# THE ROLE OF WATER STRESS IN THE INACTIVATION OF MESSENGER RNA OF GERMINATING WHEAT EMBRYOS

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In a previous communication,[1] it was shown that embryos of ungerminated wheat seeds contain masked messenger RNA (mRNA), and that newly transcribed mRNA can be detected in the embryo 24 hours after germination. Protein biosynthesis in the embryo is initiated shortly after seed hydration.[2, 3] According to earlier observations (to be published[4]), wheat embryos can be dehydrated during the first 24 hours of germination without loss of their ability to grow normally on rehydration. Dehydration of embryos that have germinated for periods longer than 48 hours leads to an irreversible growth arrest. Twenty-four-hour wheat embryos thus represent drought-resistant plants, whereas the embryos germinated for periods longer than 48 hours represent drought-sensitive plants; both stages possess the same genetic constitution. In the present work, a description is given of the effect of water stress on the protein-synthesizing system of the drought-resistant and drought-sensitive stages of the germinating wheat embryo. Dehydration of embryos in both stages of development was found to inactivate mRNA and to arrest protein biosynthesis. Rehydration results in the transcription of complementary RNA, which resembles normal mRNA in the drought-resistant embryo but is false and inactive in the drought-sensitive embryo.

*Materials and Methods.—Materials:* Wheat seeds (*Triticum durrum* var. Nursit no. 163) were obtained from the Experimental Station, Neve Yaar, Israel. The germinating medium that was used contained 0.01 *M* tris(hydroxymethyl) aminomethane (Tris) buffer, pH 7.6, 0.02 *M* KCl, chloramphenicol (50 $\mu$g/ml), and mycostatin (200 $\mu$g/ml). Methylated albumin was prepared according to Sueoka and Cheng.[5] Electrophoretically purified pancreatic deoxyribonuclease was supplied by Worthington Biochemical Corp. Carrier-free $P^{32}$ orthophosphate was obtained from the Radiochemical Centre, Amersham. Radioactivity measurements were carried out in the Packard Tri-Carb scintillation counter.

*Growth and dehydration of the wheat embryos:* Germination of the wheat seeds was carried out in the dark at 24° in Petri dishes, each of which contained 24 seeds and 5 ml germinating medium. Dehydration of the germinating seeds was accomplished by exposing them in a drought chamber to 15% relative humidity for 48 hr at 29°. At the end of each experiment, 200 embryos were separated from the endosperm, and their RNA, DNA, and ribosomes were isolated and analyzed as described below. Embryos derived from seeds that were allowed to germinate for 24 hr are designated as (24) embryos. Embryos from seeds that were allowed to germinate for 24 hr and then were dehydrated for 48 hr are designated as (24-$\overline{48}$) embryos. Embryos obtained from seeds that were germinated for 24 hr, dehydrated for 48 hr, and then reimbibed in the germinating medium for 24 hr are denoted as (24-$\overline{48}$-24) embryos. Embryos from seeds that were germinated for 72 hr and then treated as described above (i.e., dehydrated and reimbibed) are referred to as (72), (72-$\overline{48}$), and (72-$\overline{48}$-24) embryos, respectively.

*Cell-free amino acid-incorporating system:* A cell-free ribosome and supernatant system similar to that described by Marcus and Feeley[3] was utilized in these studies. Different ribosome preparations were obtained from wheat embryos treated as described above. A supernatant system derived from ungerminated embryos was used throughout.

*Preparation of RNA and DNA:* RNA was isolated from wheat embryos and purified as described previously.[1] For the preparation of labeled RNA, wheat seeds were incubated for 24 hr in a germinating medium containing 100 $\mu$c/ml $P^{32}$-orthophosphate at the conditions specified below in *Results*. Two hundred embryos were then separated from the endosperm and washed thoroughly with 200 ml of germinating medium containing 0.05 *M* unlabeled orthophosphate.

