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CRYO-PRESERVATION OF THE PARASITIC PROTOZOA

AKIRA MIYATA

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Abstract: In the present paper, about 200 literatures on the cryo-preservation of the parasitic protozoa have been surveyed, and the following problems have been discussed: cooling rate, storage periods at various temperatures, effects of cryo-protective substances in relation to equilibration time or temperatures, and biological properties before and after freezing. This paper is composed of three main chapters, and at first, the history of cryo-preservation is reviewed in details. In the second chapter, other literatures, which were not cited in the first chapter, are introduced under each genera or species of the protozoa. In the last chapter, various factors on cryo-preservation mentioned above are discussed by using the author's data and other papers in which various interesting problems were described. The following conclusions have been obtained in this study: Before preservation at the lowest storage temperature, it appears preferable that samples are pre-cooled slowly at the rate of 1 C per minute until the temperature falls to −25 to −30 C. The cooling rate might be obtained by the cooling samples for 60 to 90 minutes at −25 to −30 C freezer. For storage, however, lower temperatures as low as possible are better for prolonged storage of the samples. Many workers recommended preservation of the samples in liquid nitrogen or in its vapor, but the storage in a dry ice cabinet or a mechanical freezer is also adequate, if the samples are used within several weeks or at least several months. Cryo-protective substances such as glycerol or DMSO are highly effective to keep higher survival rate of the protozoa in frozen state. Most workers recommended to use 10% glycerol or 5 to 7.5% DMSO for this purpose. For the use of glycerol, at least 30 to 60 minutes of equilibration at temperatures as high as 37 C, is necessary to produce satisfactory results, because at 25 C or lower temperatures, the cryo-protective action of glycerol becomes insufficient. In DMSO, however, samples should be cooled as soon as possible after adding the substance into protozoan suspension. Prolonged equilibration with DMSO is apparently toxic to trichomonad. Many workers pointed out that biological properties of the protozoa such as infectivity, virulence, antigenicity, and drug resistance, were not changed by prolonged period of preservation at the low temperature. These aspects are greatly advantageous for cryo-preservation. By adopting the cryo-preservation technique, furthermore, we save expenses for the maintenance through animal passages or in vitro culture, and thus we can store much more protozoan species or strains in the laboratory. Finally, the author proposed to build the cryo-preservation center of protozoan strains, because in this country we have no adequate center to deposit our strains. Even if nobody use a certain strain for experiment at the moment, yet we can preserve them for the future need. Other laboratories also have many strains, and some of them may be used frequently, but others are not. If all strains be collected in one center, and if the center supplies each strain in case of need, we could save a lot of expense to preserve strains in each laboratory.

Dept. of Epidemiology, Institute for Tropical Medicine, Nagasaki University.

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Before the advent of cryo-preservation technique, various strains of the parasitic protozoa had been preserved by animal inoculation or by cultivation in laboratories. For instance, maintenance of *Toxoplasma gondii* (RH strain) or *Trypanosoma gambiense* necessitates storage in mice every 3 to 4 days, and *Trichomonas vaginalis* requires to sub-culture every 48 hours. In our laboratory, we preserve the following parasites by animal passage: *Plasmodium gallinaceum, P. berghei, Trypanosoma gambiense,* and two strains of *Toxoplasma gondii.* Solely to preserve those parasites, 640 mice and 120 chickens are necessary in every year, costing us about 113,200 yen (374 dollars) for the animals. Moreover, we have several strains of *Entamoeba, Leishmania,* and *Trichomonas* in culture media. At a moderate estimation the total expense for time, culture materials, cages or feeds for the animals, might cost more than 300,000 yen (1,000 dollars) a year, which is equivalent about 10% of the total budget per year for our laboratory (6 researchers).

Since the cryo-preservation was adopted widely in various laboratories, it has become possible that not only we could save money but also we could store more various species and strains of the parasitic protozoa in a frozen state, and even some of "type specimens" preserved by other workers can be re-examined in a living state. Furthermore, in many strains, it was often reported that pathogenicity and biological properties of parasite changed gradually during the long period of animal passage or sub-culture. In cryo-preservation, however, such properties of each strain can be preserved without apparent loss or change.

Lumsden and Hardy (1965) and Lumsden (1972) proposed the terms *stabilate* and *stabilation* for population of organisms stored in frozen state. According to them the term *stabilate* means a population of organisms viably preserved "on a unique occasion", and *stabilation* is the process of preparing a *stabilate.* In cryo-preservation, organisms arrest "the continuous reproduction of a population" and thus "avoid the continuous selection" without change of its biological properties such as infectivity, drug sensitivity, and antigenicity. The term *strain* which means organismal populations maintained by serial passage is distinguished from *stabilate.* A *strain* might change during a period of serial passage in animals or in culture media. It is a great advantage to adopt cryo-preservation for workers.

During past 30 years, more than 200 papers concerning cryo-preservation of the parasitic protozoa at various temperatures (−70 to −190 C) were published and most of them were reviewed by Mühlpfordt (1960), A. U. Smith (1961), Diamond (1964), and Dalgliesh (1972). Even today, however, there are still many problems to be solved. The present paper will introduce and discuss the problems in more details from a historical view point.

I. Historical Review

According to A. U. Smith (1961), from the time of Antony van Leeuwenhoek (1676–1677), who first observed cryo-resistance of the protozoa, to the beginning of this century, there are several reports concerning the life and death of the protozoa after freezing and thawing. On the parasitic protozoa, Laveran and Mesnil (1904) showed that trypanosomes survived and retained virulence after exposure for several

minutes to liquid air at −191 C. For example, *Trypanosoma lewisi* survived after 75 minutes at that temperature, and still could infect rats. Gaylord (1908) reported that *Trypanosoma gambiense* survived even after freezing in liquid air for 20 minutes, but could not survive after about 40 minutes, and De Jong (1922) also observed that *Trypanosoma equiperdum* survived for 21 days in liquid air (−191 C).

After these pioneer's trials, Coggeshall (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 to fresh hosts. His attempt was the first important step in cryo-preservation of the parasitic protozoa. Since his report, many workers have attempted to store their materials at −70 to −190 C, and approximately 200 papers have been reported on cryo-preservation of various protozoa.

1) Coggeshall's technique of cryo-preservation and its modifications

Some early trials of cryo-preservation of pathogenic microorganisms such as spirochetes, *Treponema* spp. or filtrable viruses at −75 C were carried out by several workers including T. B. Turner (1938) during the ten years from 1930 to 1940. These studies have encouraged protozoologists, and Coggeshall (1939) wrote a paper on preservation of malaria parasites. He used the following freezing and thawing technique. One m*l* of the blood containing *Plasmodium knowlesi* or *P. inui* was placed in screw-capped celluloid tube of 2 cm in diameter. With whirling motion each tube was immersed in a mixture of solid CO_2 and alcohol. Then, as soon as the material is frozen, it was rapidly transfered to a low temperature storage cabinet, which was maintained at temperatures between −72 and −80 C by solid CO_2. For testing, the tube was thawed as rapidly as possible in a water bath at 37.5 C. Monkeys were inoculated with 1 m*l* of the infected blood stored at −76 C for 70 days, and both malaria parasites were able to infect to the monkeys. According to Horsfall (1940), Coggeshall's unpublished studies proved that *P. knowlesi* had remained infectious for 140 days, *P. inui* for 151 days, *P. cathemerium* for 35 days, *Trypanosoma brucei* for 135 days, *T. duttoni* for 34 days, and *T. equiperdum* for 62 days. Furthermore, Horsfall described an ideal dry ice cabinet for preservation of microorganisms, and the cabinet had been used by Coggeshall (1939) before Horsfall's report published.

Modifications of Coggeshall's technique were adopted by Archetii (1941) to preserve *Plasmodium gallinaceum* for 50 days at −75 C, and Manwell and Jeffery (1942), and Manwell (1943) who kept avian malaria parasites of 10 different species in the infected blood at −55 to −78 C for a maximum period of 212 days. Manwell and Edgett (1943) described some modifications of cooling method in which they used the tubes with thinner wall varied between 0.018 and 0.030 inches in thickness to increase the rate of cooling and thawing in the storage of *Plasmodium lophurae*. The tubes were rotating during cooling. According to their paper, the superiority of the thinner tubes was also a logical inference from the fact that *Toxoplasma gondii* (may be RH strain) was found to be still viable after freezing and thawing in such tubes, whereas it had never been possible to preserve this species in the thicker tubes (about double thickness of the thinner tube) previously used. They concluded that the temperature for freezing was not so important as the temperature of storage, and it was possible to preserve *P. lophurae* in viable condition after freezing at a temperature as high as −10 C. For storage, lower temperatures were better because

P. lophurae survived for 244 days at −75 C. According to Manwell *et al.* (1945), *T. gondii* was kept frozen only a few minutes in a mixture of dry ice and ethyl alcohol using the thinner tube, but long period of preservation was not reported. F. Wolfson (1945) also preserved *P. lophurae, P. cathemerium,* and *P. relictum* for period varying from 2 days to one year at −75 C. Saunders and Scott (1947) were the first to preserve the human malaria parasite, *Plasmodium vivax.* About 2 to 3 m*l* of the citrated human blood infected with *P. vivax* were put into small vials and the blood was rapidly frozen at −75 C, then stored in dry ice box at −50 C. The parasites survived up to 16 days or longer and, in a case, up to 37 days, and the blood was capable to transmit malaria and induce fever in the patients who have been treated for neurosyphilis by this means. Boyd (1949) also reported that *Plasmodium falciparum* survived for 156 days at −72 to −80 C.

Weinman and McAllister (1947) have studied to preserve many species of the pathogenic protozoa at −70 C. They reported that better results were obtained when the material was first frozen at −15 C and then stored at −70 C than when it was immediately brought to a temperature of −70 C. There were survivors with both procedures, but in the case of *Trypanosoma rhodesiense* or *T. cruzi,* the number of survivors using the former procedure increased 2 to 10 times of the latter. This modified cooling technique is the first proof that slow cooling is less distructive than rapid cooling of the protozoa. According to their paper, following results were included; *Trypanosoma gambiense* survived for 561 days at −70 C, *T. rhodesiense* for 595 days, *T. cruzi* for 653 days, *T. lewisi* for 531 days, *Leishmania tropica* for 794 days, *L. donovani* for 276 days, *Pentatrichomonas hominis* for 407 days, and *Plasmodium lophurae* for 235 days, but *Trichomonas vaginalis, Chilomastix mesnili, Balantidium coli, Tetrahymena geleii* (non parasitic ciliate), and *Entamoeba histolytica* did not survive under the same condition. In the case of *Trichomonas vaginalis,* only 2 out of 10 samples frozen at −15 C and stored at −70 C contained living organisms when thawed. *Toxoplasma gondii* also could survive after freezing, but could not be stored. Later, Weinman (1958) reported that *T. rhodesiense* survived for 8 years at −70 C, and the parasite was still infectious to fresh host. Levaditi (1952) also preserved *Trypanosoma congolense* for 100 days and *Plasmodium berghei* for 15 days at −70 C. The malaria parasite was usually stored as the infected host blood in a frozen state. Only Brumpt and Bao Van Ty (1945) recorded unsuccessful experiment in which blood parasites and sporozoites of *Plasmodium gallinaceum* were rapidly frozen to −25 C, and stored at the temperature, but no viable malaria parasite was observed when thawed. The freezing and storing temperature might have been inadequate. The success of preservation of malaria sporozoites owes to Jeffery and Rendtroff (1955), who reported that viable human malaria sporozoites were obtained after cryo-preservation at −70 C. The longest survival records of each malaria parasite at this temperature were as follows: *Plasmodium vivax* for 375 days, *P. falciparum* for 180 days, and *P. ovale* for 70 days. In 1957, Jeffery again recorded longer survival of sporozoites of *P. ovale* for 997 days under the same condition described above.

