Chemical Probe of Structure and Function of Transfer Ribonucleic Acids†

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ABSTRACT: In an earlier study (Singhal, R. P. (1971), *J. Biol. Chem. 246,* 5848) it was observed that modification of six cytidine residues, including those in the anticodon loop and the C-C-A of the 3′-terminal stem, of the glutamate tRNAs of *Escherichia coli* by bisulfite abolishes the ability of the tRNAs to be aminoacylated. Several reports have confirmed the importance of those two tRNA segments for full aminoacylation. The results of the present study indicate that the reactive cytidines of the glutamate $tRNA_2$ of *E. coli* are modified at distinctly different rates. The first to react are those located in the anticodon loop and at the 3′ end of the tRNA. Aminoacylation ability is lost in parallel with the extent of modification in those two areas, and the loss precedes changes in tRNA conformation brought about by the subsequent modifications. Similarly, the modification of the 2-thiouridine derivative in the anticodon loop by either cyanogen bromide or *p*-chloromercuribenzoate is paralleled by a reduction in aminoacylation ability. It appears from these results that (a) only certain nonhydrogen-bonded cytidines that appear to be "exposed" in the conventional cloverleaf structure are unreactive ("buried"), and (b) the prime inactivation target with respect to aminoacylation is a residue that is either at the 5′ end or at the 3′ end of the anticodon. The results also indicate that the modification of cytidine in tRNA with bisulfite is very dependent upon the temperature and time of reaction. Pseudouridine is irreversibly modified under considerably more stringent conditions, yielding two anionic, alkali-unstable isomers. *p*-Chloromercuribenzoate forms a stable complex with the 2-thiouridine of glutamate tRNA, in contrast to that formed with the 4-thiouridine of formylmethionine tRNA, which dissociates on addition of magnesium ions.

Chemical modification of specific bases within a tRNA molecule is one approach to the elucidation of its three-dimensional structure, upon which may depend its biological behavior—especially interaction with the cognate ligase. The rationale is that if a reagent specific for a particular purine or pyrimidine base fails to react with certain ones of that species, the nonreactive bases are inaccessible to the reagent under those conditions and are therefore "buried." The extent to which a particular base is reactive may be influenced by conditions that influence folding or unfolding of the structure, such as temperature, detergents, and counterions. Accordingly, the study of chemical modification must take into account not only the specificity of a given reagent for one or another species of base, but also the influence of temperature, etc., upon the rates of reaction of the same base in different locations in a particular tRNA molecule. Hence, the first step is to find a reagent that reacts specifically with only one, or very few, species of bases. The second step is to ascertain the rate of modification of each one of that (or those) species within the tRNA under various conditions.

Among the reagents that have been exploited are *p*-chloromercuribenzoate (Pal *et al.,* 1972), cyanogen bromide (Saneyoshi and Nishimura, 1971; Walker and Rajbhandary, 1972; Agris *et al.,* 1973), and benzoic anhydride (Cedergren *et al.,* 1973), all of which can be used to modify relatively rare sulfur-containing bases in unique positions of the tRNA. Bisulfite, on the other hand, reacts with both common pyrimidines, but conditions can be chosen so that only cytosine is modified with loss of the 4-amino group. This reaction, introduced by Hayatsu *et al.* (1970a,b) and Shapiro *et al.* (1970), is used here to examine the reactivities, and hence the accessibilities, of the cytosine residues in the $tRNA^{Glu}_2$ of *Escherichia coli* (this tRNA is devoid of certain rare bases that might complicate the results). The rates of modification of each reactive site under various reaction conditions have been determined. In addition, since cyanogen bromide and *p*-chloromercuribenzoate react with 2-thiouridine derivatives, they are used to examine the reactivity of a 2-thiouridine derivative that occupies the "wobble position" in the $tRNA^{Glu}_2$ anticodon.

The modification of residues in the anticodon region—the cytidines by bisulfite, the 2-thiouridine derivative by either cyanogen bromide or *p*-chloromercuribenzoate—considerably reduces the aminoacylation ability of this tRNA. The results, obtained by the use of three different chemicals, indicate that the prime inactivation target of aminoacylation is a residue either at the 5′ end (Schulman and Goddard, 1973; Saneyoshi and Nishimura, 1971; Agris *et al.,* 1973; Cedergren *et al.,* 1973; Litt, 1971; Litt and Greenspan, 1972) or at the 3′ end (Kućan *et al.,* 1971; Chambers *et al.,* 1973) of the anticodon. This conclusion agrees with the conclusion drawn from nonchemical studies, such as the binding of complementary oligonucleotides to tRNA segments (Uhlenbeck *et al.,* 1970; Schimmel *et al.,* 1972). The results also indicate that certain cytosine residues that seem "exposed" in the conventional cloverleaf structure are nonreactive ("buried") under appropriate reaction conditions. A preliminary report has been published (Singhal, 1971).

Materials and Methods

Transfer RNAs. Samples of $tRNA^{Glu}_{1,2}$ and $tRNA^{fMet}_1$ (*E. coli,* K-12 MO7) were generously supplied by A. D Kelmers, Chemical Technology Division, of this laboratory, who prepared them as described by Weeren *et al.* (1970). The characteristics of these tRNA samples have been described elsewhere (Singhal and Best, 1973). In earlier studies, $tRNA^{Glu}_{1,2}$ was further purified on a benzoylated DEAE-cellulose column (Singhal, 1971); however, this method was inadequate to re-

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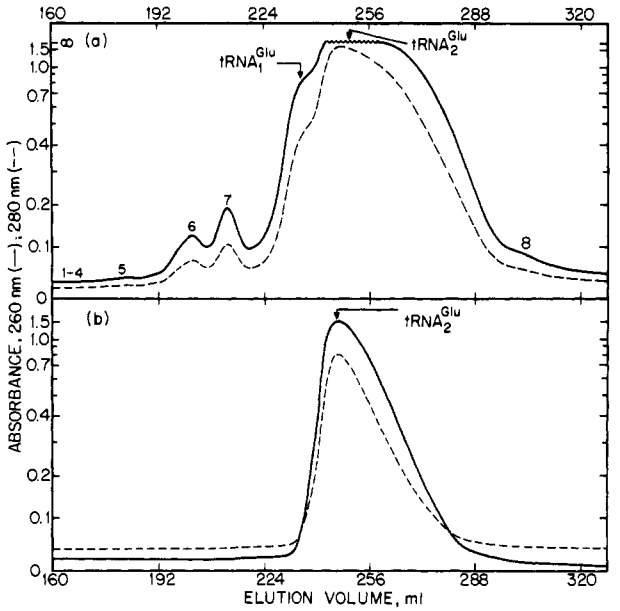

FIGURE 1: Purification of glutamate tRNAs by reversed-phase chromatography (RPC-5). The column (26 × 0.63 cm) was eluted with a linear gradient from 0.3 to 1.0 M NaCl (250 ml each), containing 2 M $Na_2S_2O_3$, 10 mM $MgCl_2$, and 50 mM NaOAc (pH 5.4). The elution was at a flow rate of 0.4 ml/min (100 psi resultant pressure) and 40°. (a) Partially purified glutamate tRNAs (80% pure, *ca.* 70 A_{260} units) in 0.5 ml of the starting buffer was applied to the column (see Singhal, 1973). The two glutamate tRNAs accepted 840 and 1500 pmol of [^{14}C]glutamic acid per A_{260} unit, respectively. The numbered peaks could not be charged with glutamic acid and so were considered to be contaminant tRNAs (peaks 1-4 appeared before 160 ml of the eluent). (b) Rechromatography of $tRNA^{Glu}_2$ (*ca.* 30 A_{260} units), obtained by pooling the appropriate fractions from (a). This tRNA sample accepted 1520 pmol of glutamate per A_{260} unit.

solve the two isoacceptors, and the tRNA recovery was only 60%. Two different methods were used to obtain $tRNA^{Glu}_2$ of *ca.* 100% purity for the present studies.

