

## On the Cryo-biological Study of the Parasitic Protozoa

(1) Studies on the freezing conditions of trichomonads  
in a  $-25^{\circ}\text{C}$  and a  $-75^{\circ}\text{C}$  freezer

**Akira MIYATA**

*Department of Epidemiology, Institute for Tropical Medicine  
Nagasaki University*

(Chief : Prof. Toshio NAKABAYASHI)

(Received for Publication, September 3, 1973)

### Abstract

Examinations were done of the cooling method, the effect of the cryo-protective substance such as glycerol (GLY) and dimethyl sulfoxide (DMSO), and of salt concentration on the low temperature preservation of *Trichomonas vaginalis* and *Tritrichomonas foetus* in a  $-25^{\circ}\text{C}$  or a  $-75^{\circ}\text{C}$  freezer.

The following results were obtained:

1. In the  $-25^{\circ}\text{C}$  freezer trichomonad survived for several days in the presence of GLY or DMSO. The longest survival record was two weeks in *T. vaginalis* and one week in *T. foetus*, whereas the survival rate in either species was quite low.
2. Both the trichomonad species in a sample with GLY or DMSO placed directly into the  $-75^{\circ}\text{C}$  freezer could survive for a month or longer, but the survival rate was low.
3. The pre-cooling method in which a sample was pre-cooled in the  $-25^{\circ}\text{C}$  freezer then transferred into the  $-75^{\circ}\text{C}$  freezer was found to give a good effect to a rise in the survival rate of trichomonads.
4. In a 5-day storage in the  $-75^{\circ}\text{C}$  freezer by the pre-cooling method, the survival rate became higher than 70% in *T. vaginalis* and nearly 10% in *T. foetus* in the presence of 2.5~7.5% DMSO, while it remained lower than 20% in *T. vaginalis* and only several percents in *T. foetus* in the presence of 10% GLY.
5. In a one-day storage in the presence of 5.0% DMSO by the pre-cooling method, the survival rate of *T. vaginalis* was higher in 0.9~1.5% NaCl than in the lower salt concentration. As the highest survival rate of *T. foetus* was 30.4% in 0.3% NaCl, the optimum salt concentration for *T. foetus* was thought to be lower than that for *T. vaginalis*.

6. As a conclusion it could be said that the pre-cooling method might be useful for the low temperature preservation of trichomonad and would be applicable to the cases of other parasitic protozoa.

### Introduction

Gaylord (1908) reported that *Trypanosoma gambiense* could resist freezing in liquid air for 20 minutes, and De Jong (1922) also mentioned that *Trypanosoma equiperdum* survived for 21 days in  $-190^{\circ}\text{C}$  liquid air.

Coggeshall (1939) reported that malaria infected blood cells which were stored for 70 days in a  $-76^{\circ}\text{C}$  dry-ice box still could be infective to a fresh host. Horsfall (1940) and Manwell *et al.* (1942, 1943 a & b) also published their reports. According to Weinman *et al.* (1947), *Trypanosoma*, *Plasmodium* and *Leishmania* could be preserved safely for several months in a  $-70^{\circ}\text{C}$  to  $-79^{\circ}\text{C}$  dry-ice cabinet, but *Entamoeba histolytica*, *Trichomonas vaginalis*, *Toxoplasma gondii* and *Balantidium sp.* could not.

Since Polge *et al.* (1949) found that glycerol could be used as a cryo-protective substance for the freezing preservation of bull semen, many workers have attempted to use glycerol for the freezing preservation of protozoa. Fulton *et al.* (1953) reported that *E. histolytica* could survive for 65 days at  $-76^{\circ}\text{C}$  in the presence of 5~10% glycerol. *T. vaginalis* by McEntegart (1954) and *T. foetus* by Levine *et al.* (1955) were found to be ca-

pable of survival for a long period at  $-76^{\circ}\text{C}$  to  $-79^{\circ}\text{C}$  in the presence of glycerol.

Lovelock *et al.* (1959) found out that dimethyl sulfoxide had a cryo-protective action similar to glycerol in the freezing preservation of the living cell. This new cryo-protective substance also has been used for the freezing preservation of parasitic protozoa by many workers.

The freezing preservation of protozoa was thought to be useful to maintain many kinds of protozoa strains in a laboratory. Therefore, since Coggeshall (1939), attempts have been made to preserve protozoa in a freezing condition safely for a long period. Those papers were reviewed by Mühlpfordt (1960), Smith (1961) and Diamond (1964) respectively. Shorb (1963) also reviewed concerning the freezing preservation of trichomonads. But very few papers have been published as to the cryo-biological study in the freezing preservation of parasitic protozoa. In the present paper an attempt was done to further advance the knowledge on the basic study of the low temperature preservation of *T. vaginalis* and *T. foetus* in a  $-25^{\circ}\text{C}$  and a  $-75^{\circ}\text{C}$  freezer.

### Materials and Methods

These experiments were carried out as the following procedure:

1. Strain TK of *Trichomonas vaginalis* originally isolated from a patient of trichomo-

niasis in Nagasaki City by the author, and strain Yamaguchi of *Tritrichomonas foetus* was given from Chugoku Branch Laboratory, the National Institute of Animal Health.

