APPENDIX D: Report for Project Year, June 30, 1975 through June 29, 1976 for FDA Contract No. 223-75-3028 entitled "Development of Resistance Parameters for Biological Indicators Used in Sterilization Processes"

SURVIVOR CURVES OF BACTERIAL SPORES HEATED IN PARENTERAL SOLUTIONS[a]

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ABSTRACT

Bacillus stearothermophilus spores and Clostridium sporogenes spores have been heated at several temperatures in parenteral solutions and in Butterfield's and Sorensen's phosphate buffer solutions. The survivor data have been analyzed by fitting a least-squares line to the data points; the intercept value of this line is reported as an intercept ratio and the slope as a D-value. The Bigelow thermal death time model was fitted to the D-value data obtained for each solution at the several temperatures, and the z-value of the thermal death time curve determined. In addition to the survivor curve parameters of intercept ratio and D-value, z-values have been calculated and survivor curve graphs have been prepared for all conditions. All tests have been carried out at least in duplicate.

The results of these studies show the effect of the buffer solutions and parenteral solutions on the survival characteristics of both the Clostridium sporogenes and Bacillus stearothermophilus spores. When D-value results were plotted on thermal death time curves, the data for Clostridium sporogenes for all parenteral and buffer solutions produce z-values that range from 9 to 13°C. When the D-value data for Bacillus stearothermophilus were plotted on thermal death time curves, the data do not in all cases fall on a straight line. For four of the six solutions two lines are required. The z-value at the higher temperatures is smaller than the z-value at the lower temperatures.

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[a] This research was reported at the Spore Group Meeting held at Leeds, England on December 16-18, 1975. It is anticipated that portions of this manuscript will be published in Spore Research, eds. Barker, A. N., Wolf, J., Ellar, D. J., Dring, G. J. and Gould, G. W., Academic Press Inc., London (1976).

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INTRODUCTION

Microbial heat destruction tests satisfy many objectives including: (1) the development of basic data on the behavior of biological systems, (2) the gathering of data for use in the design of sterilization processes, and (3) evaluating the performance of microorganisms that will be used as biological indicators.

The studies that are reported in this manuscript were carried out to assess the performance of Bacillus stearothermophilus and Clostridium sporogenes spores as biological indicator organisms. The survivor curve method of evaluation was used because it was anticipated that the final biological indicator system would be a count-reduction procedure perhaps refined to the point where a calibrated biological indicator system will be used as a biological process evaluation unit.

These studies were directed toward biological indicators for parenteral solutions; therefore, in this study four parenteral solutions were evaluated: water for injection, dextrose 5% in water, dextrose 5% in lactated Ringer's solution and dextrose 5% in saline. Two buffer solutions were also tested: Sorensen's M/15 phosphate buffer (pH 7.0) and Butterfield's phosphate buffer, APHA dilution buffer (pH 7.2), Butterfield (1933).

A single suspension of Bacillus stearothermophilus spores and a single suspension of Clostridium sporogenes spores were used. Two survivor curve tests each on a different day were carried out for each test condition. Four different temperatures were evaluated in the Bacillus stearothermophilus and three temperatures in the Clostridium sporogenes studies.

The objectives in this report are to show on the one hand, what we believe to be rather good reproducibility of the spores when tested repeatedly under the same conditions, and secondly, the wide variation in the D and z-values and in the shape of survivor curves for the same spores tested in the same system as a function of temperature and of suspending solution.

We are including in this report 24 survivor curves for Bacillus stearothermophilus and 18 survivor curves for Clostridium sporogenes. While D and IR values describe the regression line through the data points, these parameters fail to convey completely the shape characteristics of the survivor curve. We believe that the only way of communicating the shape of survivor curves is to actually show the survivor curves themselves.

MATERIALS AND METHODS

Test Solutions

Butterfield's phosphate buffer (pH 7.2). A stock solution was prepared by dissolving 34.0 g of commercially prepared dehydrated buffer (BBL, Division of Becton, Dickinson and Co., phosphate buffer, APHA, pH 7.2) in one liter of distilled water. The working buffer solution was made by adding 1.25 ml of stock solution to one liter of distilled water, The prepared buffer solution was autoclaved at 121°C for 15 minutes.

Sorensen's M/15 phosphate buffer. Sorensen's M/15 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 distilled water. The prepared buffer was autoclaved for 15 minutes at 121°C.

Commercial parenteral solutions. Four commercially prepared parenteral solutions, water for injection, dextrose 5% in water, dextrose 5% in lactated Ringer's, and dextrose 5% in saline were used in these tests. The manufacturer's specifications for the four parenteral solutions are presented in Table 1.

Table 1
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	

Test System

Preparation of the test solutions, the inoculation of spores, and the sampling and recovery procedures were carried out in a Class 100 clean room.

Preparation of test solutions. The day before a test, 18 x 150 mm screw cap test tubes with the cap liners removed and replaced with neoprene gaskets were sterilized, dryed and then transferred to the clean room for filling. Five ml of laboratory prepared buffer or commercially prepared parenteral solution were aseptically added to each tube using a 10 ml pipette. Enough tubes were prepared for all heating times including tubes for determining the initial number of microorganisms per tube. The filled tubes were stored in the clean room overnight.

Spore inoculation. Immediately before the start of a test, using an Eppendorf pipette, each tube was inoculated with either 0.01 or 0.05 ml of the spore suspension so there were about 10^6 spores per tube. Each tube was then tightly closed, shaken five times and placed in an ice water bath. The inoculated tubes in the ice water bath were transferred to the retort room. The tubes remained in the ice water until the heating test was carried out.

Heating procedure. Two different methods of heating were used in carrying out these tests. For temperatures above 100°C the miniature retort system was used, while for temperatures below 100°C a water bath was utilized.

In the miniature retort system the inoculated tubes were taken from the ice water bath, shaken, placed in the miniature retort and heated at the desired temperature for the specified time. Three replicate tubes were heated at each time. After heating the tubes were placed in the ice water bath to cool and held there until recovery procedures were initiated. When the heating times were relatively short, heating of all tubes in an experiment was completed before recovery procedures were started. When the heating times were long, recovery of the spores was carried out after each heat treatment.

For water bath tests with temperatures between 90 and 99°C, a Labline "Magnestir" water bath, model 3088, which has two magnetic stir bars to

agitate the water was used. All of the tubes for one experiment were placed in a test tube rack and the rack placed in the water bath at time zero. Three tubes were withdrawn after each specified heating time and placed in an ice water bath for cooling and holding until recovery procedures were started.

For each temperature solution spore condition, two experiments on different days were carried out. The plan was to carry out at least four heating times in each experiment.

The heating and cooling lag correction factors for the 5 ml of solution in the 18 x 150 test tubes were calculated using the method described by Jaynes et al. (1961). All heating times were corrected using this factor.

The temperature of the water in the water bath or steam in the miniature retort was recorded at regular intervals and the average temperature for the test was determined and used in the z-value calculations. Both the experimental design temperature and the actual test temperature will appear in this report.

Data Analysis

In the survivor curve experiments to evaluate the effect of a heat stress on microbial spores, all conditions except heating time were held as constant as possible. The resulting data were the number of colony forming units for each test heating time and the unheated control.

The semi-logarithmic model was used as the basis for correlating heat destruction data. The general equation is:

$$\log N = -U/D + \log N_o$$

N = Number of surviving organisms after treatment (U)

N_o = Initial number of organisms

U = Sterilizing value at test temperature

D = Heat resistance parameter

In using this model it was recognized, as discussed by Pflug and Bearman (1972), that all microbial heat destruction data will not fit the semi-logarithmic model exactly but that it is the most convenient and usable model available today.

The data were analyzed with the aid of a digital computer. In the analysis procedure the survivor data for the unheated controls (N_o) are separated from the survivor data at the several heating times. The survivor data for the several test heating times (not including the unheated controls) were correlated using the simple linear regression of the logarithm of the survivors versus sterilizing value (Draper and Smith, 1966). The slope of the regression line was used to estimate the thermal resistance parameter (D). The zero time intercept of the regression line (Y_o) was also calculated. The Y_o and N_o values were used to calculate the intercept ratio:

$$IR = \log Y_o / \log N_o$$

The statistical variation of these parameters and the point-to-point D-value of the data was calculated by the computer and printed out in appropriate tables. A survivor curve graph was also prepared by the computer.

For each test condition the D-values at each temperature were analyzed to determine the z-value.

The D-value and z-value are related in the equation of the thermal death rate curve:

$$\log D(T) = \frac{T_B - T}{z} + \log D(T_B)$$

T = Temperature

T_B = Base temperature

D(T) = D-value as a function of temperature

An appropriate computer program was used to fit the D-value data into the thermal death rate curve model using the least squares procedure. The z-value and confidence interval of the z-value were calculated as well as the D(100°C) and D(120°C) values.

BACILLUS STEAROTHERMOPHILUS STUDIES

Experimental Design

Survivor curve tests were carried out at four temperatures using Bacillus stearothermophilus spores suspended in the six solutions.

The original plan was to carry out the spore heat destruction tests in all six solutions at 105, 110, 115 and 121°C. The high heat resistance of the spores in water for injection and the two buffer solutions made tests at 105°C impractical; therefore, tests in water for injection, Butterfield's buffer and Sorensen's buffer were carried out at 110, 113, 117 and 121°C and tests in the other parenteral solutions were carried out at 105, 110, 115 and 121°C.

Spores

Bacillus stearothermophilus spores, code PBDT, suspended in water for injection, were used in all the tests. These spores were grown from the American Type Culture Collection strain No. 7953.

Recovery procedure. A one ml aliquot from each tube was diluted in an appropriate volume of Butterfield's phosphate buffer (pH 7.2), and duplicate 0.1, 1.0 or 10 ml aliquots of the diluted suspension were plated. When the expected number of survivors was low, duplicate 0.1 or 1.0 ml aliquots of the undiluted heated suspension were directly plated.

Trypticase Soy agar (TSA) manufactured by BBL and prepared according to their recommendations was used for all plate counts. When plating 1.0 or 0.1 ml of sample, 20 ml TSA was used for each pour plate, and when plating 10.0 ml quantities, 30 ml of 1.5 strength TSA was used. All plates were allowed to solidify before being incubated in an inverted position for 48 hours at 55°C. Following incubation, the plates were counted with the aid of a Bactronic colony counter.

Plastic Tube Tests

Several hundred plastic tubes were filled on the same day with Bacillus stearothermophilus spores suspended in water for injection. During the following three months tubes were selected at random and survivor curve tests were carried out at 121°C. At least three tubes were evaluated at each test time. The prepared tubes were stored at 4°C.

Results and Discussion

The results of the repeated tests over a three month period of Bacillus stearothermophilus spores in water for injection in plastic tubes are shown graphically in Figure 1. The mean number of survivors for each of the nine tests and the overall means are indicated. There appears to be no difference in the results of these nine experiments.

It seems logical that if spores from a single suspension are subjected to identical heat destruction tests, the results in terms of number of survivors should be the same for all tests. If there are differences in the results these differences will be due either to differences in the spores from the single suspension or differences in the environmental conditions of the spores during heating. The problems in obtaining reproducible results we believe stem largely from problems in assuring that the organisms in comparable tests are subjected to the same amount of heat energy and that other experimental conditions are controlled so there are no variations in the test procedure. We believe that microorganisms are sensitive to minute changes in environmental conditions. In some cases methods may not be available to measure the changes in environmental conditions that cause changes in the destruction rate of the spores.

The results of the survivor curve tests of Bacillus stearothermophilus spores PBDT suspended in dextrose 5% in saline are presented in Figure 2 and are summarized in Table 2; dextrose 5% in lactated Ringer's in Figure 3 and Table 3; dextrose 5% in water in Figure 4 and Table 4; water for injection in Figure 5 and Table 5; Butterfield's buffer (pH 7.2) in Figure 6 and Table 6; and Sorensen's M/15 phosphate buffer (pH 7.0) in Figure 7 and Table 7.

All of the Bacillus stearothermophilus survivor curves are concave downward. There is wide variation in the amount of curvature as can be observed in Figures 2 through 7. It appears that both the suspending solution and the test temperature have an effect on the shape of the survivor curve.

