On the Cryo-biological Study of the Parasitic Protozoa

(4) Comparative study of protective effects of glycerol, ethylene glycol and DMSO on prolonged cryo-preservation of *Trichomonas vaginalis* at −75 C

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ABSTRACT : In the previous papers (Miyata, 1973, 1975a and 1975b), the author reported the effect of glycerol and DMSO on cryo-preservation of *Trichomonas vaginalis* at −75 C. In those studies, survival rate of *T. vaginalis* was examined after 5 to 10 day-storage at −75 C; in the present paper, however, three kinds of protective substances (glycerol, ethylene glycol and DMSO) were examined for the effect on prolonged preservation such as one to three months. The following results were obtained: 1) The initial survival rates in the presence of each substance are equally higher than 50% respectively. 2) After prolonged preservation for one to three months at −75 C, the difference of survival rate in samples containing each substance was recognized. Ten percent glycerol is apparently superior to other two substances, and the survival rate after prolonged storage is still kept at the high level as equivalent as that of the initial 10 day-storage. Trichomonads still had a moving activity after 60 day-preservation. 3) In the presence of 7.5% DMSO, about 50 to 60% of trichomonads were alive after 10 days, but about 10% could survive after 60 days. 4) Ten percent of ethylene glycol is somewhat superior to DMSO. Three out of 5 strains still kept higher than 50% survival rates after 60 day-storage. After 30 to 90 days, however, most of living trichomonads lost their motilities. 5) From the above mentioned results, it might be concluded that glycerol is the most superior substance as a cryo-protectant among 3 chemicals examined in the present study.

In the previous papers (Miyata, 1973, 1975a and 1975b), the author reported the effect of glycerol and DMSO on cryo-preservation of *Trichomonas vaginalis* at-75 C. The survival rate of *T. vaginalis* within one or two week-preservation is higher than 50% in the presence of each cryo-protective substance. However, in a prolonged preservation, it was not reported whether or not the initial survival rate could be maintained. There are many papers concerning cryo-preservation of the parasitic psotozoa (see the review by Miyata, 1975b), but so far no worker has reported on the comparative effects of prolonged storage period on survival rate of *T. vaginalis* in the presence of different cryo-protective substances.

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Therefore, in the present study, three kinds of the protective substance, glycerol, ethylene glycol and DMSO, are examined for the effect on a prolonged storage such as one to three months. Those substances are equally useful in cryo-preservation of *T. vaginalis* in one or two week-preservation at -75 C: the survival rate of trichomonad is at least higher than 50%. Cryo-protective effect of ethylene glycol has also been studied by the author, but the results will be published elswhere.

MATERIALS AND METHODS

1. Five strains (T-22, T-36, T-40, T-58, and T-153) of *Trichomonas vaginalis* were used in the present experiment. Those strains were isolated in Nagasaki City by the author and they were maintained in V-Bouillon medium (Hamada, 1953).
2. Each strain of *T. vaginalis* was cultivated in the medium for 48 hours at 37 C, then after centrifugation (350×g for 5 minutes), a half of the supernatant was discarded. The sedimentation (trichomonads) was resuspended in the remaining half of the supernatant.
3. For the cryo-protective substance, the following three chemicals were selected: glycerol, ethylene glycol and dimethyl sulfoxide (DMSO). The cryo-protective substance was principally prepared in distilled water at double the concentration desired.
4. The diluted cryo-protective substance was mixed with an equal volume of trichomonad suspension, thus finally 10% glycerol, 10% ethylene glycol and 7.5% DMSO were obtained respectively. Each 1 ml of the mixture was distributed into a small test tube, which was sealed with a rubber cap.
5. The equilibration of samples with glycerol was carried out at 37 C for 90 minutes. For ethylene glycol and DMSO, 30 minute-equilibration at 25 C was selected. According to the results of preliminary experiments, the survival rate ot trichomonads in the presence of each substance under similar conditions was usually higher than 50% at least one or two week-preservation at −75 C.
6. After the equilibration, each sample was pre-cooled in a −30 C freezer for 90 minutes (see Miyata, 1973), then it was preserved in a −75 C freezer for one day to several months.
7. For examination, the sample was thawed in a 37 C water bath as soon as possible after taking out from the 75 C freezer. Survival rate of each sample was examined within one hour after thawing, and was expressed as follows:

