Storage and Metabolism of Poly(adenylic acid)-mRNA in Germinating Cotton Seeds†

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ABSTRACT: When the RNA from both the nucleus and polysomes of germinated cotton seeds were fractionated on poly(U)-cellulose columns it was found that both contained poly(A)-RNA. In ungerminated seeds most of the poly(A)-RNA is located in the nucleus of the cell. 3′-Deoxyadenosine was found to inhibit the synthesis of both nuclear and polysomal poly(A) by 75% in germinated seeds, but had little or no effect on protein synthesis during the first 6 hr of germination. After this time 3′-deoxyadenosine had a pronounced inhibitory effect on protein synthesis presumably due to the inhibition of poly(A) addition to newly synthesized mRNA.

Dormant plant seeds contain mRNA that was transcribed during late embryogenesis and is translated during germination (Davies et al., 1972; Dure and Waters, 1965). Most of this work has dealt with the effects of RNA inhibitors on germination and protein synthesis, but little is known about the location and the storage form of these mRNA molecules. Because most eukaryotic mRNA contains a covalently linked poly(A) segment, mRNA can be easily isolated by its affinity for immobilized oligo(deoxythymidylic acid) or poly(uridylic acid). Thus, many investigators have reported the isolation and characterization of poly(A)-mRNA from a variety of plant species (Harris and Dure, 1974; Higgins et al., 1973; Ho and Varner, 1974; Manahan et al., 1973). We report here the isolation of poly(A)-RNA from the cultivated cotton (*Gossypium hirsutum*) using affinity chromatography on poly(U)-cellulose columns.

We also used this technique to investigate the location and storage form of these poly(A)-RNAs in dormant seeds. To determine whether this poly(A)-RNA is indeed mRNA, the effects of 3′dAdo[1] on poly(A)-RNA formation and protein synthesis were examined. The results presented here indicate that most of the stored mRNA is polyadenylated and located in the nucleus.

Materials and Methods

Germination and Radioactive Labeling. Cotton seeds (*Gossypium hirsutum*) were surface sterilized in 15% hypochlorite solution and germinated in petri dishes (20 seeds/dish) containing 20 ml of distilled H_2O. Seedlings of the appropriate age were labeled by addition of [8-^{3}H]adenine (50 μCi/ml) or $^{32}PO_4$ (250 μCi/ml) for the last 3 hr of the germination period. All labeling was done in the dark at 37°. Labeling was terminated by thoroughly washing seedlings with ice-cold distilled H_2O. At this point the seed coat was removed, and the entire embryo was homogenized.

Isolation of Nuclei and Extraction of Nuclear RNA. Labeled seedlings were homogenized in a cold mortar and pestle in grinding buffer [0.1 *M* Tris-HCl (pH 8.0), 0.3 *M* $MgCl_2$, 0.06 *M* KCl, 0.2 *M* sucrose (RNase-free)]. The slurry was passed through a single layer of Miracloth and the nuclei pelleted at 1000*g*. The nuclear pellet was resuspended in grinding buffer plus 0.1 vol of 10% Triton X-100 and repelleted. This was repeated until a pure nuclear pellet was obtained, as judged by microscopic examination. The nuclei were lysed in 1% sodium pyrophosphate, 1% sodium dodecyl sulfate, 0.01 *M* NaCl, 0.01 *M* Tris-HCl (pH 7.5), plus 5 mg/ml Bentonite for 30 min at 37°, and the nucleic acid was extracted by shaking with an equal volume of chloroform + 1% isoamyl alcohol at 4°. The DNA was hydrolyzed with DNase I (20 μg/ml) for 30 min at 37°, and the RNA precipitated by addition of 2 vol of cold ethanol.

Isolation of Polysomes and Polysomal RNA. The supernatant liquid from the initial 1000*g* centrifugation was centrifuged at 30,000*g* to remove mitochondria. The polysomes were pelleted from the resultant supernatant liquid through 0.8 *M* sucrose at 100,000*g* for 90 min. This pellet was resuspended in 10 ml of lysing buffer and deproteinized by shaking with an equal volume of chloroform + 1% isoamyl alcohol.

