THE MAINTENANCE OF STOCK STRAINS OF TRICHOMONADS BY FREEZING

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One of the difficulties of studying protozoa *in vitro* is the maintenance of stock strains. Unlike bacteria and viruses most protozoa cannot simply be put aside at room temperature or in a refrigerator until next required. If cultures are to be kept in a sound healthy condition they require frequent passage, generally at intervals of a few days. Clearly, if several strains are being employed this calls for the expenditure of a great deal of time and material. The isolation of protozoa in bacteria-free culture has been greatly simplified by the antibiotics, but is still far from easy. It is, in fact, sufficiently troublesome to make it worth while keeping in continuous passage every bacteria-free strain isolated even though it is of no immediate value to the investigation in progress. Quite apart from the time and material involved, frequent passage *in vitro* may lead to degradation of the strain until it bears little resemblance to a freshly isolated one. It was with a view to overcoming these difficulties that this study was undertaken.

Weinman & McAllister in 1947 demonstrated that a number of pathogenic protozoa would survive freezing and thawing and could be stored in the frozen state without deteriorating; amongst those successfully preserved were trypanosomes, leishmania and plasmodia. The observations of these workers on trichomonads were limited to two species, *Trichomonas vaginalis* and *T. hominis*, both cultivated with bacteria. Of these *T. vaginalis* failed to survive at all and *T. hominis* was preserved in a proportion of experiments only.

No successful results were recorded with ciliates or with *Entamoeba histolytica.*

Recent work has shown that glycerol will protect a variety of living cells against injury during freezing and thawing (Smith, 1954). In 1953 Fulton and Smith were able to demonstrate for the first time that *E. histolytica* could be maintained at $-79°$ C. as a result of the protective action of glycerol. Unfortunately glycerol may be injurious to the amoeba and it is probable that the ideal conditions for long-term storage of this parasite have yet to be discovered.

These observations of Fulton & Smith were encouraging and it seemed possible that, by making use of glycerol, trichomonads also might be maintained frozen for useful periods.

MATERIALS AND METHODS

Four species of trichomonads were being maintained in the laboratory; *Trichomonas vaginalis*, *T. foetus* (Belfast) and *T. gallinae* growing without bacteria, and *T. hominis* growing with a mixed bacterial flora. All bacteria-free cultures were grown in a modification of the medium described by Feinberg (1953). This modification, which was made solely as a matter of convenience, consisted of the

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substitution of Hartley broth, which was available as a standard laboratory medium, for the Douglas double strength broth and water of the original medium.

T. hominis could not be grown without bacteria and was therefore cultured in a simple two-phase medium consisting of a one-eighth dilution of inactivated horse serum in Ringer's solution layered over an inspissated serum slope. The associated bacteria were a coliform and a *Streptococcus faecalis*.

The strains growing without bacteria reached their peak of growth about 18–20 hr. after inoculation. Very large inocula were used, 1 ml. of well-grown culture being added to 9 ml. of fresh medium.

At the height of growth the populations in these cultures varied from $2\cdot5 \times 10^6$/ml. to $4\cdot0 \times 10^6$/ml. according to the species. *Trichomonas gallinae* gave the highest counts and *T. vaginalis* the lowest. *T. hominis* under the conditions of cultivation employed gave much lower counts, the average being about $0\cdot5 \times 10^6$/ml.

Cultures for freezing were centrifuged and the deposits resuspended in a small volume of fresh medium. A measured volume of this packed cell suspension was then added to an equal volume of medium plus glycerol, so that the final suspension contained approximately 100×10^6 protozoa/ml. in the appropriate concentration of glycerol. Thus a suspension in 10% glycerol was prepared by adding a packed cell suspension containing 200×10^6 protozoa/ml. to an equal volume of medium containing 20% glycerol and so on. The suspensions were distributed in approximately 0·1–0·2 ml. volumes into small Pyrex glass ampoules which were then sealed. *T. hominis* was treated in the same way, but as the initial protozoan population was lower and the final deposit contained both bacteria and protozoa the number of protozoa per unit volume was considerably less than with the other three strains.

