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Early Growth of Wheat Embryonic Axes and the Synthesis of RNA and DNA[1]

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ABSTRACT

The requirement for the synthesis of RNA and DNA in early germination of wheat (*Triticum aestivum* var Newana) embryonic axes has been studied by incubating embryos in the presence of appropriate inhibitors and monitoring both embryo growth and the rates of specific metabolic processes. Experiments with 5-fluorouridine showed that both rRNA and DNA synthesis could be curtailed by 60 to 70% without affecting embryo growth to 24 hours. Similarly, the presence of mitomycin C and methotrexate inhibited DNA synthesis 70%, with only a small effect on growth. Experiments with a range of concentrations of cordycepin and α-amanitin indicated that mRNA synthesis could be curtailed by 30 to 40% within the first 8 hours of germination with only a small effect on embryo growth. Thus, at least the initial phases of seed embryo germination are not closely linked to the synthesis of mRNA, rRNA, or DNA. Maximal sensitivity of embryo growth was obtained with cycloheximide and 2-(4-methyl-2,6-dinitroanilino)-*N*-methyl propionamide, supporting the idea that protein synthesis is the macromolecular process most closely linked to early germination.

Synthesis of proteins and RNA occurs at a substantial rate at the earliest stages of seed embryo germination (see Ref. 1 for a recent review). The extent to which these processes are required for early embryo growth is not certain. We have shown earlier that the rapid initial increase in protein synthesis that occurred during imbibition or shortly thereafter was independent of newly synthesized mRNA. The key experimental observation was that addition of either cordycepin or α-amanitin, at a level that strongly inhibited both transcription and polyadenylation, failed to prevent the increase in polyribosome content that occurred during imbibition (13). More recently, experiments have been reported in which extending the treatment with the inhibitors for several hours resulted in a substantial decrease in the rate of protein synthesis. These experiments were interpreted to indicate a strong requirement for newly synthesized RNA in early embryo growth (2, 3). In our laboratory, we have carried out similar extended inhibitor treatments with several modifications. We add the inhibitory reagents to the embryos after an initial imbibition, a procedure that allows a more discriminating inhibition. In addition, the analyses have been done over a range of inhibitor concentrations and have included a determination of embryo growth as the primary criterion of development. Our results show that early embryo growth can occur despite a partial inhibition of the synthesis of new mRNA. In addition, both rRNA and DNA synthesis can be strongly curtailed for at least 24 h with no effect on the rate of growth.

MATERIALS AND METHODS

Incubation of Axes and Measurement of Growth. Embryonic axes (120 mg) prepared from whole seed of wheat (*Triticum aestivum* var Newana, 1978 harvest), were incubated in Petri dishes (5.5 cm in diameter) with 1.6 ml of H_2O and 10 μl/ml chloramphenicol as described previously (11). Transfer of axes to a new solution was done by removing the top layer of filter paper with the axes, blotting from below for 20 s and placing it in a new dish with two layers of paper. To measure growth, *i.e.* the change in fresh weight, the top layer of paper with the axes was blotted from below for about 30 s, the axes were transferred to a tarred weighing paper, and their weight was determined.

In vivo rates of protein synthesis were determined as previously described (11), with the labeling incubations carried out for 20 min and with an additional 10% TCA precipitation of the protein from a suspension in 0.2 N NaOH (8). Analyses of RNA synthesis were done by incubating embryos with either 2-[^{3}H]adenine or 5,6-[^{3}H]uridine and isolating poly A(+)RNA and poly A(−)RNA (5). The amounts of embryos used, the specific activities of the precursors, and the conditions of incubation are given in the legends to the tables.

DNA synthesis was determined by labeling embryos for 70 min either in 2-[^{14}C]thymidine or 2-[^{3}H]adenine. At the end of the incubation period, the filter paper was transferred to a Büchner funnel and the embryos were rinsed with 50 ml of ice-cold water. The embryos were then homogenized first in 0.5 ml of 10% TCA, then in 3.0 ml of 5% TCA, and the suspension was centrifuged at 12,000g for 20 min. An aliquot of the supernatant was counted to determine the 'uptake' of isotope into the embryos. The pellets were suspended for 24 h in 2.0 ml of 0.5 N NaOH to hydrolyze the RNA, and the DNA was then precipitated by adding 3.0 ml of 10% TCA. The TCA-insoluble pellets were suspended in 2.0 ml of 0.1 N NaOH, left at room temperature for 30 min, and a 1.0-ml aliquot was treated with 2.0 ml of 10% $HClO_4$ for 20 min at 70°C. After cooling in ice, the tubes were centrifuged for 20 min at 14,000g and a 1-ml aliquot of the supernatant was mixed with 10 ml of aqueous counting fluid and counted. The radioactivity released by the hot $HClO_4$ was taken as a measure of the incorporation into DNA.