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

RESULTS

The results obtained for 5 different strains of trichomonads were shown in Figs. 1 to 5 where the pooled survival rate of 10 samples was shown for each protective substance. The intial survival rats of 5 strains in the presence of each different cyro-protective substance are equally higher than 50% respectively, and hence it is not clear which kind of substance is most superior in cryo-preservation of *T. vaginalis*. While, after prolonged preservation for

one to three months at −75 C, the difference of survival rate in each sample was recognized. In the presence of glycerol, which is apparently superior to other two substances, the survival rate of each strain is still kept at the rate as high as in the initial 10 day-storage, whereas in the presence of other substance the survival rate gradually decreased to a lower level, and finally in some strains the rate reached even lower than 10% (Figs. 2 and 5). In the case of ethylene glycol, the survival rate of 3 strains (T-22, T-40 and T-58) after 60 day-preservation was still maintained at about 50%. This substance thus seems to be somewhat superior to DMSO. In the presence of glycerol, preserved trichomonads were still very active even after 60 day-storage at −75 C, and there were no sign of deterioration of the cells. By using ethylene glycol, however, most of living trichomonads lost their motilities and sometimes it was very difficult to determine whether trichomonad was still alive or not. Most of living trichomonads obtained from the sample frozen with DMSO were still active, but their cytoplasms were vacuolated, suggesting an apparent sign of deterioration.

DISCUSSION

Both glycerol and DMSO are famous cryo-protective substances for the low temperature preservation of the parastic protozoa. The effect of glycerol as a cryo-protectant was discovered by Polge *et al.* (1949). Since then, many workers adopted this substance for freezing storage of their materials. In 1959, Lovelock and Bishop found that DMSO had a protective action similar to glycerol. This protectant was also widely adopted in many laboratories. Walker and Ashwood-Smith (1961) and Collins and Jeffery (1963) pointed out that both substances were effective to trypanosomes or malaria paraste in cryo-preservation, and they concluded that the lower toxicity of DMSO to both the parasite and the host made it a useful alternative for glycerol.

Ethylene glycol was tested by Levine and Marquardt (1955) for preservation of *Tritrichomonas foetus* at −20 C. They reported that an average of 72% of the protozoa could be alive after 1 day in the presence of 6 % ethylene glycol, and an average 29% after 8 day storage, but maximum survival period appeared to be longer in glycerol, because there were no survival trichomonad after 64 days in tubes containing ethylene glycol.

There are many papers concrning cryo-preservation of the parasitic protozoa (Miyata, 1975b), but, in the presence of different cryo-protective substance, comparative study of survival rate after long period storage is rather few. In each samples containing a protectant, survival rate of *Trichomonas vaginalis* was higher than 50% after initial 10 days. But, in prolonged preservation at −75 C, glycerol was most superior as a cryo-protectant compared to other two. In the presence of DMSO, survival rate of *T. vaginalis* was higher than 50% after one month preservation, but the rate gradually become lower than 10% within 2 to 3 months. Ethylene glycol was also effective as a protectant, but survival rate was more gradually decreased, and living trichomonads sometimes lost their motilities. From above mentioned results, it might be concluded that glycerol is the most superior substance as a cryo-protectant among 3 substances examined in the present study.

