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Plant Desiccation and Protein Synthesis

AN *IN VITRO* SYSTEM FROM DRY AND HYDRATED MOSSES USING ENDOGENOUS AND SYNTHETIC MESSENGER RIBONUCLEIC ACID[1]

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ABSTRACT

The conditions and requirements for an *in vitro* protein synthesizing system from the moss *Tortula ruralis* are outlined. Using this system the effects of desiccation, achieved quickly or slowly, were studied. Slowly dried moss retained fewer polyribosomes on desiccation but more active ribosomes than rapidly dried moss. Even in the completely desiccated moss the polyribosomes and/or free ribosomes present have retained their synthetic capacities. On rehydration, the slowly dried moss resumed protein synthesis more quickly than moss previously desiccated rapidly. Moss ribosomes are cycloheximide sensitive and chloramphenicol insensitive and thus the major protein synthesis occurs within the cytoplasm on rehydration. Extracted polyribosomes *per se* can withstand desiccation to a significant extent, suggesting that protection by the cytoplasm might not be necessary. The aquatic moss *Hygrohypnum luridum* can retain polyribosomal and ribosomal activity during desiccation, but this decreases greatly on rehydration.

Previous studies on *Tortula ruralis* have shown that this moss can survive complete desiccation over silica gel (1, 3) and resume protein synthesis rapidly on rehydration, utilizing in part those polyribosomes conserved during desiccation (2). The structural integrity of the polyribosomes in the dried moss is dependent upon the rate at which desiccation has been reached (11)—slowly dried moss, in contrast to fast dried moss, apparently having no intact polyribosomes. Even the slowly dried moss, however, rapidly resumes protein synthesis on rehydration, with concomitant reassembly of the polyribosomes.

Since, on rehydration of the slowly dried moss, the protein synthesis observed is largely independent of any prior RNA synthesis (11), it is postulated that the conserved ribosomes and messenger RNA can quickly recombine to form polyribosomes, in a manner similar to those events taking place during the hydration of seeds (9, 16, 18, 24).

In this paper we describe the development of an *in vitro* protein synthesizing system from *Tortula ruralis* as part of our continuing studies to determine the nature of desiccation resistance in this moss. We also report on the relative capacity of the ribosomes and polyribosomes from dried and hydrated *Tortula* to conduct protein synthesis *in vitro* and we make a comparison with the protein synthesizing system from the desiccation-labile aquatic moss, *Hygrohypnum luridum* (5).

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MATERIALS AND METHODS

The moss *Tortula ruralis* ([Hedw.] Gaertn, Meyer and Scherb) was collected, stored, and processed as previously described (1). *Hygrohypnum luridum* ([Hedw.] Jenn) was collected as previously described (5) and maintained in an illuminated and humid greenhouse at 25 C until required. For experimental purposes, the 7 to 10 mm green tops of the gametophytes were removed and washed in a large volume of distilled H_2O before use.

Rapidly desiccated moss was obtained by placing the plant over activated silica gel in a Petri dish for 1 hr. Slow desiccation of the moss was achieved by first placing the moss for 1 hr in a desiccator containing a stored saturated solution of KCl and then transferring to another desiccator containing a stored saturated solution of ammonium nitrate for 2 hr (11). Rehydration of the moss was carried out by placing it in a small volume of distilled H_2O.

Extraction of Ribosomes. The technique for ribosome extraction was based on that already described (4). The dry or hydrated moss material (fresh weight 1.1 g) was placed in an ice-cold mortar and ground in 2 ml of grinding solution: 0.25 M sucrose, 40 mM KCl, 4 mM magnesium acetate, 50 mM tris-acetate (pH 8.1), and 5 mM mercaptoethanol. No detergent was used. Two further 3-ml lots of grinding solution were added and the homogenate was transferred to a Duall ground glass homogenizer (Kontes Glass Co., Vineland, N.J.) and grinding completed with five strokes of the pestle (2). The homogenate, total volume 8 ml, was cleared by centrifugation for 10 min at 15,000*g* and the supernatant was loaded over a 2.5 ml 1.5 M sucrose pad containing 40 mM KCl, 4 mM magnesium acetate, 50 mM tris-acetate (pH 8.1), and 5 mM mercaptoethanol. The ribosomal pellet was collected by centrifugation at 166,500*g* for 1.5 hr in a Beckman Ti50 rotor and then resuspended in 0.6 ml of resuspension solution, containing the same salts as above, but no sucrose. After clearing at 10,000*g* for 10 min the supernatant, containing a known amount of rRNA (estimated A_{260}) was used for the *in vitro* experiments.

