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Effects of Cryoprotective Additives on Intracellular Ice Formation and Survival in Very Rapidly Cooled HeLa Cells*

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Abstract

The effects of glycerol, sucrose and PVP as cryoprotective additives on survival of HeLa cells, which were very rapidly cooled to -196°C , were examined. The cells in physiological salt solution with and without cryoprotective additives were frozen at various rates between 30° and $1000^{\circ}\text{C}/\text{sec}$. Post-thawing survivals of rapidly frozen cells were affected in various degrees by both cooling velocity and cryoprotective additives. It seems obvious through the electron microscopic observation of the frozen cells that the effects of cooling velocity and cryoprotective additives on maintenance of viability of rapidly frozen cells were inseparably related with their efficiency on the retardation of the growth of intracellular ice crystals during freezing. Glycerol, sucrose and PVP seem to act as protective reagents for cells during rapid freezing because of the ability to inhibit the growth of intracellular ice crystals owing to their molar concentrations in suspending medium regardless of their efficiency to permeate into cells.

1. Introduction

It is well known that freezing is seriously injurious process to most living cells, while it is an effective means of preserving the living cells for a long time. Since the discovery of the cryoprotective effects of glycerol by POLGE, SMITH and PARKES in 1949¹⁾, it has been well known that many reagents including sugars, glycols and polymers protect living cells against injury caused by freezing and thawing^{2,3)}. Today, varieties of living cells

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are widely preserved in frozen state with cryoprotective additives. In such a case living cells are usually cooled to subzero temperatures slowly enough to prevent the intracellular freezing, which is lethal in most cases⁴⁻¹⁰. It has been conceived that cryoprotective reagents are effective by their buffering action to protect the living cells from injury caused by the increased concentration of solutes in and around the living cells during slow freezing^{11,12}.

On the other hand, LUYET proposed an idea in 1940¹³ that vitrification of non-dehydrated protoplasm was considered as a possible means of preserving the living organisms. In order to obtain the vitreous state, one should cool the organisms rapidly enough to pass the range of ice crystallization temperatures within a period of time shorter than those required to form the ice crystal nuclei. The vitrification procedure was assumed to allow the solidification of living organisms with probably less alteration of biological structures than any other fixing methods. LUYET¹³ therefore suggested that vitrification of living protoplasm was probably available not only for a possible means of preserving hydrated living organisms at very low temperatures but also for the applications of optical and X-ray analysis of the fine structures of biological constituents. STEERS¹⁴ applied the freeze-fixation of biological specimens for electron microscopy to eliminate the artifacts produced by chemical fixation. He demonstrated the fine structures of plant virus crystals by the use of freeze-etching technique. Then, MOOR *et al.*^{15,16} beautifully showed through the freeze-etching technique that the solidification of protoplasm to amorphous or extremely fine crystalline state obtained by very rapid freezing yielded both an appreciable survival and a great deal of new information about fine structures in yeast cells. Glycerol, which had been well known as a very efficient cryoprotectant for slowly frozen living cells, was again introduced by MOOR as a reagent for reducing the growth of intracellular ice crystals during rapid freezing. It may be safely said that some parts of recent advance in cryobiology is owing to the application of the freeze-etching techniques to the investigation of frozen state of living cells¹⁷⁻²⁰.

The present report deals with the effects of cryoprotective additives, such as glycerol, sucrose and PVP on survival of very rapidly frozen HeLa cells. In order to clarify the relationship between the formation of intracellular ice crystals and the survival of the frozen cells, fine structures of cryoprotected cells which were rapidly frozen to very low temperatures were also observed electron microscopically by the use of freeze-etching technique.

II. Materials and Methods

Materials

The cells of an established cell line, HeLa-S 3, originally derived from a human cervical carcinoma were employed as materials. The cells have been maintained by continuous subculture since 1969 in our institute. Normal growth medium consisted of 90% TC-199 medium and 10% fetal calf serum, both containing 100 U penicillin/ml and 100 μ g streptomycin/ml.

Preparation of cell suspensions for freezing

Cell sheets of HeLa grown in 800 ml glass T-flasks were disaggregated by exposure to a certain amount of 0.1% solution of trypsin containing 0.01% EDTA (ethylenediaminetetraacetate) and the solution was removed by centrifugation and decantation. The disaggregated cells were repeatedly washed with a sufficient amount of the test medium. They were finally suspended in a small amount of the test medium to yield a concentration of 1×10^7 cells/ml. Physiological salt solution (PSS, 0.15 M NaCl), PSS containing 1.25 or 2.5 M glycerol, 0.5 or 1 M sucrose or 20% PVP (K-30, mol wt 40,000) were employed as test media.

Freezing procedures

2, 5 and 10 μ l of cell suspensions were dropped on aluminum foils (5×10 mm) and they were immersed directly into liquid Freon 22 precooled to -150°C and then transferred into liquid nitrogen. Cooling rates in 2, 5 and 10 μ l specimens were 1000° , 500° and $200^\circ\text{C}/\text{sec}$ respectively. 10 μ l of cell suspensions were additionally dropped on aluminum foils, and cooled at $30^\circ\text{C}/\text{sec}$ by direct immersion into liquid nitrogen (-196°C). All of these specimens were used for survival assay.

For electron microscopic observation, 2 and 10 μ l of cell suspensions were dropped on copper disks (5×10 mm) and immersed directly into cooled liquid Freon (-150°C). Cooling rates in 2 and 10 μ l specimens were 1000° and $250^\circ\text{C}/\text{sec}$ respectively. 10 μ l drops of cell suspensions on copper disks also were cooled at $50^\circ\text{C}/\text{sec}$ by direct immersion into liquid nitrogen. These specimens for electron microscopic observation stored in liquid nitrogen until they were transferred into the freeze-etching apparatus.

The temperature changes of the specimens during cooling were estimated by a 42-gauge copper-constantan thermocouple which was inserted in the drop of cell suspension. The cooling curves of the specimens were recorded by an oscillograph (Yokogawa, photocorder 2901) connected with the thermocouple through a DC amplifier. Mean cooling rates were calculated from

the cooling curves between 0° and -50°C.

Survival assay of the frozen-thawed cells

Frozen drops of cell suspensions on aluminum foils were kept in liquid nitrogen for 10 min. They were, then, thawed very rapidly by direct immersion into normal growth medium prewarmed to 38°C. Appropriate number of frozen-thawed cells suspended in normal growth medium were taken out in plastic petri dishes. They were incubated at 37°C for 7 to 10 days in a gassed incubator (5% CO₂ in air, pH 7.4). After incubation, the colonies were fixed with 10% formalin-phosphate buffer solution and stained with 1% solution of methylene blue to count them. The percentage of survived cells was calculated as the mean ratio of the number of colonies on plates from frozen-thawed specimens to the number on unfrozen control ones.

Electron microscopic observation of the frozen cells

A general freeze-etching technique developed by MOOR^{15,16)} was applied in order to observe the frozen cells cooled to -196°C. Frozen drops of cell suspensions on copper disks were transferred to a cold stage of the freeze-etching apparatus (JEE-AFE-01) and fractured by a cold knife at a temperature around -90°C in a vacuum of 2×10^{-5} Torr. Fractured surfaces of the frozen specimens were slightly etched and replicated with platinum-palladium and carbon at the same temperature in a vacuum. Replicas and adhering frozen specimens were dipped into dilute solution of Bleach (aqueous solution containing NaOCl) to dissolve the frozen specimens. The replicas floated off were washed with distilled water and observed under the electron microscope (JEM-6 AS).