DEAE-Sephadex Chromatography. Approximately 50 mg of $tRNA^{Glu}$ (78% pure) was chromatographed (Nishimura, 1971) on a column (92 × 2.5 cm) of DEAE-Sephadex A-50 using a linear gradient of 0.5–0.75 M NaCl (containing 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, and 20 mM NaOAc buffer (pH 4.0), 2 l. each at 0.5 ml/min and 20°, and monitoring the effluent at 260 nm. One major, five smaller, and numerous minor tRNA peaks were resolved. Peaks 1, 2, and 3 represented 8, 66, and 3% of the applied material and accepted about 800, 1450, and 340 pmol of glutamate per A_{260} unit, respectively. (One A_{260} unit is the amount of substance in 1 ml of solution that gives an absorbance of 1.0 when measured at 260 nm with a path length of 10 mm.) The remaining peaks could not be charged with glutamate. This chromatographic method was found unsatisfactory, since the column shrank considerably during chromatography and up to 10 days was needed for a single run.

Reversed-Phase Chromatography. When 78% $tRNA^{Glu}$ was chromatographed on a reversed-phase-5 (RPC-5) column, the presence of two isoaccepting $tRNA^{Glu}$ species was confirmed (Figure 1). The low aminoacylation (840 pmol/A_{260} unit) found for $tRNA^{Glu}_1$ was perhaps due to contaminant tRNAs or to a high extinction coefficient of this tRNA. Further purification of the $tRNA^{Glu}_2$ peak by this method did not increase its glutamate acceptance. Since the $tRNA^{Glu}_2$ preparation contained 1520 pmol of 3′-terminal adenosine and accepted 1490 pmol of glutamate per A_{260} unit, it was considered to be 98% pure and was used in most of the experiments described here.

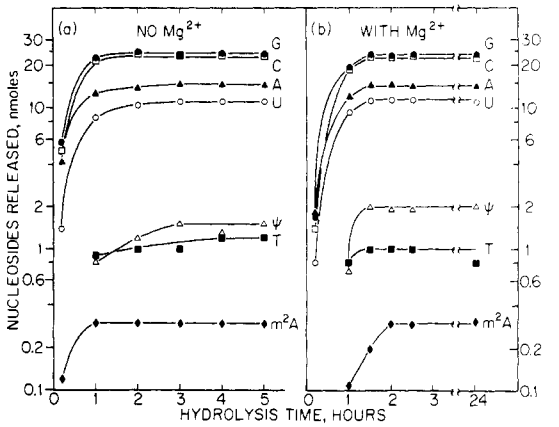

FIGURE 2: Enzymatic hydrolysis of *E. coli* tRNAs to nucleosides, and release of nucleosides with time. (a) Approximately 100 A_{260} units of mixed tRNAs was dissolved in 1.0 ml of 0.2 M ammonium carbonate (pH 8.7). Then a mixture of three enzymes—pancreatic RNase (37.5 units), phosphodiesterase I (10 potency units; Worthington), and salt-free alkaline phosphatase (0.25 unit; Calbiochem)—in 5 μl of water was added for each A_{260} unit of tRNA. The mixture was digested in a polyethylene microtube at 50°; aliquots, in triplicate, were withdrawn at various times; and each sample, containing 1.0 A_{260} unit or 75 nmol of nucleosides, was analyzed by anion-exclusion chromatography (Singhal, 1972). (b) Hydrolysis conditions were the same as described in (a), except that the tRNA was dissolved in 0.2 M ammonium carbonate containing 1 mM magnesium acetate.

Hydrolysis of tRNAs. NUCLEOSIDES. The conditions for enzymic hydrolysis of tRNA and of oligonucleotides to nucleosides in an easily volatilizable buffer were investigated. [Enzymes: alkaline phosphatase, EC 3.1.3.1; 5′-nucleotidase, EC 3.1.3.5; phosphodiesterase I (venom phosphodiesterase), EC 3.1.4.1; RNase T_1 (guanyloribonuclease), EC 3.1.4.8; pancreatic RNase (ribonuclease I, RNase A), EC 3.1.4.22; RNase T_2 (ribonuclease II), EC 3.1.4.23.] Approximately 1.0A_{260} unit (50 μg) of tRNA in 10 μl of 0.2 M ammonium carbonate (pH 8.7) containing 1 mM magnesium acetate was mixed with 5 μl of a mixture of three enzymes: pancreatic RNase (37 units), venom phosphodiesterase (10 units), and alkaline phosphatase (0.25 unit). The release of nucleosides from tRNAs at different hydrolysis times is indicated in Figure 2. Though tRNAs are hydrolyzed at a slower rate initially when Mg^{2+} is included in the digest, a complete RNA hydrolysis is achieved in 2 hr under these conditions with or without Mg^{2+}. The modified nucleosides are released slowly, *e.g.*, pseudouridine in 3 hr (Figure 2a). Hence, the hydrolysis was routinely carried out at 50° for 4 hr or at 37° for 15 hr.

3′-NUCLEOTIDES. One A_{260} unit of oligonucleotides in 10 μl of 50 mM ammonium acetate (pH 4.5) was mixed with 3 μl of three enzymes ($RNaseT_2$, 2 units; RNase T_1, 25 units; pancreatic RNase, 1.5 units). The hydrolysis was complete as no oligonucleotide was detectable under these conditions.

5′-NUCLEOTIDE. One A_{260} unit of oligonucleotides in 10 μl of 0.2 M ammonium carbonate and 1 mM magnesium acetate (pH 8.7) containing 10 potency units of phosphodiesterase I (freed of 5′-nucleotidase activity; see Killer, 1964) was incubated at 50° for 2–3 hr to produce a complete hydrolysis.

OLIGONUCLEOTIDES. (a) RNase T_1. Fifty A_{260} units of tRNA, dissolved in 200 μl of 20 mM Tris-HCl (pH 7.4), was incubated at 50° for 15 min. Thereafter, 5 μl of RNase T_1 (free of RNase T_2 activity, Calbiochem) containing 500 units was added, and the mixture was digested at 37° for 30 min. The reaction was stopped by adding 300 μl of 8 M urea. The digest was acidified, when desired, to convert cyclic phosphates into 3′-phosphates.

(b) Pancreatic RNase. The conditions were similar to those described elsewhere (Singhal, 1973). After the hydrolysis, urea was added as mentioned above. The addition of urea immediately after the hydrolysis, besides avoiding nonspecific-bond splitting, prevents aggregation of oligonucleotides and thus helps in the total recovery of large oligonucleotides from the column (see Singhal, 1973).