The attempts of cryo-preservation, before the discovery of cryo-protective action of glycerol to living cells by Polge *et al.* (1949), were discussed already. As a rule, *Plasmodium* spp., *Trypanosoma* spp., and *Leishmania* spp. are the easiest parasites to

be stored in frozen state without any cryo-protectant. The successful preservation for prolonged period seems to depend upon constant maintenance of temperature of the storage cabinet. In early days, most workers used dry ice cabinet to preserve their materials, because mechanical freezers were not available, and the temperature within the freezers sometimes fluctuated often. Even now, many workers (Lumsden, 1972, and others) recommended to use chemical refrigeration (dry ice or liquid nitrogen) for cryo-preservation of the protozoa rather than mechanical refrigeration.

2) Discovery of cryo-protective effect of glycerol

In 1938, T. B. Turner described that viruses placed in glycerol and maintained at refrigerator temperature will remain virulent for a number of weeks. In this case, glycerol have been used only as suspended media of the material. The effect of glycerol as a cryo-protectant was not recognized until Polge *et al.* (1949) found that glycerol had a protective action to the bull semen in cryo-preservation. Stimulated by their finding, many workers tried to use glycerol in cryo-preservation of the parasitic protozoa (Fulton and A. U. Smith, 1953, may be the first). At that time, one of the most important problems was how to avoid contamination of *Tritrichomonas foetus* to the bull semen, because artificial insemination may spread trichomonad infection among cattles. Since the bull semen can be stored safely at −79 C in the presence of glycerol (Polge and Lovelock, 1952), the possibility was examined by workers if *T. foetus* also can survive with spermatozoa after freezing and thawing procedure. Joyner (1954) answered that *T. foetus* did not survive at some concentrations of glycerol tolerated by bovine spermatozoa: they could be eliminated from infected semen by overnight storage at 5 C in 29% glycerol, or preferably, by freezing to −79 C in the presence of 10% glycerol (cited from Shorb, 1964). Soon McWade and Williams (1954) reported that *T. foetus* could survive in prepared milk semen extender in the presence of 7.5% glycerol at −79 C for 125 days. Leidl and Mahrla (1954) also pointed out that the trichomonads were tolerated in the horse serum broth with 10% glycerol frozen at −99 C. Joyner and Bennett (1956) again confirmed that *T. foetus* failed to survive freezing and thawing in the presence of 10% glycerol (incubation for overnight at 5 C) in egg yolk citrate diluent under the same condition which was adequate for sperm survival. They believed that *T. foetus* were particularly sensitive to the toxic effects of glycerol when suspended in other media such as egg yolk phosphate or milk.

Prior to Joyner's and other papers, McEntegart (1954) reported that the most satisfactory condition of cryo-preservation of trichomonads was found to be storage at −79 C following slow cooling recommended by Fulton and A. U. Smith (1953), the trichomonads being suspended in medium containing 10% glycerol. The "slow cooling" was carried out by the "slow addition" of powdered solid CO_2 into alcohol bath in which samples in ampoule were immersed, and from 0 to −15 C cooling was in the rate of 1 C per minute, below −15 C in the rate of 4 C per minute. According to McEntegart, *Trichomonas gallinae* was easily preserved and survived for 6 months or more even without glycerol, and *T. vaginalis* remained viable for 4 months but died out after 6 months. *Pentatrichomonas hominis* in the presence of 10% glycerol survived for 6 months, but with 5% glycerol did not survive beyond 4 months, and *Tritrichomonas foetus* could not survive under any of the conditions tested. More

recently McEntegart (1959) has reported that the survival period of *T. vaginalis* at −79 C in the presence of 5% glycerol was prolonged to 26 months, and after rapid thawing, a few sluggishly motile trichomonads were still observed microscopically.

Levine and his co-workers studied intensely on cryo-preservation of *T. foetus*, and their first paper appeared in 1954. The paper was only a short abstract (Levine and Marquardt, 1954), but soon they published its detail in the next year (Levine and Marquardt, 1955). According to their paper, they adopted slow cooling method (1 C per minute) similar to McEntegart (1954), and *T. foetus* survived more than 128 days at −76 C in the presence of 1.0 to 1.1 M glycerol (9 to 10%). They also tested the effect of several compounds related to glycerol such as ethylene glycol, 1, 2, 3, 4-butanetetrol, 1, 2-propanediol, and others, and concluded that while glycerol was the most effective compound studied, several related compounds also protected the protozoa against the injurious effects of freezing. In the next experiment, Levine *et al.* (1958) wanted to determine some of the factors which affect the protective action of glycerol both against the immediate effects of freezing and against the subsequent slow deterioration during the storage in a −20 C freezer in the presence of 1.0 M glycerol. They thought that if *T. foetus* died of slow metabolism during prolonged preservation at this temperature, it might be possible to prevent the death by adding of the antimetabolites such as malonic acid, sodium fluoride and sodium iodoacetate. For this purpose, their selected concentration of the antimetabolites failed to lower the metabolism of trichomonads at 37 C, but when the medium frozen, remaining liquid changes to a high concentration of these antimetabolites sufficient to prevent slow metabolism. However, they found that the addition of antimetabolites did not improve the survival. They also experimented the addition of lechitin to the medium in the hope of decreasing the brittleness of the cell membrane, but survival rate did not increase. Increase of the sodium chloride concentration of the medium decreased survival rate of *T. foetus*. They also examined optimal concentration of glycerol in freezing at −21 C, and reported that it was 1.5 M. According to the author's experience (Miyata, 1973a) at −20 C, the medium similar to the one used by Levine *et al.* sometimes did not freeze in the presence of 10% glycerol (about 1.0 M), and the medium maintained a super-cooled state, in which trichomonad still survived. It was not clear whether the medium of Levine *et al.* froze or not, and also even if the medium already froze, question remained whether trichomonad froze or not. Salt concentration was examined by the author (Miyata, 1973a), and somewhat different result was obtained from Levine *et al.*: in *T. vaginalis* it might be said the survival rates in salt concentration of 0.9 to 1.5% were higher than those in lower concentrations.

In other experiment, Levine *et al.* (1959) froze *T. foetus* at −21 C in the presence of 1.0 M glycerol at different phases of the growth curve, and they concluded that when the trichomonad froze during initial and logarithmic phases, they did not survive, and optimum survival came after at the peak of the growth curve.

In the papers written by Fitzgerald and Levine (1957 and 1961), the term *equilibration* was used, and they also checked the temperature fluctuation inside the tubes of frozen medium themselves with thermocouples. When equilibration

with glycerol was carried out at 24 C, survival after freezing and thawing was better following rapid equilibration (glycerol was added all at once, and one hour equilibration) than following slow equilibration (1/6 of final amount of glycerol was added each hour for 6 hours, the equilibration time, 7 hours), but survival of *T. foetus* was extremely poor following either rapid or slow equilibration at 4 C, and glycerol might become toxic at this temperature. They also reported that buffering the storage medium to pH 6.4 to 7.1 with glycylglycine increased survival upon freezing, but triethanoamine had no significant effect.

Levine *et al.* (1962) reported that *T. foetus* survived much longer in the extended storage at −95 C than at −28 C, because at −28 C trichomonads die off slowly, whereas survivors remained constant at −95 C for 128 to 256 days. The survival was much better in the original Diamond's medium (1957) in which trichomonads had been grown than when they were frozen in a physiological salt solution or in the fresh Diamond's medium. They thought that presumably some product or products of the trichomonad's metabolism have an additional protective action which supplements that of glycerol.

3) Discovery of the effect of DMSO as a cryo-protectant

In 1959, Lovelock and Bishop found that dimethyl sulfoxide (DMSO) had a protective action similar to glycerol to living cells in cryo-preservation. This finding also stimulated many protozoologists. Walker and Ashwood-Smith (1961) showed that 5% DMSO and 10% glycerol were equally effective for cryo-preservation of *Trypanosoma rhodesiense* and *T. congolense* at −79 C, and they concluded that the lower toxicity of DMSO both to the host and to the parasite, as compared with glycerol in cryo-preservation. Collins and Jeffery (1963) also compared 5% DMSO and 10% glycerol in low-temperature preservation of blood stage of *Plasmodium berghei* and *P. gallinaceum* at −70 C, and they reported that both substances were effective to those malaria parasites, and although 10% glycerol gave rise to superior survivals, the lower toxicity of DMSO to the parasites made it a useful alternative for preservation.

4) Preservation in liquid nitrogen

Although the possibility of preservation of the living protozoa in liquid nitrogen was suggested by early workers, the use of liquid nitrogen for dry ice or mechanical refrigeration is relatively new. The temperature in the dry ice cabinet or mechanical freezers was maintained from −70 to −95 C at the lowest, and at such relatively high temperatures, the cryo-preservation of the protozoa is not permanent but only for several months or a few years. The cause of death of the protozoa during preservation period is thought that ice crystal can form and grow in the protozoan cells. The ice crystal, however, does not form and grow below the recrystallization point of water (−130 C). Therefore, the temperature for preservation of living cells might be better kept below this point. Diamond *et al.* (1961 and 1963) reported that use of liquid nitrogen refrigerator for the preservation of *Entamoeba histolytica* (monoxenic culture grown in association with a *Crithidia* sp.), *Tritrichomonas foetus, Trichomonas gallinae, T. vaginalis, Pentatrichomonas hominis, Trypanosoma cruzi,* and *T. ranarum* in the presence of 5% DMSO. Before cooling, trichomonads and amoebae-*Crithidia* suspensions were allowed to equilibrate for 15 and 30 minutes, respectively, at 35 C, and the trypanosomes for 15 minutes at room temperature, the samples

were then cooled by two-step cooling technique, in which the samples were cooled at first at the rate of 1 C per minute from 0 to −35 C, then they were plunged directly into liquid nitrogen. To examine the survival, aliquots of the samples were momentarily immersed and rapidly swirled about in a 45 C water bath containing a few drops of 25% Aerosol O. T. (American Cynamid Co.) which was added to improve heat transfer. According to their results, cultures of *E. histolytica* were obtained from samples stored as long as 14 months, and also viable cultures were obtained from *T. gallinae* and *T. vaginalis* stored for 10 months and *T. foetus* and *P. hominis* stored for 5 months. *T. cruzi* and *T. ranarum* survived up to 4 months. No difference in numbers was found between samples thawed 24 hours after freezing and those thawed after the longest period of storage. The result indicated the absence of gradual death during extended period of storage in liquid nitrogen, whereas such death was commonly reported at dry ice temperature by many workers.

