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THE SEQUENCE OF INITIATION OF RNA, DNA AND PROTEIN SYNTHESIS IN THE WHEAT GRAINS DURING GERMINATION

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Abstract—The syntheses of main macromolecular substances, in a whole wheat grain allowed to germinate, are triggered in the following order: RNA, protein, DNA. The RNA synthesis, as judged by [2-^{14}C]uridine incorporation, is initiated almost immediately after the seeds are exposed to the optimal germination conditions, whereas [1-^{14}C]leucine and [2-^{14}C]thymidine incorporation begins to occur only 3 and 4 hr later, respectively. The initiation of protein synthesis is accompanied by an apparent cessation of uridine incorporation.

INTRODUCTION

THE WAY in which a mature seed, exposed to suitable germination conditions, passes from a latent to a highly active metabolic state is a topic of increasing interest. The sequence of biosynthetic events leading to embryo differentation and radicle emergence, however, still remains obscure. From the available data it is difficult to conclude how early and in which order the main macromolecular syntheses are triggered in a whole seed when appropriate changes in the environment allow it to germinate. Walton[1] found that both RNA and protein synthesis are prerequisities for the initiation of bean axis elongation. In full agreement with these findings, Marcus *et al.*[2] have established that isolated wheat embryo acquires capacity for rapid protein synthesis within the first 30 min of imbibition. With regard to RNA synthesis, however, most investigators[3–7] have found it was not essential for the immediate process of germination. Indeed, RNA synthesis has been observed only in seeds germinated for at least several hours;[4,7–12] but it should be pointed out that no attempts have so far been made to define precisely the germination time at which its biosynthesis is triggered.

The purpose of the present work is to establish and to compare the germination time necessary for the initiation of RNA, DNA and protein synthesis in wheat grains. We consider that a knowledge of the sequence of these biosynthetic events is a necessary first step toward a better understanding of the obviously complex processes leading to active development of the embryo under natural environmental conditions.

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RESULTS

Methods for the Introduction of Precursors and Triggering of Germination

To detect biosynthetic events as soon as they begin in response to the induction of the germination process, two important requirements obviously should be fulfilled. First, the germination conditions used should be kept as close as possible to those of the natural environment. And second, labelled precursors should be introduced into the seed before germination begins. For these reasons we preferred to use whole seed rather than isolated

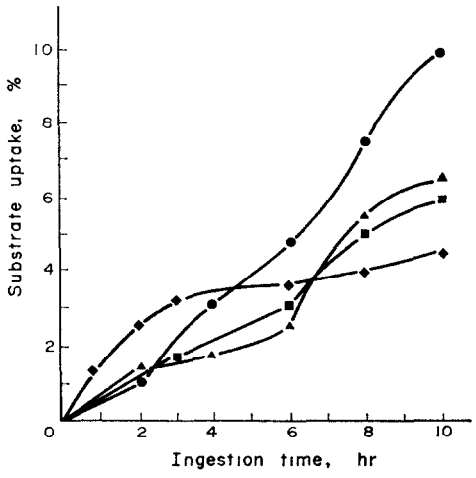

FIG. 1. RATES OF UPTAKE OF URIDINE, THYMIDINE, LEUCINE AND WATER BY WHEAT GRAINS.

The standard samples of 350 wheat grains were soaked in 10 ml solutions of either [2-^{14}C]uridine, or [2-^{14}C]thymidine, or [1-^{14}C]leucine and left at 2° for the indicated time. Total radioactivity of the substrates was 5×10^6 dis/min in each case; uptake is expressed as a percentage of this value. ▲——▲, Uridine; ■——■, thymidine; ●——●, leucine; ◆——◆, water.

