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Effects of Various Rates of Freezing on the Metabolism of a Drought-tolerant Plant, the Moss *Tortula ruralis* [1]

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ABSTRACT

The response of the drought-tolerant moss *Tortula ruralis* ([Hedw.] Gaertn., Meyer, Scherb.) to freezing and thawing at controlled rates has been studied. Slow freezing (at 3 C per hour to −30 C) of hydrated *T. ruralis* leads to only temporary, reversible changes in metabolism. These changes can be considered to result from desiccation due to extracellular ice formation. In contrast, rapid freezing in liquid N_2 and thawing in 20 C water leads to deterioration in all aspects of metabolism studied: ribosome, protein, and ATP levels decrease, and *in vivo* and *in vitro* protein synthetic activity is lost rapidly. Such changes probably result from intracellular ice formation. Following freezing and thawing at an intermediate rate (60 C per hour), only ATP levels and *in vivo* protein synthesis are reduced. The protein-synthesizing apparatus (the polyribosomes) remains intact and active in an *in vitro* protein-synthesizing system even 24 hours after one 60 C per hour freeze-thaw cycle. These metabolic responses are discussed in terms of the two-factor hypothesis of Mazur *et al.* (1972 Exp. Cell Res. 71: 345–355).

Cells of certain plants are able to withstand subzero temperatures, providing that the rate at which they are frozen is sufficiently low (*e.g.* 5 C/hr or less [12, 17]). According to the widely accepted hypothesis proposed by Mazur (18), during slow freezing ice crystals grow outside the cell drawing water from the cytoplasm. Thus, tolerance of slow freezing could be attributed to the cell's capacity to tolerate desiccation stress induced by extracellular ice formation. In plants which do sustain damage due to freezing it is not easy to distinguish whether this is due to ice-induced desiccation damage of the cells, or to some other factors such as intracellular freezing and low temperature. In this study we have examined the effects of various freezing treatments on a known desiccation-tolerant plant—the moss *Tortula ruralis* (2, 3). Our premise is that extracellular ice formation induced by slow freezing should not lead to irreversible damage to such a plant. When damage does occur, *i.e.* following faster freeze-thaw cycles, we should be able to distinguish cellular damage other than that caused by freeze-desiccation. Such an approach, using a desiccation-tolerant plant, has not previously been used.

MATERIALS AND METHODS

The moss *T. ruralis* ([Hedw.], Gaertn., Meyer, Scherb.) was collected, stored, and prepared for experiments as previously described (2).

Freezing and Thawing Treatments. Batches (300 mg) of *T. ruralis* gametophores were placed in 150-ml Corex centrifuge tubes and 2.5 ml of sterile distilled H_2O were added. Capped tubes were submerged in the cooling solution of a Haake KT52 low temperature bath equipped with a Haake PG 11 programmer. Using this apparatus, linear cooling and warming rates were obtained from 3 C/hr to 60 C/hr over the range from +20 C to −30 C. For any one treatment the same freezing and thawing rates were used, except in the experiments reported in Figure 2 (see legend). The temperature of the water in the tube with the moss was monitored using a YSI 42 SL thermistor with No. 409 probe. The water medium never supercooled to less than −6 C and the moss was not kept at the minimum temperature reached for longer than 5 min. Rapid temperature changes were obtained by plunging Corex tubes containing moss into liquid N_2 for 5 min. Thawing was carried out rapidly by placing the moss in a water bath at 20 C. It is reasonable to presume that by using this treatment the rate of thawing approximated that of freezing; we do not, however, have the appropriate equipment to test this.

The metabolic activity of the thawed moss was tested immediately upon thawing of the medium (*i.e.* at about 2 C) and at 0 hr, 2 hr, or 24 hr after the temperature had reached 20 C (subsequently referred to as the "end of treatment"). Moss maintained at 20 C for 2 hr and 24 hr after thawing to this temperature was placed in 2.5 ml of sterile distilled H_2O in 5-cm Petri dishes and kept on the laboratory bench.

Drying Treatment. Samples of partially or fully dried *T. ruralis* were obtained by periodically removing 300-mg fresh wt equivalents of the moss from a desiccator containing silica gel (0% relative humidity).

