On the Cryo-biological Study of the Parasitic Protozoa

(3) Effects of temperature and time of equilibration with glycerol or DMSO on survival of *Trichomonas vaginalis*

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ABSTRACT: By using 5 strains of *Trichomonas vaginalis*, the importance of time and temperature of the equilibration of samples with glycerol or dimethyl sulfoxide (DMSO) was studied prior to the pre-cooling at −30°C for 90 minutes followed by the preservation at−75°C. The survival rate was calculated after freezing and thawing procedure, because before the freezing there were no remarkable differences in the number of living trichomonads between the samples with or without the 3−hour equilibration with glycerol or DMSO at various temperature. (1) In the presence of 10% glycerol at 25°C, the survival rate of trichomonad increased gradually with the prolonged equilibration time. At 37°C, the survivals markably increased within the shorter equilibration time than that needed at 25°C, and the pooled survival rate of 5 strains became about 50% after the 30−60 minutes equilibration at 37°C and the survival rate was also conspicuously higher than that at 25°C. (2) In the presence of 7.5% DMSO, the survival rate was highest (more than 70%) without equilibration and then rapidly decreased with duration of the equilibration time at 25°C or 37°C, and finally the rate reached lower than 20% after 120 minutes. (3) At various temperatures the samples were allowed to equilibrate for 100 minutes in the presence of 10% glycerol or 7.5% DMSO. In the case of glycerol, the survival rate of 5 strains was about 10% at 0−5°C, but about 80% at 37°C. In the case of DMSO, however, the survival rate was highest (50−70%) at 0−25°C, whereas at 30−37°C the rate rapidly decreased to 30%. (4) The optimum concentration of the additive varied with differences of temperature or time of the equilibration; with glycerol, the optimum was about 12.5−15.0% at 25°C, and about 7.5−12.5% at 37°C after 100 minutes equilibration; and in the case of DMSO, the optimum was around 7.5% at 25°C for 60 minutes equilibration.

In my previous reports on the low-temperature preservation of *Trichomonas vaginalis* and other parasitic protozoa in the presence of glycerol or dimethyl sulfoxide (DMSO) (Miyata, 1973 a and b), samples were allowed to equilibrate, before cooling, for 10 minutes with DMSO or for 30 minutes with glycerol respectively at room temperature (about 25°C).

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The present paper deals with effects of temperature and time of the equilibration with glycerol or DMSO on survival in more detail. Some of the results obtained in this study has been reported in Japanese (Miyata, 1974 and 1975) and from which some figures have been used for the present paper.

MATERIALS AND METHODS

The experimental procedures were mostly the same as those reported in previous papers (Miyata, 1973 a and b), as follows:

1. Five strains (T−22, T−36, T−40, T−58, and T−153) of *Trichomonas vaginalis* were isolated by myself from patients in Nagasaki City, and were routinely maintained in V-bouillon (Hamada, 1953) at 37°C. Fortyeight-hour 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.

2. Glycerol or DMSO solutions were originally made up in distilled water at double the concentration desired, and mixed with an equal volume of the trichomonad suspension. Then, one ml of the mixture was distributed into each small test tube with a rubber cap. The percent concentration of glycerol or DMSO was shown as volume per volume.

3. The mixture was allowed to equilibrate at various combinations of temperature and time in each experiment.

4. After the equilibration the tubes were pre-cooled in a −30°C freezer for 90 minutes, then the samples were transfered and stored in a −75°C freezer for 1−5 days.

5. The frozen samples were directly thawed in a 37°C water bath, and the survival and dead trichomonads were counted. The survival rate was expressed as follows:

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

Before cooling, number of the survival trichomonads did not decrease during prolonged period of the equilibration at various temperature in the presence of glycerol or DMSO, and losses of living trichomonads were due to freeze-thawing, then the number was counted only after cooling and thawing in the present study.

