CRYOPRESERVATIVE STUDIES ON MOUSE SPLEEN LYMPHOCYTES, *ICHTHYOPHTHIRIUS MULTIFILIIS* AND *GIARDIA LAMBLIA*, WITH NOTES ON THE IMMUNE RESPONSE IN CHANNEL CATFISH

by

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INTRODUCTION[1]

Cryopreservation is the protection of living cells below their normal operating temperature. Cryoprotection is based on the avoidance or minimization of intracellular freezing and the minimization of damage to the cell from the environment of concentrated solutes during cooling (65). Many studies have been conducted to elucidate the optimal conditions for freezing and thawing of many cell types. A variety of penetrating and nonpenetrating cryoprotective agents have been studied and their mode of action hypothesized. As the field of cryobiology has been studied in greater depth, the development of cryobiological equipment has become more refined. The present study reviews several major cellular freezing hypotheses, the mode of action of penetrating and nonpenetrating cryoprotective agents, and currently available cryobiological equipment.

Freezing Hypothesis: In 1978 Leibo and Mazur (54, 62) summarized the physical events a cell experiences during a freezing process. When a cell suspended in an aqueous medium is cooled to temperatures slightly below 0°C, ice forms first in the extracellular solution. As a consequence, the dissolved solutes become more concentrated as water is removed in the form of ice. As the temperature of the cell suspension is lowered, more ice forms, resulting in a progressively more concentrated extracellular solution. The higher the concentration, the lower the chemical potential of water. The cell responds osmotically to equalize the chemical potential of water across its membrane. Hence, during freezing, a cell will lose water. If a cell is cooled slowly enough, it will progressively lose water as the temperature is lowered so as to remain in osmotic equilibrium with the

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[1]References are located at the end of Appendix II.

extracellular solution. Rapid cooling causes formation of intracellular ice which is considered to be a primary factor responsible for cell death during freezing (54, 61, 63, 64).

Although the freezing point of cytoplasm is usually above -1°C, cells generally remain unfrozen, and therefore supercooled, to -10°C or -15°C, even when ice is present in the external medium (63, 64). The degree of extracellular supercooling prior to the initial formation of ice is an important parameter in determining the occurrence of intracellular ice (27). The ability of cells to remain unfrozen while supercooled indicates that the cellular membrane can prevent the growth of external ice into the supercooled interior. The indication also exists that cells neither are, nor contain, effective nucleators of supercooled water.

Since intracellular freezing is precluded above about -10°C and since supercooled water has a higher vapor pressure than ice, cells begin to equilibrate by losing water (57, 63, 64). The resulting dehydration concentrates the cell's solutes, thus lowering the intracellular aqueous vapor pressure. As the temperature decreases, the amount of cell water decreases while extra- and intracellular solutes concentrate and eventually precipitate. The precipitation of solutes, known as solution effects, causes a pH change which may adversely affect vital cellular proteins.

Meryman (63, 73) proposes that injury from solute concentration is not the result of denaturation by the concentration of any specific solute but appears to be related to the osmotic reduction of cell volume. Osmotic stress, possibly through the development of a pressure gradient across the membrane, leads to an abrupt change in membrane permeability. Such a change permits an influx of extracellular solute and simultaneously causes a loss of, or alteration of, surface constituents (carbohydrate portions of glycoprotein and glycolipids), leading to a loss of stability of membrane

macromolecules. This permits further degradative events leading to irreversible damage.

Cooling velocities above optimum in terms of cell survival not only produce intracellular crystals, they also produce small crystals, which are likely to enlarge during warming because of their high surface free energy. Cooling rates below optimum cause cells to dehydrate without producing intracellular ice (63, 69). The ultimate result of slow cooling rates is the development of a few large ice crystals that have incorporated all available free water and have relegated the saturated solution of electrolyte, carbohydrate, protein and other cell constituents, with their "bound" water, to the crystal interfaces (69). The configuration of inner membrane protein and or protein-lipid interaction may be altered producing a change in the cell membrane (88). Other solution effects include the concentration and precipitation of solutes and changes in pH (64).

Other researchers (25, 30, 63, 89) report that during slow freezing cellular membranes are altered by the hypertonic solutions produced. The alteration in itself does not cause membrane leakage of normally impermeant solutes but it renders the cells susceptible to solute leakage on the application of a stress. Such stress is provided during freezing by the reduction in temperature (thermal shock) and during thawing by dilution (dilution shock). The extent of the stress of temperature reduction is independent of the cooling rate (81). Rapid freezing creates a dehydration with the same potential for denaturation as that responsible for injury following slow freezing (69).

Thawing can affect the slowly frozen cell in two ways: exposure to high electrolyte concentration at high temperatures will produce a high rate of injury, and prolonged exposure at higher temperatures may permit the growth of ice crystals by recrystallization prior to actual melting (61, 64, 69).

If a cell suspension has been frozen at a rate less than optimum, the warming rate is rather unimportant (53). If a cell suspension has been frozen at a rate greater than the optimum, then cell survival depends critically on the warming rate (53). A rapid thawing rate is essential to survival after rapid freezing (69). Cells require sufficient time to lose water as they cool in order to avoid subsequent intracellular freezing, but must be cooled rapidly enough to minimize solution effects (32).

Penetrating Cryoprotective Agents: Leibo and Mazur (54) report that when a cell is placed into a hyperosmotic solution of a permeating solute, the cell quickly shrinks by water loss to restore osmotic equilibrium across its membrane. Concurrently, the solute begins to permeate the cell, accompanied by water entry. The progressive and coupled entry of solute and water continues until the chemical potential equilibrium across the membrane has been restored. The time dependent permeation of solute into the cell is a function of the initial solute concentration and the temperature of exposure. The greater the concentration and the higher the temperature of exposure, the faster the solute permeates.

Any solute that can be elaborated in or penetrate cells will lower the vapor pressure of the aqueous solution and reduce the amount of ice formed at any temperature (73). Dimethyl sulfoxide (Me_2SO) and glycerol are the two most widely used penetrating cryoprotectants. Their use is based on their colligative properties (65, 67, 73) reducing the amount of ice formed at any temperature during cooling.

Me_2SO and glycerol permeate the cell and create the high ionic strength environment only at lower temperatures where damage to the cell develops more slowly (65).

Me_2SO was first introduced as a low temperature cryopreservative agent for mammalian cells by Lovelock and Bishop (6, 58, 63). Me_2SO was reported

to protect on a molar basis by reducing the electrolyte concentration in the residual unfrozen solution in and around a cell at any given temperature (63).

Farrant (29) reported Me_2SO postponed both the shrinkage of human erythrocytes and the onset of cation leaks to higher osmolalities, equivalent to lower temperatures during freezing.

Me_2SO and glycerol have been used as cryoprotective agents enabling the successful freezing of a variety of cells including bone marrow cells (87), mouse lymphocytes (6, 13, 99, 102, 103), peripheral blood mononuclear cells (48), chinese hamster tissue culture cells (65, 68), *Tetrahymena pyriformis* (45), *Giardia lamblia* (74) and *Ichthyophthirius multifiliis* (12), to mention but a few. In most studies greater cell viability has been associated with the use of Me_2SO as opposed to glycerol. When Me_2SO is used as the protective agent, cell survival appears to be less critically dependent on the exposure time and temperature (13, 54).

Two immediate obstacles in using penetrating agents are the penetration of cells must be uniform and achieved without the imposition of osmotic stresses which can in themselves be destructive, and the concentrations of penetrating agents necessary to achieve protection are toxic to cells (72, 99, 102, 103). Dilution damage during both thawing and post-thaw dilution may be due to osmotic swelling as Me_2SO and normally excluded solutes leave the cell (103).

Nonpenetrating Cryoprotective Agents: Nonpenetrating solutes, e.g., polyvinyl-pyrrolidone (PVP), sucrose, glycerol, and hydroxyethyl starch (HES) do act as cryoprotectants to at least some cell systems at temperatures of -80°C to -196°C (101). Protection lies in their ability to alter the permeability of the cell membrane, and then allow it to leak solute reversibly under an osmotic pressure gradient (70, 71, 72, 101).

This point has been disputed by Farrant and Woolgar who postulate that the action of a nonpermeant depends on its ability to reduce the concentration

of electrolyte in contact with frozen cells and to moderate the transcellular cation concentration gradient (31, 101).

Other researchers hypothesize nonpenetrating additives osmotically "squeeze" water from the cells primarily during the initial phases of freezing at temperatures between -10°C to -20°C when these additives become concentrated in the extracellular regions (65, 67). In the presence of nonpenetrating agents survival of cells is increased at the faster cooling rates (65).

PVP and sucrose offer a significant but low degree of protection to chinese hamster ovary cells during cooling-storage-thawing at -196°C. This protection is markedly reduced at -80°C storage temperatures (101).

Glycerol need not penetrate a cell to offer protection. As glycerol enters a cell, changes may occur in the response of cells cooled at a constant rate to and warmed from -196°C (65).

HES is responsible for an osmotic stress on cells which results in a loss of cell water at high subzero temperatures. Protection against further cooling is obtained by osmotic shrinkage due to the increased concentration of HES in the extracellular region. The ability of HES to increase the osmotic pressure of the extracellular solution is related to colligative action of polymers, particularly in high concentration (65). Increasing the concentration of HES to 20% allowed increased recovery of chinese hamster fibroblasts thawed from -196°C (65).

Cryopreservative Equipment and Methods: There are many systems on the market for regulated cooling (12). A microcomputer-based temperature and cooling/warming rate controller (66) can control temperatures from 199.9°C to -199.9°C, and rate of change of temperature from 0.01°C to 99.99°C/min. A digital thermometer is used to monitor the temperature, and the system can control a variety of heating and cooling equipment.

A microprocessor-controlled rate controller (8) differs from those currently available in that the actual temperature of the cell suspension being cooled is compared with a preselected ramp of the microprocessor. Differences between the two determine the opening of solenoid valves that permit entry of liquid nitrogen (LN_2) vapors into the freezing chamber. The heat of fusion, which is released as extracellular ice crystallizes, automates the openings of additional solenoid valves. The rapid entry of LN_2 vapors into the chambers cools the cell suspension, therefore restoring the programmed cooling rate.

A simple, inexpensive system to manipulate and accurately reproduce cooling rates was developed by modifying the system designed by Leibo and Mazur (54) and altered by Zeilmaker and Verhamme (112). Changing the volume of ethanol, the cooling bath medium, served to coarsely adjust the cooling rate. Fine adjustments were made by changing the vacuum level drawn from the Dewar flask. Beeler (12) reported that reducing the vacuum level during the phase transition increases heat flux and may reduce cell destruction from latent heat of fusion.

The use of several types of freezing containers have been studied by Simione *et al.* (95). They report the use of plastic ampoules to be unsuitable for storage directly in LN_2. Heat transfer during thawing was slower in plastic ampoules than in glass ampoules. Imperfectly heat-sealed glass ampoules, however, can shatter dangerously during rapid thawing due to leakage of nitrogen into the ampoules. Glass screw-cap ampoules eliminate the necessity for sealing but do not prevent accidental contamination of storage facilities due to breakage.

Monitoring freezing/thawing rates with a copper-constant thermocouple inserted directly into one of the vials filled with the suspension to be frozen is the preferred temperature recording method (94, 99).

Various freezing methods have been reported utilizing many different techniques. A 1-step cooling by direct immersion in -78°C coolant has been found to be far more harmful than a 2-step cooling, which prolongs the stay of the cells in the phase transition or crystallization temperature range (100).

A 2-step cooling process requires: a rapid cooling to a subzero holding temperature, remaining at that temperature for a predesignated period of time, and rapid cooling into liquid nitrogen (110). Eight-cell mouse embryos in 1.5 M Me_2SO demonstrated the effectiveness of this procedure for good survival providing that warming is rapid. Poor survival was obtained using a slow warming rate suggesting that this simpler method allows some intracellular freezing. The 2-step cooling process was reported to be a poorer method for freezing mouse lymphocytes (in a form optional for freezing sperms) by Birkeland (13).

Other variations for freezing cells include a prefreezing temperature equilibration in a constant temperature bath at a subzero setting. The sample can be seeded with ice by touching the surface of the cell suspension with the tip of a Pasteur pipet containing frozen medium (103), or be allowed to spontaneously crystallize.

In the following studies the present author used an adaptation of Beeler's apparatus (12). The adaptations were a thermocouple connected to a temperature recorder and glass screw-cap tubes. Me_2SO was chosen as the cryoprotective agent. Mouse spleen lymphocytes were used to determine the effectiveness of the modified Beeler apparatus. Cryopreservative studies were conducted on *Ichthyophthirius multifiliis*, a ciliate parsite of fresh-water fish, and *Giardia lamblia*, a flagellate parasite in the intestinal tract of man and possibly certain other mammals. The kinetics of the immune response in channel catfish (*Ictalurus punctatus*) was investigated for future studies concerning antibody production to *I. multifiliis*.

CRYOPRESERVATION OF MOUSE SPLEEN LYMPHOCYTES USING DIMETHYL SUFLOXIDE AND A CONSTANT VACUUM IN A DEWAR FLASK

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Summary

This study examined the effect of varying freezing conditions on mouse spleen lymphocyte viability using a constant vacuum in a Dewar flask and an ethanol bath to regulate the freezing rate. Lymphocyte suspensions containing 13% (v/v) Me_2SO (dimethyl sulfoxide) were frozen either by spontaneous crystallization or by seeding the medium. Optimal freezing conditions included spontaneous crystallization at -10°C with no perceptible latent heat of fusion, a controlled cooling rate of -0.828°C/min. to -70°C, and immersion into liquid nitrogen vapor to the storage temperature of -195.8°C. Optimal thawing conditions required rapid thawing while agitating in a 37°C water bath at 445.49°C/min. and Me_2SO dilutions at 5°C. The optimal lymphocyte viability after thawing was 77.4%. Me_2SO toxicity studies without freezing demonstrated that cell viability decreased 23.8%.

Dimethyl sulfoxide (Me_2SO) was first introduced as a low temperature cryopreservative agent for mammalian cells by Lovelock and Bishop (1, 7, 8). Since that time this chemical has been used successively to cryopreserve a variety of cell types utilizing varying freezing techniques. The present study utilized several freezing conditions; a constant vacuum in a Dewar flask, and an ethanol bath controlling the freezing rate to investigate their effects upon mouse spleen lymphocyte viability.

Materials and Methods

Isolation of Mouse Spleen Lymphocytes: Adult male C3H/HEN(MTV^-) mice under one year of age were used as the source of spleen lymphocytes. Individual mice were sacrificed by cervical dislocation and placed on a pegboard lying on their right side. Alcohol was applied to their abdominal-thorax region and a vertical incision made in the lower abdominal region penetrating the dermal layer. Using steril forceps the skin on either side of the incision was grasped and pulled horizontally in opposite directions thus tearing the skin and exposing the underlying muscle layer. Using sterile scissors the muscle layer was cut and laid back exposing the spleen. Sterile blunt nose forceps were used to hold the organ while trimming away connective tissue and fat. Once excised the spleen was placed in a sterile plastic Petri dish containing 10 ml buffered salt solution (BSS). Using sterile forceps and probes the spleen was emptied by grasping the capsule in the middle with the forceps, tearing open one end and forcing out the contents by stroking the capsule with the probe; then the same procedure was applied to the opposite end of the spleen and the capsule discarded. With the aid of a 10 ml pipet a single cell preparation was made by repeated aspirations of the cell clumps in BSS.

The cell suspension was centrifuged at 200xg for 8-10 minutes. The supernatant was aspirated off and the cells resuspended in 1 ml of lysing solution. The lysing solution was one part stock solution (A) consisting of 20.6 g tris buffer per liter water; pH adjusted to 7.2-7.4 with HCl, and nine parts stock solution (B) consisting of 0.83 g ammonium chloride per 100 ml water. The mixture was placed on crushed ice for 10 minutes while agitating periodically. The media in this study consisted of Delbecco's Minimal Essential Medium (DMEM) with either 10% (v/v) fetal calf serum (FCS), or 5% FCS (v/v) plus 5% (v/v) horse serum (HS). After 10 minutes, 9.0 ml of media with serum was added and the cell mixture centrifuged at 200xg for 8-10 minutes.

Following centrifugation the supernatant was removed and the cells were washed using 10 ml BSS. Finally, the cells were resuspended in 10 ml BSS, aspirated in a 10 ml pipet and then filtered through a nylon tube filter to remove tissue debris. The cells were then washed with BSS for the third time and resuspended in 1 ml media with serum for counting.

Dye Exclusion: The viability of the cells was measured by trypan blue exclusion (4, 13, 16). The cells were incubated briefly with the dye; stained cells were scored as nonviable.

Freezing Lymphocytes: The lymphocytes (solution 1) were diluted with media plus serum to a final concentration of 20-100 x 10^6 lymphocytes per ml of solution and placed on crushed ice. Solution 2, consisting of media with serum plus Me_2SO at 26% (v/v) of the total solution, was chilled on crushed ice. Varying numbers of 20 ml screw-cap tubes are individually filled with 1 ml solution 1 and 1 ml solution 2 immediately prior to the actual freezing procedure.

Freezing Equipment: The freezing equipment used in this study consisted of a 2095 Bath and Circulator (Forma Scientific) filled with anti-freeze solution pre-cooled to temperatures ranging from -5°C to -15°C. This apparatus was used to enable seeding of the tubes before placing them in the Dewar flask containing ethanol (2, 10, 16). The second apparatus consisted of a 1500 ml Dewar flask filled with 600 ml 100% ethanol (2, 10). A vacuum was drawn in the Dewar flask measured at 23.75" Hg for 1-5 tubes or 23.0" Hg for 6-10 tubes being frozen. This constant pressure gave a freezing rate of approximately 1.0°C/min. The temperature of the ethanol was measured using an alcohol thermometer. The ethanol was circulated using a motor driven stirrer. Constant sample temperature was recorded using a Honeywell Electronic 111 measuring -200°C to 50°C with a type T thermocouple (copper-constant wire) directly inserted into the cell solutions to be frozen (11, 13). The larger Dewar flask was filled with liquid nitrogen (LN_2), whose boiling point is -195.8°C (10), into which the first Dewar flask was placed keeping the LN_2 at a constant level. A schematic drawing of the cooling apparatus is shown in Figure 1. This apparatus was adapted from Beeler (2).

Freezing Procedure: Solutions 1 and 2 were mixed into varying numbers of tubes based on final lymphocyte concentration. Some tubes contained solutions 1 and 2 without cells to determine the physical freezing parameters of the media. The thermocouple was placed into the solution of one of the tubes. The cells were then frozen either by spontaneous crystallization of the medium or seeding by touching the surface of the cell suspension in the anti-freeze bath with the tip of a Pasteur pipet containing frozen medium (16). When the temperatures of the samples and the ethanol bath were the same, the samples were then transferred from the anti-freeze bath to the cooling ethanol bath. The seeded samples were cooled at approximately

1.0°C/min. to -46°C and the spontaneous crystallized samples to -65°C, -70°C, and -90°C. Temperature endpoints of at least -45°C were chosen because water desorption terminates at about -40°C (2, 6). At the aforesaid temperature endpoints, samples were placed into either LN_2 vapor or liquid (2, 16) for 1 to 20 days.

Thawing Procedure: Rapid thawing was achieved by agitation of the samples in a 37°C water-bath (2, 4, 16). Just before the last ice crystals had melted, the samples were removed and placed on ice for 5 minutes. During that time 8 ml of media with serum were mixed with the samples to dilute the 13% (v/v) Me_2SO. The cells were then centrifuged at 200xg for 8-10 minutes, the supernatant aspirated off and the cells resuspended in 1 ml media with serum. Viable cells were counted using the previously described trypan blue dye exclusion method.

Me_2SO Control: Spleen lymphocytes were prepared as stated above, solutions 1 and 2 mixed and remained in crushed ice for 5 minutes [13% Me_2SO (v/v) concentration]. The samples were then diluted with 8 ml media with serum while agitating, then placed in crushed ice for 5 minutes. These samples were centrifuged and the cells counted as described in the thawing procedure.

