IDENTIFICATION OF 5-FLUOROCYTIDINE IN RNA FROM *ESCHERICHIA COLI* GROWN IN THE PRESENCE OF 5-FLUOROURACIL

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1. Introduction

A number of organisms readily incorporate FUra into their RNA's [1, 2]. This incorporation is largely the result of FUrd replacement of uridine, although a number of minor pyrimidines are also replaced ([3] and references therein). FUra is apparently converted to FUrd triphosphate and FCyt triphosphate [4], although no evidence for the incorporation of the latter into RNA has previously been reported.

Data presented here indicate that a small amount of FCyt is present in RNA of *Escherichia coli* grown in the presence of FUra. Identification was based on (1) its mobility in thin-layer and paper chromatography systems, (2) its ultraviolet spectra, and (3) its apparent ability to be deaminated under acidic conditions giving a FUrd-like product. The amount of FCyt found in FUra-tRNA ranged from 0.6 to 1.2 mole %, while in FUra-containing rRNA the values were 0.5 to 1.0 mole %. Limited treatment of radioactive FUra-tRNA's with venom phosphodiesterase indicates that FCyt is not concentrated at the 3'-ends of the polynucleotide chains.

2. Materials

Unlabeled 5-fluorouracil and 5-fluorocytidine were kindly provided by Dr. W.E. Scott of Hoffman-LaRoche, Inc. [2-^{14}C]5-Fluorouracil (16.4 mCi/mmole) was purchased from Schwarz BioResearch and used without further purification. [6-^{3}H]5-fluorouracil (1.08 Ci/mmole) was from Amersham/Searle. About 4% of the total radioactivity did not chromatograph as 5-fluorouracil and was removed before use. Other materials were described in [3].

Abbreviations

FUra	: 5-fluorouracil
FUrd	: 5-fluorouridine
FCyt	: 5-fluorocytidine
FUra-tRNA	: 5-fluorouracil-containing tRNA.

3. Methods

3.1. *Bacterial growth and RNA isolation*

E. coli were grown under vigorous aeration at 37° in the salt medium of Davis and Mingioli [5], modified to contain 1 mg $FeCl_3$ per ℓ, at a final glucose concentration of 1% as previously described [3]. In the early exponential phase of growth unlabeled FUra and thymidine were added to the culture, followed 5 min later by the radioactive FUra. Incubation was continued for 3 hr. The final concentration of thymidine was 25 μg/ml, whereas that of unlabeled FUra was 12 and 20 μg/ml in the ^{14}C- and ^{3}H-labeled cultures, respectively. Labeling was carried out with either 30 μCi of [2-^{14}C]5-fluorouracil or 750 μCi of [6-^{3}H]5-fluorouracil. Cells were harvested by centrifugation and the tRNA prepared as described [3], except that a 2 M NaCl precipitation preceded the isopropanol treatment. The high molecular weight rRNA was prepared from the 2 M NaCl precipitate by resuspending the material and passing it over Sephacex G-75 [3]. The material eluting at the void volume was collected, precipitated with ethanol, and used without further purification. Nucleotide analyses [6] of the various samples used gave $\frac{\text{FUrd}}{\text{FUrd+Urd}} \times 100$-values of 60–70% for the rRNA's and 70–85% for the tRNA's.

3.2. *Large-scale isolation of the minor nucleoside from unlabeled FUra-tRNA*

About 3 mg of FUra-tRNA was hydrolyzed enzymically to the nucleoside level [3], applied to impregnated Whatman no. 1 paper as a streak 22 cm wide, and developed in the system of Lane [7]. A faint ultraviolet absorbing band, which migrated slightly ahead of adenosine was cut out and eluted with water. After concentrating, the eluent was applied to a small column (1 × 50 cm) of Sephadex G-10 and eluted with water. The material eluting between 33–39 ml was collected, pooled, and freeze-dried. The same fractionation procedure was carried out with a sample of nucleosides from normal tRNA. No ultraviolet-absorbing band could be detected in the region cut out for the FUra-tRNA sample. In addition, when this region was eluted and passed over Sephadex G-10, *virtually no* A_{260}-absorbing material was found eluting between 33–39 ml.

3.3. *Ultraviolet spectra*

The concentrated material (33–39 ml) from Sephadex G-10 was dissolved in 1 ml of 0.01 M potassium phosphate (pH 7.1). Exactly 0.80 ml was placed in a semi-micro silica cuvet (1-cm path length) and the spectra taken in a Gilford 240 spectrophotometer. The sample was acidified by the addition of 10 μl of 4 M HCl (final conc. of HCl, 0.05 M; pH ~ 1.75) and finally made basic by the addition of 20 μl of 4 M KOH (final conc. of KOH, 0.05 M; pH ~ 12.2). The appropriate additions were also made to the blank.

3.4. *Venom phosphodiesterase digestions*

Radioactive FUra-tRNA's were enzymically digested for 30 min at 37° as described [8]. Following this incubation the entire mixture was precipitated with cold $HClO_4$ and centrifuged. The precipitate was resuspended and subjected to complete enzymic digestion to the nucleoside level [3]. The supernatant was neutralized with KOH, centrifuged, and the nucleotides converted to nucleosides [3]. The nucleoside mixtures found in both the supernatant and precipitate were resolved by paper chromatography [3] in the presence of an unlabeled FCyt marker. The normal separated nucleosides were quantitated spectrophotometrically [6] and the radioactive FCyt and FUrd spots by either eluting first and counting an aliquot [3] or counting the entire chromatogram spot suspended directly in a toluene scintillation mixture [6].

