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Freeze Preservation of *Eimeria acervulina* Tyzzer, 1929

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Abstract. Sporozoites of *E. acervulina*, excysted in vitro, protected with 7.5% glycerol and stored in liquid nitrogen for 1, 7, 28, 56 and 172 days produced light infections in experimental chicks after inoculation directly into the duodenum. Oocysts recovered from these birds sporulated normally and produced normal infections in chicks subsequently inoculated per os.

The technique of freeze preservation is invaluable as a means of stabilising strains of organisms and of saving the trouble and expense incurred in their routine culture. The introduction of a range of laboratory equipment for use with liquid nitrogen has made the whole procedure more simple and more reliable than earlier techniques using solid carbon dioxide or electrical apparatus. Reports of the freeze preservation of free living and parasitic protozoa have been summarized by DIAMOND (1964), and by LEVINE and ANDERSON (1966). Review articles by FARRANT (1966) and WALKER (1966) have established the underlying principles of the technique as applied to parasites. Many papers have appeared on the preservation of blood parasites and flagellates but there appear to be no previous reports of the freeze preservation of coccidia.

Successful cold storage of these parasites could lead to considerable savings in labour and expense. At Weybridge, 28 species of *Eimeria* are maintained, involving serial passage in 6 species of host animal. This paper records preliminary observations with *Eimeria acervulina* Tyzzer, 1929 from the domestic fowl.

MATERIALS AND METHODS

1. Apparatus. Specimens were stored in 1 ml. borosilicate glass ampoules held on metal spines in a Union Carbide L.R. 35 liquid nitrogen refrigerator, after controlled freezing in a Union Carbide B.F. 5 biological freezer which operates in the neck of the refrigerator. The nitrogen vapour cools the specimens at a rate which can be predetermined according to the number of ampoules which are being treated and to their position in the neck of the freezer. Analytical grades of glycerol and dimethyl sulphoxide (DMSO) were employed as protective agents during cooling.

2. **Experimental birds.** Day-old White Link hybrid cockerel chicks were reared in isolation, in wire-floored, heated hovers, and transferred to the experimental unit for infection at 3 to 4 weeks of age. Chicks were fed ad lib (JOYNER and DAVIES, 1960).

3. **Oocyst cultures.** These were prepared from the faeces of 4-week old chicks inoculated with 5,000 oocysts. The oocysts were recovered from the faeces by screening and salt flotation; after washing, they were suspended in 2% potassium dichromate solution at a concentration of 10^6 oocysts/ml. and incubated at 27 °C for 72 hrs. Oocyst cultures were standardized by the method of LONG and ROWELL (1958) and stored at 4 °C.

4. **Excystation of sporozoites in vitro.** The excystation procedure was developed largely by following the techniques of DORAN and FARR (1961) and JACKSON (1964) and may be summarized as follows:

i 20 mls. oocyst culture was washed three times by centrifugation in order to remove K_2CrO_4.

ii Oocysts were suspended in 2 mls. sodium hypochlorite solution Sp. G 1.075, for 20 minutes for further cleaning. This solution sterilizes the preparation and, in order to avoid gross bacterial contamination, sterile solutions were used after this step. Aseptic precautions were regarded as unnecessary.

iii The sodium hypochlorite was removed by repeated centrifugation in sterile physiological saline until no smell of chlorine could be detected.

iv The oocysts were suspended in 10 mls. of 1.4% sodium bicarbonate which contained 0.0025% Phenol red; Carbon dioxide gas was bubbled through the preparation until the pH dropped to 6.8—6.9. The tube was then incubated for 3—4 hours at 37 °C and shaken at intervals to maintain an even suspension. Although the CO_2 treatment was devised by JACKSON (1964) for the oocysts of *E. arloingi*, a mammalian species, it was found to be equally useful in the excystation of avian species, a higher rate of sporocyst release being obtained.

v After washing in sterile saline, the oocysts were suspended in 2 mls. sterile Ringer's solution and ground in a Griffiths tube until the majority of the sporocysts was released.

vi The sporocysts were concentrated by centrifugation and suspended in 2 mls. Ringer's solution which contained 10% chicken bile and 0.25% trypsin. Excystation commenced after 10—15 minutes at 37 °C and was maximal after 30 minutes.

