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Polyribosome Formation and Protein Synthesis in Imbibed but Dormant Lettuce Seeds[1]

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ABSTRACT

Dormancy is maintained in Grand Rapids lettuce (*Lactuca sativa*) seeds imbibed on water in darkness at 25 C. Polyribosome formation and protein synthesis occur early in the imbibition phase and considerable polysomal material is also present after 24 and 48 hours, even though the seeds have failed to germinate. Incorporation of labeled leucine into protein following a 24-hour preincubation period shows that these polysomes are active in protein synthesis.

Grand Rapids lettuce seeds imbibed in darkness at 25 C fail to germinate unless supplied with a particular germination stimulus. Thus, light (11, 12, 21) or gibberellic acid (20) stimulates almost 100% germination within 24 hr, while few untreated seeds germinate within the same time period. Such water-imbibed seeds maintain their dormant condition for long periods, up to 12 weeks (27), but retain their light sensitivity for only a matter of hours (16) or a few days (7, 13), before becoming skotodormant.

Germination in darkness of these seeds is promoted by both D- and L-threo chloramphenicol and by actinomycin D (6, 7). Furthermore, both isomers, at low concentrations where germination is not promoted, prevent the onset of skotodormancy. Black and Richardson (7) suggest these inhibitors exert their effects by reducing a protein synthesis-dependent inhibitory process occurring in dark-imbibed dormant seeds, but the possibility also exists that they are acting indirectly by initially decreasing respiration (15).

Although dark-imbibed dormant Grand Rapids lettuce seeds show these responses to external stimuli, to date the status of protein synthesis in the seed tissues remains obscure. Bewley and Black (4) showed protein synthesis occurring in dark-imbibed dormant seeds from hours 5 to 15. Similarly, a ribosomal fraction extracted from seeds imbibed in darkness on water at times prior to, and including the time of germination of light-treated seeds, was shown to support *in vitro* protein synthesis without the addition of artificial mRNA, although this declined to a low level by 24 hr (10). In hazel seeds, GA_3 treatment substitutes for a chilling requirement, and a dormant condition, seemingly similar to that in dark imbibed seeds of lettuce, is imposed on the untreated seeds. *In vivo* labeling experiments indicated considerable protein synthesis occurring in dormant embryonic axes, prior to the time of germination of GA_3-treated axes (19). Similarly, dormant seeds of *Avena fatua* are capable of synthesizing protein at a rate comparable to that of nondormant seeds (8).

On imbibition the association of ribosomes and stored mRNA present in dry seeds to form polyribosomes, with resultant activation of protein synthesis, has been widely documented (18, 24–26, 30). It has been suggested, however, that in light-sensitive lettuce seeds, maintenance of the dormant condition in darkness is characterized by a complete lack of a polyribosomal complement and that polyribosome formation is initiated only when the seeds receive a germination stimulus (27). Mitchell and Villiers (27) report, furthermore, that there is no polyribosomal component in ribosomal fractions extracted from seeds incubated in darkness even after 24 hr, although Black and Richardson (7) previously reported *in vivo* synthesis of protein up to 72 hr in darkness. The following experiments were performed to clarify the apparent paradox between the claim that protein synthesis occurs in dark-imbibed but dormant lettuce seeds (4, 7, 10), and that which reports polyribosome formation, and presumably protein synthesis, only following a germination stimulus (27).

MATERIALS AND METHODS

Seeds of *Lactuca sativa* cv. Grand Rapids purchased from the Ferry Morse Seed Company were stored in a desiccator under reduced pressure at 0 to 4 C. Seeds from the 1971 harvest were used for the majority of experiments, although the polyribosome data in Figure 2 were obtained from seeds of the 1970 harvest which showed a similar low dark germination percentage.

Seeds used for the extraction of the ribosomal fraction were incubated in 5-cm Petri dishes on one layer of filter paper saturated with 2 ml of distilled water. The Petri dishes were wrapped in aluminum foil and incubated at 25 C for the required time period. When isolated embryos were used, these were dissected from the enclosing layers with the aid of a dissecting microscope in dim green light at 25 C. The number of seeds or embryos used in different experiments varied and are reported in the figure legends.