Poly(U)-Cellulose Chromatography. Poly(U)-cellulose was prepared according to Sheldon et al. (1972), formed into 0.8 × 5 cm columns, and equilibrated with binding buffer I (0.12 *M* NaCl and 0.01 *M* Tris-HCl, pH 7.5) at 4°. The RNA samples (0.1–0.2 mg) were dissolved in binding buffer I, loaded on the column, and allowed to bind for 15 min at 4°. Non-poly(A)-RNAs were eluted from the column with 35 ml of cold binding buffer I. The temperature of the column was raised to 40° and the poly(A)-RNAs eluted with 35 ml of elution buffer (0.1 *M* Tris-HCl, pH 7.5). Each of these elutions was collected in seven 5-ml fractions. The amount of RNA in each fraction was determined spectrophotometrically, and the radioactivity determined by liquid scintillation in a toluene, Triton X-100 based fluor.

Base Composition. RNA samples labeled with ^{32}P were hydrolyzed with 0.3 *M* KOH at 37° for 18 hr. After neutralization with perchloric acid the hydrolysate was subjected to two-dimensional paper chromatography as described by Vodkin et al. (1974). The ultraviolet light absorbing region of the paper was cut out and radioactivity determined directly by liquid scintillation.

Polyacrylamide Electrophoresis. RNA samples were analyzed on cylindrical acrylamide gels according to Bishop et

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[1] Abbreviation used is: 3′dAdo, 3′-deoxyadenosine.

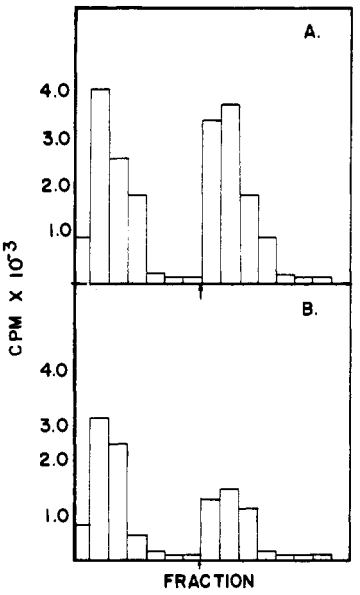

FIGURE 1: Poly(U)-cellulose chromatography of nuclear and polysomal RNA. Germinated seeds (48 hr) were labeled for 3 hr with [8-^{3}H]adenine and the RNA extracted from purified nuclei (A) and purified ribosomes-polysomes (B) as described under Materials and Methods. RNA samples containing the same amount of material were fractionated as described under Materials and Methods. Each bar in the histogram represents the total radioactivity present in that particular fraction. The arrow indicates when the low salt elution began.

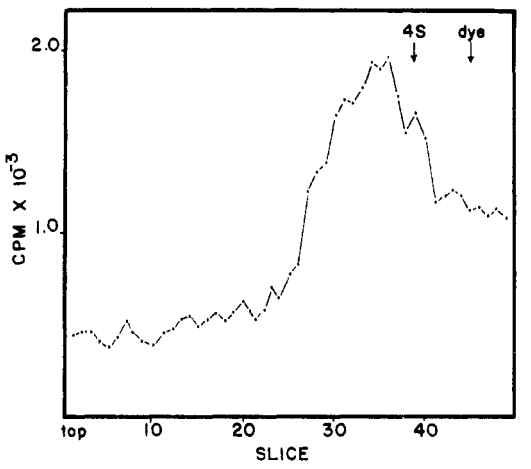

FIGURE 2: Electrophoresis of ribonuclease-resistant poly(A) segment in acrylamide gels. Total RNA was extracted from [8-^{3}H]adenine labeled seedlings and hydrolyzed with a mixture of T_1 and pancreatic ribonuclease. The enzymes were removed by shaking with chloroform, and the resultant poly(A) was isolated on poly(U)-cellulose columns. The low salt eluent was precipitated with ethanol plus cold carrier RNA. Samples were then electrophoretically analyzed in 7.5% acrylamide gels as described under Materials and Methods. tRNA was used as an optical marker.

