THE EFFECT OF POPULATION LEVELS AND EXPOSURE TIMES AND TEMPERATURES ON GROWTH CURVES OF M S 102

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THESIS

THE EFFECT OF POPULATION LEVELS AND EXPOSURE TIMES AND TEMPERATURES ON GROWTH CURVES OF MS 102

I. The lag phase of MS 102 with constant population and variable heat.

II. The lag phase of MS 102 with constant heat and variable population.

By

ORMOND CHARLES PAILTHORP

AN ABSTRACT

Submitted to the College of Agriculture
Michigan State University of Agriculture and
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of the requirements for the degree of

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Department of Dairy

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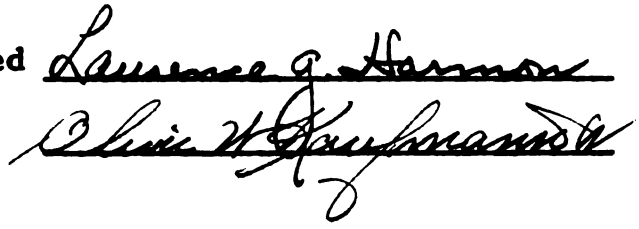
Approved 

ABSTRACT

Selected population levels of Micrococcus varians sp. MS 102 were heated at 61°C. for 30 minutes, 69°C. for 17 seconds, 76°C. for 17 seconds, 81°C. for 5 seconds and 82°C. for 5 seconds. Growth curves were constructed for survivor populations in milk; a geometrical method of lag measurement was used to estimate the lag times for all growth curves. Control lag times for the various cell levels were established simultaneously for use as norms for MS 102 in milk.

At high initial inoculum levels (100,000 to 200,000 cells per ml.) the lag times with unheated cells were comparable to those obtained after heat treatment. At the temperatures cited above, the lag phases were progressively extended beyond control lag phases by elevating the processing temperature. This temperature - lag time relationship prevailed at medium and low survival levels (10,000 to 20,000 and 1,000 to 9,000 cells per ml. respectively), but not at high populations of survivors after heating. A notable exception occurred at low initial cell populations

after Low-temperature Long-time treatment when the lag time (42 hours) equalled that obtained after 82°C. for 5 seconds.

The maximum lag times (1.5 to 2.0 days) were obtained with medium, low and very low survivor populations after heat treatment at 82°C. for 5 seconds (the highest temperature studied).

Control growth curves of MS 102 in milk revealed progressively shorter lag phases for initial populations above 1,000 cells per ml.; below this population the lag phase increased gradually as the initial cell concentration was reduced.

Lag times for high survivor levels were not significantly altered by the heat treatments explored. At high concentrations of survival the size of the initial population appeared to affect the lag times in a manner similar to that observed for unheated controls. The combined lag extending effects of lower populations and heat treatment seemed to account for the appreciable lag time extensions obtained for medium and low survivor populations.

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To Jacqueline, Nanette

and

My Parents

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INTRODUCTION

In the interest of public health, several pasteurization standards for milk have been developed and approved by the various regulatory agencies. Initially, Low-temperature Long-time (LTLT) (143°F. for 30 minutes) milk pasteurization prevailed; eventually, a High-temperature Short-time (HTST) (161°F. for 16 seconds) process was accepted. The consumer's protection from pathogens was paramount while the keeping quality or shelf life was less significant.

Continued research revealed the presence, in milk and other foods, of heat tolerant bacteria capable of propagating at post-heat storage temperatures used for these foods. The shelf life of milk and its products was shown to be partially dependent on the type and numbers of thermoduric bacteria surviving pasteurization. Instances of delayed bacterial spoilage were reported. One plausible explanation was that injury to the cell during heating was followed by a recovery or lag period and physiological adjustment which preceded logarithmic growth. Research has demonstrated the extension of the cellular recovery period by controlled exposure of the cells to physical and chemical agents.

The study reported herein was designed to supplement the information concerning the reaction of bacteria to various levels of "heat shock." The roles of population size, and time and temperature as precursors of extended bacterial latency were examined.

LITERATURE REVIEW

The Lag Phase of Bacterial Growth

Porter (18) acknowledged that Muller probably first noted the lag phase of bacterial growth in 1895. The lag phase is described by Thomas and Grainger (24) as a period during which the population level is nearly constant. Hinshelwood (12) described lag more specifically as the elapsed time between inoculation and the stage of maximum cell division. True lag was differentiated as the elapsed time between inoculation and initial cell growth; apparent lag was described as the entire period from the initial inoculation until the onset of logarithmic growth. Both of these lag phases appear to be similar. Smith and Martin (23), as well as Sarles et al. (21), also defined the bacterial lag phase as a time lapse between initial inoculation and the onset of the logarithmic growth rate. Another description of lag was presented by Dubos (5) who referred to the lag period of a culture as the time during which there was an increase in cell volume in the absence of cell division.

Several explanations for bacterial latency have been presented. Winslow (28) employed cell counts at selected time intervals to demonstrate two distinctly different phenomena of lag. He reported an adjustment period involving bactericidal or bacteriostatic action and a period during which the cell mass increased rapidly while propagation was retarded. Hershey (11) determined that bacterial latency was created by a pause in the fission of smaller cells which later would undergo division when they had attained adult proportions on the new substrate.

Experimental studies of Chesney (3) were designed to secure information on the metabolic activities of selected pneumococci. He concluded that cell alteration was reflected as a deviation in lag from the normal for a constant set of conditions. Such alterations were believed to have resulted from the lack or absence of a metabolic factor essential to normal physiological activity. Cell injury merely resulted from the direct or indirect exposure of the cells to their metabolic wastes. The extent of this injury would be measured by the lag extension. Where cell damage was great, death would probably follow. Chesney concluded that lag evidenced current or previous exposure to unsuitable nutritional sources.

Many factors have been described, any one or combination of which would conceivably alter the period of latency. Substrate constituents may be inadequate to nourish normal growth (3, 8, 12). Hydrogen ion concentration affects the growth curve (12). The cautious control of pH is advisable during any studies of growth characteristics. Topley and Wilson (26) include the size of inoculum among factors considered essential to normal lag and growth. Within certain limits, the inoculum level may show effect upon the growth characteristics. Hershey (11) observed the effect of population levels on the latent period of Escherichia coli. The lag was not affected by the size of seeding when the inoculum was small. Pure cultures involving concentrations above 10^7 cells per ml. in fresh broth resulted in an unfavorable environment which caused fission of smaller cells, thus shortening the resulting latent period. At the same time, the rate of increase in cell size was retarded.

The growth stage of parent cells, freshly transferred to a suitable medium, affects the lag phase of their progeny (26); in addition, the duration of the latent phase reportedly decreases as the frequency of transfer increases.

As the optimum growth temperature for an organism is approached, the lag portion of the growth curve more closely approximates the norm when all other controlling factors are maintained at optimum (12, 26). Appreciable variations in incubation could thus alter the results of controlled studies on bacterial growth.

Specific genera of organisms may possess a typical lag phase; that is, a time lag which is particularly long or short relative to latent phases of other bacterial genera. The coli-typhoid group has been observed to reach the logarithmic phase of growth very rapidly (26).

At least two methods of lag measurement have been proposed. Hershey (9) calculated lag as an increase in bacterial size or cell volume. He assumed an average maximum size for the adult cell of each species and derived the formula below:

$$L = \frac{1}{M \log 2} \cdot \log \frac{4Sa}{3So}$$

where L = time for 50% population increase

M = generations per hour assuming binary fission

Sa = average size of adult cells

So = average cell size initially

Aerated cultures provided stable cells when all oxidizable matter had been utilized. These stabilized cells were used to construct growth curves from which the required values were obtained.

Hinshelwood (12) proposed a method for lag measurement. This was recently evaluated by Finn (7) who considered the method comparable to that for measuring relative lag.

Changes in the lag phase of bacterial growth have been observed under various experimental conditions. Eijkman (6) heated B. coli at 125.6°F. for 0 to 35 minutes. Suspension of cells heated for 6 to 35 minutes showed no growth at 3 days; all but the 35 minute sample showed bacterial growth at 13 days. Lawton and Nelson (16) explored the influence of heat and chlorine on the growth of selected psychrophiles. Survivors of a culture partly destroyed by heat displayed increased lag when incubated at 5 or 10°C. Incubation at 25°C. or less resulted in less extension of lag. Extension of the latent period was demonstrated using Pseudomonas fluorescens and Pseudomonas geniculata isolated from milk.

The effect of plating media on the recovery of heat treated Pseudomonas fluorescens was observed by Heather

and van der Zant (8). Plate counts on more complex media yielded more survivors. The addition of glutamic acid, proline, histidine, glycine and methionine, normal chemical components of milk, to plating media enabled heat shocked cells to propagate in greater numbers on synthetic substrates. Studies of factors limiting bacterial growth led Hershey (9) to report that bacteria surviving heat showed a progressively lengthened lag phase. Unexposed cells and cells exposed to 56°C. were compared via growth characteristics. This worker concluded that extended latency resulted from injury, rather than from the survival of specially adapted cells occurring in small numbers. Extension of the lag phase of Escherichia coli was demonstrated.

Kaufmann and Andrews (14) established the thermal destruction rates of selected Pseudomonads to determine their heat resistance. Experimental results indicated that both growth rate and contamination level are important in psychrophilic spoilage of milk. It was concluded that the initial population level at contamination may be less significant than the growth rate of the organism in so far as shelf life is concerned.

Mossel and Mol (17) incubated coffee milk at 32°C. and noted that slight curdling occurred after 11 days (initial controls were negative). Obvious spoilage resulted after 14 days, at which time Bacillus coagulans was isolated.

