Biological Function of 2-Thiouridine in *Escherichia coli* Glutamic Acid Transfer Ribonucleic Acid†

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ABSTRACT: In order to study the biological functions of the modified nucleoside 5-methylaminomethyl-2-thiouridine, located in the 5′-terminal position of the anticodon of *Escherichia coli* $tRNA^{Glu}$, two types of alterations of this nucleoside in tRNA were produced. *E. coli* C6 *rel⁻met⁻cys⁻* was grown in sulfur-deficient medium. $tRNA^{Glu}$ purified from it was found to lack its usual complement of the 2-thiouridine derivative. Normal $tRNA^{Glu}$ was treated with cyanogen bromide causing reaction with the 2-thiouridine derivative. The sulfur-deficient $tRNA^{Glu}$ could be aminoacylated as well as normal $tRNA^{Glu}$. Cyanogen bromide treated $tRNA^{Glu}$ showed decreased affinity for glutamyl-tRNA synthetase indicated by a sixfold increase in apparent K_m. With increased amounts of pure glutamyl-tRNA synthetase the cyanogen bromide treated $tRNA^{Glu}$ could be aminoacylated to 70% of the level reached with normal $tRNA^{Glu}$. This suggests that the accurate fit of the cognate aminoacyl-tRNA synthetase with $tRNA^{Glu}$ requires a defined steric relationship around the anticodon region of the tRNA. Ribosome binding studies showed that $tRNA^{Glu}$ purified from the sulfur-deficient culture recognizes GpApG much better than GpApA while normal $tRNA^{Glu}$ recognized preferentially GpApA. Cyanogen bromide treated normal $tRNA^{Glu}$ showed no response to either triplet.

Transfer RNA is unique among cellular RNA species because it contains many modified nucleosides. The number and structural variety of the modified nucleosides found in tRNA are surprisingly large. Some regularities concerning the position of each kind of modified nucleoside in the primary structure of tRNA molecules have emerged from sequence studies (Hall, 1971; Söll, 1971; Nishimura, 1972). In particular, 2-thiouridine or derivatives of this nucleoside have been found in the first position (5′ end) of the anticodon in *Escherichia coli* $tRNA^{Glu}$ (Ohashi *et al.*, 1970, 1972) and $tRNA^{Gln}$ (Folk and Yaniv, 1972), yeast $tRNA^{Glu}$ (Sekiya *et al.*, 1969; Yoshida *et al.*, 1970, 1971) and $tRNA^{Lys}$ (Baczynskyj *et al.*, 1968; Madison *et al.*, 1972), and rat liver $tRNA^{Glu}$ and $tRNA^{Lys}$ (Kimura-Harada *et al.*, 1971) as shown in Table I. Trinucleotide stimulated ribosomal binding studies with *E. coli* $tRNA^{Glu}$ (Ohashi *et al.*, 1970) and yeast $tRNA^{Glu}$ (Sekiya *et al.*, 1969) have shown that these tRNAs have apparently lost the ability to pair with the cognate codon ending in G (Table I), although uridine at the 5′ end of the anticodon can pair with either A or G (Crick, 1966). Attempts to correlate the positional regularities of modified nucleosides in tRNA with the biological functions of this molecule are hampered by insufficient experimental evidence. Chemical modification of *E. coli* tRNA with BrCN has provided information on the nature of the interaction of tRNA with aminoacyl-tRNA synthetase. Treatment of the unfractionated *E. coli* tRNA with BrCN markedly decreased the level of amino acid acceptance of $tRNA^{Glu}$, $tRNA^{Gln}$, and $tRNA^{Lys}$ (Saneyoshi and Nishimura, 1971), all of which are now known to contain 2-thiouridine derivatives in their anticodons (Table I). These results suggest that derivatives of 2-thiouridine in the first position of the anticodon are involved in the interaction of the tRNA with the cognate aminoacyl-tRNA synthetase as well as in codon recognition.

With these thoughts in mind we decided to investigate further the significance of a thiolated uridine in the anticodon of tRNA with respect to aminoacylation and codon recognition. We selected *E. coli* $tRNA^{Glu}$ for our work since its full nucleotide sequence had been determined and the 2-thiouridine derivative at the first position of the anticodon characterized as 5-methylaminomethyl-2-thiouridine (s^2U^*).[1] In addition, 4-thiouridine, another chemically reactive sulfur nucleoside present in many *E. coli* tRNAs, was absent in $tRNA^{Glu}$ (Ohashi *et al.*, 1972). Furthermore, *E. coli* glutamyl-tRNA synthetase had been purified and studied extensively in our laboratory (Lapointe and Söll, 1972a–c).

We have purified $tRNA^{Glu}$, undermodified with respect to thiolation, from a sulfur-deficient culture of a cysteine-requiring, relaxed mutant of *E. coli* and have treated fully modified $tRNA^{Glu}$ with BrCN. In this paper we report that the sulfur-deficient $tRNA^{Glu}$ can accept glutamic acid normally, while the BrCN-treated $tRNA^{Glu}$ can be charged only by a