Table I: Base Composition of RNAs from 48-hr Seedlings.

	Mol %			
	AMP	GMP	CMP	UMP
Poly(A) nuclear RNA	29.0	40.3	15.7	15.0
Poly(A) polysomal RNA	28.5	38.0	15.5	18.0
Total RNA	24.5	30.9	23.6	21.0
Ribonuclease-resistant poly(A) segment	93.0	1.8	1.6	3.6

al. (1967). The RNA to be analyzed was dissolved in 0.1 *M* potassium acetate–0.1% EDTA–15% sucrose, the solution was loaded onto a 90 × 4.0 mm gel, and electrophoresis was performed for 2 hr at 5 mA/tube. When analyzing the RNase-resistant poly(A) segment a 7.5% acrylamide gel was used and the running time increased to 3.5 hr at 5 mA/tube. At the end of the run the gels were removed from the tubes, frozen in Dry Ice, and sliced into 2-mm sections. Each slice was placed into a scintillation vial, and dissolved 0.5 ml of 30% H_2O_2 at 50° for 24 hr. Radioactivity was measured by liquid scintillation.

Inhibition of Poly(A) Synthesis by 3′dAdo. Surface sterilized seeds were germinated in distilled H_2O containing 3′dAdo (20 μg/ml) for the desired length of time. Subsequently, [8-^{3}H]adenine (5 μCi/ml) was added and the incubation continued for 3 hr. Poly(A)-RNA was isolated on poly(U)-cellulose columns, and radioactivity determined by liquid scintillation.

Inhibition of Protein Synthesis. Again the seeds were germinated in distilled H_2O containing 3′dAdo for the desired length of time. The seeds were then labeled with ^{3}H-labeled protein hydrolysate (5 μCi/ml) for the final 3 hr of the germination period. The seeds were washed with cold H_2O, and the total soluble protein was extracted in 0.01 *M* Tris-HCl (pH 8.0). The proteins were precipitated by 10% Cl_3CCOOH (cold), pelleted, and resuspended in the extraction buffer. Protein concentrations were determined as described by Chaykin (1966), and radioactivity in the protein was determined by liquid scintillation on 1-ml aliquots of the protein sample. Specific activity was expressed as counts per minute per milligram of protein.

Results

Isolation and Characterization of Poly(A)-RNA. The RNAs from purified nuclei and polysomes can be separated into those which bind and those which are eluted from poly(U)-cellulose columns with high-salt binding buffer (Figure 1). In the case of polysomal RNA it can be shown electrophoretically that the nonbinding RNA is a mixture of tRNA and rRNA (unpublished results). As can be seen in Table I, the base composition of the bound RNA from both nuclei and polysomes is enriched for AMP, and RNase treatment of these RNAs produced a poly(A) segment which is over 90% AMP. Electrophoresis of the isolated poly(A) segment indicates that it ranges in size from 75 to 100 nucleotides (Figure 2). This argues that the enzymatically released poly(A) molecules are heterogeneous in size. To further characterize these poly(A)-RNAs, they were analyzed electrophoretically on 2.4% acrylamide gels. Both nuclear and polysomal poly(A)-RNA were found to be polydisperse in size (6–25 S) (Figure 3). These results are similar to those obtained for heterogeneous nuclear RNA and mRNA from other systems (Darnell et al., 1971; Ihle and Dure, 1969).

