3. The solution-holding time effect on heated spores of *Bacillus stearothermophilus* spores PCIF was markedly reduced when the spores were held at 0°C compared to 22°C.
4. For the heated spores of *Bacillus stearothermophilus* the holding time was a more important factor than the holding solution or solution concentration in the culture media. For the nonheated spores the holding solution was found to be more significant than the holding time.
5. A carry over of parenteral or buffer solutions, other than Water for Injection, into the petri plate can have an effect on the number of *Bacillus stearothermophilus* spores that grow out. The effect was not observed when the concentration of the added solution in the petri plate was 0.5% but it was measurable when it was 10%.

ACKNOWLEDGMENTS

Rebecca Gove, Mary Deziel and Yvonne Heisserer assisted in the laboratory.

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).
3. Scheffe, H., *The Analysis of Variance.*, Wiley, New York, 1959.

Discussion of Paper

DR. MICHAEL KORCYNSKI (Abbott Laboratories): Would you comment on the number of tubes you used per analysis run and the level of inoculum per tube?

DR. PFLUG: We used five in the analysis and we were running about 10^6.

DR. KORCYNSKI: Would you comment on Z value comparisons among the solutions?

DR. PFLUG: We have not done Z value comparisons among the solutions.

_____ (Ethicon): Have you noticed this dying off or this holding death in spores other than *B. stearothermophilus?*

DR. PFLUG: I don't think so; we looked for the variation and this is what we found. We are going to evaluate it in *B. sporogenes* and *B. subtilis.*

_____ : In your D value curves how many samples do you use to arrive at your point? What kind of variation did you experience around the points on the curves?

DR. PFLUG: The points are an average of three; variation would be within a log.

_____ : How might this affect the significance of the difference between the two curves?

DR. PFLUG: A measure of this would be the confidence interval. I should point out that in one test we had a wider confidence interval, one point apparently being not as precise as the other two.

MR. SUMNER KAUFMAN (Miles Laboratories): Have you evaluated other solutions and could you speculate on a 50% glycerin solution?

DR. PFLUG: We have not and I wouldn't want to speculate. I think that the spores behave in their own way and I would want to test them before I speculated. The reason we selected the five solutions above is because we felt they were in wide use. Undoubtedly, we will be checking others.

MR. JOSEPH McINTYRE (Schering Corporation — Moderator): Thank you very much, Dr. Pflug.

BULLETIN
of the
PARENTERAL
DRUG ASSOCIATION

Effect of Holding Time in Parenteral Solutions on the Outgrowth of Bacillus Stearothermophilus Spores

IRVING J. PFLUG,
MIRA SCHEYER,
GERALDINE M. SMITH,
and RICHARD G. HOLCOMB
University of Minnesota
Minneapolis, Minnesota

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Effect of Holding Time in Parenteral Solutions on the Outgrowth of Bacillus Stearothermophilus Spores*

IRVING J. PFLUG, MIRA SCHEYER, GERALDINE M. SMITH, and RICHARD G. HOLCOMB
University of Minnesota
Minneapolis, Minnesota

ABSTRACT: The effect of holding heated and unheated Bacillus stearothermophilus spores for 0, 40, 80, and 120 minutes in parenteral and buffer solutions at 0 and 22°C was determined. It was found that the holding time, holding temperature and solution affected the number of organisms that were able to form colonies. The effect of the addition of 0.5, 10, and 50% parenteral or buffer solution to Trypticase Soy agar medium at the time of plating was determined; the effect on the number of organisms that were able to form colonies varied with both the type of solution and the concentration of solution.

Introduction

Buhlmann, Gay and Schiller (2) suspended *Bacillus stearothermophilus* spores in both distilled water and Trypticase Soy broth and stored the suspensions at 4°C and at room temperature. After seven months of storage there was no decrease in either spore count or heat resistance. Brown (1) suspended *Bacillus stearothermophilus* spores in distilled water, heated them at 115°C and then plated the survivors using media adjusted to various pH's. He found that heated spores were very sensitive to the pH of the recovery medium. Maximum recovery of heated spores was obtained with the medium at a pH of 7.4. Brown (1) also heated spores in M/10 phosphate buffer pH 7.0 and plated aliquots of the buffer to recover surviving spores. He observed that phosphate buffer in the recovery medium inhibited the germination of the heated spores.

