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FOOD SUPPLY AS A FACTOR IN THE SURVIVAL OF FROZEN AND THAWED ROTIFERS*

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In an earlier communication[2] it was shown that rotifers (*Philodina acuticornis odiosa*) could survive freezing to liquid nitrogen temperatures and subsequent rapid thawing. Survivals of populations of animals varied from 40 to 60% over a cooling rate range of 1 to 10°C per min and glycerol concentrations of 1 to 5%. There were several disturbing variations and inconsistencies in this initial study, including some abnormally low survival values (10 to 20%). No definitive reasons were found to explain these low values, but it was suggested that a possible cause might be the lack of control of nutritional state among the various populations.

The present report describes survival studies in which the food supply (*Aerobacter aerogenes*) for animals recovering from freezing and thawing was maintained at a high level. In addition to repeating the earlier work with glycerol-protected animals, another series of experiments was carried out with dimethyl sulfoxide (DMSO) as the cryoprotective agent. It will be shown not only that ensuring an adequate food supply during recovery tends to reduce variability in survival over the cooling rate range studied, but that the absolute survival is significantly higher.

MATERIALS AND METHODS

The general procedures for culturing the rotifers (*P. acuticornis odiosa*) as well as the freezing techniques were similar to methods previously described.[2]

Populations of animals were placed on small copper discs (6-mm diameter, 0.25 mm thick) and frozen either at various predetermined rates from 1 to 20°C per min in a Linde BF-3, or ultrarapidly by plunging into liquid nitrogen-cooled Freon 22. Thawing was accomplished by rapidly immersing the frozen specimen discs into 1 cc of nutrient at room temperature. One experiment employed slow, controlled thawing to test the effect of this parameter on survival. Glycerol and DMSO (both Baker reagent grade) were employed as cryoprotective agents by treating animal populations with dilute solutions before freezing. Earlier experience with glycerol[2] suggested that a standard incubation time of 2½ to 3 hrs in a 2½% solution gave optimal survival results so that these values were used throughout the study. A few experiments employing a glycerol concentration of 7.2% were also included in order to compare survivals with the DMSO-treated animals on an equimolar basis. DMSO was utilized in concentrations ranging from 1 to 10%. Incubation time in DMSO was standardized to 1 hr after studying the effect of pretreatment times ranging from 10 min to 3 hrs. Glycerol and DMSO dilutions were prepared in fresh baked lettuce medium[2] containing no bacteria. After freezing and thawing, the rotifers were rinsed four times with fresh baked lettuce medium and 8 drops (about 0.33 cc) of a dense suspension of *Aerobacter aerogenes* were added to the 1 cc of medium. The mechanical process of rinsing the animals was shown to have no effect on survival. Recovery was scored by observing and counting moving or swimming animals 16 to 18 hrs after thawing took place.

A given experimental or control point was always based on a sample of more than 100 animals and in many cases involved 200 to 300 rotifers. Reproducibility of the survival and other counting data is estimated to be better than 10%.

RESULTS

Experiments were carried out designed to test the value of the addition of bacterial suspension on the survival of rotifers. These populations were glycerol-treated (2.5% for 3 hrs) and slowly frozen at 5°C per min. Bacterial suspension was added after freezing and thawing was completed

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TABLE 1

EFFECT OF BACTERIAL ADDITION ON ROTIFER SURVIVAL AFTER GLYCEROL TREATMENT

Number of Drops Added*	Controls		Frozen	
	Number of animals	Recovery	Number of animals	Recovery
		%		%
0	185	96	342	76
2	193	97		
4	205	100	308	80
6	234	100		
8	253	100	273	91
10	215	100		

* One drop = approximately 0.04 cc of suspension containing 1×10^7 to 5×10^7 cells per cc.

and after the animals had been rinsed four times with fresh baked lettuce medium. Controls were treated in the same way but not frozen. Results of a typical experiment are shown in Table 1. It is seen that bacterial addition had little or no effect on the unfrozen controls, but that a significant enhancement of survival (about 15%) resulted in the case of frozen animals when recovery took place in medium containing an adequate food supply. Plate counts of the bacterial suspensions indicated that the concentration of bacteria was in the range 1×10^7 to 5×10^7 per cc; subsequent suspensions contained approximately similar numbers. Obviously, different numbers of rotifers were fed in this way with somewhat varying numbers of bacteria which, at room temperature, probably multiplied to some degree in the 18 hrs before evaluation of survival. Therefore, precise quantitation of the bacterial addition was not attempted, nor is the exact number important as long as it is a sufficient amount. In the experiments which will now be described, an adequate amount of bacterial suspension was always added after the final rinsing process.

