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THE EFFECT OF SUBZERO TEMPERATURES ON PROTEIN METABOLISM OF A DROUGHT TOLERANT MOSS *Tortula ruralis*

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled THE EFFECT OF SUBZERO TEMPERATURES ON PROTEIN METABOLISM OF A DROUGHT TOLERANT MOSS *Tortula ruralis* submitted by Lada Malek in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The moss *Tortula ruralis* is tolerant of very low temperatures (down to -196°C), providing the initial rate at which it is frozen is slow (up to 6°C/hr). Direct liquid nitrogen immersion is lethal. The response of the moss to freezing and thawing at slow (3°C/hr), intermediate (60°C/hr) and rapid (liquid nitrogen immersion) rates is discussed in terms of the widely accepted two-factor theory of freezing damage (68, 69). The freezing tolerance observed at the slowest freezing rate is attributed to the desiccation tolerance of the moss. The damage suffered at intermediate and rapid rates of freezing is attributed to an increasing degree of intracellular ice formation. The relationship between hydration level of the moss and its tolerance of rapid freezing has been determined.

The ability of this moss to synthesize proteins is used as a criterion of survival. The decline of *in vivo* protein synthesizing capacity following intermediate freezing treatment is not due to damage to the ribosomal or polyribosomal material. Lack of energy available for protein synthesis (in the form of ATP) or damage to the protein factors involved in protein synthesis are implicated as possible sites of damage caused by 60°C/hr freezing rates.

Only slight seasonal changes in the ability of the moss to survive freezing are detectable - freezing tolerance remains high throughout the year. The moss is able to synthesize protein at -2.5°C and 36°C. The significance of these observations is related to the environmental conditions which *Tortula ruralis* encounters in its habitat.

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1. INTRODUCTION

In the temperate zones of the earth, low temperatures limit the cultivation of many crops. For this reason, increasing the low temperature tolerance of plants and understanding the mechanism(s) by which low temperature injures plants are of potentially great inportance to man.

Over the past one hundred years considerable progress has been made in the study of cold tolerance of plants, although several major questions remain to be answered. Many plants are injured by chilling temperatures, i.e. temperatures above 0°C, but the work reviewed here is concerned only with the damage suffered by more tolerant plants at temperatures below 0°C. At subzero temperatures, both low temperature and the presence of ice affect the degree of damage suffered by the plant (see Literature Review). Ice can have two effects on a living organism, depending on its location in the tissue:

1) changes in the properties of water during crystallization (eg. volume, orientation of molecules) may have a direct physical impact, and
2) ice can very effectively dry out tissues and induce damage through dehydration.

Cold hardiness research carried out over the past few years has involved the use of relatively drought-sensitive plants, and thus any freezing damage has been attributed mainly to ice-induced desiccation damage. In my investigation, a drought-tolerant plant, the

moss *Tortula ruralis* has been used. The study was initiated on the assumption that a drought-tolerant plant would not be damaged by the dessication properties of ice, but directly by the ice itself.

Various metabolic reactions of mosses have been employed as indicators of the degree and type of damage suffered during freezing. Determination of *in vivo* protein synthetic capacity (i.e. incorporation of the radioactive amino acid leucine into hot trichloroacetic acid-precipitable material) is considered to be a valuable marker of survival, since protein synthesis is essential for maintaining the functional integrity of the cell (30, 45). Therefore, *in vivo* protein synthesis and several other aspects of protein synthesis were studied. In addition, metabolic markers, eg. adenosine triphosphate (ATP), protein and chlorophyll levels, metabolite loss and tissue re-growth were monitored after the freezing treatments.

The following studies were planned to answer the questions:

a) What is the physiological response of a moss to various controlled freezing and thawing regimes?

b) How would the metabolic responses of the drought-tolerant *Tortula ruralis* to subzero temperatures contrast with previous observations on drought-sensitive species at subzero temperatures?

c) How do various components of the protein synthetic apparatus react to freezing stress?

2. LITERATURE REVIEW

2.1 Introduction

In 1937, Scarth and Levitt (108) stated: "The mode of [freezing] injury varies with conditions, such as the rate of freezing or the rate of thawing, and also with the type of plant." Forty years later, we know much more about the conditions under which freezing damages plants. However, the mechanisms (modes) by which an organism suffers injury remain unclear.

This literature review will concentrate on the more recent developments from plant cold hardiness studies - the older, "classical" studies have already been reviewed extensively (3, 22, 56, 67, 68, 73, 83, 96, 121) and only little of this work will be considered in any detail. Emphasis will be placed on studies of plants, although relevant work from animal systems will be mentioned.

2.2 The two-factor theory of freezing damage to living organisms.

In the 1950's and 1960's a major contribution to our understanding of the changes which occur during freezing of living materials was made by P. Mazur and his co-workers. On the basis of his studies on yeast, red blood cells, mouse bone-marrow cells and Chinese hamster tissue culture cells, Mazur formulated the two-factor theory of freezing damage (66, 68, 69). In essence, the theory states that as temperature of tissue slowly decreases below 0°C, extracellular water freezes. The cell membrane inhibits the growth of relatively large

ice crystals into the cell's interior. Since the vapor pressure of water is lower in the solid (ice) phase than in the liquid phase, extracellular ice draws water from the cell protoplast. Thus the cell may, at a slow rate of temperature decrease, effectively dry out. Thus, the first factor causing freezing damage is prolonged desiccation. A decrease in cell size is considered as evidence for the desiccating effect of ice. Intracellular freezing takes place during freezing at fast rates when the cell water is not lost from the cell quickly enough and the freezing point of the cell water is reached. This intracellular freezing is always lethal to the cell - presumably due to the destruction of internal cellular compartmentalization by expanding ice crystals (89). Intracellular ice formation is a second factor of freezing damage and can be observed as a sudden change in the refraction of light passing through the cell (darkening of the cell) (6). The original formulation of Mazur's two-factor theory was in mathematical terms, and since that time some of the mathematical assumptions have been questioned and modified (2, 55). Nevertheless, the core of Mazur's theory has remained untouched and is still widely accepted.

In the animal tissues and yeast used by Mazur and co-workers, not only fast freezing rates (Fig. 1a, for red blood cells, faster than 5×10^{3}°C/min.), but also the slowest freezing rates tested (Fig. 1a, for red blood cells rates slower than 10^{3}°C/min.) led to a decrease in survival. A survival optimum was obtained at

intermediate freezing rates (Fig. 1a, for red blood cells rates between 5×10^3 and 10^3°C/min.). Mazur explained this by stating that at slow rates of freezing drought-tolerant cells are exposed to drying conditions longer, thus suffering a greater damage, whereas at fast freezing rates the intracellular ice formation is the cause of damage. The optimal freezing rate is not so fast as to cause intracellular freezing, yet minimizes the exposure of the tissue to desiccating extracellular ice.

As can be seen in Fig. 1a, the optimal rate for survival varies with the specific types of cells. According to Mazur, the water permeability of the membrane, the degree to which this permeability changes with temperature, and the surface to volume ratio are factors which determine the rate of water loss from a cell. It is these properties of various types of cells which determine the optimal freezing rate for survival.

Very few freezing tolerance studies have been carried out with the intention of testing the applicability of two-factor theory of freezing damage to plants. However, the response of plant cells to a wide range of freezing rates has been tested in the following studies.

Carrot tissue culture cells (78, see Fig. 1b) respond to freezing in a manner fully explicable in terms of the two-factor theory. Mulberry cortical parenchyma sections (102, see Fig. 1b); however, deviate from the relationship between the freezing rate and survival. At slow freezing rates (less than 10°C/min.), the cells are not

damaged by prolonged exposure to ice. An explanation of this apparent inconsistency can be made within the framework of the two-factor theory: the cortical parenchyma cells are considered to be drought-hardy and thus capable of surviving the desiccating action of ice. The tolerance of many other plant tissues to slow freezing rates has also been demonstrated and explained in terms of drought tolerance (5, 56, 99, 100).

Recently, the observation that mouse embryos (122, see Fig. 1b) and lymphocytes (71) are also tolerant of slow freezing rates, under carefully controlled conditions, had cast some doubt on the interpretation of the slow freezing damage as desiccation damage. These animal tissues are not likely to be desiccation-tolerant.

Another difficulty associated with the two-factor theory is its inability to account for the contradicting observation that some slowly frozen tissues (less than 1°C/min., 67) survive better after fast thawing, while other tissues (56, 67, 122) survive better after slow thawing. These and other criticisms (84, 85, 116) have pointed out the shortcomings of the two-factor theory. These will be discussed later in relation to response of *Tortula* to freezing. Nevertheless, the two-factor theory remains the most plausible and useful one on which to base interpretations of freezing tolerance studies at the present time.

2.3 Response of plants to subzero temperatures.

The main purpose of many studies of plant responses to freezing

temperatures has been to determine the minimum temperature which allows survival of a particular species or cultivar. In these studies usually only a single freezing rate has been used (often not reported) making discussion in terms of the two-factor theory nearly impossible. Furthermore, the experimental methods and freezing conditions used (not only freezing and thawing rates but also age of plant, water content, and minimum temperature reached) have not been standardized, making comparisons between results difficult. Since a unified approach to the cold tolerance studies cannot be taken, discussion of the studies reported here will be based on the various factors and experimental conditions (mentioned above) affecting the plant freezing tolerance.

2.3.1. Methods of quantifying the freezing damage.

A method commonly used by agriculturalists to evaluate hardiness of cultivars is the "LT 50 method". Groups of plants are brought to a particular subzero temperature, thawed, and several days later visible damage is observed. The temperature at which 50% of the plants are killed is considered the tolerance limit of the cultivar. The assumption is that survival is a function only of the minimum temperature reached. As long as the freezing rate is kept constant, this relationship generally holds true (26, 90).

One severe limitation of LT 50 determinations is that it is not possible by this method to determine whether or not an organism is dead. For this, one of the criteria of damage need to be used:

a) vital staining, i.e. the ability of cells to absorb or metabolize;a dye neutral red (103) and tetrazolium salt (118) are commonly utilized;

b) intactness of a cell (its outer membrane) by plasmolyzing the cell in a hypertonic solution - here osmotically-induced plasmolysis has to be distinguished from plasmolysis caused by the freezing alone (frost plasmolysis), however;

c) leaching of metabolites determined by electrical conductivity measurements or by quantitative analysis of the leaching substances (eg. amino acids);

d) oxidative browning of the damaged tissue - as determined by Li and Weiser (58);

e) viability measurements have also resulted from studies on metabolic activity of cells. Rates of respiration, photosynthesis or protein synthesis have been measured directly, or indirectly by estimating the concentration of "vital" metabolites (protein, chlorophyll, etc.);

f) determination of the ability of cells to grow and divide (applicable only to fast growing materials).

While each of the above criteria has certain advantages, it is unwise to make conclusions about the physiological state of an organism using only one. Several criteria should be used. Unfortunately, this often has not been done. A further complication of

survival determinations arises when whole organisms are used which consist of several tissues of varying cold tolerance.

2.3.2. Effect of age on survival of subzero temperature.

Actively growing cells appear to be more sensitive to freezing than "resting" mature cells. This is most apparent in perennial plants of temperature zones which pass through annual cycles of low-temperature tolerance (56). These changes in low-temperature tolerance are often referred to as cold hardiness cycles. In the spring, the rapidly growing meristems are very susceptible to freezing, and often just to chilling. The cessation of growth in fall is accompanied by increasing tolerance of the over-wintering tissues to freezing temperatures. These hardiness changes are mainly induced by the changes in daylength and decreasing temperatures, but some change can take place in the absence of environmental stimuli, i.e. there is an endogenous annual hardiness cycle (56). Stimulation of growth by nitrogen fertilization, long days or growth regulators also leads to loss of frost tolerance. These observations support the contention that freezing tolerance is inversely related to growth. Cox and Levitt (25), however, have provided evidence that in cabbage leaves, frost hardiness is a function of not only growth rate, but also of the developmental stage. In the alga *Chlorella*, cold hardiness was also found to depend on the age of the culture (77).

2.3.3. Effects of water content and water supercooling on freezing survival.

Some plant cells can avoid intracellular freezing by dehydration. Dry seeds and spores are extremely tolerant of low temperatures because they contain no freezable water. It has also been found that rapidly droughted cabbage leaves become very frost hardy (27). A different way in which plant cells can decrease their freezable water content is by increasing the concentration of solutes within the cell. The concentration increases necessary to produce a significant decrease in the proportion of freezable water and thus significant increase in hardiness has not been measured, however.

Some attention has been given to the process of water supercooling (22) as a possible mode of protection from ice-induced damage. A truly supercooled solution is one which remains in the liquid phase at a temperature below its melting point. The degree of supercooling depends on the probability of an ice-like crystalline structure (nucleus) forming within the bulk of the solution, and the probability of this taking place increases as the volume of the solution and the period of time at subzero temperature increase. The observation that there are series of freezing points in floral buds (36) very much below the freezing point of expressed cell sap has been used as evidence of supercooling taking place within individual floral primordia. This supercooling is believed to be due to a) separation of the solution into small volumes which cannot nucleate each other, or b) due to presence of antinucleating materials in hardened buds.

The existence of antinucleating materials has been documented (117) in polar fish. The active molecules were shown to be soluble blood glycoproteins. This discovery appears to have prompted a search for similar molecules in plant cells. Williams (123, 124) isolated glycoproteins from the cytoplasm of cold-hardy and non-hardy dogwood cells but did not test their ability to prevent or interfere with ice formation.

Before supercooling is accepted as widespread mode by which cells avert ice damage, two questions will have to be answered:

a) Where in the tissue (intra- or extra-cellularly) is supercooling taking place; and

b) how is ice nucleation prevented?

2.4 Changes in metabolites and cell ultrastructure associated with cold-tolerance changes of plants.

In the previous section, changes in plant cold hardiness associated with age and growth rate were treated as one potential source of variation in determinations of absolute cold tolerance of a species. On the other hand, such changes in cold hardiness, when induced experimentally, have been used to advantage in studies correlating a change in metabolism with freezing tolerance. Presumably some aspect of metabolism should undergo a change and become less freezing sensitive as tolerance of the plant increases. Therefore, parallel changes have been sought between cold tolerance and a particular metabolite or metabolic event. To the frustration of researchers,

all molecular components of the cells examined to date (sugars, starch, amino acids, proteins, RNA, lipids) have been found to change during hardening (3) and thus it is difficult to pinpoint specific, as opposed to general, metabolic adaptations.

Protein concentration increases during the winter months (20, 41, 90). Shomer-Ilan and Waisel (110), found changes in the amino acid composition of ribulose diphosphate carboxylase of hardened cabbage leaves. Rochat and Therrien (98) observed that two new unidentified water-soluble proteins were synthesized during hardening of winter wheat. Several workers have also observed increases in RNA, particularly ribosomal RNA (rRNA) with hardening (41, 106), or changes in ribosome structure (17). Proteins and protein synthesis are considered important targets of freezing injury, mainly the ice-induced desiccation. Several hypotheses (reviewed in Ref. 22) have been proposed to explain how proteins may be damaged, though none of the hypotheses are completely satisfactory.

Increases in mono and disaccharides and a related decrease in starch have been observed during hardening (3, 90, 103) and these, in turn, have been related to changes in protein metabolism. It has been shown that sugars can protect photosynthetic activity of isolated chloroplasts from freezing damage (35, 44, 104, 105), presumably by protecting membrane proteins. Alterations in the distribution of particles (coupling factors)in chloroplast thylakoid membranes (35), in wheat cell membranes, starch grains and osmiophilic granules (97) are known to take place during hardening at low temperature.

The lipid component of cells has also been studied in relation to cold hardening. Unsaturation of fatty acids, and therefore increased fluidity of cell membranes at low temperature, is related to the maintenance of activity of membrane-bound enzymes in chilling-tolerant plants (64, 125). This observation prompted a search for similar unsaturation increases in more tolerant plants. These increases were detected in several tissues (29, 38, 40, 51, 115). Recently, it has been suggested that these increases represent not a change in unsaturation level but rather an overall increase in total concentration of phospholipids containing unsaturated fatty acids (114). Siminovitch and co-workers claim (90, 114) that an increase in all cellular contents (cytoplasmic augmentation) would decrease the proportion of freezable water in the cell and thus decrease the probability of intracellular ice formation. Returning to Mazur's two-factor theory of freezing damage, these metabolic changes would presumably have a two-fold function: a) decreasing the probability of intra- or extracellular ice formation and b) increasing the drought hardiness of the overwintering cells. More information is needed about the biophysical relationship between water and cellular components and how this relationship effects the function of the cell.

2.5 Response of mosses to low temperature.

The observation that mosses constitute an important component of the tundra vegetation has resulted in a number of studies center-

ing upon their physiological adaptations to the extreme environment.

In his review article, Lamb (52) made the following points about mosses in Antarctica: by 1961, seventy two species of mosses had been collected, and they were most abundant on seepage slopes and wetter areas,on scree and morainic detritus and in crevices of rock faces. Growth, even though slow, was observed but production of sexual structures was rare. Also in Antarctica, Greene and Longton (39), have recorded temperatures of -29°C in exposed moss turfs, although they point out that many mosses are covered by an insulating layer of snow which, depending on its depth and atmospheric temperature, can maintain the temperature of the moss at around 0°C. Similar observations of such a "greenhouse" effect of snow over mosses have been reported from northern Canada (61) and northern USSR (107).

Snow cover may not only protect plants from low temperature extremes but actually create conditions suitable for growth. Mosses have been shown by several workers to be metabolically active at sub-zero temperatures. Gannutz (34) measured photosynthetic gas exchange down to -4°C in some Antarctic mosses. Rastorfer (93) detected photosynthesis and respiration at -2°C in *Bryum argenteum*, and Atanasiu (4) measured CO_2 assimilation at -8°C to -9°C and respiration at -14°C in three other mosses.

Ample evidence exists that mosses grow vegetatively in polar regions, yet little is known about their ability to complete the sexual reproductive cycle during the short summer period. The extensive research of Greene and Longton provides some information on this

matter (39, and references within). Gametangia and sporangia fail to develop in *Polytrichum* at the end of the Antarctic summer. Also there appears to be a decreasing gradient of sporophyte production from the coastal region towards the pole (in species readily producing capsules under favorable conditions). Furthermore, there is a seasonal pattern in sporangial development, but the environmental factors controlling this have not been identified.

The belief that mosses exist successfully in cold climates because they occur in microclimatically favorable conditions (eg. under the snow) persists in the literature, yet the numerous observations that some mosses are very tolerant of temporarily unfavorable conditions (drought, extremes of temperature) are largely ignored.

An extreme example of the environmental tolerance is apparent from the observations of Biebl on cold- and drought-tolerant tropical mosses (14, 15, 16). The tropical environment is characterized by high temperatures and ample moisture, yet some species were found to be tolerant of total desiccation and others of temperatures down to -16°C (*Herberta juniperina, Rhizogonium spiniforme*). The freezing rates used in Biebl's tests were not reported but were relatively harsh, achieved by direct placement of the moss into cooling solution at -16°C. The test of survival was not rigorous either (plasmolysis in 1.5 M KNO_3).

Species found in the more temperate zones might be expected to be even more tolerant of low temperature than tropical species. Dilks and Proctor (33) screened a large number of British mosses using

assimilation and respiration as survival criteria. The freezing rate used by them was fast, about 60°C/min. Some species were found to survive at -10°C despite the fast freezing rate. The temperature limit was probably the temperature to which the moss supercooled before intracellular freezing took place.

The possibility that mosses, like higher plants, undergo seasonal changes in cold hardiness was investigated by Riedmuller - Scholm (95). He detected annual cycles in the cold hardiness of some Alaskan mosses, with survival minima below -80°C in the winter months. Unfortunately, the experimental conditions were not rigorously controlled in this study.

Much slower freezing rates were used by Ochi (81). Seven of the species tested by him were found to survive -17°C. His attempts at finding a relationship between the osmotic value (not defined by the author), permeability and cold tolerance were not conclusive. Fast thawing was more damaging than slow thawing and young parts of the plants were more sensitive than older (lower) parts. A similar observation of the greater sensitivity of young leaves to freezing was made by Hudson and Brutskern (46) in *Mnium undulatum*. At a freezing rate of 4°C/hr the mature leaves survived freezing to -30°C, and subsequently to -130°C, whereas the young leaves did not. Intracellular ice formation was noted in young leaves at only -4°C to -12°C. Modlibowska and Rogers (75) studied extracellular and intracellular ice formation in various plant materials, including a moss *Pterygophyllum lucens*. They observed that a) wounded areas provided

intracellular ice nucleation sites, and b) propagation of intracellular ice took place through plasmodesmata. In the moss, freezing of the cells was seen to take place in two steps in quick sequence - the cell walls froze first and than the protoplast.

Only mosses frozen in the hydrated state have been discussed so far. A very useful mechanism of avoiding freezing exists in desiccation-tolerant mosses and moss spores. In the dry state, these mosses tolerate liquid nitrogen temperature (-196°C), while in the remoistened state this treatment is lethal (9, 59, 76). A recent study showed that a few hours after thawing following immersion in liquid nitrogen, the undesiccated moss *Tortula ruralis* lost their ability to synthesize RNA and protein, and that ribosomes were degraded. The synthetic activity and polyribosomes recovered to control levels two hours after rehydration of the dry frozen moss (9). Curiously, respiration (O_2 uptake) appeared unaffected after liquid nitrogen freezing of moistened *Tortula ruralis* (11).