Seeds, or embryos isolated after various imbibition periods, were ground in 3 ml of a grinding solution in a prechilled mortar. The grinding solution, based on that of Jachymczyk and Cherry (18), consisted of 250 mM sucrose (RNase free, Mann Research) 50 mM tris-HCl, pH 7.6, 20 mM KCl, 10 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 1% (w/v) sodium deoxycholate. The suspension was transferred to a Duall glass tissue grinder and gently homogenized with two strokes, each with several turns, and the 9-ml total volume

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homogenate was centrifuged at 20,000*g* for 10 min. The supernatant was layered over 2.75 ml of a 1.66 M sucrose solution containing the same components as the grinding solution, with the exception of the detergent, and centrifuged at 174,600*g* (R_{av}) for 90 min in a Beckman L2-65B ultracentrifuge (type 65 rotor). The ribosomal pellet was resuspended using a glass rod in 0.75 ml of a solution containing 10 mM tris-HCl, pH 7.6, 20 mM KCl, 10 mM magnesium acetate, and 5 mM 2-mercaptoethanol and centrifuged at 20,000*g* for 10 min. The RNA concentration of an aliquot of the supernatant was estimated from the absorbance at 260 nm, and a volume containing 0.08 mg of RNA was loaded (using a prechilled pipette) onto the top of a 5.5-ml linear 15 to 35% sucrose gradient containing 10 mM tris-HCl, pH 7.6, 20 mM KCl, and 10 mM magnesium acetate. The gradients were centrifuged at 114,000*g* (R_{av}) in a Spinco SW50.1 rotor for 95 min.

After centrifugation, the distribution of the components of the ribosomal fraction was determined by monitoring the gradients at A_{254} in a modified, continuous flow ISCO UA2 analyzer. From the absorbance profile, the ribosomal peak was extrapolated to the baseline, and from triplicate copies, the relative areas of the free ribosomal and polyribosomal components were estimated by cutting and weighing.

For the *in vivo* labeling experiments, 100 seeds per replicate were taken from cold storage and surface-sterilized according to Frankland *et al.* (14) in dim green light at 25 C. The seeds were added to the outside chamber of a glass Conway unit which contained 25 μc of DL-leucine 4,5-T (purchased sterilized and in aqueous solution from the Radiochemical Centre, Amersham). Several batches were used ranging in specific radioactivity from 16.4 c/mmole to 28.0 c/mmole, but for any related series of experiments leucine of the same specific radioactivity was used. Sterile phosphate (10 mM, pH 5.0), 100 units of penicillin and streptomycin (Bio-Cult Laboratories, Glasgow) were also included, and the incubation solution was made up to 2 ml with distilled water. One milliliter of distilled water was added to the inside chamber to minimize possible evaporation from the outer well, the lid was secured, and the units were wrapped in aluminum foil before incubation for the required time period at 25 C.

For the labeling experiments requiring a 24-hr preincubation in water, surface-sterilized seeds were transferred to a sterile Petri dish and incubated on one layer of filter paper saturated with sterile water. After 24 hr at 25 C, 100 ungerminated seeds per replicate were introduced to the label as above. All subsequent operations were performed at 0 to 4 C. Following the labeling period, the seeds were washed thoroughly with distilled water and ground in a prechilled mortar in 0.1 M tris glycine buffer, pH 8.4, containing 1% (w/v) sodium deoxycholate. The suspension was homogenized in a tissue grinder and centrifuged at 12,000*g* for 15 min. The total homogenate volume was 9.0 ml. Sufficient 40% (w/v) trichloroacetic acid was added to the supernatant to make it 5% (w/v) trichloroacetic acid. After 10 min at 0 C, the precipitated protein was collected by centrifugation and resuspended in 5% trichloroacetic acid, heated to 95 C for 10 min, and cooled on ice for 10 min. The pellet following centrifugation was washed with 5% trichloroacetic acid and subjected to two lipid extractions with 5 ml of ethanol-diethyl ether (v/v). The pellet obtained from the second lipid extraction was dried and dissolved in 2 ml of 90% formic acid.

Aliquots were counted in a standard toluene-ethanol (7:3) scintillator in a Nuclear Chicago liquid scintillation spectrometer, and protein concentration relative to bovine serum albumin standards was assayed by the method of Lowry *et al.* (23) following reprecipitation from the formic acid with trichloroacetic acid and solubilization in 1 N KOH.

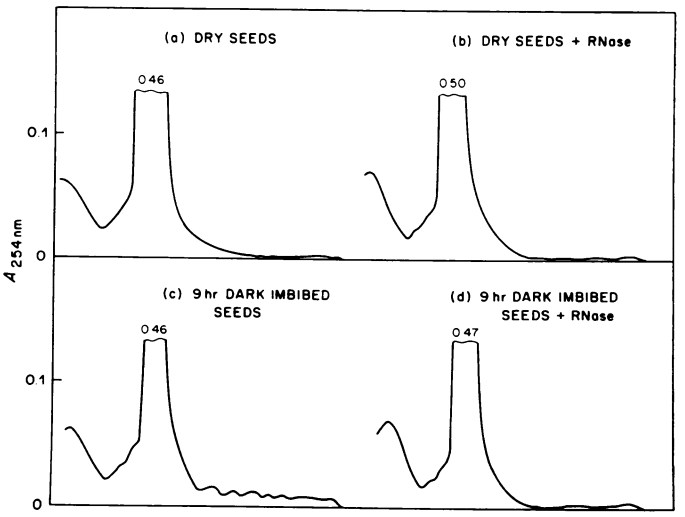

FIG. 1. Absorbance profiles of the ribosomal fraction. a: Dry seeds; b: dry seeds treated with 1 μg/ml bovine pancreatic RNase A for 2 min prior to loading on gradient; c: seeds imbibed on water in darkness for 9 hr, d: 9-hr imbibed seeds treated with 1 μg/ml RNase for 2 min. Two replicate extractions of each treatment were made from 100 mg of seeds.