Localization of Poly(A)-RNA in Dormant Seeds. Since much of the mRNA in eukaryotic cells contains a poly(A) segment we should be able to use this as an assay for locating stored polyadenylated mRNA in dormant seeds. To do this we removed the seed coats from dry dormant seeds and homogenized the embryos in grinding buffer. The resultant slurry was fractionated into a nuclear pellet and a submitochondrial supernatant fraction. The latter fraction contained ribosomes, cytoplasm, and any material released from the nucleus during homogenation. The RNA was extracted from these fractions and chromatographed: poly(A)

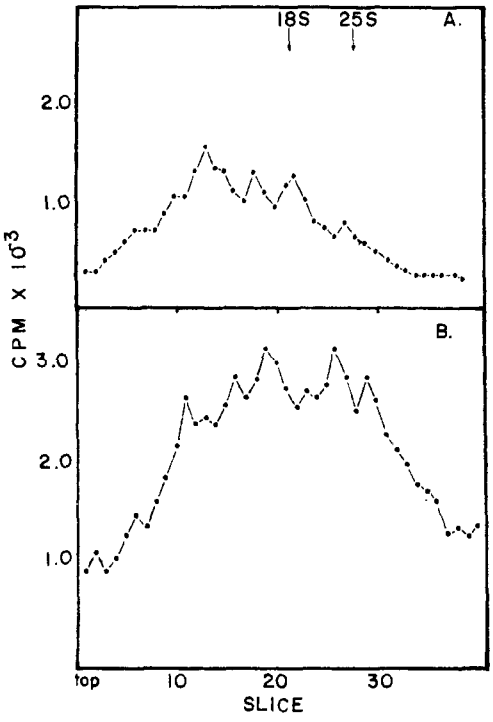

FIGURE 3: Electrophoresis of nuclear and polysomal poly(A) RNA. The poly(A)-RNA fractions obtained as described in Figure 1 were analyzed in 2.4% acrylamide gels as described under Materials and Methods. The positions of the 18S and 25S rRNA were determined by optical scanning of the gels: (A) polysomal poly(A)-RNA; (B) nuclear poly(A)-RNA.

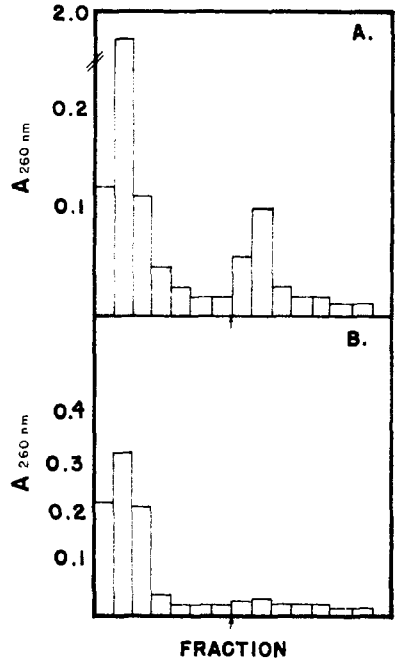

FIGURE 4: Poly(U)-cellulose chromatography of dormant seed nuclear and cytoplasmic RNA. RNA was isolated from the nuclei and submitochondrial fractions of dormant seeds as described under Materials and Methods. Samples were fractionated on poly(U)-cellulose and the amount of RNA in each fraction was determined spectrophotometrically. Each bar in the histogram represents the optical density per milliliter in that fraction. The arrow indicates the beginning of low salt elution: (A) nuclear RNA; (B) cytoplasmic RNA.

was found in the nuclear pellet (11%), and only a small amount (<1.5%) was found in the submitochondrial fraction (Figure 4). This latter amount was considerably less than the 6% found in 48-hr germinated seed.

