ACTIVATION OF PROTEIN SYNTHESIS IN THE IMBIBITION PHASE OF SEED GERMINATION

By Abraham Marcus and John Feeley

FIELD CROPS AND ANIMAL PRODUCTS BRANCH, MARKET QUALITY RESEARCH DIVISION, AGRICULTURAL MARKETING SERVICE, UNITED STATES DEPARTMENT OF AGRICULTURE, BELTSVILLE, MARYLAND

Communicated by Sterling B. Hendricks, April 7, 1964

Initiation of germination in many seeds requires only contact with water at the appropriate temperature. The nature of the metabolic phenomena occurring during water imbibition is an intriguing question. Recognizing that the ability to make new protein is certainly an early requirement during germination, we have undertaken a study of the protein synthesis system in dry and imbibed seeds. While germination manifests itself visibly only in the growth of the embryo, nevertheless studies[1, 2] have indicated that the supporting tissue (cotyledon or endosperm) also develops new enzymatic activities with germination. Thus, concern for the activation of the protein synthesis system would apply similarly to this supporting tissue.

The studies to be described indicate that the entire apparatus necessary for protein synthesis is functional in the cotyledon of the ungerminated peanut and that messenger RNA is limiting. Imbibition corrects this inadequacy. Studies with peanut and wheat embryos suggest that the formation or activation of messenger RNA during imbibition may be a general phenomenon in seed germination.

Materials and Methods.—The medium for preparation of microsomes was 0.5 *M* sucrose, 0.01 *M* $MgCl_2$, 0.05 *M* tris buffer pH 7.9, 0.025 *M* KCl, and 0.005 *M* 2-mercaptoethanol. With cotyledons from peanuts germinated 1–4 days,[3] 2 ml of medium were added per seed. With cotyledons from dry seed, an additional 0.2 ml of water was added per seed. The cotyledons were blended twice for 15 sec; the suspension was filtered through cheesecloth and centrifuged for 20 min at 22,000 × *g*. The supernatant was removed carefully (to keep out most of the upper lipid layer) and centrifuged in a Spinco ultracentrifuge for 60 min at 105,000 × *g*. Supernatant was removed from the upper third of the tube (105,000 × *g* supernatant), and the remaining supernatant was discarded. The pellet was suspended in a solution containing 0.01 *M* tris pH 7.9, 0.004 *M* $MgCl_2$, and 0.005 *M* mercaptoethanol, and centrifuged for 10 min at 23,500 × *g*. The opalescent supernatant was used as the "microsome" preparation. In some experiments the 105,000 × *g* pellets were resuspended in blending medium and recentrifuged. These preparations are referred to as "washed microsomes." In addition, "105,000 × *g* supernatant" was dialyzed for 4–5 hr against 60 vol of 0.01 *M* tris pH 7.9, 0.01 *M* KCl, 0.01 *M* $MgCl_2$, 0.005 *M* mercaptoethanol, and recentrifuged for 2 hr at 105,000 × *g*. The top third of these supernatants were pooled and stored as "dialyzed supernatant."

Peanut axes (attached to the cotyledon) were allowed to imbibe for 20 hr at 25° in the usual manner.[3] Wheat embryos imbibed for 16 hr at 20° on paper towel moistened with a solution of streptomycin sulfate (100 γ ml). In some experiments the wheat embryos were germinated on sucrose-agar containing antibiotics.[4] Both embryonic materials were ground by hand with sand in a mortar. In the peanut experiment (Table 5, expt. 1) 100 dry axes and 71 one-day axes were ground with 15 ml of sucrose medium. In the wheat experiment, 2 gm of viable wheat embryos, prepared according to Johnston and Stern,[4] were ground with 14 ml of sucrose medium.

The RNA content of all microsome preparations was determined from the optical density at 260 mμ, assuming E = 20 for 0.1% solution of RNA. Protein content was determined according to Lowry *et al.*[5] In general, the microsome preparations contained approximately two parts protein to one part RNA. The spectra were those expected for ribonucleoproteins, except for the preparations from peanut cotyledon. The latter absorbed more in the 230–250 mμ region, apparently due to occluded lipid.

