Effects of 5-Fluorouracil on Base Modification in *Escherichia coli* tRNA†

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ABSTRACT: Recent findings indicate that modified pyrimidines derived from uridine in tRNAs are reduced more extensively in 5-fluorouracil-containing tRNAs (FUra-tRNAs) than can be accounted for by random replacement of uridine by 5-fluorouridine. Examination of FUra-tRNAs from *Escherichia coli* B, by two independent methods of nucleoside analyses, shows an exponential decrease in pseudouridine, ribosylthymidine (5-methyluridine), and 5,6-dihydrouridine with increasing fluorouridine incorporation. This decrease cannot be accounted for by a preferential incorporation of fluorouridine into uridine positions destined to become modified since tRNAs with a low mole % of fluorouridine content show a total decrease *exceeding* fluorouridine incorporation and FUra-tRNAs containing 1–2 mol % of the analogue show an *increase* in uridine content. All modified pyrimidines are not affected in the same way, since 4-thiouridine content decreases linearly with increasing fluorouridine incorporation. Attempts at in vivo modification of FUra-tRNAs that contained low levels of fluorouridine but that were grossly deficient in pseudouridine and ribosylthymidine and partially deficient in 5,6-dihydrouridine were unsuccessful. These findings indicate that sufficient residual inhibitor is retained by the cells to prevent the modifying enzymes from functioning and suggest that the mechanisms used by these enzymes are similar. No changes were observed either in the content of adenosine, guanosine, and cytidine or in the modified purines. Significant accumulations of precursor tRNAs were not observed in *E. coli* treated with 5-fluorouracil.

Addition of the FUra[1] or FUrd to growing cultures of either prokaryotic or eukaryotic cells results in the replacement of Urd by the analogue (Horowitz & Chargaff, 1959; Heidelberger, 1965). Under certain conditions, this replacement can be essentially complete. In *Escherichia coli* B, a number of modified pyrimidines are also present in reduced amounts in FUra-containing tRNAs, including Ψrd (Lowrie & Bergquist, 1968; Johnson et al., 1969), rThd (Lowrie & Bergquist, 1968; Baliga et al., 1969), H_6^5Urd (Kaiser et al., 1969), s^4Urd (Kaiser, 1969), uridine 5-oxyacetate (Horowitz et al., 1974), 5-methylaminomethyl-2-thiouridine (Kaiser, 1972), and 2-thiocytidine (Kaiser, 1972). FUra-treated eukaryotic cells also show decreases in Ψrd, rThd, and H_6^5Urd in their tRNAs (Giege et al., 1969; Kaiser, 1971; Lu et al., 1976). Earlier studies from our laboratory and others suggested that, for the most part, the decreases observed in the modified pyrimidines were proportional to the amount of replacement of Urd by FUrd. There were indications, however, that the contents of some of the modified pyrimidines—namely, Ψrd, rThd, and 5-methylaminomethyl-2-thiouridine—were reduced more extensively than could be accounted for by random replacement of Urd by FUrd. It was proposed that preferential incorporation of FUrd occurred at certain Urd sites destined to be modified (Baliga et al., 1969). More recently, work from Randerath's laboratory on tRNAs from mice treated with either FUra or FUrd clearly shows that enzymes involved in modification of uridines at the 5 position of the ring are directly inhibited (Lu et al., 1976; Tseng et al., 1978). Transfer RNAs having low levels of FUra incorporation are grossly deficient in Ψrd, rThd, and H_6^5Urd.

This paper shows that tRNAs from *E. coli* containing low levels of FUra incorporation display an exponential decrease in these three modified nucleosides with increasing analogue content. The sulfur-containing pyrimidine, s^4Urd, shows a strictly linear decrease. Attempts to convert undermodified, preformed FUra-containing tRNAs in vivo have been unsuccessful. These modified pyrimidine deficiencies do not appear to affect either the trimming processes involved in tRNA maturation or purine base methylation. An abstract of this work has appeared (Frendewey & Kaiser, 1978).