Characterization of Ribosomes from Dormant and Germinating Seeds. We now consider the origin of the small amount of poly(A) contained in the submitochrondrial fraction in dormant seeds. We prepared ribosomes from the submitochondrial fraction by centrifugation, resuspended them in TMK buffer (see Figure 5 legend), and analyzed ribosomes and polysomes on 15–30% linear sucrose gradients. Only monoribosomes were found in dormant seed preparations. Polysomes did not appear until the sixth hour of germination (Figure 5). It should be noted that both the number and size of the polysomes increased through the first 48 hr of germination. These findings are similar to those made by Marcus and Feeley (1966) for dormant wheat seeds. It may be that polysomes are present in dormant seed and are reduced to monoribosomes by shearing forces or ribonuclease activity during extraction. If this is the case, it may be that mRNA remains bound to the monoribosomes. Isolation of the RNA from dormant seed monoribosomes and fractionation on poly(U)-cellulose indicates that no poly(A)-RNA is found associated with these ribosomes (data not shown). A similar examination of the subribosomal fraction (fraction after the 100,000*g* spin) indicates that this is the location of the previously mentioned submitochondrial poly(A)-RNA fraction.

Effect of 3′dAdo on Poly(A)-RNA Synthesis. 3′dAdo is generally believed to be a chain terminator in RNA synthesis. In mammalian systems it has been shown to inhibit the addition of poly(A) to mRNA (Jelnik et al., 1973). To test the effects of 3′dAdo on mRNA synthesis, seeds were germinated in 3′dAdo and labeled with [8-^{3}H]adenine for the

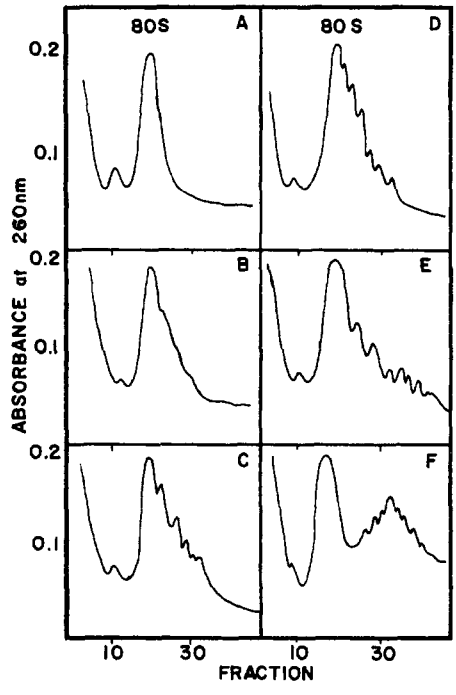

FIGURE 5: Sucrose gradient analysis of ribosome–polysomes at various times during germination. Ribosomes were isolated from seedlings using a modified procedure of Davies et al. (1972) in 0.2 *M* sucrose RNase, free 0.2 *M* Tris-HCl (pH 8.0), 0.3 *M* $MgCl_2$, 0.6 *M* KCl, 1% diethylpyrocarbonate, plus 0.1 vol of 10% Triton X-100. Ribosome pellets were resuspended in 0.04 *M* Tris-HCl (pH 8.0), 0.01 *M* $MgCl_2$, and 0.02 *M* KCl (TMR buffer), and analyzed on 15–30% linear sucrose gradients. The time periods are: (A) dormant seed; (B) 6 hr; (C) 12 hr; (D) 18 hr; (E) 24 hr; and (F) 48 hr. These curves are representative of three or more analyses.

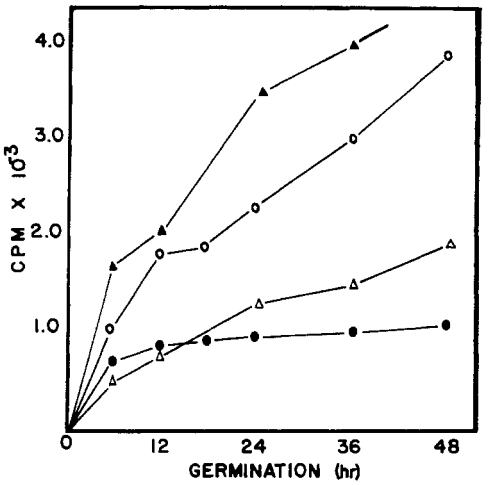

FIGURE 6: Effect of 3′dAdo on poly(A)-RNA synthesis. Seeds were germinated in the presence of 3′dAdo and labeled with [8-^{3}H]adenine as described under Materials and Methods. Samples from various times were fractionated on poly(U)-cellulose and the radioactivity in the poly(A) fraction was measured by liquid scintillation. At each time point the same amount of material was analyzed: (▲) nuclear poly(A)-RNA from untreated seeds; (△) nuclear poly(A)-RNA from treated seeds; (○) polysomal poly(A)-RNA from untreated seeds; (●) polysomal poly(A)-RNA from treated seeds.