V-Bouillon (Modified CPLM by Hamada, 1953)		Modified Diamond's Medium (Diamond, 1957)	
Peptone	2.0g	Trypticase	2.0 g
Yeast extract	0.2g	Yeast extract	1.0 g
Liver extract	0.2g	L-ascorbic acid	0.02g
Glucose	1.0g	Maltose	0.5 g
L-cysteine HCl	0.2g	L-cysteine HCl	0.1 g
NaCl	0.9g		

↓

Dissolve in 100 ml of boiling distilled water.

↓

Adjust pH to 6.5 with NaOH.

↓

Dispense 4.5 ml in test tube.

↓

Autoclave at 15 pounds pressure for 20 minutes.

↓

Heat in boiling water for a few minutes and cool rapidly in water, then add 0.5 ml of inactivated bovine serum to each tube just before using.

↓

Dissolve in 100 ml of boiling distilled water.

↓

Adjust pH to 6.5 with NaOH.

↓

Dispense 4.5 ml in test tube.

↓

Autoclave at 15 pounds pressure for 20 minutes.

↓

Add 0.5 ml of inactivated bovine serum to each tube just before using.

**Table 1.** V-Bouillon and Diamond's Medium for trichomonad culture.

Both strains were cultivated axenically in V-Bouillon or Diamond's Medium for 48 hours at 37C, and the culture was centrifuged at 350×g for 10 minutes. Then the upper half of the supernatant was discarded and trichomonads were resuspended in the rest of the medium. In Table 1, the components of V-Bouillon and modified Diamond's Medium were presented.

2. Twice as much as the required final concentration of a cryo-protective substance was prepared in distilled water. In these experiments, two kinds of cryo-protective substances, dimethyl sulfoxide ((CH<sub>3</sub>)<sub>2</sub>SO, DMSO) and glycerol (CH<sub>2</sub>(OH)CH(OH)CH<sub>2</sub>(OH), GLY) were used. The concentration of substance was shown as volume per volume percent.

3. The diluted cryo-protective substance was mixed into the protozoa suspension, then one ml of the mixture was distributed into each

small test tube with a rubber cap.

4. The mixture was allowed to equilibrate at room temperature for 10 minutes for DMSO or 30 minutes for GLY.

5. After equilibration was finished, the tubes were placed in a -25C freezer (HITACHI Super Freezer, -25±2C) or a -75C deep freezer (REVCO ULT-657, -75±10C). The cooling rate of the samples was recorded automatically with an electric recording thermometer (Chino Works, LTD., ET-1200-IRSP, copper-constantan thermocouple, range of measurement +50~-200C, equilibration time 1 sec.)

6. The samples frozen were rapidly thawed in a 37C water bath, and the survival and dead trichomonads were counted with Thoma's haemocytometer. The survival rate was expressed as follows:

$$\text{Survival rate (\%)} = \frac{\text{No. of survival trichomonads}}{\text{No. of dead trichomonads} + \text{No. of survival trichomonads}} \times 100$$

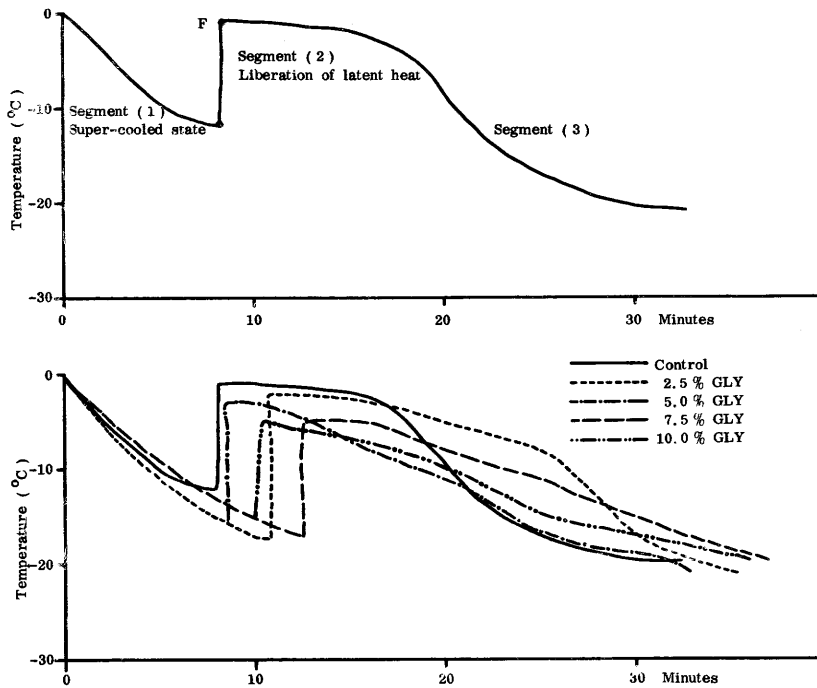


Fig. 1. The cooling curves of samples in the presence of GLY.