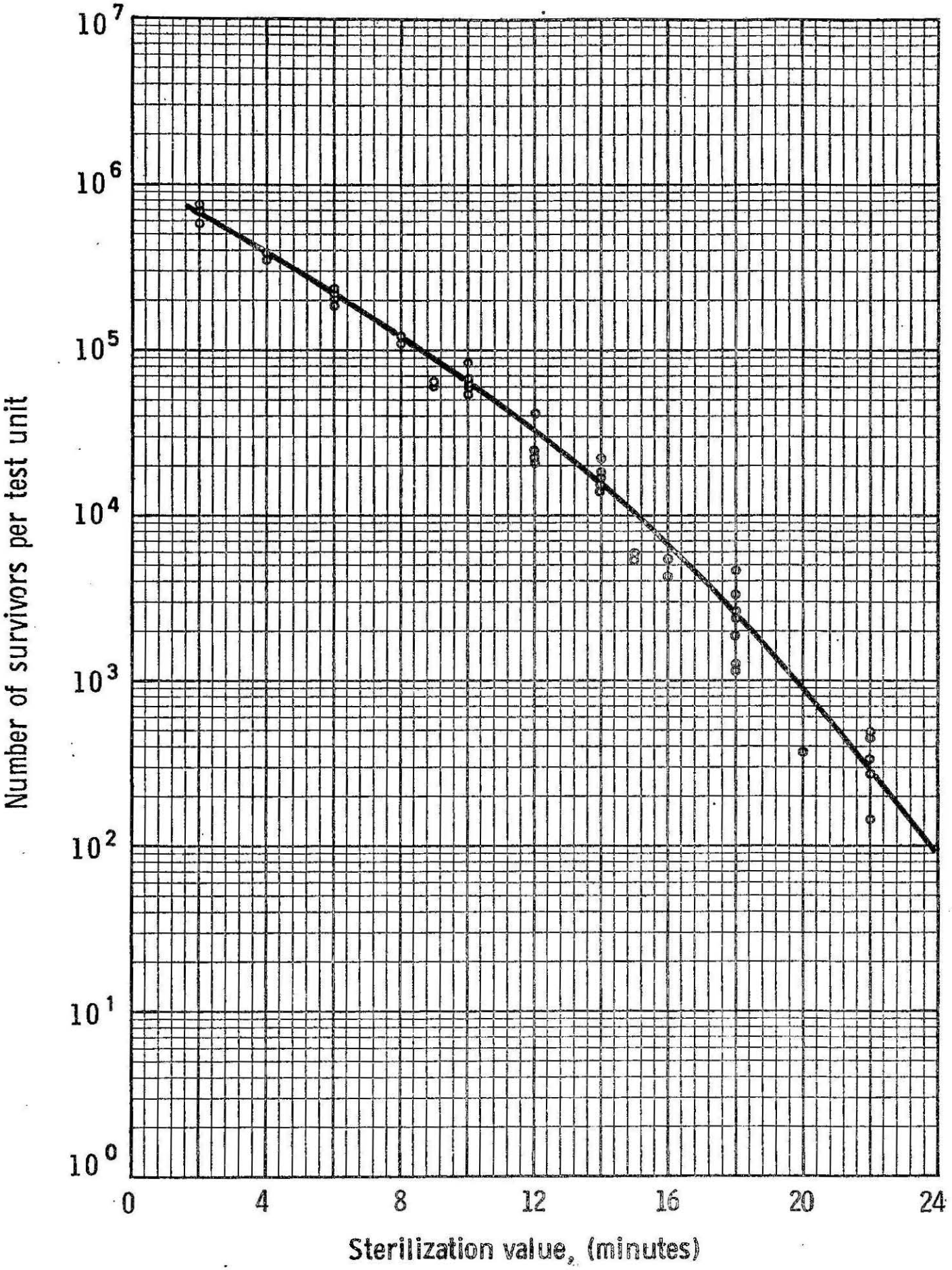

Figure 1: Mean number of survivors for nine heat destruction tests over a three-month period of Bacillus stearothermophilus spores heated at 121°C.

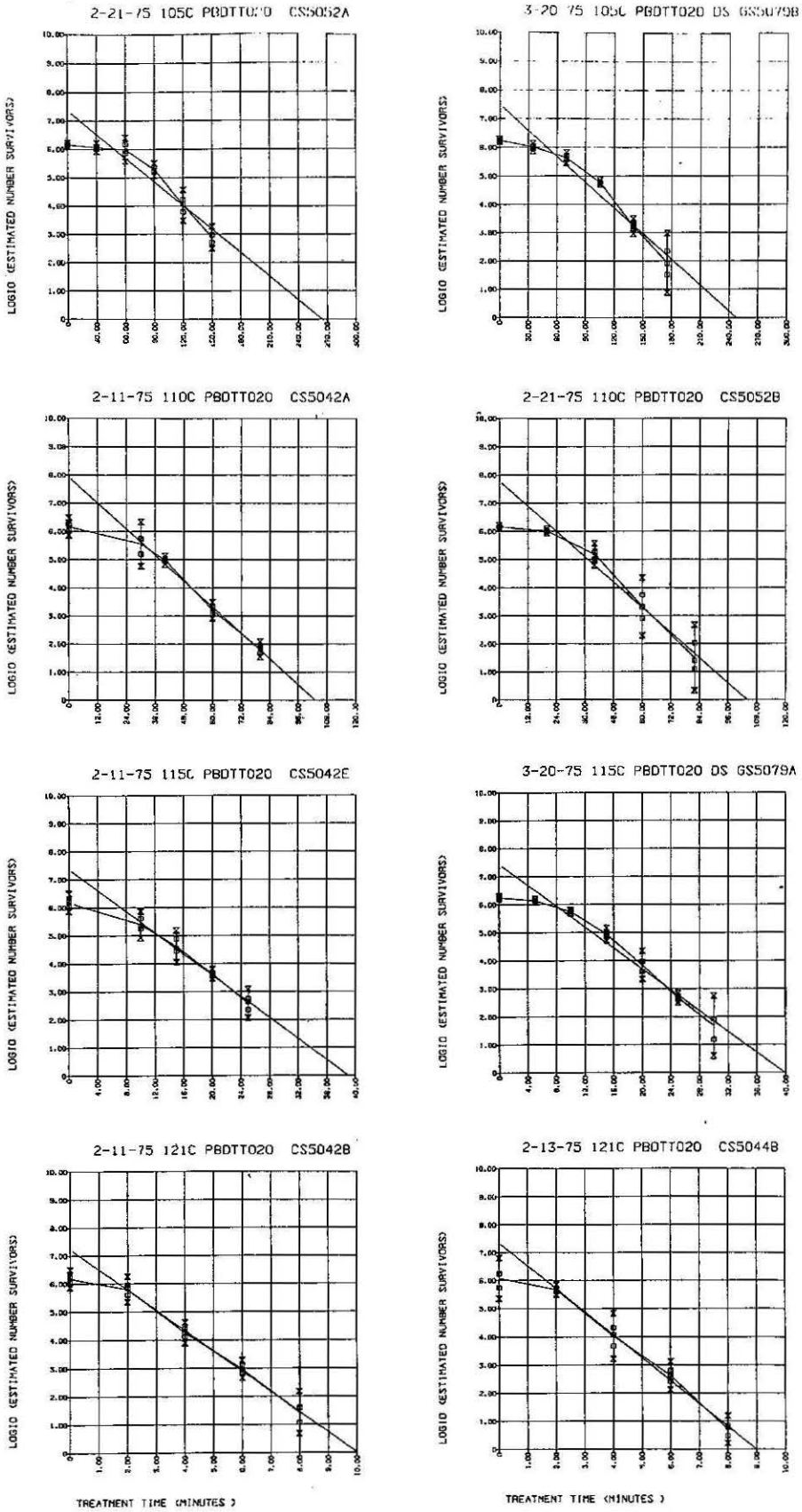

Figure 2: Bacillus stearothermophilus spores heated in dextrose 5% in saline.

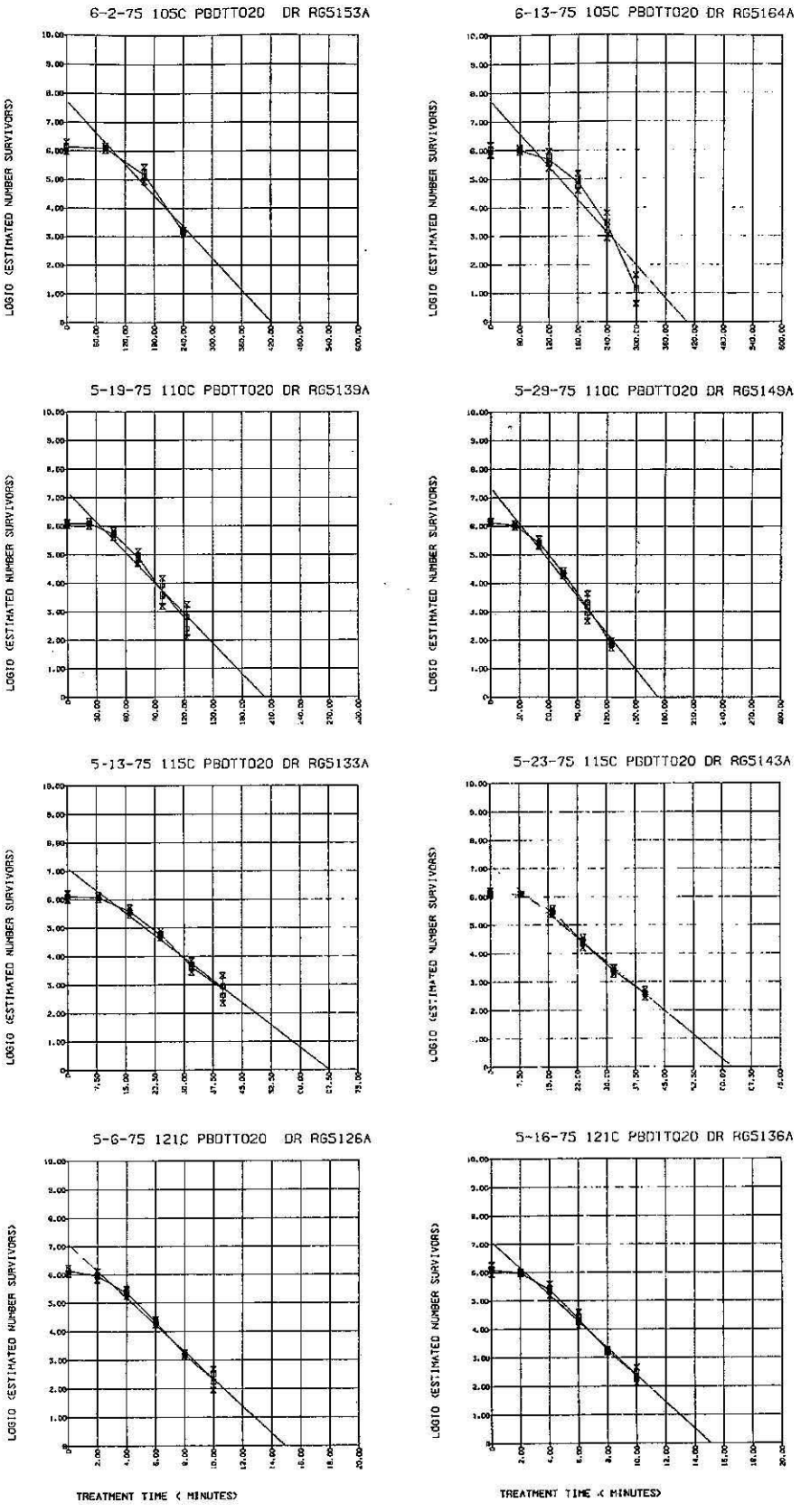

Figure 3: Bacillus stearothermophilus spores heated in dextrose 5% in lactated Ringer's solution.

