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Protein Synthesis in Imbibed Seeds III. Kinetics of Amino Acid Incorporation Ribosome Activation, and Polysome Formation

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Summary. The kinetics of development of protein-synthesizing capacity in the imbibing wheat embryo, were studied both in vivo and in vitro. During the first 30 minutes of imbibition protein-synthesizing capacity rises rapidly, lagging about 10 minutes behind water uptake. This rise in synthesizing capacity is accompanied by an increase in polysome content. As imbibition continues, both protein-synthesizing capacity and polysome content increase. With embryos from aged seed, the rate of protein synthesis is initially limited by another, presumably nonribosomal, reaction.

An earlier report (4) concluded that seed germination is accompanied by an increased capacity for protein synthesis. This conclusion was based primarily on the observation that ribosomes isolated from dry (unimbibed) embryos, in contrast to those isolated from embryos imbibed for 16 hours, are capable of only a low level of amino acid incorporation into protein. Since the desiccated state of the unimbibed embryos might have resulted in the destruction of ribosomal functional capacity during isolation, it appeared desirable to examine more closely the relationship between water uptake and ribosomal activity. Furthermore, it was observed (5) that imbibition for 16 hours resulted in the formation of polysomes and that the polysome fraction was the only ribosomal component capable of amino acid incorporation (5, 6). Such observations suggested that polysome formation might be the factor limiting protein synthesis in the early stages of germination. Should this be true, a close temporal relationship between polysome formation and ribosomal activity, would be expected. The present communication is concerned with further exploration of these points. In addition, an examination of the kinetics of in vivo incorporation of amino acids, is described. Such a study bears directly on both the question of capacity for protein synthesis and on the relevance of polysome formation.

Material and Methods

In vivo Incorporation. Assay 1. Wheat embryo samples (200 mg) were imbibed for the desired times and then transferred to a test tube where they were incubated in 2.5 μmoles potassium phosphate (pH 6.0), 40 μg chloramphenicol, 5.1 mμmoles L-leucine-^{14}C (0.25 μcuries) and water to 0.53 ml. The volume of incubation medium was such that the embryos were submerged. After incubation, 200 μmoles of L-leucine-^{12}C were added; free radioactivity was removed by washing with water, and the embryos were ground to a homogeneous suspension in 5 % trichloroacetic acid containing 200 μmoles L-leucine-^{12}C. An appropriate aliquot of the suspension was centrifuged; an aliquot of the supernatant was counted in Bray's solution (1) (trichloroacetic acid soluble). Another aliquot of the suspension was centrifuged, resuspended in 5 % trichloroacetic acid, and heated for 15 minutes at 90°. After cooling for 10 minutes in ice, the precipitate was collected on a membrane filter, washed with 5 % trichloroacetic acid and counted in toluene-PPO-POPOP (trichloroacetic acid insoluble).

Assay 2. Wheat embryo samples were imbibed for the desired times on 5.5 cm Whatman No. 1 filter circles in 1.55 ml water containing 50 μg streptomycin. The paper with the embryos was then removed, blotted for 2 to 3 min and transferred to a dish containing 2.5 μmoles potassium phosphate (pH 6.0), 40 μg chloramphenicol, 5.1 mμmoles L-leucine-^{14}C (0.25 μcuries) and water to 1.3 ml. (These conditions effectively measure incorporation during a period of continued imbibition). After incubation, the embryos were rinsed and processed as in assay 1.

[1] The decreased incorporation rate may be obtained simply by immersing the embryos in a test tube with 0.53 ml of water for 20 minutes. This treatment causes about 50 % loss of polysomes and a 50 % loss of oxygen uptake.

