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Effect of Phosphate Buffer Concentration on the Heat Resistance of *Bacillus stearothermophilus* Spores Suspended in Parenteral Solutions†

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The effect of various quantities of Butterfield phosphate buffer added to four parenteral solutions on the survival of *Bacillus stearothermophilus* spores heated at 121°C was determined. The effect of the addition of phosphate buffer on spore survival varied with the parenteral solution. Spore survival was increased or decreased, depending upon the composition of the parenteral solution and the buffer concentration. The results obtained in these experiments attest to the fact that environmental factors, including the type of ions present and ionic concentration, affect the heat destruction rate of *B. stearothermophilus* spores. Therefore, the sterilization requirements of a product such as a parenteral solution may be affected by small changes in formulation.

There are a number of factors that have been shown to affect the heat resistance of *Bacillus stearothermophilus* spores (2–4, 8–10). One important factor is the chemical composition of the solution in which the spores are suspended during heating.

We have observed during studies to determine the effect of storage time and storage solution on spore heat resistance (unpublished data) that *B. stearothermophilus* spores behaved differently when heated in solutions with different levels of phosphate buffer.

Williams and Hennessee (11) noted that the heat resistance of *B. stearothermophilus* spores was affected by the concentration of the phosphate buffer in which the spores were heated, but they did not quantify the effect other than to observe that the heat resistance of the spores measured at 120°C increased with a decrease in the concentration of phosphate in solution over the range of 0.05 to 0.067 M. Cook and Gilbert (3) concluded after studying the heat resistance of *B. stearothermophilus* spores in water in McIlvaine buffer and in Sorensen buffer that significant variation in heat resistance may occur when different buffers are used.

Apparently, the effect of ions also had been observed by Brown (Ph.D. thesis, University of London, London, England, 1962), who suspended *B. stearothermophilus* spores in 1/4-strength Ringer solution, an ionic solution. Both Ringer solution and lactated Ringer solution contain the major ions, Na^+, K^+, Ca^{2+}, and Cl^-; lactated Ringer solution contains additional sodium in the form of sodium lactate.

This is the report of a study to determine the effect of adding varying quantities of phosphate buffer to four parenteral solutions (water for injection, dextrose 5% in water, dextrose 5% in lactated Ringer solution, and lactated Ringer solution) on the heat resistance of *B. stearothermophilus* spores. To simplify the addition of the phosphate buffer to the solutions, increments of Butterfield (1) phosphate buffer were added. Standard Butterfield buffer is 3.1×10^{-4} M.

MATERIALS AND METHODS

Experimental plan. The experimental plan was to evaluate the heat resistance at 121°C of *B. stearothermophilus* spores suspended in four parenteral solutions containing various concentrations of Butterfield phosphate buffer. Three series of experiments were carried out. Series I evaluated the behavior of the spores in dextrose 5% in water and dextrose 5% in lactated Ringer solution with buffer concentrations ranging from 0 to 32×. Series II evaluated the heat resistance of the spores in water for injection and lactated Ringer solution with buffer concentrations ranging from 0 to 160×. Series III evaluated the spores suspended in water for injection and lactated Ringer solution containing buffer concentrations ranging from 0 to 30×.

Spores. Spores of *B. stearothermophilus*, grown in our laboratory from strain ATCC 7953, were used in all tests.

The spores were grown on nutrient agar supple-

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mented with 5 μg of manganese sulfate per liter. Incubation was at 55°C for 48 h. The spore crop was cleaned by repeated washings with distilled water and centrifugation. The working spore suspension was suspended in water for injection and stored at 4°C.

Preparation of solutions. Butterfield phosphate buffer was prepared from dehydrated buffer manufactured by Baltimore Biological Laboratory. The stock solution was prepared using label directions; 34 g of dehydrated buffer was combined with 1 liter of water for injection and then autoclaved for 15 min at 121°C.

A dilution of the stock solution was used to obtain the levels of buffer concentration desired for these experiments. For a standard-strength Butterfield phosphate buffer solution, 0.63 ml was added to 500 ml of commercially prepared parenteral solution.

