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Inhibition of Transfer RNA Function by Replacement of Uridine and Uridine-Derived Nucleosides with 5-Fluorouridine†

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ABSTRACT: As part of a study of the functional significance of minor nucleotides, the protein synthetic activity of *Escherichia coli* tRNA, with most of its uridine and uridine-derived residues replaced by 5-fluorouridine, was examined. Although the modified tRNA could be charged with all amino acids, the rate of aminoacylation with lysine was only 3–7% of normal. Similar, though less marked, inhibitory effects were noted with aspartate, glutamate, glutamine, and histidine; 13 other amino acids were accepted at normal rates. Incorporation of 5-fluorouridine decreased the affinity of lysine tRNA for its synthetase, as indicated by a fivefold increase in the apparent K_M. The aminoacylation of analogue-containing tRNA with lysine was incomplete and increased with increasing concentrations of synthetase; at lower enzyme levels the extent of charging was proportional to enzyme concentration. Irreversible inactivation of synthetase or degradation of tRNA was ruled out. The rates of enzymatic and nonenzymatic deacylation of lysyl-tRNA were similar for normal and fluorouridine-substituted tRNA, indicating that incomplete charging of the analogue-containing tRNA with lysine is due to the lower rate of aminoacylation. Three peaks of lysine acceptor activity, isolated from 5-fluorouridine-containing tRNA by benzoylated diethylaminoethyl-cellulose chromatography, each exhibited the low rate and extent of lysine acceptance observed with unfractionated tRNA. Analogue-substituted lysine tRNA is able to stimulate only low levels of polypeptide synthesis in a tRNA-dependent cell-free system directed by synthetic polynucleotides or phage f2 RNA. Ribosome-binding studies showed that fluorouridine-containing lysyl- and glutamyl-tRNA were bound less efficiently than normal aminoacyl-tRNA in response to poly(A), poly(A,C), or poly(A,G). It is suggested that the reduction in the initial rate of charging and of ribosome binding observed with several tRNAs is due to fluorouridine-induced changes in the anticodon region of these molecules.

E scherichia coli transfer RNA which has 90–95% of its uridine and uridine-derived nucleosides replaced by 5-fluorouridine (Horowitz and Chargaff, 1958; Horowitz and Huntington, 1967; Lowrie and Bergquist, 1958; Johnson et al., 1969; Geige et al., 1969a; Kaiser et al., 1969; Kaiser, 1969a, 1972; Horowitz et al., 1974) has been used to probe the relationship between tRNA structure and function and to study the biological role of modified nucleosides (Horowitz et al., 1974, 1977; Ofengand et al., 1974). Previous results from this and other laboratories have indicated that highly substituted, unfractionated tRNA is able to accept all amino acids (Horowitz and Huntington, 1967; Lowrie and Bergquist, 1968; Johnson et al., 1969; Kaiser, 1969b; Geige et al., 1969b), and more detailed investigations with purified FUrd[1]-containing *E. coli* $tRNA^{Val}_1$ have shown that the rate of aminoacylation of this tRNA is unimpaired (Horowitz et al., 1974). FUrd-substituted $tRNA^{Val}_1$ is also able to function as well as control tRNA in all the subsequent steps of protein synthesis. Ternary complex formation with EFTu-GTP, EFTu-dependent binding to the ribosomal A site, nonenzymatic binding to the

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[1] Abbreviations used are: BD-cellulose, benzoylated diethylaminoethylcellulose; FUrd, 5-fluorouridine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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ribosomal P site, and polypeptide formation all occur normally (Ofengand et al., 1974). Furthermore, FUrd-containing tRNA ([FUrd]tRNA) is fully functional in the tRNA-dependent synthesis of ppGpp by stringent factor-ribosome-mRNA complexes (Chinali et al., 1978).

Such studies with FUrd-substituted tRNA have helped to demonstrate that a number of the minor pyrimidine nucleotides are not essential for tRNA function but may play a modulating role, increasing the efficiency or specificity of reactions carried out by tRNA (Ofengand et al., 1974). The usefulness of analogue-substituted tRNAs lies in their ability to function normally despite the massive incorporation of 5-fluorouridine. Most of the FUrd-containing tRNAs examined do not seem to be affected by these base substitutions. In this paper, however, we show that there are anomalies in the aminoacylation of five [FUrd]tRNAs, the function of lysine-specific tRNA being inhibited the most. FUrd-substituted lysyl tRNA also has an impaired ability to bind to ribosomes and to function in polypeptide synthesis. Preliminary reports of some of these findings have appeared (Horowitz and Ramberg, 1972; Horowitz et al., 1973).

Experimental Procedure

Materials

Escherichia coli transfer RNA, radioactive amino acids, and BD-cellulose were purchased from Schwarz/Mann Bioresearch. Snake venom phosphodiesterase (code VPH) and *E. coli* alkaline phosphatase (code BAPF) were from Worthington Biochemical Corp. Ribonuclease A (type 1-A) was from Sigma Chemical Co. Tritium-labeled potassium borohydride was Amersham Searle No. TRK293. Polynucleotides were obtained from Miles Laboratories; copolymeric ribonucleotides contained the individual nucleotides in equal proportion.

Methods

Bacterial Growth and Isolation of tRNA. Growth of *E. coli* B with 5-fluorouracil and the isolation of tRNA were fully described earlier (Horowitz et al., 1974). Transfer RNA highly substituted with 5-fluorouridine was obtained by chromatography on DEAE-cellulose at pH 8.9 (Kaiser, 1969b). The nucleotide composition of tRNA was determined by one-dimensional paper chromatography (Johnson et al., 1969) or by the chemical tritium-labeling procedure of Randerath et al. (1972). All [FUrd]tRNA preparations used had more than 90% of their uridine residues replaced by 5-fluorouridine. Chromatography of tRNA on BD-cellulose was carried out as previously described (Horowitz et al., 1974).

Aminoacylation of tRNA. Aminoacyl-tRNA synthetases were prepared from *E. coli* B by the method of Muench and Berg (1966), which removes endogenous tRNA. Approximate protein concentrations were determined from the relative absorbance at 280 and 260 nm and the enzyme preparations stored in small aliquots at −60 °C.