The incubations for measurement of C^{14} incorporation into protein are detailed in the legends to the tables. After incubation, the vessels were immersed in ice; 0.6 ml of a 0.1 *M* solution of the appropriate amino acid was added, followed by 0.4 ml 30% trichloroacetic acid (TCA) and 0.3 ml 2% casein. The pellets obtained by centrifugation were washed twice with 4 ml cold 5% TCA and then extracted with 4 ml 5% TCA for 15 min at 90°. The radioactivity extracted in this fraction is designated as RNA. The pellets were then washed with 4 ml of ethanol-ether (1:1) for 30 min at 30° and with 4 ml of ether. After drying, the residue was dissolved in formic acid, and aliquots were plated. This radioactivity is designated as protein. In the experiments in which nonradioactive amino acids were added, the following L-amino acids were included: tyrosine, phenylalanine, aspartic, glutamic, leucine, isoleucine, alanine, arginine, lysine, cysteine, histidine, proline, threonine, valine, glycine, serine, tryptophan, asparagine, glutamine, methionine. The one amino acid added as the C^{14}-labeled compound was omitted from the mixture of cold amino acids. When only amino acid binding to RNA was assayed, the conditions were as described in Table 2. After incubation, 0.35 ml of a 0.1 *M* solution of the appropriate amino acid, 0.25 ml of 30% TCA, and 0.3 ml of 2% casein were added. The pellets were washed twice with cold 5% TCA, once with ethanol-ether (1:1), and dissolved in 0.1 *N* ammonium acetate pH 9. These assays are modifications of the procedures of Siekevitz[6] and Holley *et al.*[7]

Peanut sRNA was prepared from cotyledons defatted at various stages of germination. The procedures used were similar to those of Holley *et al.*[7] and will be described in detail elsewhere. Yeast sRNA was obtained from General Biochemicals, Inc. Polyuridylic acid (poly U) was obtained from Calbiochem, Inc.

Results.—The most obvious approach to ascertaining the capacity of a particular system in protein synthesis is to examine the classical microsomal-supernatant system.[8] As shown in Table 1, unwashed microsomal preparations (containing adhered supernatant) from cotyledons of imbibed peanuts (1-day) incorporate C^{14}-leucine into protein. In contrast, similar preparations from ungerminated seeds (0-day) are essentially inactive. Supplementing the microsome, either with 0- or 1-day supernatant, augments amino acid incorporation with 1-day ribosomes, while 0-day ribosomes are unaffected, suggesting that the defect with regard to protein synthesis is in the microsome.

Further evidence for the efficiency of 0-day supernatant was obtained by separate assays of the supernatant components. In Table 2, experiment 1 compares the ability of supernatant preparations from 0- and 4-day cotyledons to catalyze incorporation of leucine-C^{14} into sRNA, i.e., amino acid activation. Experiment 2 compares the capacity of sRNA from 0- and 4-day cotyledons to bind C^{14}-leucine. The data indicate little difference in these two supernatant components (amino acid activating enzyme and sRNA) whether prepared from 0- or 4-day cotyledons. Evidence for a third component (necessary for protein synthesis) in 0-day supernatant is presented in Table 3. The logic for this conclusion is as follows: addition

TABLE 1

PROTEIN SYNTHESIS IN THE MICROSOMAL SYSTEM OF IMBIBED PEANUT COTYLEDONS

Microsomes (mg RNA)	Supernatant (mg protein)	RNA (cpm)	Protein (cpm)
0 day—0.36	—	1676	15
0 day—0.36	0 day—1.6	852	12
0 day—0.36	1 day—1.0	256	0
1 day—0.40	—	184	167
1 day—0.40	1 day—1.0	132	214
1 day—0.40	0 day—1.6	200	276

The complete system included 50 μmoles tris buffer pH 7.9, 5 μmoles $MgCl_2$, 50 μmoles KCl, 20 μmoles 2-mercaptoethanol, 1 μmole ATP, 10 μmoles creatine phosphate, 50 μg creatine phosphate kinase, 120 μg of 0-day peanut sRNA, 2.2 mμmoles of leucine-C^{14} (87,500 cpm) and other additions to 1.0 ml, and was incubated for 40 min at 30°. The supernatant was obtained directly from the 105,000 × *g* centrifugation. The data are corrected for controls (TCA at 0 time) showing 52 cpm in the RNA and 33 cmp in the protein.

TABLE 2

AMINO ACID ACTIVATION IN DRY AND IMBIBED PEANUT COTYLEDONS

Experiment no.	Supernatant (mg protein)	sRNA (mg)	Incorporation (cpm)
1	—	4 day—0.4	10
...	0 day—0.10	"	220
...	" 0.21	"	685
...	" 0.42	"	1204
...	4 day—0.12	"	208
...	" 0.25	"	475
...	" 0.50	"	832
2	1 day—0.66	—	21
...	"	0 day—0.19	271
...	"	" —0.38	504
...	"	4 day—0.20	292
...	"	" —0.40	549
...	"	Yeast—0.40	183

The complete system contained 50 μmoles tris buffer pH 7.9, 5 μmoles $MgCl_2$, 30 μmoles KCl, 3 μmoles ATP, 1.25 μmoles of mercaptoethanol, 0.4 mμmoles leucine-C^{14} (15,500 cpm) in a vol of 0.59 ml and was incubated for 20 min at 30°. The supernatants were used directly from the 105,000 × *g* centrifugation. In expt. 1, both supernatants were freshly prepared while in expt. 2, the supernatant was stored for 24 hr at −10°.