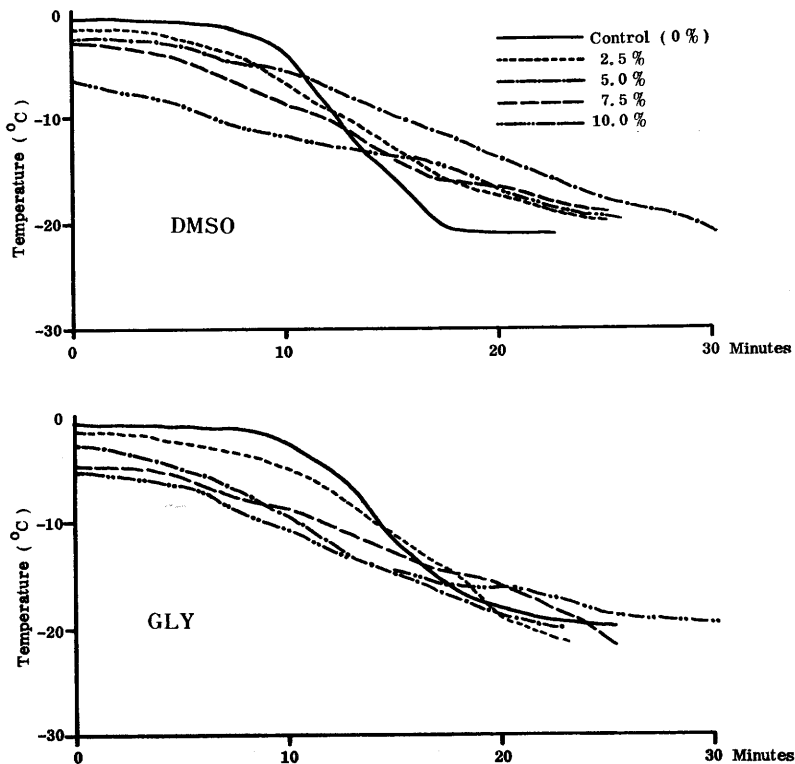


Fig. 2. The cooling curves in the -25C freezer.

## Results

### *Experiment 1. Freezing in a -25C freezer.*

In the -25C freezer, the cooling curve of the sample without cryo-protective substance was consisted of three main segments (Fig. 1 upper).

- (1) The first segment: drops without freezing, and the super-cooled state is continuing.
- (2) The second segment: goes up by the liberation of the latent heat of crystallization, and drops again gradually.
- (3) The third segment: drops more rapidly. The cooling curve of this segment has a similar tendency as that of the first segment. The cooling rate described in the paper was expressed as that in the segment 3.

In the sample with GLY, the super-cooled state continued longer, and the cooling rate and the freezing temperature were lower than those in the control sample without GLY. The cooling curve of the sample in each concentration of GLY was shown in Fig. 1 lower. The cooling curves of the samples in each concentration of GLY and DMSO after passing off the freezing point (F) were in Fig. 2.

In the control sample without a cryo-protective substance the motile *T. vaginalis* and *T. foetus* decreased rapidly in number around -20C and no motile trichomonad was found after a day. While in the sample with 2.5% DMSO the survival rates of *T. vaginalis* and *T. foetus* were higher than 90% at -20C (Fig. 3) and the longest record of storage at -25C was 2 weeks in *T. vaginalis* and one week in *T. foetus*. The motile trichomonads detected microscopically, however, were very few and incapable of multiplying in culture. Attempts were repeated several times and it was clearly understood that these two kinds of trichomonads could

not survive for longer than two weeks in the -25C freezer.

### *Experiment 2. Freezing in a -75C freezer.*

In the -75C freezer the cooling curves of the samples with DMSO or GLY were presented in Fig. 4. The super-cooled state did not continue as long as in the -25C freezer. The freezing temperature dropped in proportion to the concentration of the cryo-protective substance.

To examine the survival rate of trichomonads at several points in the cooling curves, the thermocouple was put in one of the samples with GLY or DMSO. When the temperature of the sample dropped to the point respectively, another sample was taken out from the freezer to examine the survival rate. The results were shown in Fig. 5 and

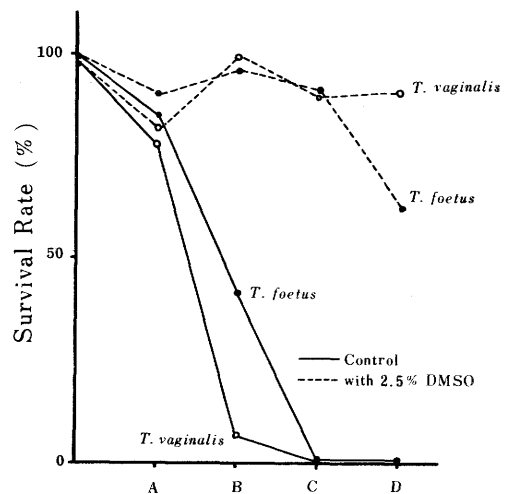


Fig. 3. The freezing preservation of trichomonads in the -25C freezer.

A : at -10C, B : at -20C,

C : stored for 30 minutes after temperature reached -20C,

D : stored for 1 hour after temperature reached -20C.

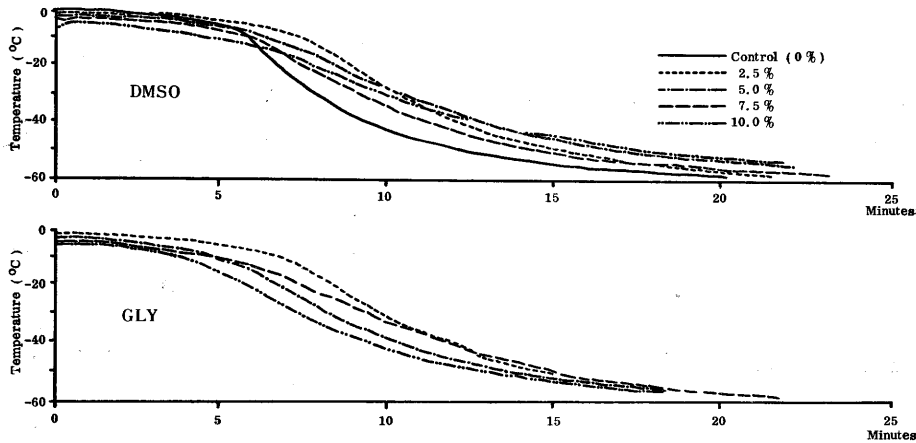


Fig. 4. The cooling curves in the  $-75^{\circ}\text{C}$  freezer.

