A Method for Locating 4-Thiouridylate in the Primary Structure of Transfer Ribonucleic Acids*

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ABSTRACT: Transformation of 4-thiouridylate residues of transfer ribonucleic acid to radioactively labeled N^4-methylcytidylate residues is achieved under mild conditions where the major bases are unaffected and the polymeric structure of transfer ribonucleic acid remains intact. The introduction of this unique, chemically stable, radioactive label at the site of 4-thiouridylate enables the use of standard sequence methods for locating this unstable residue in the primary structure of transfer ribonucleic acid. Using this labeling procedure, the distribution of 4-thiouridylate among the T-1 ribonuclease digestion products of unfractionated *Escherichia coli* B transfer ribonucleic acid has been scanned, and the sequence around the 4-thiouridylate residue of *E. coli* B valine transfer ribonucleic acid has been investigated.

Although general procedures are available for the determination of the primary structures of tRNAs (*cf.* Holley *et al.*, 1965; Sanger *et al.*, 1965), specific problems arise in locating particularly labile minor nucleotides (*cf.* Madison, 1968). Among these labile residues is 4-thioU,[1] a constituent of *Escherichia coli* tRNA (Lipsett, 1965), which is subject to desulfuration under some conditions of tRNA fractionation (*cf.* Weeren *et al.*, 1969) and of sequence analysis (Madison, 1968). In the course of investigating chemical transformations of 4-thioU residues, reactions have been developed which can incorporate a chemically stable and unique radioactive (^{14}C or ^{3}H) label at the site of 4-thioU in the primary structure of tRNA. The labeled product of this transformation, N^4-mC, may be readily located by established sequencing techniques.

The reactions employed for this transformation were previously elaborated for thionucleoside model compounds (Ziff and Fresco, 1968). Under appropriate conditions, oxidation of the 4-thiouracil moiety of these nucleosides by sodium periodate (*cf.* Scheme I) yields the corresponding 2-oxypyrimidine-4-sulfonate nucleosides. The latter compounds are highly reactive at the 4 position with a variety of oxygen, nitrogen, and sulfur nucleophiles; hydrolysis at acid or alkaline pH yields uracil nucleosides, while ammonolysis with NH_3 or CH_3NH_2 yields cytosine and N^4-methylcytosine nucleosides, respectively.

In the present work this reaction sequence has been used to transform 4-thioU in tRNA to labeled N^4-mC. Under the conditions employed, the major bases are unreactive, and the polymeric structure of tRNA remains intact. The ability to introduce this unique and stable radioactive label at the site of 4-thioU has been exploited for the purpose of locating this residue in the oligonucleotides obtained from RNase T-1 digests of unfractionated *E. coli* B tRNA, and a purified $tRNA^{Val}$ from *E. coli* B.

Materials

Unfractionated *E. coli* B tRNA was obtained from Schwarz BioResearch, Inc. It was dialyzed (4°) exhaustively *vs.* 1.0 M NaCl–0.01 M EDTA (pH 7.5) and then *vs.* H_2O, and then was lyophilized. Purified *E. coli* B $tRNA^{Val}$ (major valine-acceptor tRNA) was the generous gift of Dr. A. D. Kelmers. It accepted 1.2 nmoles of valine/A_{260} unit, and had $A_{260}/A_{335} \sim 40$. The preparation was exhaustively dialyzed (4°) *vs.* 0.01 M EDTA (pH 7.5), then *vs.* H_2O, and was then lyophilized. Unfractionated baker's yeast tRNA was prepared as previously described (Lindahl and Fresco, 1967).

Alkaline phosphatase, RNase A, and snake venom phosphodiesterase were obtained from Worthington Biochemical Corp. RNase T-1 was purified by a modification of the methods of Takahashi (1961) and Rushizsky and Sober (1962). [^{14}C]Methylamine-HCl was obtained from Nuclear Chicago Corp., and utilized at 0.78 Ci/mole unless otherwise noted. Methylamine-HCl (Matheson-Coleman, recrystallized once from H_2O) was warmed in NaOH solution in a gas-generating apparatus, and the resulting gas was bubbled through freshly boiled distilled H_2O. The concentration of methylamine solutions so obtained was determined by HCl titration to the methyl red end point. The pH of solutions of methylamine was adjusted with HCl. Solutions of sodium periodate (Fisher, reagent grade) were prepared just prior to use. Cacodylic acid (Fisher) was recrystallized from H_2O–ethanol solution to remove ultraviolet-absorbing impurities. N^4-Methylcytidine was prepared as previously described (Ziff and Fresco, 1968). Deoxyribonucleosides were obtained from CalBiochem; Sephadex G-100 and Sephadex A-25 from

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[1] Abbreviations used are: free nucleotides are indicated by the letters MP (monophosphate) preceded by the letter symbol for the base moiety, in turn preceded by the number indicating site of ribose esterification, *e.g.*, 2′-(3′)-4-thioUMP = 2′(3′)-4-thiouridylate; nucleotide residues of a nucleic acid are symbolized either by the letter symbol of the base moiety, *e.g.*, N^4-mC = N^4-methylcytidylate residue, or in conjunction with the word *residue;* PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; R_m = ratio of distance traveled by compound to that traveled by reference compound in electrophoresis.

