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Metabolism of Cytidine and Uridine in Bean Leaves[1]

Cleon Ross and C. V. Cole[2]
Department of Botany and Plant Pathology, Colorado State University, Fort Collins, Colorado 80521

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Abstract. **The metabolism of cytidine-2-^{14}C and uridine-2-^{14}C was studied in discs cut from leaflets of bean plants (*Phaseolus vulgaris* L.). Cytidine was degraded to carbon dioxide and incorporated into RNA at about the same rates as was uridine. Both nucleosides were converted into the same soluble nucleotides, principally uridine diphosphate glucose, suggesting that cytidine was rapidly deaminated to uridine and then metabolized along the same pathways. However, cytidine was converted to cytidine diphosphate and cytidine triphosphate more effectively than was uridine. Cytidine also was converted into cytidylic acid of RNA much more extensively and into RNA uridylic acid less extensively than was uridine. Azaserine, an antagonist of reactions involving glutamine (including the conversion of uridine triphosphate to cytidine triphosphate), inhibited the conversion of cytidine into RNA uridylic acid with less effect on its incorporation into cytidylic acid. On the other hand, it inhibited the conversion of orotic acid into RNA cytidylic acid much more than into uridylic acid. The results suggest that cytidine is in part metabolized by direct conversion to uridine and in part by conversion to cytidine triphosphate through reactions not involving uridine nucleotides.**

Nucleosides such as cytidine are known not to be intermediates in the usual formation of pyrimidine nucleotides (4, 5, 15, 16, 19). Nevertheless, these compounds may be important in mechanisms salvaging the purine and pyrimidine rings. Ribonuclease action upon RNA apparently produces 5′-nucleotides (22) or 2′,3′-cyclic nucleotides (3, 22). The latter are presumably then dephosphorylated by phosphomonoesterases. The resulting nucleosides could then be reutilized for RNA or DNA synthesis in the same cells (or organelles) or might be readily transported to other tissues, such as from storage tissues to embryos of germinating seeds.

We previously showed that uridine is a much better precursor of RNA in trifoliate bean leaves (16) than is uracil, and Wasilewska and Reifer (21) made the same observation in experiments with wheat, pea, and spinach leaves. We further found that cytosine was not metabolized in either bean or cocklebur leaves. These facts indicate that pyrimidine nucleosides are more important salvage products than are free pyrimidines. The mechanism of utilization of uridine has been given some attention (16, 20, 21), and although cytidine can be converted into RNA of plants (2), the reactions involved have not been studied. The present experiments were undertaken to determine whether cytidine can be readily utilized in RNA synthesis and to determine some of the intermediates in the pathway concerned.

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[2] Research soil scientist, Agriculture Research Service, USDA.

Materials and Methods

Leaflets from Idaho 111 pinto bean (*Phascolus vulgaris* L.) plants were used in these studies, since we already had information about pyrimidine nucleotide metabolism in them (16, 23). The plants were grown in soil-containing pots in a greenhouse until the first trifoliate leaflets were about 2.5 cm long. Discs (1.2 cm diameter) were cut with a cork borer from these leaflets, rinsed in water, and 10 (225 mg fr wt) placed in flasks (2-oz. Skrip ink bottles) containing 5.0 ml of 0.02 M phosphate buffer at pH 5.8. Radioactive metabolites in amounts indicated with the tables in the Results section were added to the buffer solutions. In some experiments the influence of azaserine (*O*-diazoacetyl-L-serine), an inhibitor of reactions involving glutamine (9, 17), was studied. It was added to the above-mentioned components in the main compartments of the flasks just prior to adding the leaf discs. The well at the top of each flask contained 1.0 ml of 3% KOH to collect respired CO_2. Lids of the flasks were sealed with rubber gasket material (18) to prevent gas leakage during the ensuing incubation periods. Flasks were incubated in darkness at 28 to 30° on an oscillating constant temperature water bath apparatus (Research Specialties Company) for 2 to 5 hours.

