AN ABSTRACT OF THE THESIS OF

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Title Sensitivity of Escherichia coli to Cold-Shock During the Logarithmic Growth Phase

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Abstract Approved
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The sensitivity of Escherichia coli to cold-shock was determined by adding a 1 ml. sample of a broth culture growing at 37°C. to 100 ml. of a cold-diluent held at 0°C. In the first series of experiments the sensitivity to cold-shock was observed at ten minute intervals for the entire growth phase of the culture. The decrease in the number of viable cells within the first five minutes as shown by a standard plate count, was taken as a measure of the sensitive cells. These experiments have shown that cells removed from a culture during the phases of increasing population were sensitive to cold-shock whereas cells removed from mature cultures were resistant to cold-shock. These data verify the earlier observations of Sherman and Albus (1923, 1924).

In other experiments the culture was sampled less frequently and the cold-shocked sub-culture was held continuously at 0°C. for a period of six days to determine any latent affect of the cold. These experiments have also shown that only those cells removed from a culture in the logarithmic growth phase were immediately sensitive to the cold-shock and that mature culture samples were not affected by the treatment. In addition it was found that samples removed from the culture during the lag and early logarithmic phases became sensitive after being held at 0°C. for several hours. On the basis of these data it is concluded that the phenomenon of sensitivity to cold is concerned with changes occurring within the cell as well as some stage of the cell division.

The "physiological rejuvenation" ascribed to Escherichia coli by Sherman and Albus (1923) on the basis of the sensitivity to cold cannot be confined to one phase of the growth curve, since the sensitivity to cold-shock extends through the entire growth phase.

Data were also obtained supporting the contention of earlier workers that the growth curve proceeds in a step-like manner.

SENSITIVITY OF *ESCHERICHIA COLI* TO COLD-SHOCK DURING THE LOGARITHMIC GROWTH PHASE

by

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Introduction

This investigation was undertaken to determine the resistance of a normal broth culture of *Escherichia coli* to cold-shock during its entire growth phase and to establish a relationship between the age of the culture and its sensitivity to cold. Sherman and Albus (1923) found that young cultures of *Escherichia coli* were sensitive and old cultures were resistant to cold-shock but they did not extend their observations throughout the population curve.

Historical Review

The increase of bacterial populations may be demonstrated by plotting the number of microorganisms against time. This curve will show numerical increase or decrease but the minute scale required obscures small and important changes occurring as the population increases. These small differences may be made apparent by plotting the logarithms of the numbers of bacteria against time. The logarithmic plotting gives proportional increase or decrease during the periods of rapid growth and the slope of the line is a measure of the growth rate. The logarithmic plotting of bacterial populations is commonly referred to as the growth curve.

Different investigators have divided the bacterial growth curve into specific phases. Muller (1895) recognized three of the early phases, first a period of slight numerical increase or lag, second a period during which the numbers increased logarithmically, and last a phase of slackened growth. Later (1906) Rahn and (1909) Lane-Claypon proposed a division of the growth curve into four periods, viz., lag, logarithmic increase, maximum stationary population, and decreasing population. A more thorough study led Buchanan (1918) to subdivide the growth curve into seven specific phases based upon the growth rates during successive time intervals. The lag phase previously described was further subdivided into the initial stationary phase in which the generation time, i.e., the time required for a bacterial cell to divide was infinity, and the lag phase during which the numbers of bacteria increased progressively with time. The more and more rapid increase during the lag phase continued until a majority of the cells were dividing at a constant rate corresponding to the average minimal generation time, and this continued through the logarithmic growth phase. This latter period was characterized by the numbers of cells increasing in geometrical progression. Following the logarithmic phase, there began an accelerating decrease in the rate of growth which terminated in a

period during which the numbers of bacteria neither increased or decreased appreciably. The period of gradual decrease in growth rate was called the phase of negative acceleration and the subsequent period the phase of maximum stationary population. Buchanan divided the final portion of the growth curve, which was marked by a decreasing population, into the phases of accelerated death and "logarithmic death". During the former period the death rate gradually increased and during the latter the decrease progressed in a logarithmic manner. The last period is not always apparent, and it has been attacked by some workers as non-existent (Knaysi 1930). Henrici (1928) has suggested that a phase of negative acceleration in death be added since the growth curve tends to flatten out as the abscissa is approached.

Winslow (1928) in a study of the rise and fall of bacterial populations proposed five periods: adjustment, increase, crisis, decrease, and readjustment. The first period listed by Winslow corresponds to the initial stationary and lag phases of Buchanan and the second division to the period of logarithmic increase. The phase of crisis included the phases of negative acceleration, maximum stationary population, and accelerated death. The period of decrease would coincide with Buchanan's phase of "logarithmic death" and the phase of readjustment

to Henrici's suggested period of negative acceleration in death. In addition to listing specific growth phases Buchanan (1928) and Winslow (1928) have reviewed the influencing factors. A complete discussion of these has been eliminated since the following work is concerned with certain characteristics of the growth curve rather than a study of the separate divisions.

The literature on differential characters of the earlier growth periods has been comprehensively reviewed by Winslow and Walker (1939) who have listed five of these in separating the specific growth periods. The five characters given were: the influence of the age of the inoculum on the length of the initial stationary phase, the chemical activity shown during the various growth periods, the varying resistance to environmental changes, the change in cell size, and the differences in electric charge as seen in the changing resistance to acid agglutination and electrophoretic mobility.

The influence of the age of the inoculum on the length of the initial stationary phase was demonstrated indirectly by Muller (1895) who found the generation time of a "typhoid bacillus" inoculated into a new medium varied with the age of the source culture. Barber (1908), using direct microscopic methods, showed that the initial stationary phase and the lag phase would disappear if

transfer was made from a culture growing in the logarithmic phase to an identical medium, the growth continuing in the logarithmic phase. Buchanan (1928) stressed the fact that growth tends to continue in the same phase when transfers are made from a culture to a new medium.

A consideration of the fourth differential character listed by Winslow and Walker (1939), the change in cell size, precedes the discussion of varying chemical rates and resistance to environmental change since these are frequently correlated with the change in cell size. Clark and Ruehl (1919) studied seventy strains of bacteria representing thirty seven species and found a marked increase in cell size during early growth stages of all species except the Corynebacterium, and these decreased in size during this period. This increase in size was evident after two hours, and a maximum size was usually reached between four and six hours' incubation. Henrici (1928) confirmed the work of Clark and Ruehl (1919) and extended their data. Studies by Henrici with Bacillus megatherium showed that increase in cell size began during the lag phase and reached a peak shortly after maximum growth rate set in, after which the cells gradually became shorter. If cells were transferred at the moment of increasing size, the increase continued, and if they were transferred at the moment the original size was reached following the maximum growth rate, increase began again at

once. Cells transferred later passed through the initial stationary phase before the increase in size began. Henrici has also shown that these large cells were stained more deeply with basic dyes. This increased size and increased stainability associated with the susceptability of young cultures to injurious agents (Sherman and Albus 1923) caused Henrici to differentiate between young "embryonic forms" and older cells.