This study to determine the effect of the holding of spores in parenteral solutions and of various parenteral solution concentrations in the plating medium on the outgrowth of *Bacillus stearothermophilus* spores was initiated because of unexpected experimental variation in spore destruction tests in parenteral solutions. Inconsistencies in the number of surviving spores, among replicate experimental units, particularly at the longer heating times, led us to suspect that the variations were due to: 1) the length of time the spores

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were held in solution before being plated or 2) the presence of parenteral solution in the recovery medium.

The tests described and reported below were carried out to separate and define (1) the effect of holding time and suspending solution on heated and unheated *Bacillus stearothermophilus* spores suspended in Water for Injection 5% Dextrose in Water, 5% Dextrose in Saline (0.9%), M/15 Sorensen's phosphate buffer pH 7.0, and Butterfield's phosphate buffer pH 7.2; and (2) the effect of holding time in Water for Injection from the effect of parenteral solution concentration in the plating medium on the outgrowth of heated *Bacillus stearothermophilus* spores.

Materials and Methods

Spores of *Bacillus stearothermophilus* coded PCIF were used in all tests. The PCIF spores were grown from a culture of NCA1518 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 Solutions

M/15 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). Stock solutions were prepared using Water for Injection. The buffer solutions were autoclaved for 15 minutes at 121°C.

Butterfield's buffer stock solution was made by dissolving 34.0 g of commercially prepared dehydrated buffer (BBL, Division of Bioquest, phosphate buffer, APHA, pH 7.2) in one liter of distilled water. To prepare the Butterfield's phosphate buffer, pH 7.2, 1.25 ml of the stock solution was added to one liter of Water for Injection. The prepared buffer solutions were autoclaved for 15 minutes at 121°C.

Commercially prepared 5% Dextrose in Water, 5% Dextrose in Lactated Ringer's, 5% Dextrose in Saline (0.9%) and laboratory prepared M/15 Sorensen's phosphate buffer pH 7.0 were diluted into the appropriate concentrations with Water for Injection.

The proportions are listed in Table I. In test MS4213 the solutions evaluated were: Water for Injection, 5% Dextrose in Water, 5% Dextrose in Lactated Ringer's and 5% Dextrose in Saline (0.9%). In test MS4226 Sorensen's phosphate buffer pH 7.0 was evaluated instead of 5% Dextrose in Water.

TABLE I
Solution Concentrations

Solution	*Water for Injection*
10%	90%
20%	80%
100%	0%

Ten ml of each solution concentration was added to each of 8 sterile 25 x 150 mm screw-cap tubes using a Cornwall 10 ml repetitive pipetting syringe. The same procedure was followed in preparing the 24 tubes con-

TABLE II

Experiment Numbers for the Test Solution and Test Conditions Evaluated

	Unheated Spores	Heated Spores				
	Holding 22 Deg. C	Holding 22 Deg. C		Holding 0 Deg. C	Effect of Solution In Petri Plate[a]	
Water for Injection	4214	4169	4190A	4190B	4213	4226
		4213	4226			
5% Dextrose In Water	4214	4169	4190A	4190B	4213	
5% Dextrose In Lact. Ringer's					4213	4226
5% Dextrose In Saline	4214	4169	4190A	4190B	4213	4226
Sorensen's Buffer	4214		4190A	4190B		4226
Butterfield's Buffer	4214	4169	4190A	4190B		

[a] Also a holding experiment at 22 deg. C in water for injection

taining 10 ml of Water for Injection. All the solutions were prepared and the experiment carried out under aseptic conditions in a Class 100 clean room.