Ribosomal amino acid-incorporating activity was measured with leucine-^{14}C in the standard in vitro system previously described (5) with the following modifications. After a 40 minute incubation at 30°, 0.3 ml 0.1 M L-leucine, 0.1 ml bovine albumin (2.5 mg/ml), 0.4 ml 40 % trichloroacetic acid, and 2 ml 5 % trichloroacetic acid were added. After centrifugation, the pellet was resuspended in 4 ml 5 % trichloroacetic acid and heated 15 minutes at 90°. After cooling for 10 minutes in ice, the precipitate was collected on a membrane filter, washed with 5 % trichloroacetic acid and counted in a toluene mixture. Ribosomes prepared from embryos imbibed for 10 hours had a specific activity (cpm per mg ribosomal RNA) of 31,000. In some experiments the Mg^{2+} concentration of the grinding medium (for ribosome preparation) was reduced from 0.01 M to 0.001 M and in some of the assays for ribosomal activity, 50 μg of wheat embryo s-RNA were added. Both of these changes are specifically indicated. The addition of s-RNA to the assay system doubled the ribosomal specific activity.

Results

Water Uptake and Ribosomal Activity. The time course of water uptake by germinating wheat embryos for a 24 hour period is shown in figure 1.

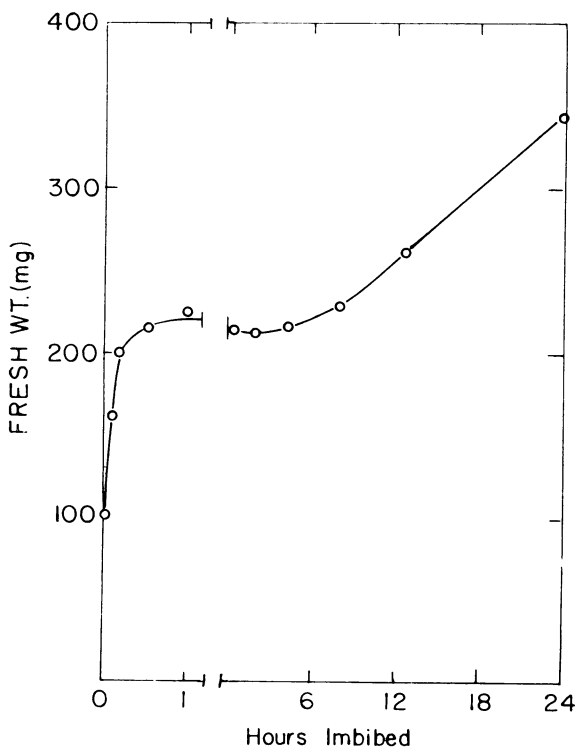

FIG. 1. Time course of water uptake. 100 mg samples of embryos were imbibed for the times shown, blotted thoroughly and weighed.

Table I. *Effect of Temperature of Imbibition on Development of Ribosomal Activity*

Embryos (200 mg) were imbibed at the indicated temperature shown, blotted on filter paper at that temperature for 2 to 3 minutes and processed for preparation of ribosomes. In the temperature-transfer experiments the filter papers were dried at the initial temperatur before transfer. The fr wt data described in the text were obtained with separate embryo samples.

Imbibition	Specific Activity cpm/mg RNA
15 min 4°	210
30 min 4°	240
15 min 23°	2175
30 min 23°	8150
15 min 4°—15 min 23°	2350
15 min 23°—15 min 4°	4600

There is a rapid initial increase in fresh weight of 120 % in 20 to 30 minutes, a plateau of 4 to 5 hours, and a secondary steady increase continuing for at least 18 additional hours. Figure 2 shows a detailed analysis of the increase in fresh weight during the first 1.5 hour of imbibition as compared with the increase in ribosomal leucine-incorporating activity. Ribosomal activity lags considerably behind water uptake. For example, at 15 minutes when the fresh weight has reached 83 % of the plateau value, ribosomal activity has only reached 15 % of its value at 1.5 hours. In another type of experiment in which water uptake was dissociated from attainment of ribosomal activity, 100 mg samples of embryo were imbibed at 4°. The fresh weight increased in 15 and 30 min to 171 and 190 mg respectively, while the ribosomal specific activity remained at the control level (table I). The cold treatment per se was not injurious, as was ascertained from a companion series of experiments (table I), in which a 15 min cold treatment did not inhibit response to a subsequent 15 min warm treatment. In addition, a 15 min warm treatment followed by a cold period resulted in continued increase in ribosomal activity.