Preparation of Wheat Germ Supernatant. The procedure was based on that of Legocki and Marcus (17): 1.1 g of germ (Ogilvie Flour Mills, Calgary) was homogenized as described for the moss ribosome preparation using the same grinding solution. After centrifugation at 166,500*g* for 1.5 hr, the upper

two-thirds of the supernatant was carefully pipetted off and an aliquot (2 ml) dialyzed for 2 hr against 500 ml of buffer solution containing 40 mM KCl, 4 mM magnesium acetate, and 50 mM tris-acetate (pH 8.1). The unused portion of the supernatant was stored at −20 C until required.

***In Vitro* Incorporation of ^{14}C-Leucine.** An incubation mixture of 0.4 ml contained: 40 mM KCl, 4 mM magnesium acetate, 50 mM tris-acetate (pH 8.1), 0.244 mM mercaptoethanol, 0.125 μCi of ^{14}C-leucine (NEN, 240 mCi/mmole), 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 (Sigma Chemical Co.), ribosomes (60–85 μg as RNA), and dialyzed wheat germ supernatant (1200 μg of protein content). Incubation was carried out at 31 C for 20 min and then the reaction was terminated by the addition of 0.2 ml of 16% and 4 ml of 5% trichloroacetic acid plus 0.3 ml of 1% BSA. The precipitate was pelleted by centrifugation at 1000*g*, resuspended in 4 ml of 5% trichloroacetic acid and heated at 95 C for 15 min to discharge amino acids from amino acyl tRNA. After cooling on ice, the precipitate was collected on Whatman GF/A filters and washed twice with 4 ml of 5% trichloroacetic acid containing 0.05 M L-leucine before drying and counting. In experiments where the same ribosomal source was used for all the variants, the results are expressed in cpm incorporated. Where different ribosomal sources were used in the same experiment, the results are expressed in relation to the amount of RNA added as ribosomes.

***In Vitro* Polyphenylalanine Synthesis.** An incubation volume of 0.4 ml contained: 80 mM KCl, 10 mM magnesium acetate, 50 mM tris-acetate (pH 8.1), 0.224 mM mercaptoethanol, ribosomes (60–80 μg as RNA), 0.1 μCi of ^{14}C-phenylalanine (NEN, 383 mCi/mmole), 200 μg of polyuridylic acid (Calbiochem), 60 μg of dialyzed wheat germ supernatant (1000 μg of protein content), 0.25 mM GTP, 1 mM ATP, 8 mM creatine phosphate, and 16 μg of creatine phosphate kinase. Incubation was at 31 C for 20 min before determination of amino acid incorporation as described above.

Since there is no previous record of an *in vitro* protein synthesizing system using moss ribosomes and endogenous or synthetic messenger RNA the determination of the above optimal conditions for incorporation is outlined under "Results and Discussion."

RESULTS AND DISCUSSION

Requirements for Optimal ^{14}C-Leucine Incorporation. In these experiments ribosomes from the undesiccated moss were used. The effects of omitting components or varying components in the incubation mixture are outlined in Table I. In the absence of ribosomes, wheat germ supernatant, or the ATP-generating system, there was little leucine incorporation. Additional wheat germ tRNA (prepared according to Zubay [25]) was not necessary for incorporation.

Attempts to replace wheat germ supernatant with that from *Tortula ruralis* were unsuccessful, even though the latter was used as a concentrated pH 5 supernatant protein fraction (21, 22). The addition of wheat germ tRNA to the moss supernatant did not improve incorporation, although amino-acyl tRNA was not tried. We are working to obtain an active moss supernatant fraction.

Addition of pancreatic ribonuclease to the incubation mixture resulted in low incorporation, indicating that the messenger fraction associated with the ribosomes was degraded. The optimal range of buffer concentrations was quite wide (50–60 mM) at the optimal K^+ concentration (6).

Leucine incorporation reached a maximum after 20 min incubation (Fig. 1). Incubation of the mixture in the presence of 25 or 37.5 μM aurintricarboxylic acid, a known inhibitor of protein chain initiation *in vitro* at these concentrations (19) resulted in little reduction in incorporation over this time period, indicating that extracted polyribosomes were indeed active. The fact that there was no increased protein synthesis after this incubation period in the presence or absence of ATA[4] suggests that there is an inherent restriction on reinitiation *in vitro* and that complete translation of the extracted polyribosomes requires about 20 min.