Analysis. The compositional analysis was determined on Aminex A-6 columns at pH 9.8 by anion-exclusion chromatography (Singhal, 1972) or on Aminex A-25 or A-28 columns at pH 3.75 by cation-exclusion chromatography (Singhal and Cohn, 1973) or on a Dowex-1 column at pH 9.8 by anion-exchange chromatography (Singhal and Cohn, 1972) of nucleosides. The 3′ and 5′ ends of oligonucleotides and tRNAs were determined by alkali hydrolysis (0.3 M KOH, 37°, 15 hr) followed by the separation of nucleosides, nucleotides, and guanosine bisphosphate on a reversed-phase column (Singhal, 1973) or on an Aminex A-28 column (Singhal, 1974), using 0.15–1.0 M NaCl (linear gradient) (pH 9.8) containing 20 mM Na_2CO_3–$NaHCO_3$ buffer, at 50°.

In the early part of these studies, oligonucleotides were resolved on DEAE-cellulose columns. However, these separations were unsatisfactory (see Singhal, 1971). Oligonucleotides obtained after digestion with either pancreatic RNase or RNase T_1 were satisfactorily resolved by anion-exchange chromatography on the RPC-5 column, using a linear salt gradient of either ammonium acetate or sodium chloride buffered at pH 9.8. Whereas ammonium acetate was removed from the eluate by repetitive freeze-drying, sodium chloride was removed on a column of either Sephadex G-10 (100 × 0.9 cm) or DEAE-cellulose (3.5 × 1 cm) (see Harada *et al.*, 1971). A typical separation of the products of pancreatic RNase digestion of tRNA-$^{Glu}_2$ has been described elsewhere (Singhal, 1973).

Exclusion Volume. The exclusion volume of tRNA was determined on a Sephadex G-100 column (40- to 120-μm beads). Approximately 2 A_{260} units of tRNAs, applied in 100 μl to the column (100 × 0.9 cm), was eluted with 20 mM phosphate buffer containing 10 mM $MgCl_2$ at pH 7.0, 20°, and 0.2 ml/min.

Melting Temperature. The melting temperatures and thermal hyperchromicities of tRNAs were assayed in a Gilford 2400 spectrophotometer. The samples were dialyzed in pretreated dialysis tubes (see Figure 2 in Singhal and Best, 1973) against 0.15 M Na^+ (phosphate) (pH 7.0), with three changes at 5°.

Aminoacylation of tRNAs. This was done according to the procedure and conditions described by Rubin *et al.* (1967, 1971). For glutamate charging, the final assay mixture was 100 mM in 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5), 20 mM in $Mg(OAc)_2$, 5 mM in KCl, 3.2 mM in ATP, 5 μM in L-[^{14}C]glutamic acid, 10 mM in β-mercaptoethanol, and contained, in 250 μl, 0.04–0.05 A_{260} unit of tRNA and *ca.* 0.05 mg of the partially purified synthetase preparation (Kelmers *et al.*, 1965). The mixture was incubated at 37° for 30 min. To aminoacylate the modified tRNAs, this reaction was also measured after the addition of purified *E. coli* adenylyl(cytidylyl)-transferase (4 μg/0.04 A_{260} unit of tRNA), which was a gift from A. N. Best (Best and Novelli, 1971). The modified reaction was carried out after CTP was added at two different concentrations, to give ATP/CTP ratios of 4 and 8. The aminoacylation under these conditions was tested at different times, ranging from 0.5 to 2 hr. The modified aminoacylation reaction was carried out to determine whether a poor aminoacylation of the chemically modified tRNA could arise from a nuclease activity that had been found (Kucan and Chambers, 1972) in crude synthetase preparations.

Modification of tRNAs. BISULFITE. The sodium bisulfite (Mallinckrodt Chemical Works, St. Louis, Mo.) solution of a desired concentration was either buffered with 50 mM phosphate buffer or mixed with a sodium sulfite solution (Singhal, 1971) to maintain the pH at *ca.* 6. Solutions were prepared just before use. In a typical reaction, 2 mg of tRNA in 100 μl of phosphate buffer in a 1-ml reaction vial (Pierce Chemical Co., Rockford, Illinois) was mixed with 900 μl of bisulfite solution and incubated in a water bath, generally for 24 hr. The sample was desalted by gel filtration on Sephadex G-100 column (100 × 1 cm). The reaction was completed by incubation of the modified tRNA at pH 9 and 20° for 15 hr. The degree of cytosine deamination was determined by hydrolyzing the tRNA enzymatically and assaying the individual nucleosides.

p-CHLOROMERCURIBENZOATE. The method of tRNA modification by this mercury compound was a modification of the procedure reported by Pal *et al.* (1972). ^{14}C-Labeled *p*-chloromercuribenzoate (Schwarz/Mann, Orangeburg, N. Y.) was dissolved in 1 M NaOH, and *ca.* 20 A_{260} units of tRNA in 0.2 ml of 0.2 M Tris-HCl (pH 8) containing 10 mM $MgCl_2$ was mixed with 20 μl of the labeled compound in 5 molar excess. The mixture was incubated at 50° for 4 hr. The product was freed of the excess reagent by gel filtration on a Sephadex G-10 column (100 × 1 cm) that was eluted with 0.2 M NH_4HCO_3–$(NH_4)_2CO_3$ containing 1 mM $MgCl_2$ (pH 8) at 2°. The tRNA was freeze-dried, and the extent of the reaction was determined both from the molar incorporation of ^{14}C label and from elemental analysis for mercury by an isotope-dilution method (Carter and Sites, 1971). When the reaction was performed in the absence of Mg^{2+}, tRNA was freed of Mg^{2+} by exhaustive dialysis, and $MgCl_2$ was omitted from all solutions.

CYANOGEN BROMIDE. The method was a modification of the procedure described by Saneyoshi and Nishimura (1970). Transfer RNA in 0.1 M Na_2CO_3–$NaHCO_3$ containing 10 mM $MgCl_2$ (pH 8.5) was mixed with a 50 molar excess of cyanogen bromide (Aldrich Chemical Co., Milwaukee, Wis.), which was dissolved in a minimal volume of ethanol–buffer (pH 8.5) mixture (1:10) just before use. The final reaction volume was 1.0 ml per 100 A_{260} units of tRNA. After the incubation at 25° for 10–15 min, excess reagent was removed by gel filtration as described above. The freeze-dried tRNA, dissolved in 0.1 M sodium carbonate buffer containing Mg^{2+} at pH 9.0, was left at 25° for 4–5 hr to convert the thiocyanate to a cyanate derivative (the thiocyanate is relatively stable at acid pH's (R. P Singhal, unpublished results)).

Results

Reaction of Bisulfite with tRNAs. REACTION SPECIFICITY. Transfer RNAs were treated with sodium bisulfite under standard reaction conditions, and changes in their base compositions were determined. Whereas the amount of adenosine, guanosine, ribothymidine, and pseudouridine remained constant for up to 6 days, deamination of cytidine into uridine was noticeable (about 0.7% within the first 30 min). A steady loss of cytidine was paralleled by an equivalent increase in uridine for several weeks.

