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The Site of Cryoprotection of Glycerol in Mouse Fibroblast (Strain L Cell)

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The cryoprotective action of glycerol on the strain L cells was studied. Glycerol, even when present only in the cell interior or only in the surrounding medium, apparently furnishes the cells cryoprotection to some extent, but glycerol in the cell interior seems to be more effective than in the medium. Its presence both in the cell interior and in the medium is a requisite for perfect protection against freezethawing injury.

Since Polge, Smith and Parks[2)] discovered that spermatozoa of the fowl and human could be frozen and thawed without loss of motility when glycerol was added to their suspending medium, the cryoprotective action of glycerol has made possible the preservation by freezing of the cells and tissues.[3–5)] The mechanism of the cryoprotection of glycerol, however, is not clear. Lovelock[3)] suggested from his experiments on hemolysis by freezing and thawing that the intracellular glycerol is essential to the survival of human erythrocyte, and he generalized that living cells can be protected from the damage by freezing, only when fully permeated by glycerol before freezing.[6,7)]

On the other hand, Doebbler and Rinfret[8)] showed that certain disaccharides and polymers which can not enter red blood cells possess greater cryoprotective action than permeable most monosaccharides, and suggested that their protective activity could be correlated with concentration of the potential hydrogen-bonding groups provided by the solutes. Sherman[9)] too, on the basis of structure of the unfertilized mouse eggs, proposed that the site of protective action of glycerol during freezethawing is extracellular. However, all the works men-

1) Location: *Katahiracho, Sendai*; *a*) Present address: *Department of Chemical Pharmacology, Tohoku University School of Medicine.*
2) C. Polge, A.U. Smith and A.S. Parkes, *Nature*, **164**, 666 (1949).
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7) J.E. Lovelock and C. Polge, *Biochem. J.*, **58**, 618 (1954).
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tioned above have given no information about the quantitative relation between the amount of glycerol in the cells and their survival rate after freezethawing.

The author investigated the effect of glycerol on the preservation by freezing of the strain HeLa and strain L cells and further whether there is a relation between the survival rate and the quantity of glycerol penetrated into the cell.

Experimental

Strain L cells derived from the mouse subcutaneous tissue have been maintained in our laboratory by continuous cultivation since 1961. In this experiment the cells were used at the early stationary phase six days after inoculation.

Procedures for Collection of Cells——After the nutrient culture medium (YLHS, culture medium containing yeast extract, lactalbumin hydrolysates and 20% bovine serum) was discarded, 0.02% trypsin solution in a Ca and Mg-free Rinardini (balanced salt solution) was added. After incubating for 5—10 minutes at 37°, the bottles were vigorously shaken to detach the cells. The cell suspension thus prepared was then centrifuged at 2000 rpm for 5 minutes. The pellet was resuspended in the buffer solution. After centrifugation at 2000 rpm for 5 minutes, the supernatant was discarded and YLHS added so as to reach a given density of $1—6\times10^7$ cells per ml. After incubation at 37° for 1—2 hr, the cell suspension was pipetted into the glass tube for experiment.

Freezing and Thawing Method——Throughout this experiments the low temperature was prepared by supercooling apparatus (subzero, Osaka Kinzoku Co. Ltd.). The freeze-thawing condition used was as follows: 1.5×10.7 cm glass tube containing 0.5 ml of the cell suspension was immersed directly in the ethanol bath, and kept there for 3 minutes after the cell suspension reached the required temperatures. The frozen cell suspension was quickly thawed in a water bath of 37°. The temperature of the suspension was recorded with a thermojunction consisting of copper and constantan wires of 50 μ in diameter and a selfrecording voltmeter.

Eosin Exclusion Technique for Cell Viability[10]——Immediately after quick thawing, 0.5 ml of 0.2% aqueous eosin-Y solution was mixed with the cell suspension and the unstained cells counted by means of a hemocytometer were regarded as the cells survived freezing and thawing.

Estimation of the Intracellular Glycerol——After washing, the cells suspended in 3 ml of the PBS (phosphate buffer salt solution) were homogenized by a tephlon homogenizer for 20 minutes. Mixture of the homogenate and of the PBS of 2 ml used for rinsing the homogenizer was centrifuged (1500 rpm for 20 min) and the supernatant was used for estimation of glycerol. The estimation was carried out colorimetrically after the procedure of Hanahan and Olley.[11]

Results and Discussion

Protective Action of Glycerol against Freeze-thawing Damage

Throughout the experiments the L cells were frozen for 3 minutes after a required temperature had been recorded. Therefore the total periods of freezing time varied according to the low temperatures required. Fig. 1 shows the representative freezing curve of the cell suspension containing 5% glycerol. In this case the cell suspension required the immersion of 7.5 minutes in the cooling bath for retaining −40° for 3 minutes.

