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TERMINATION OF RNA BY NUCLEOTIDES OF 9-β-D-XYLOFURANOSYLADENINE

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SUMMARY: Incubation of CHO cells with 21 μM [^{3}H]9-β-D-xylofuranosyladenine for 2 hr resulted in the intracellular accumulation of 9-β-D-xylofuranosyladenine 5'-triphosphate and the incorporation of radioactivity into $HClO_4$ -insoluble material. After incubation of the $HClO_4$ -insoluble material in 0.3 N KOH for 16 hr, the radioactivity associated with the nucleoside fraction coeluted with authentic 9-β-D-xylofuranosyladenine by high-pressure liquid chromatography and was resistant to oxidation with $NaIO_4$. Upon incubation with adenosine deaminase (E.C. 3.5.4.4),the radioactivity coeluted with xylosylhypoxanthine. The data suggest that termination of RNA chains by xylosyl nucleotides may be a mechanism for producing the toxicity of 9-β-D-xylofuranosyladenine.

Studies of the mechanism of cytotoxicity of xylA have revealed several routes of metabolism and sites of inhibition. The nucleoside is phosphorylated initially by adenosine kinase (E.C. 2.7.1.20) (1) and subsequently accumulates as the 5'-triphosphate, xylATP (2-4). Further metabolism of this compound to the xylosyl analogs of S-adenosylmethionine and/or S-adenosyl homocysteine (5) may contribute to the observed inhibition of nuclear RNA methylation and processing (6-8). The observation that xylATP inhibited synthesis of 5-phosphoribosyl-1-prophosphate (9) appeared to provide an explanation for the associated inhibition of nucleic acid synthesis (2-4,6). Our studies demonstrated that cells that had accumulated high concentrations

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Abbreviations used are: HPLC, high pressure liquid chromatography; xylA, 9-β-D-xylofuranosyladenine; xylATP, 9-β-D-xylofuranosyladenine 5'-triphosphate.

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of xylATP were rapidly inhibited in their nucleic acid synthesis and had lost the ability to form clones (3,4). However the fact that these cells were not measurably affected in their content of 5-phosphoribosyl-1-pyrophosphate, ribonucleosides, or deoxyribonucleosides (4) encouraged us to search for alternative mechanisms by which xylA might exert its lethal action.

We reasoned that once a xylosyl nucleotide was incorporated into the 3'-terminus of a nascent RNA chain, the configuration of the 3'-hydroxyl group would not be a suitable substrate for the formation of a 3'-5' phosphodiester linkage with a subsequent nucleotide and probably would function to terminate synthesis of that RNA chain. Therefore, the ability of CHO cells to incorporate xylosyl nucleotides into the 3'-termini of RNA was evaluated as a general mechanism of the toxicity of xylA.

MATERIALS AND METHODS

XylA and the adenosine deaminase inhibitor, 2'-deoxycoformycin, were provided by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. [^{3}H]xylA was prepared by ICN Pharmaceuticals and purified to greater than 99.7% purity by HPLC (4). Adenosine deaminase was obtained from Sigma Chemical Co.

Cell Culture. A line of CHO cells, designated AA^r-6/AG^r-7, deficient in AMP:pyrophosphate phosphoribosyltransferase (E.C. 2.4.2.7) and IMP:pyrophosphate phosphoribosyltransferase (E.C. 2.4.2.8.) was obtained from Dr. P. P. Saunders of this department. Cells were grown in 850 cm^2 roller bottles containing 75 ml of McCoy's 5a medium with 10% fetal bovine serum until 80% confluent. The cell number and the mean cell volume were determined by a model ZBI Coulter Counter equipped with a model C-1000 channelyzer.

HPLC of Nucleotides and Nucleosides. Cellular nucleotides were extracted with $HClO_4$ and separated and quantitated exactly as described (4). Standard mixtures of nucleosides and various fractions of the 3'-terminal nucleosides isolated after base hydrolysis of RNA were separated by elution from a column of μBondapak C_{18} (Waters Associates) at 2 ml/min with 5% methanol: 95% H_2O (v/v).

