# STUDIES ON THE DEATH OF BACTERIA AT LOW TEMPERATURES

## I. The Influence of the Intensity of the Freezing Temperature, Repeated Fluctuations of Temperature, and the Period of Exposure to Freezing Temperatures on the Mortality of Escherichia coli

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The preservation of cultures of microorganisms and numerous biological materials by the freezing and drying technique has lately come into such wide practice that an exhaustive study of the factors concerned with this type of preservation and the optimum conditions for its application is pertinent.

An understanding of the preservation afforded by drying from the frozen state should logically rest on a thorough knowledge of the separate action of the two major influences involved, that of freezing and of drying. The manner in which bacterial cells are injured as a result of exposure to low temperatures and drying has not been extensively studied and needs reinvestigation. The first part of the present investigation is devoted to studies on the death of bacteria at low temperatures. The second part will be devoted to death of bacteria by drying.

### GENERAL METHODS

Unless otherwise specified, the methods outlined below were used throughout the present investigation.

The cultures were grown on nutrient agar slants within the pH range of 6.9 to 7.1 at 35 C to 37 C for 15 to 20 hours. They were transferred from cultures 12 to 24 hours old. Stock cultures were subcultured at frequent intervals and stored at refrigeration temperature.

Suspensions of organisms used in the various experiments were washed twice in M/60 phosphate buffer, pH 7.0. The use of M/60 phosphate buffer at pH 7.0 for washing and dilution of bacteria has been recommended by Ordal (1945). The approximate number of organisms was judged turbidimetrically with the Klett-Summerson photoelectric colorimeter, and appropriate dilutions were made first in buffer and then in 1 per cent peptone, pH 7.0, to give between 200,000 and 400,000 organisms per ml.

The peptone suspension was distributed into sterile lyophile[1] tubes in 0.1-ml amounts with a carefully calibrated capillary pipette or with a tuberculin syringe and a 6-inch 20-gauge hypodermic needle. Samples of this small size were found to freeze very quickly in the thin-walled lyophile tubes when exposed to low temperatures. The lyophile tubes were prepared from pyrex glass tubing with an inside diameter of from 4 to 5 mm. Tubing approximately 24 cm in

[1] In referring to these tubes, the term *lyophile* is used as a matter of convenience, following the adoption of the term by Flosdorf and Mudd (1935).

length was separated in the middle by heat fusion so that no pin-point holes remained. The fused ends were heated in a small oxygen torch and thin-walled bulbs, 10 to 14 mm in diameter, were blown. The tubes were cleaned in dichromate cleaning solution and thoroughly rinsed several times with distilled water forced through capillary pipettes into the bulbs of the tubes.

Special "thermos" containers were used for low-temperature baths. The temperatures between 0 C and −20 C were carefully maintained with mixtures of ice and calcium chloride, the temperatures between −30 C and −78 C with dry ice and methyl cellosolve, and the temperatures of −190 C and −195 C with liquid air or liquid nitrogen. The lyophile tubes were agitated during the freezing of their contents so that a thin shell was formed lining the bulbs of the tubes. The mouths of the tubes were plugged with cotton and kept well above the tops of the containers to avoid the possible harmful effects which might be produced by entrance of carbon dioxide gas coming from the baths.

Recovery of the suspension from the lyophile tubes, following treatment, was accomplished by repeatedly filling and emptying the tubes with the diluting buffer by means of a capillary pipette. This also accomplished rapid thawing. Upon the recovery of material from the tubes, dilutions were promptly made in M/60 phosphate buffer, pH 7.0, from which 1-ml amounts were plated in quadruplicate on nutrient agar at pH values of 6.9 to 7.1 and incubated at 37 C for at least three days. Colony counts were made with the aid of a Quebec colony counter and a hand tally. Since a delayed germination was often noted in treated materials, a recount on a few sets of plates of each experiment was made after several days' additional incubation to make certain that the first counts were correct. In no case did the count increase after 3 days. The authors worked together in the various experiments, thus accomplishing the "paired control" so highly desirable in experiments such as these in which time is of the utmost importance. When more than one experimental set of tubes was being used, platings were made in alternating sequence to insure uniform treatment. The coefficient of variation of counts conducted to test recovery on 15 sets of 4 to 8 tubes was from 5.9 per cent to 14.0 per cent, which is well within the normal range of expected variation. Following the various low-temperature treatments, variations greater than this were often noted between tubes, as has also been observed by Sedgwick and Winslow (1902), Haines (1937–38), and others.

From the colony counts the number of viable organisms was computed and the following calculations made: the mean, the standard deviation, the standard deviation of means, the range of means, and the coefficient of variation, as outlined by Halvorson and Ziegler (1933). In cases in which the significance of the data was questionable "Student's" $t$ factor analysis was applied. The results are given as the average percentage of reduction and range of reduction in plate count, calculated from the means. The standard deviation of means ($\sigma m$) and the coefficient of variation ($C_v$) of plate counts are also given. In experiments which extended over a period of time, and during which some reduction of "initial numbers" may have taken place, a mean of the means of

counts taken at the beginning and the end of the experiment was used to calculate the percentage of reduction of viable organisms in treated tubes. In such experiments these initial control suspensions were stored at 4 to 8 C until used. In most instances no measurable reduction in viable organisms was noted in the control suspensions during the course of the experiment, and it probably did not introduce significant error in any case.

## LITERATURE

The mechanism of death of organisms due to low temperatures and freezing has held the attention of scientists for many years and has led to the publication of many hundreds of articles on the subject. Logically, the botanists have shown the most marked interest in this subject because of the great economic importance of crop destruction by low temperatures. The explanation of the death of plants by freezing has been sought largely through studies on "cold hardiness."

In spite of extensive investigation, the manner by which freezing serves to kill cells has never been satisfactorily explained. Numerous theories have been advanced, most of which certainly fall short of fitting all the known facts. It appears most probable that death by freezing involves factors which have been proposed in more than one theory and that the part played by these various factors may well vary with different cells, especially those which differ most widely in function as well as in structure.

Whereas structural differences between bacterial cells and other cells are marked, it is likely that, in a certain measure at least, knowledge of the nature of freezing injury to bacterial cells would be of value in advancing our knowledge of cold injury to other cells. The use of bacterial cells has the advantage of providing single cells which can be evenly suspended and readily subjected to varying environments so as to insure uniform treatment. Furthermore, death rates can be rather accurately determined, thus affording good quantitative data.

Extensive reviews on the nature of cell injury due to freezing have appeared in the literature from time to time. Many of these reviews pertaining especially to the cells of higher plants and animals are critical and well done, some of the most recent being those of Maximow (1929), Stiles (1930), Iljin (1933), Belehradek (1935), Scarth and Levitt (1937), Luyet and Gehenio (1940), and Levitt (1941).

Critical reviews on the death of bacteria at low temperatures are few, and many data remain widely scattered in the literature among other subject materials.

Only a brief summary of some of the most important publications will be given here as an extensive review of the subject is beyond the scope of this paper. Some of the authors who have presented the most extensive bibliographies on the subject are Prudden (1887), Sedgwick and Winslow (1902), Smith and Swingle (1905), Keith (1913), Hilliard and Davis (1918), Vass (1919), Hampil (1932), Wallace and Tanner (1933), Turner and Brayton (1939), Kyes and Potter (1939), and Luyet and Gehenio (1940).

As the result of investigations involving experiments on supercooling versus

freezing, repeated freezing, and storage of frozen water suspensions of various bacteria, Prudden (1887) concluded that the marked initial killing following freezing is due to the immediate killing of the "more feeble" bacteria. He reported that supercooling was even more destructive than freezing and that very low temperatures were more destructive than the higher freezing temperatures. He also noted a gradual storage death, a higher mortality with repeated freezing, and a low resistance of old cultures of *Staphylococcus aureus* to freezing.

Park (1901) reported a storage death in water suspensions of *Eberthella typhi* stored at −5 C and at the temperature of liquid air. The pronounced death rates he reported in suspensions stored at liquid air temperature over short periods ranging from 3 minutes to 130 minutes is surprising and may have been the result of error due to the limited number of observations made.

Sedgwick and Winslow (1902) presented an excellent review of the literature to which they added their own extensive observations. They found that repeated freezing is somewhat more lethal than a single freezing and that death rates in chilled suspensions near the freezing point and in frozen suspensions at a slightly lower temperature were essentially the same. From this they concluded that there is no mechanical crushing due to ice, and that destruction above and below the freezing point is continuous and is a function of time and temperature.

Smith and Swingle (1905) conducted extensive quantitative studies on the death of bacteria subjected to low temperatures. They observed that the mortality at various freezing temperatures, ranging from near 0 C to −195 C, was essentially the same and that the critical point at which immediate destruction took place was somewhere near 0 C. They believed that the few vegetative bacteria which survive freezing do so because of their low water content, thus behaving like endospores. Repeated freezing of a suspension for 10 times was found to destroy *Pseudomonas campestris* so completely that it was near the vanishing point.