DNA was prepared by the procedure of Marmur.[6]

*RNA-DNA hybridizations:* The hybridization experiments were carried out according to the procedure of Gillespie and Spiegelman.[7]

*Results.—Endogenous activity of the ribosomes of dehydrated wheat embryos:* The data presented in Table 1 show that ribosomes obtained from wheat embryos germinated for 24 hours and then dehydrated for 48 hours support the incorporation of only one third of the amount of amino acids incorporated by ribosomes derived from normal 24-hour germinated embryos. Rehydration of the dry (24–$\overline{48}$) seeds for 24 hours leads to a marked recovery of the endogenous activity of the ribosomes. When embryos germinated for 72 hours were allowed to dehydrate for 48 hours, the endogenous activity of the isolated ribosomes dropped to less than 10 per cent of their activity before dehydration. Seed reimbibition did not restore endogenous ribosome activity. Addition of poly U to ribosomes derived from (24), (24–$\overline{48}$), or (24–$\overline{48}$-24) embryos (see Table 1) stimulated $C^{14}$-phenylalanine incorporation. The amount of labeled amino acid incorporated was the same in all of the three cases investigated. A similar finding was recorded for $C^{14}$-phenylalanine incorporation in the presence of poly U by ribosomes derived from (72), (72–$\overline{48}$), and (72–$\overline{48}$-24) embryos. It may thus be concluded that wheat embryo dehydration causes damage at the programming level but does not affect ribosome activity *per se*.

TABLE 1. *Endogenous activity of ribosomes of dried and normal germinating embryos.*

| | Cpm/Mg Ribosomal RNA | |
|---|---|---|
| Embryo | Endogenous activity | + Poly U |
| (24) | 4,540 | 146,000 |
| (24–$\overline{48}$) | 1,600 | 145,000 |
| (24–$\overline{48}$-24) | 3,300 | 160,000 |
| (72) | 3,250 | 32,000 |
| (72–$\overline{48}$) | 230 | 31,000 |
| (72–$\overline{48}$-24) | 405 | 43,000 |

The incorporation system of 0.5 ml contained 2.2 $\mu$M $MgCl_2$, 22 $\mu$M KCl, 3 $\mu$M GTP, 1 $\mu$M ATP, 20 $\mu$g phosphoenolpyruvic kinase, 120 $\mu$g phosphoenolpyruvate, 25 $\mu$M Tris buffer (pH 7.8), 10 $\mu$M mercaptoethanol, 50 $\mu$g wheat sRNA, 105,000 $\times$ *g* purified supernatant containing 500 $\mu$g protein and 0.1 $\mu$c $C^{14}$-phenylalanine (200 $\mu$c/$\mu$M). The reaction was started by the addition of ribosomal preparations obtained from the various treatments of the wheat embryos as described in *Materials and Methods*. The reaction was carried out for 45 min at 30° and was stopped with 1 ml of 20% TCA at 0°. After centrifugation at 5000 rpm for 10 min, the precipitate was incubated at 90° for 15 min with 3 ml 10% TCA, cooled, filtered on a Millipore filter, and counted.

*mRNA in dehydrated wheat embryos:* The hybridization competition experiments illustrated in Figure 1 show that RNA obtained from (24–$\overline{48}$) wheat embryos competes with $P^{32}$-labeled RNA, transcribed in the time interval of 24–48 hours of normal germination, for the same complementary sites of wheat embryo DNA. Since it has been shown that (*a*) only 0.3 per cent of wheat embryo DNA is involved in the transcription of sRNA and rRNA, and (*b*) the mRNA transcribed during the time interval of 24–48 hours of germination resembles in its

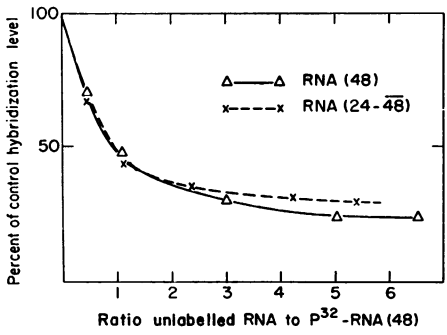


FIG. 1.—Persistence of mRNA during dehydration of 24-hr germinated embryos. The hybridization competition experiment contained in each sample 40 μg DNA, 134 μg $P^{32}$-RNA (48) (8100 cmp/μg), transcribed in the interval of 24–48 hr of germination, and increasing amounts of unlabeled RNA (24–48) derived from dehydrated 24-hr wheat embryos. Self-competition with unlabeled RNA (48) is included.

