ACTION OF 3'd ADENOSINE (CORDYCEPIN) AND 3'd CYTIDINE ON THE TRANSLATION OF THE STORED mRNA OF COTTON COTYLEDONS

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SUMMARY: Cordycepin (3'dAdo), an inhibitor of RNA synthesis and poly(A) addition to pre-mRNA, inhibits protein synthesis and the appearance of enzyme activity thought to come from stored mRNA in germinating cotton cotyledons. 3'dCyd and Actinomycin D, other RNA synthesis inhibitors, have no effect on early germination. The period of sensitivity to 3'dAdo is short, comprising the 6th-30th hour of germination. This is consistent with the idea that the stored mRNA of cotton cotyledons is not processed until germination.

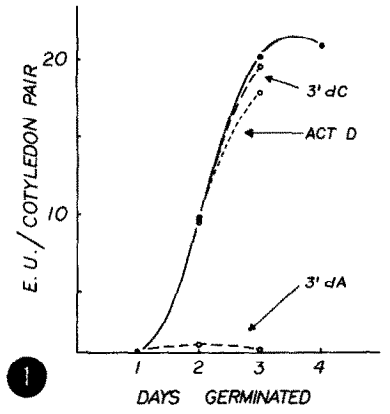
INTRODUCTION: Over the past several years we have accumulated data that suggest that much of the protein synthesis that takes place in cotton cotyledons during the first several days of germination is directed by mRNA that exists in the dry seed, i.e. that is transcribed during embryogenesis (1,2,3). At least some of this stored mRNA is for enzymes unique to the germination process and not found during the embryonic growth of this tissue (4). The premature translation of this body of mRNA during late embryogenesis when it appears to be transcribed would lead to plant vivipary -- the germination of immature seeds within the fruit structure of the parental plant, in this case the cotton boll, which generally is a lethal genetic phenomenon. This premature translation seems to be prevented naturally in cotton by the appearance of the plant growth regulator, abscisic acid, in the ovule tissues during the late stages of embryogenesis (2,3).

The manner in which the translation of this body of stored mRNA is prevented until germination begins is of interest since a temporal separation of transcription from translation appears to be a characteristic of early development in many organisms (5,6). An obvious possibility would be that this mRNA is not "processed" during embryogenesis and that this processing is delayed until germination.

The processing of mRNA, in part, has been shown to entail the post-transcriptional addition of a chain of adenylic acid residues onto the 3'OH end of RNA molecules thought to contain presumptive mRNA sequences (7) which results in the findings that most functional mRNA isolated from polysomes contain this poly(A) sequence on the 3'OH end (7). Further, it has been shown that the fungal antibiotic cordycepin, 3'dAdo, is an effective inhibitor *in vivo* of the post-transcriptional poly(A) addition to incipient mRNA in mammalian cells (8), presumably through the chain terminating action of it's 3'dATP derivative.

With this in mind we have investigated the effects of this inhibitor and of a related molecule, 3'dCyd, on *in vivo* protein synthesis and on the appearance of carboxypeptidase C activity, an indicator germination enzyme considered to arise from the stored mRNA (1,2,3,4) during the germination of cotton cotyledons.

MATERIALS AND METHODS: Cotton seeds (*Gossypium hirsutum*) were variety Coker 413. Actinomycin D was obtained from Calbiochem, cordycepin (3'dAdo) from Sigma Chemical Co., and 3'dCyd was kindly provided by the Upjohn Company. [^{14}C]-leucine, valine and isoleucine were obtained from New England Nuclear Corporation. Cotton seeds, removed from their seed coats, were germinated in petri dishes between several layers of washed Whatman # 41 filter paper wetted with distilled water or with solutions containing 5 mM 3'dAdo, 5 mM 3'dCyd or 20 µg/ml Actinomycin D in darkness at 30°C. Carboxypeptidase C activity was measured in extracts from germinating cotyledons as previously described (4). *In vivo* protein synthesis was measured by incubating cotyledons that had germinated 24 hours in a solution containing [^{14}C]-leucine, valine and isoleucine for 30 min, after which they were allowed to germinate for 8 more hours. These cotyledons were homogenized in cold 0.1 M phosphate buffer, pH 6.8, containing 5 M urea. The homogenate was centrifuged for 10 min at 10,000 g and the pellet rehomogenized and repelleted as before. The supernatants were pooled and made 10% in trichloroacetic acid (TCA) to precipitate



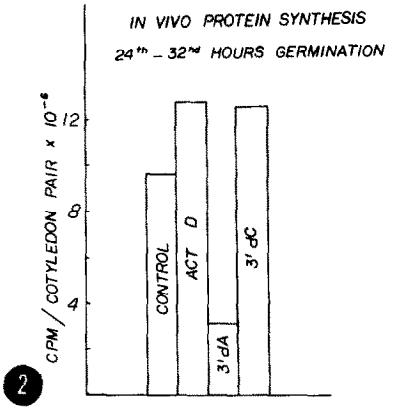


Figure 1. Effect of RNA synthesis inhibitors on the appearance of carboxypeptidase C activity during germination in cotton cotyledons. Enzyme units and assay defined in ref. 4.