In order to determine the optimal DMSO concentration for pretreatment, animals were incubated in baked lettuce medium containing 1, 5, 7.5, or 10% DMSO for periods of 1½ to 3 hrs, rinsed, treated with nutrient, and assayed for survival. No freezing was utilized in this series of experiments. Essentially 100% survival was realized for the 5 and 7.5% concentrations. At the highest concentration (10%) survival ranged between 80 and 90%, suggesting some toxicity. A surprising result was obtained for the survival of animals treated with the lowest concentration of DMSO (1%). Although 1% DMSO showed little effect on the survival of animals incubated for 10 to 30 min, successively longer incubation produced definite lethality. Only 25% of the rotifers recovered from an exposure of 3 hrs to the 1% DMSO solution. This peculiar lethality in the face of high survival at greater DMSO concentrations will be discussed below. The value of 7.5% DMSO was utilized in subsequent freezing experiments.

As mentioned earlier, the time of incubation in glycerol had been assessed in previous experiments[2] and was not reinvestigated. A 3-hr incubation in glycerol was employed routinely. Similar data, however, were not available for DMSO, and a reasonable incubation time had to be determined. Table 2 shows results of incubating rotifers, at room temperature, in 7.5% DMSO for times ranging from 10 min to 3 hrs before freezing. The survival data indicate that even at 10 min the DMSO is exerting its full protective influence and that survival is not appreciably altered within the span of incubation times studied. For experiments designed to examine other parameters, the time of incubation in DMSO was standardized at 1 hr

The survival data for frozen and thawed rotifers as a function of cooling rate are summarized in Tables 3 and 4.

Two kinds of controls were always included together with the experimental populations One of these controls involved animals which were pretreated with a cryoprotective agent and handled in the same way as the experimental group but not frozen. These populations always yielded essentially 100% survival. The second type of control involved animals which were frozen but without prior pretreatment with

TABLE 2

EFFECT OF INCUBATION TIME IN DMSO (7.5%) ON SURVIVAL AFTER FREEZING AND THAWING

Incubation Time	Number of Animals	Recovery
		%
10 min	430	86
½ hr	298	89
1 hr	191	86
1 hr	415	84
2 hrs	215	88
3 hrs	313	83
Mean		86 ± 2

cryoprotective additives. These populations yielded very low levels of survival ranging from 0 to 10%. Therefore, the experimental data presented reflect a fairly accurate measure of the value of DMSO or glycerol pretreatment. The animals pretreated with DMSO are seen to yield consistently high survivals between 90 and 100% (mean = 96, σ = 2.4) apparently independent of cooling rate. The exception is for the case of ultrarapid cooling, which yielded essentially no survivors either with or without the addition of cryoprotective agents. Animals treated with glycerol (Table 4) yielded consistently lower and more variable survivals (73 to 94%) than comparable DMSO-treated populations (92 to 99%). The experimental points appear to vary with cooling rate for the glycerol-treated populations, suggesting a dependence on this parameter over the rate studied. A consideration of inherent errors and the rather slight spread (σ = 9.5), however, makes the likelihood of a real dependence questionable. The experiment which involved slow thawing (approximately 2°C per min) yielded very small numbers of survivors for both DMSO- and glycerol-protected populations. The rapid thawing procedure was far more effective in ensuring survival of a large fraction of the frozen individuals.

When the glycerol concentration was adjusted to an equimolar basis (7.2%) to the DMSO pretreatment solution (7.5%) and utilized as cryoprotective agent, the results were as follows. Control (nonfrozen) populations experienced about a 12% reduction in survival at the higher glycerol concentration. At a cooling rate of 3°C per min, survival after freezing and thawing was 60%, and at 12°C per min, recovery fell to 34%. Obviously, the glycerol was not as effective at this higher concentration and could not be directly compared to DMSO on an equimolar basis because of the appearance of toxic factors.

TABLE 3

SURVIVAL OF DMSO-TREATED (7.5%, 1 HR) *PHILODINA* AFTER FREEZING AND THAWING AS A FUNCTION OF COOLING RATE

Cooling Rate	Number of Animals	Recovery
°C per min		%
1.5	277	98
3	436	97
5	287	98
11	495	99
12	446	93
13	362	92
16	173	95
19	450	93
20	287	96
20	318	96
Mean		96 ± 2.4
20	284	2.1*
Ultrarapid in Freon	266	0

* Slow thawing (about 2°C per min).