Jensen (1928) made a thorough study of the growth curve of Escherichia coli using a vital staining procedure. He concluded that the period of absolute latency was one of increasing cell size without fission and that the period of sub-logarithmic growth was merely a statistical characteristic of the culture as a whole. Jensen's studies point to increased cell size occurring before fission.

Bayne-Jones and Adolph (1932) observed the growth phases of Escherichia coli by means of a cinematograph and found the same early increases in cell size. They found a period during which there was no growth, a period of maximum growth after one hour's incubation at 37°C., and a final period of maximum reproduction after about two hours' incubation. They concluded that since growth rate preceded reproduction rate, growth must dictate fission.

The second differential character given by Winslow and Walker (1939) i.e., chemical activity, is manifest in increased rates of metabolism during the lag and early logarithmic periods. Bayne-Jones and Rhees (1929) recorded heat production of a culture of Escherichia coli and found an increased rate in the gram calories produced per cell during the first three hours' incubation at 37°C. This increase was followed by rapid decrease during the remainder of the growth period. These workers found an agreement between the shape of their curves and those of Henrici (1928) for the area-length ratio of Escherichia coli. Martin (1932) measured oxygen consumption of an Escherichia coli culture and found that the rate reached its maximum within thirty to ninety minutes while the greatest cell size occurred between sixty and one hundred twenty minutes of incubation.

Walker and Winslow (1932) found an increased rate of ammonia nitrogen and carbon dioxide production during the early growth phase of Escherichia coli as compared with peak stability rates. Using an aerated culture they reported 41 to 185 x 10^{-11} mgm. of CO_2 formed per cell per hour during the lag phase against 2 x 10^{-11} mgm. for the close of the logarithmic period. For ammonia nitrogen production they found from 6 to 36 x 10^{-11} mgm. per cell per hour during the lag phase compared with

0.2×10^{-11} mgm. during the phase of maximum population. Walker, Winslow, Huntington, and Mooney (1934) confirmed these data. Their calculations based upon the data of Bayne-Jones and Rhees (1929), Bayne-Jones and Sandholzer (1932), and Martin (1932) showed that increased rates of heat production, oxygen consumption, carbon dioxide and ammonia nitrogen production were explainable for the most part on the basis of increased cell size. The decreased rates during the maximum stationary phase could not be accounted for by decrease in cell size. Huntington and Winslow (1937) determined the cell volume of Escherichia coli and found, in five out of eight experiments, that the mean cell volume increased to a maximum before the point of maximum reproduction. This work confirms the data of Bayne-Jones and Adolph (1932) and Martin (1932). Rates of carbon dioxide production were determined and the workers were unable to account for the increase solely on the basis of increased cell volume.

Resistance to environmental changes is considered as the fourth differential character of specific growth phases. Reichenback (1911) found young cultures in the lag and logarithmic stages more sensitive to heat than cultures in the peak of the population curve. Schultz and Ritz (1910) noted a slight resistance to heat (25 minutes at 53°C.) during the initial stationary phase

whereas cells from a four-hour culture of the organism were entirely destroyed by the same treatment. They found an increased resistance to this heat treatment when the culture was seven to fourteen hours old.

The biological importance of this variation in resistance was first suggested by Sherman and Albus (1923) who demonstrated physiological differences between cells of Escherichia coli taken from young and old cultures. They observed an increased sensitivity to cold, to two per cent sodium chloride, and to heat in the case of cells removed from young cultures, and they suggested that the cells pass through a "physiological rejuvenation" prior to rapid multiplication. Sherman and Albus (1924) repeated this work and observed sensitivity to cold after one and one-half hours and after three hours' incubation. Sensitivity to both cold and sodium chloride was observed before cell proliferation could be demonstrated. Sherman and Cameron (1934) showed that cells of Escherichia coli from a culture one and one-half to three hours old were rendered non-viable by environmental changes within the natural growth limits. A ninety-five per cent reduction in the number of viable cells occurred within one hour when cells grown at 45°C. were transferred to a medium held at 10°C. Such environmental changes were ineffective if they took place slowly or if the growth had been retarded by incubation at low temperature.

The Schultz-Ritz phenomenon of lowered resistance to heat has been observed by many workers. Ørskov (1925) described lowered resistance to heat in young cells of certain colon-typhoid strains. Robertson (1927) noted the same thing in Microbacterium lacticum, Sarcina lutea, and Streptococcus thermophilous. Studies made by Elliker and Frazier (1938 a and b) contradict these data by indicating an increased resistance to heat during the early growth phases. These workers found an increased resistance to heat during the initial stationary phase of a culture of Escherichia coli. This increased resistance was evident after one and one-half hours' incubation for a culture grown at 28°C. and heated to 53°C. for thirty minutes. Weeks and Hegarty (1939) have verified this phenomenon for a culture of Escherichia coli grown at 37°C. and heated to 53°C. for ten minutes. The cells reached a one hundred per cent resistance during the later part of the initial stationary phase while during the logarithmic phase, the cells were usually one hundred per cent sensitive to the same treatment. This work shows a new characteristic which supports physiological differentiation of the cultures during specific growth phases.

Experimental Methods

Samples from a normal broth culture of Escherichia coli, i.e., a nutrient broth culture inoculated from a twenty-four-hour-old parent culture and incubated at 37°C., were removed at frequent intervals for simultaneous plate counts and determinations of sensitivity to cold-shock by the method of Sherman and Albus (1923). The method consisted of removing a one ml. sample of a normal broth culture for a standard plate count at the same moment that a second sample was removed for dilution in 100 ml. of a cold-shock medium held at 0°C. The difference in plate counts of the two samples was taken as a measure of the sensitivity of the cells to cold-shock. The dilutions for counting the culture sample were made in water at 37°C.; those for counting the cold-resistant cells in water at 0°C. The incubation temperature for the culture was maintained with a thermostatically controlled water bath and the cold-shock temperature was maintained with a melting ice bath. The plate counts were made in duplicate using nutrient agar media. Following the suggestions of Hershey (1938) the same lot of Bacto Difco materials were used in the preparation of all culture media.

Sherman and Albus used either a one-per cent peptone solution or distilled water as the cold-shock medium. They noted that there were lethal effects resulting from sudden changes in osmotic pressure. An experiment was designed to determine if the changes arising from the transfer of broth-grown cells to distilled water were of consequence. Nutrient broth, tap water, and distilled water were used to prepare dilutions for the plate count. These materials were held at 37°C. to eliminate effects arising from temperature change. The results, shown in Table I, indicate that with constant temperature any slight change arising on transfer of cells from nutrient broth into tap water or distilled water will not cause a significant difference in the bacterial counts obtained. In the following studies distilled water was used as the cold-shock medium if the cold-shock was to be of short duration, and for dilution purposes in all cases rather than nutrient broth, since nutrient broth foams badly upon shaking and gives rise to an appreciable sampling error.

