Table 2. Urine and serum sodium, calcium, and potassium in milliequivalents per liter in *Fundulus kansae* held in fresh water and in sea water (S.W.) (mean ±S.E.).

Treatment	Specimen No.	Urine	Serum
		Sodium	
Tap water	6	12.8 ± 0.7	160 ±2
3 days in S.W.	5	261 ±65	231 ±2
20 days in S.W.	6	140 ±16	157 ±1
		Calcium	
Tap water	6	0.4 ± 0.1	4.9 ±0.2
3 days in S.W.	5	12.8 ± 2.1	9.2 ±0.7
20 days in S.W.	6	19.9 ± 2.4	6.0 ±0.4
		Potassium	
Tap water	6	0.17 ± 0.01	3.88 ±0.31
3 days in S.W.	5	6.4 ± 3.2	2.00 ±0.42
20 days in S.W.	6	2.0 ± 0.1	1.20 ±0.10

sea water, *F. kansae* does have functional glomeruli (*5*). The levels of urine production reported in Table 1 for adapted fish has been seen repeatedly, and there seems to be no reason to suppose that the fish are diuretic. Forster and Berglund found that the plasma osmotic pressure rose slowly in the diuretic fish and also noted marked shifts in the electrolyte content of the diuretic urine. No such shifts were seen here, and the osmotic pressure of the serum taken from the adapted fish was close to that found in fish held in tap water.

Finally, it should be pointed out that in no case did the osmotic pressure of the urine exceed that of sea water. While the osmotic pressure of this fluid is largely caused by sodium salts (Table 2), the sodium concentration of the hypertonic urine was, on the average, 180 meq/lit. less than that of the sea water. Thus, the kidney would never be able to handle the salt load imposed on the animal as a result of drinking sea water. Considerable salt must still be eliminated by another route, presumably the gills.

JON G. STANLEY
WARREN R. FLEMING
Department of Zoology,
University of Missouri, Columbia

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Freezing and Viability of Tetrahymena pyriformis in Dimethylsulfoxide

Abstract. *Ciliated protozoa may be preserved at very low temperatures.* Tetrahymena pyriformis, *suspended in 10 percent dimethylsulfoxide, survived when frozen in two steps, first to −20°C, then transferring immediately to a temperature of −196°C. Motility and ability to reproduce were recovered after freezing by this method and subsequent storage for 3 months in a liquid nitrogen refrigerator.*

Some amoebae have been frozen in the presence of glycerol (*1*) and have remained viable. Of the flagellated protozoa some of the trichomonads (*2, 3*) and trypanosomes (*4, 5*) have been subjected to freezing and thawing and have remained viable, but no reports of successful attempts to freeze and store cultures of ciliated protozoa at very low temperatures have come to our attention.

The American Type Culture Collection includes in its protozoan collection two strains of *Tetrahymena pyriformis.* Because of the difficulty of maintaining and preserving protozoa in culture, a study of freezing and preserving these strains at liquid nitrogen temperatures was undertaken.

Tetrahymena pyriformis, R. H. Hall strains H (ATCC 9357) and W (ATCC 10542), deposited in the Collection (*6*) in 1952, were maintained and propagated in tetrahymena broth (5 g of Difco proteose peptone, 5 g of tryptone, and 0.2 g of KH_2PO_4 in 1 liter of water). The cultures were grown at 26°C for 50 to 60 hours. Cells for experiments were obtained by pipetting 1 ml from the upper portion of a 7-ml broth culture. Samples to be frozen were usually collected from several cultures. Five-tenths milliliter of dimethylsulfoxide, $(CH_3)_2SO$ (*7*), was added to 4.5 ml of cell suspension, thus producing a 10 percent solution, by volume, of the protective agent.

Flat-bottomed, soft-glass vials (14 by 85 mm) were used to contain the samples. A flat-bottomed vial was selected so that the cell suspension would form a layer of even thickness.

An upright deep freezer (household model) was used for the initial stage of the two-step freezing procedure. This freezer had an air temperature between −17° and −20°C and a shelf temperature of −20° to −23°C. When the specimens were placed in the freezer, the door was opened and closed rapidly so that relatively constant temperatures were maintained.

The temperatures of the air, shelf, and specimen were recorded on a four-point Honeywell Brown Electronik recorder (*8*) throughout the cooling, and the cooling rates were determined from the specimen temperatures recorded by means of a thermocouple inserted into one of the sample vials.

In the second step of the freezing process a liquid nitrogen bath was used to lower the specimen temperature from −20°C to −196°C. For long-term storage of the specimens a liquid nitrogen refrigerator (*9*) was used.

After the protective agent (dimethylsulfoxide) was added to the culture the mixture was incubated at 26°C for 30 minutes. A sample of the suspension (0.2 ml) was then pipetted into each vial. The vials were plugged with cotton but not sealed, and were placed upright on the shelf of the freezer for 20 minutes. At the end of this time the first stage of freezing was complete, and three vials were removed at random from the freezer and the material was thawed immediately. The remaining vials were plunged directly into liquid nitrogen. After 10 minutes in liquid nitrogen several specimens were thawed, and the others were stored in the gas compartment of the liquid nitrogen refrigerator where the temperature was not above −170°C.