III. Results

Survivals of frozen-thawed cells with and without cryoprotective additives

Survival curves of frozen-thawed cells obtained from the present experiments are shown in Fig. 1. Survival rates of the cells were considerably affected by cooling velocity and cryoprotective additives. When the cells were frozen without cryoprotective additives, very few cells remained alive after rapid thawing in the whole range of examined cooling rates. Living cells were scarcely observed in the specimens cooled at 30° and 200°C/sec. In the specimens cooled at 500°C/sec, only 0.012% of frozen-thawed cells was estimated to survive. As the cooling rate was increased to 1000°C/sec, survival rate approached 0.1%.

Addition of PVP to suspending medium brought an increase in number of survived cells by ten times or more in each freeze-thawing condition.

When the cells were cooled with 20% PVP at 30°C/sec, survival rate of the cells after rapid thawing was nearly 0.5%. Survivals were decreased to a minimum as cooling rate was increased from 30° to 200°C/sec. A further increase in cooling rate, however, resulted in a progressive increase in survival rate. In the specimens cooled at 1000°C/sec, about 1% of the cells remained alive after rapid thawing.

Glycerol was very effective to protect the living cells from injury caused

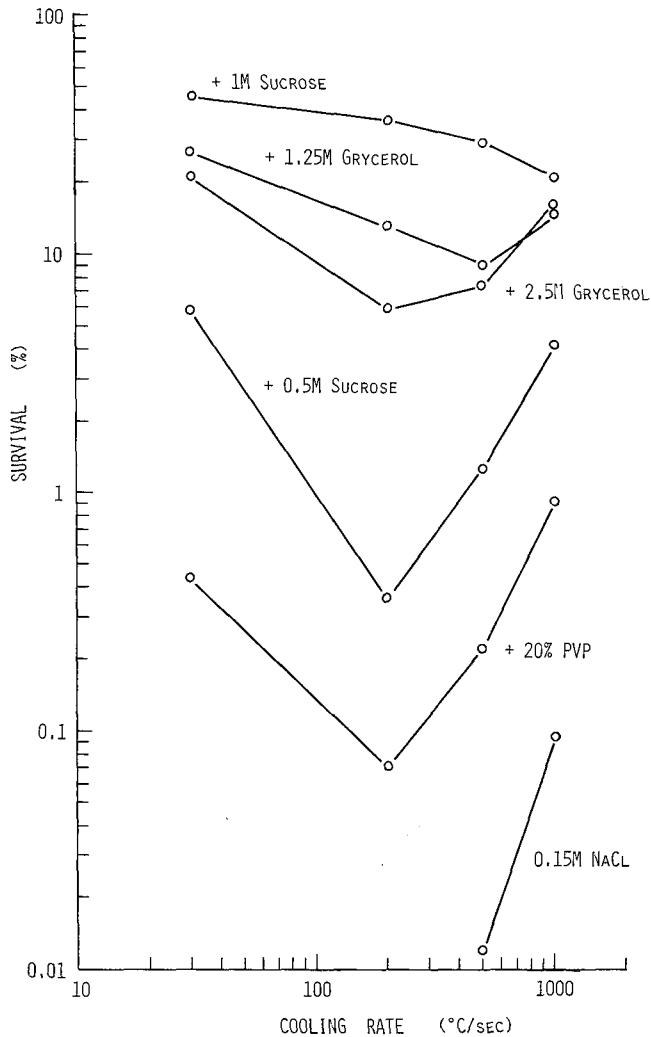


Fig. 1. Post-thawing survival curves of the rapidly frozen HeLa cells with and without cryoprotective additives

by rapid freezing. When the cells were frozen with 2.5 M glycerol at the cooling rate of 30°C/sec, mean survival rate of the cells was 21.3%. Survivals were decreased to a minimum as the cooling rate was increased from 30° to 200°C/sec, as observed in the specimens frozen with 20% PVP. However even at the minimum nearly 6% of the cells remained alive. In the specimens cooled faster than 200°C/sec, increase in cooling rate brought a progressive increase of survived cells.

In the case of the cells frozen with 1.25 M glycerol, survivals were somewhat better than those frozen with 2.5 M glycerol in the range of cooling rates from 30° to 500°C/sec. A cooling rate of 30°C/sec was optimum for cell survival among the examined cooling conditions. About 27% of the cells remained alive after rapid thawing. On the contrary, survival rate was reduced to a minimum when the specimens were cooled at 500°C/sec.

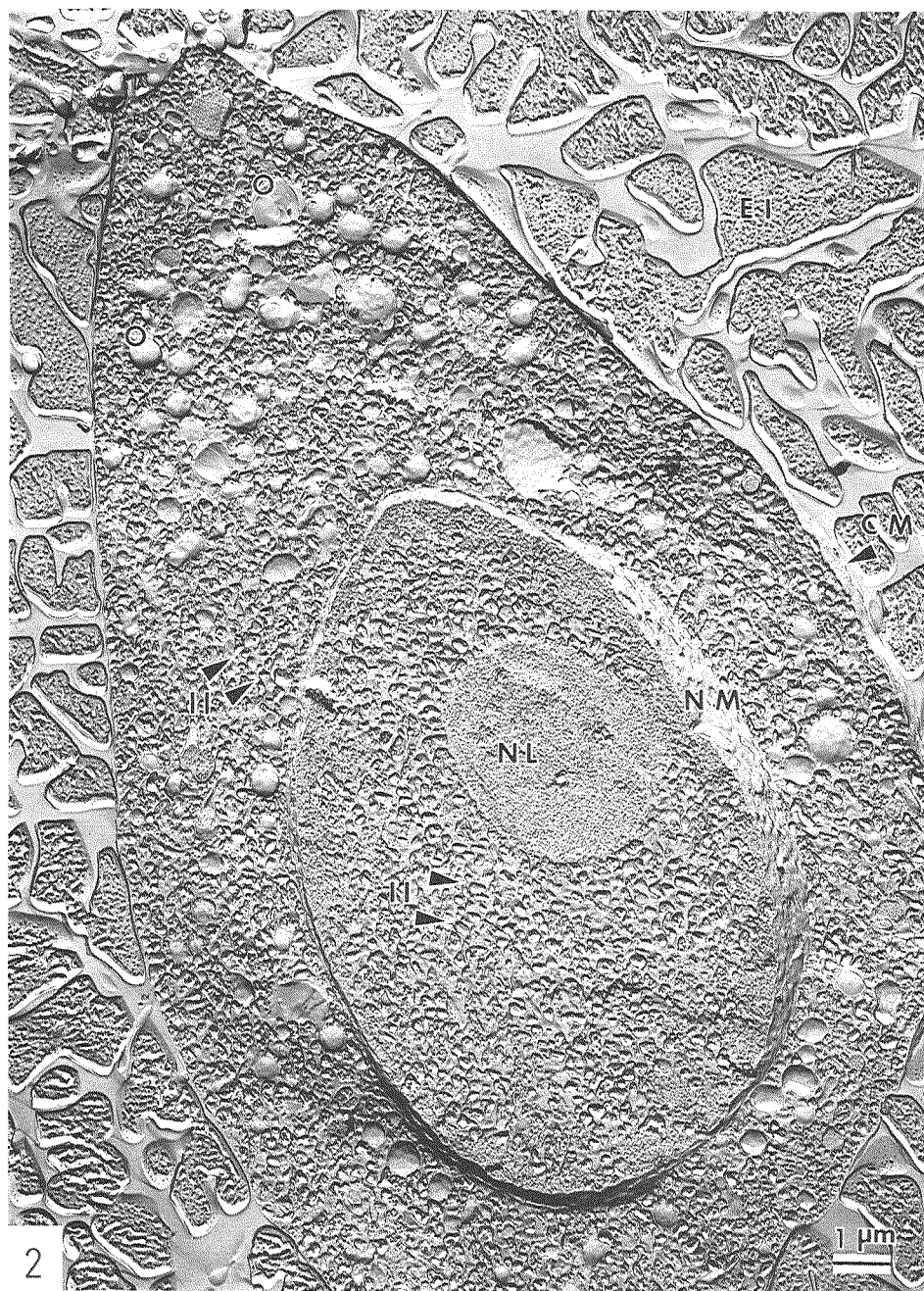
Addition of sucrose to suspending medium brought a remarkable effect on survival of frozen-thawed cells. However there was a considerable difference in survival curves between the specimens frozen-thawed with 0.5 M sucrose and those with 1 M sucrose. The cells frozen with 0.5 M sucrose were always better than those frozen with PVP and always worse than those frozen with glycerol in survival, regardless of cooling rates. Survival rate of the cells with 0.5 M sucrose was minimum when the cells were cooled at 200°C/sec, and remarkably enhanced by both relatively slow (30°C/sec) and very rapid cooling (1000°C/sec), as observed in the specimens frozen with PVP.