Pharmacia Fine Chemicals; Dowex AG 1-X4 from Bio-Rad Corp.; and Whatman DE-32, microgranular DEAE-cellulose from Reeve-Angel, Inc. The latter was washed and equilibrated with buffer according to manufacturer's directions. Paper for chromatography was Whatman No. 1, used as obtained.

Methods

Ultraviolet Absorption. Measurements were performed with either a Cary Model 14 spectrophotometer, using 1.0 and 0.1 slide wires as required (spectra), or with a Beckman DU monochromater equipped with a Gilford Model 220 optical density converter (single wavelength measurements).

Radioactivity. A Packard scintillation spectrometer was employed. Aqueous solutions were counted in 15 ml of Bray's solution (Bray, 1960), while samples retained on Millipore filters or on paper chromatogram strips were counted in a scintillation fluid containing 4 g of PPO and 200 mg of POPOP per l. of toluene. Counting efficiency was determined either by the channels ratio technique or by counting a known number of micromoles of [^{14}C]methylamine under standard conditions.

tRNA Concentration. This was determined spectrophotometrically in 0.01 M $MgCl_2$–0.01 M cacodylate (Na^+) (pH 7.0), using the relationships: A_{260} 0.1% solution tRNA = 20.5 (Lindahl and Fresco, 1967) $\cong$ 1.8 nanomoles of tRNA = 1 A_{260} unit.

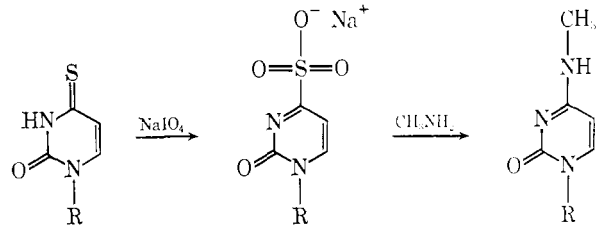
Derivatization of Unfractionated tRNA. tRNA (1.5 mg/ml) in 0.0078 M [^{14}C]methylamine (pH 10.4)–0.0078 M $NaIO_4$ (added last to initiate reaction) was incubated at 40° for 45 min. The tRNA was precipitated with 0.05 volume of 3 M NaCl and 2 volumes of ethanol at 0° (1 hr), collected by centrifugation, and dialyzed (4°) *vs.* 0.01 M NaCl–0.01 M phosphate (Na^+), pH 7 (400 volumes, 2 changes), and then exhaustively *vs.* H_2O. The course of this reaction was determined by withdrawing aliquots (100 μl) at designated times, and precipitating the tRNA at 0° with 1.5 ml of 10% trichloroacetic acid. The precipitate was collected on Millipore filters, rinsed with trichloroacetic acid, and counted.

Iodine-Oxidized tRNA. E. coli B tRNA (7 mg/ml) in 0.002 M I_2–0.002 M KI–0.01 M phosphate (Na^+, pH 7.0) was incubated for 30 min at 0°. The tRNA was freed of reagents by repeated precipitation with NaCl and ethanol, dialysis (4°) *vs.* 800 volumes of 0.15 M NaCl–0.015 M citrate (Na^+, pH 6.8, 10 hr), and exhaustive dialysis *vs.* H_2O.

Gel Filtration of tRNA. A column of Sephadex G-100 (93 × 0.9 cm) at 22° was equilibrated and eluted with 0.15 M KCl–0.01 M $MgCl_2$–0.0005 M EDTA–0.01 M cacodylate (Na^+, pH 7.0); 10-min fractions (1.8 ml) were collected and A_{260} was determined directly. Radioactivity was determined by counting 1-ml aliquots.

Alkaline Hydrolysis of tRNA. tRNA (1.0–1.5 mg/ml) in 0.3 M KOH (3–4.5 ml) was incubated at 37° for 18 hr in a sealed polyethylene tube. The digestion mixture was adjusted to pH 7 with formic acid, diluted 20-fold with H_2O, and applied to a Dowex AG 1-X4 (formate) column (25 × 0.9 cm). After a rinse with 20 ml of H_2O, the column was eluted at 0.5 ml/min with two linear gradients of formic acid (H_2O to 1.0 M formic acid, total volume 150 ml; 1.0 M formic acid to 4.0 M formic acid, total volume 400 ml) followed by 4.0 M formic acid (80–120 ml) and finally 2 M HCl. Radioactivity

SCHEME I



was determined by counting aliquots (250 μl) of fractions. (Formic acid did not decrease the counting efficiency by more than 5%.) Aliquots containing HCl were either taken to dryness or neutralized with NH_4OH before counting. The eluted mononucleotides were freed of formic acid by lyophilization, and were identified when possible by their ultraviolet spectra in acidic and basic solution. Radioactive peaks corresponding to 2′- and 3′-N^4-mCMP were dephosphorylated by incubation with alkaline phosphatase (10 μg/ml) in 0.15 M NH_4HCO_3 at 37° for 18 hr. Salts were then removed by lyophilization.

