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Phototransformation of 4-Thiouridine in *Escherichia coli* Valine Transfer Ribonucleic Acid to Uridine, Cytidine, and N^4-Methylcytidine*

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ABSTRACT: The minor nucleoside 4-thiouridine in *Escherichia coli* tRNA^{Val} is transformed selectively to uridine upon irradiation of cetyltrimethylammonium salt of tRNA^{Val} in *tert*-butanol with light of wavelengths longer than 300 mμ. The four major nucleosides and the minor nucleosides pseudouridine, 1-methyladenosine, N^6-isopentenyladenosine, 5,6-dihydrouridine, 2-thiomethyl-N^6-isopentenyladenosine, and glucosyl-2-thiouracil (used as a model for 2-thiouridine), were found to be inert under these conditions. In a *tert*-butanol solution containing methylamine or ammonia, 4-thiouridine in tRNA^{Val} is converted into a mixture of N^4-methylcytidine and uridine or cytidine and uridine, respectively. At 93% total conversion of 4-thiouridine in tRNA^{Val} in the presence of 0.57 M methylamine, 54% uridine and 46% N^4-methylcytidine had been formed; at 92% total conversion in the presence of 0.72 M ammonia, 69% uridine and 31% cytidine had been formed.

The ratio of N^4-methylcytidine and cytidine over uridine formed increases with increasing amine concentrations. Uridine formation, however, cannot be suppressed even at high amine concentrations. Transformation of 90% of the original 4-thiouridine in tRNA^{Val} to uridine led to a 19% reduction in the valine acceptance. Retention of a substantial portion of the aminoacylation activity suggests that 4-thiouridine *per se* is not required as a specific recognition site for valyl-tRNA synthetase.

While the primary structures of numerous tRNAs have been elucidated and the major biological functions of tRNA understood, surprisingly little is known about the relation between the two. To be able to carry out its multifaceted functions as the translator molecule in protein synthesis considerable information and specificity must be built into the structure (primary, secondary, and tertiary) of each individual tRNA molecule, *i.e.*, ultimately into the sequence of 75–85 nucleotide residues. A highly characteristic feature of tRNA sequences is the presence of a variety of simple derivatives of the major nucleotides, the rare or minor nucleotides. Almost any derivation of a major nucleotide will result in some change of its base-pairing and -stacking properties. A large number of subtle structural variations become possible by changing the distribution, number, and kind of minor components in the nucleotide sequence of a tRNA. Such a "fine structure" may be needed for the specific interaction of tRNA with enzymes, ribosomes, and mRNA, for the regulation of protein synthesis or for functions of tRNA which are not yet appreciated.

One such minor component, 4-thiouridine, has been identified in purified, unfractionated tRNA from *Escherichia coli* (Lipsett, 1965). Subsequent sequencing studies on several highly purified, fractionated tRNAs from *E. coli* have demonstrated that this nucleoside occupies the eighth nucleoside site removed from the 5′-terminal end (Zachau, 1969). A number of chemical procedures for the modification of 4-thiouridine have been developed. Transformation of 4-thiouridine to uridine or, in the presence of added nucleophiles such as ammonia or methylamine, to uridine plus cytidine derivatives has been accomplished with $KMnO_4$, $NaIO_4$, and OsO_4 (Hayatsu and Ukita, 1967; Hayatsu and Yano, 1969; Ziff and Fresco, 1968; Burton, 1970). The usefulness of these reactions for the modification of 4-thiouridine in tRNA is limited since the 3′-terminal ribose and possibly other components are also altered under these conditions. Iodine oxidation of 4-thiouridine to disulfides (Carbon *et al.*, 1965; Lipsett, 1966; Lipsett and Doctor, 1967) and sodium borohydride reduction of 4-thiouridine have also been investigated (Cerutti *et al.*, 1968).

More selective approaches to the study of the functional role of 4-thiouridine have emerged recently. Modification of 4-thiouridine with cyanogen bromide has been demonstrated to yield uridine as the sole transformation product (Walker and RajBhandary, 1970; Saneyoshi and Nishimura, 1970). *N*-Ethylmaleimide has also been shown to react selectively and quantitatively with 4-thiouridine in tRNA. tRNA species partially deficient in 4-thiouridine have been investigated in an attempt to implicate the nucleoside as essential for tRNA synthetase recognition (Johnson *et al.*, 1970; Kaiser, 1969), while the biological properties of mutant tRNA^{Tyr} have been studied (Abelson *et al.*, 1970; Smith *et al.*, 1970). A method for the selective photochemical transformation of 4-thiouridine in *E. coli* tRNA to uridine or uridine plus cytidine (or N^4-methylcytidine) has been developed in our laboratory and

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is described here in detail.[1] This reaction induces a transition of a minor to a major nucleotide; no "unnatural" component with unpredictable effects on the properties of the tRNA is formed. The modified tRNA obtained by the selective transformation of 4-thiouridine to uridine may be considered a biosynthetic precursor. The product obtained from the transformation of 4-thiouridine to cytidine, on the other hand, may be considered a mutated tRNA. The effect of the modification on the amino acid acceptance of highly purified $tRNA^{Val}$ is being investigated.