final 3 hr of the germination period. The results indicate that after 36 hr poly(A) addition is inhibited by 75% in both the nuclear and polysomal RNA. To increase inhibition we tried doubling the concentration of 3′dAdo to 40 μg/ml, but found little increase in the percent inhibition. We were also unable to determine if all the poly(A) addition occurs in the nucleus, or if cytoplasmic polyadenylation takes place as is found in sea urchin embryos (Wilt, 1973).

Effect of 3′dAdo on Protein Synthesis. When seeds were germinated in the presence of 3′dAdo it was found that protein synthesis was also inhibited. Seeds were germinated for the desired length of time and labeled with a mixture of ^{3}H-labeled amino acids (protein hydrolysate). As is shown in Figure 7, 3′dAdo has little or no effect on protein synthesis during the first 6 hr of germination although the data points for the first 6 hr indicate a very low specific activity. It must be remembered that dormant cotton embryos contain a great deal of nonlabeled soluble protein to which labeled material is added. The total counts per minute in these samples was approximately 15,000 cpm. Since the increase in total amount of protein during the period indicated is small, the fact that we express our results as specific activity merely normalizes the variations in the amount of extracted protein from sample to sample. These experiments also indicate that protein synthesis continues in the treated seeds for 12 hr at which time there is a drastic reduction in the rate of protein synthesis. This slowdown in protein synthesis after 18 hr corresponds to the approximate time when we observe a rapid increase in the synthesis of poly(A)-RNA in the nucleus and its subsequent appearance in the cytoplasm (Figure 6). In untreated control seeds the rate of protein synthesis continues to increase throughout the first 48 hr of germination.

Discussion

In the present study we have isolated and characterized the poly(A)-RNA as a preliminary to an examination of the storage form of mRNA in dormant cotton seed. The finding of a poly(A) sequence of 75–100 adenine residues in length on some RNA in cotton is similar to that reported by Harris and Dure (1974). The importance of this is that we employed different isolation regimes and analysis procedures. We were able to isolate this poly(A)-RNA from both the nucleus and polysomes of germinated seed, and found that they were very similar in both size and base composition. Our results also indicate that the rates of synthesis of nuclear and cytoplasmic poly(A)-RNA are approximately the same (Figure 6). These results are similar to those for other eukaryotic systems (Darnell et al., 1971; Latorre and Parry, 1974; Sheiness and Darnell, 1973) in which the investigators have postulated a precursor–product relationship between nuclear poly(A)-RNA and cytoplasmic poly(A)-RNA. Our results do not provide any direct evidence concerning a similar situation in cotton.

It should be noted that a considerable heterogeneity exists in the size of the poly(A) sequence. Since very high concentrations of ribonuclease are used in the isolation it does not seem likely that this is due to incomplete hydrolysis. It has been suggested by Sheiness and Darnell (1973) that length of the poly(A) is related to the longevity of the mRNA. In other words, the most long-lived molecules are those in the population with the longest poly(A) segment. In our hands, enzymatic isolation of the poly(A) segment does not reveal any difference in the size between nuclear and polysomal poly(A). It would be of interest to label in vitro the isolated poly(A) from dormant seed to see if it is any larger than poly(A) from germinated seed.