2.6 Effects of low temperature on protein synthesis.

As discussed previously, changes in ribosomes and proteins take place during the fall hardening. Brown (19) studied alterations in the polyribosomes (the protein synthesizing complex) during hardening of mimosa seedlings. The ribosome and polyribosome fractions remained the same during nine weeks of cold tolerance induction. The presence of polyribosomes during the study period indicated the continuing potential for protein synthesis. The amount of extractable

polyribosomes increased in potato plants grown at low temperature (119). A similar increase in polyribosome concentration was detected in the moss *Tortula ruralis* kept at 2°C (Bewley, unpublished). The significance of this finding is difficult to assess but possible interpretations are that a) at lower temperatures there is an increased potential for protein synthesis or b) a decreased ability for ribosomes to run off the messenger RNA, i.e. restricting the termination reaction. Evidence partially supporting the former interpretation was provided by Kenefick et al. (48), who detected an increase in the incorporation of ^{75}Se methionine into protein during low temperature induced acclimation (increase in cold tolerance) of barley. Furthermore, polyribosome absorption patterns from hardy and non-hardy barley were obtained and differences between them were ascribed to higher activity or ribonuclease (RNase) in the non-hardy barley.

Polyribosome and RNase levels were studied by Lefler (53) in chilling-sensitive cotton seedlings. Low temperatures caused a number of morphological and metabolic lesions, including a decrease in the ability to form polyribosomes and to synthesize ribonuclease. At 30°C, a burst in polyribosome formation was observed on day 3 of seedling development. This burst was reduced by a 6°C treatment on days 1 and 2. In contrast, when the low temperature treatment was given on day 5, a further <u>increase</u> in the amount of polyribosomes relative to 30°C control took place. The concentration (activity) of RNase increased gradually with seedling development.

When given on days 2 and 3, the chilling treatment was most effective in reducing the increase in RNase activity. Only seedlings chilled on, or after day 3 were capable of recovering the control RNase levels when brought to 30°C. Lefler's study demonstrated a close relationship between the decrease in polyribosome concentration, the decrease in RNase activity and chilling on the second and third day of seedling development.

The only study available on the effects of subzero temperature on protein synthesis has already been discussed in section 2.5 (9). More information on this subject is needed.

3. METHODS

3.1 Collection and preparation of plant material.

Mats of moss *Tortula ruralis* (Hedw.), Gaertn., Meyer, Scherb., were collected west of Calgary on north-facing banks of the Bow River. Collections were made in July and January throughout the study period. The material was dried on a laboratory bench before storage in plastic bags at 20°C. Twenty four hours before each experiment, the required amount of moss was placed in a glass dish, saturated with deionized water and excess water poured off. The rehydrated moss was kept covered under diffuse light at 20°C unless specified otherwise.

For each experiment, the green tops of the gametophores were harvested, washed initially with deionized water, then with sterile distilled water. Excess moisture was blotted off with paper towels. Samples were weighed out and placed into experimental vessels.

3.2 Freezing and thawing treatments

Three hundred mg batches of *Tortula ruralis* were placed into 150 ml Corex centrifuge tubes and 2.5 ml of sterile distilled water were added to prevent dehydration. The control moss batches were kept at the room temperature (20°C) in 2.5 ml of sterile distilled water in 5 cm Petri dishes. The Corex tubes to be frozen were capped with parafilm and placed in a test tube rack almost completely sub-

merged in the cooling solution (ethylene glycol, or methanol-water mixtures) of a Haake KT 52 low temperature thermostat bath equipped with the Haake PG 11 programmer. Using this apparatus, linear cooling and warming rates could be obtained from 3°C/hr to 60°C/hr over the temperature range from 20°C to -30°C (Fig. 4). Temperature changes of the water medium containing the moss were monitored using a YSI 42 SL thermistor with No 409 type temperature probe. Temperature change was recorded on a Metrohm E478 chart recorder.

Rapid temperature changes were obtained by plunging Corex tubes with moss material into liquid nitrogen at -196°C for five minutes and thawing rapidly in a water bath at 20°C. In the initial studies, moss was placed in a cheesecloth bag in contact with the liquid nitrogen and the thawing water. Both of these treatments were found to give the same results.

Since the various freezing treatments lasted anywhere from five minutes to thirty four hours, it was necessary to choose standard times at which the responses to the treatments could be tested. Initially, the moss was left to recover for 2 hours after the end of the thawing process (i.e. 2 hours after reaching 20°C). Later, two more time-points were chosen, the point of complete thawing of the medium and a point 24 hours after the end of the treatment.

3.3 Drying treatments.

3.3.1. Over silica gel

Three hundred mg samples of moss were placed in weighed

5 cm glass Petri dishes and enclosed in a large desiccator containing silica gel. At intervals, samples were removed, quickly weighed and frozen. Percentage water loss was expressed as $\frac{\text{g dried moss}}{\text{g fresh moss}} \times 100$.

3.3.2. In polyethylene glycol (mol.wt. 6000 to 7500) (PEG 6000)

Three hundred mg samples of moss were placed into Petri dishes containing 5 ml of PEG 6000 solution at appropriate concentrations for one hour equilibration. Before freezing, moss was removed from the PEG 6000 and excess solution blotted off with paper towels.

3.4. Incorporation of radioactively labeled leucine *in vivo*.

3.4.1. Incubation of the moss in radioactive leucine

Control and freeze-thawed samples of moss were blotted and placed into 5 cm glass Petri dishes with 2.5 ml of sterile distilled water. Ten microcuries of L-[4,5-^{3}H] leucine (Amersham Co.; specific activity 60 Ci/mmole, or New England Nuclear, specific activity 40 - 60 Ci/mmole) or one microcurie of L-[^{14}C(U)] leucine (New England Nuclear, specific activity greater than 270 mCi/mmole) was applied and the solution swirled to ensure mixing. After 20, 40, or 60 minutes the moss was washed 3 times with distilled water to remove excess radioactivity. Washing with dilute cold leucine solution and fifteen minute exchange at 4°C with cold leucine were not found to remove any additional radioactive leucine (Figs. 2 and 3). These figures also show the effect of changing the amount of applied radioactive leucine on the amount of leucine uptake and incorporation into protein.

Doubling the amount of radioactivity to 20μCi/300 mg moss did not eliminate the plateau in uptake observed after only 20 minutes of incubation. For this reason absolute linearity in incorporation of leucine into protein was not achieved - presumably due to limiting amounts of available leucine.

3.4.2. The extraction of radioactive protein.

The following protein extraction steps were carried out at approximately 4°C. Blotted moss was ground in 10 ml of Tris-glycine buffer (0.1 M, pH 8.4) (all chemicals were reagent grade obtained from standard sources) in a Duall ground glass homogenizer (Kontes Glass Co., Vineland, New Jersey). The homogenate was centrifuged at 20,000 x g for 10 minutes. A 0.1 ml aliquot of the supernatant was placed on a glass fiber disk (diameter 2.4 cm - Whatman GF/A) and radioactivity determined as described in Part 3.2.3. These measurements were considered to be the total radioactive leucine taken up into the tissue and were expressed as DPM/300 mg fresh weight of moss. A 1.5 ml aliquot of 40% trichloroacetic acid (TCA, W/V) was added to the remaining supernatant and left on ice for 15 minutes. The TCA precipitate was collected by a 7 minute centrifugation at 500 x g. The pellet was resuspended in 5 ml of 5% TCA using a teflon motor-driven pestle. The resuspended material was heated to 95°C in hot water bath for 15 minutes, cooled on ice and cetrifuged at 500 x g for 7 minutes. The TCA precipitate containing radioactive protein was dissolved overnight in 2N NaOH in capped tubes.

3.4.3. Liquid scintillation counting.

Aliquots (0.1 ml) of the radioactive material were soaked onto a glass fibre disk and dried under an infrared lamp for 15 minutes. Dry disks were placed in the bottom of scintillation vials. Ten ml of scintillation cocktail (4 g 98% PPO 2% bis MSB IV fluor mixture - Kent Biochemicals - dissolved in 1l of reagent grade toluene) was added to each vial. The vials were kept in darkness for one hour before counting in a Nuclear Chicago Isocap/300 - PDS/3 liquid scintillation counter. The radioactive content of the protein was expressed as DPM/mg protein.

3.4.4. Protein determination (modified from Ref. 62).

To a 1 ml sample of protein solution was added 10 ml of the following mixture - 100:1:1 (volumes) of sodium carbonate (2% W/V in 0.1N NaOH) : copper sulfate (1% W/V) : sodium-potassium tartrate (2% W/V). After ten minutes, 0.5 ml of Folin phenol reagent (2N, Fisher Chemicals) was added with stirring. The color was allowed to develop for 30 minutes and the absorbance of 1 ml samples was determined at 500 nm using a Perkin-Elmer double beam spectrophotometer. Protein concentration was determined from a calibration curve obtained using fraction V bovine serum albumin.

3.5. Determination of $^{14}CO_2$ release during incubation of the moss in L-[^{14}C-(U)] leucine.

From measurements of the distribution of the radioactive leucine

in the moss and in the incubation medium, it became apparent that not all the applied radioactivity could be accounted for at the end of an experiment (Tables 1 and 2). The possibility was tested that radioactive $^{14}CO_2$ was being formed by oxidation of the applied leucine and then released into the atmosphere. A flow apparatus was constructed to collect any $^{14}CO_2$ given off by the moss into solutions of 2N KOH. Three hundred milligrams of moss were placed in 2.5 ml of distilled sterile water in a 50 ml Erlenmeyer flask. One microcurie of L-[^{14}C(U)]leucine (New England Nuclear, specific activity greater than 270μCi/mmole) was added and moist air was passed over the moss at a rate of 0.2 SCFH. The radioactive CO_2released was carried in the stream of air out of the incubation vessel and collected by bubbling the air through 5 ml of 2N KOH solution. Fresh KOH solution was supplied every half hour to avoid its saturation with CO_2. Radioactivity in 0.1 ml aliquots containing absorbed $^{14}CO_2$ was determined as described in section 3.4.3. The results were expressed as DPM released per hour per 300 mg of moss (see Tables 1 and 2).

3.6 Polyribosome extraction, separation and quantitative determination.

Polyribosomes were extracted at 4°C by a method previously reported by Bewley (7). Five hundred milligrams of moss were ground in 10 ml of grinding solution (0.25 M RNase free sucrose - Schwartz Mann - 40 mM KCl, 5 mM magnesium acetate, 50 mM Tris-HCl, pH 8.1, 5 mM mercaptoethanol) in an ice-cold Duall ground glass homogenizer.

After a clearing by centrifugation for 10 minutes at 20,000 x g, the ribosomal pellet was collected through a 1.0 M sucrose pad containing the salts and mercaptoethanol (at the same concentrations as above) by centrifugation (Beckman L5-50 ultracentrifuge) for 1.5 hours at 200,000 x g (Beckman Ti50 rotor). The ribosomal pellet was resuspended in 0.6 ml of above medium (minus sucrose) and a 0.2 ml aliquot (containing 0.3 mg of rRNA material as determined according to Ref. 65)was layered on top of a 5.6 ml linear (10% to 32% W/V) sucrose gradient containing the same salts and mercaptoethanol as above. The gradients were centrifuged at 120,000 x g for 60 minutes (in Beckman SW 50.1 rotor).

The gradients were analysed using an ISCO UA-2 continuous flow spectrophotometer set at a wavelength of 254 nm, and the absorption patterns recorded. The relative proportion of free ribosomes to polyribosomes was determined by cutting and weighing copies of the recorded patterns. The free ribosome region was taken as that area between the lines extrapolated from the monoribosome peak to the baseline (7). The results were expressed as per cent 254 nm absorbance in the monoribosomal region relative to the polyribosomal region (see Fig. 11a).

The addition of 1% BSA, 1% PVP (polyvinyl pyrrolidone) or 1mM phenyl-thiourea to the grinding medium was found to have no effect on the total yield or quality of the polyribosomes extracted.

3.7. *In vitro* incorporation of radioactive amino acids.

The ability of extracted polyribosomes and free ribosomes to incorporate amino acids into polypeptides was tested using the system developed by Gwozdz and Bewley (42).

3.7.1. Ribosome extraction.

Ribosomes were extracted as already described in section 3.6., except that more moss material was used (1.0 g) and the final ribosomal pellet was resuspended in 1.0 ml of salt medium and cleared by a slow (500 x g) centrifugation for 10 minutes. Aliquots (0.08 ml) containing 100 to 120 µg of RNA were used in the assays.

3.7.2. Preparation of wheat germ supernatant.

The procedure was that based on Legocki and Marcus (54). One gram of wheat germ (Maple Leaf Co., Calgary) was homogenized as described for the moss ribosome preparation using the same grinding solution. After centrifugation at 166,000 x g for 1.5 hours, the upper two-thirds of the supernatant was pipetted off and a 2 ml aliquot dialysed for 2 hours against 500 ml of extraction buffer at 4°C. The unused portion of the supernatant was frozen at -20°C until required.

3.7.3. *In vitro* activity of extracted polyribosomes.

An incubation mixture of 0.4 ml contained: 40 mM KCl, 4mM

magnesium acetate, 50 mM Tris-acetate buffer (pH 8.1, 0.244 mM mercaptoethanol, 0.125 µCi of L-[^{14}C(U)]leucine, 0.125 mM amino acid mixture lacking leucine, 1 mM ATP, 0.25 mM GTP, 8 mM creatine phosphate, 16 µg of creatine phosphate kinase, ribosomes (100 - 120 µg RNA) and 60 µl of dialysed wheat germ supernatant (600 µg of protein). Incubation was carried out at 31°C for 20 minutes in a constant temperature water bath. The reaction was terminated by the addition of 0.2 ml of 16% (W/V) TCA and 4 ml 5% TCA plus 0.3 ml of 1% (W/V) BSA. The precipitate was pelleted by centrifugation at 500 x g for 7 minutes. After cooling on ice, the precipitate was collected on Whatman GF/A filters and washed three times with 4 ml of cold 5% TCA. Filters were dried and counted as described before. Results were expressed as per cent incorporation (DPM/20 min./0.4 ml mixture) relative to control (per cent of control).

3.7.4. *In vitro* activity of free ribosomes.

An incubation mixture of 0.4 ml contained 80 mM KCl, 10 mM magesium acetate, 50 mM Tris-acetate buffer (pH 8.1), 0.244 mM mercaptoethanol, ribosomes (100 - 120 µg as RNA), 0.1 µCi of L-^{14}C phenylalanine (New England Nuclear, specific activity 383 mCi/mmole), 200 µg of polyuridylic acid, 60 µl of dialysed wheat germ supernatant (600 µg protein), 0.25 mM GTP, 1 mM ATP, 8 mM creatine phosphate 16 µg of creatine phosphate kinase. Incubation, and radioactivity determination was carried out as above. Results were expressed as incorporation (DPM/20 min./0.4 ml mixture) relative to the control (per cent of control).

3.8 ATP content determination.

3.8.1. ATP extraction

A method developed in our laboratory by Krochko (personal communication) was used. Lots of moss (200 mg) were extracted with 9 ml of cold 35% (V/V) perchloric acid in a mortar cooled to -20°C. The following steps were carried out at about 4°C. The crude homogenate was further homogenized in a Duall ground glass homogenizer, left on ice for 15 minutes and finally centrifuged at 20,000 x g for 35 minutes. The supernatant was titrated (over ice) to pH 7 with 6N KOH (both containing 50 mM K_2HPO_4) and left on ice for further 15 minutes. The resulting precipitate was removed by 15-minute centrifugation at 20,000 x g. The supernatant was made up to constant volume of 30 ml with distilled water and diluted 20 x before assaying. Unused extract was frozen at -20°C.

3.8.2. ATP assay

The firefly extract assay for ATP was employed (1). Small flint glass vials were placed inside borosilicate glass scintillation vials, cooled to 0°C, and kept at this temperature throughout the assay. Distilled water (0.9 ml) and 0.1 ml of cold firefly extract (Sigma; prepared according to manufacturer's instructions and filtered through Whatman #1 filter paper) were mixed in the small vials. The scintillation counter (refrigerated, Packard 3320), was used in the low energy (tritium) setting, counting in both A - B channels

with discriminator set at 50-1000, at 52% gain and the coincidence switch off. The counting was for 6 seconds. The sample (0.1 ml) was injected with force and the vial lowered into the counting chamber. Background luminescence of the enzyme alone was determined for each vial before ATP extract injection, and subtracted from the total counts.

The ATP concentration in the extracts was obtained using a set of standard ATP solutions at 1 pmole to 15 pmoles per 0.1 ml of solution. The ATP content was expressed as nmoles/200 mg of fresh moss.

3.9. Chlorophyll content.

Moss (200 mg) was extracted in cold 80% (V/V) acetone (10 ml) in a darkened laboratory. The chlorophyll content was determined from a nomogram produced by Kirk (50)using spectrophotometer readings at 663, 645, and 480 nm. Concentration was expressed in µg chlorophyll/ /200 mg of moss and as per cent of control levels.

3.10. Loss of conductive materials from the moss into the medium

The loss of electrolytes from the moss after freezing treatments was determined as follows: 300 mg of moss in 2.5 ml of distilled water were frozen in 50 ml Kimax culture tubes capped with parafilm. After the treated material had thawed, a further 17.5 ml of double-distilled water were added and stirred. The moss was allowed to

settle to the bottom of the solution which was then decanted into a glass tube. The glass electrode of a conductivity meter (Radiometer, Copenhagen) was submerged in the solution without touching the walls of the glass tube. Conductivity (in reciprocal micro ohms - μmho) was directly read on the instrument. The background conductivity of the distilled water was subtracted from all readings. The controls were kept at 20°C for the duration of the freeze-thawing treatment and the conductivity was determined as described. Results were expressed as per cent increase in conductivity relative to the controls.

3.11. Culture of the moss leaves (phyllidia) and protonema growth.

The five largest green leaves of the control and freeze-thaw treated moss were gently detached from the stem using forceps. Preliminary experiments indicated that the best orientation for optional protonema growth was with the adaxial surface of the leaf up, with the base pressed gently into the nutrient medium. Five leaves were planted in each plastic Petri dish (9cm) containing a thin layer of 1% (W/V) agar with the following salts (Knopp's medium): 0.8 g/l $Ca(NO_3)_2$, 0.2 g/l $MgSO_4$, 0.2 g/l KH_2PO_4 and 0.2 g/l KNO_3. Sterilization of leaves in 1% (V/V) sodium hypochlorite was lethal; therefore this was not done.

The cultures were kept for 3 weeks on the laboratory bench in diffuse light at room temperature (20°C). Appearance and protonematal growth were checked periodically under a compound microscope.

4. OBSERVATIONS AND RESULTS

4.1. Field observations

Temperature measurements and observations were made in January 1976 on the moss *Tortula ruralis* in its habitat along the banks of the Bow River, west of Calgary, Alberta. On January 6th, 1976 the ambient air temperature was -25°C. Mats of moss, dry in appearance and devoid of snow cover, were at the same temperature as the air. The moss mats covered by 5 cm of snow were at -20°C. When the snow cover was removed, the moss plantlets were observed to be in the expanded, hydrated form, frozen within a layer of granular snow changing into ice. Another observation was made on January 20th, 1976 when the ambient air temperature was at 3°C. On this day, the moss *Tortula ruralis* was observed in three different forms. The mats, not covered with snow, were in the dry form. In contrast, those mats at least partially covered with melting snow were rehydrated and their temperature was 0°C. In the central portions of the mats, under the snow cover, the temperature was also 0°C but the rehydrated plantlets were still encrusted in ice.

Unfortunately, the actual rates of temperature change at moss level could not be determined in the field with the available instrumentation. In Alberta, the rates of air temperature change can be as high as 30°C/hr from above 0°C temperature to about -30°C (60).

The following laboratory studies were carried out to determine temperature conditions injurious to *Tortula* and metabolic responses of this moss to subzero temperature stress.

4.2. Response of protein metabolism to freezing treatments.

In vivo protein synthesis was used as a criterion of survival of freezing stress. If maintained in the dry state, moss can survive extremely rapid freezing in liquid nitrogen on rehydration and re-warming to room temperature, whereas fresh moss frozen in liquid nitrogen can only conduct protein synthesis at 6% of the control value on re-warming (Table 3). Survival capacity did not improve until a very low water content was reached (33% of fresh weight) before freezing.

Dehydration of the moss in osmoticum (PEG 6000) at very high concentration (1 kg PEG 6000/1 kg H_2O) also partially protected the moss from liquid nitrogen induced damage (Table 4). Lower concentrations of PEG 6000, however, were not effective in preventing the freezing damage.

As an extension of the initial studies done by Bewley (9), it was found that immersion of hydrated moss in liquid nitrogen inhibited subsequent uptake and incorporation of radioactive leucine (Figs. 5g and 6g). No difference in survival was achieved by employing a faster thawing rate (thawing in 20°C water) than used previously (9).

Freezing to -30°C and thawing of hydrated *Tortula* at slow rates (Fig. 4) was less damaging than rapid freezing in liquid nitrogen (Figs. 5b-e and 6b-e). From Figures 5f and 6f it can be seen that freezing and thawing of hydrated moss at 60°C/hr was almost as destructive as immersion in liquid nitrogen, at least as far as uptake of radioactive leucine and its incorporation into protein is concerned. The slower freeze-thaw treatments of 30°C/hr, 18°C/hr, 6°C/hr and 3°C/hr were survived progressively better by the moss.

Freeze-thaw at the rate of 3°C/hr and 6°C/hr to -30°C appeared innocuous to the moss with respect to radioactive leucine uptake. However, only the 3°C/hr treatment was not significantly different from the control with respect to its capacity to incorporate labelled leucine. This apparent greater sensitivity of protein synthesis compared to that of uptake is expressed more clearly in Table 5. With increasing harshness of the freezing treatment (i.e. increasing the freezing rate) the proportion of radioactive leucine incorporated into protein decreased in relation to the total radioactive leucine taken into the tissue. For example, in the control, up to 30% of the leucine taken up was incorporated into protein after 60 minutes, while after a 60°C/hr freeze-thaw cycle only 5% of the total leucine taken up was found in protein.