Experimental Section

I. General

Materials. Purified, unfractionated tRNA (*E. coli* B; 22.1 A_{260} units/mg of tRNA in distilled water) was obtained from Schwarz BioResearch, Inc. Purified, fractionated $tRNA^{Val}$ (*E. coli* K12 MO7; lot no. 15-179; valine acceptance, 1310 pmoles/A_{260}; alanine acceptance, 160 pmoles/A_{260}; terminal adenosine, 1590 pmoles/A_{260} unit) was provided by Oak Ridge National Laboratory. *E. coli* bacterial alkaline phosphatase (35 units/mg; 10 mg/ml) was obtained from Worthington Biochemical. 4-Thiouridine was prepared according to Fox *et al.* (1958). For spectral characteristics of 4-thiouridine, see Kochetkov *et al.* (1963). Technical grade cetyltrimethylammonium bromide (CTA-bromide,[2] 80% active) and *tert*-butyl alcohol were obtained from Schwarz BioResearch, Inc. [^{14}C]-Methylamine hydrochloride (6.55 mCi/mmole) and [^{3}H]-methylamine hydrochloride (34.16 mCi/mmole) were from New England Nuclear.

Radioactivity Measurements. Counting efficiency for tritium on nitrocellulose filters (Schleicher und Schüll, type B-6) under 10 ml of scintillation fluid (PPO, 4 g; POPOP, 0.2 g; toluene to 1.0 l.) in a Beckman LS200 liquid scintillation system was 25.4%. Developed Whatman No. 41 paper chromatograms were cut into 1 × 4 cm strips about the N^4-[^{3}H]methylcytidine region; each strip was shredded, placed in a counting vial containing 1.0 ml of distilled water and, after standing 30 min, covered with liquid scintillation fluid (naphthalene, 60 g; PPO, 4 g; methanol, 100 ml; and dioxane to 1.0 l.). Under these conditions, carbon-14 counting efficiency was 85% while tritium counting efficiency was 25.2%.

Irradiation Conditions. Photolysis was effected with a 200-W Hanovia high-pressure mercury lamp, used in conjunction with a Pyrex sleeve filter. Unless otherwise noted the entire apparatus was contained in a thermostated water bath and the lamp positioned 7.5 cm from the photolysis solution. All CTA-tRNA solutions were irradiated in a 1-cm path-length cuvet at 10° and were stirred with a magnetic microspin bar. In the experiments on the monomer the 4-thiouridine solutions were irradiated in a 100-ml capacity quartz vessel (1-cm path length) at 25° and stirred with a magnetic spin bar.

II. Photochemical Transformation of 4-Thiouridine to Uridine and Cytidine or Uridine and N^4-Methylcytidine. To a solution of 4-thiouridine (1.49 μmoles, 0.3 A_{327} unit/ml) in 10 ml of *tert*-butyl alcohol was added 33 ml of approximately 1.8 M ammonia in *tert*-butyl alcohol. Sufficient *tert*-butyl alcohol was added to give a final volume of 100 ml (0.298 A_{327} unit of 4-thiouridine/ml) and the final ammonia concentration was determined as 0.56 M by titration. The solution was irradiated at 25.0° under conditions described above until less than 10% of the original absorbance at 327 mμ remained. The solution was then evaporated to dryness *in vacuo*, the residue was dissolved in 1.0 ml of distilled water, and applied to a Dowex 50W-X4 (H^+) column (200–400 mesh; 1.5-ml bed volume). Uridine and unmodified 4-thiouridine were eluted quantitatively from the column in five 1.0-ml fractions of distilled water, and then the cytidine was eluted in 7.0 ml of 2 N HCl. The uridine and cytidine content of the fractions was determined spectrophotometrically. In a similar manner, 4-thiouridine (1.49 μmoles, 0.3 A_{327} unit/ml) in 100 ml of 0.61 M methylamine was irradiated. Uridine and N^4-methylcytidine were separated on Dowex 50W-X4 (H^+) and quantitated.

III. Photochemical Transformation of 4-Thiouridine in E. coli $tRNA^{Val}$ to Uridine, Uridine and Cytidine, or Uridine and N^4-Methylcytidine. 1. PREPARATION OF CETYLTRIMETHYLAMMONIUM-tRNA SALT AND REGENERATION OF SODIUM tRNA SALT. To a solution of *E. coli* tRNA (29.3 A_{260} units; 3.9 × 10^{-3} mequiv of phosphate) in 1.0 ml of distilled water was added slowly with stirring 1.0 ml of 5 × 10^{-3} M cetyltrimethylammonium bromide (4.0 × 10^{-3} mequiv). The cetyltrimethylammonium tRNA salt (CTA-tRNA) precipitated immediately (Weil and Ebel, 1962). After stirring 10 min, the contents were centrifuged at low speed and the supernatant was discarded. The salt was suspended in 0.8 ml of *tert*-butyl alcohol, the contents stirred for 2 hr, centrifuged at low speed, and the clear supernatant was collected. Usually 20–30 A_{260} units of CTA-tRNA/ml (0.4–0.6 A_{330} unit/ml) was contained in the supernatant. These solutions were quite stable and could be stored at 5° without solidification or significant precipitation of the salt occurring.

For regeneration of sodium tRNA the *tert*-butyl alcohol solution containing CTA-tRNA was evaporated to dryness and the residue dissolved in a small amount of 3 M NaCl at 4°. After 15 min the tRNA was precipitated by the addition of an equal volume of cold ethanol. This procedure was repeated three times to ensure complete exchange of CTA for sodium. Finally, the precipitated tRNA was washed once with ethanol–water (3:1, v/v) to free the precipitate of excess NaCl. All supernatants were assayed for absorbance at 260 mμ and radioactivity where applicable. The regenerated $tRNA^{Val}$ was stored at −20° in 0.01 M bicine buffer (pH 7.8). Alternatively the *tert*-butyl alcohol solution containing CTA-tRNA was evaporated *in vacuo*, and the residue was dissolved in a small amount of 1 M NaCl and applied to a Dowex 50W-X4 (Na^+) column (200–400 mesh). The sodium salt of the tRNA was eluted with water. The tRNA-containing fractions were evaporated *in vacuo*. The residue was taken up in a small amount of 10^{-3} M Tris buffer (pH 7) and tRNA freed of excess NaCl on a Sephadex G-50 column.