Keith (1913) held the opinion that the immediate death of bacteria by freezing results from the crushing action of extracellular ice. This view was based on the results of quantitative studies of bacteria suspended in a number of different mediums. The greatest mortality was observed to occur in water. Keith believed that the protective effect of suspending mediums, such as sugar, glycerine, milk, peptone, etc., is due to the protection these nonaqueous substances afford the bacteria being extruded between the forming ice crystals. He regarded the higher mortality in water as resulting from a complete crystallization which offers no refuge from the crushing action of growing ice crystals, as do other menstra. Slow dying at low temperatures was explained as being caused by starvation or destructive metabolism.

The views of Hilliard and Davis (1918) were essentially the same as those of Keith (1913). These authors were convinced that the crushing action of extracellular ice is the major factor responsible for the death of bacteria by freezing. This was based upon an observed protective effect of colloids, greater mortality in suspensions frozen in water than in supercooled glucose solutions,

and enhanced mortality with repeated freezing. These authors did not believe the intensity of the freezing temperature to be important and stated: "There is no critical temperature below freezing where the germicidal effect is greatly accelerated."

The excellent work of Haines (1937–38) affords good quantitative data relative to the mechanism of death of bacteria at low temperatures. Haines found that freezing temperatures of −5 C, −20 C, and −70 C produced essentially the same mortality in a suspension of *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*), whereas prolonged storage at various temperatures from −1 C to −20 C resulted in the greatest death rate at the higher temperatures, −2 C being the most lethal. Native cellular proteins of *B. pyocyaneus* were found to denature most rapidly at −2 C. The author found no evidence of cytological change following freezing and stated: "It is suggested either that two factors are responsible for the death of the bacterial cell on freezing: one unknown, but apparently not mechanical, and the other, some change leading to flocculation of the cellular proteins; or else that there is in reality one process leading to coagulation, but with a time lag, so that coagulation is not found on immediate freezing and thawing." He believed this flocculation of the cellular proteins to be due to the high salt concentration and change in pH obtained in freezing.

Dubos (1937) made the interesting observation that freezing of suspensions of *Diplococcus pneumoniae* resulted in a rapid disruption of the cells which only took place when the temperature of the thawing bath was high. He suggested that this lysis is due to the action of certain autolytic enzymes liberated to act by the process of freezing and that freezing destroys some structure in the cell which normally prevents cell enzymes from attacking a substrate of cell architecture.

Turner (1938) determined the survival of *Treponema pallidum*, *Treponema pertenue*, and the viruses of human influenza, yellow fever, and spontaneous encephalomyelitis of mice by pathogenicity tests following prolonged storage at low temperature. The viruses showed no loss of titer after 6 months' storage at −78 C, and the treponemas were not appreciably altered after periods of at least 1 year. On the other hand, it was found that at −10 C and −20 C the treponemas survived the immediate effects of freezing but died during subsequent days or weeks so that none survived so long as 2 months. This suggested to the author that "the damage to the organism which occurs at these temperatures is not due to physical alterations in the cell protoplasm at the time of freezing but to changes which occur during the maintenance period."

In a subsequent investigation, Turner and Brayton (1939) studied the spirochete of relapsing fever, *Borrelia swellengrebel*, using the mouse pathogenicity test as a measure of viable organisms. No storage death was noted at −78 C even after storage for 6 months. By varying the rates of cooling to −78 C and by subsequent warming, etc., the authors obtained evidence that greater injury occurs during thawing than during freezing. They suggested that there are two factors which produce injury: one acts rapidly and is associated with freezing and thawing; the other is a storage injury acting at higher freezing temperatures,

but not at −78 C, and is caused by changes detrimental to cell metabolism or to proteolytic or other enzymes.

A perusal of the literature reveals that only a few observations have been made which appear to be certain enough to justify their acceptance as a contribution toward our knowledge of the mechanism of death of bacteria by freezing, namely, that freezing and thawing per se exerts an "immediate" and marked lethal action, that a "storage death" occurs at the higher freezing temperatures, that colloids and many other nonaqueous materials serve to protect against freezing injury, and that repeated freezing is more destructive than a single freezing or prolonged storage at freezing temperatures.

## THE INFLUENCE OF THE INTENSITY OF THE FREEZING TEMPERATURE UPON THE MORTALITY OF BACTERIA IN SUSPENSION

In attempting to determine the mechanism of low-temperature injury of bacteria due to freezing, it is important to ascertain, if possible, all of the changes of physical state which may occur, including the sites of ice formation.

In higher plant and animal tissues it has been noted that ice may form either extracellularly or both extracellularly and intracellularly. Death of the cell may or may not occur under either of these circumstances depending upon the resistance of the cell and the quantities of ice formed. Whereas a considerable quantity of extracellular ice may often be tolerated by cells, the formation of any appreciable amount of intracellular ice invariably results in death. In only a few instances have cells been observed to survive the formation of even small quantities of intracellular ice.

Levitt (1941) believes that cold hardiness in some plants is due, in part at least, to their increased resistance to the formation of intracellular ice as well as to an increased resistance to the injurious effects of both intracellular and extracellular ice. In the case of bacteria subjected to low temperatures, we have no direct evidence that intracellular ice forms. Nevertheless, indirect evidence may go far toward settling this important question. If it is assumed that bacteria possess no more resistance to the formation of intracellular ice than do plant cells, the indications are that intracellular ice does not form in quantity in bacterial cells, since many of them survive exposure to extremely low temperatures. Several investigators, including Molisch (1897) and D'Arsonval (1901), have suggested that ice may not form in bacterial cells at low temperatures because of the strong capillarity effects which cells of such small size should exert to promote supercooling.[2]

Levitt and Scarth (1936) have shown that the increased frost hardiness of certain plant cells correlates to a marked degree with the reduction in cell size, and that in some instances the cold hardiness of these small cells is due, in part at least, to their greater tendency to supercool. However, the factors which may contribute to the supercooling of cells are so difficult to evaluate that it is rather

[2] The term *supercooling* is used to signify low temperatures which may be attained before freezing occurs regardless of the causes involved, and hence does not differentiate between subcooling and true supercooling.

hazardous even to speculate as to what extent bacteria may be capable of supercooling. In plant cells the ability to supercool is thought by Levitt (1941) to be due principally to the high initial osmotic pressure of the cell contents and the ability of the cell to increase its osmotic pressure by rapid loss of water to the accumulating extracellular ice. Levitt has also suggested that this loss of water is extremely rapid in very small cells, because of the large cell surface per unit volume of cytoplasm. In addition, he has pointed out that small cells should suffer less distortion per unit reduction in volume attending such water loss.

In plant and animal tissues it is well established that at low temperatures, so long as free water in the cells remains unfrozen, fluid passes to the exterior with the formation of extracellular ice. There is no reason to suppose that bacteria behave differently from other cells in this respect, and hence they may be expected to possess a marked capacity to dehydrate and supercool.

In the case of plant cells which succumb to the formation of intracellular ice, it is observed that the cells die whenever the temperature is reduced to a point at which such ice forms. This critical temperature at which ice forms varies, of course, with conditions which alter the freezing point of the cell contents.

If, in a similar manner, the lethal injury to bacteria which is observed following exposure to low temperature is due in any measure to the formation of intracellular ice, it is to be expected that passage through the point or range of temperature at which such a change takes place would result in an abrupt increase in mortality of the suspended organisms. Furthermore, this increase in mortality might be expected to be greatly accentuated by repeated passage, even though narrowly limited to that particular point or temperature range. It is also to be expected that if the resistance of bacteria to low temperature is due to their marked ability to dehydrate and supercool rapidly, quick freezing, which might exceed in rate the ability to dehydrate, would result in greater killing than slow freezing.

Several investigators have studied the influence of the intensity of the freezing temperature upon the death of bacteria. In most instances, however, the times the suspensions remained frozen were so long that storage death may have been so great, especially at the higher temperatures, as to make a direct comparison of the immediate effects of freezing impossible.

Haines (1937–38) has presented critical quantitative work to determine the effect of different freezing temperatures on the mortality of bacteria. By using small quantities of water suspensions of 20-hour cultures of *Bacillus pyocyaneus*, placed both on cover slips and in tubes, he found that no marked difference in mortality occurred when the preparations were rapidly frozen and thawed at temperatures of −70 C, −20 C, and −5 C. Haines believed this result to be in opposition to the mechanical destruction theory. His belief was based on two assumptions: first, that intracellular ice forms in bacteria, and, second, that large ice crystals obtained by slow freezing at the higher temperatures should be expected to exert a more marked disruptive effect than small ice

crystals formed at the lower temperatures. It is doubtful, however, that one can correctly assume that intracellular ice forms in bacteria, especially at the higher temperatures, since factors which favor supercooling, such as small cell size and high osmotic pressure, are more pronounced in bacteria than in certain plant and animal cells which have been observed to supercool to temperatures much lower than those used by Haines. As evidence of this, Morrison (1924–25) has presented data which indicate that the cell contents of bacteria do not freeze at −20 C. His findings are based on the observation that light, which is dependent upon metabolic activity, is produced by luminous bacteria at this temperature.