The possibility was investigated that the differences in the rate of uptake and incorporation in Figures 5 and 6 were due to the increasing period of time the moss remained at above 0°C temperatures during rewarming to 20°C (i.e. 20 minutes for 60°C/hr treatment, but

7 hours for the 3°C/hr treatment). To determine if the 3°C/hr treated moss had recovered within the 7 hours while above 0°C, the rate of uptake and incorporation of radioactive leucine was followed after the 3°C/hr frozen (to -30°C) moss was thawed at 3°C/hr and subsequently brought to 20°C as quickly as possible. No decrease in uptake or incorporation was detected early after the 3°C/hr treatment and both were at control levels (as in Figs. 5 and 6, data not presented). The reverse possibility that faster frozen-thawed (60°C/hr) moss would recover with time above 0°C was tested by measuring the rate of uptake and incorporation 24 hours after the end of the treatment (thawing to 20°C). The results presented in Figures 7 and 8 indicate that the ability for leucine uptake deteriorated with time but that rate of incorporation actually increased)compare to Fig. 5f). The incorporation data, however, is presented on the basis of protein content and, as can be seen from Table 9, this was decreasing over 24 hours - by about 33%. If this decrease is taken into account in Figure 7, then the rate of incorporation 24 hours after the freeze-thaw treatment becomes almost as low as that measured only 2 hours after the treatment. These results show that hydrated moss does not recover from 60°C/hr freeze-thawing cycles after a longer time of rewarming and that the results in Figures 5 and 6 indeed reflect the response of the moss to freeze-thaw treatments.

It appears from Figure 7 that the ability of the control moss to incorporate leucine (at 20°C) decreases with time, although

variability in the results suggests non-significant differences. However, a similar trend towards a decrease in the ability of controls with time at 20°C to take up and incorporate leucine was observed in a separate experiment (Table 6). As may be noted in Table 9, the protein content of the control moss did not change with time and thus the decrease in incorporation at 20°C can be ascribed to its continually deteriorating metabolic state at this temperature. This observation is relevant to Figures 5a and 6a, which are averages of controls for all treatments used. For, while the control of the fast freezing experiments remained at 20°C only for about 4 hours, the control of the 3°C/hr treatment was kept at 20°C for 36 hours. Thus the averages might be expected to have a low value. The controls which represent the original state of the moss, at 20°C for only 4 hours, (Figs. 5h and 6h), are probably a better basis for comparison.

Upon establishing a relationship between survival (uptake and incorporation of amino acid into protein) and freezing and thawing at equal rates, it was then determined whether fast freezing or fast thawing alone could affect metabolism during the 60°C/hr treatment. For this purpose, the moss was frozen at 60°C/hr to -30°C and thawed at 3°C/hr, and *vice versa*, before protein synthesis at 20°C was determined (Figs. 9 and 10). The fast freezing and slow thawing (Figs. 9d and 10d) treatment was similar in its effect on uptake and incorporation as that of the 60°C/hr freeze-thaw treatment. The reciprocal treatment (3°C/hr freeze and 60°C/hr thaw, Figs. 9c, 10c)

caused some damage to the tissue as indicated by the 20% decrease in incorporation relative to the control. Uptake, however, appeared unaffected by this treatment.

The effect of long storage of the moss in the frozen state at -20°C is also presented in Figures 9 and 10. The moss was brought to the storage temperature and subsequently thawed at 3°C/hr to prevent damage. Both 15-day and 30-day storage periods at -20°C had no damaging effect on uptake or incorporation of leucine by the moss (Figs. 9b and 10b).

To further investigate the status of the protein synthesizing apparatus in the moss after freezing, polyribosomes were extracted and their capacity to synthesize protein *in vitro* was tested. Results of polyribosome extractions are presented in Figure 11 and Table 7. The absorbance peaks of polyribosomes extracted from the control and 60°C/hr frozen-thawed moss showed no significant differences (Figs. 11, A and C). The moss frozen-thawed at 3°C/hr and 60°C/hr (kept at 20°C for 24 hours after reaching this temperature, Figures 11B and D) had a slightly increased polyribosome content (73% relative to control at 62%). The total amount of ribosomal material extracted after the 3°C/hr and 60°C/hr treatments did not change appreciably (Table 7). In contrast, liquid nitrogen freezing led to marked changes in the ribosomal material. A decrease in the total ribosomal content down to only 26% of the original amount occurred after 24 hours, at which time the polyribosomal peaks could not be resolved on a sucrose

gradient. Even two hours after the liquid nitrogen treatment, 20% of the ribosomal material was degraded and resolution of the polyribosome peaks was poor. Interestingly, the single ribosome to polyribosome ratio did not change 2 hours after the liquid nitrogen treatment. This indicated that degradation of both polyribosomes and single ribosomes was taking place at about the same rates.

The polypeptide synthesizing capacity of the cell-free ribosomal fraction, extracted at periods after freeze-thawing, related well to the extractable levels of ribosomal material (Tables 7 and 8). The shift to more polyribosomes after 3°C/hr freeze-thaw was paralleled by increased *in vitro* incorporation of ^{14}C leucine by polyribosomes and a decreased ^{14}C phenylalanine poly-U directed incorporation by the unpolymerized ribosomal units. After the 60°C/hr treatment, the incorporation of amino acids by both ribosomal fractions was almost equivalent to the control. The slight decrease and variability in *in vitro* incorporation after 60°C/hr treatment may reflect the slightly lower amount of ribosomal material (by about 10%) extracted.

Following the liquid nitrogen treatment in the *in vitro* synthetic activity of both the polyribosomes and single ribosomes closely followed the decrease in ribosomal material (Table 7). After 24 hours, only a slight incorporation of ^{14}C phenylalanine (5% of control) could be measured, possibly reflecting the activity by remaining ribosomes detected in the small absorption peak (Fig. 11 F).

4.3. Protein and ATP levels after freezing treatments.

The results of the experiments described above indicate that the protein synthesizing complex itself is not greatly affected even by the 60°C/hr freeze-thaw treatment. The freezing, however, may affect other aspects of protein synthesis: a) the activity of some proteins necessary in the reactions of protein synthesis (eg. initiation, elongation and termination factors, or the transfer RNA charging enzymes) or b) decrease the energy available for some of the vital reactions. A detailed study of some of the specific proteins is beyond the scope of this thesis but would be of interest. Such a study may be difficult, however, because it has not yet been possible to make the components of the moss supernatant catalyse *in vitro* protein synthesis (42). To gain some information about the status of proteins after freezing, changes in total protein concentration were measured.

Protein concentration decreased very rapidly after liquid nitrogen treatments (to 50% of the control in 2 hours - Table 9). Following a 60°C/hr treatment there was also a significant decrease in protein with time after rewarming, although this occured more slowly than after liquid nitrogen treatment. A similar trend in protein concentration changes after freezing at other rates was observed - see Table 10.

The hydrolysis of phosphate bonds of the adenosine triphosphate

(ATP) molecule provides the energy for numerous metabolic reactions, including ion and amino acid uptake and some reactions of protein synthesis (tRNA charging and possibly initiation). Thus ATP levels could affect the rate of leucine uptake and incorporation into protein and for this reason, ATP levels were determined after the freeze-thaw treatments (Table 11). There was a decrease in the ATP concentration to only 37% of the control level during the 3°C/hr freezing treatment. Within two hours after the end of this treatment normal levels of ATP were regained. Immediately after the 60°C/hr freeze-thaw cycle a marked decrease in ATP level was measured. Twenty-four hours later, ATP concentration did not recover but decreased further, to 44% of the control. Immediately upon thawing of the moss after immersion in liquid nitrogen only 32% of the control ATP levels were present and, after 24 hours, only 3% of ATP remained.

Control moss maintained at 20°C for the duration of the 3°C/hr treatment (36 hours) contained somewhat less ATP than controls left for only a short time. A similar, but nonsignificant decrease in ATP levelswas detected in moss stored at 20°C for 48 hours (27 ± 2nmoles/ /200 mg of moss) relative to moss stored at 2°C (31 ± 7 nmoles/200 mg of moss) for the same period.

4.4. Other responses of *Tortula ruralis* to freezing.

Chlorophyll content, leakage of conductive materials from the cells,

appearance (browning) of the moss and the ability of cultured moss leaves (phyllidia) to produce protonema growth were tested to see what other aspects of metabolism were damaged by the freezing treatments previously shown to affect protein synthesis.

There was no decrease in chlorophyll content during freezing itself in either a liquid nitrogen treatment or 60°C/hr treatment and only 24 hours after thawing was there a substantial decrease in chlorophyll content in liquid nitrogen - frozen moss. The chlorophyll content of the moss frozen and thawed at 60°C/hr decreased as the controls (Table 12). The change in chlorophyll content after 60°C/hr treatment was not as great as was expected from microscopic observations of cultured moss leaves (Table 13). The degree of browning of leaves after either of the fast freezing treatments appeared visually to be much greater than could be accounted for by chlorophyll loss alone (i.e. all liquid nitrogen frozen-thawed moss leaves and about 50% of the 60°C/hr frozen-thawed leaves were brown).

To further clarify the condition of the freeze-treated moss, particularly with respect to the age of the leaves, a second culture experiment was carried out. The frozen and thawed leaves were removed from the plant, in approximate order of age, and placed on moistened filter paper at room temperature for several days. Observations on liquid nitrogen frozen-thawed moss confirmed the results of the first experiment. The youngest leaves, however, became lighter ("colorless") in appearance, but not brown. The 60°C/hr treated moss responded in two ways: a) On some plants, all of the leaves appeared to be

similarly affected as the liquid nitrogen treated plants, i.e. the young leaves were light and old leaves brown; however, in some 60°C/hr plants the degree of color change was not as marked as that observed after liquid nitrogen treatment. b) On other plants only the youngest leaves were affected and the remaining leaves appeared same color as the control moss. Quantification of the browning response was not carried out.

The ability of the young leaves to continue growth after freezing and thawing (as the ultimate criterion of survival) could not be tested due to their slow growth rate. Instead, the growth of protonema filaments from the base of the leaves was determined, Unfortunately, protonema growth reflects only the survival of certain cells, not the whole leaf. The ability to produce protonema filaments was significantly reduced only after liquid nitrogen treatment (Fig. 12a). Also, the single protonema filament which appeared after liquid nitrogen treatment did not grow (Fig. 12b). The protonema number and growth of 3°C/hr and 60°C/hr treated moss was not significantly impaired (Figs. 12a and 12b).

Lastly, the degree of leakage of electrolytes from leaf cells was measured. Leakage is believed to reflect the degree of semi-permeability loss in the cell membrane. Some electrolytes were lost even from the control moss (Table 14). The moss frozen and thawed at 3°C/hr and 60°C/hr lost almost equal amount of electrolyte which was 170% greater than loss from control moss. The liquid nitrogen treatment proved very damaging with a loss in excess of 600% of control.

4.5. Seasonal acclimatization of the moss.

Moss collected at the beginning of July was used in experiments described above. It was of interest to determine if moss collected in winter (at the beginning of January) responded differently to freezing treatments than moss collected in the summer. To resolve this, two approaches were used. Firstly, the response of July and January collected moss to an 18°C/hr freeze-thaw cycle was studied. Secondly, the ability to take up leucine and to synthesize protein at low temperature (2°C) was compared in both types of moss.

For the first series of experiments, an 18°C/hr freezing treatment was selected because it was not so harsh as to destroy the tissue, but severe enough to allow recognition of any differences between the summer and winter moss. As indicated in Figures 13 and 14, there was no appreciable difference between the control moss collected in the summer or winter. However, in respect to incorporation after 18°C/hr freezing, the winter moss was able to synthesize protein slightly, but significantly better than summer moss. There was not a significant difference in uptake.

On testing the ability of the winter and summer collected moss to take up and incorporate leucine at 2°C, a slightly greater rate of incorporation by the January collected moss was found (Fig. 15). This difference between the two types of moss became even more significant when the uptake data was compared (Fig. 16). After 60 minutes there was actually less leucine available for incorporation in the winter

moss, than in the summer moss, yet the rate of incorporation was greater than in the latter. The previously described "trend" (albeit statistically insignificant) toward decreased ability with time (compare moss kept at 2°C and 20°C for 24 or 48 hours, Figs. 15 and 16) to take up and incorporate radioactive leucine was detected again in these experiments. This deterioration took place not only at 20°C but also at 2°C.

Protein concentrations were determined in above experiments and they are reported in Table 14. There were no significant differences in the protein content of the winter and summer moss.

4.6. Metabolic activity of *Tortula ruralis* at extreme temperatures.

As discussed in the literature review, several workers have detected metabolic activity in mosses at very low, even subzero temperatures. The rate of uptake and incorporation of radioactive leucine was measured at -2.5°C. The incubation medium was found to supercool (remain liquid) to about -5°C for at least 2 hours. An incubation of the moss in liquid radioactive medium at -2.5 ± 0.5°C was therefore possible. Although the rate of leucine uptake was lower at -2.5°C than at 20°C the final level of radioactive material in the tissue was not significantly less (Fig. 18). The rate of leucine incorporation into protein was very low at -2.5°C (Fig. 17) despite the availability of the amino acid in the tissue. After 2

hours of incubation there was no further increase in the amount of radioactive leucine incorporated at -2.5°C which after 1 hour was only about 10% of the control level.

While investigating the capacity of *Tortula ruralis* to synthesize protein at subzero temperatures, it was of interest to also determine the upper temperature limit for protein synthesis in the moss, i.e. the extremes. While at 36°C incorporation and uptake proceeded at a rate only slightly faster than in the control at 20°C, at 48°C both events were greatly inhibited (Figs. 19 and 20). The upper temperature limit for protein synthesis in this moss thus appears to be between 36° and 48°C.

Figure 1

a) Effects of cooling rate on the survival of cells frozen to -196°C and thawed rapidly. The yeast (dark circles) and human red cells (light circles) were frozen in distilled water and blood plasma, respectively. The marrow stem cells (squares) and hamster cells (triangles) were suspended in salt solutions containing 1.25 M glycerol. (From Mazur, Ref. 69)

b) Comparative effects of cooling rate on the survival of various cells, Circles - survival of mulberry cortical parenchyma cooled to -75°C at indicated rates and rewarmed rapidly in 30°C water (light symbols) or slowly in the air (dark symbols); from Sakai & Yoshida (102). Triangles - survival of eight cell mouse embryos in 1M DMSO frozen to -196°C at indicated rates, and warmed at 4°C/min.;from Whittingham et al. (122) Squares - survival of carrot root cell suspension cultures (in the presence of 5% DMSO) frozen to -196°C at indicated rates, stored for 7 days at -196°C and thawed at 120°C/min.; from Nag and Street (78). Inverted triangles - survival of *Tortula ruralis* frozen at indicated rates to -30°C and thawed at the same rates; no cryoprotectants added. The rate of freezing in liquid nitrogen was not determined in the cases of carrot root cell cultures and the moss and was arbitrarily placed at 100°C/min.

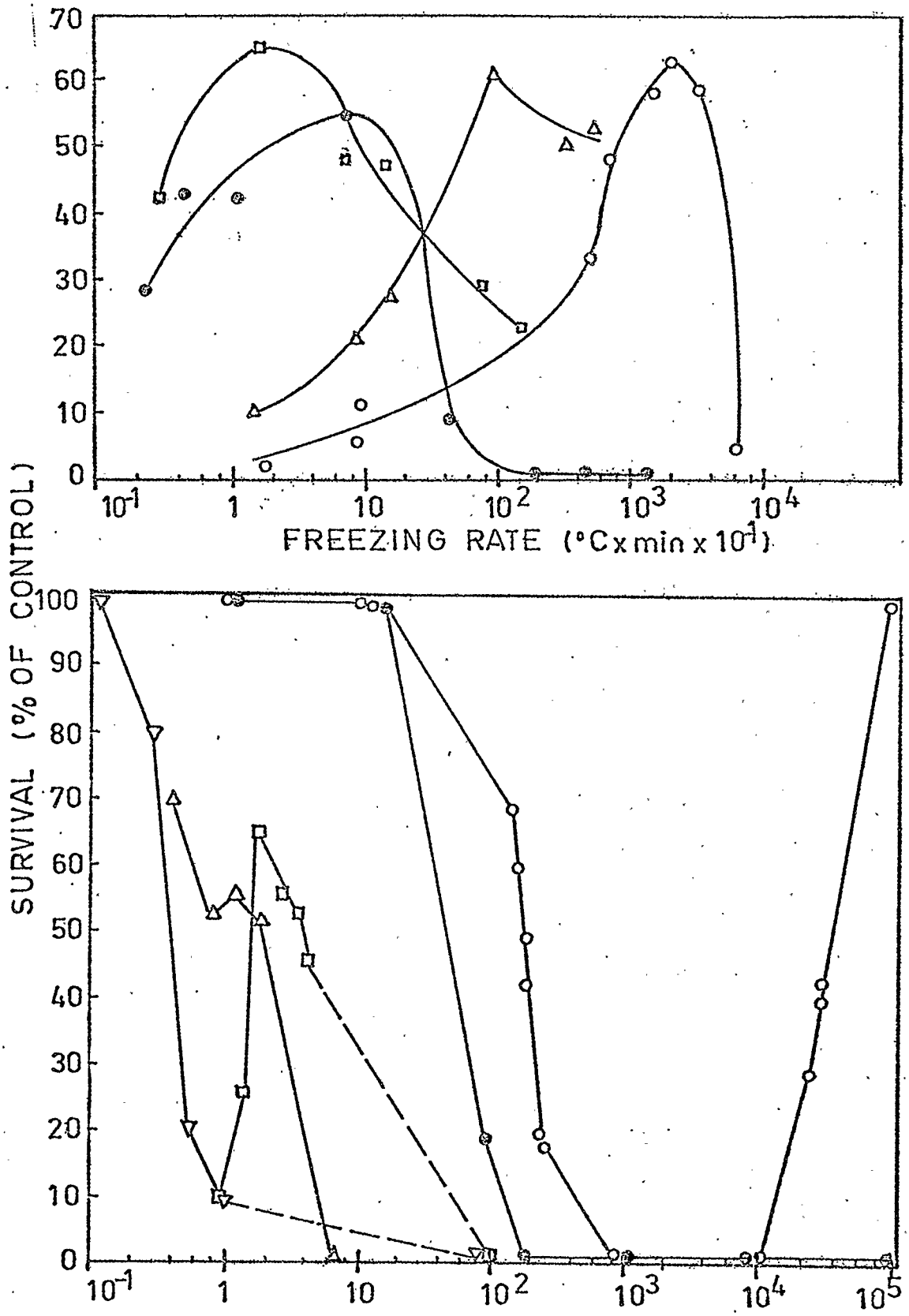

SURVIVAL (% OF CONTROL)
FREEZING RATE (°C x min x 10⁻¹)

Figure 2

Effect of changing the amount of radioactive ^{3}H leucine added to moss on its incorporation into a hot TCA precipitate.

a) 20μCi/2.5 ml

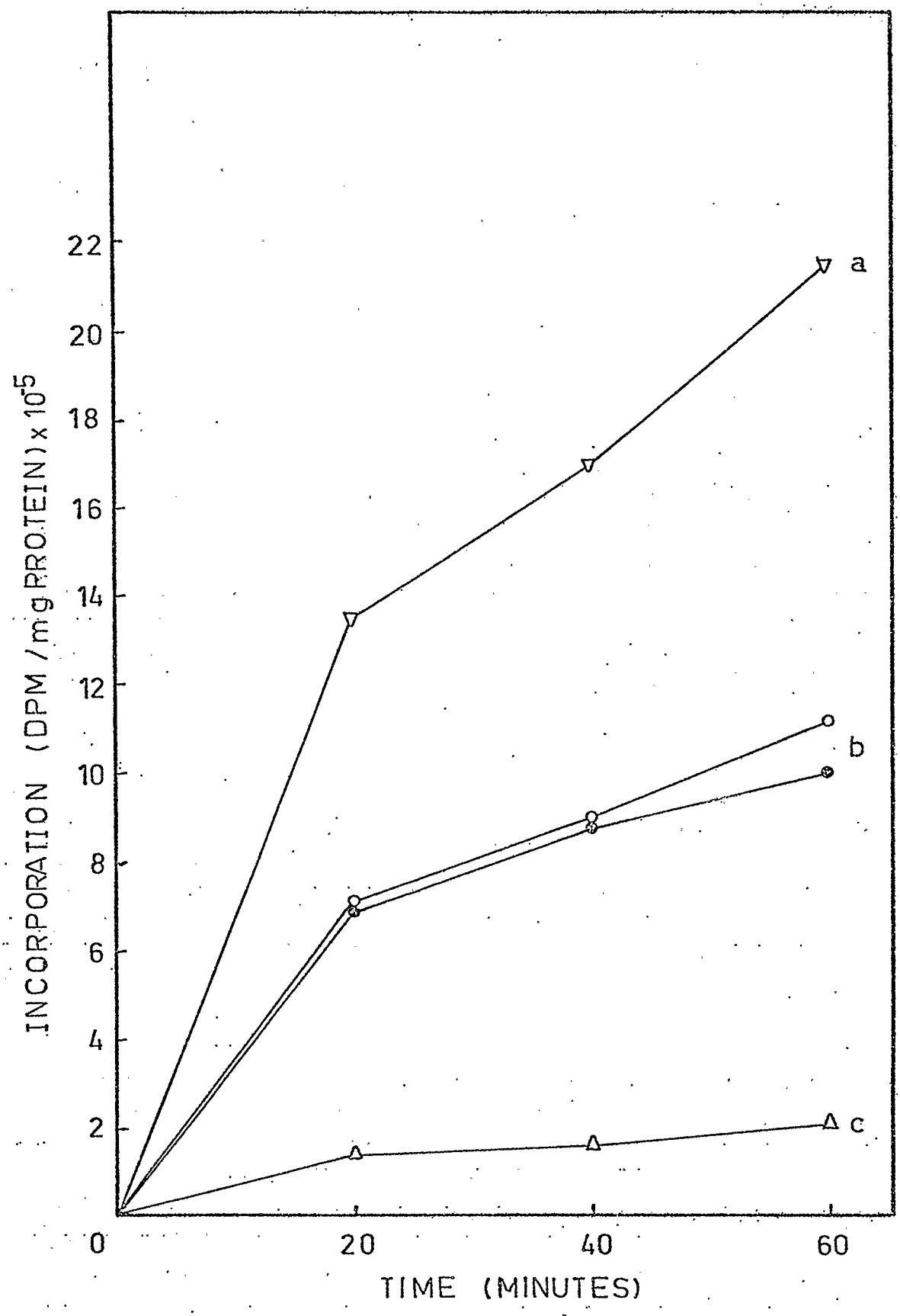
b) 10μCi/2.5 ml

c) 2μCi/2.5 ml

Dark symbols in b) indicate the effects of 15 minute exchange of radioactive leucine with cold leucine (220 mg/l) at $0^{o}C$.