It has been known for some time that mRNA is stored in cotton embryos during late embryogenesis (Dure and Waters, 1965). This has been concluded indirectly from inhibition studies with actinomycin D. If the stored mRNA is polyadenylated during embryogenesis then it should be possible to isolate it from dormant seed. Our results indicate that polyadenylated RNAs are indeed present in dormant seed with most of it located in the nuclear pellet. We also found a small amount of poly(A)-RNA in the cytoplasmic material from dormant seed. An examination of the ribosomes indicated that only monoribosomes are present and no poly(A)-RNA is associated with these ribosomes. Although a cytoplasmic origin of this poly(A)-RNA cannot be ruled out it may be that this small amount is released from the nucleus during homogenation. It is also apparent that any mRNA which is not polyadenylated will not be recognized as such, and will be overlooked by our procedures.

A further examination of poly(A)-RNA was made in experiments employing 3′dAdo as an inhibitor of poly(A) synthesis. We found that 65% inhibition was achieved for both nuclear and polysomal poly(A)-RNA within 6 hr after germination began. This inhibition reached a maximum of 75% after 36 hr, and we were unable to increase this inhibitory value. This is similar to the recent results of Harris and Dure (1974) with the exception that they used much higher concentrations of 3′dAdo to achieve the same results. Thus, we were able to substantially inhibit the formation of poly(A)-RNA very early in germination.

Walbot et al. (1974) have also shown that the de novo synthesis of carboxypeptidase is dependent on the addition of poly(A) to stored mRNA prior to the 30th hr of germination. Our results indicate that 3′dAdo has no effect on total protein synthesis during the first 6 hr of germination. Remembering that poly(A) addition is inhibited during this time period leads to two possible conclusions. First, protein synthesis is supported by mRNA which has been polyadenylated during embryogenesis, or protein synthesis is not de-

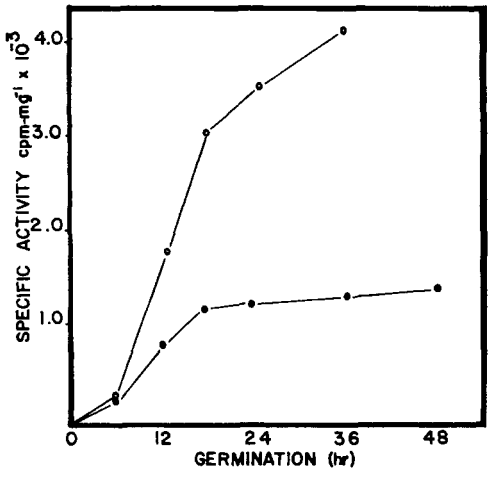

FIGURE 7: Effects of 3'dAdo on protein synthesis. Seeds were germinated in 3'dAdo and labeled for the last 3 hr of each time period with 0.2 mCi of ^{3}H-labeled protein hydrolysate. Control seeds were germinated and labeled in the same manner with 3'dAdo. Total soluble protein was extracted and the specific activity calculated (counts per minute per milligram of protein): (○) control seeds; (●) seeds treated with 3'dAdo.

pendent on polyadenylation of stored mRNA. This latter possibility seems to be ruled out by both Walbot's results and those presented here. The first of these two conclusions is supported by the fact that we are able to isolate poly(A)-RNA from the nuclei and possibly the cytoplasm of dormant seed. Further support comes from an examination of the kinetics of protein synthesis (Figure 7). After 18 hr of germination in 3'dAdo there is a very rapid decrease in protein synthesis; this is the approximate time when we observe a very rapid increase in the synthesis of poly(A)-mRNA.

In conclusion, there is a substantial amount of poly(A)-RNA found in the nuclei of dormant cotton embryos, and there is no indication that further adenylation of mRNA is necessary for the synthesis of total soluble protein during the first 6 hr of germination. When poly(A) synthesis is inhibited it has a dramatic inhibitory effect on protein synthesis after 18 hr of germination indicating that the embryos are beginning to utilize newly transcribed mRNAs, and poly(A) addition is necessary for these mRNAs to function in the synthesis of total soluble protein.

Acknowledgments

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