The Mechanism of Cellular Injury by Freezing in Microorganisms*

Tokio NEI, Tadashi ARAKI and Tadao MATSUSAKA
根井外喜男　荒木　忠　松坂理夫
The Institute of Low Temperature Science
Hokkaido University, Sapporo, Japan

Abstract

The mechanism of cell injury by freezing in microorganisms was investigated from a morphological and physiological point of view.

A water suspension of *Escherichia coli* cells was frozen slowly at −25°C and rapidly at −150°C. Thin-sectioned and non-sectioned specimens prepared by freeze-drying were observed with the electron microscope. Viable cell counts were made to determine the survival rate of the cells after freeze-thawing.

The cells became extremely shrunken and showed a high survival rate in freezing at −25°C, while the other cells retained their original size and shape but contained many small cavities which have a high possibility of being previously occupied by ice in freezing at −150°C and they also showed a lower survival rate. From these results, it was assumed that the main cause of cell injury in rapid freezing might be the intracellular ice formation.

A comparison of aerobic and anaerobic cells, which are quite different in resistivity against rapid freezing, was made to account for the relationship between cell water and intracellular freezing. It was confirmed from the data which showed a high susceptibility to rapid removal of cell water that the anaerobic cells have a low permeability to water. The high possibility of intracellular ice formation and death of the anaerobic cells in freezing at −150°C can thus be explained by such a low permeability of these cells.

After freeze-thawing, no morphological change was found in the fine structure of cytoplasm or membrane of the cells, even in the specimens which previously showed intracellular freezing, regardless of the cultural conditions.

Introduction

Tiny cells such as microorganisms are, in general, more resistant to freezing than larger plant or animal cells. In microorganisms, extremely minute cells such as bacteria show a higher resistivity as compared to yeast or other larger cells. Even in such resistant cells, it has been ascertained that a certain percentage of cells are killed under certain freezing conditions. To investigate the mechanism of cellular injury or resistance in freezing microorganisms is a part of our long range research program. Studies for this aim should be done from various points of view and covered by a wide range of research.

For microorganisms, a considerable number of studies on the survival of cells subjected to low temperatures have been reported, but only a few studies on the morphology

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of the frozen cells can be found (Smith, 1961). Electron microscopy is generally used to observe the fine structure of microorganisms but there are some difficulties in electron microscopic observation of changes in such minute cells in the frozen state. This may be the cause of the scarcity of morphological investigations. Although the problem is very important, evidence as to whether or not ice crystals can be formed in bacterial cells has not yet been given. Recently, Rapatz and Luyet (1963) reported an electron microscopic study of formation of ice in bacteria.

The present experiment was carried out to examine the relationship between the morphology of *E. coli* cells, both in the frozen and after freeze-thawing, and the post-thawing survival of the cells. Since it was concluded from the results obtained that intracellular freezing might be the main cause of cell death in rapid freezing, further examinations on the factors affecting the possibility of intracellular ice formation were achieved by several means.

I. Methods

Materials. *E. coli* cells were used in the present experiment. The cells were collected from cultures on a nutrient agar or in a broth, contained in a flask with a narrow neck, which was non-aerated but shaken for obtaining a rich yield, for 6 or 24 hours, or in an aerated broth for 24 hours at 37°C and washed three times in deionized water and finally suspended in water with a concentration of 300 mg cell/ml.

Freezing and thawing. 0.02 ml of the cell suspension was spread between two copper plates or two sheets of aluminum foil of 20×25 mm. The following three types of freezing conditions were chosen as rather extremely different cooling rates: one was a slow freezing in a freezer at −25°C, and the other two were a moderately rapid freezing and very rapid freezing by abrupt immersion in iso-pentane baths previously cooled to −25 and −150°C, respectively. Those cooling rates were approximately 50, 2,000 and over 10,000°C/min. After completion of freezing at each temperature for more than 10 minutes, the specimen was thawed in water at room temperature and used for viable count.

For examination at rather slow cooling rates, 0.2 ml of the cell suspension put in a test tube was frozen to desired temperatures at a constant rate of cooling controlled by adding liquid nitrogen into the alcohol bath.

Morphological observation. For electron microscopic observations of cells in the frozen state or after freeze-thawing, various procedures were employed. Observations were made with a JEM 6 AS electron microscope.

1. Observations of the cells in the frozen state.

(A) Non-sectioned specimens. Specially devised cooling attachment incorporated into the electron microscope was used for this experiment (Nei, 1966). The specimens were sprayed directly on the grid of the specimen holder which was previously cooled to the desired temperatures in vacuum and then freeze-dried in a specimen chamber. In this type, individual cells and cell clumps were observed without sectioning.

