SENSITIVITY OF ESCHERICHIA COLI TO COLD-SHOCK DURING THE LOGARITHMIC GROWTH PHASE[1]

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Physiological differences between cells of *Escherichia coli* taken from young and mature cultures were observed by Sherman and Albus (1923). These observers demonstrated an increased sensitivity to cold, to two per cent sodium chloride, and to heat, for cells removed from young cultures and they suggested that the cells pass through a "physiological rejuvenation" prior to rapid multiplication. In 1924 Sherman and Albus repeated this work and observed sensitivity to cold after one and one half hours and after three hours incubation. Susceptibility 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 rate of growth of the cell had been retarded by incubation at low temperature.

The following investigation was undertaken to study the resistance of bacteria to cold during the entire growth range of a culture. Samples from a broth culture of *Escherichia coli* 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).

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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 in cultures of young cells. An experiment was designed using nutrient broth, distilled water, and tap water to prepare dilutions for the plate count. All materials were held at 37°C. The results are shown in table 1. Table 1 shows that with constant temperature any slight change resulting from the transfer of cells from nutrient broth into tap water or distilled water does not cause a significant difference in the counts obtained. Distilled water was used as the cold-shock medium and for dilution purposes rather than nutrient broth which foamed badly upon shaking, giving rise to an appreciable sampling error.

TABLE 1

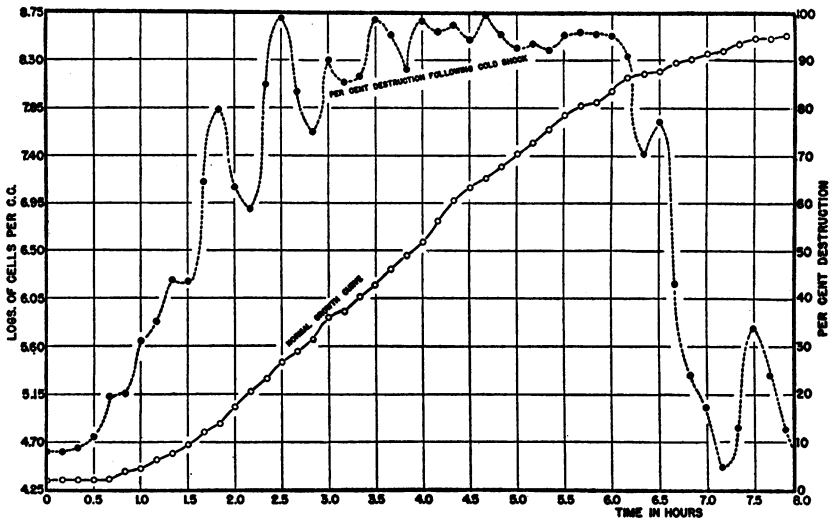
Plate counts obtained using nutrient broth, distilled, and tap water for dilutions

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

The relation between total and sensitive cells was established by making two sets of plate counts from a broth culture of *Escherichia coli* incubated at 37°C. The dilutions for counting the total viable cells were made in water at 37°C.; those for counting the cold-resistant cells were made with water at 0°C. This latter temperature was maintained with a melting ice bath. Following the suggestion of Hershey (1938) the same lots of Difco materials were used in the preparation of all media used in these studies.

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 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 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 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



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

FIG. 1

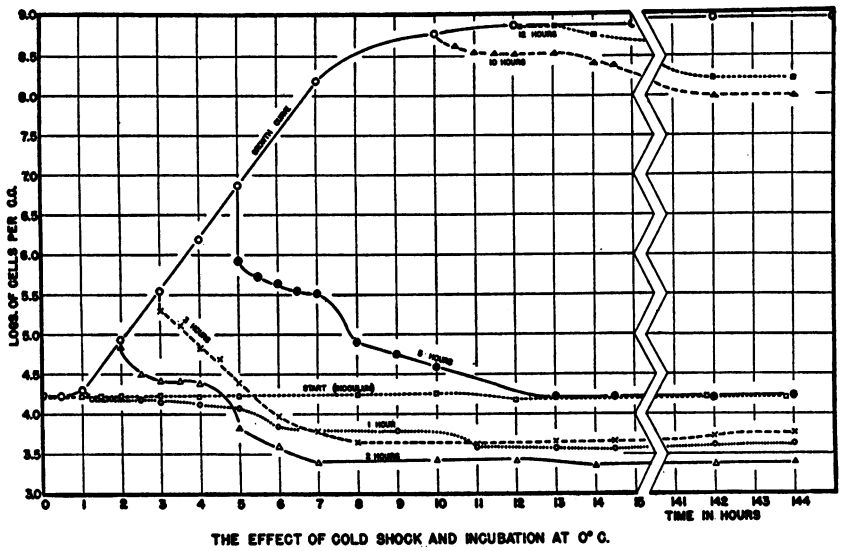
and during the first twenty minutes of the period there was no increase in the number of cold-sensitive cells. During the next twenty minutes of the stationary period twelve per cent more of the total viable cells became susceptible to cold-shock. Sherman and Albus (1924) observed a similar increased 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 viable cells were destroyed by the

