Some Metabolic Properties of Nucleotides of 1-β-D-Arabino-
furanosylcytosine*

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SUMMARY

Arabinosylcytosine1 is a compound markedly inhibitory to the multiplication of many animal tumor cells and of viruses containing deoxyribonucleic acid. 5'-Nucleotides have been prepared and studied for possible incorporation into polynucleotides. Arabinosylcytosine mono-, di-, and triphosphates were synthesized by both chemical and enzymatic methods. Labeled and unlabeled arabinosylcytosine nucleotides were tested for their capacity to serve as substrates for purified polynucleotide phosphorylase, deoxyribonucleic acid-dependent ribonucleic acid polymerase, deoxyribonucleic acid polymerase, and the enzyme which incorporates adenosine monophosphate and cytidine monophosphate into amino acid acceptor ribonucleic acid. The inhibitory effects of these nucleotides were also determined on these enzymes. Arabinosylcytosine diphosphate was found to be completely inert as a substrate for polynucleotide phosphorylase, although it strongly inhibited the polymerization of adenosine diphosphate and cytidine diphosphate into polyadenylate and polycytidylate, respectively. In mixtures having 15 moles of arabinosylcytosine diphosphate to 85 moles of adenosine diphosphate or cytidine diphosphate, polymerization of the diphosphate substrates catalyzed by polynucleotide phosphorylase was inhibited by more than 95 per cent. No incorporation of tritium-labeled arabinosylcytosine nucleotides was detected in the ribonucleic acid or deoxyribonucleic acid synthesized in the presence of the labeled compounds. Thus, tritiated arabinosylcytosine triphosphate was found to be less than 1 per cent as effective a substrate as cytidine triphosphate for ribonucleic acid polymerase, deoxycytidine triphosphate for deoxyribonucleic acid polymerase, and cytidine triphosphate for the enzyme incorporating adenosine monophosphate and cytidine monophosphate into amino acid acceptor ribonucleic acid. No marked inhibition of these enzymes was produced by arabinosylcytosine triphosphate in amounts equal to the cytidine triphosphate or deoxycytidine triphosphate present. The absence of incorporation of arabinosylcytosine into polynucleotides tends to eliminate some significant potential toxicities in the development of chemotherapy with this compound.

This laboratory has previously described some of the metabolic properties of the D-arabinosylnucleosides of uracil (24, 33), adenine (10), and thymine (6). The properties and possible metabolic significance of these compounds, including arabinosylcytosine,1 have also been summarized earlier (7). The biological activity of 1-β-D-arabinofuranosylcytosine (arabinosylcytosine) in the inhibition of multiplication of tumor cells (5, 8) and of DNA viruses (28, 34) has now led us to a more detailed study of this compound. Arabinosylcytosine is a synthetic nucleoside (35) whose structure, as presented in Chart 1, closely resembles the natural nucleosides, cytidine and deoxycytidine; it has been shown that the inhibitory activity of the analog in animal systems is reversed by deoxycytidine (5, 28). This activity has been postulated to occur by phosphorylation of the nucleoside to a nucleotide which inhibits the conversion of cytidine diphosphate (CDP) to deoxycytidine diphosphate (dCDP) (5, 7). It should be noted that, since this reduction is markedly inhibited by a variety of deoxynucleoside triphosphates (27), it is not
clear whether the inhibition would be effected by the arabinosyl nucleoside diphosphate (ara-CDP) or the triphosphate (ara-CTP).

This laboratory has previously prepared the 5'-monophosphate of arabinosylcytosine (ara-CMP) by an enzymatic method (24) and has described the relative inactivity of this compound as a substrate for the virus-induced enzyme, deoxycytidylate hydroxymethylase (24). In this paper we describe the synthesis by chemical and enzymatic methods of ara-CDP and ara-CTP.

We have used these nucleotides to determine whether they can act as substrates for enzymes capable of polynucleotide synthesis. We wished to know if nucleotides of arabinosylcytosine are inhibitory in such syntheses or can be incorporated into nucleic acid and thereby introduce a potential hazard as a mutagen or other toxicity in the development of the chemotherapy of virus disease or of tumors with the nucleoside. Four partially purified enzymes were tested in these respects: these were polyribonucleotide phosphorylase, the DNA-dependent RNA polymerase, DNA-dependent DNA polymerase, and the enzyme which adds CTP and ATP as the monophosphate to the ends of transfer RNA. We have shown that arabinosylcytosine is not incorporated to any detectable extent into polynucleotides made by these mechanisms. Furthermore, ara-CTP is not highly inhibitory in syntheses requiring ribo- or deoxyribonucleoside triphosphates. However, ara-CDP is markedly inhibitory in the synthesis of polyadenylate or polycytidylate by polynucleotide phosphorylase.