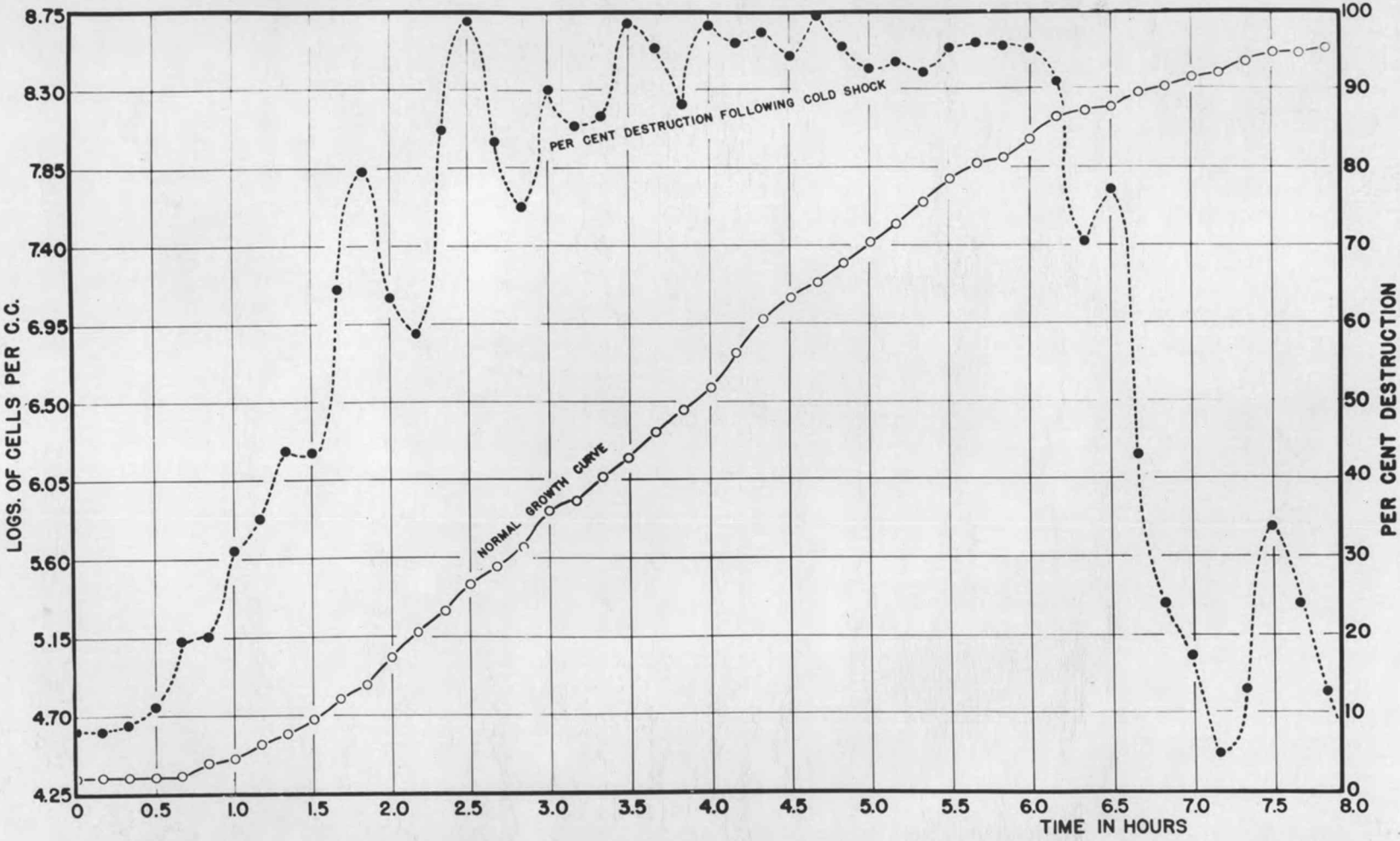
A series of experiments was undertaken to determine the number of cells sensitive to a cold-shock of only five minutes' duration at ten minute intervals throughout the entire growth range of the culture. A flask containing 200 ml. of nutrient broth at 37°C. was inoculated

TABLE I

PLATE COUNTS OBTAINED USING NUTRIENT BROTH, DISTILLED, AND TAP WATER FOR DILUTIONS

Age of culture	Plate count following dilution in		
	Nutrient broth	Distilled water	Tap water
Hours	Cells per ml.	Cells per ml.	Cells per ml.
2.5	162,000	164,500	161,000
5.0	29,300,000	28,500,000	29,300,000

with cells from a twenty-four hour culture to contain approximately 20,000 bacteria per ml. This flask was shaken fifty times and duplicate one ml. portions were removed for the total count, and the differential cold-resisting count. The cold-shocked samples were held for five minutes in distilled water at 0°C. before plating. In a similar manner the total number of cells and the number of cold-resisting cells were established at each ten-minute interval during an eight hour period. These data (Table II) are presented in Figure 1. Only eight per cent of the mature cells used for inoculation displayed sensitivity to cold. The initial stationary phase lasted for forty minutes and during the first twenty minutes of the period there was no increase in the number of cold-sensitive cells. During the next twenty minutes of the initial stationary period twelve per cent more of the total viable cells became susceptible to cold-shock. Sherman and Albus (1924) observed a similar increased sensitivity prior to the first indication of cell division. Immediately after the first apparent multiplication, the number of sensitive cells increased rapidly, and throughout the logarithmic phase a majority of the cells were susceptible to cold-shock. At certain points ninety-nine per cent of the cells were destroyed by the initial cold-shock. When the point of inflection of the



PER CENT DESTRUCTION EFFECTED BY COLD SHOCK AT TEN MINUTE INTERVALS DURING THE NORMAL GROWTH CYCLE

FIG. 1

TABLE II

PER CENT DESTRUCTION EFFECTED BY COLD SHOCK
DURING THE NORMAL GROWTH CURVE OF
Escherichia coli

Age culture (minutes)	Culture count (thousands/cc)	Cells surviving cold shock (thousands/cc)	Per Cent killed
Start (24 hr cells)	21.35	19.6	8.2
10	21.75	20.1	7.9
20	22.25	20.35	8.6
30	22.25	19.9	11.0
40	22.70	18.25	19.6
50	26.45	21.15	20.1
60	28.1	19.4	31.0
70	34.0	22.1	35.0
80	38.65	21.7	43.9
90	47.9	27.05	43.6
100	61.2	21.65	64.6
110	73.9	15.2	79.5
120	117.	42.95	63.3
130	149.	61.7	58.6
140	197.5	30.0	84.8
150	281.	2.75	99.06
160	354.	59.95	83.1
170	464.	115.0	75.2
180	740.	72.5	90.2
190	850.	123.45	85.5
200	1150.	154.5	86.6
210	1460.	21.5	98.6
220	2070.	89.0	95.7
230	2850.	330.0	88.5
240	3770.	67.0	98.3
250	5850.	226.0	96.2
260	9450.	237.5	97.5
270	12750.	58.5	94.4
280	15250.	5.5	99.9
290	19600	240.0	96.1