Tissue extraction methods, analysis of ^{14}C in respired CO_2, and other radioactivity measurements were performed as described previously (16), except that ethanol and perchloric acid extracts were freeze-dried prior to chromatography and radioactivity in metabolites separated by paper chromatography was

measured with a Nuclear-Chicago Mark I liquid scintillation counter. Chromatography of nucleotides produced by KOH digestion of RNA was as described before, but separation of metabolites in the 80 % ethanol soluble fractions involved the newer methods of Cole and Ross (7). These methods give far better resolution of nucleotides than those used earlier (16) and largely prevent nucleotide destruction during chromatography. Whatman 3 MM papers (46 × 57 cm) were used. The first solvent (IBA) was a mixture of isobutyric acid – NH_4OH-H_2O (57:4:39 v/v). This was followed in the short dimension of the paper with MAA (methanol-1 M ammonium acetate, 7:3 v/v). Compounds thus separated were eluted in water and further purified and identified using other chromatographic solvents and by paper electrophoresis, as described earlier (7, 16). UTP[3], CTP, UDP, and UDPG were partially dephosphorylated during the elution from the papers upon which they were originally separated. Thus, when rechromatographed, autoradiograms showed that labeled compounds were present which had chromatographed exactly with the nucleotides containing 1 or 2 less phosphate groups. For example, when the compound believed to be CTP was rechromatographed, labeled CDP and CMP were also found. When UDGP was rechromatographed, labeled compounds having the same mobilities as UDP, UMP, and uridine were detected. These observations left little doubt as to the identity of the 5 above-mentioned nucleotides. UMP, CMP, uridine, and uracil were identified only by paper co-chromatography and electrophoresis, but their positions matched exactly those of the corresponding knowns in 3 chromatographic solvents in addition to IBA and MAA.

All ^{14}C-labeled compounds were purchased from the New England Nuclear Corporation. Uridine-2-^{14}C and cytidine-2-^{14}C solutions were checked for impurities by chromatography in IBA and MAA at the time they were first used. Thereafter they were kept frozen. The only detectable contaminant in the uridine was uracil (1.6 %), while cytidine contained 3 contaminants (1 of which was cytosine), each having about 2 % as much ^{14}C as did cytidine itself. None of these 3 contaminants was ever found on chromatograms of extracts from plants fed this cytidine. We did not check the purity of the orotic acid-6-^{14}C.

Azaserine was a gift of Parke-Davis and Company. TMP, TDP, TTP, deoxyuridine, and dihydrouridine were obtained from Sigma Chemical Company. Dihydrouracil, TDGP, and all uridine and cytidine nucleotides were purchased from Calbiochem. β-Ureidopropionic acid and ribosyl-β-ureidopropionic acid were prepared by KOH hydrolysis of dihydrouracil and dihydrouridine, respectively.

Results and Discussion

In table I are data from 1 of 3 experiments comparing the effectiveness of incorporation of cytidine and uridine into RNA. Totals of approximately 14 % of the added cytidine and 19 % of the added uridine were recovered in the various tissue fractions, including respired CO_2. The rates that the 2 nucleosides were broken down into CO_2 and converted into RNA were very similar. Since uridine and orotic acid were previously shown (16) to be more efficiently converted into RNA than other compounds labeling the pyrimidine ring, it is clear that cytidine is also an efficient RNA precursor.

The percent ^{14}C distribution data in table I suggest that cytidine and uridine are metabolized along similar pathways. If cytidine were quickly converted to uridine by a plant deaminase which acts upon nucleosides but not upon nucleotides (1, 10), distribution of radioactivity should be very similar to that which was found. However, analysis

[3] Abbreviations used: UMP, CMP, TMP, UDP, CDP, TDP, UTP, CTP, and TTP are the 5′-mono, -di, and -triphosphates of uridine, cytidine, and thymidine; UDPG, uridine diphosphate glucose; TDPG, thymidine diphosphate glucose.