MATERIALS AND METHODS

Sources of compounds.—1-β-d-Arabinofuranosylcytosine and 1-β-β-arabinofuranosylcytosine-H3 were gifts from Dr. J. Hunter of the Upjohn Company. CMP-H3, ATP-8-C14, CMP-2-C14, and dCMP-2-C14 were purchased from Schwarz Laboratories. CMP, 6-azauridinediphosphate (aza-UDP), phosphoenolpyruvate (cyclohexylammonium salt), and pyruvic kinase were purchased from the California Corporation for Biochemical Research. 6-Thioinosine diphosphate (MDP) was a gift from Dr. J. A. Carbon, Abbott Laboratories. Nucleotides not listed above and γ-nitrophosphosphate were purchased from the Sigma Chemical Company. Lyophilized venom of Crotalus adamanteus was purchased from Ross Allen's Reptile Farms. DNase was obtained from Worthington Biochemical Corporation. Protamine sulfate was purchased from Eli Lilly and Company, and streptomycin sulfate was obtained from Nutritional Biochemicals.

Enzyme preparations.—Nucleoside phosphotransferase was purified from wheat germ as described by Barner and Cohen (1), CMP kinase from E. coli as described by Hurwitz (11), polynucleotide phosphorylase from E. coli by the method of Littauer and Kornberg (17), phosphodiesterase from rattle snake venom by the method of Korner and Sinshheimer (15), DNA-dependent RNA polymerase from E. coli as described by Chamberlin and Berg (4), DNA polymerase from E. coli according to the method of Hurwitz et al. (12), and the enzyme from E. coli incorporating CMP and AMP into RNA after the procedure of Preiss et al. (26). Unfractionated rattlesnake venom was used as a source of the 5'-esterase, as described by Potter et al. (25).

Amino acid-acceptor RNA, prepared by a phenol extraction procedure, as described by Nirenberg and Matthaei (23), was isolated by Dr. M. Sekiguchi in this laboratory. The cytidylate (3'-5'-cytidylate) 3'-5'-adenosine terminal grouping at the 3'-hydroxyl end of the sRNA was removed as described by Preiss et al. (26). DNA was prepared by the method of Kay et al. (13).

Analytical methods.—Spectrophotometry was carried out with a Beckman Model DU spectrophotometer. Phosphate was determined by the method of King (14). Protein was determined by the biuret reaction (16), by the method of Lowry et al. (18), and by the method of Sutherland et al. (32) with a standard of twice-recrystallized bovine plasma albumin. Descending paper chromatography was carried out on Whatman No. 1 filter paper with the isobutyric acid-ammonia solvent (19). The papers were developed for 16 hours at 25°C, dried in air, and the nucleotides were located by the quenching of fluorescence in ultraviolet light. Radioactivity was determined either by counting planchets in a windowless gas flow counter or by a liquid scintillation counter (2).
with a Packard Tri-Carb liquid scintillation spectrometer.

Chemical preparation of nucleotides.—Arabinosylcytosine was phosphorylated, and the 5'-isomer was separated from the isomeric cytidylates by the method of Weiss et al. (36). The yield for ara-CMP was 6 per cent, as compared with 30 per cent for CMP. The starting material is deaminated, and the arabinosyl linkage is cleaved to a greater extent than that observed in phosphorylation of cytidine by this method. The separation of the products of such a synthesis is presented in Chart 2.

Ara-CDP was prepared by the method described by Moffatt and Khorana (21) for the synthesis of CDP through the phosphomorpholidate. Both ara-CDP and CDP were obtained in a 70 per cent yield from ara-CMP and CMP, respectively.

Ara-CTP was prepared by a general method with dicyclohexylcarbodiimide used for the synthesis of nucleoside triphosphates, as described by Smith and Khorana (31). The yield of ara-CTP starting from ara-CMP was 10 per cent.

Ara-CMP, ara-CDP, and ara-CTP had ultraviolet absorption spectra and molar absorbancies (calculated from phosphorus content) in 0.1 N HCl identical (within the listed permissible error) with the values given for ara-CMP. Both ara-CDP and ara-CTP had 1 and 2 moles, respectively, of phosphorus per mole of ara-C. Ara-CDP and ara-CTP had 1 and 2 moles, respectively, of inorganic phosphate liberated after 10 minutes at 100° C. in N perchloric acid. The chemically synthesized nucleotides of arabinosylcytosine were considered to be ara-CMP, ara-CDP, and ara-CTP because of the above analyses and the methods of synthesis and isolation. Such materials were used as standards for the chromatographic identification of nucleotides of arabinosylcytosine prepared enzymatically.