RESULTS

Wheat embryonic axes exposed to water undergo a period of minimal increase in fresh weight before sustained growth begins (7). The variety of wheat embryo used in the present study

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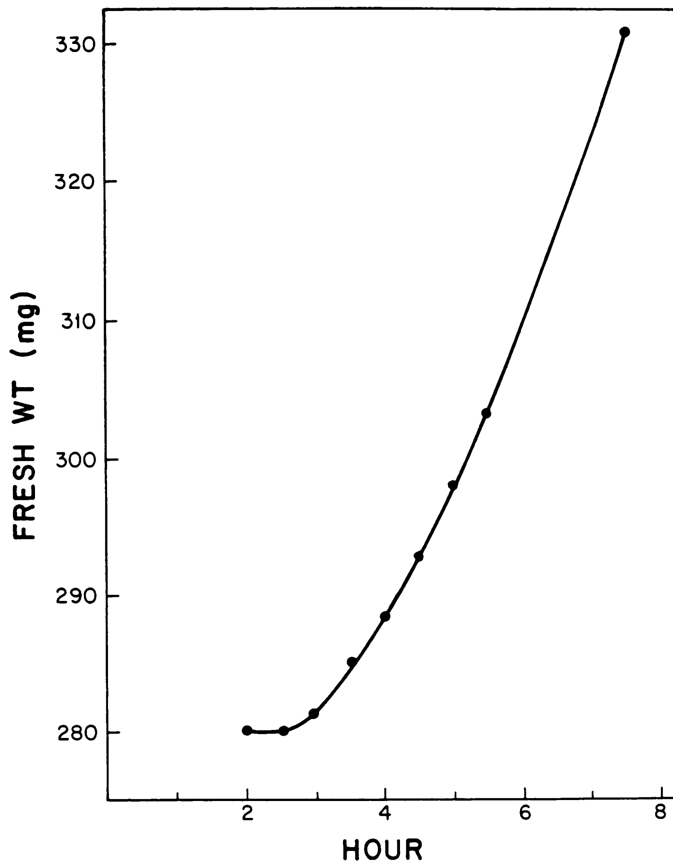

FIG. 1. Growth of wheat embryo var Newana. Fresh weights of 120-mg samples of embryo were determined at the time points shown.

(Newana) had a shortened lag period, thus making it possible to obtain significant growth data within the first 8 h of germination. Figure 1 shows a time course of the increase in fresh weight. In contrast to other embryo varieties that have only a minimal rise in fresh weight before 5 to 5.5 h, Newana embryos begin substantial growth shortly after 3 h. Typical fresh weight data for 120 mg of dry embryo are 281 mg at 3 h, 303 mg at 5.5 h, and 331 mg at 7.5 h.

With these embryos, we have tested the effect of several inhibitors on embryo growth, monitoring at the same time their effect on specific metabolic processes. The rationale in these studies is that, to the extent that a particular process can be eliminated without strongly affecting the increase in fresh weight, the process can be considered irrelevant to early embryo growth. Prevention of growth by the inhibitor suggests a metabolic area that may be more closely related to early growth. In preliminary tests, we observed that addition of inhibitors to embryos after the initial rapid water uptake generally reduced the inhibitory effect on embryo growth and on the particular metabolic process being considered. Overall, the delayed exposure to inhibitors increased the specificity of the inhibition. Several typical experiments showing the relative specificity of RNA synthesis inhibitors are presented in Table I. FUrd[3] treatment causes a strong inhibition of incorporation of radioactive adenine into cytoplasmic poly A(−)RNA with only a small effect on poly A(+)RNA. Cordycepin, 3′-deoxyadenosine, also inhibits precursor incorporation into poly A(−)RNA, but in addition, it inhibits incorporation into poly A(+)RNA. The third RNA synthesis inhibitor, α-amanitin, also decreases incorporation into both poly A(−)RNA and poly A(+)RNA, but its effect is greater on the poly A(+)RNA.