Table I. *Distribution of ^{14}C From Uridine and Cytidine Among Various Tissue Fractions*

Ten leaf discs were incubated in flasks containing 5.0 ml of 20 mM phosphate buffer (pH 5.8), 0.15 ml (1.06×10^6 cpm) labeled cytidine (24.5 mc/mmole) or uridine (30 mc/mmole). Flasks were incubated 2.8 hours at 28° in darkness.

RNA Precursor	Radioactivity recovered	^{14}C Distribution[1]				
		CO_2	80 % EtOH soluble	$HClO_4$ soluble	RNA	C/U[2]
	cpm	%	%	%	%	*Ratio*
Cytidine-2-^{14}C	148,000	15.4	49.9	12.4	22.3	1.50 ± 0.13
Uridine-2-^{14}C	194 800	16.3	53.9	10.6	19.2	0.39 ± 0.03

[1] Based on percent of total counts/min recovered. Values are means from results of 4 flasks.
[2] C/U is the ratio of radioactivity in RNA cytidylic/uridylic acid.

of ^{14}C in cytidylic and uridylic acids of the RNA showed that anabolic pathways of the two are, to some extent, different (note C/U ratio in table I). While uridine resulted in C/U ratios considerably less than unity as predicted by its conversion into UTP prior to CTP, such ratios were consistently greater than unity following cytidine incorporation. These results suggest that cytidine was not deaminated to form uridine, or deaminated only in part, and that it was converted into CTP more effectively than into UTP.

If cytidine was indeed phosphorylated and converted into CMP, CDP, and CTP, these nucleotides should have been labeled in these experiments. To determine whether this was the case, the 80% ethanol extracts were chromatographed in IBA and MAA, autoradiographs made, and ^{14}C in each compound thus detected was measured. Figure 1 shows a typical separation, but in most cases uridine and uracil were completely resolved. Table II lists the average percent distribution of ^{14}C among these metabolites when either cytidine or uridine was incorporated. The values should not be considered entirely representative of the tissue contents, since improved methods used to prevent phosphatase activity during extraction (7) were not used when these experiments were performed. Thus, the values for some of the phosphorylated compounds listed are probably too low.

Table II. *Distribution of ^{14}C Among Ethanol Soluble Metabolites After Feeding Labeled Uridine or Cytidine*

Ten leaf discs were incubated in flasks containing 5.0 ml of phosphate buffer (pH 5.8), 0.15 ml (1.06×10^6 cpm) cytidine-2-^{14}C (24.5 mc/mmole) or uridine-2-^{14}C (30 mc/mmole). Incubation proceeded 5.0 hours at 28°.

Metabolite[1]	Percent ^{14}C distribution Precursor	
	Cytidine-2-^{14}C	Uridine-2-^{14}C
UTP	32	3.6
UDP	8.8	10.6
UMP	7.4	8.3
UDPG	55.8	59.2
Uridine	2.0	1.5
Uracil	8.7	7.0
UK-a	2.1	2.2
UK-b	4.6	5.1
UK-c	Undetected	0.2
CTP	3.5 ± 0.8[2]	0.3 ± 0.5
CDP	3.3 ± 0.8	1.3 ± 0.2
CMP	0.5	0.6

[1] Total ^{14}C in all metabolites chromatographed was approximately 30,000 cpm.
[2] Standard deviations.