(B) Thin-sectioned specimens. Specimens, placed between two sheets of aluminum foil, were frozen at −25 or −150°C. After completion of freezing, the aluminum foils were

separated at those temperatures and thin-layered specimens adhering to each foil sheet were dried by connecting their containers to the freeze-dryer and by keeping them at −25°C or below −50°C. The specimens thus freeze-dried were fixed with osmium tetroxide-alcohol solution and embedded in Epon. They were then thin-sectioned with a Porter-Blum ultra-microtome.

2. Observations of the cells after freeze-thawing. Specimens, sandwiched between two metal plates or placed in a test tube, frozen at −25 or −150°C and thawed at room temperature, were also used for electron microscopic observation. For the non-sectioned specimens among them, a drop of the thawed cell suspension was placed on the grid and dried in air. Other specimens were thin-sectioned by following the ordinary preparation method.

Determination of viability. The specimens, frozen in the manner described above, were rather rapidly thawed at room temperature by immersion in an adequate amount of water for dilution. The number of viable cells in the frozen and thawed specimens was determined by a dilution-plating procedure and a colony count.

Permeability of the cells and removal of cell water. In order to investigate the permeability of *E. coli* cells and the removal of cell water by freezing, physiological and morphological alterations in the cells caused by exposure to hypertonic solutions were examined. Specimens prepared by fixing in state as exposed to 1 M sucrose solution were observed with an electron microscope. From the results obtained, it was ascertained that plasmolysis in *E. coli* cells can be observed with this method. Viable count was also done on those specimens. Velocities of cell water removal, *i.e.*, dehydration and rehydration, were controlled under the following conditions for examination of the permeability of the cells: rapid dehydration by abrupt addition of 0.3 ml of the cell suspension into 5.7 ml of 1 M sucrose solution, slow dehydration by dropwise addition of 2 M sucrose solution into 2.0 ml of the cell suspension (one drop per 5 sec), rapid rehydration by an abrupt addition of 0.3 ml of the cell suspension into 5.7 ml of distilled water and slow rehydration by dropwise addition of 9.5 ml of distilled water into 0.5 ml of the cell suspension.

II. Results

COMPARISON OF FREEZING CONDITIONS

Physiology and morphology of *E. coli* cells, which had been cultured on agar, frozen under the three kinds of conditions as described above, were investigated.

1. *Viability of the frozen and thawed cells*

As shown in Table 1, the survival rate of the cells frozen at −25°C was around

Table 1. Survival rates of cells, cultured on agar, subjected to −25 or −150°C

Incubation time (hrs)	Survival rates after freeze-thawing		
	Frozen at −25°C (slow freezing) (%)	Frozen at −25°C (moderately rapid freezing) (%)	Frozen at −150°C (very rapid freezing) (%)
6	86.5±8.1	—	36.4± 8.8
24	87.8±9.7	76.7±7.6	53.2±11.1

87%, regardless of the incubation time. The cells frozen at −150°C showed different values in survival rate, depending upon the incubation time; approximately 36 and 53% in 6 and 24 hour cultures, respectively. In all cases, viability in the cells frozen at −150°C was distinctly lower than that found in the cells frozen at −25°C. The cells, frozen at a moderately rapid rate by immersion in an iso-pentane bath at −25°C, resulted in an intermediate value between slow freezing at −25°C and rapid freezing at −150°C.

2. *Morphology of the cells in the frozen state*

As described above, it was ascertained that the cell death resulting from freeze-thawing depends upon the freezing conditions. As one of the means to clarify the factors affecting the cell viability in freezing, electron microscopic observation of the cells in the frozen state was first made.

(A) Non-sectioned cells. Normal *E. coli* cells show a rod-like shape, having a smooth contour and a homogeneous internal structure. They often contain several small transparent areas or dense particles (Fig. 1-1). Cells subjected to −25°C by slow freezing were extremely shrunken and wrinkled and sometimes twisted. Some areas within the cells were dense, but others were transparent (Fig. 1-2). In rapid freezing at −150°C, the morphological finding was quite different from that found at −25°C. Three kinds of cells were found in the specimen: (1) apparently intact cells, (2) shrunken cells similar to those observed in slow freezing, and (3) cells retaining their original size and shape but containing numerous small cavities (Fig. 1-3 and 4). A mixture of those three types of cells were scarcely found in the same droplet, because generally one droplet contained one type of cells. The third type was observed only in rapidly frozen specimens. The cavities within these cells were very probably loci previously occupied by ice crystals (Fig. 1-5 and 6).

(B) Thin-sectioned cells. Unfrozen normal cells showed the characteristic internal structure, *i.e.*, a double-structured outer envelope and cytoplasm, filled with homogeneous and moderately transparent materials, including dense particles and fine filaments (Fig. 2-1). Cells subjected to −25°C tended to make clumps. The cells, contained in the clumps and became quite shrunken, were bordered with rather transparent but rugged cell walls. The original fine structure of cytoplasm became obscure and dense (Fig. 2-2). In the specimens frozen at −150°C, two types of cells were found: (1) extremely rugged and dense cells resembling confetti, (2) cells having slightly rough margins and an internal fine network, formed by many small or a few large loci (Fig. 2-3). The structure of the cytoplasm which is found in normal cells lost its original configuration and showed such a network, when squeezed by the occupation of ice crystals (Fig. 2-4).