TABLE 4

SURVIVAL OF GLYCEROL-TREATED (2.5%, 3 HRS) *PHILODINA* AFTER FREEZING AND THAWING AS A FUNCTION OF COOLING RATE

Cooling Rate	Number of Animals	Recovery
°C per min		%
1.5	188	88
5	185	80
11	263	73
16	75	73
20	228	94
20	214	85
Mean		82 ± 9.5
20	205	0*
Ultrarapid in Freon 12	81	0

* Slow thawing (about 2°C per min).

DISCUSSION

Several unambiguous conclusions can be reached from examination of the foregoing data, particularly when they are taken together with the earlier studies. First of all, it is quite clear that the nutritional environment of recovering rotifers (at least the species here studied) is important in the ultimate survival statistics. Whereas the earlier, nutritionally uncontrolled populations (glycerol-protected) yielded only 40 to 60% survival, well fed populations, reported here, yielded an average survival of 82% (σ = 9.5) with no values below 73%. Equally important is the observation that apparent variations of survival as a function of cooling rate were much reduced by food addition. Thus, variations of 20 to 30% in survival ascribed earlier to either the cooling rate parameter or unknown causes are seen to disappear under the present controlled nutrition conditions. Cooling rate, at least in the restricted range of 1 to 20°C per min,

appears to exert little influence on final survival values.

The enhancement of survival due to feeding is not manifest in the data on animals not subjected to cryoprotective treatment. Indeed, whereas an average, over the cooling rate range, of 10% of the animals survived after no pretreatment in the earlier studies, only about 3% survived after feeding. One cannot draw any conclusions from a comparison of these two small figures, particularly when we recall that errors of the order of 10% or higher must be invoked in such studies. However, it is probable that the typical unprotected animal is so severely traumatized by freezing and thawing that the addition of food material has little beneficial action. Indeed, it is conceivable that a high density bacterial culture might infect and destroy a seriously damaged, but potentially viable, individual. On the other hand, it may be speculated that the beneficial effect of high nutrient concentration during recovery in cryoprotected populations has a metabolic basis. Conceivably, energy production and biosynthetic activity in well-fed populations can aid in the recovery process.

The present study also demonstrates that DMSO is highly successful as a cryoprotective agent for rotifers of this species. An average value of 96% ($\sigma = 2.4$) survival for a combined total of over 3500 animals was obtained over the slow cooling rate range (1° to 20°C per min). Such exceedingly high viability is surprising when we consider that rotifers are rather complex multicellular animals possessing a high degree of structural organization. Indeed, such consistently high survival after freezing and thawing is rarely encountered even in such frost-hardy cell types as erythrocytes or spermatozoa.

As mentioned with reference to the DMSO incubation time studies, very low concentrations (1%) as well as high concentrations (10%) of this reagent show definite toxic effects on populations of *Philodina*. The reason for the effect at low concentrations may lie in the behavior and physiological response of the animals to DMSO addition. An actively swimming and feeding population goes into a state of inactivity or "hibernation" when treated with DMSO concentrations of 5% or higher. This kind of response was also noted in the earlier work on glycerol protection.[2] This hibernative state is not induced by 1% DMSO solutions, so that the animals continue their usual pattern of feeding and swimming. It is possible that the toxic properties of DMSO are potentiated or amplified in animals in an active physiological state. This speculation is consistent with our observations and, more importantly, can be tested by a number of experimental approaches.

The very low survival after slow, controlled thawing (2°C per min) suggests that highly lethal events are occurring during this period since the animals were frozen at rates giving high survival for rapidly thawed populations. Consideration of recrystallization phenomena during thawing may not be sufficient to account for such lethality under these conditions (slow cooling, slow thawing).

The finding that an exposure to DMSO of only 10 min affords essentially full protection is consistent with observations on the permeation kinetics of this reagent in ascites cells. It appears that the rotifer cuticle provides no appreciably greater barrier to the penetration of DMSO than typical cell membranes.

Summary

The provision of an adequate food supply (bacteria) after freezing and thawing the rotifer *Philodina* is shown to increase the percentage survival. Furthermore, variations in the survival statistics as a function of cooling rate appear to be minimized by the addition of food after thawing. Both glycerol and DMSO provide high levels of cryoprotection, DMSO being slightly more effective. Recovery of close to 100% of a population can be achieved with proper attention to pretreatment, cooling, and thawing procedures.

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