The Heat Tolerant Micrococcus varians sp. MS 102

Barber (1, 2) reviewed the history and characteristics of Micrococcus varians sp. MS 102. The organism was isolated from pasteurized milk at the National Dairy Research Laboratories. Studies revealed the ability of MS 102 to resist 180°F. for 15 seconds. Growth on N-Z-Amine Agar produced easily observed golden-yellow colonies, possessing a thermal death time curve slope (in ice cream mix), of 11.4°F. compared to 12.6°F. for Mycobacterium tuberculosis. Barber also discussed the stability of MS 102, its uniform resistance to heat, and ease of enumeration as the reasons for its use in the experimental heat treatment of bacteria.

Speck et al. (22) used MS 102 to test the efficiency of High-temperature Short-time pasteurization of ice cream mix. The results of these heat treatment evaluations follow: 180°F./2 seconds... 82.9 per cent survival,

185°F./1 second ... 94.1 per cent survival, 190°F./1 second ... 20.9 per cent survival. Tryptone Glucose Meat Extract Agar was employed as the recovery medium, and incubation was at 35°C. for 48 hours. The work of Read et al. (19) showed clearly the effect of "come-up time heat" on MS 102. At 88.8°C. for 0.25 second 99.9 per cent destruction was obtained. An equivalent kill occurred at 88.1°C. for 0.5 second. More recent studies by Read et al. (20) indicated 99.9 per cent destruction of MS 102 at 191.9°F. and 190.6°F. for 0.25 and 0.50 second, respectively, using milk as the suspensory medium. Within the 190.6° to 191.9° F. range, the pH of the milk was not significantly altered, and little protein denaturation was noted.

EXPERIMENTAL PROCEDURES

Normal Growth Curves at Adjusted Population Levels

Preparation of MS 102 Cells. Actively propagating cells of Micrococcus varians species MS 102 (1) from a 24-hour culture were transferred to N-Z-Case agar slants prepared according to Tobias et al. (25). The composition of the medium was as follows:

Yeast Extract	1.0 g.
N-Z-Case*	0.5 g.
Glucose	0.5 g.
K_2HPO_4	0.4 g.
KH_2PO_4	0.1 g.
Agar	1.5 g.
Distilled Water	100 ml.

Slants were incubated 24 hours at 32°C. ± 1°C. and then refrigerated. After 48 hours at 6°C. ± 1°C. the culture was transferred to 150 ml. prescription bottles and incubated for 24 to 26 hours at 32°C. prior to harvesting cells for growth studies in milk. Thirty ml. of agar were dispensed into each bottle to provide a reasonably constant surface area.

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*This preparation was obtained from Sheffield Chemical, Norwich, New York.

The 24 to 26 hour cultures of MS 102 were harvested using two 20 ml. portions of sterile, buffered distilled water. An inoculating needle was employed to tease cells away from the slant surface. The cell suspension was filtered through sterile cotton to remove clumps of agar and transferred to a chilled Waring blendor jar. Following 30 seconds of blending, the suspension was transferred to a dilution bottle. Cell loss during decantation was minimized by rinsing the blendor with sterile water. The final volume of suspended cells was adjusted to 100 ml. and refrigerated at 6°C. ± 1°C. until appropriate dilutions were made for normal growth curve studies in milk.

Dilution Scheme. Cell dilution methods were devised to yield desired populations per ml. Flow diagrams for all dilution procedures are given in Figure 2. Control growth studies were made in 150 ml. prescription bottles containing sterile, homogenized milk (3.5 per cent butterfat).

Some difficulty was encountered when the milk tended to display caramelization after autoclaving. This problem was avoided by autoclaving the milk at 121°C. for 10 minutes under 15 pounds of pressure.

Water for dilution blanks and cell washing was buffered to pH 7.0 and sterilized at 121°C. for 15 minutes

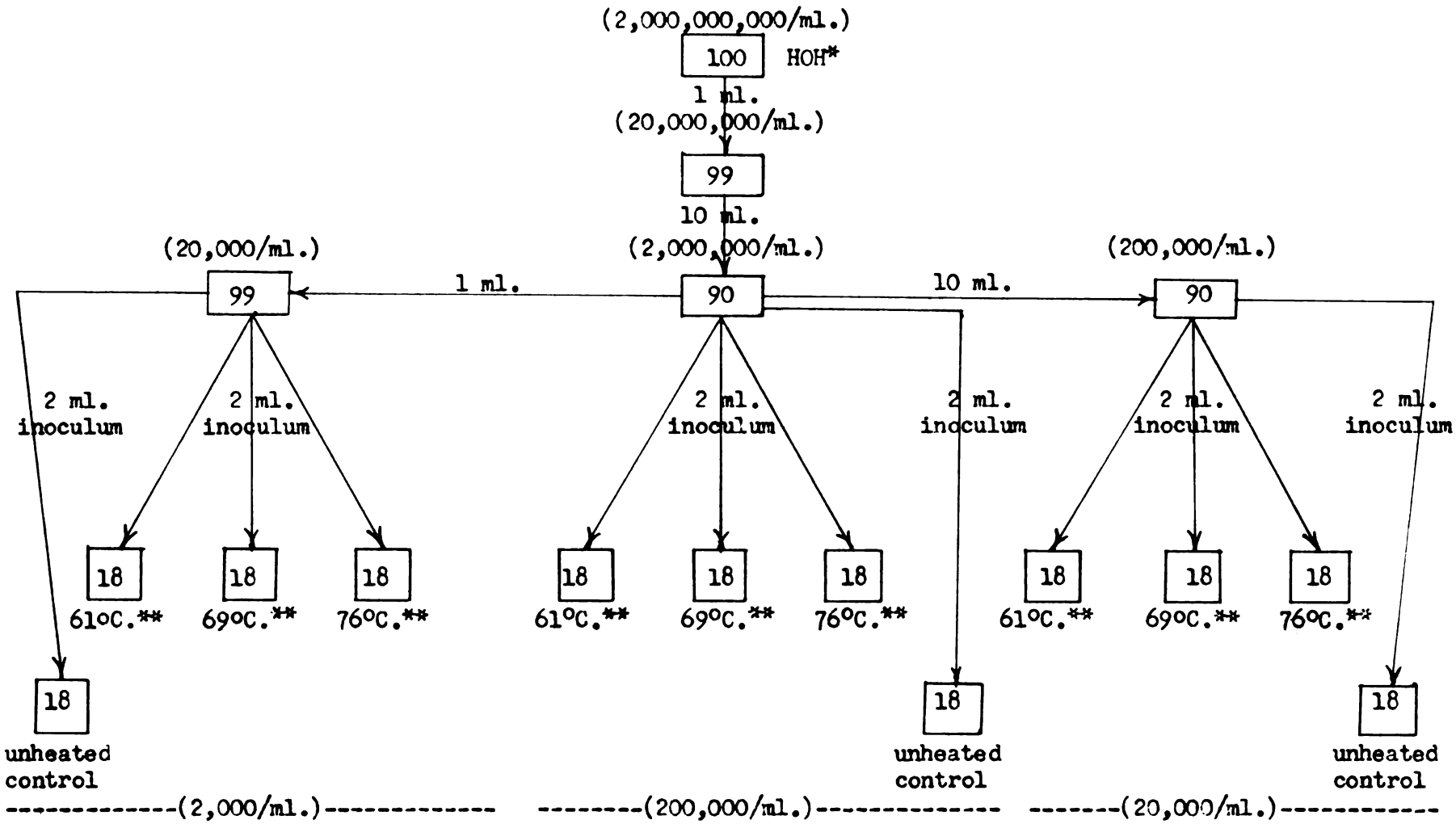

Figure 1. The procedure used to secure the desired cell concentrations before heat treatment.

*Sterile milk was used in dilutions below this level.

