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Differential Effects of 3′-Deoxy Nucleosides on RNA Synthesis in Cotton Cotyledons†

Barry Harris and L. S. Dure III*

ABSTRACT: SDS and formamide polyacrylamide gel electrophoresis of RNA synthesized by germinating cotton cotyledons shows that the syntheses of the putative products of RNA polymerase I and III are inhibited by 3′dCyd and 3′dAdo *in vivo.* The synthesis of mRNA, considered the product of RNA polymerase II, is not inhibited by 3′dCyd. No mRNA-poly(A) is formed in the tissue treated with 3′dAdo, but its effect on mRNA synthesis distinct from its effect on polyadenylation of pre-mRNA is not measurable in these experiments.

In the course of investigating the processing of the putative stored mRNA in germinating cotton cotyledons (results to be published elsewhere), we have had occasion to test the effects of 3′dAdo[1] and 3′dCyd on the synthesis of different classes of RNA in this plant tissue. It has been reported previously (Siev *et al.,* 1969; Penman *et al.,* 1970; Abelson and Penman, 1972) that in HeLa cells both these inhibitors, probably by way of their triphosphorylated derivatives, truncate nucleolar rRNA synthesis. Yet neither affect the synthesis of heterogeneous nuclear RNA (HnRNA), although 3′dAdo prevents the appearance of mRNA in the cytoplasm, presumably through an inhibition of the poly(A) addition phase of mRNA processing. We report here the effects of these inhibitors on rRNA, 5S RNA, tRNA, and mRNA-poly(A) synthesis in a higher plant tissue, which, in view of the results obtained with HeLa cells, suggest that the difference in response of the cotton RNA polymerases to the 3′d nucleotide triphosphates *in vivo* may be a general characteristic of these enzymes.

The effect of the inhibitors on the synthesis of the several classes of RNA was determined by measuring the *in vivo* incorporation of radioactive precursors into RNA which had been fractionated by salt precipitation and chromatography on poly(U)-Sepharose and separated by gel electrophoresis.

Materials and Methods

Sepharose 4B was obtained from Pharmacia Fine Chemicals; poly(U), from Miles Laboratories; pancreatic and T_1 RNases were obtained from Worthington and Calbiochem, respectively. 3′dAdo was obtained from Sigma Chemical Co. and 3′dCyd was the kind gift of Dr. John C. Babcock of the Upjohn Company.

Germination and Radioactive Labeling of Cotton Cotyledons. Cotton embryos were removed from their seed coats, and placed in solutions of gramacidin D (0.5 mg/ml, used to prevent bacterial growth) with and without 3′dCyd or 3′dAdo (5 mM). These solutions were then shaken 30 min; the embryos were removed and placed in petri dishes between filter paper wetted with the gramicidin D solution with and without 3′dCyd or 3′dAdo. After 8 hr of germination at 25°, the embryos were transferred to identical milieu but containing $^{32}PO_4$ (100 μCi/ml) and [2-^{3}H]adenosine (100 μCi/ml) and incubated an additional 12 hr. At this point the embryos were removed and washed well with water, the axes were removed from cotyledons and discarded, and the cotyledons were homogenized.

Purification and Fractionation of RNA. Cotyledons were homogenized in an ice-cold solution of 0.1 M Tris-HCl (pH 7.8), 0.1 M NaCl, 0.001 M EDTA, and 0.5% SDS (2 ml/cotyledon pair) in a motor driven Duall homogenizer. The homogenate was diluted 1:1 with a 1:1 mixture of phenol and chloroform and shaken for 30 min at 4°. The phases were separated by centrifugation, and the phenol-chloroform layer along with the interface material was extracted with one-half volume of a solution containing 0.1 M Tris-HCl (pH 9), 0.001 M EDTA, and 0.5% SDS by shaking 30 min at room temperature. The phases were again separated,

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[1] Abbreviations used are: SDS, sodium dodecyl sulfate; 3′dAdo, 3′-deoxyadenosine; 3′dCyd, 3′-deoxycytidine; HnRNA, heterogeneous nuclear RNA.