Enzymatic synthesis of nucleotides containing cytosine.—

Monophosphates: Ara-CMP and ara-CMP-H³ were synthesized by a slight modification of an enzymatic synthesis (1, 24) involving the transfer of an esterified phosphate from p-nitrophenyl phosphate to the 5'-hydroxyl of a recipient nucleoside. The reaction mixture contained sodium acetate buffer, 0.20 M pH 4.0; arabinosylcytosine, 0.140 M; sodium p-nitrophenylphosphate, 0.225 M; and 1.1 mg/ml of wheat shoot extract protein. Incubation of the reaction mixture at 30° C. was continued until the formation of p-nitrophenol had stopped (spectrophotometrically determined at pH 12, λ 18,200, λ 400). Production of p-nitrophenol usually continued for about 6 hours and reached a concentration of approximately 0.10 M.

Yields of ara-CMP could be increased by repeated incubation periods after removal of p-nitrophenol by three extractions with 2 volumes of ether, addition of fresh enzyme, and adjusting the p-nitrophenylphosphate concentration to its original level. More than 97 per cent of the p-nitrophenol could be removed by three successive extractions each with 2 volumes of ether. The volume was reduced by evaporation under reduced pressure at 30° C. in order for additions of enzyme and p-nitrophenol to be made. After removal of p-nitrophenol the reaction mixture was applied to a Dowex-1-formate column, and ara-CMP was selectively eluted with 0.05 M formic acid. The formic acid was removed by evaporation in vacuo at 40° C., and ara-CMP was crystallized as the free acid from a concentrated aqueous solution at 0° C. after the addition of one volume of acetone.

Diphosphates: Ara-CDP-H³ and CDP-H³ were synthesized by a transfer of phosphate catalyzed by CMP kinase from ATP to ara-CMP-H³ and CMP-H³ (11). A typical reaction mixture used for the synthesis of diphosphates contained 0.1 M potassium phosphate buffer (pH 7.0), 0.0 M MgCl₂, 3 X 10⁻⁴ M ATP, 7 X 10⁻⁴ M ara-CDP-H³, and 168 units of CMP kinase per ml.; the mixture was incubated at 37° C. for 30 minutes. The reaction mixture was diluted 40-fold with water, adjusted to pH 8, and applied to a Dowex-1-formate column. Nucleotides were separated in the following sequence: ara-CMP, ara-CDP, and ara-CTP with 0.05 M formic acid, AMP with 0.2 M formic acid, and CDP or ara-CDP with 0.25 M ammonium formate, pH 4.9. The diphosphate fraction was then diluted, adjusted to pH 8 with NH₄OH, and applied to a Dowex-1-bicarbonate column. After the column was washed with water, the nucleotide was eluted with 0.2 M ammonium bicarbonate. The pH of the mixture was adjusted to 4.5 with Dowex 50-H⁺, and the resin was removed by filtration. The pH was adjusted to 7.0 with 0.1 N KOH at 0° C., and the volume was reduced by evaporation in vacuo at 30° C.

Triphosphates: Enzymatic synthesis of ara-CTP-H³, CTP-H³, CTP-H³, and dCTP-2-C⁴ and dCTP-2-C⁴ was similar to the method used for the synthesis of diphosphates except that an ATP-generating system of phosphoenolpyruvate and pyruvic kinase was added (26). A typical reaction mixture contained 52 mM Tris buffer, pH 7.4; 6 mM MgCl₂; 10 mM KCl; 0.4 mM ATP, CMP, dCMP, or ara-CMP at less than 1.0 mM; phosphoenolpyruvate, 6 mM; pyruvic kinase, 0.3 mg/ml; and 168 units of CMP kinase per ml. The reaction mixture was incubated at 37° C. until an analysis of an aliquot of the reaction mixture by means of column chromatography (using Dowex-1-formate as

<table>
<thead>
<tr>
<th>Marker spot</th>
<th>Rp*</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCMP</td>
<td>0.53-0.60</td>
</tr>
<tr>
<td>dCDP</td>
<td>0.35-0.43</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.18-0.24</td>
</tr>
<tr>
<td>Ara-CMP</td>
<td>0.42-0.50</td>
</tr>
<tr>
<td>Ara-CDP</td>
<td>0.28-0.33</td>
</tr>
<tr>
<td>Ara-CTP</td>
<td>0.14-0.22</td>
</tr>
<tr>
<td>CMP</td>
<td>0.42-0.50</td>
</tr>
<tr>
<td>CDP</td>
<td>0.21-0.30</td>
</tr>
<tr>
<td>CTP</td>
<td>0.12-0.18</td>
</tr>
</tbody>
</table>

* Descending paper chromatography of enzymatically synthesized phosphates in the isobutyric acid-ammonia solvent.