vii After dilution with sterile borate buffered solution of salts (BS-2, LUMSDEN, CUNNINGHAM, WEBBER, VAN HOEVE and WALKER 1963) the preparation was lightly centrifuged and the supernatent fluid containing the sporozoites was carefully removed, leaving the majority of the unruptured oocysts and empty sporocysts in the sediment.

viii The sporozoites were concentrated by centrifugation, resuspended in 5 mls. sterile BS-2 + 10% bovine serum and counted in a Neubauer haemacytometer. The concentration of sporozoites was adjusted to 5,000,000 per ml. by the addition of BS-2+ serum, and the preparation was then ready for subsequent treatment; either inoculation into chicks or controlled freezing with protective agents for storage in liquid nitrogen.

5. **Freezing and thawing suspensions of sporozoites.** In the first 5 experiments, a number of variables such as the type and concentration of freeze protective, the time and temperature for equilibration and different routes for the inoculation of chicks were investigated. With the knowledge thus gained, the following technique was used for experiment 6.

a) **Freezing**

i Freshly excysted sporozoites were suspended in BS-2+ 10% bovine serum to give a concentration of 5×10^6 sporozoites per ml.

ii An equal volume of a solution of 15 % glycerol in BS-2+ serum was added drop by drop over a period of 5 minutes in order to minimise osmotic effects.

iii A disposable syringe and needle were used to dispense 1 ml. amounts of the protected sporozoite suspension into ampoules which were heat-sealed.

iv Equilibration, at room temperature, was allowed for 20 minutes from the moment when the glycerol solution was first added to the sporozoite suspension.

v The ampoules were cooled to —70 °C at 1 °C per minute, calculated according to the calibration chart supplied for the apparatus. They were then quickly clipped to pre-cooled metal spines which were stored in the liquid nitrogen refrigerator.

b) Thawing

Sporozoite suspensions were thawed by plunging the ampoule into 4 litres of water at 37 °C.

6. **Inoculation of chicks.** Immediately after thawing, 0.2 ml amounts of the sporozoite suspension were inoculated into 5 chicks using a 1 ml. all glass syringe. Sporozoites were injected either orally, intravenously, intraperitoneally or directly into the duodenum. The latter route gave the best results and was readily accomplished through a half-inch incision in the body wall after the local subcutaneous injection of 0.2 mls of lignocaine. The peritoneum, muscle layers and skin were closed with cotton sutures and a suspension of penicillin and streptomycin was injected into the wound.

7. **Assessment of results.** In the first 5 experiments the birds were killed and examined at 96 hours when infections were detected by the presence of lesions in the intestine, the presence of gametocytes in fresh smears and the presence of oocysts in salt-flotation preparations of the intestinal contents. In the 6th experiment the oocyst output of the infected chicks was recorded on the 4th, 5th and 6th days, using the technique of Long and Rowell (1958). The chicks were killed on the 6th day when the characteristic lesions of *E. acervulina* were expected to be still visible in the small intestine. Oocysts recovered on the 5th day were set to sporulate, and subsequently inoculated into a further batch of chicks under the standard conditions used for routine culture preparation. The number of oocysts recovered from this infection, and their percentage sporulation were compared with those of routine cultures.

RESULTS

Five preliminary experiments were conducted to see if standard protective agents and prodecures could be employed for the freeze preservation of sporozoites of *E. acervulina*. The results are summarized in Table 1.

Expt. 1. The optimum route for inoculation of freshly excysted sporozoites. The birds which were inoculated intravenously all remained negative. One of those inoculated intraperitoneally showed a light infection which was detected only by salt-flotation of the intestinal contents. All the chicks inoculated orally became infected as did 5 of those which received the inoculum directly into the duodenum. The one failure in this group was thought to be due to unfamiliarity with the technique, the inoculum being injected either into the lower intestine or the peritoneal cavity. Infections produced after oral and intra-duodenal inoculation were equally heavy, but the sporozoite suspension contained a small number of intact oocysts which would have excysted and infected the birds in the usual way after inoculation per os.