Descending Paper Chromatography. This was performed at 22° as described under Results. Major spots were located by ultraviolet quenching, and radioactive components by counting 1-cm wide strips of the chromatogram.

4-ThioU Content of tRNA. This was determined from the difference spectrum (300–360 mμ) between nuclease digests of untreated *E. coli* B tRNA and nuclease digests of *E. coli* B tRNA whose 4-thioU had been removed ($tRNA_{IO_4^-}$). $tRNA_{IO_4^-}$ was prepared by incubating tRNA (2.5 mg/ml) in 0.01 M $NaIO_4$ for 45 min at 40°. The tRNA was freed of periodate by repeated precipitation with NaCl and ethanol, then dialyzed (4°) first *vs.* 0.01 M acetate (Na^+), pH 4.5 (500 volumes, 2 hr), then exhaustively *vs.* H_2O, and finally lyophilized. Nuclease digests were prepared by incubating tRNA (1.5 mg/ml) at 37° with both RNase A (10 μg/ml) and RNase T-1 (6 μg/ml) in 0.01 M Tris-HCl (pH 7.2). After 12 hr, the pH was adjusted to neutrality with several microliters of 1 M NaOH, and the incubation was continued for an additional 6 hr. Final digests of both tRNA and $tRNA_{IO_4^-}$ exhibited hyperchromic changes of 32% at 260 mμ relative to the Mg^{2+}-containing starting polymers, indicating digestion to very short oligonucleotides. While the spectrum of the digested tRNA revealed the 4-thioUMP absorption band with maximum at 330 mμ (A_{330}-digested tRNA = 2.13% A_{260} Mg^{2+} containing undigested tRNA), this band was absent from the spectrum of the digest of $tRNA_{IO_4^-}$. The only absorption at 330 mμ observed with the latter was contributed by the tail of the (main) 260-mμ absorption band (A_{330}-digested $tRNA_{IO_4^-}$ = 0.40% A_{260} Mg^{2+}-containing undigested $tRNA_{IO_4^-}$). The difference between these two spectra was a spectrum characteristic of 4-thioUMP, *i.e.*, it was symmetrical and gaussian between 300 and 360 mμ, with a maximum at 332 mμ equal to 1.75% of A_{260} of the Mg^{2+}-containing undigested tRNA. Using the extinction coefficient $\epsilon_{331} = 21.5 \times 10^3$ (determined at pH 6.5 for 4-thiouridine (Kochetkov *et al.*, 1963)) this absorption difference is equivalent to 0.45 mole of 4-thioU/mole of tRNA.

Sensitivity of Major Bases to Derivatization. Individual 2′-deoxyribonucleosides (concentration indicated in Table II) in 12 ml of the reagent solution used to derivatize tRNA

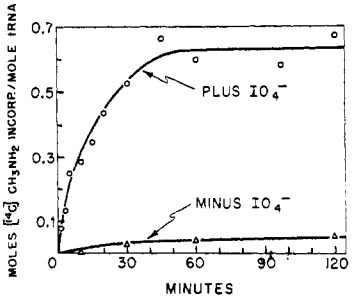


FIGURE 1: Kinetics of incorporation of ^{14}C from methylamine into *E. coli* B tRNA. Standard derivatization conditions were employed, except that the control lacked periodate. Assay was for trichloroacetic acid insoluble radioactivity.

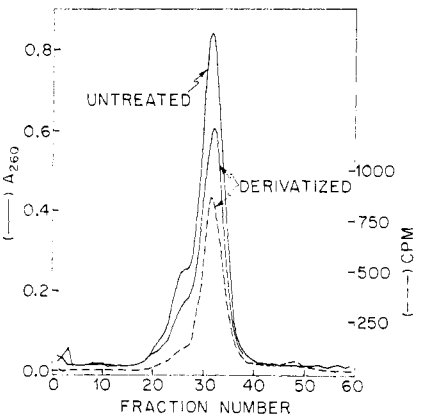


FIGURE 2: Gel filtration chromatography of *E. coli* B tRNA before (9.7 A_{260} units) and after derivatization (7.6 A_{260} units). Heating of an aliquot of the derivatized tRNA for 2 min at 70° in 0.01 M cacodylate (Na^+, pH 7) had no effect on its profile.

were incubated for 45 min at 40°. After neutralization with 0.1 M HCl, the solutions were freed of periodate on passage through a Dowex AG 1-X4 (acetate) column (volume = 2 ml). Unreacted nucleosides were recovered after an H_2O wash (40 ml) (exception: 2′-deoxyguanosine was isolated with a 40-ml wash of 30% methanol (v/v)). Spectral measurements at pH 1 (exception: 2′-deoxyguanosine at pH 7) enabled identification and estimates of recovery.