Thirty-five milliliters of each parenteral buffer solution was distributed into screw-cap test tubes (25 by 150 mm). Each tube was then inoculated with 0.35 ml in series I and 0.2 ml in series II and III of the same crop of *B. stearothermophilus* spores that were suspended in water for injection. The initial spore concentration was 7.7×10^6 per ml for series I and 4.4×10^5 per ml for series II and III experiments.

The inoculated tubes were allowed to equilibrate at 4°C for at least 5 days before heating tests were carried out.

Test procedures. Heating tests were carried out in screw-cap test tubes; 3 ml of the equilibrated inoculated substrate was transferred into the tubes (18 by 150 mm) immediately before carrying out the heating tests. The tubes were heated in a miniature retort at 121.1°C. Two replicate tubes were heated at each time in series I and II, and three replicate tubes were heated at each time in series III. One tube was analyzed to obtain the N_0 count in the series II and III experiments.

Heating times were corrected for the lag in heating and cooling. A lag correction factor of 2.0 min was used for the 18- by 150-mm screw-cap test tubes containing 3.0 ml of solution.

At the end of the heating time, the tubes were removed from the miniature retort, immersed in an ice-water bath for cooling, and held in the ice water until the spore recovery procedures were initiated.

In the spore recovery operation, the substrate in the tubes was first agitated to insure uniformity. Where required, dilutions were made in Butterfield phosphate buffer (pH 7.2). Duplicate 0.01-, 0.1-, or 1.0-ml aliquots were plated; about 30 ml of Trypticase soy agar was added to each petri plate. The plates were incubated at 55°C for 48 h, and the colony-forming units were counted with the aid of a Bactronic colony counter. Solution preparation, spore inoculation, and recovery procedures were carried out in a class 100 clean room.

Data analysis. The data, in terms of the number of surviving organisms per tube as a function of heating time, were analyzed with the aid of a survivor curve computer program. The *D*-value, the 95% confidence interval of the *D*-value, and the intercept ratio (IR) were calculated (6). The IR is defined as the log of the zero-time intercept of the regression line divided by the log of the N_0 value.

RESULTS

Series I experiments. Tests to determine rates of spore survival were carried out in dextrose 5% in water and dextrose 5% in lactated Ringer solution with added Butterfield phosphate buffer in amounts of 0, 1, 2, 4, 8, 16, 24, and 32× standard strength.

The results for the series I experiments, in terms of the mean logarithm of the number of survivors estimated from plate counts in dextrose 5% in water for injection and dextrose 5%

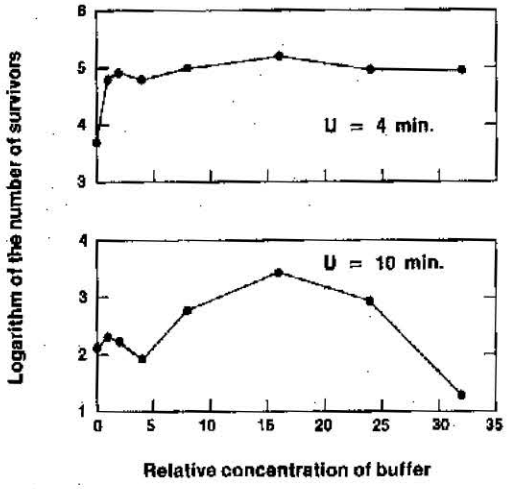

FIG. 1. *Logarithms of the number of survivors at two heating times for spores suspended in various concentrations of Butterfield phosphate buffer in dextrose 5% in water (series I).*

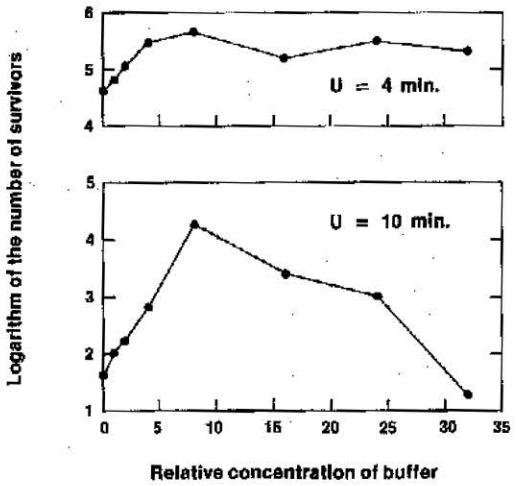

FIG. 2. *Logarithms of the number of survivors at two heating times for spores suspended in various concentrations of Butterfield phosphate buffer in dextrose 5% in lactated Ringer solution (series I).*

in lactated Ringer solution, are shown graphically for each heating time and buffer concentrations from 0 to 32× in Fig. 1 and 2, respectively.