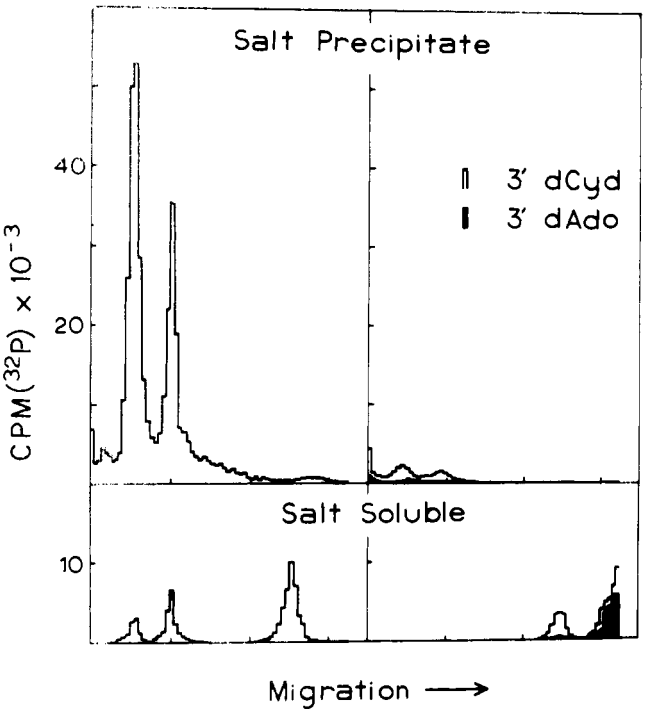

FIGURE 1: Radioactivity profiles (^{32}P) of SDS polyacrylamide gels using 2.5% polyacrylamide. Electrophoresis carried out as described by Loening (1967). Top panels show profiles of RNA precipitated by 2 M NaCl from the control preparation (left) and the 3'dCyd and 3'dAdo treated preparations (right). Bottom panels are of RNA soluble in 2 M NaCl from the control preparation (left) and the 3'dCyd and 3'dAdo treated preparations (right). Profiles have been normalized to show radioactivity extracted from 0.2 cotyledon pair.

the aqueous phase was combined with the initial aqueous phase, and total nucleic acid was precipitated from this solution with 2.5 volumes of ethanol kept at −20°. The precipitate was dissolved in a solution of 0.01 M Tris-HCl (pH 7.6), 0.001 M EDTA, and 0.005 M NaCl, reprecipitated with ethanol, and redissolved in the above solution which was then made 2 M in NaCl. High molecular weight RNA was allowed to precipitate from this solution overnight at 4° after which it was collected by centrifugation and dissolved in 0.036 M Tris-PO_4 (pH 7.6), 0.001 M EDTA, and 0.2% SDS. After an aliquot was removed for SDS polyacrylamide electrophoresis, the RNA was reprecipitated with ethanol and dissolved in 0.01 M Tris-HCl (pH 7.4), 0.4 M NaCl, 0.01 M EDTA, and 0.2% SDS for chromatography on poly(U)-Sepharose.

The DNA and RNA remaining soluble in the 2 M NaCl solution were precipitated with ethanol and dissolved in the SDS polyacrylamide electrophoresis buffer.

Poly(U)-Sepharose Chromatography. Poly(U)-Sepharose was prepared by the method of Wagner *et al.* (1971) and the column (0.9 × 5 cm) formed in a solution of 0.01 M Tris-HCl (pH 7.4), 0.1 M NaCl, 0.01 M EDTA, and 0.2% SDS. The 2 M NaCl precipitated RNA in 2 ml was loaded on to the column in the above solution that was 0.4 M in NaCl at 1 ml/min and 15 min allowed for the formation of poly(U):poly(A) double- and triple-stranded complexes. RNA not retained by the column was eluted in 25 ml at room temperature with the 0.4 M NaCl buffer solution, and the column was washed next with 15 ml of the same buffer solution containing no NaCl. A small amount of RNA elutes in this fraction. To elute the RNA containing appreciable poly(A), the temperature of the column was raised to 50° and the column was washed with 15 ml of buffer solution lacking NaCl. Elution was monitored at 253 nm with an ISCO flow-through monitor. Carrier amounts of yeast RNA were added to each of these three fractions, the RNA was precipitated with ethanol, and the precipitates were dissolved in 0.5 ml of 0.02 M phosphate buffer (pH 7). Aliquots of these fractions were electrophoresed on 99% formamide gels.