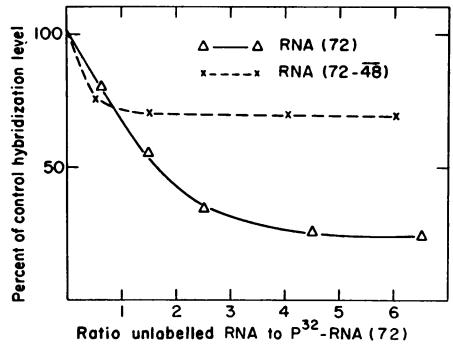


FIG. 2.—Changes in mRNA population during the dehydration of 72-hr germinated embryos. The hybridization competition experiment contained in each sample 10 μg DNA, 125 μg $P^{32}$-RNA (72) (11,500 cpm/μg), transcribed in the interval of 48–72 hr of germination, and increasing amounts of cold RNA (72–48) from embryos germinated for 72 hr and dehydrated for 48 hr. Self-competition of RNA (72) is included.

base sequence the mRNA present in 24-hour germinated embryos,[1] it is plausible to assume that the mRNA of the (24) embryo survives the 48-hour dehydration period. The competition experiment illustrated in Figure 2, in which unlabeled RNA of (72–48) embryos competes with $P^{32}$-labeled RNA from 72-hour germinated embryos for complementary sites on wheat embryo DNA, shows that about 70 per cent of the messenger population present in the normal (72) embryo cannot be displaced by the RNA of the (72–48) embryo. The mRNA in the (72) embryo, in contradistinction to the mRNA of the (24) embryo, seems therefore to be markedly affected by the 48-hour dehydration period.

*Synthesis of mRNA during reimbibition:* Germinated and dehydrated wheat seeds (24–48) were reimbibed for 24 hours in a medium containing $P^{32}$-orthophosphate. The separated embryos yielded total $P^{32}$-RNA of a specific activity of 30,000–40,000 cpm/μg RNA. A similar specific activity was recorded for the total RNA obtained from normal embryos labeled during 24–48 hours of germination. Reimbibition of (72–48) wheat seeds in medium containing $P^{32}$-orthophosphate as described above resulted in RNA of a specific activity of 600–1000 cpm/μg RNA. Labeling of normally germinated (72) embryos under similar conditions yielded an RNA of a specific activity of 30,000 cpm/μg RNA. Dehydration of (72) embryos thus leads to a marked decrease in the rate of RNA synthesis. For further characterization of the RNA transcribed after reimbibition, the saturation experiments shown in Figures 3 and 4 were carried out. The present results show that the $P^{32}$-RNA isolated from (24–48–24) embryos and from (72–48–24) embryos are complementary to 1.4–1.6 per cent of wheat embryo DNA. The RNA transcribed during reimbibition seems, therefore, to represent a messenger type of RNA.

*Characterization of the mRNA transcribed during reimbibition:* No growth of

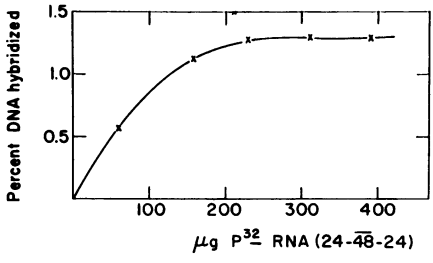


FIG. 3.—Saturation curve with $P^{32}$-RNA (24–48–24). Each sample contained 10 μg of DNA and increasing amounts of $P^{32}$-RNA (24–48–24) (38,000 cpm/μg), transcribed during 24 hr of reimbibition of embryos germinated for 24 hr and dehydrated for 48 hr.

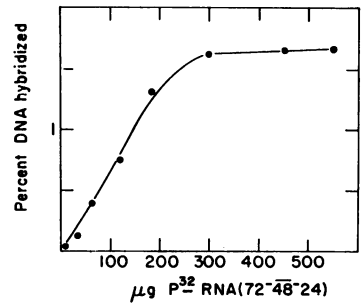


FIG. 4.—Saturation curve with $P^{32}$-RNA (72–48–24). Each sample contained 150 μg DNA and increasing amounts of $P^{32}$-RNA (72–48–24) (160 cpm/μg), transcribed during 24 hr of reimbibition of embryos germinated for 72 hr and dehydrated for 48 hr.

embryo occurs during the 48 hours of dehydration. It was therefore of interest to compare the RNA transcribed during the 24 hours of reimbibition of the (24–48) embryo with the RNA transcribed during the 24–48 hours of normal germination. The hybridization competition experiments illustrated in Figure 5 show clearly that both RNA's compete for similar sites of wheat embryo DNA.