RESULTS

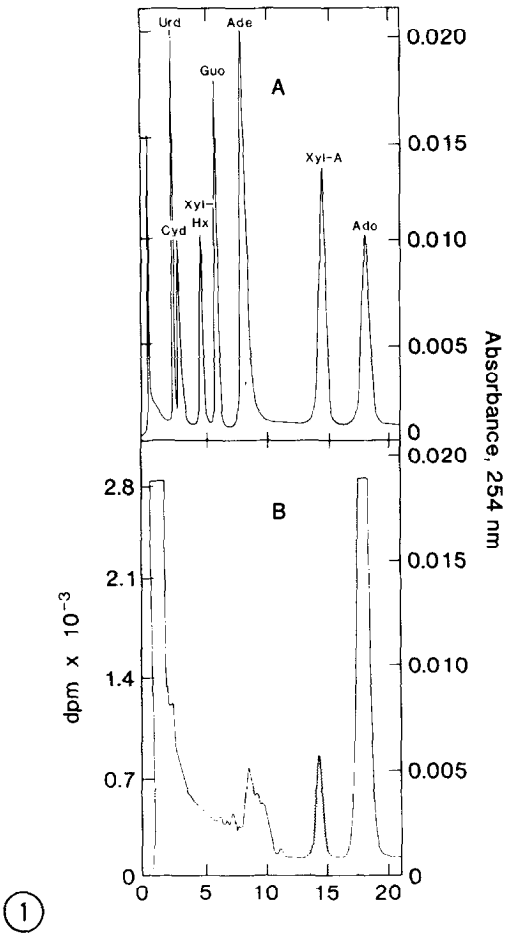
After a 30-min preincubation with 10 μM 2'-deoxycoformycin, [^{3}H]xylA was added to the cell culture at a final concentration of 20.6 μM. Soluble nucleotides were extracted with $HClO_4$ after 2 hr and nucleoside triphosphates were separated by HPLC and quantitated as described (4). The cellular concentrations or ribonucleoside triphosphates were: CTP, 1.08 mM; UTP, 2.32 mM; ATP, 4.96 mM; GTP, 1.61 mM. These concentrations are

comparable to those determined in wild-type CHO cells (4). The intracellular concentration of xylATP was calculated to be 1.27 mM from comparison of the uv absorbance with that of standards, and by determining the amount of radioactivity associated with xylATP in the column eluate. The radioactivity associated with ATP was less than 0.5% of that of xylATP.

$HClO_4$-insoluble material was washed and RNA was hydrolyzed by incubation with 0.3 N KOH for 16 hr at 37°. The alkali resistant material was removed by precipitation with $HClO_4$. After washing, no radioactivity greater than background was detected in the $HClO_4$-precipitable, alkali stable material that contained the DNA. The 3'-terminal nucleosides liberated by base hydrolysis were separated from 2'(3') nucleotides by retention on a cartridge of Sep-Pak μC_{18} (Waters Associates). After washing with H_2O to remove 2'(3') ribonucleotides, adenine nucleosides were eluted with 1 ml of 50% methanol (v/v) and reduced to dryness before separation by HPLC. The separation of a mixture of standard nucleosides and adenine is shown in Fig 1A, for reference, and that of the 3'-terminal nucleosides from the hydrolyzed RNA in Fig 1B. The major radioactive fraction (2219 dpm) coeluted with unlabeled xylA that had been added as an internal marker. Only 362 dpm were associated with adenosine, and no radioactivity was detected with adenine.

The following experiments were carried out to provide more evidence for the identity of the radioactive material associated with xylA in Fig 1B. The remaining volumes of each fraction eluting with xylA in Fig 1B were pooled and evaporated to dryness. After dissolving in phosphate buffer, the material was incubated with adenosine deaminase. Separation of the products of a control deamination reaction mixture containing authentic [^{3}H]xylA carried out in parallel is shown in Fig 2A. Conversion of authentic xylA to xylosylhypoxanthine was quantitative. Identical HPLC analysis of the deamination products of the 3'-terminal nucleoside that eluted with xylA in Fig 1B resulted in a 90% conversion to a compound that coeluted with xylosylhypoxanthine (Fig 2B).

To investigate the nature of the carbohydrate portion of the radioactive 3'-terminal nucleosides, a fraction of this material, purified, through the