Figure 3

Effect of changing the amount of applied radioactive ^{3}H leucine on its uptake into the moss tissue. Legend as in Fig. 2.


INCORPORATION (DPM / mg PROTEIN) x 10^{-5}
22
20
18
16
14
12
10
8
6
4
2
0
20
40
60
TIME (MINUTES)
a
b
c

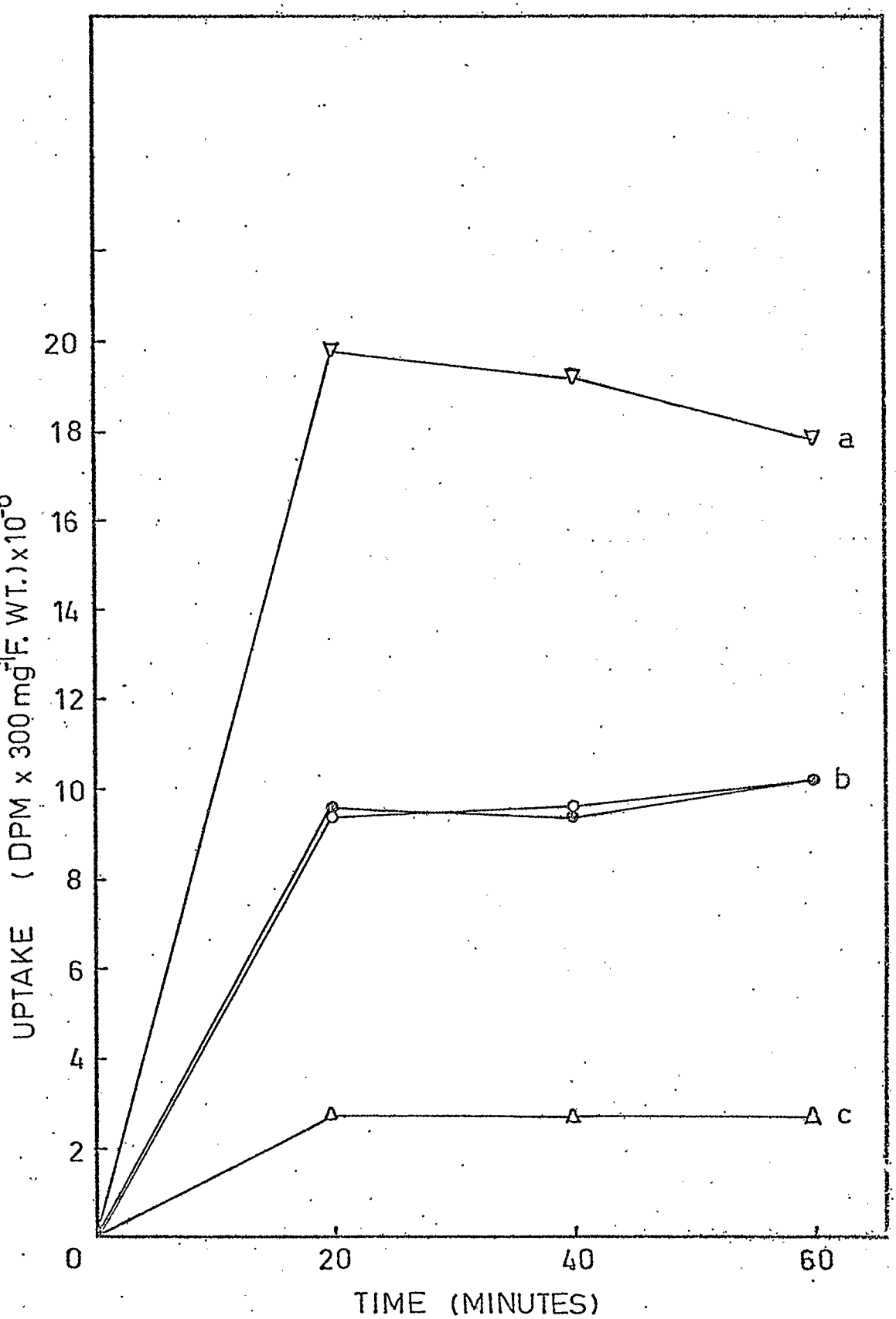

UPTAKE (DPM x 300 mg⁻¹F. WT.) x10⁻⁶
0
2
4
6
8
10
12
14
16
18
20
20
40
60
TIME (MINUTES)
a
b
c

Figure 4

Representative freezing curves of 300 mg of moss in 2.5 ml of distilled sterile water.

a) 60°C/hr

b) 3°C/hr

The rate of freezing and thawing during the liquid nitrogen treatment could not be determined using the available apparatus.

Arrows indicate points at which metabolic response of *Tortula* was studied.

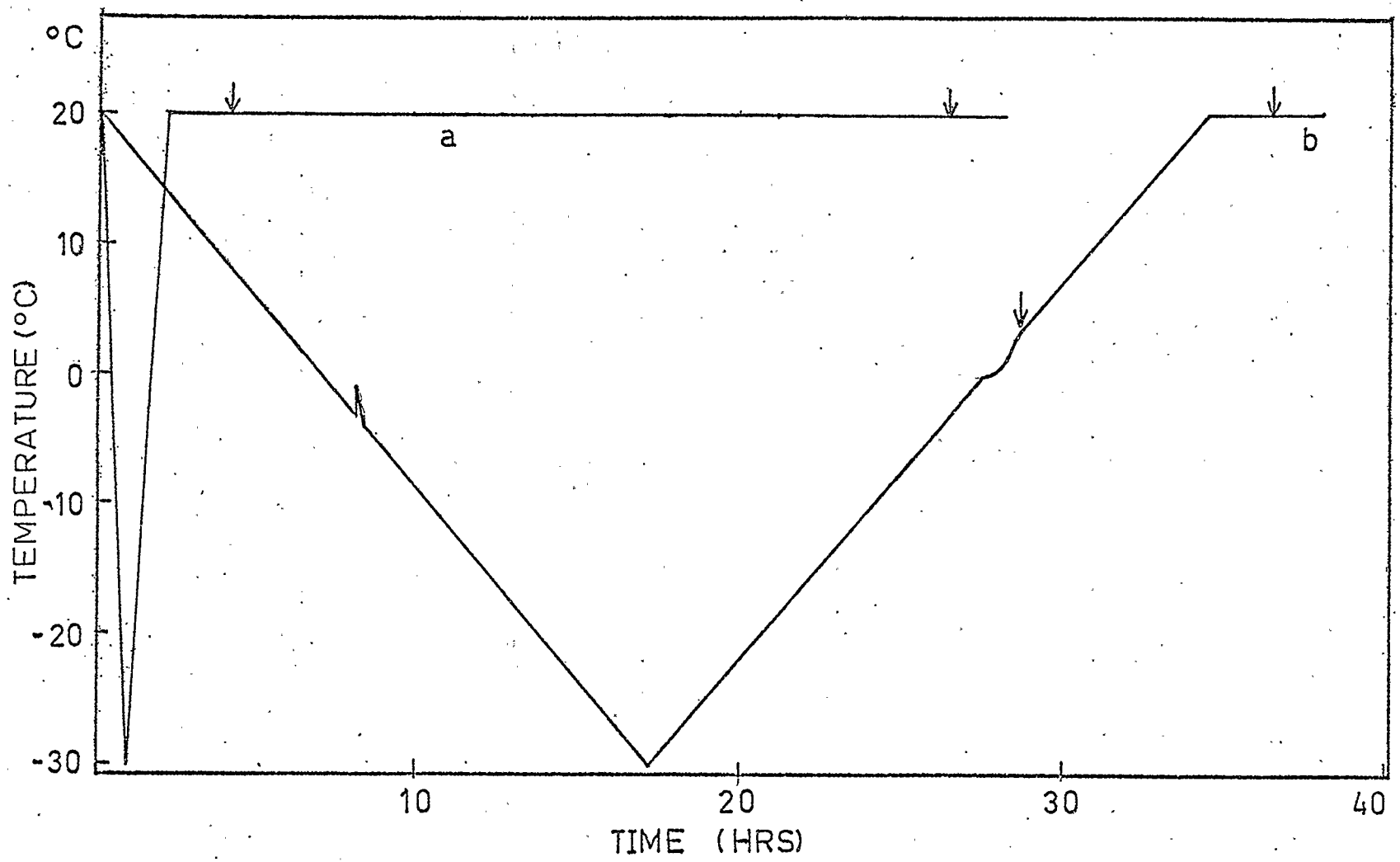

°C
20
10
0
-10
-20
-30
TEMPERATURE (°C)
a
b
10
20
30
40
TIME (HRS)

Figure 5

Incorporation of ^{3}H leucine into a hot TCA precipitable fraction (soluble protein) after freezing and thawing of hydrated moss at the same rates.

a) average of controls for each experiment

b) 3°C/hr

c) 6°C/hr

d) 18°C/hr

e) 30°C/hr

f) 60°C/hr

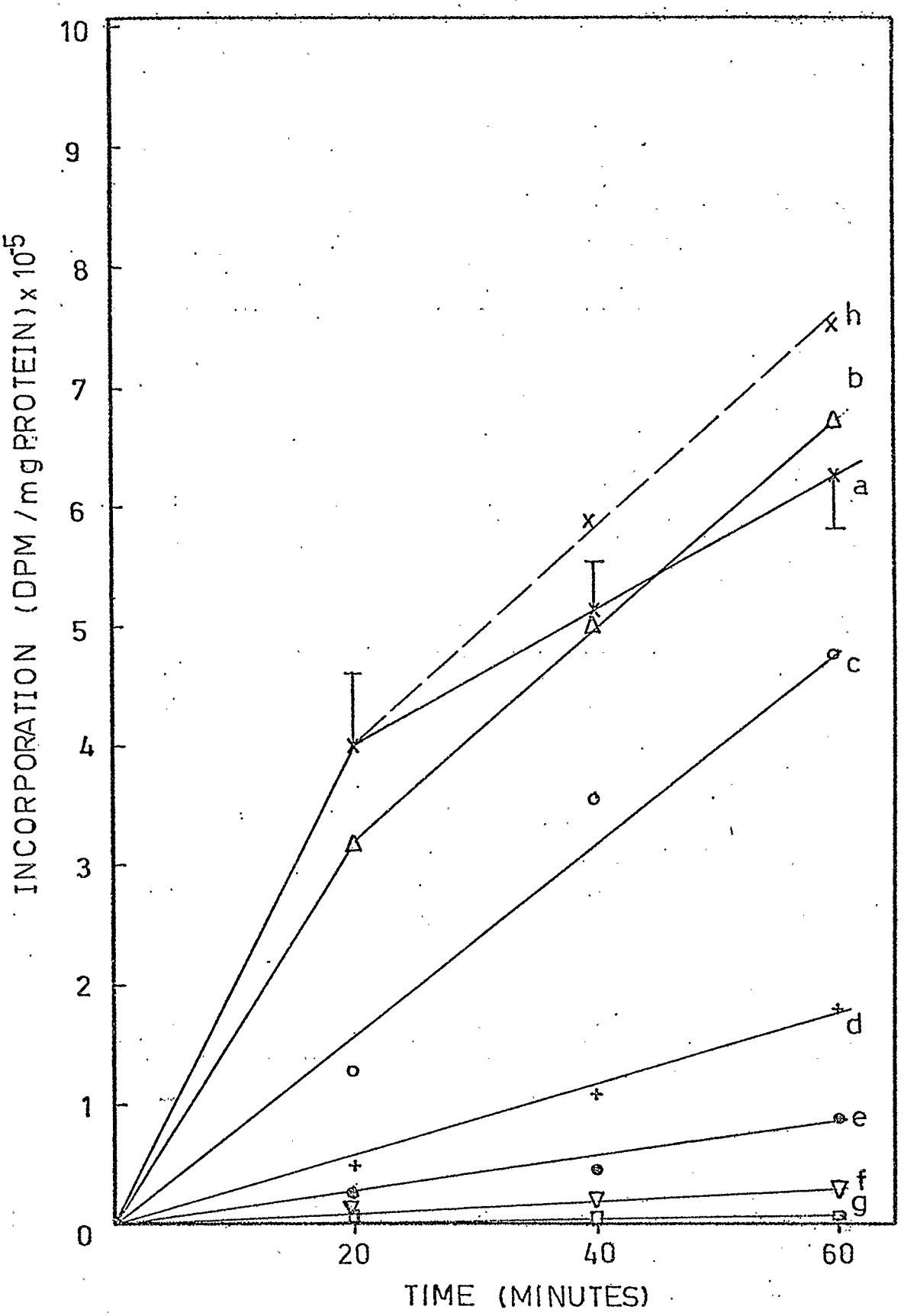
g) liquid nitrogen submersion and fast thaw in water at 20°C

h) control of the liquid nitrogen and 60°C/hr treatments

Figure 6

Entry of ^{3}H leucine into the moss tissue with time (uptake) after freezing and thawing at equal rates. Legend as in Figure 3.

Variability: Standard error of the mean.


INCORPORATION (DPM / mg PROTEIN) x 10^{-5}
TIME (MINUTES)
0
1
2
3
4
5
6
7
8
9
10
20
40
60
a
b
c
d
e
f
g
h

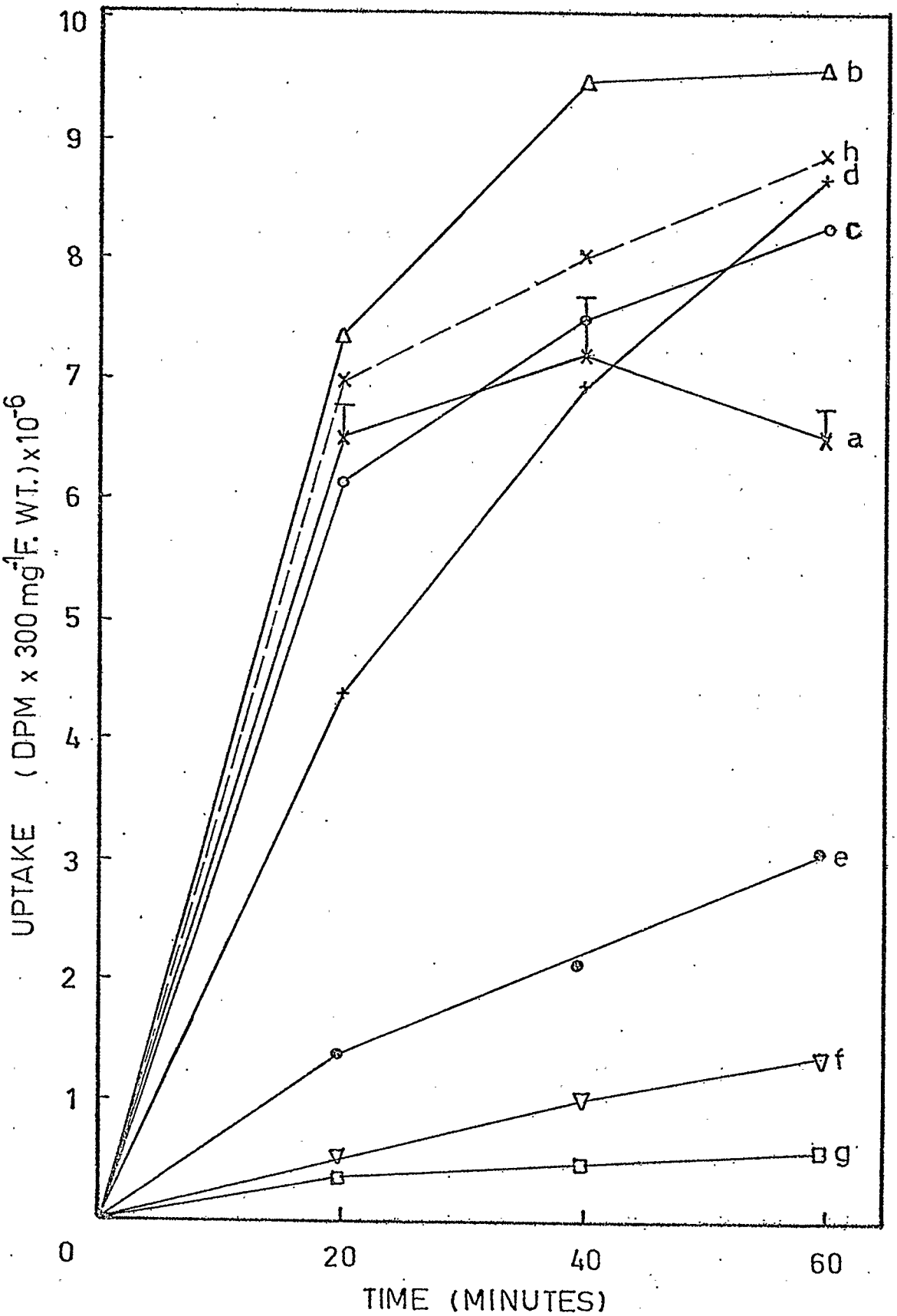

10
9
8
7
6
5
4
3
2
1
0
20
40
60
TIME (MINUTES)
UPTAKE (DPM x 300 mg⁻¹F. WT.) x10⁻⁶
b
h
d
c
a
e
f
g

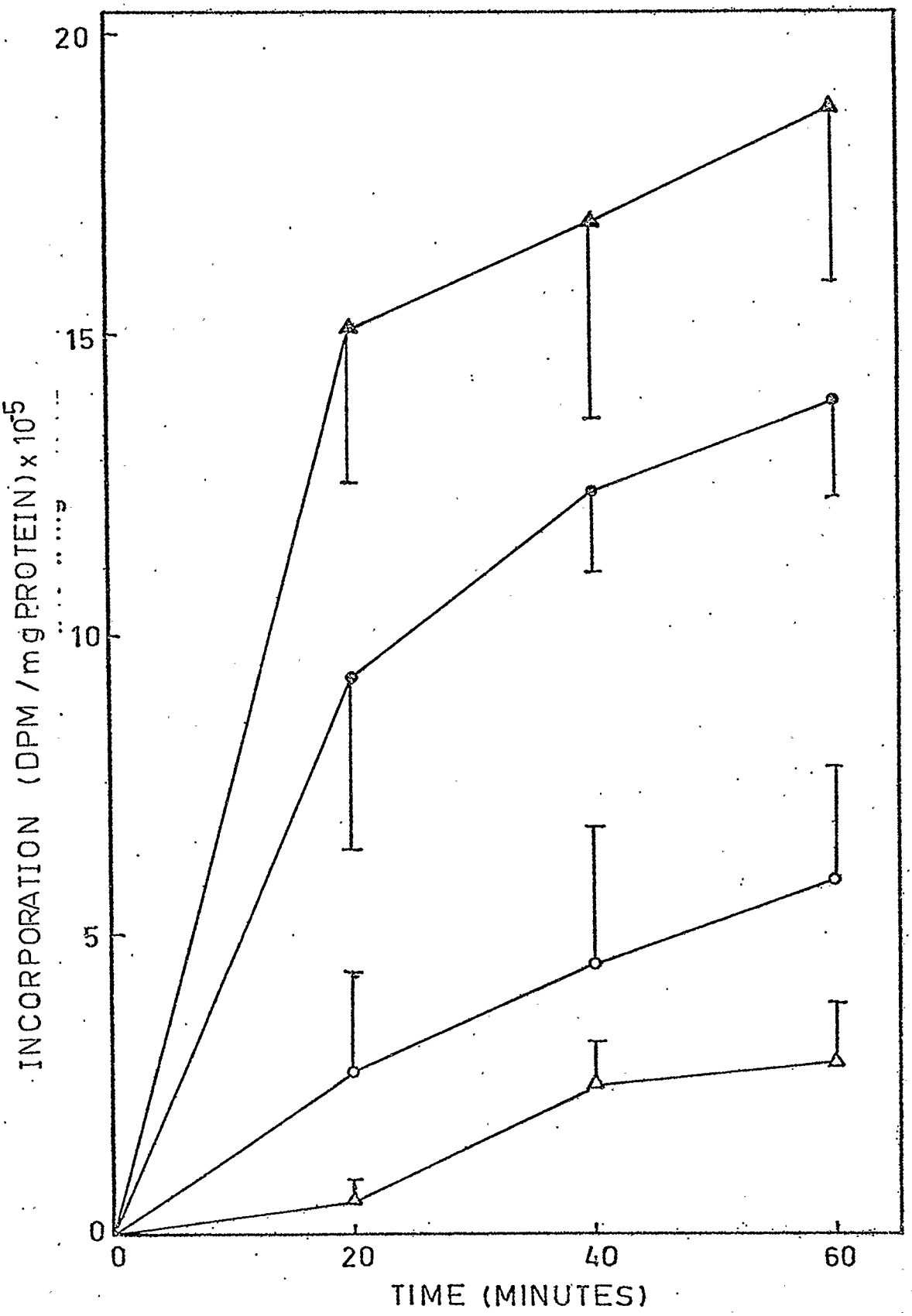
Figure 7

Recovery of the moss from 60°C/hr freezing and thawing. Incorporation of ^{3}H leucine into a hot TCA precipitate. Dark symbols - controls. Light symbols - 60°C/hr frozen and thawed moss. Triangles - 2 hours after end of treatment. Circles - 24 hours after end of treatment.