A comparison of the hybridization characteristic of $P^{32}$-labeled RNA transcribed during 24-hour reimbibition of (72–48) embryos with that of unlabeled RNA of (96) normally germinated embryos, is shown in Figure 6. The results of the hybridization competition experiment reveal that the labeled RNA formed during the reimbibition of the injured embryo differs markedly from the RNA transcribed during the corresponding time of a normally germinated wheat embryo.

*Damage to the DNA of drought-sensitive embryos upon dehydration:* The marked change in RNA synthesis upon rehydration of the (72–48) wheat embryos induced us to investigate the effect of dehydration of the DNA template for RNA synthesis. When DNA was prepared from normal 24-hour embryos and sedimented on a sucrose density gradient, the sedimentation profile shown in Figure 7 was obtained. A similar distribution pattern was obtained from 72-hour germinated wheat embryos. When the DNA was labeled with $H^3$-thymidine during the first 24 hours of germination and the embryos were then dried for 48 hours and allowed to germinate in an unlabeled germinating medium for another 24 hours, the resulting $H^3$-DNA gave a sedimentation profile similar to the normal DNA preparation. However, when the DNA was labeled with $H^3$-thymidine during the 24 hours between 48 and 72 hours of germination and the embryos were dried for 48 hours and reimbibed for an additional 24 hours, and Marmur's procedure for DNA separation[6] was applied, no DNA could be spooled out. A small amount of DNA could be collected by alcohol precipitation and centrifugation. Analysis on sucrose gradient of this DNA revealed the disappearance of the highly polymerized component and the appearance of a substance of relatively low molecular weight.

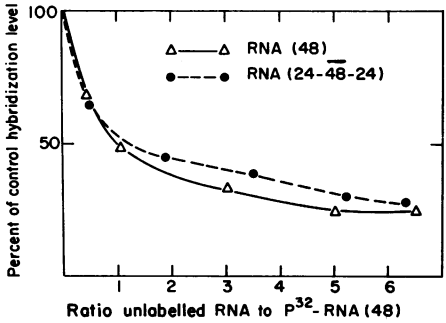


FIG. 5.—Competition experiment comparing the mRNA population transcribed during rehydration of (24–48) wheat embryos with normal RNA transcribed in the interval of 24–48 hr of germination. Each sample contained 29 $\mu$g DNA, 66 $\mu$g $P^{32}$-RNA (24–48–24) (14,260 cpm/$\mu$g), transcribed during 24 hr of the rehydration of embryos germinated for 24 hr and dehydrated for 48 hr, and increasing amounts of unlabeled RNA prepared from 48-hr germinated embryos. Self-competition with unlabeled RNA (24–48–24) is included.

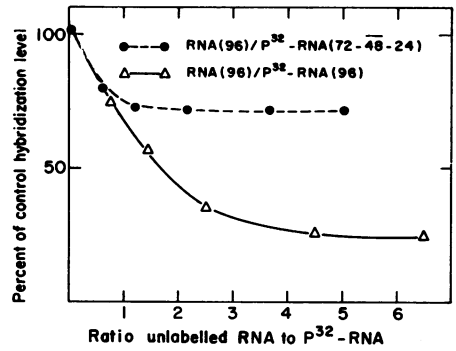


FIG. 6.—Competition experiments in which the mRNA population from 96-hr germinated embryos is compared with the RNA population transcribed in the rehydrated drought-sensitive embryo (72–48–24).

(●—●) Each sample contained 150 $\mu$g DNA, 240 $\mu$g $P^{32}$-RNA (72–48–24) (650 cpm/$\mu$g), transcribed during 24 hr of rehydration of (72–48) embryos.

(△——△) Each sample contained 10 $\mu$g DNA, 125 $\mu$g $P^{32}$-RNA (72) (11,500 cpm/$\mu$g), transcribed during 48–72 hr of germination, and increasing amounts of unlabeled RNA derived from (96) embryos.