Incorporation of [^{3}H]Leucine into Protein. Thawed samples (300 mg fresh wt) of the moss were washed and placed in 2.5 ml of sterile distilled H_2O. Ten μCi of L-[4,5-^{3}H]leucine (Amersham/Searle 60 Ci mmol^{-1}) were added with mixing. After incubation the moss was washed three times with distilled H_2O to remove excess radioactivity. Neither washing with dilute nonradioactive leucine (220 mg ml^{-1}) nor a 15-min exchange at 4 C with nonradioactive leucine was found to remove additional free radioactive leucine.

Radioactively labeled protein was extracted as previously described (3) except that the moss was ground in 10 ml of cold Tris-glycine buffer (0.1 M, pH 8.4). The protein was dissolved in 2 N NaOH and 0.1-ml aliquots were dried on Whatman GF/A glass filter discs, placed in scintillation vials with scintillation cocktail, and counted in Nuclear-Chicago Isocap/300-PDS/3 counter. Also, soluble radioactivity in 0.1-ml aliquots of the initial tissue homogenate supernatant was determined. This radioactivity is subsequently referred to as the basis for our uptake measurements. Protein concentration was determined by the Folin method.

Extraction of Moss Ribosomes. The ribosome extraction technique was based on that described previously (4, 10), with the following modifications. *T. ruralis* (500 mg) was ground in 10 ml

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of grinding buffer and the final ribosomal pellet was resuspended in 0.6 ml of the same buffer without sucrose. A 0.2-ml aliquot (controls containing 0.3 mg of rRNA as determined according to Marcus *et al.* [15]) was layered on top of a 5.6-ml linear (10–32% w/v) sucrose gradient.

For the purpose of *in vitro* incorporation studies, ribosomes were extracted from 1 g of *T. ruralis* and the final ribosomal pellet was resuspended in 1 ml of buffer and cleared by slow (500*g*) centrifugation for 10 min. Aliquots (0.08 ml) containing 100 to 120 μg of rRNA were used in the assays.

In Vitro Protein Synthesis. The assay method for *in vitro* incorporation of [^{14}C]leucine as previously reported by Gwóźdź and Bewley (10) was employed. The L[U-^{14}C]leucine specific radioactivity was greater than 270 mCi mmol^{-1} (New England Nuclear) and the protein content of the wheat germ dialysate was only 10 μg/μl. *In vitro* polyphenylalanine synthesis was carried out as described in reference 10. Wheat germ supernatant was prepared according to references 10 and 11, except that 1 g of wheat germ (Maple Leaf Co., Calgary, 1975 harvest) was used.

ATP Extraction and Determination. These procedures were based on those previously reported (1, 5) and modified in our laboratory by Krochko (personal communication).

T. ruralis (200 mg) was ground in 9 ml of cold perchloric acid (35% v/v). The homogenate was kept on ice for 15 min and then centrifuged at 20,000*g* for 35 min. The supernatant was titrated (over ice) to pH 7 with 6 N and 0.1 N KOH (both containing 50 mM K_2HPO_4) and left on ice for a further 15 min. The resulting precipitate was removed by a 20,000*g* centrifugation for 15 min and the supernatant diluted to a constant assayable volume.

For the firefly extract assay for ATP, small vials were placed inside glass scintillation vials, cooled to 0 C and maintained at this temperature throughout the assay. Distilled H_2O (0.9 ml) and cold firefly extract (0.1 ml, Sigma, prepared according to manufacturer's instructions and filtered through Whatman No. 1 filter paper) were mixed in the small vials. A refrigerated Packard 3320 scintillation counter was used in the low energy (tritium) setting, counting in both A and B channels with discriminator set at 50 to 1,000, at 52% gain and with the coincidence switch off. The sample (0.1 ml) was injected with force from a syringe and the vial lowered into the counting chamber immediately for a 6-sec counting. Background luminescence was subtracted and ATP concentration was determined using standard ATP (Sigma) solutions at 1 to 15 pmol/0.1 ml of solution.