6. DMSO was found more effective in cryo-protective activity than GLY. In a concentration of the cryo-protective substance higher than 10%, the survival rate was at a low level. These cryo-protective substances might be toxic to trichomonad cells in high concentration.

In the control sample without any cryo-protective substance, the survival trichomonads were detected at temperatures higher than  $-45^{\circ}\text{C}$ , but at the lower temperatures no survival trichomonad was detected microscopically.

Both trichomonad species could survive for a month or more by use of this direct cooling method in the  $-75^{\circ}\text{C}$  freezer in the presence of DMSO or GLY, but the survival rate was low. The same experiment was repeated with *T. foetus*, but the survival rate of this species was very low in comparison with *T. vaginalis* even in the presence of DMSO or GLY.

#### Experiment 3. Effect of pre-cooling [in a $-25^{\circ}\text{C}$ freezer.

In this experiment the samples with 5.0% DMSO were stored in the  $-75^{\circ}\text{C}$  freezer after pre-cooling in the  $-25^{\circ}\text{C}$  freezer, and the effect

of the pre-cooling period was compared. The samples were divided into 5 groups (A, B, C, D, and E) and pre-cooled in the  $-25^{\circ}\text{C}$  freezer as described below.

A) pre-cooled until the temperature of the samples reached  $0^{\circ}\text{C}$ .

B) pre-cooled until the temperature of the samples reached  $-10^{\circ}\text{C}$  after passing off the freezing point.

C) pre-cooled until the temperature of the samples reached  $-20^{\circ}\text{C}$  after passing off the freezing point.

D) pre-cooled for one hour after the temperature of the samples reached  $-20^{\circ}\text{C}$ .

E) pre-cooled for two hours after the temperature of the samples reached  $-20^{\circ}\text{C}$ .

After pre-cooling, the samples were stored in the  $-75^{\circ}\text{C}$  freezer for 5 days. Then, the samples were thawed rapidly in a  $37^{\circ}\text{C}$  water bath and examined for the survival rate of trichomonad. Each group was composed of 10 samples in *T. vaginalis* and 5 samples in *T. foetus* and the range of survival rate were presented respectively in Fig. 7. In both the species the survival rate of trichomonad in group C, D and E were higher than that in group A.

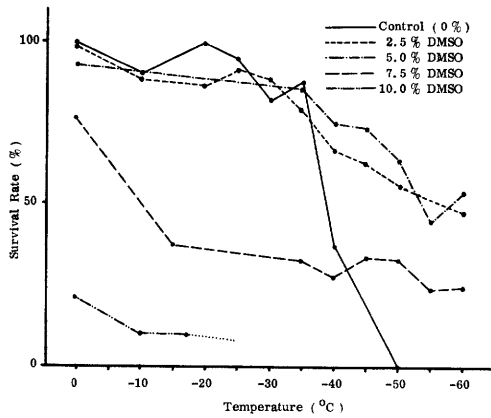


Fig. 5. The survival rate of the sample cooled directly in the -75C freezer in the presence of DMSO. (*T. vaginalis*)

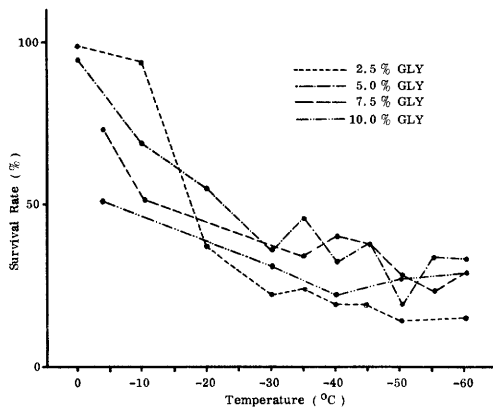


Fig. 6. The survival rate of the sample cooled directly in the -75C freezer in the presence of GLY. (*T. vaginalis*)

Experiment 4. Effect of the concentration of GLY or DMSO.

In this experiment, the effect of [the concentration of DMSO and GLY for the survival of trichomonad in a frozen state was examined by the pre-cooling method used in experiment 3, D. Ten samples with different concentrations of DMSO or GLY were stored in the -75C freezer for 5 days, then thawed in a 37C water bath.