When the specimens frozen at −150°C were stored for three days in a freezer kept at −25°C, a few large cavities were found within the cells. These cavities were definitely larger than those in the cells found immediately after freezing at −150°C (Fig. 2-5). According to this finding, it is clear that those cavities are a result of ice crystal formation and not artifacts produced during the preparation procedure. This fact also suggests that intracellular ice crystals formed in the rapid freezing at −150°C grew in size by storage at higher temperatures and left larger cavities after sublimation.

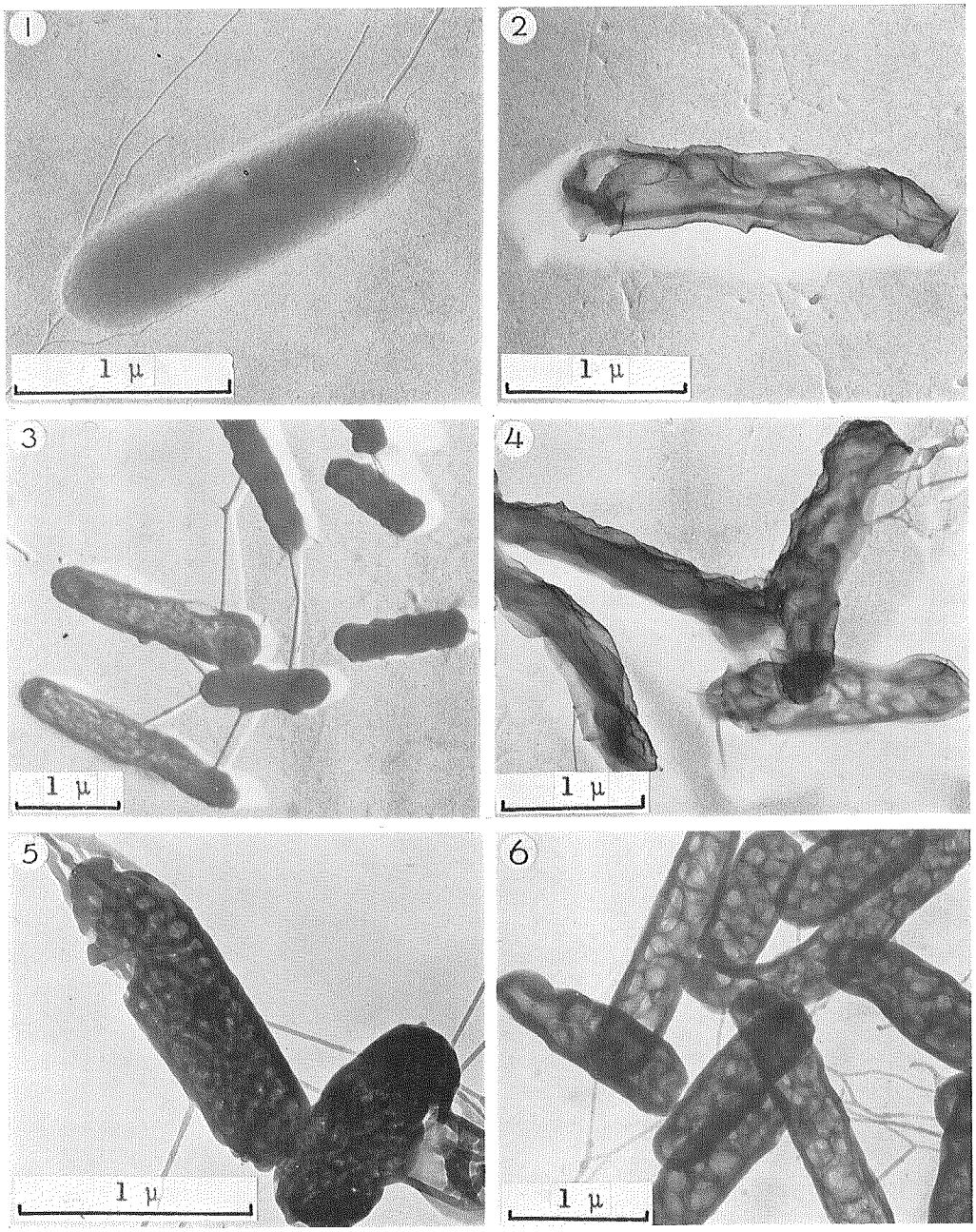

Fig. 1. Cells in the specimens prepared with the attachment for spray freezing

1. A control cell unfrozen but air-dried
2. A cell frozen at −25°C and shrunken
3 to 6. Cells frozen at −150°C
3 and 4. Note three kinds of cells; apparently intact, shrunken and intracellularly frozen cells
5 and 6. Cells showing numerous small cavities