Figure 8

Uptake of ^{3}H leucine into the moss tissue. Legend as in Figure 7.

Variability: Standard error of the mean.


20
15
10
5
0
INCORPORATION (DPM / mg PROTEIN) x 10^{-5}
0
20
40
60
TIME (MINUTES)

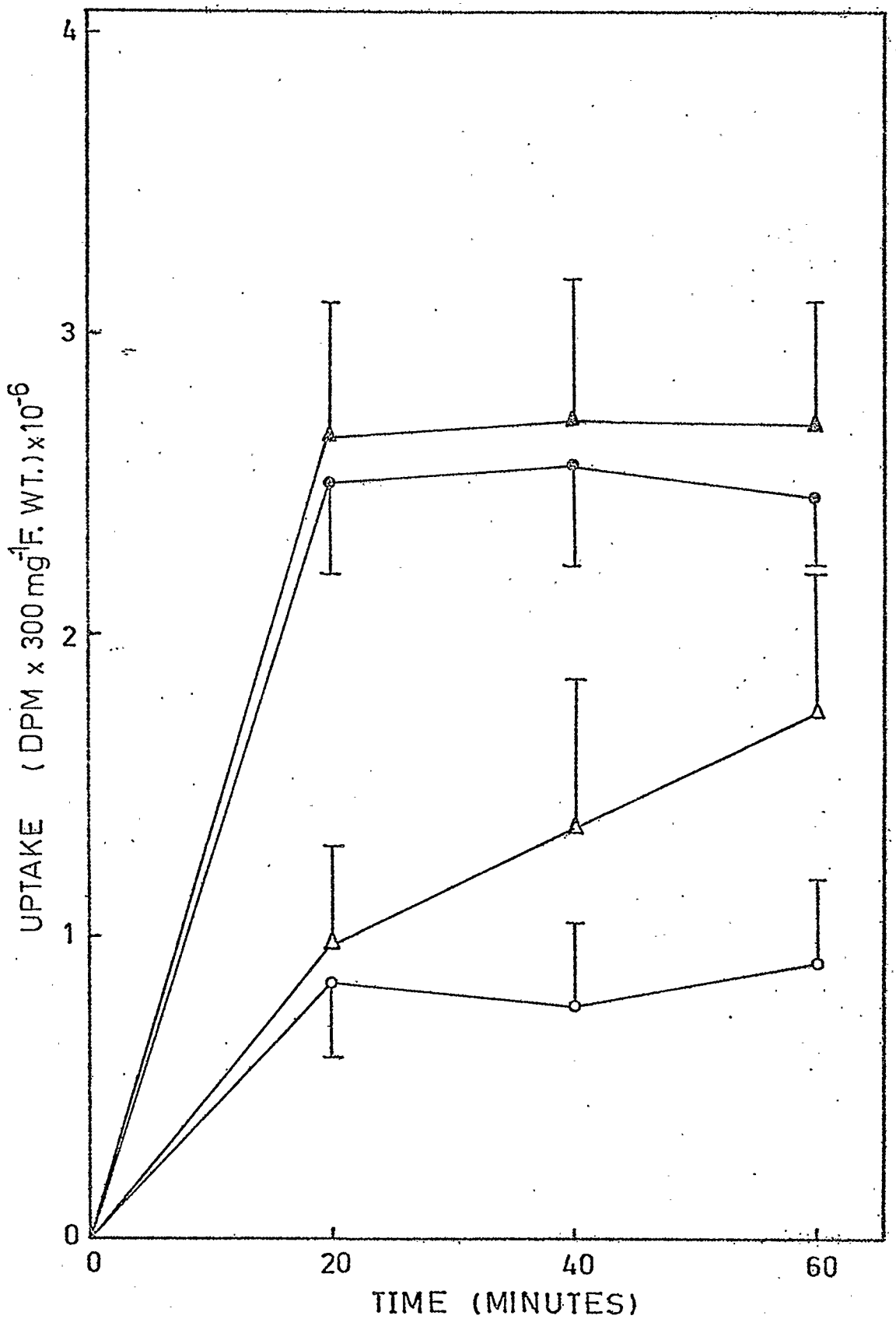

4
3
2
1
0
UPTAKE (DPM x 300 mg^{-1}F. WT.) x10^{-6}
0
20
40
60
TIME (MINUTES)

Figure 9

^{3}H leucine incorporation into a hot TCA precipitate after uneven freeze-thaw treatments and effect of long term storage after 3°C/hr freeze on protein synthesis.

a) control - unfrozen moss

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

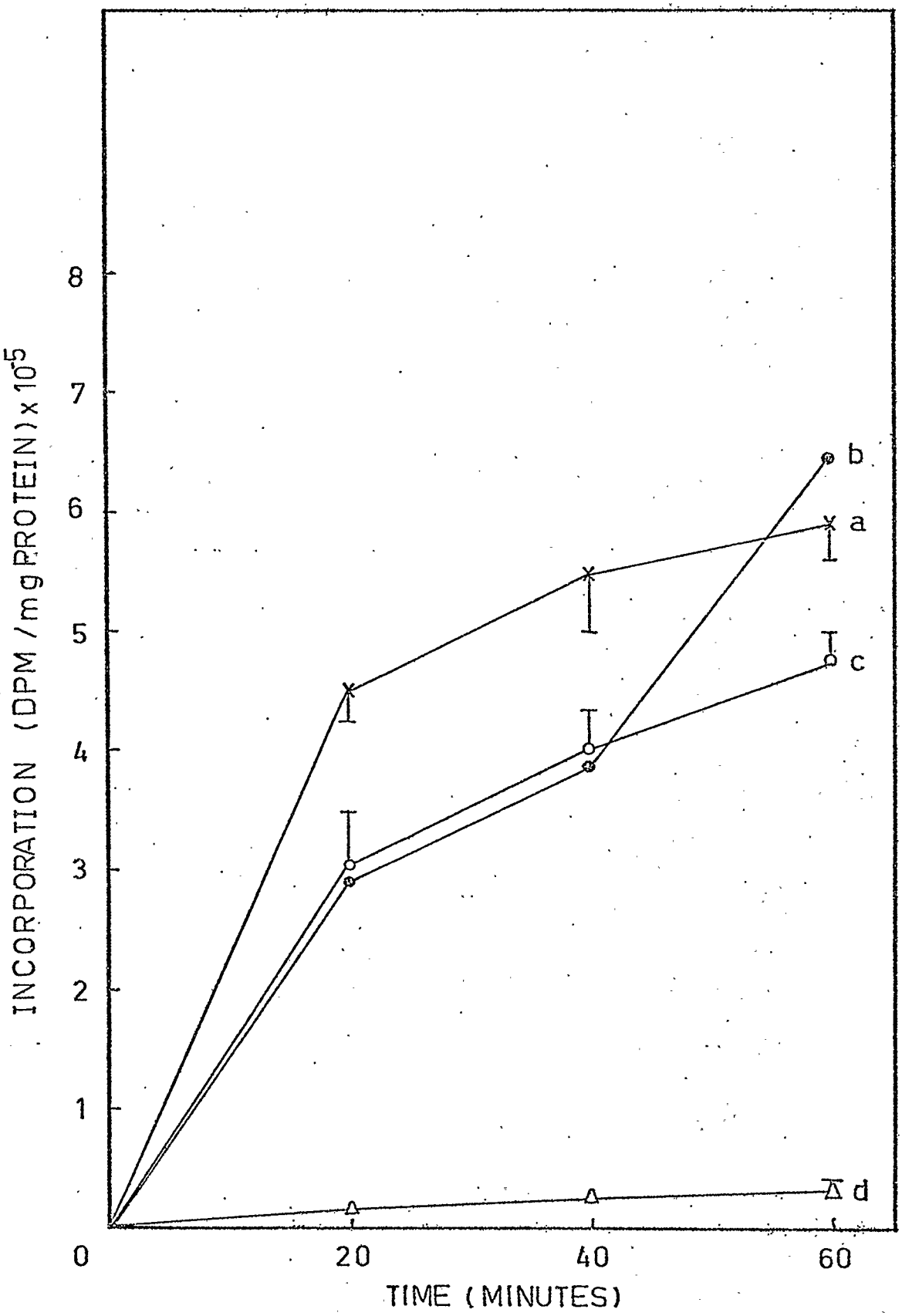
c) moss frozen at 3°C/hr, thawed at 60°C/hr

d) moss frozen at 60°C/hr, thawed at 3°C/hr

Figure 10

^{3}H leucine uptake into moss tissue after uneven freeze-thaw treatments and following a long term storage after 3°C/hr freeze. Legend as in Figure 9.

Variability: Standard error of the mean.


INCORPORATION (DPM / mg PROTEIN) x 10^{-5}
8
7
6
5
4
3
2
1
0
20
40
60
TIME (MINUTES)
a
b
c
d

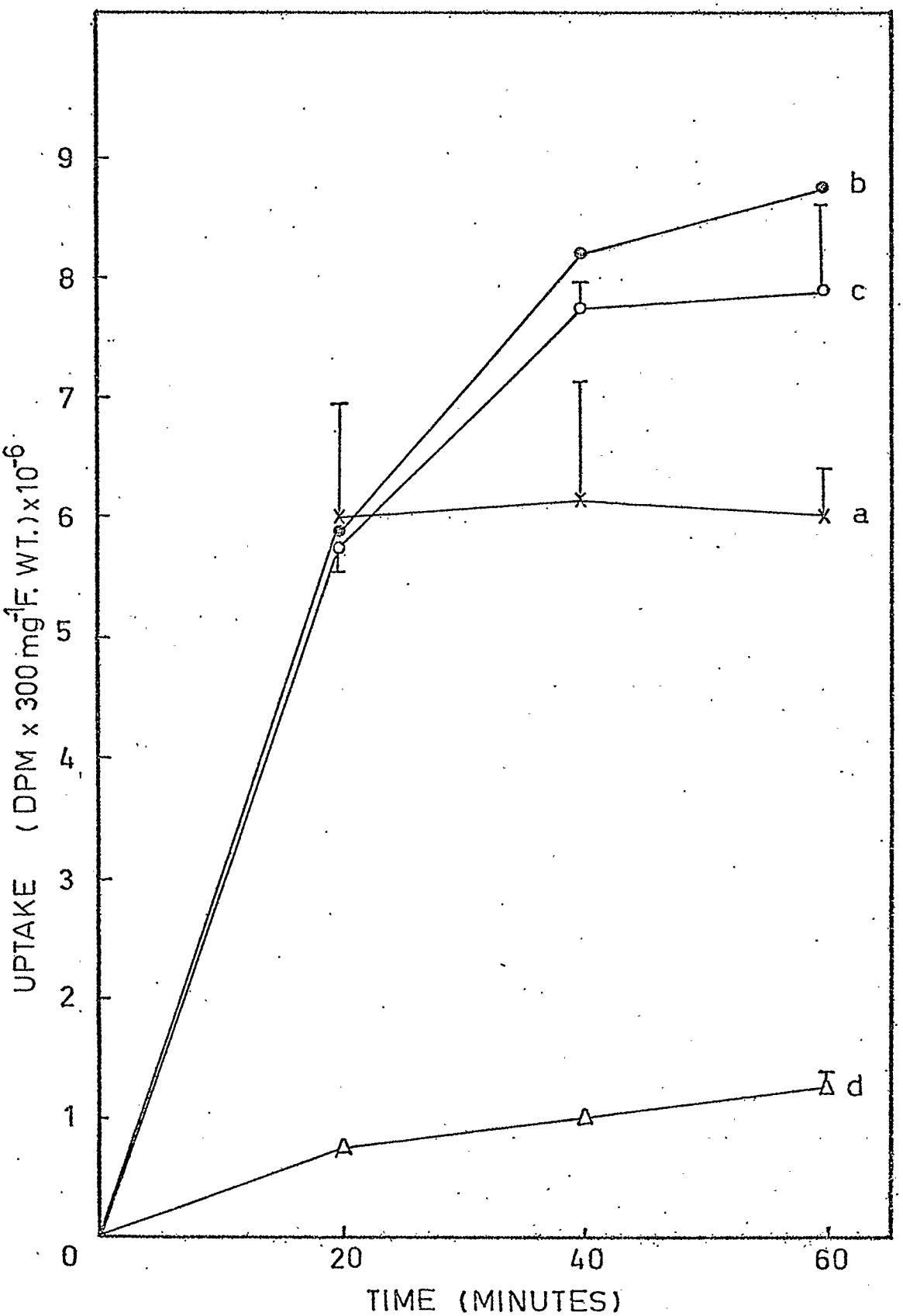

UPTAKE (DPM x 300 mg^{-1}F. WT.) x 10^{-6}
TIME (MINUTES)
0
1
2
3
4
5
6
7
8
9
20
40
60
a
b
c
d

Figure 11

The effect of freezing treatments on ribosomal material. The absorbance patterns of ribosomes and polyribosomes separated on a 10% to 32% sucrose gradient.

A. control (untreated moss), (R = ribosomes, P = polyribosomes)

B. 2 hours after freezing and thawing at 3°C/hr

C. 2 hours after freezing and thawing at 60°C/hr

D. 24 hours after freezing and thawing at 60°C/hr

E. 2 hours after freezing and thawing in liquid nitrogen

F. 24 hours after freezing and thawing in liquid nitrogen

0.2 ml (0.94 mg rRNA equivalent) of ribosomal material were applied to the top of the gradient in all cases except E and F, where 0.4 ml aliquots were applied containing greatly decreased amounts of rRNA.

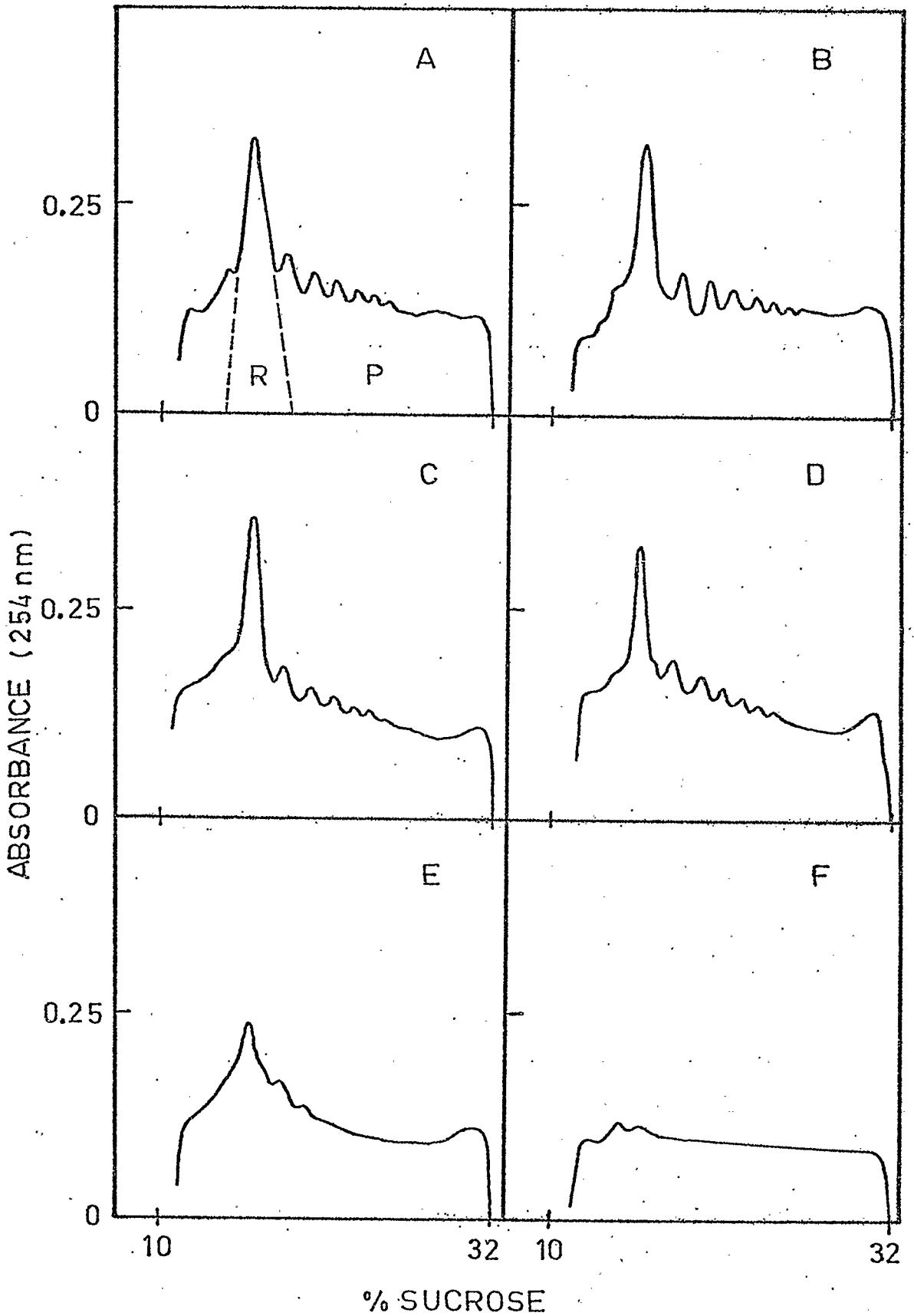

A
B
C
D
E
F
R
P
0.25
0
0.25
0
0.25
0
ABSORBANCE (254 nm)
10
32
10
32
% SUCROSE

Figure 12

a) Average number of protonema filaments produced per leaflet (phyllidium) cultured for 3 weeks after the freezing and thawing treatment

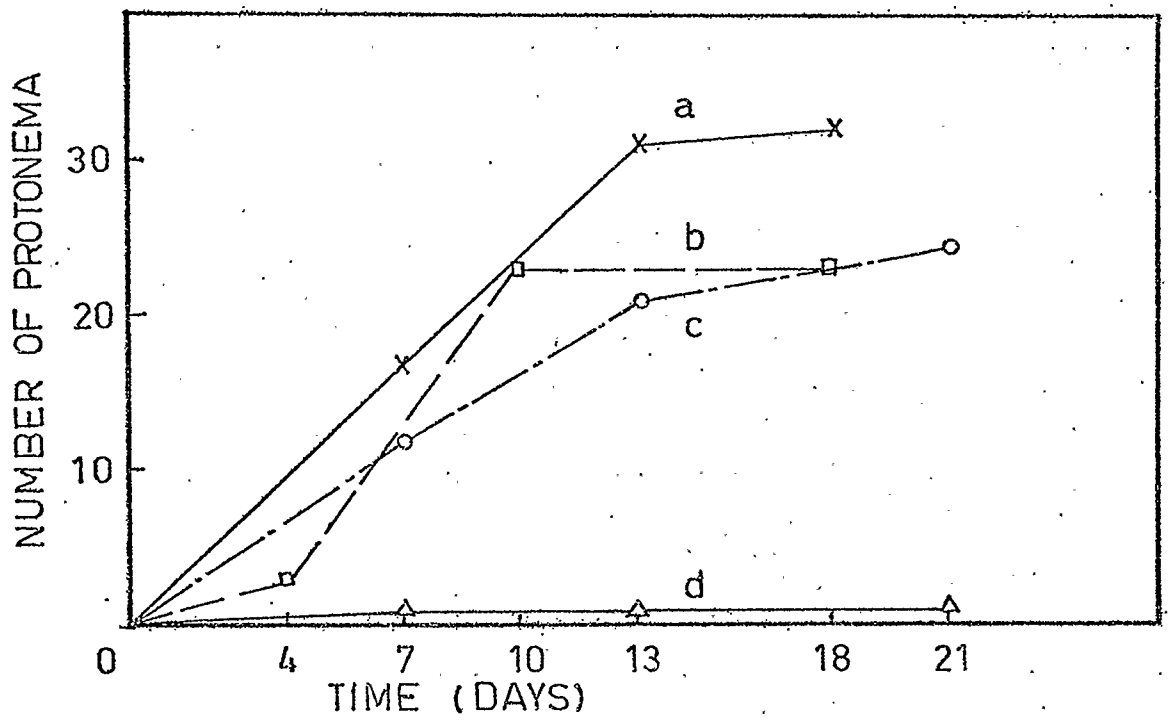
   a) control

   b) $60^{o}C$/hr freeze and thaw

   c) $3^{o}C$/hr freeze and thaw

   d) liquid nitrogen treatment

b) The growth of protonema filaments in culture leaflets frozen and thawed at various rates. See legend for Figure 12a.