Test Procedures

The experiments that were performed and the factors that were examined are summarized in Table II.

Spore inoculum. In MS4169, MS-4190, MS4213 and MS4226 heated spores were tested. The same spore suspension was used in all of these tests; the ratio of the volume of spore suspension to the volume of the carrier solution was 1:1000, to give a spore concentration of 10^7 spores per tube before the heat treatment. In MS4214 unheated spores were plated and a more dilute spore suspension was used. The ratio of the volume of spore inoculum to the spore carrier solution was 1:1000, to give a spore concentration of 10^5 spores per tube.

MS4169, holding heated spores at 22°C. Five minutes before the start of heating, 100 µl of a water suspension of *Bacillus stearothermophilus* (PCIF) spores were added to a 18 x 150 mm screw-cap test tube containing 10 ml Water for Injection. The tube and contents were heated for 8 minutes in a miniature retort at 121°C. After heating the tube was cooled in an ice bath for 1.5 minutes and then taken to the clean room.

In the clean room the heated spore suspension was agitated to insure uniformity and 1 ml was transferred to Erlenmeyer flasks containing 100 ml of each of the following sterile solutions: Water for Injection, 5% Dextrose in Water, 5% Dextrose in Saline (0.9%), and Butterfield's phosphate buffer pH 7.2. Immediately after the addition of the spores, the solutions were mixed and five replicate 0.1 and 1.0 ml aliquots of each solution were pipetted into 100 mm diameter petri plates and

25 ml of single strength Trypticase Soy agar (TSA) was poured into each plate.

The flasks of solution containing spores were then placed on magnetic stir plates at 22°C and aliquots were plated after 40, 80, and 120 minutes. The processing for all solutions at any one holding time was completed within 3 minutes of the specified holding time.

The plates were incubated at 55°C for 48 hours and the colonies were counted with the aid of a Bactronic colony counter.

MS4190 holding heated spores at 0 and 22°C. This test duplicated and expanded the holding time conditions investigated in MS4169. The procedures that were changed are described below:

Five minutes before heating was started, 150 μl of a water suspension of *Bacillus stearothermophilus* (PCIF) spores were added to 15 ml of Water for Injection. In addition to the four solutions tested in MS4169, M/15 Sorensen's phosphate buffer pH 7.0 was also included in this test. Duplicate flasks of each of the five solutions were prepared. After the 2 sets of solutions had been inoculated with the heated spores and the zero time aliquots had been plated, one set of solutions was held at 22°C and the other set of solutions was placed in an ice bath at 0°C.

MS4214, holding unheated spores at 22°C. In this test we investigated the effect of holding unheated *Bacillus stearothermophilus* (PCIF) spores in Water for Injection. 5% Dextrose in Water, 5% Dextrose in Saline (0.9%), M/15 Sorensen's phosphate buffer pH 7.0, and Butterfield's phosphate buffer pH 7.2 for 0, 40, 80, and 120 minutes at 22°C. One hundred μl of a water suspension of spores were added to a 18 x 150 mm screw-cap test tube containing 10 ml of Water for Injection.

MS4213 and MS4226, effect of holding time and concentration of solution inculture media. These tests were carried out to determine the effect of the concentration of the solution in the culture medium on the outgrowth of heated *Bacillus stearothermophilus* spores and also to evaluate the effect of holding spores suspended in Water for Injection at 22°C for periods of 0, 40, 80, and 120 minutes.

For the effect of solution tests, four sets of test tubes were prepared; each set contained 24 tubes. In each set there were 6 tubes of 10 ml Water for Injection and 2 tubes of 10 ml of each concentration (1%, 20%, 100%) of the three different test solutions. In MS4213 the solutions were 5% Dextrose in Lactated Ringer's, 5% Dextrose in Saline and M/15 Sorensen's phosphate buffer pH 7.0. In MS4226 5% Dextrose in Water replaced Sorensen's phosphate buffer.