Inhibitors of RNA and Protein Synthesis and Embryo Growth. A compilation of the effects of several inhibitors of RNA and protein synthesis on embryo growth is presented in Table II. The data with FUrd (exp. I) present the simplest conclusion and provide a basis for interpreting the effects of the other inhibitors. Presence of FUrd curtails the synthesis of poly A(−)RNA (primarily rRNA) by about 75% without affecting embryo growth indicating clearly that much of the rRNA synthesis is not necessary for early embryo growth. With cordycepin and α-amanitin (exps. II and III), the most striking data were obtained at low levels of these inhibitors. At 75 μM cordycepin and at 8 μg/ml α-amanitin, 40% of the A(+)RNA synthesis was inhibited with an effect on growth of less than 15%. At higher concentrations of these inhibitors, there was a greater effect on embryo growth, concomitant with an increased inhibition of the synthesis of both poly A(−)RNA and poly A(+)RNA. Inasmuch as the data with FUrd indicated that curtailing poly A(−)RNA synthesis has no effect on growth, the inhibition of growth by cordycepin and α-amanitin are probably due to their effect on mRNA (poly A(+)RNA) synthesis.

Data with the protein synthesis inhibitors cycloheximide and

[3] Abbreviations: FUrd, 5-fluorouridine, MDMP, 2-(4-methyl-2,6-dinitroanilino)-*N*-methyl propionamide.

Table I. *Inhibition of RNA Synthesis by FUrd, Cordycepin, and α-Amanitin*

Embryos (75 mg) were first imbibed in water either for 40 min or for 1 h and then transferred to the inhibitor solutions for the time period indicated. The embryos were then assayed for the ability to incorporate either 2-[^{3}H] adenine (exp. I, 50 μCi and 5 × 10^{-5} M, 40 min incubation) or 5,6-[^{3}H]uridine (exp. II, 50 μCi and 5 × 10^{-5} M, 40 min incubation; exp. III, 25 μCi and 5 × 10^{-5} M, 60 min incubation) into RNA.

Exp.	Treatment	Total Uptake	Incorporation into RNA	
			poly A(+)	poly A(−)
		cpm × 10^{-6}	*cpm*	
I	Control	6.0	18,899	30,965
	2 mM FUrd (1.0–5.2 h)	6.6	16,642	9,161
	% inhibition		12	70
II	Control	5.7	85,513	131,421
	75 μM cordycepin (0.7–4.7 h)	5.5	43,222	51,882
	% inhibition		49	61
III	Control	6.6	40,225	112,760
	12 μg/ml amanitin (0.7–4.2 h)	6.1	18,904	92,832
	% inhibition		53	18

Table II. *Effects of FUrd, Cordycepin, α-Amanitin, Cycloheximide, and MDMP on Embryo Growth, and on the Synthesis of RNA and Protein*

Embryos were imbibed and analyzed for growth as in Figure 1. RNA synthesis was determined as in Table I, while protein synthesis was assayed in incubations of 12 to 20 min in 8 mM [^{14}C]leucine as described in "Materials and Methods." Growth analyses were at 7.5 or 8 h with the inhibitor regimes continued until this time. Protein synthesis analysis in experiment II was done at 5.5 h with the embryos kept in the cordycepin solution until this time. Otherwise, all analyses were carried out at the end of the described period of exposure to the inhibitor.

Exp.	Inhibitor	Concn.	Growth	Synthesis		
				Protein	poly A(−)RNA	poly A(+)RNA
			% of control		*% of control*	
I	FUrd					
	1.0–5.5 h	2 mM	100	90	30	97
		4 mM	100	85	26	90
		5 mM	84			
II	Cordycepin					
	0.7–4.7 h	75 μM	97	88	39	60
	0.7–4.7 h	150 μM	78	77	29	48
	0.7–7.7 h	100 μM	77		16	35
III	Amanitin					
	0.7–4.2 h	8 μg/ml	89		86	57
	0.7–7.2 h				80	51
	0.7–4.2 h	12 μg/ml	73		74	41
	0.7–7.2 h				63	33
IV	Cycloheximide					
	1.0–5.0 h	0.5 μg/ml	51	60		
	1.0–5.0 h	0.8 μg/ml	28	20		
	MDMP					
	1.0–5.0 h	0.1 μM	76	44		
	1.0–5.0 h	0.2 μM	28	10		

Table III. *Effect of FUrd on DNA Synthesis in 19-Hour Germinated Wheat Embryos*

Embryos (75 mg) were incubated for 18 h 25 min and then transferred to a 1-ml solution containing 5×10^{-5} M 2-[^{3}H]adenine for 70 min. The embryos were then processed for the analysis of DNA synthesis as described in "Materials and Methods." In the FUrd-treated samples, the embryos were transferred to a 4 mM solution of the inhibitor at 1 h.

	DNA Synthesis
	cpm
Control	6,836
FUrd	2,665

MDMP are presented in experiment IV. In contrast to the RNA synthesis inhibitors, with these reagents there is a close correlation between the extent of inhibition of amino acid incorporation and the inhibition of embryo growth.