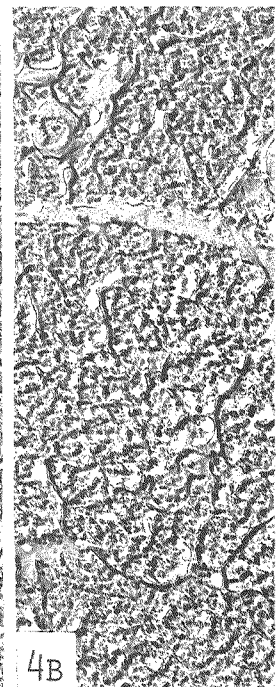
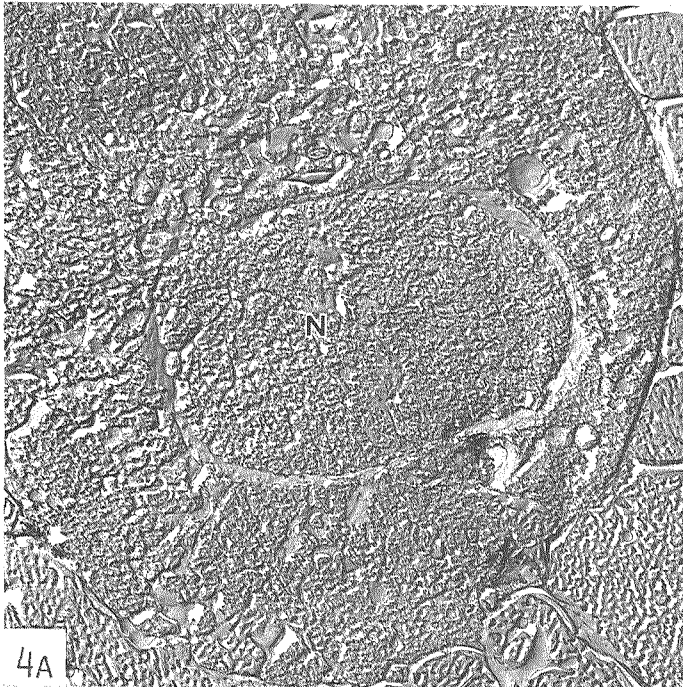
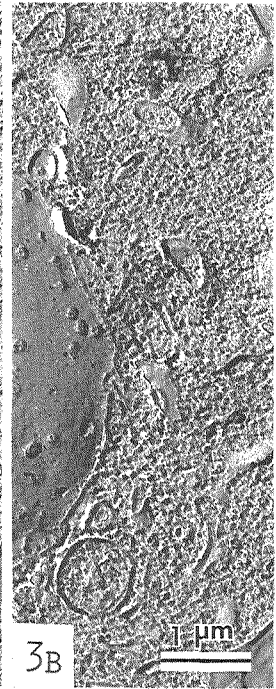
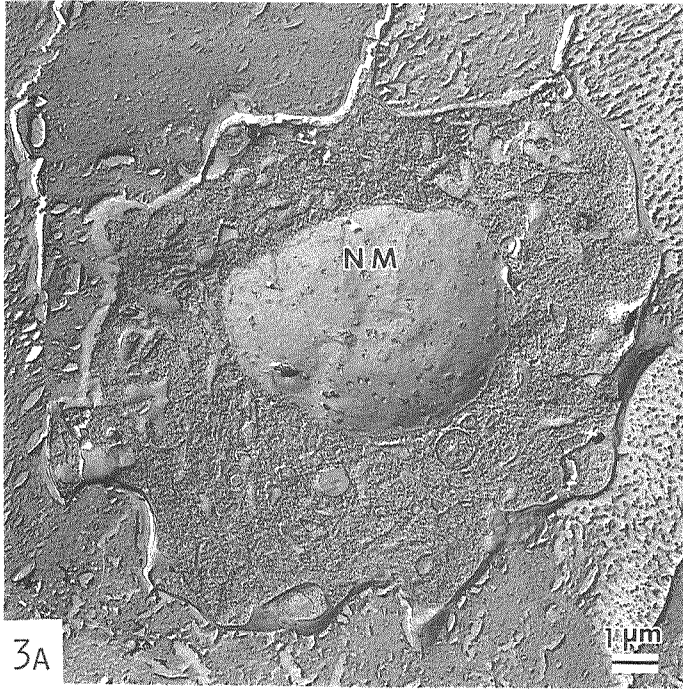
In contrast to the above mentioned results, addition of 1 M sucrose to suspending medium brought a remarkable protection of frozen-thawed cells without any minimal survival at an intermediate rate of cooling. Among the cryoprotective media employed in the present experiments, 1 M sucrose exhibited the best protection against freezing injury throughout the whole range of examined cooling rates. In this case, when the cooling rate was 30°C/sec, survival rate of the frozen-thawed cells was maximum reaching about 45%. Increase of cooling rate brought a gradual decrease in survival rate.