TABLE II

(Cont'd)

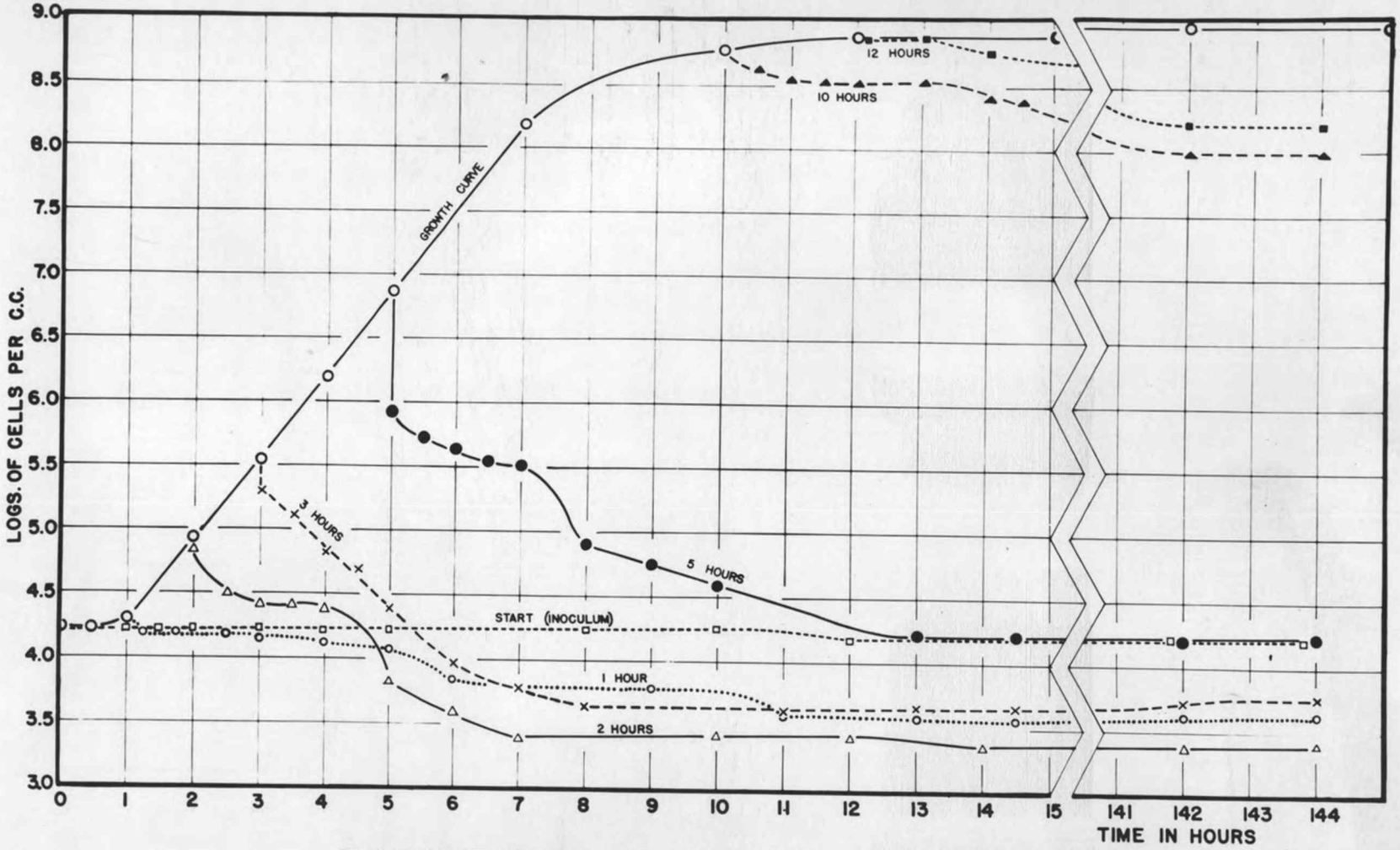
Age culture (minutes)	Culture count (thousands/cc)	Cells surviving cold shock (thousands/cc)	Per Cent killed
300	25700	1840.0	92.9
310	32850	2600.	93.6
320	44850	3450.	92.4
330	60000	2790.	95.5
340	74500	2850.	96.2
350	79500	3400.	95.8
360	123000	5850.	95.3
370	139000	1270.	90.9
380	149000	4400.	70.4
390	156000	3590.	77.0
400	189000	108000.	43.1
410	206000	156000.	24.1
420	248000	206000.	16.8
430	245000	232000.	4.9
440	284000	247000.	13.0
450	291000	193000.	33.5
460	321000	245000.	23.9
470	342000	305000.	12.7
480	342000	330000.	3.4

growth curve was reached, after about six hours, the number of sensitive cells decreased rapidly until at seven and one-half hours less than twenty-five per cent of the total viable cells were susceptible to cold-shock.

The first experiments show only the number of cells rendered non-viable by the cold-shock within five minutes. It seemed probable that prolonged exposure to cold might still further reduce the number of viable cells. This was ascertained by sampling for cold-shock less frequently and making counts, at various intervals, of the sub-culture held at 0°C. The technique used for these experiments was essentially that described in the previous experiment. Since distilled water might have affected viability on prolonged standing (Sherman and Albus 1924) nutrient broth was used as the cold-shock medium. Duplicate one ml. samples were removed from a normal broth culture for total viable count, and for a count of the cold-resistant cells at the time of the inoculation (start). Counts were made in the same manner after 1, 2, 3, 5, 12, 24, 28, 48, 72, and 144 hours' incubation of the normal broth culture. The cold diluent bottles contained ninety-nine ml. of nutrient broth and one ml. of the culture sample, and were held continuously in a melting ice bath. Plate counts of these sub-cultures

were made every thirty minutes for the first three hours and then at frequent intervals for the next six days. The data (Table III) are presented in Figure 2.