2. TRANSFORMATION OF 4-THIOURIDINE IN *E. coli* $tRNA^{Val}$ TO URIDINE. A 1.3-ml aliquot of CTA-tRNA in *tert*-butyl alcohol (15 A_{260} units/ml; 0.3 A_{330} unit/ml) was irradiated as described above in a stoppered 1-cm path-length cuvet. The extent of transformation of 4-thiouridine was obtained from the loss of absorbance at 330 mμ as a function of exposure (see Results).

3. PREPARATION OF SOLUTIONS OF CETYLTRIMETHYLAMMONIUM-tRNA SALT IN AMMONIA OR METHYLAMINE CONTAINING *tert*-BUTYL ALCOHOL. Solutions of ammonia or methylamine (1.5–2.0 M) in *tert*-butyl alcohol were prepared by the addition of ammonia or methylamine gas to 5 ml of *tert*-

[1] A preliminary account of part of this research has been published previously (Pleiss *et al.*, 1969).

[2] Abbreviations used are: CTA, cetyltrimethylammonium ion; CTA-tRNA, cetyltrimethylammonium salt of tRNA; 4-thio-U, 4-thiouridine; m^4C, N^4-methylcytidine; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

butyl alcohol. Ammonia was vented directly from a cylinder. Methylamine, generated by the action of 3 g of 50% aqueous KOH on 0.925 g (0.014 mole) methylamine hydrochloride, was collected and then distilled directly into *tert*-butyl alcohol. Final molarities were determined by titration of aliquots contained in 10 ml of distilled water, with standard HCl (methyl red end point). Solutions containing radioactive methylamine were prepared in the following manner. To 2.58 mg of [^{14}C]-methylamine hydrochloride (3.8×10^{-2} mmole; 6.55 mCi/mmole), dissolved in 0.35 ml of water contained in a 5-ml flask, was added 0.45 ml of 2.3 M methylamine hydrochloride (1.04 mmoles; recrystallized). A short column, indented at the lower end to contain about 0.8 g of KOH pellets, was fitted atop the flask, followed in turn by a condenser and a short right-angle drying tube (KOH pellets). Short sections of tygon tubing were used to connect the drying tube to a small (5-ml capacity) gas U trap (T1) which was connected in series to a second small (5-ml capacity) gas U trap (T2). Screw clamps were positioned between the drying tube and trap T1 (C1), between trap T1 and trap T2 (C2) and between T2 and the high vacuum line (C3). Clamp C2 was closed, trap T1 was cooled with liquid nitrogen, and the pressure was then adjusted to 400 mm. The flask was carefully heated with a microburner such that the water vapor continuously dissolved the KOH pellets in the indented column at a slow controlled rate (Spinder, 1962). After the gas was generated, the flask was cooled slightly and transfer of the gas to trap T1 was completed by further evacuation to 20 mm. After 45 minutes clamps C1 and C3 were closed, trap T2 (containing 0.75 ml of distilled *tert*-butyl alcohol) was cooled with liquid nitrogen, clamp C2 was opened, and trap T1 was immediately surrounded with a Dry Ice–acetone bath. The methylamine was then distilled from trap T1 into trap T2 by further evacuation to 3 mm. Distillation was complete within 45 min. Clamp C2 was closed, trap T2 was warmed to room temperature, and clamp C3 was opened momentarily to adjust the internal pressure. The contents in trap T2 were equilibrated for 30 min prior to use.

In a similar manner [^{3}H]methylamine hydrochloride (9.7 mg; 0.143 mmole; 34.8 mCi/mmole) was dissolved in 0.3 ml of distilled water contained in a 5-ml flask and diluted with 0.49 ml of 2.3 M methylamine hydrochloride (1.13 mmoles; recrystallized). *tert*-Butyl alcohol solutions containing [^{3}H]-methylamine were prepared as described above.

To a swirling solution of CTA-tRNA in 1.2 ml *tert*-butyl alcohol (30 A_{260} units/ml; 0.6 A_{330} unit/ml) was added dropwise, 0.8 ml of 1.85 M ammonia (or methylamine) in *tert*-butyl alcohol. This procedure was necessitated by the fact that although *tert*-butyl alcohol solutions of CTA-tRNA were quite stable in themselves, the addition of ammonia (or methylamine) had a detrimental effect on this stability. The direct addition of ammonia (or methylamine) did not, consistently, give an optically clear solution, and in extreme cases gross precipitation occurred. (At high amine concentrations (0.8 M) incipient precipitation occurred, independent of the method of preparation.) The final molarity (0.72 M) was determined by titration.