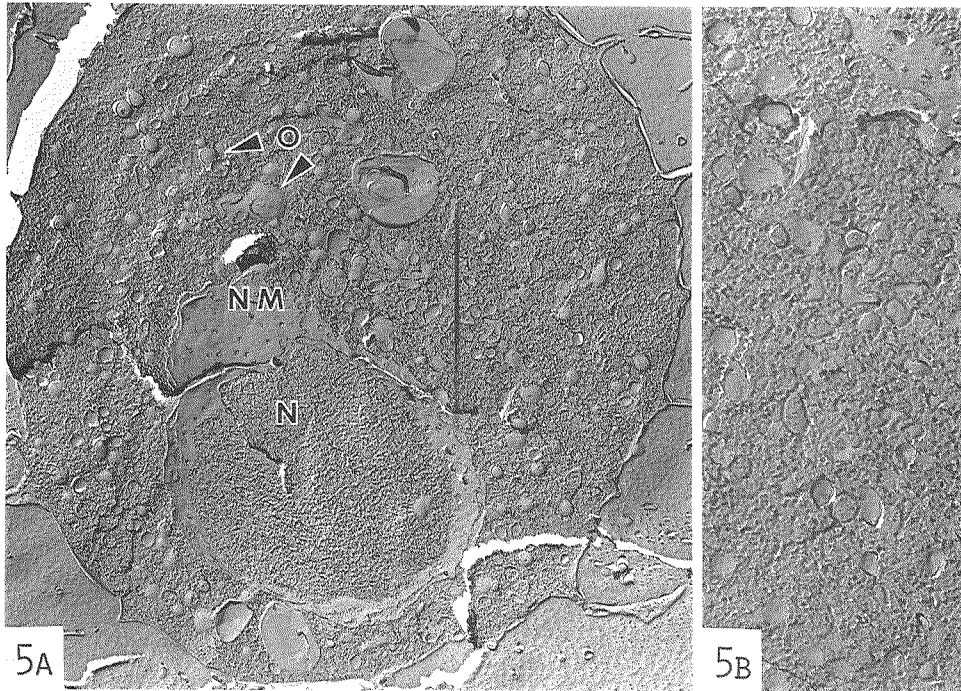
Key to Abbreviations

CM	cell membrane
EI	extracellular ice crystal
II	intracellular ice crystal
N	nucleus
NL	nucleolus
NM	nuclear membrane
O	cytoplasmic organelle

Fig. 2. Cross-fractured surface of a frozen HeLa cell. $\times 7,500$







Figs. 3-5. Cross-fractured surfaces of the frozen cells observed in the specimens frozen without cryoprotective additives. A, lower magnification ($\times 6,000$); B, higher magnification ($\times 12,000$)

Fig. 3. A cell cooled to -196°C at $50^{\circ}\text{C}/\text{sec}$. The cell was contracted by extracellular freezing

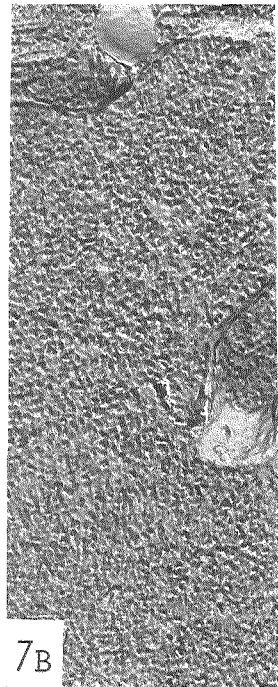
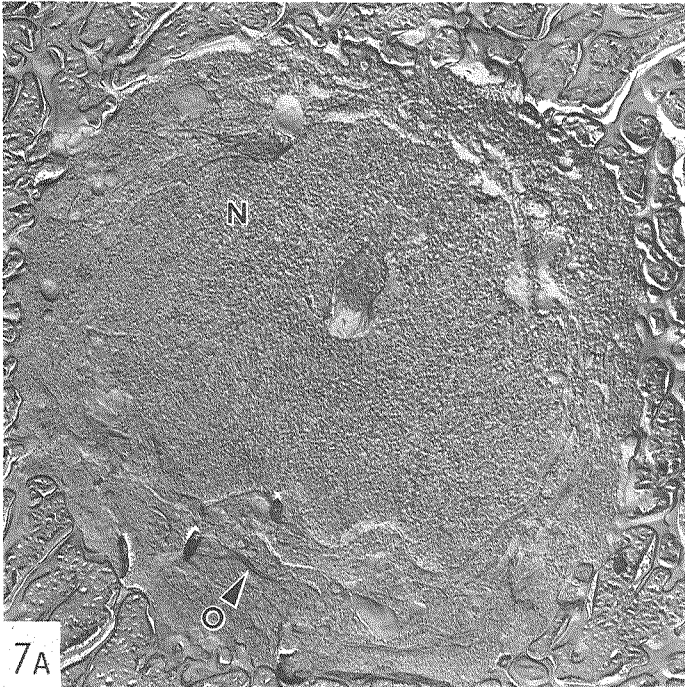
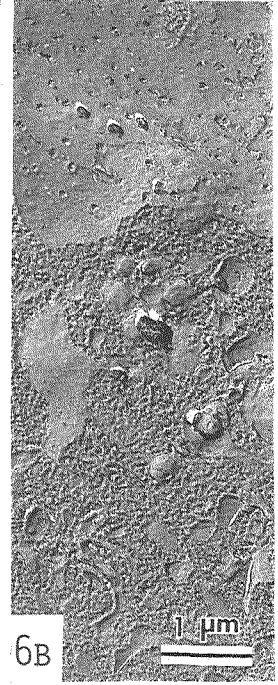
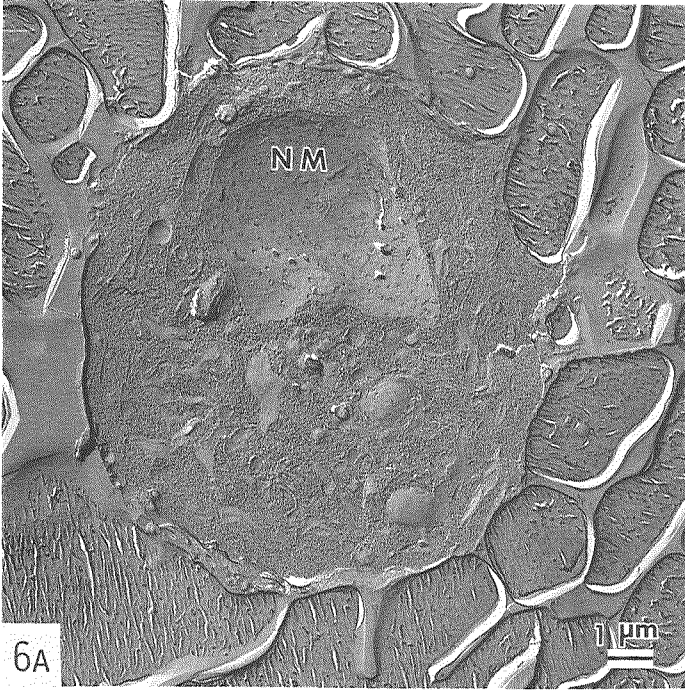
Fig. 4. A cell cooled to -196°C at $250^{\circ}\text{C}/\text{sec}$