Two methods of freezing were investigated: first, snap freezing by plunging the sealed ampoules into an alcohol-CO_2 mixture until they reached the temperature of the mixture; and secondly, slow freezing carried out at the rate recommended by Fulton & Smith (1953) in their studies on *Entamoeba histolytica*. The ampoules were first cooled to 0° C. in a refrigerator, about 30 min. being taken to reach this temperature. They were then transferred to alcohol at 0° C. and further reduction in temperature was effected by the slow addition of powdered solid CO_2. From 0° to −15° C. cooling was at the rate of 1° C. per min., below −15° C. at the rate of 4° C. per min. Once the desired temperature was reached the ampoules were transferred to a storage cabinet and maintained at the same temperature.

Samples for examination were thawed rapidly by agitation in a water bath at +40° C. The average time for thawing was about 15–20 sec. All samples were examined microscopically immediately after thawing for the presence of intact and actively moving cells, the remainder of the sample being used to inoculate a tube of the appropriate culture medium.

In preliminary experiments attempts were made by various means to reduce the concentration of glycerol within the protozoa. The cultural results following these attempts were all less successful than those following the direct inoculation of the glycerinated suspension into fresh medium. The latter method of cultivation was therefore adopted in all subsequent experiments.

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In the interpretation of results only samples giving rise to positive cultures were recorded as having survived. With very few exceptions growth only occurred when active protozoa were seen in direct preparations, the rate of growth being directly related to the proportion of actively motile protozoa observed in the thawed specimen. Preparations which appeared to be fully active gave full growth as rapidly as cultures which had not been cooled.

EXPERIMENTAL RESULTS

Before investigating the protective action of glycerol the possibility that it has an injurious effect on the protozoa had to be excluded. The effect of cultivating the four species in the presence of various concentrations of glycerol was therefore examined, and the results of this experiment are given in Table 1, which shows that all strains were able to grow in the presence of 5% glycerol, some to a limited extent in 10% and none in 15%.

Table 1. *Growth of trichomonads in the presence of various concentrations of glycerol*

Strain of *Trichomonas*	Glycerol concentrations in medium %					
	15	10	5	2½	1	Control 0
T. vaginalis	−	±	+	+ + +	+ + +	+ + +
T. foetus	−	±	+	+ + +	+ + +	+ + +
T. gallinae	−	+	+ +	+ + +	+ + +	+ + +
T. hominis	−	−	+	+ + +	+ + +	+ + +

+ + + = Optimum growth. − = No growth

As storage space for long-term survival experiments was limited, an attempt was made to reduce the number of tubes to be stored by determining concentrations of glycerol which could be expected to give the optimum protection. The immediate effect on the protozoa of concentrations of from 2½ to 25% was studied, the higher concentrations being reached by a series of additions in order not to subject the cells to too great a stress. As a result of these tests it was concluded that 2½% gave inadequate protection, whereas concentrations of 15% and above were likely to damage the protozoa if subcultures were made without reducing the concentration within the cell, a process which had already been shown to jeopardize the organism's survival.

The concentrations chosen for investigation were 5 and 10% plus a control series without glycerol. In retrospect the omission of a 15% series was probably ill-judged; some of the failures recorded might perhaps have survived in the presence of this concentration.

Freezing was investigated in the following three ways:

(1) Snap freezing to −79° C. followed by immediate thawing.
(2) Slow freezing to −20° C. with storage at that temperature, survival being investigated after 3 weeks and 6 weeks.
(3) Slow freezing to −79° C., storage at that temperature with sampling at intervals for 6 months.

Tables 2, 3 and 4 show the results of these experiments.

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As was expected, snap freezing caused greater destruction than slow freezing. This has been a fairly general finding with most living cells, particularly where the volumes frozen are small. Slow freezing and storage at $-20°$ C. compared unfavourably with storage at $-79°$ C. This was disappointing as storage cabinets to

Table 2. *Survival of trichomonads following snap freezing to* $-79°$ C. *and immediate thawing*

Strain of *Trichomonas*	Percentage glycerol in suspension		
	0	5	10
T. vaginalis	–	–	+
T. foetus	–	–	–
T. gallinae	+	+	+
T. hominis	–	–	–

+ = Growth on subculture. – = No growth on subculture.