NUMBER OF PROTONEMA
30
20
10
0
4
7
10
13
18
21
TIME (DAYS)
a
b
c
d

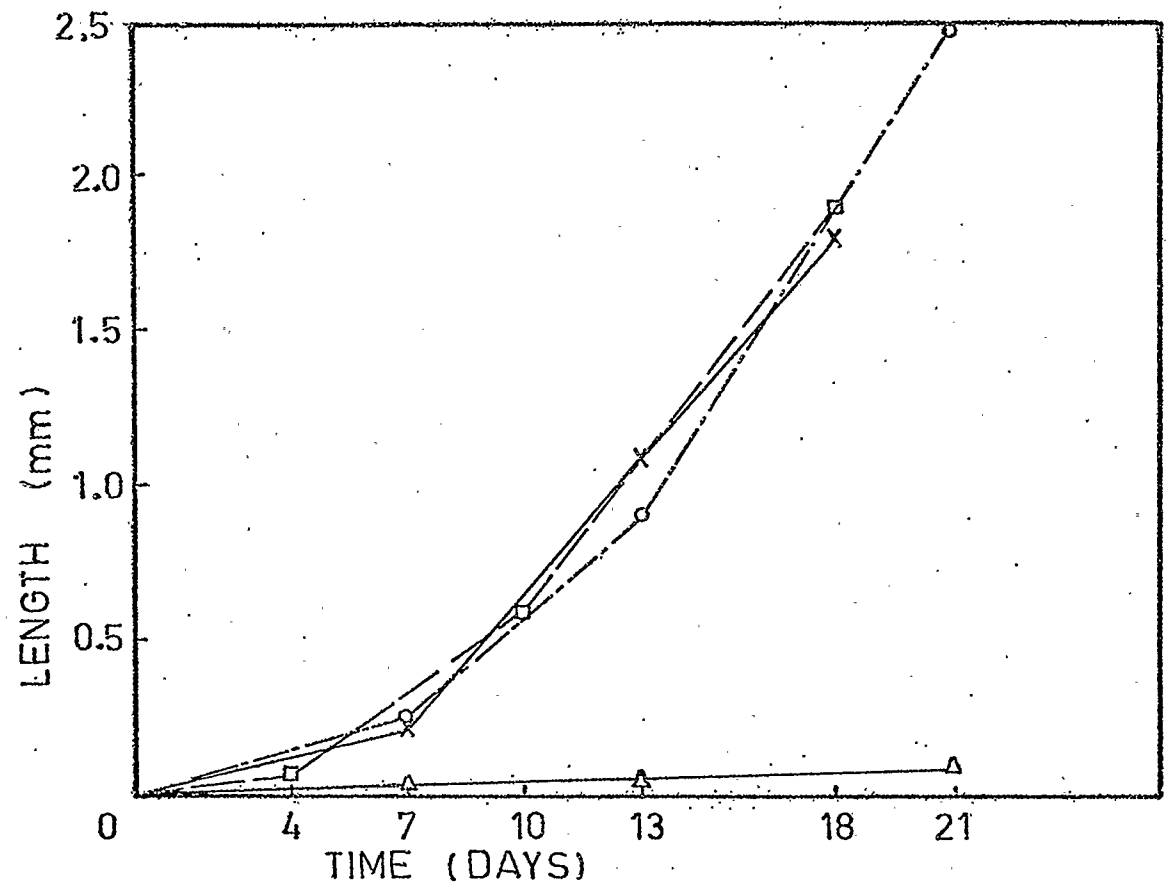

LENGTH (mm)
2.5
2.0
1.5
1.0
0.5
0
4
7
10
13
18
21
TIME (DAYS)

Figure 13

Adaptation to low temperatures - recovery from freezing stress. Incorporation of ^{3}H leucine into a hot TCA precipitate 2 hours after the 18°C/hr freeze-thaw cycle. Moss collected in January (triangles) and July (circles) was compared.

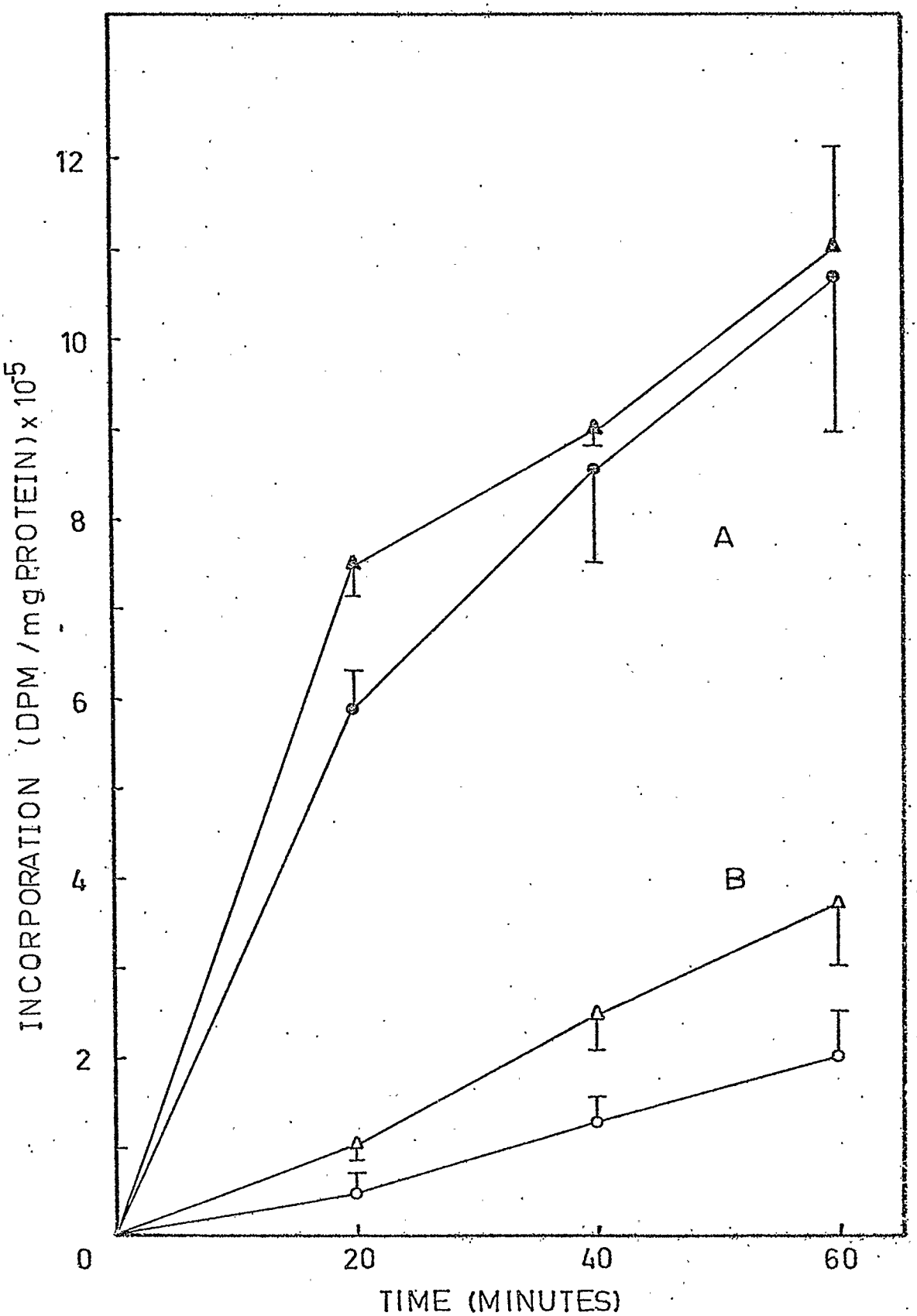
A. untreated controls (solid symbols)

B. treated material (open symbols)

Figure 14

Uptake of ^{3}H leucine into moss tissue 2 hours after the 18°C/hr freeze-thaw cycle. Legend as in Figure 13.

Variability: Standard error of the mean.


INCORPORATION (DPM / mg PROTEIN) x 10^{-5}
12
10
8
6
4
2
0
20
40
60
TIME (MINUTES)
A
B

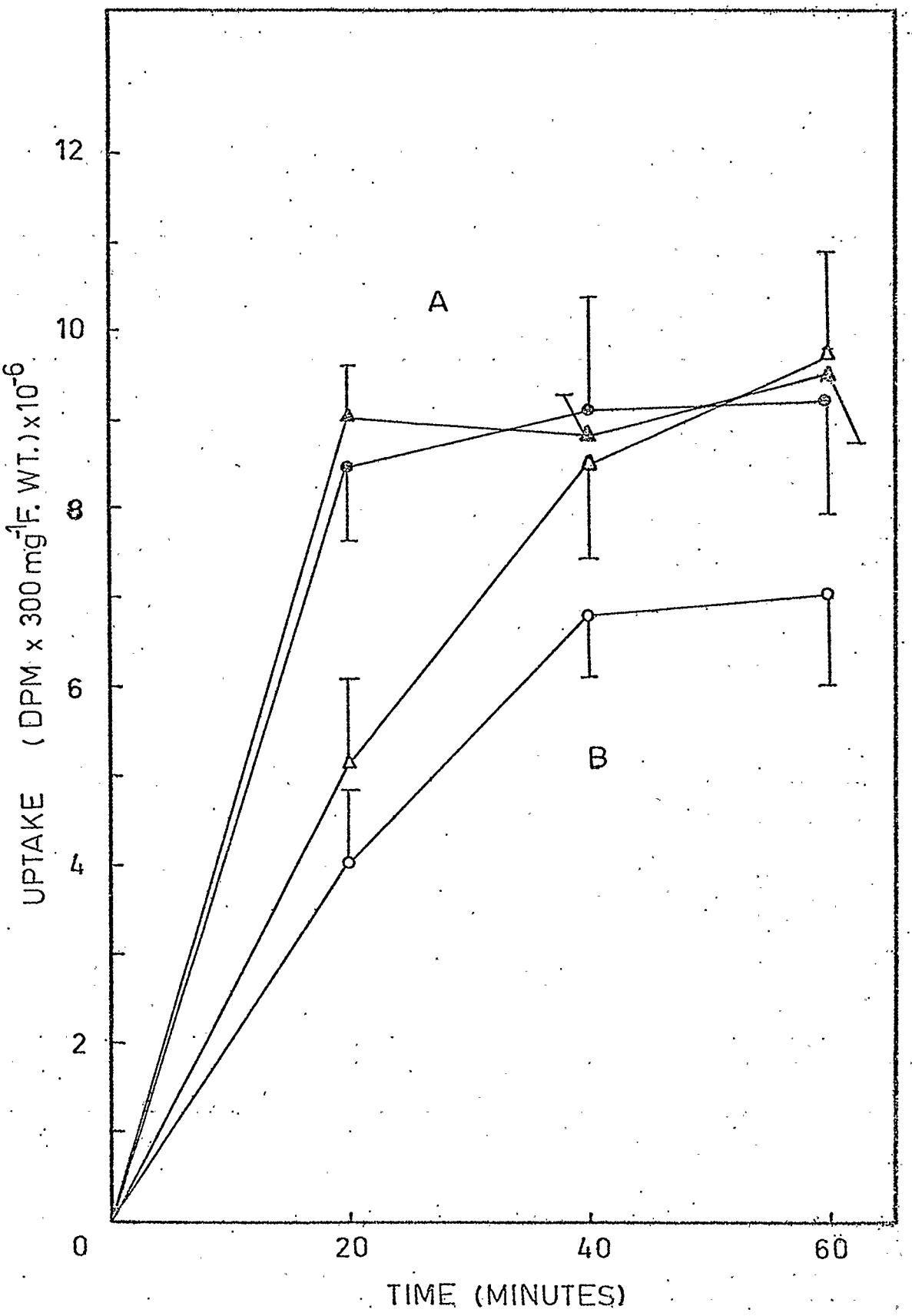

12
10
8
6
4
2
0
20
40
60
A
B
UPTAKE (DPM x 300 mg^{-1}F. WT.) x10^{-6}
TIME (MINUTES)

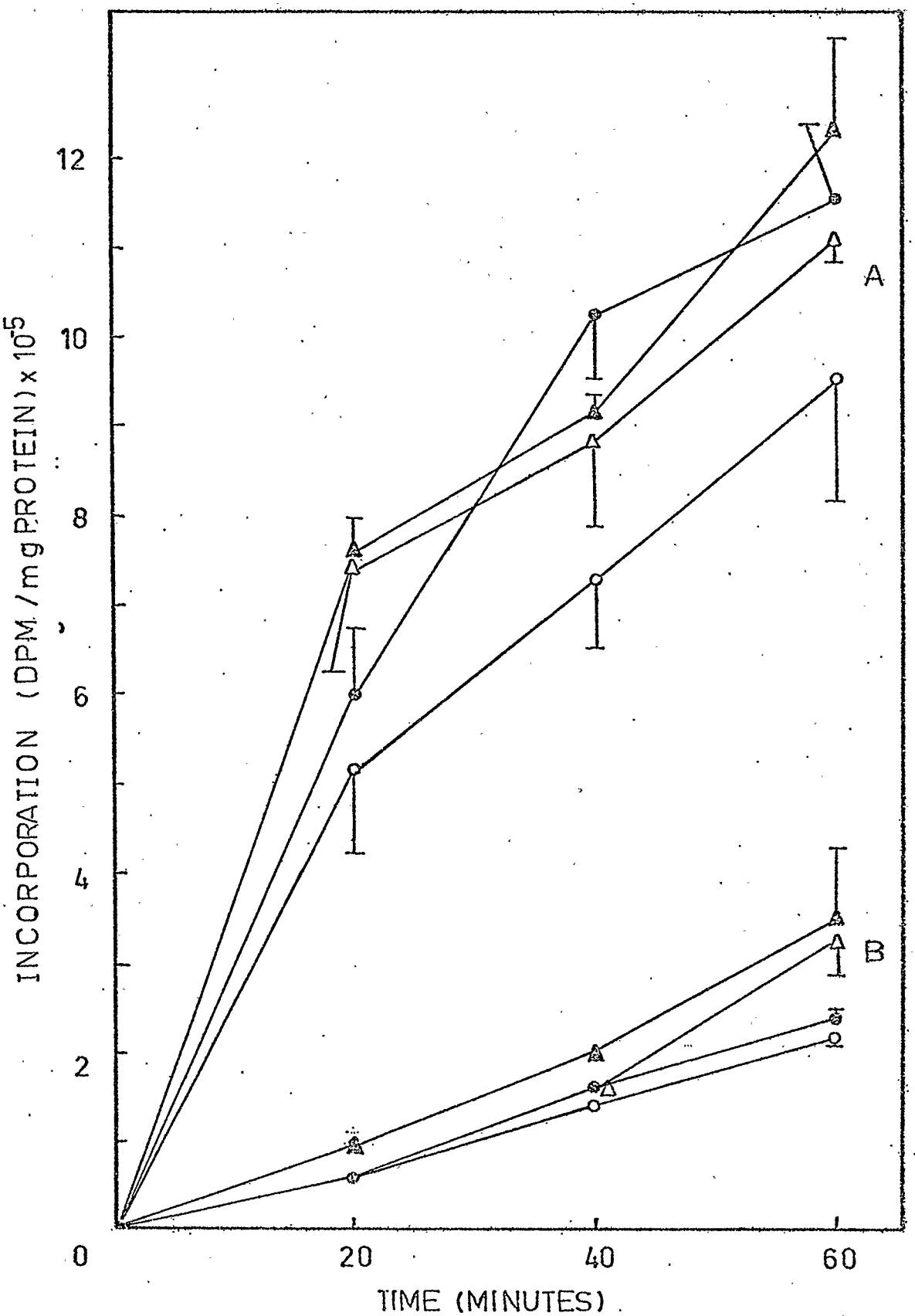
Figure 15

Adaptation to low temperatures - protein synthesis at low temperatures. Incorporation of ^{3}H leucine into a hot TCA precipitate by moss kept at 2°C or 20°C for 24 hours or 48 hours. The moss was collected in July and in January. Group A - control moss stored and tested for incorporation at 20°C. Group B - moss stored and tested for incorporation at 2°C. Triangles - January collected moss, circles - July collected moss. Solid symbols - after 24 hours at the storage temperature, empty symbols - after 48 hours at the storage temperature.

Figure 16

Uptake of ^{3}H leucine into moss tissue after storage at 2°C or 20°C for 24 or 48 hours by moss collected in July and in January. Legend as in Figure 15.

Variability: Standard error of the mean.


INCORPORATION (DPM/mg PROTEIN) x 10^{-5}
12
10
8
6
4
2
0
20
40
60
TIME (MINUTES)
A
B

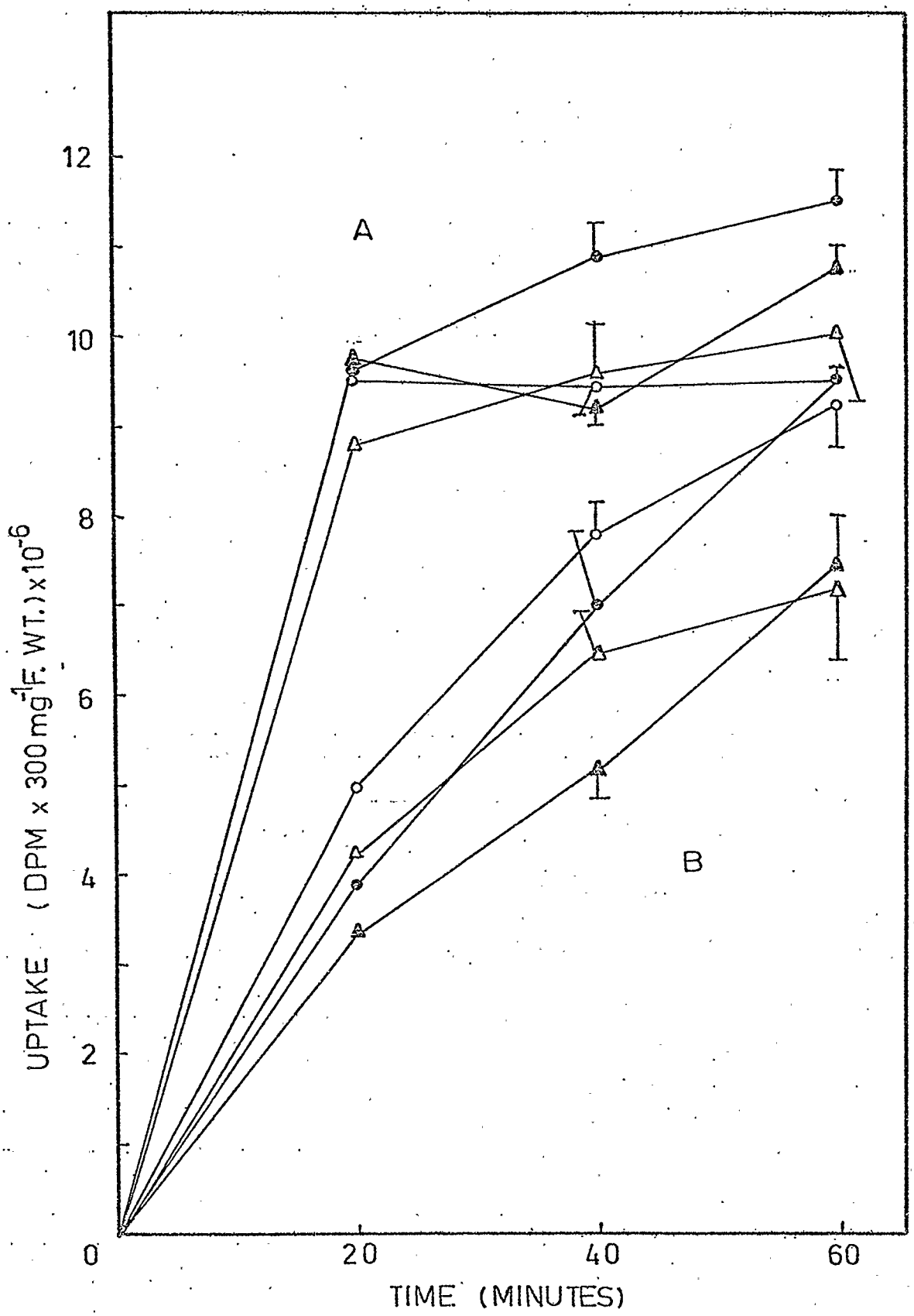

12
10
8
6
4
2
0
A
B
UPTAKE (DPM x 300 mg^{-1}F. WT.) x10^{-6}
20
40
60
TIME (MINUTES)

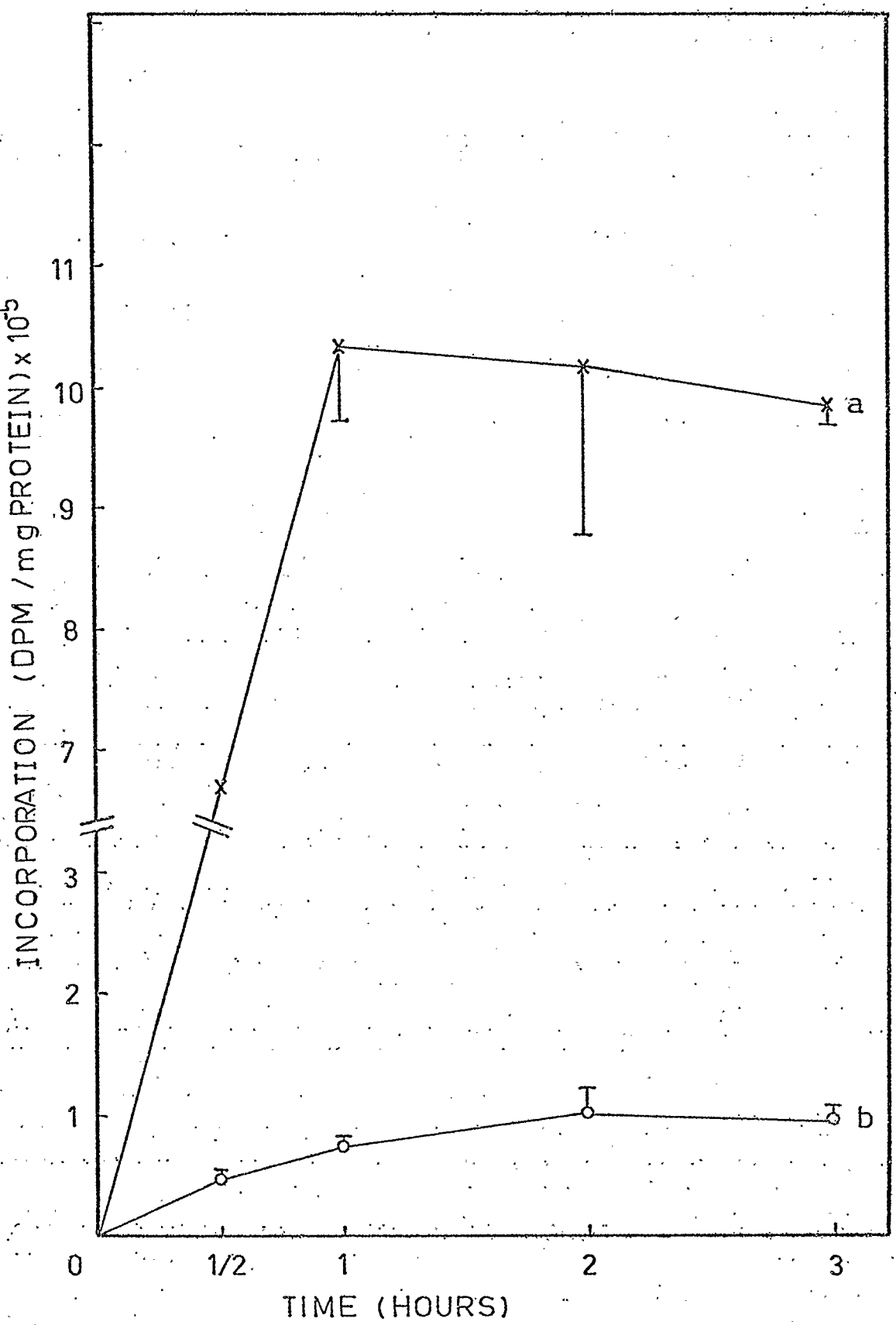
Figure 17

Incorporation of ^{3}H leucine into a hot TCA precipitate by moss kept at subzero (-2.5 ± 0.5°C) temperature (b) vs. control at 20°C (a).