Aminoacylation of tRNA was carried out in a reaction mixture containing 100 mM Tris-HCl (pH 7.4), 20 mM KCl, 1 mM dithiothreitol, 10 mM ATP, 0.1 mM CTP, 20 mM magnesium acetate, 0.02–0.05 mg of tRNA, 0.025–0.05 mM ^{14}C-labeled amino acid, 0.04 mg of bovine serum albumin, and 0.002–0.12 mg of enzyme protein, in a final volume of 0.1 mL. Variations in buffer and other components of the mixture are detailed in the text. Reactions were run at 25 °C, unless otherwise noted. Samples were preincubated for 3 min, and the reaction was initiated by the addition of enzyme. The reaction was terminated by the addition of ice-cold 5% trichloroacetic acid, which contained 2.4–5 mM unlabeled amino acid. The samples were collected on Millipore filters, washed three times with 5% trichloroacetic acid, dried, and counted in a Packard liquid scintillation spectrometer in a toluene/PPO/POPOP mixture. Optimum Mg^{2+} concentrations were determined for each amino acid in separate experiments. Initial rates of aminoacylation were calculated from duplicate samples taken 30, 60, and 90 s after the addition of enzyme. Under the reaction conditions described, the initial velocity of the aminoacylation reaction was proportional to tRNA concentration. The amount of a given amino acid specific tRNA present was determined from the plateau level of aminoacylation at high enzyme concentration, which gave maximum charging of the tRNA.

Aminoacyl-tRNA, for ribosome binding and deacylation studies, was prepared by scaling up a standard aminoacylation reaction mixture. After incubation at 37 °C for 30 min, the reaction was stopped by phenol extraction. The aminoacyl-tRNA was recovered by ethanol precipitation, desalted on Sephadex G-25 equilibrated with 0.02 M sodium acetate (pH 5.2) containing 0.5 M NaCl, and stored at −20 °C in 0.02 M sodium acetate buffer (pH 5.2).

Nonenzymatic Binding of Aminoacyl-tRNA to Ribosomes. Aminoacyl-tRNA binding to ribosomes was determined at 10 mM Mg^{2+} by the procedure of Leder (1968). Ribosomes were prepared from preincubated S-30 extracts derived from *E. coli* Q 13, as described by Nirenberg and Matthaei (1961).

Polypeptide Synthesis. Polypeptide synthesis from free amino acids was measured in a reaction mixture containing, per 0.1 mL: 50 mM Tris-HCl (pH 7.4), 7.5 mM NH_4Cl, 15 mM magnesium acetate, 3 mM ATP, 1 mM dithiothreitol, 3 μg of pyruvate kinase, 5 mM phosphoenolpyruvate, 0.225 mM GTP, 0.2 mM CTP, 50–150 μg of normal or FUrd-containing tRNA, 150–250 μg of preincubated S-30 (tRNA depleted), 125 μg of S-100 (tRNA-depleted), 0.05 mM of each amino acid except the radioactive amino acid to be incorporated, 0.05 mM ^{14}C-labeled amino acid, and 25 μg of poly(U) or 5–8 μg of poly(A). Reaction was initiated by the addition of the S-30 and S-100 preparations, and incubation was at 37 °C for the times indicated. The reaction was stopped by adding cold 5% trichloroacetic acid [a trichloracetic acid–tungstic acid mixture was used to precipitate polylysine (Gardner et al., 1962)] and heating at 90 °C for 15 min. After cooling to room temperature, the samples were collected on Millipore filters, washed three times with trichloroacetic acid, dried, and counted in a Packard liquid scintillation spectrometer in a toluene/PPO/POPOP mixture.

Amino acid incorporation into polypeptides was dependent on the addition of exogenous tRNA and messenger RNA. The S-30 fraction was preincubated as detailed by Hung et al. (1966) for depletion of endogenous messenger. Endogenous tRNA was removed from the preincubated S-30 fraction by chromatography on Sephadex G-100 (Hung et al., 1966). In our hands, this preparation was inactive unless supplemented with tRNA-depleted S-100 (Muench and Berg, 1966).

Polypeptide synthesis from aminoacyl-tRNA was determined in a reaction mixture containing, per 0.1 mL: 40 mM Tris-HCl (pH 7.4); 160 mM NH_4Cl; 10–15 mM magnesium acetate; 1 mM GTP; 12 mM dithiothreitol; 4 μg of poly(U), 5.5 μg of poly(A), or 9–10 μg of poly(G,U); 60–300 μg of preincubated ribosomes (Nirenberg and Matthaei, 1961); 100 μg of tRNA-depleted S-100, and ^{14}C-labeled aminoacyl-tRNA as indicated in the legends to the figures. Reaction mixtures were preincubated for 5 min at 30 °C, and the reaction was initiated by the addition of tRNA-depleted S-100. The reaction was stopped and prepared for counting as described for the incorporation from free amino acids.

Polypeptide synthesis with f2 RNA as messenger was measured as described by Webster et al. (1967) with the modifications of Model et al. (1969). One microgram of leucovorin (Lederle Labs) was added to each 0.1 mL of reaction mixture to stimulate protein formation. Bacteriophage f2 was the gift of Dr. R. E. Webster, Duke University. Phage f2 RNA was prepred by the procedure of Webster et al. (1967).

Results

Kinetics of Aminoacylation of Normal and 5-Fluorouridine-Substituted tRNA. Previous studies from this laboratory, as well as from others, have shown that the *extent* of amino acid acceptance by FUrd-containing tRNA is comparable to that of control tRNA despite extensive replacement of uridine and uridine-derived minor bases by the pyrimidine analogue (Horowitz and Huntington, 1967; Lowrie and Bergquist, 1968; Johnson et al., 1969; Kaiser, 1969b). These experiments were carried out at relatively high enzyme concentrations to ensure rapid and complete aminoacylation. A more detailed investigation of the charging reaction, at enzyme levels which permit kinetic studies, has revealed some anomalies in the acylation of FUrd-substituted tRNA with several amino acids.