*Discussion.*—Wheat embryos germinated for 24 hours are drought-resistant, i.e., dehydration does not impair their ability to grow normally after rehydration. Wheat embryos germinated for 72 hours, on the other hand, are drought-sensitive, i.e., dehydration inhibits their growth irreversibly. Since it could be expected that water stress affects protein biosynthesis, the effect of dehydration on the transcription and translation mechanisms in drought-resistant and drought-sensitive embryos was investigated.

When measured *in vitro* in a cell-free system, the ribosomal endogenous activity, of 24-hour or of 72-hour embryos decreased markedly with prior dehydration of the embryos for 48 hours. The ribosomes derived from the (24–48), as well as the (72–48) embryos, were not damaged during the dehydration process; they supported phenylalanine incorporation in the presence of poly U to an extent similar to that of ribosomes derived from the corresponding normal embryos. These results are in accord with the findings of Marre[2] and Sturrani and Marre[8] concerning the activity of ribosomes obtained from water-stressed castor bean seedlings. Hybridization competition experiments revealed that most of the mRNA population of 72-hour wheat embryos disappears upon dehydration for 48 hours. The mRNA of 24-hour embryos, on the other hand, is preserved under similar conditions but is rendered inactive; it thus resembles the latent mRNA of ungerminated wheat embryos.

Only trace amounts of complementary RNA were transcribed during 24-hour rehydration of (72–48) embryos. The transcribed RNA differed from the mRNA formed in normal germinating embryos since each complemented different

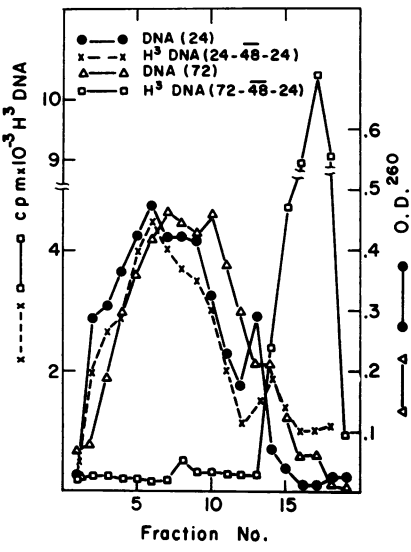


FIG. 7.—Sedimentation pattern of wheat embryo DNA. One thousand embryos were germinated for 24 or 72 hr in germinating solution at 24°. The embryos were then separated from the endosperm and the DNA was prepared according to the procedure of Marmur.[6]

In a parallel experiment, the embryos were either germinated in germinating solution containing $H^3$-thymidine (10 $\mu$c/ml, 120 mc/mmole) for 24 hr (X---X), or transferred to the labeled solution for 24 hr after 48 hr of germination in an unlabeled solution (□——□). The embryos were then thoroughly washed with an 0.05 *M* solution of unlabeled thymidine, dried for 48 hr at 15% relative humidity, and reimbibed for 24 hr in a germinating solution. $H^3$-DNA was prepared as described above; 0.2 ml containing 500 $\mu$g DNA was layered over a 5–20% sucrose gradient and centrifuged in the SW 39 Spinco rotor for 2 hr at 36,000 rpm. Eighteen fractions from each sample were collected and analyzed spectrophotometrically at 260 m$\mu$, or measured in the Packard Tri-Carb scintillation counter.

sites of the genome. It seems to represent a false message, since it cannot be translated *in vivo*. Rehydration of (24–$\overline{48}$) embryos for 24 hours led to the transcription of new and active mRNA resembling in its complementary characteristics the mRNA found in normal 48-hour germinating embryos.

The lack of normal active mRNA in the (72–$\overline{48}$–24) embryos prevents the formation of enzymes and other proteins essential for normal growth and differentiation. The breakdown of the DNA of 72-hour embryos upon dehydration might explain the slow rate of transcription *in vivo* as well as the formation of false messages due to the lack of proper initiation points, or a frame transition, on the broken template. DNA is actively duplicated and transcribed in 72-hour wheat embryos and is relatively inactive during the early stage of germination.[4] One is thus prompted to assume that the drought-sensitive stage of germinating wheat embryos might be correlated with DNA replication and mRNA transcription.

The abbreviations used are: mRNA, messenger RNA; Tris, tris(hydroxymethyl)aminomethane; GTP, guanosine 5′-triphosphate; ATP, adenosine 5′-triphosphate; TCA, trichloroacetic acid.

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