacillus stearothermophilus* spores for the monitoring of antimicrobial treatment in steam autoclaves," *Pharmaceutica Acta Helvetiae, 48,* (1973).