Digestion of Derivatized E. coli B tRNA with RNase T-1. tRNA (41 A_{260} units) was digested with RNase T-1 (12 μg/ml) for 18 hr at 37° in 0.01 M Tris-HCl (pH 7.2), total volume 2.0 ml. After lyophilization, the digest was taken up in 0.2 ml of H_2O and applied to a DE-32 (DEAE) column (103 × 0.5 cm) equilibrated with 0.05 M acetate (NH_4^+) (pH 8.3). The oligonucleotides were eluted under pressure, at 0.2 ml/min using a linear gradient of ammonium acetate (pH 8.3; 0.05–0.75 M, total volume 760 ml), and located by scanning for A_{260} and radioactivity (which was determined by counting 1.0-ml aliquots of fractions).

Locating 4-ThioU in E. coli B tRNA^{Val}. $tRNA^{Val}$ (14.7 A_{260} units) was incubated with 31 μmoles of [^{14}C]methylamine (pH 10.4; 0.97 Ci/mole) and 15 μmoles of $NaIO_4$ (total volume 2.0 ml) at 40° for 50 min. Such conditions were shown in preliminary experiments to give maximal yield of derivative. After addition of 0.2 ml of 3 M NaCl and 4 ml of cold ethanol and storage at −20° for 10 hr, the precipitated tRNA was collected by centrifugation, dialyzed (4°) *vs.* 400 volumes of 0.01 M phosphate (Na^+, pH 7.0) overnight, then exhaustively *vs.* H_2O, and then lyophilized. The sample was taken up in 1.0 ml of 0.01 M Tris-HCl (pH 7.2) and 25 μl of toluene (preservative) and digested with RNase T-1 (60 μg/ml) in a sealed tube for 22 hr at 37°. After lyophilization, the digestion mixture was fractionated on a column of DE-32 as described above, and fractions were scanned for A_{260} and radioactivity (determined on 250-μl aliquots). The pooled fractions of the major radioactive oligonucleotide peak were desalted by dilution to an ammonium acetate concentration of 0.05 M, and passage onto a column of Sephadex A-25 (HCO_3^-) (1.5 × 1.0 cm). After rinsing the column with 10 volumes of 0.05 M ammonium carbonate and 15 volumes of H_2O, the oligonucleotide was eluted with 15 ml of 1.0 M ammonium bicarbonate (later removed by lyophilization).

Digestion of this oligonucleotide with RNase A, alkaline phosphatase, or snake venom phosphodiesterase was carried out in capillary tubes according to Sanger *et al.* (1965). Alkaline phosphatase was removed by phenol extraction. The dinucleotide standard ApCp was prepared by digesting poly (A,C) kindly prepared by A. Lomant and A. Lesk with polynucleotide phosphorylase (input diphosphate ratios, ADP: CDP, 1:4), with RNase A and extracting the resulting hydrolysate repeatedly with phenol. ApCp was resolved electrophoretically as described in Results.

Electrophoresis was carried out with a Savant water-cooled flat-plate electrophoresis apparatus at 50 V/cm applied voltage on 50-cm long strips of Whatman No. 3MM paper, with a buffer of 1% acetic acid titrated to pH 4.1 with concentrated NH_4OH. Samples were adjusted to this pH prior to application. Spots were located as for paper chromatorgrams.

Results

Derivatization of tRNA. The chemical transformation of 4-thioU (*cf.* Scheme I) in tRNA to N^4-mC residues involves two successive reactions (Ziff and Fresco, 1968). The rate of the first step, the oxidation of 4-thioU by $NaIO_4$ (in large excess) to 2-oxypyrimidine-4-sulfonate residues, was shown previously (Ziff and Fresco, 1967) to be very substantially slowed by conditions that stabilize the secondary and tertiary structure of tRNA. To minimize these structural influences, tRNA was freed of divalent cations prior to derivatization by exhaustive dialysis *vs.* EDTA, and oxidation was carried out in a solvent of low ionic strength at a temperature near T_m for tRNA (in that solvent).