Table I. ^{14}*C-Leucine Incorporation by Polyribosomes from Fresh, Undesiccated Tortula ruralis*

The moss was rehydrated for 24 hr prior to ribosome extraction and 80 μg were used. The dialyzed post-ribosomal wheat germ supernatant contained 1200 μg of protein. Moss supernatant I represents a dialyzed post-ribosomal fraction (40 μg of protein), while the moss supernatant pH 5 fraction contained 600 μg of protein after dialysis. When moss supernatants were used, 12 μg of wheat germ tRNA were added to the incubation mixture, and wheat germ supernatant was omitted.

Components	Incorporation	
	cpm	%
Complete	1150	100
Without wheat germ supernatant	30	3
Without tRNA	1240	108
Without ribosomes	170	15
Without ATP and generating system	50	4
With moss supernatant I	60	5
With moss supernatant pH 5 fraction	40	4
With tris-acetate pH 8.1		
30 mM	550	48
40 mM	780	68
60 mM	1050	91
With RNase (10 μg)	120	10

The incorporation system showed a clear dependence upon Mg^{2+} concentration (Fig. 2a), with an optimal range of 4 to 5 mM and a sharp drop at concentrations above and below this level. At a K^+ concentration of 40 mM there was optimal incorporation with a less pronounced decrease on either side (Fig. 2b).

Requirements for Poly U Directed ^{14}C-Phenylalanine Synthesis. For these experiments ribosomes extracted from slowly desiccated moss were used. Phenylalanine incorporation was absolutely dependent upon the presence of poly U, dialyzed wheat germ supernatant, and ribosomes, whereas the absence of all the other amino acids but phenylalanine had no effect on the level of incorporation (data not presented). Addition of wheat germ ribosomes to the system, instead of moss ribosomes, increased incorporation by nearly 50%.

The incorporation of phenylalanine shows a strict dependence upon the concentration of poly U (Fig. 3a). It has been observed that the amount of ribosomes in the incubation mixture limits further increase of phenylalanine incorporation at higher poly U concentrations (data not presented). The kinetics of phenylalanine incorporation was linear over a 40-min incubation period. The optimal Mg^{2+} concentration for phenylalanine incorporation lies between 8 and 10 mM in the presence of 80 mM K^+. The Mg^{2+} concentrations above and below this level result in dramatic decreases in incorporation (Fig. 3b). A shift in the optimal K^+ concentration was noted for different

[4] Abbreviation: ATA: aurintricarboxylic acid.

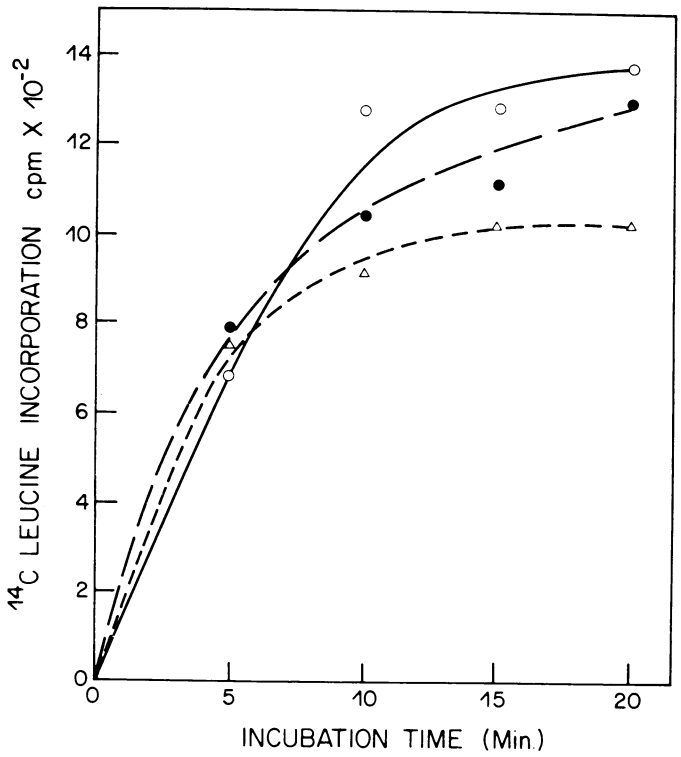

FIG. 1. Time course of ^{14}C incorporation into protein by polyribosomes from *Tortula ruralis*. Complete incubation medium (○); + 2.5 × 10^{-5} M ATA (●); + 3.75 × 10^{-5} M ATA (△).