Since very small quantities of RNA were handled in some of the fractions, all glassware was treated with a 2% solution of dichlorodimethylsilane in benzene and autoclaved before use.

SDS and Formamide Polyacrylamide Electrophoresis. Aqueous gel electrophoresis in 0.2% SDS utilizing 2.5 and 10% polyacrylamide gels was performed as described by Loening (1967); 99% formamide gels were prepared as described by Duesberg and Vogt (1973) and electrophoresis was carried out as prescribed by Haines *et al.* (1974). The gels were sliced into 1-mm sections to determine the distribution of radioactivity.

Preparation of Poly(A) and Nucleotide Analysis. Aliquots of the RNA retained on poly(U)-Sepharose (low salt eluate and 50° eluate) were digested with pancreatic and T_1 RNase as described by Adesnik and Darnell (1972). Nuclease resistant polynucleotides were ethanol precipitated with the aid of carrier yeast nucleate RNA, and the purity of the poly(A) chains was determined by the electrophoretic separation of nucleotides produced by alkaline hydrolysis. The average chain length of the poly(A) was determined by measuring the adenosine:AMP ratio by electrophoresis and from its migration on 10% SDS polyacrylamide gels. The molar base ratio was also determined after alkaline hydrolysis for all the eluate fractions from the poly(U)-Sepharose column and for the enzyme digested mRNA from the 50° eluate.

Scintillation Counting. Aqueous samples (1 ml) were dissolved in 10 ml of a cocktail comprised of toluene and Triton X100 (2:1) containing the fluor. Gel slices were dissolved in 0.5 ml of 30% hydrogen peroxide, kept tightly capped overnight at 70°, and counted with the above cocktail. Nonaqueous samples (paper strips, Millipore filters) were counted in a 100% toluene solution containing the fluor. The efficiency of counting ^{3}H in these systems was measured and corrections were made for quenching.

Throughout the fractionation of RNA ^{32}P and ^{3}H were monitored with considerable bookkeeping of radioactivity maintained to ensure that no RNA was lost to nucleases or by adherence to glass, etc. If considerable care was taken, no RNA appeared to be degraded and all the radioactivity of the initial total nucleic acid fraction could be accounted for throughout the subsequent fractionations.

Results

Figure 1 (top) shows the profile of ^{32}P radioactivity obtained when an aliquot of the 2 M NaCl precipitated RNA from control and inhibitor-treated cotyledons is electrophoresed in the SDS system. Essentially identical quantities of RNA and DNA are obtained (about 7.5 A_{260} units/cotyledon pair) from each set of cotyledons regardless of treatment. The radioactivity found in the control sample is predominately in the 25 and 18S rRNA species. Very little radioactivity is associated with the RNA from the 3'dCyd cotyledons and essentially none in that from the 3'dAdo treated sample. Table I gives the percentages of control radioactivity found in this and other fractions from inhibitor-

TABLE I: RNA Synthesis in Inhibitor-Treated Cotyledons (Per Cent of Control).

