The Isolation of 4-Thiouridylic Acid from the Soluble Ribonucleic Acid of Escherichia coli

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Although 4-thiouracil was synthesized chemically as long ago as 1908 (1) and the preparation of the nucleoside was described in 1958 (2), no previous report of the natural occurrence of either compound has been made. The present work describes the isolation and identification of 4-thiouridylic acid as a normal minor component of the soluble ribonucleic acid of Escherichia coli.

MATERIALS AND METHODS

E. coli S-RNA1 was obtained from General Biochemicals. Other preparations were available in this laboratory. A sample of S-RNA prepared from Salmonella typhiimurium was the gift of Dr. David Smith. The 4-thiouridine disulfide was a gift of Dr. J. Fox, and another portion was obtained from Cyclo Chemical Company. Phosphomonoesterase from E. coli was kindly provided by Dr. Leon Heppel, and samples of xylose, lyxose, and arabinose were the gift of Dr. Gilbert Ashwell. Rhodium-alumina catalyst was obtained from Baker and Company.

Paper electrophoresis was carried out with the apparatus of Markham and Smith (3) and a potential of 20 volts per cm applied for 1 to 11 hours. Descending paper chromatography was done on Whatman No. 1 or 3MM paper, with either System I, 86% butanol-water (4), or System II, saturated (NH4)2SO4, pH 8.6. Paper electrophoresis in 0.05 M NaHCO3, pH 8.6, where the 4-thioUMP has a mobility of 19.6 cm per hour, 1.17 times greater than the fastest major mononucleotide, VUMP. The band is grossly a light yellow, and is readily eluted with water. Paper chromatography in System II removes the remaining impurities. This solvent often separates the 2'- and 3'-phosphate esters of nucleosides, and the 4-thioUMP was also found to travel as two bands (RUMP = 0.65 and 0.73). Elution of these two bands yielded materials which had matching spectra. Infrared spectra were run on a Perkin-Elmer model 21 spectrometer.

Column Separation of Alkaline Hydrolysates—S-RNA (in 1-g portions) was hydrolyzed with 0.3 M KOH at 37° for 18 hours, neutralized with Dowex 50 (H form) to pH 9 to 10, centrifuged, and adjusted to pH 8.6 with HCl. The solution, with an approximate volume of 250 ml, was brought to 0.02 M NH4HCO3, pH 8.6, and applied to a DEAE-cellulose column (4 × 24 cm) which had been equilibrated with 0.01 M NH4HCO3, pH 8.6. The column was washed with 200 ml of 0.025 M NH4HCO3, pH 8.6, and eluted with a linear gradient consisting of 750 ml of 0.05 M NH4HCO3 in 7 M urea in the mixing chamber and 750 ml of 0.25 M NH4HCO3 in 7 M urea in the reservoir, according to the method of Tomlinson and Tener (6). Fractions (20 ml each) were collected. Urea was removed from the pooled 4-thioUMP peak by the use of a smaller DEAE-cellulose column (7).

RESULTS

Isolation of 4-Thiouridylic Acid

Eight different noncommercial preparations of E. coli S-RNA, prepared by varying procedures, including hot detergent, phenol, and Ecteola column fractionation, were all found to have absorptions at 330 mp amounting to between 1.5 and 2% of that at 260 mp. A representative spectrum is shown in Fig. 1. This peak was found in S-RNA from strains B, K, and W, as well as in a sample of S-RNA obtained from the related organism, S. typhiimurium. Preparations of S-RNA derived from yeast and rat liver, on the other hand, lacked this peak. Their spectra curved smoothly down to the base line in the region of 310 mp.

Treatment of the E. coli S-RNA by prolonged dialysis against water, passage through columns of Sephadex G-75, chromatography on Ecteola columns (8), or adsorption with Norit or XE-64 resin failed to change the spectrum of the recovered RNA. It was concluded to be an integral part of the molecule.

Therefore, the S-RNA was digested with alkali, and the digests were fractionated chromatographically on urea-DEAE-cellulose columns at pH 8.6. At this pH, the absorption maximum of the minor peak in the whole digest had shifted to 320 mp. A peak which absorbed at 320 mp was eluted from the column between the major mononucleotide peak and the nucleoside diphosphates (Fig. 2). This peak was pale yellow in color. Further purification was carried out by paper electrophoresis in 0.05 M NH4HCO3, pH 8.6, where the 4-thioUMP has a mobility of 19.6 cm per hour, 1.17 times greater than the fastest major mononucleotide, UMP. The band is grossly a light yellow, and is readily eluted with water. Paper chromatography in System II removes the remaining impurities. This solvent often separates the 2'- and 3'-phosphate esters of nucleosides, and the 4-thioUMP was also found to travel as two bands (RUMP = 0.65 and 0.73). Elution of these two bands yielded materials which had matching spectra. Dephosphorylation with E. coli phosphomonoesterase (which released all the phosphate present) gave products which were apparently identical. They had the same spectrum (identical with that of the nucleotide, shown in Fig. 3) and the same mobilities on electrophoresis at pH 8.6 (6.6 cm per hour) and in paper Systems I (Rart = 2.17) and II (RUMP = 0.49). The formation of the 2'- and 3'-phosphate isomers of 4-thioUMP is good evidence that the nucleotide is indeed covalently bound in the ribonucleic acid chain.