In Vivo Incorporation. The time course of development of protein-synthesizing capacity was also ascertained by assaying incorporation in vivo. Two assays were developed in which embryos were incubated with leucine ^{14}C; subsequently radioactivity was determined both in the acid-soluble and acid-insoluble fractions. The conditions of the first assay (Materials and Methods, Assay 1) allow a rapid uptake of a large quantity of radioactivity into the embryos and are, therefore, convenient for short incubation periods. The kinetics of incorporation in this assay (table II) show a rate of incorporation that decreases continuously with incubation time, probably due to oxygen deficiency resulting from the submerged state of the embryos.[1] The second assay (Materials and Methods, Assay 2) is in effect a period of continued imbibition in the

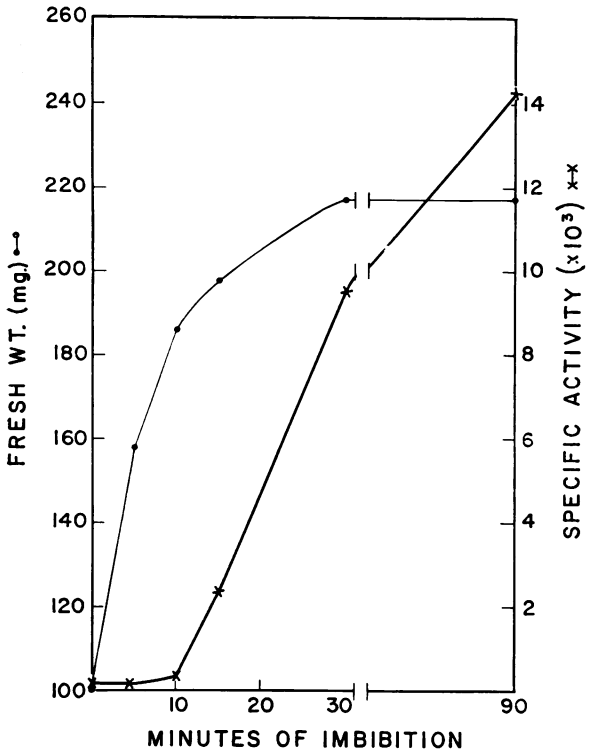

FIG. 2. Comparison of water uptake and development of ribosomal activity in the first phase of imbibition.

presence of leucine-^{14}C. The kinetics of this assay show an increased rate of incorporation after 20 minutes (table II).

When embryo incorporation capacity was assayed by either of these procedures, a marked difference between lots of embryos, was noted (table III). Thus, in a comparison of samples imbibed for 0.5 and 6 hours, embryos from freshly harvested seed (represented by Pennoll 1965) showed a 3 to 5 fold increase in incorporating capacity while embryos from aged seed (represented by Durum 1963-aged) increased 14 to 20 fold. In general, after 6 hours of imbibition, the relative capacities did not differ markedly. It was, rather, the low incorporating capacity of aged embryos during the first 3 hours which provided the primary difference. Ribosomes isolated from both fresh and aged embryos had essentially identical incorporation rates in vitro, indicating that the differences in vivo are not at the ribosomal level.

Attempts to employ the in vivo assay during the first 30 minutes of imbibition were only partially successful. The difficulty lies in the low rate of incorporation obtained in the short incubation periods (see table II) and the lack of an accurate method for ascertaining non-specific low-level adsorption of radioactivity. In a typical experiment with Pennoll 1965 embryos, in incubations of 5 and 10 minutes (conditions of Assay 2), unimbibed embryos had activity values of 0.014 and 0.048 in comparison with embryos that were imbibed for 30 minutes which had rates of 0.042 and 0.13.