The second step, the nucleophilic displacement of the sulfonic acid moiety by methylamine, is facilitated by the good leaving-group character of SO_3^- (Suter, 1944; Bunnett and Zahler, 1951), and the electron-withdrawing effect of the azine ring nitrogens, which activates the 4 position of the pyrimidine ring for nucleophilic attack (Shepherd and Fedrick, 1965). The aqueous solvent, of course, constitutes a competing nucleophile which could lead to the formation of uracil residues at the expense of N^4-mC residues (Ziff and Fresco, 1968). To minimize this possibility, the desired nucleophilic derivatizing agent, methylamine, was included together with the periodate oxidizing agent so that the sequential reactions could take place concurrently. Since the unprotonated (free base) form

TABLE I: Paper Chromatography and Electrophoresis of Various Compounds.[a]

Compound	R_F Solvent A	R_F Solvent B	R_m pH 4.1
2′-(3′)-N^4-mCMP	0.62	0.58	1.14
N^4-Methylcytidine	0.50	0.81	
Cytidine	0.57		
2′-(3′)-CMP	0.63	0.39	1.00
ApCp			1.70
Digest 2 fragment—presumed N^4-mCpAp			1.98
Digest 1 fragment—presumed ApUpN^4-mCpAp			2.6

[a] Solvent A, saturated $(NH_4)_2SO_4$–isopropyl alcohol–1 M sodium acetate (pH 6.0, 80:2:18); solvent B, ethanol–1 M ammonium acetate (pH 7.5, 7:3). R_m values were determined (see Methods) using 2′-(3′)-CMP as the reference compound.

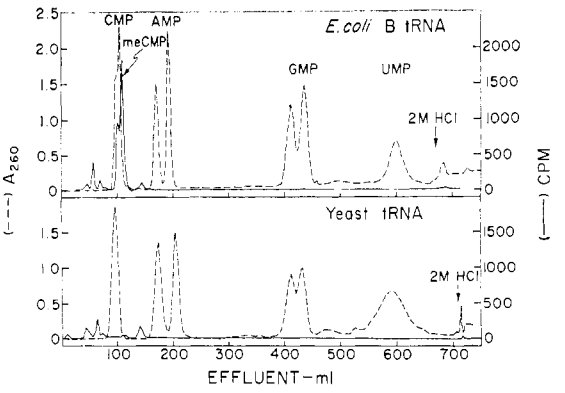


FIGURE 3: Dowex 1 (formate) chromatograms of alkaline digests of derivatized tRNA. Before digestion, *E. coli* B tRNA (87 A_{260} units) contained 0.62 mole of trichloroacetic acid precipitable methylamine-derived radioactivity per mole of tRNA, and yeast tRNA (96 A_{260} units) 0.08 mole/mole of tRNA.

of methylamine is the active nucleophile, derivatization was carried out near its pK, at pH 10.4.

Incubation of *E. coli* B tRNA in such a derivatization reaction mixture resulted in periodate-dependent incorporation of ^{14}C from methylamine into trichloroacetic acid insoluble material (Figure 1) that reached a plateau when 0.62 mole of ^{14}C per mole of tRNA was bound. Gel filtration of this derivatized tRNA (after purification), both before and after thermal denaturation (Figure 2), showed that over 90% of the radioactivity cochromatographed with the tRNA, indicating that label from [^{14}C]methylamine was covalently bound to the polynucleotide. Furthermore, the ultraviolet elution profiles of derivatized tRNA before and after thermal treatment closely resembled that of untreated tRNA, suggesting that chain cleavage did not occur during derivatization. Since the native conformation of tRNA is stable under the chromatography conditions (Fresco *et al.*, 1966), this result also shows that the transformation of 4-thioU to N^4-mC residues does not grossly alter the conformation of native tRNA.

Identification and Yield of the Transformation Product. The 335-mμ band of the 4-thioU residues of untreated *E. coli* B tRNA (Lipsett, 1965) was absent from the spectrum of derivatized tRNA. This loss of absorption could not be reversed by reducing agents, distinguishing it from the qualitatively similar spectral change that occurs when disulfide bridges are formed as a result of iodine oxidation of 4-thioU residues of tRNA (Lipsett, 1967) or model 4-thiouracil nucleosides (Ziff and Fresco, 1968). Thus, under conditions that reduce such disulfide bridges (11 A_{260} units of tRNA/ml, 0.05 M $Na_2S_2O_3$–0.01 M phosphate (Na^+), pH 7, 22°) and regenerated 25% of the 4-thioU absorption at 335 mμ of an I_2-oxidized sample of tRNA within 60 min, *no* increase of A_{335} occurred for periodate–methylamine-derivatized tRNA during 200 min. This result is consistent with the observed incorporation of radioactivity from the methylamine nucleophile into the derivatized tRNA.