Initial velocities of charging normal and [FUrd]tRNA with 18 amino acids are compared in Table I. The two tRNA preparations accepted 13 amino acids at comparable rates. Significant reductions were observed, however, in the rates of aspartate, glutamate, glutamine, histidine, and lysine acceptance by [FUrd]tRNA compared to control tRNA (italicized in Table I). The largest effect was noted with lysine; [FUrd]-tRNA was acylated with lysine at a rate only 3–7% that of normal.

TABLE I: Rate of Aminoacyl-tRNA Formation with Normal and 5-Fluorouridine-Substituted Transfer RNA.[a]

amino acid	initial velocity (pmol of amino acid (pmol of tRNA)$^{-1}$ min^{-1})		[FUrd]tRNA / normal tRNA
	normal tRNA	[FUrd]tRNA	
Ala	0.15	0.11	0.73
Arg	0.33	0.33	1.00
Asn	0.46	0.35	0.76
Asp	*0.41*	*0.19*	*0.46*
Glu	*0.036*	*0.019*	*0.53*
Gln	*0.17*	*0.050*	*0.29*
Gly	0.13	0.13	1.00
His	*0.51*	*0.19*	*0.37*
Ile	0.040	0.045	1.13
Leu	0.18	0.18	1.00
Lys	*1.01*	*0.056*	*0.06*
Met	0.16	0.16	1.00
Phe	0.10	0.10	1.00
Pro	0.21	0.16	0.76
Ser	0.73	0.50	0.68
Thr	0.22	0.17	0.77
Tyr	0.52	0.41	0.79
Val	0.18	0.17	0.94

[a] Aminoacylation reactions were carried out as described under Experimental Procedure, at a Mg^{2+} concentration optimal for each amino acid. Enzyme levels varied between 2.4 and 11 μg/0.1 mL of reaction mixture; identical enzyme concentrations were used when comparing normal and [FUrd]tRNA preparations with a given amino acid. The concentration of each amino acid specific tRNA was determined from the plateau level of aminoacylation at high enzyme concentration.

This reduced rate of aminoacylation cannot be ascribed to damage of the tRNA as the result of exposure to the high pH used in preparing highly substituted [FUrd]tRNA. Samples of [FUrd]tRNA that had not been subjected to the final purification on DEAE-cellulose at pH 8.9 also accepted lysine at a reduced rate. Furthermore, tRNA in the first peak eluted from the DEAE-cellulose column by the pH 8.9 salt gradient, which contains little or no 5-fluorouridine, exhibits normal acylation kinetics.

Aminoacylation reactions were routinely carried out in the presence of 0.1 mM CTP to ensure the integrity of the 3′ terminus of the tRNA, thus eliminating this as a possible cause of the reduced rate of aminoacylation with lysine or other amino acids. The reactivity of several, but not all, [FUrd]-tRNA preparations was stimulated by the addition of CTP.

It is unlikely that misacylation of a tRNA other than $tRNA^{Lys}$ is responsible for the low rate of charging [FUrd]-tRNA with lysine, because the addition of a 50-fold molar excess of 19 unlabeled amino acids caused no change in the rate of lysine acceptance by either normal or [FUrd]tRNA (data not shown). Competition from the added nonradioactive amino acids would be expected to reduce any mischarging, since amino acids are preferentially esterified to their cognate tRNAs (Yarus, 1972; Strickland and Jacobson, 1972). Incorporation of FUrd into tRNA, therefore, does not change the specificity of the aminoacylation reaction.

Because the function of lysine-specific tRNA was inhibited most by the incorporation of 5-fluorouridine, further study focused on this tRNA. To gain a better understanding of the interaction of FUrd-containing lysine tRNA with its cognate synthetase, a more detailed kinetic analysis of lysyl-tRNA formation was undertaken. Apparent K_M and V_{max} values, determined from Lineweaver–Burk double-reciprocal plots (results not shown), were, respectively, 1.5×10^{-6} M and 20 nmol of lysine min^{-1} (mg of synthetase)$^{-1}$ for normal tRNA and 7.2×10^{-6} M and 4.6 nmol of lysine min^{-1} (mg of enzyme)$^{-1}$ for analogue-containing tRNA. 5-Fluorouridine incorporation, thus, increased the apparent K_M approximately fivefold, indicating a less efficient interaction of [FUrd]-$tRNA^{Lys}$ with its cognate synthetase; V_{max} is decreased approximately 75%.

Incomplete Aminoacylation of 5-Fluorouridine-Substituted tRNA. [FUrd]tRNA could be charged with lysine, but high concentrations of synthetase were needed for complete aminoacylation. At lower enzyme concentrations, the acylation reaction was incomplete and the steady-state (plateau) level of charging was proportional to synthetase concentration (Figure 1B). Similar results were obtained with glutamic acid (results not shown); aspartate, glutamine, and histidine were not tested. The extent of charging control tRNA with lysine was much less dependent on synthetase concentration (Figure 1A).

Incomplete aminoacylation of [FUrd]tRNA with lysine could be due to an inactivation of lysine tRNA or of lysyl-tRNA synthetase. Both possibilities are unlikely, as shown by the results in Figure 2. In this experiment [FUrd]tRNA was acylated with [^{14}C]lysine at a synthetase concentration known to give approximately 50% of the maximum charging level. Addition of more enzyme after the plateau of charging had been reached (40 min) resulted in the formation of additional lysyl-tRNA (Figure 2, curve c), indicating that the [FUrd]-tRNA present in the reaction mixture had not been degraded. In a similar experiment, addition of more [FUrd]tRNA also led to an increase in the charging level (Figure 2, curve b). This rules out the type of irreversible enzyme inactivation recently observed with *E. coli* isoleucyl-tRNA synthetase (Marashi and Harris, 1977). It also argues against formation of a stable adduct between the synthetase and a 5-fluorouridine residue in [FUrd]tRNA, resulting in enzyme inactivation by a mechanism analogous to the inactivation of thymidylate syn-