initial cold-shock. At the point of inflection of the growth curve, 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 killed by 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 at less frequent intervals and making counts of the sub-culture held at 0°C. at various intervals. The technique used for these experiments was essentially that described in the previous experiment. Nutrient broth was used as the cold-shock medium rather than distilled water which might have affected viability on prolonged standing (Sherman and Albus 1924). Duplicate one-ml. samples were removed for a total viable count and a count of cold-sensitive cells at the time of inoculation from a twenty-four hour culture, (start) and after 1, 2, 3, 5, 10, 12, 24, 28, 48, 72, and 144 hours. The cold diluent bottles contained 99 ml. of broth and 1 ml. of the culture and were held continuously in a melting ice bath, and plate counts were made every thirty minutes for the first three hours and then at frequent intervals for the next six days. The data are presented in figure 2.

Cells from the twenty-four hour old culture used for inoculation were not sensitive to cold-shock and no decrease in numbers resulted from holding the sub-culture at 0°C. for the 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 samples from a one-hour culture contained cells which were not immediately sensitive to cold but after holding 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 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 interesting to note that the period of tolerance preceding the increased sensitivity was several hours shorter than with cells from a culture one hour old. Cells removed from a culture after three and five hours contained large numbers of bacteria which were rendered non-viable within two minutes after the sample of culture was added to the cold diluent. The numbers of cells



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

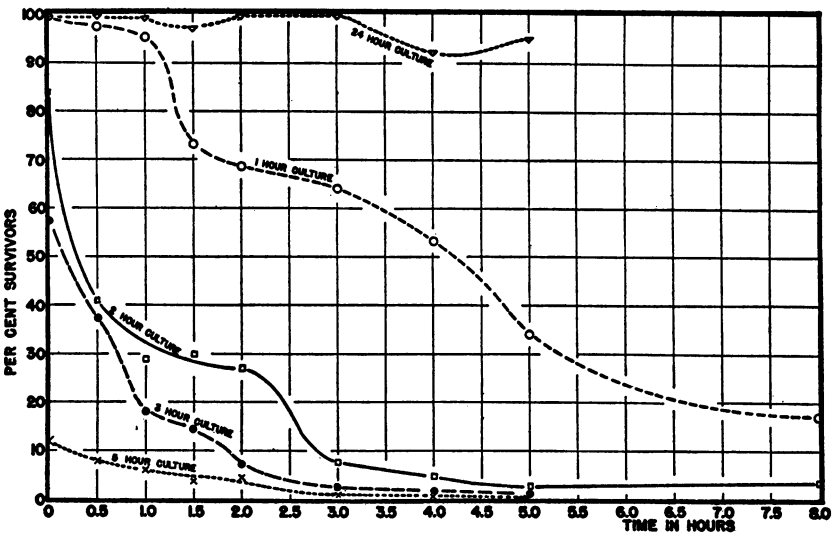
FIG. 2

which showed this initial sensitivity increased as the culture progressed in the logarithmic growth phase. After five hours ninety per cent of the viable cells were instantly destroyed by the initial cold-shock. A slight increase in sensitivity continued after one-hour incubation at 0°C. These results have been confirmed by three experiments which yielded superimposable curves.

The data from a typical experiment have been recomputed and are presented in figure 3. The percentage of surviving cells, those cells resistant to cold, is plotted against time as the abscissa

in order to eliminate the confusion of 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 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.