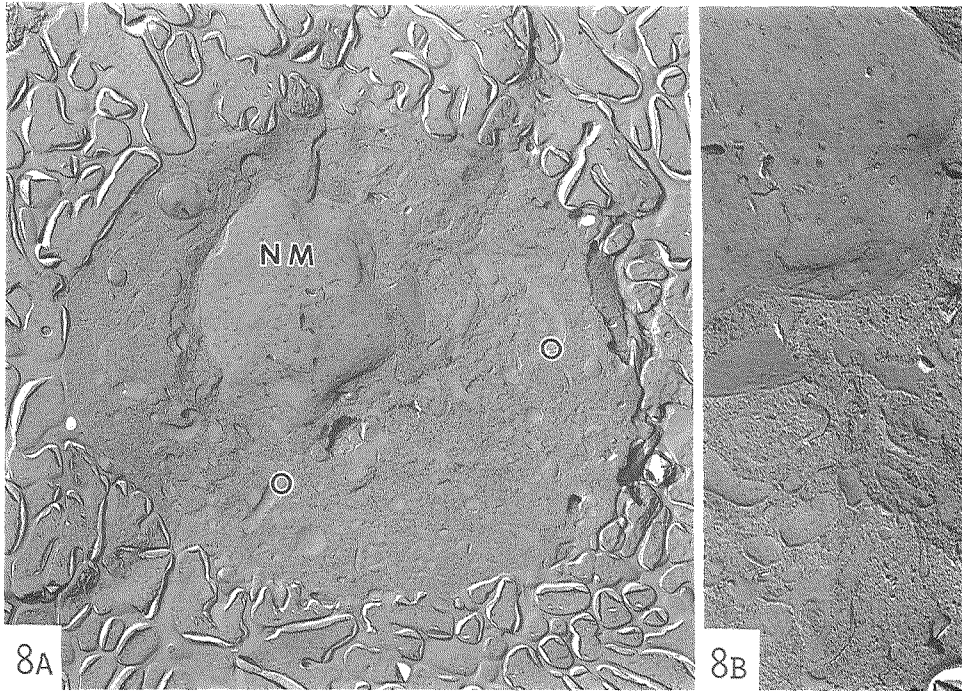
Fig. 5. A cell cooled to -196°C at $1000^{\circ}\text{C}/\text{sec}$. The cell contained fine intracellular ice crystals smaller than $0.05\ \mu\text{m}$ in each individual size

Electron microscopic observation of frozen cells cooled to -196°C with and without cryoprotective additives

Electron microscopic structure of frozen cells, which were cooled at various rates to -196°C with and without cryoprotective additives, was observed focussing attention on the effects of cryoprotective additives on the formation of intracellular ice crystals.

There was a considerable difference in morphological appearance between the freeze-etched cells with and without cryoprotective additives. In general at the cross-fractured surfaces of frozen cells, various kinds of vacuolar, capsular and sometimes membranous structures were recognized being surrounded by various sizes of granulous or grain structures (Fig. 2). Former





Figs. 6-8. Cross-fractured surfaces of the frozen cells observed in the specimens frozen with 20% PVP. A, lower magnification ($\times 6,000$); B, higher magnification ($\times 12,000$)

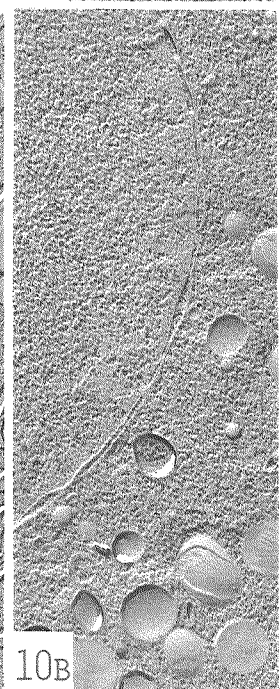
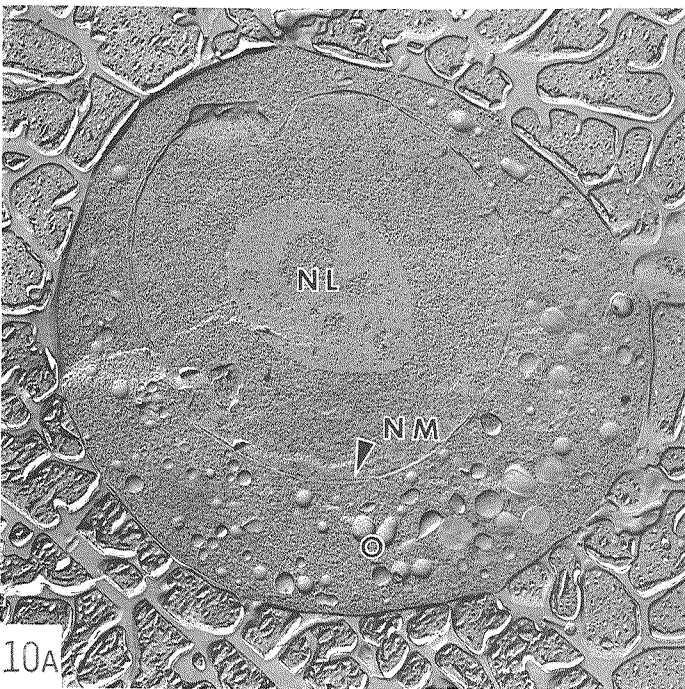
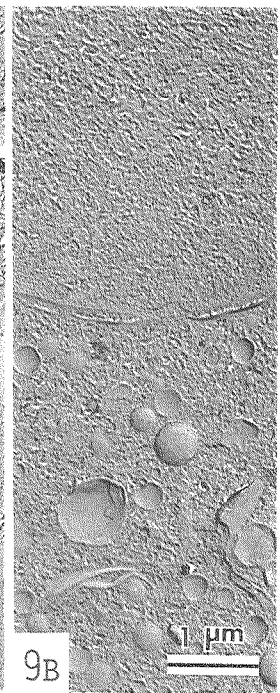
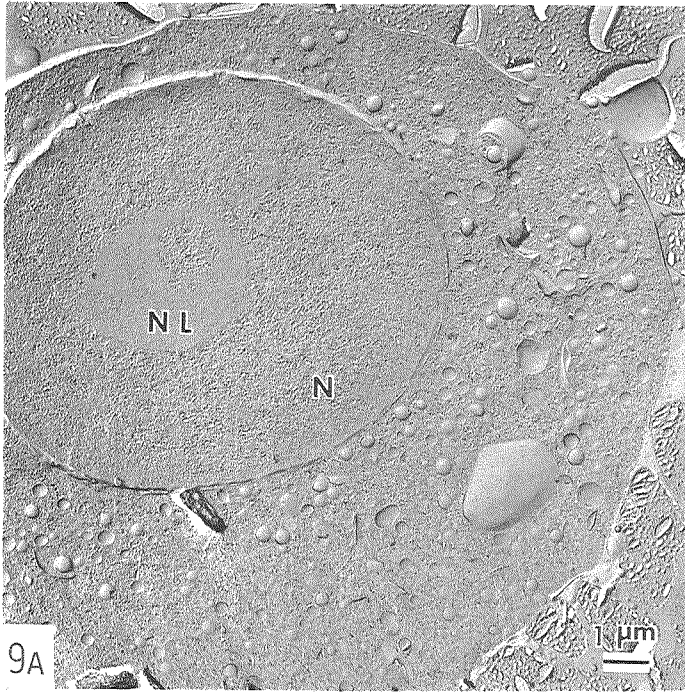
Fig. 6. A cell cooled to -196°C at $50^{\circ}\text{C}/\text{sec}$. The cell was contracted by extracellular freezing