Figure 18

Uptake of ^{3}H leucine into moss at subzero (-2.5 ± 0.5°C) temperature (b) vs. control at 20°C (a).

Variability: Standard error of the mean.


INCORPORATION (DPM / mg PROTEIN) x 10^{-5}
11
10
9
8
7
3
2
1
0
1/2
1
2
3
TIME (HOURS)
a
b

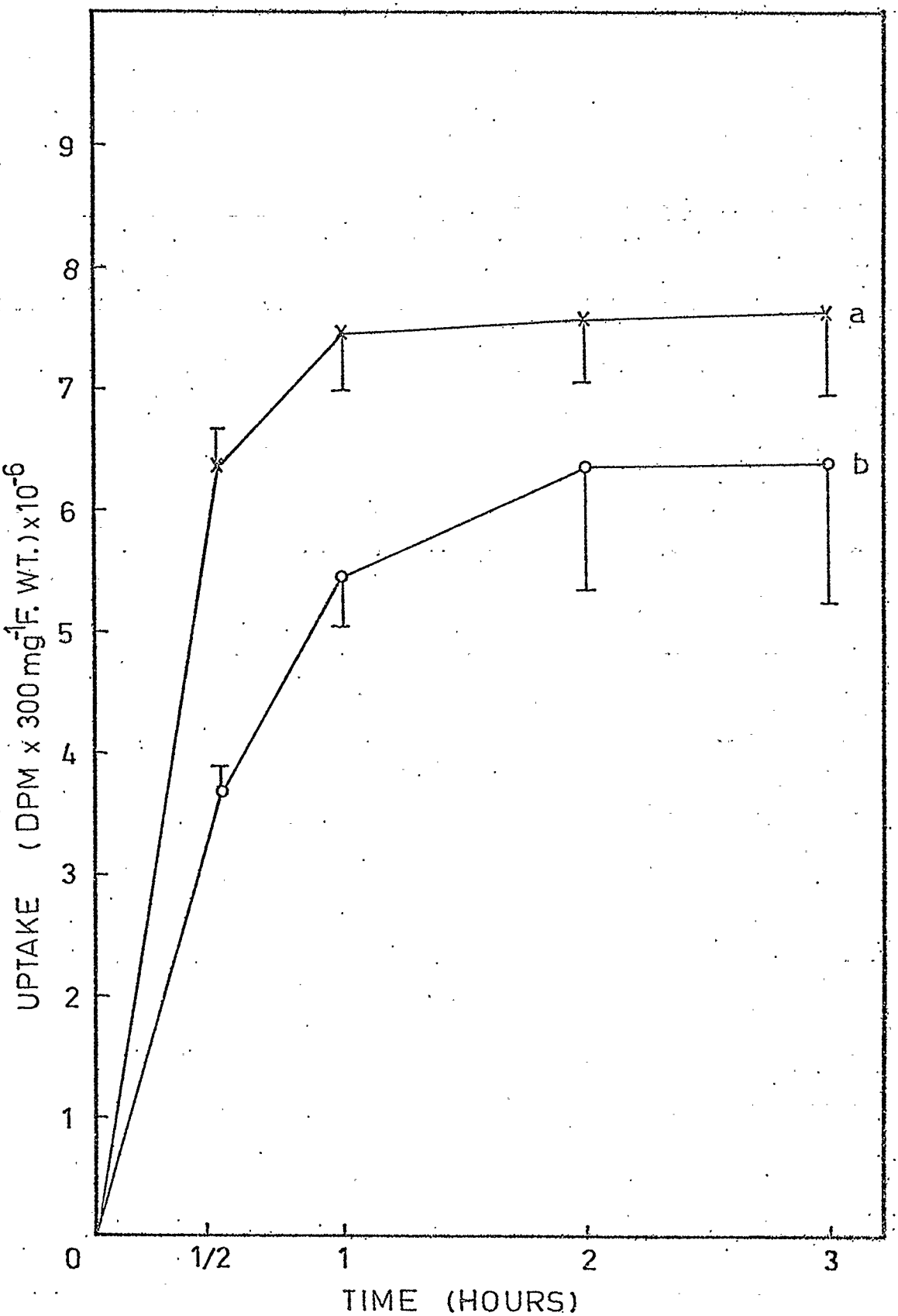

UPTAKE (DPM x 300 mg^{-1}F. WT.) x10^{-6}
9
8
7
6
5
4
3
2
1
0
1/2
1
2
3
TIME (HOURS)
a
b

Figure 19

Incorporation of ^{3}H leucine into a hot TCA precipitate from moss kept at high temperatures.

a) 20°C control

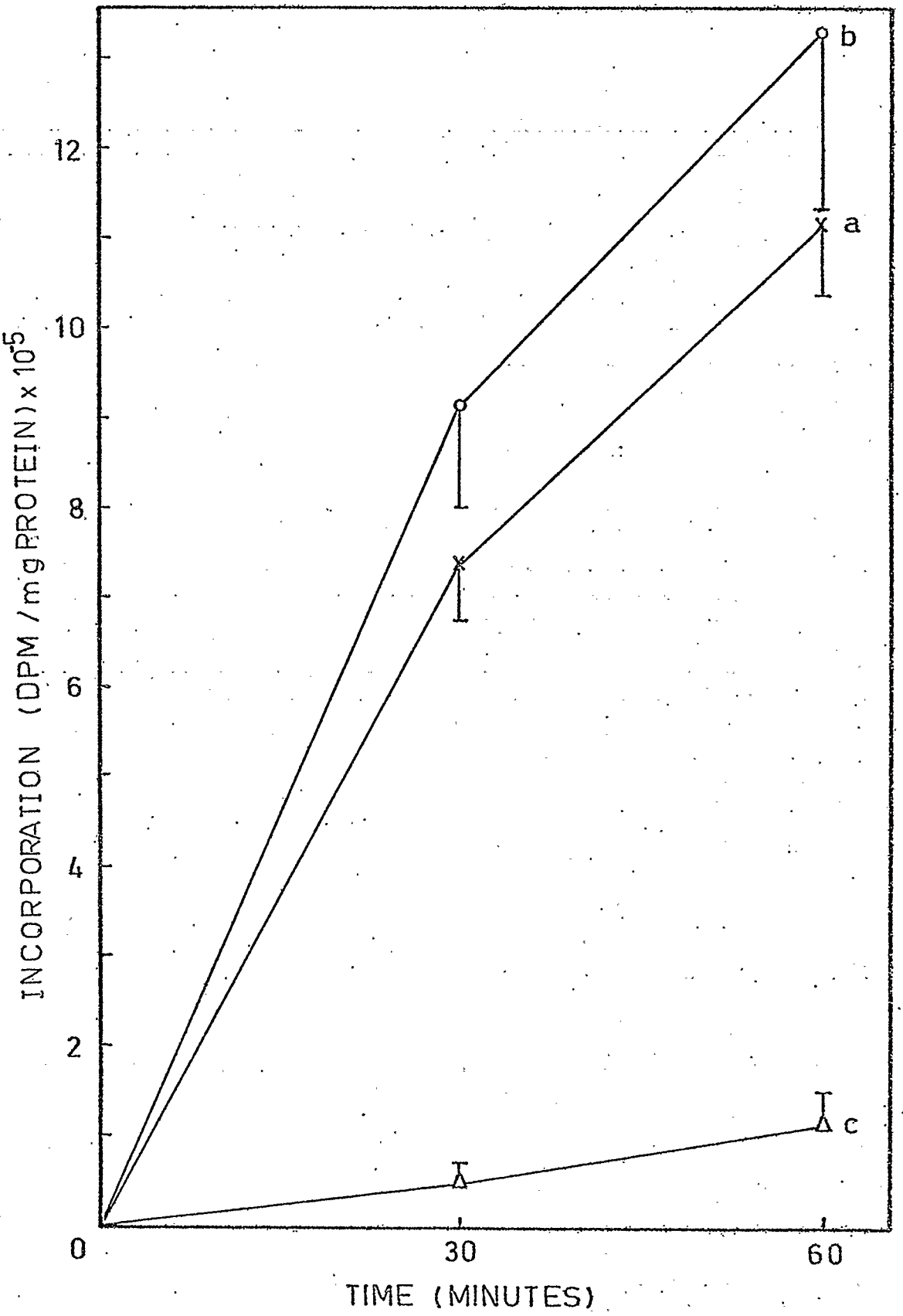
b) 36°C

c) 48°C

Figure 20

Uptake of ^{3}H leucine into moss tissue at high temperatures. Legend as in Figure 19.

Variability: Standard error of the mean.


b
a
c
12
10
8
6
4
2
0
30
60
INCORPORATION (DPM / mg PROTEIN) x 10^{-5}
TIME (MINUTES)

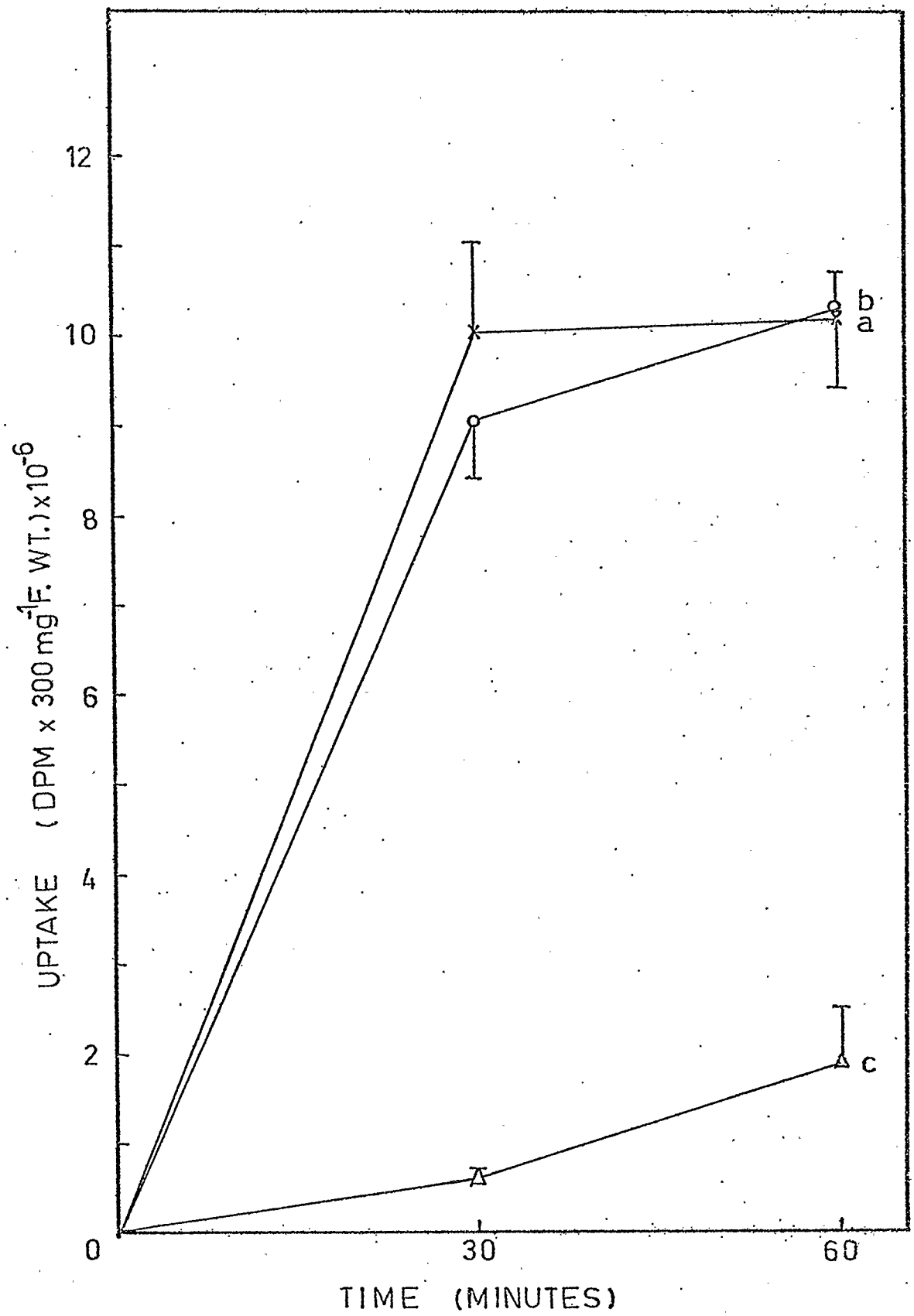

12
10
8
6
4
2
0
30
60
b
a
c
UPTAKE (DPM x 300 mg^{-1}F. WT.)x10^{-6}
TIME (MINUTES)

TABLE 1

Distribution of radioactive (^{14}C) leucine in 300 mg of moss tissue after one hour of incubation

(1μCi applied = 2.2×10^6 DPM, 2.1×10^6 DPM actually counted)

	Total Tissue Homogenate	In Protein*	Lost as $^{14}CO_2$	Remaining in the Medium
DPM	1.54×10^6	3.94×10^5	9.3×10^4	1.6×10^5
% of Total DPM	86%	22%	5%	9%
% of DPM in Tissue Homogenate		25%	5%	

* Note that these values are a component of values in the preceeding column.

TABLE 2

Distribution of radioactive (^{3}H) leucine in 300 mg of moss tissue after one hour of incubation

($10\mu Ci$ applied = 22×10^6 DPM, 12×10^6 DPM actually counted)

	Total Tissue Homogenate	In Protein*	Lost as $^{14}CO_2$	Remaining in the Medium
DPM	10×10^6	2×10^6	0.5×10^6	1.5×10^6
% of Total DPM	83%	17%	4%	13%
% of DPM in Tissue Homogenate		20%	5%	

* Note that these values are a component of values in the preceeding column.

Table 3

Incorporation of ^{3}H leucine into a hot TCA precipitate for one hour by moss dried to various levels, frozen in liquid nitrogen and thawed in water at 20°C.

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

*Control moss was dried and rehydrated, but not frozen.

Table 4

Incorporation of ^{3}H leucine into a hot TCA precipitate by moss dehydrated in polyethylene glycol (Av. M.W. 6000), frozen in liquid nitrogen and thawed in water at 20°C.

Polyethylene glycol (g/l)	Incorporation (% of control)
400	3
600	4
800	4
1000	34

Table 5

The relationship between the amount of ^{3}H leucine taken into tissue and ^{3}H leucine actually incorporated into TCA precipitate after freezing treatments of fresh moss. Freezing and thawing rates were the same, incorporation was tested 2 hours after the end of each treatment.

Treatment	Period of Incorporation (Minutes)	DPM in protein x 10^3/300mg moss	DPM in tissue x 10^3/300mg moss	$\frac{\text{DPM in protein}}{\text{DPM in tissue}}$ x 100 (%)
Control	20	1304	6550	20
	40	1674	7200	23
	60	2029	6500	31
3°C/hr	20	1073	7400	15
	40	1683	9500	18
	60	2228	9600	23
6°C/hr	20	518	6200	8
	40	1037	7500	14
	60	1539	8300	19
18°C/hr	20	186	4400	4
	40	372	7000	5
	60	543	8700	6
30°C/hr	20	80	1400	6
	40	146	2250	6
	60	226	3050	7
60°C/hr	20	25	500	5
	40	51	1000	5
	60	76	1400	5
Liquid Nitrogen	20	4	500	1
	40	14	550	3
	60	19	600	3

Table 6

Decrease in the ability of controls to take up and to incorporate radioactive leucine with time at 20°C.

Uptake (DPM/300 mg F.W T./hr) x 10^{-3} (% of control)		
2 hours	7 hours	24 hours
7900 ± 1044 (100%)	6175 ± 1308 (78%)	4377 ± 425 (60%)
Incorporation (DPM/mg protein/hr) x 10^{-3} (% of control)		
2 hours	7 hours	24 hours
1137 ± 226 (100%)	885 ± 191 (78%)	683 ± 162 (60%)

TABLE 7

Changes in polysome levels and rRNA content at times after the freezing treatments.

	Hours after end of treatment	Relative absorbances of: Polysomes	Ribosomes	rRNA content (% of control)
Control	0 to 24	62%	38%	100%
3°C/hr	2	73%	27%	94%
60°C/hr	0	-	-	89%
	2	65%	35%	88%
	24	73%	27%	92%
Liquid Nitrogen	0	-	-	102%
	2	64%	36%	84%
	24	not measurable	not measurable	26%

Table 8

In vitro incorporation of ^{14}C leucine and ^{14}C phenylalanine by polyribosomes and ribosomes, respectively, extracted from moss at different periods after freezing treatments (thawed at the same rate as frozen).

	Hours after the end of treatment	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.)
3°C/hr	2	138%	65%
60°C/hr	0 2 24	108% 89% 102%	88% 94% 80%
Liquid nitrogen	0 2 24	102% 3% 0%	90% 20% 5%

Loss of protein after fast freezing treatments, expressed as mg protein/300 mg fresh moss (% of control).

	Hours after end of treatment		
	2	7	24
Control	3.34±± 0.08*	3.48 ± 0.08	3.06 ± 0.14
60°C/hr	2.60 ± 0.08 (78%)	2.46 ± 0.12 a** (71%)	2.02 ± 0.08 a (66%)
Liquid nitrogen	1.96 ± 0.12 a (59%)		

* Standard error of the mean

** Using confidence limits at P = 0.05, the controls are not significantly different from each other. Letter a denotes a significant difference of the treatment in relation to the control at P = 0.05.

Protein content of fresh moss 2 hours after the end of freezing treatments.

Treatment (Freezing + thawing rate)	mg protein/ 300 mg FWT.	% of control
Control (a)	3.22 ± 0.10* abcde**	100
3°C/hr (b)	3.30 ± 0.18 abcd	102
6°C/hr (c)	3.24 ± 0.20 abcdef	101
18°C/hr (d)	3.10 ± 0.18 abcdef	96
30°C/hr (e)	2.66 ± 0.14 acdef	83
60°C/hr (f)	2.54 ± 0.16 cdef	79
Liquid nitrogen (g)	1.94 ± 0.16 g	60

* Standard error of the mean

** The treatments designated by the same letter are not significantly different at P = 0.05 level.

Adenosine triphosphate levels (nmoles ATP/200 mg of moss) after freezing and thawing at controlled rates. Numbers in brackets represent per cent of control.

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

* Standard error of the mean

** The time of extraction is indicated as hours after reaching 20°C. It should be noted that due to different rates of thawing the points of extraction differ in the length of time spent at above zero temperatures before 20°C was reached.

Chlorophyll content (µg/200 mg moss) after freezing and thawing at indicated rates (% of control).

	2 hours after end of treatment	24 hours after end of treatment	Negative change (µg/200 mg moss)
Control	60.5	40.0 (100%)	20.5
60°C/hr	62.5	38.0 (95%)	24.5
Liquid nitrogen	65.5	25.0 (62.5%)	40.5

Table 13

Appearance of leaflets during culture after freezing treatment.

Scoring Classes:	I	II	III	IV
% Area green (vs. brown)	100 - 75%	75 - 50%	50 - 25%	25 - 0%

Days after treatment	4	7	10	13	18	21
Control		I		I	I	
3°C/hr freezing	I		I		I	
60°C/hr		II		II		II
Liquid nitrogen		IV		IV		IV

Electrolyte leakage from moss frozen and thawed at indicated rates.

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

Table 15

Protein content (mg protein/300mg of moss) of moss collected in July and in January kept rehydrated for 48 hours at 2°C or 20°C.

	July moss	January moss
2°C	3.84 ± 0.16*	3.38 ± 0.16
20°C	3.62 ± 0.18	3.52 ± 0.16

* Standard error of the mean.

Note: Applying the Student's T test to the original data, any combination of treatments and moss material does not contain significantly different amount of protein at the P = 0.10 level.