Cells from the twenty-four hour culture used in inoculation were not sensitive to cold-shock and no decrease in numbers resulted from holding the sub-culture at 0°C. for six days. Similar results were obtained on samples tested for sensitivity after 24, 28, 48, 72, and 144 hours' incubation of the mother culture. The curves from these determinations are not shown on Figure 2, since they are identical with that labeled "start" (inoculum). Cells transferred for cold-shocking after 1, 2, 3, and 5 hours' incubation demonstrate varied sensitivity. The sample from a one-hour culture contained cells which were not immediately sensitive to cold but after holding for several hours at 0°C. there was a sudden decrease of twenty-five per cent in the number of viable cells. Following this sudden period of sensitivity there were slight changes which ultimately ceased; the numbers amounting to fifteen per cent of the original sub-culture population of viable cells then remained constant for the duration of the experiment (six days). Cells removed from the culture when it was two hours old showed a similar type of increased sensitivity several hours after the initial cold-shock, but it is



THE EFFECT OF COLD SHOCK AND INCUBATION AT 0° C.

FIG. 2

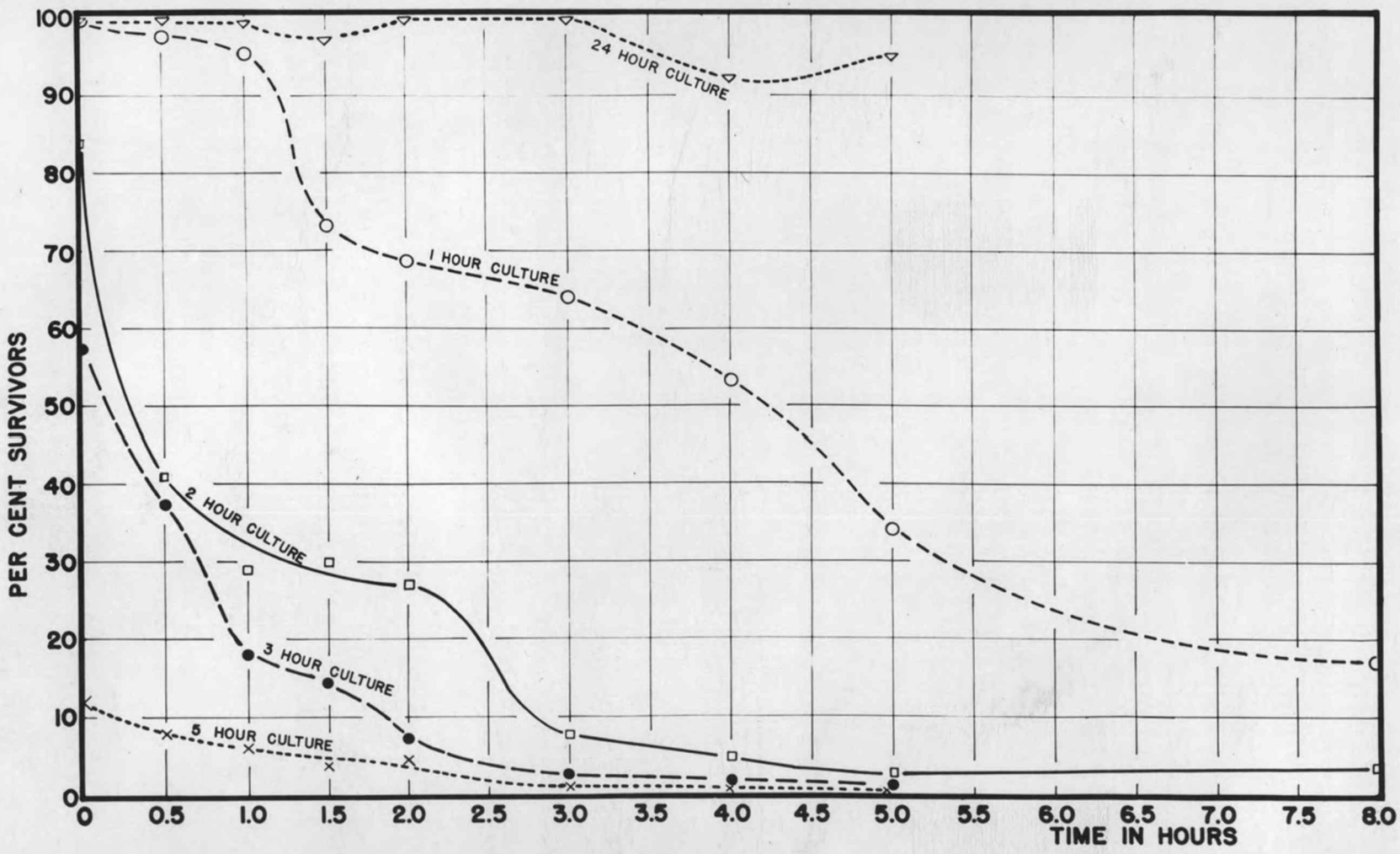
TABLE III

EFFECT OF COLD SHOCK AND PROLONGED EXPOSURE TO 0°C
ON CELLS OF *Escherichia coli*

Normal parent culture		Cold shocked sub-cultures										
Age (hrs.)	Plate count (thousands/ml.)	Number of cells surviving cold-shock and subsequent holding at 0°C (thousands/ml.)										
		No. hrs. held at 0°C	Age of culture when subjected to cold shock (hrs.)									
0	17.5		0 (inoculum)	1	2	3	5	10	12	24	28	48	72
1	20.1	0	17.50	25.20	71.5	196	870	468,000	810,000	1,690,000	3,080,000	2,110,000	1,130,000
2	85.5	0.5	17.65	19.85	35.3	127	559.5	443,500		2,420,000	2,790,000	2,010,000	
3	340.5	1.0	17.45	19.15	25.5	63.5	432	475,000	915,000	1,940,000	2,470,000	1,990,000	915,000
4	1,470.0	1.5	17.10	14.80	26.5	50.15	234.5	345,000		2,080,000	2,770,000	1,670,000	
5	7,150.0	2.0	19.30	14.00	23.0	24.45	337	336,000	550,000	1,770,000	2,290,000	2,070,000	
7	153,000.0	3.0	17.70	13.05	6.7	8.95	79	355,000	590,000	2,020,000	2,550,000	2,040,000	
10	595,000.0	4.0	16.15	11.80	3.95	6.18	58.5	258,000		2,120,000	2,450,000	2,090,000	
24	1,855,000.0	5.0	16.80	6.85	2.50	4.35	38.0	255,000		2,150,000	2,310,000	1,850,000	
28	2,720,000.0	8.0	18.05	3.80	2.95	4.20	19.5				2,450,000		
48	2,175,000.0	10.0	19.05	4.20	2.65	4.55	20.6			2,250,000		1,700,000	686,000
72	1,200,000.0	12.0	15.15	3.95	2.25	4.52			375,000	1,860,000			
108	203,500.0	24.0	28.25	5.20	2.5	7.4	9.5	228,000	200,000	1,300,000	2,000,000	1,700,000	
		48.0	21.75	6.10	3.4	6.1	4.35	116,000	232,000	1,400,000	1,450,000	770,000	
		72.0	16.40	6.75	3.95	7.15	5.18	104,000	204,000	900,000	940,000		
		108.0	9.20	2.80	2.50	1.83	0.98	81,700	160,000				

interesting to note that the period of tolerance preceding the increased sensitivity was several hours shorter than with the cells from a culture one hour old. Cells removed from the culture after three and five hours respectively contained large numbers of bacteria which were rendered non-viable within two minutes after the sample of the culture was added to the cold diluent. The number of cells which showed this initial sensitivity increased as the culture progressed in the logarithmic phase. After five hours, ninety per cent of the cells were instantly rendered non-viable by the initial cold-shock. A slight increase in sensitivity continued for the first hour of incubation at 0°C.; following this there was no further change for the remainder of the experiment. These results have been confirmed by two additional experiments which yielded superimposable curves. These data are given in Tables V and VI. Inspection of these data reveals the correlation between individual experiments.