Table 2

Results of Survivor Curve Tests for Bacillus stearothermophilus Spores PBDT Suspended in 5 ml of Dextrose 5% in Saline

Temp. °C	Test No.	D-Value (min)	95% C. I. (min)	Width of 95% C. I. (min)	IR
105.0	CS5052A	36.0	30.4 - 44.0	13.6	1.19
104.8	GS5079B	33.1	28.9 - 38.7	9.8	1.20
	Mean	34.5			1.20
110.0	CS5042A	13.0	12.0 - 14.2	2.2	1.28
109.9	CS5052B	13.4	11.6 - 15.7	4.1	1.26
	Mean	13.2			1.27
115.0	CS5042E	5.30	4.8 - 6.0	1.2	1.19
114.9	GS5079A	5.35	4.9 - 5.9	1.0	1.19
	Mean	5.32			1.19
120.9	CS5042B	1.39	1.3 - 1.5	0.2	1.17
121.0	CS5044B	1.21	1.1 - 1.3	0.2	1.21
	Mean	1.30			1.19

Table 3

Results of Survivor Curve Tests for Bacillus stearothermophilus Spores PBDT Suspended in 5 ml of Dextrose 5% in Lactated Ringer's Solution

Temp °C	Test No.	D-Value (min)	95% C. I. (min)	Width of 95% C. I. (min)	IR
105.0	RG5153A	54.4	45.5 - 67.6	22.1	1.26
105.0	RG5164A	52.1	42.9 - 66.4	23.5	1.28
	Mean	53.2			1.27
109.9	RG5139A	28.1	25.1 - 32.0	6.9	1.19
110.0	RG5149A	23.5	21.8 - 25.6	3.8	1.20
	Mean	25.8			1.19
114.9	RG5133A	9.50	8.76 - 10.4	1.64	1.16
115.1	RG5143A	8.77	8.31 - 9.28	0.97	1.16
	Mean	9.14			1.16
120.9	RG5126A	2.11	1.98 - 2.25	0.27	1.15
120.9	RG5136A	2.13	2.01 - 2.27	0.26	1.16
	Mean	2.12			1.16

Table 4

Results of Survivor Curve Tests for Bacillus stearothermophilus

Spores PBDT Suspended in 5 ml of Dextrose 5% in Water

Temp °C	Test No.	D-Value (min)	95% C. I. (min)	Width of 95% C. I. (min)	IR
105.0	RG5153B	91.1	70.7 - 127.8	57.1	1.21
105.0	RG5167A	84.4	65.8 - 117.8	52.0	1.24
	Mean	87.8			1.22
110.0	RG5149B	31.4	27.0 - 37.6	10.6	1.23
110.0	RG5139B	32.5	27.8 - 39.2	11.4	1.21
	Mean	32.0			1.22
114.9	RG5133B	12.0	10.8 - 13.4	2.6	1.17
115.0	RG5140A	11.4	10.3 - 12.6	2.3	1.18
	Mean	11.7			1.18
120.9	RG5126B	2.44	2.30 - 2.61	0.31	1.16
120.9	RG5136B	2.40	2.2 - 2.61	0.40	1.16
	Mean	2.42			1.16

Table 5

Results of Survivor Curve Tests for Bacillus stearothermophilus

Spores PBDT Suspended in 5 ml of Water for Injection

Temp. °C	Test No.	D-Value (min)	95% C. I. (min)	Width of 95% C. I. (min)	IR
109.9	CS5050A	59.0	54.2 - 64.8	10.6	1.22
109.8	CS5055A	63.2	58.8 - 68.3	9.5	1.22
	Mean	61.1			1.22
112.8	CS5036A	33.4	29.8 - 38.1	8.3	1.13
112.7	CS5042H	34.5	32.2 - 37.1	4.9	1.13
	Mean	34.0			1.13
117.0	CS5034A	9.9	8.9 - 11.3	2.4	1.16
117.0	CS5042F	10.5	10.2 - 10.7	0.5	1.12
	Mean	10.2			1.14
120.9	CS5042C	2.92	2.6 - 3.3	0.7	1.16
121.0	CS5044C	3.03	2.8 - 3.2	0.4	1.14
	Mean	2.98			1.15

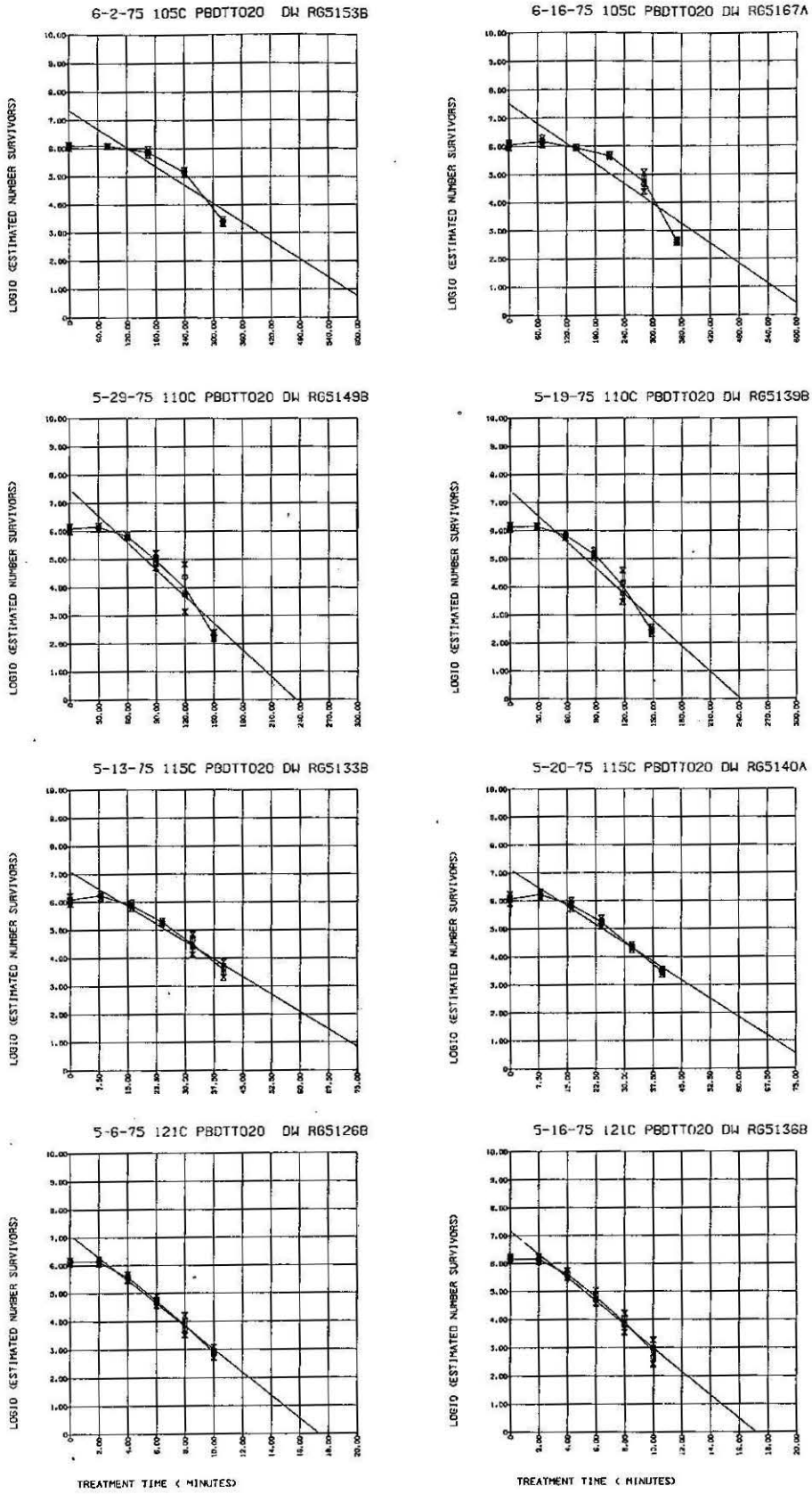

Figure 4: Bacillus stearothermophilus spores heated in dextrose 5% in water.

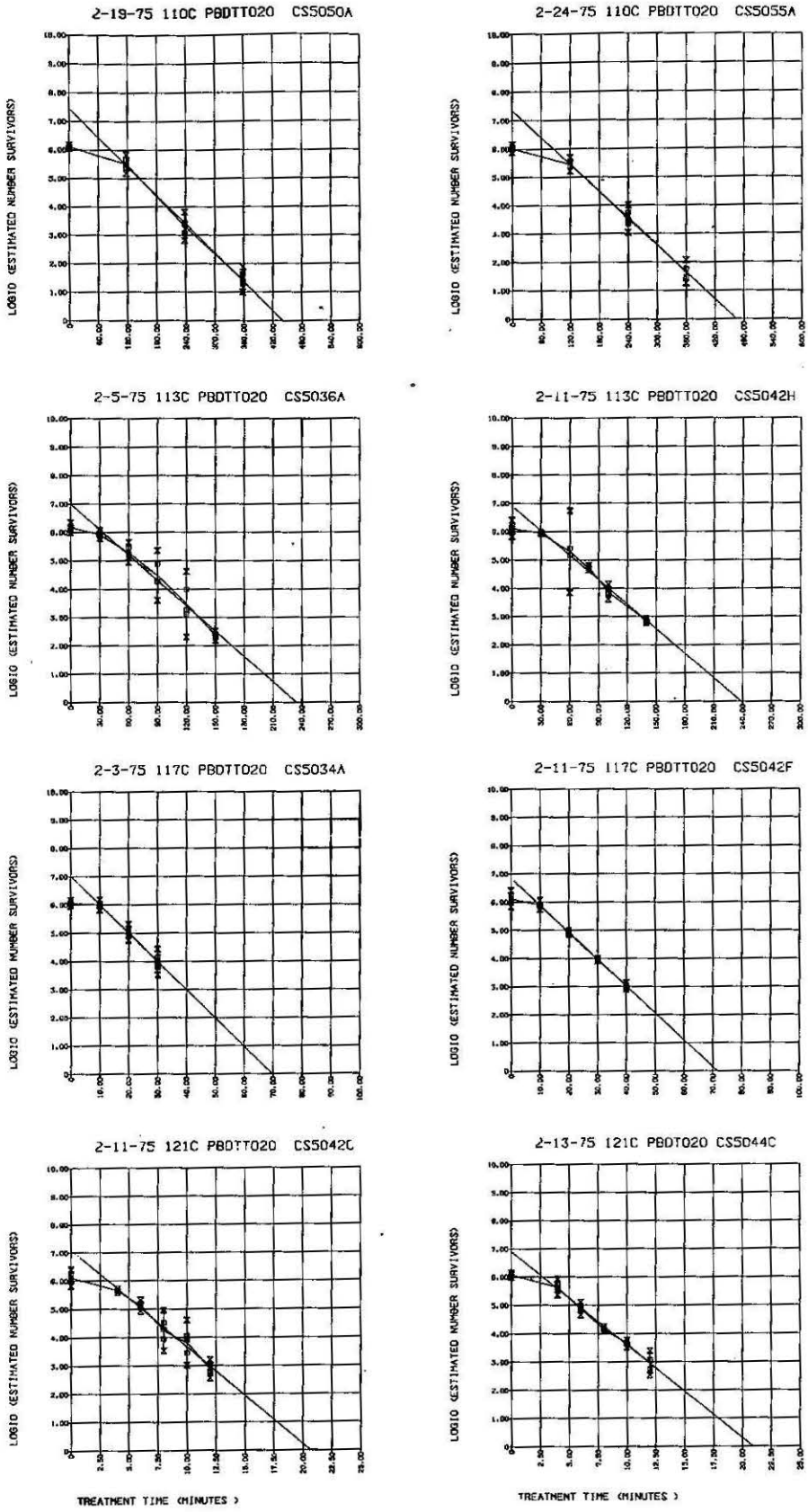

Figure 5: Bacillus stearothermophilus spores heated in water for injection.