Herbert *et al.* (1968) also reported the storage of *Trypanosoma brucei* subgroup at −196 C by using rapid cooling method, and without glycerol or DMSO they obtained the excellent result. Reather and Seidenath (1972) successfully stored a total of 28 species of the parasitic protozoa containing 10 genera, *Entamoeba, Pentatrichomonas, Trichomonas, Tritrichomonas, Crithidia, Leishmania, Trypanosoma, Plasmodium, Babesia,* and *Eimeria,* for 720 to 2,662 days at −196 C. Their cooling procedure is as follows: The samples containing DMSO or glycerol are stored in plastic ampoules for 30 minutes at 4 C, then they cooled at 0.4 C per minute from 4 to −10 C, at 2 C per minute from −10 to −30 C, at 5 C per minute from −30 to −60 C, and finally when reached −60 C, the samples are stored in liquid nitrogen. Their paper shows the possibility of extremely prolonged preservation in liquid nitrogen, and this is the longest storage record in the frozen state reported in the literature. The record, however, does not mean the maximum and more extension may be possible in future.

II. Other Literatures

1) Amoebae

In the history of cryo-preservation of the parasitic protozoa, glycerol as a cryo-protectant was used at first by Fulton and A. U. Smith (1953) for the storage of *Entamoeba histolytica* at −79 C. Their success stimulated many workers. Fulton and A. U. Smith used *E. histolytica* which cultured monoxenically with *Bacillus coli,* and in the presence of 5 to 10% glycerol, the amoebae were cooled by two step technique; 0 to −15 C for 20 minutes, −15 to −70 C for 30 minutes, and storage at −79 C. Before cooling, the samples were allowed to equilibrate with glycerol for 30 minutes at 37 C. Thawing after 65 days of freezing, the samples were inoculated to fresh media, and the survived amoebae multipled in the media.

Kasprzak and Rydzewski (1970) reported that *E. histolytica* (3 strains), *E. invadens,* and free-living *E. moshkovskii* survived for 80 days, 30 days, and 450 days, respectively, at −75 C in the presence of 10% glycerol by slow cooling.

Gordon *et al.* (1969) using DMSO, preserved axenic *E. histolytica* for 5 months at −196 C in liquid nitrogen. They cooled the samples at the rate of 1 C per minute untill temperature reached −70 C, then the samples were stored in liquid nitrogen.

Bosh and Frank (1972) observed that *E. invadens* and *E. histolytica* could survive freezing in liquid nitrogen using 10% DMSO. Neal *et al.* (1974) also reported their successful storage of 6 species of the genus *Entamoeba* in liquid nitrogen in the presence of DMSO. Other papers concerning the preservation of *Entamoeba* spp. in liquid nitrogen were introduced in page 167 of this review.

In the papers introduced above, trophozoite stage samples were used for cryo-preservation, but Halpern and Dolkart (1954) and Chang (1955) examined the cyst of *E. histolytica* at about −30 C without cryo-protectant, but their results were unsuccessful except for several hour survival after freezing.

Molinari (1955, 1956a, and 1956b) wrote several papers on the effect of low temperature (4, −20, −79, or −180 C) on survival of trophozoites of *E. histolytica* without using cryo-protectant.

According to the author's own experiments (Miyata, 1973b), the cryo-preservation of Laredo strain of *E. histolytica* at −75 C did not succeed even in the presence of glycerol or DMSO, and the main reason of this failure could be explained that rice powders which were contained in the medium became injurious, because under freezing condition, amoeba decreased the cell volume, and rice powders ingested inside the cell might be destroyed by mechanical pressure to organelles such as nucleus or cell membrane.

2) Trypanosomatids

Polge and Soltys (1957) studied cryo-preservation of trypanosomes with or without glycerol by two different cooling methods; (a) *Quick freezing*: the samples were frozen directly in a mixture of dry ice and absolute alcohol at −79 C. (b) *Two stage freezing*: samples were first placed in alcohol at room temperature, then cooled slowly (2 C per minute) by adding small pieces of dry ice untill the temperature reached −20 C. After the desired temperature was obtained, the samples were continued to cool at 5 C per minute to −79 C.

According to their paper, both the whole horse serum and the yolk citrate diluent proved unsatisfactory without glycerol. In Alsever's solution (glucose 4.66 g, sodium chloride 1.05 g, sodium citrate 2.0 g, and distilled water 200 m*l*) and sperm-Ringer or other diluents, but the best results (about 50% survival) were obtained with the fastest rate of cooling without glycerol, and survived trypanosomes decreased when the cooling rate was slower. In contrast, in the media containing glycerol, the viability after thawing was poor after quick freezing and was much better after slow cooling. The best results in all diluents (about 80% survival) were obtained with slow cooling and with 5 or 10% glycerol in the medium.

Lymph tubes, instead of test tubes or vials, were used by Cunningham *et al.* (1963b) for preservation of trypanosomes at −80 C. The trypanosome suspensions containing 7.5% glycerol were distributed in "about 25 mg quantities" to the tubes, and after sealing, they were cooled slowly. By the use of these small tubes, many materials could be preserved in a small space.

The following authors also studied cryo-preservation or cryo-resistance of trypanosomes at various temperatures: Reuße (1956), Molinari and Montézin (1956a and 1956b), Molinari (1960b), Gordon (1961), Flück (1962), Berson (1962), Cunningham and Harley (1962), Cunningham *et al.* (1963a and 1965), Resseler *et al.*

(1965), Mieth (1966), Lapierre and Vinh Hien (1968a and 1968b), Dar *et al.* (1972), Miyata (1973b), and Filardi and Brener (1975).

Leishmania spp. were easily preserved by Foner (1963), Most *et al.* (1964), and Heymen and Monsour (1962). Foner reported that *Leptomonas culicidarum* and *Herpetomonas muscidarum* survived for 13 months at −60 C in the presence of 10% glycerol.

3) Trichomonads

The following papers are not cited in this review: Blackshaw and Beattie (1955), Honigberg and King (1962), Honigberg *et al.* (1965), Resseler *et al.* (1965), Lumsden *et al.* (1965 and 1966), Müller (1966), Kasprzak and Rydzewski (1970), and Miyata (1973a, 1974, 1975a, and 1975b).

4) *Giardia* spp.

As far as the author knows, there are only three papers containing cryo-preservation of *Giardia* spp. Bemrick (1961) reported that trophozoites of *Giardia muris* could survive for 40 days in frozen state (at −38 C?) in the presence of glycerol. At −20, −38 and −70 C, the parasites would survive at least 24 hours if glycerol were used. Slow freezing in cotton insulated tubes (a modification of Levine and Marquardt, 1955) resulted in a higher survival rate than quick freezing, and if 1% Tween 80 was added into basal medium containing glycerol, the number of survived parasites after freezing and thawing was greater than that where the Tween 80 was not present. The temperature of −38 C appeared to be the most favorable for the survival of *G. muris,* and at the temperautre, optimum concentration of glycerol was 14.2 percent. But *G. muris* may survive quick freezing at −70 C for several months.

Meyer and Chadd (1967) preserved trophozites of *Giardia* spp. obtained from culture at −70 C for at least 2 years in the presence of 7% glycerol. They compared various concentrations of glycerol (1, 3, 5, 7, 9, and 15%) and DMSO (1, 3, 5, 7, and 9%). All samples were frozen slowly in four steps: room temperature for 15 minutes, −12 to −15 C for 30 minutes, and −22 C until frozen (45 to 90 minutes); then they were stored at −70 C. The parasites survived after freezing and thawing (in a 37 C water bath) at every glycerol and DMSO concentration examined, but most viable parasites were recovered after using 7% DMSO. The parasite did not survive in cultures frozen without cryo-protectants.

The paper written by Le Corroller *et al.* (1970) also dealt with preservation of *G. muris* as cited in page 177 of this review.

5) *Dientamoeba fragilis*

According to Dwyer and Honigberg (1971), *Dientamoeba fragilis* could survive for 6 months after storage in liquid nitrogen by using slow-cooling technique in the presence of DMSO.

6) Ciliates

As far as the author knows, only one paper (Weinman and McAllister, 1947) was published on the cryo-preservation of *Balantidium coli* at −70 C with negative result cited in page 164 of this review. After discovery of the effect of cryo-protectants such as glycerol or DMSO, there are still few papers on the study of low-temperature preservation of ciliate.

Hwang *et al.* (1964) reported that *Tetrahymena pyriformis* could survive for 3 months

after freezing at −196 C in the presence of 10% DMSO. The samples were cooled for 20 minutes in a −20 C freezer (2 C per minute), then they were stored in liquid nitrogen. The number of survived ciliate after freezing and thawing reduced to about one fourth of that before cooling.

Wang and Marquardt (1966) also preserved *T. pyriformis* for 112 days in a −95 C deepfreezer using 10% DMSO, and the ciliate still survived. They also adopted two step freezing; the samples were first cooled in a −27 C freezer for 20 minutes (4.5 C per minute), and those were transfered into a −95 C alcohol bath, then stored in the freezer. Furthermore, they reported that *Paramecium aurelia* survived for 7 days at −27 C or −196 C in the presence of 6.0 to 7.5% DMSO. Unfortunately the survival ciliates, however, did not multiply in the inoculated fresh media, except for the storage for 20 minutes at −27 C. According to Marquardt *et al.* (1963), ciliates (*Colpoda steinii*) and amoebae (*Vahlkampfia* sp.) were found from sands or dusts collected in an ice tunnel of Greenland, and cyst of both species could survive at the low temperature of −28 or −95 C, but trophozoites of either protozoa did not.

C. Wolfson (1935) observed *Paramecium* by using a cryo-microscope which was designed by himself. Other papers on the study of cryo-biology of ciliate are as follows; Efimoff (1924), Altavilla *et al.* (1971), Matsusaka (1971), and Tanno (1972).

7) Malaria parasites

Plasmodium spp. seem to be one of the easiest parasites for cryo-preservation in dry ice, mechanical freezers, or liquid nitrogen in the presence or absence of cryo-protectants already mentioned above. The freezing methods adopted by various workers are simillar to each other, and it is needless to repeat the details of the papers written by following authors: Vargues (1952), Molinari and Montézin (1956b), Molinari (1960a and 1961), Molinari and Tebibzadeh (1961), Jeffery (1962 and 1965), Shute and Maryon (1962), Yoeli *et al.* (1963), Collins *et al.* (1963), Vincke *et al.* (1965), Jacobs (1965), Warhurst (1966), Bafort *et al.* (1966), Bafort (1968), Killick-Kendrick and Bruce-Chawatt (1969), Booden and Geiman (1970 and 1973), Allen (1970), Miyata (1973b), and so on.

Among them, Molinari (1961), Shute and Maryon (1962), and Bafort (1968), reported on cryo-preservation of sporozoites in the salivary gland of vector mosquitoes.

Estimation of the number of alive malaria parasites before and after freezing was rather difficult. Warhurst (1966), however, pointed out that percentage survival of 2 strains of *P. berghei* after freezing at −78 C in 7% DMSO might be measured by application of a lineal relationship between log inoculum size and *pre-two percent period* (the period between intravenous inoculation of parasites and infection of two percent of the red cells). The measurement of the effect of low-temperature on the protozoa by titration was reported by Overdulve and Antonisse (1970a, 1970b and 1970c).

8) *Toxoplasma gondii*

Toxoplasma gondii was one of the difficult species to preserve at low temperature without cryo-protectants. Recently, Fabio *et al.* (1967), however, described that *T. gondii* (RH strain) which was prepared in physiological saline could survive for 47 days at −70 C. Miyata (1973b) also reported that *T. gondii* (RH starin) was

easily preserved in a −75 C deepfreezer for at least 30 days without using any cryo-protectant.