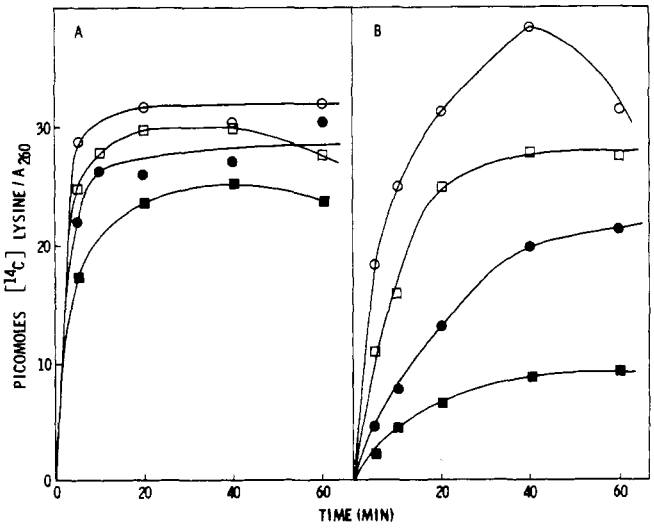

FIGURE 1: Effect of synthetase concentration on the extent of lysyl-tRNA formation. Reactions were carried out as described under Experimental Procedures. (A) Normal tRNA, 42 μg/0.1-mL assay. (B) [FUrd]tRNA, 43 μg/assay. The amount of tRNA-depleted enzyme preparation used per assay was: (○-○) 60, (□-□) 24, (●-●) 12, and (■-■) 2.4 μg.

thetase by 5-fluorodeoxyuridylic acid (Santi et al., 1974; Dannenberg et al., 1974). Formation of a transient adduct is still a possibility; transient formation of a covalent bond between synthetases and the uridine-8 residue of tRNAs has recently been suggested on the basis of tritium-exchange studies (Schoemaker and Schimmel, 1977).

To determine whether FUrd-substituted lysine tRNA was partially inactive as the result of denaturation, the tRNA was heated for 10 min at 50 °C in a 100 mM Tris-HCl buffer (pH 7.5) containing 10 mM Mg^{2+} (Ishida et al., 1971). This treatment did not increase the extent of charging with lysine at low enzyme concentrations; denatured $tRNA^{Glu}$ was renatured to full activity under these conditions.

Addition of more ATP or [^{14}C]lysine had no effect on the extent of lysine acceptance, indicating that these substrates were not limiting.

Effect of Reaction Conditions on the Lysine Acceptance of tRNA. Aminoacylation of [FUrd]tRNA is influenced more strongly than control tRNA by reaction conditions such as magnesium ion concentration, buffer composition and concentration, pH, and ionic strength. For example, the rate and extent of lysine acceptance by [FUrd]tRNA were sharply inhibited by NaCl concentration in the range 0.1–0.3 M, while the aminoacylation of normal tRNA was unaffected. Variations in pH influence lysine acceptance by normal and fluorouridine-substituted tRNA differently. Both the rate and the extent of aminoacylation of control tRNA increased with increasing pH in the range 6.5–8.5 (Table II; the extent of lysyl-tRNA formation with normal tRNA was the same at high and low enzyme concentrations). Generally, the reverse was true for [FUrd]tRNA (Table II); the extent of aminoacylation dropped with increasing pH. There was a small increase in the rate of aminoacylation between pH 6.5 and 7.5, but the rate decreased when the pH was increased further to 8.5. Similar effects of pH on the aminoacylation of [FUrd]-tRNA with lysine were recently reported by Moore and Kaiser (1977). The pK_a of 5-fluorouracil, 8.1–8.3 in poly(FU) (Szer and Shugar, 1963; Massoulie et al., 1966), is considerably lower than the pK_a of uracil, 9.6–9.8 in poly(U). Evidently, ionization of fluorouridine residues in tRNA, at pH values near the pK_a of FUrd, inhibits the aminoacylation reaction either by altering the secondary or tertiary structure of the tRNA or

TABLE II: Effect of pH on the Rate and Extent of Lysyl-tRNA Formation.[a]

tRNA prep	pH	initial velocity (pmol of Lys (pmol of tRNA)$^{-1}$ min^{-1})	extent of aminoacylation (nmol of Lys/mg of tRNA) high [E]	low [E]
normal tRNA	6.5	0.32	0.79	
	7.5	0.60	0.85	
	8.0		0.87	
	8.5	0.78	0.98	
[FUrd]tRNA	6.5	0.046	1.24	0.43
	7.0		1.18	0.36
	7.5	0.057	1.09	0.31
	8.0		0.83	0.20
	8.5	0.019		0.16

[a] Aminoacylation reactions were run as described under Experimental Procedures, at a Mg^{2+} concentration of 20 mM. For kinetic studies the reaction mixtues (0.1 mL) contained 11 μg of enzyme and 36 μg of normal tRNA or 45 μg of [FUrd]tRNA; 100 mM Tris-HCl buffer was adjusted to the indicated pH values. Plateau levels of aminoacylation were determined in reaction mixtures (0.1 mL) containing either 120 μg of enzyme (high [E]) or 2.4 μg of enzyme (low [E]) and 36 μg of normal tRNA or 49 μg of [FUrd]tRNA; 50 mM Tris-HCl buffer was adjusted to the indicated pH values.

as the result of dissociation of a residue at the site of tRNA-enzyme interaction. Although pH affects the charging of tRNA with lysine, dissociation of FUrd residues does not account for the lower rate or extent of aminoacylation of [FUrd]tRNA, because differences from normal tRNA are evident at all pH values examined, even at pH 6.5 where the ionization of FUrd is not a factor.

Deacylation of Lysyl-tRNA. It has been reported that the plateau level of an aminoacylation reaction is a function of the rates of acylation and deacylation (Bonnet and Ebel, 1972). To determine whether an increased rate of deacylation is, in part, responsible for the reduced steady-state level of charging [FUrd]tRNA with lysine, the rate of lysyl-tRNA deacylation was examined, first under standard conditions of aminoacylation. Transfer RNA was acylated with [^{14}C]lysine. After the charging plateau had been reached, excess nonradioactive lysine was added and the loss of tRNA-bound radioactivity was followed (Figure 3A). The rates of deacylation of normal and FUrd-substituted tRNA were essentially equal. Normal lysyl-tRNA had a $t_{1/2}$ of 25 min under these conditions, while FUrd-containing lysyl-tRNA had a half-life of 27 min.