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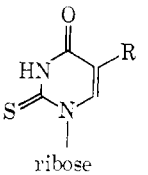
[1] Nucleotides and nucleosides are abbreviated according to the suggestions of the IUPAC–IUB Commission on Biochemical Nomenclature (*Progr. Nucl. Acid Res. Mol. Biol. 9*, ix, 1969). Abbreviations used are: ms^2i^6A, 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine; i^6A, N^6-(Δ^2-isopentenyl)adenosine; s^2U, 2-thiouridine; s^2U^*, 5-methylaminomethyl-2-thiouridine; s^2U^{**}, 5-aminomethyl-2-thiouridine (tentative identification); s^4U, 4-thiouridine; BD-cellulose, benzoylated DEAE-cellulose; GluRS, glutamyl-tRNA synthetase; tlc, thin layer chromatography; A_{260} unit, that amount of material having an absorbance of 1.0 at 260 nm when dissolved in 1 ml of water at neutral pH measured in a 1-cm light path. Sulfur-deficient tRNA denotes tRNA isolated from *E. coli* C6 *rel⁻met⁻cys⁻* grown under conditions of cysteine deprivation. [^{35}S]tRNA denotes radioactive tRNA isolated from *E. coli* K12 (CA244) which was cultured in medium containing $Na_2{}^{35}SO_4$.

TABLE I: Presence of 2-Thiouridine Derivatives as the First Nucleosides of tRNA Anticodons.



ribose
s^2U derivative

Organism	tRNA	R =	Codon	Reference
E. coli	Glu_2	Methylaminomethyl	GA*A*	Ohashi *et al.*, 1970, 1972
Yeast	Glu_3	Acetic acid methyl ester	GA*A*	Sekiya *et al.*, 1969; Yoshida *et al.*, 1970, 1971
Rat liver	Glu_3	Methyl	GA*A*	Kimura-Harada *et al.*, 1971
E. coli	Gln_1	Not known	CA*A*	Folk and Yaniv, 1972
Yeast	Lys	Acetic acid methyl ester	AA*A*	Baczynskyj *et al.*, 1968; Madison *et al.*, 1972
Rat liver	Lys_2	Methyl	AA*A*	Kimura-Harada *et al.*, 1971

large amount of pure glutamyl-tRNA synthetase. Template directed binding of the sulfur-deficient $tRNA^{Glu}$ to ribosomes was stimulated more by the triplet GpApG than by the triplet GpApA.

Materials and Methods

General. Cyanogen bromide was obtained from Matheson Coleman and Bell. Sodium [^{35}S]sulfate (200 mCi/mmol) was purchased from New England Nuclear. Labeled amino acids were obtained commercially with the following specific activities (milliCuries/millimole): [U-^{14}C]glutamic acid, 269 and 209; [3-^{3}H]glutamic acid, 27,300. DEAE-cellulose (DE52) was purchased from Whatman; BD-cellulose and the 2-naphthoxyacetyl ester of *N*-hydroxysuccinimide from Boehringer-Mannheim, DEAE-Sephadex (A-50) from Pharmacia, and tlc plates of microgranular cellulose (250 μ) from Analtech. Trinucleotides GpApA and GpApG which were chemically synthesized (Lohrmann *et al.*, 1966) were kindly supplied by Joyce Heckman and Dr. H. G. Khorana of Massachusetts Institute of Technology. Ribonucleases-T_1 and -T_2 (Sankyo) were obtained from Calbiochem. *E. coli* $tRNA_2^{Glu}$ which accepted 1450 pmol of glutamic acid per A_{260} unit of tRNA, was a gift of Dr. A. D. Kelmers of the Oak Ridge National Laboratory. *E. coli* Q13 ribosomes prepared according to the method of Anderson *et al.* (1967) were generously provided by Dr. Mohan Sopori, Yale University.

Nucleotide Analysis of tRNA. tRNA was digested with T_2 ribonuclease and the resulting nucleotides were analyzed by two-dimensional thin layer chromatography (Agris *et al.*, 1973). The separated nucleotides were detected by visual inspection under an ultraviolet lamp or by autoradiography. Radioactive nucleotides were quantitated by scintillation counting of the cellulose scrapings corresponding to the exposed areas of the autoradiogram.

Chromatography. Two-dimensional thin layer chromatography in solvent I, isobutyric acid–concentrated ammonia–H_2O (199:3:98), and solvent II, isopropyl alcohol–12 N HCl–H_2O (68:17.6:14.3), has been described (Agris *et al.*, 1973).

Polyacrylamide Gel Electrophoresis. Analytical gel electrophoresis of tRNA and [^{14}C]- or [^{3}H]aminoacyl-tRNA was conducted using 8.5% polyacrylamide slab and disc gels containing 7 M urea at pH 5.8 according to the methods of Varricchio and Seno (1973).

Aminoacylation. Amino acid acceptance was measured according to the procedures of Lapointe and Söll (1972a). Routine assays were performed at 37° and kinetic experiments at 30°.

Growth of Bacteria. E. coli C6 *rel⁻met⁻cys⁻* (Harris *et al.*, 1969), a kind gift of Dr. E. B. Titchener, was grown in the minimal medium described by Harris *et al.* (1969) except that $MgSO_4$ was replaced by $MgCl_2 \cdot 6H_2O$ (0.2 g/l.) and that methionine (150 mg/l.) and cysteine (1.8 mg/l.) were added. The inoculum (2 l.) was a culture grown at 37° to stationary phase in the same medium but containing a ten times higher concentration of cysteine. The 100-l. culture was grown at 37° in a Fermacell fermentor (New Brunswick Scientific) for 13 hr—including 7.5 hr at stationary phase. The cells were harvested by centrifugation in a Sharples Super Centrifuge, washed with a solution of 0.5% NaCl–0.5% KCl, quickly frozen, and stored at −20°.