Loss of Conductive Materials from *T. ruralis*. The moss (300 mg in 2.5 ml of sterile distilled H_2O) was frozen and thawed in capped 50-ml Kimax tubes. One hr after reaching 20 C, a further 17.5 ml of sterile distilled H_2O were added. Conductivity of the solution was measured using a conductivity meter (Radiometer, Copenhagen). Background conductivity of the distilled H_2O was subtracted from all readings.

RESULTS

***In Vivo* Uptake and Incorporation of [^{3}H]Leucine.** Freezing to and thawing from −30 C of hydrated *T. ruralis* at progressively faster rates lead to increased cellular damage, as determined by the decreasing ability of the moss to take up and incorporate [^{3}H]leucine into protein (Fig. 1, A and B). Freezing and thawing at 3 C/hr do not affect leucine incorporation and slightly enhance uptake. Two hr after a 60 C/hr freezing and thawing treatment little leucine enters the moss or is incorporated into protein. It is apparent that both uptake and synthetic processes are affected, for following a 3 C/hr freeze-thaw cycle 23% of the leucine in the moss tissue is found in protein but after 60 C/hr only 5%. Furthermore, moss frozen and thawed at 60 C/hr does not recover its ability to take up and incorporate leucine 24 hr after thawing (data not presented). The effects of 6, 18, and 30 C/hr freeze-thaw cycles are also evident from Figure 1, A and B. Freezing in liquid N_2 followed by rapid thawing in water at 20 C irreversibly impairs both uptake and incorporation.

We then determined whether it was the events taking place during 60 C/hr freezing, or the thawing, which result in reduced uptake and protein synthetic capacity. As shown in Figure 2, A and B, freezing at 60 C/hr and thawing at 3 C/hr are as inhibitory as 60 C/hr freezing and thawing (Fig. 1, A and B). On the other hand freezing at 3 C/hr and thawing at 60 C/hr only slightly affect both uptake and incorporation. Thus, it is the fast rate of freezing, not the thawing, which results in the greatest cellular damage.

We also found that following 3 C/hr freezing, moss could be stored for at least 1 month in the frozen state before thawing (Fig. 2, A and B); moss frozen to −30 C at 3 C/hr could be plunged into liquid N_2 for 10 min, replaced into the cold water bath at −30 C, and then brought to 20 C at 3 C/hr without reduction in either uptake or incorporation of [^{3}H]leucine into protein (data not presented).

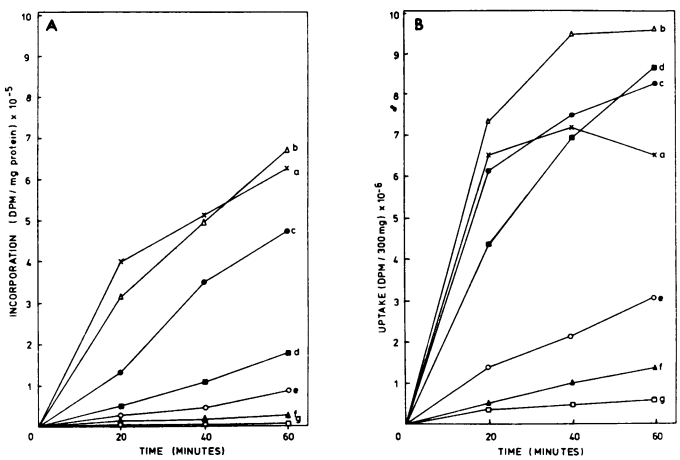

FIG. 1. A: incorporation of [^{3}H]leucine into a hot trichloroacetic acid-precipitable fraction (soluble protein) 2 hr after freezing and thawing of hydrated moss at the same rates. The numbers in parentheses below indicate the percentage radioactivity taken into the tissue which was incorporated into protein after 60 min: a: average of controls (unfrozen moss kept at 20C) (31%); b: 3 C/hr (23%); c: 6 C/hr (19%); d: 18 C/hr (6%); e: 30 C/hr (7%); f: 60 C/hr (5%); g: liquid N_2 submersion and fast thaw in water at 20 C (3%). B: entry of [^{3}H]leucine into the moss tissue with time (uptake) after freezing and thawing at equal rates. Legend as in A.