Hydrolysis of lysyl-tRNA in the absence of AMP and PP_i was examined by isolating normal and FUrd-substituted lysyl-tRNA and measuring the deacylation with and without added synthetase. Figure 3B shows that the rates of deacylation, in the absence of AMP and PP_i, were similar for normal and [FUrd]tRNA.

BD-Cellulose Chromatography of Normal and FUrd-Substituted tRNA. To determine whether [FUrd]tRNA contains two or more isoaccepting species of $tRNA^{Lys}$, which differ in their rate of aminoacylation, the chromatographic behavior of normal and FUrd-substituted tRNA on BD-cellulose was examined (Figure 4). Normal tRNA exhibited one major peak of lysine-accepting activity which eluted at approximately 0.55 M NaCl (Figure 4A). A trailing shoulder may represent a second species of $tRNA^{Lys}$; some question remains as to whether there are one or two isoaccepting species

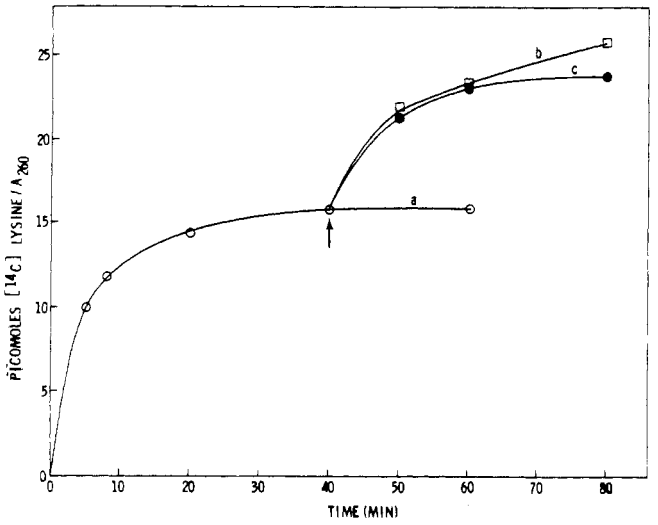

FIGURE 2: Effect of additives on the extent of lysyl-tRNA formation. The reaction was carried out under standard conditions, with 12 μg of synthetase and 43 μg of [FUrd]tRNA/0.1 mL reaction mix. At 40 min, after net aminoacylation had ceased, the following additions were made: (a) none; (b) 21 μg of [FUrd]tRNA; (c) 60 μg of enzyme.

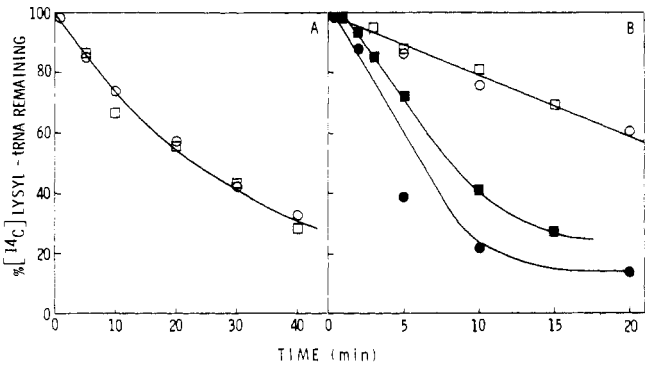

FIGURE 3: Enzymatic deacylation of lysyl-tRNA. (A) Deacylation under standard aminoacylation conditions. Aminoacylations were performed as described under Experimental Procedure, at a synthetase concentration of 110 μg/mL. The incubation mixture contained either 470 μg of [FUrd]tRNA (□-□) or 380 μg of normal tRNA (○-○) per mL. When the plateau was reached, a tenfold excess of unlabeled lysine was added (zero time), and samples were removed at intervals for measurement of radioactivity. (B) Deacylation of lysyl-tRNA in the absence of AMP and PP_i. Normal (●-●) or 5-fluorouridine-containing (■-■) [^{14}C]lysyl-tRNA was incubated in 0.1 M Bicine buffer (pH 7.5) containing 20 mM magnesium acetate, all 20 unlabeled amino acids (2.5 mM), and 369 μg/mL tRNA-depleted synthetase. Controls (○-○, normal tRNA; □-□, [FUrd]tRNA) had no synthetase added.

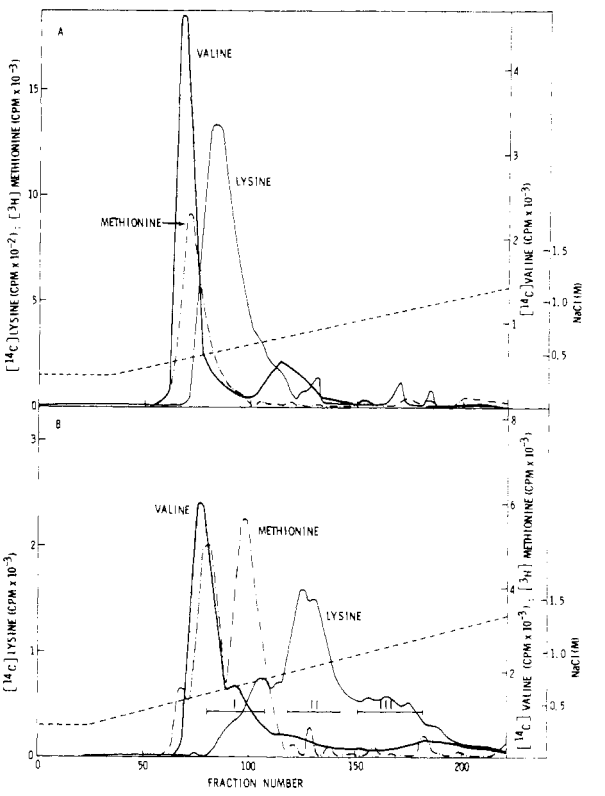

FIGURE 4: Chromatography of tRNA on benzoylated DEAE-cellulose. 550 A_{260} units of unfractionated tRNA was chromatographed on a 0.9 × 28 cm column of BD-cellulose as described under Experimental Procedure. The column was run at 4 °C and 3-mL fractions were collected at a flow rate of 35 mL/h. Amino acid acceptance was assayed as described in the text: (A) Normal tRNA; (B) 5-fluorouridine-substituted tRNA: (—) [^{14}C]lysine; (—) [^{14}C]valine; (- • -) [^{3}H]methionine; (- - -) NaCl molarity.