E. coli K12 (strain CA244, Brenner and Beckwith, 1965) was grown in 500 ml of the same medium as described above except that the amino acid supplement contained only tryptophan (40 mg/l.). $Na_2{}^{35}SO_4$ (25 mCi) was added to the medium in order to label the thionucleosides present in the bacterial tRNA. The radioactive medium was inoculated with 5 ml of stationary phase culture grown in the same medium but containing nonradioactive Na_2SO_4. After incubation for 12 hr at 37° the cells were harvested, washed with 0.5% NaCl–0.5% KCl, quickly frozen, and stored at −20°.

Extraction of tRNA. E. coli tRNA was isolated according to the method of Zubay (1962) including a final DEAE-cellulose step (Söll *et al.*, 1967). For the extraction of tRNA from the ^{35}S-labeled *E. coli* K12 (CA244) cells, 120 A_{260} units of nonradioactive unfractionated tRNA from the same strain were added before phenol treatment.

Purification of [^{35}S]*tRNA^{Glu} and Sulfur-Deficient tRNA^{Glu}.* Unfractionated tRNA obtained from the sulfur-deprived culture or from the ^{35}S-labeled cells were subjected to column chromatography on BD-cellulose at pH 5 in the presence of Mg^{2+} according to Roy *et al.* (1971). Those fractions having glutamic acid acceptor activity were combined and the tRNA was concentrated by ethanol precipitation. The tRNA (400 A_{260} units/ml of [^{35}S]tRNA or 800 A_{260} units/ml of sulfur-deficient tRNA) was aminoacylated with glutamic acid (labeled with ^{3}H for the [^{35}S]tRNA and with ^{14}C for the sulfur-deficient tRNA) in the presence of large amounts of purified GluRS to secure full charging, derivatized with the 2-naphthoxyacetyl ester of *N*-hydroxysuccinimide and subjected to chromatography on BD-cellulose columns (Roy *et al.*, 1971).

Those fractions containing ^3H or ^{14}C radioactivity were combined and concentrated by ethanol precipitation. The tRNAs were deacylated by incubation for 30 min at 37° in 0.5 M Tris-HCl (pH 9) and then dialyzed against glass distilled water. The final purification step was chromatography of the tRNAs on DEAE-Sephadex A-50 at pH 4.0 (S. Nishimura, personal communication) as described in the legend to Figure 1.

Preparations of Glutamyl-tRNA Synthetase. Crude aminoacyl-tRNA synthetase from *E. coli* K12 (CA244) was prepared as described previously (Söll *et al.*, 1967). Pure GluRS was obtained by preparative polyacrylamide gel electrophoresis as a final step in the procedure described by Lapointe and Söll (1972a).

Reaction of tRNA with BrCN. The reaction was carried out according to the procedure of Saneyoshi and Nishimura (1971). Approximately 1 A_{260} unit of $tRNA^{Glu}$ in 0.1 ml of 0.1 M $NaHCO_3$ (pH 8.9) was shaken with 250 μg of BrCN for 10 min at room temperature. The solution was then adjusted to pH 6.0 and dialyzed against 0.5 mM EDTA at 4° for 14 hr.

Assay for Binding of tRNA to Ribosomes. The general procedure of Nirenberg and Leder (1964) was used. Incubation mixtures (0.05 ml) contained 0.1 M Tris-HCl (pH 7.5), 0.05 M KCl, 0.01 M mangesium acetate, 1.0 A_{260} unit of ribosomes, 0.1 A_{260} unit of trinucleotide, and [^{35}S]$tRNA^{Glu}$ or [^{14}C]glutamyl-$tRNA^{Glu}$. Incubations were carried out for 15 min at 25°.

Results

Preparation of Sulfur-Deficient $tRNA^{Glu}$ and [^{35}S]$tRNA^{Glu}$. When *E. coli* C6 *rel⁻met⁻cys⁻* was grown under cysteine starvation conditions, sulfur-deficient tRNA was obtained (Harris *et al.*, 1969). The decrease in the amount of s^4U, the major sulfur-containing nucleoside, was judged from the ratio of the ultraviolet (uv) absorbance of the tRNA at 335 nm to that at 260 nm (Lipsett, 1965). Unfractionated tRNA, which we extracted from *E. coli* C6 *rel⁻met⁻cys⁻* grown under conditions of sulfur starvation, had an absorbance at 335 nm which was equal to only 0.7% of that at 260 nm. On the other hand, unfractionated tRNA from cells of the same strain grown in fully supplemented medium had an absorbance at 335 nm which was 1.7% of that at 260 nm (Table II). These values agree with the results of Harris *et al.* (1969) for tRNA from the same mutant and indicate that the sulfur-deficient tRNA lacks the normal complement of s^4U. In addition, this tRNA contained only one-third the normal amount of the thiolated nucleoside, ms^2i^6A, and ten times the normal amount of the nucleoside, i^6A (Table II). The lack of s^4U and ms^2i^6A in the unfractionated sulfur-deficient tRNA indicated that the tRNA might also be deficient in other thiolated nucleosides such as 2-thiocytidine and derivatives of 2-thiouridine. Since these nucleosides occur in small amounts in tRNA we did not, for lack of tRNA, analyze their exact amounts.