4. TRANSFORMATION OF 4-THIOURIDINE IN *E. coli* tRNA^{Val} TO URIDINE AND N^4-METHYLCYTIDINE; KINETICS AND QUANTITATION. A solution of highly purified *E. coli* tRNA^{Val} in *tert*-butyl alcohol (15 A_{260} units/ml; 0.37 A_{327} unit/ml) and 0.57 M in [^{3}H]methylamine (3.94 mCi/mmole) was prepared and irradiated as described above. Aliquots were removed after 0, 56, 80, and 93% transformation of 4-thiouridine as judged from the loss in absorbance at 327 mμ as a function of exposure time. Each sample was concentrated with a nitrogen purge, dried *in vacuo*, and dissolved in molar aqueous NaCl. The extent of transformation was obtained from the kinetics of loss in absorbance at 327 mμ in methylamine *tert*-butyl alcohol. The sodium salt of the irradiated tRNA was then prepared by precipitation from 3 M NaCl with an equal volume of ethanol (see section III (1)). An aliquot of each sample of modified tRNA^{Val} was diluted with sufficient *E. coli* B tRNA to give approximately 7 A_{260} units and hydrolyzed with 0.3 N KOH to the mononucleotides. After neutralization with perchloric acid, the supernatant was collected, diluted with an equal volume of 0.1 M HCl, and applied to a Dowex 50W-X4 (H^+) column (200–400 mesh; 1 × 5 cm). Uridine 2′(3′)-phosphate and guanosine 2 (3′)-phosphate were eluted with 7.0 ml of 0.05 M HCl and 7.5 ml of water, respectively. The remaining mononucleotides were rinsed from the Dowex 50W column directly onto a Dowex 1-X8 ($HCOO^-$) column (200–400 mesh; 1 × 1 cm) with 27.5 ml of water and then eluted simultaneously from the Dowex 1 column in 30 ml of 0.5 M HCOOH (Katz and Comb, 1963). (Insignificant amounts of radioactivity were present in either the UMP or GMP eluates; no radioactivity was observed in the water eluate.) The nucleoside monophosphates obtained from the formic acid eluate were evaporated to dryness and treated with 3 μl (1.0 unit) of bacterial alkaline phosphatase in 0.6 ml of 0.1 M ammonium carbonate buffer (pH 8.5) for 18 hr at 37°. The enzyme digest was concentrated after addition of authentic N^4-methylcytidine as carrier, applied quantitatively to Whatman 41 paper, and developed by descending chromatography (1-butanol–water, 86:14, v/v; 18 hr). The absorption spot corresponding to N^4-methylcytidine was located and 1 × 4 cm strips were cut from the chromatogram in this region. Each strip was assayed for radioactivity; the total radioactivity served to quantitate the amount of 4-thiouridine transformed to N^4-[^{3}H]methylcytidine (see Table II).

5. SELECTIVITY OF THE TRANSFORMATION OF 4-THIOURIDINE IN UNFRACTIONATED *E. coli* tRNA. The selectivity of the photochemical transformation of 4-thiouridine in CTA-tRNA was further demonstrated on a preparative scale. The photolysis solution was prepared by the addition of [^{14}C]methylamine hydrochloride (2.5 mg; 6.55 mCi/mmole) in 50 μl of water to 11.0 ml of 0.2 M methylamine in *tert*-butyl alcohol containing the CTA-tRNA salt (unfractionated *E. coli* B tRNA). Addition of the aqueous hydrochloride solution resulted in some precipitation of the CTA-tRNA salt. After irradiation the modified, radioactively labeled tRNA (200 A_{260} units) obtained by chromatographic regeneration was hydrolyzed in alkali and the resultant nucleotides were separated by ion-exchange chromatography as described in the preceding section (Katz and Comb, 1963). Recovery of the major nucleotides was identical for both irradiated and unirradiated tRNA. Major amounts of radioactivity were obtained in the water eluate of the Dowex 50W-X4 (H^+) column and the 0.05 M formic acid eluate of the Dowex 1-X8 ($HCOO^-$) column. The latter radioactivity peak was identified as N^4-[^{14}C]methylcytidine 2′(3′)-phosphate by chromatographic comparison (Whatman 1, 1-butanol–water, 86:14, v/v; R_F 0.35), after dephosphorylation with bacterial alkaline phosphatase, with an authentic sample of N^4-methylcytidine. The unambiguous identification of the radioactive material(s) in the Dowex 50W water eluate proved more difficult. All of our results indicated that this radioactivity was associated with non-nucleotide material. The compound, *N*-ribosyl-*N*-(*N*-methyl-β-propionamidyl)urea, a plausible hydrolysis product of dihydrouridine in the presence of methylamine, could not be detected in this eluate. In addition, an undetermined amount

of radioactive material was present in the water wash which passed through both ion-exchange columns. The chromatographic behavior of this material was also suggestive of nonnucleotide material. The presence of unspecific, nonnucleotide radioactivity has been attributed to contaminants in the commercial preparation of [^{14}C]methylamine hydrochloride previously (Ziff and Fresco, 1969). Significantly, subsequent studies using [^{14}C]methylamine and [^{3}H]methylamine as prepared from the corresponding hydrochloride by trap-to-trap distillation, did not generate significant quantities of radioactivity in either the water eluate or the water wash.