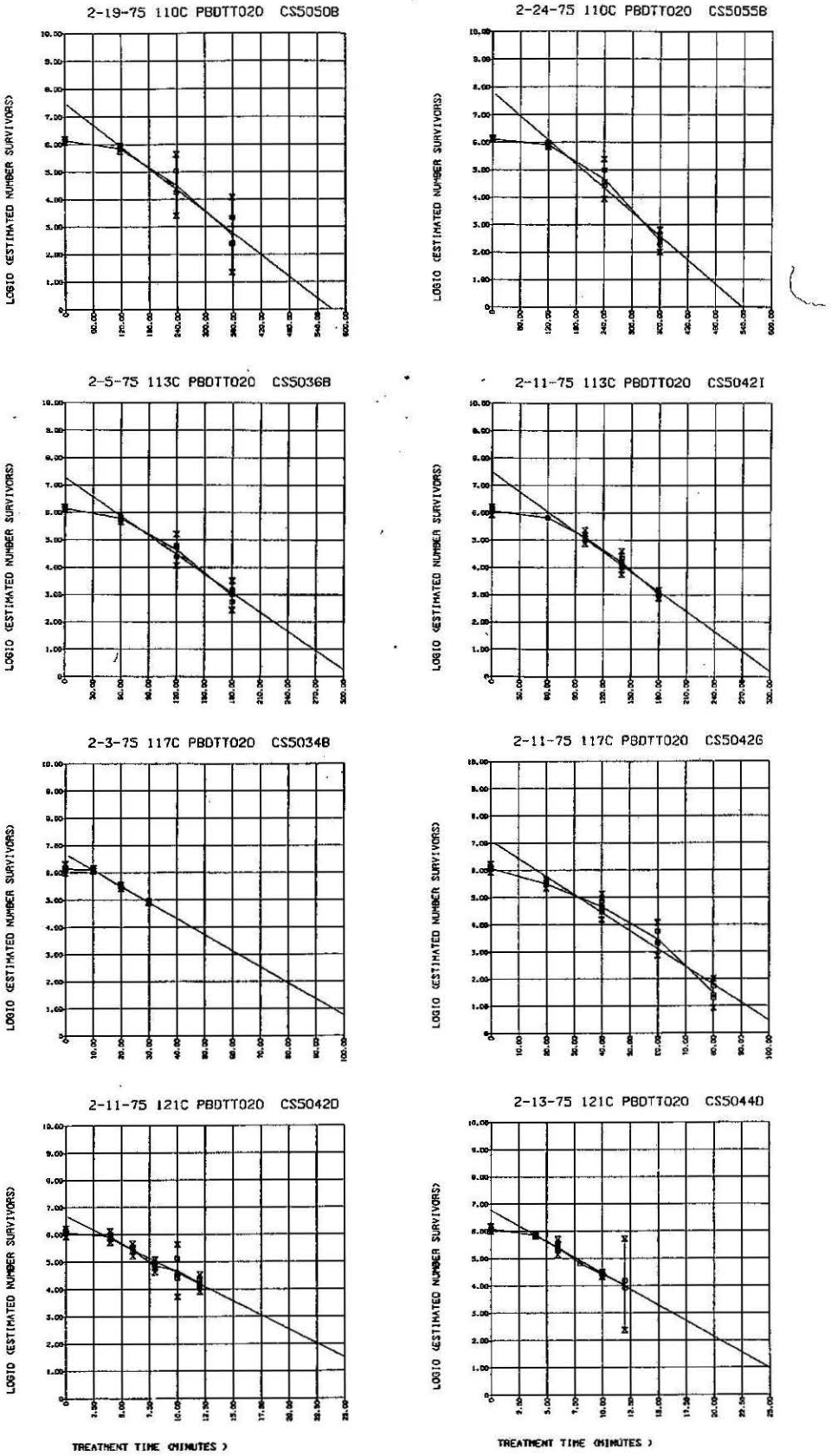

Figure 6: Bacillus stearothermophilus spores heated in Butterfield's phosphate buffer.

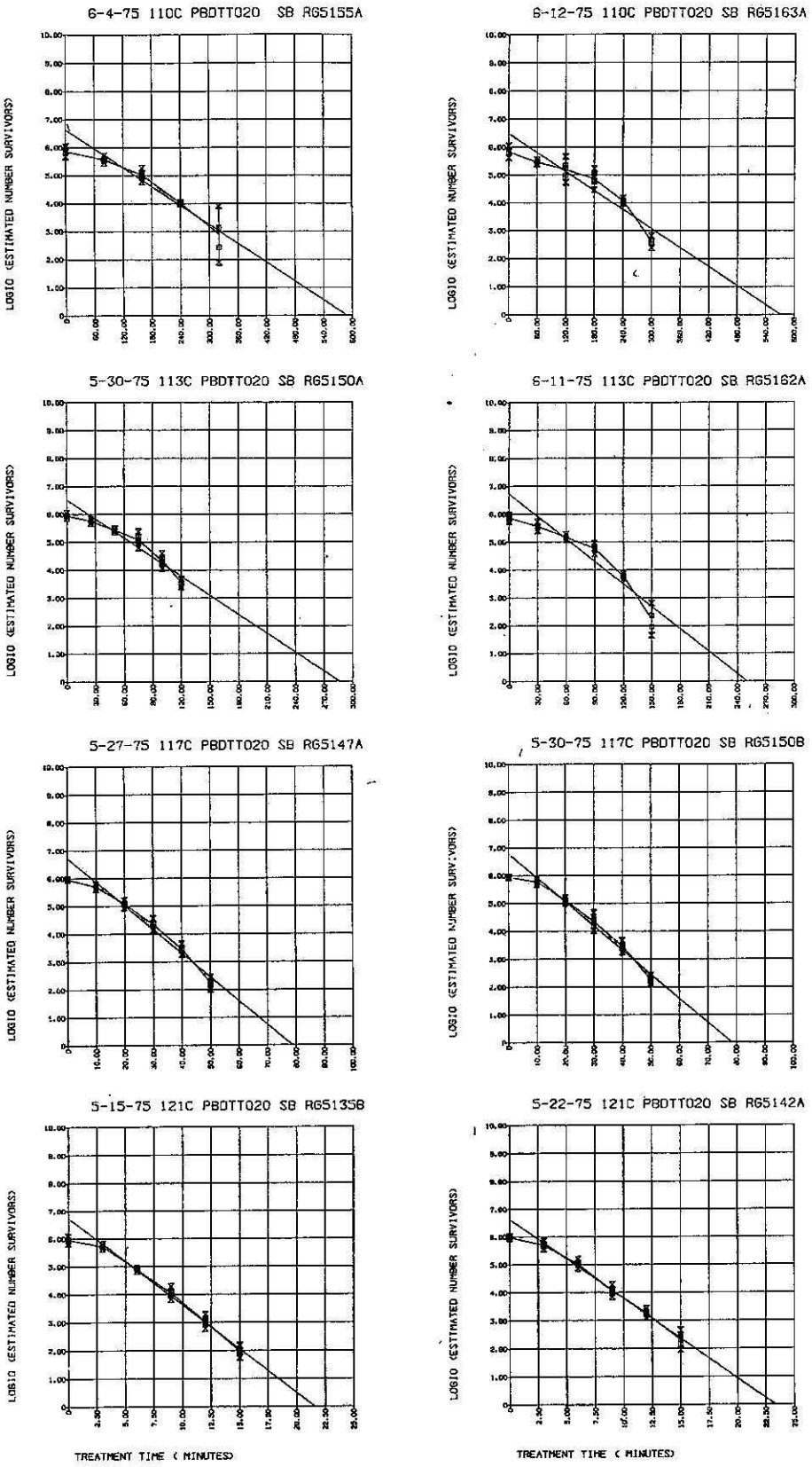

Figure 7: Bacillus stearothermophilus spores heated in Sorensen's M/15 phosphate buffer.

Table 6

Results of Survivor Curve Tests for Bacillus stearothermophilus Spores

PBDT Suspended in 5 ml of Butterfield's Phosphate Buffer (pH 7.2)

Temp. °C	Test No.	D-Value (min)	95% C. I. (min)	Width of 95% C. I. (min)	IR
109.9	CS5050B	76.2	61.2 - 101.0	39.8	1.22
109.9	CS5055B	68.6	58.3 - 83.4	25.1	1.28
109.9	GS5085A	72.7	62.4 - 87.1	24.7	1.22
	Mean	72.5			1.24
112.9	CS5036B	42.7	37.1 - 50.4	13.3	1.18
112.8	CS5042I	41.1	36.8 - 46.6	9.8	1.23
	Mean	41.9			1.20
117.0	CS5034B	16.9	16.1 - 17.7	1.6	1.09
116.8	CS5042G	15.1	13.0 - 17.9	4.9	1.17
117.0	GS5076A	14.3	13.1 - 15.6	2.5	1.15
		15.4			1.14
120.9	CS5042D	4.82	4.1 - 5.9	1.8	1.10
121.0	CS5044D	4.31	4.0 - 4.7	0.7	1.11
120.9	GS5076B	4.97	4.5 - 5.5	1.0	1.08
	Mean	4.70			1.10

Table 7

Results of Survivor Curve Tests for Bacillus stearothermophilus Spores

PBDT Suspended in 5 ml of Sorensen's M/15 Phosphate Buffer (pH 7.0)

Temp. °C	Test No.	D-Value (min)	95% C. I. (min)	Width of 95% C. I. (min)	IR
110.0	RG5155A	88.8	76.6 - 105.3	28.7	1.13
110.0	RG5163A	88.0	71.7 - 113.8	42.1	1.11
	Mean	88.4			1.12
113.0	RG5150A	43.8	37.9 - 52.0	14.1	1.09
112.8	RG5162A	37.2	31.2 - 46.1	14.9	1.15
	Mean	40.5			1.12
117.0	RG5147A	11.7	10.8 - 12.8	2.0	1.13
117.0	RG5150B	11.5	10.7 - 12.5	1.8	1.14
	Mean	11.6			1.14
121.0	RG5135B	3.20	3.06 - 3.35	0.29	1.13
120.8	RG5142A	3.51	3.35 - 3.69	0.34	1.10
	Mean	3.36			1.12

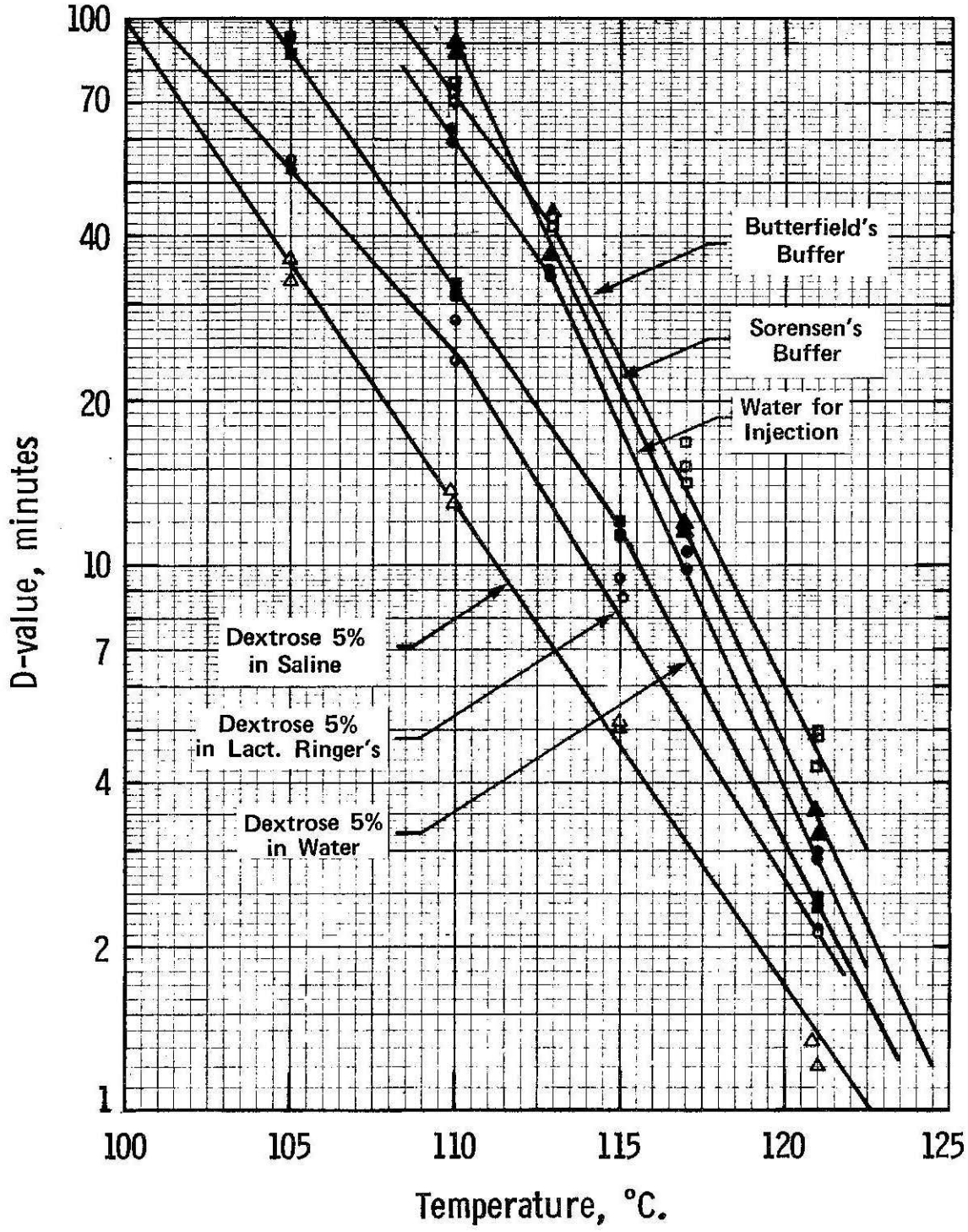

Figure 8: z-Value analysis for Bacillus stearothermophilus spores heated in six solutions.