About 5 minutes before the start of a heating test 50 μl of a water suspension of *Bacillus stearothermophilus* PCIF spores were added to a tube containing 5 ml Water for Injection. The tube and contents were heated for 8 minutes in a miniature retort at 121°C. After heating, the tube was cooled in ice water for 1.5 minutes. The tube was then taken to the clean room; 2.5 ml of the heated suspension was transferred to a sterile Erlenmeyer flask containing 250 ml of Water for Injection.

Immediately one ml aliquots of the suspension were pipetted into each of 24 petri plates. Each of the 24 test tubes in one set of prepared solutions (6 tubes Water for Injection, 2 tubes of the 3 solutions at concentrations of 1, 20, and 100%) was poured into one of the 24 petri plates. Fifteen ml of double strength TSA media were added to each plate and the plate was swirled to mix the contents. About 3 minutes were required to prepare the 24 plates. To maintain an uniform suspension, the contents of the flask were mixed using a magnetic stirrer during the entire test.

The zero time procedure was repeated at the end of 40, 80 and 120 minutes holding time. The resulting 4 sets of plates were incubated at 55°C for 48 hours and the colonies were then counted with the aid of a Bactronic colony counter.

Statistical Analysis

Experiments MS4169, MS4190, MS-4213, MS4214, and MS4226 were analyzed using an analysis of variance (ANOVA) procedure. The experimental designs were all of the factorial type (2 or 3 factors) with the treatments given considered as fixed effects. The original plate count data was transformed to equalize the expected variances in the treatment cells. Since plate count data is thought to follow a Poisson distribution model, the appropriate variance equalizing transformation was the square root transformation. The analysis was carried out on the square roots of the original plate count data.

Since there was significant interaction between treatment of factors in almost all cases the difference between treatment means for any given factor must be thought of as an averaged difference over all other factors. There were many comparisons made between treatment means. The overall error levels within the analysis of a given experiment was kept at .001 for these contrasts by using Scheffe's (3) procedure to adjust the size of the critical difference for testing significance.

Results

MS4169. The average plate counts and the percent change from the zero time counts for heated *Bacillus stearothermophilus* spores held in the various solutions at 22°C for different time intervals are presented in Table III.

Holding heated spores in Water for Injection and 5% Dextrose in Water produced a large decrease in the number of spores recovered at all holding times. The reduction in the number of spores recovered from 5% Dextrose in Saline was small after 40 and 80 minute holding times, but after 120 minutes the counts were reduced by 50%. In Butterfield's buffer the counts were decreased by 15% after 40 minutes and by 25% after both 80 and 120 minutes of holding at 22°C.

The analysis of variance presented in Table IV gives highly significant tests for the effects of suspending solution and holding time on the plate counts. There was also a highly significant interaction effect between the solution used and the holding time. This interaction indicates that the effects of solution and holding time are not simply additive.

As we might suspect from the table

TABLE III

Effect of Holding Heated Spores at 22 Deg. C (Test MS4169)

Solution	Minutes in Solution	Mean Plate Count [a]	Percent change from zero time counts
Water for injection	0	300	
	40	113	-62
	80	56	-81
	120	29	-90
5% Dextrose in Water	0	268	
	40	119	-56
	80	69	-74
	120	71	-74
5% Dextrose in Saline (0.9%)	0	241	
	40	215	-11
	80	231	-4
	120	117	-52
Butterfield's Buffer pH 7.2	0	295	
	40	250	-15
	80	224	-24
	120	226	-23

[a] Average of 5 plates

of treatment means, Table V, there was a highly significant difference between Water for Injection and Butterfield's buffer, and between Water for Injection and 5% Dextrose in Saline. Both Butterfield's buffer and the 5% Dextrose in Saline had the higher plate counts averaged across holding times than did the other two solutions.

There was a highly significant difference ($P < .001$) between the 0 time holding treatment and the other three holding times (40, 80, and 120 minutes) averaged across all solutions.