	3′dCyd		3′dAdo	
	^{32}P	^{3}H	^{32}P	^{3}H
Total RNA	15	16	4	3
25, 18S RNA[a]	9	7	4	3
5S RNA	0	0	0	0
tRNA[b]	22	18	5	6
mRNA-poly(A)[c]	100	100	*f*	*f*
mRNA[d]	100	100	*f*	*f*
poly(A)[e]	100	100	9	8

[a] Determined from the "not retained" fraction from poly(U)-Sepharose chromatography. [b] Values may include radioactivity from CCA turnover. [c] Determined from the "50° eluate" fraction from poly(U)-Sepharose chromatography. [d] Determined from radioactivity from "50° eluate" fraction rendered soluble in 70% ethanol after digestion with T_1 and pancreatic RNases. [e] Determined from radioactivity from "50° eluate" fraction precipitated by 70% ethanol after digestion with T_1 and pancreatic RNases. Precipitate shown to be 98–99% A by electrophoresis of alkaline digest. [f] mRNA synthesis cannot be determined in these experiments, since its separation from other RNA depends on retention on poly(U)-Sepharose.

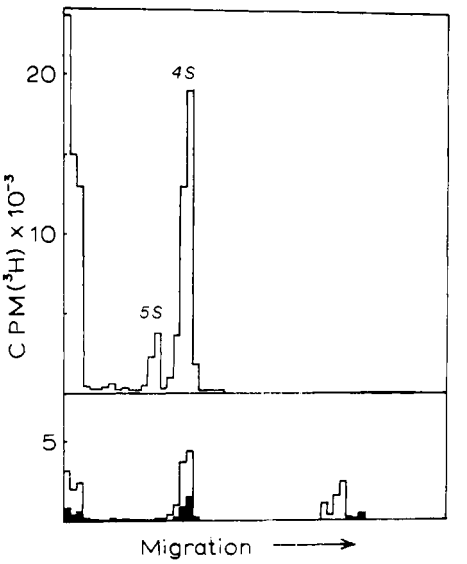

FIGURE 2: Radioactivity profiles (^{3}H) of 2 M NaCl soluble RNA from SDS polyacrylamide gels using 10% polyacrylamide. Top panel gives profile obtained with the control preparation and the bottom panel those obtained with the 3′dCyd and 3′dAdo treated preparations. 3′dCyd is open histogram; 3′dAdo is solid histogram. Profiles have been normalized to show radioactivity extracted from 0.4 cotyledon pair.

treated cotyledons. When the nucleic acid from the control and treated preparations not precipitated by 2 M NaCl is precipitated with ethanol and dissolved in buffer, and aliquots (equivalent to the same amount of starting material) are electrophoresed in the SDS system, ^{32}P radioactivity profiles shown in Figure 1 (bottom) are obtained. In the control preparation a small amount of rRNA is found and the 4S and 5S RNAs are present. These profiles of inhibitor-treated preparations indicate that a small amount of radioactivity is present in the 4S and 5S region of the gel of the 3′dCyd-treated material and almost none in the gel of the 3′dAdo-treated material. Some radioactivity in oligonucleotides of very short chain lengths is observed in both of the gels. This radioactivity may represent oligonucleotide fragments the further synthesis of which has been aborted by the putative 3′CTP and 3′ATP derivatives of the inhibitors.

From these gels it is apparent that the synthesis of the two large rRNA species is prevented by the two 3′d nucleosides at 5 mM concentration in this tissue, which synthesis is generally attributable to RNA polymerase I (Roeder and Rutter, 1970). To examine further the effect of the inhibitors on tRNA and 5S rRNA synthesis, aliquots of the 2 M NaCl soluble fraction were electrophoresed on 10% gels in the SDS system (Figure 2). The radioactivity profiles presented in this figure are of ^{3}H which is shown to distinguish RNA from ^{32}P containing material that does not contain ^{3}H. We have determined that the ^{3}H label is entirely in adenylic acid by determining the molar base ratios of nucleotides in all RNA fractions. Our exclusive use of [2-^{3}H]adenosine precludes the appearance of [^{3}H]GMP in RNA although there is an extensive conversion of adenosine to GMP in this tissue during the time course of the experiment (shown with [^{14}C]adenosine). In Figure 2 the 5S and 4S (tRNA) molecules are resolved as shown by the radioactivity profile obtained from the control preparation (top panel). The relative amounts of 5S to 4S radioactivity are what would be expected were there about 14 tRNA molecules per ribosome in this tissue, a value we have previously reported (Merrick and Dure, 1972), and suggest that these RNAs have been labeled to the same specific activity. The radioactivity profiles obtained from the inhibitor treated preparations show no radioactivity associated in either case with the 5S rRNA. A small amount of radioactivity is associated with tRNA, more in the 3′dCyd-treated preparation than in the 3′dAdo, and again some evidence of ^{3}H-containing oligonucleotide fragments is seen. Whether this small amount of radioactivity in tRNA represents C-C-A turnover exclusively is not known. The existence of ^{3}H labeled oligonucleotide fragments in the 3′dAdo preparation is trivial compared with that discernible with ^{32}P (Figure 1, bottom), which would be anticipated. Thus both 5S and tRNA synthesis, which have recently been reported to be the products of RNA polymerase III (Weinmann and Roeder, 1974), are seen to be sensitive to derivatives of the two 3′d nucleosides.