MODIFICATION OF PSEUDOURIDINE. Although pseudouridine does not react under our standard reaction conditions (2 M bisulfite, 20°, 24 hr), stronger treatment, such as 3 M bisulfite at 60°, transforms this minor component of tRNA into two reaction products, which are resolved on an anion-exchange column (Figure 3). Both pseudouridine and pseudouridylates are desorbed before the modified species; the modified pseu-

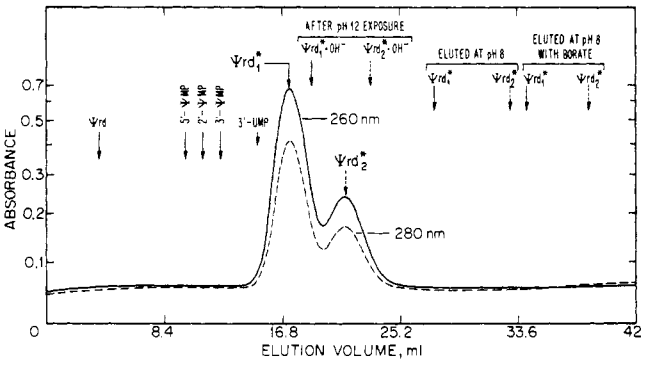

FIGURE 3: Separation of bisulfite-modified pseudouridines. One milligram of pseudouridine (C isomer; see Cohn, 1960) was reacted with 1.0 ml of bisulfite solution, pH 6, at about 65° for 24 hr and then was desalted on a Sephadex G-10 column (100 × 0.9 cm). The modified pseudouridines were eluted as one peak between the unreacted pseudouridine and sodium bisulfite peaks. Approximately 2 A_{260} units of the modified pseudouridines (ψrd*$_1$ and ψrd*$_2$) was applied to a column (25 × 0.5 cm) of Dowex 1-X8 (400 mesh) and eluted with 0.1 M Cl^- at pH 4.5, 22°, and 0.21 ml/min. The two peaks contained roughly 150 and 50 nmol of the modified isomers, respectively. Though the two peaks were completely resolved in 0.1 M HCl, the peak positions remained essentially unchanged from pH 1 to 7. The peak positions of pseudouridine (ψrd), 2′-, 3′-, and 5′-pseudouridylates (ψMPs), and 3′-uridylate (3′-UMP) under similar separation conditions are indicated by arrows. After a brief alkali treatment (pH 12, 20°, 1 hr), the two modified pseudouridylates appeared in this chromatography system as indicated by ψrd*$_x$-OH^-. When the two isomers were chromatographed in 0.1 M Cl^- with 20 mM Tris-HCl buffer at pH 8, they were strongly anionic, as indicated by their retarded peak positions. When 20 mM sodium borate was added to the pH 8 eluent, the two peaks were further retarded on this column.

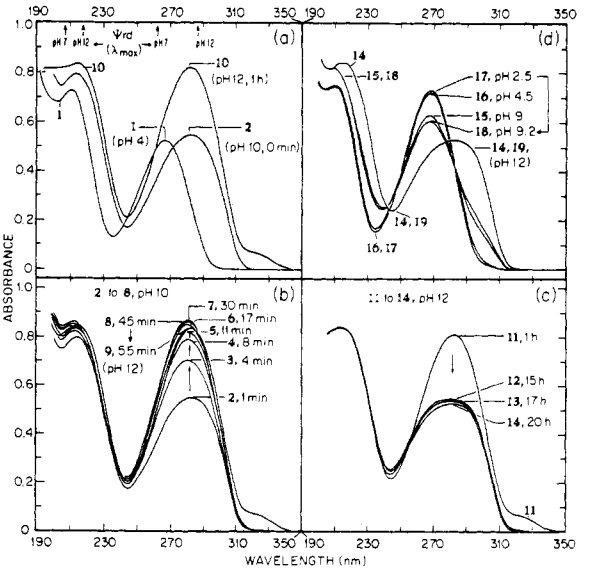

FIGURE 4: Spectral study of bisulfite-modified pseudouridine isolated as the first peak (ψrd*$_1$ in Figure 3) by anion-exchange chromatography: spectrum 1, modified pseudouridine in 3.0 ml of NH_4Cl at pH 4 (an identical initial spectrum was obtained at neutral pH); spectrum 2, 5 μl of 5 M NH_4OH added to raise the pH to 10; spectra 3–8, the pH 10 solution assayed at different times (note the increase in absorbance with time); spectrum 9, 25 μl of 2.5 M NaOH added to raise the pH to 12; spectra 10–14, the pH 12 solution assayed at various times (note the decrease in absorbance with time); spectra 15–17, 2.5 M HCl added (50, 5, and 5 μl) to lower the pH to 9, 4.5, and 2.5, respectively (note the increase in absorbance on acidification); spectrum 18, 10 μl of 2.5 M NaOH added to reduce the pH to 9.1 (note the decrease in absorbance and the similarity to spectrum 15); spectrum 19, 25 μl of 2.5 M NaOH added to restore the pH to 12. All spectra were determined with a light path of 10 mm in a Cary 19 spectrophotometer in the same cuvet at 22°. Initial and final solution volumes were 3.0 and 3.125 ml, respectively. No corrections were made for the change in absorbance due to evaporation or dilution.

douridines are stronger anions than their corresponding nucleotides. After exposure to alkaline pH, their chromatographic positions were changed (compare positions of ψrd* and ψrd*OH^-). The similarity in chromatographic and spectral properties (see below) leads to the conclusion that they are isomers.

When the spectra of either of the two isomers were examined at different pH's, starting with an acid or neutral solution, the following differences from unmodified pseudouridine were noted (Figure 4): (a) λ_{max} values at pH 4–7 were 210 and 267 nm (ψrd, 207 and 263 nm, respectively) and at pH 12 were 215 and 281 nm (ψrd, 217 and 286 nm, respectively); (b) when the solution was brought to pH 10, the absorbance increased with time and reached a maximum after 30 min (no such change was found below pH 9); (c) on the other hand, when the solution was made strongly alkaline (pH 12), the absorbance decreased slowly and reached a stable value after about 15 hr; (d) when this alkali-reacted solution was returned to an acid pH, although the λ_{max} remained unchanged, the extinction coefficient was increased by 40%. The original spectrum at pH 12 could not be achieved by making this solution alkaline again. No further change in its spectrum at pH 12 was noted.

The bisulfite-modified pseudouridines eluted later at pH 8 than at pH 4.5, indicating a second pK_a. Both peaks were further retarded in the presence of 20 mM borate (Figure 3). They consumed about 1.8 mol of periodate per mol of the modified pseudouridines (Cohn, 1960). The complexing with borate and the oxidation with periodate clearly indicate that the 2′ and 3′ positions of the ribose remained unsubstituted after the reaction with bisulfite. With [^{35}S]bisulfite, the radioactivity was associated with both modified pseudouridines in about equimolar quantities—taking 10,000 as the molar extinction coefficient for the two species at pH 6 and 260 nm.

To compare the reactivity of pseudouridine with other bases substituted at the 5′ position, thymine was treated with bisulfite under similar conditions. The results (position on an ion exchange column and spectral data) indicate that thymine does not react with bisulfite even under these conditions.

EFFECTS OF TIME AND TEMPERATURE. The modification of cytidine in mixed tRNAs was truly linear in the initial stages of the reaction, *e.g.*, up to 6 hr. However, the reaction rate reached a plateau under these conditions (Figure 5) after 3 days, when about 23% of the cytidine was deaminated. Nevertheless, the reaction could be reactivated by merely raising the

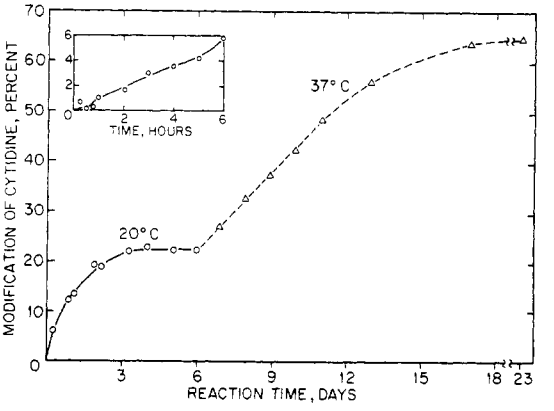

FIGURE 5: Modification of *E. coli* tRNAs with 2 M bisulfite at 20°, effects of time and temperature. The extent of cytidine modification was determined by nucleoside analysis by anion-exclusion chromatography (see Materials and Methods).