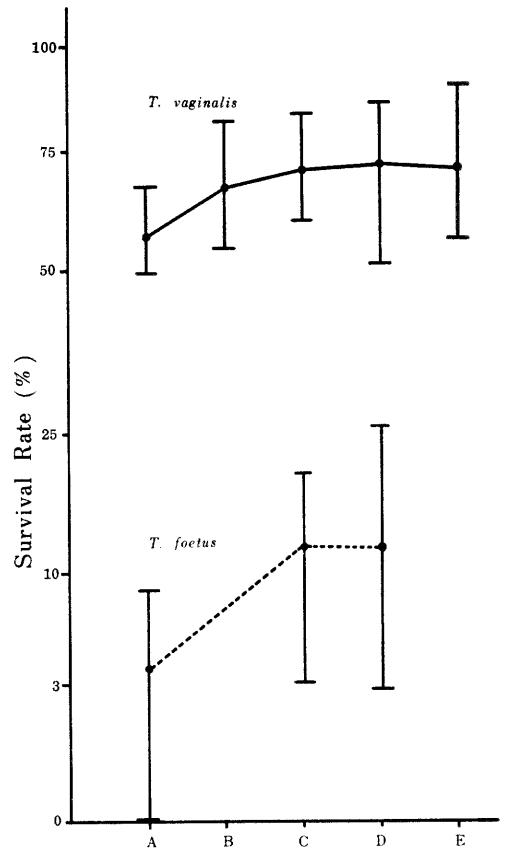


Fig. 7. The effect of the pre-cooling in the -25C freezer.

The following 5 groups, each composed of 10 samples, were compared in the survival rates. The average and the range of the survival rates were shown in each result.

- A) 0C → -75C freezer\*
- B) -10C → -75C freezer\*
- C) -20C → -75C freezer\*
- D) -20C → 1 hour → -75C freezer\*\*
- E) -20C → 2 hours → -75C freezer\*\*

\*When the temperature of the sample reached to required degrees (0C in A, -10C in B and -20C in C) in the -25C freezer, the sample was transferred into the -75C freezer.

\*\*The sample in the -25C freezer was stored for 1 hour (D) or 2 hours (E), after cooled to -20C, then transferred into the -75C freezer.

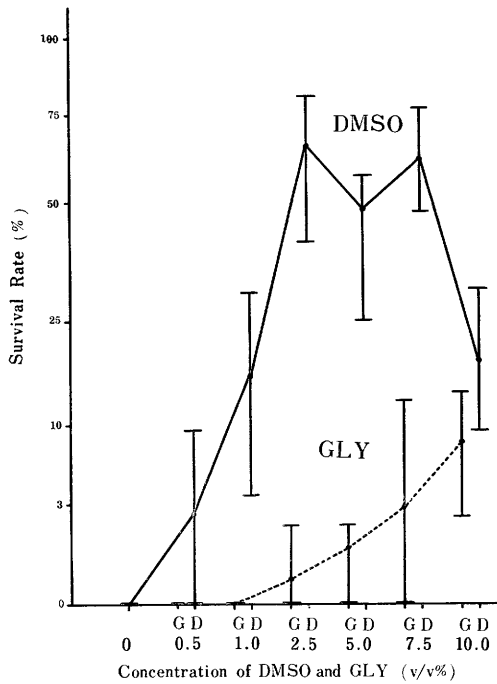


Fig. 8. The effect of the concentration of cryo-protective substances (*T. vaginalis*).

According to the results shown in Fig. 8, DMSO was more cryo-protective than GLY in the same concentration. In the control group without any cryo-protective substance, no motile trichomonad was observed microscopically from all of ten samples.

In this experiment the survival rate of trichomonad in 5.0% DMSO might be somewhat lower than that in 2.5% or 7.5% DMSO, although the difference was not significant statistically at 95% confidence level. In the case of *T. foetus*, the survival rate in every concentration was very low, then no clear result could be obtained.

#### Experiment 5. Effect of salt concentration.

The effect of salt concentration in the samples was examined comparatively and the result was shown in Fig. 9.

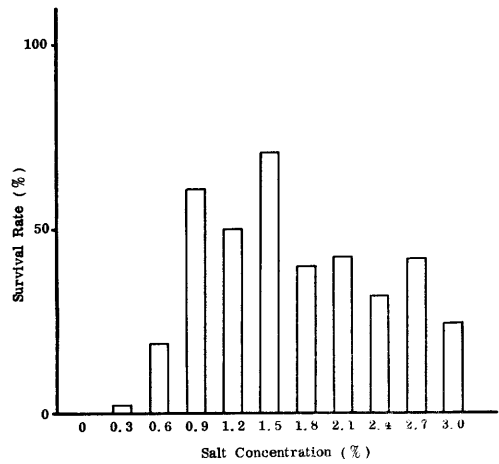


Fig. 9. The effect of the salt concentration (*T. vaginalis*).

The experimental procedure was described as follows: The 48-hour cultivation of *T. vaginalis* in V-Bouillon was centrifuged at  $350\times g$  for 10 minutes. The supernatant was discarded completely and the sediment was suspended separately in different concentrations of NaCl solutions with 5.0% DMSO. After one hour pre-cooling in the  $-25^{\circ}\text{C}$  freezer, all samples were stored for 24 hours in the  $-75^{\circ}\text{C}$  freezer. In this experiment, only one sample was prepared for each salt concentration.

According to the result, in *T. vaginalis* it might be said that the survival rates in the salt concentration of 0.9~1.5% were higher than those in lower salt concentration.

In *T. foetus*, the survival rate was generally very low in the salt concentrations ranging from 0.3 to 3.0%. The highest survival rate, 30.4%, was obtained in the sample of 0.3% NaCl, whereas survival rate was less than 0.01% in the sample of 0.9% NaCl.