Unfractionated tRNA extracted from *E. coli* K12 (CA244) *rel⁺* grown in the presence of sufficient $Na_2{}^{35}SO_4$ to give a fully supplemented medium contained approximately 0.6 nmol of ^{35}S per A_{260} unit of tRNA. This indicates the presence of approximately 5.5 ^{35}S-containing nucleosides per tRNA molecule. After digestion of this tRNA with ribonuclease-T_2 and two-dimensional tlc of the resulting nucleotides (Nishimura, 1972), an autoradiogram showed that the nucleotides ms^2i^6Ap, s^2Cp, s^4Up, $s^2U^{**}p$, and s^2U^*p had been radioactively labeled (data not shown).

TABLE II: Amount of Thiolated Nucleosides in Normal and Sulfur-Deficient tRNAs.

tRNA[a]	Amount[b] of s^4U	Amount[c] of ms^2i^6A	Amount[c] of i^6A
Normal	1.74	8.00	2.25
Sulfur-deficient	0.71	2.81	25.00

[a] Normal tRNA was extracted from *E. coli* C6 *rel⁻* grown in fully supplemented medium. Sulfur-deficient tRNA was extracted from the same strain, grown in medium deficient in any source of sulfur. [b] Amount of s^4U is the absorbance of equivalent samples of tRNA at 335 nm expressed as a per cent of that at 260 nm. [c] The tRNAs were assayed in the laboratory of Dr. F. Skoog for the presence of ms^2i^6A and i^6A by a method dependent on the cytokinin activities of the nucleosides (Bartz *et al.*, 1970). Amounts of ms^2i^6A and i^6A are expressed as ng of kinetin required to give the same growth response in the cytokinin assay as 1 mg of tRNA. Complete results of these cytokinin assays will be published elsewhere (P. F. Agris, D. J. Armstrong, K. Schäfer, F. Skoog, and D. Söll, manuscript in preparation).

$tRNA^{Glu}$ was purified from the sulfur-deficient and ^{35}S-labeled tRNA by column chromatography on BD-cellulose and DEAE-Sephadex (see Materials and Methods). The elution profile for the final DEAE-Sephadex chromatography is given in Figure 1. As shown by S. Nishimura (personal communication) $tRNA^{Glu}$ elutes very early from this column in contrast to its behavior during DEAE-Sephadex chromatography at pH 7.5 (Nishimura, 1971). A similar pattern was obtained in the purification of the [^{35}S]$tRNA^{Glu}$. Both tRNA preparations accepted 1400 pmol of glutamic acid per A_{260} unit of tRNA. When subjected to polyacrylamide gel electrophoresis both samples migrated as single bands with the same mobility as pure *E. coli* $tRNA_2^{Glu}$. Two-dimensional chromatographic analysis of the nucleotides obtained from a ribonuclease-T_2 digest of sulfur-deficient $tRNA^{Glu}$ indicated, as determined by visual inspection of the tlc plates, that this tRNA had approximately one-third its normal complement of s^2U^* compared to [^{35}S]$tRNA^{Glu}$ and pure $tRNA_2^{Glu}$. The existence of a fractional amount of s^2U^* in this tRNA was confirmed when only a partial loss of acceptor activity was observed after the tRNA had been treated with BrCN (see below). However, sulfur-deficient $tRNA^{Glu}$ did contain the other modified nucleotides (Tp, ψp, and m^2Ap) expected from the known sequence of *E. coli* $tRNA_2^{Glu}$ (Ohashi *et al.*, 1972).

Reaction of Cyanogen Bromide with $tRNA^{Glu}$. [^{35}S]$tRNA^{Glu}$ was treated with BrCN at pH 8.9. The extent of the reaction was analyzed by quantitation of the radioactive nucleoside 3′-phosphates of ribonuclease-T_2 digests of the treated tRNA. Figure 2 shows chromatographic mobilities of the radioactive nucleotides present in the [^{35}S]$tRNA^{Glu}$ before and after treatment with BrCN. In the untreated tRNA only two radioactive nucleotides were found. They were found to be in equal amounts and were tentatively identified as $s^2U^{**}p$ and s^2U^*p. Since we do not have standards for these nucleotides we are not certain of their identities. However, we have found that the ^{35}S-labeled nucleotide denoted s^2U^*p in Figure 2 has the same chromatographic mobility as the thiolated uridine present in the ribonuclease-T_2 digest of pure *E. coli* $tRNA_2^{Glu}$ which was identified as 5-methylaminomethyl-2-thiouridine.