of $tRNA^{Lys}$ in *E. coli* (see discussion by Chakraburtty et al., 1975). Lysine-accepting activity in FUrd-substituted tRNA showed a much different elution profile (Figure 4B). Several peaks were observed, all of which were eluted at salt concentrations higher than 0.65 M. For comparison, the isoaccepting tRNA profiles for valine and methionine are also shown in Figure 4. Three peaks of methionine-accepting activity were observed in [FUrd]tRNA. As previously reported, the first two of these were formylatable (Horowitz et al., 1974). Both the valine- and methionine-accepting activities in FUrd-substituted tRNA were eluted at higher salt concentrations than their normal counterparts.

Fractions containing lysine-accepting tRNA were pooled as shown in Figure 4B. Each of the pooled fractions exhibited the same low rate and extent of lysine acceptance observed with unfractionated [FUrd]tRNA (data not shown).

Transfer RNA Dependent Polypeptide Synthesis. The ability of [FUrd]tRNA to stimulate polypeptide synthesis in a tRNA-depleted, cell-free reaction mixture was examined. Activity in the in vitro protein-synthesizing system used in these studies was linearly dependent on the amount of added tRNA, over a wide range of tRNA concentrations; synthetic polynucleotide messengers or phage f2 RNA stimulated incorporation 20- to 25-fold.

There was essentially no difference in the ability of normal and [FUrd]tRNA to stimulate poly(U)-dependent phenylalanine incorporation (Table III). [FUrd]tRNA was, however, considerably less effective than control tRNA in supporting poly(A)-dependent lysine incorporation. After 30 min at 37 °C, only one-third (and often less) as much lysine was transferred into polypeptide from [FUrd]tRNA than from normal tRNA.

The effect of added tRNA on protein synthesis directed by a natural message is shown in Table III. In contrast to the results with poly(U), stimulation of phenylalanine incorporation by added [FUrd]tRNA in the phage f2 RNA coded system was only half that observed with normal tRNA. [FUrd]tRNA was even less effective in supporting lysine incorporation in the phage RNA-directed system; it stimulated only 25% as much incorporation as normal tRNA. The lower efficiency of [FUrd]tRNA in stimulating phenylalanine incorporation in the f2 RNA directed system may be a consequence of the inhibition of lysine incorporation. Both amino acids occur in the polypeptides coded by phage RNA. The activity of [FUrd]-tRNA cannot be due to residual unsubstituted tRNA, since polypeptide synthesis is proportional to the concentration of added tRNA, and the analogue-containing tRNA preparations

TABLE III: Stimulation of Amino Acid Incorporation into Polypeptide by Normal and 5-Fluorouridine-Substituted Transfer RNA.[a]

expt	mRNA	tRNA prep	amino acid incorp (pmol/0.1 mL)	[FUrd]tRNA / normal tRNA
		lysine		
1	poly(A)		0.41	
	poly(A)	normal tRNA	11.8	0.35
	poly(A)	[FUrd]tRNA	4.1	
2	f2 RNA		0.5	
	f2 RNA	normal tRNA	20.7	0.26
	f2 RNA	[FUrd]tRNA	5.3	
		phenylalanine		
3	poly(U)		0.5	
	poly(U)	normal tRNA	72.4	1.2
	poly(U)	[FUrd]tRNA	83.9	
4	f2 RNA		0	
	f2 RNA	normal tRNA	25.1	0.51
	f2 RNA	[FUrd]tRNA	12.8	

[a] Methods used are described under Experimental Procedures. The enzyme preparation was depleted of endogenous tRNA as detailed in the text. Fifty micrograms of normal or 5-fluorouridine-substituted tRNA was added to each 0.1-mL reaction in expts 1, 2, and 4; 11 μg of tRNA was used in expt 3. The tRNA preparations had similar specific activities, approximately 40 pmol of Phe/A_{260} and 50 pmol of Lys/A_{260}. Reaction mixtures were incubated at 37 °C for 30 min. The specific activities of ^{14}C-labeled lysine and phenylalanine were 315 and 50 Ci/mol, respectively.

used in all experiments had 90–95% of their uridine residues replaced by 5-fluorouridine.

Variation of the Mg^{2+}, ribosome, or polynucleotide concentration had no effect on the difference in the ability of normal and [FUrd]tRNA to stimulate incorporation of lysine into polypeptides.

The S-100 preparations used in these studies contained enough aminoacyl-tRNA synthetase to permit efficient charging of [FUrd]tRNA with lysine, but, to rule out any possibility that the low level of lysine incorporation was due to an inhibition of lysyl-tRNA formation, polypeptide synthesis from preformed aminoacyl-tRNA was examined (Figure 5). Approximately equal molar amounts of normal and FUrd-containing aminoacyl-tRNAs were added to the reaction mixture in each experiment. Figure 5A shows the poly(A)-dependent incorporation of lysine from lysyl-tRNA. Only a low level of lysine transfer into polypeptide was observed with [FUrd]tRNA, a clear indication that the poor incorporation of lysine in the [FUrd]tRNA-dependent system was not due to the slow rate of [FUrd]tRNA aminoacylation. Phenylalanine was incorporated equally well from normal and FUrd-substituted Phe-tRNA using either poly(U) or poly(G,U) as the messenger (Figure 5B).

