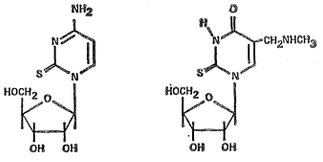
Thiobases in Escherichia coli Transfer RNA: 2-Thiocytosine and 5-Methylaminomethyl-2-thiouracil

Abstract. *Two new sulfur-containing pyrimidine nucleotides have been isolated from hydrolyzates of* Escherichia coli *transfer RNA. The structures, 2-thiocytosine and 5-methylaminomethyl-2-thiouracil, have been assigned to the bases as a result of study of ultraviolet and mass spectra. An acid-degradation product, 5-methylaminomethyluracil, has been synthesized and is identical to that derived from the natural product.*

Sulfur-containing bases are minor constituents of *Escherichia coli* tRNA (*1–3*). Although four or more different labeled bases can be demonstrated in hydrolyzates of *Escherichia coli* S^{35}-tRNA, the structure of only one of these has been elucidated. The major thiobase (*3*) is present as 4-thio-2′(3′)-uridylic acid in alkaline hydrolyzates of tRNA. Two other thionucleotides have now been isolated in pure form from such hydrolyzates and are the nucleotides derived from 2-thiocytidine (structure I) and 5-methylaminomethyl-2-thiouridine (structure II). The latter compound is apparently the result of several enzymatic modifications of the uracil nucleus, including thiolation, aminomethylation, and methylation. It represents the first example of a nucleotide from tRNA in which a strongly basic side chain is present. Another 2-thiouracil derivative, 2-thio-5(or 6)-uridine acetic acid methyl ester, was identified as a minor constituent of yeast tRNA (*4*).



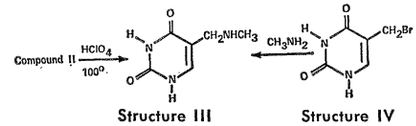
Structure I Structure II

A sample of tRNA (5 g), isolated from *Escherichia coli* B by the method of Zubay (*5*), was mixed with 6 mg of S^{35}-tRNA (*2*) containing 8.2×10^6 count/min, and hydrolyzed overnight in 0.3*M* KOH (1100 ml) at 37°C. After neutralization to *p*H 8 with Dowex 50 (H^+ form), the hydrolyzate was fractionated on a column (3.5 by 42 cm) of Dowex 1 (formate) (*2*). Under these conditions, approximately 10 percent of the radioactivity is eluted in a small peak [labeled peak 3 in reference (*2*)] just ahead of 2′(3′)-CMP, while an additional 3 to 5 percent of the radioactivity appears as a peak [labeled peak 6 in reference (*2*)] between 2′-(3′)-AMP and 2′(3′)-GMP. The remainder of the radioactivity is associated with 4-thio-2′(3′)-uridylic acid and is retained by the column.

The radioactive nucleotide in peak 3 was purified by preparative paper chromatography on Whatman 3MM paper, with solvent A (*6*) for 20 hours (descending). Ultraviolet-absorbing bands were present at $R_F = 0.24$, 0.47, 0.54, 0.60, and 0.65. The band at $R_F = 0.24$, which contained over 80 percent of the radioactivity and 56 percent of the A_{260} units (absorbancy at 260 nm) applied to the paper, was isolated by elution with water and evaporation to obtain approximately 5 to 6 mg of the 2′(3′)-nucleotide of compound II. The ultraviolet absorption spectra of this substance in acidic, neutral, and basic solutions are similar to those given by 2-thiouridine (*2*). The presence of a rather basic group on the molecule was indicated by the early position of elution from the Dowex-1 (formate) column and the low mobility displayed in cellulose thin-layer electrophoresis at *p*H 3.5 ($R_{Up} = 0.05$ to 0.06).

The presence of a phosphomonoester grouping on the molecule was shown by the direct isolation of P^{32}-labeled material from a similar fractionation of a hydrolyzate of P^{32}-tRNA. Furthermore, analysis of the nucleotide for total phosphorus (*7*) gave 1 μmole of P per 11.3 A_{270} units. 2-Thiouridine has an extinction at 270 nm of about 15 per micromole (*8*). The nucleotide (3 mg) derived from II was readily converted to the nucleoside by incubation at 37°C overnight with 1 unit of bacterial alkaline phosphatase (Worthington) in 2.5 ml of 0.05*M* triethylammonium carbonate (*p*H 8.9), and it was isolated by paper chromatography in solvent D, $R_F = 0.29$ (Table 1).