Figure 2. Effect of RNA synthesis inhibitors on incorporation of [^{14}C]-amino acids into protein by cotyledons during time of germination indicated.

macromolecules. This precipitate was washed with cold 5% TCA, with 5% TCA at 80°C for 5 min to discharge tRNA, and finally with cold ethanol ether (3:1). The precipitate was dissolved in the phosphate buffer urea solution and the radioactivity in aliquots determined by aqueous counting procedures and by the paper disc method of Bollum (9).

RESULTS: Fig. 1 shows the effect of the three compounds on the appearance of carboxypeptidase activity in cotyledons during germination. As has been reported previously, the appearance of this enzyme is the result of de novo synthesis and is insensitive to high levels of Actinomycin D (4). This figure shows that the enzyme's appearance is sensitive to 3'dAdo but not to 3'dCyd. Fig. 2 shows the effect of these inhibitors on the in vivo incorporation of radioactive amino acids into protein in cotyledons between the 24th and 32nd hour of germination. This time period was chosen because the activity of the carboxypeptidase (and presumably many other germination enzymes) first appears and rapidly increases during this period. Again Actinomycin D and 3'dCyd have

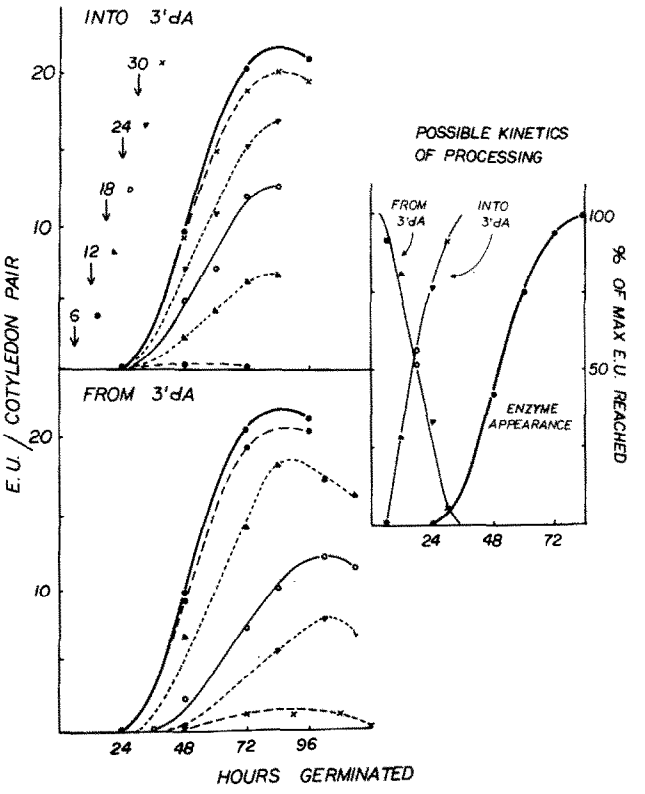


Figure 3. Effect on appearance of carboxypeptidase activity of transferring germinating cotyledons to solutions of 3'dAdo (into 3'dA) or removing them from solutions of the inhibitor (from 3'dA). Right panel described in text.

a similar effect, here a stimulation of incorporation, whereas 3'dAdo inhibits it about 70%. Fig. 3 (upper left) shows the effects on the time course of carboxypeptidase appearance of placing germinating cotyledons in 3'dAdo (into 3'dA) at different time points after the commencement of germination. A family of curves of enzyme appearance is obtained indicating that if cotyledons are transferred to a solution of the inhibitor after 6 hours of germination, no appreciable carboxypeptidase activity subsequently develops; however, if cotyledons are not transferred until the 30th hour, essentially all enzyme activity develops with the same time course as found in normal germination. Transferring cotyledons at intermediate time points results in intermediate levels of carboxypeptidase activity developing. Fig. 4 shows that these