RESULTS AND DISCUSSION

Typical polyribosomal patterns obtained from dark-imbibed lettuce seeds are shown in Figure 1c. Although the yield of polyribosomes appears to be lower than from other seeds, as extracted by other workers, *e.g.* peanut (18), wheat (34), the technique employed here for lettuce resulted in extraction of maximal polyribosomal material and any changes in pH or salt concentration in the medium resulted in poorer polyribosomal yields. The methods described by Davies *et al.* (9) for pea stem internodes, Lin *et al.* (22) for soybean roots, and Bewley (3) for moss also resulted in poor yields of the polysome fraction with a high yield of free ribosomal material. The unmodified technique of Jachymczyk and Cherry (18) produced yields only slightly lower than the present method.

That the UV-absorbing material in our sucrose gradients corresponds to polysomal material was verified in two ways. Figure 1c shows the components of the ribosomal fraction extracted from seeds incubated in darkness for 9 hr. Figure 1d shows the result of incubating an aliquot of the same fraction with 1 μg/ml ribonuclease which removes the heavier sedimenting peaks.

The second set of experiments utilized the compound D-MDMP,[2] which is an inhibitor of the initiation process of protein synthesis in plant systems (2, 33) by preventing the association of ribosomes with mRNA. The outer seed layers were carefully slit from 100 seeds per treatment, 1 hr from the start of imbibition, the slit seeds were transferred to either water at 10^{-3} M D-MDMP and incubated for a further 8 hr. Slit seeds incubated in D-MDMP yielded no absorbance peaks associated with the polysomal area of the gradients, although peaks were present over the same time period in water-imbibed seeds. A similar experiment involving intact seeds was performed, but D-MDMP was ineffective in preventing polyribosome formation. It seemed likely that, in this case, the inhibitor was not reaching the embryo, since the endosperm surrounding it is known to act as a restrictive barrier to various organic compounds (1, 32).

[2] Abbreviation: D-MDMP: [2-(4 methyl-1,6-dinitroanilino)-N-methyl propionamide].

The inhibitory action of D-MDMP in plant tissues, by interfering with the proper combination of message with ribosome, verified that in these experiments the material sedimenting at a faster rate than the ribosome peak is most likely polyribosomal in nature.

Ribosomal Fraction of Dry Seeds. Results shown in Figure 1a indicate that the ribosomal fraction prepared from dry lettuce seeds is almost exclusively in the form of free ribosomes with no peaks in the denser portion of the sucrose gradient. There is, however, some limited absorbance at 254 nm in this latter half of the gradient relative to the baseline, although the majority of it probably represents tailing of the single ribosome peak. Treatment of the dry seed fraction with bovine pancreatic ribonuclease (Fig. 1b) did not reduce this value, indicating the absorbance is not polyribosomal.

Polyribosome Levels and Protein Synthesis during Imbibition. The changes in the polyribosome complement and *in vivo* protein synthesis of seeds incubated for varying periods of time after the introduction of water in darkness are summarized in Figure 2. Accompanying and following the increase in fresh weight due to water uptake, there is an increase in the amount of polyribosomal material and a corresponding increase in the specific activity of extracted protein. Up to 12 hr no germination has occurred in these seeds and by 24 hr a maximum level of 10% is attained. By contrast, seeds imbibed in 0.1 mg/ml GA_3 begin to germinate by 10 hr and are 98% germinated by 24 hr.

Similar levels of dark incorporation of labeled amino acids into protein in Grand Rapids lettuce in the period preceding germination of GA_3- (4) or light- (7) treated seeds have been reported. The question now arises as to whether the polyribosome complement present in the earlier stages of incubation in darkness disappears by 24 hr and therefore corresponds with the observation of Mitchell and Villiers (27), or if the early protein synthesis is maintained in dormant seeds as indicated by the earlier report of Black and Richardson (7).