According to this result, the author employed Diamond's Medium for *T. foetus* and V-Bouillon for *T. vaginalis*, because the salt



concentration in Diamond's Medium was quite low as compared with that in V-Bouillon.

### Discussion

The conditions for preservation for a long period of *T. vaginalis* and *T. foetus* in a  $-25^{\circ}\text{C}$  freezer and a  $-75^{\circ}\text{C}$  freezer were studied in the present experiments.

In the preservation at  $-25^{\circ}\text{C}$ , the cryo-protective action of DMSO and GLY to living trichomonads was clearly recognized, but the survival rate decreased every day. The longest survival period was 2 weeks in *T. vaginalis* and one week in *T. foetus*. It could be thought that in the sample frozen completely at  $-25^{\circ}\text{C}$ , trichomonad cells might be still maintained in the unfrozen state without sufficient dehydration and once the state was broken, an intracellular ice-formation might occur to destroy the cells mechanically. The freezing temperature of the cells might be lowered by addition of the cryo-protective substance, and as a result, the unfrozen state of the cells might continue for one or two weeks. According to Levine (1962), 15% of *T. foetus* survived for a 128-day storage in a  $-28^{\circ}\text{C}$  freezer. The reason of the difference between Levine's result and the author's might depend upon the difference of the freezers and trichomonad strains used for experiment. For clear understanding, the freezing process of cells must be observed by a low temperature microscope according to the reports of Asahina (1962) and Nei *et al.* (1967), which the present author could not use in this experiment.

When temperature of the samples placed directly in the  $-75^{\circ}\text{C}$  freezer dropped to  $-20^{\circ}\sim-40^{\circ}\text{C}$ , the survival rate of trichomo-

nads markedly decreased. If the trichomonad cells could safely pass through this temperature zone, they could survive at  $-75^{\circ}\text{C}$ .

The trichomonads which were transferred into the  $-75^{\circ}\text{C}$  freezer after pre-cooling in the  $-25^{\circ}\text{C}$  freezer showed higher survival rates than those placed directly in the  $-75^{\circ}\text{C}$  freezer. Asahina (1958, 1959) pointed out the importance of the pre-cooling procedure, and in this experiment the pre-cooling method was likewise effective on the survival of *T. vaginalis* and *T. foetus* in the low temperature storage.

In comparing cryo-protective effect of DMSO and GLY, it was found that DMSO was more effective on the trichomonad survival in the frozen sample than GLY. The optimum concentration of DMSO was from 2.5% to 7.5%, and that of GLY was 7.5~10.0%. In either substance, more than 10% concentration was somewhat toxic to the trichomonad cell. Joyner *et al.* (1956) and Fitzgerald *et al.* (1961) pointed out already that 10% GLY had toxicity to *T. foetus*. Then the present author used 5% DMSO for the long period preservation of trichomonads in the  $-75^{\circ}\text{C}$  freezer.

In a sample subjected to cool temperatures such as  $-25^{\circ}$  or  $-75^{\circ}\text{C}$ , the salt concentration in the solution of a sample became high by dehydration which was caused by the ice crystal-formation in the sample, and the high salt concentration might be injurious to the living trichomonad cells. Therefore, the survival rates of trichomonads in the samples with different percents of NaCl

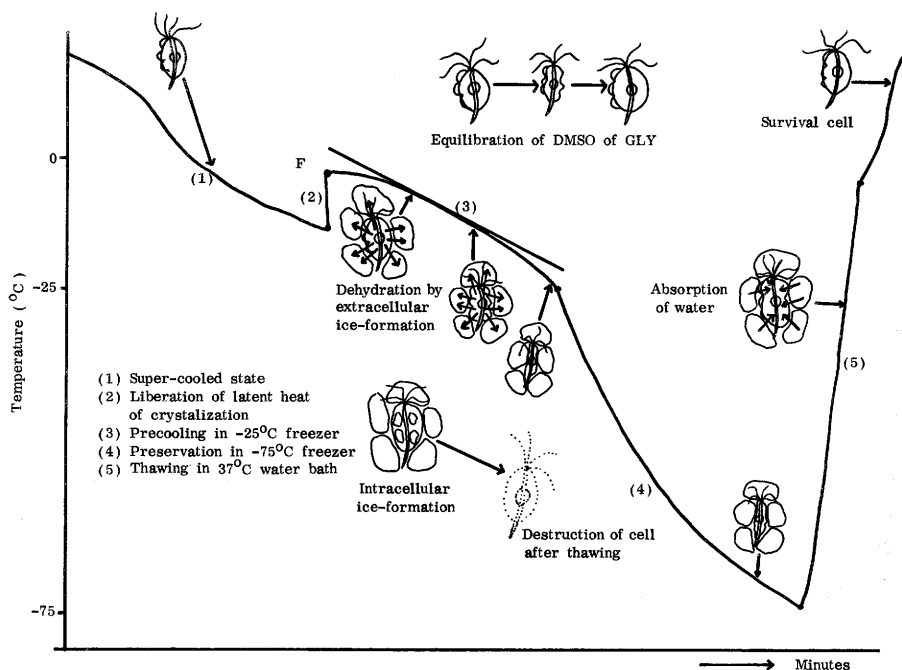


Fig. 10. Schematic explanation of the pre-cooling method.

were examined comparatively. From the result it was found that 0.9~1.5% NaCl concentration did not do damage to *T. vaginalis* but did to *T. foetus*. Levine *et al.* (1962) observed that for preservation of *T. foetus* at low temperatures, Diamond's Medium, which did not contain NaCl, was better than CPLM medium containing 0.9% NaCl.