Results

After Me_2SO treatment 6.07×10^7 cells/ml were viable indicating a 74.2% cell recovery from the initial 8.18×10^7 cell concentration (Table 1). The difference was presumably due to Me_2SO treatment and cell processing.

The results shown in Table 2 indicate that samples of medium A, with or without seeding, have close thawing rates - a difference of 10.9°C. Likewise, medium B with and without seeding have thawing rates which differ only by 15.39°C. The addition of HS and the decrease of FCS in medium B decreased the thawing rate by approximately 130°C/min. when compared with

medium A (Table 2). The temperature of spontaneous crystallization for media A and B were -11°C and -15°C, respectively. The temperature at latent heat of fusion for both media was -6°C. The latent heat of fusion given off into the crystallizing media for media A and B increased the temperature 5°C and 9°C, respectively. Correlation coefficients for all cooling and thawing rates ranged between 0.961 and 0.998 where temperature was the ordinate and time the abscissa indicating a straight line relationship.

Table 3 displays the physical parameters of the media with serum plus cells measured under varying freezing conditions. The 0.728 correlation coefficient associated with the 503.10°C/min. thawing rate indicated a marginal straight line relationship.

Table 4 displays cell viability data according to the freezing parameters in Table 3. One can correlate the results in the two tables using the time frozen column. The greatest viability both after thawing and in relation to the initial cell concentration was the mixture frozen for two days. The physical parameters for those samples were a controlled freezing rate of -0.828°C/min. in the ethanol, -22.36°C/min. in LN_2 vapor and a thawing rate of 445.49°C/min. in the 37°C water bath (Table 3). The temperature recorder did not indicate any perceptible latent heat of fusion which may have contributed to the increased cell survival through equilibration with the surrounding ethanol temperature.

Discussion

Me_2SO has been used for freezing a variety of cell types. Tate and Cram (13) used a variety of Me_2SO concentrations to preserve bovine lymphocytes. They reported an optimal (72%) survival of lymphocytes following freezing and thawing of whole blood protected by 7.3% (v/v) Me_2SO. Furthermore, reduction of Me_2SO to 2% (v/v) resulted in a substantial decrease in the number of lymphocytes recovered.

Karpovitch *et al.* (4) reported a final concentration of 10% (v/v) Me_2SO resulted in a mean viability of 90% human peripheral blood lymphocytes, but a mean recovery of 71% in comparison to the initial concentration.

Thorpe *et al.* (15, 16) determined that during freezing and thawing, the cooling rate giving optimal survival of 75% mouse mesenteric lymph node lymphocytes was 8-15°C/min. for cells in 5% (v/v) Me_2SO and 1-3°C/min. for 10% (v/v) Me_2SO. Cell survival after exposure to 10% (v/v) Me_2SO for 20 minutes was reduced by 50% at room temperature, and by 20% at 0°C (15). Studying Me_2SO toxicity Thorpe *et al.* later found an 80% viability of the lymphocytes following 10% (v/v) Me_2SO treatment for 2 hours at 20°C using the trypan blue dye exclusion technique for cell counting (16). They further determined that a 5%-15% (v/v) fetal calf serum present in the freezing medium produced a 4-5 fold improvement in mouse lymphocyte survival. Dilution of thawed cells at 25°C or 27°C gave a much improved cell survival (75%) compared with dilution at 0°C (30%).

The results on Me_2SO toxicity substantiate the results presented by other groups as the 13% (v/v) Me_2SO concentration used in this study decreased cell viability by 23.8%. The optimum lymphocyte viability obtained after thawing in this study was 77.4% as depicted in Table 4. By decreasing the concentration to around 8-10% (v/v) Me_2SO and perhaps limiting the number of washings used in the lymphocyte preparations, a greater number of cells would survive. It appears that the loss in cell viability was not due to the freezing and thawing process as much as to the Me_2SO concentration and the rigors of lymphocyte handling.

This study indicated that a slow controlled 2-step freezing process is desirable. A cooling rate of 0.828°C/min. to a temperature endpoint of -70°C seems to absorb the latent heat of fusion with subsequent immersion of the

sample into LN_2 vapor and storage at -195.8°C. A very rapid thawing in a 37°C water bath at 445.49°C/min. and Me_2SO dilution around 5°C also contributes to optimal cell viability. Any fixed period of exposure to Me_2SO prior to freezing appears to be unnecessary in the present study (Table 3) which substantiates Birkeland(3). Birkeland however found that a 2-step freezing procedure seemed to be a poorer method for freezing lymphocytes (in a form optimal for freezing sperms). Taylor (14) reported that a 1-step cooling by direct immersion in 78°C coolant was far more harmful than 2-step cooling, because the latter method prolongs the stay of the cells in the phase - transition or crystallization temperature range. The 2-step cooling procedure has been used on various cell types under a variety of conditions (9, 17).

Controlling cooling by a variable vacuum in a Dewar flask was first described by Leibo and Mazur (5) and altered by Zeilmaker and Verhomme (18) and Beeler (2). The particular freezing apparatus used in this study was adapted from Beeler (2) and used a thermocouple connected to a temperature recorder. The use of an ethanol bath is recommended above a solid matrix because liquids with high specific heat (ethanol: specific heat of 2.29 J/g°C) are better as heat transfer media. An alcohol bath cooling unit is valuable for accurate temperature control and for minimizing the latent heat effect (10).

Glass screw-cap tubes were chosen for use over glass or plastic ampoules on the basis that Simione *et al.* (12) found plastic ampoules unsuitable for storage directly in LN_2, heat transfer during thawing was slower in plastic ampoules than in glass ampoules (with agitation in 35°C water bath the contents of plastic ampoules took four times longer to thaw than glass ampoules), and imperfectly heat-sealed glass ampoules can shatter dangerously during rapid thawing due to leakage of LN_2 into the ampoules.

Glass screw-cap tubes eliminate the necessity for sealing but do not prevent accidental contamination of storage facilities due to breakage.

Follow up studies using a slow controlled cooling rate and rapid thawing rate are required to increase the mouse spleen lymphocyte viability. Particular attention is to be paid to the Me_2SO concentration and the lymphocyte preparation protocol. An apparatus such as the constant vacuum in a Dewar flask/ethanol bath described in this study offers rigid control over the critical cooling rate and is recommended to be used in the cryo-preservation of spleen lymphocytes.

Figure 1. Schematic drawing of the cooling apparatus.

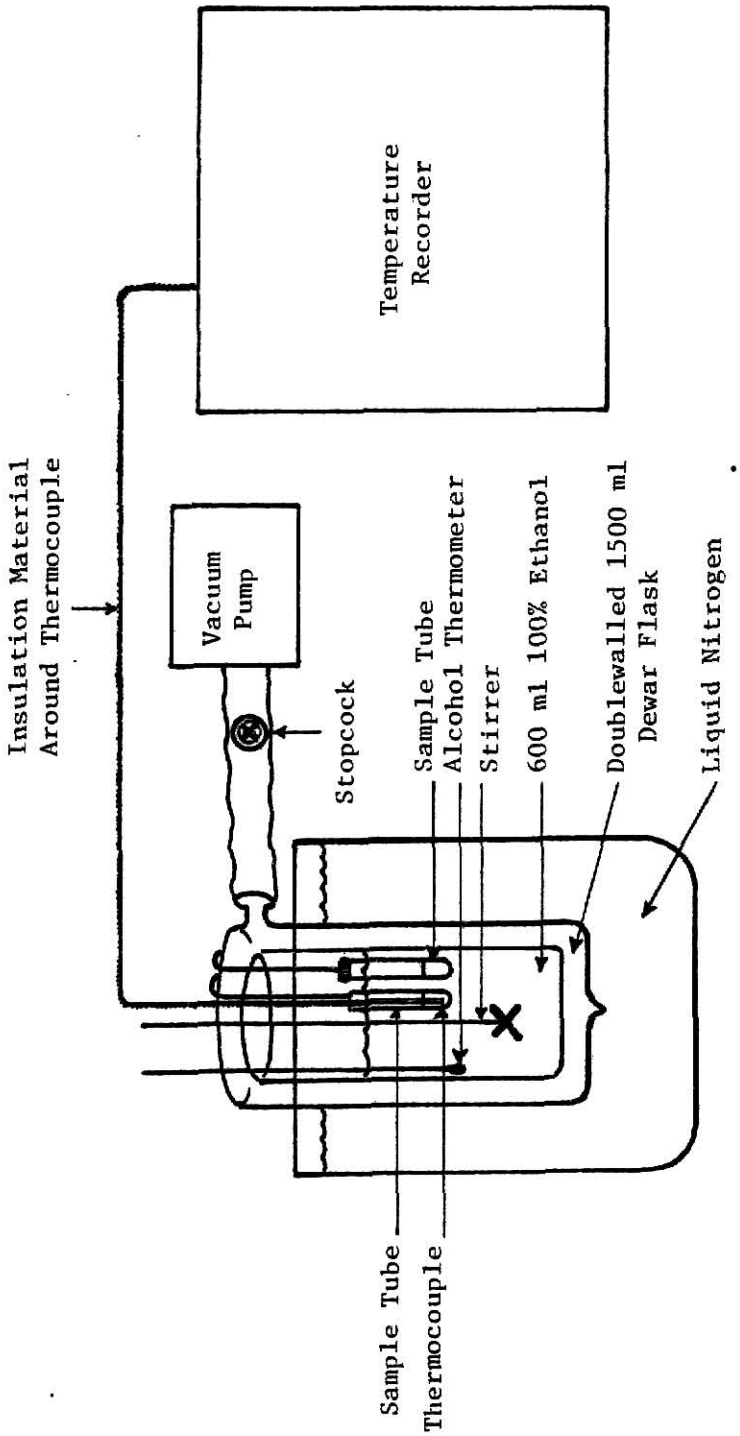
Temperature Recorder
Insulation Material Around Thermocouple
Vacuum Pump
Stopcock
Sample Tube
Alcohol Thermometer
Stirrer
600 ml 100% Ethanol
Doublewalled 1500 ml Dewar Flask
Liquid Nitrogen
Sample Tube
Thermocouple

Table 1. Viability of Spleen Lymphocytes Following Treatment With 13% (v/v) Me_2SO[a]

	Cell Count		Concentration	Percent
	Viable	Total	Cells/ml	Viable
Before Me_2SO	409	440	8.18×10^7	92.9
After Me_2SO	60.75	64.50[b]	6.07×10^7	94.2

[a] Medium with serum = DMEM + 5% HS + 5% FCS

[b] Average from 4 tubes due to final lymphocytes concentrations

Table 2. The Physical Parameters of the Cell Media Under Varying Freezing Conditions

Media With Serum	Seeding °C	Spontaneous Crystallization °C	Temperature at Latent Heat of Fusion °C	Temperature Endpoint °C	Cooling Rates °C/min Ethanol	Cooling Rates °C/min LN_2	Thawing Rate °C/min
A	-5	NA	NA	-50	-1.32 r= -0.993	-30.05(v) - 0.996	+506.82 + 0.998
A	NA	-11	-6	-50	-1.38 r= -0.991	-26.30(v) - 0.995	+495.9 + 0.998
B	-5	NA	NA	-65	-1.15 r= -0.995	-27.77(v) - 0.996	+378.23 + 0.985
B	NA	-15	-6	-65	-1.45 r= -0.992	-71.57(1) - 0.961	+362.84 + 0.983

A = 1 ml DMEM + 10% FCS plus 1 ml DMEM + 10% FCS + 26% Me_2SO

B = 1 ml DMEM + 5% FCS + 5% HS plus 1 ml DMEM + 5% FCS + 5% HS + 26% Me_2SO

r = correlation coefficient

(v) = vapor

(1) = liquid

NA = not applicable

Table 3. Spleen Lymphocyte Plus Media Physical Parameters Under Varying Freezing Conditions

Media With Serum	Seeding °C	Spontaneous Crystallization °C	Temperature at Latent Heat of Fusion °C	Temperature Endpoint °C	Time Frozen (days)	Cooling Rates °C/min Ethanol	Cooling Rates °C/min LN_2	Thawing Rate °C/min
A	-5	NA	NA	-46	20	-1.19 r= -0.994	-24.27(v) - 0.994	ND
B	-5	NA	NA	-46	4	-1.18 r= -0.998	-26.84(v) - 0.998	ND
B	NA	-14	-6	-65	6	-1.09 r= -0.989	-20.43(v) - 0.993	ND
B	NA	-10	N	-70	2	-0.828 r= -0.996	-22.36(v) - 0.997	+445.9 + 0.991
B	NA	-14	-7	-90	1	-0.880 r= -0.990	-20.78(v) - 0.999	+503.10 + 0.728
B	-4	NA	-45	-45	1	-1.04 r= -0.997	-33.03(v) - 0.999	+457.27 + 0.991

A = 1 ml DMEM + 10% FCS + cells plus 1 ml DMEM + 10% FCS + 26% Me_2SO
B = 1 ml DMEM + 5% FCS + 5% HS +cells plus 1 ml DMEM + 5% FCS + 5% HS + 26% Me_2SO
r = correlation coefficient
(v) = vapor
NA = not applicable
ND = not done
N = no measurement indicated

Table 4. Viability of Spleen Lymphocytes Following Different Freezing Conditions.

Time Frozen (days)	Thawing: Average Cell Count Viable	Thawing: Average Cell Count Total	Thawing: % Viability	Cell Concentration (cells/ml): Before Me_2SO	Cell Concentration (cells/ml): After Thawing	Cell Concentration (cells/ml): % Viability
20	NR	NR	NR	2.46×10^7	6.13×10^6	24.9
4	23.26	39.15	59.41	7.44×10^7	2.30×10^7	30.9
6	38.25	54.06	70.75	6.75×10^7	3.82×10^7	56.6
2	34.25	44.25	77.40	3.54×10^7	2.48×10^7	70.05
1	23.17	32.50	71.29	3.96×10^7	1.86×10^7	47.0
1	34.00	52.25	65.07	4.25×10^7	2.04×10^7	48.0

NR = not recorded

References

1. Ashwood-Smith, M. J. Low temperature preservation of mouse lymphocytes with dimethyl sulfoxide. Blood 23, 494-501 (1964).

2. Beeler, C. R. Controlling cooling rates with a variable vacuum in a Dewar flask. Cryobiology 18, 79-81 (1981).

3. Birkeland, S. A. The influence of different freezing procedures and different cryoprotective agents on the immunological capacity of frozen-stored lymphocytes. Cryobiology 13, 442-447 (1976).

4. Karpovitch, X. L., Rosenkovitch, E., Ben-Basset, H., and Izak, G. Structure and functional alterations in lymphocytes induced by cryopreservation. Cryobiology 17, 12-17 (1980).

5. Leibo, S. P., and Mazur, P. Methods for the preservation of mammalian embryos by freezing. In "Methods in Mammalian Reproduction" (J. C. Daniel, Jr., Ed.), p. 179-201. Academic Press, New York, 1978.

6. Litvan, G. G. Mechanism of cryoinjury in biological systems. Cryobiology 9, 182-191 (1972).

7. Lovelock, J. W., and Bishop, M. W. H. Prevention of freezing damage to living cells by dimethyl sulfoxide. Nature (London) 183, 1394 (1959).

8. Mazur, P. Cryobiology: The freezing of biological systems. Science 168, 939-949 (1970).

9. McGann, L. E., and Farrant, J. Survival of tissue culture cells frozen by a two-step procedure to -196°C. II. Warming rate and concentration of dimethyl sulfoxide. Cryobiology 13, 269-273 (1976).

10. Pegg, D. E., Hayes, A. R., and Kingston, R. E. Cooling equipment for use in cryopreservation. Cryobiology 10, 271-281 (1973).

11. Schwabe, K. G., and Terracio, L. Ultrastructural and thermocouple evaluation of rapid freezing techniques. Cryobiology 17, 571-584 (1980).

12. Simione, F. P., Daggett, P., McGrath, M. S., and Alexander, M. T. The use of plastic ampoules for freeze preservation of microorganisms. Cryobiology 14, 500-502 (1977).

13. Tate, E. H., and Cram, L. S. A method for simultaneous isolation and cryopreservation of bovine lymphocytes. Cryobiology 19, 136-146 (1982).

14. Taylor, A. C. The physical state transition in the freezing of living cells. Ann. N.Y. Acad. Sci. 85, 595-609 (1960).

15. Thorpe, P. E., Knight, S. C., and Farrant, J. Cryopreservation of mouse lymphocytes. Cryobiology 11, 543 (1974).

16. Thorpe, P. E., Knight, S. C., and Farrant, J. Optimal conditions for the preservation of mouse lymph node cells in liquid nitrogen using cooling rate techniques. Cryobiology 13, 126-133 (1976).

17. Wood, M. J., and Farrant, J. Preservation of mouse embryos by two-step freezing. Cryobiology 17, 178-180 (1980).

18. Zeilmaker, G. H., Verhamme, C. M. P. M. A simplified method for freezing mouse embryos. Cryobiology 16, 6-10 (1979).

ATTEMPTS TO CRYOPRESERVE *ICHTHYOPHTHIRIUS MULTIFILIIS* USING DIMETHYL SULFOXIDE AND A CONSTANT VACUUM IN A DEWAR FLASK

by

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Summary

This study examined the effect of varying freezing conditions on the freshwater fish parasite *Ichthyophthirius multifiliis* using a constant vacuum in a Dewar flask and an ethanol bath to regulate the freezing rate. The cryopreservation of all three life cycle stages (trophozoite, cyst and tomite) were investigated. A variety of freezing media and procedures were studied. Dimethyl sulfoxide (Me_2SO) was the cryoprotective agent of choice. Toxicity studies were conducted on the freezing media, viability assay and Me_2SO concentrations. This study resulted in the unsuccessful cryopreservation of all three parasite stages. These results are in direct conflict with those of another author who reported the successful freezing of trophozoites and cysts using the same protocol and freezing media as used in this study.

Dimethyl sulfoxide (Me_2SO) was first introduced as a low temperature cryopreservative agent for mammalian cells by Lovelock and Bishop (1, 11, 12). Since that time this chemical has been used successively to cryopreserve a variety of cell types utilizing varying freezing techniques. The present study utilized several freezing conditions; a constant vacuum in a Dewar flask, and an ethanol bath controlling the freezing rate to investigate their effects on the viability of *Ichthyophthirius multifiliis*.

Materials and Methods

Isolation of *I. multifiliis* Trophozoites and Cysts: The mature feeding stage (trophozoites) was obtained from infected channel catfish (*Ictalurus punctatus*) 10-14" (25.4-35.6 cm) in length. Pre-moribund fish were removed from aquaria and placed in bowls to allow the parasite to leave the host. Alternatively, the hosts were pithed by driving a needle between the eyes thus severing the spinal chord; the parasites left the fish within 24 hours after death. As the trophozoites are between 250-1000 µm in diameter, they were readily observed leaving the gills, fins and epithelium of the host.

Upon leaving the host the trophozoites moved freely about for one to twelve hours before encysting upon the floor of the container. A Pasteur pipet was used to collect the motile and sessile forms of the parasite. Use of the pipet afforded avoidance of mucus and epithelial aggregations commonly sloughed-off by the moribund host. When such aggregations interferred with trophozoite and cyst collection, 710 µm mesh-screen filters were used to separate the organisms. The organisms were concentrated using a combination of decanting and pipeting techniques. Use of a mild vacuum system to remove the mucus and parasites by suction from the surface of the fish was attempted (9) but proved futile. The majority of the mucus that was removed remained clogged in the glass tubing; thus the apparatus was not

used in this study. A portion of the collected organisms were introduced to naive hosts (catfish that had never been exposed to the parasite) thus perpetuating the infection. The preponderance of the organisms were stored in tubes in crushed ice.

Isolation of *I. multifiliis* Tomites: Encysted trophozoites were allowed to multiply and produce actively motile, infective tomites 30-40 µm in size. Aquaria water containing the tomites was filtered using 50 cc. syringes and 8.0 µm 25 mm nucleopore filters in the 25 mm nucleopore filter holder. The tomites were trapped and concentrated on the filter which was placed in 20 ml filtered aquaria water. This procedure was conducted several times in order to obtain numerous organisms. The 20 ml filtered aquaria water plus tomites was then placed in a refrigerator.