The data from Table III have been recomputed and are presented in Figure 3 (Table IV). The percentage of surviving cells, those cells resistant to cold, is plotted against time to eliminate any possible misinterpretation resulting from the logarithmic plotting. The curves show the number of cells surviving the exposure to 0°C. during the prolonged holding periods. It is evident that as the



THE EFFECT OF COLD SHOCK UPON SURVIVORS DURING INCUBATION AT 0° C.

FIG. 3

TABLE IV

PER CENT CELLS SURVIVING COLD SHOCK AND PROLONGED EXPOSURE TO 0°C

Hours held at 0°C	Per cent survival				
	Age of culture when subjected to cold shock (hrs)				
	0 (inoculum)	1	2	3	5
0	100	100	84.0	57.5	12.0
0.5	100	98.7	41.0	37.3	7.8
1.0	99	95.2	29.0	18.0	6.0
1.5	97	73.1	30.0	14.5	3.2
2.0	100	69.0	27.0	7.1	4.7
3.0	100	64.0	7.8	2.6	1.1
4.0	92	53.0	4.6	1.7	0.8
5.0	95	34.0	2.9	1.2	0.5
8.0		18.0	3.4		0.2

TABLE V

EFFECT OF COLD SHOCK AND PROLONGED EXPOSURE TO 0°C
ON CELLS OF ESCHERICHIA COLI

Normal parent culture		Cold shocked sub-cultures					
Age (hrs)	Plate count (thousands/ml)	Number of cells surviving cold shock and prolonged holding at 0°C (thousands/ml)					
		No. hrs. held at 0°C	Age of culture when subjected to cold shock (hrs) 0 (inoculum)	1	3	5	8
Start	23.95	0	21.45	30.4	389	690	161,000
1	30.3	0.5	25.75	22.85	233.5	31.55	122,000
2	97.5	1.0	24.45	25.25	108	25.55	125,000
3	301	1.5	22.75	20.95	74.5	12.35	122,000
5	500	2.0	22.85	14.55	73	9.55	106,000
8	164,000	2.5	23.75	15.50	53.75		101,000
10	246,000	3.0	24.80	10.25	44.5	46.5	121,000
24	1,430,000	3.5	22.30	6.65	40.5	22.1	
		4.0	21.20	8.1	49.5	19.8	
		4.5		6.1	57.5	19.25	
		5.0	22.1	5.85	46.1	2.85	
		5.5		5.90	37.95	8.15	
		6.0		8.90	36.40	15.6	
		6.5		9.90			
		7.0		9.70			
		8.0	23.15	9.15	35.1		
		10.0	20.5	7.75			
		24.0	21.9	6.20	30.5	6.55	6,400

TABLE VI

EFFECT OF COLD SHOCK AND PROLONGED EXPOSURE TO 0°C
ON CELLS OF ESCHERICHIA COLI

Normal parent culture		Cold shocked sub-cultures				
Age (hrs)	Plate count (thousands/ml)	Number of cells surviving cold shock and prolonged holding at 0°C (thousands/ml)				
Start		No. hrs. held at 0°C	Age of culture when subjected to cold shock (hrs) 0 (inoculum)	1.5	3	5	10
1		0	16.65	36.0	286	2340	735,000
1.5		0.5	17.5	21.05	45	297	770,000
2.0		1.0	17.3	21.3	38	78	550,000
3.0		1.5	18.85	14.45	10.9	47	150,000
4.0		2.0	15.55	17.25	3.55	55	187,000
5.0		3.0	15.75	13.7	0.95	23.5	140,000
6.0		4.0					182,000
10.0		4.5	16.70				
12.0		5.0		12.6	1.45	38	
25.0		8.0	11.15		0.50		
		10.0	15.0	7.4	0.40	11.25	
		25.0	11.25	6.65	0.65	8.8	

culture proceeds into the logarithmic phase the initial sensitivity to cold-shock increases greatly and, at the same time, there is a marked decrease in the number of cells which become sensitive after several hours' incubation at 0°C.

Sherman and Albus (1923) noted a sensitivity in young cultures of Proteus vulgaris to two per cent sodium chloride and to heat similar to that demonstrated by them for Escherichia coli. This might be expected since the two organisms are closely related physiologically. They made no attempt to apply their tests to organisms of an entirely different group. In the following experiments Streptococcus lactis was grown in dextrose tryptone phosphate-buffered broth at 30°C. and was subjected to the cold-shock treatment and to a prolonged holding at 0°C. in the manner outlined in the second series of experiments. The data are given in Table VII. The mature cells used for inoculation were one hundred per cent resistant to the cold-shock. Culture samples removed during the logarithmic phase displayed a slight initial sensitivity to the cold-shock but there was no further change upon prolonged holding at 0°C. Culture samples of Escherichia coli removed during the logarithmic growth phase were from ninety to one hundred per cent sensitive to the same treatment. The average

TABLE VII

EFFECT OF COLD SHOCK AND PROLONGED EXPOSURE TO 0°C
ON *STREPTOCOCCUS LACTIS*

Normal parent culture		Cold shocked sub-cultures					
Age (hrs)	Plate count (thousands/ml)	Number of cells surviving cold shock and prolonged holding at 0°C (thousands/ml)					
		No. hrs. held at 0°C	Age of culture when subjected to cold shock (hrs) 0 (inoculum)	1	3	5	8
Start	9.75	0	11.85	11.25	30.15	298	1890
1	12.2	0.5	11.55	11.65	30.2	293	1510
2	17.5	1.0	11.95	10.35	27.5	339	1460
3	49.5	1.5	11.20	10.7	32.4	332.5	2070
5	325	2.0	10.8	10.7	32.75	350	1680
8	2080	2.5	10.85	11.6	32.70	447.5	
10	187,000	3.0	10.95	10.95	33.7	353	
24	288,000	3.5	12.75	10.80	34.35	321	
		4.0	11.15	10.0	35.95	352	
		4.5	9.7	10.65	35.5	412	
		5.0	10.6	10.0	36.55	397	
		5.5	11.55	11.05	40.85		
		6.0	11.25	12.5	44.55		
		8.0	12.60	11.35	36.7		
		24.0	12.35	10.7	41.45	376	2030

generation time of the cells in the streptococcus culture was thirty minutes, whereas the average generation time for cultures of Escherichia coli was twenty-five minutes. The culture of Escherichia coli was grown at 37°C., while Streptococcus lactis was grown at 30°C. Sherman and Cameron (1934) have shown that cells of Escherichia coli were not affected by cold-shock if the growth was retarded by a lower incubation temperature. This, taken in conjunction with the decreased magnitude of the temperature change in the case of the streptococcus, might explain the discrepancies. Further studies were not made; however, Hegarty (1938) has demonstrated a physiological difference in cells of Streptococcus lactis in the formation of adaptive enzymes (see discussion).