The D-value data for the spores heated in the four parenteral solutions and the two buffer solutions are shown graphically in the z-value analysis form in Figure 8. It is obvious from the data shown in Figure 8 that the thermal resistance curves of Bacillus stearothermophilus in parenteral and buffer solutions are not always straight lines. Two point z-values have been calculated for all of the data as well as the z-value determined from a regression line through all of the D-value points and these data are summarized in Table 8. Since there appear to be abrupt changes in z-values we have grouped D-values at two or three temperatures together and calculated what we believe are the most appropriate z-values. These results along with their confidence intervals are summarized in Table 9.

Four of the six solutions evaluated produced a non-linear thermal destruction line. The range of z-values for segments of the thermal destruction curve of the spores across all solutions varied from 7.3 to 15.6°C. There appears to be a general tendency for the z-value to decrease with increasing temperature. The two solutions that yielded spore destruction data that produced a straight z-value line, Sorensen's M/15 phosphate buffer (pH 7.0) and dextrose 5% in saline appear to have little else in common. The D-values are at the opposite ends of the range of values obtained for the six solutions. Also, Sorensen's M/15 phosphate buffer has a low z-value with a large D-value whereas the dextrose 5% in saline has a large z-value and a relatively low D-value. The one similarity is that they both have high ionic concentrations.

It is obvious that there is a measurable effect of the six different suspending solutions evaluated on heat destruction rates of spores PBDT. It is also obvious that the effects are not the same at different temperatures.

The D and IR values for the Bacillus stearothermophilus spores in the six solutions at 110 and 121°C are summarized in Table 10. The spores were the least resistant in the dextrose 5% in saline solution. At 121°C the spores were most resistant in Butterfield's buffer; however, at 110°C they were most resistant in Sorensen's buffer. This change in relative resistance between 110 and 121°C was due to the difference in the z-value of the spores in two buffer solutions (Figure 8).

Table 8

z-Values (°C) for Bacillus stearothermophilus Spores PBDT Calculated Using Least Squares Fit With Four Temperatures and Two Point z-Values for the Three Sub-Intervals

Solution	z-Value °C Least Square Line For Four Temp.	Two point z-values °C Lowest Temp. Interval	Middle Temp. Interval	Highest Temp. Interval
Dextrose 5% in Saline	11.4	12.1	13.7	9.3
Dextrose 5% in Lact. Ringer's	11.3	15.6	11.2	9.3
Dextrose 5% in Water	10.3	11.4	11.3	8.7
Water for Injection	8.4	11.4	8.1	7.3
Butterfield's Buffer	9.3	12.4	9.4	7.8
Sorensen's Buffer	7.6	8.6	7.6	7.3

Table 9

Estimate of z-Values for Bacillus stearothermophilus Spores for The Specified Temperature Ranges for the Six Solutions Tested

Solution	D-Value Temp. °C	z-Value °C	95% C. I. °C
Dextrose 5% in Saline	110 - 121	11.4	10.6 - 12.3
Dextrose 5% in Lact. Ringer's	105, 110	15.6	10.4 - 31.2
	110, 115, 121	10.1	9.2 - 11.2
Dextrose 5% in Water	105, 110, 115	11.4	10.9 - 11.9
	115, 121	8.7	8.4 - 9.1
Water for Injection	110, 113	11.4	9.6 - 13.9
	113, 117, 121	7.7	7.3 - 8.2
Butterfield's Buffer (pH 7.2)	110, 113	12.4	9.9 - 16.8
	113, 117, 121	8.4	7.7 - 9.3
Sorensen's Buffer (pH 7.0)	110 - 121	7.6	7.2 - 8.1

Table 10

Summary of D and IR Values for *Bacillus stearothermophilus* Spores at 110°C and 121°C

	110°C		121°C	
Solution	D(min)	IR	D(min)	IR
Dextrose 5% in Saline	13.2	1.27	1.30	1.19
Dextrose 5% in Lact. Ringer's	25.8	1.19	2.12	1.16
Dextrose 5% in Water	32.0	1.22	2.42	1.16
Water for Injection	61.1	1.22	2.98	1.15
Butterfield's Buffer (pH 7.2)	72.5	1.24	4.70	1.10
Sorensen's Buffer (pH 7.0)	88.4	1.12	3.36	1.12

CLOSTRIDIUM SPOROGENES STUDIES

Experimental Design

Survivor curve tests using Clostridium sporogenes spores (GCDF) were carried out at three temperatures with the spores suspended in six different solutions. It was hoped that the tests could be carried out at 105, 110 and 115°C; however, the heat resistance of the spores varied widely with the solution in which the spores were suspended. The resistance of the spores in dextrose 5% in water, dextrose 5% in lactated Ringer's, and dextrose 5% in saline was so low that it was necessary to reduce the test temperatures to about 95, 99 and 105°C for spores in these solutions. When the spores were suspended in water for injection, Butterfield's phosphate buffer (pH 7.2) and Sorensen's M/15 phosphate buffer (pH 7.0), the heat resistance tests were carried out at 105, 110 and 115°C.

Spores

Clostridium sporogenes, PA 3679, spore code GCDF were used in these tests. This spore crop was grown from spores received from Dr. C. F. Schmidt, Continental Can Co., Chicago, Ill. The spores were cultured in December 1972 in beef heart infusion medium as described by Wheaton and Pratt (1961). The spores were stored in distilled water at 4°C.

Plating Procedure

Supplemental yeast extract agar (Augustin and Pflŭg, 1967) was used as the recovery medium. Yeast extract: yeast extract (BBL) 10.0 g; starch, soluble (BBL), 1.0 g; K_2HPO_4, 2.0 g; agar, 15 g; distilled water, 1,000 ml; pH 7.2. Supplements for one liter of basic yeast extract medium: 12.5 ml of a 10% sodium thioglycollate solution, 12.5 ml of a 40% dextrose solution, 25 ml of a 4% sodium bicarbonate solution. Dextrose was sterilized by filtration, and the other two compounds were sterilized by heat. The basic medium without the supplements was made up one to five days before a test,

dispensed in 300 ml amounts in 500 ml Erlenmeyer flasks, sterilized at 121°C and stored at 4°C. The day of the test the flasks of medium were heated in the autoclave at 121°C for five minutes and than cooled to 60°C. Immediately prior to pouring the inoculated plates the sterile supplements were added to each flask of medium.

A 1.0 ml sample of the heated spore suspension was diluted in Butterfield's buffer and duplicate 0.1 and 1.0 ml aliquots were plated. When the expected number of survivors was low, duplicate 0.1 and 1.0 ml aliquots of the heated spore suspension were directly plated. Approximately 25 ml of supplemented yeast extract agar were added to each plate. The plates were incubated in BBL GasPak anaerobic jars, using hydrogen and carbon dioxide Gaspak generators, at 32°C for 72 hours. The colonies were counted with the aid of a Bactronic colony counter.

Results and Discussion

The results of the survivor curve tests for Clostridium sporogenes spores GCDF suspended in dextrose 5% in saline are presented in Figure 9 and summarized in Table 11; dextrose 5% in lactated Ringer's in Figure 10 and Table 12; dextrose 5% in water in Figure 11 and Table 13; water for injection in Figure 12 and Table 14; Butterfield's buffer (PH 7.2) in Figure 13 and Table 15 and Sorensen's M/15 phosphate buffer (pH 7.0) in Figure 14 and Table 16.

The D-value data for the spores in the six solutions are shown graphically in the z-value analysis form in Figure 15. The z-value results are tabulated in Table 17. The z-values vary from a low value of 9.0°C for Sorensen's M/15 phosphate buffer to 12.4°C for water for injection. In Table 18 two point z-values for high and low temperature points are shown.

The thermal destruction data for spores in a specific solution appear to have a general tendency to form a z-value curve rather than a straight line; the curve would be concave downward in four solutions and concave upward in two solutions. Since the degree of curvature is relatively small (for all solutions except water for injection) and since both concave upward and concave downward curves were obtained, we believe that a straight line z-value correlation, results shown in Table 17 and Figure 15, is warranted for the limited data available for Clostridium sporogenes spores GCDF.

Table 11

Results of Survivor Curve Tests for *Clostridium sporogenes*

Spores GCDF Suspended in 5 ml of Dextrose 5% in Saline

Test No.	Mean Test Temp. °C	D-Value (min)	95% C. I. (min)	Width of 95% C. I.	IR
MD5080B	94.6	20.2	16.4 - 26.3	9.90	1.03
MD5091B	94.8	18.1	15.5 - 21.7	6.20	1.08
Mean		19.2			1.06
MD5098B	99.7	7.92	7.09 - 8.96	1.87	1.02
MD5262B	100.0	6.88	6.50 - 7.31	0.81	1.08
Mean		7.40			1.05
MD5171B	105.1	2.70	2.54 - 2.87	0.33	1.13
MD5279B	105.1	2.65	2.46 - 2.87	0.41	1.17
Mean		2.68			1.15

Table 12

Results of Survivor Curve Tests for *Clostridium sporogenes* Spores

GCDF Suspended in 5 ml of Dextrose 5% in Lactated Ringer's

Test No.	Mean Test Temp. °C	D-Value (min)	95% C. I. (min)	Width of 95% C. I.	IR
MD5080A	94.6	16.3	12.0 - 25.5	13.5	0.99
MD5084A	93.9	13.1	10.6 - 17.2	6.60	1.09
Mean		14.7			1.04
MD5098A	99.7	5.33	4.55 - 6.41	1.86	0.99
MD5101B	99.7	4.05	3.68 - 4.49	0.81	1.18
Mean		4.69			1.08
MD5171A	105.1	1.12	1.01 - 1.28	0.27	1.39
MD5175A	105.1	1.17	1.06 - 1.29	0.23	1.36
Mean		1.14			1.38

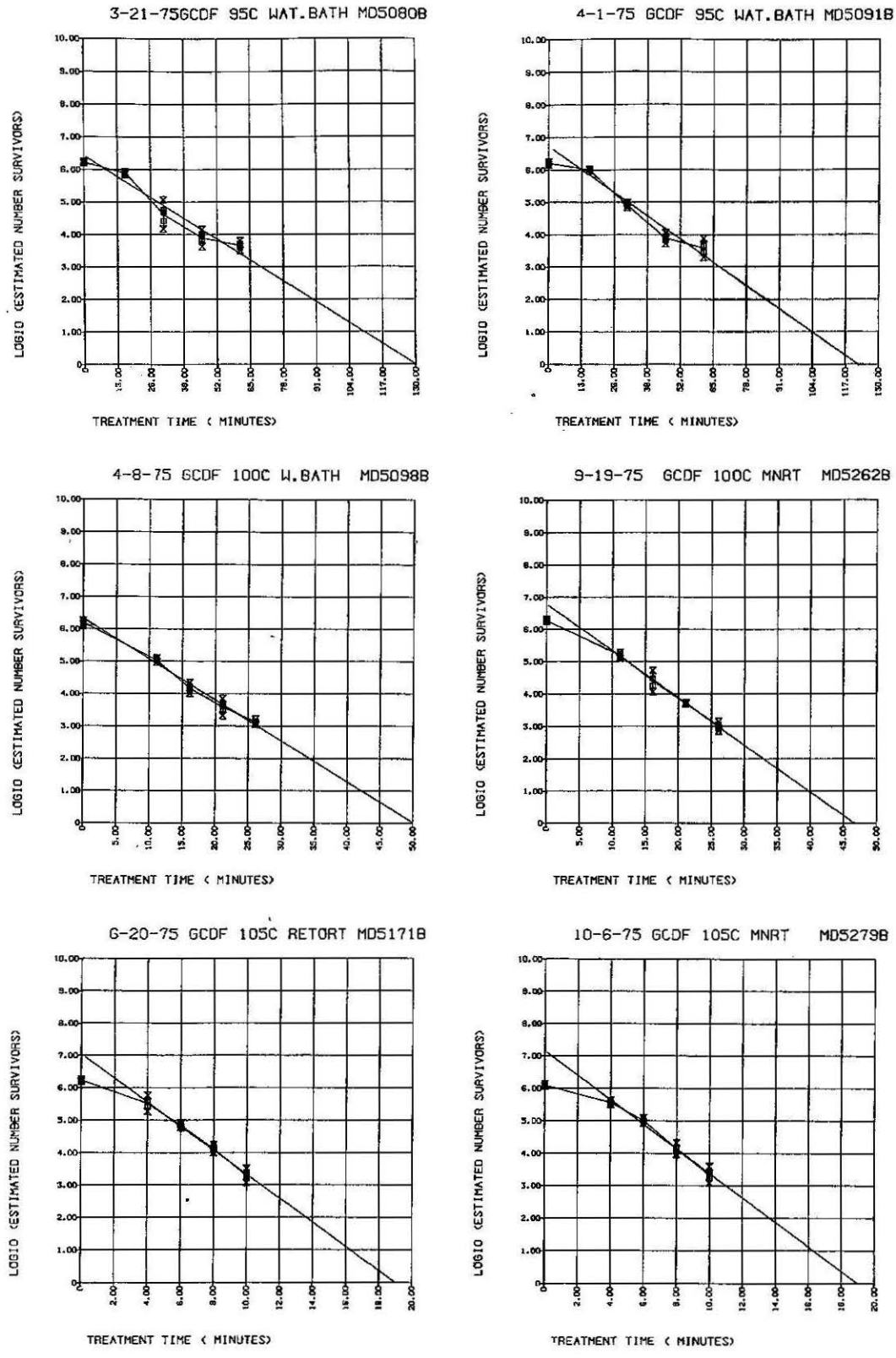

Figure 9: Clostridium sporogenes spores heated in dextrose 5% in saline.