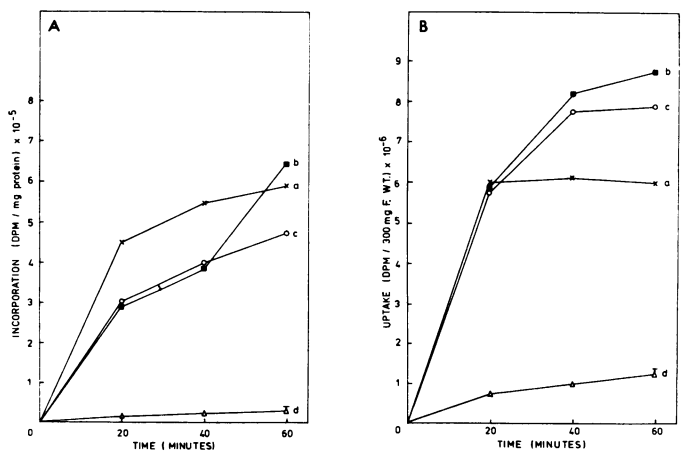

FIG. 2. A: [^{3}H]leucine incorporation into a hot trichloroacetic acid precipitate after freezing and thawing at dissimilar rates and effect of long term storage after 3 C/hr freezing on protein synthesis. a: control, unfrozen moss kept at 20 C; b: moss frozen at 3 C/hr to −30 C, stored for 15 or 30 days at −20 C and thawed at 3 C/hr; c; moss frozen at 3 C/hr, thawed at 60 C/hr; d: moss frozen at 60 C/hr, thawed at 3 C/hr. B: [^{3}H]leucine uptake into moss tissue after uneven freeze-thaw treatments and following a long term storage after 3 C/hr freezing. Legend as in A.

Effect of Freezing on Integrity of Ribosomes and Polysomes. Ribosomal material (containing single ribosomes and polysomes) was extracted from frozen and thawed moss and analyzed on a linear sucrose gradient. Freezing in liquid N_2 followed by fast thawing causes a decrease in total extractable ribosomal material after 24 hr, and degradation of both ribosomes and polysomes after 2 hr and particularly after 24 hr (Fig. 3, E and F). Following 60 C/hr freezing and thawing extractable polysomes are comparable with those of unfrozen, control moss (Fig. 3, C and D). Thus, while this freeze-thaw treatment causes loss of *in vivo* protein synthetic activity, this is not due to degradation of the polysomes, even 24 hr after the end of the treatment. After a 3 C/hr treatment, polysomes, as expected, are conserved.

Next we determined if the polysomes and ribosomes in moss frozen and thawed at different rates had retained their capacity for protein synthesis. This we tested using two *in vitro* systems: one for polysome activity (involving [^{14}C]leucine incorporation into polypeptides using the endogenous mRNA), and one for ribosome activity (involving [^{14}C]phenylalanine incorporation catalyzed by the synthetic message, poly(U)). Neither 3 C/hr nor 60 C/hr treatments reduced polysome activity, nor in the case of 60 C/hr did they substantially reduce ribosome activity. In fact after 3 C/hr treatment polysome activity increased at the expense of ribosome activity (Table I). This is probably a consequence of the accumulation of larger polysomes during the prolonged period that the moss was at low (but above zero) temperatures during temperature decrease and increase. We have observed that moss maintained at 2 to 5 C contains larger polysomes than moss at 15 to 20 C (data to be presented elsewhere). The rapid decline in *in vitro* ribosome and polysome activity after liquid N_2 treatment is in agreement with the loss of these components as visualized on sucrose gradients.

Table I. In vitro incorporation by polyribosomes and ribosomes extracted from moss at different periods after freezing treatments.

	Hours after the end of treatment, i.e. time at 20C	Polysome directed ^{14}C-leucine incorporation as % of control	Poly-U directed ^{14}C-phenylalanine incorporation as % of control
Control	0	100% (900 dpm/20 min)	100% (4600 dpm/20 min)
3C/hr	2	138	65
60C/hr	0	108	88
	2	89	94
	24	102	80
Liquid nitrogen	0	102	90
	2	3	20
	24	0	5

Rate of thawing was the same as that of freezing.

