In Vivo Synthesis of 6-Azauridine 5'-Triphosphate and Incorporation of 6-Azauridine into RNA of Germinating Wheat Embryonic Axes*

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The cytostatic effect of 6-aza uridine on cell growth is generally regarded to be a consequence of the inhibition of de novo pyrimidine biosynthesis by the metabolite, 6-aza uridine 5'-monophosphate. We show here that wheat embryonic axes further metabolize 6-aza uridine to the 5'-triphosphate and incorporate the analogue into RNA, thus offering an alternative mechanism for growth inhibition. At a level of 6-aza uridine required to maximally inhibit UTP biosynthesis, the ratio of 6-azaUTP to UTP is about 2:1 and substitution of 6-aza uridine for uridine in new RNA is on the order of 1 in 18. The new metabolites of 6-aza uridine are identified by high pressure and thin layer chromatography coupled with enzyme treatments.

The mode of action of 6-aza uridine in its capacity as a growth inhibitor and antineoplastic agent (1) has been generally attributed to an inhibition of UTP biosynthesis by 6-aza uridine 5'-monophosphate (2-4). During a recent study of the effect of reduced UTP levels on growth and protein synthesis in wheat embryonic axes, we observed that maximal inhibition by 6-aza uridine occurred after withdrawal of the analogue, at a time when the UTP level had returned to normal. This result indicated that the decrease in UTP biosynthesis per se was not the specific cause of the inhibition and prompted a search for an alternative explanation. This report describes the identification of 6-aza uridine 5'-triphosphate and the incorporation in vivo of the analogue into embryonic RNA.

EXPERIMENTAL PROCEDURES

Incubation and Extractions—Wheat embryonic axes (120 mg) were germinated as described previously (5) for 4.5 h in 1.6 ml of 7.3 mM 6-aza Urd containing 10 μg/ml of chloramphenicol. Embryos were homogenized with 5% trichloroacetic acid (5) and the trichloroacetic acid-soluble fraction was subsequently treated with 1 volume of a fresh solution of 1,1,2-trichloro-1,2,2-trifluoroethane (3 parts) and Alamine 336 (General Mills Chemicals, 1 part) in order to remove the trichloroacetic acid (6). The aqueous phase was neutralized with Tris base, centrifuged, and stored frozen. The trichloroacetic acid-insoluble pellet was extracted for RNA (7), with the modification that the first phenol extraction was performed at room temperature and the second for 10 min at 40°C. The ethanol-precipitated RNA was dissolved in 2 ml of H2O and reprecipitated with 2 volumes of ethanol and 0.1 M NaCl. Alkaline hydrolysis of the RNA was by 0.3 M KOH for 18 h at 37°C followed by cooling, neutralization with perchloric acid, and centrifugation at 4°C to remove potassium perchlorate.

Separation Methods—High pressure liquid chromatography was performed on a column of Partisil 10-SAX (Whatman). In Method 1, the sample was eluted at a flow rate of 1.5 ml/min with a linear gradient (45 min) from 0 to 100% 0.44 M KCl, 0.22 M KH2PO4, pH 4.5, then elution at 100% was continued for another 30 min. On occasion, the sample was first eluted by H2O for 10 min before initiating the gradient to improve resolution of the early compounds.) Method 2 used the SAX column as a reverse phase system. The column was equilibrated with 1 M KH2PO4, pH 2.9, washed extensively with H2O, and then equilibrated with acetonitrile (87 parts) mixed with 10 mM KH2PO4, pH 2.9 (13 parts). The sample was applied and pyrimidine nucleosides were resolved by elution at a flow rate of 0.5 ml/min with the 87:13 mix (40 min). A linear gradient from 0 to 100% 10 mM KH2PO4, pH 2.9, was imposed (20 min) and elution was continued with the 10 mM KH2PO4, pH 4.5, then was used to ensure elution of azaUMP. This procedure was sufficient to resolve uridine, azaUrd, and cytidine (Fig. 3).