**Temperatures after the addition of 2 ml. of 43°C. inoculum.

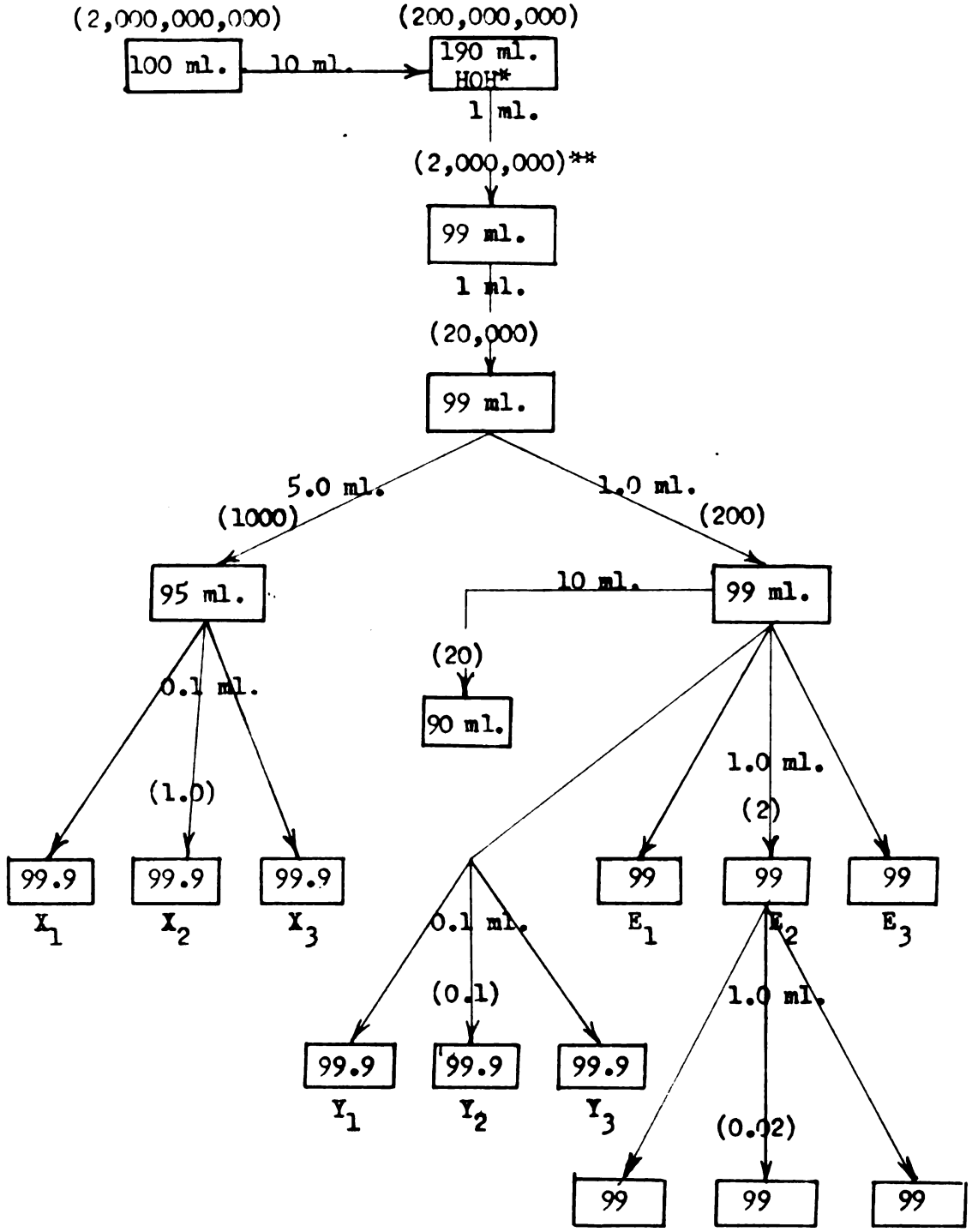

Figure 2. A diagram of the dilution procedure utilized to obtain extremely low cell concentrations prior to establishing unheated control growth studies.

*Buffered water. Except where otherwise noted, sterile milk was the diluent.

**Numbers in parentheses indicate the calculated cells per ml. at the various levels of dilution.

under 15 pounds of pressure. Cell yields per slant approximated two billion cells per ml. after dilution to 100 ml.

To assure uniform, accurate counts, cell suspensions were shaken 25 times in accordance with accepted methods before appropriate dilutions were made.

Incubation Procedure for Growth Curves. MS 102 was grown in milk which was tempered to 32°C. prior to inoculation. Incubation was interrupted only to take samples for plating at predetermined intervals. Since the lag was measured according to the graphical method proposed by Hinshelwood (12) and supported by Finn (7), it was necessary to obtain well defined lag segments of all growth curves.

Plating Procedure. Plate count agar was selected for plating. Samples containing less than one organism per ml. were plated in adequate volume to yield final counts of one or more cells per 10 ml. whenever possible. If less than one cell per ml. was encountered in low population studies, the result was converted to a whole number. Plates were poured in duplicate. When exceptionally low counts were encountered, the milk was distributed in several plates. The colonies counted per group of plates were divided by the volume plated to determine the count per ml. When

necessary, as previously discussed, counts of less than one cell per ml. were converted to whole numbers which represented larger aliquots. For example, 0.5 cell per ml. would convert to 5 cells per 10 ml.

Post Heat Shock Growth Curves

Procedures used for the preparation of cells, incubation of heated cells, plating, incubating and counting were the same as described under the previous section. Uniform procedures were selected so that growth curves of heated and unheated MS 102 populations could be compared.

Dilution Scheme. A flow diagram outlining the procedures employed to obtain cell dilutions of 1:10 and 1:100 is presented in Figure 1. The method diagrammed was also used in obtaining dilutions for growth curves after heat treatments at 81°C. and 82°C. Sterile, homogenized milk was used in the dilution procedure to obtain desired initial bacterial populations for all growth curves. Whenever survivor growth trials were made on heat treated cells, corresponding control growth curves were established on unheated cells.

Method of Inoculation for Heat Treatment. Eighteen ml. of sterile milk were added to sterile test tubes. The tubes and contents were brought to the required temperature before the organisms were injected. A thermometer well, containing 18 ml. of milk, permitted evaluation of the product temperature prior to inoculation and timing. Come-up time was further minimized by tempering the inoculum to 43°C. before injection into the heating medium.

Two ml. of inoculum were injected into the heated milk using a 5 ml. syringe with a six inch, 18 gauge needle. By ejecting the bacterial suspension while moving the needle toward the bottom of the tube, efficient cell dispersion was possible. Sterile gauze pads were utilized to wipe inoculum from the outside of the needle as bubbles were expelled from the syringe barrel. Timing commenced at the instant the inoculum was added. At the conclusion of the desired holding time, each tube was promptly plunged into a solution of 29 per cent calcium chloride which had been stored at -23°C. for 24 hours prior to use, as recommended by Kaufmann et al. (15). Hand agitation for 15 seconds hastened cooling of the heated contents of the tubes. The samples were held at -12°C. for 5 minutes before plating. All specimens (Figure 1) were brought to

room temperature prior to sampling to assure accurate measurement.

Heating Equipment. A Magni Whirl Full Visibility Jar Bath* was used to maintain the required temperatures.

Methods of Measurement. Holding times are estimated to be accurate to within ± 1 second. Manual operations were standardized to yield uniform tests. Temperatures were measured with two factory calibrated Centigrade thermometers. The relatively large volume of inoculum at 43°C. caused a demonstrable reduction in holding temperatures. A comparison of minimum temperatures calculated calorimetrically, and experimental values obtained each 5 seconds with a recording potentiometer, showed close agreement. Since the minimum temperature for all treatments was evidenced at 5 seconds, the minimum temperatures were selected for reporting and discussion.

The minimum and maximum temperatures for each treatment follow:

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*Manufactured by the Blue M Electric Company, Blue Island, Illinois.

Temperature of Milk before Inoculation	Minimum Calculated Temperature after adding 2 ml. of Inoculum
± 1°C.	
87°C.	82°C.
86°C.	81°C.
80°C.	76°C.
72°C.	69°C.
61°C.	60°C.

The recording potentiometer employed in this work first registered the temperature 5 seconds after the addition of the inoculum. That part of the heating curve which occurred between injection of the inoculum and 5 seconds of holding may be highly significant at the higher temperatures of treatment. Instruments were not available which would give a temperature response in less than 5 seconds, therefore it was impossible to evaluate this segment of the heating curve.

TABLE I

Plate counts of milk samples containing a high initial cell concentration of MS 102 after heat treatment at 61°C., 69°C. or 76°C.

TRIAL I*

Time (hrs.)	Unheated (control)	69°C. 17 sec.	76°C. 17 sec.	Time (hrs.)	61°C. 30 min.
	Av. count per ml. (x1000)	Av. count per ml. (x1000)	Av. count per ml. (x1000)		Av. count per ml. (x1000)
0	180	230	220	0	180
9	170	210	180	13	180
18	290	210	220	21	200
24	680	300	410	27	260
31	9200	700	6800	33	1300
36	17000	3900	8300	37	2100
46	19000	5300	3900	44	3400
-	-	-	-	50	52000
	TRIAL II				
0	210	240	250	0	190
10	230	270	260	13	260
15	390	310	270	21	330
20	450	500	290	27	370
25	5500	1600	260	33	1100
32	9000	14000	380	37	2800
37	26000	15000	1700	44	8700
42	16000	19000	1800	50	35000
48	840	960	810	-	-

*Trials I and II represent separate tests run on different generations of MS 102.