The freezing injury of L cells suspended in the YLHS free from glycerol commenced with about −6.5°, and then the survival rate sharply declined as clearly shown in Fig. 2. On the contrary, the injury of the cells suspended in the same medium but containing 20% glycerol increased gradually with lowering temperature, and the survival rate appeared to settle on about 50% below −50° (Fig. 2). Hence the protective action of glycerol against freeze-thawing injury for L cells was well demonstrated.

The Relation between the Survival Rate and the Quantity of the Glycerol penetrated into the Cell Interior

Whether there exist a relation between the survival rate and the amount of glycerol penetrated into the cell was next examined. About 7.5×10^6 L cells were suspended in the

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11) D.J. Hanahan and J.N. Olley, *J. Biol. Chem.*, **231**, 813 (1958).

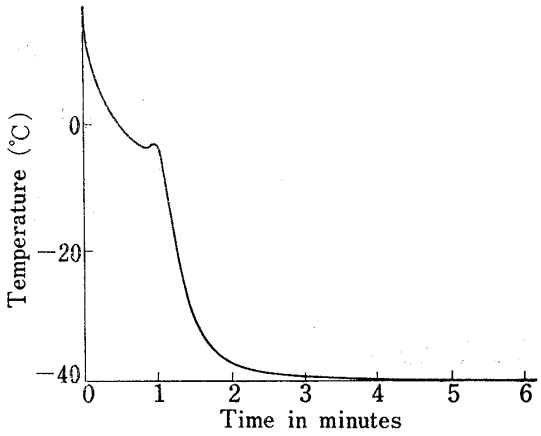

Fig. 1. Freezing Curve of Cell Suspension

cell suspension: 1.7×10^7 cells/0.5 ml of 5% glycerol–YLHS medium
test tube: 1.5 cm×17 cm
cooling rate: 12.6°/minute
final temperature : −40°
freezing point: −3.2°

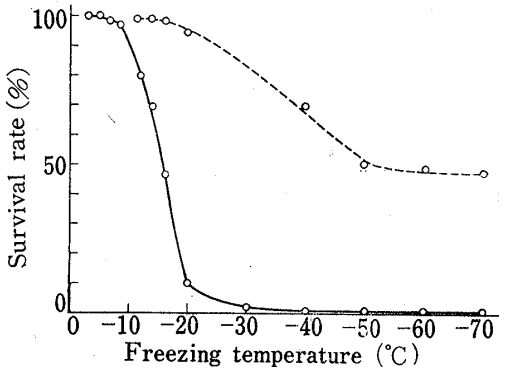

Fig. 2. The Relation between the Survival Rate of the Strain L Cell and the Freezing Temperature

thawing temperature: 37°
The cells were suspended in YLHS medium containing 20% glycerol for 30 minutes at room temperature (···O···) and in YLHS medium without glycerol (—O—), then they were frozen at each temperatures for 3 minutes after a required temperature had been recorded.

YLHS containing glycerol of 20% by volume. After incubation for given period of time at room temperature (18°), the cells were washed once with the glycerol-free PBS and then homogenized. The homogenate was centrifuged at 1500 rpm for 20 minutes and the amount of glycerol in the supernatant was estimated. In parallel, the replicated cell suspension incubated in all the same way was frozen at −40° and thawed quickly in a water bath at 37°, and then the survival rate of the cells was estimated.

The results shown in Fig. 3 indicates the intimate correlation between the survival rate and the intracellular amount of glycerol, suggesting that intracellular glycerol may play a role for cryoprotective action.