Expt. 2. The optimum route for inoculation of sporozoites after preservation in liquid N_2. After a little experience had been gained with the freezing techniques, Expt. 1 was repeated with sporozoites which had been stored in liquid N_2 for 14 days. Two groups of 5 chicks were inoculated orally, one group after overnight starvation and the other group with full crops. Only 3 of these 10 birds were positive when examined 5 days later. Five chicks which received the inoculum directly into the duodenum however, all became infected.

Table 1. The protective effects of Glycerol and DMSO in the freeze preservation of sporozoites of *E. acervulina*

Expt.	Protective agent	Equilibration Time Mins	Equilibration Temp. °C	No. of days at —196 °C	Inoculation route	No. of birds infected	Remarks
1	Nil	Nil	Nil	Nil	i/v	0	
	Nil	Nil	Nil	Nil	i/p	1/5	
	Nil	Nil	Nil	Nil	per os	5/5	
	Nil	Nil	Nil	Nil	i/duod	5/6	
2	7.5 % Glycerol	30	0	14	per os	2/5	starved overnight
	7.5 % Glycerol	30	0	14	per os	1/5	
	7.5 % Glycerol	30	0	14	i/duod	5/5	
3	5 % Glycerol	30	22	14	per os	3/5	
	7.5 % Glycerol	30	22	14	per os	4/5	
	10 % Glycerol	30	22	14	per os	3/5	
	12.5 % Glycerol	30	22	14	per os	4/5	
	15 % Glycerol	30	22	14	per os	4/5	
4	5 % DMSO	10	22	14	per os	0	
	7.5 % DMSO	10	22	14	per os	0	
	10 % DMSO	10	22	14	per os	2/5	
	12.5 % DMSO	10	22	14	per os	0	
	15 % DMSO	10	22	14	per os	0	
5	7.5 % Glycerol	10	22	14	per os	4/5	
	7.5 % Glycerol	20	22	14	per os	5/5	
	7.5 % Glycerol	30	22	14	per os	—	Broken ampoule
	7.5 % Glycerol	40	22	14	per os	4/5	
	7.5 % Glycerol	10	0	14	per os	5/5	
	7.5 % Glycerol	20	0	14	per os	5/5	
	7.5 % Glycerol	30	0	14	per os	4/5	
	7.5 % Glycerol	40	0	14	per os	5/5	

Expt. 3. The optimum concentration of glycerol. The results, summarized in Table 1, show that the concentration of glycerol is not a critical factor. Oral inoculation of sporozoites was much more successful than in the previous experiment, but the infections were very light. In order to minimise any difficulties which might arise from osmotic lysis, the lowest satisfactory concentration of glycerol, 7.5 %, was chosen for subsequent experiments.

Expt. 4. The optimum concentration of DMSO. The results, summarised in Table 1, show that DMSO was ineffective as a freeze protective for the sporozoites of E. acervulina. After removal from the liquid nitrogen, the sporozoites presented a very poor appearance, only an occasional motile parasite was seen, the majority being vacuolated and degenerate. Two birds became infected, but these infections were very light and only detected by the presence of a few oocysts after salt flotation of the contents of the small intestine.

Expt. 5. The optimum length of time for equilibration with 7.5 % glycerol at 0 °C and 22 °C. The results summarized in Table 1, indicated that neither the time alloted for equilibration, nor the temperature at which it was carried out were critical factors in the successful preservation of sporozoites.

The infections were very even throughout the seven groups of birds which were inoculated, and the most convenient procedure therefore, was adopted for subsequent experiments. This was equilibration for 20 minutes at room temperature, which allowed a comfortable time for filling and heat-sealing a batch of ampoules.

Expt. 6. Long term storage of sporozoites and the re-establishment of the parasites in routine culture. The first 5 experiments indicated that glycerol, added slowly to the sporozoite suspension to give a final concentration of 7.5 %, equilibration for 20 minutes at room temperature, and cooling at approximately 1 °C per minute would protect the parasites during storage in liquid N_2 for periods up to 14 days. Injection into the duodenum was the optimum route for subsequent inoculation. In order to test the efficiency of this routine over a longer period, a batch of ampoules was prepared and stored in the liquid nitrogen refrigerator.