Mg^{2+} concentrations. At 6.5 mM Mg^{2+}, the optimal K^+ concentration was 50 mM, but at 10 mM Mg^{2+}, the K^+ optimum shifted to 80 mM, with the total incorporation being almost twice as high as at 6.5 mM Mg^{2+} (Fig. 3c).

Effect of Desiccation and Desiccation Rate on Amino Acid Incorporation. Previous studies have already indicated that the structural integrity of polyribosomes in the desiccated moss is dependent upon the rate at which desiccation is achieved. Using sucrose gradient analysis it has been shown that rapidly dried moss conserves some polyribosomes (1, 2, 11), but none could be observed in the slowly dried moss (11). These results are borne out by those shown in Table II. The experiments involving ^{14}C-leucine incorporation were carried out in the presence of 25 μM aurintricarboxylic acid to preclude any initiation of possible extracted messenger RNA. Rapid desiccation of the moss resulted in some reduction in extractable polyribosomes, whereas slowly desiccated moss had negligible capacity for ^{14}C-leucine incorporation, showing the complete absence of polyribosomes from the moss in this state. On the other hand, there was an opposite effect on the capacity of the slowly dried moss to conduct poly U directed protein synthesis. With a decrease in polyribosomes in the moss there was presumably an increase in available free ribosomes for polyphenylalanine synthesis and thus the slowly desiccated moss showed an increased incorporation of phenylalanine of over 100%. It should be noted that under the conditions for optimal polyphenylalanine synthesis there is little incorporation utilizing the endogenous messenger RNA attached to polyribosomes. Thus, slow desiccation of the moss results in fewer polyribosomes and more free ribosomes that, even in the completely dried moss, have retained their capacity for conducting protein synthesis upon rehydration.

^{14}C-Leucine Incorporation on Rehydration. The ribosomal pellet was extracted from the moss at different times of rehydration following slow or rapid desiccation and its activity was determined *in vitro* by following ^{14}C-leucine incorporation into protein. The pellet from the slowly desiccated moss had much less activity at zero time (*i.e.* before rehydration) than the rapidly desiccated moss (Fig. 4 and Table II). After rehydrating the moss for 2 hr, the ribosomal pellet extracted from the moss which had previously been dried slowly was more active than that extracted from the moss which had been dried quickly (Fig. 4). *In vivo* incorporation studies also appear to confirm this observation (11). Moss which is dried slowly contains fewer polyribosomes than that dried quickly, and yet on rehydration the former appears to form polyribosomes more quickly than the latter. Perhaps on rehydration the ribosomal run-off from the conserved polyribosomes in the rapidly dried moss occurs more slowly than reformation of new polyribosomes on rehydration of the slowly dried moss, or even limits formation of new polyribosomes. The reason for this is not apparent.

Effects of Inhibitors on *in Vitro* ^{14}C-Leucine Incorporation. Previous studies on the ribosomes of this moss by analytical ultracentrifugation have shown that the major species present in the extracted polyribosomes is 80S (23). The effects of cycloheximide (a specific inhibtor of protein synthesis by 80S ribosomes) and chloramphenicol (an inhibitor of 70S ribosomes) were tried on the *in vitro* ^{14}C-leucine incorporating system using rehydrated moss. Cycloheximide (150 μg in the incubation) inhibited leucine incorporation by 84% (370 cpm incorporation compared with 2100 cpm by the uninhibited complete system) whereas chloramphenicol (150 μg) had only a slight inhibitory effect (1820 cpm incorporated), suggesting that the major protein synthesis on rehydration takes place within the cytoplasm.