TABLE IV

Results of the Analysis of Variance of the Transformed Data of Test MS4169

Source	SS	DF	MS	F
Solution	407.4	3	135.8	49.8[a]
Holding time	490.6	3	163.5	278.4[a]
Interaction	218.7	9	24.3	335.3[a]
Error	31.2	64	.5	
Total	1147.9	79		

[a] $P < .001$

TABLE V
Treatment Means for Transformed Data of Test MS4169

Solution	*Mean*
Water for Injection	10.17
5% Dextrose in Water	10.98
5% Dextrose in Saline	14.04
Butterfield's Buffer, pH 7.2	15.73
Holding Time	
0 minutes	16.58
40 minutes	12.99
80 minutes	11.45
120 minutes	9.90

In fact, all holding times were significantly different from each other with an overall error rate for all contrasts held at the .001 level.

MS4190. The mean plate counts and the percent change from the zero time counts for heated *Bacillus stearothermophilus* spores held in the various solutions at 22 and 0°C are presented in Table VI.

The results at 22°C for Water for Injection, 5% Dextrose in Water, 5% Dextrose in Saline (0.9%), and Butterfield's phosphate buffer pH 7.2 are similar to the results in test MS4169. M/15 Sorensen's phosphate buffer, pH 7.0 was also tested in this experiment and heated spores suspended in this buffer behaved in the same manner as when suspended in Butterfield's phosphate buffer pH 7.2.

The results of the analysis of variance are summarized in Table VII. All treatment variables (solution, holding time, and holding temperature) and their interaction effects had significant tests at the .001 error level. The most significant treatment variable tested was holding temperature, followed by holding time and solution.

There was a high significant difference (Table VIII) between the mean for Water for Injection averaged overall holding times and temperatures, and the other solutions: Butterfield's buffer, Sorensen's M/15 buffer, and 5% Dextrose in Saline.

There was a highly significant difference ($P < .001$) between the effect of 0 minutes holding time and any of the other three holding times (40, 80, 120 minutes). The 40 minute holding time effect was significantly different also from the 80 to 120 minute holding times.

No significant difference was found at the .001 error level between any pair of holding times (at 0°C) treatment means.

The 0°C holding temperature was significantly different ($P < .001$) from the 22°C holding temperature as might be inferred from the holding temperature contribution in the ANOVA table.

MS4214A. The mean plate counts and the percent change in plate count from the zero time counts for nonheated spores held in the various solutions are presented in Table IX.

For nonheated spores held in Water for Injection and 5% Dextrose in Water there was a large decrease in the number of spores per plate. In Water for Injection after 40 minutes of holding the reduction was 65% and after 120 minutes the decrease was 75%. In 5% Dextrose in Water after 40 minutes the number of spores recovered had decreased by 10%. The largest decrease (88%) occurred after

TABLE VI

Effect of Holding Heated Spores at 0 and 22 Deg. C (Test MS4190A & B)

Solution	Minutes in Solution	22°C Mean Plate[a] Count	22°C Percent Change From Zero Time Counts	0°C Mean Plate[a] Count	0°C Percent Change From Zero Time Counts
Water for Injection	0	164		137	
	40	64	-61	139	+1
	80	18	-89	133	-3
	120	16	-90	120	-12
5% Dextrose in Water	0	135		138	
	40	75	-44	133	-4
	80	15	-89	127	-8
	120	7	-95	130	-6
5% Dextrose in Saline (0.9%)	0	134		125	
	40	98	-28	117	-6
	80	68	-49	115	-8
	120	64	-52	118	-6
Sorensen's Buffer (M/15) pH 7.0	0	143		136	
	40	123	-14	145	+6
	80	91	-36	139	+2
	120	93	-35	130	-4
Butterfield's Buffer pH 7.2	0	157		132	
	40	131	-17	131	-1
	80	127	-19	121	-8
	120	114	-27	100	-24