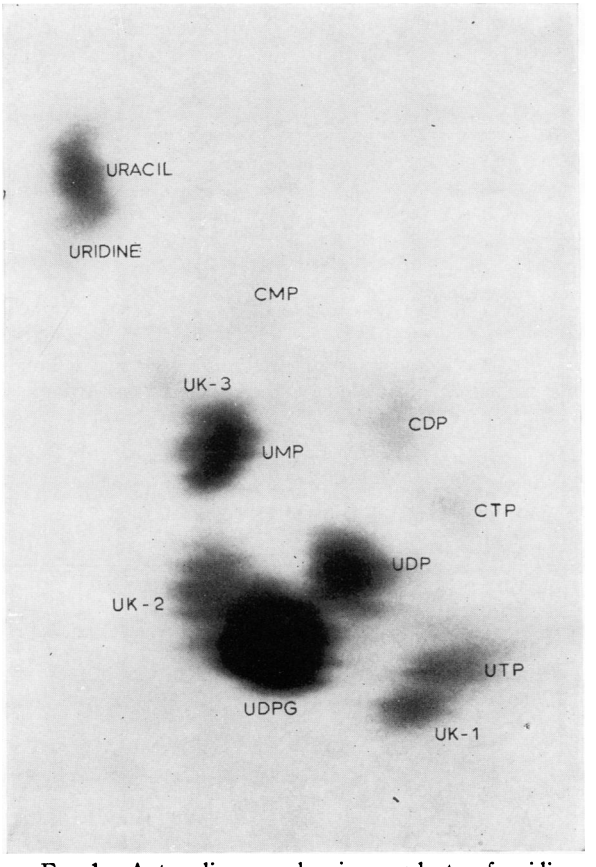

FIG. 1. Autoradiogram showing products of uridine-2-^{14}C metabolism in bean leaf discs.

In general, the same metabolites were labeled and in about the same relative amounts following addition of either uridine or cytidine. This result is consistent with the interpretation that much of the cytidine is initially deaminated to uridine and therefore metabolized in the same way. UDPG contained most of the ^{14}C in both cases. Three unknown compounds (UK-a, UK-b, and UK-c in table II) were found. We suspected that one might be β-ureidopropionic acid, since this compound was identified as a minor product of uridine metabolism when other chromatographic solvents had been used (16). However, β-ureidopropionate chromatographs just above uracil in these solvents and we have not found ^{14}C in it using these solvents. Whether this means that it was only an artifact resulting from non-enzymatic breakdown of dihydrouracil in earlier experiments has not been determined. None of the unknowns chromatographed with deoxyuridine, deoxy-UMP, TDPG, TTP, or TDP. UK-c ran in the same or very nearly the same position in these solvents as ribosyl-β-ureidopropionic acid (which might be formed from uridine via dihydrouridine in the same manner that uracil is converted to dihydrouracil and β-ureidopropionate) and TMP, but it was not cochromatographed with them. UK-b is probably a sugar derivative of UDP, since during elution and cochromatography with various known compounds it was sometimes partially converted into UDP and UMP. No clues about the identity of UK-a were obtained.

Cytidine was converted to uridine, presumably by direct deamination (1, 10), and labeled uracil was also a detectable metabolite of cytidine. No unmetabolized cytidine was found nor was any detectable cytosine produced, suggesting that cytidine was catabolized via uridine and uracil.

A small but important difference among the metabolites formed from uridine and cytidine was the higher radioactivity in CTP and CDP after feeding cytidine. This result supports the interpretation based upon labeling of RNA cytidylic acid and uridylic acid, that some of the cytidine was converted to CTP by reactions not involving uridine nucleotides.

The enzyme catalyzing the conversion of UTP to CTP using glutamine as an amino donor, CTP synthetase, has been demonstrated in *Escherichia coli* (13, 14) and there is evidence for a similar one in mammals (11). These enzymes are inhibited by the glutamine analogue DON (6-diazo-5-oxo-L-norleucine) (8, 11) and apparently also by azaserine (12). DON becomes tightly bound to the mammalian enzyme, resulting in an irreversible ability to combine with glutamine (6, 11). It has apparently not been demonstrated that azaserine binds in the same manner to this enzyme, but French *et al.* (9) demonstrated that azaserine alkylated phosphoribosylformylglycineamidine synthetase, an enzyme similarly dependent upon glutamine. If an enzyme interconverting UTP and CTP occurs in plants, the RNA cytidylic and uridylic acid labeling patterns of table I would be explainable (see fig 2). Uridine is presumably converted to UTP, some of which is incorporated directly into RNA and some into CTP, followed by incorporation of CTP into RNA. Thus the RNA C/U ratios from uridine should be and were less than one. Following similar reasoning, if part of the cytidine is converted into CTP without involvement of UTP as an intermediate RNA C/U ratios should initially be greater than one, as was observed. Demonstrations of reversibility (*i.e.* conversion of CTP to UTP) by CTP synthetase have apparently not been reported, yet the present tracer data suggest that this occurs.