Thin layer chromatography (in closed tanks) employed Brinkmann plastic sheets coated with 0.1 mm of cellulose MN 300 polyethylene-imine with fluorescence indicator. After identification of marker positions by quenching the azaUMP, the polyethyleneimine-cellulose was removed in 5-mm strips which were counted in liquid scintillation fluid. Solvent A was H2O-saturated sec-butyl alcohol with the plate run twice with drying in between. Solvent B was 1 N acetic acid until the solvent front was 2 cm past the origin, then 9.1 v/v 1 N acetic acid, 3 M LiCl without intermediate drying. Solvent C was 0.65 M KH2PO4, pH 3.4.

Trichloroacetic acid-soluble compounds were separated preparatively at 4°C on a column of Whatman DE52 cellulose (15.0 × 0.9 cm), eluted with 20 ml of 5 M triethylamine-carbonate, pH 7.8, followed by a linear, 80-ml gradient of 50 mM to 500 mM triethylamine-carbonate. The flow rate was 10 to 12 ml/h. Pooled fractions were concentrated under vacuum by evaporation at 37°C over a dry ice trap. Each residue was re-evaporated five times after washing with methanol and the residues were dissolved in 0.4 to 1 ml of H2O. 6-Aza[5-3H]uridine was obtained from Moravek Biochemicals (City of Industry, Calif.).

RESULTS AND DISCUSSION

When incubated with 6-azaUrd, wheat embryonic axes accumulate a compound which has now been identified as 6-azaUTP. This unusual metabolite of azaUrd was first observed as a new ultraviolet-absorbing peak in the nucleoside triphosphate region upon high pressure liquid chromatography of the trichloroacetic acid-soluble extract (Fig. 1). Incubation of axes in 7.3 mM azaUrd reduced the levels of both UTP and CTP (the usual consequence of azaUrd incubation) and allowed the synthesis of putative azaUTP. The new compound had a retention time about 2 min longer than that of CTP, using HPLC* Method 1.

To determine whether the new compound was indeed an azaUrd metabolite, wheat axes were incubated with 6-aza[5-3H]Urd and the acid-soluble fraction was again fractionated by HPLC (Fig. 2). The radioactivity ascribed to azaUMP was coincident with authentic (marker) azaUMP, while a distinct *This work was supported by Grant PCM79-00268 from the National Science Foundation, by Grants GM-15122, CA-06927, and RR-08539 from the National Institutes of Health, and by an appropriation from the Commonwealth of Pennsylvania. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Wheat axes require 1 mM and 10 mM azaUrd to inhibit net UTP biosynthesis by 50% and 80%, respectively, when supplied between 0 and 4.5 h of incubation.

2 The abbreviation used is: HPLC, high pressure liquid chromatography.
peak of radioactivity co-eluted with the putative azaUTP seen by ultraviolet absorbance as in Fig. 1. Neither [H]orotic acid nor [H]uridine was able to label the new metabolite.

Co-chromatography of known markers and H-metabolites on polyethyleneimine-cellulose plates substantiated the proposed identity of azaUTP. First, the metabolites of [H]-azaUrd were separated preparatively on a column of DE52-cellulose with 95% of the radioactivity being eluted in three distinct peaks by 0.16, 0.26, and 0.37 \text{m} buffer. Alkaline phosphatase (from calf intestinal mucosa) converted over 85% of the total radioactivity in each peak back to [H]-azaUrd as was shown using Solvent A in a thin layer system where uridine, azaUrd, and cytidine are clearly separated. Without enzyme treatment, 89% of the label in the first (0.16 \text{m}) peak co-migrated with marker azaUrd. This peak thus represented unmetabolized [H]azaUrd. Seventy-two per cent of Peak II chromatographed with marker azaUMP. Radioactivity (90%) in the smallest peak (III) chromatographed between UTP and CTP using Solvent C. Thus, this latter metabolite is a triphosphate of azaUrd, most likely azaUTP.