To test the effects of the inhibitors on mRNA synthesis, the 2 M NaCl precipitated preparations were passed over poly(U)-Sepharose columns. In our hands this procedure yields three fractions of RNA; the first of which passes through the column when applied in 0.4 M NaCl, the second elutes when the column is washed with the buffer solution without NaCl, and the third eluting with the same buffer solution when the elution temperature is raised to 50°. No RNA is retained by the column after this high temperature fraction is eluted. [Actually, the RNA eluted in this last fraction can be subfractionated by temperature into increments that elute from 30 to 50°. The average poly(A) chain length contained in the RNA eluting at the different temperatures is linearly related to elution temperature (B. Harris and L. S. Dure, unpublished data).]

When the RNA of these three elution fractions is ethanol precipitated and redissolved aliquots run on polyacrylamide

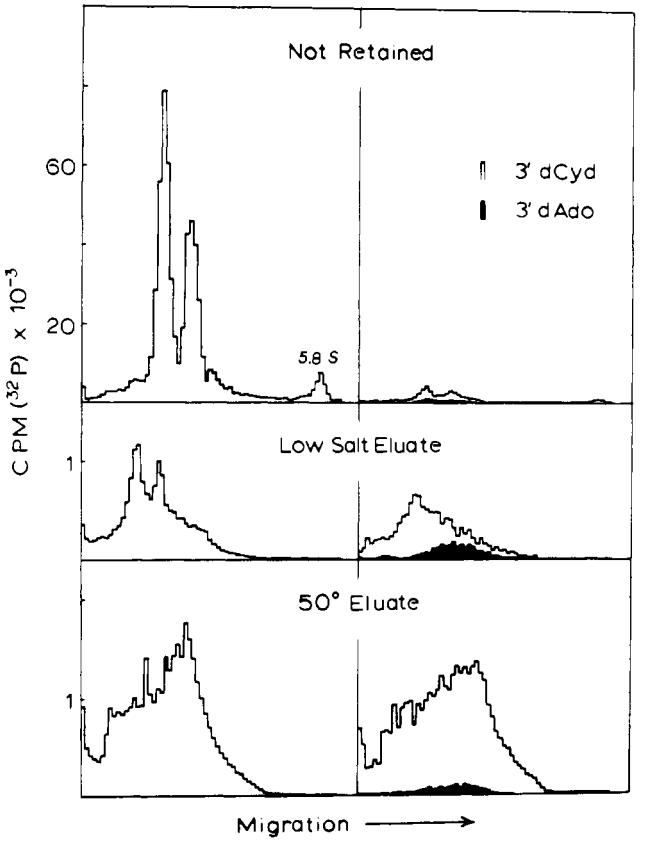

FIGURE 3: Radioactivity profiles (^{32}P) of 99% formamide polyacrylamide gels. Electrophoresis carried out as described by Duesberg and Vogt (1973). Top panels are profiles of RNA not retained on poly(U)-Sepharose columns, the middle panels are profiles of RNA eluted when the column is washed with the buffer solution not containing NaCl, and the bottom panels are profiles of RNA eluted from the column in the buffer solution without NaCl at 50°. In each case the RNA from the control preparation is on the left and that from the 3'dCyd and 3'dAdo treated on the right. The profiles have been normalized to show radioactivity extracted from 0.3 cotyledon pair, but the ordinate has been expanded 25-fold in the middle and bottom panels, since the radioactivity in these fractions is very low compared to the fraction not retained on the column.