In dextrose 5% in water, there was an increase in the number of survivors at the 4-min heating time with an increase in buffer concentration up to 2×; then the number of survivors remained stable through 32×. After 10 min of heating, the increase in the number of survivors with increasing buffer concentration was greater than at 4 min. The number of surviving spores was greatest at 8× buffer and then decreased through 32×. The number of surviving spores in 32× buffer, after 10 min of heating, was lower than in dextrose 5% in water with no added buffer.

B. stearothermophilus spores heated in dextrose 5% in lactated Ringer solution exhibited the same general pattern of heat resistance as in dextrose 5% in water. There was an increase in the number of survivors with increasing concentrations of buffer after both 4 and 10 min of heating. The highest heat resistance occurred at 8× buffer. After 4 min of heating, the heat resistance remained fairly stable through 32× buffer, whereas after 10 min, the number of survivors decreased until at 32× buffer there were fewer survivors than when no buffer was added.

Series II and III experiments. In series II experiments, spore survival tests were performed in 20 buffer concentrations: 0, 1, 2, 4, 8, 12, 16, 20, 25, 30, 35, 40, 45, 50, 60, 80, 100, 120, 140, and 160× in both water for injection and lactated Ringer solutions. In series III experiments, spore survival tests were performed in 10 buffer concentrations: 0, 1, 2, 4, 8, 12, 16, 20, 25, and 30× buffer in both water for injection and lactated Ringer solution.

The results for series II and III experiments, in terms of the mean logarithm of the number of survivors for each heating time and buffer concentrations from 0 to 30× in water for injection and lactated Ringer solution, are shown graphically in Fig. 3 and 4, respectively.

The results of the survivor experiments with various buffer concentrations in both water for injection and lactated Ringer solution were essentially the same in experimental series II and III. Survivor curves for *B. stearothermophilus* spores in both water for injection and lactated Ringer solution at 121°C for buffer concentrations of 0, 1, 2, 4, 8, and 16× for experimental series II are shown in Fig. 5.

The number of spores surviving in both water for injection and lactated Ringer solution for the 4-min heating time at 121°C was relatively constant for all concentrations of buffer greater than 4× (Fig. 3 and 4).

The critical nature of low concentrations of buffer, 1 to 30×, on the survival of spores in water for injection, is apparent at heating times greater than 4 min (Fig. 3); spore survival increased with an increasing concentration of buffer in water for injection. The survival of spores in water for injection at the 12- and 20-min heating times in both series II and III (Fig. 3) increased when the buffer was increased from 0 through 30×. The maximum number of survivors was found for buffer concentrations between 12 and 30×. In series II, the number of survivors varied with buffer concentration and with heating time between concentrations of 30 and 120×. Buffer concentrations between 120 and 160× seemed to have little effect on the number of survivors in water for injection.

In lactated Ringer solution, after 12 and 20 min of heating at 121°C, a major decrease in the