RESULTS

1. Equilibration time

a. Glycerol

Samples prepared with 10% glycerol were allowed to equilibrate for 0−180 minutes at 25°C or 37°C. After the equilibration, each group of 10 samples was cooled and stored as mentioned above, and 1−5 days later, the samples were thawed and examined microscopically for the survival rate of trichomonads. At 25°C the survival rate increased gradually with time up to 60−90 minutes and then reached some equilibrium (Fig. 1). At 37°C, on the other hand, the speed required to reach the equilibrium was significantly rapid, usually within 30 minutes (Fig. 2). Furthermore, the survival rates at 37°C were remarkably higher

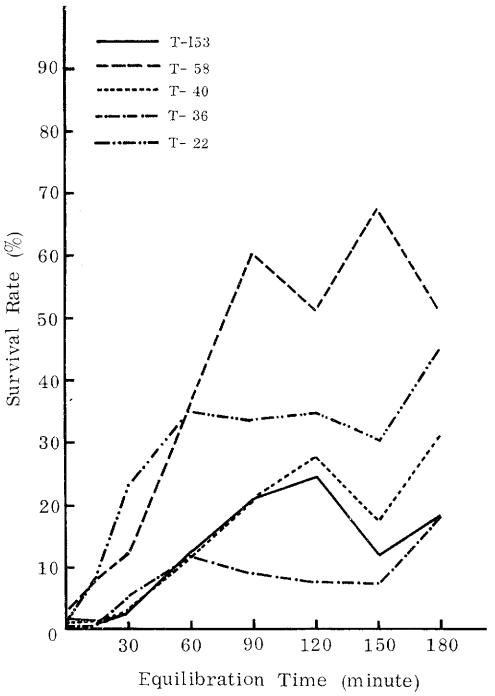

Fig. 1. The effect of the equilibration time on trichomonad survival with 10% glycerol at 25°C

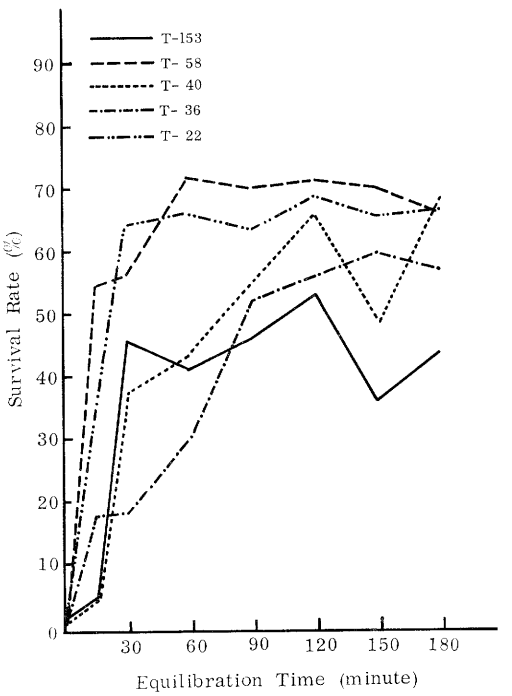

Fig. 2. The effect of the equilibration time on trichomonad survival with 10% glycerol at 37°C

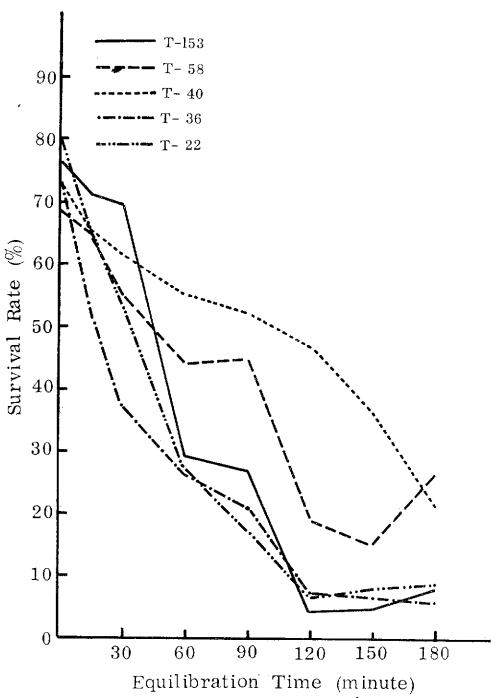

Fig. 3. The effect of the equilibration time on trichomonad survival with 7.5% DMSO at 25°C