By using such cryo-protectants, at least RH strain of the parasite is more easily stored in dry ice, deepfreezers, or liquid nitrogen. For example, Chandler and Weinman (1956) preserved two toxoplasma strains at −70 C for 184 days by using mouse peritoneal exudate for freezing in the presence of 10% glycerol. Eyles *et al.* (1956) also preserved *T. gondii* by slow freezing in glycerol solution for 209 days at −70 C. They observed that very slow cooling using 5 to 10% glycerol was the best, but even with the optimum methods, the initial loss of viability after freezing was great, whereas storage at below −70 C resulted in little further loss of the viability over the period examined. Recently, Stewart and Feldman (1955) described the use of rollar-tube tissue cultures of *T. gondii* as a source of parasites for cryo-preservation by two step cooling in dry ice, and after 360 day preservation, the cultures became positive after one blind passage.

In liquid nitrogen, according to Franchi and Hahn (1968a and 1968b), *T. gondii* survived in the presence of 10% glycerol after slow cooling. The samples were kept at 4 C for 2 to 3 hours, then they were cooled at the rate of 1 C per minute. After pre-cooling, the samples were stored at −196 C. R. Smith (1973) stored safely RH strain of *T. gondii* which was obtained from tissue culture, in liquid nitrogen with the fetal calf serum as a cryo-protectant. He preserved samples directly in liquid nitrogen. Before reaching this ideal method, he attempted to use DMSO or glycerol by slow cooling at the rate of 1 C per minute to −70 C, then they were cooled and stored in liquid nitrogen.

All the authors of the papers cited above used proliferative form of *T. gondii*, but following two authors showed the possibility of preservation of cysts or parasites within cysts. Kwantes *et al.* (1967) preserved cyst-cyst type strains which were isolated from various human tissues or tissue fluid. After storage at −70 C the strains produced cysts in mice and their virulence did not change during the preservation. Roble (1965) also observed that cysts survived for 200 days in fresh milk at −76 C. Miyata (1973b), however, reported a negative results on cysts of Beverley strain in preservation at −75 C with or without glycerol. According to this paper, even in those negative samples, a few cysts of normal appearance was detected microscopically without damage, but they have already lost the infectivity to mouse.

Frenkel and Dubey (1973) observed that sporulated oocysts of *T. gondii* could survive for 28 days at −20 C without using cryo-protectants.

Gartner and Theile (1970) studied on deep-frozen toxoplasm with electron microscope. According to them, in the process of rapid cooling, water penetrated into cell through the conoid by pressure differences between the outside and the inside of the cell, and after freezing the water changed to ice which destroyed the cell. They also described that in slow cooling, dehydration of the cell was a cause of cell damage, while mechanical destructions were diminished and the parasite could survive. Before the publication of their interesting paper, this kind of study has not been reported by protozoologists, but the direction suggested by Gartner and Theile would be followed by other workers in near future. Other papers on cryo-preservation of *T. gondii* were as follows; F. and A. Roger (1957), Fabio *et al.* (1967), Bugiar-

dini *et al.* (1967), Bollinger *et al.* (1974), and Dumas (1974a, 1974b and 1974c).

9) *Eimeria, Leucocytozoon, Babesia, Eperythrozoon, Nosema, Anaplasma,* and *Theileria*

Cryo-preservation of above genera was studied by following workers: *Babesia berbera* by Pipano and Senft (1966), *B. bigemina* by Waddell (1963), Pipano and Senft (1966), and Barnett (1964); *B. trautmani* by Barnett (1964); *B. calballi* by Frerichs *et al.* (1968) and Wayne *et al.* (1968); *B. equi* by Frerichs *et al.* (1968); *B. rodhaini* by Overdulve and Antonisse (1970b), and Mieth (1966); *B. canis* by Reuße (1956) and Mieth (1966); *Leucocytozoon simondi* by Kocan *et al.* (1967); *Eimeria tenella* by Kouwenhoven (1967), Doran and Vetterling (1968 and 1969), Doran (1969a and 1970), and Norton and Joyner (1968); *E. acervulina* by Norton *et al.* (1968); *E. meleagrimitis* by Doran and Vetterling (1968 and 1969) and Doran (1969a); *E. adenoeides* by Doran (1970) and Norton and Joyner (1968); *E. ahsata, E. arloinigi, E. duodenalis, E. maxima, E. phasiani,* and *E. stiedae* by Norton and Joyner (1968); *Eimeria* spp. by Landers (1953); *Eperythrozoon wenyoni* by Ishihara and Minami (1968); *Anaplasma centrale* and *A. marginale* by Barnett (1964), Pipano and Senft (1966), and Ishihara and Minami (1968); *Theileria* sp. (2 strains) by Ishihara and Minami (1968); *Nosema cuniculi* by Bedrnik and Vavra (1971).

According to Doran and Vetterling (1969), and Doran (1969a), oocysts, sporocysts released from oocysts, and excysted sporozoites of *E. meleagrimitis* and *E. tenella* in media containing 7% DMSO were frozen at −80 C (1 C per minute), and stored in liquid nitrogen vapor for 4 months. After thawing, oocysts could not infect fresh hosts, although these samples appeared in an excellent condition under microscope. After 3 months, sporocysts of both species could infect, but in a less extent than those infected by fresh oocysts. Frozen sporozoites caused infection to fresh hosts comparable to those caused by unfrozen fresh oocysts. Those results are somewhat similar to those of the cyst of Beverey strain of *Toxoplasma gondii* (Miyata, 1973b). According to the author's opinion, since water in the inside of the oocyst is unable to pass the oocyst membrane during the freezing procedure, the water would form ice crystals within the oocyst, and the ice becomes injurious to sporozoites inside of the oocyst. Water penetration to the sporocyst membrane appeares faster than that to the oocyst membrane.

Doran (1969b) studied freezing of excysted sporozoites of *Eimeria* spp. According to his results, survival of sporozoites was better at the lower concentration of DMSO before freezing, but after freezing and thawing, survival was better at the higher concentration of DMSO. With 10 to 12.5% DMSO, various equilibration periods and cooling rates were compared, and the best results were obtained where cooling rate from the freezing point to −30 C was 1 C per minute. In the concentrations of 2.5, 5.0 and 7.5% DMSO, various equilibration periods up to 2.5 hours were examined, but the survival rate of sporozoites was not improved.

10) Other protozoa

The following authors studied on cryo-biology of the protozoa: A. U. Smith *et al.* (1951), Gehenio and Luyet (1953), Sharf (1954), Altavilla *et al.* (1971), and Klein (1972).

Annear (1956) preserved *Strigomonas oncopelti* for 12 months in dried state *in vacuo* at 4 or 20 C. The contents of ampoules yielded heavy growths of the flagellates

after 12 month storage.

11) Helminths

Weinman and McAllister (1947) studied on the cryo-preservation of helminth at −70 or −15 C. Microfilariae of *Wuchereria bancrofti* and *Dirofilaria immitis* could survive at −70 C without cryo-protectant, and infective larvae of *Ancylostoma caninum* also survived for 107 days at −15 C and 44 days at −70 C, respectively.

Recently, several papers were published on the attempts to preserve helminth larvae at low temperatures in the presence of cryo-protectant. Microfilariae or mosquito stages are easily preserved. The following authors published their studies on cryo-preservation or cryo-biology of helminths including some papers concerning free living nematodes: De Coninck (1951), J. H. Turner (1953), Gustafson (1953), Poole (1956), Asahina (1959), Taylor (1960), Beye and Lawless (1961), Bemrick *et al.* (1965), Restani (1968), Ogunba (1969), Parfitt (1971), Obiamiwe and Macdonald (1971), Campbell *et al.* (1972 and 1973), Isenstein and Herlich (1972), Campbell and Thomson (1973), McCall *et al.* (1975), and James (1975).

III. Various Factors on Cryo-preservation

Various factors involved in cryo-preservation of the parasitic protozoa were introduced and discussed briefly in this chapter.

1) Cooling rate

Before preserving the parasitic protozoa in the frozen state, most workers cooled the samples at a rate of about 1 C per minute to temperature around −30 C. To obtain the cooling rate, the samples were pre-cooled at −20 to −30 C for 30 to 90 minutes, then they were stored at −70 to −80 C or −170 to −190 C. The author compared five different cooling rates between 0.25 to 5 C per minute in preservation of *Trichomonas vaginalis* (Miyata, 1974). Each group of 10 samples in the presence of 10% glycerol was pre-cooled in each freezer of 5 different temperatures for 90 minutes, then the samples were stored in a −75 C freezer. According to the results, the highest survival rate of *T. vaginalis* was obtained at the cooling rate of 0.78 C per minute by the use of a −30 C freezer (Figs. 1 and 2). The survival rate at 5 C per minute was about 20 percent lower than the highest survival rate.

Diamond (1964) reported that 30 to 40 percent of *Entamoeba invadens* protected with 15% DMSO and 4.5% glucose have survived when cooled at 1 C per minute, but there were almost no survivors in the samples cooled at 8 C per minute.

The cooling and thawing process of the parasitic protozoa in cryo-preservation can be explained in Fig. 3 (after Miyata, 1973a). The sample is cooled at about −30 C for one to two hours. The temperature of the sample drops without freezing, and the super-cooled state continues for several minutes. The survival rate of the cells does not change during super-cooled state, but when the state is broken, the temperature slightly goes up by liberation of the latent heat in crystallization, then the temperature of the sample again drops slowly. The point at the beginning of freezing (F in Fig. 3) is called the freezing point or the freezing temperature of the sample. The freezing temperature is lower in higher concentration of the cryo-protectant as shown in Fig. 4. From the point to −30 C or lower, dehydration of the cells is carried out by

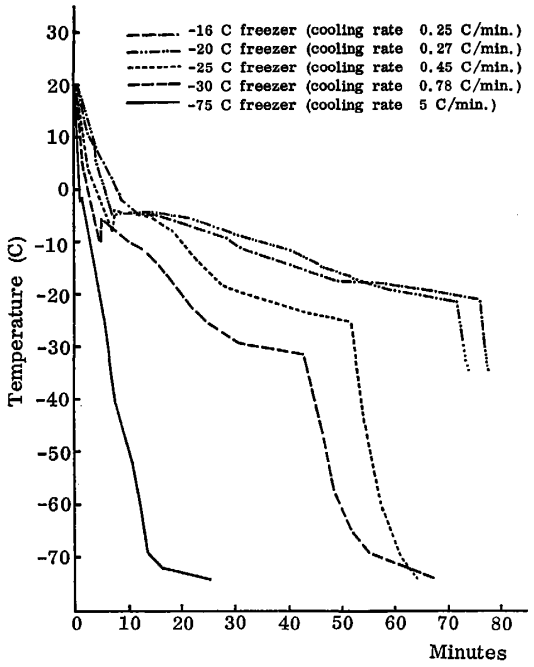

Fig. 1 The cooling curves of pre-cooling in various freezers (Miyata, 1974).
Note: Cooling curves were only traced until temperature reached the lowest temperature in each freezer and after transfered into the −75 C freezer.