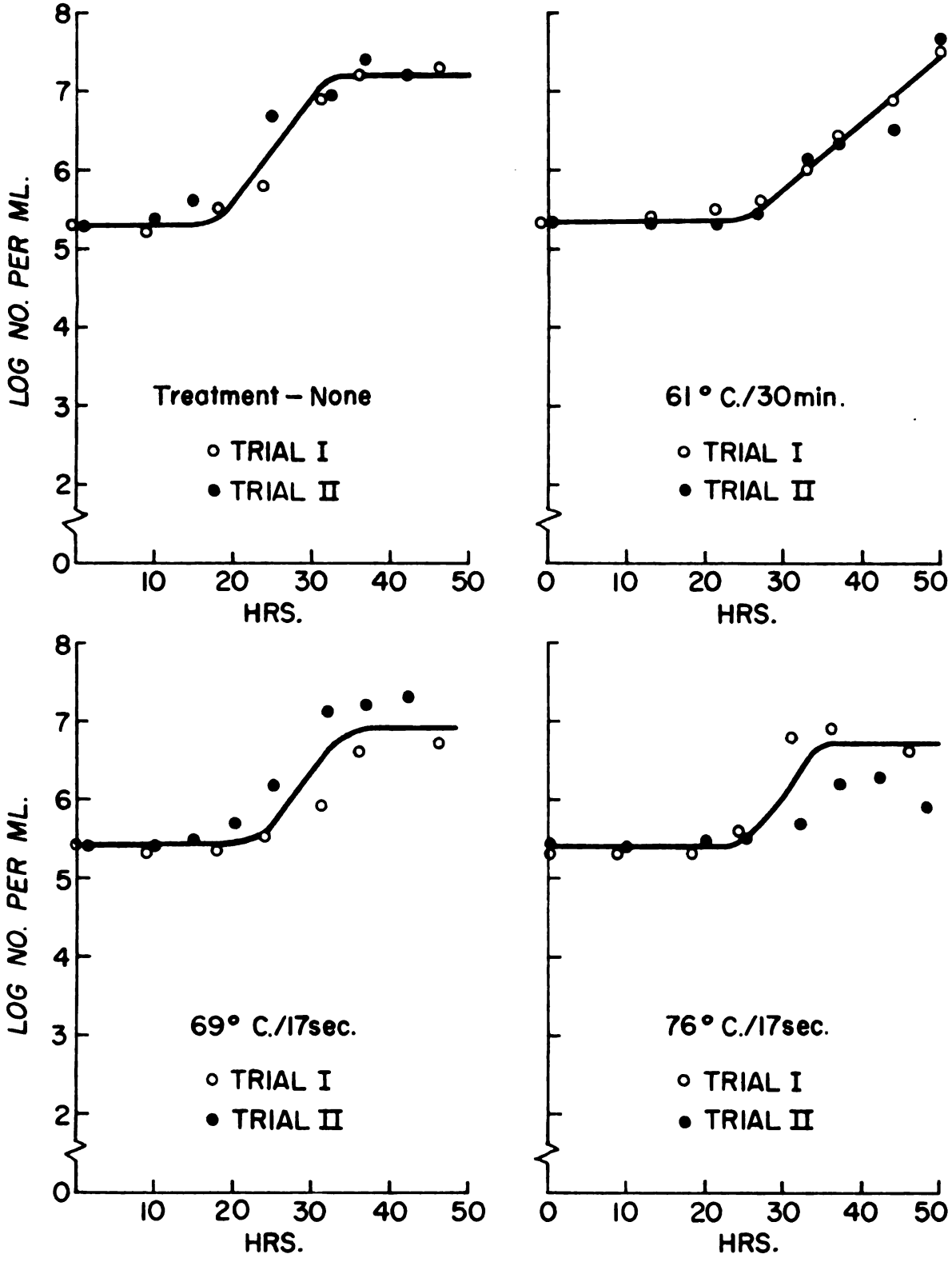


Figure 3. Effect of heat shock on the growth curve of survivors. (High initial cell concentration).

TABLE II

Plate counts of milk samples containing a medium initial cell concentration of MS 102 after heat treatment at 61°C., 69°C. or 76°C.

TRIAL I*

Time (hrs.)	Unheated (control)	69°C. 17 sec.	76°C. 17 sec.	Time (hrs.)	61°C. 30 min.
	Av. count per ml. (x1000)	Av. count per ml. (x1000)	Av. count per ml. (x1000)		Av. count per ml. (x1000)
0	14	21	17	0	17
9	13	19	14	13	37
18	30	16	9	21	18
24	80	49	200	27	720
31	5600	274	1500	33	2600
36	8400	1600	4300	37	8700
46	14000	3000	8300	44	11000
-	-	-	-	50	9200
	TRIAL II				
0	19	19	18	0	13
10	19	19	9	13	17
15	29	22	10	21	15
20	140	250	12	27	600
25	460	410	206	33	2500
32	5600	5400	4800	37	6200
37	7200	5400	19000	44	7600
42	14000	6500	9700	50	8100
48	3400	6900	11000	-	-

*Trials I and II represent separate tests run on different generations of MS 102.

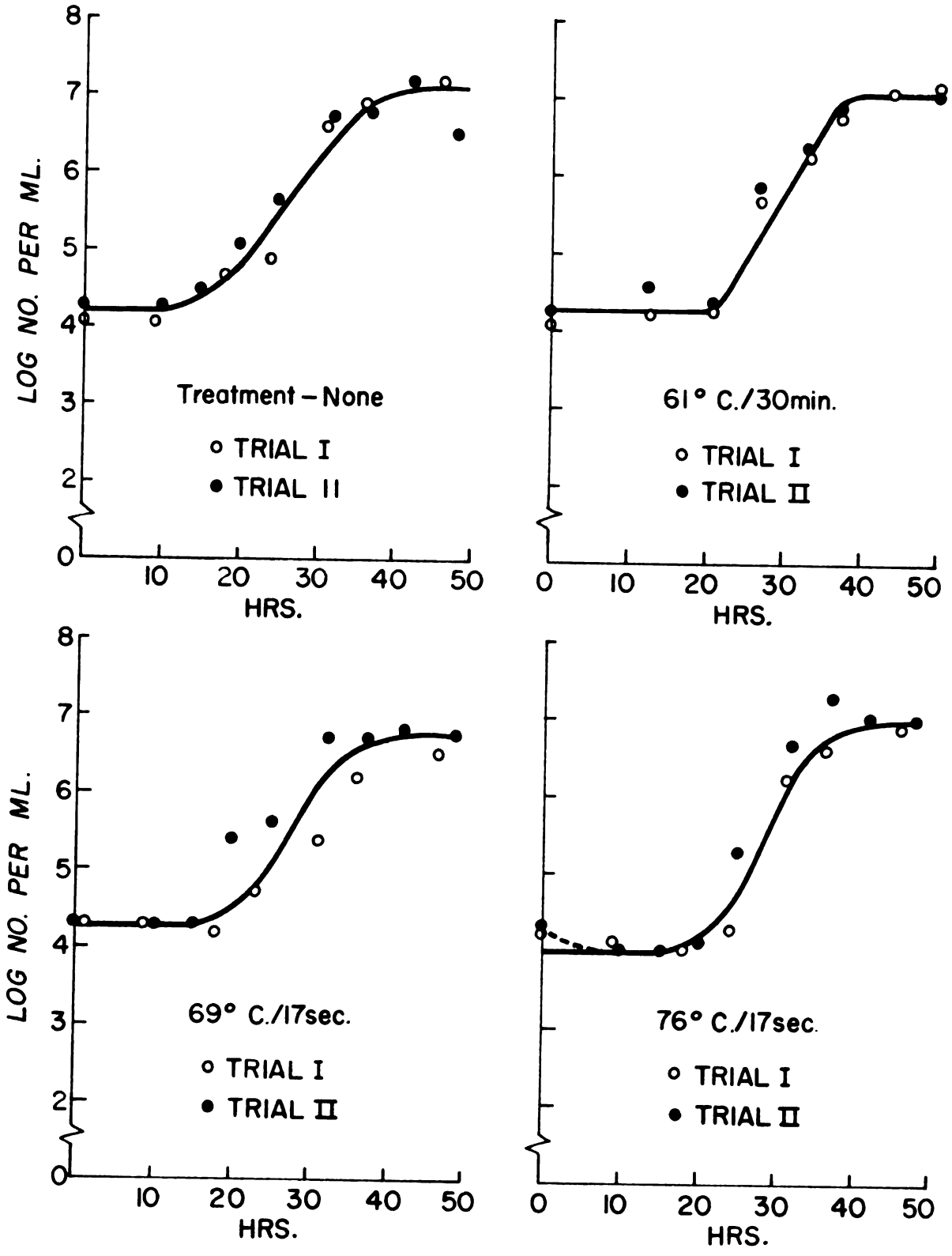


Figure 4. Effect of heat shock on the growth curve of survivors. (Medium initial cell concentration).

TABLE III

Plate counts of milk samples containing a low initial cell concentration of MS 102 after heat treatment at 61°C., 69°C. or 76°C.

TRIAL I*

Time (hrs.)	Unheated (control)	69°C. 17 sec.	76°C. 17 sec.	Time (hrs.)	61°C. 30 min.
	Av. count per ml. (x1000)	Av. count per ml. (x1000)	Av. count per ml. (x1000)		Av. count per ml. (x1000)
0	2.0	1.1	1.8	0	2.2
9	1.7	1.8	1.5	13	1.9
18	1.9	1.7	1.4	21	2.6
24	40.0	1.8	1.5	27	3.0
31	3200.0	19.0	7.0	33	3.1
36	3700.0	420.0	400.0	37	2.9
46	15000.0	1400.0	5500.0	44	4.5
-	-	-	-	50	1400.0
	TRIAL II				
0	2.4	2.3	1.7	0	1.8
10	2.0	2.1	1.9	13	1.5
15	2.7	2.6	1.9	21	1.7
20	2.2	20.0	2.1	27	1.5
25	57.0	280.0	1.7	33	2.1
32	2400.0	1100.0	3.7	37	1.8
37	5100.0	1100.0	360.0	44	2.5
42	3300.0	6500.0	2700.0	50	1700.0
48	41000.0	2900.0	2300.0	-	-

*Trials I and II represent separate tests run on different generations of MS 102.