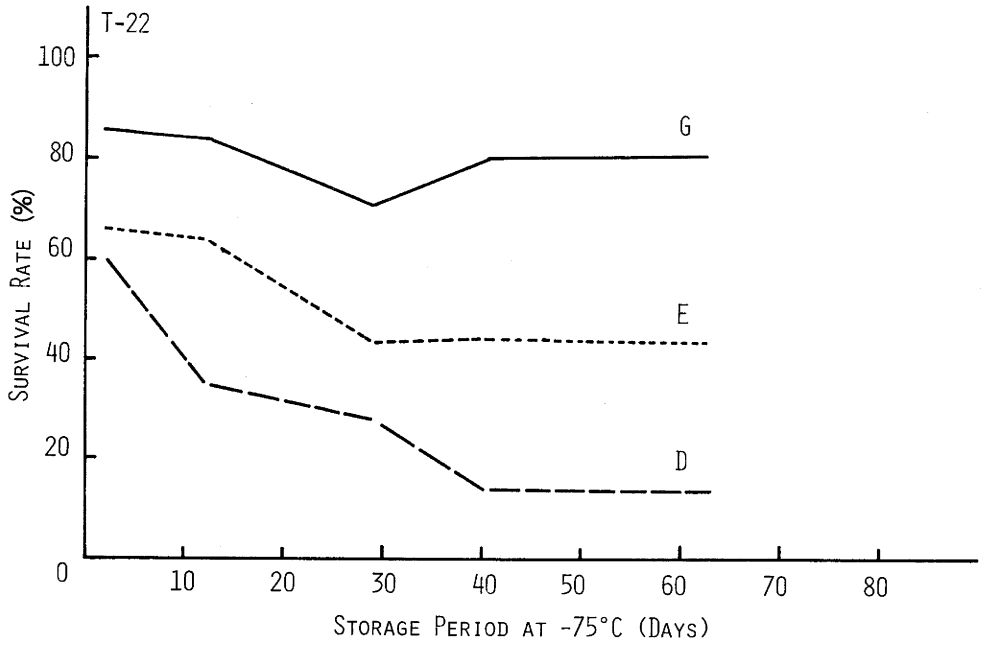

Fig. 1 The effect of preservation period on trichomonad survival with glycerol (G), DMSO (D) or ethylene glycol (E). (1) Strain T-22.

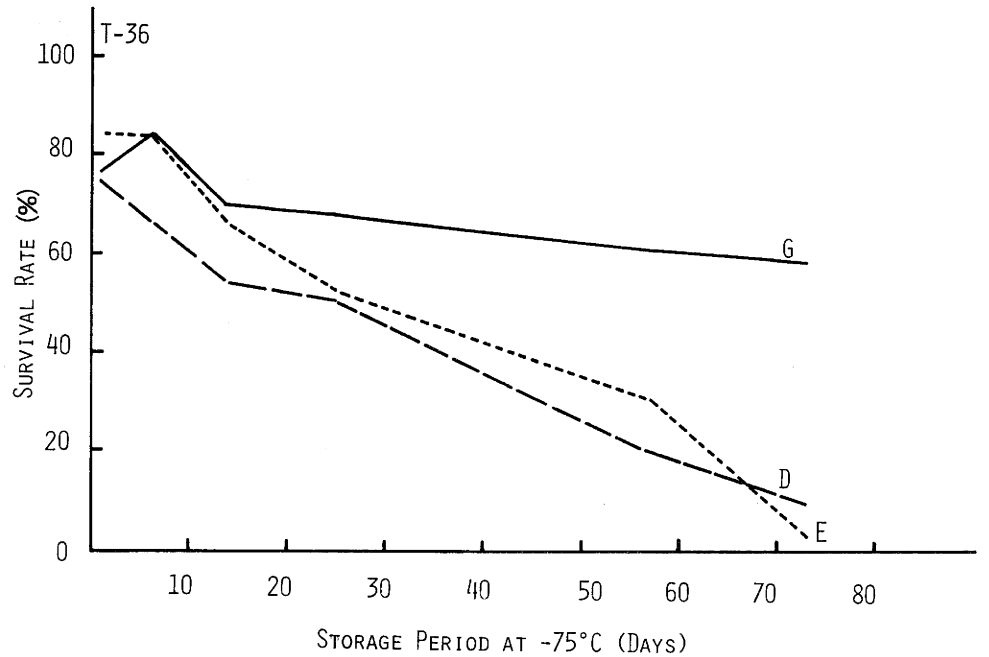

Fig. 2 The effect of preservation period on trichomonad survival with glycerol (G), DMSO (D) or ethylene glycol (E). (2) Strain T-36.