Single ampoules were removed after 1, 7, 28, 56 and 172 days; 3 ampoules remained for subsequent testing at longer intervals. The results of the experiment are summarized in Table 2. All the birds inoculated became infected, and produced

Table 2. The infectivity of sporozoites of *E. acervulina* after preservation for different periods at —196 °C.

No. of days at —196 °C	No. of chicks infected	No. of oocysts per bird $\times 10^6$, day 4 to day 6	Sporulation Rate
1	5/5	10	84 %
7	5/5	12	92 %
28	5/5	49	93 %
56	5/5	9	95 %
172	5/5	51	90 %

similar numbers of oocysts from the 4th to the 6th days. There was no evidence of a decline in the viability or virulence of the sporozoites during the 6 month storage period.

Satisfactory cultures of oocysts were prepared on the 5th day, and these were subcultured by inoculation to further chicks. The results obtained from these

Table 3. A comparison of subcultures of *E. acervulina* after storage of the sporozoites for different periods at —196 °C, with those obtained from cultures routinely passaged and stored at +5 °C

Culture	No. oocysts per bird $\times 10^6$	Sporulation rate
Subculture after 1 day at —196 °C	74	86 %
Subculture after 7 days at —196 °C	31	94 %
Subculture after 28 days at —196 °C	51	92 %
Subculture after 56 days at —196 °C	103	90 %
Subculture after 172 days at —196 °C	53	88 %
Average routine culture	84	91.5 %
Lowest routine culture	38	82 %
Highest routine culture	205	97 %

infections were similar to those from routine cultures serially passaged and stored as sporulated oocysts at +5 °C (Table 3).

DISCUSSION

Infection of the rat following the parenteral inoculation of oocysts of *Eimeria nieschulzi* was recorded by LANDERS (1960). Similarly, DAVIES and JOYNER (1962) and SHARMA and REID (1962) infected fowls with various species of *Eimeria* by the parenteral inoculation of oocysts. This method of infection was also found to be possible using suspensions of sporozoites excysted in vitro. LONG and ROSE (1965) produced infections of *E. tenella* following intravenous and intraperitoneal injection of sporozoites, but subcutaneous and intramuscular injections were unsuccessful. In the present study, sporozoites of *E. acervulina* did not produce infections after intravenous injection, but 1 out of 5 birds became infected following intraperitoneal injection. Although oral inoculation of sporozoites was successful, the resulting infections were very slight and the best results were obtained after injection into the duodenum by laparotomy.

The excystation of sporozoites in vitro has been an established laboratory procedure for some years and has proved a valuable technique for certain investigations into coccidiosis. Such preparations have been used to infect chick embryos (LONG and TANIELIAN 1965, LONG 1966) and tissue cultures (PATTON 1965). When the sporozoites of fowl coccidia are injected into unusual development sites, certain species can complete their life-cycles in these sites (HORTON—SMITH and LONG 1965, 1966). Sporozoites of *E. tenella*, injected intravenously, have been used to test active and passive immunity in chicks which had previously been given oocysts orally, or had received injections of disintegrated schizonts or serum globulin fractions from resistant birds (LONG and ROSE 1965).

Sporozoites are similar in nature to many of the parasitic and free-living protozoa which have been stored successfully at very low temperatures. The present study has demonstrated that the sporozoites of *E. acervulina* can be preserved in liquid nitrogen by a simple procedure. There was no decline in viability during a period of 172 days at —196 °C, the variation in oocyst production of the groups of birds which received sporozoites after different lengths of storage (Table 2) probably being referable to the small number of chicks involved. Three ampoules from the same batch remain in liquid N_2 for further testing at longer intervals. There should be no deterioration over a period of years. *Trichomonas foetus* has survived for 5.6 years at —95 °C (LEVINE and ANDERSEN 1966). The slow decline in viability of the organisms over this period was thought to be due to protein denaturation and slow metabolism, both of which should cease at approximately —130 °C. There is little doubt that similar techniques can be applied with equal success to the sporozoites of other species of coccidia. The suspending medium, the protective agent and the speed at which it is added, and the rate of cooling all have a bearing on the