TABLE 1

CHROMATOGRAPHY OF NUCLEOSIDE PHOSPHATES

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<table>
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<th>Marker spot</th>
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<tr>
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<td>Ara-CMP</td>
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<td>Ara-CTP</td>
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<tr>
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<td>0.21-0.30</td>
</tr>
<tr>
<td>CTP</td>
<td>0.12-0.18</td>
</tr>
</tbody>
</table>
previously described under Diphosphates) indicated that more than 69 per cent of the nucleoside monophosphate had disappeared. The products were separated on a column of Dowex-1-formate. The cytosine nucleoside monophosphates, AMP, and cytosine nucleoside diphosphates were removed as described under the synthesis of diphosphates. ADP was removed with 3.0 M formic acid, and cytosine nucleoside triphosphates were eluted with 0.5 N ammonium formate, pH 4.9. Formate was removed from the triphosphate fraction as previously described for diphosphates with absorption on a Dowex-1-bicarbonate column and elution with 0.3 M ammonium bicarbonate. The bicarbonate was decomposed with Dowex-50-H+, and the resin was filtered. The pH of the solution was adjusted to 7.0 with 0.1 N KOH at 0°C, and the volume was reduced by evaporation in vacuo at 30°C.

The enzymatically synthesized radioactive nucleotides were identical to the chemically synthesized standards when analyzed by paper chromatography (Table 1) and, as will be seen below, by enzymatic reactivities as well.

RESULTS

STUDIES WITH POLYNUCLEOTIDE PHOSPHORYLASE

The incorporation of nucleoside diphosphate into polynucleotides catalyzed by polynucleotide phosphorylase was carried out as described by Littauer and Kornberg (17). The reaction mixture of 0.5 ml. contained glycylglycine buffer, 0.2 M, pH 7.4; MgCl₂, 4 mM; nucleoside diphosphate, approximately 3 mM; and 36 /µg of of polynucleotide phosphorylase (1 mg. of our preparation of polynucleotide phosphorylase catalyzed the polymerization of 7 mmole of ADP per hour). The reactions were stopped by adding perchloric acid to a concentration of 1.4 per cent and 0.8 mg. of bovine plasma albumin. The precipitate was collected by centrifugation, washed twice with 1 per cent perchloric acid, and once with 0.01 N HCl. The polynucleotide was hydrolyzed by heating for 15 hours at 37°C in 1.0 N KOH. Polynucleotide phosphorylase used in these experiments incorporated ADP or CDP into an acid-insoluble polynucleotide (polyadenylate or polycytidylate) which increased in amount in a linear fashion until about 50 per cent of the diphosphate substrate had disappeared. At this point the amount of acid-insoluble polynucleotide remained constant for several hours and decreased slowly upon prolonged incubation. During the course of polymerization, the acid-soluble fraction of the reaction mixture was assayed for the appearance of inorganic orthophosphate and the disappearance of ADP, while the appearance of organic phosphorus and adenine or cytosine was determined spectrophotometrically in the acid-insoluble material.

The inhibition of polynucleotide phosphorylase by ara-CDP.—As can be seen in Tables 2 and 3, in the absence of the analogs approximately 1 mole of inorganic phosphate appeared for each mole of adenine or cytosine which disappeared in the acid-soluble fraction and for each mole of organic phosphorus and adenine or cytosine which appeared in the acid-insoluble material. Even after prolonged incubation periods most of the nucleoside diphosphate which had not been incorporated into polynucleotide chains could be recovered from the reaction mixture. Thus, these materials had not been eliminated by deg-

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor (µM)</th>
<th>µmoles PO₄ formed</th>
<th>µmoles CDP polymerized</th>
<th>Per cent inhibition</th>
<th>Inhibitor X 100 total initial nucleoside diphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0.370</td>
<td>0.300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MDP</td>
<td>3.0</td>
<td>0.252</td>
<td>0.118</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>MDP</td>
<td>5.0</td>
<td>0.074</td>
<td>0.018</td>
<td>94</td>
<td>14</td>
</tr>
<tr>
<td>MDP</td>
<td>10.0</td>
<td>0.090</td>
<td>0.007</td>
<td>97</td>
<td>25</td>
</tr>
<tr>
<td>Aza-UDP</td>
<td>3.0</td>
<td>0.200</td>
<td>0.150</td>
<td>48</td>
<td>7</td>
</tr>
<tr>
<td>Aza-UDP</td>
<td>5.0</td>
<td>0.152</td>
<td>0.039</td>
<td>86</td>
<td>14</td>
</tr>
<tr>
<td>Aza-UDP</td>
<td>10.0</td>
<td>0.110</td>
<td>0.018</td>
<td>94</td>
<td>25</td>
</tr>
<tr>
<td>Ara-CDP</td>
<td>1.3</td>
<td>0.323</td>
<td>0.201</td>
<td>30</td>
<td>4</td>
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<tr>
<td>Ara-CDP</td>
<td>3.8</td>
<td>0.184</td>
<td>0.104</td>
<td>64</td>
<td>11</td>
</tr>
<tr>
<td>Ara-CDP</td>
<td>5.4</td>
<td>0.136</td>
<td>0.055</td>
<td>81</td>
<td>14</td>
</tr>
</tbody>
</table>