[a] Average of 5 plates

TABLE VII

Results of the Analysis of Variance of the Transformed Data of Test MS4190A

	Source	SS	DF	MS	F
1)	Solution	156.62	4	39.15	110.53[a]
2)	Holding Time	250.69	3	83.56	235.90[a]
3)	Holding Temperature	248.80	1	248.80	702.36[a]
	Interaction 1, 2	86.80	12	7.23	20.42[a]
	Interaction 1, 3	197.65	4	49.41	139.49[a]
	Interaction 2, 3	161.74	3	53.91	152.19[a]
	Interaction 1, 2, 3	94.96	12	7.91	22.34[a]
	Error	56.68	160	.35	
	Total	1253.93	199		

[a] $P < .001$

TABLE VIII

Treatment Means for Transformed Data of Test MS4190A

Solution	*Mean*
5% Dextrose in Water	9.07
Water for Injection	9.35
5% Dextrose in Saline	10.15
Sorensen's Buffer	11.14
Butterfield's Buffer	11.21
Holding Time (minutes)	
at 22 and 0 deg. C	
0	11.79
40	10.65
80	9.37
120	8.93
at 0 deg. C	
0	11.546
40	11.495
80	11.247
120	10.910
Holding Temperature	
0 deg. C	11.30
22 deg. C	9.07

80 minutes of holding, and did not change significantly at 120 minutes.

In 5% Dextrose in Saline, Sorensen's buffer and Butterfield's buffer the counts did not change or the differences were very small. In Butterfield's buffer at 40, 80 and 120 minutes there was a small increase in the number of recovered spores.

The results from the analysis of variance (Table X) are similar to those found in experiment MS4169A. The effect of solution, holding time, and interaction between solution and holding time were all found to be highly significant.

The transformed treatment means are presented in Table XI. A highly significant difference was found between Water for Injection and 5% Dextrose in Water and three other solutions: Butterfield's buffer, Sorensen's buffer and 5% Dextrose in Saline. These solutions had significantly higher plate counts, averaged

TABLE IX

Effect of Treatment on Non-Heated Spores, 22 Deg. C (Test MS4214)

Solution	Minutes in Solution	Mean Plate[a] Count	Percent Change From Zero Time Counts
Water for Injection	0	63	
	40	22	-65
	80	21	-67
	120	16	-75
5% Dextrose In Water	0	58	
	40	54	-7
	80	7	-88
	120	4	-93
5% Dextrose In Saline (0.9%)	0	60	
	40	61	+2
	80	64	+6
	120	60	0
Sorensen's Buffer pH 7.0	0	66	
	40	61	-8
	80	61	-8
	120	66	0
Butterfield's Buffer pH 7.2	0	62	
	40	73	+15
	80	67	+8
	120	69	+10

[a] Average of 5 plates

TABLE X

Results of the Analysis of Variance of the Transformed Data of Test MS4214

Source	SS	DF	MS	F
Solution	214.0	4	53.5	193.29[a]
Holding Time	58.6	3	19.5	70.60[a]
Interaction	136.1	12	11.3	40.97[a]
Error	22.1	80	.3	
Total	430.9	99		

[a] $P < .001$

across holding times, than did the Water for Injection.

There was a highly significant difference ($P < .001$) between the 0 holding time and the 80 and 120 minute holding times averaged across all suspending solutions.

MS4213. The mean plate counts for heated spores held in Water for Injection at 22°C for various time intervals and then plated with various concentrations of solution in the culture medium are summarized in Table XII.

The results of the analysis of variance (Table XIII) indicates highly significant effects on plate counts due to both the solution in the media and the holding time in Water for Injection. The effect due to interaction was not shown to be significant at the .01 error level.