gels in the 99% formamide system [to minimize hydrophilic aggregation of RNA molecules (Duesberg and Vogt, 1973)], the radioactivity profiles shown in Figure 3 are obtained. As anticipated, the fraction that is not retained is predominately rRNA (top panels). Notice in the profile from the control preparation (top left panel) the separation of the largest rRNA species from the 18S RNA species is not as great as is found with SDS gels, nor is the amount of the largest rRNA species quite 1.8 times that of the 18S species (the anticipated relationship based on molecular weights). This is due to the release of the 5.8S fragment, which is found in the gel profile, from the 25S rRNA species in formamide. As was seen previously the two 3'd nucleosides have essentially stopped this synthesis in this tissue (top right panel). The radioactivity profile of the RNA fraction from the control preparation that elutes with buffer solution alone (middle left panel) suggests that this RNA fraction contains heterogeneous RNA and perhaps precursor rRNA species, judging from their range of apparent *s* values. Very little poly(A) is obtained from this RNA fraction when it is digested with T_1 and pancreatic RNase, and it has an average chain length of between 15 and 20 AMP residues. This fraction contains about 1.5% of the total radioactivity (^{32}P) of the 2 M NaCl precipitated RNA of the control preparation, thus the ordinate of this and the bottom panels of this figure have been expanded 25-fold relative to the top panel. The nature of the forces causing the retention at high salt of this RNA fraction, whether hydrogen bonding or hydrophobic forces, is not known. Whatever the nature of the RNA in this fraction, its synthesis is only partially prevented by 3'dCyd (middle, right panel). It is tempting to speculate, in view of the effect of 3'dCyd on the appearance of mature rRNA, that the profile obtained with the 3'dCyd-treated preparation in comparison with the control profile results from the uninhibited synthesis of HnRNA but the absence of rRNA precursor synthesis. 3'dAdo seems to have allowed the synthesis of much less material in this fraction. Formamide electrophoresis of the RNA from the 50° poly(U)-Sepharose eluate yields the radioactivity profiles shown in Figure 3, bottom panel. The apparent molecular weight of this RNA in formamide is rather large having a mean of about 20S, although it is much smaller than is seen when electrophoresed in the SDS system (data not shown). This RNA, which contains about 4% of the total radioactivity in the RNA of the control preparation, contains poly(A) chains on the 3'OH ends of the molecules that average 100–120 nucleotides in length (B. Harris and L. S. Dure, unpublished data) and is considered the functioning mRNA of the tissue. The synthesis of this RNA is not inhibited by 3'dCyd as shown in Figure 1 (bottom right panel). Essentially no radioactive RNA is obtained in this fraction from the 3'dAdo-treated preparation. However, this may not indicate an inhibition of mRNA synthesis, but simply its nonretention on the column due to the lack of poly(A) chains, the addition of which to pre-mRNA has been inhibited by the putative 3'dATP.

The amount of radioactivity (both ^{3}H and ^{32}P) in the poly(A) portion of the mRNA-poly(A) of this fraction from control and 3'dCyd-treated material is about twice that expected from a knowledge of the poly(A) chain length, of the percentage of AMP in mRNA and in mRNA-poly(A), and the assumption of an average chain length of mRNA of about 1000–1500 nucleotides. This is due to the fact that some of the poly(A) chains are attached to preexisting mRNA (the stored mRNA) during the first day of germination. Hence there are molecules of mRNA-poly(A) that have radioactive poly(A) but unlabeled mRNA (B. Harris and L. S. Dure, unpublished data).