Polysome Content. Earlier studies (5) had suggested that polysome formation might be a limiting factor in development of capacity for protein synthesis during germination. These studies were based solely on in vitro experiments. To confirm the significance of the polysome fraction in amino acid incorporation, the ribosomal fraction was isolated after labeling in vivo and was separated in a sucrose gradient. Almost all the radioactivity

Table II. *Kinetics of Incorporation with Increased Incubation Time*

The conditions of the assays are described in the text. Incorporation rate for the given time period is calculated by dividing the radioactivity incorporated into the trichloroacetic acid-insoluble fraction during that period by the trichloroacetic acid-soluble radioactivity found at the end of the period. In the case of a 20-minute period (assay 1), the rate is halved. Thus in the 3 hour imbibed sample the rate for the 20 to 40 minute period is 800/50,400 or 1.6 % giving a rate of 0.8 % for 10 minutes. The experiment shown for assay 1 used embryos from aged Durum 1963 wheat while that for assay 2 used Pennoll 1965 (See Results). Similar data were obtained with other embryo preparations from both fresh and aged wheat seeds.

	Imbibition	Incubation	Trichloroacetic acid soluble	Trichloroacetic acid insoluble	Incorporation rate
	hr	min	cpm	cpm	% per 10 min
Assay 1	3	10	34,200	810	2.3
		20	42,500	1400	1.4
		40	50,400	2200	0.8
	14	10	69,000	12,050	14.9
		20	113,000	20,100	7.1
		40	149,000	30,000	3.4
Assay 2	0.5	10	2500	328	13.1
		20	4150	845	12.5
		30	4860	1790	19.4
	6	10	3070	2120	69
		20	5540	5830	67
		30	6970	13,100	104

Table III. *Effect of Imbibition Time on Capacity for Incorporation as Measured In Vivo*

		Durum–1963–aged			Pennoll–1965		
	Imbibition	Trichloroacetic acid soluble	Trichloroacetic acid insoluble	Activity	Trichloroacetic acid soluble	Trichloroacetic acid-insoluble	Activity**
	hr	cpm	cpm		cpm	cpm	
Assay 1	0.5	26,700	272	1.0	26,700	1690	6.0
	1.5	28,600	472	1.6	26,500	3570	11.0
	6	52,500	8480	13.9	66 600	13.500	16.9
Assay 2	0.5	6700	324	0.05	4580	1340	0.29
	1.5	6650	480	0.07	5180	3650	0.70
	6	7750	6950	0.90	5630	7750	1.38

* Incubation time is 19 min in assay 1 and 24 min in assay 2.

** Activity in assay 1 is calculated as % of total radioactivity (Trichloroacetic acid soluble + trichloroacetic acid insoluble) in the trichloroacetic acid insoluble fraction. In assay 2, where the soluble trichloroacetic acid radioactivity maintains a steady state (see also table II), activity is calculated as units (trichloroacetic acid insoluble radioactivity divided by trichloroacetic acid soluble radioactivity).

was confined to the polysomal region (fig 3A). When the ribosomal fraction was treated with deoxycholate (fig 3B) and immediately layered on the gradient (8) a considerable amount of radioactivity shifted to the low molecular weight region (tubes 21–24) with no transfer to the monosomal area (tubes 16–20). Increasing the time or temperature of incubation with deoxycholate did not increase the quantity of radioactivity shifted from the polysomal region. However, treatment with ribonuclease prior to addition of deoxycholate (fig 3C), resulted in complete removal of radioactivity from the polysomal area, and the increment due to ribonuclease shifted to the monosomal area. In similar experiments (not shown) ribonuclease treatment, without addition of deoxycholate, caused radioactivity to shift from the polysomal to the monosomal region, with no change in the low molecular-weight region.