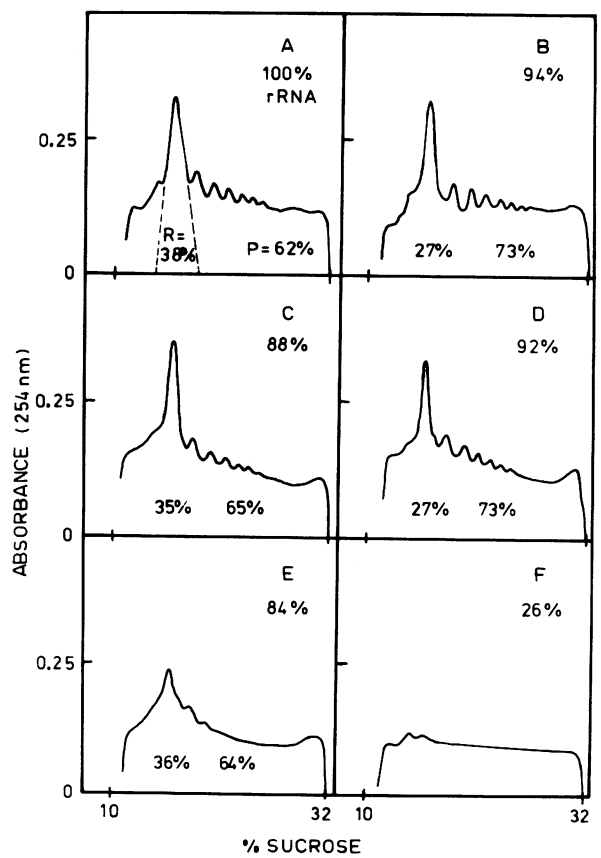

FIG. 3. Effect of freezing treatments on ribosomal material. Absorbance patterns of ribosomes and polyribosomes separated on a 10 to 32% sucrose gradient. Per cent values within the profiles indicate relative proportions of the ribosomal and polyribosomal fractions. Ribosomal material (0.2 ml or 0.94 ml mg rRNA equivalent) was applied to the gradient in all cases except E and F, where 0.4-ml aliquots were applied containing decreased amounts of rRNA. Per cent values in top right corner of each graph represent per cent of control rRNA levels extracted. A: control (untreated moss) (R = ribosomes, P = polyribosomes); B: 2 hr after freezing and thawing at 3 C/hr; C: 2 hr after freezing and thawing at 60 C/hr; D: 24 hr after freezing and thawing at 60 C/hr; E: 2 hr after freezing in liquid N_2 and thawing in 20 C water; F: 24 hr after freezing in liquid N_2 and thawing in 20 C water.

Effects of Freezing and Thawing upon ATP Levels. Because it has not been possible to extract the cytoplasmic components involved in protein synthesis from this moss (10), we were unable to test the effect of freeze-thawing treatments upon their integrity. We did consider the possibility that *in vivo* protein synthesis following the 60 C/hr treatment could be affected by limited supply of ATP energy, inasmuch as this is supplied to our *in vitro* systems.

Following freezing in liquid N_2, ATP levels decline sharply (Table II) and 24 hr after thawing are only 3% of those of control moss. Immediately after 60 C/hr freezing and thawing ATP is 56% of control levels and this is maintained during warming to 20 C. There is a slow decline thereafter over the next 24 hr. At the point of thawing during 3 C/hr warming (from freezing at the same rate) only 37% of the ATP is present in the moss cells compared with that in unfrozen moss. By the time the moss reaches 20 C more ATP has already been synthesized and normal levels are reached within 2 hr thereafter. Thus, in moss which does not recover its ability to conduct *in vivo* protein synthesis ATP levels do not return to those observed in controls. After a 3 C/hr freeze-thaw cycle both protein synthesis and ATP levels recover. It is tempting to speculate that there is a causal relationship between ATP availability and protein-synthesizing capacity, but we cannot exclude the possibility that other factors may be involved also.