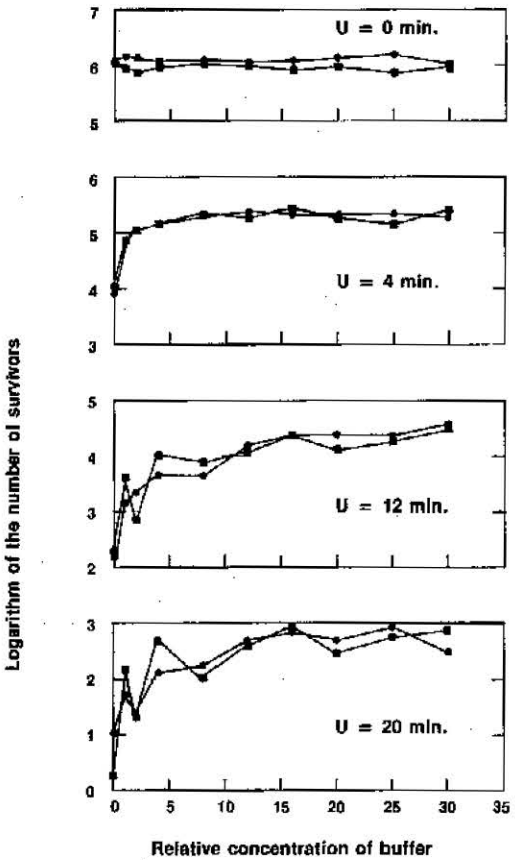

FIG. 3. *Logarithms of the number of survivors at four heating times for spores suspended in various concentrations of Butterfield phosphate buffer in water for injection:* (■) *series II;* (●) *series III.*

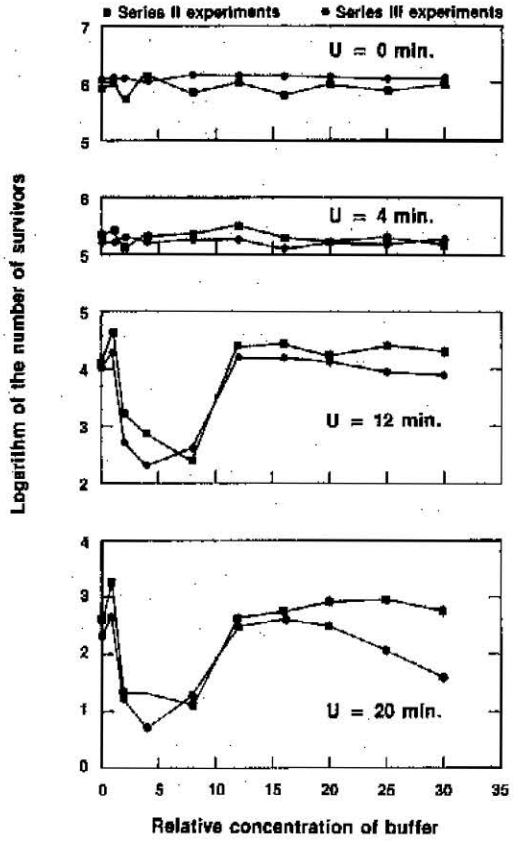

FIG. 4. *Logarithms of the number of survivors at four heating times for spores suspended in various concentrations of Butterfield phosphate buffer in lactated Ringer solution:* (■) *series II; and* (●) *series III.*

spore survival level occurred with an increase from 1 to 2× buffer strength. However, after the initial decrease at lower buffer concentrations, the number of surviving spores increased between the 8 and 12× buffer concentrations and approached the survival levels detected at the 1× buffer concentration (Fig. 4). In series II, for buffer concentrations greater than 30 to 35×, the spore survival pattern varied after 12 and 20 min of heating. After 12 min of heating, the number of surviving spores decreased slowly with increasing buffer concentrations from 30 to 160×. For spores heated at 20 min, the survival pattern was variable; the general trend was for the number of survivors to decrease with increasing buffer concentration.

D-values and IRs were calculated from the regression line through the data points, excluding the N_0 data; the *D*-values and IRs for series II and III experiments for water for injection and lactated Ringer solution, respectively, are shown in Tables 1 and 2. For spores heated in water for injection, the relative change in the *D*-value was small after the initial increase from 0 to 1× buffer over the entire range of concentrations evaluated. However, the IR (log Y_0/log N_0), which is a measure of the shape of the curve and its location relative to N_0, changed. The mean intercept ratios varied from a low of 0.92 for standard-strength buffer to a high value of 1.09 for 30× standard-strength buffer, which indicates that the shape of the survivor curve for water for injection gradually changed from concave upward for standard-strength buffer to a straight line for 4× buffer to concave downward for 8 through 30× buffer.

The effect of buffer concentration on the calculated *D*-value was quite different for lactated Ringer solution than for water for injection. With no added buffer, the mean *D*-value was 5.4 min, with an increase to 5.7 min for standard-strength buffer. The mean *D*-value then gradually decreased to a low of 2.9 min at 4× standard-strength buffer and then gradually increased at 8 and 12× buffer to a high value of 5.3 min at 16× buffer. The mean *D*-value remained at about 5 min from 20 through 30× buffer concentrations. The shape of the semilogarithmic survivor curves for spores of *B. stearothermophilus* in lactated Ringer solution varied from slightly concave downward (0× buffer concentration) to sigmoidal (3 to 30× buffer concentrations).