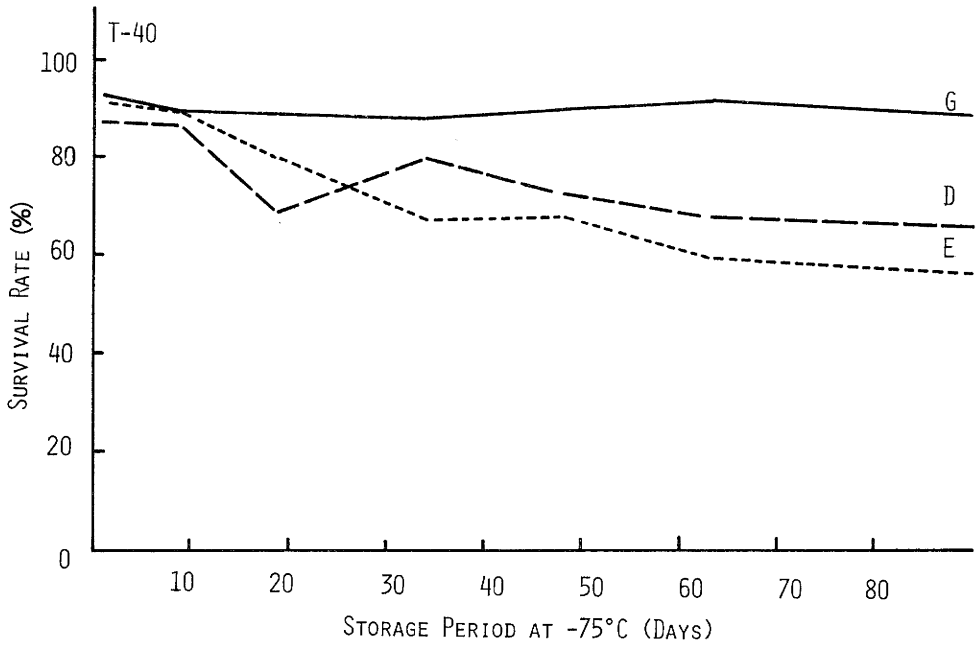

Fig. 3 The effect of preservation period on trichomonad survival with glycerol (G), DMSO (D) or ethylene glycol (E). (3) Strain T-40.

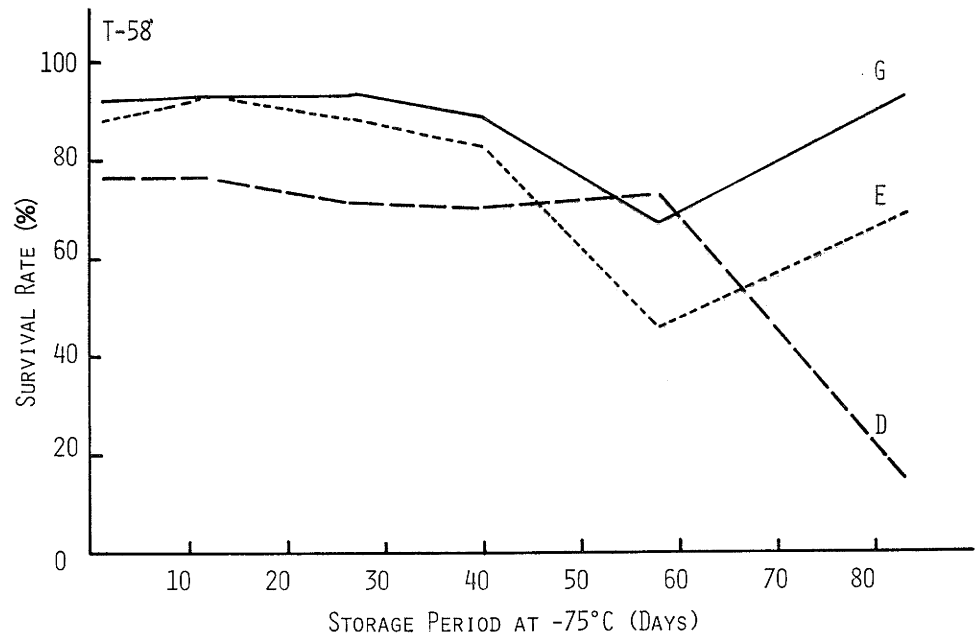

Fig. 4 The effect of preservation period on trichomonad survival with glycerol (G), DMSO (D) or ethylene glycol (E). (4) Strain T-58.