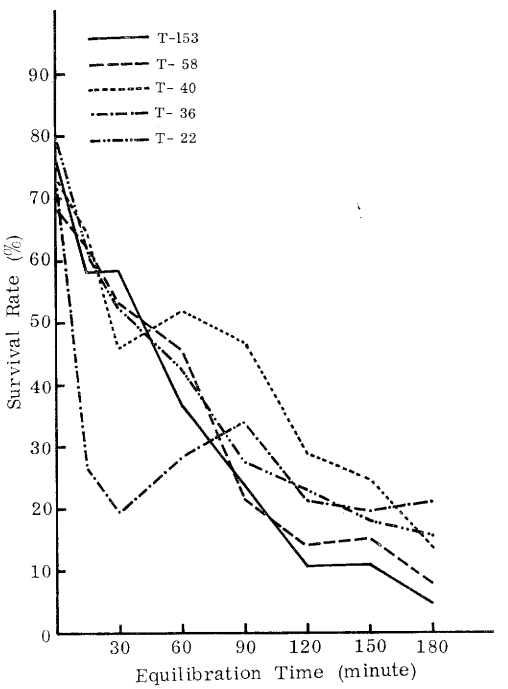

Fig. 4. The effect of the equilibration time on trichomonad survival with 7.5% DMSO at 37°C

than those at 25°C.

b. DMSO

Similar experiments were carried out in the presence of 7.5% DMSO. The results obtained were conspicuously different from those with glycerol. Namely, the survival rate decreased rapidly with time either at 25°C or at 37°C (Figs. 3 and 4). The average survival rate of 5 strains was higher than 70% without the equilibration, but the rate down to 40% after 60 minutes equilibration and finally the rate reached to lower than 20% after 120 minutes.

2. Equilibration temperature

As mentioned above the importance of the equilibration temperature was noted in the case of glycerol whereas no significant difference was observed in the case of DMSO. Then, in the next experiment, incubations at 0°, 10°, 20°, 25°, 30°, and 37°C were attempted. The samples in the presence of 10% glycerol or 7.5% DMSO were allowed to equilibrate for 100 minutes at each designed temperature. After the equilibration the samples were preserved at low temperature. The survival rates in equilibrations with 10% glycerol and 7.5%

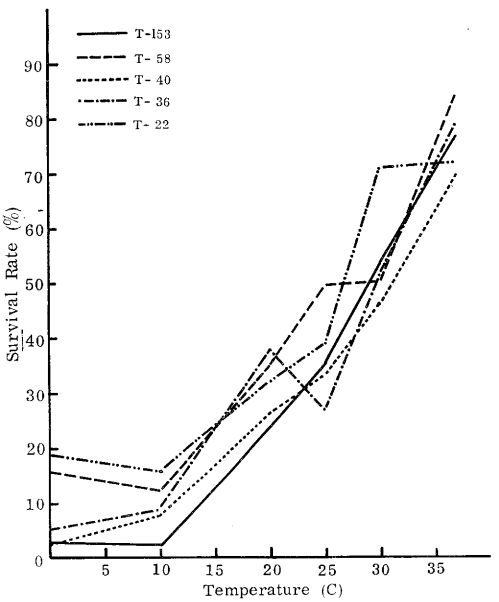

Fig. 5. The effect of the equilibration temperature on trichomonad survival with 10% glycerol for 100 minutes

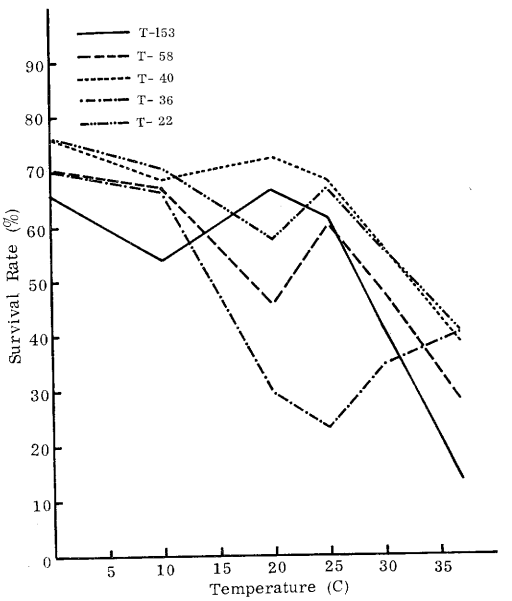

Fig. 6. The effect of the equilibration temperature on trichomonad survival with 7.5% DMSO for 100 minutes

DMSO at the variety of temperature were shown in Figs. 5 and 6, respectively. In the case of glycerol, the survival rate increased at higher temperature and the highest rate was observed at 37°C in all the 5 strains tested (Fig. 5). In the case of DMSO, however, the survival rate was higher in a range from 0°C to 25°C, and at 30−37°C the rate rapidly decreased (Fig. 6). In the case of glycerol, the survival rate of 5 strains was about 10% at 0−5°C, but the rate became about 80% at 37°C. In the case of DMSO, however, the survival rate was 50−70% at 0−25°C, and at 30−37°C the rate rapidly decreased to 30%.