The curve portraying the normal growth of Escherichia coli in Figure 1 has not been drawn as a straight line. Irregularities which reoccur at the same points in several experiments indicate that the variations are consistent phenomena. This irregular growth curve is in agreement with the observations of Sherman and Cameron (1934). Bayne-Jones and Adolph (1932) found that cells of Bacillus megatherium reproduce more or less simultaneously, resulting in sudden increases of the population. Following such a population increase a stationary period occurred, which was apparently the time necessary for the cells to prepare

for division. They refer to this step-like series of increases as "fission waves". Rogers and Greenbank (1930) observed that a long tube of sterile medium held at a constant temperature and inoculated at one end showed turbidity progressing as a series of spurts rather than as a gradual process. These experiments could not be duplicated by Bibb (1932) using a different technique. It is evident from Figure 1 that there is a fair correlation between the peaks of sensitivity and the "fission waves" of the curve.

Discussion

Sherman and Albus (1923, 1924) determined the sensitivity of Escherichia coli to cold-shock using cells from cultures of different ages. These investigators showed that cells from young cultures were sensitive to the cold-shock while cells from old cultures were resistant. This initial work was interpreted as indicating physiological differences between cells from young and old cultures. The observations of Sherman and Albus have been extended in this investigation to cover the entire growth phase of Escherichia coli using essentially the same technique. The results of sampling the culture at frequent intervals (Figure 1) show that the sensitivity to cold-shock continues through the period of active proliferation and

ceases almost entirely during the subsequent periods of stable and declining population. These data indicate differences in susceptability of the cells from young and old cultures of Escherichia coli to cold-shock and confirm the original observations of Sherman and Albus.

The data secured by holding samples of a normal broth culture of Escherichia coli at 0°C. show three distinct reactions of the sub-culture to the cold, i.e., an initial sensitivity, a delayed sensitivity, and a resistance to cold-shock. Cells removed from a culture one and two hours old and placed in broth at 0°C. exhibit slight initial sensitivity, but upon being held at 0°C. for a period of time the culture suddenly becomes more and more sensitive during a short period. When this secondary sensitivity ceases, the remaining cells show resistance to the cold for at least six days (Figures 1 and 2). The latent period in destruction is probably the time necessary for the cells to undergo changes concerned with cell division, and is a continuation of a process already under way at the time of the cold-shock but progressing at a retarded rate due to the low temperature.

Jensen (1928) concluded that the lag in the growth of Escherichia coli was merely a statistical characteristic of the culture as a whole. According to this concept all stages leading up to fission and the fission

process itself would be progressing during the lag phase. In the early logarithmic period more and more of the cells begin to divide, and once the culture has passed into the logarithmic phase of growth a majority of the cells appear to divide and in an apparently simultaneous manner (Bayne-Jones and Adolph 1932). Indirect evidence for this simultaneous fission may be seen in the wave-like progression of the growth curve in Figure 1 (loc. cit.). During the early lag period, before all of the cells have begun to reproduce, the initial sensitivity is slight, and the time of holding at 0°C. which precedes the secondary sensitivity extends for four hours. As the culture approaches logarithmic growth, initial sensitivity gradually increases and the time of holding which precedes secondary death decreases. Cultures which are in the logarithmic phase show an initial sensitivity which increases in magnitude for about six hours, i.e., until the point of inflection of the growth curve is reached. The phenomenon of secondary sensitivity almost entirely dissappears at this point. Immediately after the point of inflection the initial sensitivity decreases rapidly, and during the period of maximum stationary population the culture approaches a one hundred per cent resistance to the cold-shock. These data would seem to indicate that only those cells in a

certain state of division are instantly destroyed by the cold-shock and that cells in stages preceding fission are not immediately affected by the cold but upon holding at 0°C. changes occur which render them sensitive. This is illustrated by the following hypothetical consideration in which three cell stages are assumed on the basis of the three reactions exhibited by cold-shock and prolonged holding at 0°C. The first stage represents cells which are resistant to the cold-shock and to the prolonged holding. The second or intermediate stage describes cells which resist the initial cold-shock but become sensitive upon holding at 0°C; and the third stage includes cells initially sensitive to cold-shock. The exact nature of cells when they show sensitivity to cold is not known, nor is it known that the cells show this sensitivity at a definite point, but the assumption is made that the cells are sensitive at some point immediately before fission. The assumption is also made that daughter cells are at least momentarily resistant to cold-shock. These resistant cells are capable of reproduction providing they find a suitable environment, and in this event the cells grow and pass into the second stage. It seems logical to suppose that once cell growth has begun it will continue until the cell divides. Henrici (1928) and Buchanan (1928) stress the fact that growth will continue to

progress once it has been iniated even if a transfer is made to a new medium. From this it is assumed that the cells showing a delayed sensitivity to cold exhibit this reaction because of a change to a sensitive stage, and that this change is probably retarded by the low temperature. This concept shows that cells in the initial stationary phase and the early part of the lag phase exist largely in the intermediate stage, and that the holding time at 0°C. preceding the delayed sensitivity is a measure of the time required for the cells to pass into the sensitive stage, delayed because of the low temperature of the sub-culture. As the culture progresses in the lag phase and through the logarithmic phase, the initial sensitivity increases, and the delayed sensitivity decreases; here the third stage predominates in a progressive manner. Figure 1 shows that immediately following a point of great initial sensitivity, there occurs a slight increase in the resistance of the culture to the cold-shock. This might indicate a momentary resistance of the daughter cells. The resistant stage is most apparent during the early part of the initial stationary phase and in the phases following logarithmic growth. During the early growth periods before any apparent increase in the culture population occurs, the cells of Escherichia coli are known to enlarge apparently at the

expense of division (Bayne-Jones and Adolph 1932), ultimately, however, these cells divide. During this period delayed sensitivity is most apparent, and it would appear that this intermediate stage is associated with the increase in cell substance. In the subsequent period of rapid division Knaysi (1938) has shown that there is a drastic reduction in the length of cells during the successive generations; here the cells appear to be dividing at the expense of their size. If delayed sensitivity is associated with increase in cell substance, it should be less and less apparent as the logarithmic period progresses. Reference to Figures 1 and 2 shows that the initial sensitivity increases and the delayed sensitivity decreases as this phase progresses. Through continued reproduction at the expense of cell size the cells should reach a point at which a further decrease would result in destruction, it seems logical to suppose that the cells would cease reproduction before such a limit is reached and undergo physiological changes which would fit them for further reproduction. These changes do not become apparent until the cells are transferred to a new medium; instead the culture becomes resistant to cold-shock. This resistance has been associated with cells that are not preparing to divide.