Results and Discussion

Photochemistry of Monomeric 4-Thiouridine in tert-Butyl Alcohol. The mild photochemical transformation of the minor nucleoside 4-thiouridine to uridine occurred virtually quantitatively upon irradiation in air-saturated *tert*-butyl alcohol with light from a high-pressure mercury lamp transmitted through a Pyrex sleeve filter ($\lambda > 300$ mμ) (Pleiss *et al.*, 1969). The nature of the product was suggested by the increase in the absorption at 262 mμ concomitant with the loss in the spectrum at 330 mμ during ultraviolet exposure. The product was further characterized by thin-layer chromatography and paper chromatography comparison with authentic uridine, its chromatographic behavior on ion-exchange resin and by its absorption spectrum subsequent to isolation. Isosbestic points around 240 and 280 mμ were obtained in the absorption spectra measured as a function of ultraviolet exposure. Linear plots of the logarithm of the absorbance at 330 and 260 mμ as a function of exposure time afforded nearly identical pseudo-first-order rate constants for disappearance of 4-thiouridine (0.10 min^{-1}) and formation of uridine (0.08 min^{-1}), respectively. The presence of oxygen in the reaction medium was obligatory; sufficient oxygen was present in a *tert*-butyl alcohol solution of 4-thiouridine equilibrated at ambient conditions, however, to allow the photochemical reaction to proceed at close to a maximal rate. While the presence of 2% of water in *tert*-butyl alcohol did not influence the transformation of 4-thiouridine to uridine, irradiation of 4-thiouridine in aqueous solution at wavelengths greater than 300 mμ resulted in a complex mixture of products, derived in part from photohydration of the 5,6-double bond in 4-thiouridine (P. Cerutti and H. Ochiai, 1969, unpublished results). Although irradiation of 4-thiouridine in deaerated *tert*-butyl alcohol led to the gradual loss in absorbance at 330 mμ, the nature of this reaction has not been established.

The mechanism of the photooxidative transformation of 4-thiouridine in *tert*-butyl alcohol is not clearly understood. The chemical oxidation of thiopyrimidines to sulfinate or sulfonate intermediates and their subsequent hydrolysis to the corresponding pyrimidines has been proposed previously (Brown, 1962; Taylor and Chang, 1960). Stable intermediates sulfinates and sulfonates have been isolated in some cases. Sodium periodate oxidation of 2-deoxy-4-thiouridine gave the corresponding *N*-(2′-deoxyribosyl)-2-oxypyrimidine-4-sulfonate, while potassium permanganate oxidation of 4-thiouridine gave *N*-ribosyl-2-oxypyrimidine-4-sulfonate (Ziff and Fresco, 1968). Both sulfonate intermediates were susceptible to nucleophilic substitution reactions in aqueous solution. The formation of sulfenic acids as one of the intermediates derived from the hydrolytic cleavage of disulfides has been postulated to account for products obtained. More recently, 1-methyluracil-4-sulfenic acid has been isolated from the bis(1-methyl-4-thiouracil) disulfide (Pal *et al.*, 1969). No direct evidence for the formation of any of these sulfur-containing intermediates was obtained during the photochemical oxidation of 4-thiouridine nucleoside in *tert*-butyl alcohol. However, sulfite ions were detected in the photolysis solution, consistent with the intermediacy of *N*-ribosyl-2-oxypyrimidine-4-sulfonate, its sulfinate or its sulfenate. In contrast to chemical oxidation, mild photooxidation may preclude attainment of appreciable concentrations of an intermediate and would therefore go undetected by the spectrophotometric techniques employed. Moreover, oxygen-deficient solutions of 4-thiouridine in *tert*-butyl alcohol containing ammonia (see below) exhibited an absorbance maximum at 293 mμ in addition to a maximum at 322 mμ (A_{293}/A_{322} 1.3) after prolonged irradiation. No further change in the spectrum was noted when the solution was allowed to stand in the dark. Chromatography of the reaction residue on Dowex 50W (H^+) gave mostly recovered 4-thiouridine; however, fractions eluted with water after 4-thiouridine contained an absorption maximum at 362 mμ (5% of the original A_{327} value). These observations are not inconsistent with disulfide and sulfenic acid formation, respectively.

The presence of additional nucleophilic reagents had a pronounced influence on the nature of the photochemical reaction products. Irradiation of 4-thiouridine in *tert*-butyl alcohol in the presence of ammonia or methylamine resulted in mixtures of cytidine and uridine, or N^4-methylcytidine and uridine, respectively. In all cases the kinetics of transformation followed pseudo-first-order reaction rates. The nature of the products formed in the presence of added nucleophiles was established by thin-layer chromatography and DEAE paper chromatographic comparisons to authentic material, chromatographic behavior on ion-exchange resin and by their absorption spectra subsequent to isolation. The ratio of uridine to cytidine formed was dependent on the concentration of the added nucleophile. Uridine represented the major transformation product in *tert*-butyl alcohol solutions containing ammonia. Uridine formation could not be completely suppressed either at high amine concentrations or by irradiation of 4-thiouridine in liquid ammonia at $-78°$. On the other hand, N^4-methylcytidine was the major product at moderately high concentrations of methylamine. The relative percentages of uridine and cytidine and uridine and N^4-methylcytidine formed from the nucleoside under experimental conditions analogous to those used for transformation of 4-thiouridine in tRNA were determined. At 0.56 M ammonia 69% uridine and 31% cytidine were formed from 4-thiouridine in a pseudo-first-order reaction with a rate constant of 0.017 (at 25°); at 0.61 M methylamine, 48% uridine and 52% N^4-methylcytidine were formed and the pseudo-first-order rate constant was 0.005 (at 10°). The total conversion of 4-thiouridine was 90% in these experiments.

The selectivity of this photochemical transformation for 4-thiouridine was demonstrated. The major nucleosides, adenosine, guanosine, cytidine, and uridine were inert under the reaction conditions employed, as judged by thin-layer chromatography and absorption spectroscopy. Similarly, the following minor nucleosides were judged to be inert: pseudouridine, 1-methyladenosine, N^6-isopentenyladenosine, 5,6-dihydrouridine, 2-thiomethyl-N^6-isopentenyl-adenosine, and glucosyl-2-thiouracil.