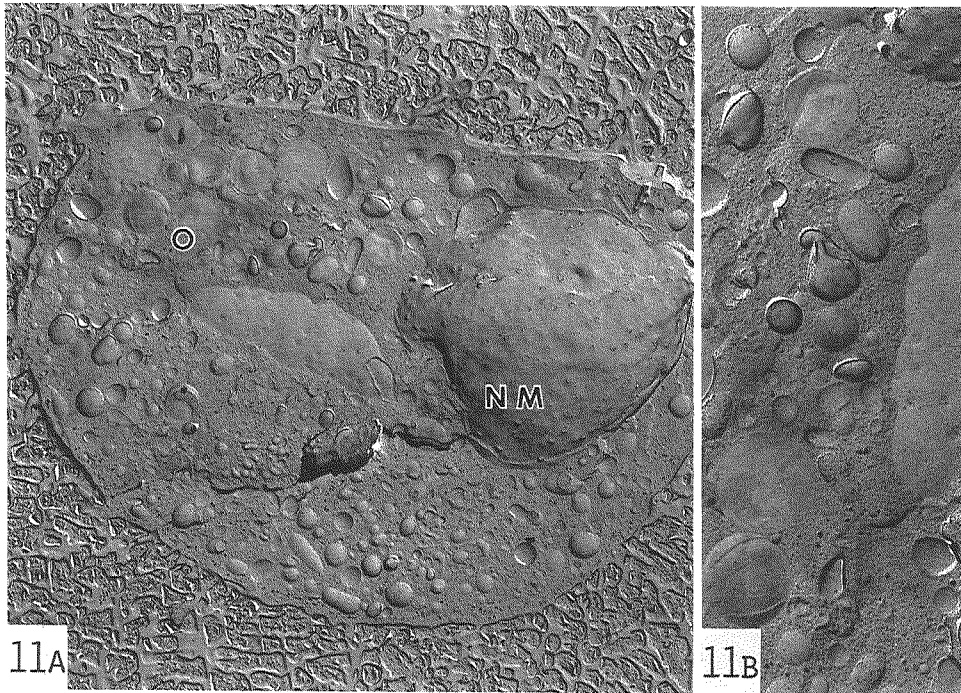
Fig. 7. A cell cooled to -196°C at $250^{\circ}\text{C}/\text{sec}$. The cell contained intracellular ice particles larger than $0.1\ \mu\text{m}$

Fig. 8. A cell cooled to -196°C at $1000^{\circ}\text{C}/\text{sec}$. The cell contained fine intracellular ice crystals smaller than $0.05\ \mu\text{m}$

structures probably indicated the presence of cytoplasmic organelles, although it was difficult to identify them with the exception of nuclear membrane. Nuclear membrane was easily distinguishable from other organelles and identified by its unit membrane system and membrane surface peculiarity. Latter structures, on the other hand, appeared to indicate the presence of intracellular ice crystals. Ice crystals formed in rapidly cooled cells were differed in size in each cell even in the same frozen specimen.

The cells suspended in PSS without any additives scarcely contracted when they were cooled at rates higher than $250^{\circ}\text{C}/\text{sec}$ (250° and $1000^{\circ}\text{C}/\text{sec}$). The majority of frozen cells maintained their external spherical form





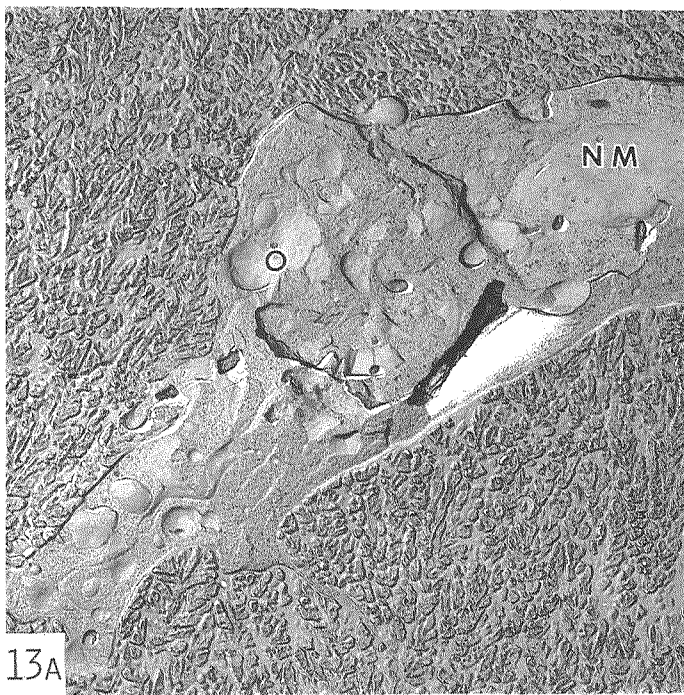
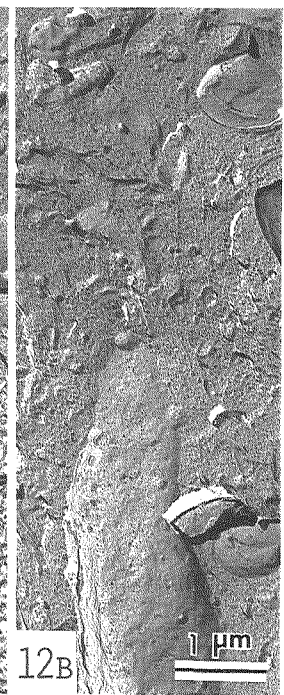
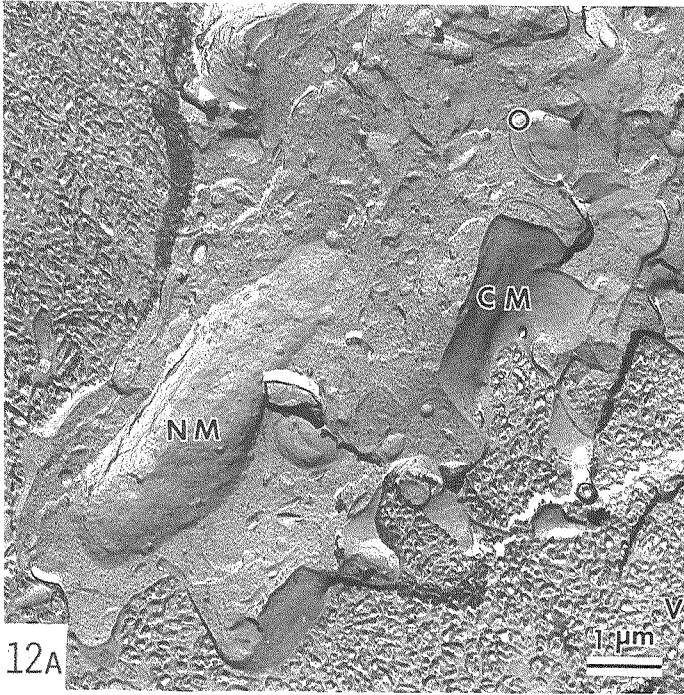
Figs. 9-11. Cross-fractured surfaces of the frozen cells observed in the specimens frozen with 2.5 M glycerol. A, lower magnification ($\times 6,000$); B, higher magnification ($\times 12,000$)