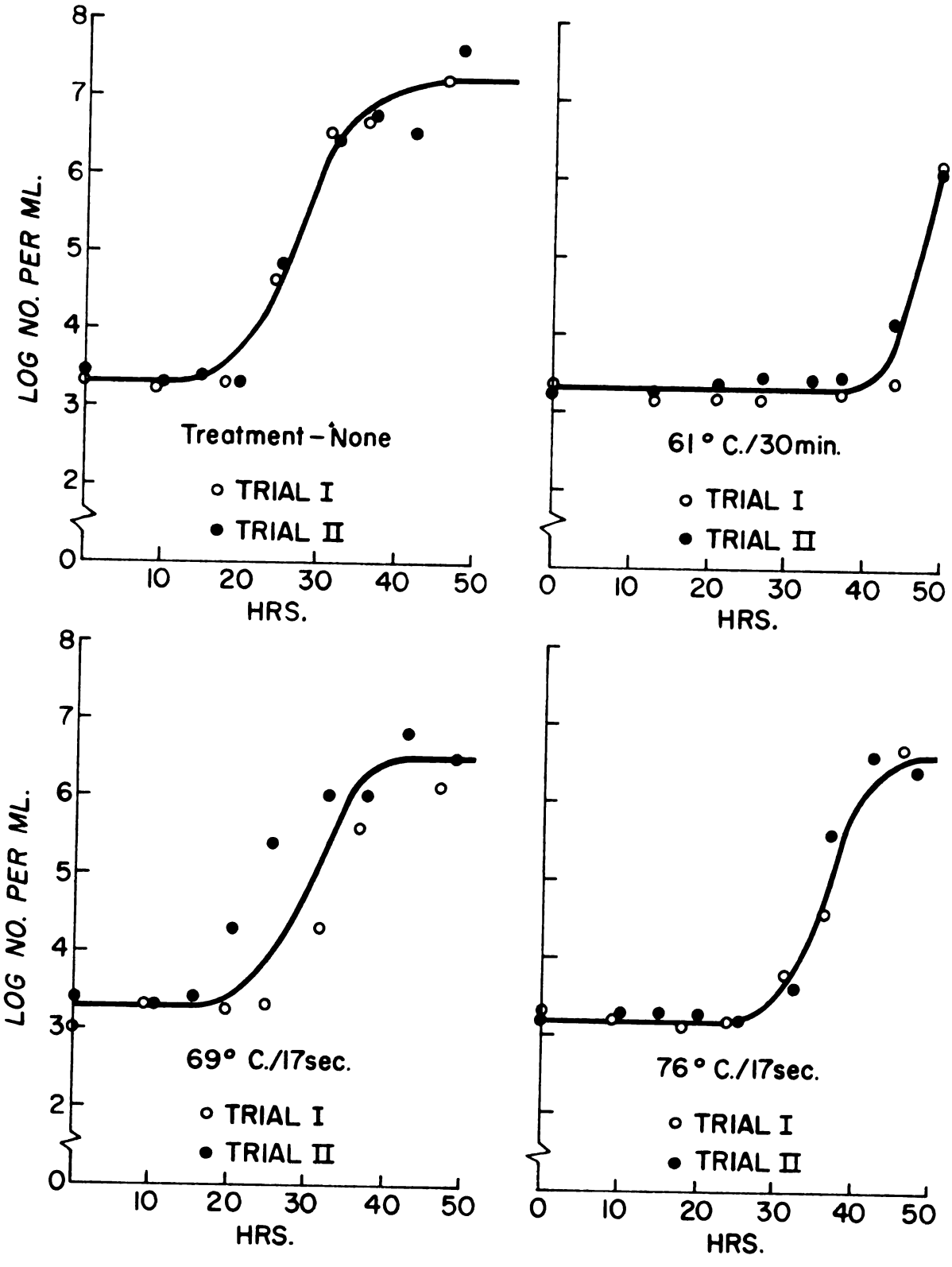


Figure 5. Effect of heat shock on the growth curve of survivors. (Low initial cell concentration).

TABLE IV

Plate counts of milk samples containing a low or medium initial cell concentration of MS 102 after heat treatment at 81°C. or 82°C.

TRIAL I*

	Unheated control	81°C./5 sec.	82°C./5 sec.
Time (hrs.)	Av. count per ml. (x1000)	Av. count. per ml. (x1000)	Av. count per ml. (x1000)
0	210	21.0	2.7
12	200	6.4	1.9
18	590	6.7	1.6
25	1400	7.4	2.0
30	1900	7.6	1.8
35	2300	960.0	22.0
39	2800	3000.0	28.0
43	7400	14000.0	31.0
50	17000	12000.0	240.0
55	-	15000.0	1000.0
	TRIAL II		
0	(same as above)	140	130
12		250	38
18		180	88
25		1100	200
30		1900	1400
35		2300	1800
39		2600	3000
43		5000	11000
50		4000	11000
55		8500	13000

*Trials I and II represent separate tests run on different generations of MS 102.

TABLE V

Plate counts of milk samples containing a low initial cell concentration of MS 102 after heat treatment at 81°C. or 82°C.

TRIAL I*

	Unheated control	81°C./5 sec.	82°C./5 sec.
Time (hrs.)	Av. count per ml. (x1000)	Av. count per ml. (x1000)	Av. count per ml. (x1000)
0	16	3.7	1.4
12	20	1.3	.63
18	870	1.2	.82
25	3500	1.1	.81
30	L.A.**	1.3	.70
35	8100	1.5	.72
39	40000	3.2	1.0
43	-	9.6	3.3
50	-	370.0	16.0
55	-	1700.0	890.0
	TRIAL II		
0	(same as above)	5.9	.59
12		1.7	.38
18		1.4	.22
25		1.4	.31
30		1.4	.25
35		4.4	.64
39		42.0	.58
43		540.0	.60
50		sample	sample
55		depleted	depleted

*Trials I and II represent separate tests run on different generations of MS 102.

**Laboratory Accident.

TABLE VI

Plate counts of milk samples containing a very low initial bacterial population after heat treatment at 81°C. or 82°C.

TRIAL I*

	Unheated control	81°C./5 sec.	82°C./5 sec.
Time (hrs.)	Av. count per ml. (x1000)	Av. count per ml. (x1000)	Av. count per ml. (x1000)
0	1.9	.19	.35
12	1.9	.04	.012
18	1.5	.13	.019
25	32.0	.07	.015
30	720.0	.06	.012
35	2900.0	.22	.020
39	3600.0	.30	.016
43	5800.0	.50	.030
50	-	.46	-
55	-	-	-
	TRIAL II		
0	(same as above)	.055	L.A.**
12		.038	
18		.043	
25		.032	
30		.031	
35		.039	
39		.050	
43		.052	
50		.020	
55		.010	

*Trials I and II represent separate tests run on different generations of MS 102.

**Laboratory Accident.

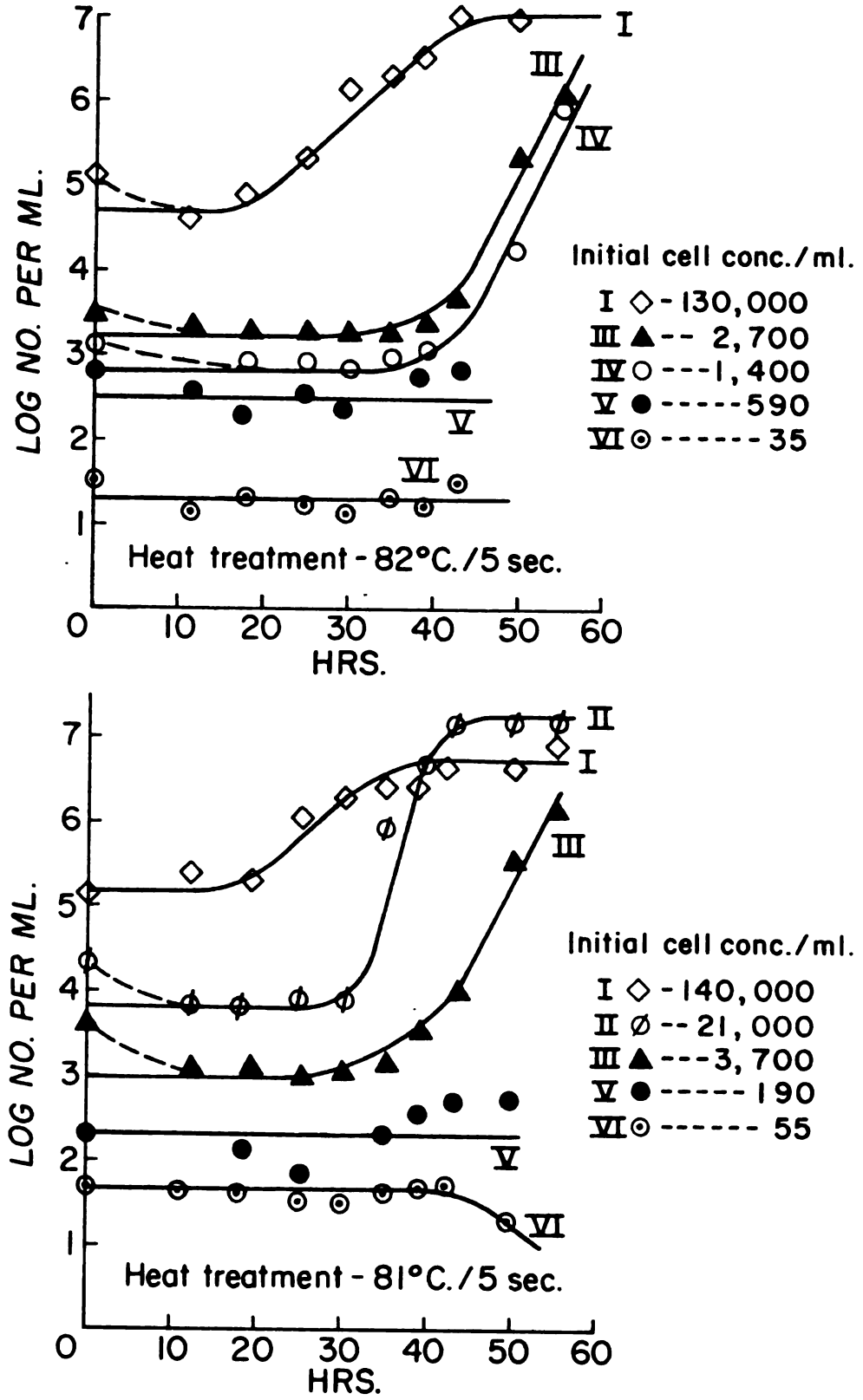


Figure 6. Growth curves of MS 102 cells which survived 81°C. or 82°C. for 5 seconds.