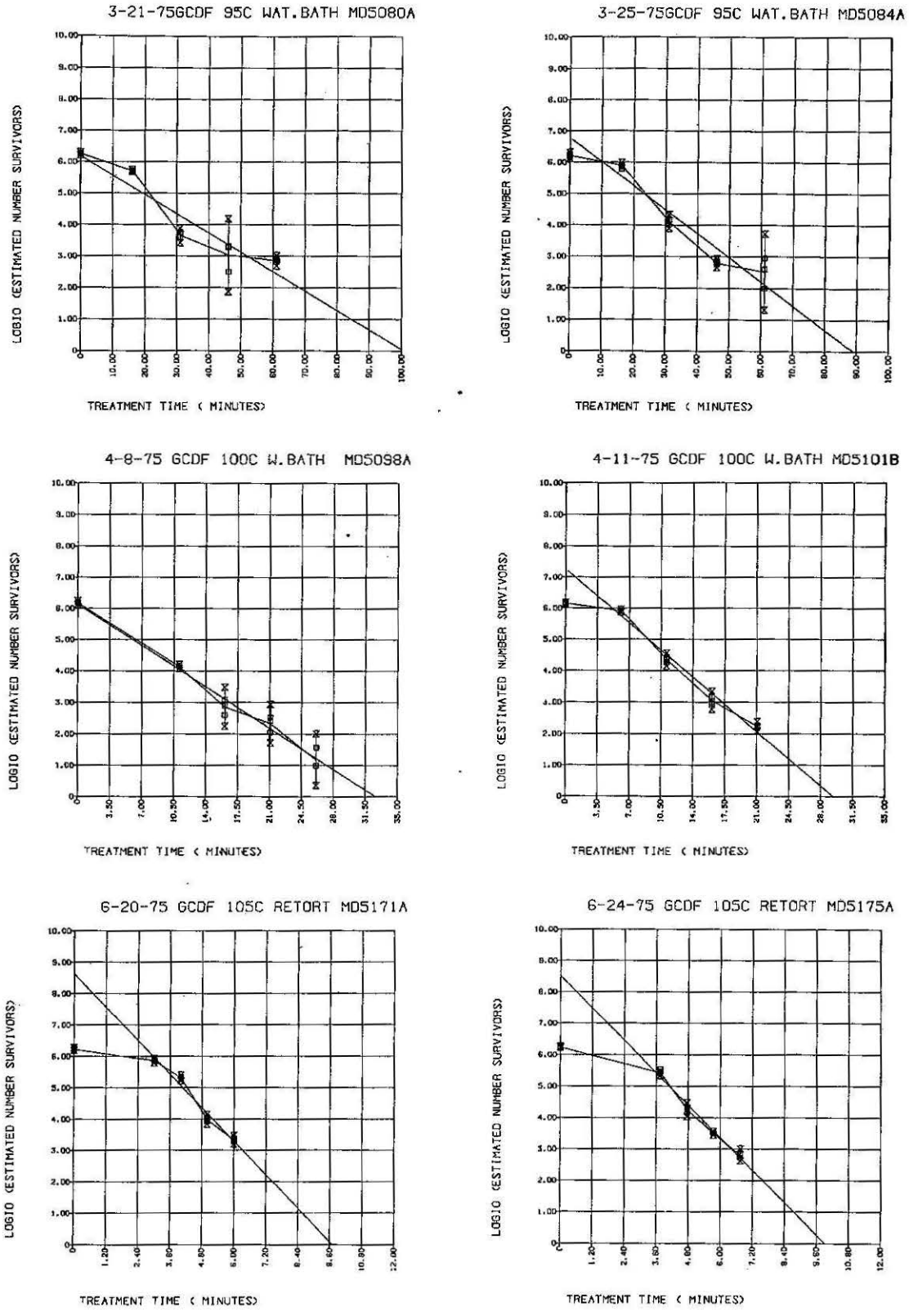

Figure 10: Clostridium sporogenes spores heated in dextrose 5% in lactated Ringer's solution.

Table 13

Results of Survivor Curve Tests for *Clostridium sporogenes*

Spores GCDF Suspended in 5 ml of Dextrose 5% in Water

Test No.	Mean Test Temp. °C	D-Value (min)	95% C. I. (min)	Width of 95% C. I.	IR
MD5084B	93.9	14.0	11.8 - 17.3	5.50	1.21
MD5091A	94.8	13.4	11.4 - 16.3	4.90	1.18
Mean		13.7			1.20
MD5101A	99.7	4.29	3.88 - 4.77	0.89	1.20
MD5262A	100.0	4.38	3.96 - 4.89	0.93	1.21
Mean		4.34			1.20
MD5063A	105.1	1.28	1.16 - 1.41	0.25	1.44
MD5171C	105.1	1.39	1.33 - 1.46	0.13	1.38
Mean		1.34			1.41

Table 14

Results of Survivor Curve Tests for *Clostridium sporogenes*

Spores GCDF Suspended in 5 ml of Water for Injection

Test No.	Mean Test Temp. °C	D-Value (min)	95% C. I. (min)	Width of 95% C. I.	IR
MD5087A	105.0	14.9	12.4 - 18.7	6.3	1.08
MD5168B	105.0	12.5	10.3 - 15.8	5.5	1.09
Mean		13.7			1.08
MD5269A	110.1	3.99	3.43 - 4.78	1.35	1.15
MD5273A	110.2	4.16	3.81 - 4.59	0.78	1.11
Mean		4.08			1.13
MD5239A	115.2	2.01	1.87 - 2.17	0.30	1.03
MD5247B	115.0	2.23	1.93 - 2.64	0.71	1.00
Mean		2.12			1.02

Table 15

Results of Survivor Curve Tests for *Clostridium sporogenes* Spores

GCDF Suspended in 5 ml of Butterfield's Buffer (pH 7.2)

Test No.	Mean Test Temp. °C	D-Value (min)	95% C. I. (min)	Width of 95% C. I.	IR
MD4094A	104.7	22.8	20.5 - 25.8	5.30	1.12
MD5168A	105.0	19.6	17.3 - 22.7	5.40	1.14
Mean		21.2			1.13
MD5070A	110.0	7.35	6.74 - 8.09	1.35	1.09
MD5269B	110.2	6.95	6.49 - 7.47	0.98	1.03
Mean		7.15			1.06
MD5247A	115.0	2.07	1.90 - 2.28	0.38	1.00
MD5252B	115.1	2.11	1.96 - 2.28	0.32	1.02
		2.09			1.01

Table 16

Results of Survivor Curve Tests for *Clostridium sporogenes* Spores

GCDF Suspended in 5 ml of Sorensen's Buffer (pH 7.0)

Test No.	Mean Test Temp. °C	D-Value (min)	95% C. I. (min)	Width of 95% C. I.	IR
MD5087B	105.0	42.7	40.5 - 45.2	4.70	1.12
MD5094B	105.0	42.4	38.4 - 47.3	8.90	1.15
Mean		42.6			1.14
MD5234B	110.1	9.87	8.96 - 11.0	2.02	1.11
MD5273B	110.2	8.47	7.39 - 9.91	2.52	1.11
		9.17			1.11
MD5252A	115.0	3.13	2.61 - 3.90	1.29	1.16
MD5279A	115.0	3.42	2.96 - 4.06	1.10	1.07
Mean		3.28			1.12

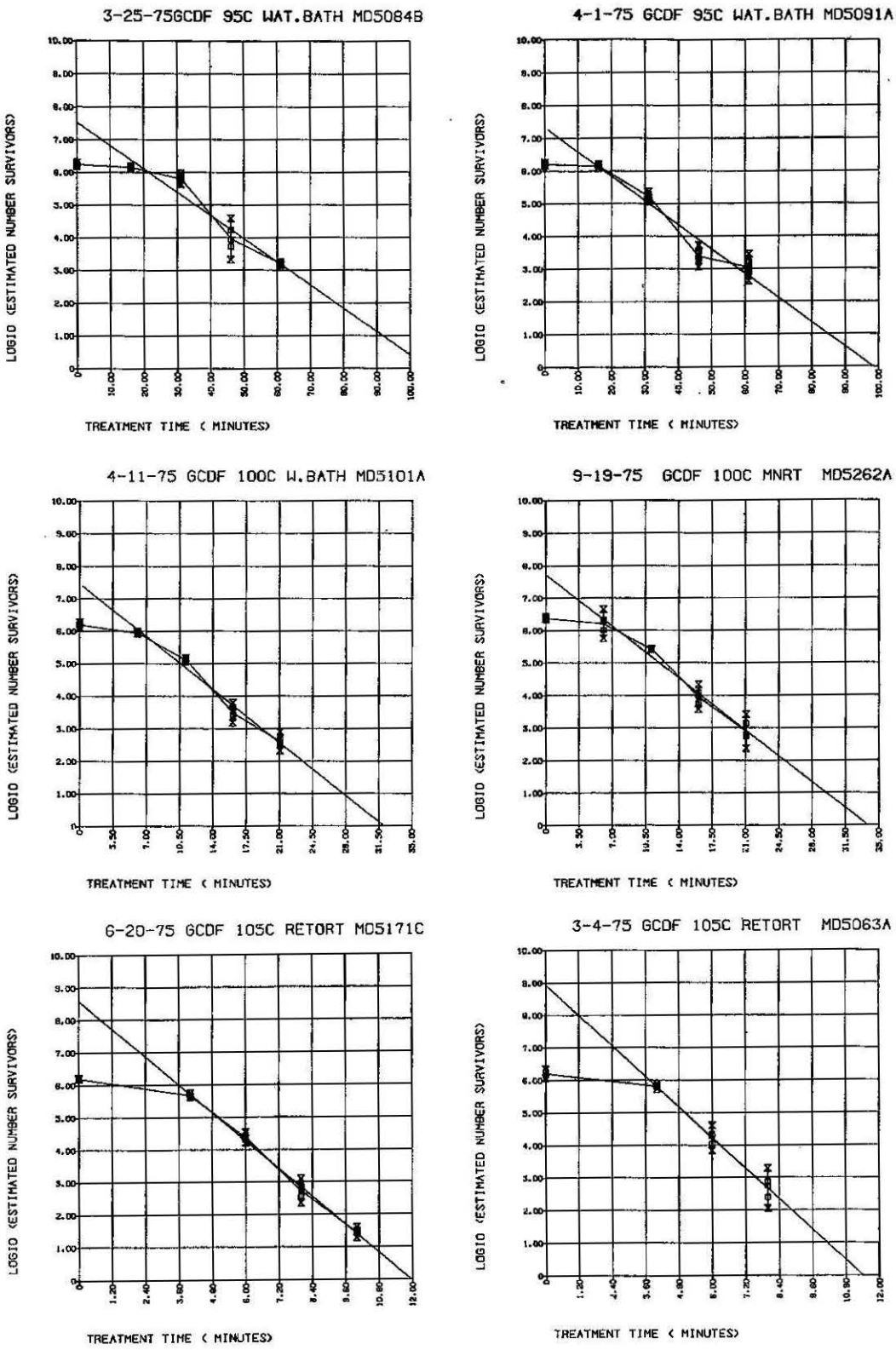

Figure 11: Clostridium sporogenes spores heated in dextrose 5% in water.