Freezing Media: Aquaria water passed through 0.22 µm or 0.45 µm filters (Millipore) and allowed to stand for an hour is referred to in this study as biological control water (BCW) (3). A 20% glucose solution was prepared using reagent grade glucose and BCW. A 40% Me_2SO solution was prepared by mixing 3 parts 20% glucose in BCW with 2 parts reagent grade Me_2SO. The 40% Me_2SO solution was cooled in crushed ice as was a volume of fetal bovine serum (FBS).

A second freezing medium consisted of Delbecco's Minimal Essential Medium (DMEM) with 5% (v/v) fetal calf serum (FCS) plus 5% (v/v) horse serum (HS). Reagent grade Me_2SO was combined with this medium (v/v) to yield various Me_2SO concentrations.

Freezing *I. multifiliis* Trophozoites and Cysts: Various combinations and concentrations of the aforesaid media were investigated. Into 20 ml screw-cap glass tubes were placed: 1) 1 ml BCW with cells + 0.75 ml FBS + 0.25 ml 40% Me_2SO in 20% glucose-BCW solution (a final Me_2SO concentration of 5%).

2) 1 ml BCW with cells + 0.77 ml FBS + 0.23 ml 40% Me_2SO in 20% glucose-BCW solution (a final Me_2SO concentration of 4.6%). 3) 1 ml DMEM + 5% FCS + 5% HS with cells plus 1 ml DMEM + 5% FCS + 5% HS + 5% (v/v) Me_2SO. 4) 1 ml DMEM + 5% FCS + 5% HS with cells plus varying volumes of DMEM + 5% FCS + 5% HS and Me_2SO yielding 1.0 ml. Varying numbers of 20 ml screw-cap tubes were individually filled with the previously described media combinations immediately prior to the actual freezing procedure.

Freezing *I. multifiliis* Tomites: Into 20 ml screw-cap glass tubes were placed: 1) 1 ml BCW with cells + 1 ml 20% glucose-BCW solution. 2) 1 ml BCW with cells + 0.77 ml 20% glucose-BCW solution + 0.23 ml Me_2SO [4.6% (v/v) final concentration]. 3) 1 ml BCW with cells + 0.54 ml 20% glucose-BCW solution + 0.46 ml Me_2SO [9.2% (v/v) final concentration]. 4) 1 ml BCW with cells + 0.77 ml FBS + 0.23 ml Me_2SO [4.6% (v/v) final concentration]. 5) 1 ml BCW with cells + 0.54 ml FBS + 0.46 ml Me_2SO [9.2% (v/v) final concentration].

Freezing Equipment: The freezing equipment used in this study consisted of a 2095 Bath and Circulator (Forma Scientific) filled with anti-freeze solution pre-cooled to temperatures ranging from -5°C to -15°C. This apparatus was used to enable seeding of the tubes before placing them in the Dewar flask containing ethanol (2, 15, 22). The second apparatus consisted of a 1500 ml Dewar flask filled with 600 ml 100% ethanol (2, 15). A vacuum was drawn in the Dewar flask measured at 23.75" Hg for 1-5 tubes or 23.0" Hg for 6-10 tubes being frozen. This constant pressure gave a freezing rate of approximately 1.0°C/min. The temperature of the ethanol was measured using an alcohol thermometer. The ethanol was circulated using a motor driven stirrer. Constant sample temperature was recorded using a Honeywell Electronic 111 measuring -200°C to 50°C with a type T thermocouple (copper-constant wire) directly inserted into the cell solutions to be frozen (13, 17, 19, 21). The larger Dewar flask was filled with liquid nitrogen (LN_2), whose boiling point

is -195.8°C (15), into which the first Dewar flask was placed keeping the LN_2 at a constant level. A schematic drawing of the cooling apparatus is shown in Figure 1. This apparatus was adapted from Beeler (2).

Freezing Procedure: The previously described freezing media combinations were mixed together. Some tubes contained the media combinations without cells to determine the physical freezing parameters of the media. The thermocouple was placed into the solution of one of the tubes. The cells were then frozen either by spontaneous crystallization of the medium or seeding by touching the surface of the cell suspension in the anti-freeze bath with the tip of a Pasteur pipet containing frozen medium (22). When the temperatures of the samples and the ethanol bath were the same, the samples were transferred from the anti-freeze bath to the cooling ethanol bath. The seeded samples were cooled at approximately 1.0°C/min. to -45°C and the spontaneous crystallized samples to -65°C. Temperature endpoints of at least -45°C were chosen because water desorption terminates at about -40°C (2, 10). At the aforesaid temperature endpoints, samples were placed into either LN_2 vapor or liquid (2, 22) for 5 to 218 days.

Trophozoite and Cyst Thawing Procedure: Rapid thawing was achieved by agitation of the samples in a 37°C water-bath (2, 4, 8, 22). Just before the last ice crystals had melted, the samples were removed and placed on ice. The Me_2SO in samples that were frozen using BCW, glucose and FBS was then diluted drop-wise with 2 ml cold 20% glucose-BCW solution and mixed by vortexing gently, 2 ml cold FBS and vortexed, and then 4 ml 20% glucose-BCW solution and vortexed. The Me_2SO in samples that were frozen using DMEM + 5% FCS + 5% HS was diluted drop-wise with 8 ml of the media with serum (DMEM + 5% FCS + 5% HS) and vortexed. The samples in both cases were then centrifuged at 5000 rpm for 10 min. at 4°C (Beckman Model J-21 Centrifuge). Following centrifugation, sample media was decanted and the cells resuspended with 10 ml cold BCW.

The procedure was repeated for a third time, resuspended in 10 ml BCW and warmed to room temperature.

Tomite Thawing Procedure: Rapid thawing was achieved as previously described. The cell suspension was filtered using the technique described for tomite concentration. The filter containing the tomites was then placed in a watch-glass containing 3 ml BCW.

Trophozoite and Cyst Viability Assay: Upon warming to room temperature, the cells in 10 ml BCW were then assayed for viability. Into 5 ml disposable plastic tubes were placed; 100 µl 0.01 molar fluorescein diacetate (also known as diacetyl fluorescein), 2 ml BCW and 2 ml of the cell suspension. This mixture was then incubated in the dark for 20-30 min. The cells were then placed in depression slides and examined under an ultra-violet microscope. The ability of living cells to cleave the diacetate groups and fluoresce a green color was used as the viability criterion. Viable and total cell numbers were tallied and the percent viability determined.

Tomite Viability Assay: The contents of the watch-glass containing 3 ml BCW plus tomites were examined under a dissecting microscope (30 X magnification). Observation of motile tomites was used as the viability criterion since motility is required for a tomite to infect its host.

Toxicity Controls: Toxicity controls were conducted on trophozoite and cyst media components. Viability was based on the ability of the living cells to cleave the diacetyl groups from diacetyl fluorescein thus exhibiting a green fluorescence under ultra-violet radiation.

Me_2SO medium toxicity studies were conducted on tomite freezing media. Viability was based on the observation of tomite motility.

Diacetyl fluorescein toxicity on trophozoites and cysts was investigated in addition to studies on diacetyl fluorescein concentration and incubation time.

Results

Table 1 presents the physical parameters of the cell media used in this study under varying freezing conditions. A definite latent heat of fusion was recorded for seeding and spontaneous crystallization of all media. The change in temperature due to the latent heat of fusion was 3°C and 4°C for media A, 1.5°C and 5°C for media B, and 3°C and 9°C for media C for seeding and spontaneous crystallization, respectively. Cooling rates in the ethanol bath were between -1.05 and -1.45°C/min. Correlation coefficients ranging between -0.986 and -0.993 indicated a good straight line relationship where temperature was the ordinate and time the abscissa. Cooling rates in the LN_2 vapor or liquid were varied. LN_2 vapor cooling rates of -26.38°C/min. for media A and -28.60°C/min. for media B were close. LN_2 liquid cooling rates ranged from -275.93°C/min. up to -415.62°C/min. for media B. Media C exhibited the low cooling rate of -71.51°C/min. in LN_2 liquid presumably due to the media components. Thawing rates in a 37°C water-bath were high, ranging from 362.84°C/min. for media C to 579.82°C/min. for media B. Again all LN_2 cooling rates and media thawing rates exhibited a straight line relationship.

Table 2 presents data for the physical parameters and viability of trophozoites and cysts under varying freezing conditions. Media A resulted in 0% viability regardless of the cell storage time in LN_2 or the approximately 1°C/min. cooling rate. All cell samples were seeded and most samples exhibited a greater temperature change due to the latent heat of fusion than exhibited by that media without cells (Table 1). The pH values of the BCW ranged between 7.1 and 8.6 which were tolerated by the parasite and the fish host. The ethanol cooling rates and correlation coefficients were consistant with the values in Table 1. The LN_2 cooling rates were consistant with the value in Table 1 except for a high rate of -877.10°C/min. and a moderately low rate of -170.0°C/min. The accompanying correlation coefficients were

-0.379 and -0.893 indicating a very poor and a marginal straight line relationship, respectively. All other cooling rate correlation coefficients showed straight line relationships. No thawing rates were conducted for media A. Neither media B nor C exhibited any trophozoite or cyst viability. Media B exhibited a slower cooling rate for both ethanol and LN_2 than in Table 1, but cannot be compared under spontaneous crystallization. Media C-I showed a low LN_2 liquid cooling rate as depicted in Table 1.

Table 3 presents data concerning media component toxicity on trophozoites and cysts. These data indicate that no mortality was due to any of the media components. A 100% cell viability resulted for each assayed component.

Table 4 presents data of the toxicity of diacetyl fluorescein on trophozoites and cysts indicating no mortality was due to the diacetyl fluorescein concentration, volume or incubation time. A decrease in cell viability of 2.4% was found when using 10 µl of 0.0050 moles/l diacetyl fluorescein.

The physical parameters and viability of tomites under varying freezing conditions is presented in Table 5. Zero percent viability was found using various concentrations of Me_2SO with or without FBS, and for one sample without FBS and Me_2SO. The physical parameters were measured using media B components. A spontaneous crystallization temperature of -11°C was obtained as opposed to -6°C in Table 1. Slower ethanol and LN_2 cooling rates were measured with higher correlation coefficients. A thawing rate comparable to that in Table 1 was obtained.

Me_2SO medium toxicity on tomites was assayed in Table 6. These data indicated no mortality was due to BCW, 20% glucose in BCW, FBS or a 4.6% (v/v) final concentration of Me_2SO. Mortality occurred when Me_2SO was used at a final concentration of 9.2% (v/v).

Discussion

There have been may parasitic and nonparasitic organisms that have been

cryopreserved using the protocol: Me_2SO as the cryopreserving agent, approximately a 1°C/min. cooling rate followed by a rapid cooling in LN_2, and rapid warming at 37°C. Organisms that have been successfully cryopreserved using the aforesaid protocol are microfilariae of *Onchocerca gutturosa* (6), *Naegleria gruberi* and *N. fowleri* (18), *Didinium nasuterm* cysts (14), *Babesia rodhaini* (5), *Tetrahymena pyriformis* (7, 19), *Paramecium aurelia* (20) and *Ichthyophthirius multifiliis* (3).

The cryopreservation of *T. pyriformis* offers the possibility for freezing *I. multifiliis* tomites due to the size similarity of the two organisms. One author obtained a 13.5% viability of strain H and a 21.0% viability of strain W using a 10% (v/v) Me_2SO concentration and a cooling rate of 2°C/min. (7). Another author obtained an 85-90% viability based on motility and multiplication using a 20% (v/v) Me_2SO concentration and a cooling rate between 2.5-3.0°C/min. (19).

The cryopreservation of *P. aurelia* offers a similar size correlation to *I. multifiliis* trophozoites and cysts. A 16% viability was obtained for syngen 1 and a 1.3% viability for syngen 4 using a 15% (v/v) Me_2SO concentration and a cooling rate between 2.3°C/min. followed by immersion into LN_2 (20).

The cooling rates and Me_2SO concentrations previously described are suggested by Dalgliesh (4). Dalgliesh reviewed the theoretical and practical aspects of freezing parasitic protozoa and reported five general principles. 1) A cryoprotectant should be used. Me_2SO at a concentration of about 10% is recommended because it is an effective protectant well-tolerated by most protozoa. Me_2SO rapidly passes through the cell membrane, thereby eliminating the need for lengthy equilibration periods, and reducing possible osmotic shock. 2) Most parasites survive cooling rates of approximately 1°C/min. when a cryoprotectant is used. 3) The storage temperature should be as low as is practicable, and no higher than -60°C. Storage in LN_2 is recommended.

4) Thawing should be rapid, performed by agitating the frozen sample in a water-bath at about 40°C. 5) There is evidence to suggest that parasites thawed after frozen storage require a period for structural reconstitution before inoculation. Nevertheless, when Me_2SO is used, the period between thawing and inoculation should be kept to a minimum because toxicity to Me_2SO for some species has been demonstrated.

Beeler (2, 3) reported the successful cryopreservation of *I. multifiliis* trophozoites and cysts at a 60-65% viability. These optimal results were achieved using a 1°C/min. cooling rate obtained by the apparatus used in this study, a freezing medium consisting of 1 ml BCW + 0.25 ml 40% (v/v) Me_2SO in 20% (v/v) glucose-BCW solution + 0.75 ml FBS. Beeler determined cell viability using the diacetyl fluorescein assay described in this study. Enzymatic hydrolysis of the fluorogenic ester substrate diacetyl fluorescein results in the intracellular accumulation of the fluorescent product fluorescein in undamaged cells. There is free passage of the non-polar ester substrate into the cell where enzymatic hydrolysis removes the acetyl radicals thereby releasing fluorescein. Fluorescein emits a bright green color when radiated with light in the excitation wavelength range of 450-500 nm. Fluorescein is a polar compound and cannot readily escape the cell membrane unless the membrane is damaged (13, 16).

In a personal communication Beeler stated that glucose in BCW lowers the freezing temperature of BCW by raising the solute concentration. Me_2SO penetrates the cell and causes a step-wise dehydration to occur. As the temperature decreases, the Me_2SO concentration increases thus maintaining a gradient across the cell membrane thus drawing intracellular water out. BCW was used because the organisms appear to exist better in that medium. FBS is nonpenetrating and effects the solute concentration of the cell different from that of Me_2SO; how exactly is not known.

Upon repetition of Beeler's experiment (3) zero percent viability of *I. multifiliis* trophozoites and cysts were obtained (Table 2). Media component and diacetyl fluorescein toxicity studies were then conducted in the attempt to explain the above results. Table 3 and 4 present data showing 100% viability was obtained in those studies. Those results directly implicated the freezing process or medium as used was inadequate for the cryopreservation of trophozoites and cysts. The zero viability in Table 2 was based upon the absence of a green fluorescence using diacetyl fluorescein, an apparent cellular membrane disruption, and the absence of cytoplasmic streaming; all of which occur in live trophozoites and cysts. Other freezing media and conditions were investigated (Table 2) all resulting in zero viability.

The cryopreservation of *I. multifiliis* tomites was investigated using a variety of freezing media and recommended cooling and thawing rates (Table 5). Those studies resulted in zero viability. Me_2SO medium toxicity studies were then conducted. Based upon motility as the viability criterion a final Me_2SO concentration of 9.2% (v/v) was lethal to tomites. A 4.6% (v/v) final Me_2SO concentration was not lethal, nor were the other media components (Table 6). Those results also implicate the freezing process of media as inadequate for the successful cryopreservation of tomites.

Since the results in this study are in direct conflict with those obtained by Beeler (2, 3), further investigation is recommended. It is this author's opinion that a revised protocol include a 1°C/min. cooling rate followed by immersion into LN_2, and a rapid thawing of the cell suspension utilizing a 37°C water-bath. A constant vacuum in a Dewar flask as used in this study offers an excellent control over the cooling rate. A modification in the freezing medium is recommended. The use of BCW as the major portion of the freezing medium appears detrimental to the survival of the organisms.

Increasing the concentration of Me_2SO and buffering media (FBS, FCS, etc.) is recommended. The present author postulates the size of the trophozoite and cyst as a possible deterrent to successful cryopreservation. *Paramecium aurelia*, as previously presented, is comparable in size to *I. multifiliis* trophozoites and cysts. Viability of *P. aurelia* was 16% and 1.3% for syngens 1 and 4 respectively (20). The 60-65% viability of *I. multifiliis* trophozoites and cysts obtained by Beeler (2, 3) may have occurred due to an unawareness of a procedural change in freezing protocol or media preparation.

Future studies should incorporate the xenodiagnostic technique used for *Trypanosoma cruzi*. Exposure of naive hosts to thawed *I. multifiliis* (regardless of the viability assay conclusions) will further substantiate any viability results. The possibility exists that a few organisms may have survived the freezing protocol but when assayed appeared nonviable or escaped observation by the researcher.

Figure 1. Schematic drawing of the cooling apparatus.

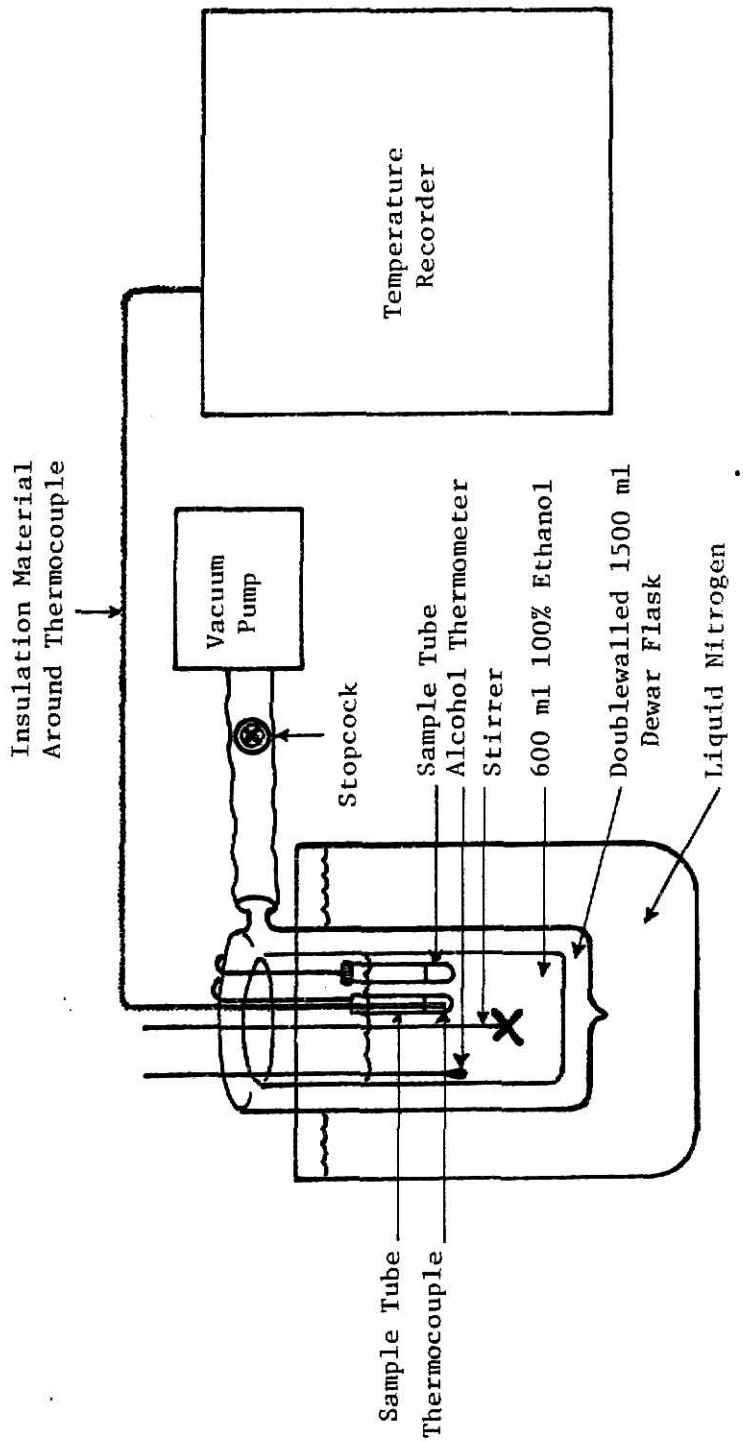
Temperature Recorder
Insulation Material Around Thermocouple
Vacuum Pump
Stopcock
Sample Tube
Alcohol Thermometer
Stirrer
600 ml 100% Ethanol
Doublewalled 1500 ml Dewar Flask
Liquid Nitrogen
Sample Tube
Thermocouple

Table 1. The Physical Parameters of the Cell Media Under Varying Freezing Conditions

Media With Serum	Seeding °C	Spontaneous Crystallization °C	Temperature at Latent Heat of Fusion °C	Temperature Endpoint °C	Cooling Rates °C/min. Ethanol	Cooling Rates °C/min. LN_2	Thawing Rate °C/min.
A	NA	-5	-1	-46	-1.31 r= -0.989	- 26.38(v) - 0.997	+404.10 + 0.976
A	-4	NA	-1	-45	-1.05 r= -0.992	-341.10(1) - 0.984	+578.08 + 0.978
B	NA	-6	-1	-45	-1.31 r= -0.986	-415.62(1) - 0.953	+579.82 + 0.990
B	-2	NA	-0.5	-45	-1.23 r= -0.992	-275.93(1) - 0.999	+535.96 + 0.997
B	-4	NA	-1	-44	-1.09 r= -0.993	- 28.60(v) - 0.988	+509.31 + 0.989
C	NA	-15	-6	-65	-1.45 r= -0.992	- 71.51(1) - 0.961	+362.84 + 0.983

A = 1 ml BCW plus 0.25 ml (40%) Me_2SO in 20% glucose - BCW solution plus 0.75 ml FBS
B = 1 ml BCW plus 0.23 ml (40%) Me_2SO in 20% glucose - BCW solution plus 0.77 ml FBS
C = 1 ml DMEM + 5% FCS + 5% HS plus 1 ml DMEM + 5% FCS + 5% HS + 5% Me_2SO
r = correlation coefficient
(1) = liquid
(v) = vapor
NA = not applicable

Table 2. The Physical Parameters and Viability of *I. multifiliis* Trophozoites and Cysts Under Varying Freezing Conditions.