The curve portraying the normal growth of *Escherichia coli* in figure 1 has not been drawn as a straight line. Irregularities



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

FIG. 3

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 referred to this series of step-wise increases

as "fission waves." Rogers and Greenbank (1930) observed that a long tube of sterile media held at a constant temperature and inoculated at one end showed turbidity progressing through the tube as a series of spurts rather than as a gradual process. These experiments could not be duplicated by Bibb (1932). 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

The experiments on cold-shock confirm the work of Sherman and Albus (1923, 1924) and present further data. Cells removed from a culture one and two hours old and placed in broth at 0°C. showed slight initial death, but mortality increased with time when the cells were held at 0°C. This phenomenon of an increased number of sensitive cells developing upon holding at 0°C. indicates that the rôle of cold in the destruction of certain cells is probably concerned with some change occurring within the cell and is not due to a physical effect alone. The latent period in destruction (figs. 2 and 3) is probably the time necessary for the cells to undergo physiological changes concerned with cell division, and is a continuation of a process already under way at the time of the initial cold-shock, but progressing at a retarded rate due to the low temperature. Those samples removed from the mother culture in the later stages of logarithmic growth showed marked initial sensitivity and less latent sensitivity. Mature, non-proliferating cells showed but slight initial or latent sensitivity. This would seem to indicate that only those cells in a specific state of cell division are instantly destroyed by cold-shock.

The changes in physiological activity and in the size of the cell during the time referred to as "physiological youth" have been investigated by many workers. Clark and Ruehl (1919) and Henrici (1928) noted an increased cell size during the early growth stages of *Escherichia coli*. Bayne-Jones and Adolph (1932) observed an initial period during which there was no growth or increase in cell substance, and a period of maximal increase in cell size after one hour's incubation. They concluded that since

the growth of the cell precedes reproduction of the cell, that growth must dictate fission.

Increases in the rates of metabolism have been noted by Bayne-Jones and Rhees (1929), Martin (1932), Walker and Winslow (1932) and others. Walker, Winslow, Huntington and Mooney (1934) calculated that increased rates of heat production, oxygen consumption, carbon dioxide production, and ammonia-nitrogen production were explainable for the most part on the basis of increased cell size. They point out however that while maximal rates were accountable on the basis of increased cell size, decreased cell size could not account for the decreased metabolic rates during the stable growth phases. Huntington and Winslow (1934) were unable to explain increased rates of carbon dioxide production entirely on the basis of cell size. They concluded that this increased cell size was a factor to be considered in conjunction with increased metabolism in defining physiological youth, but that it did not serve as a means of elucidation.

Elliker and Frazier (1938) have found that for *Escherichia coli* there is a period of increased resistance to heat during the initial stationary phase of a culture. 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.

Hershey and Bronfenbrenner (1938) and Hershey (1939) have made nephelometric measurements, viable counts, and a determination of bacterial nitrogen and of oxygen consumption. Oxygen consumption per unit of bacterial nitrogen gave a constant value throughout the growth period, indicating that increased oxygen consumption was entirely *accountable* on the basis of increased cell size. They concluded that the lag in multiplication rate could be explained by the increase in cell size. Calculations of the rate of growth from nephelometric readings support this contention. Hershey (1939) presents data which substantiate this work. On the basis of these experiments, Hershey discarded physiological differences between young and old cells. Regardless of whether increased cell size can account for these various maxima, it is apparent that there exists in *Escherichia coli* a period during the latter part of the initial lag phase in which

maximum rates are reached, and that these appear as a succession of events rather than occurring simultaneously. Hegarty (1939) observed that for *Streptococcus lactis* adaptive enzymes could be produced only during a limited time between the lag phase and the logarithmic phase of growth. This indicates that, for this organism, there are actually physiological changes which occur only during the latter part of the lag phase, and supports the contention that "physiological youth" is a definite state of the culture, and not an artifact as has been suggested by Hershey (1939).

The fact that increased sensitivity to cold-shock is evident slightly before increases in population occur indicates that this phenomenon is concerned in some manner with the synthesis of new protoplasm or with an early stage of the division process itself. Cells of *Escherichia coli* show great sensitivity to a cold-shock only during the logarithmic phase of growth indicating that it is associated with some definite stage of the fission process.

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 to cold-shock extends throughout the entire logarithmic phase of growth.

3. Mature cells 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 effect. Upon holding at 0°C., subsequent to a stationary period, there is a marked increase in the numbers of cold-susceptible cells. This indicates that the phenomenon is concerned with changes within the cell, rather than to a physical effect alone.

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

6. The sensitivity of *Escherichia coli* to cold seems to be related

in some manner to cell division and to changes within the individual cell.

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