*Mortality of Escherichia coli following a single freezing of 3 to 10 minutes at −15 C, −20 C, −30 C, −50 C, and −78 C.* The lyophile tubes containing suspensions prepared from a 19-hour culture were stored in a water bath at 8 C to 10 C until used. The freezing temperatures were maintained by carbon dioxide and methyl cellosolve and were held constant to within plus or minus

TABLE 1

*Percentage of reduction in plate counts of E. coli following a single freezing of 3 to 10 minutes at various temperatures*

Suspension of 19-hour culture in 0.5% peptone M/120 buffer mixture, pH 7.0

| TEMPERATURE | AVERAGE REDUCTION | RANGE OF REDUCTION | $\sigma$ m | $C_v$ |
|---|---|---|---|---|
| −15 C | 76.3 | 73.4–79.4 | 5.06 | 17.5 |
| −20 C | 70.0 | 64.0–76.0 | 11.60 | 25.3 |
| −30 C | 62.4 | 57.3–67.4 | 9.28 | 22.3 |
| −50 C | 60.1 | 56.8–62.5 | 4.16 | 9.5 |
| −78 C | 62.4 | 58.2–64.0 | 5.18 | 12.0 |

one degree. Initial numbers were determined by plating the contents of 4 untreated tubes in appropriate dilutions. The platings were done in triplicate. Similar untreated controls were plated after an interval of 100 minutes and after 180 minutes, the termination of the experiment. The influence of the intensity of freezing on mortality was determined by shell-freezing a set of 4 tubes at each of the various freezing temperatures. After 3 minutes in the freezing bath, rapid thawing of the first 2 tubes of each set was effected by adding buffer at room temperatures. They were then simultaneously diluted and plated in triplicate. Seven minutes later, the 2 remaining tubes of the set were similarly thawed and plated.

There was no significant change in the count of initial controls during the course of the experiment. The mean of the initial count was 381,800, the range 380,000 to 383,600, $\sigma$ m 0.09, and $C_v$ 14. The results are shown in table 1 and figure 1.

The results revealed no significant difference in the reduction in plate count of suspensions frozen at temperatures of −30 C and below. On the other hand, with increasing temperatures above −30 C the counts were sharply and progressively reduced.

It is probable that this sharp reduction in count at temperatures above −30 C is the result of a more rapid storage death at these higher temperatures, which becomes manifest within a few minutes of storage. Further data will support this interpretation. A possible explanation for the uniform mortality observed at −30 C and below is that the eutectic point of intercrystallic fluid may have been reached near −30 C which, due to the separation of the solid phases of ice and salt, removed some rapidly acting lethal influence operating at the higher freezing temperatures. This influence is presumed to be associated with concentrated solutes in the intercrystallic films into which the bacteria are extruded.

It is well recognized that as aqueous solutions freeze, the ice crystals which form are of pure water substance. This results in an increasing concentration of solutes in the unfrozen film between the crystals. Apparently all water substance behaves in this manner, even that containing only the minutest amount of foreign material. This behavior is well described by N. Ernest Dorsey (1940) in his monograph *Properties of Ordinary Water-Substance*, as follows:

> When each small volume of water crystallizes, it rejects the impurities it originally contained. Each minute crystal is surrounded by a layer of water more impure than that from which it is formed. As the crystal grows, this layer is continuously pushed out, becoming ever more impure, but always hugging the crystal until it meets a similar layer surrounding a neighboring crystal. Further growth in that direction means a thinning of the combined layer and an increase in its concentration. Thus each crystal becomes enclosed in a material having a lower melting-point than itself. Diffusion will, of course, tend to equalize at each instant the concentration of the impurities throughout the volume that is still liquid; and adsorption may tend to retain at the crystal surface more of one type of impurity than of another.
>
> The actual existence of an intercrystallic material differing in properties from the crystals is conclusively shown by the fact that melting always begins at the boundaries between the crystals. Furthermore, E. K. Plyler observed that infrared radiation is much more strongly absorbed in the region between the crystals than in the crystals themselves; in some cases the boundary region was found to be less than 8 $\mu$ (0.0008 cm) thick. Ice cleaves most readily between crystals, that is, along the cell walls.
>
> The purer the water, the smaller is the amount of intercrystallic material, but that one is ever justified in considering that material as negligible seems improbable, especially when one realizes that the action of this material in separating the crystals is a surface phenomenon, and recalls how minute an amount of an impurity may suffice to produce a profound change in the properties of a surface.

Whether intercrystallic films would become thin enough to exert a crushing action upon bacteria or remain thick enough to exert no crushing action appears difficult to predict from existing data. However, it is quite certain that bacteria in aqueous suspension frozen at temperatures above the eutectic point of the intercrystallic material would be greatly affected by the concentrated solutes surrounding them in the intercrystallic film. It is also to be expected that the rate of action of any resulting lethal effect of concentrated solutes would be greatly influenced by temperature. This could explain the higher mortality noted at −15 C than at −20 C even though the solutes in the intercrystallic film may have been more concentrated at the latter temperature.

The indications are that the effect of intercrystallic solutes is only one of

the lethal influences operating in frozen suspensions of bacteria since further data shown in tables 5 and 6 reveal that a markedly altered storage death occurs at temperatures of −30 C and below, which is also a function of both time and temperature.

*Mortality of E. coli following a single freezing of 2 minutes at −12 C, −30 C, −78 C, and −195 C.* A second experiment similar to the first was performed by freezing a washed suspension from a 19.5-hour agar slope culture at −12 C,

TABLE 2

*Percentage of reduction in plate counts of E. coli following a single freezing of 2 minutes at various temperatures*

Suspension of 19.5-hour culture in 0.5% peptone at pH 7.0

| TEMPERATURE | AVERAGE REDUCTION | RANGE OF REDUCTION | σ m | $C_v$ |
|---|---|---|---|---|
| −12 C | 62.50 | 56.0–59.0 | 2.04 | 7.1 |
| −30 C | 52.60 | 41.7–63.5 | 7.35 | 20.4 |
| −78 C | 52.65 | 44.2–61.1 | 2.47 | 6.1 |
| −195 C | 52.10 | 42.6–61.6 | 4.65 | 12.7 |

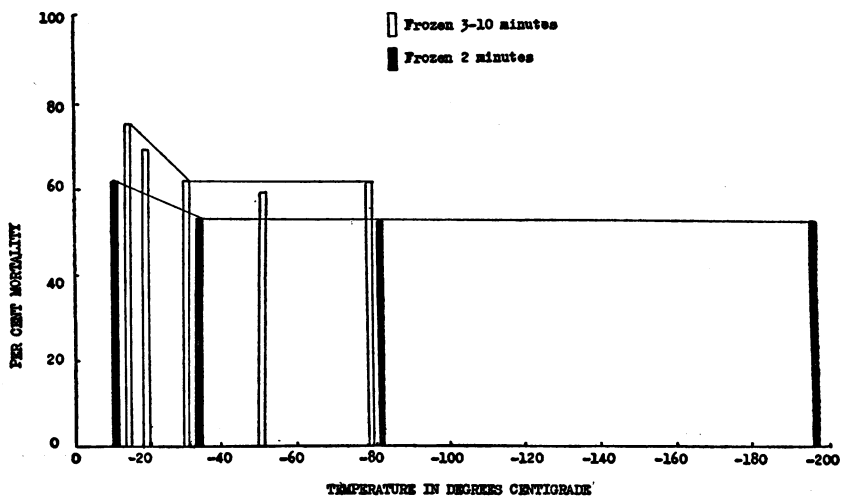


FIG. 1. THE EFFECT OF THE INTENSITY OF THE FREEZING TEMPERATURE ON THE MORTALITY OF ESCHERICHIA COLI

−30 C, −78 C, and −195 C. The first three temperatures were again maintained by the use of methyl cellosolve and carbon dioxide and the latter by use of liquid nitrogen. The procedure differed from that of the first experiment with respect to two items, the suspending menstrum and the time frozen. The suspending menstrum was 0.5 per cent peptone, instead of the peptone buffer mixture. Suspensions in the 4 tubes in each group were shell-frozen (2 at a time) and allowed to remain in the freezing bath for 2 minutes. They were then removed and simultaneously thawed, diluted, and plated in triplicate. Initial and final stored controls were plated as in the preceding experiment. The results are given in table 2 and figure 1.