Survivor curves for *B. stearothermophilus* spores in both water for injection and lactated Ringer solution at 121°C for buffer concentrations of 0, 1, 2, 4, 8, and 16× are shown in Fig. 5 for experimental series II. The shape of the survivor curve has usually been considered to be an attribute of the spores, but in these results with water for injection and lactated Ringer solution plus buffer, it appears to be at least partially an attribute of the concentration of ions in the solution.

DISCUSSION

At this time we can only speculate as to why the survival of *B. stearothermophilus* spores varied with relative buffer concentration in the four parenteral solutions. The concentrations of major ions in the three parenteral solutions and in standard-strength Butterfield phosphate buffer are shown in Table 3.

In the case of water for injection and dextrose 5% in water, where few ions are present in the base solution, the combined effect of increasing

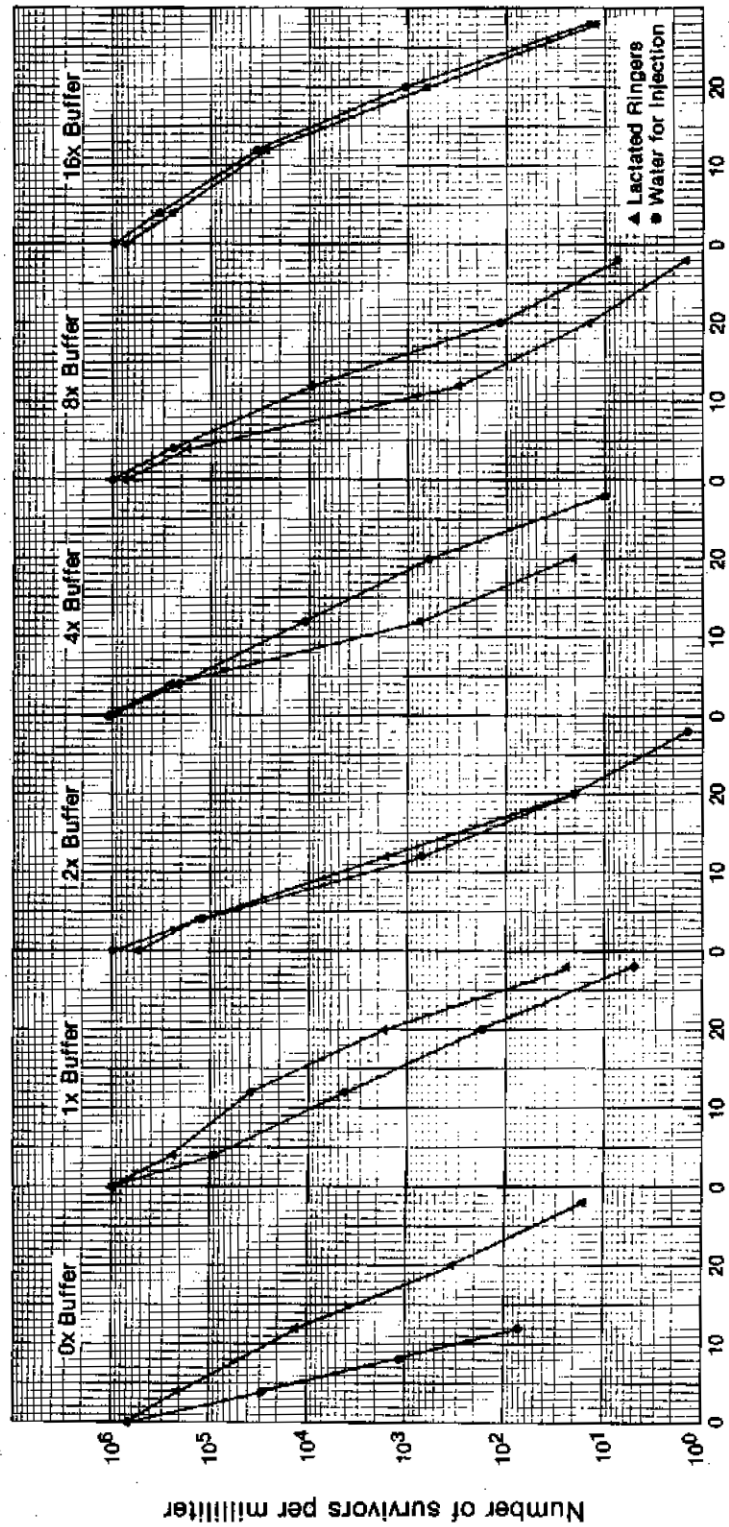

FIG. 5. *Survivor curves for both water for injection and lactated Ringer solution at 0, 1, 2, 4, 8, and 16× standard-strength Bufferfield phosphate buffer (series II).*