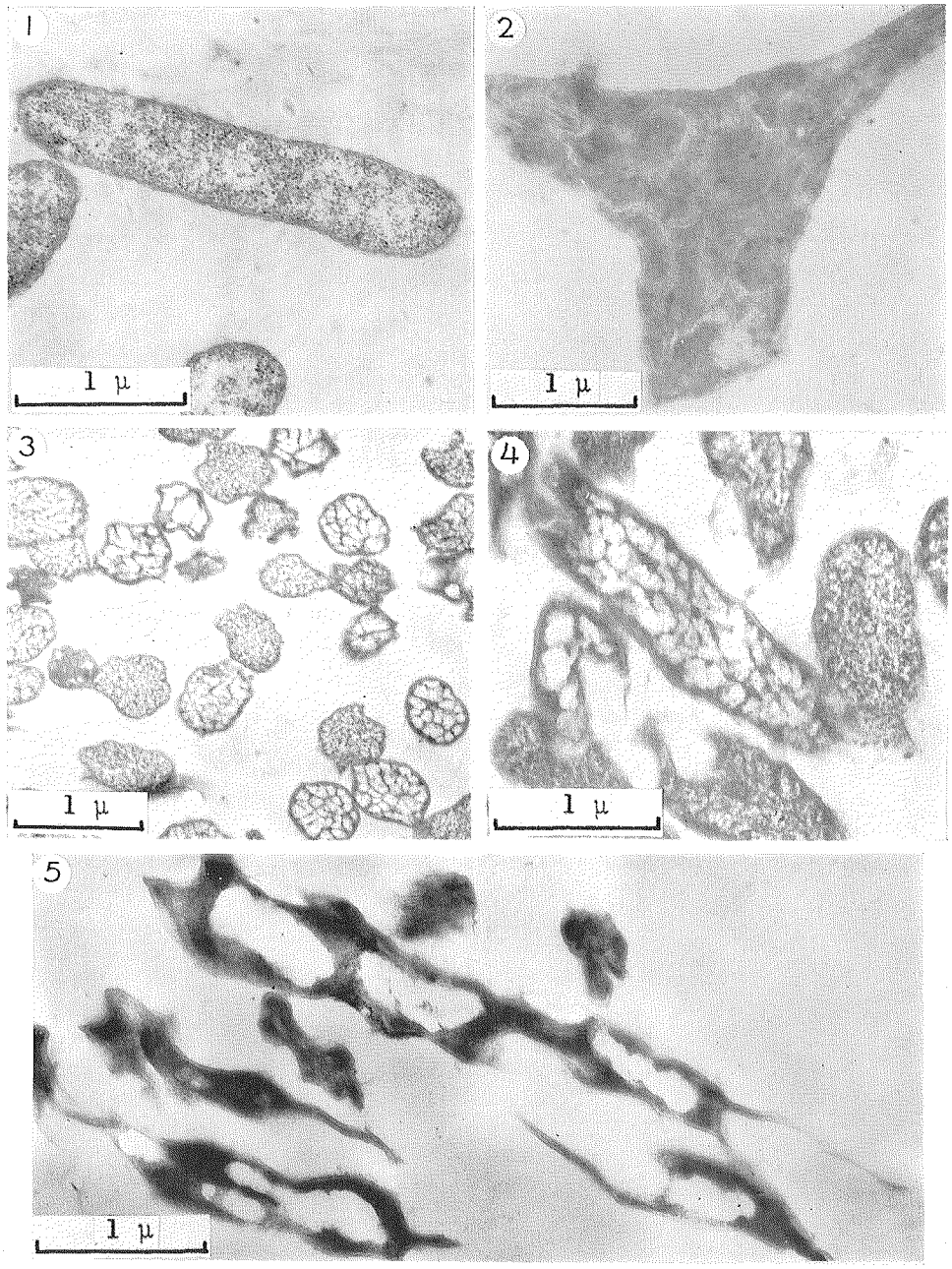

Fig. 2. Cells in the specimens prepared by freeze-drying and thin-sectioning

1. Unfrozen control cells prepared by conventional methods
2. Cells, slowly frozen at −25°C, which show clumping. Individual cells, bordered with transparent cell walls, are shrunken and dense
3. Two kinds of cells frozen at −150°C; shrunken and dense cells like confetti and cells having many small cavities
4. Cells frozen at −150°C. Note the variety of the number and size of intracellular cavities
5. Cells, frozen at −150°C and then stored at −25°C for 3 days, showing a few large intracellular cavities

3. *Morphology of the cells after freeze-thawing*

Both in the non-sectioned and thin-sectioned cells, no remarkable change in the fine structure of the cytoplasm and the membrane could be found when compared with the unfrozen normal controls. Even in the specimens frozen at −150°C, showing intracellular cavities and a low rate of survival, cells looked apparently intact after thawing (Fig. 3-1 and 2).