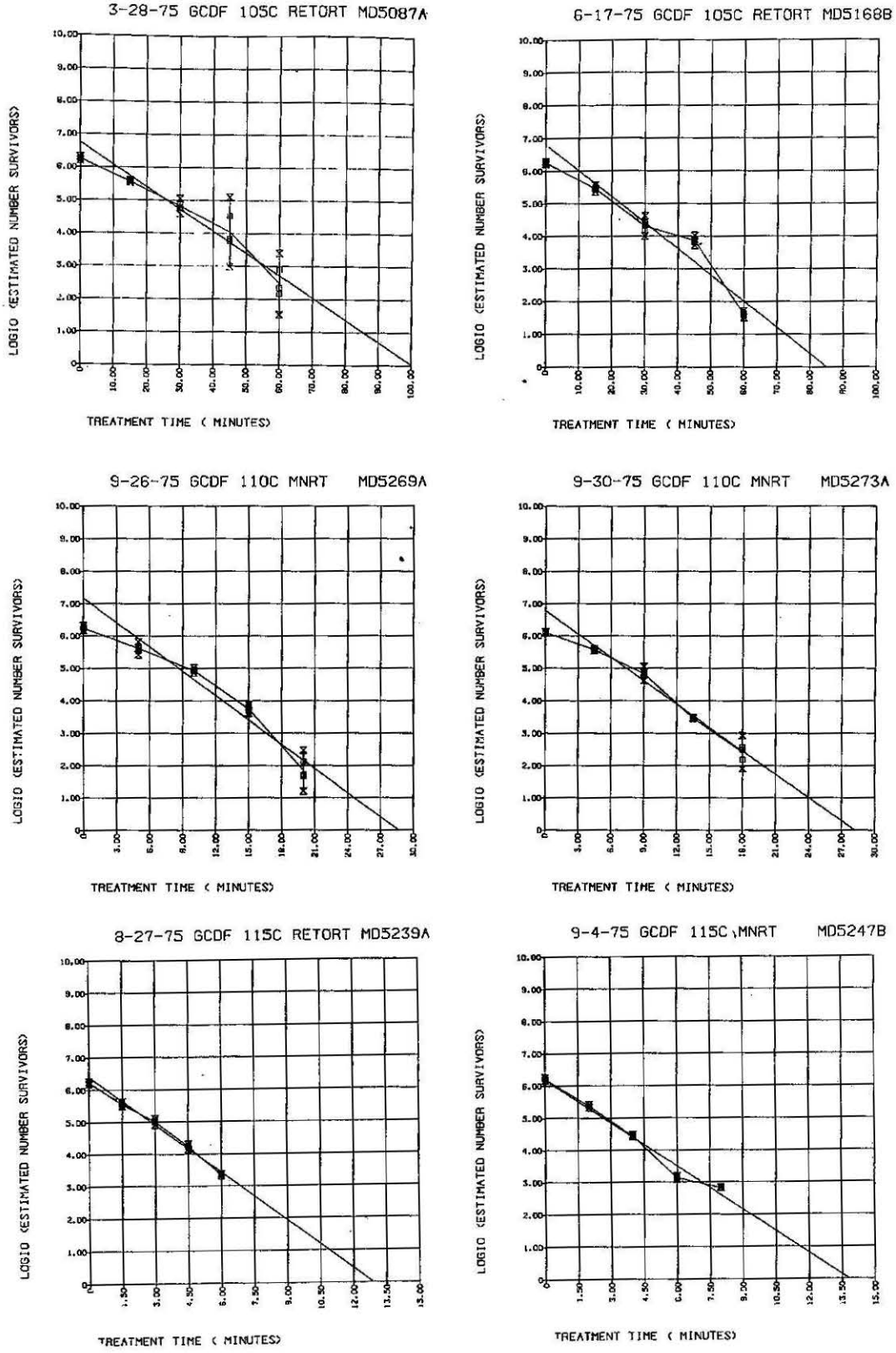

Figure 12: Clostridium sporogenes spores heated in water for injection.

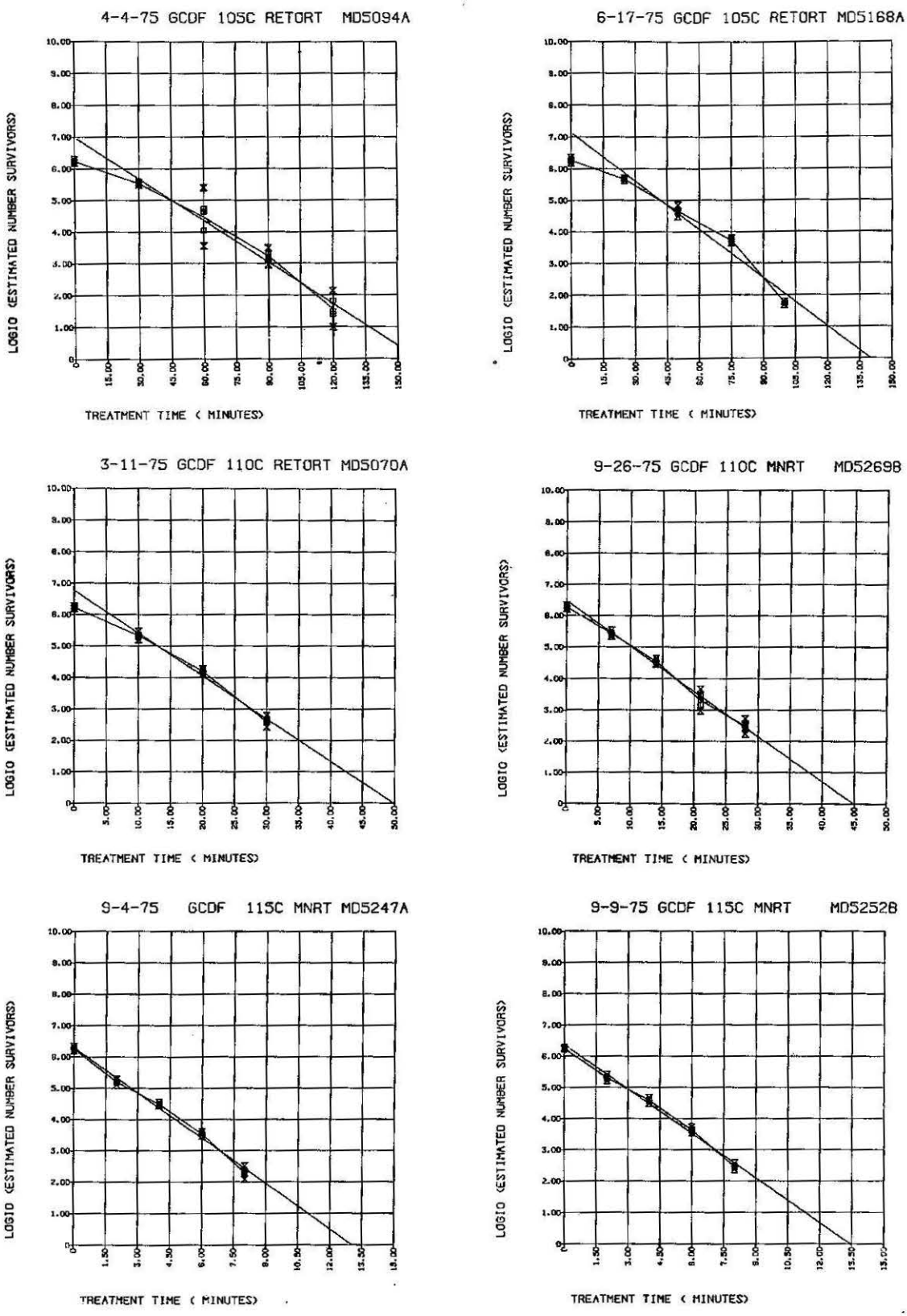

Figure 13: Clostridium sporogenes spores heated in Butterfield's phosphate buffer.

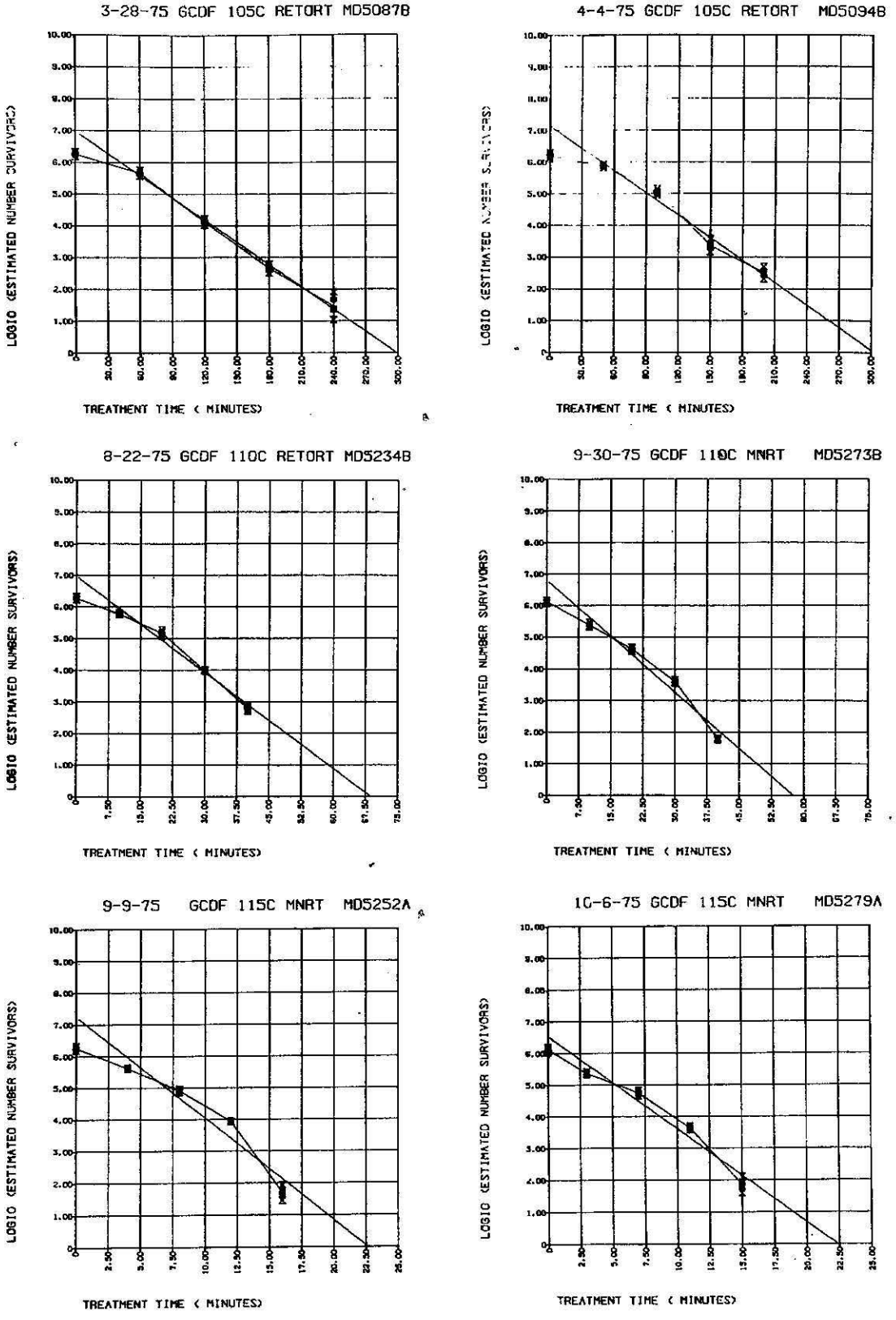

Figure 14: Clostridium sporogenes spores heated in Sorensen's M/15 phosphate buffer.

Table 17

z-Value Results for Clostridium sporogenes Spores in Six Solutions

Solution	z-Value °C	95% C. I. of z, °C	Width of 95% C. I.
Dextrose 5% in Saline	12.2	11.5 - 12.9	1.4
Dextrose 5% in Lact Ringer's	9.9	8.2 - 12.4	4.2
Dextrose 5% in Water	10.7	9.8 - 11.7	1.9
Water for Injection	12.4	10.0 - 16.5	6.5
Butterfield's Buffer (pH 7.2)	10.2	9.4 - 11.1	1.7
Sorensen's Buffer (pH 7.0)	9.0	7.8 - 10.5	2.7

Table 18

z-Values (°C) for Clostridium sporogenes Spores GCDF Calculated Using a Least Squares Fit With Three Temperatures and Two Point z-Values for The Two Sub-Intervals

Solution	z-Value °C Least Square Line For Three Temp.	Two-Point z-Value, °C	
		Lowest Temp. Interval	Highest Temp. Interval
Dextrose 5% in Saline	12.2	12.4	11.9
Dextrose 5% in Lact. Ringer's	9.9	11.2	8.9
Dextrose 5% in Water	10.7	11.1	10.3
Water for Injection	12.4	9.8	17.4
Butterfield's Buffer	10.2	11.1	9.3
Sorensen's Buffer	9.0	7.7	10.8

The two-point z-values shown in Table 18 are all within the z-value 95% confidence interval in Table 17, except for water for injection and Sorensen's buffer. This suggests that the use of a straight z-value line for these two solutions is open to question. The z-value line for Clostridium sporogenes spores in water for injection and Sorensen's buffer may be a curve. It is interesting that the Clostridium sporogenes spores in these solutions form concave upward curves whereas the trend for Bacillus stearothermophilus z-value lines is to be concave downward (Figures 15 and 8 respectively).