TABLE 3

STIMULATION OF C^{14}-LEUCINE INCORPORATION INTO PROTEIN BY 0-DAY SUPERNATANT

Microsomes (mg RNA)	Supernatant (mg protein)	sRNA (mg)	RNA (cpm)	Protein (cpm)
2 day—0.42	—	—	112	205
" —0.42	—	4 day—0.07	212	300
" —0.42	0 day—0.8	—	152	714
" —0.42	" —0.8	4 day—0.07	1356	1030

The incubation was identical to that of Table 1 except that the microsomes were washed.

of supernatant, in the absence of exogenous sRNA (line 3), results in a 3.5-fold increase in the incorporation into protein, with only a small increase in the incorporation into RNA. If the increased protein incorporation were due solely to the presence of additional amino acid activating enzyme, one would expect to find a greater increase in the RNA count. Furthermore, increasing the count in the RNA by adding sRNA in the absence of supernatant (line 2) has only a small effect in increasing the incorporation into protein. These data suggest that 0-day supernatant contains another component, necessary for protein synthesis, in addition to amino acid activating enzyme and sRNA—presumably the enzyme catalyzing amino acid transfer from amino acyl-sRNA to the microsome.[9]

The above observations clearly implicated the microsome as the limiting component in the 0-day protein synthesizing system. Microsomes were then prepared from 0-day and 1–4 day cotyledons and examined by sucrose density gradient centrifugation.[10] One peak was observed at the same position for all preparations. Microsomal RNA (prepared from isolated microsomes) showed the usual two-peak system[11] on sucrose density gradient centrifugation, with no differences between RNA from cotyledons of germinated and ungerminated seed. It thus appeared that the deficiency in the 0-day microsome was due either to a missing microsomal component or to the presence of an inhibitor of protein synthesis. Experiments involving mixed 0-day and 1-day microsomes gave incorporation equal to the sum of their respective activities, indicating that the 0-day microsomes do not have an easily dissociable inhibitor. Attempts were then made to augment protein synthesis by adding 1-4 day microsomal RNA, as well as various types of partially

damaged 1–4 day microsomes, to the 0-day microsomes. Consistent stimulation was not obtained.

The possibility that messenger RNA[11] was the missing component in the 0-day microsome was examined more critically in studies involving polyuridylic acid and the incorporation of phenylalanine C^{14}.[12] Such a system has been used by Nemer[13] to demonstrate ribosomal activation during fertilization of the sea urchin egg. As may be seen in Table 4, again with phenylalanine-C^{14} as with leucine-C^{14} previously, there is little incorporation with unsupplemented 0-day microsomes, in contrast to similar preparations from germinated cotyledons (expt. 1). However, when poly U was added to the system (expt. 2), 0-day microsomes incorporated phenylalanine-C^{14} to the same extent as microsomes prepared from germinated (1–4) day cotyledons. Similar observations were made in experiments with peanut axes (germinated while attached to the cotyledon and subsequently dissected) and with wheat embryo (separated from the endosperm prior to imbibition). As may be seen in Table 5, both of these embryonic systems require poly U for the incorporation of C^{14}-phenylalanine in the unimbibed condition (0-day). After imbibition (1-day), considerable incorporation is obtained in the absence of poly U.

In tests with commercial wheat embryo under similar conditions, the dry embryo was found to provide an excellent poly U-requiring, C^{14}-phenylalanine incorporating system. However, after 16 hr at 20°, the microsome system still showed a requirement for poly U, in contrast to the system prepared from viable wheat embryo (discussed above).