TABLE XI

Treatment Means for Transformed Data of Test MS4214

Solution	*Mean*
5% Dextrose in Water	4.81
Water for Injection	5.28
5% Dextrose in Saline	7.82
Sorensen's Buffer, pH 7.0	7.98
Butterfield's Buffer, pH 7.2	8.22
Holding Time	
0 minutes	7.87
40 minutes	7.23
80 minutes	6.22
120 minutes	5.97

The treatment means for the transformed data are presented in Table XIV. There was a highly significant difference between the counts for Water for Injection and the 50% level of the other three solutions averaged across all holding times. A significant

TABLE XII

Plate Count Results[a] in Experiment MS4213, Effect of Holding Time at 22 Deg. C in Water for Injection and Concentration of Various Solutions in Plate, Heated Spores

Solution	Concentration of Solution in Petri Plate in Percent	Holding Time, Minutes 0	40	80	120
Water for Injection	50.0	712	358	390	290
	50.0	641	482	364	293
	50.0	585	468	377	282
5% Dextrose in Water	0.5	766	403	340	302
	10.0	628	326	329	246
	50.0	541	256	262	207
5% Dextrose in Lact. Ringer's	0.5	716	420	362	268
	10.0	613	460	340	244
	50.0	333	141	202	168
5% Dextrose in Saline (0.9%)	0.5	678	480	386	290
	10.0	562	366	275	270
	50.0	256	164	154	116

[a] Average of two plate counts

TABLE XIII

Results of the Analysis of Variance of the Transformed Data of Test MS4213

Source	SS	DF	MS	F
Solution in media	568.1	11	51.6	39.45[a]
Holding Time	915.3	3	305.1	233.06[a]
Interaction	85.5	33	2.6	1.98
Error	62.8	48	1.3	
Total	1631.7	95		

[a] $P < .001$

difference was not demonstrated at this error level between the mean count for Water for Injection and the .5 or 10% level of the other solutions.

There was a highly significant ($P < .001$) difference between the 0 holding time and the other three holding times (40, 80, and 120 minutes) averaged across all solutions. There was also a highly significant difference between the 40 minute and the 120 minute holding times.

MS4226. This test was a replicate of MS4213 except that M/15 Sorensen's phosphate buffer, pH 7.0 was tested instead of 5% Dextrose in Water. The mean plate counts are presented in Table XV.

The results of the analysis of variance (Table XVI) indicates that the concentration of the solution in the plating media, the holding time, and their interaction all have a highly significant effect on plate count results.

The treatment means for the transformed data are presented in Table XVII. As in MS4213A there was a highly significant difference, averaged across all holding times, between Water for Injection and the 50% levels of the other solutions.

There was a highly significant ($P < .001$) difference between each of the four holding times (0, 40, 80, and 120 minutes) compared pair-wise.

Discussion

In Table II are listed the experiment numbers as a function of all of the solutions that we have evaluated in these experiments and the different conditions to which the spores have

TABLE XIV

Treatment Means for Transformed Data of Test MS4213

Solution	Concentration of Solution of Petri Plate in Percent	Mean
Water for Injection	50.0	20.62
5% Dextrose in Water	0.5	20.89
	10.0	19.22
	50.0	17.44
5% Dextrose in Ringer's	0.5	20.67
	10.0	20.06
	50.0	15.55
5% Dextrose in Saline	0.5	21.14
	10.0	19.07
	50.0	12.95
Holding Time		
0 minutes		23.98
40 minutes		19.09
80 minutes		17.60
120 minutes		15.62

TABLE XV

Plate Count Results[a] in Experiment MS4226, Effect of Holding Time at 22 Deg. C in Water for Injection and Concentration of Various Solutions in Plate, Heated Spores

Solution	Concentration of Solution in Petri Plate in Percent	Holding Time, Minutes 0	40	80	120
Water for Injection	50.0	400	249	118	76
	50.0	434	268	137	88
	50.0	452	287	148	98
Sorensen's Buffer (M/15) pH 7.0	0.5	444	250	168	85
	10.0	378	230	125	69
	50.0	1	2	5	3
5% Dextrose in Lact. Ringer's	0.5	433	233	142	89
	10.0	386	232	130	68
	50.0	214	123	67	41
5% Dextrose in Saline (0.9%)	0.5	435	266	140	80
	10.0	356	232	112	66
	50.0	161	105	56	26