The residue end product of the derivatization, labeled N^4-mC, was isolated from alkaline digests of derivatized tRNA. Ion-exchange chromatography of the resulting mononucleotides (Figure 3) completely separated the four major nucleotides and partially fractionated the 2′ and 3′ isomers of CMP, AMP, and GMP. Of the radioactivity applied to the column, 15% eluted with an H_2O wash and was judged to be nonnucleotide in character. (A similar proportion was found to be acid soluble, although nondialyzable, prior to digestion with alkali. A radioactive fraction with such characteristics was encountered in each experiment and may represent methylamine bound to tRNA by an acid-labile linkage; *e.g.*, Schiff base formation with the dialdehyde resulting from periodate oxidation of the 3′ terminus; *cf.* Khym, 1963.) Over 70% of the radioactivity retained by the column after H_2O wash (the mononucleotide fraction) eluted in a double peak overlapping the partially resolved 2′ and 3′ isomers of CMP; another 15% eluted as minor peaks in the same region of the gradient, and the remainder was scattered throughout the chromatogram. Treatment of aliquots of each member of the major double radioactive peak with alkaline phosphatase yielded a single radioactive component chromatographically identical with authentic N^4-methylcytidine (*cf.* Table I). On this basis, the double radioactive peak was identified as 2′- and 3′-N^4-mCMP.

From the amount of radioactivity in this double peak, it was estimated that the derivatized tRNA contains >0.4 mole of N^4-mC per mole of tRNA, which agrees well with the spectrophotometrically determined value of 0.45 mole of 4-thioU/mole of tRNA for the same sample prior to derivatization. Essentially quantitative transformation of 4-thioU to N^4-mC is thereby indicated.

Maximum derivatization of purified $tRNA^{Val}$ (see below and Methods) required a lower concentration of tRNA and a higher concentration of methylamine in the derivatization mixture, in comparison with conditions used to derivatize unfractionated tRNA. These more strenuous conditions for derivatization may reflect some unusual stability conferred upon the 4-thioU residue by the secondary structure of $tRNA^{Val}$, or some property particular to homogeneous preparations of tRNA, such as their tendency to aggregate (Schleich

TABLE II: Stability of Major Nucleosides under Derivatization Conditions.

Nucleoside	$A_{\lambda max}$ Units		Absorption Ratios		
	Incubated	Recovered	Wavelengths	Initial	Final
2′-Deoxyuridine	104	102	260/280	2.92	2.93
2′-Deoxycytidine	130	128	280/260	2.10	2.10
2′-Deoxyadenosine	137	138	258/230	4.93	4.85
2′-Deoxyguanosine	135	139	253/280	3.42	3.30

and Goldstein, 1964; Zachau, 1968; D. Henley, T. Lindahl, and J. R. Fresco, 1969, manuscript in preparation). Derivatization of $tRNA^{Val}$ under the modified conditions empolyed incorporated 0.8 mole of methylamine/mole of tRNA into a trichloroacetic acid insoluble form.

Specificity of Derivatization. The reactivity of the four major bases under the conditions of derivatization was examined using their 2′-deoxyribonucleosides as model compounds. After exposure to derivatizing conditions, each deoxyribonucleoside was quantitatively recovered in an unchanged form (Table II).

The specificity of derivatization was also examined (see Table III) using preparations of *E. coli* B tRNA with altered 4-thioU residues, and yeast tRNA, which contains little or no 4-thioU (Lipsett, 1965). *E. coli* B tRNA oxidized with periodate at pH 7 and then dialyzed at pH 4.3 (a procedure which eliminates the 335-mμ absorption band, and converts the 4-thiouracil model nucleosides into uracil counterparts (Ziff and Fresco, 1968)) accepted only 15% as much methylamine as an untreated tRNA sample. Pretreatment with I_2 to form disulfide bridge between 4-thioU residues (Lipsett, 1967) also reduced the capacity of *E. coli* B tRNA to accept methylamine. This would be expected in view of the previous finding (Ziff and Fresco, 1968) that disulfides of model nucleosides are much more resistant to periodate oxidation at neutral pH than the corresponding 4-thiouracil nucleosides. Under the alkaline conditions of derivatization (pH 10.4) some reaction of these disulfides would be expected, however, as has been observed here with tRNA. Such disulfides dismutate in alkali to the corresponding 4-thiouracil and 4-sulfenic acid moieties (Pal *et al.*, 1969), which in turn can be readily oxidized by periodate to the intermediate reactive with methylamine. Incubation of untreated yeast tRNA under the standard derivatization conditions led to the incorporation of low but significant levels of methylamine. Chromatography of alkaline digests of such derivatized yeast tRNA (Figure 3) revealed radioactive peaks qualitatively similar to the *minor* components observed in comparable chromatograms of derivatized *E. coli* B tRNA (Figure 3). However, less than 0.02 mole of radioactive mononucleotide per mole of yeast tRNA eluted in the position expected for 2′-(3′)-N^4-mCMP. These minor components could be due to low levels of other reactive nucleotides, possibly thionucleotides, in both these tRNAs.