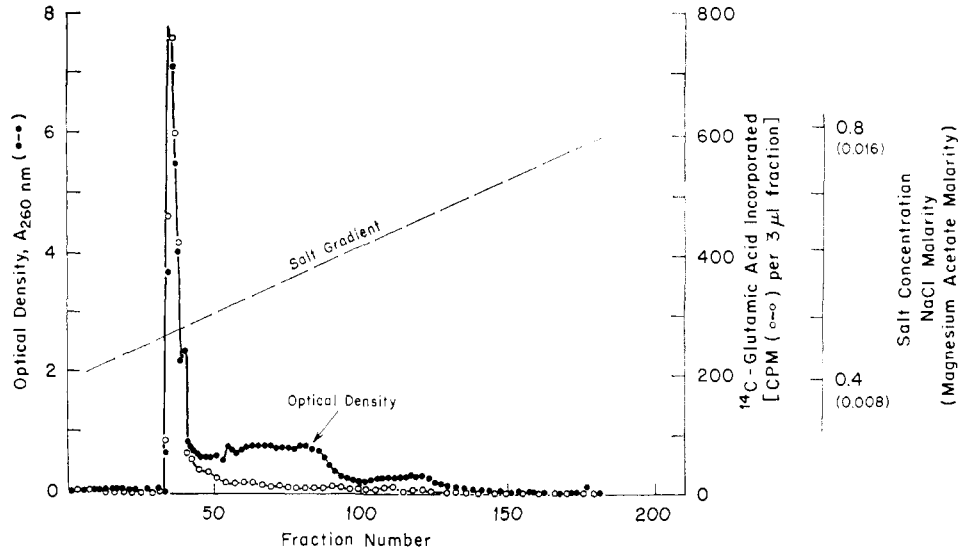


FIGURE 1: Final purification of $tRNA^{Glu}$ by DEAE-Sephadex column chromatography at pH 4.0. Partially purified tRNA (50 A_{260} units; see Materials and Methods) was applied to a DEAE-Sephadex A-50 column (0.6 × 25 cm) preequilibrated with 0.05 M sodium acetate (pH 4.0)–0.4 M NaCl–0.008 M $MgCl_2$–0.04% NaN_3. Elution was carried out at room temperature with a linear gradient of 60 ml of the equilibration buffer and 60 ml of 0.04 M sodium acetate (pH 4.0)–0.8 M NaCl–0.016 M $MgCl_2$–0.04% NaN_3. Fractions were collected every 10 min. The volumes fluctuated between 0.5 and 1 ml. The absorbance (A_{260} nm, ●) of every second fraction was measured and the glutamic acid acceptance (○) of 3 μl of every third fraction was assayed using a crude aminoacyl-tRNA synthetase preparation.

After BrCN treatment of the $[^{35}S]tRNA^{Glu}$ >90% of the radioactivity moved to the positions designated in the figure as NC-s^2U**p and NC-s^2U*p. The amount of radioactivity in each of the modified nucleotides was the same. The remaining radioactivity migrated as [^{35}S]sulfate. We do not know whether the radioactive areas represent the thiocyanate derivatives (Saneyoshi and Nishimura, 1970) of the original thiolated uridines in the tRNA. However, we believe this to be the case because of their parallel migration on tlc relative to each other and to s^2U**p and s^2U*p, because of the absence of any other radioactive nucleotides in the tRNA after the BrCN reaction and because NC-s^2U**p and NC-s^2U*p contained the same relative amounts of radioactivity as did s^2U**p and s^2U*p (~1:1). Thus we conclude that reaction of BrCN with the thiolated uridines in $[^{35}S]tRNA^{Glu}$ was 90% complete.

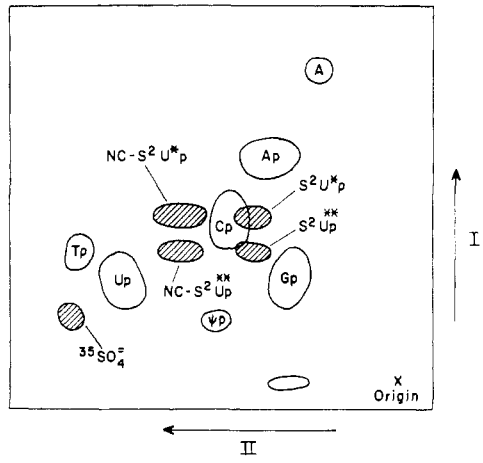


FIGURE 2: Chromatographic analysis of nucleotides in $[^{35}S]tRNA^{Glu}$ before and after cyanogen bromide treatment. A mixture of $[^{35}S]tRNA^{Glu}$ (~20,000 cpm, 0.1 A_{260} unit) and nonradioactive *E. coli* CA244 tRNA (2 A_{260} units) was digested with ribonuclease-T_2 and the resulting nucleotides subjected to two-dimensional (I, first dimension; II, second dimension) tlc and then autoradiography. An equivalent amount of $[^{35}S]tRNA^{Glu}$ which had been reacted with BrCN was treated similarly. The drawing is a composite of the two tlc plates and the two autoradiographs. The positions of those nucleotides (Ap, Cp, Gp, Up, ψp, and Tp) which occur in sufficient quantities to allow their presence to be easily detected visually (with a uv lamp) are indicated. The cross-hatched areas show the locations of the radioactive nucleotides and of [^{35}S]sulfate. The nucleotides s^2U**p and s^2U*p were not detected by autoradiography of the tlc plate containing the BrCN-treated material. The radioactivity located in the areas designated NC-s^2U**p and NC-s^2U*p was detected only in the case of the BrCN-treated tRNA. The radioactive nucleotides were quantitated by scintillation counting.

Walker and RajBhandary (1972) have modified the s^4U in $tRNA^{Tyr}$ with BrCN and subsequently hydrolyzed the resulting NC-s^4U derivative to U without any other detectable change in the tRNA. We tried, similarly, to convert the s^2U* in $tRNA^{Glu}$ to U. However, we found that the alkaline or acidic conditions needed to hydrolyze the BrCN adduct of s^2U* in $tRNA^{Glu}$ caused a large loss of glutamic acid acceptor activity in the control $tRNA^{Glu}$, which had not been reacted with BrCN (data not shown). Therefore, we did not achieve a removal of sulfur from the s^2U derivative of $tRNA^{Glu}$.