3. Optimum concentration

From the results obtained above, it is assumed that optimum concentration of glycerol might change at different temperature or time for the equilibration. Then, the next attempt was carried out. The samples contained 0−20% of glycerol were allowed to equilibrate for 100 minutes either at 25°C or at 37°C. The results obtained were shown in Figs. 7 and 8, respectively. The optimum concentration of glycerol was about 12.5−15.0% at 25°C and

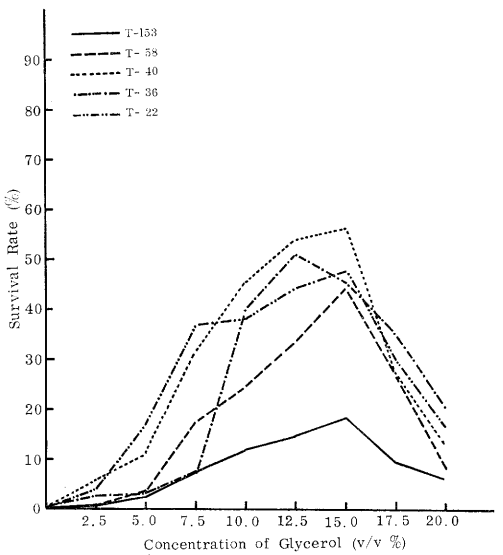

Fig. 7. The effect of the concentration of grycerol on trichomonad survival after 100 minutes equilibration at 25°C

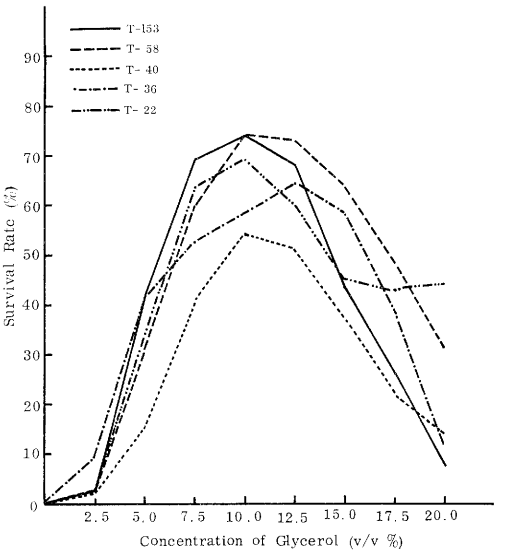

Fig. 8. The effect of the concentration of glycerol on trichomonad survival after 100 minutes equilibration at 37°C

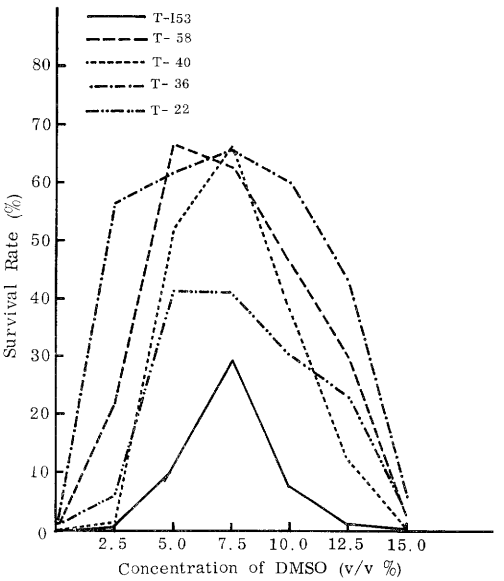

Fig. 9. The effect of the concentration of DMSO on trichomonad survival after 60 minutes equilibration at 25°C

7.5−12.5% at 37° C, respectively. In the case of DMSO the equilibration was tested only at 25°C for 60 minutes. With this condition, the optimum concentration of DMSO was within a range from 5.0% to 10.0% (Fig. 9).

DISCUSSION

Gaylord (1908) reported that *Trypanosoma gambiense* survived even after freezing in liquid air for 20 minutes, and De Jong (1922) also observed that *T. equiperdum* survived for 21 days in liquid air (−190°C). After these pioneer's trials, Cogeshall (1939) succeeded to preserve *Plasmodium knowlesi* and *P. inui* in a −76°C dry-ice box for 70 days, and the parasites were still infective.