While bacteria, animal, and plant cells all metabolize azaUrd to azaUMP, the wheat embryonic axis is the first system in which azaUTP biosynthesis has been shown. At 7.3 \text{mM} azaUrd, the ratio of azaUTP to UTP reaches about 2:1. Both UMP and azaUMP probably use nucleoside monophosphate kinase and nucleoside diphosphate kinase to synthesize their triphosphates. Under conditions used here, we calculate an internal concentration of azaUMP of 2 \text{nM}, a level at least 40- to 100-fold higher than that of endogenous UMP which might explain the competitive ability of azaUMP in the wheat axis.

The presence of azaUTP in the wheat axes, coupled with our biological studies, suggested that azaUrd might be incorporated into RNA. After tissue incubation with [H]azaUrd, total RNA was therefore extracted and analyzed for the incorporation of azaUrd. Nucleoside monophosphates (mixed 2' and 3') were released by alkaline hydrolysis and then subjected to HPLC separation by one of two methods, designed to identify either azaUMP (Method 1) or azaUrd (Method 2). By Method 1, 80 to 95% of the \text{H} radioactivity of the RNA hydrolysate was associated specifically with azaUMP. When treated with \text{P}1 nuclease (a 2',3'-phosphomonoesterase), the radioactivity was converted to azaUrd, which was identified by Method 2 (see Table I and Fig. 3). As

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**Table I**

Identification of [H]azaUrd in [H]RNA

<table>
<thead>
<tr>
<th>Analysis</th>
<th>F1 nuclease</th>
<th>HPLC position</th>
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<tr>
<td>azauridine</td>
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<td>91</td>
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<td>azauridine(5')UMP</td>
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In Vivo Synthesis of 6-Azauridyl-RNA

Fig. 3. Identification of aza[^H]uridine in the RNA of wheat embryonic axes. The alkaline hydrolysate of wheat RNA, which had been extracted at 4.5 h, was treated with P1 nuclease, then mixed with (marker) unlabeled azaUrd, and the sample was chromatographed on Partisil SAX by HPLC Method 2 (87% acetonitrile with phosphate buffer). The bars indicate the position of this radioactivity. These data are superimposed over a UV absorption graph (continuous line) formed by elution of a mixture of uridine, azaUrd, and cytidine in a separate run. The retention time of the azaUrd was identical in both cases. The[^H]azaUrd peak from the hydrolysate had a broader spread because of mixing in the larger diameter tubing between the absorbance cell and the fraction collector.

a control, aza(5')UMP, which also had been synthesized by the axes, was treated with the P1 nuclease and no ^[^H]nucleoside was released. From the specific activity of[^H]azaUrd, the amount of actual azaUMP in 13.7 µmol (nucleotide residues) of total RNA was 1.76 nmol of azaUMP. Using data from a study of the incorporation of uridine into RNA (5), we can calculate that during the first 4.5 h of germination, about 31 nmol of Urd are added to RNA. This provides an average mole ratio of azaUrd to Urd in newly synthesized RNA of about 1 in 18 (or higher if the rate of RNA synthesis is affected). At this stage of development, germinating wheat embryos do not synthesize DNA and, indeed, no[^H]azaUrd radioactivity was detected in the DNA fraction.

In earlier studies, both azaUDP and azaUTP were shown to be recognized in vitro by enzyme systems involved with RNA biosynthesis. Synthetic azaUDP inhibits polynucleotide phosphorylase and CCA turnover at 3' ends of tRNA (8, 9), and azaUTP can inhibit RNA polymerase in vitro (10). The present findings that both azaUTP and azaUrd-RNA are synthesized by the wheat embryonic axis extend this conclusion demonstrating the recognition in vivo of the azaUrd metabolites.

REFERENCES