4-ThioU Distribution among E. coli B tRNA Sequences. Unfractionated tRNA was derivatized, digested with RNase T-1, and the oligonucleotides were fractionated. Radioactive

TABLE III: Dependence of Incorporation of Methylamine Radioactivity (during Derivatization) upon Integrity of 4-thioU Residues of tRNA.

Sample and Pretreatment	Results after Derivatization[b]	
	TCA-[a] Precipitable Methylamine	N^4-mCMP Recovered
E. coli B tRNA, untreated	0.62	0.40
E. coli B tRNA, periodate treated at pH 7.0 and then exposed to pH 4.3	0.10	
E. coli B tRNA, iodine treated	~0.3	
Yeast tRNA, untreated	<0.1	<0.02

[a] TCA = trichloroacetic acid. [b] In moles per mole of tRNA.

oligonucleotide peaks were scattered throughout the chromatogram (Figure 4), indicating heterogeneity in the nucleotide sequences surrounding 4-thioU residues in the many tRNA species of the unfractionated mixture. However, the pattern of such labeled peaks suggests a nonrandom distribution of the 4-thioU residue among a limited number of oligonucleotides.

Location of 4-ThioU in an E. coli B $tRNA^{Val}$. The small sample available of purified $tRNA^{Val}$ permitted only a limited number of experiments to further evaluate the derivatization procedure for sequence studies. This sample (~75% pure) was derivatized, and two aliquots were separately digested with different preparations of RNase T-1 (digests 1 and 2) and then fractionated. As expected, these digests displayed much simpler radioactive profiles, with one predominant radioactive oligonucleotide in each case. The predominant oligonucleotide of digest 2 (*cf.* Figure 4) was present in >0.3 mole/mole of tRNA, while several minor ones each represented <0.05 mole/mole of tRNA. None of these minor peaks was present in an amount sufficient, relative to the major one, to indicate a second 4-thioU residue in the (major) $tRNA^{Val}$ component in the sample. However, these minor peaks could contain the same 4-thioU residue, and result from imperfect specificity by T-1 RNase, nuclease contaminants in the T-1 RNase, incomplete digestion, or else they could be derived from tRNA contaminants in the sample. These minor peaks were not further investigated.

From its position in the chromatogram, the major oligonucleotide in digest 2, which overlapped a nonradioactive peak, was judged to be at least a tetranucleotide, and it was likely terminated by Gp. After removal of its 3′-terminal phosphate with alkaline phosphatase, digestion with snake venom phosphodiesterase yielded 5′-N^4-mCMP, indicating that this residue is internal. Digestion of the untreated oligonucleotide with RNase A yielded 2′-(3′)-N^4-mCMP; the internal N^4-mC therefore had to be adjacent to a pyrimidine residue on its 5′ side.

The major radioactive oligonucleotide from digest 1 was judged by its position on the chromatogram to be a trinucleotide, again terminated by Gp. In this case, however, while digestion with RNase A also yielded 2′-(3′)-N^4-mCMP, this residue was released as the nucleoside after removal of the 3′-terminal phosphate from the oligonucleotide with alkaline phosphatase followed by digestion with snake venom phosphodiesterase; the N^4-mC residue was therefore in the 5′-terminal position.

Consistent with these differences, removal of the 3′-terminal Gp by alkaline phosphatase, followed by periodate oxidation and lysine cleavage (Neu and Heppel, 1964), gave different products for the oligonucleotides obtained from digests 1 and 2. That from digest 1 yielded a radioactive fragment with an electrophoretic mobility at pH 4.1 just greater than that of ApCp (Table I). This is as expected if Ap were the only internal residue of the trinucleotide, since N^4-mC has a lower pK than C, as verified by the greater electrophoretic mobility of 2′-(3′)-N^4-mCMP *vs.* 2′-(3′)-CMP at pH 4.1 (Table I). The sequence thereby indicated, N^4-mCpApGp, was further substantiated by the detection of only these residues in a microanalysis for nucleosides, kindly performed by Dr. M. Uziel. When the oligonucleotide of digest 2 was similarly treated to remove its 3′-terminal Gp, the resulting radioactive fragment exhibited an electrophoretic mobility at pH 4.1 very substantially greater than that of the corresponding fragment from the oligonucleotide of digest 1 (Table I), indicating its larger size and/or U content. Since both oligonucleotides were derived from the same tRNA sample and were the major N^4-mC-containing components in their respective digests, it seems likely that they both derive from the single 4-thioU residue of the $tRNA^{Val}$, so that their sequences overlap. The oligonucleotide from digest 1 presumably suffered additional degradation from some extraneous enzymatic contaminant since specific chemical cleavage at the site of derivatization of the tRNA has been excluded (*cf.* Figure 2).

Taken together, then, these results indicate that both N^4-mC-containing oligonucleotides were derived from the tRNA sequence .... *Pyrp* 4-thioUpApGp...., with the 5′ terminus indeterminate. This sequence is compatible with the pentanucleotide .... ApUp 4-thioUpApGp.... obtained from T-1 RNase digests of the same tRNA by Yaniv and Barrell (1969).