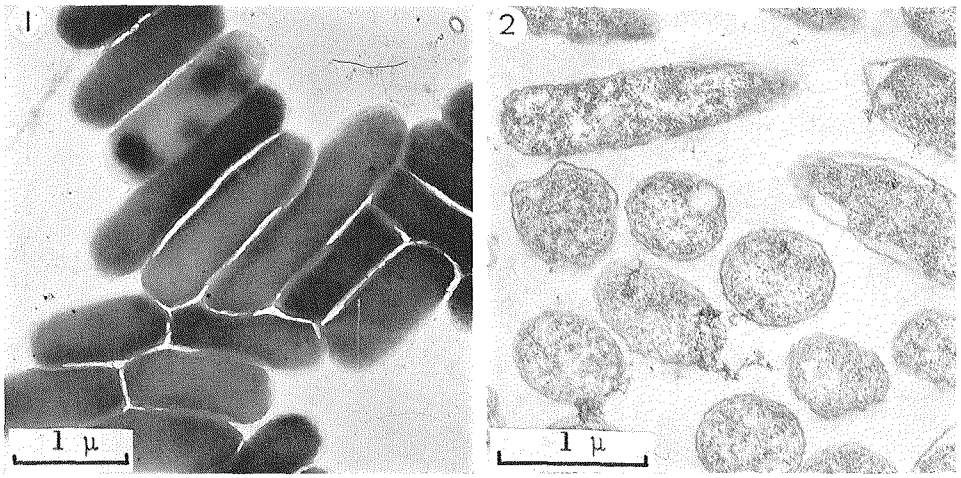

Fig. 3. Cells frozen at −150°C and thawed

1. Non-sectioned cells. No destruction in morphology
2. Thin-sectioned cells. No change in the fine structure. Compare with the cells in Fig. 2-1

4. *Permeability of the cells after freeze-thawing*

In order to examine the effect of freeze-thawing on the semipermeability of cells, plasmolysis was determined on the thin-sectioned specimens exposed to 1 M sucrose solution with electron microscope. Untreated control cells showed typical plasmolysis. Most cells frozen slowly at −25°C were also plasmolysed, but about half of the total cells frozen at −150°C retained their original internal structure without plasmolysis. It was confirmed, therefore, that the cells subjected to −150°C suffer injury in their membrane function as well as in viability, despite no observable change in morphology (Fig. 4-1 to 3).

COMPARISON OF AEROBIC AND ANAEROBIC CELLS

From the results obtained hitherto, it was concluded that morphological and physiological changes observed in the frozen and thawed cells depended upon the freezing conditions and also that the main cause of freezing injury of the cells might be intracellular ice formation. As the next step of the investigation, a comparison of two kinds of cells, originating from the same strain but cultured under different conditions (Harrison and Cerroni, 1956), was made to clarify the mechanism of freezing injury and, in particular, to examine the relationship between cell water and intracellular ice formation.