DNA Synthesis and Embryo Growth. The observation that FUrd did not inhibit embryo growth at 8 h prompted a test of the effect of this reagent at a later time period. Analysis of rRNA synthesis at 17 h showed that the FUrd treatment still resulted in an inhibition of at least 75% (data not shown). Furthermore, the absolute amount of rRNA in embryos (as determined by the extractable A_{260}) treated with FUrd for 24 h decreased to 75 to 80% of that of control embryos. Nevertheless, the fresh weight of the embryos at 17.5 h was unaffected, control embryos weighing 438 mg, and FUrd-treated embryos (transferred at 1 h to 4 mM FUrd) 440 mg.

In addition to its effect on RNA synthesis, FUrd is known to inhibit DNA synthesis (4). Since the FUrd did not inhibit growth even at the later time points, we could tentatively conclude that DNA synthesis is not critical to early embryo growth. To verify that FUrd-inhibited DNA synthesis, we incubated embryos germinated for 19 h (a time at which the rate of DNA synthesis is substantially increased; Ref. 9) with [^{3}H]adenine, and analyzed the radioactivity that is incorporated into alkali-stable acid-insoluble material, and can be released by hot $HClO_4$. Table III shows that incubation of the embryos in FUrd resulted in a reduction in incorporation of more than 60%. The total radioactivity taken up into the embryos and the absolute levels of ATP (data not shown) were not affected by the FUrd treatment. The difference in incorporation seems therefore to be an authentic effect on DNA synthesis.

Table IV. *Effects of Mitomycin C and Methotrexate on Early Embryo Growth and DNA Synthesis*

Embryos were incubated for 17.5 h either in water or in solutions of the indicated inhibitors as described in Table III. The embryos were then either weighed as in Figure 1 or incubated with 0.15 μCi of 2-[^{14}C] thymidine (5×10^{-5} M) and analyzed for DNA synthesis as in Table III.

Inhibitor Added	Concn.	Growth	[^{14}C]Thymidine Incorporated into DNA
		% of control	
Mitomycin C	2 μg/ml	83	
	5 μg/ml		21
	10 μg/ml	70	
Methotrexate	1.2 mM	76	27

To examine further the relationship between DNA synthesis and early embryo growth, we tested embryo growth in the presence of mitomycin C and methotrexate, two known inhibitors of DNA synthesis. Table IV shows that addition of these compounds at levels causing better than 70% inhibition of thymidine incorporation into DNA, inhibited growth less than 25%. These data agree

with the conclusion of the FUrd experiment and indicate that embryo growth during the initial 20 h of wheat embryo germination is essentially independent of DNA synthesis.

DISCUSSION

Our results allow three conclusions. (a) The synthesis of rRNA can be strongly curtailed for at least 20 h even to the point of a decrease in the absolute level of ribosomes in the embryos, without any effect on the rate of growth. Observations of this type have been reported in earlier studies (10), but the particular RNA affected was not determined. (b) The synthesis of DNA can be strongly inhibited for at least 20 h again with little effect on the rate of growth. (c) The rate of mRNA synthesis can be significantly reduced with only a minor effect on early embryo growth (8 h). The experimental basis for this conclusion is the ability to treat embryos with levels of cordycepin and α-amanitin sufficient to inhibit partially the synthesis of mRNA, with little effect on embryo growth.

Attempts to extend the mRNA synthesis inhibitor treatments to 17 h have resulted in equivocal results; in general, showing a relatively greater inhibition of growth. These observations suggest that preexistent mRNA can maintain the onset of growth, but that new mRNA synthesis may be important when the more rapid rate of growth sets in. An unanswered question is whether the need for new mRNA synthesis is simply to maintain a steady-state level of mRNA against a background of turnover of preexistent mRNA or whether the synthesis of mRNA serves a more specific function, *i.e.* the synthesis of special growth-linked protein(s) (*e.g.* Ref. 12). Finally, the results of Jendrisak (6), showing that, at 0.1 μg/ml of α-amanitin, synthesis of mRNA is inhibited 80% while embryo growth up to 36 h is inhibited only 20%, extend our conclusion considerably in terms of the extent of growth that is independent of mRNA synthesis. We have not been able to obtain such an extensive distinction nor can we get the strong selectivity of α-amanitin inhibition towards poly A(+)RNA relative to poly A(−)RNA. Nevertheless, our studies agree qualitatively that early embryo growth can occur at the normal rate despite a significant diminution in the rate of synthesis of mRNA.

The strong sensitivity to inhibition of protein synthesis remains an intriguing contrast to the lesser effect of the RNA synthesis inhibitors. A possible approach to understanding the protein synthesis requirement is to seek metabolic reactions critical to the growing embryo. Analysis of the turnover of proteins that regulate these reactions might provide a clue to the strong requirement for ongoing protein synthesis.

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