Polyribosome Levels and Protein Synthesis in Seeds Maintained in the Dormant Condition. Ribosomal fractions were extracted from ungerminated seeds maintained on water at 25 C in darkness for 24 hr. Figure 3a shows the absorbance profile of this fraction and a significant polyribosomal complement is evident. An identical proportion of polyribosomal material

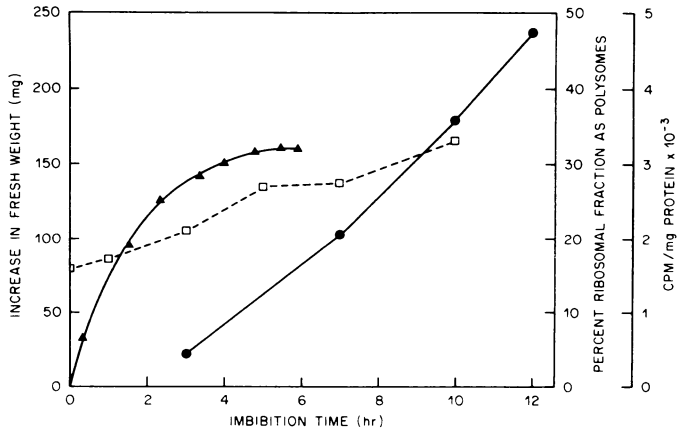

FIG. 2. Changes in fresh weight (▲), polyribosome levels (□), and protein synthesis (●) in dormant seeds imbibed in darkness on water. Triplicate ribosomal fractions were extracted from 200 mg of seeds at the times indicated. Incorporation data are based on triplicate extractions of protein from 100 mg of seeds incubated in labeled leucine. Percentage of ribosomal fraction as polyribosomes was calculated as indicated in "Materials and Methods."

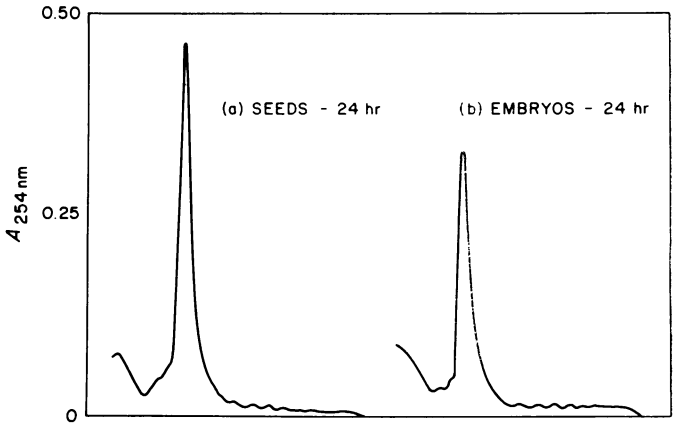

FIG. 3. Absorbance profiles of the ribosome fraction. a: Seeds imbibed on water and incubated at 25 C for 24 hr in darkness; b: embryos dissected from seeds following imbibition on water at 25 C for 24 hr in darkness. 100 seeds or embryos were extracted in duplicates.

was obtained from seeds extracted from a 48-hr preincubation.

The reported absence of a polyribosome fraction at 24 hr (27) was based on extractions from embryos dissected from seeds following the 24-hr incubation period. Our experiments, however, utilized whole seeds and since lettuce seeds contain living tissue other than the embryo (the thin sac-like endosperm), the possibility that this tissue was supplying the polyribosome component to the extracts was considered. The endosperm in lettuce seeds in metabolically active (7), and its integrity has been suggested as a controlling factor in the germination of these seeds (5, 17, 28, 29, 31). We therefore dissected embryos from ungerminated seeds following 24-hr preincubation in darkness and extracted the ribosomal fraction from them. The results, as illustrated in Figure 3, a and b, show that most, if not all, of the polyribosome fraction is to be found in the embryos, and in the presence of the surrounding layers more free ribosomes are extracted.

Protein extracted from 75-seed replicates, incubated with 25 μc of tritiated leucine (specific synthetic activity 28 c/mmole) for 12 hr following a 24-hr preincubation (all in darkness), showed incorporation of 41,280 cpm/mg protein, indicating considerable protein synthetic activity over this period.

With regard to polysome formation, the situation described above appears similar to that in isolated embryos of Grand Rapids lettuce whose germination is prevented by abscisic acid in darkness (5). Here the dormant state is also characterized by considerable polyribosome formation up to 28 hr and increases in *in vivo* protein synthesis up to 30 hr (Fountain, unpublished).

In conclusion, therefore, we have established that polyribosomes are formed in seeds imbibed for up to 24 hr in darkness, even though these seeds are not going to germinate. This polyribosome formation is always accompanied by considerable protein synthesis, confirming the studies of previous workers (4, 7). Even isolated embryos whose germination can be permanently inhibited by abscisic acid were observed to synthesize polyribosomes and proteins for up to 30 hr (Fountain, unpublished). Since our experiments have produced consistent results, and on three different harvests of Grand Rapids, we find it difficult to explain the inconsistency between the results of Mitchell and Villiers (27) and our own.

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