The physiological changes which precede fission are most evident before the culture begins to increase in numbers and were first observed by Sherman and Albus (1923) who suggested that bacterial cells undergo physiological rejuvenation prior to cell division and proposed the term "physiological youth" for the period during which these changes occur. Many of the data discussed in the literature review show that "physiological youth" extends as a series of events through the logarithmic growth phase.

The early increase in cell size and high rates of metabolism have been advanced in support of "physiological youth". Increased cell size has been observed to reach a maximum during the lag phase before maximum reproduction (Clark and Ruehl, 1919; Henrici, 1938; Bayne-Jones and Adolph, 1932; and Huntington and Winslow, 1937). Increased cell size has not entirely explained the high rates of metabolism which exist during the latter part of the initial stationary phase and the lag phase (Walker, Winslow, Huntington, and Mooney, 1934; Huntington and Winslow, 1937). Simultaneous occurrence of increased cell size and high rates of metabolism would support such an explanation; however, the events appear as a sequence. The data of Martin (1932) demonstrate that oxygen consumption reached a maximum rate before the point of

maximum cell size. Huntington and Winslow (1937) found that maximum carbon dioxide production preceded maximum rate of fission. This sequence of events continues into the logarithmic phase. Data from cold-shock experiments support this statement inasmuch as the sensitivity to cold, which is probably associated with physiological changes preceding fission, extends through the logarithmic growth phase.

Hershey and Bronfenbrenner (1938) and Hershey (1939) have explained increased oxygen consumption entirely on the basis of increased cell size. The oxygen consumption of Escherichia coli per unit of bacterial nitrogen gave a constant value throughout the growth period. They concluded that a lag in multiplication rate could be explained by increased cell size and their calculation from nephlometric data support this contention. The initial measurements of oxygen consumption were made at the time of inoculation and the next observation after three hours' incubation. The maximum rate of oxygen consumption for Escherichia coli has been observed to occur between one and two hours' incubation (Martin 1932). It is possible then that Hershey did not observe the actual period of maximum oxygen consumption. Further, Martin (1932) has shown that this maximum occurs before the increase in cell size. It is noteworthy that Hershey in his attack

on "physiological youth" has selected a character whose increased rate has previously been shown to be entirely accounted for by the increase in cell size (Walker, Winslow, Huntington, and Mooney 1934). Such explanations do not take into consideration the increased oxygen consumption occurring before the increase in cell size. Hershey (1939) determined the initial rate of growth of transplants from parent cultures of different ages. Here again he found that there was no lag in the rate of growth if cell size was considered. Hershey concludes that it is not necessary to postulate "obscure" physiological differences between young and old cultures to explain difference in growth rates. Absolute lag in growth can be taken as a measure of the adaption of a culture to a new medium and may disappear if favorable conditions for growth are used (Walker and Winslow 1939). Hershey has used large inocula and favorable conditions for growth in all of his work which might explain the failure of this worker to observe absolute lag in the growth rate of Escherichia coli. Regardless of whether or not increased cell size can account for these various maxima it is at once apparent that there exists in cultures of Escherichia coli a period during the lag phase, proceeding into the logarithmic phase, in which maximum rates are reached, and

that these appear as a succession of events rather than simultaneously.

A third concept of "physiological youth" has been advanced by Hegarty (1939) who observed that for Strepto coccus lactis adaptive enzymes could be formed only for a limited time during the lag and early logarithmic phases of growth. He states that, for this organism, there are actually physiological changes which occur only during the latter part of the lag phase, and that this supports the contention that "physiological youth" is a definite state of the culture and not an aritfact as has been suggested by Hershey (1939). Physiological youth defined on the basis of adaptive enzyme formation would limit the phenomenon to the latter part of the lag phase.

"Physiological Youth" was originally defined by Sherman and Albus (1923) on the basis of their cold tolerance tests for Escherichia coli. These cold-tolerance tests do not give comparable data with Streptococcus lactis which differs metabolically and morphologically from Escherichia coli. On the other hand, the adaptive enzymes demonstrated for Streptococcus lactis (Hegarty 1939) have not as yet been investigated for Escherichia coli. If adaptive enzyme formation, which is confined to the lag and early logarithmic periods, defines the state of physiological youth, then sensitivity to cold

demonstrated for Escherichia coli cannot be taken as a measure of the phenomenon, because the sensitivity to cold extends through the entire growth phase. Adaptive enzyme formation supports a definition of "physiological youth" based upon the reaction of the culture as a whole. At any one moment the culture represents the average of individual cell activity, and so the attempt has been made in discussing the cold-shock data to consider the individual cell, showing that if the individual cell is considered, the physiological changes preceding each cell division extend through the entire growth period of the culture.

Conclusions

The experiments on cold-shock show an increase in sensitivity before any apparent increase in the culture population occurs as well as extreme sensitivity for cells from a rapidly growing culture. The cells from a mature culture do not exhibit a sensitivity to the same treatment. These data, for Escherichia coli, indicate that only those cells which are in a certain stage of the fission process are affected by the cold-shock. These data also demonstrate physiological differences between cells from young and old cultures and point to the fact that these differences cannot be limited to any one phase but extend through the entire growth period.

Summary

1. The observations of Sherman and Albus (1923, 1924) that young cells of Escherichia coli are susceptible to an initial cold-shock from 37°C. to 0°C. have been confirmed.

2. It has been found that this sensitivity extends through the entire logarithmic phase.

3. Cells from mature cultures are not affected by either an initial cold-shock, or prolonged holding at 0°C.

4. During the period between lag and the very early part of logarithmic growth, sudden cold-shock has but slight initial affect. Upon holding at 0°C. subsequent to a latent period, there is a marked increase in the number of cold-susceptible cells. This indicates that the phenomenon is dependent upon changes within the cell, which render it susceptible to cold.

5. Frequent plate counts have shown the growth curve to progress in a step-wise manner which is probably due to simultaneous division of many cells.

6. The sensitivity of Escherichia coli to cold is related in some manner to cell division and to changes within the cell.

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