maximal survival of protozoan parasites at very low temperatures. Rapid thawing and immediate inoculation by the optimum route are equally important. There is probably a wide choice of suspending media for sporozoites of *Eimeria* species. They will survive for a considerable period in excystation medium, which consists of enzymes and bile, and will withstand cooling to 5 °C for a period of 2 days (LOTZE and LEEK 1967). In the present study they survived equally well in physiological saline or balanced salts solution with or without 10% serum added. The effect of different pH values has not been assessed, but when suspensions were stored in liquid Nitrogen, parasites in borate buffered solution, pH 8, gave only slightly heavier infections than those in balanced salts solution/serum alone. Ten per cent serum was added to the freezing medium because its presence has proved beneficial in a number of other systems for the low temperature preservation of protozoa (WALKER 1966). Because of the danger of immunological reactions, bovine serum was used for avian parasites. Glycerol appears to be more satisfactory than DMSO as a freez-protective for *E. acervulina* (Table 1, Expts. 3 and 4). By contrast many trichomonads and trypanosomes may be successfully preserved with both these substances (LEVINE and ANDERSEN 1966), but the slow addition of the freeze protective appears to be essential in order to minimise osmotic shock (JEFFRIES and HARRIS 1967). Rapid freezing was not attempted, but satisfactory survival was obtained with the relatively slow cooling rate of 1 °C per minute to —70 °C which has proved successful with a number of other cells. The apparatus used in the present study is very convenient but allows a wide variation in the cooling rate of plus or minus 40% with a full load of ampoules set to cool at 1 °C/min. This error is acceptable when reproducibility is not an essential requirement; it does, however, necessitate a test of the viability of each batch which is stored. For more accurate work a simple apparatus may be constructed, which employs the principle of conducting heat away from 96% Ethanol by aluminium rods of various cross-sectional area (WALKER and WILÉN 1967).

JEFFRIES and HARRIS (1967) have drawn attention to the need for slow dilution of the protective agent after thawing in order to minimise osmotic effects. It is possible that heavier infections might be obtained by slowly adding 15 mls. of BS-2 to the thawed sporozoite suspension and then concentrating the parasites by centrifugation. However, any advantage gained, might be lost in the length of time taken, which would not be less than 20 minutes; most authors stress the need for immediate inoculation after thawing. Rather light infections followed the inoculation of freeze-preserved sporozoites. Sufficient oocysts were produced, however, to ensure the successful re-establishment of the strain, and subcultures were well up to the usual standards, both in numbers of oocysts and percentage sporulation.

The extension of freeze preservation techniques to other species of coccidia will lead to savings in the labour and expense which would otherwise be incurred in their routine subculture. Although the oocysts of most species will survive for a year at refrigerator temperatures, occasionally we have encountered loss of viability with some types and prefer to make subcultures at more frequent intervals. The

presence of a bank of deep-frozen sporozoites of these difficult species would be reassuring. Deep-frozen material would be the most economical way of maintaining pure cultures of those species of coccidia which are rarely required for experimental work.

A second advantage in freeze preservation of coccidia is the stabilisation of strains. The establishment of "stabilates" has become a standard practise in some fields of protozoology and this may now be extended to the coccidia. Most laboratory cultures originate from the isolation of a single oocyst. Repeated passage through experimental animals inevitably imposes selection which may result in alterations in the properties of the strain. With coccidia, the effects of selection have been demonstrated in the development of drug-fast strains (JOYNER 1957), but the effects due to other factors can be demonstrated only by reference to the reliable stabilates which low temperature storage provides. During the preparation of this manuscript, KOUWENHOVEN (1967) reported the low temperature freezing of sporocysts of *E. tenella*. DMSO proved in this case to be an effective freeze protective.

During the present series of experiments with *E. acervulina*, a single trial with oocysts and sporocysts was carried out, using 7.5% glycerol as a protective. The results were not encouraging. After 3 days at —196 °C very few sporozoites excysted in vitro. Only very light infections occurred in 3 out of 5 chicks inoculated orally with 800,000 sporocysts after storage for 80 days. Inoculation of 450,000 oocysts stored for the same period did not result in any infection.

It was felt that the successful transmissions could have been due to excysted sporozoites rather than intact sporocysts which had survived the freezing process. The conditions required for successful freeze preservation of *E. acervulina* and *E. tenella* may well be very different.

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