Solutions of 4-thiouridine are not completely stable at neutral or acid pH. A slow decomposition has been noted which results

1 Abbreviations used are as follows: S-RNA, soluble ribonucleic acid; 4-thioUMP, the 2'-(3')-phosphate of 4-thiouridine.
in a loss of absorption at 330 m\(\mu\), an increase at 260 m\(\mu\) and in
the appearance of uridine on chromatography in System I. This
acid instability precludes the use of Dowex 1 (formate) columns
for isolation, since the bulk of the 4-thioUMP is degraded before
it can be eluted. Traces of the intact material may be detected
just after the elution of GMP.

**Yield**—The yield of 4-thioUMP from the DEAE-cellulose col-
umn, with the use of an average \(E_{260}\) of 12,000 for the alkaline
digest of S-RNA and the known \(E_{260}\) of 17,000 for the 4-thioUMP
peak, is calculated to be about one 4-thioUMP for 140 S-RNA
nucleotides.

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**Fig. 1.** Spectrum of S-RNA from *E. coli* B. A solution of
S-RNA prepared in 0.01 m \(\text{NH}_4\text{HCO}_3\), pH 8.6, and having an \(A_{260}\)
of 74.5, was diluted 100-fold in the same buffer. The insert shows
the spectrum of the undiluted solution in the 300 to 380 m\(\mu\) region.

**Fig. 2.** Chromatography of an alkaline digest of *E. coli* S-RNA
on a urea-DEAE-cellulose column. For details, see under “Ma-
terials and Methods.”

**Identification of 4-Thiouridyllic Acid**

**Conversion to UMP**—A solution of the isolated nucleotide was
hydrolyzed in 1 n \(\text{HCl}\) at 100\(^\circ\) for 1 hour. Chromatography of
the hydrolysate in System II indicated that the original sub-
stance had been partially changed to a second nucleotide, migrat-
ing with UMP and CMP. More vigorous HCl hydrolysis, heating in 5 n \(\text{HCl}\) at 100\(^\circ\) for 2 hours, was carried out. No re-
ducing sugars were liberated, as measured by the orcinol test.
Electrophoresis of the neutralized hydrolysate indicated a single
major nucleotide, migrating with the mobility of UMP. The
yield, based on phosphate, was about 80\% of that in the original
unknown nucleotide. The infrared spectrum, run in \(\text{D}_2\text{O}\),
agreed with those for UMP (10).\(^2\) Dephosphorylation of the
UMP and chromatography in System I, which will separate
uridine from thymidine, ribothymidine, 5-hydroxyuridine, and
5-hydroxymethyluridine, confirmed the identification as uridine.

**Identification of Ribose**—A sample of the UMP resulting from
the preceding hydrolysis in 5 n \(\text{HCl}\) was hydrogenated in 0.05 m
phosphate, pH 8.0, with a rhodium-alumina catalyst (11).
After filtration through glass wool, the filtrate gave a quantita-
tive orcinol test for pentose in 60\% yield, approximately that
obtained from known UMP.\(^3\) A second sample of the reduced
solution was dephosphorylated with phosphomonoesterase, hy-
drolyzed in 1 n \(\text{HCl}\) at 100\(^\circ\) for 1 hour, dried, and electrophoresed
in 0.05 m borate buffer, pH 9.1, against samples of the four pos-

\(^2\) We are indebted to Dr. H. T. Miles for this spectrum.

\(^3\) G. Ashwell, personal communication, and M. N. Lipsett, un-
published observation.
Identification of Sulfur—The isolated 4-thioUMP was dephosphorylated, and on exploratory examination of this material in the mass spectrometer revealed a peak of mass 128.002. This mass corresponded well with that of a thiouracil, 128.003, and the observation prompted qualitative tests for sulfur. Oxidation of the nucleoside with nitric acid resulted in a positive test for sulfate by the β-chloranilate method (13). Vigorous reduction of the nucleotide at pH 8 with solid KBH₄ carried out until the 330 m to absorption was gone, was followed by acidification, which led to a noticeable odor of H₂S.