Discussion

From these data it would seem that *in vivo* RNA synthesis by RNA polymerase I and perhaps III is sensitive to derivatives of 3'dCyd and 3'dAdo. That these derivatives are 3'dCTP and 3'dATP seems likely in view of the findings of Horowitz *et al.* (1974) that RNA synthesis by RNA polymerase I, II, and poly(A) polymerase from yeast is terminated by 3'dATP in a competitive manner in cell-free assays. However, the cotton RNA polymerase II appears to discriminate against 3'dCTP since mRNA-poly(A) synthesis continues in the 3'dCyd-treated material. Conceivably RNA polymerase II discriminates against 3'dATP also, since an inhibition of poly(A) addition to pre-mRNA by 3'dATP would preclude the retention on the poly(U)-Sepharose column of mRNA synthesized in the 3'dAdo-treated cotyledons. The observation in HeLa cells that neither 3'dCyd nor 3'dAdo prevents the synthesis of HnRNA (Siev *et al.*, 1969; Abelson and Penman, 1972) supports this notion.

However, failure of 3'd nucleosides to bring about an inhibition of mRNA synthesis *in vivo* is at odds with the reports that in cell-free systems both RNA polymerases I and II from calf thymus are equally sensitive to 3'dATP (Blatti *et al.*, 1970) and that RNA polymerase II from yeast is even more sensitive to 3'dATP inhibition than RNA polymerase I (Horowitz *et al.*, 1974). Possibly RNA polymerase II has different catalytic specificities *in vivo* and *in vitro*, or perhaps it draws from a distinct nucleotide triphosphate pool which is not readily accessible to nucleotide triphosphate produced from nucleosides.

Very high levels (substrate levels) of 3'dCyd and 3'dAdo are necessary to inhibit RNA polymerase I and perhaps III in this tissue. This high concentration of 3'dAdo is that required to prevent the appearance during germination of carboxypeptidase C activity (Walbot *et al.*, 1974), which we believe to be an activity resulting from the putative stored mRNA of cotton cotyledons (Ihle and Dure, 1969,1970,1972a,b). This is consistent with the notion that these inhibitors act competitively through their 3'd triphosphates. That is, 3'dATP and 3'dCTP must compete with endogenous levels of ATP and CTP. The endogenous concentration of ATP in this tissue at this developmental stage is about 10^{-3} M. Thus the high inhibitor concentration required may not be unreasonable.

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In Vitro Iodination of DNA. Maximizing Iodination While Minimizing Degradation; Use of Buoyant Density Shifts for DNA–DNA Hybrid Isolation†

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ABSTRACT: Conditions have been determined for the two stages of the $TlCl_3$ catalyzed *in vitro* iodination reaction of denatured DNA which maximize iodination while minimizing degradation. Of the cytosine in DNA 50% may be converted to 5-iodocytosine with a melting temperature depression of less than 3°. Chromatography of enzymatic digests of heavily iodinated DNA shows that 5-iodocytosine is the only stable, identifiable DNA base modification. Absorbance at 310 nm, buoyant densities of native and denatured DNA in CsCl and Cs_2SO_4, melting temperatures, and renaturation rates are reported as a function of the degree of iodination. Iodinated λcI 857S7 DNA is used with *Escherichia coli* K12 (λ) DNA to demonstrate DNA–DNA hybrid isolation in a buoyant density gradient.

Commerford (1971) introduced an *in vitro* iodination method for nucleic acids involving the use of $TlCl_3$ and KI. His system is capable of producing a high conversion of cytosine to 5-iodocytosine in denatured DNA and RNA. Furthermore, use of $^{125}I^-$ in the same system leads to DNA or RNA which may be used as a radioactive probe. The documented uses of his method are varied, but have usually dealt with radioisotope labeling. Prensky *et al.* (1973), Scherberg and Refetoff (1973), and Getz *et al.* (1972) have explored the use of ^{125}I labeled RNA in DNA–RNA hybridization experiments. Schmidt *et al.* (1973) have explored the use of iodination as a structural probe in tRNAs. Robertson *et al.* (1973) have used the technique for finger-

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