The poly(G,U)-dependent incorporation of valine proceeded well with either tRNA preparation, but normal valyl-tRNA was utilized somewhat more effectively (Figure 5C). Uridine-5-oxyacetic acid (cmo^{5}U) is located in the first (5′) position of the anticodon of normal *E. coli* tRNA$^{Val}_1$ (Murao et al., 1970), the major valine isoaccepting species present in *E. coli*. This nucleoside is replaced by FUrd, in [FUrd]tRNA$^{Val}_1$ (Horowitz et al., 1974). Nishimura (1972) has suggested that cmo^{5}U is able to base pair with U, permitting tRNA$^{Val}_1$ to recognize the codon GUU in addition to GUA and GUG. Absence of cmo^{5}U in [FUrd]tRNA$^{Val}_1$ could reduce the efficiency with which this tRNA recognizes poly(G,U) as a messenger and might account for the lower level of valine

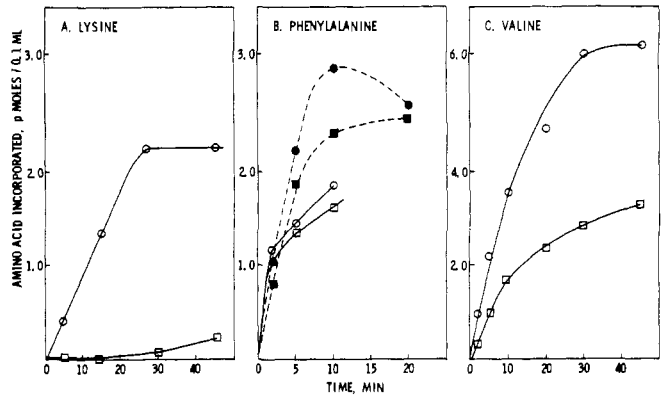

FIGURE 5: Time course of polypeptide synthesis from aminoacyl-tRNA. Polypeptide synthesis was assayed as described under Experimental Procedure. The values shown have been corrected by subtracting the incorporation observed in the absence of polynucleotide. (A) Poly(A)-dependent incorporation of lysine. Twenty-five pmoles of normal or 24 pmol of 5-fluorouridine-substituted [^{14}C]Lys-tRNA was added to each reaction mixture. (B) Incorporation of phenylalanine dependent on poly(U) (open symbols) or poly(G,U) (closed symbols). Three pmoles of normal or 3.4 pmol of FUrd-substituted [^{14}C]Phe-tRNA was used in each assay with poly(U); 6.3 pmol of normal and 6.7 pmol FUrd-substituted [^{14}C]Phe-tRNA were added to each reaction mixture in the assays with poly(G,U). (C) Poly(G,U)-dependent incorporation of valine. Per assay, 19 pmol of either normal or FUrd-containing [^{14}C]Val-tRNA was used: (○-○ and ●-●) normal tRNA; (□-□ and ■-■) [FUrd]tRNA.

transfer from analogue-containing valyl-tRNA. Our previous results (Ofengand et al., 1974) had shown that purified FUrd-containing valyl-tRNA$^{Val}_1$ was fully functional in polypeptide synthesis. In those experiments, the rate and extent of valine incorporation into the copolypeptide (Phe$_n$, Val), dependent on poly(G_1U_3), were unchanged when FUrd-containing *E. coli* tRNA$^{Val}_1$ was used in place of the normal control.

Ribosomal Binding of Normal and FUrd-Substituted Aminoacyl-tRNA. The results of polynucleotide-directed ribosomal binding studies with several aminoacyl tRNAs are presented in Figure 6. The binding of lysyl-tRNA in response to poly(A), poly(A,C), and poly(A,G) is shown in Figure 6A,B. In each case, the binding of the FUrd-substituted lysyl-tRNA was less than 20% as efficient as that of normal lysyl-tRNA. It has been suggested that FUrd may on occasion base pair with guanosine, leading to transcriptional and translational errors (Champe and Benzer, 1962). Although it is unlikely that such errors occur on a massive scale (Bujard and Heidelberger, 1966; Horowitz and Kohlmeier, 1967), some such misrecognition might account for the poor ribosome binding of [FUrd]lysl-tRNA in response to poly(A) or poly(A,C). However, our results show no evidence of such misreading; only low levels of [FUrd]lysyl-tRNA bind to ribosomes coded by poly(A,G), (Figure 6B).

No marked differences were noted between normal and [FUrd]tRNA in the polynucleotide-dependent binding of phenylalanyl-, valyl-, and glycyl-tRNAs (Figure 6D,E,F). As previously reported (Ofengand et al., 1974), [FUrd]valyl-tRNA binds somewhat more tightly than normal valyl-tRNA (Figure 6E). The poly(A,G)-dependent binding of [FUrd]-glutamyl-tRNA is, however, less efficient than the binding of normal glutamyl-tRNA (Figure 6C). tRNA^{Glu} thus resembles tRNA^{Lys} in that incorporation of 5-fluorouridine inhibits ribosome-binding capabilities as well as the rate and extent of aminoacylation.

Discussion

Surprisingly, the ability of most fluorouridine-substituted

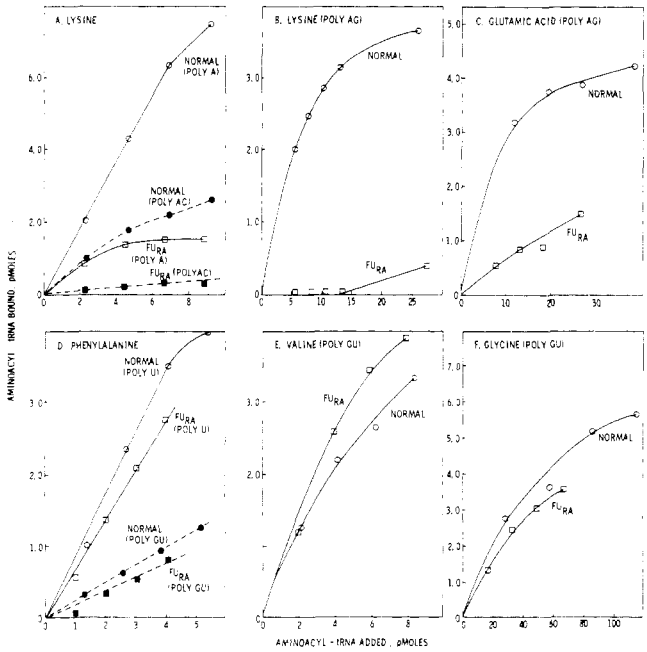

FIGURE 6: Ribosomal binding of normal and [FUrd]tRNA. Assay conditions were described under Experimental Procedure. Total binding was corrected for the binding observed in the absence of polynucleotide. (A) Poly(A)- and poly(A,C)-dependent binding of [^{14}C]Lys-tRNA. (B) Poly(A,G)-dependent binding of [^{14}C]Lys-tRNA. (C) Poly(A,G)-dependent binding of [^{14}C]Glu-tRNA. (D) Poly(U)- and poly(G,U)-dependent binding of [^{14}C]Phe-tRNA. (E) Poly(G,U)-dependent binding of [^{14}C]Val-tRNA. (F) Poly(G,U)-dependent binding of [^{14}C]Val-tRNA. (F) Poly(G,U)-dependent binding of [^{14}C]Gly-tRNA.