The control plate count at the beginning of the experiment ranged from 350,000 to 400,000, $\sigma$ m 1.23, and $C_v$ 14, and the final count on the stored controls at the end of the experiment was 300,000 to 344,000 per ml, $\sigma$ m 1.04, and $C_v$ 14. The average of the means was 349,000. This experiment corroborates the results of the previous experiment. The mortality, which is lower than in the first experiment, is undoubtedly due to the shorter freezing period used.

*Mortality of E. coli following a single freezing of 2 minutes at −2 C, −5 C, and −10 C.* A third experiment, similar to the second, was conducted. All conditions were similar except that 8 tubes were used in each treatment and platings were in quadruplicate. The tubes were also supercooled for 2 to 3 minutes before freezing and the freezing induced by the introduction of a small ice-coated glass bead chilled to −12 C.

The control plate count at the beginning of the experiment ranged from 612,000 to 643,760 per ml, $\sigma$ m 7.92, and $C_v$ 6.2; and the final count of the stored controls at the end of the experiment ranged from 600,000 to 620,000 per ml, $\sigma$ m 10.52, and $C_v$ 8.6. The results of this experiment are given in table 3.

TABLE 3

*Percentage of reduction in plate counts of E. coli following a single freezing of 2 minutes at various temperatures*

Suspension of 19.5-hour culture in 0.5% peptone at pH 7.0

| TEMPERATURE | AVERAGE REDUCTION | RANGE OF REDUCTION | $\sigma$ m | $C_v$ |
|---|---|---|---|---|
| −2 C | 15.79 | 12.51–19.07 | 10.30 | 10.90 |
| −5 C | 57.69 | 55.87–59.51 | 5.71 | 12.10 |
| −10 C | 54.73 | 52.58–56.88 | 6.75 | 13.30 |

The small difference in mortality observed in the samples frozen at −5 C and −10 C was in all probability due to a slightly higher storage death at −5 C. The very low mortality at −2 C is striking and is due to the fact (as shown in later experiments) that ice formation was not completed at this temperature in the time interval used, 2 minutes.

The evidence gained from these three experiments shows that immediate death due to freezing does not vary with the temperature of freezing. This strongly supports the theory that ice does not form within bacterial cells.

## THE INFLUENCE OF REPEATED FREEZING UPON THE MORTALITY OF BACTERIA IN SUSPENSION

Repeated freezing of suspensions of bacteria is generally regarded to exert a greater lethal action than a single freezing. Weiser and Lief (1940) found that when repeated freezing and thawing is rapidly carried out at −78 C and 20 C death proceeds logarithmically. There appears to be no good quantitative data on a comparison of the effects of repeated freezing at various freezing temperatures. In order to gain evidence on this point, an experiment was conducted in which suspensions of bacteria were repeatedly frozen at the two temperatures, −20 C and −78 C.

*The influence of repeated freezing at −20 C and −78 C on the mortality of E. coli.* The tubes were alternately placed in freezing baths for 2½ minutes and in the thawing bath at 20 C for only the minimum time necessary for thawing. Hence the temperatures of the suspensions during thawing probably never rose much above 0 C. Five sets of 6 tubes each were used. They included initial unfrozen controls, controls stored at −78 C, controls stored at −20 C, treated suspensions repeatedly frozen at −20 C, and treated suspensions repeatedly frozen at −78 C. Pairs of tubes from the sets subjected to repeated freezing were plated after each 2 successive freezings. The plate count of the initial controls ranged from 396,250 to 405,990, $\sigma$ m 4.87, and $C_v$ 6.04. The average percentage of mortality of suspensions stored at −78 C was 54.08, the range 52.53 to 55.63, $\sigma$ m 3.13, and $C_v$ 7.0. The average percentage of mortality of suspensions stored at −20 C was 67.2, the range 64.94 to 69.46, $\sigma$ m 4.48, and $C_v$ 13.8. The mortalities in the treated tubes are given in table 4.

TABLE 4

*Percentage of reduction in plate counts of E. coli following repeated freezing at −20 C and at −78 C*

Suspension of 22-hour culture in 1% peptone, pH 7.0

| TEMPERATURE | NUMBER OF FREEZINGS | | | | | |
|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 12 |
| −20 C | 83.1 | 95.7 | 99.1 | 99.6 | 99.7 | 100 |
| −78 C | 73.7 | 92.3 | 98.4 | 98.8 | 99.4 | 99.8 |

Although the numbers of tubes plated at each interval were insufficient to permit the construction of precisely accurate death curves, the results clearly show that repeated freezing exerts a much greater lethal action than a single freezing and storage for a similar interval. They also indicate that death may be somewhat greater following repeated freezing at −20 C than at −78 C. This greater death at −20 C is probably due to a high storage death rate at this temperature rather than to any difference in the immediate lethal effects of freezing at the two temperatures.

## THE INFLUENCE OF REPEATED FLUCTUATIONS OF TEMPERATURE WITHIN VARIOUS SUBFREEZING RANGES UPON THE MORTALITY OF BACTERIA IN SUSPENSION

It was previously stated that any lethal action which might result from a physical change at a given temperature would be expected to be greatly accentuated by repeated passage through that temperature range. For example, if intracellular ice were to form and exert injury only at −30 C, it would be expected that repeated fluctuations of temperature between −30 C and some higher temperature at which such intracellular ice would thaw (but the extracellular menstrum remain frozen) would result in greater mortality than a single passage through this or other ranges. If the lethal effect of freezing were due to several causes it is even more probable that only by repeated ex-

posure to any particular injurious physical change would injury become sufficient to produce a significantly greater mortality than a single exposure. Accordingly, experiments were planned in which frozen suspensions of bacteria were subjected to repeated alternating changes in temperatures over various low-temperature ranges at which the menstrum remained frozen.

*The influence of repeated fluctuation of temperature on the mortality of E. coli at subfreezing ranges of −5 C to −15 C, −15 C to −30 C, and −30 C to −78 C.* An ice-salt mixture was used for the −5 C bath. Dry ice and methyl cellosolve were used for the −15 C, −30 C, and −78 C baths. Four tubes which served as stored controls were shell-frozen and stored at each of the 4 temperatures. The controls stored at −5 C were frozen momentarily at −15 C and immediately transferred to the −5 C bath. The other storage controls were frozen at the temperatures at which they were stored. A set of 6 of the fluctuated tubes were shell-frozen at −15 C and a set of 3 at −30 C. Three of the former were

TABLE 5

*Percentage of reduction in plate counts of E. coli following repeated fluctuations at the subfreezing ranges of −5 C to −15 C, −15 C to −30 C, and −30 C to −78 C*

Suspension of 20.5-hour culture in 1.0% peptone, pH 7.0

| TREATMENT | TIME | NUMBER OF FLUCTUATIONS | AVERAGE REDUCTION | RANGE OF REDUCTION | $\sigma$ m | $C_v$ |
|---|---|---|---|---|---|---|
| | *min* | | | | | |
| Stored at −5 C | 90–150 | 0 | 94.0 | 94.8–95.3 | 1.20 | 27.5 |
| Stored at −15 C | 90–150 | 0 | 88.4 | 86.0–90.7 | 2.90 | 22.7 |
| Stored at −30 C | 90–150 | 0 | 64.8 | 59.7–67.6 | 4.26 | 12.2 |
| Stored at −78 C | 90–150 | 0 | 53.2 | 44.2–59.5 | 9.10 | 17.3 |
| Fluctuated at − 5 C to −15 C | 120–135 | 22 | 93.4 | 91.6–94.8 | 1.98 | 26.6 |
| Fluctuated at −15 C to −30 C | 120–135 | 23 | 75.8 | 73.3–78.4 | 1.75 | 6.7 |
| Fluctuated at −30 C to −78 C | 120–135 | 23 | 38.6 | 31.6–44.7 | 4.20 | 6.3 |

fluctuated between −5 C and −15 C, and 3 between −15 C and −30 C. The second set of 3 tubes were fluctuated between −30 C and −78 C. All fluctuated tubes were allowed to remain at one extreme of the temperature range for 5 minutes and then transferred to the other extreme for 5 minutes. This was repeated for 22 fluctuations. Following treatment the tubes were thawed rapidly by the addition of buffer, diluted, and plated in triplicate. The procedure was conducted on pairs of tubes in an alternating sequence so that all sets underwent uniform treatment with respect to time and plating. The controls were plated at the beginning and at the end of the experiment.

The average initial count of untreated control tubes was 336,000 per ml, the range 314,000 to 358,000 per ml, $\sigma$ m 1.11, and $C_v$ 10. The results are given in table 5.

The mortality values for the storage tubes showed that the mortality became progressively greater with increasing temperatures of storage. Following storage at the high extreme of −5 C the reduction in count was 94 per cent, whereas at the low extreme of −78 C the reduction was 53 per cent.