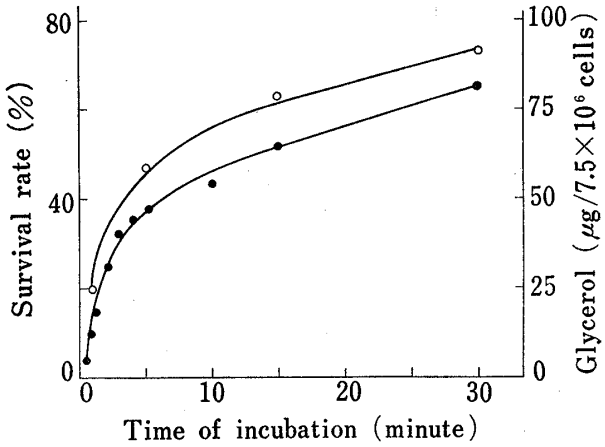

Fig. 3. The Relation between the Survival Rate of the Strain L Cell and the Quantity of the Glycerol permeated into the Cell

freezing temperature: −40°
thawing temperature: 37°
After the incubation in YLHS medium containing 20 % glycerol at room temperature (18°) for each time the cells were frozen. Glycerol estimation was carried out after the cells had been washed with saline solution (PBS) at room temperature for 5 minutes.
The open circles show the glycerol found in the homogenized cells and the closed circles show the survival rate of the cell.

The Role of Glycerol Inside or Outside the Cells for Cryoprotective Action

In the above experiments, on the one hand, the exact amount of glycerol within the cells could not be found as yet because of possibly insufficient washing. Through the period of incubation, on the other hand, glycerol in a high concentration was in the medium. Thus, the results mentioned above can not be said to be conclusive of decisive role of intracellular glycerol for cryoprotective action. To clearify this point, the following attempts were made. First, the time course of permeation of glycerol at 37° was studied by suspending the cells in the YLHS containing 5% glycerol. The amount of intracellular glycerol was found to reach the maximum within about 10 minutes. Therefore in the following experiments the incubation time was settled on 10 minutes.

After the cells were incubated in the YLHS containing 5% glycerol for 10 minutes at 37°, they were washed with the PBS of 2° and of 18° to remove the extracellular glycerol. After 8 times' washings no glycerol was found in the washing solution, while a small amount of glycerol (45 μg/4.85$\times 10^7$ cells at 2° and 15 μg at 18°) was still found within the cell. Assuming that the L cells are columnar (20 μ in diameter and 2 μ in height) and contain 70% water by volume, the intracellular glycerol concentrations may be calculated roughly at about 23 mM and 7.7 mM, respectively. Hence in the next experiments, the washing of the cells was carried out with saline solution of 2°.

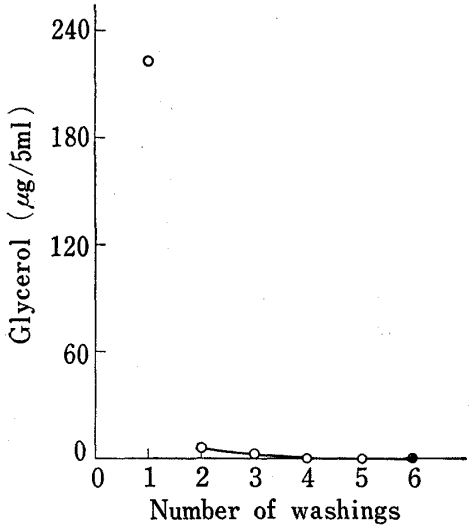

Fig. 4. The Penetration of Glycerol into the Cell at Low Temperature (2°) for 1 Minute

The cells were washed with saline solution (PBS) at 2° for 3 minutes per one time after the incubation in the YLHS medium containing 5% glycerol for 1 minute at 2°.

Open circles (○) show the glycerol contained in the suspending medium (PBS) when the cells were washed, and the closed circle (●) the glycerol contained in the homogenized cells (2.6×10^7) after the cells were washed 6 times. Almost all glycerol found in the suspending medium at the first time are that adhered to the outer surface of the cell membrane and to the wall of the test tube.

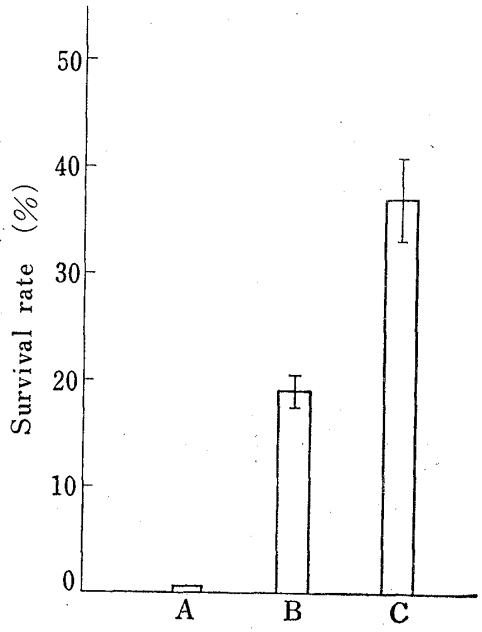

Fig. 5. Survival Rate of Strain L Cells after Freeze–thawing

freezing temperature: −40°
thawing temperature: 37°

A) The cells were frozen in the YLHS medium free from glycerol.