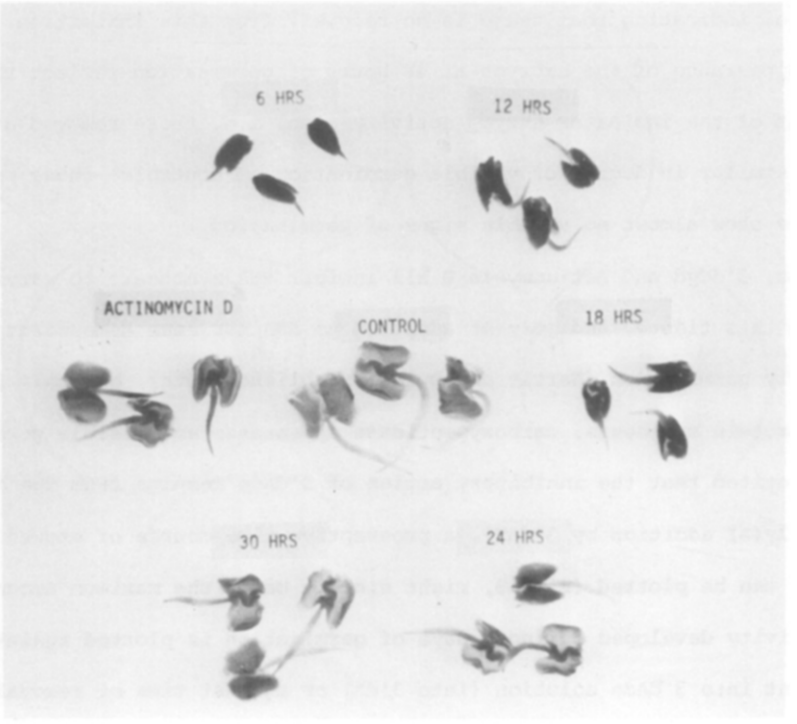


Figure 4. Appearance of embryos after 48 hours germination that have been transferred to solutions of 3'dAdo at the times indicated. Actinomycin D embryos have been treated with this inhibitor for the entire 48 hours.

data on enzyme activity are reflected in the degree of germination visible 48 hours after inhibition. Embryos placed in 3'dAdo solutions early are visibly inhibited, whereas those transferred after 30 hours have visibly germinated to almost the same extent as have normally germinated embryos. Also shown are embryos that have been continuously in Actinomycin D for the entire 48 hours. Although the development of the root-shoot axis is inhibited, the appearance of the cotyledons compares to that of controls. Fig. 3 (lower left) shows the time course of enzyme appearance if the transfer is performed in the opposite fashion, i.e. cotyledons are germinated in the presence of 3'dAdo and removed to distilled water at subsequent intervals (from 3'dA). Here it is seen that cotyledons that have been in the inhibitor only for the first 6 hours of germination subsequently develop normal amounts of enzyme activity. Cotyledons removed after 30 hours in inhibitor solution develop

almost none, indicating that there is no recovery from this inhibition. The physical appearance of the embryos at 48 hours of germination reflect the development of the indicator enzyme activity also, i.e. those removed at 6 hours are similar in degree of visible germination to controls; those removed at 30 hours show almost no visible signs of germination.

3'dAdo, 3'dCyd and Actinomycin D all inhibit RNA synthesis to varying degrees in this tissue, and poly(A) addition to RNA has been demonstrated during early germination (Harris and Dure, unpublished data), but only 3'dAdo inhibits protein synthesis, carboxypeptidase appearance and visible germination. If it is posited that the inhibitory action of 3'dAdo results from the inhibition of poly(A) addition by 3'dATP, a presumptive time course of stored mRNA processing can be plotted (Fig. 3, right side). Here, the maximum amount of enzyme activity developed during 5 days of germination is plotted against time of placement into 3'dAdo solution (into 3'dA) or against time of removal from 3'dAdo solution (from 3'dA). This plot shows that the inhibitory action of 3'dAdo begins about 6 hours of germination but is terminated by the 32nd hour. The time course of enzyme appearance, which is also shown, is removed in time from the period of susceptibility to 3'dAdo.

The data shown in Fig. 3 is from a single experiment. The time axis may vary somewhat from experiment to experiment -- but the pattern is always the same. Ideally the presumptive time course of processing should be determined by plotting the rate of enzyme appearance against time of placement into inhibitor solutions or removal from it, since this rate is probably a more valid indicator of functional mRNA concentration than is percent of maximum activity reached. However, it would require many more time points to establish accurate rate measurements and the data in Fig. 3 indicate that the pattern would not be essentially different.

The inhibitory effect of 3'dAdo could possibly be explained by an inhibition of protein synthesis *per se* by 3'dATP, rather than by an inhibition of mRNA processing. It is conceivable that 3'dATP inhibits the formation of

aminoacyl-tRNA by inhibiting synthetases or by participating in the turnover of the CCA end of tRNA bringing about the formation of tRNA that is possibly nonfunctional (3'dtRNA). This possibility seems unlikely because of the temporal separation of 3'dAdo susceptibility and enzyme synthesis. One would be forced to conclude that once 3'dAdo had been incorporated into tRNA, the tRNA ceased to participate in the CCA turnover. Otherwise once removed from the 3'dAdo solution the 3'dtRNA should be converted back to functional tRNA. Furthermore if 3'dATP brings about the formation of nonfunctional tRNA, 3'dCTP formed from 3'dCyd should also have this effect. However, to test the possibility that 3'dAdo brings about the accumulation of nonfunctional tRNA, tRNA was prepared by procedures previously described (10) from cotyledons that had been germinated for 48 hours ± the inhibitor. A crude aminoacyl-tRNA synthetase preparation was also obtained from cotyledons germinated 48 hours in water and the percentage acceptance of the tRNA preparations for 5 amino acids was determined by previously described procedures (10). The inhibitor had no effect on the acylation capacity of tRNA for the amino acids tested, nor was the yield of tRNA per cotyledon pair appreciably different in the two preparations.

There remains a final possible site of direct inhibition of protein synthesis by 3'dAdo derivatives which entails the conversion of 3'dAdo ultimately to 3'dGTP which conceivably could inhibit the GTP requiring reactions of protein synthesis. Adenosine conversion to guanine derivatives has been observed in cotyledon tissue; thus, this alternative explanation of 3'dAdo inhibition remains a possibility.

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