[a] Average of two plate counts

been subjected to. Some of our conditions have been replicated four times and approximately one-half have been replicated two times. The spores of *Bacillus stearothermophilus* have yielded consistent results in this series of experiments. The experiments in which we evaluated the holding time of heated spores in Water for Injection at a temperature of 22°C were evaluated in four experiments: MS4169, MS4190, MS4213, and MS4226. The results were similar in all four experiments. The holding time of heated spores in 5% Dextrose in Water, 5% Dextrose in Saline, and in Butterfield's buffer at 22°C were evaluated in two experiments: MS4169 and MS4190A. The results were almost identical.

The effect of "concentration of solutions in the petri plate" experiment which was combined with a holding

TABLE XVI

Results of the Analysis of Variance of the Transformed Data of Test MS4226

Source	SS	DF	MS	F
Solution in media	1337.3	11	121.6	400.73[a]
Holding Time	1306.2	3	435.4	1321.40[a]
Interaction	187.0	33	5.7	18.67[a]
Errors	14.6	48	.3	
Total	2845.1	95		

[a] $P < .001$

TABLE XVII

Treatment Means, for Transformed Data of Test MS422c

Solution	Concentration of Solution in Petri Plate In Percent	Mean
Water for Injection	50.0	14.48
Sorensen's Buffer	0.5	14.77
	10.0	13.51
	50.0	1.51
5% Dextrose in Ringer's	0.5	14.34
	10.0	13.62
	50.0	10.07
5% Dextrose in Saline	0.5	14.48
	10.0	13.20
	50.0	9.88
Holding Time		
0 minutes		17.55
40 minutes		13.74
80 minutes		10.20
120 minutes		7.76

experiment at 22°C in Water for Injection was duplicated in experiments MS4213 and MS4226. The pH of mixtures of parenteral solution and double strength Trypticase Soy agar was determined. The pH did not change by more than 0.1 pH unit as the concentration of parenteral or buffer solution increased from 0.5 to 50%. If we look at the effect of solution concentration at each holding time, we find that the number of organisms recovered decreased with increasing concentrations of the buffer or parenteral solution in 11 of the 12 conditions tested in MS4213, and in 12 out of 12 in MS4226. Sorensen's buffer produced the largest count reduction and the effect was very marked at the 50% concentration level. Both the 5% Dextrose in Lactated Ringer's and 5% Dextrose in Saline (.9%) had a definite effect at the 50% level and probably a small effect at the 10% level; the 5% Dextrose in Water produced the least effect. The overall effects were similar in the duplicate experiments; however, there were some differences in relative magnitude of the effects.

The relative contribution of the factors (holding time, solution, holding temperature) in explaining the variation observed in the data can be seen in the analysis of variance (ANOVA) tables. The contributions to the variation in the data can be compared by looking at relative sizes of the F-test statistics. In Table VII for MS4190 the most important effect is seen to be the holding temperature factor. In the ANOVA tables for the heated spores (MS4169, MS4190, MS4213, MS4226) the holding time factor is seen to be more important than the holding solution or the solution concentration in the culture media. For the nonheated spores of MS4214, however, the ANOVA in Table X shows that holding solution here was a more important factor than the holding time.

We have not carried out any experiments to try to isolate further the cause for the change in the number of *Bacillus stearothermophilus* spores that grow out. It is very possible that the spores that are failing to grow out are not dead but have reverted into a dormant state.

CONCLUSIONS

1. The holding of either heated or unheated *Bacillus stearothermophilus* PCIF spores in parenteral solutions or in buffer solutions at a temperature of 22°C for 40, 80, or 120 minutes caused measurable reduction in the number of spores that grew out when the solutions were plated.
2. The holding time effect varied with solution. It was greater in the solutions without ions, *i.e.* Water for Injection and 5% Dextrose in Water than in the solutions with ions, *i.e.* the phosphate solutions and 5% Dextrose in Saline.