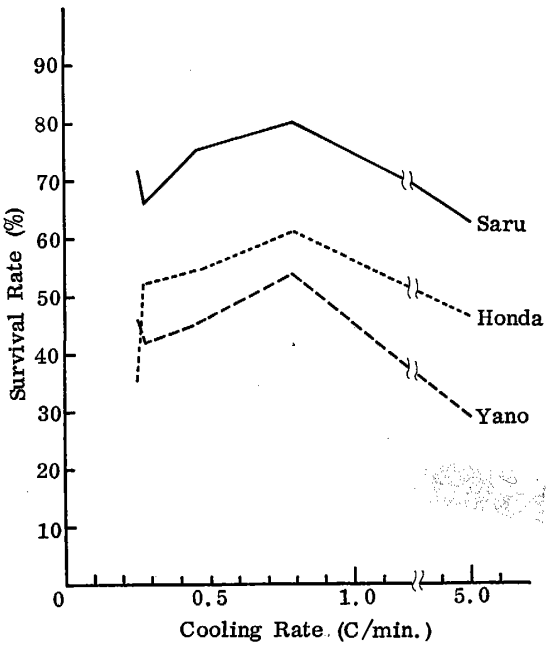

Fig. 2 The survival rate of trichomonad in each cooling rate after 1 to 5 day frozen storage in −75 C freezer (modified from Miyata, 1974).
Note: Three strains (Saru, Honda, Yano) of *Trichomonas vaginalis* were used in this experiment.

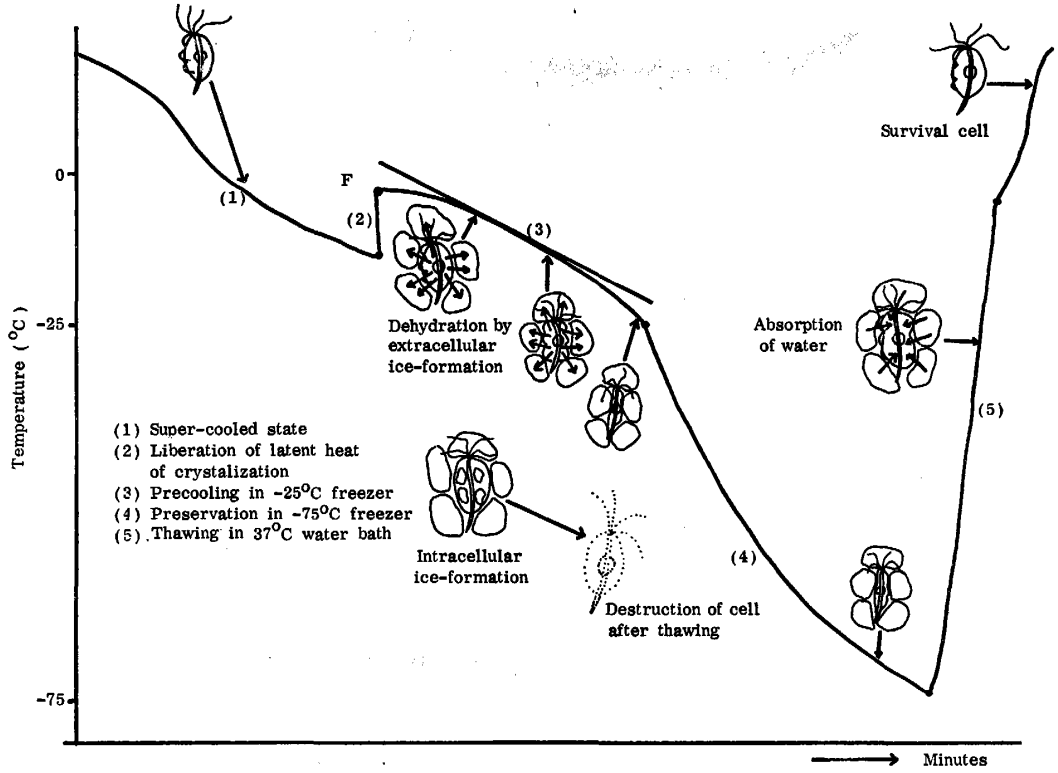

Fig. 3 The schematic explanation of the pre-cooling method (Miyata, 1973a).

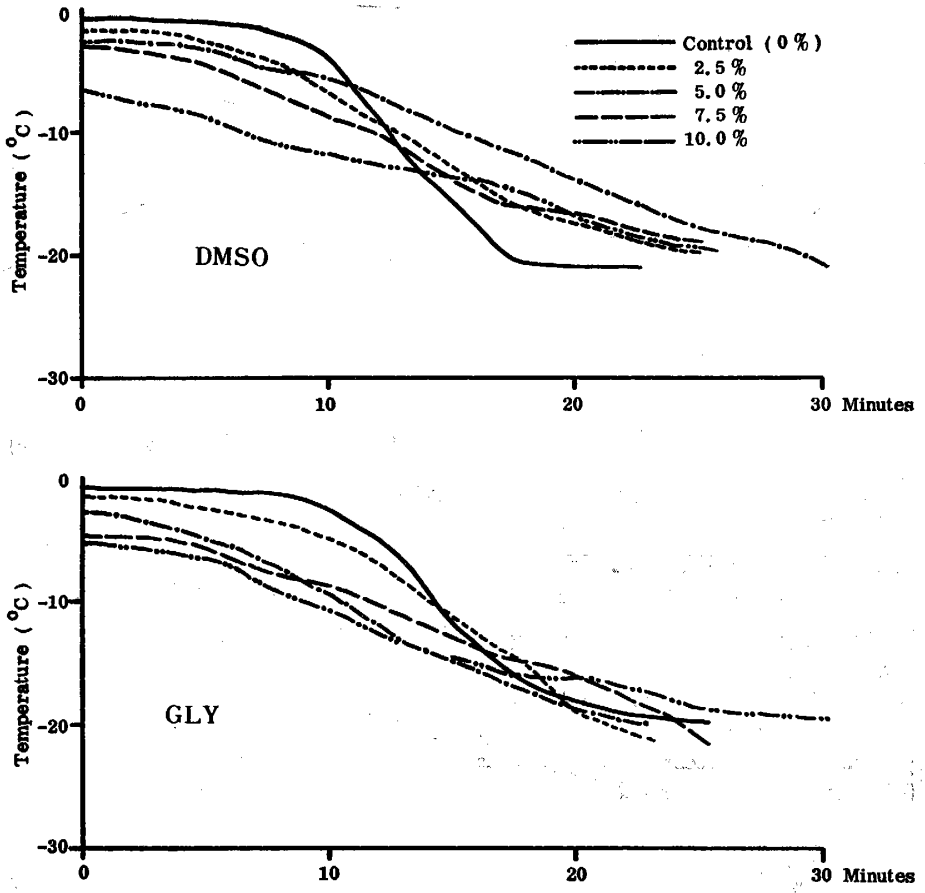

Fig. 4 The cooling curves of various concentrations of glycerol (GLY) or DMSO in a −25 C freezer (Miyata, 1973a).
Note: Each curve is shown from the beginning of freezing, and super-cooled state is excluded.

formation and growth of extracellular ice crystals. Dehydration of the cells must be thorough, because if the water remains in the cell, the water may be crystallized later and destroy the cell mechanically. After sufficient dehydration, the sample must be cooled rapidly to the storage temperature. In the rapidly cooled samples without pre-cooling, the water in the cells can not go out, and changes to ice crystal in the cell. This is the reason to explain why slow cooling is better than rapid cooling. In the case of very slow cooling, salt concentration could be effective. Several workers reported that some species or strains of the parasitic protozoa could survive after freezing directly in dry ice alcohol or liquid nitrogen. In such cases, many ice crystals may be formed within the cell, but the size of each crystal is too small to injure the cell.

Most workers recommended that the frozen samples must be thawed in a water bath at about 40 C as soon as possible after taking out from a storage cabinet, because recrystallization of ice can be avoided by such rapid thawing. Diamond (1964) and Dalgliesh (1972) pointed out that the survival rate increased in proportion to the warming rate.

2) Storage period at various temperatures

In Table 1, storage periods of the parasitic protozoa (24 genera including about 90 species) at various temperatures were summarized. The longest preservation period of each species at different temperatures in the presence or absence of cryo-protectants was sought from many literatures as far as the author knows. Several species could survive after preservation at relatively high temperatures as −20 to −30 C, but the storage period is rather short. According to such authors, gradual death occured during preservation period at such a high temperatures, and finally all organisms died. At −70 to −90 C, survival period prolonged to months or often years, but at this temperature still living parasites decreased gradually with the increase of storage period. At the temperatures of liquid nitrogen itself or its vapor, the protozoa could survive for an extremely long period. At present, the longest survival record so far tested is longer than 2,500 days (Raether and Seidenath, 1972), but the period might have been extend infinitely. It was said by many workers that before cooling and after storage for several years in liquid nitrogen, there were no significant loss of survival number and of their infectivities to the host.

3) Use of chemical compounds for cryo-preservation

Both glycerol and DMSO are widely used by workers as cryo-protectants, whereas recently, DMSO instead of glycerol was recommended by several workers (Dalgliesh, 1972; Walker and Ashwood-Smith, 1961; Collins and Jeffery, 1963; Diamond, 1964; and Miyata, 1973a), because of its rapid equilibration and lower toxicity. In addition to these two chemicals, some other substances were reported having the cryo-protective action to living cells, for example, dimethylacetamide by Djerassi *et al.* (1971) and pyridine N-oxide by Nash (1961). Dobbler (1966) reviewed and discussed of structure and function of cryo-protective compounds.

As a protozoologist, O'Connel and his co-workers (1968) examined the cryo-protective action of 83 chemicals such as alcohol, sugars, amines, and others, for preservation of *Crithidia fascilatus* at −20 C. According to their results, the best protectant among the chemicals tested was glycerol at 10 percent (w/v), and as a group the alcohols were most effective, and several sugars also showed some protective activities. Levine and Marquardt (1954) also tested several compounds related to glycerol in cryo-preservation of *Tritrichomonas foetus* introduced in page 166 of this review.

The protective action of the serum in cryo-preservation of the protozoa was reported by several workers (Jeffery and Rendtroff, 1955; Polge and Soltys, 1957; R. Smith, 1973; and Miyata, 1973b).