Media With Serum	Seeding °C	Spontaneous Crystallization °C	Temperature at Latent Heat of Fusion °C	Temperature Endpoint °C	Time Frozen (days)	Cooling Rates °C/min. Ethanol	Cooling Rates °C/min. LN_2	Thawing Rate °C/min.	BCW pH	%* viability
A	-6	NA	-2.0	-45.0	28	-1.21	-877.10(1)	ND	7.5	0
						r= -0.998	- 0.379			
A	-6	NA	-1.0	-43.0	30	-1.20	-170.0(1)	ND	7.4	0
						r= -0.997	- 0.893			
A	-4	NA	-2.0	-46.0	198	-1.24	-379.48(1)	ND	8.1	0
						r= -0.996	- 0.986			
A	-7	NA	-2.5	-46.0	201	-1.14	-326.76(1)	ND	7.1	0
						r= -0.997	- 0.992			
A	-2	NA	-1.0	-45.5	201	-1.22	-276.34(1)	ND	8.3	0
						r= -0.997	- 0.985			
A	-6	NA	-2.0	- 4.0	218	ND	-209.74(1)	ND	8.2	0
							r= - 0.995			
B	NA	-6	-1.0	-70.0	5	-0.885	-238.21(1)	+504.50	8.0	0
						r= -0.990	- 0.954	+ 0.994		
C	-5	NA	ND	ND	41	ND	ND	ND	8.6	0
C-I	-6	NA	-3.0	-48.0	54	-1.06	- 98.92(1)	+496.78	8.6	0
						r= -0.996	- 0.977	+ 0.992		

A = 1 ml BCW + cells plus 0.25 ml (40%) Me_2SO in 20% glucose - BCW solution plus 0.75 ml FBS
B = 1 ml BCW + cells plus 0.23 ml (40%) Me_2SO in 20% glucose - BCW solution plus 0.77 ml FBS
C = 1 ml DMEM + 5% FCS + 5% HS + cells plus 1 ml DMEM + 5% FCS + 5% HS + 5% Me_2SO
D = 1 ml DMEM + 5% FCS + 5% HS + cells plus 0.5 ml DMEM + 5% FCS + 5% HS + 0.5 ml Me_2SO (25%)
E = 1 ml DMEM + 5% FCS + 5% HS + cells plus 0.6 ml DMEM + 5% FCS + 5% HS + 0.4 ml Me_2SO (20%)
F = 1 ml DMEM + 5% FCS + 5% HS + cells plus 0.7 ml DMEM + 5% FCS + 5% HS + 0.3 ml Me_2SO (15%)
G = 1 ml DMEM + 5% FCS + 5% HS + cells plus 0.8 ml DMEM + 5% FCS + 5% HS + 0.2 ml Me_2SO (10%)
H = 1 ml DMEM + 5% FCS + 5% HS + cells plus 0.9 ml DMEM + 5% FCS + 5% HS + 0.1 ml Me_2SO (5%)
I = 1 ml DMEM + 5% FCS + 5% HS + cells plus 0.95 ml DMEM + 5% FCS + 5% HS + 0.05 ml Me_2SO (2.5%)
r = correlation coefficient
(1) - liquid
NA = not applicable; ND = not done
* = 100 µl diacetylfluorescein + 2 ml BCW + BCW with cells incubated 30 min in dark; cells examined under ultraviolet scope for green fluorescence.

Table 3. Media Component Toxicity on *I. multifiliis* Trophozoites and Cysts

BCW + cells (ml)	BCW (ml)	FBS (ml)	Me_2SO (40% v/v) in 20% (v/v) Glucose with BCW (ml)	20% (v/v) Glucose with BCW (ml)	Cell Count		% Viability
					Viable[a]	Total	
1.0	1.0	-	-	-	113	113	100
1.0	-	0.75	0.25	-	157	157	100
1.0	0.25	0.75	-	-	103	103	100
1.0	0.75	-	0.25	-	81	81	100
1.0[b]	1.0	-	-	-	97	97	100
1.0	0.75	-	-	0.25	111	111	100

[a] 100 µl diacetylfluorescein + 2 ml BCW + 2 ml BCW with cells incubated 30 min. in dark, cells examined under ultra-violet scope for green fluorescence

[b] mixture vortexed to determine any effect

Table 4. Diacetylfluorescein Toxicity on *I. multifiliis* Trophozoites and Cysts

Weight (g)	moles/l	Volume[a] Used (µl)	Incubation Time (min.)	Cell Count		% Viability
				Viable[b]	Total	
0.41639	0.0100	10	20	28	28	100.0
0.20819	0.0050	10	30	37	38	97.4
0.10410	0.0025	100	30	43	43	100.0

Diacetylfluorescein molecular weight = 416.39 g/mole

[a]Mixed with 2 ml BCW plus 2 ml BCW + cells

[b]Viability is based on the observation of green fluorescence by cells examined with a ultra-violet microscope

Table 5. The Physical Parameters* and Viability of *I. multifiliis* Tomites Under Varying Freezing Conditions

Media	Spontaneous Crystallization °C	Temperature at Latent Heat of Fusion °C	Temperature Endpoint °C	Time Frozen (days)	Cooling Rates °C/min. Ethanol	Cooling Rates °C/min. LN_2	Thawing Rate °C/min	% Viability
A-E	-11	-4	-60	8	-0.984 r= -0.990	-13.36(v) - 0.992	+537.06 + 0.994	0

A = 1 ml BCW + cells plus 1 ml 20% glucose in BCW.
B = 1 ml BCW + cells plus 0.23 ml Me_2SO (4.6% final v/v) plus 0.77 ml 20% glucose in BCW.
C = 1 ml BCW + cells plus 0.46 ml Me_2SO (9.2% final v/v) plus 0.54 ml 20% glucose in BCW.
D = 1 ml BCW + cells plus 0.23 ml Me_2SO (4.6% final v/v) plus 0.77 ml FBS.
E = 1 ml BCW + cells plus 0.46 ml Me_2SO (9.2% final v/v) plus 0.54 ml FBS.
* = as measured using media B components

Table 6. Me_2SO-Medium Toxicity on *I. multifiliis* Tomites

Media	Motility*
A	+
B	+
C	-
D	+
E	-

A = 1 ml BCW + cells plus 1 ml 20% glucose in BCW
B = 1 ml BCW + cells plus 0.23 ml Me_2SO (4.6% final v/v) plus 0.77 ml 20% glucose in BCW.
C = 1 ml BCW + cells plus 0.46 ml Me_2SO (9.2% final v/v) plus 0.54 ml 20% glucose in BCW.
D = 1 ml BCW + cells plus 0.23 ml Me_2SO (4.6% final v/v) plus 0.77 ml FBS
E = 1 ml BCW + cells plus 0.46 ml Me_2SO (9.2% final v/v) plus 0.54 ml FBS
+ = motility detected
- = no motility detected
* = time span of 5 min before observation

References

1. Ashwood-Smith, M. J. Low temperature preservation of mouse lymphocytes with dimethyl sulfoxide. Blood 23, 494-501 (1964).

2. Beeler, C. R. Controlling cooling rates with a variable vacuum in a Dewar flask. Cryobiology 18, 79-81 (1981).

3. Beeler, C. R. Studies on *Ichthyophthirius multifiliis*, freezing apparatus, cryopreservation, laboratory culture, and interspecific transfer. Masters Thesis, Kansas State University, Manhattan, Kansas. 67 pages (1981).

4. Dalgliesh, R. J. Theoretical and practical aspects of freezing parasitic protozoa. Aust. Vet. J. 48, 233-239 (1972).

5. Dalgliesh, R. J., Mellors, L. T., and Blight, G. W. Comparisons of glucose, sucrose, and dimethyl sulfoxide as cryoprotective agents for *Babesia rodhaini*, with estimates for survival rates. Cryobiology 17, 410-417 (1980).

6. Ham, P. J., James, E. R., and Bianco, A. E. *Onchocerca* spp: cryopreservation of microfilariae and subsequent development in the insect host. Exptl. Para. 47, 384-391 (1979).

7. Hwang, S., Davis, E. E., and Alexander, M. T. Freezing and viability of *Tetrahymena pyriformis* in dimethyl sulfoxide. Science 144, 64-65 (1964).

8. Karpovitch, X. L., Rosenkovitch, E., Ben-Basset, H., and Izak, G. Structure and functional alterations in lymphocytes induced by cryopreservation. Cryobiology 17, 12-17 (1980).

9. Kozel, T., and Dobra, K. W. Rapid vacuum method for removal of mucus from the epidermis of fishes. Progr. Fish Cult. 14, 18-19 (1979).

10. Litvan, G. G. Mechanism of cryoinjury in biological systems. Cryobiology 9, 182-191 (1972).

11. Lovelock, J. W., and Bishop, M. W. H. Prevention of freezing damage to living cells by dimethyl sulfoxide. Nature (London) 183, 1394 (1959).

12. Mazur, P. Cryobiology: The freezing of biological systems. Science 168, 939-949 (1970).

13. McGrath, J. J., Cravalho, E. G., and Huggins, C. E. An experimental comparison of intracellular ice formation and freeze-thaw survival of Hela S-3 cells. Cryobiology 12, 540-550 (1975).

14. McGrath, M. S., Daggett, P-M., and Nerad, T. A. Studies on the preservation of the ciliate *Didinum nasutum*. Trans. Am. Micro. Soc. 96, 519-525 (1977).

15. Pegg, D. E., Hayes, A. R., and Kingston, R. E. Cooling equipment for use in cryopreservation. Cryobiology 10, 271-281 (1973).

16. Rotman, B., and Papermaster, B. W. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. PNAS. U.S.A. 55, 134-141 (1966).

17. Schwabe, K. G., and Terracio, L. Ultrastructural and thermocouple evaluation of rapid freezing techniques. Cryobiology 17, 571-584 (1980).

18. Simione, F. B., and Daggett, P. Freeze preservation of pathogenic and nonpathogenic *Naegleria* species. J. Parasitol. 62, 49 (1976).

19. Simon, E. M. Freezing and storage in liquid nitrogen of axenically and monoxenically cultivated *Tetrahymena pyriformis*. Cryobiology 9, 75-81 (1972).

20. Simon, E. M., and Schneller, M. V. The preservation of ciliated protozoa at low temperature. Cryobiology 10, 421-426 (1973).

21. Tate, E. H., and Cram, L. S. A method for simultaneous isolated and cryopreservation of bovine lymphocytes. Cryobiology 19, 136-146 (1982).

22. Thorpe, P. E., Knight, S. C., and Farrant, J. Optimal conditions for the preservation of mouse lymph node cells in liquid nitrogen using cooling rate techniques. Cryobiology 13, 126-133 (1976).

CRYOPRESERVATION OF *GIARDIA LAMBLIA* USING DIMETHYL SULFOXIDE AND A CONSTANT VACUUM IN A DEWAR FLASK

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Summary

This study examined the effect of varying freezing conditions on the human intestinal parasite *Giardia lamblia* (Portland-1 strain) using a constant vacuum in a Dewar flask and an ethanol bath to regulate the freezing rate. The cryopreservation of the trophozoite stage was investigated. A variety of freezing media and procedures were studied. Dimethyl sulfoxide (Me_2SO) was the cryoprotective agent of choice. Me_2SO toxicity assays were conducted on those concentrations used in the freezing protocol. The results of this study indicated a 6.5% (v/v) Me_2SO concentration yields a 90% survival based upon organism motility. A 30.9% cell viability was obtained by freezing without a cryoprotective agent. Recommendations are offered concerning alternate viability criteria.

Dimethyl sulfoxide (Me_2SO) was first introduced as a low temperature cryopreservative agent for mammalian cells by Lovelock and Bishop (1, 13, 14). Since that time this chemical has been used successively to cryopreserve a variety of cell types utilizing varying freezing techniques. The present study utilized several freezing conditions; a constant vacuum in a Dewar flask, and an ethanol bath controlling the freezing rate to investigate their effects on the viability of *Giardia lamblia*.

Materials and Methods

Source of *G. lamblia* Trophozoites: *G. lamblia* trophozoites (Portland-1 strain) were obtained from the laboratory of Dr. L. Diamond through Dr. G. Marchin (Kansas State University, Manhattan, KS). This particular strain was originally axenized by Dr. E. Meyer.

G. lamblia Trophozoite Culture Medium: The TPS-1 (tryptone panmede serum) media used to culture *G. lamblia* trophozoites was developed by Diamond to culture *Entamoeba histolytica* (7). The TPS-1 media contained trypticase, panmede (ox-liver digest), glutamine, cysteine, ascorbic acid, and salts supplemented with fetal calf serum complement inactivated at 56°C for 30 min. and vitamin mixture 107. The TPS-1 media was prepared by Dr. Marchin according to Diamond (7). The culture media was sterilized by passage through a 0.2 µm millipore filter. The sterilized media was inoculated with purified *G. lamblia* trophozoites in 20 ml screw-cap tubes and incubated at 37°C. Trophozoites are known to divide by binary fission and grow exponentially. Progress of trophozoite growth was followed by microscopic examination of the screw-cap glass tube wall. The presence of trophozoites attached to the glass tube wall indicated viable organisms. Dr. Marchin's laboratory cryopreserved cultured trophozoites by mixing 0.7 ml glycerol

and 9.3 ml of a culture of *G. lamblia* containing approximately 1.2 x 10^6 organisms per ml. After mixing, 1.6 ml aliquots were distributed to 16 x 125 mm screw-cap pyrex culture tubes. Tubes were placed in a Haake refrigerated water bath containing 95% ethanol at room temperature. At approximately -14°C the cultures were removed and incubated overnight in a -20°C freezer. The next morning cultures were placed at -70°C for long term storage (17). Meyer and Chadd originally reported consistently less than 2% of the protozoa initially present survived using the aforesaid protocol (17). The remaining organisms are used to repopulate new culture tubes.

G. lamblia trophozoites used in this study were previously cryopreserved in Dr. Marchin's laboratory. The cells were thawed in 37°C water-bath, 1.5 ml cell inoculum mixed with thawed sterile TPS-1 media, and incubated at 37°C for four days.

Freezing *G. lamblia* Trophozoites: Various combinations and concentrations of TPS-1 media and Me_2SO were investigated. Into 20 ml screw-cap tubes were placed: A) 1.50 ml TPS-1 media with cells plus 0.50 ml Me_2SO (a final Me_2SO concentration of 25.0%); B) 1.75 ml TPS-1 media with cells plus 0.25 ml Me_2SO (a final Me_2SO concentration of 12.5%); C) 1.80 ml TPS-1 media with cells plus 0.20 ml Me_2SO (a final Me_2SO concentration of 10.0%); D) 1.87 ml TPS-1 media with cells plus 0.13 ml Me_2SO (a final Me_2SO concentration of 6.5%); E) 1.94 ml TPS-1 media with cells plus 0.06 ml Me_2SO (a final Me_2SO concentration of 3.0%); F) 1.97 ml TPS-1 media with cells plus 0.03 ml Me_2SO (a final concentration of 1.5%); G) 2.00 ml TPS-1 media with cells.

Freezing Equipment: The freezing equipment used in this study consisted of a 1500 ml Dewar flask filled with 600 ml 100% ethanol (2, 18). A vacuum

was drawn in the Dewar flask measured at 23.75" Hg for 1-5 tubes or 23.0" Hg for 6-10 tubes being frozen. This constant pressure gave a freezing rate of approximately 1.0°C/min. The temperature of the ethanol was measured using an alcohol thermometer. The ethanol was circulated using a motor driven stirrer. Constant sample temperature was recorded using a Honeywell Electronic 111 measuring -200°C to 50°C with a type T thermocouple (copper-constant wire) directly inserted into the cell solutions to be frozen (15, 20, 22, 24). The larger Dewar flask was filled with liquid nitrogen (LN_2), whose boiling point is -195.8°C (18), into which the first Dewar flask was placed keeping the LN_2 at a constant level. A schematic drawing of the cooling apparatus is shown in Figure 1. This apparatus was adapted from Beeler (2).

Freezing Procedure: The previously described freezing media combinations were mixed together and placed on crushed ice. The thermocouple was placed into the solution of one of the tubes. The cells were then frozen by spontaneous crystallization of the sample media. When the temperature of the samples and the ethanol bath were the same, the samples were transferred from the crushed ice to the cooling ethanol bath. The samples were cooled at approximately 1.0°C/min. to -60.0°C for media A and -40.0°C for media B-G. Temperature endpoints of at least -40°C were chosen because water desorption terminates at about -40°C (2, 12). At the aforesaid temperature endpoints, samples were placed into LN_2 (2, 25) for three hours.

Trophozoite Thawing Procedure: Rapid thawing was achieved by agitation of the samples in a 37°C water-bath (2, 4, 11, 25). Just before the last ice crystals had melted, the samples were removed and warmed to room temperature.

Trophozoite Viability Assay: Upon warming to room temperature, the cells were assayed for viability. Three viability criteria were investigated. Trypan blue exclusion (11, 24, 25) proved futile as both motile and non-motile cells excluded the dye. Fluorescein diacetate (also known as diacetyl fluorescein) also proved futile (2, 15, 19). Into 5 ml disposable plastic tubeswere placed 100 µl 0.01 molar fluorescein diacetate, 2 ml TPS-1 media and 2 ml of the cell suspension. This mixture was incubated in the dark for 20-30 min. The cells were placed on slides and examined under an ultra-violet microscope. The ability of living cells to cleave the diacetate groups and fluoresce a green color was used as this assay's viability criterion. No fluorescence was observed from either motile or nonmotile cells. Therefore, the viability criterion as used in this study was the microscopic observation of gross organismic and/or flagellar movement of individual trophozoites. Enumeration of trophozoites was conducted using a hemocytometer (8).