TABLE 1. *D-values and IRs for B. stearothermophilus spores heated at 121°C in various concentrations of phosphate buffer in water for injection*

Relative concn of buffer (×)	Molar concn of buffer ($\times 10^{-3}$)	*D*-value (min)	95% CI[a] *D*-value (min)	IR
0	0	3.12	2.86–3.43	0.97
1	0.31	5.68	5.06-6.47	0.94
1	0.31	5.06	4.24-6.27	0.89
Mean		5.37		0.92
2	0.62	4.25	3.37-5.74	0.99
2	0.62	4.44	3.97-5.04	0.96
Mean		4.34		0.98
4	1.2	5.65	5.10-6.35	1.01
4	1.2	5.05	4.44-5.84	0.98
Mean		5.35		1.00
8	2.5	5.18	4.47-6.17	1.03
8	2.5	4.96	4.47-5.24	1.00
Mean		5.07		1.02
12	3.7	4.92	4.18-5.97	1.05
12	3.7	5.05	4.62-5.58	1.05
Mean		4.98		1.05
16	4.9	5.46	4.76-6.41	1.08
16	4.9	5.00	4.34-5.80	1.07
Mean		5.23		1.08
20	6.2	4.94	4.25-5.90	1.01
20	6.2	5.30	4.82-5.89	1.03
Mean		5.12		1.02
25	7.8	5.53	4.64-6.85	1.04
25	7.8	5.37	4.82-6.02	1.04
Mean		5.45		1.04
30	9.3	5.15	4.28-5.47	1.09
30	9.3	4.65	4.09-5.38	1.09
Mean		4.90		1.09

[a] CI, Confidence interval.

amounts of the K^+ and $H_2PO_4^-$ ions may cause the rather parallel increase in spore survival.

Ordal and Lechowich (7) heated spores of *Bacillus coagulans* subsp. *thermoacidurans* at 98.5°C in different concentrations of phosphate buffer and found maximum heat resistance in 0.25 M buffer. When the buffer concentration was increased or decreased from this level, the death rate was accelerated. Cook and Gilbert (3) evaluated spores of *B. stearothermophilus* NCJB 8919 at 115°C in water and in 0.025 and 0.067 M phosphate buffer. They reported that the spores exhibited less resistance in the two buffer solutions than in water. Pflug and Smith (9) heated *Clostridium sporogenes* spores suspended in water for injection and in 0.0003 and 0.067 M phosphate buffers at 105, 110, and 115°C. The survival rate of the spores at 105 and 110°C increased with increasing molarity. However, at 115°C, spore survival was the same in water and 0.003 M buffer but increased in the 0.067 M buffer.

When spores were heated in lactated Ringer solution, increasing the concentrations of the K^+ and $H_2PO_4^-$ ions by adding phosphate buffer first increased, then sharply decreased, and then again increased spore survival, as shown by the

TABLE 2. *D-values and IRs for B. stearothermophilus spores heated at 121°C in various concentrations of phosphate buffer in lactated Ringer solution*