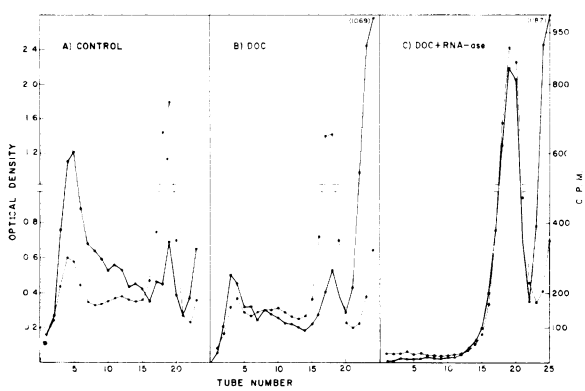

FIG. 3. Polyribosome labeling by in vivo incorporation. 400 mg of Pennoll embryo were imbibed for 12 hours and then incubated under the conditions described for assay 2 (in vivo) with the addition of 11.2 mμmoles of leucine ^{14}C (5.0 μ/curies). After 25 minutes the embryos were washed and the ribosomes prepared in the usual manner. The ribosomal pellet was taken up in 1.3 ml of suspending medium (5) and divided into 3 portions. One portion was layered directly on the sucrose gradient (3A), a second portion was treated with deoxycholate (DOC) (final concentration 0.75 %) (3B) just prior to layering, while a third portion (3C) was first incubated for 10 minutes at 20° with 2 μg of ribonuclease and then treated as in 3B. The gradient was linear from 5 to 25 % with an under-layer of 60 % (W/V) sucrose (3). After centrifugation in the SW39 Spinco rotor for 40 minutes at 36,000 RPM, the tubes were punctured and successive 12-drop samples were analyzed for absorbance (●—●) at 260 mμ and for trichloroacetic acid-insoluble radioactivity (x—x).

The general approach of figures 3B and 3C was used to estimate the time course of polysome formation. Thus, ribosome samples obtained at various times of imbibition were divided into 2 portions. Both were treated with deoxycholate to a final conc of 0.75 % and 1 portion was also treated with ribonuclease. After centrifuging through the usual gradient, the polysome area (tubes 2–11) was collected as a single fraction and the absorbance at 260 mμ was determined. The decrease in absorbance due to ribonuclease was taken as a measure of the polysome content. The data (table IV) show an initial rapid increase in polysomal content (15–30

Table IV. *Relation of Ribosomal Activity and Polysome Content to Imbibition Time*

Ribosomes were prepared in blending medium containing 0.001 M Mg^{2+} acetate. The general procedure for determining polysome content is described in the text. The specific data shown are from a determination of the absorbance at 260 mμ of the polysome fraction (tubes 2–11) diluted with 1.0 ml H_2O for 300 mg samples of embryo. The ribosomal activity data were obtained by the standard assay with the addition of 50 μg of wheat-embryo s-RNA.

Imbibition	Ribosomal activity	Polysomal content
	cpm/mg RNA	OD units
...	288	0.01
15 min	6680	0.10
30 min	23,200	1.61
1.5 hr	31,900	2.42
6 hr	50,300	3.66

min) with a slower sustained increase to 6 hours. The time course of polysome formation correlates well with the increase in ribosomal activity with the exception of the sample imbibed for 15 minutes.

Discussion

The onset of protein synthesis in the germinating wheat embryo may be divided into 2 phases. The first phase, occurring directly upon imbibition of water, (0–0.5 hrs) is apparently controlled by an activation of the ribosome-messenger system or, more specifically, by the formation of functional polysomes. This phase of protein synthesis, while it may be observed qualitatively by in vivo assay, is best studied quantitatively by in vitro assay of amino acid incorporation with isolated ribosomes (fig 2). That the increased incorporating capacity with imbibition is not an artefact of desiccation follows from several lines of evidence. As shown in table I and figure 2, ribosomal activity may be temporally dissociated from water uptake. Thus, at room temperature there is a 10 minute lag between water uptake and ribosome activation, while at 4°, water is taken up with little or no development of ribosomal activity.