Me_2SO Toxicity Assay: The toxic effect of Me_2SO was investigated at concentrations used in this study. Media A-G were prepared, allowed to stand in an ice slush for 5 min. followed by a 5 min. incubation at room temperature. Trophozoite viability was determined as previously reported.

Results

Table 1 presents the physical parameters of *G. lamblia* media plus trophozoites under varying freezing conditions. The temperature at which spontaneous crystallization occurred increased as the concentration of Me_2SO in the media decreased. A definite latent heat of fusion was recorded for crystallization of all media. The change in temperature due to the latent heat of fusion was 5°C for media A, 5.2°C for media E, 6.0°C for

media B, C and G, 6.5°C for media F and 8.5°C for media D. Cooling rates in the ethanol bath ranged between -0.848 and -1.45°C/min. Correlation coefficients ranging between -0.975 and -0.991 indicated a good straight line relationship where temperature was the ordinate and time the abscissa. Cooling rates in the LN_2 were varied, ranging from -222.69 to -331.99°C/min. The accompanying correlation coefficients ranged between -0.936 to -0.993 indicating a good straight line relationship. Thawing rates in a 37°C water-bath were high, ranging from 383.66 to 569.96°C/min. Again all thawing rates exhibited a straight line relationship as indicated by the 0.960 to 0.999 correlation coefficients.

Table 2 exhibits data on the viability of *G. lamblia* trophozoites following different freezing conditions as reported in Table 1. The average percent viability before Me_2SO was ascertained as was the percent viability after thawing. In general, the percent viability after thawing fluctuated between 51.2 for 10.0% (v/v) Me_2SO and 73.0% for 12.5% (v/v) Me_2SO. Percent viabilities of 53.6, 63.4 and 64.7 were reported for 25.0, 3.0 and 1.5% (v/v) Me_2SO, respectively. The highest percent viability of 89.2 was achieved using 6.5% (v/v) Me_2SO. When no Me_2SO was used, the lowest percent viability of 30.9 was achieved. The gross percent viability (thawing percent viability divided by percent viability before Me_2SO multiplied by 100) showed 97.3% of the trophozoites survived freezing using 6.5 and 12.5% (v/v) Me_2SO in media B and D. A 33.7% gross viability was obtained using no Me_2SO.

Table 3 presents Me_2SO toxicity data at the concentrations used in this study. A concentration of 25.0% (v/v) Me_2SO rendered 20.0% of the trophozoites immotile, and 12.5% (v/v) Me_2SO rendered 3.0% of the trophozoites immotile. As the concentration of Me_2SO decreased in media C-F, the percent of trophozoites rendered immotile decreased. A 6.7% immotility value was reported for media G into which no Me_2SO was mixed.

Discussion

There have been many parasitic and nonparasitic organisms that have been cryopreserved using the protocol: Me_2SO as the cryopreserving agent, approximately a 1.0°C/min. cooling rate followed by rapid cooling in LN_2, and rapid warming at 37°C. Organisms that have been successfully cryopreserved using the aforesaid protocol are microfilariae of *Onchocerca gutturosa* (9), *Naegleria gruberi* and *N. fowleri* (21), *Didinium nasuterm* cysts (16), *Babesia rodhaini* (5), *Tetrahymena pyriformis* (10, 22), *Paramecium aurelia* (23), *Ichthyophthirius multifiliis* (3) and *Giardia lamblia* (17).

The cooling rates and Me_2SO used in this study were recommended by Dalgliesh (4). Dalgliesh reviewed the theoretical and practical aspects of freezing parasitic protozoa and reported five general principles. 1) A cryoprotectant should be used. Me_2SO at a concentration of about 10% is recommended because it is an effective protectant well-tolerated by most protozoa. Me_2SO rapidly passes through the cell membrane, thereby eliminating the need for lengthy equilibration periods, and reducing possible osmotic shock. 2) Most parasites survive cooling rates of approximately 1°C/min. when a cryoprotectant is used. 3) The storage temperature should be as low as is practicable, and no higher than -60°C. Storage in LN_2 is recommended. 4) Thawing should be rapid, performed by agitating the frozen sample in a water-bath at about 40°C. 5) There is evidence to suggest that parasites thawed after frozen storage require a period for structural reconstitution before inoculation. Nevertheless, when Me_2SO is used, the period between thawing and inoculation should be kept to a minimum because toxicity to Me_2SO for some species has been demonstrated.

This study was conducted to verify the cryoprotective properties of Me_2SO and improve upon the results reported by Meyer (17). Meyer obtained

consistently less than 2% of the trophozoites initially frozen using Me_2SO percentiles of 1, 3, 5, 7, and 9 (v/v). He reported no viable *Giardia* were recovered from cultures frozen without Me_2SO. Meyer further reported all the Me_2SO concentrations he used were toxic to the trophozoites within 5 days. A Me_2SO concentration of 3.5% (v/v) killed more than 99% of the trophozoites in 2 days. Meyer recommends immediate Me_2SO dilution following thawing.

Results in this study indicated a 97.3% gross viability (73.0% and 89.2% post-thaw viability, respectively) for media B and D. Both media possessed similar temperatures for spontaneous crystallization, -14.0°C for media B and -13.8°C for media D. Similar ethanol cooling rates of -1.15 and -1.19°C/min. for media B and D, respectively were present. Media B possessed 12.5% (v/v) Me_2SO while media D possessed 6.5% (v/v) Me_2SO. One might expect the percent viability for cells in media C to be similar to those in media B and D due to the physical parameters exhibited by media C: 10.0% (v/v) Me_2SO, a spontaneous crystallization temperature of -12.0°C, and an ethanol cooling rate of -1.20°C/min. Media C showed a post-thaw viability of 51.2% and a gross viability of 55.8%. Table 2 presents a trend of increased cell survival as Me_2SO concentration decreased from 12.5% to 6.5% followed by a decrease in cell survival with continued decreasing Me_2SO concentrations. On the basis of this study, this author recommends a Me_2SO concentration of 6.5% (v/v), and ethanol cooling rate of -1.19°C/min., a rapid freezing rate in LN_2 and thawing rate in a 37°C water-bath as optimal conditions for cryopreserving *G. lamblia* trophozoites.

This study differs with those results reported by Meyer (17) in that Me_2SO is a superior cryoprotective agent at a concentration of 6.5% yielding approximately 90% viable organisms. Freezing without Me_2SO resulted in poor trophozoite survival (30.9%), but survival just the same. This study

corroborates Meyer's results on Me_2SO toxicity and recommends immediate Me_2SO dilution using sterile culture media.

An alternate viability criterion is necessary other than motility. The lack of motility in this organism does not indicate mortality. Within the host (man and animal), *G. lamblia* lives attached by the adhesive disc to the epithelial surface of the duodenum, jejunum, and upper ileum. Several organisms may have been attached to the surface of the hemocytometer thus exhibiting no motility and were regarded as nonviable. Gillin and Diamond (8) have developed a semi-solid agarose medium that permits clonal growth of *G. lamblia*. The reported percent colony forming efficiency was between 20-40%. The use of other dye exclusion or metabolic assays may prove fruitful. The use of the most probable number technique (95% confidence limits) may prove useful in obtaining an approximation of organism survival following cryopreservation (6).

Figure 1. Schematic drawing of the cooling apparatus.

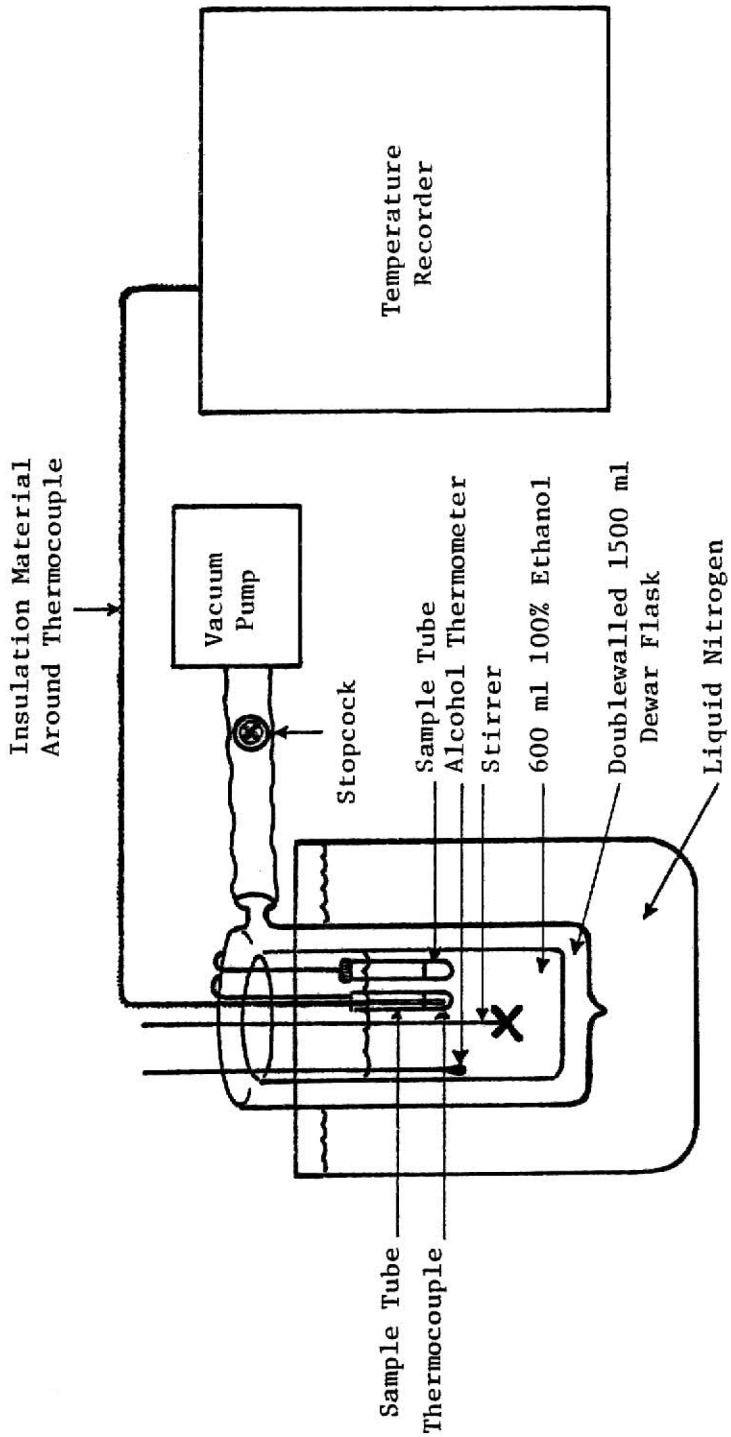
Temperature Recorder
Insulation Material Around Thermocouple
Vacuum Pump
Stopcock
Sample Tube
Alcohol Thermometer
Stirrer
600 ml 100% Ethanol
Doublewalled 1500 ml Dewar Flask
Liquid Nitrogen
Sample Tube
Thermocouple

Table 1. The Physical Parameters of *Giardia lamblia* Media Plus Trophozoites Under Varying Freezing Conditions.

Media With Me_2SO	Spontaneous Crystallization °C	Temperature at Latent Heat of Fusion °C	Temperature Endpoint °C	Time Frozen (hours)	Cooling Rates °C/min. Ethanol	Cooling Rates °C/min. LN_2	Thawing Rate °C/min
A	-25.0	-20.0	-60.0	3	-0.848	-240.30	+441.09
					r= -0.991	- 0.993	+ 0.985
B	-14.0	- 8.0	-40.0	3	-1.15	-229.98	+383.66
					r= -0.986	- 0.936	+ 0.960
C	-12.0	- 6.0	-40.0	3	-1.20	-222.69	+529.92
					r= -0.990	- 0.993	+ 0.999
D	-13.8	- 5.3	-40.0	3	-1.19	-257.39	+541.18
					r= -0.975	- 0.988	+ 0.995
E	- 8.2	- 3.0	-40.0	3	-1.18	-331.99	+477.87
					r= -0.988	- 0.976	+ 0.962
F	- 9.0	- 2.5	-40.0	3	-1.23	-324.99	+569.96
					r= -0.987	- 0.975	+ 0.971
G	- 8.0	- 2.0	-40.0	3	-1.45	-269.56	+512.48
					r= -0.983	- 0.973	+ 0.991

A = 1.50 ml TPS-1 media with cells + 0.50 ml Me_2SO (25.0% v/v)
B = 1.75 ml TPS-1 media with cells + 0.25 ml Me_2SO (12.5% v/v)
C = 1.80 ml TPS-1 media with cells + 0.20 ml Me_2SO (10.0% v/v)
D = 1.87 ml TPS-1 media with cells + 0.13 ml Me_2SO (6.5% v/v)
E = 1.94 ml TPS-1 media with cells + 0.06 ml Me_2SO (3.0% v/v)
F = 1.97 ml TPS-1 media with cells + 0.03 ml Me_2SO (1.5% v/v)
G = 2.00 ml TPS-1 media with cells
r = correlation coefficient

Table 2. Viability* of *Giardia lamblia* Trophozoites Following Different Freezing Conditions.

Media With Me_2SO	Thawing: Average Cell Count Viable	Thawing: Average Cell Count Total	Thawing: % Viability	Average % Viability Before Me_2SO	Gross† % viability
A	8.03	14.97	53.6	100.0	53.6
B	7.18	9.83	73.0	75.0	97.3
C	5.60	10.80	51.2	91.7	55.8
D	8.25	9.25	89.2	91.7	97.3
E	6.50	10.25	63.4	91.7	69.1
F	8.25	12.75	64.7	91.7	70.6
G	5.25	17.00	30.9	91.7	33.7

A = 1.50 ml TPS-1 media with cells + 0.50 ml Me_2SO (25.0% v/v)
B = 1.75 ml TPS-1 media with cells + 0.25 ml Me_2SO (12.5% v/v)
C = 1.80 ml TPS-1 media with cells + 0.20 ml Me_2SO (10.0% v/v)
D = 1.87 ml TPS-1 media with cells + 0.13 ml Me_2SO (6.5% v/v)
E = 1.94 ml TPS-1 media with cells + 0.06 ml Me_2SO (3.0% v/v)
F = 1.97 ml TPS-1 media with cells + 0.03 ml Me_2SO (1.5% v/v)
G = 2.00 ml TPS-1 media with cells
* = based upon microscopic observation of gross organismic and/or flagellar movement
† = thawing % viability ÷ % viability before Me_2SO x 100

Table 3. Viability* of *Giardia lamblia* Trophozoites Following Treatment With Varying Me_2SO Concentrations†.

Media With Me_2SO	% Me_2SO (v/v)	Average Cell Count		% Viability	Average % Viability Before Me_2SO
		Viable	Total		
A	25.0	6.0	7.5	80.0	100.0
B	12.5	8.0	3.0	72.0	75.0
C	10.0	14.0	19.0	73.7	91.7
D	6.5	12.0	14.0	85.7	91.7
E	3.0	13.0	14.0	92.8	91.7
F	1.5	10.0	10.0	100.0	91.7
G	0.0	17.0	20.0	85.0	91.7

A = 1.50 ml TPS-1 media with cells + 0.50 ml Me_2SO
B = 1.75 ml TPS-1 media with cells + 0.25 ml Me_2SO
C = 1.80 ml TPS-1 media with cells + 0.20 ml Me_2SO
D = 1.87 ml TPS-1 media with cells + 0.13 ml Me_2SO
E = 1.94 ml TPS-1 media with cells + 0.06 ml Me_2SO
F = 1.97 ml TPS-1 media with cells + 0.03 ml Me_2SO
G = 2.00 ml TPS-1 media with cells
* = based upon microscopic observation of gross organismic and/or flagellar movement
† = 5 min in ice slush followed by 5 min. at room temperature

References

1. Ashwood-Smith, M. J. Low temperatures preservation of mouse lymphocytes with dimethyl sulfoxide. Blood 23, 494-501 (1964).

2. Beeler, C. R. Controlling cooling rates with a variable vacuum in a Dewar flask. Cryobiology 18, 79-81 (1981).

3. Beeler, C. R. Studies on *Ichthyophthirius multifiliis*, freezing apparatus, cryopreservation, laboratory culture, and interspecific transfer. Masters Thesis, Kansas State University, Manhattan, Kansas. 67 pages (1981).

4. Dalgliesh, R. J. Theoretical and practical aspects of freezing parasitic protozoa. Aust. Vet. J. 48, 233-239 (1972).

5. Dalgliesh, R. J., Mellors, L. T., and Blight, G. W. Comparisons of glucose, sucrose, and dimethyl sulfoxide as cryoprotective agents for *Babesia rodhaini*, with estimates for survival rates. Cryobiology 17, 410-417 (1980).

6. Diebel, R. H., and Lindquist, J. A. General Food Microbiology Laboratory Manual. University of Wisconsin, Madison, Wisconsin. Burgess Publishing Company, Minneapolis, Minnesota. pp. 7-9 (1981).

7. Diamond, L. S. Techniques of axenic cultivation of *Entamoeba histolytica* Schaudinn, 1903 and *E. histolytica*-like amoeba. J. Parasitol. 54, 1047-1056 (1968).

8. Gillin, F. D., and Diamond, L. S. Clonal growth of *Giardia lamblia* trophozoites in a semisolid agarose medium. J. Parasitol. 66, 350-352 (1980).

9. Ham, P. J., James, E. R., and Bianco, A. E. *Onchocerca* spp: cryopreservation of microfilariae and subsequent development in the insect host. Exptl. Para. 47, 384-391 (1979).

10. Hwang, S., Davis, E. E., and Alexander, M. T. Freezing and viability of *Tetrahymena pyriformis* in dimethyl sulfoxide. Science 144, 64-65 (1964).

11. Karpovitch, X. L., Rosenkovitch, E., Ben-Basset, H., and Izak, G. Structural and functional alterations in lymphocytes induced by cryopreservation. Cryobiology 17, 12-17 (1980).

12. Litvan, G. G. Mechanism of cryoinjury in biological systems. Cryobiology 9, 182-191 (1972).

13. Lovelock, J. W., and Bishop, M. W. H. Prevention of freezing damage to living cells by dimethyl sulfoxide. Nature (London) 183, 1394 (1959).

14. Mazur, P. Cryobiology: The freezing of biological systems. Science 168, 939-949 (1970).

15. McGrath, J. J., Cravalho, E. G., and Huggins, C. E. An experimental comparison of intracellular ice formation and freeze-thaw survival of Hela S-3 cells. Cryobiology 12, 540-550 (1975).

16. McGrath, M. S., Daggett, P-M., and Nerad, T. A. Studies on the preservation of the ciliate *Didinium nasutum*. Trans. Am. Micro. Soc. 96, 519-525 (1977).

17. Meyer, E. A., and Chadd, J. A. Preservation of *Giardia* trophozoites by freezing. J. Parasitol. 53, 1108-1109 (1965).

18. Pegg, D. E., Hayes, A. R., and Kingston, R. E. Cooling equipment for use in cryopreservation. Cryobiology 10, 271-281 (1973).

19. Rotman, B., and Papermaster, B. W. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. PNAS. U.S.A. 55, 134-141 (1966).

20. Schwabe, K. G., and Terracio, L. Ultrastructural and thermocouple evaluation of rapid freezing techniques. Cryobiology 17, 571-584 (1980).

21. Simione, F. P., and Daggett, P. Freeze preservation of pathogenic and nonpathogenic *Naegleria* species. J. Parasitol. 62, 49 (1976).

22. Simon, E. M. Freezing and storage in liquid nitrogen of axenically and monoxenically cultivated *Tetrahymena pyriformis*. Cryobiology 9, 75-81 (1972).

23. Simon, E. M., and Schneller, M. V. The preservation of ciliated protozoa at low temperature. Cryobiology 10, 421-426 (1973).

24. Tate, E. H., and Cram, L. S. A method for simultaneous isolation and cryopreservation of bovine lymphocytes. Cryobiology 19, 136-146 (1982).

25. Thorpe, P. E., Knight, S. C., and Farrant, J. Optimal conditions for the preservation of mouse lymph node cells in liquid nitrogen using cooling rate techniques. Cryobiology 13, 126-133 (1976).