After several attempts by Horsfall (1940), Manwell and his coworkers (1942, 1943 a and b), Weinman and McAllister (1947) showed that *Trypanosoma*, *Plasmodium*, and *Leishmania* could be preserved safely for several months in a −70～−79°C dry-ice cabinet, but trichomonads and entamoebae could not survive in the same condition. Since Polge *et al.* (1949) found that the addition of glycerol to bull semen diluents protected spermatozoa from injury during freezing, many workers used glycerol as a cryo-protective substance for the preservation of parasitic protozoa. By the addition of glycerol, trichomonads also could survive for several months at −75～−79°C, for example *Trichomonas vaginalis* reported by McEntegart (1954 and 1959) and *Tritrichomonas foetus* by Levine and Marquardt (1954 and 1955). Ten years later from the finding by Polge *et al.* (1949), Lovelock and Bishop (1959) observed that DMSO had a cryo-protective action similar to glycerol in freezing of living cells.

Now, method for the low temperature preservation of various parasitic protozoa in deep freezers or liquid nitrogen was adopted widely in medical and veterinary laboratories throughout the world. In such laboratories, they may use cryo-protective substances such as glycerol or DMSO for agent to protect the protozoa from damage by freezing. Almost 200 papers have been published on the cryo-preservation of the parasitic protozoa for past 30 years. Those papers were reviewed by Mühlpfordt (1960), Smith (1961), Diamond (1964), and Dalgiesh (1972). Most of the papers reported on the length of survival period of the protozoa under freezing condition. Despite a number of published papers, only few studies have delt with the basic study from the cryo-biological view point, such as cooling rate, optimum concentration of cryo-protectants under various conditions, and time and temperature for the equilibration with the cryo-protectants, etc.

According to the theory of Lovelock (1953), glycerol must be present within the cell as well as in surrounding fluid to exert its full protective effect. DMSO also probably protects cells in a similar way and has the advantage of passing through cell membranes more rapidly than glycerol (Lovelock and Bishop, 1959). If that is the case, it is likely that in the presence of glycerol the survival rate of trichomonads becomes higher in prolonged equilibration time at a constant temperature of 25°C. The result shown in Fig. 1, may lead us to conclusion that at least 60−90 minutes are necessary to equilibrate with glycerol at 25°C. Furthermore, when the temperature was kept at 37°C during the various equilibration times, the survival rate markably increased in comparison with that obtained at 25°C, and only the 15−30 minutes period was enough to equilibrate the samples. It was clearly concluded that the temperature for the equilibration time was important, and at lower temperature such as 0−20°C, the survival rate was very low, but at higher temperature such as 30−37°C, the rate was conspicuously high. Optimum concentration of glycerol to preserve trichomonads at −75°C was also variable at different temperature during the equilibration time. According to literatures surveyed, very few of experiments of this kind have been carried out. Joyner and Bennett (1956) reported that *T. foetus* with glycerol was left at +5°C overnight before cooling, but they did not mention why they selected the temperature and the time. Fitzegerald and Levine (1961) observed that glycerol equilibration at room temperature was better than that at 4°C, and Jeffries and Harris (1967) follwed the identical condition for the equilibration in their experiments without further re-examination. Fulton and Smith (1953)

also studied on cryo-preservation of *Entamoeba histolytica* at −79°C, and before cooling the amoeba was allowed to equilibrate with glycerol (5−15%) at 37°C for 1 hour. In their study, however, they did not mention concerning to the equilibration at other temperatures, except 37°C. According to my previous experience (Miyata, 1973 b), *E. histolytica* was not an adequate material to examine the survival rate, because number of the amoeba obtained from cultures was not many as the case of trichomonads. Levine and Marquardt (1955) reported the effect of glycerol and related compounds on survival of *T. foetus* at freezing temperature, but they did not mention on time or temperature for the equilibration. Levine *et al.* (1958) showed a fine figure on effect of glycerol concentration on survival of *T. foetus* which was frozen at −21°C in CPLM medium and was stored at this temperature, but again they did not described any about the equilibration. In the additional paper by Levine *et al.* (1959), still they did not consider on the condition of equilibration with glycerol. After Fitzgerald and Levine's report (1961), quoted above, however, Levine and his co-workers adopted the method in which *T. foetus* was preserved after the equilibration with glycerol for 2−3 hours at room temperature (about 24°C) (Levine *et al.*, 1962). Furthermore Levine and Andersen (1966) recorded the storage of *T. foetus* for 2048 days (5.6 years) at −95°C. According to Smith (1961), the importance of time and temperature of the equilibration with glycerol was known for the preservation of spermatozoa and other living cells. As far as I know, however, the details of the condition for the equilibration was not studied up to now in the case of the parasitic protozoa except few trials mentioned above. Then, it might be concluded that the present observation is of great importance for improving methods of cryo-preservation of the parasitic protozoa.