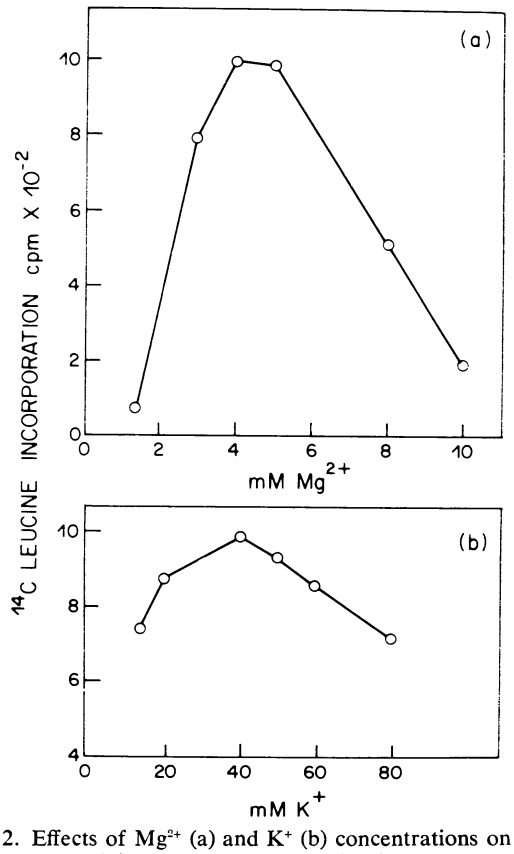

FIG. 2. Effects of Mg^{2+} (a) and K^+ (b) concentrations on *in vitro* polyribosomal activity. The K^+ concentration in (a) was 40 mM and the Mg^{2+} concentration in (b) was 4 mM.

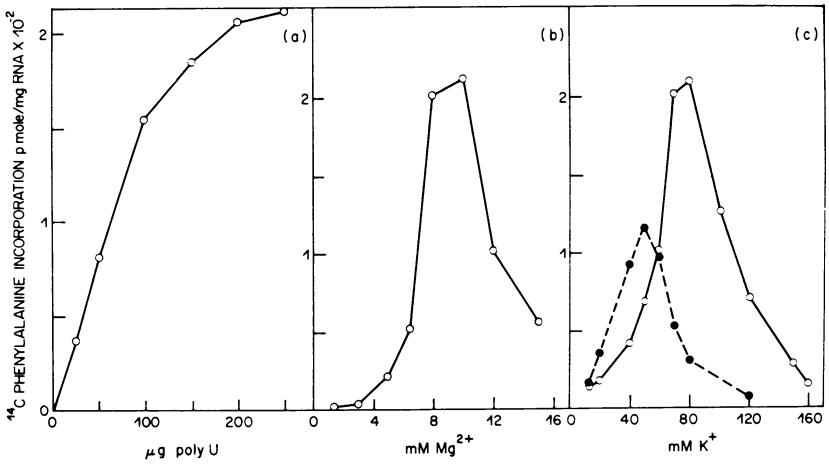

FIG. 3. a: Effect of polyuridylic acid concentration on ^{14}C-phenylalanine incorporation; b: effects of Mg^{2+} concentration on polyphenylalanine synthesis at 80 mM KCl; c: effects of varying the K^{+} concentration on polyphenylalanine synthesis at two Mg^{2+} concentrations. 6.5 mM Mg^{2+} (●); 10 mM Mg^{2+} (○).

Table II. *Effects of Slow and Rapid Desiccation on Protein Synthesis in Vitro in a Poly U and Endogenous mRNA Directed System*

The moss was desiccated rapidly by placing it over silica gel for 1 hr, or slowly in saturated atmospheres for 3 hr. After desiccation the ribosomes were extracted from the dried moss. A control incubation without ribosomes was carried out at the same time, and that incorporation value was subtracted.

Desiccation Rate	Incorporation	
	^{14}C-leucine	^{14}C-phenylalanine
	cpm × 10^{-2}/*mg rRNA*	*pmole/mg rRNA*
Undesiccated	142	104.5
Slow	13	221.5
Rapid	110	119.0

It has been reported that approximately 50% of the chloroplast ribosomes can be released from the thylakoid membranes only in the presence of detergent (8), and this was not used in our ribosomal extraction procedure. Nevertheless, no differences were found between the profiles of ribosomes on sucrose gradients extracted in the presence or absence of detergent (data not presented). Furthermore, for the analytical studies (23) Triton X-100 was included in the extraction medium and still only 80S ribosomes were observed, with the major ribosomal RNA species being 17S and 25S. Additionally, the lack of inhibition by chloramphenicol also indicates that bacterial contamination was not making a major contribution to our *in vitro* system.