The reduction in count for the fluctuated tubes, with but one significant exception, showed values lying in each case between the values of the stored controls at each extreme of the fluctuated range. The one unexpected irregularity was in the case of the −30 C to −78 C fluctuated tubes which showed a lower mortality than either of the control sets of tubes stored at −30 C and −78 C.

Otherwise, the results of the experiment are as would be expected provided no marked injurious influence due to changes in the physical state were to occur at the various ranges, and indicate that, among the various possible changes, intracellular ice does not form with fluctuation within these ranges of temperature in sufficient quantity to produce lethal effects.

TABLE 6

*Percentage of reduction in plate counts of E. coli following repeated fluctuations at the subfreezing ranges of −1.5 C to −15 C, −15 C to −30 C, −30 C to −78 C, and −78 C to −195 C*

Suspension of 15-hour culture in 1.0% peptone, pH 7.0

| TREATMENT | TIME | NUMBER OF FLUCTUATIONS | AVERAGE REDUCTION | RANGE OF REDUCTION | $\sigma$ m | $C_v$ |
|---|---|---|---|---|---|---|
| | *min.* | | | | | |
| Stored at −1.5 C | 180–210 | 0 | 99.3 | | | |
| Stored at −15 C | 180–225 | 0 | 85.2 | 82.7–87.4 | 3.02 | 30.5 |
| Stored at −30 C | 225–270 | 0 | 55.3 | 51.4–59.1 | 3.47 | 10.2 |
| Stored at −78 C | 270–315 | 0 | 47.6 | 42.8–51.6 | 3.76 | 9.4 |
| Stored at −195 C | 285–330 | 0 | 39.7 | 34.7–44.4 | 3.91 | 8.5 |
| Fluctuated at −1.5 C to −15 C | 180 | 35 | 98.4 | 98.1–98.7 | 0.67 | 26.9 |
| Fluctuated at −15 C to −30 C | 220 | 44 | 57.0 | 52.8–61.1 | 3.96 | 12.1 |
| Fluctuated at −30 C to −78 C | 220 | 46 | 41.5 | 37.1–45.8 | 3.24 | 7.3 |
| Fluctuated at −78 C to −195 C | 220 | 46 | 50.8 | 45.7–55.8 | 5.06 | 13.3 |

*The influence of repeated fluctuation of temperature on the mortality of E. coli at the subfreezing ranges of −1.5 C to −15 C, −15 C to −30 C, −30 C to −78 C, and −78 C to −195 C.* An experiment similar to that reported above was conducted in which 4 tubes were stored at each of the following temperatures, −1.5 C, −30 C, −78 C, and −195 C, and 6 tubes at −15 C. Additional sets of 4 tubes were used for each of the fluctuation ranges. The −1.5 C bath was an ice-salt bath maintained at −1.5 C to −2 C. The temperatures of the baths at −15 C, −30 C, and −78 C were maintained by dry ice and methyl cellosolve and did not vary more than plus or minus 2 C. The fluctuation of 4 tubes at each temperature range and the diluting and plating procedures were similar to those of the preceding experiment except that 5 plates were made of each dilution. The tubes stored at −1.5 C were frozen at −15 C just before storage. The contents of all other tubes were frozen at the temperature of storage.

The average initial count in untreated control tubes was 340,000 per ml, the range 327,000 to 354,000 per ml, $\sigma$ m 6.87, and $C_v$ 6.96. Calculations were made as in the previous experiment. The results are given in table 6 and figure

2. The results of this experiment are similar to those of the preceding experiment. However, the two experiments are not exactly comparable, since in the latter the culture was several hours younger and the time intervals employed were longer.

The mortality curve for the stored tubes as seen in figure 2 shows a flattening at −30 C which indicates that an additional lethal effect operated at temperatures above −30 C which was not active at lower temperatures. Since the previous experiments on the intensity of the freezing temperature showed that a uniform mortality due to immediate freezing injury occurs at −30 C and

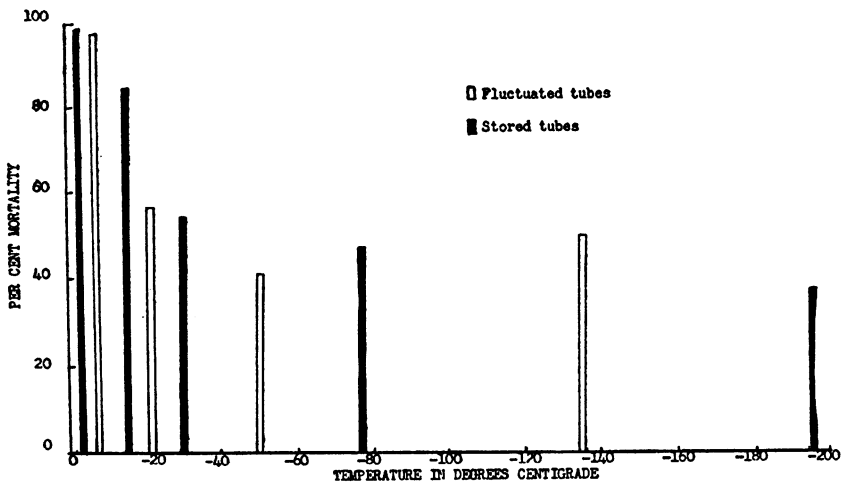


FIG. 2. THE EFFECT OF REPEATED FLUCTUATION THROUGH VARIOUS SUBFREEZING TEMPERATURE RANGES ON THE MORTALITY OF ESCHERICHIA COLI

below, the results of the present experiment, which show a markedly greater mortality in the tubes stored at −78 C than in those stored at −195 C, clearly establish that a storage death occurs at −78 C. This observation was confirmed and extended in subsequent experiments (see tables 8 and 9).

The mortalities in tubes stored at −78 C, and especially at −195 C, are considerably lower (table 6) than those observed in other experiments (tables 2 and 8) and indicate that the 15-hour cultures may be more resistant to freezing than the older cultures. The mortality in the tubes fluctuated between −78 C and −195 C is a little higher than that of the tubes stored at −78 C. However, the ranges overlap markedly and there is doubt that the difference is significant.

The mortality in the tubes fluctuated between −30 C and −78 C in this second experiment was again lower than the mortality in either of the control sets stored at these temperatures. In order to maker certain that this unexpected finding was correct, still another experiment in which suspensions of *E. coli* were fluctuated over this range of temperature was conducted. The procedure was essentially identical with that of the previous experiments. The age of the culture used was 19.5 hours. Four sets of 8 tubes each served as

initial untreated controls, controls stored at −30 C, controls stored at −78 C, and fluctuated tubes, respectively. The results are presented in table 7.

There is no doubt concerning the significance of these results, which confirm those of the two preceding experiments. They strongly suggest that either there must exist some point or range of temperature between −30 C and −78 C at which the storage death rate is lower than at these extremes or that the "dynamics" of the temperature fluctuation afford some obscure protective influence. A subsequent experiment (table 9) supports the latter explanation. This unexpected finding is the subject of further investigation.

*The influence of repeated fluctuation of temperature on the mortality of E. coli at the subfreezing range of −2 C to −195 C.* Since in the preceding experiments temperature fluctuations over the narrow ranges employed may conceivably have failed to include both the point at which a physical change may occur and the point at which it may be reversed, another experiment was conducted in

TABLE 7

*Percentage of reduction in plate counts of E. coli following repeated fluctuations at the subfreezing range of −30 C to −78 C*

Suspension of 19.5-hour culture in 1.0% peptone, pH 7.0

| TREATMENT | TIME | NUMBER OF FLUCTUATIONS | AVERAGE REDUCTION | RANGE OF REDUCTION | σ m | $C_V$ |
|---|---|---|---|---|---|---|
| | *min* | | | | | |
| Stored at −30 C..... | 140–220 | 0 | 72.7 | 70.3–75.0 | 1.56 | 12.6 |
| Stored at −78 C..... | 140–220 | 0 | 60.4 | 56.9–63.6 | 2.18 | 11.7 |
| *Fluctuated at −30 C to −78 C... | 140 | 27 | 50.2 | 46.1–54.1 | 2.52 | 11.1 |

* Fluctuated at 5-minute intervals for 140 minutes, then stored at −78 C until plated.

which the temperature fluctuation range was extended to cover the entire range from −2 C to −195 C.

A suspension of *E. coli* in 0.5 per cent peptone was prepared from 18-hour agar slope cultures in the same manner as in the previous experiment. Six of a total of 26 tubes were frozen and stored at −195 C, 4 at −20 C, and 10 at −2 C to serve as stored controls. The remaining 6 tubes were frozen at −195 C and fluctuated 26 times between −2 C and −195 C at 5-minute intervals. The temperature of the −2 C bath was maintained between −2 C and −3 C with an ice-salt mixture. The tubes stored at −2 C were induced to freeze at −17 C and immediately transferred to the storage temperature. The tubes stored at −20 C and −195 C were placed directly in their respective storage baths to freeze. All tubes were thawed rapidly by the addition of buffer.