Vigorous acid hydrolysis of 1 mg of compound II (as the nucleotide or nucleoside) was carried out in 25 μl of 70 percent perchloric acid at 100°C for 1 hour. The reaction mixture was chromatographed on paper in solvent B, and the major ultraviolet-absorbing band at $R_F = 0.28$ was isolated by elution with water. Minor reaction products at $R_F = 0.17$ and 0.45 were not investigated. The ultraviolet spectra of this acid-degradation product (structure III) at *p*H 1, 7, and 13 were typical of a uracil-like compound: maxima at 262 nm (*p*H 1 or 7); 288 nm (*p*H 13); minima at 230 nm (*p*H 1 or 7); 247 nm (*p*H 13). This material (III) was free of radioactivity, an indication of complete hydrolytic removal of the thio group.



Structure III Structure IV

The mass spectra of compounds II (nucleoside) and III were determined on a modified Bendix time-of-flight instrument capable of producing well-defined spectra with as little as 1 μg of material (*9*). The test substances were transferred directly from paper chromatograms onto a platinum filament and introduced into the source of the instrument (*10*). Compound III gave well-defined peaks at *m/e* of 155 (M), 154 (M-H), 140 (M-CH_3), 126 (M-NCH_3), and 111 (M-CH_2NHCH_3) (Fig. 1A). The nucleoside, compound II, gave peaks at 171 (M-ribose), 170 (M-ribose-H), 156 (M-ribose-CH_3), 142 (M-ribose-NCH_3), 127 (M-ribose-CH_2-$NHCH_3$) (Fig. 1C).

Final proof of the structure of the degradation product (III) (and indirectly of compound II) was obtained by direct synthesis of III by the reaction of 5-bromomethyluracil (structure IV) with aqueous methylamine. To a well-stirred 20-percent methylamine solution (20 ml) 450 mg (2.2 μmole) of 5-bromomethyluracil (*11*) was gradually added. The clear solution was stirred at room temperature for 10

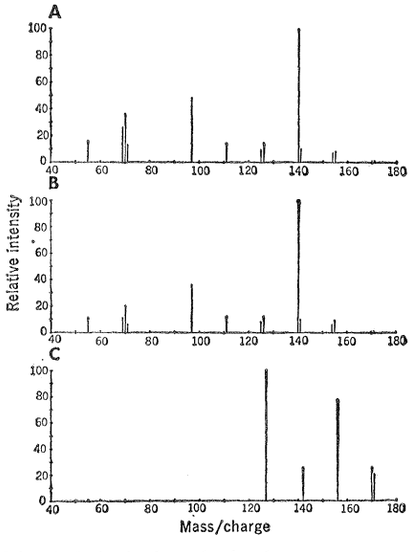


Fig. 1. Principal peaks in the mass spectra of (A) perchloric acid-hydrolysis product of the 2-thiouridine derivative (II); (B) synthetic 5-methylaminomethyluracil (III); and (C) 5-methylaminomethyl-2-thiouridine (II) from *E. coli* tRNA hydrolyzates. Spectra obtained at 70 ev.

Table 1. R_F values on cellulose thin-layer chromatograms. Values marked with an asterisk (*) were determined on Whatman 3MM paper.

Compound	R_F in solvent systems (6)					
	A	B	C	D	E	F
I, 2′(3′)-Nucleotide	0.31*		0.08	0.23		
$HClO_4$-degradation product of I		0.49				0.28
Cytosine		.49				.28
II, 2′(3′)-Nucleotide	.24*	.46	.06	.22		
II, Nucleoside		.29	.25	.37		
$HClO_4$-degradation product of II		.25		.22*	.43	
5-Methylaminomethyluracil (III)		.25			.43	

minutes and was evaporated to dryness in a vacuum; the residue was suspended in 2 ml of H_2O, and the colorless solid (160 mg) was isolated by suction filtration. This material was apparently the product of disubstitution onto the methylamine, that is, *N,N*-dithyminylmethylamine, $R_F = 0.11$ (solvent B). The aqueous filtrate, containing the main product of $R_F = 0.25$ (solvent B), was neutralized to *p*H 7 and applied to a column (1.8 by 25 cm) of IR120 (H^+ form). The column was washed with 200 ml of water and eluted with 0.5*N* HCl. After evaporation to dryness in a vacuum, and recrystallization from aqueous ethanol, the hydrochloride salt of compound III (m.p., 230° to 232°C) was obtained in 40 percent yield. The analysis showed (percent) C, 37.60; H, 5.52; N, 21.82. Calculated for $C_6H_{10}ClN_3O_2$: C, 37.61; H, 5.26; N, 21.93.

The synthetic sample of 5-methylaminomethyluracil (III) was identical in all respects to the acid-degradation product of compound II, including thin-layer chromatography (Table 1); ultraviolet-absorption spectra at *p*H 1, 7, and 13; and mass spectra (Fig. 1B).