Effects of Temperature and Drying on Extracted Polyribosomes. It was of interest to determine if the extracted polyribosomes *per se* could withstand desiccation outside the cell. Polyribosomes extracted from hydrated moss were subjected to freeze-drying or drying over ice in an air stream before addition of water to make up the original volume. The remaining polyribosomes were frozen in their final resuspension solution at −22 C, or heated to 60 C for 10 min. The results of such treatments are shown in Table III. Freezing the polyribosome suspension had little effect, whereas warming to 60 C was irreversibly destructive. Freeze-drying of the ribosomes resulted in a considerable loss of activity, but drying the ribosomes in a stream of air in the resuspension medium resulted in only a partial loss of activity. Thus, even the polyribosomes outside the cell can withstand drying to some extent.

A Comparison between Effects of Desiccation on *in Vitro* Protein Synthesizing Capacity of an Aquatic and a Terrestrial Moss. For this comparison, the terrestrial moss *Tortula ruralis* and the aquatic moss *Hygrohypnum luridum* were used. Ribosomal pellets were extracted from the moss before drying, in the dry state after rapid drying, and 2 hr after rehydration, and their capacity to incorporate ^{14}C-leucine and ^{14}C-phenylalanine *in vitro* was determined (Table IV). In both *in vitro* systems the capacity of the ribosomal pellet from hydrated *Hygrohypnum luridum* to catalyze protein synthesis was lower than that from *Tortula ruralis* under the same conditions. Fol-

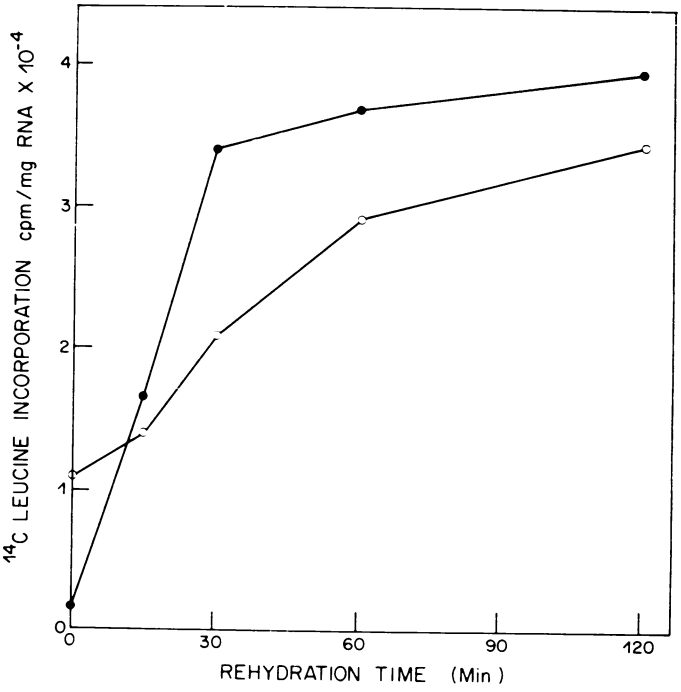

FIG. 4. Protein synthesis directed by polyribosomes extracted at various times from rehydrated moss, which had previously been desiccated rapidly or slowly. Rapidly desiccated (○); slowly desiccated (●).

Table III. *Protein-synthesizing Activity of Tortula Ribosomes being Subjected to Desiccation and Temperature in Vitro*

Polyribosomes extracted from 24 hr hydrated moss were suspended in 0.6 ml of resuspension medium containing 40 mM KCl, 4 mM magnesium acetate, 50 mM tris-acetate (pH 8.1), and 5 mM mercaptoethanol. Aliquots (0.08 ml) of this suspension (84 μg of RNA) were placed into incubation tubes and, except for the control, treated as described in the table. In the case of both the air- and freeze-dried samples, as soon as the drying was completed, water was added to the original volume. After addition of remaining components into each tube, the samples were incubated for 20 min at 31 C to allow for incorporation of ^{14}C-leucine into protein.

Treatment to Polyribosomes	Incorporation	
	cpm × 10^{-2}	%
Untreated (control)	15.7	100
Freeze-dried	3.4	22
Air-dried at 4 C	9	57
Frozen (−22 C) and thawed	13.6	86
Warmed to 60 C for 10 min	0	0

Table IV. *Amino Acid Incorporation into Protein by Ribosomes from Hygrohypnum luridum and Tortula ruralis*

Ribosomes were isolated from 1 g (fresh weight) of each moss. For both mosses desiccation was achieved rapidly over silica gel. The conditions used for measuring *in vitro* protein synthesis by *Hygrohypnum luridum* were identical to those used for *Tortula ruralis*.