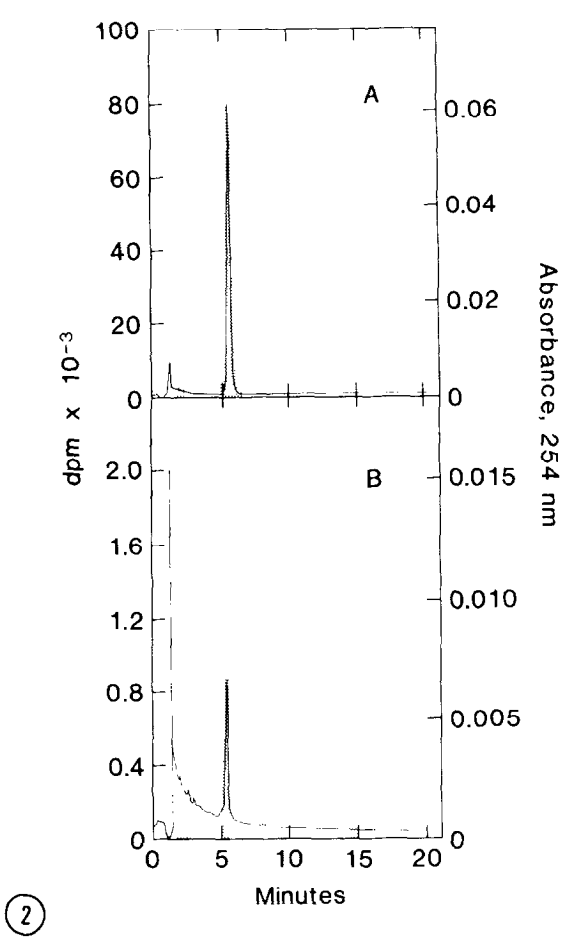


Figure 1. HPLC separation of 3'-terminal nucleosides of RNA liberated by KOH hydrolysis. A. Separation of standard nucleosides and adenine as described in MATERIALS AND METHODS. B. Separation of 3'-terminal nucleoside fraction from KOH-hydrolyzed RNA extracted from the equivalent of 1.5 x 10^8 cells after incubation with [^{3}H]xylA (specific activity, 3.19 x 10^5 dpm/nmol) for 2 hr. The radioactivity in 0.5 ml of each 1 ml fraction collected at 0.5-min intervals is shown by the shaded rectangles.

Figure 2. HPLC separation of 3'-terminal nucleosides after incubation with adenosine deaminase. A. Separation of the deamination products of authentic [^{3}H]xylA by HPLC as described in MATERIALS AND METHODS. B. Remaining portions of the radioactive fractions that eluted with xylA in Fig. 1B were pooled, evaporated to dryness, and incubated in 0.05 M K_2HPO_4 pH 7.5, with 0.3 unit of adenosine deaminase for 30 min at 37°. After HPLC separation of the reaction products, the radioactivity in 1 ml fractions collected at 0.5-min intervals is shown by the shaded rectangles.

Sep-Pak step, was incubated first with $NaIO_4$ and subsequently with cyclohexylamine to destroy nucleosides with cis-hydroxyl groups (10). HPLC analysis of this material before $NaIO_4$ treatment (Table 1) indicated that adenosine and adenine predominated in this mixture and a small amount of radioactivity

TABLE 1

	nmole recovered	
	before $NaIO_4$	after $NaIO_4$
Adenosine	1.96	0.20
Adenine	0.62	2.86
xylA	0.018 (2219 dpm)	0.018 (2172 dpm)

$NaIO_4$ oxidation of 3'-terminal nucleosides from RNA of cells incubated with [^{3}H]xylA. The 3'-terminal nucleoside fraction from 1.5×10^8 cells incubated with [^{3}H]xylA was incubated with 0.03 M $NaIO_4$ for 30 min at 22^0. Cyclohexylamine was added to a final concentration of 0.22 M and the mixture was incubated at 45^0 for 90 min. The remaining $NaIO_4$ was consumed with an equimolar quantity of glycerol and the pH was adjusted to 6 with formic acid (10). The reaction products were separated by HPLC and quantitated as described in MATERIALS AND METHODS.

eluted with xylA, as shown in Fig 1B. After $NaIO_4$ treatment, 95% of the adenosine had been converted to adenine as expected from this procedure, but the radioactivity that eluted with xylA was undiminished. These results indicate the absence of cis-hydroxyl groups on the carbohydrate of the liberated radioactive terminal nucleoside, a conclusion that is consistent with the identity of the compound being xylA.

DISCUSSION

Three articles of evidence support the conclusion that nucleotides of xylA terminate cellular RNA. 1) The radioactivity released from RNA with the 3'-terminal nucleosides upon base hydrolysis coelutes with authentic xylA, 2) the material associated with this radioactivity is converted to a compound with the chromatographic properties of xylosylhypoxanthine after incubation with adenosine deaminase, 3) the radioactive compound in the 3'-terminus fraction is stable to $NaIO_4$ oxidation, whereas adenosine is not. Calculations based on the amount of radioactivity associated with xylA in the terminal nucleoside fraction, the number of cells extracted, and the specific activity of exogenous [^{3}H]xylA indicate that the 2-hr incubation resulted in the termination of 7.2×10^4 RNA chains per cell. Future experiments will be designed to discern whether the observed terminal incorpora-

tion results in premature halting of the synthesis of nascent transcripts or if it is a posttranscriptional addition. The rapid inhibition of uridine incorporation into RNA is consistent with the former possibility (3,4), and recent investigations suggest that the transcription of ribosomal precursor RNA may be terminated prematurely (8). In contrast, in vitro studies demonstrated that xylATP was not a substrate for terminal addition to $tRNA^{phe}$-ApCpC by yeast ATP(CTP): tRNA nucleotidyl transferase (E.C.2.7.7.25) (11). Furthermore, the more rapid and extensive inhibition of the incorporation of thymidine into DNA (3,4) raises the possibility that the synthesis of primer RNA required for the initiation of Okazaki fragment formation (12) may be prematurely terminated. Thus, the data indicate that the terminal incorporation of xylA into nascent RNA chains could prevent the addition of subsequent nucleotides, resulting in the observed inhibition of RNA and DNA synthesis.

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