More recently, Le Corroller *et al.* (1970) examined macromolecular, *extracellular* cryo-protectants such as polysaccarides and polyvinyl pyrrolidone instead of micromolecular, *intracellular* cryo-protectants such as glycerol and DMSO, because the toxicity of *intracellular* cryo-protectants were pointed out by various workers. They prepared a mixture containing 15 g of dextran sorbitol (Rheomacrodex) and 15 g of polyvinyl pyrrolidone in 100 m*l* distilled water. Protozoan suspensions were mixed in equal volume of the cryo-protectant mixture. The samples which distributed in one to 4 m*l* in ampoules were cooled and stored at −70 C. *Trypanosoma lewisi, T. cruzi, T. congolense, T. brucei, T. gambiense, Tritrichomonas muris, Trichomonas tenax, T. vaginalis, Pentatrichomonas hominis, Giardia muris, G. intestinalis, Toxoplasma gondii,* and

TABLE 1 The longest preservation records in each parasitic protozoa at various temperatures with or without cryo-protectant

Species	Stage of parasite	Storage temperature (C)	Cryo-protectant	Storage period (days)	Author
Entamoeba coli	trophozoite	−170	DMSO	115*	Neal *et al.*, 1974
E. hartmanni	〃	−170	DMSO	1,043*	〃
E. histolytica	cyst	−28	none	(7.5 hrs)	Chang, 1955
〃	trophozoite	−75	glycerol	80	Kasprzak & Rydzewski, 1970
〃	〃	−170	DMSO	2,675*	Neal *et al.*, 1974
E. invadens	〃	−75	glycerol	30	Kasprzak & Rydzewski, 1970
〃	〃	−170	glycerol or DMSO	105	Diamond, 1964
〃	〃	−170	DMSO	1,909*	Neal *et al.*, 1974
E. moshkovskii	〃	−75	glycerol	405	Kasprzak & Rydzewski, 1970
〃	〃	−170	DMSO	1,902*	Neal *et al.*, 1974
E. ranarum	〃	−170	DMSO	504*	〃
E. terrapinae	〃	−170	DMSO	1,162*	〃
Dientamoeba fragilis	〃	−170	DMSO	1,435*	〃
〃	〃	−196	DMSO	180*	Dwyer & Honigberg, 1971
Tritrichomonas augusta	〃	−79	glycerol	91	Müller, 1966
Tritrichomonas foetus	〃	−16	glycerol	196	Reuße, 1956
〃	〃	−28	glycerol	128	Levine & Andersen, 1966
〃	〃	−95	glycerol	2,048	〃
〃	〃	−196	DMSO	1,383	Raether & Seidenath, 1972
Tritrichomonas muris	〃	−70	P+P†	730*	Le Corroller *et al.*, 1970
Tritrichomonas sp. (*suis* ?)	〃	−79	glycerol	91	Müller, 1966
Trichomonas gallinae	〃	−19	glycerol	133*	Stabler *et al.*, 1964
〃	〃	−72	glycerol	364*	〃
〃	〃	−196	DMSO	1,383	Raether & Seidenath, 1972

Trichomonas tenax	trophozoite	−75	glycerol	450	Kasprzak & Rydzewski, 1970
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
Trichomonas vaginalis	〃	−25	DMSO	14*	Miyata, 1973a
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	〃	−75	glycerol	780*	McEntegart, 1959
〃	〃	−170	DMSO	1,154	Diamond, 1964
Trichomitus fecalis	〃	−196	DMSO	1,383	Raether & Seidenath, 1972
Pentatrichomonas hominis	〃	−70	none	407	Weinman & McAllister, 1947
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	〃	−79	glycerol	487	Müller, 1966
〃	〃	−196	DMSO	1,383	Raether & Seidenath, 1972
Monocercomonas sp.	〃	−170	DMSO	196	Diamond, 1964
Giardia intestinalis	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
Giardia muris	〃	−38	glycerol	27	Bemrick, 1961
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
Giardia spp.	〃	−70	glycerol	730*	Meyer & Chadd, 1967
Herpetomonas muscidarum	culture	−60	glycerol	390*	Foner, 1963
Leptomonas culicidarum	〃	−60	glycerol	390*	〃
Crithidia fasciculata	〃	−20	glycerol	21*	O'Connell *et al.*, 1968
〃	〃	−170	DMSO	155	Diamond, 1964
Crithidia sp. (ReF–1: PRR)	〃	−196	DMSO	1,260	Raether & Seidenath, 1972
Crithidia sp. (ReF–2)	〃	−196	DMSO	1,260	〃
Leishmania adleri	〃	−60	glycerol	390*	Foner, 1963
Leishmania agamae	〃	−60	glycerol	390*	〃
Leishmania braziliensis	〃	−60	glycerol	390*	〃
〃	〃	−196	DMSO	420*	Resseler *et al.*, 1965
Leishmania donovani	tissue	−64	glycerol	365*	Allain, 1964
〃	〃	−70	none	276	Weinman & McAllister, 1947

Leishmania donovani	culture	−64	glycerol	180*	Allain, 1964
〃	〃	−196	DMSO	420*	Resseler *et al.*, 1965
〃	tissue	−196	glycerol	2,643	Raether & Seidenath, 1972
Leishmania enrietti	〃	−196	glycerol	2,484	〃
〃	culture	−196	DMSO	420*	Resseler *et al.*, 1965
Leishmania infantum	〃	−60	glycerol	390*	Foner, 1963
Leishmania tarentolae	〃	−60	glycerol	390*	〃
Leishmania tropica	〃	−70	none	794	Weinman & McAllister, 1947
〃	〃	−60	glycerol	390*	Foner, 1963
〃	〃	−196	DMSO	450*	Resseler *et al.*, 1965
Trypanosoma brucei	blood	−76	none	135	Horsfall, 1940
〃	〃	−80	glycerol	850*	Cunningham *et al.*, 1963b
〃	〃	−80	glycerol	850*	〃
〃	fly	−80	glycerol	850*	〃
〃	blood	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	〃	−196	DMSO	330*	Resseler *et al.*, 1965
〃	〃	−196	glycerol	2,661	Raether & Seidenath, 1972
Trypanosoma congolense	〃	−70	none	100	Levaditi, 1952
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	〃	−80	glycerol	852*	Cunningham *et al.*, 1963b
〃	〃	−196	DMSO	90*	Resseler *et al.*, 1965
〃	〃	−196	glycerol	2,650	Raether & Seidenath, 1972
Trypanosoma conorhini	culture	−20	glycerol	28*	O'Connell *et al.*, 1968
Trypanosoma cruzi	blood	−70	none	234	Weinman & McAllister, 1947
〃	culture	−70	none	653	〃
〃	〃	−80	glycerol	852*	Cunningham *et al.*, 1963b
〃	blood?	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	blood	−196	glycerol	2,627	Raether & Seidenath, 1972
〃	culture	−170	DMSO	987	Diamond, 1964
Trypanosoma duttoni	blood	−76	none	34	Horsfall, 1940
〃	culture	−64	glycerol	540*	Allain, 1964

Trypanosoma equinum	blood	−196	glycerol	2,640	Raether & Seidenath, 1972
Trypanosoma equiperdum	〃	−76	none	62	Horsfall, 1940
〃	〃	−75	glycerol	520	Kasprzak & Rydzewski, 1970
〃	〃	−190	none	21	De Jong, 1922
〃	〃	−196	DMSO	270*	Resseler *et al.*, 1965
〃	〃	−196	glycerol	2,656	Raether & Seidenath, 1972
Trypanosoma evansi	〃	−76	none	62	Horsfall, 1940
〃	〃	−79	glycerol	618	Ishihara & Minami, 1968
〃	〃	−196	glycerol	2,654	Raether & Seidenath, 1972
Trypanosoma gambiense	〃	−20	glycerol	3	Miyata, 1973b
〃	culture	−70	none	189	Weinman & McAllister, 1947
〃	blood	−70	none	561	〃
〃	〃	−79	glycerol	250	Polge & Soltys, 1957
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	〃	−196	DMSO	90*	Resseler *et al.*, 1965
〃	〃	−196	glycerol	2,662	Raether & Seidenath, 1972
Trypanosoma lewisi	culture	−70	none	185	Weinman & McAllister, 1947
〃	blood	−70	none	531	〃
〃	blood?	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	blood?	−196	DMSO	30*	Resseler *et al.*, 1965
〃	blood	−196	glycerol	2,648	Raether & Seidenath, 1972
Trypanosoma pipistrelli	blood?	−196	DMSO	420*	Resseler *et al.*, 1965
Trypanosoma ranarum	culture	−170	DMSO	987	Diamond, 1964
Trypanosoma rangeli	blood?	−196	DMSO	7	Resseler *et al.*, 1965
Trypanosoma rhodesiense	blood	−70	none	2,920*	Weinman, 1958
〃	〃	−79	DMSO	30	Walker & Ashwood-Smith, 1961
〃	〃	−196	DMSO	420*	Resseler *et al.*, 1965
〃	〃	−196	glycerol	2,650	Raether & Seidenath, 1972
Trypanosoma rotatorium	culture?	−196	DMSO	1	Resseler *et al.*, 1965
Trypanosoma theileri	culture?	−196	DMSO	7	〃
Trypanosoma vivax	culture?	−80	glycerol	852*	Cunningham *et al.*, 1963b

Tetrahymena pyriformis	trophozoite	−95	DMSO	112	Wang & Marquardt, 1966
〃	〃	−196	DMSO	90*	Hwang *et al.*, 1964
Paramecium aurelia	〃	−27	DMSO	7	Wang & Marquardt, 1966
〃	〃	−196	DMSO	7	〃
Plasmodium berghei	blood	−20	none‡	1	Miyata, 1973b
〃	〃	−20	serum‡	7	〃
〃	〃	−20	glycerol‡	49	〃
〃	〃	−70	none	15	Levaditi, 1952
〃	〃	−75	glycerol	520	Kasprzak & Rydzewski, 1970
〃	〃	−70	DMSO	168	Collins & Jeffery, 1963
〃	〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	sporozoite	−70	DMSO	45	Bafort, 1968
〃	blood	−170	DMSO	302	Diamond, 1964
〃	〃	−196	glycerol	2,622	Raether & Seidenath, 1972
Plasmodium cathemerium	〃	−78	none	156	Manwell, 1943§
Plasmodium circumflexum	〃	−78	none	137	〃
Plasmodium falciparum	〃	−70	none	1,921	Collins *et al.*, 1963
〃	sporozoite	−70	none	183	Jeffery & Rendtroff, 1955
〃	blood	−190	DMSO	52	Booden & Geiman, 1970
Plasmodium gallinaceum	〃	−75	none	50	Archetti, 1941
〃	〃	−70	glycerol	281	Jeffery, 1962
〃	〃	−70	DMSO	168	Collins & Jeffery, 1963
〃	sporozoite	−196	none	767	Weathersby & McCall, 1967
Plasmodium hexamerium	blood	−78	none	185	Manwell, 1943§
Plasmodium inui	〃	−76	none	151	Horsfall, 1940
Plasmodium knowlesi	〃	−76	none	140	〃
〃	〃	−190	DMSO	52	Booden & Geiman, 1970
Plasmodium lophurae	〃	−75	none	244	Manwell & Edgett, 1943
Plasmodium malariae	〃	−70	none	60	Jeffery & Rendtroff, 1955
Plasmodium nucleophilum	〃	−78	none	212	Manwell, 1943§

Plasmodium oti	blood	−78	none	127	〃
Plasmodium ovale	〃	−70	none	234	Jeffery & Rendtroff, 1955
〃	sporozoite	−78	none	997	Jeffery, 1957
Plasmodium relictum	blood	−78	none	158	Manwell, 1943§
〃 (*P. praecox*)	〃	−196	glycerol	2,641	Raether & Seidenath, 1972
Plasmodium routix	〃	−78	none	175	Manwell, 1943§
Plasmodium vaughani	〃	−78	none	46	〃
Plasmodium vinckei	〃	−75	?	414	Bafort *et al.*, 1966
Plasmodium vivax	sporozoite	−70	none	958	Jeffery & Rendtroff, 1955
〃	blood	−75	none	37	Saunder & Scott, 1947
Leucocytozoon simondii	〃	−196	glycerol	210*	Kocan *et al.*, 1967
Babesia argentina	〃	−80	DMSO	1,225	Dalgliesh, 1972‖
Babesia berbera	〃	−70	glycerol	215	Pipano & Senft, 1966
〃	〃	−70	glucose	15	〃
Babesia bigemina	〃	−79	glycerol	626	Ishihara & Minami, 1968
〃	〃	−70	glucose	240	Pipano & Senft, 1966
Babesia caballi	〃	−196	glycerol	1,045	Frerichs *et al.*, 1968
Babesia canis	〃	−76	glycerol	weeks	Reuße, 1956
〃	〃	−196	glycerol	2,621	Raether & Seidenath, 1972
Babesia equi	〃	−196	glycerol	603	Frerichs *et al.*, 1968
Babesia rodhaini	〃	−196	glycerol	2,627	Raether & Seidenath, 1972
Eimeria adenoides	sporozoite	−196	DMSO	853	〃
Eimeria brunetti	〃	−196	DMSO	1,120	〃
Eimeria dispersa	〃	−196	DMSO	853	〃
Eimeria gallopavonis	〃	−196	DMSO	853	〃
Eimeria meleagrimitis	〃	−196	DMSO	853	〃
Eimeria tenella	〃	−196	DMSO	1,112	〃
Toxoplasma gondii (RH)	prolif.	−20	glycerol	75	Miyata, 1973b
〃 (RH)	〃	−20	none	1	〃
〃	oocyst	−20	none	28	Frenkel & Dubey, 1973