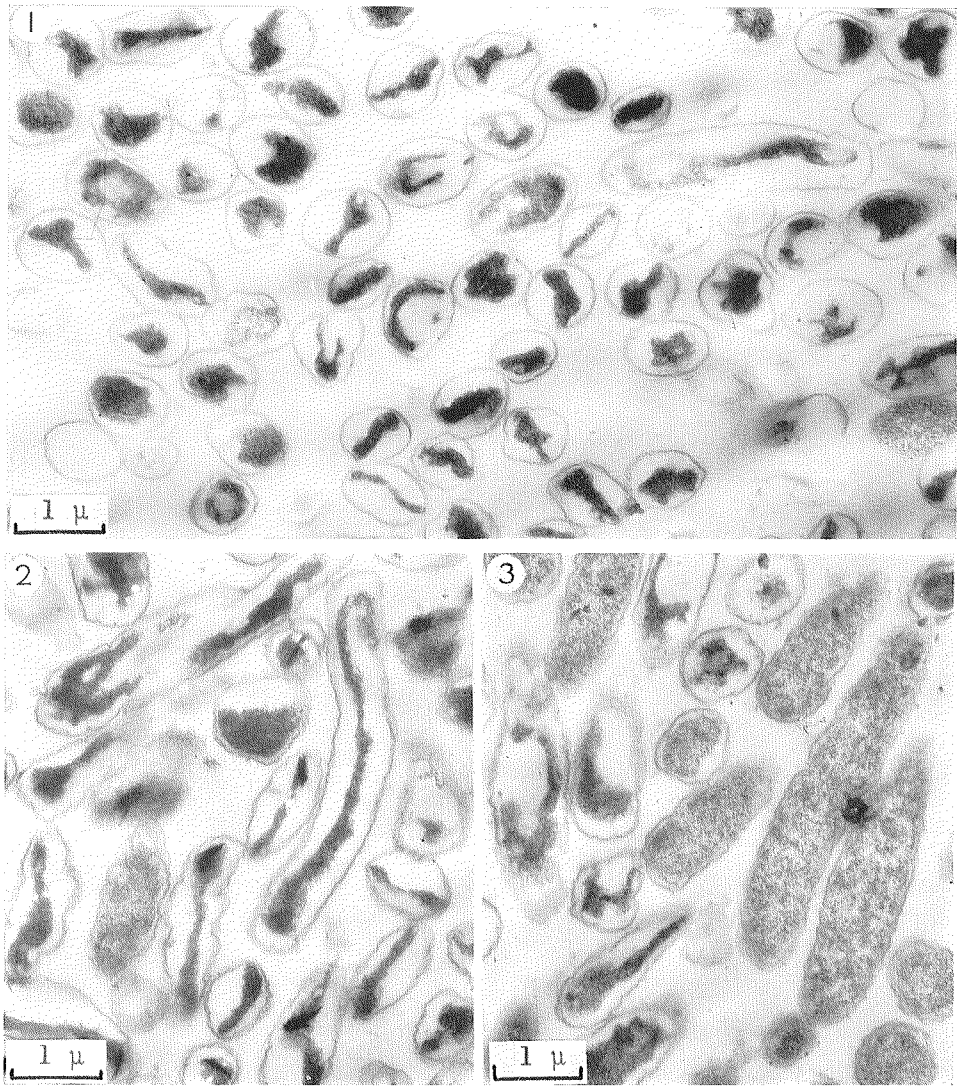

Fig. 4. Plasmolysis of frozen and thawed cells, examined by exposing to 1 M sucrose solution and thin-sectioning

1. Unfrozen control cells
2. Cells frozen at −25°C and thawed. Almost all of the cells are plasmolysed
3. Cells frozen at −150°C and thawed. About half of the cells are plasmolysed

1. *Viability of the frozen and thawed cells*

Aerobic cells cultured in aerated broth showed similar results as those cultured on agar as indicated in Table 1. In the anaerobic cells cultured in non-aerated broth, however, there was a characteristic difference. Cells frozen at −150°C showed a much lesser value, a mere 13% or less, than those in the aerated or agar cultures, whereas the cells in those cultures frozen at −25°C showed the same value, around 85%. Cells rapidly frozen at −25°C indicated an intermediate value of 27%. This may show that

Table 2. Survival rates of aerated and non-aerated cells subjected to −25 or −150°C

Specimens		Survival rates after freeze-thawing		
Culture	Incubation time (hrs)	Frozen at −25°C (slow freezing) (%)	Frozen at −25°C (moderately rapid freezing) (%)	Frozen at −150°C (very rapid freezing) (%)
Aerated	24	88.5 ± 3.5	—	50.4 ± 4.4
Non-aerated	6	83.4 ± 11.1	26.6 ± 3.4	13.6 ± 3.3
	24	85.5 ± 3.4	—	7.8 ± 3.1