The average percentage mortality of controls stored at −2 C was 97.5; of controls stored at −20 C, 72.7; of controls stored at −195 C, 53.1; and of the fluctuated tubes, 89.3. The results were statistically significant, and, as in the previous experiments, the average mortality value in the fluctuated tubes was found to be between those of the tubes stored at the extremes of the temperature range. Consequently, the evidence gained from these experiments in-

dicates that there is no point or range of temperature between −2 C and −195 C at which physical change (such as the formation of intracellular ice) occurs to produce immediate injury in frozen suspensions of bacteria.

*Mortality of E. coli following storage at −195 C for 10 hours.* In order to determine whether a storage death can be detected at −195 C, an experiment was conducted using 4 sets of 10 tubes each made up of initial unfrozen controls, controls frozen at −195 C and immediately thawed and plated, tubes stored at −195 C for 5 hours, and tubes stored at −195 C for 10 hours. The plate

TABLE 8

*Percentage of reduction in plate counts of E. coli following storage at −195 C for 5-hour and 10-hour intervals*

Suspension of 20-hour culture in 1.0% peptone, pH 7.0

| TREATMENT | AVERAGE REDUCTION | RANGE OF REDUCTION | $\sigma$ m | $C_v$ |
|---|---|---|---|---|
| Controls frozen at −195 C and immediately thawed | 55.10 | 53.91–56.29 | 5.54 | 8.40 |
| Stored 5 hours at −195 C | 56.06 | 45.82–66.30 | 4.75 | 7.35 |
| Stored 10 hours at −195 C | 57.65 | 47.00–68.35 | 4.94 | 7.90 |

TABLE 9

*Percentage of reduction in plate counts of E. coli following storage at −30 C, −45 C, −60 C, and −78 C*

Suspension of 20-hour culture in 1.0% peptone, pH 7.0

| TREATMENT | AVERAGE STORAGE PERIOD | AVERAGE REDUCTION | RANGE OF REDUCTION | $\sigma$ m | $C_v$ |
|---|---|---|---|---|---|
| | *min* | | | | |
| Control frozen at −78 C and immediately thawed | | 49.74 | 47.95–51.53 | 4.18 | 9.90 |
| Stored at −30 C | 157.5 | 68.29 | 67.30–69.28 | 2.32 | 8.70 |
| Stored at −45 C | 157.5 | 63.02 | 61.72–64.32 | 3.00 | 9.61 |
| Stored at −60 C | 157.5 | 58.50 | 57.20–59.80 | 3.00 | 8.30 |
| Stored at −78 C | 157.5 | 52.59 | 51.15–54.03 | 3.37 | 8.54 |

count of the initial unfrozen controls was 929,800, the range 924,100 to 935,500, $\sigma$ m 5.73, and $C_v$ 3.2. The results are given in table 8.

The reduction in count of the control suspensions frozen and immediately thawed is almost identical with that following storage for 5 and 10 hours. The ranges overlap so completely that the differences observed are within experimental error and establish the fact that if storage death does occur at −195 C it is extremely slow and not measurable within the periods of storage used.

*Mortality of E. coli following storage at −30 C, −45 C, −60 C, and −78 C.* This experiment was conducted to measure storage death at temperatures of −30 C to −78 C, and to ascertain whether storage death at these temperatures is directly proportional to temperature throughout the range.

Six sets of 8 tubes each were employed and comprised the initial unfrozen controls, controls frozen at −78 C and immediately thawed and plated, and treated sets stored at −30 C, −45 C, −60 C, and −78 C. The times stored ranged from 110 to 205 minutes, with an average of 157.5 minutes. The plate count of the initial unfrozen controls ranged from 452,890 to 480,600, $\sigma$ m 6.93, and $C_v$ 8.3. The results are shown in table 9.

The results show that a slow storage death occurs throughout the temperature range of −30 C to −78 C. The rate of storage death which is greatest at −30 C is progressively and markedly reduced with reduction in storage temperature. At −78 C the death rate is only 15 per cent of that at −30 C.

### A COMPARISON OF THE INFLUENCE OF SHORT AND PROLONGED PERIODS OF FREEZING AND SUPERCOOLING AT VARIOUS TEMPERATURES ON THE MORTALITY OF E. COLI IN SUSPENSION

One of the most certain means of determining whether changes in the physical state associated with freezing of the suspending menstrum exert lethal effects, exclusive of temperature effects per se, is that of comparing the mortality of bacteria in suspensions subjected to supercooling and to freezing at a given temperature.

There are very few reports of such comparisons in the literature and most of these are neither extensive nor exacting. Furthermore, the times involved have been so prolonged that the secondary effects of storage death are likely to have obscured immediate lethal effects. Prudden (1887) compared the mortality of *Staphylococcus aureus* and other organisms stored in water suspensions at 15 F to 28 F in both frozen and supercooled states. He came to the conclusion that death was more rapid in the supercooled suspensions.

Sedgwick and Winslow (1902) compared death rates in tap water suspensions of *Bacillus typhosus* (*Eberthella typhosa*) held frozen at near 0 C with similar suspensions held at 1 C. The reduction in count after a storage period of 3 hours was very nearly the same following either treatment, which led the authors to conclude: "Evidently there is nothing mysterious about the act of freezing, no mechanical crushing of bacteria, the process of destruction is continuous above and below the freezing point, depending upon the two main factors of time and temperature."

Hilliard and Davis (1918) compared death rates of *E. coli* frozen in water with those of suspensions of the same organism in undercooled glucose solutions. These authors concluded that the formation of ice exerts a lethal effect greater than that of undercooling.

*A comparison of freezing with supercooling on the mortality of E. coli stored at −3 C for 2 minutes and for 90 to 120 minutes.* The suspension of organisms was distributed into each of 34 lyophile tubes, which were then stored in a water bath at 3 C until used.

Twelve of the tubes were promptly removed and placed in an ice-salt bath maintained between −3 C and −3.5 C and allowed 3 minutes to attain the temperature of the bath. Freezing was then induced in 6 of the 12 tubes by

introducing a small sterile glass bead coated with ice. These glass beads had been previously moistened, allowed to freeze at −20 C, and then stored in a bath at −3 C. Thus, they caused no change in temperature when introduced into the supercooled tubes other than that which, of course, accompanies the formation of ice. The remaining 6 unfrozen suspensions served as the supercooled tubes. Both frozen and supercooled suspensions were stored for 90 to 120 minutes. They were then removed from the bath in pairs (1 supercooled sample and 1 frozen sample) and simultaneously diluted with buffer and plated in quadruplicate.

The effects of rapid freezing and supercooling were also investigated by supercooling pairs of tubes to −3 C for 3 minutes and then inducing one to freeze by the addition of an ice-covered bead. The entire mass was observed to solidify the instant the bead reached the suspension. It was at first glassy in appearance but after about 1 minute became milky, signifying a more complete freezing.

TABLE 10

*A comparison of freezing with supercooling on the percentage of reduction in plate counts of E. coli stored at −3 C for 2 minutes, and for 90 to 120 minutes*

Suspension of 16-hour culture in 1.0% peptone, pH 7.0

| TREATMENT | TIME | AVERAGE REDUCTION | RANGE OF REDUCTION | $\sigma$ m | $C_v$ |
|---|---|---|---|---|---|
| | *min* | | | | |
| Briefly supercooled..... | 5 | 3.8 | 0–9.87 | 7.28 | 8.0 |
| Briefly frozen..... | * | 43.3 | 37.2–50.0 | 9.30 | 14.1 |
| Stored supercooled..... | 90–120 | 2.6 | 0–8.95 | 9.40 | 9.0 |
| Stored frozen..... | 90–120 | 98.2 | 97.7–98.6 | 0.84 | 42.4 |

* Supercooled 3 minutes, then frozen 2 minutes.

Two minutes following the introduction of the bead the tubes were removed from the bath and buffer quickly added. This served to thaw the contents of the frozen tube within 2 to 3 seconds. Six pairs of tubes were treated in this manner.

The untreated controls stored at 3 C were plated in quadruplicate as follows: 4 at the beginning of the experiment, 2 after an interval of 95 minutes, and 4 at the termination of the experiment at 140 minutes. The results of the experiment are given in table 10.

Counts of the controls stored at 3 C revealed no significant change in numbers during the period of the experiment. The average plate count of these controls was 466,000 per ml, the range 450,000 to 481,000, $\sigma$ m 1.55, and $C_v$ 9.6.

The results demonstrate that supercooling to −3 C for either short or extended periods does not cause any significant reduction in numbers over that of controls stored at 3 C. On the other hand, suspensions subjected to freezing at −3 C for as short a period as 2 minutes showed a marked mortality. Even following storage for 120 minutes no reduction in count was evident in the supercooled samples, whereas the reduction in count in the frozen samples was marked.