Toxoplasma gondii		cyst	−20	none	200	Robl, 1965
〃		prolif.	−70	glycerol	711	Mackie, 1972
〃		〃	−70	P+P	730*	Le Corroller *et al.*, 1970
〃	(RH)	〃	−75	none	30	Miyata, 1973b
〃		cyst	−75	DMSO	730*	Kwantes *et al.*, 1967
〃		prolif.	−75	DMSO	730*	〃
〃		〃	−196	DMSO	450*	Resseler *et al.*, 1965
〃		〃	−196	serum	160	Smith, 1973
Nosema cuniculi		spore	−196	DMSO	60*	Bedrnik & Vavra, 1971
Eperythrozoon wenyoni		tissue	−79	glycerol	764	Ishihara & Minami, 1968
Anaplasma centrale		blood	−70	glycerol	439	Pipano & Senft, 1966
Anaplasma marginale		〃	−79	glycerol	495	Ishihara & Minami, 1968
Theileria sp.		〃	−79	glycerol	764	〃
Borrelia hispanica		culture ?	−196	DMSO	330*	Resseler *et al.*, 1965

* roughly estimated from date shown in weeks, months, or years

† polysaccarides+polyvinyl pyrrolidone (see page 177 in this paper)

‡ none: blood diluted in same volume of physiological saline
serum: blood diluted in same volume of bovine serum
glycerol: blood diluted in same volume of distilled water containing 15% glycerol

§ temperature varied between −55 to −78 C

‖ temperature varied between −70 to −80 C

trophozoite: trophozoite stage (mostly culture form)

culture: culture form

blood: stage in blood

fly: metacyclic form or other forms found in tsetse fly

sporozoite: sporozoites (in *Eimeria,* excysted sporozoites)

cyst: cyst itself or, in *Toxoplasma,* parasites which can produce cyst.

prolif.: proliferative form, parasites obtained from peritoneal exudate or from tissue culture

Plasmodium berghei survived for more than 2 years at the temperature without losing their virulence.

(a) Equilibration

In the use of cryo-protectants, samples should be kept or incubated for several minutes at least at temperatures higher than sub-zero temperature before cooling. The adequate temperature and period in the incubation was reported by some workers (Levine *et al.*, 1958; Dalgliesh, 1972; Miyata, 1974, 1975a and 1975b). They preferred the term *equilibration* or *to equilibrate* to *incubation* or *to incubate*. As one of the authors using the term, the reason of the choice should be explained. In the previous papers (Miyata, 1974, 1975a, and 1975b), the author showed several figures, and in the present paper, some of them are presented in Figs. 5 and 6. In the Fig. 5, after incubation at 25 C or 37 C for various intervals between 0 to 180 minutes, the samples (*Trichomonas vaginalis*, 5 strains) were cooled in a −30 C freezer (cooling rate, 1 C per minute) in the presence of glycerol, then the samples were stored at −75 C. The survival rate of *T. vaginalis* increased gradually with the prolonged incubation period, and after several minutes, the maximum survival rate has appeared, and no more incubation was needed. The effect of glycerol as a cryo-protectant on survival is enhanced with prolonged incubation, which is necessary to equilibrate protozoan cells to glycerol.

The temperature for equilibration is also important, as shown in Fig. 6. In this experiment, the samples were allowed to equilibrate with 10% glycerol for 100 minutes at various temperatures, and they were stored at −75 C, after pre-cooling in the −30 C freezer. The survival rate was markedly improved at higher temperatures. Incubation at 0 C was not effective, but even at this temperature, the survival rate was still very high, if compared with the samples without glycerol. The survival rate for non-glycerol samples was less than one percent, or sometimes no survivor was observed microscopically. Fitzgerald and Levine (1961), and Dalgliesh (1972) pointed out that glycerol was toxic at around 4 C. The author's opinion, however, is quite different from them, and he believes that glycerol is not toxic at 4 C, but at this temperature, cryo-protective action does not work sufficiently.

These observations clearly show the importance of the temperature and the period for the equilibration between cells and glycerol. According to Lovelock (1953), glycerol must penetrate into the cells, and for the penetration, time and temperature must be important. At least, in the present time, the author is uncertain whether or not glycerol is really present within trichomonad cells. Therefore, the volume of glycerol within cells must be measured at various temperatures and in various equilibration periods. According to literatures surveyed so far, there has been no report dealing with such problems in the protozoological field.

The action of DMSO is very different from that of glycerol as shown in Figs. 6 and 7. These experiments were carried out by the same method mentioned above. The good effectiveness of DMSO as a cryo-protectant was demonstrated at lower temperatures (Fig. 6), and the lowest survival rate of *T. vaginalis* was obtained from the samples which were incubated at 37 C. The effect of equilibration time at 25 C or 37 C on the survival was also very different. At either temperature, survival rate decreased with the prolonged equilibration time, and the highest survival rate was

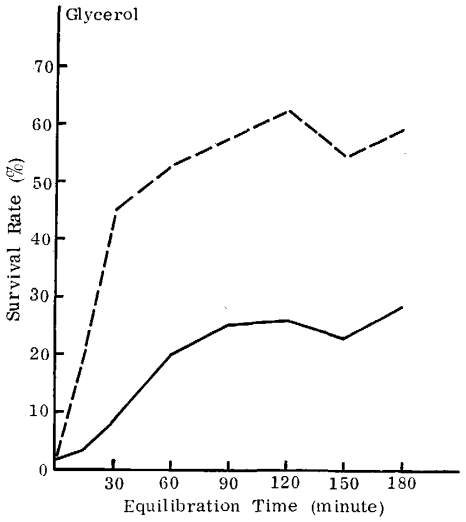

Fig. 5 The effect of the equilibration time on trichomonad survival with 10% glycerol at 25 C or 37 C (Miyata, 1975a).

Notes for Figs. 5 to 9:
---- at 37 C
—— at 25 C
After the equilibration, samples were pre-cooled at a −30 C freezer for 90 minutes, then they were stored in a −75 C freezer for 1 to 5 days. After thawing in a 37 C water bath, survival rate was examined. The pooled survival rate of 5 strains of *Trichomonas vaginalis* was shown in the figures. The details of these experiments were seen in Miyata, 1975b.

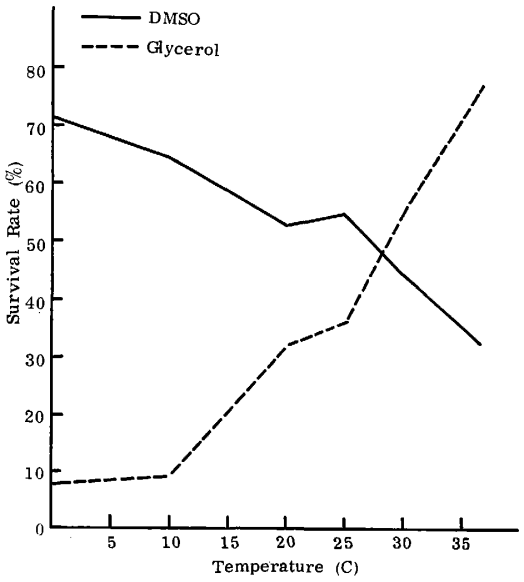

Fig. 6 The effect of the equilibration temperature on trichomonad survival with 10% glycerol or 7.5% DMSO for 100 minutes (modified from Miyata, 1975a).

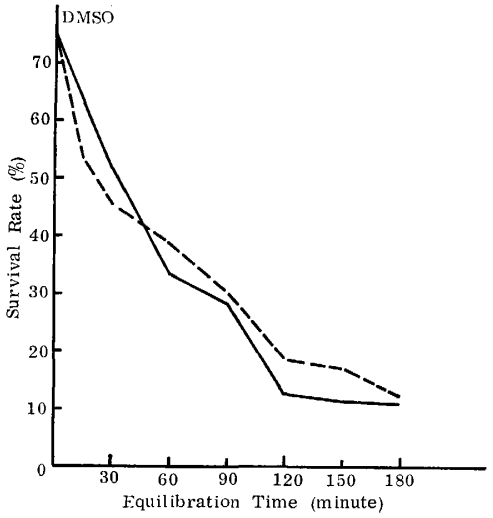

Fig. 7 The effect of the equilibration time on trichomonad survival with 7.5% DMSO at 25 C or 37 C (Miyata, 1975a).

obtained in the sample without equilibration. According to Lovelock's theory (1953), the time for penetration of DMSO into cells must be required. The author's results, however, show that penetration of DMSO is rather injurious, and if this substance is used, the samples should be cooled as soon as possible after addition of DMSO. In the samples without cooling, living trichomonads did not change in

the number during equilibration period, even after 3 hour incubation. This shows that injurious action of DMSO to cell is caused not by prolonged incubation but by cooling and thawing after prolonged incubation. The action of DMSO and glycerol varied among the species or strains used as reported by various workers.

Meyer and Chadd (1967) also pointed out that both glycerol and DMSO proved toxic to the parasites in cultivation at 37 C. DMSO are clearly more toxic; 3.5% DMSO killed more than 99% *Giardia* in 2 days while 3.5% glycerol killed about 50% of the parasites in the same period. Therefore, they recommended that the protective agent should be diluted as quickly as possible after thawing.

(b) Optimal concentration of cryo-protectants

The optimal concentration of glycerol or DMSO were studied by most workers who attempted to preserve the protozoa in a frozen state. Their opinion varied, but most of them recommended to use 10% glycerol and 5 to 7.5% DMSO. According to the author's experiment (Miyata, 1975a and 1975b), the optimal concentration of glycerol in *Trichomonas vaginalis* changed with equilibration period and temperature. For example, the optimal concentration was higher at 25 C than 37 C in equilibration for 100 minutes (Fig. 8). In the case of DMSO, the optimal concentration is fixed between 5 to 10% at various temperatures (Fig. 9). The incubation period is important, and in higher concentration many parasites may die during prolonged equilibration as pointed by Doran (1969b).