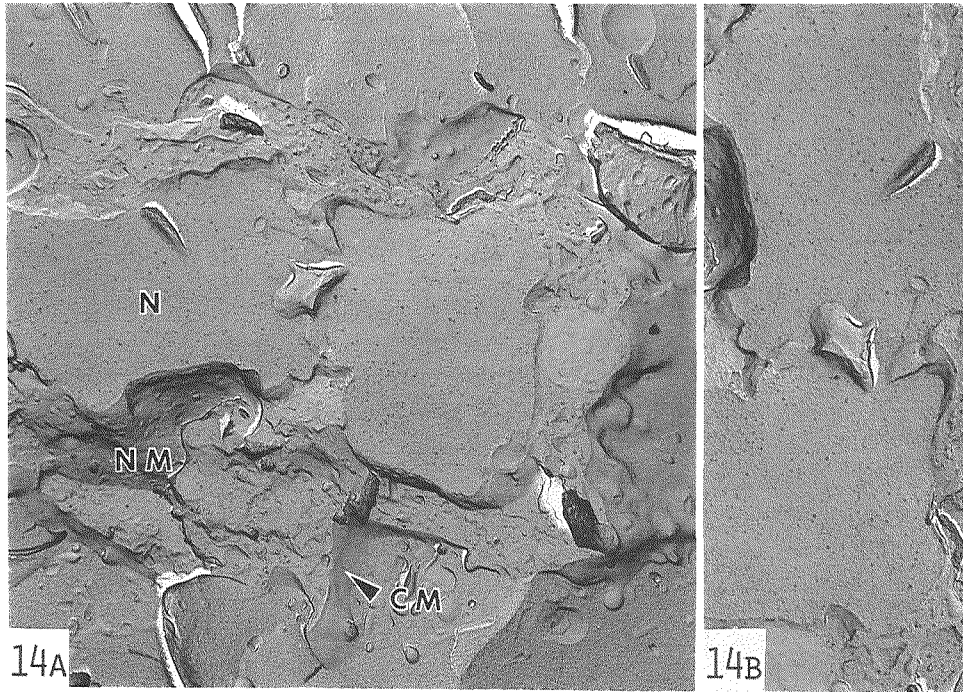
Fig. 9. A cell cooled to -196°C at $50^{\circ}\text{C}/\text{sec}$

Fig. 10. A cell cooled to -196°C at $250^{\circ}\text{C}/\text{sec}$. The cell contained fine intracellular ice crystals smaller than $0.05\ \mu\text{m}$

Fig. 11. A cell cooled to -196°C at $1000^{\circ}\text{C}/\text{sec}$. The cell contained very fine intracellular ice crystals far smaller than $0.05\ \mu\text{m}$. In the cytoplasm of the glycerolated cells, many vacuolar structures, probably originated from cytoplasmic membranous structures, were observed

during freezing. In the specimens cooled at $250^{\circ}\text{C}/\text{sec}$, intracellular ice crystals were usually larger than $0.05\ \mu\text{m}$ in individual grain size (Fig. 4). In some of the frozen cells, they were larger than $2\ \mu\text{m}$. Increasing the cooling rate to $1000^{\circ}\text{C}/\text{sec}$ generally decreased the grain size of intracellular ice crystals, although ice crystals showed a wide variety in grain size and form as observed in the specimens cooled at $250^{\circ}\text{C}/\text{sec}$. However, some of the cells cooled at $1000^{\circ}\text{C}/\text{sec}$ contained fine granulous ice crystals smaller than $0.05\ \mu\text{m}$ within their cytoplasm and nuclei (Fig. 5). In the specimens cooled at $50^{\circ}\text{C}/\text{sec}$, on the other hand, contracted cells were observed together





Figs. 12-14. Cross-fractured surface of the frozen cells observed in the specimens frozen with 1M sucrose. A, lower magnification ($\times 10,000$); B, higher magnification ($\times 12,000$). The cells were remarkably contracted by the addition of sucrose. Growth of intracellular ice crystals in each frozen cell was strongly inhibited

Fig. 12. A cell cooled to -196°C at $50^{\circ}\text{C}/\text{sec}$

Fig. 13. A cell cooled to -196°C at $250^{\circ}\text{C}/\text{sec}$

Fig. 14. A cell cooled to -196°C at $1000^{\circ}\text{C}/\text{sec}$. Ice crystal growth within the cell was completely impeded

with scarcely contracted cells. Contraction of the cells might probably indicated an occurrence of a partial extracellular freezing previous to the following intracellular freezing. Ice crystals formed in the contracted cells were generally smaller than those in scarcely contracted cells. Some of the contracted cells contained intracellular ice crystals of around $0.05\ \mu\text{m}$ in grain size (Fig. 3). Maintenance of the fine structure of the frozen cells appeared to be closely related with the grain size of intracellular ice crystals. Cytoplasmic organelles in the cells cooled at both 250° and $1000^{\circ}\text{C}/\text{sec}$, were observed to increasingly maintain their native structures with the decrease of grain size of intracellular ice crystals. On the contrary, in the cells

having relatively large ice particles, cytoplasmic organelles were deformed and obliterated by these intracellular ice crystals, except for the nuclear membranes (Fig. 4).

Addition of cryoprotective additives to suspending medium had various effects on the situation of the frozen cells, particularly on the formation of ice crystals in and around the cells. Addition of PVP was not so effective for intracellular ice crystals to decrease the grain size. The frozen cells observed were similar in morphological aspect to the cells frozen without additives, but they were somewhat contracted after very rapid cooling even at 1000°C/sec (Fig. 8). Intracellular ice crystals in the frozen cells with 20% PVP did not range in grain size so widely as compared with those formed in the frozen cells without additives. In the specimens cooled at 1000°C/sec, many of the frozen cells usually contained ice crystals of around 0.1 μm in grain size, while a few cells indicated intracellular ice crystals smaller than 0.05 μm (Fig. 8). In the cells cooled at 250°C/sec, ice crystals were usually larger than those in the cells cooled at 1000°C/sec (Fig. 7), and the intracellular ice crystals smaller than 0.05 μm were rarely observed in the specimens. By further decrease in cooling rate to 50°C/sec, some of the cells distinctly contracted during freezing, as observed in the specimens cooled at 50°C/sec without additives. In the contracted cells, grain growth of intracellular ice crystals was frequently impeded (Fig. 6). Although the cells containing intracellular ice crystals exclusively smaller than 0.05 μm were rarely observed in the specimens frozen with 20% PVP at any cooling rate, in such cells cytoplasmic organelles such as nucleus, mitochondria and endoplasmic reticulum, as a rule, maintained their native structures. Particularly, in a few cells cooled at 1000°C/sec, nuclear membrane, mitochondria and endoplasmic reticulum appeared to remain intact (Fig. 8).