APPENDIX I

Description:[1] *Ichthyophthirius multifiliis* (Fouquet, 1876) (33) is the sole parasitic genus in the family *Ophryoglenidae* (84, 85). This parasite attacks the epidermis of the skin and fins, the gill filaments, and cornea of a number of species of freshwater fish (85).

Adult trophozoites [also referred to as trophonts (97)] of *I. multifiliis* measure up to 1 mm long, are oval and covered with numerous rows of cilia. Interspersed among the ciliar rows are many circular micropores about 1 μm in diameter located in the bilayer pellicle. The pellicle contains an agranular ectoplasmic layer, but no subtending myoneme. Structures believed to be instrumental in extracellular digestion have been observed in the pellicular region, including elongate bodies with darkened cores and small ovoid vacuoles. An unciliated ring-like cytostome is located at the anterior end of the body. The macronucleus is a large horseshoe-shaped body with the small micronucleus lying in the concavity of the former. Numerous contractile vacuoles are scattered throughout the cytoplasm. The contractile vacuoles together with cytoplasmic organelles form an anatomical-physiological complex of significance. The general cytoplasm contains multioculate lipid bodies; various stages of digestive vacuoles, bacteria, polyribosomes, and large accumulations of rough endoplasmic reticula.

Each contractile vacuole is connected through a discharge canal about 1 μm in length and diameter with an external micropore. The base of the discharge canal is closed by a double membrane consisting of the thick inner pellicular layer on the one side and the very thin vacuolar membrane on the other side. These membranes rupture to empty the contractile vacuole through the discharge canal and then reform. Tubular fibrils, presumed to be contractile in function, surround the discharge canals and cover the outer

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[1]References are located at the end of Appendix II.

surface of the contractile vacuoles.

In a state of relaxation the contractile vacuoles are spheres about 35μ in diameter. Contracted vacuoles assume a multilobular figure with many fingerlike tubular extensions into the cytoplasm. Numerous minute injection canals extend from the cytoplasm to and empty through the wall of the contractile vacuoles. Granular endoplasmic reticulum and many mitochondria are intermingled with the injection canals. The mitochondria have been shown to contain tubular cristae, typical of many ciliates. These highly differentiated canalicular-vesicular zones represent drainage systems from the cytoplasm into the contractile vacuoles. A permanent cytopyge is located at the posterior end of the trophozoite. Adult trophozoites on the bottom of ponds encyst, forming large gelatinous cysts with thick, clear walls (21, 75, 76, 78, 85).

Post excystment tomites (also referred to as either theronts, swarmers or ciliospores) are pear-shaped, translucent ciliated protozoans 30-50μ long with a spherical nucleus and single pulsating vacuole (75, 78, 85, 97). The pointed, unciliated anterior end possesses a characteristic filamentous projection from a depression in the pellicular structure (49, 50). This depression is surrounded by a raised truncated conical area of the pellicle corresponding to the location of the perforatorium. This structure develops after excystment and differs from the normal somatic infraciliature. The filamentous projection differs from somatic cilia in its greater length (3x), and characteristically straight, rigid orientation. Development of the filamentous projection preceeds development of the oral apparatus in the tomite, and has not been observed in the trophozoite or in the tomies prior to excystment. Since this structure is only observed during the infective phase of development it may serve a function in facilitating infectivity of tomites (50). The filament is always seen oriented toward, or actually

contacting, host epithelium. Cells partially burrowed into host tissue have never been observed to possess a trailing filament.

Tomite penetration occurs at host epithelial cell boundaries and is characterized by a loss of squamous epithelium once the parasite has disappeared beneath the surface. Bacilli bacteria have been observed in desquamated regions. Ctyochemical techniques have demonstrated the presence of a capsule-like structure in tomites. Acid phosphatase and nonspecific esterases have been found to be localized in and around the capsule. The capsule and filament are located at the same pole of the tomite suggesting that either separately or in concert they may facilitate host penetration (49).

Life Cycle: Mature trophozoites are found in epidermal pustules of the skin, fins, tail and gill filaments of fish. Upon rupture of the pustules, they are liberated and swim about feebly. Upon coming to rest on aquatic plants, snail shells or other objects on the bottom of the pond or aquarium, each trophozoite secretes a thick-walled, clear, gelatinous cyst about itself. Within an hour after encystment, the mother trophozoite begins to divide by simple transverse division, first with 2, then 4, 8 and so on, until numerous daughter trophozoites, or tomites, are produced. The number of tomites may be as many as 1000, depending on the size of the mother trophozoite.

Within 7-8 hours, after detachment of the mother trophozoites from the fish host in water 18-20°C, they have encysted and completed their development and are leaving the cysts and attaching to fish. Unattached individuals die during the second day due to starvation (22, 75, 78, 97).

Upon attaching to a fish, the tomites bore by whirling movements into and locate under the epidermis. Once under the epidermis, the tomites move

about forming galleries that soon become occupied by additional tomites so that many of them occur together, forming pustules. Division with multiplication does not occur in the epidermis. 2-3 days after burrowing under the epidermis, a cytostome is recognizable in the tomites. The boring apparatus diminishes to form the center point of the ciliated area. Through ingestion of blood cells, body fluids and nutritive substances in the skin, growing tomites appear opaque, dark-colored granules. As growth continues, the number of vacuoles in the parasites increases and the macronucleus becomes increasingly U-shaped (22, 75, 77, 78, 80, 83, 84, 85, 107).

Ichthyophthiriasis exhibits a seasonal incidence in warm water fish. A decline occurs from January to July, peaking in April. A low level of incidence is reported through September and October, peaking again in November (108).

The initiation of the cyclic stages, and hence the onset of an epizootic, is dependent on the temperature tolerance and degree of resistance of the host fish (83). Each phase of the parasite's development is correlated with its sensitivity to changes in temperature. Suzuki (97) reported the optimal temperature for ichthyophthiriasis in carp varied from 14-17.5°C. Optimal temperature range for cold water species (trout and salmon) is 12.8-16.1°C; the range for temperate fish (carp, minnow, catfish) being 7.2-10.6°C. Encystment occurred between 13.1°C and 25°C. Division within the cysts were completed in 24 hours at 16.2-25°C; Cross (22) reports 18-20°C. The efficiency of encystment increased with length of time on the host. At 25-26°C, 50% of the trophozoites were capable of encysting in 40 hours; 100% of the trophozoites were capable of encysting in 96 hours.

The number of tomites produced from a single cyst also varied with temperature and time (97). Cysts dividing at 4°C produced 8-cell stage cysts in 8 days. At 25-27°C cysts produced 500 tomites within 48 hours, and 1,000 tomites within 48-96 hours.

Course of Infection: Hines and Spira (38) divided the course of *I. multifiliis* infection in mirror carp (*Cyprinus carpio*) into six stages, based upon a 400 parasite/fish concentration. Stage 1 (days 1-3) - fish appear normal but parasites are already in close association with the fish or have penetrated the epithelium of the skin, gills and fins. Stage 2 (days 4-7) - fish often jump and surface. The superficial vessels of the integument becomes visible. The epithelium and mucus of the skin begin to thicken. Stage 3 (days 8-12) - the superficial vessels of the integument are engorged, giving the fish a peach-red color on its lateral and ventral surfaces. Parasites are visible over the entire body. Stage 4 (days 12-14) - fish are no longer hyperactive. They swim more slowly than normal, and congregate at the bottom of the tank. The fins begin to fray. The epithelium of the skin is thickened and gelatinous in texture. Stage 5 (days 15-19) - the fish are noticeably weaker. They rest at the bottom of the tank. The fins, especially the caudal fin, are very frayed. The fish are very dark in color. The epithelial and mucus coat is thick, often clumped or patchy. The epidermis and corium on the gill opercula and over the ventral process of the postcleithrum are often eroded, leaving this bone exposed. Fish in this condition have never been observed to recover. Stage 6 (days 20-26) - fish hardly move when disturbed. No mucus whatsoever can be scraped from the body surface. The eyes are sunken, the corneas often opaque. Gills are pale. White, circumscribed areas of necrosis are often present in the gills leaving only the cartilaginous shafts of the filaments intact. Many scales have been lost, or can be detached easily. The epidermis and corium also detach easily from the underlying muscle. The fish will die if undisturbed.

Hines and Spira further reported the growth rate of the parasite was linear. Parasites were more numerous in the dorsal surface of the fish than in the lateral or ventral surface; an observation that could not be explained

by differences in skin structure. The increase in parasite numbers during the disease was greater in the gills than in the skin. Changes in behavior (congregate near the source of incoming water, scratching themselves against bottom gravel, or hiding in corners and exhibiting frequent twitching of the fins), reduced vitality, failure to feed, and the presence of pustules have been symptoms substantiated by other authors (75, 78, 80, 108).

Pathology: Hines and Spira (39) and others (85) have reported the microscopical pathology of ichthyophthiriasis as studied in a group of mirror carp (*Cyprinus carpio*) infected with 400 mature (seventh day) parasites/fish. The primary pathology consisted of inflammatory, proliferative and degenerative changes in the epidermis and hyperaemia and oedema of the spongiose dermis. This was accompanied by changes in the amount and distribution of pigment in the spongiose dermis. Proliferation in interlamellar epithelial tissue of the gills in response to the parasite resulted in partial or complete filling of the interlamellar spaces. Secondary changes in the diseases were devacuolization of hepatocytes, leukocytic depletion of the mesonephric kidneys, and leukocytic metaplasia or hyperplasia in the spleen.

Hines and Spira concluded the loss of the upper layers of the skin and exposure of the vascular network of the dermis in the later stages of infection must severely interfere with osmoregulation. The changes observed in the liver of infected fish were primarily related to mobilization of fat reserves. Fish suffering from ichthyophthiriasis die if the level of dissolved oxygen drops slightly resulting from the reduced area for diffusion of gases through the cornified gil filaments and increased thickness of the lamella.

Physiological Dysfunction: Hines and Spira (40) reported the disease, at a parasite concentration of 400/fish, to be characterized by a drop in serum sodium and magnesium and increase in serum potassium. The ability of infected fish to absorb oxygen was reduced as was their ability to tolerate dissolved oxygen concentrations under 5 ppm. Blood urea--ammonia level was elevated during the disease but blood haemoglobin level remained unchanged. Olsen (85), however, reported haemoglobin concentration declines in infected fish.

Control: Many methods for control of ichthyophthiriasis have been suggested over the years. It is important to realize chemical treatment for this parasite must be used daily or the chemical must be in contact until the parasite disappears (5-10 days) (43). If all fish are heavily infected, usually no known treatment will save them. It is best to examine fish regularly and start treatment at the first finding of the parasite (43). Any treatment in ponds is futile if the ponds can not be drained and refilled (4).

Formalin at 200 ppm. daily for 1 hour (79), at 250 ppm. daily for 1 hour (24, 37, 42, 76), or at 15 ppm. (3) or 25 ppm. (37) as a single treatment for pond fish have all been reported as effective against the parasite (78, 80). Formalin at 100-250 ml/1 (100-250 ppm.) administered as a short-term bath is not completely effective. 15 ml/1 on alternate days controls the disease in 5-7 days. Oxygen depletion may result during warm weather if formalin kills the algae. It is suggested not to use formalin below 18°C as detrimental effects to the mucus coat have been observed (22). Formalin administered 1:4000 for 1 hour daily if the water temperature is 10°C or colder is recommended by Hoffman and others (43, 82). They further suggest 1:5000 for 1 hour daily if water temperature is 10-15°C, and 1:6000 for 1 hour daily if water temperatuer is warmer than 15°C. For pond or aquariums 15-25 ppm. added every other day for 5-7 days is effective, but must be used with caution as oxygen depletion may result.

Malachite green oxalate (MGO) is recommended at a dosage of 0.1 ppm. (treatment schedule not given) (11, 76), or 1.25 ppm. daily for 30 min. (47). A dosage of 0.5 mg/1 (0.5 ppm) or 0.05-0.10 mg/1 has been found effective in controlling ichthyophthiriasis. It is recommended to use much less MGO if it is formulated with zinc chloride due to enhanced toxicity (4, 22). A 1:15,000 mixture in water as a dip for 10-30 seconds, 1-5 ppm. in water for 1 hour, or 0.1 ppm. for ponds or aquaria for an indefinite period of time have also been recommended (37, 82). Hoffman (43) recommends 0.1-0.15 ppm. two times weekly in aquariums and ponds, and 2 ppm. for half an hour daily for 5-10 days. Caution is warranted on catfish smaller than 10 cm (4"); bass are easily killed. MGO is a carcinogen in high concentration and has not been approved for use on food fish by the Food and Drug Administration and the Department of Agriculture (55).

A mixture of MGO and formalin has given excellent control (108). Schachte (92) reported a combination of 0.1 ppm. MGO and 15 ppm. formalin. He recommends a 4 day treatment schedule for periods of 3, 4, 5 and 5 hours followed by flowing water. Peak mortalities occurred on day 2, little mortality on days 1, 3 and 4. Leteux and Meyer (55) recommend mixtures of 0.05 ppm. MGO plus 15 ppm. formalin, 0.05 ppm. MGO plus 25 ppm. formalin, and 0.05 ppm. MGO plus 50 ppm. formalin. All combinations were found to be safe and effective in treatment of external protozoans including ichthyophythyriasis on channel catfish. Levels up to 0.2 ppm. MGO plus 33 ppm. formalin can be used on goldfish. In ponds, a treatment of 0.1 ppm. MGO with 24 ppm. formalin is urged since degradation occurs more rapidly under pond conditions.

Copper sulfate (blue stone) is suggested at a dosage of 0.5 ppm. weekly in ponds (76, 78). 1.5 and 0.5 mg/1 in softwater are concentrations close to maximum concentrations tolerated by many fish species (22). A 1 min.

dip at 1:2000 (500 ppm.) in hard-water plus 1 ml glacial acetic acid/1 (82). If water carbonate is less than 50 ppm. use less than 1 ppm., with extreme caution; if 50-200 ppm. carbonate, do not exceed 1 ppm.; if over 200 ppm. carbonate try 2 ppm. but effective control may be difficult. Three parts citric acid to 2 parts copper sulfate (at 2 ppm. copper sulfate) has been used in hard-water ponds (43).

Pyridylmercuric acetate (PMA) is suggested at a dose of 2 ppm. daily (18, 76) for 1 hour for the duration of the parasite life cycle, and 0.14 mg/1 (0.14 ppm.) as beneficial to fish (22). At 75°F treatment should continue 10 days, at 50°F treatment should continue 30 days or longer (85). All the chemical after each treatment should be completely flushed and the water replaced (18).

Methylene blue is recommended at 2 ppm. daily (37, 109) or 5 mg/1 (5 ppm.) (22). Allison (4) reports 4 daily treatments with 1 ppm. methylene blue results in an average of 70% survival of fingerling catfish over untreated controls. Two treatments with 2 ppm. on the first and fourth days results in 50% survival. A single earthen pond of 0.02 hectare stocked with 7,000 fingerling catfish was treated 6 times with 1 ppm. methylene blue. The treatment resulted in 46.6% survival of the population. 1 ppm. methylene blue controls ichthyophthiriasis on rainbow trout (*Salmo gairdnerii*) and golden shiners (*Notemigonus crysaleucas*) in commercial hatcheries (4). Methylene blue has a low toxicity (4, 22). Allison (4) conducted toxicity tests of methylene blue to fingerling carp (*Cyprinus carpio*) and bluegills (*Lepomis macrochirus*). No mortality occurred in carp at 3.5 and 10 ppm. methylene blue for 213 hours. No significant mortality occurred in bluegills at 3 and 5 ppm. methylene blue. An average of 20% mortality among bluegills occurred in 3 replications of 10 ppm.

Sodium chloride is suggested at a dose of 7000 ppm. for several days (10), 0.6% sea salt or a mix of NaCl and magnesium chloride 7:3 (0.6% solution) (22), 2.0% for 1 hour daily (15, 22) or 1 ppt. or greater (85). Butcher (15) reported salt treatments effective in controlling ichthyophthiriasis on rainbow and brown trout. Salt treatment must be maintained throughout the period of the parasitic phase and preferably for a few days longer, as all the parasites may not have infected the host on the same day. The 2% salt concentration is effective in destruction of free-swimming forms and thus prevents excystment. Allen and Avault (2) observed a decreased incidence of ichthyophthiriasis in brackish water. Studies of salinities ranging from 0 ppt. to 14 ppt. resulted in no infection in 9 ppt. salinity and in brackish water. Salinity of 5 ppt. resulted in a clearing of the disease in 9 days.

Acriflavine is recommended by Schaperclaus (37, 93) at a dose of 10 ppm. between 3-20 days, or 2-4 ppm. for 24-48 hours (37). There are reports of temporary sterility and possible genetic aberrations when using acriflavine (22).

Trypaflavine is recommended at 10 ppm. for 3-20 days by Bauer (9) and other authors (22, 37). Similar temporary sterility and genetic aberrations as reported with acriflavine has been reported for trypaflavine (22).

Quinine hydrochloride has been suggested at doses of 20 ppm. for 3-20 days (9, 109) and at 10 mg/l (10 ppm.) raised to 30 mg/l at pH 6.5 (22). The concentration should be built up by the addition of 3 equal doses at 12 hour intervals, and extended for at least 24 days. Quinine hydrochloride is inactivated by organic detritus.

Other chemical controls include atabrine at 1 ppm. daily for effective control (109), penicillin, mepacrine hydrochloride (quinacrine hydrochloride)

at 3 mg/l (3 ppm.), mercurochrome at 1 mg/l (1 ppm.), and wiping the fish surface with paraffin oil and dipping fish in saturated sodium carbonate (15, 22).

Non-chemical controls include a layer of sieved rotted sod on the bottom of the aquarium, separating the motile stage of the parasite from the host (limits the incidence of reinfection), and temperature (22). Periodic raising and lowering of water temperature is useful (22). For tropical fish that can resist high temperatures, raising the temperature to 90°F for 6 hours daily for 3-5 days has been found effective (43, 75, 78). Repeated daily fish transfer from contaminated aquariums to clean aquariums removes most of the free-living forms (43). A rapid flow of water sometimes helps greatly by flushing away the free-living forms.

Ichthyophthiriasis continues to cause problems almost worldwide (44, 86) and causes an annual loss of fish estimated to value about $1,000,000 (4). Monetary losses due to ichthyophthiriasis are encountered in the United States' 5000 commercial fish farmers, 4000 fee fish-out operations and 150,000 farmers who raise fish for recreational use (106). Research on immunodiagnosis and xenodiagnosis is needed and prevention through better surveillance is recommended. Studies on cryopreservation, the development of culture media and the elucidation of other species of the parasite are required.

The existence of strains of the parasite which differ from each other in host preference as well as in virulence to different hosts has been reported (86). Roque and dePytorse in 1968 reported a new Ich-like organism (*Ichthyophthirius browni*) from the skin of guppies (*Poecilia reticulata*) which lacks the watch glass organelle present in *Ichthyophthirius*, and possess a more rod-shaped macronucleus and minor variations in buccal morphology (83, 91). Another Ich-like ciliate possess a spiral shaped macronucleus.

Hlond (41, 108) reported several media for culturing *Ichthyophthirius*; dried, powdered and pasteurized fish slime appear to be most suitable. Slime powder in the proportion 0.3 g/100 ml water was used for successful culturing. He observed a gradual increase in the number of parasites (tomite stage).

Kozel et al. (51) reported trophozoites were maintained longest in enriched protease peptone media inoculated with cells less than 100 in diameter. Cells resided at, or slightly below, the boundary between liquid overlay and semi-solid agar. Trophozoites encysted and produced ineffective tomites in all overlays containing host tissue homogenate. Unencysted trophozoites survived longest in host epidermal overlays.

APPENDIX II

THE KINETICS OF THE IMMUNE RESPONSE IN THE CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)[1]

by

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[1]References are located at the end of Appendix II.