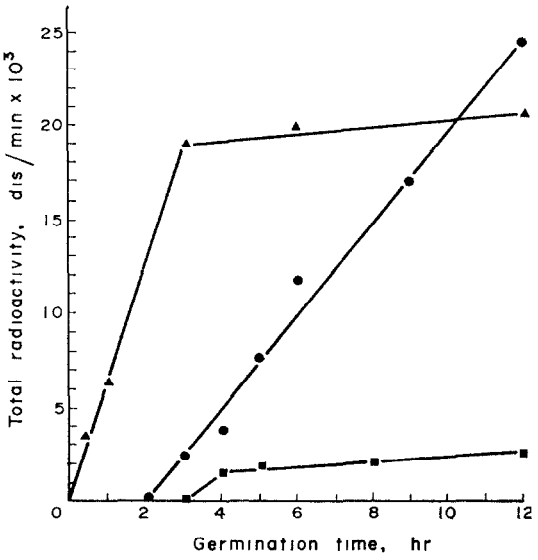

FIG. 2. RATES OF INCORPORATION OF LABELLED PRECURSORS INTO RNA, DNA AND PROTEIN BY GERMINATING WHEAT GRAINS.

[2-^{14}C]Uridine, [2-^{14}C]thymidine and [1-^{14}C]leucine were used as the RNA, DNA and protein precursors, respectively. The grains were fed for 8 hr at 2° (see Fig. 1), and then allowed to germinate at 22°. ▲——▲, RNA; ■——■, DNA; ●——●, protein

embryos, aleurone layers or subcellular fractions. Introduction of a substrate solution into seeds, without damage, can be achieved only through the natural input of imbibed water. Unfortunately however, water imbibition triggers germination, which makes efficient substrate uptake before the beginning of germination impossible. To overcome this difficulty, we have taken advantage of the fact that, under natural conditions, seed germination may be also influenced by changes in soil temperature. Thus, to introduce a labelled precursor, seeds may be soaked in the appropriate solution at a subminimal germination temperature. After ingestion, which is allowed to proceed long enough for sufficient substrate to be taken up, germination may then be triggered by exposure of the seeds to optimal temperature.

Experiments have shown that the precursors of RNA, DNA and protein are readily taken up by wheat grains under the conditions used. Figure 1 illustrates the rates of uridine, thymidine and leucine uptake at 2°. An 8-hr ingestion period was found adequate to introduce any precursor tested in a quantity large enough to follow its further conversions under

the experimental conditions examined. The substrate uptake did not fully parallel water input. The reasons for this observation were outside our interest. The substrates taken up by a wheat grain sample could be quantitatively reisolated in essentially unchanged form at the end of the ingestion period. None of them were incorporated into the acid-insoluble fraction under these conditions (see Tables 1 and 2).

Thus, in all experiments, germination was preceded by an 8-hr ingestion at 2° and zero time of germination in our system corresponds to the moment at which the samples were transferred to 22°. No inhibitory effect of such a pretreatment on subsequent germination was observed.

Many reports have underlined the caution that must be applied in assessing the results of incorporation experiments carried out under non-sterile conditions. In particular, Lonberg-Holm[13] has demonstrated that even slight contamination with bacteria can alter the labelling patterns of macromolecules of lettuce seedlings. These studies showed the obvious need to test for the possible contributions of microorganisms to the results of any investigation of this type. Preliminary series of experiments were therefore made under both sterile and non-sterile conditions. As it is commonly recommended, for the purpose of aseptic germination, the wheat grain samples were dipped in 2% sodium hypochlorite for 2 min, rinsed with sterile water and then given the ingestion and germination treatments, using autoclaved solutions and sterile vessels throughout. In experiments with labelled uridine, thymidine and leucine, radioactivities of the total RNA, DNA and protein, as well as the acid-soluble fractions, after 0, 3 and 12 hr germination were essentially the same for the grain samples grown under sterile and non-sterile conditions. Probably, the undesirable effects of microbial contaminations were appreciably reduced by the low temperature during the ingestion period, and limited hereafter mainly to endosymbionts, which are not eliminated by the generally accepted sterilization methods. In view of these results, the aseptic precautions were omitted from the standard experimental procedure.