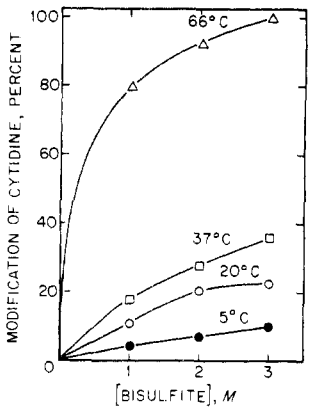

FIGURE 6: Modification of *E. coli* tRNAs with bisulfite for 24 hr, effects of temperature and concentration of bisulfite. See the legend to Figure 5 for the method of analysis.

temperature (from 20 to 37°), starting again linearly and ending as a plateau at about 65% completion. Thus, the reaction is biphasic and is strongly dependent on time and temperature.

EFFECTS OF CONCENTRATION AND TEMPERATURE. These two parameters influenced the reaction rate independently when tRNAs were reacted with 1, 2, and 3 M bisulfite solutions, each at four different temperatures and for 1 day (Figure 6). The reaction rate was enhanced only slightly by an increase in the bisulfite strength at any given temperature. The reactivity of cytidine with 2 and 3 M bisulfite was about the same at 20°. The reaction rate was significantly enhanced at 37°. Approximately 80–100% of the cytidines of the tRNAs were reactive when the reaction was performed above the first melting temperatures of the tRNAs, *e.g.*, at 66°.

MODIFICATION OF $tRNA^{Glu}_2$ WITH DIFFERENT REACTION PARAMETERS. Reaction conditions were sought to achieve modification of all cytidines at reactive positions in the tRNA. The results (Figure 7) can be summarized as follows. (a) At 20° a plateau was reached after the modification of six cytidines, *i.e.*, after 24 hr. (b) At 37° six cytidines were modified in only 6 hr, whereupon the reaction progressed linearly with time at a much slower rate. (c) At 46° the reaction was clearly biphasic; it first reached a plateau at seven or eight modified residues (in 1 day), then progressed in a strictly linear manner. (d) At 70°, although 21 cytidines were modified in the first 3 hr, another 21 hr was required for the remaining six residues to be modified.

A reaction of 5-methylaminomethyl-2-thiouridine, a minor component of $tRNA^{Glu}_2$, with bisulfite (2 M, 20°, 24 hr) did

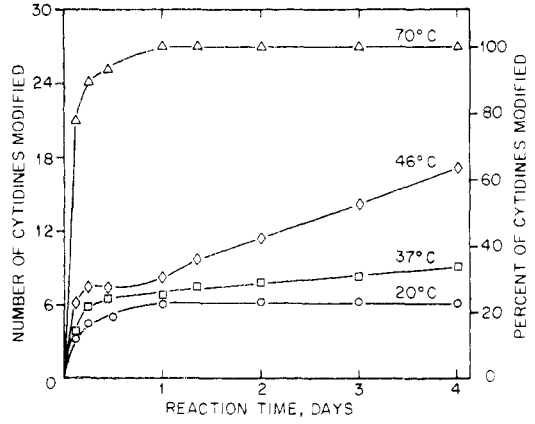

FIGURE 7: Modification of *E. coli* $tRNA^{Glu}_{1,2}$ with 2 M bisulfite at pH 6, effects of time and temperature; see the legend to Figure 5 for the method of analysis.

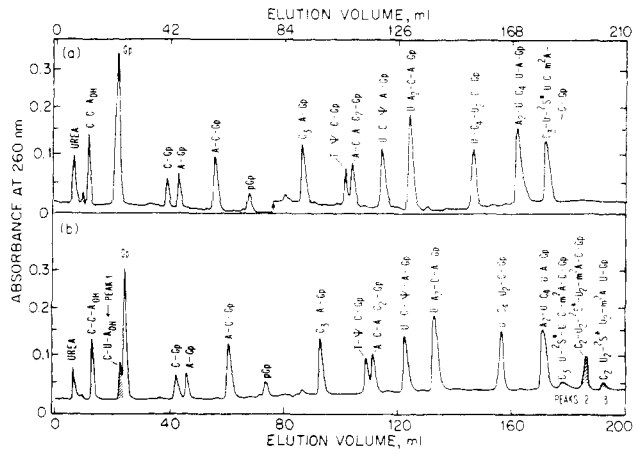

FIGURE 8: Comparison of oligonucleotides from RNase T_1 digests of (a) normal and (b) bisulfite-treated $tRNA^{Glu}_2$ (2 M bisulfite at 20° for 3 hr). Approximately 4 A_{260} units of tRNA was hydrolyzed with RNase T_1 in 25 μl, and 75 μl of urea was added to the digest (see Materials and Methods). The mixture was applied to a column (25 × 0.63 cm) of RPC-5 (Singhal, 1973). The column was eluted with a linear gradient of 0.1–0.75 M NaCl, pH 9.8 (20 mM Na_2CO_3–$NaHCO_3$ buffer), 100 ml each, at 0.5 ml/min and 50° (100 psi resultant pressure). The oligonucleotides in peaks 1, 2, and 3 of (b) are characteristic of the modified $tRNA^{Glu}_2$.

not occur, as judged from the unaltered position (and 260/280 nm ratio) of the peak in anion-exclusion chromatography. The authentic 5-methylaminomethyluridine, the expected product, was eluted at a different position.

IDENTIFICATION OF BISULFITE-REACTIVE SITES IN $tRNA^{Glu}_2$. Since the modifidation of what appear to be the six most reactive cytidines was completed in 1 day and no further modifications took place at 20°, this temperature was chosen for an examination of reaction rates. The modification of $tRNA^{Glu}_2$ apparently takes place in a linear fashion between 3 and 24 hr at 20°, reaching an end point after 24 hr; no more cytidines in $tRNA^{Glu}_{1,2}$ were modified up to 4 days (see Figure 7).

To localize the modified cytidines, aliquots were hydrolyzed separately by pancreatic RNase and by RNase T_1. The oligonucleotides were resolved by reversed-phase chromatography as described elsewhere (Singhal, 1973). The results of a 3-hr modification are described here. The two chromatograms, from control and modified $tRNA^{Glu}_2$ (Figure 8), are similar except for the presence of three extra peaks (marked as 1, 2, and 3 in Figure 8b) in that of the modified tRNA hydrolysate (peaks are characterized on the basis of base composition, and from earlier work (Singhal, 1971). Peak 1 gave equimolar quantities of cytidine, 5′-uridylate, and 5′-adenylate after phosphodiesterase digestion, and of 3′-cytidylate, 3′-uridylate, and adenosine after alkali or pancreatic RNase digestion. Hence, this peak is characterized as C-U-A_{OH}, a modification product of the C-C-A_{OH} at the 3′ end of the tRNA. The chromatographic analysis of nucleosides derived from peak 3 is shown in Figure 9. Peaks 2 and 3 are decanucleotides. Besides mononucleotides, m^2A-Cp and m^2A-Up were obtained from peaks 2 and 3, respectively, after pancreatic RNase digestion. Hence, these two peaks in Figure 8b are derived from the anticodon loop; further, cytidines 33 and 37 are modified faster than cytidine 39 (see Discussion).