There was considerable variation in the stored frozen samples, so that the values given for the percentage of mortality for this group lacks close accuracy. However, the reduction in the stored frozen samples is so much greater than in the supercooled samples that there is not the slightest doubt that the differences are significant.

These results show that storage of organisms in a frozen menstrum, at −3 C (where presumably no intracellular ice should form), results in a much greater mortality than storage in the supercooled state at the same temperature. They do not establish that any killing necessarily resulted from the immediate effects of freezing. In fact, the storage death as indicated by these and previous experiments was so rapid that, in a matter of minutes or even seconds, sufficient reduction in numbers resulting from storage death may conceivably have taken place to account for all of the killing observed.

TABLE 11

*A comparison of freezing with supercooling as to the percentage of reduction in plate counts of E. coli stored at −9 C for 2 minutes and for 110 to 150 minutes*

Suspension of 19.5-hour culture in 1.0% peptone, pH 7.0

| TREATMENT | TIME | AVERAGE REDUCTION | RANGE OF REDUCTION | $\sigma$ m | $C_v$ |
|---|---|---|---|---|---|
| | *min* | | | | |
| Briefly supercooled..... | 5 | 4.7 | 0–11.3 | 5.58 | 5.9 |
| Briefly frozen..... | * | 78.6 | 72.6–84.0 | 11.54 | 53.0 |
| Stored supercooled..... | 110–150 | 4.7 | 0–11.8 | 6.72 | 7.1 |
| Stored frozen..... | 110–150 | 96.0 | 95.4–96.7 | 1.10 | 28.2 |

* Supercooled 3 minutes, then frozen 2 minutes.

*A comparison of freezing with supercooling on the mortality of E. coli stored at −9 C for 2 minutes and for 110 to 150 minutes.* This experiment was similar to the preceding experiment except that a temperature of −9 C to −9.5 C was employed and the beads used were held at −9 C. The suspension of organisms was distributed into 30 lyophile tubes which were then stored at 8 C until used. The tubes included in the experiment were 4 untreated controls plated at the beginning of the experiment, 6 stored frozen, 6 stored supercooled, 6 supercooled for 5 minutes, 6 supercooled 3 minutes and frozen for 2 minutes, and 2 untreated controls plated at the termination of the experiment. They were all plated in quadruplicate in an orderly paired sequence.

The average count of the untreated controls was 489,000 per ml, the range 466,500 to 513,000, $\sigma$ m 2.24, and $C_v$ 13.13. The results are given in table 11.

The results of this experiment, in which a lower temperature was used, were similar to those of the preceding experiment. The supercooled suspensions showed no appreciable reduction in numbers after short or long periods of storage, whereas frozen suspensions held at the same temperature for the same lengths of time showed marked death with storage. The variation between tubes was greater in the frozen than in the supercooled samples, which was probably due,

in part at least, to the smaller numbers of surviving organisms and the variation in freezing conditions. Nevertheless, the differences are statistically significant. The results of this experiment cannot be compared directly with those of the previous experiment because of a difference in age of the cultures. However, it is likely that the low mortality observed at −3 C as compared to that at −9 C was due to the fact that ice formation was not completed at −3 C in the 2-minute period used. From these early experiments it became evident that, in order to detect any possible immediate lethal effect accompanying ice formation, determinations were necessary using shorter time intervals. Although freezing appeared to be initiated almost instantaneously in the suspensions which were frozen at −9 C, under the conditions of the foregoing experiment, ice formation was only completed after an interval judged visually to be some 20 to 40 seconds. This delay in the completion of crystallization results from the heat of crystallization, which tends to raise the temperature. This occurs even though the materials are supercooled and the transfer of heat from such small samples is rapid. It appeared desirable to study death both during and after the completion of the formation of ice in order to determine whether any accompanying changes in death rate take place. If the theory of the mechanical effect of extracellular ice is correct, there should be a sharp temporary increase in death rate as ice formation is being completed and the intercrystallic film becomes so reduced in thickness that pressure is exerted upon the cells.

*A comparison of freezing with supercooling on the mortality of E. coli stored at −9 C for 3 to 4 seconds.* At the conclusion of the preceding experiment, 3 samples were maintained at −9 C for 3 minutes, at which time the contents were frozen by introducing an ice-covered bead. As soon as ice was seen to form (and this occurred almost the instant the bead reached the supercooled suspension) the contents were thawed by rapidly adding buffer, at 20 C, with a capillary pipette. Thawing occurred in less than 3 to 4 seconds after the freezing had taken place. The contents were then diluted and plated in quadruplicate. Two tubes were supercooled for 3 minutes to serve as controls. Although the mass in the frozen tubes appeared solid throughout, it was still glassy when buffer was added, so that freezing was obviously incomplete.

The results showed that no significant lethal action had taken place in these frozen suspensions in spite of the fact that a rather large amount of ice had formed. The average mortality in the supercooled samples was 6.96 per cent, and in the frozen samples 8.45 per cent. Although the data are limited they indicate that neither the partial formation of ice nor the associated physical change exerts any appreciable instantaneous lethal action.

*A comparison of freezing with supercooling on the mortality of E. coli stored at −9 C for 10 seconds.* The procedures used in this experiment were similar to those of the preceding trial except that the time of freezing was increased to 10 seconds.

The suspension of organisms was distributed in 0.05-ml amounts into 34 lyophile tubes which were placed in a water bath at 8 C until used. Ten of these samples served as untreated controls and were plated as follows: 4 initially,

2 after 70 minutes, and 4 at the end of the experiment after 135 minutes. The treated samples included 12 supercooled and 12 frozen. The temperature of the bath was maintained between −9 C and −10.5 C. Pairs of the treated samples were placed in the bath for 3 minutes. Freezing was then induced in one of the samples by the introduction of an ice-coated bead chilled to −14 C to −15 C. Exactly 10 seconds later (as timed by a stop watch) the tubes were removed from the bath. Buffer was rapidly added and agitated, which always resulted in the thawing of the frozen material within 2 seconds. At the time the buffer was added the ice mass was still glassy, indicating that freezing was incomplete. The results of the experiment are shown in table 12.

The average mortality in the supercooled samples was 1.1 per cent, and in the frozen samples 5.78 per cent. This remarkably low mortality in the frozen samples is similar to that observed in the previous trial and confirms the observation that no marked immediate lethal action is exerted during the early stages of ice formation.

TABLE 12

*A comparison of freezing with supercooling as to the percentage of reduction in plate counts of E. coli stored at −9 C for 10 seconds*

Suspension of 18.5-hour culture in 1.0% peptone, pH 7.0

| TREATMENT | TIME | AVERAGE REDUCTION | RANGE OF REDUCTION | $\sigma$ m | $C_v$ |
|---|---|---|---|---|---|
| | *sec* | | | | |
| Supercooled | 190 | 1.10 | 0–6.9 | 2.76 | 7.6 |
| Frozen | * | 5.80 | 0–12.0 | 2.45 | 7.1 |

* Supercooled for 180 seconds, then frozen for 10 seconds.

*A comparison of freezing with supercooling on the mortality of E. coli stored at −9 C for 20, 40, and 80 seconds.* The procedures used in this experiment were similar to those of the preceding experiments except for the treatment periods employed.

The suspension of organisms was distributed in 0.05-ml amounts into 40 lyophile tubes which were then placed in a water bath at 8 C until used. Eight of these served as untreated controls and were plated as follows: 4 initially, 2 after 100 minutes, and 2 at the end of the experiment after 165 minutes. The treated samples included 8 supercooled, and 3 sets of 8 each, frozen for 20, 40, and 80 seconds, respectively. The temperature was maintained between −9 C and −10 C. The supercooled samples were placed in the bath for 260 seconds prior to plating. They were supercooled and plated during the experiment as follows: 2 initially, 2 after 57 minutes, and 4 at the end of the experiment after 150 minutes.

The samples which were frozen were treated by first supercooling for 3 minutes. This was followed by the introduction of an ice-coated bead chilled to −14 C to −15 C. They were timed with a stop watch, thawed, diluted, and plated as in the previous experiment. The average count of the untreated controls

was 248,000 per ml, the range 239,000 to 257,000, $\sigma$ m 2.25, and $C_v$ 10.4. The results are given in table 13 and figure 3.

In the process of freezing (following the introduction of the ice-coated bead) it was observed that the ice mass was at first glassy. In about 20 seconds

TABLE 13

*A comparison of freezing with supercooling as to the percentage of reduction in plate counts of E. coli stored at −9 C for 20, 40, and 80 seconds*

Suspension of 17.5-hour culture in 1.0% peptone, pH 7.0

| TREATMENT | TIME | AVERAGE REDUCTION | RANGE OF REDUCTION | $\sigma$ m | $C_v$ |
|---|---|---|---|---|---|
| | *sec* | | | | |
| Supercooled..... | 260 | 0 | 0–5.5 | 1.63 | 7.5 |
| Frozen*..... | 20 | 10.2 | 3.4–16.7 | 1.99 | 10.3 |
| Frozen*..... | 40 | 35.2 | 29.1–40.9 | 2.21 | 15.8 |
| Frozen*..... | 80 | 58.1 | 53.6–62.5 | 1.72 | 19.0 |

* These tubes were supercooled for 3 minutes prior to freezing.