Photochemical Transformation of 4-Thiouridine in E. coli $tRNA^{Val}$ to Uridine, Uridine and Cytidine, and Uridine and N^4-Methylcytidine. The selective transformation of the minor nucleoside 4-thiouridine to uridine in *E. coli* $tRNA^{Val}$ was accomplished by irradiation of the cetyltrimethylammonium salt of $tRNA^{Val}$ (CTA-$tRNA^{Val}$) in a photochemically inert,

nonaqueous medium at a wavelength longer than 300 mμ. In the presence of ammonia, uridine and cytidine were formed from 4-thiouridine in $tRNA^{Val}$ and in the presence of methylaminouridine and N^4-methylcytidine. *tert*-Butyl alcohol was chosen as the solvent, since it lacked a photochemically reactive proton on the α-carbon atom. *tert*-Butyl alcohol also had a decided advantage in that it excluded photohydration of pyrimidine nucleotides. Cyclobutane-type photodimerization was also avoided under our conditions.

The procedures developed for the preparation of CTA-tRNA soluble in *tert*-butyl alcohol, the regeneration of the sodium salt of tRNA, and experiments establishing the selectivity of the reaction for the modification of 4-thiouridine in *E. coli* tRNA have been described under Experimental Section. Physical and biological properties of unirradiated, regenerated sodium tRNA were identical with those of untreated material. No fragmentation of tRNA upon irradiation in *tert*-butyl alcohol could be detected from the elution profile on Sephadex G-200.

Kinetics of the Transformation of 4-Thiouridine in $tRNA^{Val}$. Uridine was the exclusive product formed from monomeric 4-thiouridine upon photolysis in the absence of added nucleophiles. The transformation kinetics of 4-thiouridine in $tRNA^{Val}$ to uridine could be deduced from the loss in the absorption spectrum as a function of ultraviolet exposure, at the 4-thiouridine maximum of 330 mμ. For the determination of the reaction kinetics, the following difficulty had to be overcome. Solutions of CTA-$tRNA^{Val}$ in *tert*-butyl alcohol retained a substantial portion of the original absorbance value at 330 mμ even after prolonged irradiation. This phenomenon may be attributable to the presence of material(s) other than 4-thiouridine with appreciable absorbance at this wavelength. A intramolecular reaction of 4-thiouridine with a topographically close residue (*e.g.*, cytidine number 13) under formation of a product with absorption in the 330-mμ region (Yaniv *et al.*, 1969; Shulman, 1970) appears unlikely for CTA-$tRNA^{Val}$ in *tert*-butyl alcohol but cannot be excluded. The uncertainty in the initial and final absorbance values attributable to the 4-thiouridine moiety in the CTA-$tRNA^{Val}$ salt precluded a conventional approach to an analysis of the reaction kinetics. However, an apparent pseudo-first-order rate constant could be obtained from analysis of the rate data by a method described by Guggenheim (see legend to Figure 1; Frost and Pearson, 1961). This method is applicable to a "simple" first-order reaction followed by a physical measurement, such as absorbance, where measurement of the final absorbance is not practical. As shown in Figure 1, an apparent pseudo-first-order rate constant for the transformation of 4-thiouridine to uridine in $tRNA^{Val}$ as determined by this method was 0.058 min^{-1} at 10°. (See section I of Experimental Section for irradiation conditions.) Linear Guggenheim plots were also obtained for the phototransformation of 4-thiouridine in $tRNA^{Val}$ in the presence of ammonia to uridine plus cytidine, and in the presence of methylamine to uridine plus N^4-methylcytidine (see Figure 1). The apparent pseudo-first-order rate constant for the loss of 4-thiouridine in the presence of ammonia or methylamine was 0.023 min^{-1} at 10°. Thus the transformation of 4-thiouridine to uridine alone proceeds at greater than twice the rate for transformation to uridine plus cytidine or uridine plus N^4-methylcytidine. Moreover, the rate constants for the phototransformation of monomeric 4-thiouridine and 4-thiouridine in $tRNA^{Val}$ for the conversion to uridine under similar conditions are comparable (0.046 min^{-1} for the monomer at ambient temperature; 0.058 min^{-1} for 4-thiouridine in $tRNA^{Val}$ at 10°) and to uridine plus cytidine (0.017 min^{-1} for the monomer at 25°; 0.023 min^{-1} for 4-thiouridine in $tRNA^{Val}$ at 10°), but different for the conversion into uridine plus N^4-methylcytidine (0.005 min^{-1} for the monomer at 25°; 0.023 min^{-1} for 4-thiouridine in $tRNA^{Val}$ at 10°). As mentioned below, the relative amounts of uridine and N^4-methylcytidine formed from the monomer and polymer were quite comparable on the other hand.