CHARACTERISTICS OF BISULFITE-MODIFIED tRNAS. The reaction rate of each reactive cytidine and the loss in glutamate acceptance of this $tRNA^{Glu}_2$ at various times are shown in Figure 10. Whereas cytidines 33 and 37 were modified almost completely, and cytidines 39 and 75 were modified

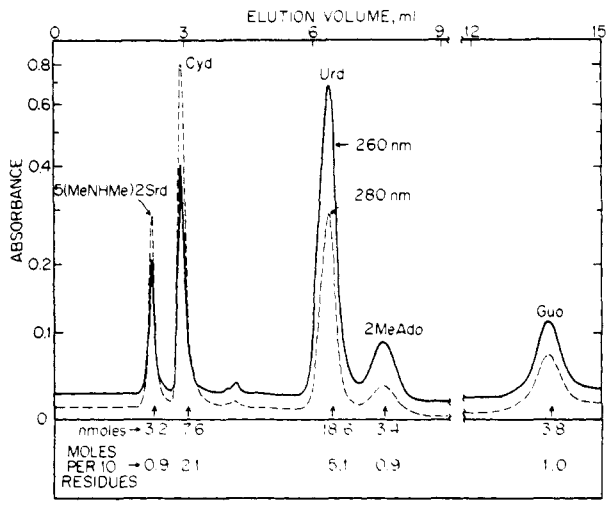

FIGURE 9: Analysis by cation-exclusion chromatography of nucleosides of the decanucleotide (peak 3 in Figure 8b) derived from bisulfite-modified $tRNA^{Glu}_2$. An enzymatic hydrolysate of the decanucleotide (0.36 A_{260} unit) was applied to a column (18 × 0.63 cm) of Aminex A-28 (7–11-μm beads). The column was eluted with 1 mM NH_4OAc (pH 3.8) at 50° and 0.2 ml/min (150 psi resultant pressure). The quantities indicated for each component at the bottom of the figure (in nmol) were calculated from their millimolar extinction coefficients at pH 3.8 (see Figure 3 in Singhal and Cohn, 1973).

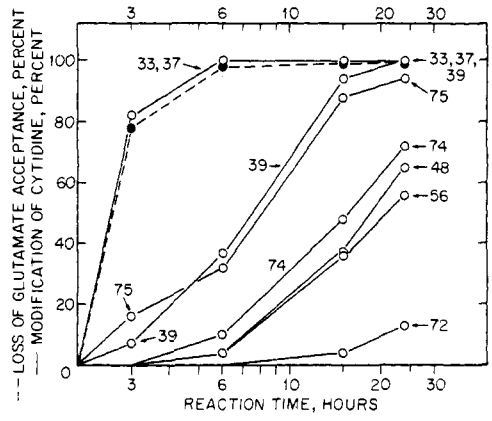

FIGURE 10: Modification of cytidines at each reactive site of *E. coli* $tRNA^{Glu}_2$ with bisulfite. Left ordinate, per cent modification of cytidines of the tRNA with 2 M bisulfite (pH 6) at 20°. Numbers indicate location of cytidines in the $tRNA^{Glu}_2$ structure (see Figure 12). The cytidine at position 48 is located in the "small extra loop," where it is supposed to pair with the guanosine at position 15 ("dihydrouridine loop") in the Levitt model of tRNA (Levitt, 1969).

up to 35% in brief reactions (3 and 6 hr), very little or no deamination of other 24-hr reactive cytidines was noticeable. However, after 15 and 24 hr, cytidines 39 and 75 were modified completely and cytidines 74, 48, and 56 were modified substantially. Nevertheless, cytidine-72, which is base paired in the cloverleaf structure, was only modified 12% after 24 hr. Though partially modified cytidines (24-hr reaction) may be expected to continue to react until completion, the results, obtained with $tRNA^{Glu}_{1,2}$ (80% pure) (Figure 7), indicate that this is not the case at 20°.

The properties of the native and bisulfite-modified $tRNA^{Glu}_2$ species are listed in Table I. The results can be summarized as follows. (a) The extent of modification of cytidines 33 and 37 parallels the loss in ability to become aminoacylated. (b) The change in the first melting temperature (54°) begins at 6 hr, and the t_m is lowered by 8° after the 24-hr modification. (c) The second melting temperature, which is around 70°, is changed by only 2° after a 15–24-hr modification. (d) The

TABLE I: Properties of $tRNA^{Glu}_2$.

	Reaction Time (hr)				
	0	3	6	15	24
Modification of cytidines of anticodon loop (%)					
33		82	100	100	100
37	None	82	100	100	100
39		7	37	94	100
Melting temp (°)	54, 72	54, 70	50, 71	48, 68	46, 68
% Thermal hyperchromicity (90° *vs.* 10°)	25	24	22	18	15
Exclusion volume (ml)	42	42	40	38	38
Glutamate acceptance (pmol/A_{260} unit)	1490	330	30	14	9
Loss in glutamate acceptance (%)	0	78	98	99	99

tRNA shows less and less thermal hyperchromicity (structure) as more and more cytidines are modified (*cf.* 24 *vs.* 15% hyperchromicity at 3 and 24 hr, respectively); however, only 1% loss in the hyperchromicity is produced in the first 3 hr of modification. (e) The exclusion volume of tRNA on a gel column begins to increase after a 6-hr modification.

Modification of $tRNA^{Glu}_2$ with Cyanogen Bromide. The common minor component, 4-thiouridine, is absent in this tRNA. A 2-thiouridine derivative is located in the "wobble" position of the anticodon. Cyanogen bromide was selected to transform this compound into its uridine derivative. As judged by both anion- and cation-exclusion chromatography, free 2-thiouridine is transformed completely into uridine under our reaction conditions.

Figure 11 shows the nucleosides derived from $tRNA^{Glu}_2$. The peak marked as 5(MeNHMe)2Srd, which corresponds to 5-methylaminomethyl-2-thiouridine, disappears completely after the cyanogen bromide treatment (Figure 11b). No new peak appears after the modification, but guanosine is increased by 7%, and the 260/280 nm ratio of the guanosine peak is changed from 0.66 to 0.50. In a separate experiment, it was found that 5-methylaminomethyluridine and guanosine elute as one peak. Hence, the 2-thiouridine derivative, after desulfuration, appears with the guanosine peak in the chromatogram. A high value of this modified compound (1.6 residues) in the guanosine peak is perhaps due to its extinction coefficient, which is higher than that used here for guanosine.

Properties of glutamate tRNAs are compared in Table II. The presence or absence of Mg^{2+} in the modification medium does not appear to affect the nature of the product nor the rate of the cyanogen bromide reaction. Thus, 20% of the 2-thiouridine derivatives remain unreacted, and the aminoacylation of $tRNA^{Glu}_2$ is reduced to 23% after cyanogen bromide treatment in the experiments cited in Table II.

TABLE II: Modification of $tRNA^{Glu}_2$ with Cyanogen Bromide.