The reaction mixture and abbreviations are the same as those for Chart 3.
Ethanol I fraction of polynucleotide phosphorylase per ml. An incubation period of 2 hours at 37° C. was used.

The reaction mixture contained ADP or CDP, 3.2 × 10^{-3} M; MgCl₂, 4.0 × 10^{-3} M; glycylglycine buffer, 0.2 M, pH 7.4; and 0.24 units of an ethanol I fraction of polynucleotide phosphorylase per ml. An incubation period of 2 hours at 37° C. was used.

These results indicate that the polynucleotide phosphorylase used in these experiments has the properties previously reported for this enzyme (9, 17).

Per cent inhibition = moles of adenine or cytidine in polymer of inhibited reaction
1 - moles of adenine or cytidine in polymer of uninhibited reaction

▲ ara-CDP (arabinosylcytosine-5'-diphosphate).
△ aza-UDP (6-aza-uridine-5'-diphosphate).
○ MDP (6-thioinosine-5'-diphosphate).
× dCDP (deoxyctydine-5'-diphosphate).

Chart 3.—Inhibitory effect of ara-CDP, aza-UDP, and MDP on the polymerization of ADP to polyadenylate and CDP to polycytidy late catalyzed by polynucleotide phosphorylase. The reaction mixture contained ADP or CDP, 3.2 × 10^{-3} M; MgCl₂, 4.0 × 10^{-3} M; glycylglycine buffer, 0.2 M, pH 7.4; and 0.24 units of an ethanol I fraction of polynucleotide phosphorylase per ml. An incubation period of 2 hours at 37° C. was used.

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These results indicate that the polynucleotide phosphorylase used in these experiments has the properties previously reported for this enzyme (9, 17).

Although about 50 per cent of the ADP and CDP in the reaction mixture would readily be polymerized by polynucleotide phosphorylase, no polymerization of ara-CDP could be detected. The addition of ara-CDP to reaction mixtures containing ADP or CDP produced a marked inhibition in their polymerization, as can be seen in Chart 3. When ara-CDP was compared with aza-UDP (30) and MDP (3) (two other nucleoside diphosphates reported to inhibit polynucleotide phosphorylase), the degree of inhibition produced, as shown in Chart 3, was virtually identical for the same concentration of inhibitor.

However, one exception to this can be noted in the effect of MDP on the polymerization of ADP. MDP appeared to be incorporated into the polynucleotide resulting from polymerization of nucleoside diphosphate mixtures containing ADP and small amounts of MDP. The evidence for this incorporation rests on spectrophotometric identification—i.e., by the absorption at λ 312 of the mercaptopurine nucleotide in hydrolysates of the polyadenylate synthesized in the presence of MDP. This absorption could not be detected in control tubes to which MDP was added at the end of the incubation period. Although MDP has previously been reported to be a more potent inhibitor of polynucleotide phosphorylase (3) than was found to be the case in this work, the enzymes used in the two investigations originated from different sources, and polynucleotide phosphorylases from different sources have been reported to give different results with nucleotide analogs (9). The apparent incorporation in our system may account for the diminished inhibitory effect toward the polymerization of ADP shown by MDP. As can be seen from Tables 2 and 3, a molar ratio of inhibitor to substrate of 15/85 inhibited polynucleotide formation by greater than 95 per cent. The per cent inhibition of the polymerization of CDP and ADP was calculated by the following equation:

1 - moles nucleotide polymerized in an inhibited reaction
moles nucleotide polymerized in an uninhibited
× 100 = % inhibition.