Table 3. *Survival of trichomonads following slow freezing to* $-20°$ C. *and storage at that temperature*

Strain of *Trichomonas*	Percentage glycerol in suspension		
	0	5	10
T. vaginalis	–	+ 3/52	+ 6/52
T. foetus	–	–	–
T. gallinae	–	+ 6/52	+ 6/52
T. hominis	–	–	–

+ 3/52 = subculture positive after 3 weeks storage.
+ 6/52 = subculture still positive after 6 weeks.
– = no growth on subculture.

Table 4. *Survival of trichomonads following slow freezing to* $-79°$ C. *and storage at that temperature*

Strain of *Trichomonas*	Percentage glycerol in suspension		
	0	5	10
T. vaginalis	± 2/52	+ 4/12*	+ 4/12
T. foetus	–	–	–
T. gallinae	± 6/12	± 6/12	+ 6/12
T. hominis	–	+ 4/12	+ 6/12

2/52 = Growth on subculture after 2 weeks.
4/12 = Growth on subculture after 4 months.
6/12 = Growth on subculture after 6 months—longest period tested.
+ = Good active sample, normal rate of growth; ± poor; very few survivors, slow growth.
– = no growth on subculture.

* Owing to breakage of ampoules the 5 % *T. vaginalis* series was not examined later than 4/12.

operate at $-20°$ C. are commoner and less costly than CO_2 cabinets or other means of maintaining a temperature of $-79°$ C. Despite the differences shown, there is the same general pattern of survival in all the experiments. *Trichomonas gallinae* is clearly the most resistant to damage by freezing and is the only strain which

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survives without glycerol, although even with this species the cultures with glycerol were more active and showed a far higher proportion of surviving cells towards the end of the experiment. *T. foetus* is the least resistant and failed to survive under any of the conditions examined. *T. vaginalis* and *T. hominis*, although less resistant to damage than *T. gallinae*, nevertheless survive for a useful period.

DISCUSSION

Three of four species of *Trichomonas* examined remained viable for periods of 4 months or more under the conditions tested. The results suggest that the survivors did not remain entirely static during storage but slowly deteriorated. Under ideal conditions of storage there should be little or no deterioration. Perhaps this ideal could be achieved with higher concentrations of glycerol, or by storage at lower temperatures such as $-190°$ C. Despite its obvious imperfections the method makes possible the storage of cultures for useful periods. Indeed, during the course of the experiments a frozen culture was taken to replace a current one which had died out owing to bacterial contamination.

The saving of time and material effected by the storage of strains not immediately required is considerable, particularly when it is remembered that these strains would otherwise be passed at least four times each week. This saving also means that the number of stock strains maintained in the laboratory can be increased, whereas at present every new strain is an added burden.

Another interesting facet of the results is the way in which they emphasize the wide differences which can exist between closely related and morphologically similar members of a genus.

No explanation can be offered for the absolute failure of *T. foetus* to survive freezing. This species differs from other trichomonads in building up very large storage deposits of glycogen (Feinberg & Morgan, 1953), but there is no obvious connexion between this characteristic and the failure to survive freezing.

An interesting practical application of this failure of *T. foetus* to survive freezing was pointed out by Joyner in a personal communication. Bull semen from an infected animal will become free from *T. foetus* if stored frozen with glycerol. This may not be desirable as a deliberate policy but does provide an added safeguard against infection occurring as a result of artificial insemination.

Apart altogether from the observations on trichomonads it seems clear that a great deal more use could be made of freezing methods in the preservation of protozoa.

Further investigations into optimum methods of storage would not only save time and material but, by making the maintenance of cultures less laborious, might help to stimulate greater interest in *in vitro* work with protozoa.

SUMMARY

The ability of four species of trichomonads to withstand freezing was investigated with a view to storage of stock cultures. Of the conditions investigated the most satisfactory was found to be storage at $-79°$ C. following slow freezing, the protozoa

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being suspended in medium containing 10% glycerol. *T. gallinae* was the most easily preserved and survived even without glycerol for 6 months. No observations on survival beyond this period have yet been made so the possible maximum time of survival is not known.

T. vaginalis remained viable for four months but deteriorated in storage and had died out after 6 months. The sample of *T. hominis* containing 10% glycerol was still viable after 6 months at $-79°$ C., but in the presence of 5% glycerol survival was not observed beyond 4 months. *T. foetus* could not be preserved under any of the conditions tested. The possible implications of these results are discussed.

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