Initiation of RNA Synthesis

When wheat grains, administered a radioactive RNA precursor for 8 hr at 2°, are exposed to the suitable germination temperature a radioactive, acid-insoluble, alkali-labile

TABLE 1. INITIATION OF INCORPORATION OF LABELLED PRECURSORS INTO RNA BY WHEAT GRAINS DURING GERMINATION

Precursor	Uptake* (dis/min × 10^3)	Total activity of RNA (dis/min) at germination time† (Time min) 15	30	60
[6-^{14}C]Orotate	225	900	2950	5125
[2-^{14}C]Uracil	255	1625	2400	4200
[2-^{14}C]Uridine	280	1100	3300	5550
[2-^{14}C]5′-UMP	270	1225	3050	5450

* For the precursor uptake the standard wheat grain sample was soaked in the appropriate substrate solution for 8 hr at 2°. Then, the sample was allowed to germinate at 22° for the indicated time. Experimental details are given in the text.

† < 50 dis/min in each case at 0 and 5 min.

[13] K. K. LONBERG-HOLM, *Nature, Lond.* **213**, 454 (1967).

product is formed almost immediately. Radioactivity in the total RNA fraction can be detected after a 15 min germination period and rises quickly thereafter (Table 1). All the precursor tested, [6-^{14}C]orotate, [2-^{14}C]uracil, [2-^{14}C]uridine and [2-^{14}C]5′-UMP, were incorporated into the total RNA fraction with similar efficiency. Independently of the precursor used, two radioactive products, 2′(3′)-UMP and uridine, were identified in the alkaline hydrolysate of the acid-insoluble fraction.

TABLE 2. INITIATION OF INCORPORATION OF LABELLED PRECURSORS INTO DNA AND PROTEIN BY WHEAT GRAINS DURING GERMINATION

Germination time (hr)	Total activity (dis/min) of	
	DNA	Protein
3	< 50	1920
4	1650	3850
5	1850	7770

[2-^{14}C]Thymidine and [1-^{14}C]leucine were used as the DNA and protein precursors, respectively. Ingestion, carried out under the standard conditions, resulted in the uptake of 250×10^3 dis/min of the former and 375×10^3 dis/min of the latter precursor. < 50 dis/min total activity was detected in each case at 0, 1 and 2 hr.

Initiation of DNA and Protein Synthesis

The technique used in experiments on the initiation of RNA synthesis was followed to establish how early the germinating wheat grain begins to synthesize DNA and protein. Typical precursors of these macromolecules, [2-^{14}C]thymidine and [1-^{14}C]leucine, respectively, were used. Unlike the RNA precursors, thymidine incorporation was detected only after a 4 hr germination period (Table 2). Somewhat earlier, but still distinctly later than RNA synthesis, the initiation of protein synthesis was found to occur.

Thus, the results obtained (Tables 1 and 2) indicate that during germination macromolecular syntheses are triggered in the following order: RNA, protein, DNA. To examine further the relationships between RNA, DNA and protein synthesis, the kinetics of uridine, thymidine and leucine incorporation during the germination period extended to the time of radicle emergence were studied.

Comparison of Kinetics of Uridine, Thymidine and Leucine Incorporation

Rates of incorporation of labelled uridine, thymidine and leucine into the corresponding acid-insoluble fractions by wheat grains during the first 12 hr of germination are shown in Fig. 2. Immediately after the onset of the incubation, radioactivity of the total RNA fraction rises linearly and, after reaching a high level at the end of the third hour, remains at a nearly constant level. A quite different pattern was found for leucine incorporation. Radioactivity of the protein fraction, after a 3-hr lag phase, rises linearly with the germination time over all the period investigated. Thymidine incorporation, although less extensive, follows a pattern similar, in general, to that found for leucine.