Treatment	Loss of 5(MeNHMe)2Srd (mol/tRNA)	Glutamate Acceptance	
		pmol/A_{260}	% Loss
None	None	1490	0
CNBr	0.80	350	77
CNBr + Mg^{2+}	0.80–0.85	365–400	73–76

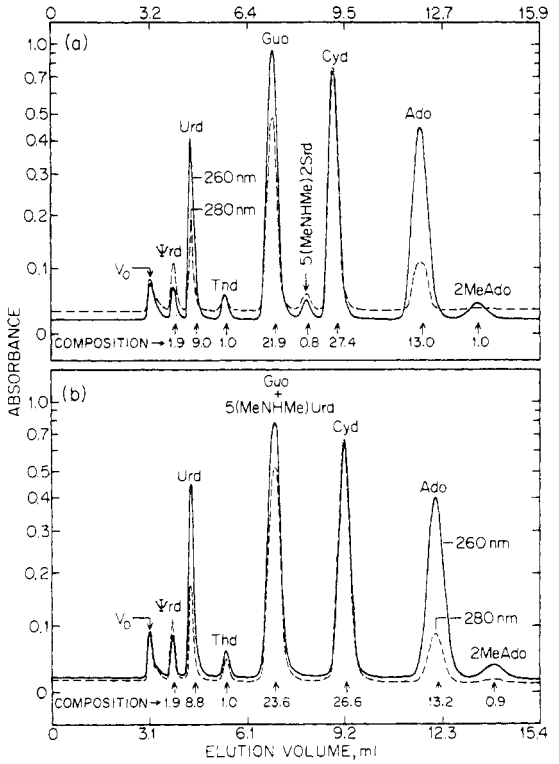

FIGURE 11: Comparison of nucleosides derived from (a) untreated and (b) CNBr-modified $tRNA^{Glu}_2$ by anion-exclusion chromatography. Approximately 0.8 A_{260} unit of the tRNA was hydrolyzed enzymatically to nucleosides in about 13 μl (see Materials and Methods). The digest was applied to a column (28.5 × 0.63 cm) of Aminex A-6 (18-μm beads). The column was eluted with 20 mM $(NH_4)_2CO_3$ ($+NH_4OH$) (pH 9.8) at 50° and 0.21 ml/min (160 psi resultant pressure). The quantities of the components were calculated from their revised millimolar extinction coefficients at pH 9.8 and 260 nm (*cf.* Singhal, 1972); Ado, 14.9; 2MeAdo, 14.7; Cyd, 7.55; Guo, 11.43; 5(MeNHMe)2Srd, 10.9; Urd, 7.92, ψrd, 4.04; Thd, 7.79.

Modification of tRNAs with p-Chloromercuribenzoate. Two tRNAs were selected as ideal candidates for this reaction, $tRNA^{fMet}_{1a,1b}$ and $tRNA^{Glu}_2$, which contain 4-thiouridine and 5-methylaminomethyl-2-thiouridine, respectively. The tRNA modification was carried out both in the presence and in the absence of Mg^{2+}. However, the aminoacylation activity was followed in a reaction mixture that contained Mg^{2+}. The extent of tRNA modification was determined by two independent methods: by analysis of mercury by isotope dilution (Carter and Sites 1971) and by measurement of incorporation of ^{14}C-labeled *p*-chloromercuribenzoate of known specific activity.

TABLE III: Modification of tRNAs with ^{14}C-Labeled *p*-Chloromercuribenzoate.

tRNA	Mg^{2+}	^{14}C Incorporation: pmol Hg/A_{260} unit	^{14}C Incorporation: %	Acceptance: pmol/A_{260} unit	Acceptance: % Loss
$tRNA^{Glu}_2$	——Untreated——			1480	0
	−	835	56	720	51
	+	710	50	635	57
$tRNA^{fMet}_{1A,1B}$	——Untreated——			1720	0
	−	517	30	1728	0
	+	92	5	1737	0

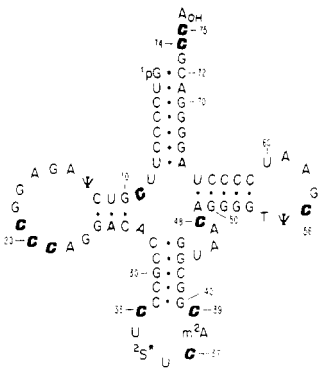

FIGURE 12: Structure of $tRNA^{Glu}_2$ of *E. coli* (Ohashi *et al.*, 1972).

The values obtained by the two methods varied from 5 to 8%. The results (Table III) indicate about 50% modification of $tRNA^{Glu}_2$ and are in fair agreement with the loss in aminoacylation activity. Magnesium ion in the reaction mixture does not appear to influence the rate of modification of $tRNA^{Glu}_2$; however, $tRNA^{fMet}$ modification is enhanced by 25% when Mg^{2+} is omitted from the reaction medium. It appears that 4-thiouridine ($tRNA^{fMet}$) is less reactive than the 2-thiouridine derivative ($tRNA^{Glu}_2$), and the mercury complex in the former case becomes unstable on the addition of Mg^{2+}. Hence, the treated $tRNA^{fMet}$ retains full aminoacylation activity.

Aminoacylation of Modified tRNAs. About 50 μg of partially purified tRNA synthetase was used in the standard aminoacylation reaction, which contained 0.04–0.05 A_{260} unit of tRNA. Considering the possibility of a lower affinity of the synthetase (*i.e.*, high K_m) for the chemically modified tRNAs, the aminoacylation was also tested with increased concentrations (50–350 μg) of synthetase at 37° for 60 min. The aminoacylation changed only within the experimental error, and the values reported in Tables I–III did not change significantly with higher quantities of the enzyme.

Discussion

Modification of $tRNA^{Glu}_2$. In an earlier report (Singhal, 1971) it was shown that only six of the 27 cytidine residues in $tRNA^{Glu}_2$ of *E. coli* can be modified in 24 hr by bisulfite under mild reaction conditions, although essentially all cytidine residues are so changed in a progressive fashion as the reaction temperature is increased (Figures 6 and 7). Hence, all cytidines are potentially reactive and need only the "exposure" that is caused by molecular unfolding (melting) for the modification. For this reason, the residues that do not react in a given set of conditions, or that react slowly, must be considered to be "buried" (sterically hindered) in the three-dimensional structure that exists under those conditions. Modification of two residues (3-hr reaction) gives no apparent change in conformation but a large loss in aminoacylation ability (Table I).

From the kinetic evidence presented in this paper, it appears that the six or seven rapidly modified cytidines are modified at distinctly different rates. The first to react are those at positions 33 and 37 (37 being at the 3′ end of the anticodon loop (Figure 12)); which is faster could not be determined. Residue 33 is probably reactive more than C-37: as shown by Cashmore *et al.* (1971), C-33, a base at the 5′ end of the anticodon of tyrosine tRNA of *E. coli su*$^+$, is relatively resistant to a methoxyamine modification. Residues 39, farther up the anticodon loop, and 75 react considerably more slowly and, from the sigmoid shape of their curves, are influenced in reactivity by the more rapid modifications of 33 and/or 37, as if a conformational change were taking place to "expose" them. Nevertheless, the drop in aminoacylation ability precedes the changes in

conformation (Table I) and correlates with the changes in residues 33 and 37 (anticodon area) and not with the later changes in residues 39, 75, 74, 46, 56, and 72. Hence it seems that aminoacylation can be blocked by the loss of NH_2 from a cytidine (or two) occurring exclusively in the anticodon loop, perhaps from the one at the 3′ end of the anticodon itself. (Chang (1973) and Chang and Smith (1973) find that the uridine at 34 is appreciably less reactive with a soluble carbodiimide than that at 37 (3′-terminal residue of the anticodon) in *E. coli* $tRNA^{fMet}$.)