Diamond (1964) used 5% DMSO, because this concentration gave him satisfactory results for many protozoa. By using 5% DMSO, *Entamoeba invadens* survived by slow cooling and storage at −170 C, but recoveries of viable organisms were

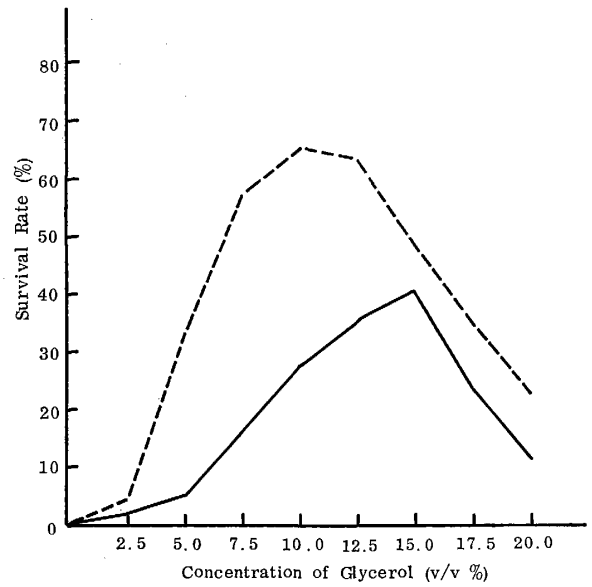

Fig. 8 The effect of the concentration of glycerol on trichomonad survival after 100 minutes equilibration at 25 C or 37 C (original).

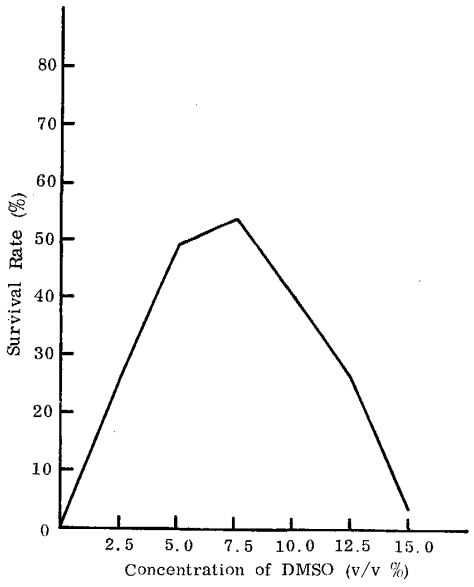

Fig. 9 The effect of the concentration of DMSO on trichomonad survival after 60 minute equilibration at 25 C (original).

only one percent. Then, the concentration of DMSO was increased to 15 percent, and this gave rise to a maximum recovery of about 25 percent. Stimulated by Djerassi and Roy (1963) who employed DMSO and sugar in freezing preservation of blood platelets of the rat, Diamond attempted to add 4.5% glucose to sample containing 15% DMSO, and 45 percent of amoebae recovered, whereas no protection was shown with glucose alone at concentrations from 2.25 to 9% (w/v).

4) Maintenance of biological properties of the parasitic protozoa in frozen state

It is well known that biological properties such as virulence, antigenicity, and drug resistance of various parasites change gradually by prolonged passage in animals or culture media. Some workers reported that several strains have lost their pathogenicity to inoculated mice after prolonged storage in a frozen state. For example, Allain (1964) observed the loss of pathogenicity in one of 9 strains of *Trypanosoma cruzi* stored at −64 C for 6 months, but other 8 strains retained their original virulences and viabilities. Many workers, however, claimed that the properties did not change by using cryo-preservation.

(a) Virulence

According to Stabler *et al.* (1964), the highly virulent strain of *Trichomonas gallinae* (Jones' Barn strain) survived for 52 weeks at −72 C by addition of 1.0 M glycerol (1 hour equilibration at room temperature), and its virulence did not change during the prolonged preservation period, but the continued in vitro cultivation at 37.5 C resulted in the gradual loss of pathogenicity of the trichomonad to domestic pigeons.

Lindgren and Ivey (1964) also reported that the virulence of *Trichomonas vaginalis* (12 strains) did not change during a period of 8 week preservation at −43 C in the presence of 10% glycerol, whereas the virulence decreased in after 2 to 3 months cultivation at 37 C. Jeffries and Harris (1967) obtained the result similar to Lindgren and Ivey on *T. vaginalis* and *Tritrichomonas foetus*. Diamond *et al.* (1965) examined the virulence of *T. vaginalis* to mice after 730 day storage at −170 C by using 5% DMSO, they found that the virulence did not change singnificantly by such long period storage.

Weathersby and McCall (1967) reported that sporozoites of *Plasmodium gallinaceum* were preserved in the vector, *Aedes aegypti,* for 767 days in liquid nitrogen without apparent loss of viability or infectivity. Minter and Goedbloed (1971) obtained live trypanosomatid parasites from the frozen tsetse flies and sandflies kept in liquid nitrogen with no obvious loss of viability or infectivity. Filardi and Brener (1975) pointed out that prolonged preservation at −196 C apparently did not change the biological characteristics of different strains of *Trypanosoma cruzi.*

(b) Gametogenesis

Many workers observed the disappearance of gametocytes of certain malaria parasites such as *Plasmodium* spp. by serial blood transferes in vertebrate hosts. By the application of cryo-preservation, the original nature to produce gametocytes in various blood parasites might be maintained. This is another advantage of cryo-preservation. Furthermore, according to Bafort *et al.* (1966), the ability to reproduce gametocytes in *P. vinckei* which had lost the power by continuous animal passages recovered in the parasites which were kept in frozen state at −75 C.

(c) Drug resistance

Schneider *et al.* (1968) examined the survival time and retention of antimalarial resistance of malaria parasites (*Plasmodium berghei* and *P. gallinaceum*) in liquid nitrogen in the presence of 10% glycerol. The samples were cooled at 1.3 C per minute for 60 minutes, then rapidly cooled in liquid nitrogen. One of the strain of *P. berghei* survived for 639 days and *P. gallinaceum* also for 62 days. There was no remarkable difference of antimalarial resistance between frozen samples and unfrozen ones. Since the antimalarial resistance of the malaria parasite is reduced if the parasites were maintained from host to host without medication, the cryo-preservation might be useful to keep such characters of the parasites.

(d) Antigenicity

According to Gordon *et al.* (1969), no permanent antigen change had occured as a result of 5 month preservation of *Entamoeba histolytica* at −196 C.

(e) Loss of kinetoplast

According to Overdulve *et al.* (1970c), one strain of *Trypanosoma evansi* lost its kinetoplast after storage at −76 C and at −196 C, and it did not reappear in subsequent mouse passage. This kind of observation, however, was not reported by other workers who preserved trypanosomes in a frozen state.

IV. Conclusion

In the introduction of this paper, the author has already pointed out the advantages of cryo-preservation of the parasitic protozoa, and he would not repeat the subject again. In cryo-preservation, biological properties such as infectivity, virulence, antigenicity, drug resistance, or to produce gametocytes, might be maintained without apparent loss or change as reported by many workers. Of course, several undesirable results were described by some workers, for example, loss of pathogenicity or even loss of kinetoplast permanently in parasites after freezing. Even considered those minus results, still cryo-preservation is a useful method to maintain our materials because during prolonged period of animal passages or in vitro cultivation, original natures of parasites frequently changes in infectivity, virulence, or other properties as pointed out by many workers. To avoid such undesirable changes, we must improve our preservation technique furthermore, and there is no doubt that problems will be solved in future. The advantages of cryo-preservation are apparently greater than those in animal passage or serial cultivation, and especially the preservation period is extremely prolonged by the use of liquid nitrogen. At present, however, the following problems are not yet solved clearly, for instance, fate of ice crystals in the frozen cell, optimal cooling rates, action of cryo-protectants, effect of salt concentration (or other substances present in suspended media), or penetration rates of cryo-protectants into the cells.

Today, cryo-preservation of the protozoa is popular all over the world, and various new techniques were proposed and discussed by many workers, but the standard methods might be summarized as follows:

(1) For most parasitic protozoa, cryo-protectants such as DMSO or glycerol are useful. Several workers recommended to use DMSO (5 to 7.5%) rather than

glycerol (about 10%) because equilibration is unnecessary. Glycerol, however, seems also an useful protectant because DMSO is rather toxic than glycerol in higher concentration and in prolonged equilibration. The equilibration with glycerol must be carried out for 30 to 60 minutes at a higher temperature as 37 C. DMSO, however, should be added at a lower temperature and the materials must be cooled as soon as possible after adding the substance.

(2) Slow cooling in the rate of 1 C per minute is ideal in most parasites, and this cooling rate can be obtained by cooling at −25 to −30 C, or by slow addition of small pieces of dry ice into alcohol bath.

(3) For cryo-preservation, the lower temperature is better, and storage in liquid nitrogen or its vapor is the best.

(4) Frozen materials should be thawed as rapidly as possible to avoid recrystallization of the ice during warming, and for that purpose, 37 to 40 C water bath is useful.

In our country, we have no center in which various protozoa strains are stored to supply to other researchers. In one laboratory, some strains might be used routinely for experiments, but other many strains are not. For example, in our laboratory, nobody uses *Trypanosoma gambiense, Entamoeba histolytica, Leishmania donovani,* and *Toxoplasma gondii* (2 strains) at present. Other laboratories may have some strains out of use. If those strains were deposited certain center, the center could supply the strains soon whenever the need for them arose. For these reason, the author would like to propose to build such a preservation center of protozoan strains in this country. If we could have the center, it would benefit all researchers who have interest in protozoology and tropical medicine.

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寄生原虫類の凍結保存

宮　田　　彬

最近30年間に発表された原虫の凍結保存に関する論文は，200篇を越えている。そこでこの論文では，それらのうち主な論文を紹介するとともに凍結保存が可能な原虫類の保存方法や保存期間などを総括し，さらに今後の問題点を論じた。また今までに十分な検討を加えずに用いられていた凍害保護剤について，特にグリセリンと DMSO の用い方，平衡時間などについて，著者の研究を中心に紹介した。原虫類の最適保存法及びこの論文の論旨は次の通りである。1）原虫は，適当な保護剤を含む溶液あるいは培地中に攪拌し，試験管またはアンプルに分注する。2）保護剤の濃度は，グリセリンは10% 前後，DMSO は，7.5%前後が適当である。グリセリンの場合は，比較的高い温度（例えば 37 C）で 30—60 分平衡させる。高温に耐えない原虫は，25 C 前後で60—90分平衡させる。DMSO は，低い温度（例えば 0C）で加え，平衡時間をおかず直ちに凍結する。3）凍結は2段階を用いる。すなわち，−30 C 前後のフリーザー中で約90分予備凍結し（この時冷却率は約 1 C, 1 分），ついで保存温度へ移す。4）保存温度としては，液体窒素のような超低温が好ましいが，−75 C でも数カ月程度は保存可能である。5）凍結材料は，37〜40 C の恒温槽中で急速融解し，融解後は，すみやかに動物あるいは培地へ接種する。6）原虫の種類によっては，もっと簡単に保存できる。原虫ごとに予備試験を行い，目的の保存温度に数日保存して高い生存率の得られる方法を採用するとよい。7）今後の問題点としては，保存原虫の性質（薬剤耐性，抗原性，感染性など）の長期保存における安定性を検討することと純低温生物学的な立場から超低温下における細胞の生死のメカニズムを解明することである。前者については，多くの研究者が凍結保存による実験株の性質の変化は認められないと指摘している。8）終りに数多くの実験株を保存し，研究者に提供する低温保存センターの設置の必要性を提案した。

長崎大学熱帯医学研究所疫学部門