In other experiments (not shown) we desiccated embryos to their original weight after allowing a 0.5 hour imbibition. Ribosomes prepared from these embryos had essentially the same activity as those from nondesiccated controls, again indicating that desiccation per se does not affect the functional capacity of subsequently isolated ribosomes. Lastly, and perhaps of most significance, we have now succeeded in obtaining an in vitro system (not requiring an external messenger) catalyzing the activation of ribosomes of unimbibed embryos.

The second phase of increased capacity for protein synthesis (0.5–6 hrs of imbibition) is superficially similar to phase 1. Thus it is accompanied by an increase in the in vitro activity of isolated ribosomes and a simultaneous increase in polysome content (table IV). In addition, phase 2 may be followed, at least semi-quantitatively, by in vivo assay (table III). Our inclination to consider this phase separately from phase 1 derives from the relative rates of increased capacity in the two time periods. Thus, the slower rate of increase in phase 2 suggests that synthesis of messenger-RNA or some analogous synthetic process on the ribosomal level may be restricting.

Embryos from aged seed apparently contain a control over amino acid incorporation that is operative at a level other than ribosomal. This follows from the fact that the relative increase in the in vivo incorporation rate (table III) is far greater than the increase in ribosomal activity or polysomal content (table IV). The water-uptake curve (fig 1) is identical for embryos of both aged and fresh seed. In considering the nature of the rate-limiting component in the aged embryo, there are a host of potential candidates. Thus, the trivial possibilities of a relatively larger pool of endogenous unlabeled amino acid (effectively lowering the specific activity of the added ^{14}C amino acid), as well as a deficiency of any 1 amino acid (without which protein synthesis could not occur) must be considered. Alternative possibilities include delayed energy production, a limiting synthesis of any aminoacyl-*s*-RNA, or another, as yet uncharacterized, intermediate phase (e.g. 2). Experimentally, we have examined the free amino acid content of embryos from aged seed at 0.5 and 6 hours of imbibition and have found no differences supporting the trivial possibilities suggested above. Respiration of intact embryos increased only slightly with time during a 6 hour imbibition period, suggesting that energy production (presumably coupled to respiration) is not limiting. Preliminary examination of radioactivity incorporated into a fraction, insoluble in cold-trichloroacetic acid but extractable into hot-trichloroacetic acid (presumably aminoacyl-*s*-RNA), indicated no rate-limiting differences during the 0.5 to 6 hour period of imbibition. Such analyses have been performed with embryos from aged seed in incorporation experiments with ^{14}C-labeled leucine, arginine, and threonine, all of which showed qualitatively the same kinetic pattern of incorporation into protein. Thus, to date we have no inkling of the nature of this second limiting component. Our interest in this component lies in its possible relevance to the many limited-type controls evinced by plant systems (e.g. dormancy).

With preparations from embryos imbibed for 12 hours, approximately 45 % of the ribosomal absorbance (after deoxycholate treatment, fig 3B) is in the polysomal region. If one assumes that the radioactivity in the monosome region is due to polysomes cleaved during isolation, the polysome content rises to 69 %. Such a high proportion of polysomes is suggestive of a relatively long-lived messenger (10). In contrast to the situation in liver (3) the polysome to monosome conversion (both absorbance and radioactivity) requires only ribonuclease (fig 3 and unpublished observations). Further addition of deoxycholate shifts the remaining absorbance and radioactivity exclusively to the low molecular-weight region. These observations suggest that, in germinating wheat embryos as in the reticulocyte (9), polysomes are functional in a form not bound to a lipoprotein membrane. Setterfield (7) reached an essentially similar conclusion based on electron micrograph studies.

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