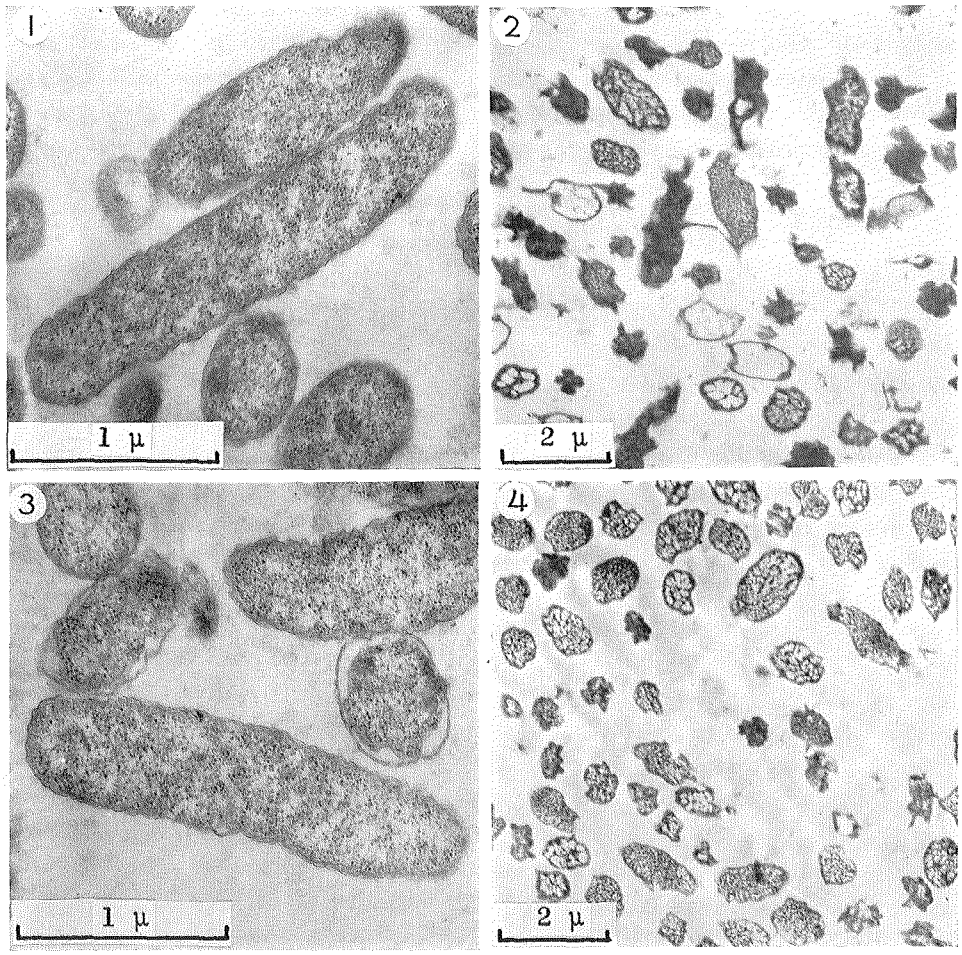

Fig. 5. Comparison of aerobic and anaerobic cells

1. Unfrozen control of aerobic cells
2. Aerobic cells frozen at −150°C. The number of shrunken and intracellularly frozen cells are almost equal
3. Unfrozen control of anaerobic cells. No difference in the fine structure of aerobic and anaerobic cells
4. Anaerobic cells frozen at −150°C. Intracellularly frozen cells are more numerous than shrunken cells in number

the anaerobic cells are more sensitive to rapid freezing than the aerobic cells (Table 2).

2. *Morphology of the cells in the frozen state*

Only thin-sectioned specimens were used for this experiment. In the control specimens, there was no difference between aerobic and anaerobic cells. The general appearance and internal structure of the cells in both cultures were almost the same as those described in the control cells cultured on agar (Fig. 5-1 and 3).

In the cells subjected to −25°C, shrinkage and wrinkling were also remarkably observable. Other findings were similar to those of the agar culture which was subjected to the same conditions. In rapid freezing at −150°C, however, there was a distinct difference in aerobic and anaerobic cells: while only some of the cells in the former contained intracellular cavities as found in the cells cultured on agar, almost all of the cells in the latter showed cavities (Fig. 5-2 and 4). From this morphological finding and also from the above-mentioned result concerning viability, a higher sensitivity to rapid freezing of the anaerobic cells is recognizable as compared to the aerobic or agar cultured cells.

3. *Morphology of the cells after freeze-thawing*

No remarkable change was found in the fine structure of the cytoplasm and the membrane of the frozen and thawed cells, as those of agar cultured cells.

4. *Effect of the cooling rates on the cell survival*

With the intent to examine the problem concerning the dehydration of cell water, a more detailed experiment on the effect of cooling rates on the cell survival was carried out. As shown in Fig. 6, the survival rate of the frozen and thawed cells tended to decrease when the cooling rate exceeded 10°C/min or thereabout. As the cooling rate increased, a remarkable reduction of the survival rate was observed in the anaerobic cells. According to the morphological findings described previously, almost all of the cells cooled at a rate of approximately 50°C/min to −25°C were shrunken probably due to extracellular freezing. A slight decrease in the survival of such shrunken cells allows for the

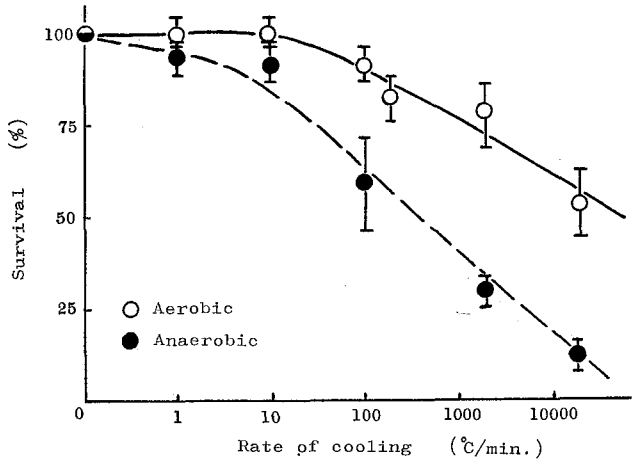

Fig. 6. Effect of cooling rates on the survival rates of aerobic and anaerobic cells

assumption that some injury is caused by considerably rapid dehydration without intracellular freezing. It is also assumed that a high cooling rate beyond 1,000°C/min may increase the cell injury resulting from intracellular ice formation and rapid dehydration.

5. *Removal of the cell water*

Concerning the cell water which will be closely correlated with intracellular ice crystal formation in freezing, a comparison of aerobic and anaerobic cells was drawn on their permeability to water. For this purpose, the effect of dehydration and rehydration velocities of cell water on the cell viability was investigated by exposing the cells to 1 M sucrose solution and returning them to distilled water under certain controlled conditions, which were followed by viable count.