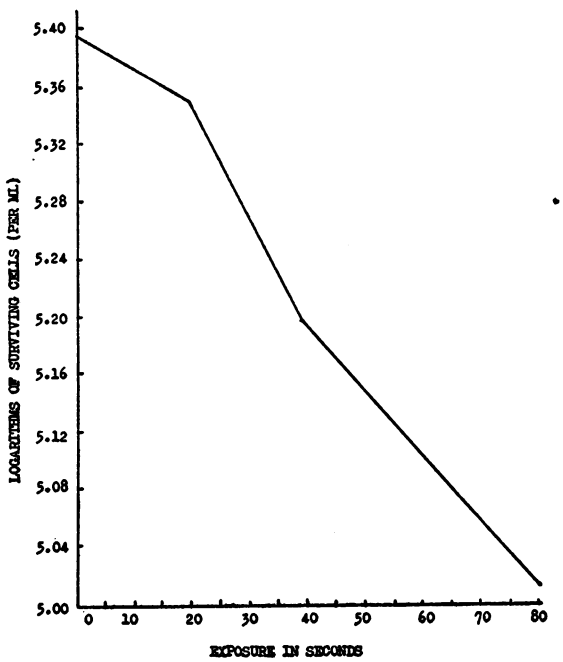


FIG. 3. THE DEATH RATE OF ESCHERICHIA COLI DURING ICE FORMATION AT −9 C

the sample began to assume a milky appearance which apparently reached its maximum at 35 seconds, giving evidence that ice formation was completed, or nearly completed, at this time. The beginning of this change in appearance probably signified the onset of the final stages of ice formation. At present we are attempting to check this by means of temperature measurements with a thermocouple.

The results show that the death rate during ice formation (figure 3) was not constant. In the early stages of ice formation, from 0 to 20 seconds while the ice mass was still glassy and freezing incomplete, the death rate was low. Between 20 and 40 seconds a marked increase in death rate occurred. This was followed by a subsequent decrease in death rate between 40 and 80 seconds. Although this decrease was not great, the ranges of the counts did not overlap and the results are statistically significant. The high rate of death observed between 20 and 40 seconds parallels the phase during which ice formation was apparently being completed and indicates that a marked lethal effect not operating at earlier or later intervals was exerted at this time.

It is rather certain that the death observed between 40 and 80 seconds (after the completion of ice formation) is largely a storage death, presumably due to conditions existing in the unfrozen intercrystallic films.

The most obvious interpretation of this is that the formation of ice, per se, accounts for the greater lethal effect observed between 20 and 40 seconds than between 40 to 80 seconds, since any indirect lethal effects, such as increased concentration of salts, should serve to maintain the death rate at a constant value during subsequent intervals. The only apparent alternative explanation which might account for the decrease in death rate after 40 seconds is that the reduction in temperature attending the completion of ice formation may decrease storage death rate as compared to that occurring at earlier intervals when the temperature is higher.

The data strongly indicate that immediate death and storage death involve different lethal factors. This concept is further supported by the observation that even the very rapid rates of freezing attained by extremely low temperatures result in mortalities essentially as high as at higher temperatures.

## DISCUSSION

The evidence presented favors the theory that ice does not form within bacterial cells. The first finding in support of this is that the intensity of the freezing temperature has no influence on immediate death due to freezing. If the formation of intracellular ice were to contribute a lethal effect it is to be expected that a sharp increase in mortality would occur when the temperature at which such ice would form was reached. The observation that marked immediate death occurs with freezing at temperatures just below 0 C, at which intracellular ice should not form, mediates against the theory of intracellular ice being the cause of death. Moreover, our observation that repeated fluctuation of temperature of frozen suspensions through ranges from $-1.5$ C to $-195$ C does not exert lethal action is further indication that ice does not form within bacterial cells, for, if it did, the repeated formation of such ice which would take place under these conditions should increase the mortality over that of stored controls. This is based on the somewhat questionable assumption that cells which survive the first exposure to low temperature would succumb to subsequent exposures due either to repeated formation of ice within each cell or to its formation in increasing numbers of cells with each fluctuation of temperature.

If intracellular ice is excluded as a lethal factor operating to produce immediate death due to freezing, there appears to be but one alternative explanation, that of the action of extracellular ice either by its direct mechanical effects or by indirect effects such as concentration of solutes.

The evidence gained from the present experiments in which the death rate during the process of ice formation was studied, as shown in figure 3, lends support to the theory of the mechanical action of extracellular ice as the principal cause of immediate death due to freezing. This is based on the observation that the most marked death occurs only during the final stages of ice formation when the intercrystallic films may presumably become so limited as no longer to accommodate the cells. This marked immediate death is apparently not due to the indirect effects of extracellular ice, such as the concentration of solutes, since in the latter case the maximum death rate reached at the completion of ice formation should continue at a constant rate. Contrary to this, the death rate was observed to diminish upon the completion of ice formation.

It has been commonly observed that the presence of certain substances in the suspending medium, such as colloids, sugar, etc., exerts a protective effect against death of bacteria by freezing. This observation also appears to conform with the theory of the destructive mechanical action of extracellular ice, the explanation of this protection being that the resulting intercrystallic films are sufficiently thick to accommodate the cells. Actually, if the freezing of such solutions is sufficiently slow, considerable unfrozen material from the intercrystallic films is extruded to form macroscopic areas of liquid in which bacteria may escape any mechanical action of extracellular ice. McFarlane (1938), in a study of the redistribution of microorganisms following slow freezing at the higher freezing temperatures, found that they become concentrated in core areas.

The fact that prolonged storage death occurs at temperatures at least as low as −78 C also favors extracellular rather than intracellular ice as being the cause of the death of bacteria in frozen menstra. The observation that a sharp decrease in storage death occurs at temperatures of −30 C and below is also in conformity with the theory of the action of extracellular ice, since it is likely that the eutectic point is reached at −30 C, which, because of the separation of ice and salt in the intercrystallic film, would remove the lethal action of concentrated solutes. If this concept is correct, the temperature or temperatures at which sharp changes in death rate may occur would depend upon the eutectic point or points of the solutes of the suspending medium. Whether storage death is the result of a single process or several processes all varying directly with temperature cannot be judged from the present data. Storage death probably begins in the very early stages of ice formation and increases in rate as the solutes in intercrystallic films become more concentrated, finally to reach a constant maximum at the completion of ice formation.

Our observation that repeated fluctuations of temperature of frozen suspensions, between −30 C and −78 C, exert a protective action was an unexpected but significant finding. Since it was shown to be dependent upon the dynamics of the temperature fluctuation, rather than upon the existence of

some intermediate temperature with a slower storage death rate, it is to be assumed that the fluctuation of temperature may affect physical processes within the cell or between the cell and its surroundings, such as gas exchange, necessary for its metabolism and continued survival. The fact that this protective effect was not noted at temperature ranges above −30 C indicates that lethal influences operate at freezing temperatures above −30 C which are not operative at −30 C and below, and lends further support to the concept that storage death at the higher freezing temperatures is due largely to the concentration of solutes in the intercrystallic film.

## SUMMARY

The purpose of this investigation was to assemble reliable quantitative data on the death of bacteria following low-temperature treatments which would be of value in elucidating the manner in which low-temperature injury is produced. The present study was limited to *Escherichia coli*. The suspending medium was 1 per cent peptone or a peptone buffer mixture at pH 7.0.

Death by freezing involves rapidly acting or "immediate" death, caused by freezing and thawing per se, and a "storage death" which is a direct function of time and temperature.

Mortality due to immediate death by freezing is marked but does not vary with the intensity of the freezing temperature.

Immediate death occurs at a brief stage in the freezing process during which extracellular ice formation is being completed.

The rate of storage death at the higher freezing temperatures is very rapid and is much greater at temperatures above −30 C than at temperatures of −30 C and below.

Repeated freezing is more lethal than a single freezing or storage in the frozen state for a similar interval of time.

Freezing is much more lethal than supercooling.

Repeated fluctuations of temperature of frozen suspensions do not exert a lethal action additional to that of storage.

Repeated fluctuations of temperature of frozen suspensions between −30 C and −78 C appear to result in a lower mortality than storage at either temperature, but this protective effect was not noted at temperature ranges above −30 C nor below −78 C.

Storage death at −195 C either does not take place or is so slow that it is difficult to detect within the storage period of ten hours studied.

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