Experiments were conducted with azaserine to determine whether it influenced the RNA C/U labeling ratios when cytidine was metabolized. Orotic acid-6-^{14}C, known to be converted into RNA via uridine nucleotides (15, 16, 23), was used to obtain RNA labeling patterns with which those from cytidine could be compared. The results from 1 of 2 such experiments are listed in table III. Azaserine inhibited somewhat the absorption of both orotic acid and cytidine and caused pronounced but unexplainable inhibitions upon their breakdown to CO_2. It also inhibited ^{14}C conversion into RNA in both cases. With orotic acid as the RNA precursor, the primary inhibition was upon incorporation of ^{14}C into cytidylic acid, so the C/U ratio was decreased to 0.14 from 0.42 in the controls. When cytidine was the precursor, azaserine caused increased C/U ratios, the effect resulting primarily from a decrease in labeling of uridylic acid. In a separate experiment, the influences of azaserine, glutamine, and asparagine upon the conversion of orotic acid-6-^{14}C into cytidine and uridine nucleotides in the ethanol and perchloric acid soluble fractions were measured. Azaserine markedly decreased labeling of the cytidine nucleotides, the percent of ^{14}C in known cytidine compounds compared to the total ^{14}C on the chromatograms being 2.5 % in the controls and 0.51 % in tissues treated with azaserine.

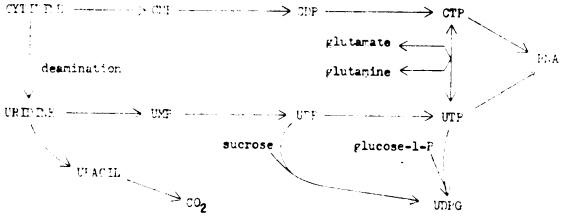

FIG. 2. A summary of reactions which may account for metabolism of cytidine and uridine in bean leaf discs.

Table III. *Influence of Azaserine Upon Metabolism of Orotic Acid and Cytidine*

Ten leaf discs were incubated in flasks containing 5.0 ml of 20 mM phosphate buffer (pH 5.8), 0.15 ml (1.2×10^6 cpm) labeled orotic acid (4.9 mc/mmole) or cytidine (24.5 mc/mmole) and 1.0 ml H_2O (controls) or 1.0 ml of 62 mM azaserine. Incubation proceeded in darkness at 27° for 3 hours.

Treatment	Radioactivity recovered	^{14}C Distribution[1]				
		CO_2	80 % EtOH soluble	$HClO_4$ soluble	RNA	C/U[2]
	cpm	%	%	%	%	*Ratio*
Orotic acid-6-^{14}C						
Controls	147,900	0.43	66 0	15.3	18.3	0.42 ± 0.03
Azaserine	107,600	0.23	81.2	13.1	5.4	0.14 ± 0.02
Cytidine-2-^{14}C						
Controls	177,800	11.0	49.6	18.4	21.0	1.76 ± 0.36
Azaserine	122,700	6.5	58.4	22.9	12.2	3.50 ± 0.82

[1] Based on percent of total cpm recovered. Values are means of 2 flasks.
[2] C/U is the ratio of radioactivity in RNA cytidylic/uridylic acid (± standard deviation).

Asparagine at a final concentration of 4.2 mM had no influence upon this percentage, while glutamine at 4.2 mM significantly increased it to 5.82%. These results suggest that an enzyme, presumably glutamine dependent, interconverts UTP and CTP in bean leaves and that azaserine may combine with it such to prevent its catalyzing the reaction in either direction.

Figure 2 summarizes the major reactions which we believe will account for the metabolism of cytidine and uridine in the tissue studied.

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