Discussion

Several reactions have recently been described for specific modification of the 4-thioU residues of tRNA (Ziff and Fresco, 1967, 1968; Ofengand, 1967; Carbon and David, 1968; Reid, 1968; Burton, 1967; Nishimura *et al.*, 1967; Cerutti *et al.*, 1968; Pleiss *et al.*, 1969). Of these, only the specific conversion of 4-thioU into N^4-mC has been exploited for the purpose of locating 4-thioU residues in tRNA sequences. This conversion represents a particular application of a more general series of transformations of the 4-thioU moiety studied in model nucleosides (Ziff and Fresco, 1968). The results reported here demonstrate that some of these reactions are, in fact, applicable to the specific chemical modification of tRNA structure at the site of 4-thioU, and that reaction of the 2-oxypyrimidine-4-sulfonate residue (formed by periodate oxidation of tRNA) with nucleophiles is a route for introducing into tRNA many different substituents on the 4 position of this residue. (In view of this reactivity, caution should be exercised in the use of nucleophilic biochemicals (*e.g.*, Tris, glutathione, cysteine, and lysine) in conjunction with periodate oxidation of thiol-containing tRNAs.) The effects of such modifications on the biological activity of tRNA will be described elsewhere.

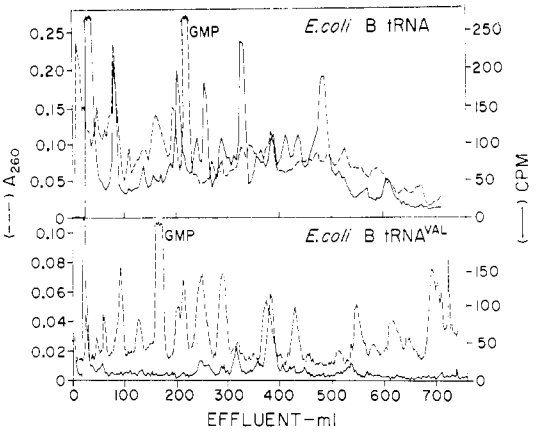


FIGURE 4: DEAE-cellulose chromatograms of RNase T-1 digests of derivatized tRNA. Before digestion unfractionated *E. coli* B tRNA (41 A_{260} units) contained 0.62 mole of trichloroacetic acid precipitable methylamine-derived radioactivity per mole of tRNA, and *E. coli* B $tRNA^{Val}$ (14.7 A_{260} units, — digest 2) 0.77 mole/mole of tRNA.

In providing a stable radioactive site label for 4-thioU in near-quantitative yield, the derivatization procedure meets the requirements of a sequence method for high yield, specificity, and sensitivity. While higher values for the 4-thioU content of unfractionated *E. coli* tRNA have been reported (Lipsett, 1966; Cerutti *et al.*, 1968), this difference may reflect sample variations due to the instability of the thiol group. Specificity derives from the limited origins possible for the radioactively labeled N^4-mC end product; *i.e.*, 2-oxypyrimidines with good leaving groups at the 4 position. Sensitivity is limited only by the specific radioactivity of the methylamine reagent. The derivatization method can therefore be used in conjunction with the method for sequence analysis of radioactive nucleic acids developed by Sanger *et al.* (1965) in which high specific radioactivity is essential for detection of sequence components. Furthermore, the method obviates the need for introducing isotopic sulfur by biosynthetic means into the 4-thioU position, where it is labile and subject to loss during purification or subsequent analytical procedures.

Other possible sites of attack by periodate or methylamine, alone or in combination, are present on the tRNA molecule. The *cis*-glycol of the 3′-nucleoside terminus of an RNA is readily oxidized to the dialdehyde by periodate (Whitfield and Markham, 1953); and the reaction conditions used to transform 4-thioU closely resemble those used to remove completely the 3′-terminal nucleoside of tRNA (Neu and Heppel, 1964). It is likely, therefore, that the 3′ terminus is liberated in the course of the derivatization procedure. While the dialdehyde provides a site for methylamine attachment through Schiff base formation, such linkages are labile at acid pH (Khym, 1963).

A systematic investigation of the reactivity of the minor bases with periodate and methylamine under derivatization conditions has not been performed. However, the lability of N^7-methylguanylate residues at alkaline pH (M. Uziel, personal

communication), and the oxidation of 2-thiomethyladenosine derivatives by periodate (Howard *et al.*, 1945) (a related residue has recently been demonstrated in tRNA (Burrows *et al.*, 1968)) suggest that these and perhaps other residues may be attacked (leading in some cases to labeled products). A slow oxidation of pseudo-uridylate by periodate has been reported (Tomasz *et al.*, 1965), but requires more vigorous conditions.

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