In the case of DMSO, many workers recommended to use it as a cryo-protective substance instead of glycerol, because of its rapid equilibration and lower toxity. For example, Walker and Ashwood-Smith (1961) pointed out as follows: Five percent DMSO and 10% glycerol are effective for low-temperature preservation of trypanosomes isolated from blood. The lower toxity of DMSO both to the host and to the parasite, as compared with glycerol, suggests that DMSO may be a useful alternative to glycerol for low-temperature preservation of trypanosomes. Collins and Jeffery (1963) also pointed out similar results in the preservation of *Plasmodium berghei* and *P. gallinaceum*. According to Doran (1969), in the cryo-preservation of excysted sporozoites of *Eimeria adenoeides*, *E. mivati*, and *E. tenella*, the best results (more than 70% survival) were obtained when the period of equilibration was 45 to 50 minutes with 10−15% DMSO, then the cooling rate was 1°C per minute. In an unfrozen condition, however, the survival of sporozoites during the prolonged equilibration was better at the lower concentrations as 2.5−5.0%. In my experience with 7.5% DMSO, in unfrozen samples motile trichomonads never decreased markably even after 3 hours equilibration at 25° or 37°C, but once the samples were frozen, the survival trichomonads decreased in number by the prolonged equilibration time. As a general rule, DMSO is useful as a cryo-protective substance, but according to results of the present study, it might be said that the survival rate higher in the sample which was allowed to equilibrate with DMSO for shorter time at lower temperature on the contrary to the results obtained in glycerol.

From the results of the present experiments, the great difference in the mechanism of cryo-protective action between glycerol and DMSO has been indicated, and hence Lovelock's theory must be re-examined, although I have not yet examined whether or not glycerol or DMSO is really present within cell. Further studies to confirm this point are being carried out.

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寄生性原虫類の低温生物学的研究(3)グリセリンまたはDMSOの平衡中の温度及び時間が膣トリコモナスの生存に及ぼす影響

宮田　彬（長崎大学熱帯医学研究所疫学部門）

膣トリコモナス5株を用い，グリセリンまたは，ジメチル・スルホキサイド（DMSO）の平衡時間及び平衡温度が，凍結保存に及ぼす影響を検討した．方法は−30°C で90分予備凍結後，−75°Cで，1〜5日間保存後，原虫生存率をしらべた．(1)10%グリセリン添加による，生存率は25°Cでは，時間の経過に従って，少しずつ高くなった．37°Cでは，25°Cの場合とくらべ，生存率は短い平衡時間(30〜60分)で，著しく高くなり，25°Cの場合よりも，はるかに高い生存率が得られた．(2)7.5%DMSO の添加では，平衡させなくても70%以上の高い生存率が得られ，25°C及び37°C のいずれの場合も平衡時間を長くするほど生存率が低下し，120分平衡させた場合生存率は 20%以下にまで下った．(3)様々の温度条件下で，10%グリセリンまたは，7.5% DMSOを添加した材料を，100分間平衡させた場合，グリセリンでは温度が高いほど生存率が高くなり，0〜5 °Cでは，約10%の生存率であったのに対し，37°Cでは80%に達した．DMSO では，グリセリンの場合とは反対に，0〜25°C では，生存率が50〜70%と高く，30〜37°Cでは，生存率は 30%近くまで急速に低下した．(4)保護剤の最適濃度は，平衡時間及び温度の変化により，かわり，グリセリンでは25°C，100分平衡では，最適濃度は12.5〜15.0%であり，また 37°Cでは，7.5〜12.5%であった．DMSO を60分間25°C で平衡させた時の最適濃度は，7.5%であった．(5)この実験を通じて，今まであいまいにされていた，保護剤の添加と，平衡中の温度及び時間と原虫生存率の関係が，詳しく解明されたことを特に強調しておきたい．

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