Before consideration of the possible mechanisms involved in the inactivation of $tRNA^{Glu}_2$, it should be noted that the actions of cyanogen bromide and *p*-chloromercuribenzoate, which also modify at the 5′ side of the anticodon, also block aminoacylation. Cyanogen bromide was used here to convert the 2-thiouridine moiety at position 35 to a uridine residue (using conditions for releasing the thiocyanate residue that do not change the aminoacylating ability of control samples of $tRNA^{Glu}_2$); aminoacylating ability was lost, although the only change was of sulfur to oxygen in the residue at position 35. Similarly, *p*-chloromercuribenzoate, which forms a stable complex with 2-thiouridine (in contrast to that with 4-thiouridine, which dissociates upon addition of Mg^{2+}) and thus also affects only the residue in position 35, blocks aminoacylation. In this case, we have the addition of a bulky side chain rather than a substitution of oxygen for NH_2 (deamination) or for sulfur (desulfuration) in the other cases.

The fact that all three types of modification of the anticodon area lead to loss of aminoacylation ability without observable change in conformation when only this area has been modified leads to the postulate that the loss of aminoacylation consequent to such modification is not a consequence of conformational changes, as no such changes are apparent in the tRNA modified only to this degree. Further, the sigmoidal shape of the reactivities of residues 39 and 75 (Figure 10) seems to indicate that further modification, leading to the conformational changes observed, involves most if not all of the molecule and results in progressively enhanced reactivity (unfolding leads to accessibility). However, this has little to do with aminoacylation, which is already lost as a result of changes in the anticodon. Hence, the enzyme (ligase) must combine with (recognize) both the anticodon area and the acceptor stem, whose integrity is also necessary for proper aminoacylation (Schulman and Goddard, 1973; Chambers *et al.*, 1973). This is not implausible; the distance is *ca.* 80 Å (Kim *et al.*, 1973), not great for an enzyme of molecular weight 102,000 (Lapointe and Söll, 1972).

It is difficult to determine whether cytidines 19, 20, and 21 (in the "dihydrouridine loop") are totally unreactive or simply inaccessible to bisulfite even after the tRNA has acquired a new conformation (15–24-hr reaction). The lack of cytidine modification in this area of the $tRNA^{Glu}_2$ is due either to the unique nature of this tRNA, which lacks the usual dihydrouridine and 4-thiouridine residues, to the reaction conditions (2 M bisulfite, 20°, no Mg^{2+}), to an unusual conformation (Willick and Kay, 1971), or to a combination of these factors.

It should be noted that the modification with bisulfite are performed in a high salt solution. This reaction medium may not represent the native environment, hence the native conformation of $tRNA^{Glu}$. Since both modification and conformation studies were performed in solutions containing 0.15 M or more sodium ion, the results should relate to an ionically "frozen" state of the macromolecule—an environment where the presence or absence of anions like magnesium is presumed not to influence the tRNA conformation.

Specificity of the Bisulfite Reaction and the Reaction with Pseudouridine. Bisulfite reacts specifically with cytidine at pH 6 and with uridine at pH 7 under mild conditions (20°). The uridine–bisulfite adduct formed from either substance readily loses the sulfite radical in a slightly alkaline solution (pH 9). However, Furuichi *et al.* (1970) observed that the isopentenyladenosine residue in the $tRNA^{Tyr}$ of yeast reacts irreversibly with bisulfite at pH 7 and 37°. Also, 4-thiouridine reacts with bisulfite in the presence of oxygen at pH 7 (Hayatsu and Inoue, 1971). Neither of these two nucleosides is present in the $tRNA^{Glu}_2$ used in this work.

The present author has now found that pseudouridine is irreversibly modified under more stringent conditions (3 M bisulfite, pH 6, 60°), yielding two anionic isomers. The results indicate that bisulfite does not add to the 2′ or 3′ position of ribose; rather, it appears that it adds, in this case as in the others, to the pyrimidine moiety. The two isomers are more anionic than pseudouridylate and 3′-uridylate, and thus appear to retain the bisulfite residue (confirmed with $^{35}SO_3^{2-}$). The ultraviolet spectra (pH 4–7) are appreciably different from that of pseudouridine. The isomeric adducts appear to react with alkali irreversibly, in that the spectra at pH 9 cannot be regained by neutralization after exposure to pH 12. Budowsky *et al.* (1972) obtained two isomers of 5,6-dihydrocytidine-N^4-methoxy-6-sulfonate as a result of the reaction of 5′-cytidylate with bisulfite in the presence of *O*-methylhydroxylamine (methoxyamine), and they identified these as diastereoisomers at the position of sulfonation. With the exception that the irreversibility of the product formed at highly alkaline pH was not shown, their results are parallel to those presented here and lead to the postulate that the pseudouridine sulfonates described here are similar diastereoisomers. The nature of the alkali-produced derivative is not known.

Although the exact nature of the two isomers may be somewhat uncertain at present, the presence of a sulfite radical and the intactness of the cis-diol structure of the ribose moiety are certain. Interestingly, thymine, which is also a 5-substituted uracil, does not react with bisulfite (pH 6, 60°). The reactivity of pseudouridine under these conditions is perhaps due to the difference in the nature of the 5-substituted group.

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3-(3-Amino-3-carboxy-*n*-propyl)uridine. The Structure of the Nucleoside in *Escherichia coli* Transfer Ribonucleic Acid That Reacts with Phenoxyacetoxysuccinimide†

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ABSTRACT: The nucleoside that reacts with the *N*-hydroxysuccinimide ester of phenoxyacetic acid in *Escherichia coli* tRNA has been isolated and its structure has been determined. The phenoxyacetylated trinucleotide containing this nucleoside has been purified from combined pancreatic and T_1 ribonuclease digests of crude *E. coli* tRNA. It was found to occur in the sequence m^7GXC and is probably identical with the X nucleoside found in *E. coli* $tRNA^{Arg}$, $tRNA^{Ile}$, $tRNA^{Phe}$, $tRNA^{Met}_m$, and $tRNA^{Val}_{2A\&B}$. The structure has been determined by its nuclear magnetic resonance spectrum and high-resolution mass spectrum to be 3-(3-amino-3-carboxy-*n*-propyl)uridine.

We have previously reported that several species of *Escherichia coli* B and rat liver tRNA react with the *N*-hydroxysuccinimide ester of phenoxyacetic acid (HSP)[1] to form a phenoxyacetyl-tRNA adduct. The phenoxyacetylated species of tRNA can be identified by their altered chromatographic properties on BD-cellulose. The reactive rat liver tRNA species are $tRNA^{Tyr}$, $tRNA^{Thr}$, $tRNA^{Ile}$, $tRNA^{Asn}$, and $tRNA^{Cys}$ (Friedman, 1972). The reactive *E. coli* tRNAs are $tRNA^{Arg}$,

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[1] Abbreviations used are: HSP, phenoxyacetoxysuccinimide; BD-cellulose, benzoylated-DEAE; A_{260} unit, that quantity of material which, when dissolved in 1 ml of water, has an absorbance of 1.0 at 260 nm in a 1-cm light path; X = an unknown nucleoside.