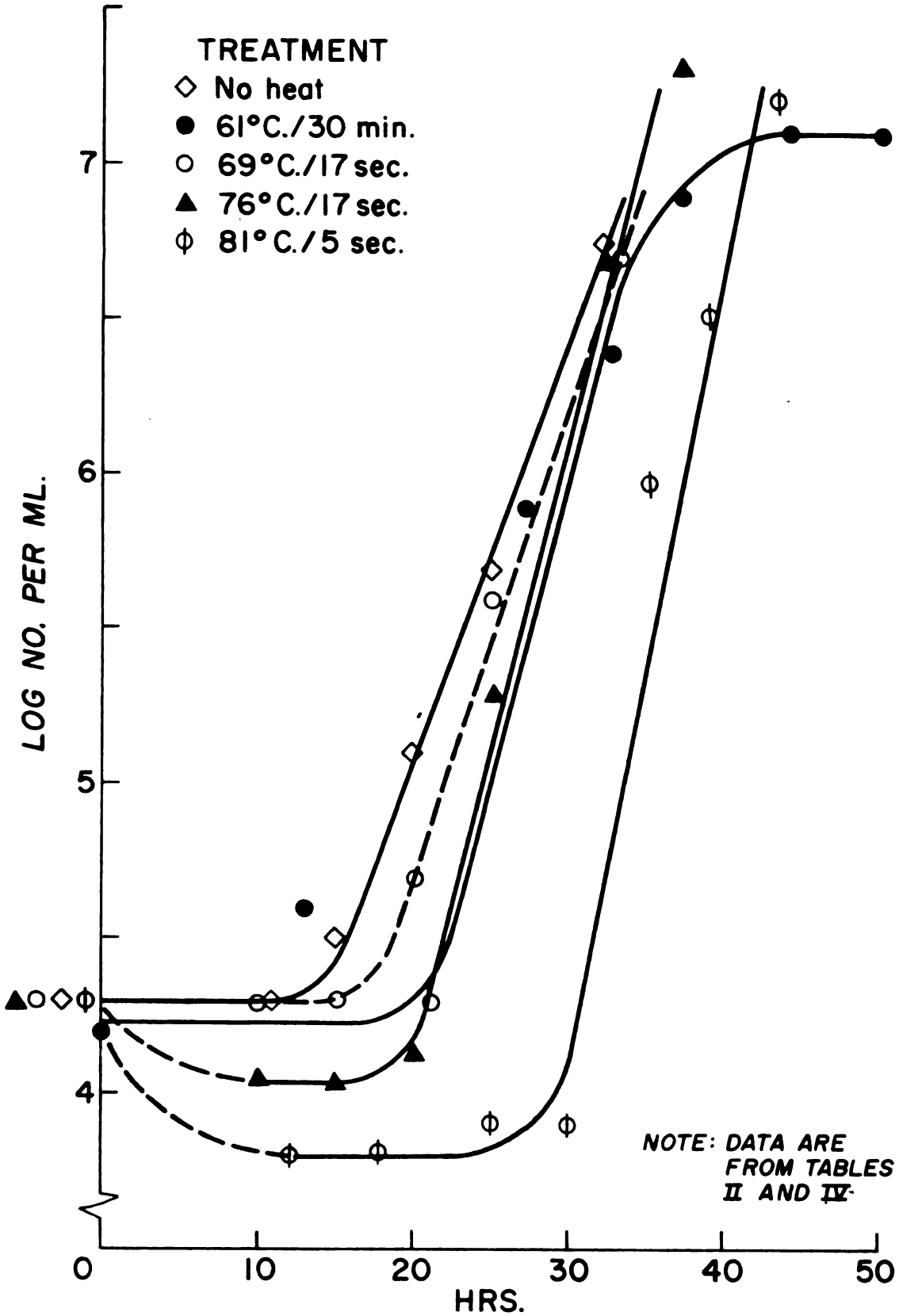


Figure 7. Effect of heat on the lag phase of MS 102. (Average initial population = 19,000/ml.)

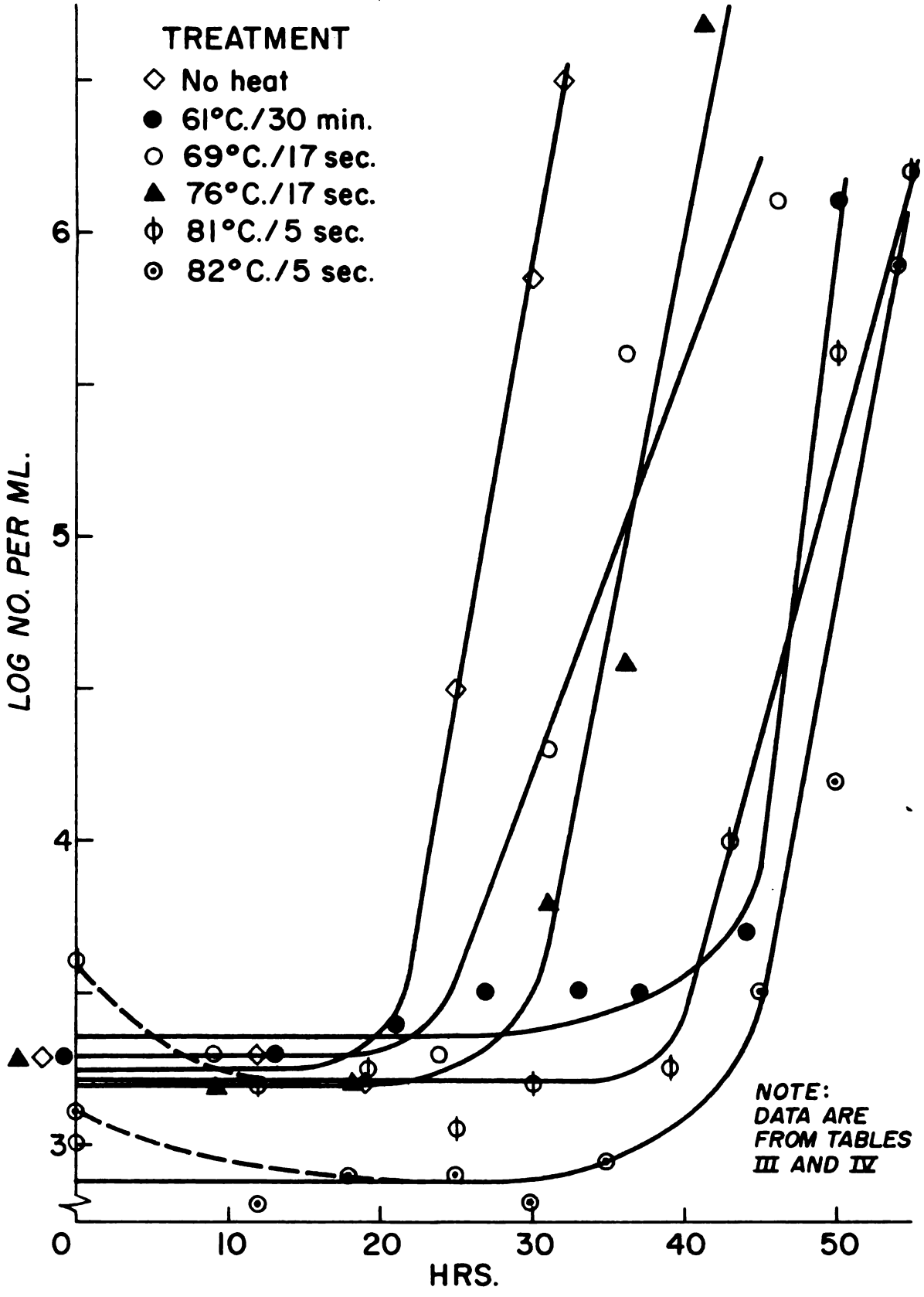
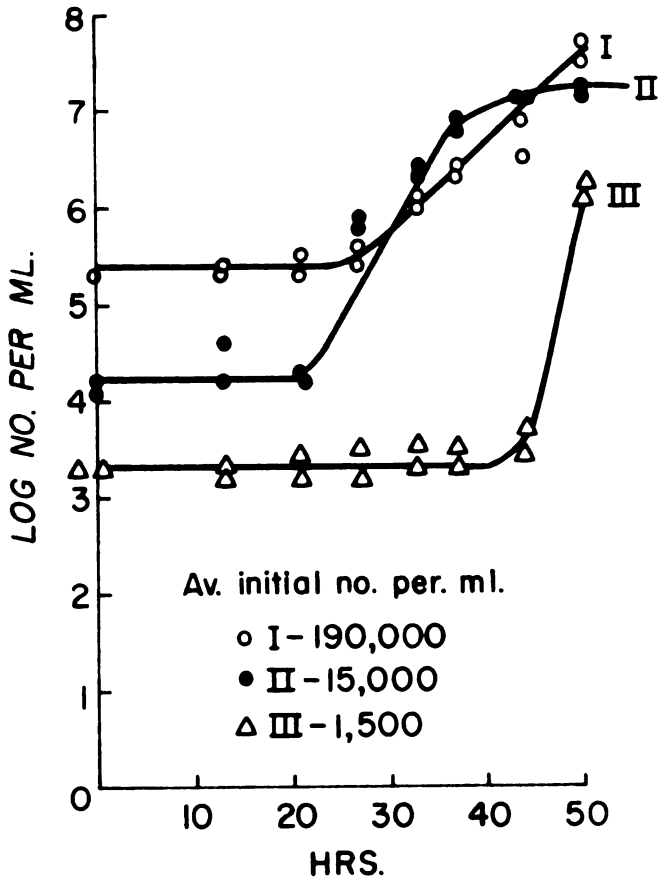
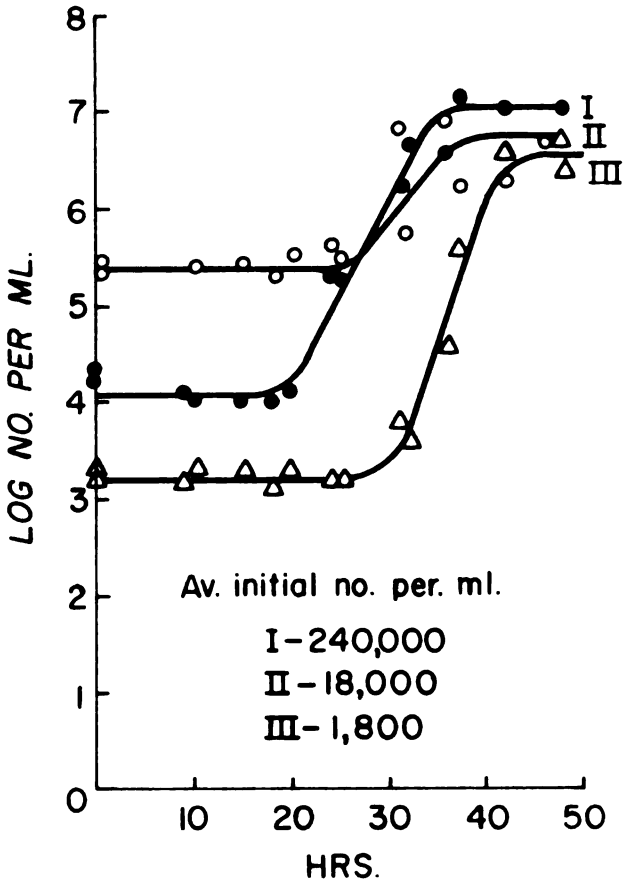