Summary

Sheep red blood cells and burro red blood cells were injected intraperitoneally into channel catfish (*Ictalurus punctatus*) to determine the time required to elicit a primary humoral response. Mucus and blood-sera samples were collected, heat-inactivated, and analyzed for antibody titre using hemolysis and hemagglutination assays. Zero mucus antibody titres were obtained. Varying and unrepeatable sera antibody titres were elicited. A review of fish immunobiology is presented accompanying possible explanations for the obtained results. Suggestions for further investigations are offered.

The kinetics of the immune response has been investigated in a variety of boney fish. Rainbow trout (*Salmo gairdneri*) elicit a humoral primary response to sheep red blood cell (SRBC) antigen (Ag) in 2-6 days peaking around day 14, as measured by plaque formation (17). Goldfish (*Carassius auratus*) elicit a primary humoral response peaking between 12-28 days when injected with either SRBC or horse red blood cells. A weak secondary response has been observed in red blood cell-primed fish boosted during the end-phase of the primary antibody (Ab) production (26). Carp have been reported to possess a 9 day latent period prior to detection of a primary humoral response using bovine serum albumin. Maximal Ab titres have been recorded between 25-35 days (60). Channel catfish (*Ictalurus punctatus*) produced detectable titres of serum Ab to the bacteria *Chondrococcus columnaris* in 1 week, and achieve a maximal Ab titre in 4 weeks. Channel catfish elicit a very pronounced and rapid secondary response (36).

This study was undertaken to gain experience prior to investigating possible primary and/or secondary reactions to *Ichthyophthirius multifiliis*.

Materials and Methods

Animals: Adult channel catfish were divided into 3 groups of 5 fish and kept in 70-l glass aquaria. Aquaria water was tested daily for pH, temperature, alkalinity, chlorine, oxygen and water hardness.

Antigens: SRBC and burrow red blood cells (BRBC) in Alsever's solution were obtained commercially. Each Ag was separately washed three times in 0.01 M Sorenson's phosphate buffer in 0.15 M NaCl, pH 7.2 (PBS), and adjusted to a final 20% suspension in PBS (16).

Immunization: The channel catfish were captured and partially immobilized in a "mesh-like" dip-net. Before the Ag was administered, blood was

collected by cardiac puncture; the sera was separated, heat-inactivated by immersion into a 56°C water-bath for 30 min., and stored in a freezer (16, 17, 36, 96). Individuals were injected intraperitoneally with 0.5 ml of a 20% SRBC suspension (group 1) or 0.5 ml of a 20% BRBC suspension (group 2). Control fish received the same volume of PBS (16). SRBC and BRBC were used to compare their antigenicity in eliciting a primary humoral response.

Sera and Mucus Preparation: The fish were bled by cardiac puncture every two days. 0.8 cc of blood was withdrawn, the sera collected, heat-inactivated, and stored as outlined above. Heat-inactivation of the sera renders the natural hemolysins and complement (C') normally found in fish sera non-functional. Alternate to blood collections were mucus removals using a mild vacuum system as reported by Kozel and Dobra (52). Collected mucus was heat-inactivated and stored as outlined above.

Hemolysis Assays: Samples of sera and mucus were analyzed for Ab titre using the hemolysis assay. Twenty-five µl of a 1:100 dilution (v/v) fetal bovine serum (FBS) in PBS was placed in each well of the round-bottom well microtitre plate. Twenty-five µl collected-prepared sera or mucus was then placed in the first well of each row and sequentially diluted using 25 µl microtitre dilution rods. Twenty-five µl of a 2% SRBC in FBS-PBS solution or BRBC in FBS-PBS solution (corresponding to group 1 or 2 sera) was added to each well followed by 25 µl channel catfish C' absorbed with SRBC and BRBC. Each microtitre plate was then covered with its accompanying lid and the solutions mixed using a gentle swirling motion. C' was obtained from non-immunized channel catfish. Titre controls consisted of separate amounts of 50 µl FBS, PBS and C' plus 50 µl SRBC or BRBC. Microtitre plates were incubated at room-temperature and the sera or mucus titres

determined. Presence of lysed SRBC or BRBC indicated a positive dilution titre. Presence of a grouped "button" of RBCs was recorded as a negative titre.

Hemagglutination Assays: Samples of sera and mucus were analyzed for Ab titre using the hemagglutination assay. Twenty-five µl of a 1:100 dilution (v/v) FBS in PBS was placed in each well of the round-bottom well microtitre plate. Twenty-five µl collected-prepared sera or mucus was then placed in the first well of each row and diluted as in the hemolysis assay. Twenty-five µl FBS-PBS solution was then added to each well followed by 25 µl of a 2% (v/v) SRBC or BRBC in FBS-PBS solution. The microtitre plates were covered, the solutions mixed and the plates incubated as described in the hemolysis assay. Control titres consisted of separate amounts of 50 µl FBS and PBS plus 50 µl SRBC or BRBC. Presence of a diffuse layer of RBC coating the bottom of a well indicated a positive titre. Presence of a grouped "button" of RBCs was recorded as a negative titre.

Note - the entire experiment was conducted twice.

Results

Aquaria water conditions are reported in Table 1. The water temperature ranged between 22.0-23.5°C reflecting the daily fluctuations in room-temperature. Oxygen measurements ranged between 5.2-7.4 ppm. A dissolved oxygen concentration below 5.0 ppm. has been determined to be undesirable for warmwater pond fish (14). Chlorine concentration greater than 1.0 ppm. was present in the tap water; the source of the aquaria water. Passing the tap water through the activated charcoal dechlorinator resulted in no detectable concentration of chlorine. A range of 0.0-0.1 ppm. chlorine was found in the aquaria water presumably due to the pellet fish food administered bi-weekly. Boyd (14) summarizes if measurable concentrations of chlorine

residuals are present in water, the water should not be considered safe for holding fish. Alkalinity values of 80 mg $CaCO_3$/l in the form of bicarbonate and water hardness values between 60-100 mg EDTA as $CaCO_3$/l were reported in the aquaria. pH values between 7.1-7.7 were reported and adjusted as needed using 3 meq/l sodium bicarbonate. Boyd (14) reports pH values between 6.5-9.0 as suitable for pond fish.

Results of the first-run hemolysis assay titres are presented in Table 2. Inconsistent titres were shown using SRBC Ag and the PBC control groups. Various SRBC sera titres in fish sera samples 1-6 and 8 were present on day 1 following Ag injection. On days 2 and 4 the titres dropped to 0 for the same fish sera samples, then increased on days 6 and 8. BRBC sera titres were inconclusive due to the death of the fish on either the second or eighth days. PBS sera titres for samples 4 and 8 were present in the control fish on days 1 and 4. Ag mucus titres for all three groups were 0.

Table 3 presents the data of the first-run hemagglutination assay titres. SRBC Ag for fish sera samples 3, 6 and 10 showed a positive titre of 64 on day 0. Sudden drops in titre to 0 occurred after 2 days for sera sample 6 and 4 days for sera samples 3, 5 and 8. Similar inconsistent results were present using BRBC Ag and PBS controls. Ag mucus titres for all three groups were 0.

Table 4 presents the data of the second-run hemolysis and hemagglutination assay titres. BRBC was chosen as possessing a greater antigenicity potential than SRBC as indicated by the higher titres for BRBC in Tables 2 and 3. Similar inconsistencies were present in Table 4 as were described in Tables 2 and 3. No mucus was assayed due to the 0 titres reported in Tables 2 and 3.

Discussion

Extensive research has been conducted on the immunobiology of fish. Heartwell (36) has drawn the following generalities concerning fish immunobiology: 1) The immune response of bony fishes is directly dependent on environmental temperature and the nature of the Ag. 2) The species of fish and route of Ag injection are of secondary importance in the mechanism of the immune response. 3) Teleost fishes are capable of a rapid and strong primary response under optimum conditions for the immune response. 4) The secondary immune response in fishes can be highly variable and is often weaker than that found in mammals. 5) The primary and secondary responses of fishes are often shorter than those of mammals. 6) Oral immunization of fishes has met with only limited success.

Various authors have reported immunized fish held at different temperatures eventually produce similar Ab titre levels but at different rates (7, 20, 23, 35, 98, 105). Further studies report the stage of acquisition of the potential for Ab production is distinct and separable from that of production of Abs and their release into the circulation. The second stage is more affected by temperature in cold-blooded animals. Thus, it is possible for cold-blooded animals to acquire immunity at low temperatures, although it is not manifested until the temperature is raised (60).

Heartwell (36) has also drawn generalities concerning Ab characterization: 1) The characteristics of teleost immunoglobulins (Igs) depend partly on the species of fish and type of Ag used. 2) Teleost Igs are heterogeneous; most fish species produce at least two types of specific Igs. 3) Most fish Igs are macroglobulins with the caracteristics of IgM of higher animals (46, 59). 4) Almost no teleost demonstrates the Ab shift from heavy to light molecular weight Igs that is characteristic in mammals.

5) Fish Igs are more heat labile than most mammalian Igs, and most are susceptible to partial reduction and alkylation with 2-mercaptoethanol.
6) Fish Igs exhibit a wide range of electrophoretic mobility (28, 104).

Other studies have revealed "natural Abs" - naturally-occurring, relatively nonspecific, lectin-like proteins or glycoproteins, which are distinct from Igs, and which react with a wide variety of Ags. Natural Abs include "acute phase" proteins, lysozyme and chitinase, interferon, agglutinins, lysins, complement and properdin, precipitins, and non-Ig, lectin-like molecules (1, 46). Secretory Abs have been reported in the mucus of teleosts (20) along with other biochemical agents capable of reaction against infective organisms (46).

There have been several reports indicating the presence of a cellular immune response in bony fish. Lewis et al. (56) report two types of cells in immune channel catfish similar to T and B lymphocytes of higher vertebrates. One type - referred to as "small lymphocyte" possessed surface erythrocyte receptors and appeared to be analogous to mammalian T cells. The second type of lymphocyte analogous to mammalian B cells possessed surface Igs and was capable of cytoadherence with *Aeromonas hydrophila* cells.

Yocum et al. (111) report the sea robin (*Prionotus evolans*) to undergo a hapten-carrier effect, suggestive of T-B cell cooperativity. In contrast to mammalian systems, the secondary anti-hapten response in the sea robin was found to involve exclusively high molecular weight Abs.

Cooper (19) reports fish to possess two fundamental types of allograft reactions. The more primitive fishes show a prolonged, chronic reaction to first-set allografts indicating a short-lived, weak immunological memory. Holosteans or near relatives elicit an acute second-set reactions indicating a sharper and better developed immunological memory. Telosts are presumed to be comparable to the Holosteans.

A system of lymph nodes comparable to those of mammals do not occur in fish. Various authors have reported well developed lymphoid tissue in the thymus, spleen, pharyngeal submucosa, pronephrose (head kidney) and mesonephrose (middle kidney) (16, 17, 90, 96).

The results in this study were varied, unreliable and unrepeatable. Tables 2 and 4 presented hemolysis titres for the control group of fish injected with PBS. These fish were never exposed to SRBC or BRBC and thus should not have produced Ab to either of the aforesaid Ags. Either the PBS sera was insufficiently heat-inactivated thus containing functional natural lysins, or the fish contained Ab to an Ag that had similar antigenic determinants as those present in SRBC and BRBC. The same explanations apply to Table 3 and 4 hemagglutination assay titre results. Presence of hemagglutination titres on day 0 may have been due to the presence of functional natural hemagglutinins in the sera.

Those fish that elicited 0 titre throughout the experiment may have been in the process of eliciting the primary response but dies before it could be detected. Another explanation may be those fish elicited a primary humoral response but Ab concentrations were too low to be detected by the assays used in this study. A third explanation for no detection of any Ab titre may be the eradication of the heat-labile Abs during sera heat-inactivation.

Tables 2 and 3 show zero mucus Ab titres for all Ags. These results oppose those reported earlier (20, 46). The explanations may be applied to a zero mucus Ab titre as have been presented towards the zero serum Ab titres obtained for sera samples throughout the time course.

Death of the fish was presumably due to stress imposed during handling, blood and mucus removal and varying water conditions. Blood removal via cardiac puncture is not recommended unless one can anaesthetize the fish to diminish excess movement. A smaller volume of blood removal is suggested

such as 0.5 cc and a greater amount of time allotted for the fish to replenish the withdrawn blood.

This author recommends the repetition of this study followed by the use of an organism such as *Ichthyophthirius multifiliis* as Ag. By knowing the peak humoral response time to Ags such as SRBC and BRBC, one could approximate the peak humoral response time for *I. multifiliis* Ab production. One may then collect Ab specific for the organism and develop possible immunization schemes for fish.

Immunization of *Ictalurus punctatus* against *I. multifiliis* has been reported using *Tetrahymena pyriformis* ciliary Ag (34) and *I. multifiliis* trophozoite Ag (5). No studies concerning the kinetics of the aforesaid responses have thus far been reported.

Table 1. Water Conditions Used in Fish Aquaria

	°C	O_2 ppm	Cl ppm	Alkalinity* mg $CaCO_3$/l	ph+	Hardness mg EDTA as $CaCO_3$/l
Tapwater	-	-	>1.0	40	9.0	120
Decholorinated Water	-	-	0.0	40	6.4	40
Aquaria Water	22.0-23.5	5.2-7.4	0.0-0.1	80	7.1-7.7	60-100

* = in the form of bicarbonate
+ = pH adjusted using sodium bicarbonate at 3 meg/l

Table 2. Hemolysis Assay Titres - I

Antigen - SRBC	Day	Fish Sera Samples									
		1	2	3	4	5	6	7	8	9	10
	1	8	4	4	16	4	8	0	4	0	0
	2	-	-	-	-	-	0	-	0	0	-
	4	-	0	0	0	0	-	-	-	-	-
	6	-	8	-	8	0	-	-	-	-	-
	8	-	8	-	16	0	-	-	-	-	-

Fish mucus samples all 0

Antigen - BRBC	Day	Fish Sera Samples									
		1	2	3	4	5	6	7	8	9	10
	0	0	0	0	0	0	0	0	0	0	0
	1	0	0	0	0	0	8	0	8	0	0
	2	0	0	0	0	0	-	-	-	-	-
	4	0	0	0	0	0	-	-	-	-	-
	6	0	0	0	0	0	-	-	-	-	-
	8	-	16	0	-	8	-	-	-	-	-

Fish mucus samples all 0

Antigen - PBS	Day	Fish Sera Samples				
		1	2	3	4	5
	0	8	4	8	4	4
	1	4	8	0	0	-
	4	4	0	4	4	-

Fish mucus samples all 0

SRBC - sheep red blood cells
BRBC - burro red blood cells
PBS - phosphate buffered saline

Table 3. Hemagglutination Assay Titres - I

Antigen - SRBC	Day	Fish Sera Samples 1	2	3	4	5	6	7	8	9	10
	0	0	0	64	0	0	64	0	0	0	16
	1	0	0	16	0	0	32	4	16	0	-
	2	-	0	16	0	16	0	-	32	0	-
	4	-	0	0	0	0	-	-	0	0	-
	6	-	0	-	0	0	-	-	-	-	-
	8	-	0	-	0	0	-	-	-	-	-

Fish mucus samples all 0

Antigen - BRBC	Day	Fish Sera Samples 1	2	3	4	5	6	7	8	9	10
	0	0	0	0	8	0	32	0	4	0	0
	1	16	0	0	16	64	-	-	-	-	-
	2	64	0	0	4	0	-	-	-	-	-
	4	64	16	0	0	0	-	-	-	-	-
	6	0	0	0	4	16	-	-	-	-	-
	10	0	-	-	-	-	-	-	-	-	-
	12	0	-	-	-	-	-	-	-	-	-

Fish mucus samples all 0

Antigen - PBS	Day	Fish Sera Samples 1	2	3	4	5	6	7	8	9	10
	0	0	8	0	0	0	8	0	-	0	4
	1	0	8	0	0	0	-	-	-	-	-
	4	0	0	0	0	0	-	-	-	-	-
	2	0	-	0	16	-	-	-	-	-	-
	6	0	-	0	-	-	-	-	-	-	-

Fish mucus samples all 0

SRBC - sheep red blood cells
BRBC - burro red blood cells
PBS - phosphate buffered saline

Table 4. Hemagglutination and Hemolysis Assay Titres - II

Hemagglutination Assay Titres

	Antigen - BRBC				
	Fish Sera Samples				
Day	1	2	3	4	5
0	0	0	0	0	0
1	0	0	0	0	0
3	0	-	0	-	0
5	0	-	0	-	-

	Antigen - BRBC				
	Fish Sera Samples				
Day	1	2	3	4	5
0	0	0	0	0	0
2	0	0	0	0	0
4	-	0	0	0	0
6	-	4	0	0	0
8	-	0	0	-	0
10	-	-	-	-	0

	Antigen - PBS				
	Fish Sera Samples				
Day	1	2	3	4	5
0	0	0	0	0	4
2	0	0	0	0	0
4	0	0	-	0	-
6	0	64	-	0	-
8	-	0	-	-	-
10	-	-	-	-	-

Hemolysis Assay Titres

	Antigen - BRBC				
	Fish Sera Samples				
Day	1	2	3	4	5
0	0	0	4	4	0
1	0	0	0	0	0
3	4	-	4	-	4
5	8	-	4	-	-

	Antigen - BRBC				
	Fish Sera Samples				
Day	1	2	3	4	5
0	0	0	0	0	0
2	0	0	0	0	0
4	-	8	8	0	8
6	-	4	8	-	16
8	-	-	-	-	4
10	-	-	-	-	-

	Antigen - PBS				
	Fish Sera Samples				
Day	1	2	3	4	5
0	0	0	0	0	0
2	0	0	0	0	0
4	8	8	-	32	-
6	0	0	-	0	-
8	-	4	-	-	-
10	-	0	-	-	-

BRBC - burro red blood cells
PBS - phosphate buffered saline

References

1. Alexander, J. B. Precipitins in the serum of the atlantic salmon. Devl. Comp. Immunol. 4, 641-651 (1980).

2. Allen, K. O., Avault, J. W. Effects of brackish water on ichthyophthiriasis of channel catfish. Progr. Fish Cult. 32, 227-230 (1970).

3. Allison, R. Some new results in the treatment of ponds to control some external parasites of fish. Progr. Fish Cult. 19, 58-63 (1957).

4. Allison, R. New control methods for *Ichthyophthirius* in ponds. FAO World Symposium On Warm Water Pond Fish Culture. Rome. FR:1X/E-9, 1-4, 18-25 (1966).

5. Areerat, S. The immune response of channel catfish, *Ictalurus punctatus (Rafinesque)*, to *Ichthyophthirius multifiliis*. Master's Thesis, Kansas State University, 47 pages (1974).

6. Ashwood-Smith, M. J. Low temperature preservation of mouse lymphocytes with dimethyl sulfoxide. Blood 23, 494-501 (1964).

7. Avtalion, R. R. Temperature effect on antibody production and immunological memory, in carp (*Cyprinus carpio*) immunized against bovine serum albumin (BSA). Immunology 17, 927-931 (1969).

8. Baartz, G., and Brock, M. A. A microprocessor-controlled rate controller for use in cryopreservation. Cryobiology 16, 497-505 (1979).

9. Bauer, O. N. Parasitic diseases of cultured fishes and methods of their prevention and treatment. In "Parasitology of Fishes" (Ed. Dagul, Petrushevski, and Polyonski) p. 265-298 (1958). Translated by Z. Kabata. Oliver & Boyd, Edinburgh & London, 1961.

10. Bauer, O. N., and Uspenskaya, A. V. New curative methods in the control of fish diseases. In "Proceedings of the Conference on Fish Diseases". Translated from Russian by Z. Kabata, Academy of Science of the USSR; Published for the National Science Foundation & Department of the Interior by the Israel Program for Scientific Translation, 19-25 (1963).

11. Beckert, H., and Allison, R. Some host responses of white catfish to *Ichthyophthirius multifiliis*, Fouquet. Proceedings, 18th annual meeting of Southeastern Association of Game and Fish Commissioners, 438-441 (1964).

12. Beeler, C. R. Controlling cooling rates with a variable vacuum in a Dewar flask. Cryobiology 18, 79-81 (1981).

13. Birkeland, S. A. The influence of different freezing procedures and different cryoprotective agents on the immunological capacity of frozen-stored lymphocytes. Cryobiology 13, 442-447 (1976).