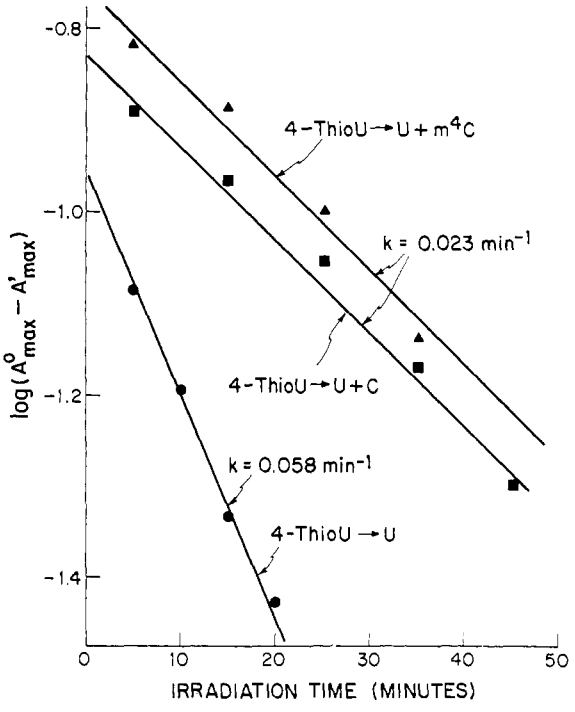

FIGURE 1: Kinetics of the transformation of 4-thiouridine in *E. coli* $tRNA^{Val}$ to uridine, uridine plus cytidine, and uridine plus N^4-methylcytidine. For experimental details, see Experimental Section, section III. The rate constants were determined by the method of Guggenheim (Frost and Pearson, 1961). Measurement of absorbance values A_i and A_i' at times t_i and $t_i + \Delta$, where Δ is some constant increment of time, relate to the apparent pseudo-first-order rate constant (k) in the following manner: $2.303 \log (A_i - A_i') + kt_i = 2.303 \log [(A_0 - A_\infty)(1 - \exp(-k\Delta))]$, where A_0 and A_∞ represent the initial absorbance and final unknown absorbance, respectively. Provided the entity giving rise to the residual absorbance at the 4-thiouridine maximum after prolonged irradiation was chemically inert under the reaction condition, that is, its contribution to the total absorbance at the 4-thiouridine maximum remained constant throughout the modification of 4-thiouridine, then a linear plot of $\log (A_i - A_i')$ *vs.* time of ultraviolet exposure ensues, with a slope equal to the apparent pseudo-first-order rate constant (after multiplication by 2.303). The intercept of this plot yields a constant equivalent to the logarithm $[(A_0 - A_\infty)(1 - \exp(-k\Delta))]$. Provided the absorbance prior to ultraviolet exposure is known, the residual absorbance (A_∞) can be determined.

Quantitation of the Transformation of 4-Thiouridine in $tRNA^{Val}$. Determination of an apparent pseudo-first-order rate constant for the transformation of 4-thiouridine in $tRNA^{Val}$ pursuant to formation of uridine only, uridine plus cytidine, or uridine plus N^4-methylcytidine permitted the quantitation of residual 4-thiouridine in $tRNA^{Val}$ under each set of conditions at any given exposure. In the absence of added nucleophiles, the amount of uridine formed in $tRNA^{Val}$ could be equated to the amount of 4-thiouridine lost during the transformation, for the reasons cited above. Transformation of 4-thiouridine to uridine plus N^4-methylcytidine was determined from the amount of N^4-[^{3}H]methylcytidine present in the digests of the $tRNA^{Val}$ modified in *tert*-butyl alcohol

TABLE I: Kinetics and Quantitation of the Transformation of 4-ThioU in *E. coli* $tRNA^{Val}$ to U plus [^{3}H]m^4C in 0.57 M [^{3}H]Methylamine–*tert*-Butyl Alcohol.[a]

Irradiation Time (min)[b]	Dpm/A_{260} Units of $tRNA^{Val}$ in [^{3}H]m^4C	pmoles of Nucleoside/A_{260} Unit of $tRNA^{Val}$[c]		
		4-ThioU[d]	[^{3}H]m^4C[e]	U[f]
Control	0	1570 (100)	0	0
0	0	1570 (100)	0	0
35	2050	690 (44)	235 (15)	645 (41)
70	4360	315 (20)	500 (32)	755 (48)
115	5800	100 (7)	665 (42)	795 (51)

[a] For experimental details, see Experimental Section, section III. [b] Control: unirradiated, unprocessed sample; 0 min: unirradiated sample carried through the same purification steps as for irradiated samples. [c] At the location of 4-ThioU (eighth nucleotide from the 5′ end); *E. coli* $tRNA^{Val}$: 1570 pmoles of terminal adenosine/A_{260} unit as measured in 0.1 M bicine buffer (pH 7.8). [d] Calculated on the basis of a pseudo-first-order rate constant for the transformation of 4-ThioU of 0.023 min^{-1}; values in parentheses give the percentage residual 4-ThioU. [e] 8.75 dpm/pmole of [^{3}H]m^4C; values in parentheses give the percentage [^{3}H]m^4C formed. [f] Obtained by difference; values in parentheses give the percentage uridine formed.

TABLE II: Kinetics and Quantitation of the Transformation of 4-ThioU in *E. coli* $tRNA^{Val}$ to U plus C in 0.72 M Ammonia–*tert*-Butyl Alcohol.[a]

Irradiation Time (min)[b]	pmoles of Nucleoside/A_{260} Units of $tRNA^{Val}$[c]		
	4-ThioU[d]	C[e]	U[f]
Control	1570 (100)	0	0
0	1570 (100)	0	0
45	565 (36)	310 (20)	695 (44)
110	125 (8)	450 (29)	995 (63)

[a] For experimental details, see Experimental Section, section III. [b] Control: unirradiated, unprocessed sample; 0 min: unirradiated sample carried through the same purification steps as for irradiated samples. [c] At the location of 4-ThioU (eighth nucleotide from 5′-end); *E. coli* $tRNA^{Val}$: 1570 pmoles of terminal adenosine/A_{260} unit as measured in 0.1 M bicine buffer (pH 7.8). [d] Calculated on the basis of a pseudo-first-order rate constant for the transformation of 4-ThioU of 0.023 min^{-1}; values in parentheses give the percentage residual 4-ThioU. [e] Based on the relative amounts of U and C formed from monomeric 4-ThioU under analogous conditions. Values in parentheses give the percentage C formed. [f] Values in parentheses give the percentage U formed.