The survival rates of *T. vaginalis* and *T. foetus* preserved at low temperature were much different. This might be understood by the assumption that *T. foetus* would be more fragile in the frozen sample at -25°C and -75°C than *T. vaginalis*. Joyner (1954) also pointed out that the survival rate of *T. foetus* was very low in a -79°C freezer.

The cooling and thawing procedure of trichomonad in the low temperature preservation might be outlined in Fig. 10. The cryo-protective substance such as GLY or

DMSO was mixed in a trichomonad suspension, and allowed to equilibrate for 10~30 minutes at room temperature. After dehydration from the cells had progressed the cryo-protective substance penetrated into the cells gradually to equilibrate to the concentration. Fitzgerald *et al.* (1961) observed that GLY equilibration at room temperature was better than that at 4°C. The sample was placed in the -25°C freezer for one hour for pre-cooling. The survival rates of trichomonad did not change during the super-cooled state for several minutes at -25°C. When the super-cooled state was broken, the temperature went up by liberation of the latent heat of crystallization. The temperature again slowly dropped and the extracellular solution became frozen. But the intracellular solution did not freeze and the dehydration of the cells continued by the extracellular ice-formation.

The trichomonad cell was reduced in size by the dehydration. In this segment of the cooling curve, some researchers already pointed out that the slow cooling at a rate of 1C/min appeared to be appropriate for the dehydration of cells. But if the intracellular ice-crystal was formed in a trichomonad cell, the cell might be destroyed, as many workers already had pointed out. When trichomonad cells were dehydrated sufficiently in the  $-25^{\circ}\text{C}$  freezer, the sample containing the trichomonads was transferred into the  $-75^{\circ}\text{C}$  freezer. The temperature of the sample dropped rapidly to  $-75^{\circ}\text{C}$ . One hour was enough to pre-cool for dehydration at

$-25^{\circ}\text{C}$ . After preservation in the  $-75^{\circ}\text{C}$  freezer, the sample was thawed rapidly in a  $37^{\circ}\text{C}$  water bath. During thawing of the sample the survival trichomonads absorbed water to regain the original size. Many workers pointed out that the survival rate in the sample thawed rapidly in a  $37^{\circ}\text{C}$  water bath was higher than that in the sample warmed slowly at room temperature.

As a conclusion it could be said that the pre-cooling method attempted by the author might be useful for the low temperature preservation of trichomonad and would be applicable to the cases of other protozoa.

#### Acknowledgement

The author wish to express his deepest thanks to Dr. Toshio Nakabayashi and Dr. Masuhisa Tsukamoto, Professor and Associate Professor of the Department, for their guidance in these experiments and also to Dr. Eizo Asahina, Professor of the Institute

of Low Temperature Science, Hokkaido University, for his advice.

This paper was reported at the 38th annual meeting of the Japanese Society of Parasitology held on 5-6 April, 1969, in Nagasaki, Japan.

#### References

- 1) **Asahina, E. & Aoki, K.** : A method by which frost-hardy caterpillars survive freezing at a super-low temperature. *Low Temperature Science, Ser. B*, 16 : 55-63, 1958.
- 2) **Asahina, E.** : Frost-resistance in a nematode *Aphelenchoides ritzemabosi*. *Low Temperature Science, Ser. B*, 17 : 51-62, 1959.
- 3) **Asahina, E.** : A mechanism to prevent the seeding of intra-cellular ice from outside in freezing living cells. *Low Temperature Science, Ser. B*, 20 : 45-56, 1962.
- 4) **Coggeshall, L. T.** : Preservation of viable malaria parasites in the frozen state. *Proc. Soc. Exptl. Biol. Med.*, 42 : 499-501, 1939.
- 5) **De Jong, D. A.** : Micro-organismes et basses températures. *Arch. Néerland. Physiol.*, 7 : 588-591, 1922.
- 6) **Diamond, L. S.** : The establishment of various trichomonads of animals and man in axenic cultures. *J. Parasitology*, 43 : 488-490, 1957.
- 7) **Diamond, L. S.** : Freeze-preservation of Protozoa. *Cryobiology*, 1 (2) : 95-102, 1964.
- 8) **Fitzgerald, P. R. & Levine, N. D.** : Effect of storage temperature, equilibration time, and buffers on survival of *Tritrichomonas foetus* in the presence of glycerol at freezing temperatures. *J. Protozool.*, 8 : 21-27, 1961.