Relative concn of buffer (×)	Molar concn of buffer ($\times 10^{-3}$)	*D*-value (min)	95% CI[a] *D*-value (min)	IR
0	0	5.73	5.29–6.23	1.04
0	0	5.15	4.83–5.51	1.02
Mean		5.44		1.03
1	0.31	6.01	5.00–7.54	1.05
1	0.31	5.39	4.83–6.09	1.03
Mean		5.70		1.04
2	0.62	4.22	3.69–4.91	1.06
2	0.62	3.93	3.32–4.83	1.02
Mean		4.08		1.04
4	1.2	3.20	2.27–5.43	1.08
4	1.2	2.67	2.43–2.95	1.12
Mean		2.94		1.10
8	2.5	3.91	3.02–5.23	1.02
8	2.5	4.06	3.42–5.00	0.97
Mean		3.98		1.00
12	3.7	5.00	4.43–5.73	1.09
12	3.7	4.85	4.34–5.56	1.06
Mean		4.92		1.08
16	4.9	5.52	4.74–6.60	1.08
16	4.9	5.07	4.48–5.86	1.02
Mean		5.30		1.05
20	6.2	5.38	4.63–6.41	1.07
20	6.2	4.74	4.18–5.47	1.06
Mean		5.06		1.06
25	7.8	5.40	4.50–6.74	1.09
25	7.8	4.85	4.51–5.26	1.02
Mean		5.12		1.06
30	9.3	5.63	4.86–6.72	1.03
30	9.3	4.39	3.66–5.48	1.05
Mean		5.01		1.04

[a] CI, Confidence interval.

TABLE 3. *Ionic concentrations of the four parenteral solutions and standard-strength (3.1 × 10^{-4} M) Butterfield phosphate buffer solution*

Solution	Major ion concn (meq/liter)
Dextrose 5% in lactated Ringer solution	Na^+, 130 K^+, 4 Ca^{2+}, 3 Cl^-, 109 Lactate$^-$, 28
Dextrose 5% in water	
Lactated Ringer solution	Na^+, 130 K^+, 4 Ca^{2+}, 3 Cl^-, 109 Lactate$^-$, 28
Water for injection	
Standard-strength Butterfield buffer (pH 7.2)	K^+, 0.31 $H_2PO_4^-$, 0.31

data in Fig. 4. The changing effect on spore survival with increasing concentration of Butterfield phosphate buffer in lactated Ringer solution may be caused by the effect of the ions present in lactated Ringer solution (Table 3) interacting with the ions present in the buffer. However, since there are large amounts of K^+ ions in lactated Ringer solution, the variation in the number of survivors may be due to some effect or interaction of the $H_2PO_4^-$ radical.

When the lactated Ringer solution contained 5% dextrose, the decrease in the number of survivors at 2, 4, and 8× standard-strength buffer was not seen (Fig. 2). The differences observed in spore survival between lactated Ringer solution and dextrose 5% in lactated Ringer solution could be due to interaction between the ions in the lactated Ringer solution and the dextrose, or to interaction between the dextrose and molecules in the spore cortex.

Gould and Dring (5) report that spore survival is due to the stabilization of vital molecules in the protoplast of the spore brought about by osmotic dehydration. The spore cortex contains peptidoglycan groups and counterions that can vary the osmotic pressure in the cortex. They suggest that heat resistance occurs when expanded electronegative peptidoglycan molecules and mobile counterions exert a high osmotic pressure on the spore protoplast that leads to osmotic dehydration. Heat sensitization of the spore is explainable in terms of electrostatic cross-linking of peptidoglycan electronegative groups and loss of counterions that cause a decrease in osmotic pressure in the cortex and consequent rehydration of the protoplast.

Apparently, the ionic makeup of the sporulation medium, the solution in which the spores are stored, and even the solution in which the spores are heated all contribute to the composition of the spore cortex and its ability to regulate or influence conditions in the spore protoplast that in turn determine the heat resistance of the spore.

The results (of these studies) indicate that both the chemical composition of the parenteral solution and the phosphate buffer concentration affect the survival rates of *B. stearothermophilus* spores. This finding that the addition of minute amounts of simple ions to a parenteral solution may have a dramatic effect on spore survival has importance in two ways: (i) since *B. stearothermophilus* is used extensively as a wet-heat biological indicator organism, a change in the response of a biological indicator inoculated into a product should be anticipated if there is any change in solution composition, and (ii) changes in the chemical composition of the solution will also affect the heat resistance of the normal microflora of the product. Therefore, a reevaluation of the sterilization process design and monitoring program must be carried out when new chemicals are added to parenteral solutions.

These results show the extreme sensitivity of bacterial spores to environmental factors and substantiate the conventional wisdom that microbial spores must be evaluated in a specific system in order to know the survival characteristics of the organisms in that system.

ACKNOWLEDGMENTS

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