Other spectrophotometric and counting methods were as described [3].

Table 1

R_f values of the unknown component and related compounds

	Thin-layer				Paper
	A	B	C	D	E
Uridine	0.58	0.70	0.64	0.15	0.56
Cytidine	0.62	0.54	0.42	0.19	0.22
Authentic 5-fluorocytidine	0.67	0.49	0.39	0.25	0.49
Unknown radioactive nucleoside	0.68	0.53	0.40	0.25	0.49
5-Fluorouridine	–	–	–	–	0.62

Ascending thin-layer chromatography was run on Eastman (6064) pre-coated cellulose plates in solvent systems A through D. A, t-butanol:methyl ethyl ketone:H_2O:conc. NH_4OH (40:30:20.10) [9]; B, isopropanol:H_2O:conc. HCl (65:18.4:16.6) [9]; C, 2-propanol:conc. HCl:H_2O (85:22:18) [10]; D, n-butanol:NH_4OH:H_2O (86:5:14) [10]; E, Ethanol:H_2O (75:25) on $(NH_4)_2SO_4$ impregnated paper [7]. For R_f-calculations the unlabeled nucleosides were located under ultraviolet light and their positions measured. The chromatogram was then cut into 0.5 × 2 cm strips and each strip was counted in toluene scintillator [6].

4. Results and discussion

During studies on RNA isolated from *E. coli* treated with radioactive FUra, a minor radioactive component in addition to FUrd was observed. This unidentified component was found migrating near adenosine in the paper chromatographic system of Lane [7] after a total enzymic digestion to the nucleoside level of both tRNA and higher molecular weight rRNA (see peak 5', fig. 4 in [3]).

[^{14}C]FUra-tRNA was hydrolyzed to the nucleoside level enzymically and the relative chromatographic mobilities of the unknown nucleoside, now characterized as FCyt, and related nucleosides determined (table 1). The mobilities of the unknown nucleoside and authentic FCyt were essentially identical in all solvent systems. Isolation of the ^{14}C-labeled minor

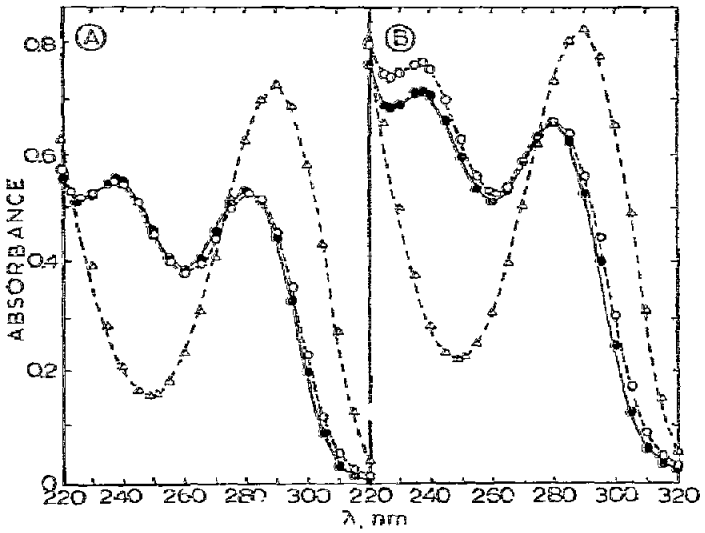

Fig. 1. Spectra of authentic FCyt (A) and the unknown (B) nucleoside isolated from FUra-tRNA after enzymic digestion (●—●—●), pH 7.1; (o--o--o), 0.05 M KOH; (Δ--Δ--Δ) 0.05 M HCl.

component and treatment with 1 M HCl for 13 hr at 95°, resulted in the majority of the label now migrating with FUrd in solvent E. An authentic sample of FCyt, subjected to the same acidic conditions, gave a product having an R_f in solvent E identical to that of authentic FUrd. This product also had an ultraviolet spectra at pH 2, 7, and 12 essentially identical to authentic FUrd.

Large-scale isolation of the unidentified component from unlabeled FUra-tRNA gave sufficient material to run an ultraviolet spectra. Fig. 1 shows the spectra of the unidentified compound and that of authentic FCyt. The two are virtually identical at all three pH-values.

The presence of FCyt in tRNA suggested that the incorporation occurred during polynucleotide biosynthesis and was not merely the result of incorporation into the CCA-end group of tRNA. To confirm this, ^{3}H- and ^{14}C-labeled FUra-tRNA was partially digested with venom phosphodiesterase and the acid soluble and insoluble products examined. The results are shown in table 2. The ratios of the cpm of FCyt per nmole of cytidine are similar for the soluble and insoluble fractions of both tRNA preparations. These results indicate that FCyt *is not* preferentially incorporated into the CCA-end group of tRNA.

Table 2
Ratio of FCyt to cytidine in different fractions of venom phosphodiesterase-treated tRNA's.

	(cpm FCyt/nmole cytidine)	
	Acid soluble	Acid precipitable
[^{3}H]FU-tRNA	5.46	5.49
[^{14}C]FU-tRNA	22.5	19.8

The cytidine/adenosine molar ratios in the soluble fractions were 1.25 to 1.32.

The amounts of FCyt found in FUra-tRNA ranged from 0.6 to 1.2 mole%, while in FUra-rRNA the values were slightly lower or 0.5 to 1.0 mole%. Assuming FCyt replaces cytidine in RNA the lower values for the rRNA's are consistent with the lower mole% of cytidine present in these species relative to the tRNA's.

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