Addition of glycerol to suspending medium was observed to be very effective to prevent the grain growth of ice crystals formed in rapidly cooled cells. As soon as the cells were suspended in PSS containing 2.5 M glycerol, they began to be dehydrated osmotically and contracted. Then, they nearly recovered their initial volume with gradual permeation of glycerol across the cell membranes previous to freezing. The frozen cells observed were scarcely contracted when they were cooled at rates higher than 250°C/sec. At any cooling rate, grain growth of intracellular ice crystals was strongly depressed by glycerol permeated into the cells. In the range of cooling rates between 250° and 1000°C/sec, intracellular ice crystals formed in glycerolated cells invariably decreased their grain size with the increase of cooling rate (Figs. 10, 11), as observed in the specimens with and without PVP. It was

not difficult to find frozen cells having intracellular ice crystals smaller than $0.05\ \mu\text{m}$ in every specimens frozen with glycerol. In some of the cells cooled at $1000^\circ\text{C}/\text{sec}$, intracellular ice crystals were very fine and their grain sizes were far smaller than $0.05\ \mu\text{m}$ (Fig. 11). In the specimens cooled at $50^\circ\text{C}/\text{sec}$, both contracted cells and non contracted cells were observed and not only the former but also some of the latter contained fine intracellular ice crystals smaller than $0.05\ \mu\text{m}$ (Fig. 9). Cytoplasmic organelles within glycerolated frozen cells were observed to be somewhat different in shape from those in the cells frozen with and without 20% PVP. Many vacuolar structures of various sizes were observed in the cytoplasm (Figs. 9, 10, 11). These vacuolar structures seemed to be the transformed cytoplasmic organelles such as mitochondria, endoplasmic reticulum, Golgi-apparatus, and other membranous structures. Transformation of these cytoplasmic membranous structures was assumably caused by glycerol in the cells. In addition, nucleoli were clearly recognized in the nuclei of glycerolated cells. Nucleolus was easily distinguished from others by its oval shape, in which ice crystals were extremely smaller than surrounding ones in grain size (Figs. 9, 10).

Sucrose had a remarkable effect on frozen cells and formation of intra- and extracellular ice crystals as compared with other cryoprotective additives. When the cells were suspended in PSS containing 1 M sucrose, they considerably contracted before freezing as a result of the loss of cellular water due to the increase of extracellular osmolality by the addition of sucrose. After freezing, the cells with 1 M sucrose remained contracted. There was little difference in degree of contraction among the cells frozen at various cooling rates. The peripheries of frozen cells appeared irregular being surrounded by extracellular ice particles (Figs. 12, 13, 14). In the specimens frozen with 1 M sucrose at various cooling rates, grain growth of intracellular ice crystals was strongly depressed (Figs. 12, 13, 14). Ice crystals formed in these cells gradually decreased their grain size with increase in cooling rate from 50° to $1000^\circ\text{C}/\text{sec}$. In some of the cells cooled at $1000^\circ\text{C}/\text{sec}$, growth of intracellular ice crystals appeared to be completely impeded (Fig. 14). In addition, some of the frozen cells in every specimen contained fine granulous ice crystals smaller than $0.05\ \mu\text{m}$ in diameter. The extracellular ice crystals formed in sucrose solutions were always smaller in grain size than those formed in any other suspending medium.

IV. Discussion

Freezing injury and action of cryoprotective additives in living cells have been investigated by many authers^{5,10-12,23-28}. It was assumed from these

investigations that injury in animal cells caused by freezing probably correlated with increased concentration of solutes produced in and around cells as ice formed. Increased concentration of solutes, which means increased ionic strength, increased osmolality, is closely associated with water release across cell membrane and decreased cell volume. Each of these factors is considered to be responsible for causing cell injury during freezing. Cryoprotective additives including sugars, glycols and polymers, therefore, seem to protect living cells during freezing by reducing the concentration of solutes in unfrozen liquid phase at any given temperatures.

On the other hand, it is well known that living cells are very apt to freeze intracellularly, when they are cooled rapidly. Formation of intracellular ice crystals is considered as another main factor of freezing injury⁴⁻¹⁰. Recently some authors electron microscopically observed the frozen cells using the freeze-etching techniques. These results^{15-19,21,22} with post-thawing survival assays in rapidly frozen cells lead to an assumption that the cause of fatal injury in rapidly frozen cells is the growth of intracellular ice crystals rather than their initial formation. MOOR *et al.*¹⁵ already demonstrated the effect of glycerol on the formation of intracellular ice crystals in yeast cells under various cooling conditions. They reported that the addition of 20% glycerol and the cooling higher than 100°C/sec were effective to maintain viability of the cells, thereby helping the vitrification of the cells during freezing.

Survival curves obtained from the present experiments revealed that the post-thawing survivals of rapidly frozen HeLa cells were affected in various degrees by both cooling velocity and cryoprotective additives. It seems obvious through the electron microscopic observation of frozen cells that the effects of cooling velocity and cryoprotective additives on maintenance of viability of rapidly frozen cells were closely related with their efficiency on the retardation of intracellular ice crystal growth during freezing. In the specimens frozen with moderately hypertonic solutions (20% PVP and 0.5 M sucrose), post-thawing survivals were strongly affected by cooling velocity. Cooling at very rapid and relatively slow rates was beneficial for cell survival rather than at intermediate rates. That is, V-shaped survival vs. cooling velocity curves were obtained. This may be attributed to the decrease in the grain size of intracellular ice crystals promoted by the increase of cooling velocity and by the decrease of cellular water during extracellular freezing in relatively slow cooling. On the contrary, in the specimens frozen with more hypertonic solutions (1.25, 2.5 M glycerol and 1 M sucrose), post-thawing survivals were affected by the addition of cryoprotective reagents itself rather

than by cooling velocity. The electron microscopic observations revealed that the grain growth of intracellular ice crystals was strongly inhibited regardless of cooling velocity probably by sufficient substitution or dehydration of cellular water before freezing. In the case of rapid freezing, therefore, glycerol, sucrose and PVP seem to act as inhibitory reagents against intracellular ice crystal growth depending upon their molar concentrations in suspending medium regardless of their permeability. In the case of relatively slow freezing, on the other hand, it may be noted that these reagents plainly protect the cells from salt injury results from the extracellular ice formation.

ASAHINA and SHIMADA²⁰⁻²²⁾ reported that in rapidly frozen cells the vitrification of protoplasm was not always necessary for survival, but the formation of intracellular ice crystals smaller than a certain critical size was certainly tolerable. They demonstrated in rat ascites tumor cells that intracellular ice crystals appeared invariably fatal to the cells whenever they grew to larger particles than $0.05\ \mu\text{m}$ in diameter, although the actual dimension of critical size remained uncertain. According to MOOR¹⁶⁾, the yeast cells cooled rapidly to temperatures below -150°C abruptly lost their viability at a temperature above -40°C during slow rewarming process by recrystallization or devitrification of intracellular ice. He suggested that if there was a certain critical size of innocuous intracellular ice crystals it would be $100\ \text{\AA}$ ($0.01\ \mu\text{m}$) in diameter at maximum. Similar results were obtained later by NEI and ASADA¹⁷⁾, and BANK^{18,19)}.

As a result of the present experiments, it appears reconfirmed that the vitrification of protoplasm is not always necessary for cell survival of rapidly frozen cells. Although a clear quantitative estimation was very difficult, a rough number of the cells having fine intracellular ice crystals smaller than $0.05\ \mu\text{m}$ seemed to well correspond to the post-thawing survival rate of the frozen cells in each specimen.

The physico-chemical meaning of the lethal injury by the grain growth of intracellular ice crystals is not known in detail. However, the mechanical force produced by the growth of intracellular ice crystals itself seems primarily lethal to delicate and complicate cellular membrane systems²⁹⁾, since extracellular freezing in the same cells was already proved to be much more harmless than intracellular freezing³⁰⁾.

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