E. coli tRNAs to accept their cognate amino acid remains essentially unchanged by extensive incorporation of the base analogue. However, the rate and extent of aminoacylation with lysine, and to a lesser degree charging with aspartate, glutamate, glutamine, and histidine, are decreased as a result of FUrd incorporation (Table I); the specificity of the charging reaction is not affected. Incorporation of 5-fluorouridine decreases the affinity of lysine tRNA for its synthetase approximately fivefold, as judged by the K_M values. It is not yet known whether this is due to replacement of one or more essential uridine or uridine-derived minor nucleosides or to perturbations in the secondary or tertiary structure of the tRNA as a result of the massive substitution of the analogue for normal nucleoside constituents.

Lowrie and Bergquist (1968) observed no difference in the rate of lysine acceptance by control and [FUrd]tRNA. This may have been due to the use of relatively high enzyme concentrations in their experiments or because their tRNA preparations were less highly substituted with FUrd than those in the present study (50% replacement of Urd by FUrd in their experiments compared with more than 90% replacement in ours). We have observed that the initial velocity of lysine acceptance is inversely proportional to the FUrd content of a tRNA preparation.

The aminoacylation of lysine tRNA modified by the incorporation of 5-fluorouridine is incomplete at low enzyme concentrations (Figure 1B). This effect is not the result of inactivation of synthetase or degradation of tRNA (Figure 2) and can be attributed to the decreased efficiency with which [FUrd]tRNA is aminoacylated with lysine. Incomplete aminoacylation has previously been observed by others, most frequently in instances of misacylation of tRNA with a noncognate amino acid (Jacobson, 1971; Dietrich et al., 1976), but also in correct aminoacylation reactions. The phenomenon has been interpreted to be the result of an equilibrium between aminoacylation and competing synthetase-catalyzed and nonenzymatic deacylations of aminoacyl-tRNA (Bonnet and Ebel, 1972; Dietrich et al., 1976). Factors which reduce the rate of aminoacylation compared to deacylation lead to a new lower steady-state (plateau) level of charging, at low enzyme concentrations.

The aminoacylation of the majority of tRNAs is not affected by the substitution of FUrd for Urd and Urd-derived minor nucleosides; this is especially clear in studies with purified *E. coli* tRNA$^{Val}_1$ reported earlier (Horowitz et al., 1974; Ofengand et al., 1974). Because many fluorouridine-substituted tRNAs remain fully functional, they should prove useful in probing the relationships between tRNA structure and function. One such study currently underway is using ^{19}F nuclear magnetic resonance spectroscopy to investigate the recognition sites of *E. coli* [FUrd]tRNA$^{Val}_1$ by its cognate synthetase (Horowitz et al., 1977).

One can only speculate as to why aminoacylation with lysine, aspartate, glutamate, glutamine, or histidine is inhibited by the incorporation of 5-fluorouridine into tRNA. It is interesting to note, however, that each of these tRNAs recognizes codons having adenosine in the second position. Their anticodons contain one or more uridine or modified uridine residues (Chakraburtty et al., 1975; Barrell and Clark, 1974) that are replaced by 5-fluorouridine in [FUrd]tRNA. The anticodon is known to be involved in the interaction with synthetase in several tRNAs (see discussion in Rich and Schimmel, 1977). Available evidence indicates that in *E. coli* tRNA^{Glu} (Agris et al., 1973), *E. coli* tRNA^{Gln} (Seno et al., 1974), and *E. coli* tRNA^{Lys} (Saneyoshi and Nishimura, 1971) the anticodon is part of the synthetase recognition site. Perturbation of the anticodon region by the incorporation of FUrd may account for the reduced rate of aminoacylation. Of all the affected [FUrd]tRNAs, one would expect tRNA^{Lys} to be the most inhibited because this tRNA will have three fluorouridines in its anticodon replacing two uridine residues and one 5-methylaminomethyl-2-thiouridine residue in the wobble position (Chakraburtty et al., 1975).

Several anticodon base modifications have previously been shown to inhibit aminoacylation. Modification of the 2-thiouridine derivatives in the wobble position of the anticodons of *E. coli* tRNA^{Glu} (Agris et al., 1973) and *E. coli* tRNA^{Gln} (Seno et al., 1974) with cyanogen bromide increases the apparent K_M and decreases the extent of aminoacylation of these tRNAs. Addition of bisulfite to the uridine at the 3′ end of the anticodon of *E. coli* tRNA$^{Met}_f$ also results in a reduced rate and extent of aminoacylation (Schulman and Pelka, 1977). The observation that FUrd-substituted lysyl- and glutamyl-tRNAs bind to ribosomes less efficiently than the corresponding normal aminoacyl-tRNAs (Figure 6) supports the argument that the ability of the anticodon region to function properly is affected by incorporation of FUrd.

Asparagine- and tyrosine-specific tRNAs also recognize codons having adenosine in the second position. The initial charging kinetics of these tRNAs, however, are not affected by FUrd incorporation. It may be that the anticodons of these tRNAs are not involved in synthetase recognition. Consistent with this supposition is the finding that mutational replacement of a nucleotide in the anticodon of *E. coli* tRNA^{Tyr} leads to the formation of *su*$_3^+$ tRNA^{Tyr}, which is still recognized by tyrosyl-tRNA synthetase (Goodman et al., 1968).

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