14. Boyd, C. E. Water quality in warm water fish ponds. Auburn University; Agricultural Experiment Station, Auburn, Alabama, 67-76 (1979).

15. Butcher, A. D. Ichthyophthiriasis in an Australian trout hatchery. Progr. Fish Cult. 9, 21-26 (1947).

16. Chiller, J. M., Hodgins, H. O., Chambers, V. C., and Weiser, R. S. Antibody response in rainbow trout (*Salmo gairdneri*). 1. Immunocompetent cells in the spleen and anterior kidney. J. Immun. 102, 1193-1201 (1969).

17. Chiller, J. M., Hodgins, H. O., and Weiser, R. S. Antibody response in rainbow trout (*Salmo gairdneri*). 11. Studies on the kinetics of development of antibody-producing cells and on complement and natural hemolysin. J. Immun. 102, 1202-1207 (1969).

18. Clemens, H. P., and Sneed, K. E. The chemical control of some diseases and parasites of channel catfish. Progr. Fish Cult. 20, 8-15 (1958).

19. Cooper, E. L. Comparative Immunology; Prentice-Hall Inc., Englewood Cliffs, N. J., 164-173 (1976).

20. Corbel, M. J. The immune response in fish: a review. J. Fish Biol. 7, 539-563 (1975).

21. Crilley, R. V., and Buckelew, T. B. The ultrastructure of *Ichthyophthirius multifiliis*. Proc. P. A. Acad. Sci. 51, 98 (1977).

22. Cross, D. G. A review of methods to control ichthyophthiriasis. Progr. Fish Cult. 34, 165-170 (1972).

23. Cushing, J. E. An effect of temperature upon antibody production in fish. J. Immun. 45, 123-126 (1942).

24. Davis, H. S. Culture and diseases of game fishes. Univ. of Calif. Press, Berkeley and Los Angeles (1953).

25. Daw, A., Farrant, J., and Morris, G. J. Membrane leakage of solutes after thermal shock or freezing. Cryobiology 10, 126-133 (1973).

26. Desvaux, F.-X., and Charlemagne, J. The goldfish immune response. I. Characterization of the humoral response to particulate antigens. Immunology 43, 755-762 (1981).

27. Diller, K. R. Intracellular freezing: effect of extracellular supercooling. Cryobiology 12, 480-485 (1975).

28. Everhart, D. L. Antibody response of goldfish to a protein immunogen and to haptenic determinants. Immunology 22, 503-507 (1972).

29. Farrant, J. Human red cells under hypertonic conditions; a moded system for investigating freezing damage. 3. Dimethyl sulfoxide. Cryobiology 9, 131-136 (1972).

30. Farrant, J., and Morris, G. J. Thermal shock and dilution shock as the causes of freezing injury. Cryobiology 10, 134-140 (1973).

31. Farrant, J., and Woolgar, A. E. Human red cells under hypertonic conditions. A model system for investigating freezing damage. Cryobiology 9, 16-21 (1972).

32. Foreman, J., and Pegg, D. E. Cell preservation in a programmed cooling machine: the effects of variation in supercooling. Cryobiology 16, 315-321 (1979).

33. Fouquet, D. Note sur une éspèce d'infusoires parasites des poissons d'eau douce. Arch. Zool. Exp. Gen. 5, 159-165 (1876).

34. Goven, B. A., Dawn, D. L., and Gratzek, J. B. Protection of channel catfish (*Ictalurus punctatus*) against *Ichthyophthirius multifiliis* (Fouquet) by immunization with varying doses of *Tetrahymena pyriformis* (Lwoff) cilia. Aquaculture 23, 269-273 (1981).

35. Harris, J. E. The immune response of dace *Leuciscus leuciscus* (L.) to injected antigenic materials. J. Fish Biol. 5, 261-276 (1973).

36. Heartwell, C. M. Immune response and antibody characterization of the channel catfish (*Ictalurus punctatus*) to a naturally pathogenic bacterium and virus. Technical Papers of the U.S. Fish and Wildlife Service #85, 31-34 (1975).

37. Herman, R. L. Chemotherapy of fish diseases: a review. J. Wildl. Dis. 6, 31-34 (1970).

38. Hines, R. S., and Spira, D. T. *Ichthyophthirius multifiliis* (Fouquet) in the mirror carp, *Cyprinus carpio* L. I. Cause of infection. J. Fish Biol. 5, 385-392 (1973).

39. Hines, R. S., and Spira, D. T. Ichthyophthiriasis in the mirror carp *Cyprinus carpio* (L.). III. Pathology. J. Fish Biol. 6, 189-196 (1974).

40. Hines, R. S., and Spria, D. T. Ichthyophthiriasis in the mirror carp *Cyprinus carpio* (L.). IV. Physiological dysfunction. J. Fish Biol. 6, 365-371 (1974).

41. Hlond, S. Experiment in in vitro culture of *Ichthyophthirius*. FAO World Symposium On Warm Water Pond Fish Culture. Rome, 18-25 (1966).

42. Hoffman, G. L. Recommended treatment for fish parasite diseases. U. S. Bureau of Sport Fisheries and Wildlife, Fishery Leaflet 486, (1959).

43. Hoffman, G. L. Control and treatment of parasite disease of freshwater fishes. U. S. Bureau of Sport Fish and Wildlife, Fish Disease Leaflet 28, (1970).

44. Hoffman, G. L. Protozoan diseases of freshwater fishes: advances and needs. Proc. Int. Wildl. Dis. Conf. 3, (1976).

45. Hwang, S. W., Davis, E. E., and Alexander, M. T. Freezing and viability of *Tetrahymena pyriformis* in dimethyl sulfoxide. Science 144, 64-65 (1964).

46. Ingram, G. A. Substances involved in the natural resistance of fish to infection - a review. J. Fish Biol. 16, 23-60 (1980).

47. Johnson, A. K. Ichthyophthiriasis in a recirculating closed-water hatchery. Progr. Fish Cult. 23, 79-82 (1961).

48. Karpovitch, X. L., Rosenkovitch, E., Ben-Basset, H., and Izak, G. Structure and functional alterations in lymphocytes induced by cryopreservation. Cryobiology 17, 12-17 (1980).

49. Kozel, T. Contact and penetration of *Ictalurus punctatus* by *Ichthyophthirius multifiliis*. 55th Annual meeting Am. Soc. Parasitologists. Abstract 201, (1980).

50. Kozel, T., and Dobra, K. W. Scanning electron microscopy of *Ichthyophthirius multifiliis*. 53rd Annual meeting Am. Soc. Parasitologists. Abstract 251, (1978).

51. Kozel, T., Dobra, K. W., and Givens, L. S. Low temperature maintenance of *Ichthyophthirius multifiliis* in vitro. 54th meeting Am. Soc. Parasitologists. Abstract 114, (1979).

52. Kozel, T., and Dobra, K. W. Rapid vacuum method for removal of mucus from the epidermis of fishes. Progr. Fish Cult. 14, 18-19 (1979).

53. Leibo, S. P., and Mazur, P. The role of cooling rates in low temperature preservation. Cryobiology 8, 447-452 (1971).

54. Leibo, S. P., and Mazur, P. Methods for the preservation of mammalian embryos by freezing. In "Methods in Mammalian Reproduction" (J. C. Daniel, Jr., Ed.), p. 179-201. Academic Press, New York, 1978.

55. Leteux, F., and Meyer, F. A. Malachite green-formalin mixtures for the control of *Ichthyophthirius* and other protozoan parasites of fish. Progr. Fish Cult. 34, 21-26 (1972).

56. Lewis, D. H., Eural, T. E., Cannon, M. S., and Grumbles, L. C. T and B cell analogues from peripheral blood of immune channel catfish, *Ictalurus punctatus*. J. Fish Biol. 14, 31-37 (1979).

57. Litvan, G. G. Mechanism of cryoinjury in biological systems. Cryobiology 9, 182-191 (1972).

58. Lovelock, J. W., and Bishop, M. W. H. Prevention of freezing damage to living cells by dimethyl sulfoxide. Nature (London) 183, 1394 (1959).

59. Marchalonis, J. J. Isolation and partial characterization of immunoglobulins of goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*). Immunology 20, 161-173 (1971).

60. Marchalonis, J. J. (Ed). Comparative Immunology. John Wiley and Sons. New York. 227-275 (1976).

61. Mazur, P. Physical factors implicated in the death of microorganisms at subzero temperatures. Ann. N. Y. Acad. Sci. 85, 610-629 (1960).

62. Mazur, P. Causes of injury in frozen and thawed cells. Fed. Proc. 24, S-175-S-182 (1965).

63. Mazur, P. Cryobiology: the freezing of biological systems. Science 168, 939-949 (1970).

64. Mazur, P., Leibo, S. P., and Chu, E. H. Y. A two-factor hypothesis of freezing injury. Evidence from chinese hamster tissue-culture cells. Exp. Cell. Res. 71, 345-355 (1972).

65. McGann, L. E. Differing actions of penetrating and non-penetrating cryoprotective agents. Cryobiology 15, 382-390 (1978).

66. McGann, L. E. A versatile microcomputer-based temperature and cooling/warming rate controller. Cryobiology 16, 97-100 (1979).

67. McGann, L. E. Optimal temperature ranges for control of cooling rate. Cryobiology 16, 211-216 (1979).

68. McGann, L. E., and Farrant, J. Survival of tissue culture cells frozen by a two-step procedure to -196°C. II. Warming rate and concentration of dimethyl sulfoxide. Cryobiology 13, 269-273 (1976).

69. Meryman, H. T. Mechanics of freezing in living cells and tissues. Science 124, 515-521 (1956).

70. Meryman, H. T. Modified model for the mechanics of freezing injury in erythrocyte. Nature (London) 216, 333 (1968).

71. Meryman, H. T. The exceeding of a minimum tolerable cell volume in hypertonic suspension as a cause of freezing injury. In "The Frozen Cell" (G. E. W. Wolstenholent & M. O'Conner, Eds.) pp. 51-64. Churchill, London (1970).

72. Meryman, H. T. Cryoprotective agents. Cryobiology 8, 173-183 (1971).

73. Meryman, H. T. Freezing injury and its prevention in living cells. Annual Rev. Biophy. Bioeng. 3, 341-363 (1974).

74. Meyer, E. A., and Chadd, J. A. Preservation of *Giardia* trophozoites by freezing. Research Notes 53, 1108-1109 (1967).

75. Meyer, F. P. Parasites of freshwater fishes. II. Protozoa. 3. *Ichthyophthirius multifiliis*. Bureau of Sport Fisheries and Wildlife. Fish Disease Leaflet 2, (1966).

76. Meyer, F. P. Parasites of freshwater fishes. IV. Miscellaneous. 6. Parasites of catfishes. Bureau of Sport Fisheries and Wildlife. Fish Disease Leaflet 5, (1966).

77. Meyer, F. P. A review of parasite and disease of fishes in warm water ponds in North America. Proceedings of the FAO World Symposium On Warm Water Pond Fish Culture, FAO Fish Rep. 44, 290-313 (1968).

78. Meyer, F. P. Parasites of freshwater fishes. II. Protozoa. 3. *Ichthyophthirius multifiliis*. Bureau of Sport Fisheries and Wildlife. Fish Disease Leaflet 2 (revised), (1974).

79. Meyer, F. P., and Collar, J. D. Description and treatment of a Pseudomonas infection in white catfish. Applied Microbiology 12, 201-203 (1964).

80. Meyer, F. P., and Hoffman, G. L. Parasites and diseases of warmwater fishes. U.S. Dept. of the Interior, Fish and Wildife Service. Circular 127, 8-9 (1976).

81. Morris, G. J., and Farrant, J. Effects of cooling rate on thermal shock hemolysis. Cryobiology 10, 119-125 (1973).

82. National Academy of Sciences. Guidelines for the breeding, care and management of laboratory animals. 68-75 (1974).

83. Nigrelli, R. F., Pokorny, K. S., and Ruggieri, G. D. Notes on *Ichthyophthirius multifiliis*, a ciliate parasite on freshwater fish, with some remarks on possible physiological races and species. Trans. Amer. Micros. Soc. 95, 6-7-613 (1976).

84. Noble, E. R., and Noble, G. A. Parasitology: the biology of animal parasites. Lea & Febiger, Philadelphia. p. 151-152 (1961).

85. Olsen, O. W. Animal parasites: their life cycles and ecology. Univ. Park Press; Baltimore. p. 194-197 (1974).

86. Paperna, I. Infection by *Ichthyophthirius multifiliis* of fish in Uganda. Progr. Fish Cult. 34, 162-164 (1972).

87. Persidsky, M. D., and Ellett, M. H. Lysosomes and cell cryoinjury. Cryobiology 8, 345-349 (1971).

88. Pribor, D. B. Inner membrane protein: site of freeze-thaw damage. Cryobiology 9, 319 (1972).

89. Pribor, D. B. Biological interactions between cell membranes and glycerol or DMSO. Cryobiology 12, 309-320 (1975).

90. Rijkers, G. T., Frederix-Wolters, E. M. H., and Von Muiswinkel, W. B. The immune system of cyprinid fish. Kinetics and temperature dependence of antibody-producing cells in carp (*Cyprinus carpio*). Immunology 41, 91-97 (1980).

91. Rogue, M., and Puytoiac, P. DE. Infraciliature d'un nouvel Ophryoglenidae *Ichthyophthiriaides browni* n. g., n. sp. Protistologica 3, 465-473 (1968).

92. Schachte, J. H. A short term treatment of malachite green and formalin for the control of *Ichthyophthirius multifiliis* on channel catfish in holding tanks. Progr. Fish Cult. 36, 103-104 (1974).

93. Schaperclaus, W. Fischkrankheiten Akademie Verlag, Berlin. (1954).

94. Schwabe, K. G., and Terracio, L. Ultrastructural and thermocouple evaluation of rapid freezing techniques. Cryobiology 17, 571-584 (1980).

95. Simione, F. P., Daggett, P-M., McGrath, M. S., and Alexander, M. T. The use of plastic ampoules for freeze preservation of microorganisms. Cryobiology 14, 500-502 (1977).

96. Smith, A. M., Potter, M., and Merchant, E. B. Antibody-forming cells in the pronephrose of the teleost *Lepomis macrochirus*. J. Immun. 99, 876-882 (1967).

97. Suzuki, J. On the reproduction of *Ichthyophthirius multifiliis* Foquet in relation to water temperature. Bull. Jap. Soc. Sci. Fish 3, 265-272 (1935).

98. Tait, N. N. The effect of temperature on the immune response in cold-blooded vertebrates. Physiol. Zool. 42, 29-35 (1969).

99. Tate, E. H., and Cram, L. S. A method for simultaneous isolation and cryopreservation of bovine lymphocytes. Cryobiology 19, 136-146 (1982).

100. Taylor, A. C. The physical state transition in the freezing of living cells. Ann. N. Y. Acad. Sci. 85, 595-609 (1960).

101. Taylor, R., Adams, G. D. J., Boardman, C. F. B., and Wallis, R. G. Cryoprotection: permeant vs. nonpermeant additivies. Cryobiology 11, 430-438 (1974).

102. Thorpe, P. E., Knight, S. C., and Farrant, J. Cryopreservation of mouse lymphocytes. Cryobiology 11, 543 (1974).

103. Thorpe, P. E., Knight, S. C., and Farrant, J. Optimal conditions for the preservation of mouse lymph node cells in liquid nitrogen using cooling rate techniques. Cryobiology 13, 126-133 (1976).

104. Trump, G. N. Goldfish immunoglobulins and antibodies to bovine serum albumin. J. Immun. 104, 1267-1275 (1970).

105. Trump, G. N., and Hildermann, W. H. Antibody responses of goldfish to bovine serum albumin. Immunology 19, 621-627 (1970).

106. U. S. Dept. og Agriculture: Economics and Statistics Service. Aquaculture: Outlook and Situation. AS-1. 1-22 (1980).

107. U. S. Dept. of the Interior: Fish and Wildlife Service. Common parasites of fishes. Circular 144, 3-4 (1962).

108. U. S. Dept. of the Interior: Fish and Wildlife Service. Second report to the fish farmers: the status of warmwater fish farming and progress in fish farming research. Publication 113, 123 (1973).

109. Van Duijn, C. Diseases of fish. Water life. Dorset House, London (1956).

110. Wood, M. J., and Farrant, J. Preservation of mouse embryos by two-step freezing. Cryobiology 17, 178-180 (1980).

111. Yocum, D., Cuchers, M., and Clem, H. W. The hapten-carrier effect in teleost fish. J. Immun. 114, 925-927 (1975).

112. Zeilmaker, G. H., and Verhamme, C. M. P. M. A simplified method for freezing mouse embryos. Cryobiology 16, 6-10 (1979).

CRYOPRESERVATIVE STUDIES ON MOUSE SPLEEN LYMPHOCYTES, *ICHTHYOPHTHIRIUS MULTIFILIIS* AND *GIARDIA LAMBLIA*, WITH NOTES ON THE IMMUNE RESPONSE IN CHANNEL CATFISH

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Cryopreservation is the protection of living cells below their normal operating temperature. Cryoprotection is based on the avoidance or minimization of intracellular freezing and the minimization of damage to the cell from the environment of concentrated solutes and ice crystal formation during cooling. Cryopreservative studies were conducted on mouse spleen lymphocytes, *Ichthyophthirius multifiliis* and *Giardia lamblia*. Dimethyl sulfoxide (Me_2SO) was the cryoprotective agent of choice. A constant vacuum in a Dewar flask and an ethanol bath were used to obtain a controlled freezing rate of approximately -1.0°C/min.

Mouse spleen lymphocyte suspensions containing 13% (v/v) Me_2SO were frozen either by spontaneous crystallization or by seeding the medium. Optimal freezing conditions included spontaneous crystallization at -10°C with no perceptible latent heat of fusion, a controlled cooling rate of -0.828°C/min. to -70°C, and immersion into liquid nitrogen (LN_2) vapor to the storage temperature of -195.8°C. Optimal thawing conditions required rapid thawing while agitating in a 37°C water bath at 445.49°C/min. and Me_2SO dilutions at 5°C. The optimal lymphocyte viability after thawing was 77.4%. Me_2SO toxicity studies without freezing demonstrated that cell viability decreased 23.8%.

The effect of varying freezing conditions on the freshwater fish parasite *I. multifiliis* was investigated. Attempts to cryopreserve the parasites' three life cycle stages (trophozoite, cyst and tomite) using a variety of freezing media and procedures were studied. Toxicity assays were conducted on the freezing media, Me_2SO concentrations and the diacetyl fluorescein viability assay. This study resulted in the unsuccessful cryopreservation of all three parasitic stages. These results are in direct conflict with those of another author who reported the successful freezing of trophozoites and cysts using the same protocol and freezing media as used in this study.

The effect of varying freezing conditions on the human intestinal parasite *G. lamblia* (Portland - 1 strain, trophozoite stage) was investigated. A variety of freezing media and procedures were studied. Me_2SO toxicity assays were conducted on those concentrations used in the freezing protocol. The results of this study indicated a 6.5% (v/v) Me_2SO concentration yields a 90% survival based upon organism motility. A 30.9% cell viability was obtained by freezing without a cryoprotective agent.

The kinetics of the immune response in channel catfish (*Ictalurus punctatus*) was investigated for future studies concerning antibody (Ab) production to *I. multifiliis*. Sheep red blood cells (SRBC) and burro red blood cells (BRBC) were injected intraperitoneally into channel catfish to determine the time required to elicit a primary humoral response. Mucus and blood-sera samples were collected, heat-inactivated, and analyzed for Ab titre using hemolysis and hemagglutination assays. Zero mucus Ab titres were obtained. Varying and unrepeatable sera Ab titres were elicited. The repetition of this study is suggested, followed by the use of an organism such as *I. multifiliis* as antigen (Ag). By knowing the peak humoral response time to Ags such as SRBC and BRBC, one could approximate the peak humoral response time for *I. multifiliis* Ab production. One may then collect Ab specific for the organism and develop possible immunization schemes for fish.