Aminoacylation Studies. BrCN-treated $[^{35}S]tRNA^{Glu}$ could be aminoacylated with glutamic acid in the presence of purified GluRS, but high concentrations of the enzyme were needed (Figure 3). The extent of aminoacylation of the BrCN-treated $tRNA^{Glu}$ was about 70% of that of the untreated tRNA. This result indicates that at least 60% of the tRNA which reacted with BrCN could be aminoacylated since we have shown that only 10% of the BrCN-treated $tRNA^{Glu}$ did not react with BrCN. BrCN treatment of $tRNA^{Glu}$ may have caused the observed lower steady-state level of charging by increasing the rate of enzymatic deacylation as has been observed by Schrier and Schimmel (1972) and discussed further by Bonnet and Ebel (1972). As mentioned above BrCn treatment of sulfur-deficient $tRNA^{Glu}$ leads to only a 40% loss of amino acid acceptor activity, whereas, under analogous aminoacylation conditions (0.5 μg of GluRS per incubation mixture), BrCN-treated $[^{35}S]tRNA^{Glu}$ was observed to have more than a 90% loss of glutamic acid acceptance (Figure 3). This substantiates the finding of only one-

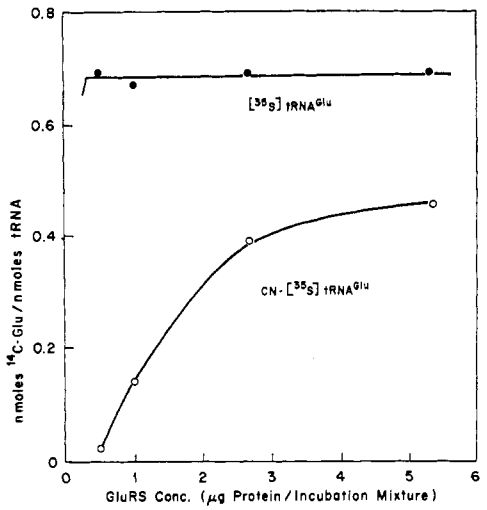


FIGURE 3: Extent of aminoacylation of BrCN-treated $tRNA^{Glu}$. BrCN-treated [^{35}S]$tRNA^{Glu}$ and untreated [^{35}S]$tRNA^{Glu}$ were aminoacylated with [^{14}C]glutamic acid. The reaction mixtures (0.05 ml) contained ~0.01 A_{260} unit of tRNA and were incubated at 37° for 60 min with varying amounts of pure GluRS. The amount of glutamic acid accepted by BrCN-treated $tRNA^{Glu}$ (○) and by untreated $tRNA^{Glu}$ (●) is plotted as a function of GluRS concentration. Radioactivity due to [^{35}S]tRNA was subtracted from the aminoacylation results using as controls incubations in the absence of glutamic acid but in the presence of [^{35}S]$tRNA^{Glu}$ with varying amounts of enzyme. In this experiment untreated [^{35}S]-$tRNA^{Glu}$ was aminoacylated to the extent of 0.7 nmol of glutamic acid/nmol of tRNA although in most experiments with this tRNA the acceptance was 0.85 nmol of glutamic acid.

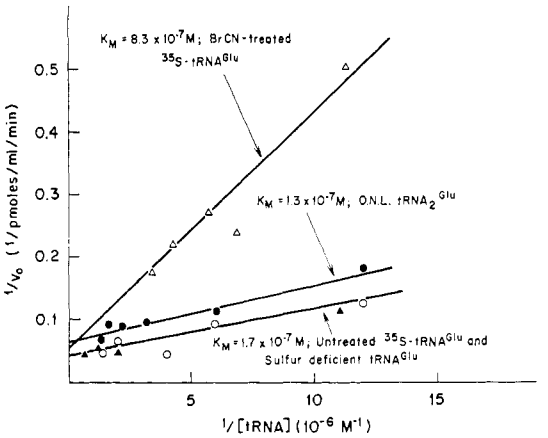


FIGURE 4: Determination of the K_m values of GluRS for BrCN-treated $tRNA^{Glu}$ and sulfur-deficient $tRNA^{Glu}$. Aminoacylations of various amounts of BrCN-treated [^{35}S]$tRNA^{Glu}$, untreated [^{35}S]-$tRNA^{Glu}$, $tRNA_2^{Glu}$, and sulfur-deficient $tRNA^{Glu}$ were carried out at 30° with [^{14}C]glutamic acid and pure GluRS as described earlier (Lapointe and Söll, 1972a). The enzyme:tRNA ratio in these experiments varied from 4.2 to 0.32 pmol of pure GluRS/pmol of $tRNA^{Glu}$ calculated on the basis that GluRS has a mol wt of 102,000 (Lapointe and Söll, 1972a) and $tRNA^{Glu}$ has a mol wt of 22,400 (Ohashi *et al.*, 1972). Initial velocities (V_0) were determined from plots of acceptor activity *vs.* incubation time. Values of the K_m of GluRS for BrCN-treated [^{35}S]$tRNA^{Glu}$ (△), untreated [^{35}S]-$tRNA^{Glu}$ (○), $tRNA_2^{Glu}$ (●), and sulfur-deficient $tRNA^{Glu}$ (▲) were determined from the Lineweaver–Burk, double reciprocal plots shown here.

third the normal complement of s^{2}U* being present in sulfur-deficient tRNA.