The attempted incorporation of ara-CDP-H³ into chains of polyadenylate and polycytidy late.—In these experiments ara-CDP-H³ (250 × 10⁶ counts/min/µmole) at 40 or 80 µM concentration was added to the reaction mixtures previously described, which contained either 3.3 mm ADP or 2.3 mm CDP. The reaction mixtures were incubated in the presence of 144 µg. of polynucleotide phosphorylase per ml. for the desired lengths of time, as shown in Table 4, and the radioactivity present in the washed acid-insoluble polynucleotide chains was determined. In none of the experiments could more than 10 counts/min above background be detected in the polynucleotide chains, although at this concentration of ara-CDP-H³, inhibition to the polymerization of ADP and CDP was not severe. As shown in Table 4, prolonged incubation periods indicated that as much as 50 per cent of a true diphosphate substrate could be incorporated into polynucleotide chains.
chains. A control sample of CDP-H₃, prepared and purified in the same manner as the ara-CDP-H₃, was used to check experimental manipulations and counting procedures. Such a control was a normal noninhibitory substrate.

As can be seen in Table 4, these experiments exclude the possibility that ara-CDP-H₃ was incorporated to any measurable extent within the polynucleotide chains. In order to exclude the incorporation of ara-CDP-H₃ into terminal positions of polycytidylic acid, a relatively large amount of the polynucleotide was synthesized in the presence of ara-CDP-H₃ at a 16 µM concentration. After a 2-hour incubation period less than 10 counts/min above background could be detected in the polynucleotide fraction in which 5.7 µmoles (38 per cent) of the CDP had been incorporated into polycytidylic acid. Thus, the polycytidylic acid chains contained 5.7 µmoles of cytidine and less than 4 × 10⁻⁴ µmoles of arabinosylcytosine or 142,000 moles of CMP for each mole of ara-CMP. This result requires that the polycytidylic acid chains must have an average molecular weight greater than 460,000 before even 1 per cent of the chains could be terminated with an arabinosylcytosine residue. The highest average molecular weight reported for polycytidylic acid synthesized in a similar manner is 500,000 (9).

Ara-CTP as a substrate for the enzyme incorporating AMP and CMP into amino acid-acceptor RNA.—Experiments were carried out with an ammonium sulfate fraction of the enzyme (26). The enzyme at this stage of purification did not degrade the unreacted nucleoside triphosphate substrate. All procedures, including the purification of the enzyme, were carried out essentially as described by Preiss et al. (26). The 0.5-ml. reaction mixture contained potassium phosphate buffer, 50 mM, pH 7.5; MgCl₂, 10 mM; 2-mercaptoethanol, 10 mM; diesterase-treated RNA, 1.4–2.0 mM (with respect to individual nucleotide units); ATP-8-C¹⁴, 6.5 × 10⁴ counts/min/µmole; 0.2 mM; CTP-2-C¹⁴ (3.5 × 10⁴ counts/min/µmole) 0.06 mM; 2.0 mM phosphoenol pyruvate; 50 µg. pyruvic kinase; and enzyme. Omission of RNA, Mg⁺⁺, or enzyme resulted in a 99 per cent loss of the ability to incorporate CTP-2-C¹⁴ into acid-insoluble material. The incorporation of nucleotide was stimulated in a linear fashion by the addition of amino acid acceptor RNA. All procedures, including the purification of the enzyme, were carried out essentially as described by Preiss et al. (26). The CTP-H₃ control, synthesized and purified in the same manner as the ara-CTP-H₃, served as a check on experimental manipulations and counting procedures. In these experiments no incorporation of ara-CTP-H₃ could be detected by radioactivity in the polymer fraction; it can be stated that ara-CTP-H₃, if active as a substrate at all, is not more than 1 per cent as effective as CTP-H₃ as a substrate.

Failure of the DNA-Dependent RNA Polymerase To Incorporate Ara-CTP-H₃ into RNA

The reaction mixture contained 40 µM labeled ribonucleoside triphosphates (270–650 counts/min/µmole), 80 µM for each of the other three ribonucleoside triphosphates, 8 µM MgCl₂, 2 mM MnCl₂, 50 µM Tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.6), calf thymus DNA equivalent to 150 µmole deoxynucleotides, and 32 µg. of the Fraction 4 enzyme described by Chamberlin and Berg (4). Table 5 shows that the Fraction 4 enzyme had an absolute DNA dependence and that removal of CTP from the reaction mixture decreased the ATP-8-C¹⁴ incorporation by approximately 83 per cent. The remaining activity was perhaps due to formation of polyadenylate (4). Magnesium and manganese were required for highest activity. At the end of the 20-minute incubation period at 37° C., most of the unreacted nucleoside triphosphates would be recovered from the reaction mixture. The requirements for RNA synthesis summarized in Table 5 are those previously reported for the DNA-dependent RNA polymerase (26).