in the presence of [^{3}H]methylamine. The chromatographic procedures which were used for the isolation of N^4-[^{3}H]-methylcytidine have been described in detail (see Experimental Section, section III (4)). The amount of uridine formed under these conditions was equated to the difference between the amount of 4-thiouridine lost and the amount of N^4-[^{3}H]-methylcytidine generated. The data are summarized in Table I. Since the secondary and tertiary structure of CTA-tRNA in *tert*-butyl alcohol should be minimal, the photochemical reactivity of monomeric 4-thiouridine and 4-thiouridine in $tRNA^{Val}$ was expected to be similar under these conditions. As expected, the relative percentage of uridine and N^4-methylcytidine formed in $tRNA^{Val}$ after 93% total conversion of 4-thiouridine (55% uridine and 45% N^4-methylcytidine) approximated that obtained from monomeric 4-thiouridine under analogous conditions (48% uridine and 52% N^4-methylcytidine.

Direct quantitation of the amount of uridine and cytidine formed from 4-thiouridine in CTA-$tRNA^{Val}$ in *tert*-butyl alcohol containing ammonia was not accomplished. The use of [^{15}N]ammonia did not provide a sufficient atom per cent excess of [^{15}N]N^4-methylcytosine to allow a meaningful interpretation of the mass spectral data. The relative amounts of uridine and cytidine formed from 4-thiouridine in $tRNA^{Val}$ were therefore estimated using the monomer experiments as guide lines. The data are summarized in Table II.

Preliminary studies on the effect of the transformation of 4-thiouridine in $tRNA^{Val}$ on the chargeability were carried out using partially purified valyl-tRNA synthetase from *E. coli* prepared by a modification of the procedure of Kondo and Woese (1969). Transformation of 90% of the original 4-thiouridine to uridine led to a 19% reduction in the valine acceptance. Complete modification of the 4-thiouridine residues to uridine corresponds, therefore, to a 22% loss in acceptance of L-valine. Similar results were obtained by Walker and RajBhandary (1970) in their studies of the effect of the transformation of 4-thiouridine to uridine in $tRNA^{Tyr}$ from *E coli* upon treatment with cyanogen bromide. A 15% drop in chargeability was observed. Furthermore, Johnson *et al.* (1970) have recently reported that $tRNA^{Ile}$ from *Mycoplasma* sp. (Kid) could be charged close to the theoretical maximum despite the fact that it was lacking its full complement of 4-thiouridine. Retention of a substantial portion of the aminoacylation activity suggests that 4-thiouridine, *per se*, is not required as a specific recognition site for valyl-tRNA synthetase. More likely, the functional role of 4-thiouridine relates to the maintenance of a biologically fully active conformation. The apparent ability of uridine to mimic the structural role of 4-thiouridine is consistent with the similar hydrogen-bonding properties exhibited by both 4-thiouridine and uridine (Kyogoku *et al.*, 1967).

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Interaction of Poly-L-tyrosine with Nucleic Acids. I. Formation of Complexes*

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ABSTRACT: The interaction of poly-L-tyrosine, $(Tyr)_n$, with various nucleic acids in aqueous solution was studied. $(Tyr)_n$ solutions were mixed with various nucleic acids and then slowly neutralized to pH 7.5, both components of each reaction mixture being initially at pH 10.6 (ionic strength after mixing $\simeq 0.06$). Under these conditions, insoluble complexes are formed between $(Tyr)_n$ and denatured DNA, poly(A), poly(I), and poly(U), but not with poly(C) or native DNA. As the molar ratio of Tyr:nucleotide increases from 0 to 10, formation of these complexes increases linearly. At a ratio of 10, the complex formation is essentially complete; in the case of denature DNA and poly(I), at a ratio of Tyr:nucleotide = 14 approximately 15 and 20% of the respective nucleic acids remain uncomplexed. At neutral pH and for varying ratios of Tyr:nucleotide from 1 to 0.1, the 1:1 complex of poly(A) and $(Tyr)_n$ (concentration $\leq 5 \times 10^{-4}$ M) is the only soluble complex found for the various systems studied; it migrated as a single component in sucrose gradient electrophoresis. In the case of poly(A)–$(Tyr)_n$ system, the following situations can exist, depending on the concentration of $(Tyr)_n$ and the ratio of Tyr:A: (1) formation of a soluble complex only, (2) formation of a soluble and an insoluble complex, and (3) formation of an insoluble complex only.

The general features of this nonelectrostatic base-specific polypeptide–polynucleotide interaction are described.

Although the importance of protein–nucleic acid interactions has long been recognized, it is only recently that this phenomenon has been investigated systematically. These interactions play a key role in the expression and regulation of genetic information in living cells. Thus, DNA–histone, tRNA-activating enzyme, and DNA–RNA polymerase are examples of systems exhibiting these interactions. Because of the complexity of these interactions extensive use has been made of model systems. These studies have shed some light on the importance of the various forces implicated in such interactions, and how these forces affect the conformation of the macromolecules. Systems consisting of basic polypeptides and nucleic acids have been investigated intensively (Tsuboi *et al.*, 1966; Latt and Sober, 1967a,b; Olins *et al.*, 1968; Davidson and Fasman, 1969; Shapiro *et al.*, 1969). These systems form well-defined and very stable complexes, held together primarily by electrostatic forces (Tsuboi *et al.*, 1966; Olins *et al.*, 1968). Nevertheless, some specificity is exhibited as far as the nature and size of the components of a given system are

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