It should not be overlooked that the cessation, or apparent cessation, of [^{14}C]uridine incorporation coincides exactly with the time at which the protein synthesis is initiated. It seemed possible *a priori*, that the observed decrease in the rate of uridine incorporation might be due to the exhaustion of the radioactive precursor. Further experiments showed, however, that approximately 72 per cent of [^{14}C]uridine taken up by wheat grains during the ingestion period still remained unchanged at the end of the third hour of germination. We were also unable to demonstrate any significant drop in the total RNA (amounting at zero germination time to 28 mg per standard 350 grain sample) during the period investigated.

DISCUSSION

An approach was found to establish the sequence of main biosynthetic events triggered *in vivo*, in whole wheat grains, in the course of the germination process. With this technique, outlined above, it was possible to observe that the first of the biosynthetic events to occur in wheat grains exposed to the optimal germination conditions, is the synthesis of RNA. It probably starts immediately and becomes measurable after a 15-min germination. Considerably later, at the end of the third hour of germination, protein synthesis is triggered. And finally, after 4 hr of germination, the initiation of DNA synthesis may be observed. All these syntheses start long before radicle emergence.

In attempts to compare our results with the previously published data, attention should be paid to differences in the experimental systems. In our experiments, germination was preceded by ingestion. Thus, the seeds referred to as being at zero germination time differ from dry ones. Although no germination was possible at 2°, some processes of physiological importance obviously occurred during the ingestion period at this temperature. These may include hydration, exchange of hormones between different parts of the grain, leaching away of germination inhibitors and events related to vernalisation. All these processes make the pretreated grains more ready to germinate than those which are dry. Germination then triggered by elevation of temperature may be more immediate and more synchronous than that resulting from imbibition of water by dry seeds at a constant temperature. We nevertheless believe that despite differences in experimental approach, an attempt to compare conclusions should be made. In this respect some similarities and some differences between our results and those reported by others may be pointed out. Thus, Barker and Rieber[5] concluded that enzymes necessary for RNA synthesis are present in dormant pea seeds, but they appeared not to be operative at the early stages of germination. In full agreement with the first part of this conclusion we have observed the immediate initiation of RNA synthesis in wheat grains allowed to germinate. The same observation, however, indicates further that these enzymes are not only present but also operative from the onset of the germination process. Contrary to our observations, Chen *et al.*[6] have found no RNA synthesis during the first 24 hr of wheat embryo germination. Probably, the absence of endosperm, and especially of aleurone layers[9,14,15] leads to considerable restrictions of biosynthetic capacity of the isolated embryo as compared with the whole, undamaged grain. It is also possible to explain this contradiction as resulting from the different nature of the precursors used, [^{32}P]orthophosphate in the studies of Chen *et al.*, and [^{14}C]pyrimidine derivatives in our experiments. If, for example, [^{14}C]uridine was phosphorylated at expense of ATP present in dormant seeds, then it might be possible to observe uridine but

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not orthophosphate incorporation. Such an explanation is open to some criticism, however, as it is well known that respiratory processes, converting orthophosphate to ATP, are very active in germinating seeds.

As concerns protein synthesis, we were unable to detect it at as early a stage of germination as was reported by Marcus *et al.*[2] The lag period, observed in our experiments, did not result from the cold pretreatment which, as was demonstrated by Marcus *et al.*, is not injurious for subsequent development of protein-synthesizing capacity by the imbibing wheat embryo. In this respect our results are closer to the data of Abdul-Baki[16] who observed rapid incorporation of [1-^{14}C]leucine after 5 hr of barley grain germination. DNA synthesis, as observed under our experimental conditions, is initiated at a somewhat earlier germination stage than that indicated by earlier reports.[8,17,18]