The Effect of ara-CTP and dCTP on the Incorporation of Labeled Nucleoside Triphosphates into RNA.—The addition of dCTP and ara-CTP in amounts equal to the labeled CTP present in the reaction mixture did not have a marked effect upon the incorporation of the CTP into RNA as can be seen in Chart 4. Replacing CTP by dCTP or ara-CTP did not stimulate ATP-8-C¹⁴ incorporation into RNA (Table 5). From these results it was apparent that

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**TABLE 5**

**Requirements for RNA Synthesis**

<table>
<thead>
<tr>
<th>Component</th>
<th>µmoles AMP-8-C¹⁴ incorporated</th>
</tr>
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<tbody>
<tr>
<td>Complete system</td>
<td>0.510</td>
</tr>
<tr>
<td>Minus DNA</td>
<td>0.005</td>
</tr>
<tr>
<td>Minus Mn⁺⁺⁺</td>
<td>0.130</td>
</tr>
<tr>
<td>Minus CTP</td>
<td>0.090</td>
</tr>
<tr>
<td>CTP replaced by dCTP</td>
<td>0.125</td>
</tr>
<tr>
<td>CTP replaced by ara-CTP</td>
<td>0.045</td>
</tr>
</tbody>
</table>

*Mg⁺⁺ could not be completely eliminated, because it was in the solution containing the enzyme. The reaction mixture is described in the text. 32 µg. Fraction 4 were used in a 20-minute incubation period at 37°C.

In this experiment CTP in the reaction mixture was replaced with ara-CTP-H₃ at 0.08 mM concentration, and the reaction mixture was incubated for 60 minutes at 37° C. At the end of the incubation period the acid-insoluble polynucleotide fraction was washed and the radioactivity determined by liquid scintillation spectrophotometry. The CTP-H₃ control, synthesized and purified in the same manner as the ara-CTP-H₃, served as a check on experimental manipulations and counting procedures. In these experiments no incorporation of ara-CTP-H₃ could be detected by radioactivity in the polymer fraction; it can be stated that ara-CTP-H₃, if active as a substrate at all, is not more than 1 per cent as effective as CTP-H₃ as a substrate.
ara-CTP was unable to replace CTP in stimulating the incorporation of ribonucleoside triphosphates into RNA.

**Attempted incorporation of ara-CTP·H³ into RNA.**—The capacity of RNA polymerase to incorporate ara-CTP·H³ and dCTP-2-C¹⁴ into RNA was tested by replacing CTP with these two substances in the reaction mixture. The reaction mixture was the same as previously used, except that all four ribonucleoside triphosphates were present at 100 μM concentration. Several different incubation periods were used as indicated in Chart 4.

Although some dCTP-2-C¹⁴ incorporation was observed (about 20 per cent as much as the CTP-2-C¹⁴ control), no incorporation of ara-CTP·H³ was detected. The reason for incorporation of dCTP into the polynucleotide under these conditions is unexplained. From these experiments it was determined that ara-CTP·H³ was incorporated into RNA less than 0.3 per cent as effectively as CTP·H³.

**Ara-CTP·H³ as a substrate for DNA polymerase.**—The ASII fraction of DNA polymerase and the experimental procedures are the same as those described by Hurwitz et al. (12). The reaction mixture contained dCTP-2-C¹⁴, 10 μM; 15 μM each of dATP, dGTP, and dTTP; calf thymus DNA equivalent to 100 μmole of deoxynucleotides; MgCl₂ 8 mM; 2-mercaptoethanol, 4 mM; calf thymus DNA equivalent to 100 μmole deoxynucleotides and 50 μg of the ammonium sulfate fraction of DNA polymerase in 0.5 ml. Ara-CTP and dCTP additions were made as indicated at 10 μM concentration.

- ▲ dCTP-C¹⁴ incorporation, no additions.
- ▼ dCTP-C¹⁴ incorporation, no additions.
- ▼ ara-CTP·H³ incorporation, no additions.
- ▲ ara-CTP·H³ incorporation, ara-CTP added.
- ▼ ara-CTP·H³ incorporation, CTP added.

**TABLE 6**

<table>
<thead>
<tr>
<th>Additions</th>
<th>μmole incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system with “activated” DNA (17)</td>
<td>1.13</td>
</tr>
<tr>
<td>Complete system with “native” DNA</td>
<td>0.5</td>
</tr>
<tr>
<td>Omit DNA</td>
<td>0.03</td>
</tr>
<tr>
<td>Omit dATP or dGTP or dTTP</td>
<td>0.03</td>
</tr>
<tr>
<td>Omit dATP and dGTP and dTTP</td>
<td>0.03</td>
</tr>
<tr>
<td>Omit Mg⁺⁺</td>
<td>0.03</td>
</tr>
<tr>
<td>Replace dATP, dGTP, dTTP with ATP, GTP, UTP</td>
<td>0.03</td>
</tr>
</tbody>
</table>

The complete system is described in the text. "Activated" calf thymus DNA has been described (17).