Non-linear z-values have been previously reported: Esselen and Pflug (1956) evaluated the thermal resistance of Putrefactive Anaerobe No. 3679 (a strain of Clostridium sporogenes) in vegetables in the temperature range of 121 to 143°C and found a trend for the z-value to increase with increasing temperature. The average z-value for the temperature range 121 to 132°C was 8.8°C and for 132 to 143°C was 12.2°C. Licciardello and Nickerson (1963) observed a z-value line for Clostridium sporogenes spores in phosphate buffer that was concave downward, the z-value was 12°C between 90 and 105°C and 9°C between 105 and 120°C. Wang et al. studied the heat destruction of Bacillus stearothermophilus spores and found that the thermal resistance curve was concave upward. These results taken together suggest that the z-value is a function of the particular organism being evaluated and that both Bacillus stearothermophilus and Clostridium sporogenes can produce straight concave upward and concave downward z-value lines depending on the test system.

Calculated D-values for 100 and 120°C are presented in Table 19. The spores GCDF have relatively high resistance in three solutions and low resistance in three solutions. The three high resistance solutions are: Sorensen's M/15 phosphate buffer (pH 7.0), D(120°C) value of 0.84 minutes; water for injection, D(120°C) value of 0.78 minutes; and Butterfield's phosphate buffer (pH 7.2), D(120°C) value of 0.70 minutes. The low resistance solutions are dextrose 5% in saline, in water, or in lactated Ringer's. At 100°C the D-values in dextrose 5% in water and in dextrose 5% in lactated Ringer's are nearly the same, whereas the D-value in dextrose 5% in saline is more than 50% greater than for dextrose 5% in water or dextrose 5% in lactated Ringer's solution.

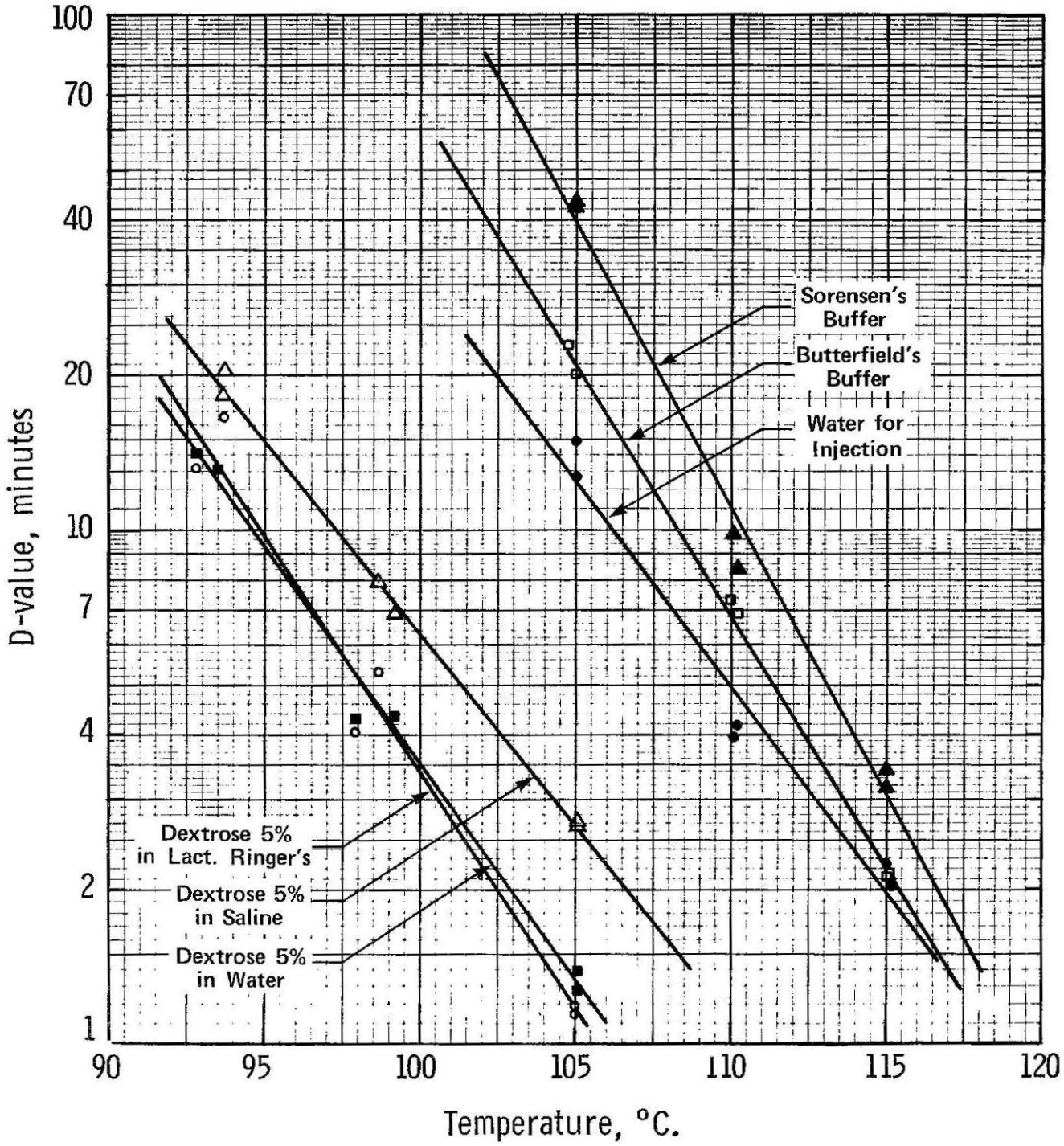

Figure 15: z-Value analysis for Clostridium sporogenes spores heated in six solutions.

Table 19

D_T-Values for Spores GCDF in the Six Solutions as Part of the z-Value Analysis for 100°C and 120°C

Solution	Calculated D_T-Value, (min) T = 100°C	T = 120°C
Dextrose 5% in Saline	7.07	0.16
Dextrose 5% in Lact. Ringer's	3.96	0.037
Dextrose 5% in Water	4.08	0.054
Water for Injection	31.6	0.78
Butterfield's Buffer	65.8	0.70
Sorensen's Buffer	143	0.84

Discussion of the Shape of the Clostridium sporogenes Survivor Curves

The shape of the survivor curves for Clostridium sporogenes spores GCDF in the six different solutions was found to vary with solution and test temperature.

Survivor curves for spores heated in dextrose 5% in saline are shown in Figure 9. There appears to be a significant temperature effect on survivor curve shape. At the middle temperature the survivor curves were approximately straight lines, at the lowest temperatures the curves were sigmoidal in shape while at the highest temperature the curves were concave downward.

The survivor curves for spores heated in dextrose 5% in lactated Ringer's solution shown in Figure 10 are sigmoidal in shape at the lowest temperature. At the higher temperatures they are concave downward.

The survivor curves for spores heated in dextrose 5% in water shown in Figure 11 are sigmoidal in shape at the lower temperatures and concave downward at the higher temperatures. There is much similarity among the survivor curves of spores in dextrose 5% in saline, dextrose 5% in lactated Ringer's solution and in dextrose 5% in water.

The survivor curves for spores heated in water for injection shown in Figure 13 are slightly concave downward at 105 and 110°C but are generally straight lines through N_o at 115°C.

The survivor curves for spores heated in Butterfield's buffer shown in Figure 14 are somewhat similar to the survivor curves for water for injection in that at 105°C the curves are concave downward but at 110 and 115°C the survivor curves are straight lines.

The survivor curves for spores heated in Sorensen's M/15 phosphate buffer shown in Figure 15 are all generally similar in shape and all are concave downward.

When Clostridium sporogenes spores GCDF were heated in a solution containing dextrose, not only was the heat resistance lower in these solutions but the survivor curves were all sigmoidal in shape at the lower test temperatures. The curves were straighter at the higher test temperatures.

It appears that the carbohydrate in the solution (the 5% dextrose is responsible for the decrease in heat resistance of the spores. The D(100°C) value of the spores GCDF is 31.6 minutes in water for injection and 3.51 minutes in dextrose 5% in water. The only difference between these two solutions is the dextrose; however, the dextrose does lower the pH from approximately 5.7 to perhaps 5.0. In further tests it would be interesting to change the pH of the buffer solutions to find out if it is the ionic strength and nature of the buffer solution that gives the high D-values or if pH is also involved.

The reproducibility of these uniquely shaped survivor curves and the rather small confidence interval of the data all add validity to these observations.

From these results, it is obvious that if spores of Clostridium sporogenes similar to GCDF are used as biological indicators in parenteral solutions, the resistance of these spores in the specific solutions must be evaluated before they can be used in a meaningful way to monitor heat processes.

We believe that it is very important that additional z-value studies be carried out using another strain of Clostridium sporogenes to determine if this pattern of resistance variation with the suspending solution holds for other strains of this organism. In carrying out additional tests it is suggested that perhaps only three or four solutions be evaluated; they are: Sorensen's M/15 phosphate buffer, water for injection, dextrose 5% in water and perhaps dextrose 5% in saline.

SUMMARY AND CONCLUSIONS

In this report we have presented the results of survivor curve tests using spores of two common test organisms, Bacillus stearothermophilus and Clostridium sporogenes. The tests were carried out in a single system using six different solutions in which the spores were suspended during the heat treatment. Bacillus stearothermophilus tests were carried out at four different temperatures and Clostridium sporogenes tests at three different temperatures. All tests were carried out at least two times on different days. Data have been included for Bacillus stearothermophilus where a single spore crop has been evaluated over a three month period.

We conclude from the repeated test series using Bacillus stearothermophilus that when a single spore suspension is tested repeatedly in the same test system, the spores will produce essentially identical survivor data for all of the tests.

The spores of Bacillus stearothermophilus and of Clostridium sporogenes were suspended in six different solutions in this test program. From the results we observe that the shape of the semi-logarithmic survivor curve changes with solution and test temperature. The shape of the survivor curve appears to be a unique property of the microorganisms being evaluated, the solution in which it is suspended and the temperature at which it is being tested.

The temperature coefficient of heat destruction also appears to be a unique property of the spores being evaluated and the test solution. It is often assumed that when the logarithm of the D-value is plotted versus temperature, a straight line will result. The data that we have presented suggests that in general over reasonably narrow ranges of temperatures, this is a practical assumption. However, the D-value data do not produce a straight z-value line in all cases. The shape of the z-value curve appears to vary with solution and microorganism; for Bacillus stearothermophilus spores the z-value line varies from being straight to being concave downward, whereas for Clostridium sporogenes spores the z-value line varies from being straight to being concave upward.

Two tools, the semi-logarithmic survivor curve and the semi-logarithmic thermal destruction curve have been used in this analysis. We believe that it is important to emphasize that these are empirical tools to aid the

researcher and the user of microbial destruction data. These tools offer a standard form for analyzing the data so that variations from this form can be observed. In this report large numbers of survivor curves have been included to visually communicate the effect of the solutions and test temperature on the survival of the Bacillus stearothermophilus and Clostridium sporogenes spores. A wide range of survivor curve shapes have been obtained; only a few of these semi-logarithmic survivor curves are straight lines through N_o. We conclude that the precise shape of the survivor curve and the precise shape of the thermal destruction curve is a unique properly of the microorganisms being tested, the suspending solution, and the test system that includes all environmental factors that can impinge on microbial survival. We believe that the effect of measurable and non-measurable environmental conditions on microorganisms manifest themselves as changes in the destruction rate which appear as changes in the shape of the survivor curve.

Since only a few of the semi-logarithmic survivor curves are straight lines through N_o, it must be concluded that the heat destruction of Bacillus stearothermophilus and Clostridium sporogenes does not exactly follow the semi-logarithmic model. However, there is no good reason why we should not use the semi-logarithmic model as a framework for evaluating heat destruction data. The semi-logarithmic model can be used efficiently as a standard form for the comparison of the performance of the same spores under different test conditions or different spores under similar test conditions.

The results of these experiments suggest strongly that the only way to determine how a specific spore will perform under a specific set of environmental conditions is to evaluate the organism under these conditions. We believe that there are so many factors involved in microbial spore destruction that we will have to wait for major advances in the understanding of spore death before we can generate the elaborate destruction model that will be necessary to take into account all of the factors that impinge on the microbial destruction rate.

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