Discussion.—The data presented (Tables 4 and 5) suggest that a major aspect

TABLE 4

PHENYLALANINE INCORPORATION IN THE PRESENCE AND ABSENCE OF POLY U WITH PEANUT COTYLEDON PREPARATIONS

Experiment no.	Microsomes (mg RNA)	Protein Incorporation (cpm) 0-day	1-day	4-day
(1) No poly U				
Unwashed microsomes	0.4	3	57	78
	0.8	6	90	109
Washed microsomes	0.2	2	75	84
	0.4	1	125	—
(2) Poly U added				
Unwashed microsomes	0.05	576	478	—
	0.10	876	523	543

The complete system was identical to that of Table 1 except that 0.5 μmoles GTP, 200 μg yeast sRNA (in place of peanut sRNA), and 2.1 mμmoles phenylalanine (101,000 cpm) (in place of leucine-C^{14}) were added. In the incubations with poly U, 100 μg were added, while in all other incubations 125 mμmoles of each of the amino acids (except phenylalanine) were included. In all experiments, 0.27 mg of 0-day "dialyzed supernatant" was added. The data given are corrected for incorporation in the absence of ribosomes (24 and 19 cpm with and without poly U, respectively).

TABLE 5

C^{14}-PHENYLALANINE INCORPORATION WITH EMBRYO PREPARATIONS FROM PEANUT AND WHEAT

Experiment no.	Microsomes (mg RNA)	Protein Incorporation (cpm) 0-day	1-day
(1) Peanut axis —no poly U	0.17	0	54
	0.60	0	155
" —with poly U	0.05	699	175
(2) Wheat embryo—no poly U	0.17	2	120
	0.85	9	881
" —with poly U	0.02	240	408
	0.16	3681	3522

The complete system was identical to that of Table 4 except that 62.5 mμmoles of the 19 amino acids (except phenylalanine) were added in the nonpoly U tubes.

of the passage from the quiescent to the active stage in seed germinaton is the formation or activation of messenger RNA. The fact that nonviable wheat embryos do not show this response during imbibition lends further support to the physiological nature of this process. Presumably, other conditions which prevent germination might be examined for their effect on this process.

The mechanism of the formation of an "active" messenger RNA has not been considered in this study. Clearly, several possibilities exist to explain the situation in the ungerminated seed. These include spatial separation of messenger RNA from the microsomes, lack of one of the components necessary for its synthesis, or presence of an inhibitor. Studies are currently in progress to obtain further information on these points.

The earlier studies of Nemer,[13] involving the fertilization of sea urchin eggs, coupled with the present data for both embryo, which undergoes cell division, and cotyledon, which does not, suggest that the formation of an "active" messenger RNA may be a broad physiological control mechanism. Thus, the quiescent state would be characterized by a fully active protein synthesizing system, needing only the "messenger RNA" to trigger its function. Such a hypothesis should at least suggest experimental approaches to the study of other control phenomena manifested by living systems.

Summary.—Preparations from the cotyledon of the unimbibed peanut seed show a low level of amino acid incorporation into protein. Similar preparations after imbibition are greatly increased in activity. Evidence presented suggests that the soluble enzymes required for protein synthesis are active prior to imbibition but that microsomal activity requires imbibition. Experiments with polyuridylic acid indicate that during imbibition an active messenger RNA is formed. Additional studies with peanut and wheat embryos suggest that the activation or formation of messenger RNA during imbibition may be a general phenomenon in seed germination.

[1] Toole, E. H., S. B. Hendricks, H. A. Borthwick, and V. K. Toole, *Ann. Rev. Plant Physiol.*, **7**, 299 (1956).

[2] Koller, D., A. M. Mayer, A. Poljakoff-Mayber, and S. Klein, *Ann. Rev. Plant Physiol.*, **13**, 437 (1962).

[3] Marcus, A., and J. Velasco, *J. Biol. Chem.*, **235**, 563 (1960).

[4] Johnston, F. B., and H. Stern, *Nature*, **179**, 160 (1957).

[5] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

[6] Siekevitz, P., *J. Biol. Chem.*, **195**, 549 (1952).

[7] Holley, R. W., J. Apgar, B. P. Doctor, J. Farrow, M. A. Marini, and S. H. Merrill, *J. Biol. Chem.*, **236**, 200 (1961).

[8] Hoagland, M. B., M. L. Stephenson, J. F. Scott, L. I. Hecht, and P. C. Zamecnik, *J. Biol. Chem.*, **231**, 241 (1958).

[9] Fessenden, J. M., and K. Moldave, *J. Biol. Chem.*, **238**, 1479 (1963).

[10] Britten, R. J., and R. B. Roberts, *Science*, **131**, 32 (1960).

[11] Gros, F., H. Hiatt, W. Gilbert, C. G. Kurland, R. W. Rosebrough, and J. D. Watson, *Nature* **190**, 581 (1961).

[12] Nirenberg, M. W., and J. H. Matthaei, these PROCEEDINGS, **47**, 1588 (1961).

[13] Nemer, M., *Biochem. Biophys. Res. Commun.*, **8**, 511 (1962).