Site of Attachment of Sulfur—Although the ultraviolet spectrum of the isolated nucleotide agreed with that for known 4-thiouridine (see above, Fig. 3), further proof was sought for the site of attachment of the sulfur. The isolated 4-thioUMP (0.3 μmole) was sealed in a tube with 0.2 ml of ethanol which had been saturated with ammonia gas at 0°C. The tube was heated for 5 hours in a water bath at 65°C, after which the contents were dried and separated by paper electrophoresis at pH 8.6. There was only one major band, which migrated with the CMP marker and which on elution had the acid and alkaline spectra of CMP. This result definitely places the sulfur in position 4 of the pyrimidine nucleoside, according to the reaction shown in Scheme 1.

Infrared Spectrum—As a final check on the identification of this nucleotide as 4-thioUMP, a sample was dephosphorylated and chromatographed in System II, along with a known sample of 4-thiouridine. Infrared spectra were run on KBr pellets prepared from the eluted materials and were essentially identical. The band ascribed to a 4-carbonyl, usually seen at 6.05 μ (10), is lacking, further evidence of thiolation in this position. Infrared spectra run in D₂O solution gave essentially the same results.

Properties of 4-Thiouridylic Acid

The spectral characteristics of 4-thiouridine are given in Table I. Neither the Ellman (15) nor the nitroprusside (16) reaction occurs to any noticeable extent at usual sulfur concentrations. The qualitative test with the iodine-sodium azide reaction (17), which is positive with catalytic amounts of sulfide or sulfhydryl compounds, was found to be positive with intact S-RNA and with each step of the purification. This reagent may be used as a paper spray for quick location of sulfide-containing compounds, since they decolorize the iodine instantly and leave a white spot on the brown background.

A spectrophotometric titration of the isolated 4-thiouridine was carried out, with use of the shift in absorption maximum from 331 to 317 m to as the criterion. Both the known and the isolated 4-thiouridine showed a pHₕ of enolization of 8.2. This pH explains the behavior of 4-thioUMP during the preliminary separations on washed DEAE-cellulose and electrophoresis. Both methods depend upon the molecular charge, and at pH 8.6, where they were carried out, 4-thioUMP is more highly charged than the other nucleotides.

The formation of a disulfide compound is easily carried out in solutions of 4-thiouridine with 10⁻² M iodine in KI (18). The reaction, accompanied by the shift in ultraviolet maximum to 311 m to and a marked hypochromicity, proceeds more rapidly in alkaline (pH 8 to 9) solution than at neutrality, although it can be observed at pH 7. On chromatography in System I, thiouridine disulfide migrates with an Rₕ of 0.06, as compared with 0.15 for uridine and 0.35 for 4-thiouridine. On paper electrophoresis at pH 8.6, its mobility is 0.9 cm per hour, compared to 2.7 cm per hour for uridine and 7.0 cm per hour for 4-thiouridine.

The instability of 4-thioUMP in neutral or acid solutions, evidenced by desulfuration to UMP, has been mentioned above. Oxidation of 4-thiouridine with periodate will also cause desulfuration, yielding uracil and the ribose fragments.

Preliminary observations on S-RNA from E. coli which has been partially fractionated on DEAE-cellulose columns indicate that the 330 m to absorption characteristic of 4-thioUMP is not present in the same proportion over the entire spectrum of amino acid acceptor species. No attempts at correlation of occurrence with species have yet been made.

**DISCUSSION**

In general, the unusual nucleotides in S-RNA have been found to contain either methylated varieties of the common bases or ribose attachments through a carbon to carbon bond, as in pseudouridine. While the work reported here was being completed, a report by Carbon, Hung, and Jones (19) described the occurrence of 2-thiouridylic acid in the S-RNA of rabbit liver and E. coli. The thionucleotides, therefore, represent a third class of unusual bases present in S-RNA.

The presence of 4-thioUMP in S-RNA could confer some unique properties on the molecule. First, the unusually low pK reported here for the nucleotide, namely, 8.2, makes possible a marked shift in the hydrogen-bonding potential of the base within a pH range attainable in a cell. Secondly, the possibility of the formation of disulfide bridges is introduced. Carbon et al. (19) have shown that such bonds can be produced chemically in S-RNA preparations. Further, they have shown that in lysine acceptor S-RNA, for example, only one species of that amino acid acceptor can form disulfides. Whether the thionucleotide responsible in this case is the 2 or the 4 isomer has not yet been determined. The product could even be a mixed disulfide, since the occurrence of 4-thiouridine in unfractonated E. coli S-RNA.

We are indebted to Dr. Bruce Ames and Dr. David Smith for these samples.
is approximately 4 times that of 2-thiouridine (19). In any case, it is certainly within the realm of possibility that such disulfide bonds, easily formed and easily reversible, may play a role in the stereochemistry of S-RNA.

SUMMARY

A sulfur-containing nucleotide, 4-thiouridylic acid, has been isolated as a minor constituent from normal soluble ribonucleic acid of Escherichia coli.

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