Moss Species	State of Hydration	Incorporation	
		^{14}C-leucine	^{14}C-phenylalanine
		cpm × 10^{-2}/*mg rRNA*	*pmole/mg rRNA*
Hygrohypnum luridum	Before drying	23	32.4
	Dry	14.5	41.4
	2 hr rehydrated	4.7	16.0
Tortula ruralis	Before drying	142	104.5
	Dry	120	119.0
	2 hr rehydrated	139	89.5

lowing desiccation the activity of the ribosomal pellet from *Hygrohypnum* was reduced with respect to ^{14}C-leucine incorporation, but increased in its capacity to synthesize polyphenylalanine. Sucrose gradient analysis of the ribosomal pellet following rapid desiccation of *Hygrohypnum* showed a reduction in polyribosomes and a greatly decreased capacity for protein synthesis *in vivo* on rehydration (5). On rehydration of *Hygrohypnum* for 2 hr, there was a large reduction in the capacity of the ribosomal pellet to incorporate in either *in vitro* system, showing that it is during rehydration that the major damage to the protein synthesizing system occurs rather than during the desiccation process itself. By contrast, the activity of the polyribosomal pellet of the terrestrial moss *Tortula* increased on rehydration, as shown by the levels of ^{14}C-leucine incorporation, and decrease in polyphenylalanine production, indicating an enhanced utilization of the free ribosomes to form polyribosomes in the cell.

CONCLUSIONS

An *in vitro* protein synthesizing system has been obtained from mosses, utilizing wheat germ as the source of supernatant factors. Moss supernatant did not catalyse *in vitro* synthesis using endogenous or synthetic message, although the reason for this is not apparent.

Tortula that had been dried rapidly retained more polyribosomes than *Tortula* that had been dried slowly and these polyribosomes retained their capacity to conduct protein synthesis when extracted and utilized in the *in vitro* system. There was increased activity of the ribosomal pellet from slowly dried moss when poly U was used as the message, showing not only that there are increased free ribosome levels in the slowly dried moss, but also that these retain their activity even though the cells they are contained in are completely dried (3). Activity of the polyribosomes increased rapidly on rehydration—more rapidly in moss that had been previously dehydrated slowly than in more that had been dried quickly. This indicates a rapid reassociation of messenger RNA and ribosomes within the moss cells on reintroduction to water. The major part of the protein synthesis by the extracted ribosomes appears to be cycloheximide sensitive and chloramphenicol insensitive, indicating that the site of protein synthesis is cytoplasmic. Electron microscope studies of this moss (7, and unpublished results of E. B. Tucker) have shown that a major part of the cell volume is occupied by several large chloroplasts.

The fact that polyribosomes themselves can be desiccated and retain some synthetic activity when removed from the cell and air dried is an interesting phenomenon. It suggests that the polyribosomes have some form of natural stability of their own and may not have to be stabilized by modifications of the cytoplasmic matrix.

The aquatic moss, *Hygrohypnum luridum*, does not retain its capacity for protein synthesis on rehydration following desiccation. A reduction in polyribosomal activity was observed following rapid desiccation, but this was accompanied by an increase in the activity of the released ribosomes to catalyze poly U directed polyphenylalanine synthesis. On rehydration the protein synthesizing capacity directed by endogenous or synthetic message was greatly reduced, showing that it is the rehydration process and not the desiccation process which is lethal.

This ability of the moss *Tortula ruralis* to survive desiccation is a remarkable phenomenon; such a level of tolerance is rare in higher plants (10, 12), although the latter can withstand limited stress (14, 20–22). These and previous studies (1–3, 7, 11, 23) illustrate that the capacity for protein synthesis can be retained in *Tortula* during complete desiccation. In certain respects, this moss system resembles that of the dormant seed, where slow desiccation following maturation results in the separation of ribosomes from the endogenously stored messenger RNA (15, 16, 24). The onset of seed dormancy and desiccation is a slow process however, and this moss system appears to offer several advantages for studying drought tolerant metabolic changes during desiccation, particularly since desiccation can be achieved under controlled conditions within hours.

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