Figure 8. Effect of heat on the lag phase of MS 102.
(Average initial population = 2000/ml)



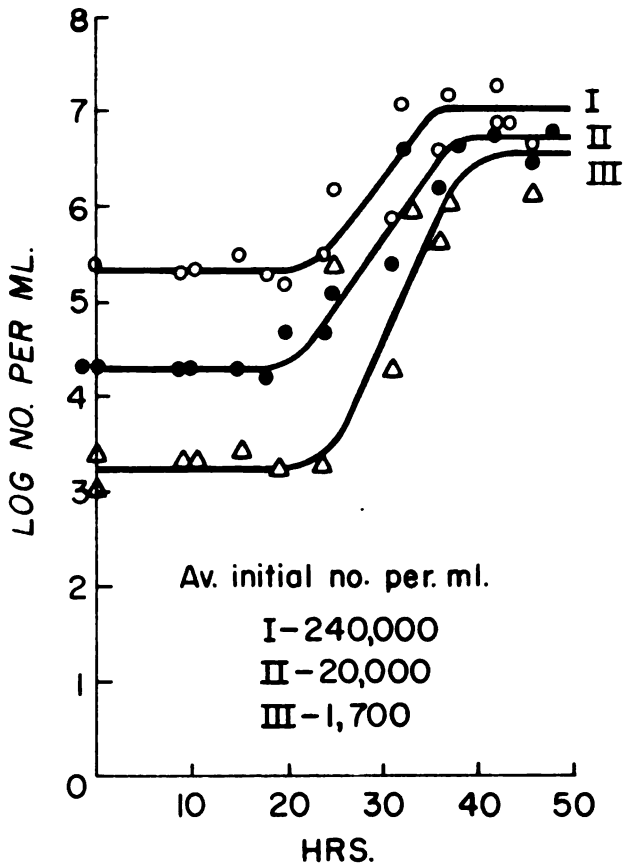
Note: Data are from Tables I, II and III

Figure 9. Effect of the survivor cell concentration on the growth curve after 61°C. for 30 minutes.



Note: Data are from Tables I, II and III

Figure 10. Effect of the survivor cell concentration on the growth curve after 76°C. for 17 seconds.



Note: Data from Tables I, II, III

Figure 11. Effect of the survivor cell concentration on the growth curve after 69°C. for 17 seconds.

TABLE VII

Plate counts of milk samples containing initial cell concentrations selected for the construction of control growth curves showing rate of growth in milk

Time (hrs.)	Count per ml. (x1000) C*	Count per ml. (x1000) D*	Count per ml. E*	Count per ml. F*
0	12	1.1	150	18
4	14	1.0	150	18
9	16	1.2	170	14
12	19	1.2	200	16
18	41	1.1	200	14
22	630	2.4	200	10
26	5000	4.8	260	40
33	13000	700	1000	260
36	17000	3500	2900	720
42	-	8000	18000	3100

	B*	A*
0	210	1800
10	230	1600
15	390	3600
20	450	3500
25	5500	3300
32	9000	4900
37	2600	6200
39	L. A.**	4800
42	16000	-
48	840	-

*Refers to the correspondingly designated growth curve of Figure 12.

**Plates at 39 hours were contaminated.

TABLE VIIb

Plate counts of milk samples containing extremely low initial cell concentrations selected for the construction of control growth curves in milk

Time (hrs.)	Count per ml. G*	Count per ml.	Count per 10 ml.	Count per 10 ml.
0	4	4	4	4
10	4	3	8	no recovery
21	5	4	8	"
25	5	6	4	"
30	4	4	no recovery	"
38	3	3	"	"
48	no recovery	no recovery	"	"
53	"	"	"	"
72	"	"	"	"
78	"	"	"	"
84	"	"	"	"
96	"	"	"	"
99	"	"	"	"
104	"	"	"	"

Note: The dilution scheme followed to obtain these extremely low initial cell concentrations is presented in Figure 2.

*Refers to the correspondingly designated growth curve of Figure 12.

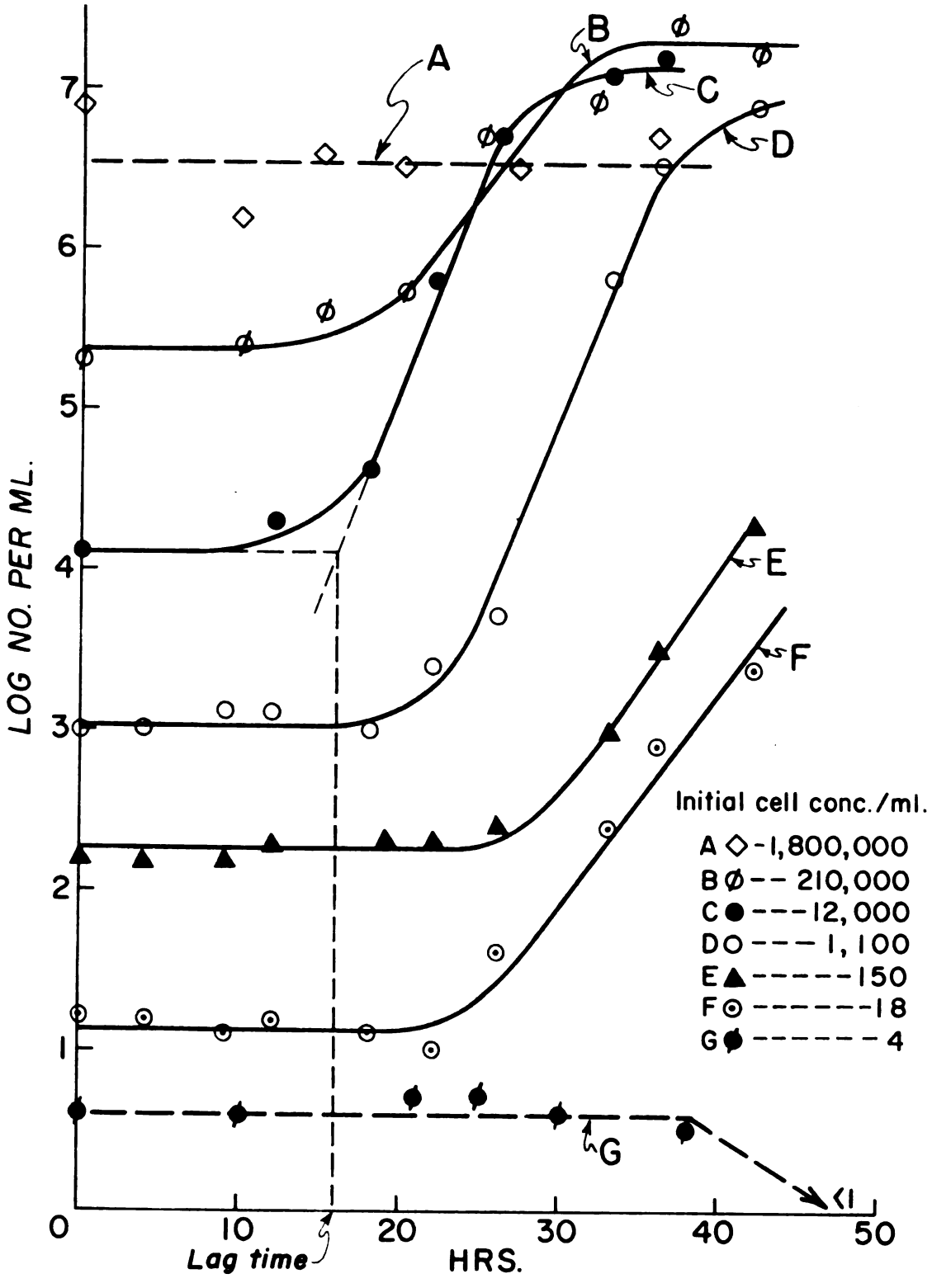


Figure 12. Effect of initial cell concentration on the lag phase of control growth curves of unheated cells.

TABLE VIII

Plate counts of milk samples containing extremely low initial cell populations

Time in hours	Triplicate samples								
	E_1	E_2	E_3	X_1	X_2	X_3	Y_1	Y_2	Y_3
	Colonies per 10 ml. plated								
0	5	7	3	4	4	3	no recovery		
12	0	1	1	0	1	1	"		"
22	2	0	0	0	0	1	"		"
33	6	2	0	no recovery			"		"
42	20	0	2	"		"	"		"
60	10	0	0	"		"	"		"
72	no recovery			"		"	"		"
96	"		"	"		"	"		"
13 days	"		"	"		"	"		"

Note: Samples E, X and Y were designed to contain 2, 1 and 0.1 cell per ml. as shown in Figure 2.

TABLE IX

The effect of selected heat treatments on the total lag phase of MS 102

Initial population range per ml.	Unheated control	61°C. 30 min.	69°C. 17 sec.	76°C. 17 sec.	81°C. 5 sec.	82°C. 5 sec.
	Lag in Hours					
210,000	17	25	23	25	19	21
18,000	16	21	20	21	31	42
1,800	20	42	22	30	35	42
190	28	-	-	-	-	50*
100	25	-	-	-	52*	44*

*The lag phase was longer than the time indicated. Depletion of the sample prevented continuation of the growth curve.