Experiments designed to study the kinetics of the aminoacylation of BrCN-treated $tRNA^{Glu}$ showed that the K_m of GluRS for the BrCN-treated [^{35}S]$tRNA^{Glu}$ was about six times higher than that for the untreated [^{35}S]$tRNA^{Glu}$ (Figure 4). However, the K_m of GluRS for untreated sulfur-deficient $tRNA^{Glu}$ was found to be very similar to the K_m of GluRS for untreated [^{35}S]$tRNA^{Glu}$ and for untreated, unlabeled $tRNA_2^{Glu}$ (Figure 4). Therefore *in vitro* chemical alteration of $tRNA^{Glu}$ in the anticodon decreases the interaction of GluRS with its cognate tRNA, whereas lack of the naturally occurring modification in the anticodon does not affect this interaction. The absence of the naturally occurring thiolation of uridine in the anticodon did not change the specificity of the aminoacylation reaction because, in the presence of crude aminoacyl-tRNA synthetase and each one of the 20 common amino acids, sulfur-deficient $tRNA^{Glu}$ is charged only with glutamic acid (data not shown).

Ribosomal Binding Studies. In ribosomal binding studies with GpApA and GpApG, the two glutamic acid coding triplets (Khorana *et al.*, 1966), sulfur-deficient [^{14}C]Glu-$tRNA^{Glu}$ was preferentially bound to ribosomes in the presence of GpApG, whereas normal [^{14}C]Glu-$tRNA_2^{Glu}$ and [^{35}S]$tRNA^{Glu}$ were preferentially bound in the presence of GpApA (Table III). BrCN-treated [^{35}S]$tRNA^{Glu}$ did not bind to ribosomes in the presence of either GpApA or GpApG. Therefore, the reaction of the anticodon nucleoside, s^{2}U*, with BrCN disrupts codon–anticodon interaction.

The preferential recognition of GpApG by the sulfur-deficient $tRNA^{Glu}$ represents a change in the specificity of the base pairing of the modified uridine in the anticodon from A to G. Since the nature of this nonthiolated nucleoside in the sulfur-deficient $tRNA^{Glu}$ is not known it is difficult to interpret this result.

Discussion

We have been able to isolate and purify from cultures of *E. coli* grown in $Na_2{}^{35}SO_4$, a tRNA which is specific for glutamic acid. This $tRNA^{Glu}$ contains [^{35}S]s^{2}U* and the incompletely modified nucleoside [^{35}S]s^{2}U**. Purified [^{35}S]$tRNA^{Glu}$ was subsequently altered by reaction with BrCN to yield tRNA containing the thiocyanate derivatives of s^{2}U* and s^{2}U**. By growing an approprirate cysteine auxotroph of a relaxed strain of *E. coli* in a sulfur-deficient medium we were able to obtain and purify sulfur-deficient $tRNA^{Glu}$. This second preparation of $tRNA^{Glu}$ is a mixture of thiolated and nonthiolated molecules because the cells from which it was isolated were grown in medium first containing and then lacking a source of sulfur. Base analysis showed that approximately 60% of the sulfur-deficient $tRNA^{Glu}$ molecules did not contain 5-methylaminomethyl-2-thiouridine. We do not know the nature of the nonthiolated nucleoside present since an insufficient amount of pure sulfur-deficient $tRNA^{Glu}$ was obtained to allow sequence analysis. If s^{2}U* is derived from a uridine or cytidine in $tRNA^{Glu}$ we might expect the nonthiolated nucleoside to be either modified or unmodified U or C. We may note in this context that Kaiser (1972) has recently reported findings which suggest that U, not C, is the precursor to derivatives of s^{2}U in tRNA.

We have examined the interaction of pure GluRS with sulfur-deficient $tRNA^{Glu}$, with [^{35}S]$tRNA^{Glu}$, and with BrCN-treated [^{35}S]$tRNA^{Glu}$. The absence of the thiol modification in the anticodon of sulfur-deficient $tRNA^{Glu}$ had no effect on the rate, extent or specificity of the charging reaction. Squires and Carbon (1971) have also reported that a change in the nucleoside at the first or "wobble" position of the anticodon of tRNA, in this case $tRNA^{Gly}$, does not affect the rate or

TABLE III: Triplet-Stimulated Ribosomal Binding of $tRNA^{Glu}$.

tRNA	Triplet	tRNA Bound to Ribosomes[a] (Δcpm/tube)
[^{14}C]Glu-$tRNA_2^{Glu}$	None	(75)
	GAA	160
	GAG	52
[^{35}S]$tRNA^{Glu}$	None	(40)
	GAA	140
	GAG	80
BrCN-treated [^{35}S]$tRNA^{Glu}$	None	(60)
	GAA	−10
	GAG	10
Sulfur-deficient [^{14}C]Glu-$tRNA^{Glu}$	None	(90)
	GAA	70
	GAG	222

[a] The reaction mixtures contained approximately 3000 cpm of either [^{35}S]$tRNA^{Glu}$ or [^{14}C]Glu-$tRNA^{Glu}$ and were incubated at 25° for 15 min as described under Materials and Methods. Since complete charging of BrCN-treated $tRNA^{Glu}$ was impossible and since both the BrCN-treated [^{35}S]$tRNA^{Glu}$ and the untreated [^{35}S]$tRNA^{Glu}$ contained radioactive labels, binding studies were conducted with uncharged tRNA. Sulfur-deficient $tRNA^{Glu}$ and $tRNA_2^{Glu}$ were aminoacylated with [^{14}C]glutamic acid and then used in this experiment. The radioactivity bound to ribosomes in the absence of triplet is shown in parentheses and has been subtracted from that bound in the presence of triplet to give Δcpm/tube.

specificity of aminoacylation. Furthermore, a change in the nucleoside at the first position of the anticodon of $tRNA^{Tyr}$, producing the amber suppressor, su_{III}^{+}, does not affect the rate or specificity of the aminoacylation of this tRNA (Goodman *et al.*, 1968; Abelson *et al.*, 1970). However, we have found, as judged by K_m values, that GluRS has a sixfold lower affinity for BrCN-treated $tRNA^{Glu}$ than for untreated $tRNA^{Glu}$. Since the cyanate group is present in 90% of the BrCN-treated $tRNA^{Glu}$ yet 70% of the tRNA is chargeable at very high enzyme concentrations, the K_m value obtained obviously did not result from the amino acid acceptor activity of the 10% of the tRNA which did not react with BrCN. The increase in the K_m value indicates that the cyanate group may cause a local distortion of the aminoacyl-tRNA synthetase–tRNA complex in the vicinity of the anticodon.