The nucleotide derived from 2-thiocytidine (structure I) was isolated from the radioactive peak 6 of the Dowex-1 (formate) chromatography (*2*). In this peak, eluted immediately after 2′-(3′)-adenylic acid, apparently there are at least two sulfur-containing nucleotides, along with some unlabeled nucleotides. The concentrated material from peak 6 was chromatographed on Whatman 3MM paper in solvent A for 15 hours (descending). The radioactive band at $R_F = 0.31$ was eluted and purified further by paper chromatography in solvent C for 48 hours (descending). Three ultraviolet-absorbing bands and one ultraviolet-fluorescent band were obtained by this method. The fastest-moving band (12 cm from the origin), containing approximately 50 percent of the radioactivity applied to the paper, was eluted and concentrated; approximately 600 μg of material with the ultraviolet-absorption spectra typical of a 1-substituted-2-thiocytosine derivative (Fig. 2) was obtained. These spectra were also identical to the spectra of synthetic 2-thiocytidine (*13*). Treatment of this S^{35}-nucleotide with 70 μl of 70 percent perchloric acid at 100°C for 1 hour gave a non-radioactive base with mobility identical to cytosine when chromatographed on thin-layer plates (Table 1). This material was unequivocally identified as cytosine by ultraviolet and mass spectra.

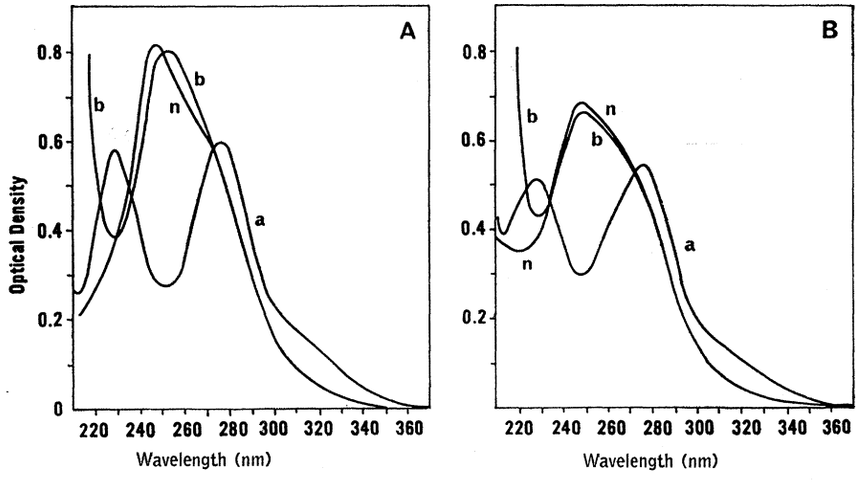


Fig. 2. Ultraviolet spectra of (A) synthetic 2-thiocytosine arabinoside (*12*) and (B) 2-thio-2′(3′)-cytidylate from *E. coli* tRNA hydrolyzates. Spectra were determined in water (*n*); 0.1*N* HCl (*a*); and 0.1*N* NaOH (*b*).

Although our findings allow reasonable certainty of the structures of the heterocyclic bases in these minor nucleotides, we have no information on the nature of the sugar portion of the molecules. It is assumed that the sugar consists of the usual β-D-ribofuranosyl group, but further work is necessary to substantiate this assumption.

The function of these unusual bases in tRNA remains unknown. The occurrence of the basic 5-methylaminomethyl side chain in tRNA also raises an interesting biosynthetic problem, especially if one assumes that this minor base is produced by enzymatic modification of specific uracil residues in the intact polynucleotide chain.

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References and Notes

1. Abbreviations used are as follows: tRNA, transfer ribonucleic acid; R_{TP}, electrophoretic mobility relative to 2′(3′)-uridylic acid; A_{270} units, optical density (1-cm cell) at 270 nm multiplied by the total volume of solution in milliliters; CMP, cytidylic acid; AMP, adenylic acid; GMP, guanylic acid; *m/e*, mass-to-charge ratio.
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6. The solvent systems used were as follows: solvent A, isopropyl alcohol, water (7 : 3); solvent B, isopropyl alcohol, conc. HCl, water (170 : 41 : 39); solvent C, *n*-butanol, formic acid, water (77 : 10 : 13); solvent D, *n*-butanol, acetic acid, water (5 : 1 : 4), upper layer; solvent E, *n*-butanol, acetic acid, water (5 : 3 : 2); solvent F, *n*-butanol saturated with water, ammonia (100 : 1).
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14. The mass spectra were obtained under the auspices of the U.S. Atomic Energy Commission. We thank L. Moore for technical assistance.
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