Leakage of Conductive Materials. It is an accepted practice to measure the leakage of conductive materials from cells in order to gain some insight into the degree of damage sustained by membranes within tissues subjected to stress treatments. Conductivity measurements of solutions in which *T. ruralis* was bathed during 3 C/hr and 60 C/hr freezing and thawing show a comparable degree of leakage (Table III). A much greater amount of material leaked from cells following freezing in liquid N_2 and thawing to 20 C. As far as the 3 C/hr and 60 C/hr treatments are concerned, there appears to be little relationship between the ability of the moss to take up radioactive precursor and the degree of leakage of conductive materials (compare Fig. 1B and Table III).

It is pertinent at this stage to record briefly some visual observations on the moss following freeze-thaw treatments. Following freezing and thawing at 3 C/hr the moss showed no changes in coloration over a 24-hr period. After fast freezing and thawing to and from liquid N_2 temperatures, the moss turned brown within 24 hr with some loss of Chl. Some degree of browning was observed within 1 day after a 60 C/hr freeze-thaw cycle, although

the Chl content was equal to that of unfrozen control (data not presented). Initiation and growth of protonema from the basal portions of detached phyllidia of *T. ruralis* was not impaired by freezing to −30 C at either 3 or 60 C/hr. No protonema developed following freezing in liquid N_2.

Total protein content of the moss was found to decline following a 60 C/hr treatment to 78% of controls 2 hr after the end of treatment and to 66% after 24 hr. Two hr after liquid N_2 treatment only 59% of control protein levels was extracted. A 3 C/hr treatment was without effect.

Degree of Hydration at which *T. ruralis* Can Withstand Freezing in Liquid N_2. A number of workers have shown that dry moss spores or dry vegetative moss pieces can survive at temperatures below −100 C (*e.g.* 3, 6 and references therein). We conducted an experiment to establish more closely the relationship between water content and survival at very low temperatures brought on by a fast rate of freezing. As seen in Table IV, moss must reach a very low water content (less than 29% of original fresh wt) before it can withstand harsh freezing treatments.

DISCUSSION AND CONCLUSIONS

Mazur and co-workers (16, 18) have proposed that two factors are important in freezing-induced cellular damage: desiccation by extracellular ice formed during "slow" temperature decrease, and disruptive intracellular ice formation during "rapid" temperature changes. In *T. ruralis* it has not been possible to establish the location of ice crystals during freezing, but on the basis of the various metabolic criteria we have studied we can discuss our results in relation to this two-factor hypothesis.

Freezing at slow rate (3 C/hr) does not appear to have any permanent effect on the metabolism of drought-tolerant *T. ruralis*. Some reversible changes (ATP levels, electrolyte leakage) may be taken as indirect evidence for extracellular ice formation leading to desiccation. For example, the decline in ATP during slow freezing and its subsequent resynthesis after thawing also occur during drying and rehydration of the moss at room temperature (5). Moreover, the degree of electrolyte leakage from freeze-thawed tissue compares favorably with that occurring on rehydration of *T. ruralis* desiccated at 20 C (8). In both cases the leakage is likely to be associated with reconstitution of cellular membranes on rehydration, and not with membrane damage (which occurs following freezing of hydrated moss in liquid N_2). The lack of cellular damage (as measured by *in vivo* protein synthesis) in moss frozen to −30 C at 3 C/hr before plunging into liquid N_2 and thawing slowly (3 C/hr) from −30 C is also indicative that the cells were largely dehydrated by extracellular ice formation. Others have reached similar conclusions from work with less tolerant plant tissues (23).

Loss of the moss cell's capacity for protein synthesis following liquid N_2 treatment probably results from destruction of cell compartmentalization by intracellular ice formation resulting in release of degradative enzymes into the cytoplasm (21). Loss of capacity for *in vivo* and *in vitro* protein synthesis, degradation of ribosomes and protein, loss of ATP, massive leakage of electrolytes, browning and lack of protonemal growth on phyllidia, all strongly suggest irreversible damage by intracellular ice. Only at very low water contents (dehydration to less than 29% of original fresh wt) is loss of protein-synthesizing capacity prevented. Luyet and Gehenio (13) claim to have obtained high survival (though not using a metabolic marker) upon fast thawing (in 20 C water) of hydrated *Mnium* sp. frozen in liquid air. Nondamaging water vitrification, which they claim to have achieved by using rapid temperature changes, does not appear to take place during comparable freezing of *T. ruralis*.