Triplet-mediated ribosomal binding of yeast (Sekiya *et al.*, 1969) and *E. coli* (Ohashi *et al.*, 1972) $tRNA^{Glu}$ showed preferential recognition by the tRNA of the glutamic acid codon ending in A. Since these tRNAs contain derivatives of s^2U in the first position of the anticodon the authors concluded that this thiolated nucleoside is unable to pair with G and thus recognizes only one codon. Our results (Table III) confirm their observation since normal $tRNA^{Glu}$ preferentially binds to ribosomes in the presence of GAA. Modification of the s^2U derivative with BrCN greatly affects ribosomal binding. We found that BrCN-treated $tRNA^{Glu}$ did not bind to ribosomes in the presence of triplets coding for glutamic acid. This is in agreement with the work of Saneyoshi and Nishimura (1971) with BrCN-treated, unfractionated tRNA aminoacylated with histidine, lysine, and tyrosine. Sulfur-deficient $tRNA^{Glu}$ did bind to ribosomes but with altered specificity, preferring the GpApG triplet to the normal GpApA triplet. We cannot draw any definite conclusions from this result since we do not know the nature of the nucleoside now present in the first position of the anticodon of sulfur-deficient $tRNA^{Glu}$. However, we feel that sulfur-deficient $tRNA^{Glu}$ may be used to advantage in the detection of the enzyme(s) responsible for the biosynthesis of s^2U derivatives in tRNA.

The basis for the interactions of tRNAs with their cognate synthetases and the functions of the great variety of modified nucleosides in tRNA are two of the most challenging problems in tRNA biochemistry. Our work has shown that the interaction of $tRNA^{Glu}$ with its cognate synthetase may require a defined steric relationship between the enzyme and the tRNA at the anticodon region. We have also shown that a lack of modification of the "wobble" nucleoside in the anticodon of $tRNA^{Glu}$ does not affect the aminoacylation of this tRNA but does alter the coding specificity in trinucleotide-stimulated ribosomal binding.

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Transcription of Chromatin. Initial and Terminal Nucleotides of Ribonucleic Acid Synthesized by Calf Thymus and *Escherichia coli* Ribonucleic Acid Polymerases†

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ABSTRACT: Initial and terminal nucleotides of RNA transcribed from chromatin were compared with RNA transcribed from native and denatured DNA by an RNA polymerase obtained from calf thymus and *Escherichia coli* RNA polymerase. Enzymically transcribed RNA was hydrolyzed in alkali and the hydrolysate resolved by electrophoresis into initial nucleoside tetraphosphate, internal nucleoside monophosphate, and terminal nucleoside. With native calf thymus DNA as template, calf thymus RNA polymerase initiates over 90% with ATP and GTP rather than UTP and CTP. With denatured DNA, initiation by GTP relative to ATP increases over twofold and the RNA is one-fourth as long. With calf thymus chromatin as template, initiation using both calf thymus and *E. coli* RNA polymerases is over 95% with ATP and GTP. The amount of initiation by ATP relative to GTP for the two enzymes is significantly different when chromatin is used as template. These results indicate that calf thymus RNA polymerase initiates predominantly with purine ribonucleoside triphosphates and at sites which may differ from those at which *E. coli* RNA polymerase initiates. The results suggest that the structure of RNA polymerase, in addition to chromatin control factors, may determine the sites at which transcription occurs. Total termination values are two- to fourfold higher than total initiation values. Using calf thymus enzyme, termination with adenosine is significantly higher when chromatin, rather than DNA, is the template. Using *E. coli* enzyme, there is a smaller increase in termination with adenosine. These results suggest preferential termination with adenosine when chromatin is used as template or, alternatively, the presence of an enzyme in chromatin which adds adenosine to the 3′-OH end of RNA.

Differentiation is thought to involve the transcription of only a selected set of genes in a given cell. Control factors which govern this selection of genes are only poorly understood. Non-histone proteins (Paul and Gilmour, 1968) or RNA (Bekhor *et al.*, 1969; Huang and Huang, 1969) may interact with histones to derepress specific genes, *i.e.*, to allow RNA polymerase (RNA nucleotidyltransferase, EC 2.7.7.6) to interact with selected segments of the total DNA. This interaction may also depend upon the structure of the RNA polymerase. We have recently observed that an RNA polymerase preparation obtained from calf thymus and *Escherichia coli* RNA polymerase interact differently with calf thymus chromatin, at least in terms of the kinetic parameters of transcription (Keshgegian and Furth, 1972). One of the predictions of such a difference is that mammalian and bacterial polymerases may transcribe at different sites on the chromatin template. If transcription does occur at different sites, the initial nucleotides of the RNA synthesized may differ. We have therefore investigated the initiation of RNA synthesis by calf thymus RNA polymerase, and compared the initiation on a chromatin template with initiation by *E. coli* RNA polymerase.

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