B) The cells were washed 8 times with cold saline solution (PBS, 2°) after incubation in 5% glycerol–YLHS for 10 minutes at 37°, then frozen in YLHS. The glycerol was contained only in the cell interior (about 23 mM).

C) The cells suspended in cold 5% glycerol–YLHS medium were frozen after the same treatments as B). About 23 mM glycerol was contained in the cell interior and about 0.5M in the suspending medium.

Next, the permeation of glycerol into the cells at low temperature (2°) for 1 minute was estimated. After the cells were incubated in the cold YLHS containing 5% glycerol for 1 minute, almost all the glycerol was found in the washing solution and no glycerol in the homogenized cells (Fig. 4). This indicates that a little glycerol permeates into the cell at 2° for 1 minute. Taking the result into consideration, then, the survival rate of the cells after freeze–thawing was investigated under the following conditions.

(A) The cells were frozen in the YLHS free from glycerol.

(B) The cells were washed 8 times with the cold saline solution (PBS, 2°) after incubation in the 5% glycerol–YLHS at −40°. The glycerol was contained only within the cell (about 23 mM).

(C) The cells suspended in the cold 5% glycerol–YLHS were frozen at −40° within 1 minute after the same treatment as (B). About 23 mM glycerol was contained in the cell

and about 0.5M glycerol in the suspending medium. The results obtained are presented in Fig. 5.

From the results of experiment (B) it was clearly shown that the intracellular glycerol had apparently the cryoprotective capacity, but the extracellular glycerol also produced good cryoprotection as shown in (C).

To ascertain this fact more precisely, an aqueous solution of cupric chloride was used as a washing solution for preventing the leakage of glycerol from the cell during washing. First, suitable cupric chloride concentration and duration of washing were determined, and it was found that 7 times' washing of the cells at room temperature (18°) for 5 minutes at a time with PBS containing 10^{-4}M cupric chloride was sufficient. Under these conditions, glycerol of 60 μg per 5.7×10^7 cells was retained in the cell after washing 7 times (Fig. 6). This amount of the intracellular glycerol corresponds to about 26 mM. For this reason, in the following experiments PBS containing 10^{-4}M cupric chloride was used for washing the cells.

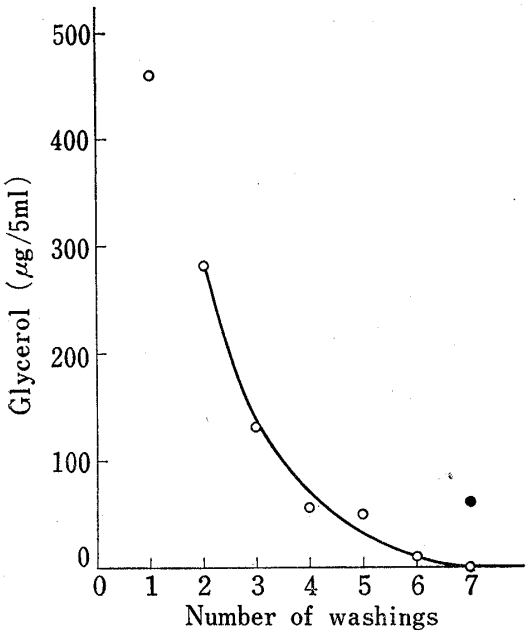

Fig. 6. The Course of Exit of Glycerol from the Cells

The cells were washed with saline solution (PBS) containing 10^{-4}M $CuCl_2$ at room temperature (18°) for 5 minutes per one time after incubation in the YLHS medium containing 5% glycerol for 10 minutes at 37°. Open circles (○) show the glycerol contained in the suspending solution (PBS) when the cells were washed, and the closed circle (●) the glycerol contained in the homogenized cell after the cells (5.7×10^7) were washed 7 times. This glycerol with the closed circle corresponds to about 26 mM. Almost all glycerol found in the suspending solution at the first time are that adhered to the outside of the cell membrane and the wall of the test tube.