**Chart 5.**—The effect of ara-CTP and CTP on the incorporation of cytosine nucleotides into DNA catalyzed by DNA polymerase. The reaction mixture contained dCTP-2-C¹⁴ (500 counts/min/μmole), CTP-2-C¹⁴ (500 counts/min/μmole), or ara-CTP·H³ (280 counts/min/μmole), 10 μM; dATP, 15 μM; dGTP, 15 μM; MgCl₂ 18 mM; 2-mercaptoethanol, 4 mM; calf thymus DNA equivalent to 100 μmole deoxynucleotides and 50 μg of enzyme. The requirements of the enzyme preparation for the synthesis of DNA are given in Table 6. The enzyme at this stage of purification has the properties described for DNA polymerase and does not degrade the nucleoside triphosphates which are not incorporated into DNA.

The effect of ara-CTP and CTP on the incorporation of dCTP-2-C¹⁴ into DNA.—As can be seen in Chart 5, CTP or ara-CTP added to the reaction mixture at the same concentration as dCTP-2-C¹⁴ had no effect on the incorporation of dCTP-2-C¹⁴ into DNA.

**Attempted incorporation of CTP-2-C¹⁴ and ara-CTP·H³ into DNA.**—The attempted incorporation of CTP-2-C¹⁴ and ara-CTP·H³ into DNA was studied by replacing CTP-2-C¹⁴ in the reaction mixture with these two nucleoside triphosphate isomers. The reaction mixture was incubated for 80 minutes at 37° C. The polynucleotide was precipitated and washed, and the radioactivity was determined by liquid scintillation counting. Under these conditions no incorporation of CTP-2-C¹⁴ or ara-CTP·H³ into the polynucleotide fraction could be detected. It can be stated that if ara-CTP·H³ serves as a substrate it is less than 0.2 per cent as effective as dCTP-2-C¹⁴.
DISCUSSION

It is of interest that arabinosyleytosine and its derivatives do participate actively in many enzymatic reactions—e.g., deamination of arabinosyleytosine by cytidine deaminase of E. coli (24), phosphorylation of arabinosyleytosine by wheat phosphotransferase (24), phosphorylation of ara-CMP by kinases of E. coli to ara-CDP and ara-CTP, etc. It is clear then that the epimerization of the 2'-OH in the sugar does not so distort the structure as to prevent the arabinosynucleosides from approaching a variety of catalytic sites in several enzymes. Indeed, the pK of the amino group of ara-CMP approaches that of CMP, and since this value is affected considerably by the relative proximity of the 5'-phosphate it may be supposed that distances between amino group and phosphate are similar in the two compounds.

On the other hand, arabinosyluracil is not a good substrate for nucleoside phosphorylase (33), and ara-UMP and ara-CMP are poor substrates for thymidylate synthetase and dCMP hydroxymethylase, respectively (24). Polynucleotide phosphorylase is incapable of using ara-CDP, which is nevertheless as good an inhibitor of this enzyme as are other analogs. Actually, ara-UDP, described by Michelson and Grunberg-Manago (20), and ara-CDP described in this paper are the only known inhibitors of this enzyme which contain an apparently unnatural sugar.

The capacity of these diphosphates to inhibit polyribonucleotide synthesis by polynucleotide phosphorylase but not to be incorporated highlights the importance of the 2' position in all polynucleotide synthesis. Thus, under normal conditions of growth and reproduction the productivity of RNA devoid of deoxynucleotides and of DNA devoid of ribonucleotides appears to be the rule. The exclusion of incorporation of ara-CTP into polynucleotides by the variety of enzymes tested above, enzymes which are concerned with synthetic reactions, further emphasizes this point. The fact that ara-CTP is not even markedly inhibitory in these reactions perhaps emphasizes the rigorous steric requirement at the 2' position for enzymes recognized to play a synthetic role in contrast to the action of ara-CMP with polynucleotide phosphorylase whose major role appears to be degradative (29).

In any case, the exclusion of nucleotides of arabinosyleytosine from polynucleotides suggests rather strongly that these compounds will not present some of the toxic hazards when used in chemotherapy, hazards which appear with compounds which do enter nucleic acid. Fluorouracil, which is incorporated into RNA, can lead to phenotypic changes in protein structures. Bromouracil, which is incorporated into RNA, can lead to the compound is reversible by deoxycytidine. The control of pathology provoked by long-term administration of the drug is, of course, a continuing problem, as indeed it is for many compounds, such as fluorodeoxyuridine, which produce a relatively specific inhibition of DNA synthesis and thereby affect both rapidly dividing normal and pathological cells.

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Cancer Res 1964;24:1595-1603.

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