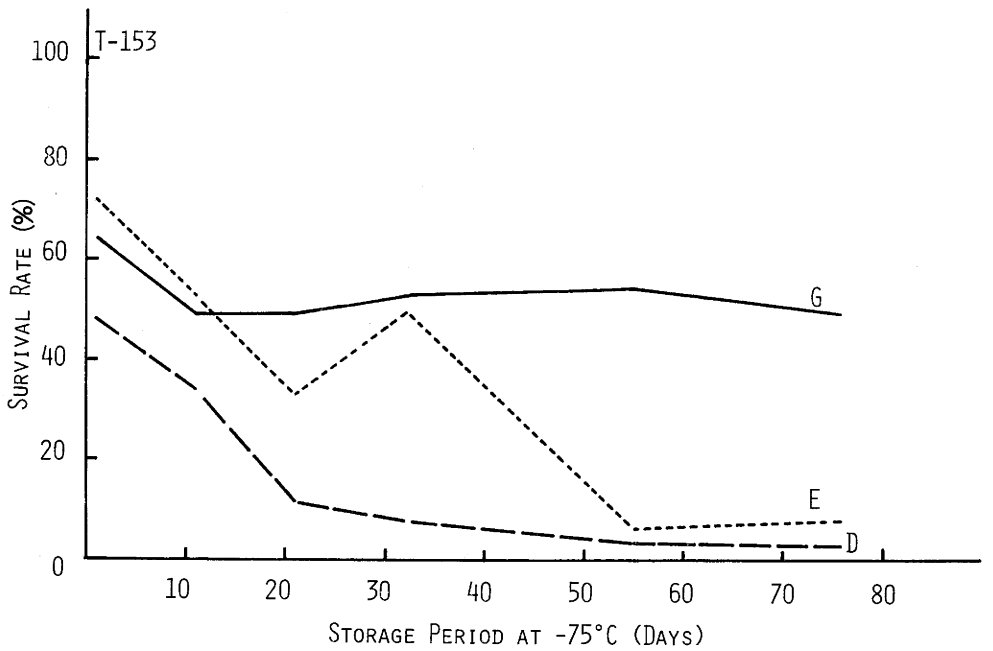

Fig. 5 The effect of preservation period on trichomonad survival with glycerol (G), DMSO (D) or ethylene glycol (E). (5) Strain T-153.

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寄生性原虫類の低温生物学的研究(4). －75度における膣トリコモナスの長期保存におけるグリセリン，エチレン・グリコールおよび DMSO の保存効果の比較研究

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グリセリンおよび DMSO が，寄生性原虫類の凍結保存に有効な凍害保護剤であることは，先に報告した通りである（Miyata, 1973, 1975a および 1975b）．しかしそられの実験では，保存期間がせいぜい10日までであり，その程度の期間内では，両保護剤はともに膣トリコモナスの－75度における保存では高い生存率をしめした．またエチレン・グリコールもやはり有効である（未発表資料）．しかし１～３ケ月のような長い期間の保存でも果して以上の３保護剤の効果に変化がないものかどうか，まだ明確な結論は出ていず，引用できる論文もない．そこでその点について検討した．得られた成績は次の通りである．－75度における保存初期（10日前後）の膣トリコモナスの生存率は，３保護剤とも少なくとも50％は越えており，どの保護剤がすぐれているか，この時点でははっきりしない．しかし１～３カ月保存後では，各保護剤ごとに生存率に大きな差異が生じてくる．10％グリセリンを用いた群では，明らかにもっとも生存率が高く，ほぼ初期の生存率が維持される．また生存虫体は，活発な運動性をもっている．7.5％ DMSO を用いた群では，保存初期ではグリセリンとかわらない生存率をしめすが，60日保存後には，生存率10％に低下する．エチレン・グリコールを用いた群は，供試膣トリコモナス株により異なるが，DMSO 群よりは生存率が高い．しかしどの株も明らかにグリセリン群に劣っている．またエチレン・グリコール群では，長期保存後，生きていると思われる虫体が殆んど運動性をなくしているものが多く，この点でもグリセリン群に劣っている．以上の成績から，－75度における長期保存では，グリセリンが最もすぐれた保護剤であると考えられる．

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