- 9) **Fulton, J. D. & Smith, A. U.** : Preservation of *Entamoeba histolytica* at  $-79^{\circ}\text{C}$  in the presence of glycerol. *Annals of Tropical Medicine and Parasitology*, **47** (3) : 240-246, **1953**.
- 10) **Gaylord, H. R.** : The resistance of embryonic epithelium, transplantable mouse cancer, and certain organisms to freezing with liquid air. *J. Infect. Dis.*, **5** : 443-448, **1908**.
- 11) **Hamada, Y.** : Biological studies of *Trichomonas vaginalis*. I. Bacteria-free culture of *T. vaginalis*. *Osaka Daigaku Igaku Zassi*, **5** (5) : 429-235, **1953**.
- 12) **Horsfall, F. L.** : A low temperature storage cabinet for the preservation of viruses. *J. Bacteriology*, **40** : 559-568, **1940**.
- 13) **Joyner, L. P. & Bennett, G. H.** : Observations on the viability of *Trichomonas foetus* during the process of freezing to  $-79^{\circ}\text{C}$  and thawing in presence of glycerol. *J. Hyg.*, **54** : 335-341, **1956**.
- 14) **Levine, N. D. & Marquardt, W. C.** : The effect of glycerol and related compounds on survival of *Trichomonas foetus* at freezing temperatures. *J. Protozool.*, **2** : 100-107, **1955**.
- 15) **Levine, N. D., Andersen, F. L., Lodch, M. B., Notzold, R. A. & Mehra, K. N.** : Survival of *Trichomonas foetus* stored at  $-28$  and  $-95^{\circ}\text{C}$  after freezing in the presence of glycerol. *J. Protozool.*, **9** (3) : 347-350, **1962**.
- 16) **Lovelock, J. E. & Bishop, M. W. H.** : Prevention of freezing damage to living cells by dimethyl sulphoxide. *Nature*, **183** : 1394-1395, **1959**.
- 17) **Manwell, R. D. & Jeffery, G.** : Preservation of avian malaria parasites by low temperature freezing. *Proc. Soc. Exp. Biol. Med.*, **50** : 222-224, **1942**.
- 18) **Manwell, R. D.** : The low temperature freezing of malaria parasites. *Amer. J. Trop. Med.*, **23** : 123-131, **1943a**.
- 19) **Manwell, R. D. & Edgett, R.** : The relative importance of certain factors in the low temperature preservation of malaria parasites. *Amer. J. Trop. Med.*, **23** : 551-557, **1943b**.
- 20) **McEntegart, M. G.** : The maintenance of stock strains of trichomonads by freezing. *J. Hygiene*, **52** : 545-550, **1954**.
- 21) **Mühlfordt, H.** : Der einfluss tiefer Temperaturen auf Protozoen. *Ztschr. f. Tropenmed. u. Parasit.*, **11** (4) : 481-507, **1960**.
- 22) **Nei, T. & Okada, J.** : Designing of a new cryomicroscope. *Low Temperature Science, Ser. B*, **25** : 149-153, **1967**.
- 23) **Polge, C., Smith, A. U. & Parkes, A. S.** : Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature*, **164** : 666, **1949**.
- 24) **Shorb, M. S.** : Storage and survival of trichomonads under various conditions. 2. Preservation by freezing of trichomonad culture. *Biochemistry and Physiology of Protozoa, III*: 438-439, **1963**.
- 25) **Smith, A. U.** : Biological Effect of Freezing and Supercooling. *Monographs of the Physiological Society*, **9** : 1-462, **1961**. (Protozoa p. 110-137)
- 26) **Trussell, R. E.** : *Trichomonas vaginalis* and trichomoniasis. Blackwell Scientific Publications, Oxford, 1-277, **1947**.
- 27) **Weinman, D. & McAllister, J.** : Prolonged storage of human pathogenic protozoa with conservation of virulence: observations on the storage of helminths and leptospiras. *Amer. J. Hyg.*, **45** : 102-121, **1947**.

## 寄生性原虫類の低温生物学的研究

1. -25度および-75度のフリーザー中におけるトリコモナス類の凍結条件に関する研究.

宮 田 彬

長崎大学熱帯医学研究所疫学部門 (主任: 中林敏夫教授)

## 摘 要

この実験は、トリコモナス類を-25度および-75度フリーザー中で、低温保存するための冷却方法、凍害保護剤(ジメチル・スルホキサイド(DMSO)、およびグリセリン(GLY))の影響、および至適塩濃度について検討したものである。

得られた成績は、以下の通りである。

1. -25度のフリーザー中では、トリコモナスは、DMSOやGLYを添加しても、数日間しか生存できなかった。このフリーザー中での最長生存期間は *T. vaginalis* では2週間、*T. foetus* では1週間であった。しかし生存率は、どちらもきわめて低かった。
2. GLYやDMSOの存在下で、-75度のフリーザーにトリコモナスを直接冷却し保存した場合は、両方の種とも1ヶ月あるいは、それ以上生存した。しかし生存率は低率であった。
3. あらかじめ材料を-25度のフリーザーで予備凍結し、-75度のフリーザーに移して保存する場合には、トリコモナスの生存率は高くなった。
4. 予備凍結法を用い、-75度フリーザー中で5日間保存後しらべると、2.5~7.5%DMSOを加えた材料では、*T. vaginalis* は、生存率70%以上、*T. foetus* は10%前後であった。一方GLYを加えた材料では *T. vaginalis* は、生存率20%、*T. foetus* は、わずかに数パーセントであった。
5. 5%DMSOを加えた材料を予備凍結し、1日間、-75度フリーザー中に保存後の各塩濃度におけるトリコモナスの生存率は、*T. vaginalis* では塩濃度0.9~1.5%の場合が、それよりも低い塩濃度よりも高い生存率をしめした。一方 *T. foetus* の場合、最も生存率が高かったのは、塩濃度0.3%の場合で、30.4%であった。
6. 結論として、予備凍結法は、トリコモナス類の低温保存に有用で、その他の寄生性原虫類の場合にも応用できるものと考えられる。