Note: An initial cell concentration of 2700 per ml. was included in the medium range since, of the data obtained, it most nearly approximated the limits defined.

TABLE X

The effect of selected heat treatments on the extension of the lag phase of MS 102

Temperature and time of treatment	Initial population per ml. after heating				
	210,000	18,000	1,800	190	100
	Extension of the lag in hours (beyond control)				
69°C./17 sec.	6	4	2	*	*
61°C./30 min.	8	5	22	*	*
76°C./17 sec.	8	5	10	*	*
81°C./5 sec.	2	15	15	*	27
82°C./5 sec.	4	26	22	22	19

*No determinations were made.

RESULTS AND DISCUSSION

Effect of Heat Treatment on the Lag Phase at Selected Cell Concentrations

The initial populations obtained after heat treatment are referred to as high in the range of 100,000 to 200,000 cells per ml., medium in the range of 10,000 to 20,000 per ml., low in the range of 1,000 to 9,000 per ml. and very low in the range of 100 to 900 cells per ml.

Individual growth curves of survivor populations after the various heat treatments are shown in Figures 3, 4, 5, 6, 7 and 8; the average curves are shown in Figures 9, 10 and 11. The total lag times calculated from the growth curves of survivor and unheated control populations are summarized in Table IX. The hours of lag extension, beyond their respective controls, are presented in Table X.

With high initial populations, the total lag times after heating at 61°C. for 30 minutes or 69°C. for 17 seconds were 25 and 23 hours respectively, as compared to 17 hours for the control. It is apparent that these two treatments only extended the lag phase of MS 102 about 6

to 8 hours. It is interesting to note that both of these procedures are approximately equivalent in their effect on the lag time when high initial cell concentrations are used. Little or no lag extension over the unheated control occurred on heating to 81°C. or 82°C. for 5 seconds. The effect of massive initial inoculation on the lag phase of growth (26) may have limited the effects of "heat shock" at the temperatures used in this instance.

The lag time for medium survivor populations was slightly greater than the control after all heat treatments (Figures 4, 6 - curve II). At 81°C. for 5 seconds and at 82°C. for 5 seconds the total lag periods were 31 and 42 hours respectively. This represents an extension of the lag time by at least 26 hours at the higher temperature. At this population level the total lag times of the samples subjected to 61°C. for 30 minutes and 69°C. for 17 seconds were 20 and 21 hours; this represents an insignificant increase in lag of 4 and 5 hours, respectively. Since the population was constant, the only variable factor which might account for the obvious increase in lag time would be the heat treatment; the increase in lag phase appears to be a direct result of "heat shock." Conditions which approximated the standard pasteurization process for milk failed

to induce an appreciable extension of the lag phase; treatments which tended slightly in the direction of ultra high temperature processing yielded a lag phase of approximately 31 to 42 hours.

Very low post-heat populations (Figure 6 - curves V and VI), showed lags of 44 to 52 hours after treatment at 82°C. and 81°C. respectively for 5 seconds. Undoubtedly longer lag phases would have been obtained had it been possible to continue the experiment.

At low population levels the total lag time was 42 hours after heat treatment at both 82°C. for 5 seconds and 61°C. for 30 minutes, as compared to 20 hours with the unheated control; this is an increase of 22 hours. At 69°C. for 17 seconds the increase was very slight. No definite explanation can be given at this time for the very long (22 hour) extension of the lag phase observed for low concentrations of survivors after 61°C. for 30 minutes and 82°C. for 5 seconds, particularly in view of the relatively low lethality of the LTLT treatment. A temperature of 69°C., which is higher than 61°C., did not induce an appreciably extended lag phase. This would suggest a minimum temperature for the occurrence of the "heat shock" phenomenon under certain conditions; the minimum temperature would probably

vary with different organisms. The lack of any appreciable extension of the lag time at 69°C. for 17 seconds at low population levels would tend to minimize the shelf life of a product.

Following heat treatment at 81°C. and 82°C., a gradual decrease in cell count was noted during the first 12 hours of the lag phase (Figures 6, 7 and 8). With the former, the populations obtained by the standard plate counts were reduced about 60 per cent between 0 time and 12 hours of incubation (Tables IV, V and VI); at the latter temperature the average population decrease was 57 per cent in a similar period of time. This post-heat-death may have resulted from a nutritional difference between the standard plating agar and the growth medium (milk). Post-heat cell counts were made immediately after heat treatment using Plate Count Agar (PCA). If the essential recovery factors of a nutritional or physio-chemical nature were supplied by PCA, but not by milk, then greater recovery of injured cells would be expected on plates poured at zero time. During the first 12 hours of incubation in milk, some of the injured cells may have gradually lost their viability due to the absence of essential recovery factors. The PCA may have provided the readily available amino acids essential

to the recovery of the heat injured cells. Although milk proteins contain the essential amino acids, the amino acids, per se, may not be as readily available for assimilation. Injury to the semipermeable membrane may also be involved in the gradual death of cells due to abnormal osmotic balance.

Effect of Survivor Cell Concentrations on the Lag Phase

Normal growth curves of MS 102 at selected cell concentrations are presented in Figure 12. Unheated control growth curves with initial cell populations above 1,100 per ml. showed progressively shortened lag phases; below this level the lag phase appeared to be progressively extended. Results of studies for extremely low initial levels of inoculum are presented in Tables VIIb and VIII. Uniform growth was not obtained at initial populations below 10 cells per ml.

The results pertaining to the effect of population on the lag phase after heat treatment are given in Figures 9, 10, 11 and Table X. At high and medium levels of survival the lag times of samples heated to 61°C. for 30 minutes, 69°C. for 17 seconds and 76°C. for 17 seconds were similar. At all high levels of population the heat

treatment did not materially increase the duration of the lag phase; at medium cell concentrations the effect of heat was slight with respect to lag extension. Exceptionally short lag times occurred in samples heated to 81°C. and 82°C. for 5 seconds at high initial cell concentrations, as compared to the lag obtained at medium and low populations. Since the lag times for high cell levels at these temperatures (19 and 21 hours) are similar to the 17 hour lag time of the unheated control, perhaps the lag reduction effect of high populations overbalanced the lag extension effect of the heat treatment employed.

Among samples heated to 61°C. for 30 minutes the longest lag time occurred at the low initial cell concentration. In all cases, the lag times for high and medium cell levels after 61°C. for 30 minutes were similar. Lag times for MS 102 organisms surviving 69°C. for 17 seconds at high and medium population levels were also similar (23 and 20 hours respectively). A marked contrast occurred at 82°C. for 5 seconds at which the lag time was much shorter for high concentrations of cells than for medium or low surviving populations (21, 42 and 42 hours respectively). At 81°C. and 82°C. for 5 seconds the effect of "heat shock" on the lag phase with high populations seems small compared

to the effect on lower concentrations of cells.

General Considerations Evolving from the Lag Extension Studies

The keeping quality of food products depends, to some extent, on the duration of the lag phase of the bacteria which survive the thermal process to which the product was exposed. According to Williams (27) aerobic spore formers, heat resistant micrococci, streptococci and corynebacteria are the four genera most likely to cause post-pasteurization spoilage in milk and related foods.

The results reported herein show that the lag phase of growth of MS 102, subjected to 82°C. for 5 seconds, is considerably longer than that obtained in whole milk after exposure at 69°C. for 17 seconds (conditions approximating HTST). The limitations of equipment prevented a study of the "heat shock" phenomenon at temperatures between 82°C. and 143°C. If the lag phase increases considerably at these higher temperatures, the storage-life of milk might be materially extended. Preliminary studies by Kaufmann (13) on whole milk pasteurized at high temperatures (97°C.) indicate that the lag period, per se, may be as long as 2 or 3 weeks when milk is stored at 4 to 7°C. On the basis

of the studies reported here, and those of Kaufmann (13), it is conceivable that processing at ultra high temperatures might result in long lag periods for spoilage bacteria which survive and increased storage life for thermally processed foods.

The literature contains several reports of unexplained, delayed bacterial growth after heat treatment. Day (4) reported false negative tests for psychrophiles unless the milk was held at least 3 days. Such delays after HTST processing may represent the normal growth process for certain psychrophiles from post-heat contamination, or may indicate an extended lag time after heat treatment. Eijkman (6) reported that a suspension of B. coli cells, heated for 6 to 35 minutes at 125.6°F. showed no growth after 3 days, but all except the sample heated for 35 minutes contained viable cells after incubation for 15 days. Heather and van der Zant (8) demonstrated increased lag for some pseudomonads after heat treatment at 135°F. for 1 to 8 minutes. Mossel (17) observed the curdling of coffee milk (processed at 110°C. for 30 minutes) after 11 days at 32°C. The delays observed in these instances may be associated in part with an extension of the lag phase which exists after exposure to heat.

SUMMARY

The Effect of Heat Treatment on the Lag Phase of MS 102

Selected cell concentrations of MS 102 were heated in milk at 61°C. for 30 minutes, 69°C. for 17 seconds, 76°C. for 17 seconds, 81°C. for 5 seconds and 82°C. for 5 seconds. The lag phases of growth curves of surviving bacteria, propagated in whole milk at 32°C., were extended beyond the lag times observed in unheated controls. The lag extension effect was observed at medium and low survivor populations (10,000 to 20,000 and 1,000 to 9,000 cells per ml. respectively), but not at high survivor cell concentrations (100,000 to 200,000 cells per ml.).

Medium population levels subjected to 61°C. for 30 minutes or 69°C. for 17 seconds showed lag times of 21 and 20 hours respectively. These periods of lag were approximately one half as long as the lag time (42 hours) observed after heating cells at 82°C. for 5 seconds.

At low population levels subjected to 82°C. for 5 seconds or 61°C. for 30 minutes the lag periods (42 hours) were much greater than the lag time (22 hours) after 69°C. for 17 seconds.

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