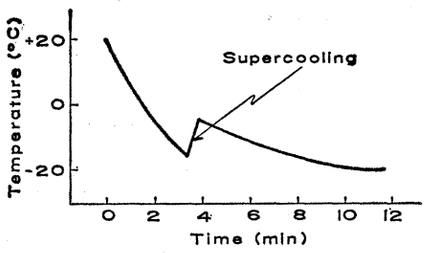

Fig. 1. Cooling rates for *Tetrahymena pyriformis* during the first step of the freezing procedure (+20°C to −20°C).

The frozen material in the vials was thawed by agitating in a water bath at 35°C. Approximately 18 or 36 seconds were needed for a 0.2-ml ice sample to thaw from −20°C or −196°C, respectively, at which time 2 ml of tetrahymena broth were added to the thawed material. After 10 minutes at ambient temperature samples were removed from the vial and examined microscopically for motility. Motility was used as the criterion of survival and was determined quantitatively by counting the number of motile cells in 0.05 ml of the sample distributed in droplets on a slide. The thawed specimens were incubated at 26°C to determine whether the motile cells would reproduce and whether normal subcultures would develop.

As shown in Table 1, the motility of *T. pyriformis* was affected by both steps in the freezing procedure. For strain W the loss in motility was greatest during the first step of freezing and least during the rapid drop from −20°C to −196°C. Regardless of the effect on the motility of the cells during the first freezing step, the number of motile cells in the sample after cooling to −196°C was fairly consistent in all experiments with this strain.

Strain H was more sensitive to the cooling temperatures. It had a greater loss of motility during both stages of the freezing procedure. Also with this strain the total number of motile cells recovered from samples at −20°C and −196°C was less constant from experiment to experiment. Nevertheless, viable cells recovered from samples of both strains developed to apparently normal cultures after 48 hours of incubation at 26°C.

Table 1. Effect of freezing on the motility of *Tetrahymena pyriformis*. Results are based on the number of motile cells per milliliter. Strain W is ATCC No. 10542; strain H is ATCC No. 9357.

Strain	Motile cells × 10^3		
	Before freezing	At −20°C	At −196°C
W	40.0	14.6	9.6
W	40.0	12.0	11.0
W	57.2	30.0	12.2
W	40.0	14.5	12.5
H	51.6	17.7	10.0
H	60.0	21.4	5.0
H	60.0	13.3	7.6

Figure 1 represents a typical curve of cooling rates for the first step of the freezing from +20° to −20°C. At −15°C the cell suspension appeared to be supercooled. The rate of cooling to this point was approximately 9°C per minute. The temperature of the cell suspension then rose rapidly to −5°C and cooled slowly at a rate of approximately 2°C per minute to −20°C, at which point the suspension seemed to be frozen solid. The temperature of the specimen, as recorded, did not go lower than −20°C during the first step of freezing.

Thus, *T. pyriformis* can be frozen to −20°C, then rapidly cooled further to −196°C, and recovered after thawing. Preliminary results of experiments to determine viability of samples stored in the gas phase of liquid nitrogen indicate that after cooling to −196°C, these organisms can be stored between −170°C and −196°C without any further loss of viability. Records are available for 3 months' storage of both strains at these temperatures.

Apparently the critical phase for these protozoa is the initial step from +20°C down to −20°C, and surviving this phase seems to depend on the rate at which the temperature falls. Several different rates of cooling were tried: for example, 1°C or 3°C per minute to −20°C, then directly into liquid nitrogen at −196°C; or rapid cooling from +20° to −76°C by placing the culture directly into dry ice slush; and from +20°C to −196°C by plunging it into liquid nitrogen. However, no viable cells were recovered from samples so treated. Viable cells were recovered only under the conditions reported. Nevertheless, even under the optimum conditions for survival, the proportion of cells that maintained their motility was not more than one-half, and in many cases was as low as one-fifth, of the initial population (Table 1).

The lethal effect upon these living cells seems to take place at the time the liquid phase of the suspending fluid changes into the solid. In other experiments, in which the cooling rate was 1°C per minute, observations made from samples taken out at 1°- to 2°-intervals during the cooling indicated that the loss of motility occurred after the cultures reached the frozen state (between −20°C and −30°C).

If ice formation causes death of the cells, the question arises why these organisms do not respond uniformly at the temperature at which ice formation occurs. Only by changing the cooling rate were conditions created that had less injurious effects on the cells studied.

It is a disappointing fact that organisms do respond differently to cooling rates. Before very low temperatures can be used for the long-term preservation of fastidious microbes a cooling rate that has less injurious effects upon cells must be sought.

SHUH-WEI HWANG
E. E. DAVIS
M. T. ALEXANDER
American Type Culture Collection, Rockville, Maryland 20852

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Chromosome Number and Morphology of a Human Preinvasive Neoplasm

Abstract. *The cervical epithelium from five patients with varying degrees of histologically proven intra-epithelial neoplasia can be grown successfully in tissue culture. After four or less successive transfers, the cells are diploid and have a normal karyotype.*

If there is a relationship between chromosome morphology and neoplasia, evidence of the relationship would be obtained most readily by studying cells from early cancers, particularly neoplasms which are designated as preinvasive, rather than cells from advanced cancers (*1*). Preinva-