The natural synchronization of developmental events, ordered with respect to breaking of dormancy, makes germinating seed a particularly attractive system for studying the interrelationships between DNA, RNA and protein biosynthesis during the initiation of active differentiation processes. Our observations clearly indicate that protein synthesis is triggered only at a time when RNA radioactivity reaches a high level and stops to rise further (Fig. 2). The apparent cessation of [^{14}C]uridine incorporation after the first 3 hr of germination may neither be due to the radioactive precursor exhaustion nor to the decrease in the total RNA amount. Both possibilities are excluded by the experimental data. Instead, it appears likely that simultaneously with the initiation of protein synthesis a breakdown of the newly made RNA begins to occur. Such an explanation may be plausible in view of a possible messenger RNA degradation in the course of protein biosynthesis.[19,20] Our assumption is also consistent with the report of Jachymczyk and Cherry[21] on germination-dependent messenger RNA appearance in peanut seeds, although does not deny evidence[3-6] for the presence of long-lived messenger RNA in dormant seeds. It may be postulated that both stored and newly made messengers are essential for the wheat germ differentiation, similarly as was shown for the early embryonic development of sea urchin.[22]

Finally, the fact that [^{14}C]thymidine incorporation starts soon after both protein synthesis and probable RNA degradation begin to occur raises the question whether these two processes are prerequisities for the triggering of DNA replication in germinating seeds. Such a dependence would have implications for RNA-dependent DNA synthesis, demonstrated recently for other systems.[23-25]

EXPERIMENTAL

Plant material. The grains of winter wheat (*Triticum aestivum*, ssp. vulgare, var. lutescens Al., cv. Kutnowska) were used throughout. Main characteristics of the grain: red; medium size; weight of 1000 grains, 47·1 g; moisture content, 11 8; germination 96%.

Radioactive presursors. [6-^{14}C]orotate, [2-^{14}C]uracil, [2-^{14}C]uridine, [2-^{14}C]5′-UMP, [2-^{14}C]thymidine and [1-^{14}C]leucine were of commercial origin. The radioactive substrates were individually dissolved in

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H_2O and adjusted with their non-labelled counterparts to make 0 05 mM solutions, containing 5×10^5 dis/min/ml, in each case.

Ingestion. 350 wheat grains were placed horizontally, embryo down in Petri dishes. The sample was poured with 10 ml of one of the prepared substrate solutions (as specified for each experiment) and left at 2° for 8 hr. Then, the grains were removed from the substrate solution and washed with cold tap H_2O to remove the externally adhering substrate. The remaining substrate solution and the washings were combined and used for total radioactivity determination. The difference between initial and remained radioactivity is referred to as a quantity of the precursor taken up by the grain sample during the ingestion period.

Germination. To trigger germination, the grain sample, administered labelled precursor, was transferred from 2° to 22°. The individual samples were kept at this temperature on a filter paper moistened with tap water in a dark germinator for periods varying from 0 to 12 hr, as specified for each experiment. Germination was stopped by thoroughly grinding the grain sample in a mortar with 25 ml of 0·3 N $HClO_4$ at 4°.

Analytical methods. A slurry obtained on grinding of the grain sample was centrifuged at 9000 *g* for 10 min. The precipitate was washed 5 × cold 0·2 N $HClO_4$, containing non-labelled form of the radioactive precursor used in experiment. The washings and the original supernatant were combined and used for isolation of acid-soluble pyrimidine derivatives performed as described previously.[26] Depending on the kind of precursor used, either nucleic acids or protein were extracted from the acid-insoluble sediment. When incorporation of any of the labelled pyrimidine derivatives was studied total RNA and DNA fractions were extracted and separated according to the method of Smillie and Krotkov.[27] The sediment derived from grain samples administered [^{14}C]leucine, on the other hand, was extracted with a phenol–HOAc–H_2O mixture[28] to obtain a crude protein fraction. Total radioactivities of the RNA, DNA and protein fractions were then determined in Packard liquid scintillation counter. The RNA hydrolysate was used also for isolation of 2′(3′)-UMP and uridine performed in a manner similar to that described for acid-soluble pyrimidine derivatives. From the quantity of isolated 2′(3′)-UMP, total RNA amount was calculated, assuming 20% molar content of UMP for wheat RNA.[29]

All values reported are for the standard 350 wheat grain samples, weighing on average 16·5 g.

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