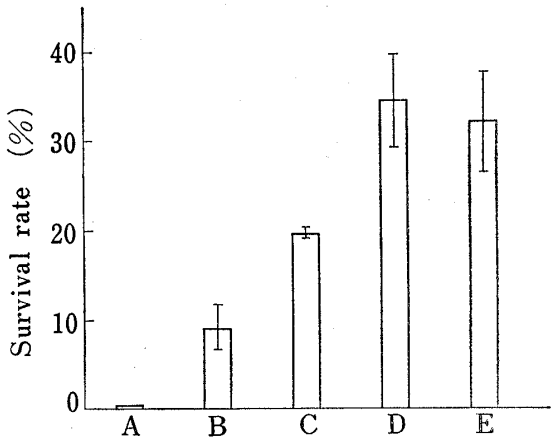

Fig. 7. Survival Rate of the Strain L Cells after Freeze–thawing

freezing temperature: −40°
thawing temperature: 37°
A) The cells were frozen in the YLHS medium.
B) The cells were frozen immediately after suspending in the cold (2°) YLHS medium containing 5 % glycerol.
C) After pre–treatment with 5 % glycerol at 37° for 10 minutes, the cells were washed 7 times with the saline solution (PBS) containing 10^{-4}M cupric chloride. Then the cells were frozen in the YLHS medium.
D) After the same treatments as C), the cells were frozen in the cold YLHS medium containing 5% glycerol.
E) After incubation at 37° for 10 minutes in the YLHS medium containing 5% glycerol, the cells were frozen in this medium.

Fig. 7 illustrates the survival rate of the strain L cells after freezing for 7.5 minutes at −40° under the following conditions.

(A) The cells were frozen in the YLHS in which no glycerol was contained.

(B) The cells were frozen immediately after being suspended in the cold (2°) YLHS containing 5% glycerol. Glycerol exists only in the suspending medium and not within the cell.

(C) After pre–treatment with 5% glycerol at 37° for 10 minutes, the cells were washed 7 times with the saline solution containing cupric chloride at room temperature. Then the

cells were frozen in the pure YLHS. Under the freezing condition, glycerol was contained only within the cell at a concentration of 26 mM.

(D) After the same treatment as (C), the cells were frozen immediately in the cold (2°) YLHS containing 5% glycerol. In this case, the glycerol concentrations within the cell and in the suspending medium were about 26 mM and 0.5M, respectively.

(E) The cells were incubated at 37° for 10 minutes in YLHS containing 5% glycerol, and then frozen in this medium. In this case, the cells were frozen under the full permeation by glycerol.

In Fig. 7, about 9% survival rate in (B) suggests that the extracellular glycerol also considerably protects the cells against freeze–thawing injury. Although this result seems to coincide with the opinion of Sherman[11] that the site of protective action of glycerol during freeze–thawing of mouse eggs is extracellular, the result shown in Fig. 7 (C) is contrary to his opinion and strongly suggests that the intracellular glycerol is more effective for the cryoprotection than the extracellular. The same results were obtained in the previous experiments shown in Fig. 5 (B). At least for the L cell the intracellular glycerol was not detrimental though Sherman[11] had so stated. The maximal survival rate obtained when glycerol existed at both the interior and exterior of the cells as clearly shown in Fig. 7 (D) and (E). These results would lead to the conclusion that the cryoprotective actions of glycerol within the cell and in the medium would be additive in the L cell.

The cryoprotective action of the particular substances may be much more complicated. For example, according to Doebbler and Rinfret[10] disaccharide to which red blood cells are impermeable afforded greater protection than many monosaccharides which are permeable to red blood cells under rapid cooling and rapid warming, whereas Lovelock[8] stated that sucrose and polyethylene glycol to which red blood cells are impermeable showed no cryoprotective action in any concentration under slow cooling and slow warming. Considering these facts and the biological variability, to give a solution of the mechanisms of cryoprotection during freeze–thawing more systematic experiments should be carried out under the same experimental conditions.

Acknowledgement The author is indebted to Prof. Kiyoshi Aoki, of the Biological Institute, Tohoku University, for his interest and valuable advice. Thanks are also due to Mr. Takato Mayahara, of the Pharmacological Institute, Showa University, for his suggestions and encouragement. The cooperation of Mr. Yoshinobu Shimizu of the Bacteriological Institute, Tohoku University School of Medicine, in maintenance of the strain L cells, is appreciated.