As indicated in Table 3, there are marked differences in the anaerobic cells under four given conditions of water removal, whereas no difference in the aerobic cells was seen. From the results indicating that rapid removal of water, both dehydration and rehydration, was highly injurious to the anaerobic cells, it was recognized that the anaerobic cells have a lower permeability to water as compared to the aerobic cells. It is accordingly assumed that the anaerobic cells still maintain a certain amount of water sufficient to produce intracellular ice formation during freezing, since the low permeability of those cells inhibits the rapid withdrawal of cell water.

Table 3. Effects of velocities of dehydration and rehydration on the cell survival by exposure to 1 M sucrose solution

Dehydration	Rehydration	Survival rates after treatment	
		Aerobic cells (%)	Anaerobic cells (%)
Slow	Slow	93.7 ± 8.3	93.1 ± 4.2
	Rapid	89.7 ± 3.7	52.5 ± 10.8
Rapid	Slow	96.0 ± 12.1	37.7 ± 20.7
	Rapid	98.3 ± 8.3	34.9 ± 18.0

III. Discussion

A large number of studies on the morphology and physiology of animal and plant cells subjected to various low temperatures have brought forth a considerable amount of knowledge for the elucidation of the mechanism of cellular injury by freezing in various kinds of living cells. It has been recognized that dehydration, solute concentration and intracellular ice formation are the three main causes involved in the freezing injury in the majority of large cells examined hitherto.

This hypothesis on the mechanism of freezing injury was also confirmed in yeast cells (Nei, 1959; Mazur, 1961), but has not been determined in extremely minute cells such as bacteria. While there was a recent report by Rapatz and Luyet (1963), this was merely concerned with morphological observations of frozen bacteria.

In the present experiment, our initial intent was to examine the formation of ice crystals within the bacterial cells subjected to low temperatures, and for this purpose,

two procedures for electron microscopic observation were employed: (1) thin-sectioned specimens were prepared by ordinary freeze-drying method, and (2) non-sectioned specimens were prepared by freeze-drying in a specially devised cooling attachment incorporated into the electron microscope. In particular, the latter specimens were made for direct observation of the frozen cells at low temperatures without the usual prehandling such as fixation or embedding.

From the results obtained in these experiments, the following evidence was established: *E. coli* cells became extremely shrunken and dense by slow freezing, whereas the cells rapidly frozen retained their original size and shape but contained numerous small cavities previously occupied by ice. These morphological alterations seem to be quite the same as those observed in yeast cells. It is assumed, therefore, that even in bacterial cells the withdrawal of intracellular water, resulting from the extracellular ice formation by slow freezing, results in the shrinkage of the cells, and, on the other hand, extremely rapid freezing at extremely low temperatures causes intracellular ice formation.

The relationship between the morphology and viability of the cells frozen at −25° and −150°C should be mentioned as an important point in this experiment. The cells which had become shrunken after freezing at −25°C showed a rather high rate of post-thawing survival, around 87%. On the contrary, the specimens which showed intracellular ice formation at −150°C indicated a lower rate of survival. From these results, it can be assumed that the main cause of the freezing injury in bacteria might be intracellular ice crystal formation.

The cells subjected to low temperatures revealed such remarkable morphological alterations in the frozen state, but they recovered their normal configuration after thawing and did not show any distinct change. It is easily understandable that the extracellularly frozen water, which is withdrawn from the cells, can return into the cells when it is thawed, and instantly the shrunken cells recover their original shape. However, from the evidence indicating that the cells frozen at −150°C showed no prominent change in morphology after thawing, despite the intracellular ice formation as well as the semi-permeability damage and the cell death, it is assumed that the cell injury might be due to submicroscopic damage. A more detailed investigation in morphology should be done as the next logical step along this line.

Another important problem in the present experiment was the comparison of aerobic and anaerobic cells as materials for elucidation of the mechanism of freezing injury. From the results obtained, the anaerobic cells showed much higher rates of intracellular freezing and cell death than the aerobic cells in rapid freezing at −150°C. Factors associated with such susceptibility to rapid freezing were then examined dealing especially with the membrane functions: the fact that the rapid removal of cell water injured the anaerobic cells proved the low permeability of those cells. It can be explained that such low permeability to water of anaerobic cells results in the high possibility of intracellular ice formation and a subsequent remarkable decrease in the survival rate. This may also substantiate the view that the dehydrating velocity of cell water is closely related with the likelihood of ice formation within the cells, as Mazur (1963) pointed out.

A comparison among the different species of cells has been made on resistivity against freezing. From the reports on the survival rate after thawing, it was noted that *E. coli*

cells are more resistant than yeast cells (Nei, unpublished). It is also of great interest that even *E. coli* cells, which are very minute in size and fairly resistant against freezing, permit intracellular ice formation under a certain freezing condition.

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