The response of *T. ruralis* to freezing and thawing at 60 C/hr does not lend itself so readily to discussion in terms of the two-factor hypothesis. The lack of ribosome and polyribosome degradation, only slight decreases in Chl and protein concentration, and the low level of leakage, appear to indicate that intracellular freezing did not take place in the majority of cells during the 60 C/hr treatment. The aspects of metabolism which were affected by this treatment were ATP levels, and uptake and *in vivo* incorporation of radioactive leucine, possibly by mechanisms similar to those leading to damage in chilling-sensitive organisms—membrane phase changes or enzyme inactivation (14). It is not easy to explain our results (of 60 C/hr treatment) on the basis of intracellular freezing, for this should lead to indiscriminate destruction of the cellular contents. To invoke this reasoning, the concept of intracellular freezing damage would have to be modified to account for the lower degree of damage in terms of differences in sensitivity of various cellular components and/or possible lower rates of degradation. Such mild, selective action of internal ice crystals, however, is difficult to conceive.

The observation that following slow (3 C/hr) freezing, fast rewarming (60 C/hr) leads to some damage could be explained as a rapid rehydration (osmotic?) or rapid temperature change shock. Such interpretation has been used in several investigations of slowly frozen tissues where fast rewarming was found to be more damaging than slow rewarming (22, 25). The exact mode by which such shocks may lead to damage is not understood.

In summary, we have established that changing the rate of temperature decrease has a marked effect on the metabolic re-

Table II. Adenosine triphosphate levels after freezing and thawing at controlled rates.

	Immediately on thawing (at about 2C)	0 hours	2 hours	24 hours
Expt. 1				
Control	27 ± 2[a]	32 ± 2	24 ± 4	27 ± 5
3C/hr	10 ± 2 (37%)	23 ± 5 (72%)	26 ± 2 (108%)	25 ± 2 (93%)
Expt. 2				
Control	24 ± 3	31 ± 1	36 ± 6	24 ± 3
60C/hr	19 ± 3 (56%)	17 ± 2 (55%)	15 ± 1 (42%)	15 ± 1 (44%)
Liquid nitrogen	11 ± 2 (32%)	-	3 ± 1 (8%)	1 ± 1 (3%)

[a]Standard error of the mean.

Numbers in brackets represent per cent of control. The time of extraction is indicated as hours (0, 2, or 24) after reaching 20C. Note that due to different rates of thawing the points of extraction differ in the length of time spent at above zero temperatures before 20C was reached. The ATP levels are given in nmol per 200 mg of moss.

Table III. Electrolyte leakage from moss frozen and thawed at indicated rates.

Treatment	Electrolytes released (μmho/300 mg fresh wt. hr)	% of control
Control	9	100
3C/hr	25	277
60C/hr	24	266
Liquid nitrogen	65	722

Measurements made one hour after reaching 20C.

Table IV. Protein synthesis by moss dried to various degrees, frozen in liquid nitrogen, and thawed in water at 20C.

Weight of Moss (% of fresh moss control)	Incorporation (% of control)[a]
100	6
65	3
61	1
49	4
47	6
33	14
29	24
21	99

[a]Control moss was dried and rehydrated, but not frozen.

Incorporation of ^{3}H-leucine into a hot trichloroacetic acid precipitate was followed for one hour starting 2 hr after the moss was returned to 20C.

sponse of a moss to low temperature. This, to our knowledge, has not been shown before, for previous studies on freezing of mosses (*e.g.* 4, 7, 9, 19) have employed only a single rate of freezing and thawing. Our initial premise that desiccation induced by extracellular ice formation during slow freezing should not inflict irreversible damage on a desiccation-tolerant plant appears to have been correct. The response of this moss to faster freezing rates can be explained on the basis of the two-factor hypothesis of Mazur (18). However, additional factors such as effect of temperature alone (14) and presence of the cell wall (20, 24) may have to be considered.

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