5. DISCUSSION

5.1. Response of *Tortula ruralis* to freezing and thawing at controlled rates.

The events taking place during freezing of organisms at different rates have been described by Mazur (67, 68, 69) and summarized in the literature review. To recapitulate , at slow rates of temperature decrease, ice forms extracellularly and subjects the cells to desiccation stress. At faster rates the freezing point of the cell water is reached before substantial desiccation takes place. On the basis of these observations, Mazur proposed that at slow freezing rates cells are damaged due to prolonged desiccation stress, while at faster rates intracellular ice formation causes the damage.

Wide ranges of freezing rates have not been used often in plants to test this hypothesis. The work of Sakai and Krasavtsev (in 67) provided evidence that intracellular ice formation leads to decrease in survival of parenchyma cells in twigs of cold-hardy woody species. Slow freezing of such material did not cause damage and such cells were apparently tolerant of the desiccating action of extracellular ice. In contrast, only extracellular ice was detected during slow freezing (less than $6^{o}C$/hr) of less hardy tissues (56) and damage in such tissues was presumably due to ice-desiccation. While there is a lack of definitive evidence for the

location of ice in plant tissues following fast or slow freezing, Mazur's theory of freezing damage due to one of the factors - intracellular freezing or ice induced desiccation, has been widely accepted and majority of recent plant freezing tolerance studies have been interpreted on its basis (21, 37, 78, 116). For this reason the discussion of my results will also be based on the predictions of Mazur's two-factor theory, but deviations have been recorded.

5.1.1. Effect of liquid nitrogen treatment.

It has been shown quite convincingly that decreased survival of many types of cells after freezing at very rapid rates (often achieved by immersion in liquid nitrogen) is associated with the formation of intracellular ice (70). Intracellular ice formation leads to extensive damage to the organelles and release of degradative enzymes into the protoplasm upon thawing (89).

The metabolic events examined in this study of *Tortula ruralis* were found to be greatly affected by liquid nitrogen treatment, thus pointing to the possibility of intracellular freezing. Ribosome content decreased to a very low value within 24 hours, possibly due to the release of RNases into the cytoplasm. The rapid decline in ATP levels may have been due to degradation by ATPase and/or due to lack of synthesis of new ATP molecules, as a consequence of damage to mitochondrial and/or chloroplast membranes and enzymes. Decrease in protein and chlorophyll concentration suggests that such

damage took place. Loss of the ability to produce new protonematal growth indicates loss of viability of all cells after this treatment. Furthermore, a large amount of the electro-conductive cellular contents escaped upon thawing, and the ability to absorb and incorporate radioactive leucine into protein declined dramatically.

It is apparent that intracellular ice formation did not preferentially damage any specific cellular component or metabolic reaction but induced an overall degradative process, probably by destroying cellular compartmentalization. Luyet and Gehenio (63) claimed that *Mnium* cells frozen rapidly in liquid nitrogen and thawed rapidly in warm water can survive this treatment by solidification of water molecules in non-crystalline form (vitrification). This phase change was not associated with disruptive volume changes characteristic of ice crystal formation. The destruction of *Tortula* observed in my experiments carried out under the same conditions indicates that vitrification is not taking place here.

5.1.2. Effect of 3°C/hr treatment

An examination of the metabolic response of *Tortula ruralis* to very slow freezing and thawing at 3°C/hr revealed that such treatment had either no effect or only a slight, reversible effect. The appearance and ability of the moss to produce protonema after slow freezing are comparable to that of the control moss. Also, uptake and incorporation of radioactive leucine into protein *in vivo*

were not lower than in the controls. The protein content also remained the same after this treatment.

The prediction made at the onset of this study that *Tortula ruralis* would survive very slow freezing appears to be borne out. However, the question remains whether this survival is due to tolerance of the desiccating effect of the extracellular ice. On the basis of theoretical considerations (67) and observations on slow freezing of other plant tissues (56), it is likely that only extracellular ice will be present around *Tortula* during the 3°C/hr freezing. But Levitt (56) and recently Burke et al. (22), have pointed out that intracellular freezing of some plant tissues (eg. ray parenchyma) may be prevented by supercooling of the cells even to -40°C. Supercooling is an unlikely mode of protection from slow freezing in *Tortula*, since this moss survives immersion into liquid nitrogen after precooling to -30°C at a slow rate. In other words, if supercooled water was present in the cells at -30°C, it would have frozen during the fast temperature decrease in liquid nitrogen, causing damage similar to that observed after direct liquid nitrogen immersion. It appears that supercooled water was not present within the cells of *Tortula*. Thus it is likely that during freezing of *Tortula* intracellular water was lost to the extracellular ice, i.e. freeze desiccation took place. Indirect evidence for this possibility can be seen from two more or less identical responses of *Tortula* metabolism to slow freezing and to air drying. These are: a) decrease in ATP levels and recovery on release from either stress -

desiccation or slow freezing and b) leakage of materials from the rehydrated or thawed moss.

A similar trend in ATP levels was observed after slow freezing and air drying of *Tortula ruralis*. As shown by Bewley and Gwozdz (13) and confirmed by Krochko (unpublished), ATP levels decrease during slow dehydration of this moss and recover to control levels within 10 minutes of rehydration. A comparable decrease in ATP concentration was measured after slow freezing (Table 11), but the rate of recovery was slower (within 2 hours). While the similarities in ATP levels during slow drying and slow freezing are striking, the possible additional effect of low temperature *per se* cannot be excluded. A decrease in mitochondrial activity in response to chilling temperatures has been well documented in various chilling-tolerant and intolerant tissues (91, 125). It is possible that prolonged exposure of *Tortula* to near 0°C temperatures during the 3°C/hr treatment caused a decrease in phosphorylating capacity of the cells due to membrane phase changes (91, 125).

The integrity of cell membranes further illustrates the similarity of the slow freezing and air drying treatments. Plasma membrane integrity has been determined by measuring the amount of materials which leak out of the cell after it is released from the stress condition (90). Two types of leakage from cells should be recognized. During imbibition of seeds, some electro-conductive materials are released into the medium before the membrane bilayer structure re-forms. A much greater amount of electrolyte escapes

from the seeds in which the membranes have been damaged by liquid nitrogen freezing (112, 113) or other stresses (111). Examination of Table 14 reveals a similar trend in electrolyte leakage from *Tortula ruralis*. There is a basal level of leakage from the control moss (100%). Following the 3°C/hr treatment, during which presumably desiccation and rehydration membrane changes took place, there was an increased electrolyte leakage (277% of control). After liquid nitrogen treatment, the membrane was severely damaged, allowing a large quantity of electrolyte to leave the cells (722% of control). Leakage of radioactive leucine during rehydration of prelabelled *Tortula* has been measured by Dhindsa and Bewley (32). Their data indicate that while only 15% of the soluble radioactivity leaks into solution from undesiccated moss, 45% of soluble radioactivity leaks from the rapidly dried and rehydrated *Tortula*. If the 15% leakage from control moss in reference (32) were considered basal amount of leakage (100%) then the 45% of soluble radioactivity leaking out on rehydration of rapidly dried *Tortula* would represent 300% of control. This amount compares favorably with the electrolyte leakage at 277% of control after the 3°C/hr freezing. It thus appears that leakage occuring after the air drying and/or 3°C/hr freezing (neither of which treatments is irreversibly damaging) may have a common origin, namely escape of solute before reconstitution of the membrane bilayer following drying.

To summarize above paragraphs, 3°C/hr freezing and thawing is not without effect on the moss *Tortula ruralis*. The changes in ATP

levels and leakage of electrolytes could be attributed to the desiccating effect of extracellular ice. The slight increase in the proportion of polyribosomes after slow freezing treatment (Table 7) on the other hand may be due to prolonged exposure of *Tortula* to near 0°C temperatures. An increase in the proportion of polyribosomal material at chilling temperatures has been reported in several tissues (53, 119) and also observed previsously in *Tortula ruralis* (Bewley, unpublished). An increase in polyribosomes after the slow freezing treatment is also reflected in the increased *in vitro* polypeptide synthesizing activity directed by polyribosomes (Table 8). Conversely, the decrease in the *in vitro* activity of the single ribosomes was not due to damage to these organelles, but reflected the decrease in their concentration relative to increased polyribosome formation.

While *in vivo* incorporation after 3°C/hr freezing and thawing was comparable to the control (Fig. 5), uptake of this amino acid appeared to be stimulated. The loss of cellular electrolytes (including leucine) during the slow freezing and thawing may decrease the size of intracellular pools of these materials, thus increasing the potential of the cells for uptake.

An assumption made at the beginning of this study, which predicted high survival of *Tortula ruralis* following a slow freezing and thawing treatment, is borne out. This prediction was based on the premise that a drought tolerant plant would be expected to

survive the desiccation induced by extracellular ice. Some events taking place during the slow freezing (ATP levels, leakage) hint at the possibility that desiccation was indeed taking place.

5.1.3. Effect of 60°C/hr treatment.

During the 60°C/hr freezing, desiccation induced by extracellular ice might be expected to be less complete than during 3°C/hr freezing. Enough water would probably remain in the protoplast for intracellular freezing to take place, but the extent of freezing would be less than that after liquid nitrogen treatment because of partial desiccation at 60°C/hr.

The experimental evidence obtained in this study appears to support this hypothesis. A decrease in the ability to take up radioactive leucine and incorporate it into protein, a significant irreversible decrease in ATP level, a decrease in protein content, and browning of the cells, all indicate extensive damage to the moss. However, this damage is never as severe as that suffered during liquid nitrogen freezing. In addition, several aspects of metabolism were not appreciably affected by the 60°C/hr treatment. The ribosomal concentration and activity (see section 5.3. for more detailed discussion), chlorophyll content and protonema growth were all comparable to the controls. Also, the electrolyte leakage was low, similar to that taking place during rehydration of the ice-desiccated, 3°C/hr treated cells. This may indicate that the plasmalemma lipid bilayer structure could be physically re-

constituted upon thawing from freezing at 60°C/hr. However, membrane functionality, i.e. uptake of leucine, was not restored.

It appears that intracellular freezing of the partially ice-dehydrated, 60°C/hr frozen moss caused damage only at more susceptible sites in the cell (possibly more hydrated cellular components), or that the rate of degradation of some cellular components was much slower than after liquid nitrogen treatment and was not detected within the 24 hour period after end of treatment.

5.2. Validity of the two-factor theory of freezing damage in respect to freezing of *Tortula ruralis* and some additional observations.

Tortula ruralis responds to freezing treatments in a manner which can be discussed in terms of the two-factor theory. While this theory provides a useful framework for discussion of freezing studies some observations have been made by other workers which cannot be easily explained in terms of this theory. My own study indicates that several degrees of intracellular damage may possibly be induced by different fast freezing rates. Reservations have also been raised by others about the effects of slow freezing and the so-called "solution effects" induced by ice-desiccation.

Firstly, no reasonable explanation can be offered for the observation that some slowly frozen tissues are damaged more by fast thawing than by slow thawing. If the damage were due to prolonged

exposure to desiccating conditions as predicted by the two-factor theory, slow thawing would allow longer exposure to such conditions and, consequently cause more damage. This does not appear to be the case in some tissues. The greater damage during fast thawing has been attributed to rapid re-entry of water into the cell (74). This interpretation could possibly be applied to the situation in *Tortula*.

Secondly, Levin et al. (55) have pointed out that while Mazur's calculations predict almost total desiccation at slow enough (equilibrium) freezing rates, only a 20% decrease in water volume can be measured in red blood cells. These authors propose that cell membrane water permeability decreases more rapidly below 0°C than above. This leads to a resistance to desiccation of the cells. Presumably, water content reached would be sufficiently low to prevent intracellular freezing injury. However, as was discussed on page 75, *Tortula* appears to avoid damage by extensive desiccation during the 3°C/hr treatment. That *Tortula* avoids freezing damage by almost complete desiccation is also indicated in Table 3: only the moss air dried to between 20% and 30% of its fresh weight survives liquid nitrogen immersion. Furthermore, slowly frozen and thawed *Tortula* appears unaffected by one month storage in frozen water at -20°C. This indicates that ice-induced desiccation is sufficient to prevent any degradative reactions which are presumably responsible for damage in more susceptible tissues.

A third difficulty associated with the two-factor theory is its inability to explain the cryoprotective effect of glycerol or dimethylsulfoxide which apparently do not penetrate into the cell to prevent the slow freezing damage (74, 79, 94). The ice-induced desiccation damage has been usually interpreted as damage within the protoplast, but the observations on non-penetrating cryoprotectants indicate that such desiccation may lead to lesions at the surface of the cell. These observations do not bear much significance to my study, since the slow freezing treatment (3°C/hr) was readily survived by *Tortula* in the absence of cryoprotectants. However, an unusual cryoprotective effect of PEG 6000 was observed in my study - PEG 6000 appeared to protect from rapid liquid nitrogen freezing, while other cryoprotectants are usually used to counteract the extracellular ice induced desiccation stress.

A water content of 50% fresh weight was achieved by immersion of *Tortula* in PEG 6000 at 1 kg/1 kg H_2O (31). Liquid nitrogen treatment was not as damaging to PEG 6000 protected moss as to air dried moss at the same water content (compare Tables 3 and 4). One explanation of this apparent cryoprotective action of PEG 6000 could be as follows: as the external medium freezes, the PEG 6000 may be excluded from the solid ice structure (i.e. lose water to the ice) and the water potential of the PEG 6000 would decrease to values below those expected for 1 kg PEG 6000/1 kg H_2O solution at that particular temperature. This "excluded", dry PEG 6000 would, in turn, lead to a greater desiccation of the moss than calculated (less than 50% fresh weight), ultimately

protecting it from intracellular freezing.

The relationship between hydration level of the tissue and the survival of liquid nitrogen treatment was interesting in view of reports that slight water stress in higher plants can increase their cold tolerance (27, 56, 58). It is possible that lower water content decreases the probability of water freezing within the cells.

The objections raised against the two-factor theory of freezing damage do not appear to lessen its applicability to the discussion of the response of *Tortula ruralis* to freezing. To present a complete discussion, however, the hypothesis advanced by Olien (83, 85, 86) should be reviewed. He proposed that it is the rate of extra-cellular ice formation and associated "thermodynamic imbalance" of the tissue which determine the degree of fast or slow freezing damage. There is not a very clear theoretical backing for this hypothesis and furthermore, it is difficult to obtain sufficient evidence for it. The observation that air desiccation is less damaging than extracellular ice-induced desiccation to the same degree (84, 116) points out that not all freezing damage can be attributed to desiccation damage. This is reasonable, since some temperature effect may be expected below 0°C, as may be the case with ATP synthesis (see page 76) in *Tortula*. Furthermore, the observations that cellulose filter paper (86) or polymers extracted from cereals (82) interfere with the ice formation indicate that

the cell wall or other polysaccharide - rich components of plant cells may be important in determining the rate of formation and location of ice.

Sufficient data was not obtained in my study to determine if the cell wall of *Tortula* is a potential barrier to ice growth and thus has some protective function.

5.3. Response of protein synthetic reactions to controlled freezing and thawing treatments.

From the previous discussion it is apparent that slow freezing and thawing (3°C/hr) had very little effect on *Tortula* metabolism, including protein synthetic reactions. The liquid nitrogen treatment on the other hand was very destructive to all aspects of metabolism examined, whereas the 60°C/hr treatment causes a more specific type of damage to protein metabolism. The polyribosomes remain unaffected by 60°C/hr treatment (Figs. 11C, 11D, Table 7) and the *in vitro* polypeptide synthesizing capacity of both polyribosomes and single ribosomes is comparable to the activity of ribosomal material extracted from the control moss (Table 8). Nevertheless, the rate of protein synthesis determined *in vivo* is significantly decreased by the 60°C/hr freezing. It appears that endogenous ATP level decreases may be responsible for the decline *in vivo* protein synthesis. ATP may be involved in initiation reactions of protein synthesis in wheat embryos (109) and is generally recognized as energy yielding molecule in the aminoacylation of tRNA. ATP can also donate a

phosphate group in GDP - GTP transphosphorylation to increase the availability of GTP for elongation and termination phases of protein synthesis. A correlation exists between reversible decrease in the rate of protein synthesis and decrease in ATP levels induced by incubation of *Tortula ruralis* in nitrogen atmosphere in the dark (13). Furthermore, the polyribosomes remain undegraded during such treatment. It appears that a similar relationship exists between low ATP levels and low rate of *in vivo* protein synthesis after the 60°C/hr treatment. Intracellular ice formation or low temperature *per se* may cause damage to the mitochondria which, in this case, is not reversible. However, relatively high (50% of control) ATP levels after 60°C/hr treatment may indicate that some synthesis and hydrolysis of ATP is taking place after such treatment. A rapid decline in ATP, comparable to that in liquid nitrogen frozen moss, did not take place after 60°C/hr treatment.

The decrease in radioactive leucine incorporation by moss after 60°C/hr treatment could also be due to damage to the protein catalysing some of the protein synthesizing reactions. For example, activities of Elongation Factors 1 and 2 and phenylalanyl-tRNA synthetase have been shown to be lower in non-viable pea seeds (18). These lesions have been considered to be partially responsible for the inability of the seeds to germinate. The lesions developed during seed storage, not in response to environmental stress.

While specific proteins were not studied in the moss, a decrease in the concentration of soluble proteins following 60°C/hr

treatment may indicate their degradation. Freezing-induced denaturation of proteins in non-tolerant tissues is a well-documented phenomenon (28, 43, 49). It should be noted, however, that a normal rate of protein degradation, in the absence of protein synthesis, would eventually lead to a decrease in total protein concentration, although in a fast growing tabacco callus tissue the rate of protein degradation was determined to be about 1% of all protein/hour (47). This mechanism would not likely account for the 11% of cell soluble protein/hour protein loss after the 60°C/hr freezing of *Tortula ruralis* (Table 9).

Other factors: ionic balance, rough endoplasmic reticulum, transport of amino acids across internal membranes, etc., may have also been upset by the 60°C/hr treatment and thus affect the rate of *in vivo* protein synthesis. These possibilities were not investigated.

The stability of the *Tortula ruralis* ribosomal fraction in respect to freezing stress perhaps should not be surprising in view of the previously reported stability of this organelle during drying of this moss (7, 10, 12, 42). In plants less tolerant of environmental extremes ribosomes may prove to be more sensitive to the effects of freezing stress.

5.4. Seasonal low temperature acclimatization of *Tortula ruralis* and protein synthesis at temperature extremes.

As is apparent from Fig. 13, the moss collected in summer was

slightly but significantly more sensitive to 18°C/hr freezing than moss collected in winter. It is difficult to assess what seasonal metabolic changes took place. Since the water content of the moss was shown to be important in determining the degree of freezing injury, it was considered possible that an increase in water permeability of membranes during the fall could lead to a better ability of the acclimated moss to lose water to extracellular ice, and diminish intracellular freezing. Changes in water permeability with cold hardening have been reported in several higher plant tissues (72 and references within).

The seasonal freezing tolerance changes of *Tortula ruralis* are small, and providing the initial freezing rate was slow (3°C/hr), even the summer collected moss was very cold tolerant and capable of survival of liquid nitrogen temperature (-196°C). It is doubtful that acclimatization studies in which very fast "unnatural" freezing rates are employed (95) can be used to evaluate cold tolerance of mosses and to predict the temperature limits for occurence of mosses. It will be necessary to determine the *in situ* temperature conditions and only then to correlate the survival of individual species with such temperature conditions. The moss *Tortula ruralis* appears to be well equipped to tolerate the freezing conditions it may encounter in its habitat. Even though *in situ* temperature changes were not determined, the moss might rarely encounter freezing rates of 60°C/hr (60). The plants were severely damaged by this

temperature change, but the surviving cells did retain the capacity to produce new growth (Fig. 12) thus ensuring continuing presence of this moss in its habitat.

Tortula ruralis not only tolerated extremely low temperatures, it was also capable of synthesizing proteins at very low and high temperatures. Synthesis of proteins was measured at -2.5°C (Fig. 17) and at 36°C (Fig. 19). At 38°C the rate of protein synthesis *in vivo* decreased, presumably due to heat denaturation of proteins (88). The rate of radioactive leucine uptake was very high even at -2.5°C. Leucine uptake has been shown in many organisms to be an active process requiring energy and membrane carrier molecules (23, 87, 92). The affinity of the uptake molecule for leucine and the ATP hydrolysing capacity was very little affected by this low temperature in *Tortula ruralis*. It appears that this moss has a wide temperature range at which metabolism can take place. In this respect it is similar to many other bryophyte species (4, 14, 16, 24, 80, 93).

Protein synthesis and leucine uptake was measured at 2°C (Figs. 15 and 16). There was slight, but not significant increase in the rate of incorporation by the winter moss, compared to the summer moss. It was expected that in winter moss, which was exposed to low temperatures in its habitat, protein synthesis at 2°C would be significantly higher (adapted to low temperature) than that measured at 2°C in summer moss. Such adaptation of protein synthesis to low temperature has been observed in wheat seedlings (12). In that

study seedlings grown at 4°C synthesized protein at 5°C twice as fast as seedling grown at 10°C and above. This difference disappeared when protein synthesis was measured at 20°C. It appears that *Tortula* protein synthesis underwent only slight adaptive changes, possibly because its metabolism is geared mainly to synthesis during colder seasons anyway. During the warm summer, the moss is often found in the dry, metabolically inactive state, except for several days following rain. During the cool and moist late fall and spring the moss can be encountered more often in the hydrated state. Some metabolism in *Tortula* may take place during winter under the protective snow layer.

There is no doubt that the moss *Tortula ruralis* is well adapted to survive and metabolize at the temperatures it encounters in its natural habitat and may have low temperature tolerance beyond the stresses to which it is normally subjected. It also possesses certain metabolic adaptations which other plants lack. A future task is to determine the cellular adaptations which allow this plant to survive slow temperature decreases and ice or air desiccation.

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