Experimental Section

Methods

Bacterial Growth and Isolation of tRNAs and Supernatants. E. coli B cells (kindly provided by Dr. P. D. Bear, Division of Microbiology, University of Wyoming) were grown in a minimal medium containing either excess phosphate (A) or low phosphate (B) if $^{32}PO_4^{2-}$ was to be used to label the cells. The growth medium consisted of (per liter) 12.1 g of Tris base, 1.5 g of KCl, 5.0 g of NaCl, 1.0 g of NH_4Cl, and 0.3 mL of 0.01 M $FeCl_3 \cdot 6H_2O$ and was adjusted to pH 7.4 with HCl. Medium A contained 1.1 mL of PO_4^{2-} stock (6 g of Na_2HPO_4 + 3 g of KH_2PO_4 per 100 mL) per 100 mL of medium and was 1.0 mM in K_2SO_4. Medium B contained 1.0 mL of PO_4^{2-} stock per 500 mL of medium and was 0.1 mM in K_2SO_4. Both media were 0.2% in glucose and 1 mM in $MgCl_2$.

Cultures were grown at 37 °C in either a constant-temperature water bath with horizontal shaking or a room maintained at 37 °C (±2 °C) with shaking on a rotary shaking table. Growth was followed by absorbance at 660 nm. Initial inoculations were from a prewarmed slant of *E. coli* B into 20 mL of medium A and grown overnight. After several successive growths, 1 drop of this culture was used to inoculate 12 mL of the medium, which was grown overnight. The 250-mL cultures of medium B were inoculated with the entire

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[1] Abbreviations used: FUra, 5-fluorouracil; FUrd, 5-fluorouridine; FCyd, 5-fluorocytidine; Ψrd, pseudouridine; rThd, ribosylthymidine (5-methyluridine); H_6^5Urd, 5,6-dihydrouridine; s^4Urd, 4-thiouridine; $NaDodSO_4$, sodium dodecyl sulfate; cpm, counts per minute; FUra-tRNA, tRNA isolated from bacteria grown in the presence of FUra; S-150, supernatant from an *E. coli* cell extract after centrifugation at 150000*g* for 3–4 h; A_{260} unit, a unit of material which in a volume of 1 mL will have an absorbance of 1 at 260 nm when measured in a cell of 1-cm path length.

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12 mL and grown for the desired period of time.

The cultures (250 mL) were grown with vigorous shaking to early exponential phase ($A_{660} \simeq 0.15$, Coleman Junior II, 1.8-cm path-length cuvettes), at which time either Ura or Ura *plus* FUra (in 0.25 M Na_2CO_3) additions were made. After 2–5 min, 1–3 mCi of ^{32}P (or 50 μCi of either [^{14}C]Ura or [^{14}C]FUra) was added to each flask, and growth was continued for an additional 3 h. The cultures were chilled on ice and harvested by centrifugation at 4 °C. The cell pellets were washed once in 0.01 M Tris-HCl (pH 7.4) and 0.01 M $MgCl_2$, recentrifuged, and stored as pellets at −70 °C until extracted.

For direct phenol extraction of whole cells, the frozen pellets were resuspended in 0.01 M Tris-HCl (pH 7.4) and 0.1 M NaCl and treated with an equal volume of 80% phenol (distilled) in buffer with shaking at room temperature for 1 h. After cooling on ice and centrifugation at 4 °C, the aqueous layer was removed and the phenol layer reextracted with 0.25 volume of buffer at room temperature. After cooling and centrifugation, the aqueous layers were combined and the RNA was precipitated by 2 volumes of 95% ethanol at −20 °C for at least 2 h. After centrifugation, the white pellet was resuspended in 1 mL of 1.8 M Tris-HCl (pH 8 at 37 °C) and incubated at 37 °C for 90 min to deacylate the tRNAs (Sarin & Zamecnik, 1964). The stripped RNA mixture was applied to either a Sephadex G-75 superfine column (1.5 × 100 cm, flow rate $\simeq$ 7 mL/h) or a Sephacryl S-200 superfine column (1.0 × 100 cm, flow rate $\simeq$ 15 mL/h) at room temperature. Fractions (3.0 mL) were collected and their absorbances monitored at 260 nm. The tRNA peak was pooled, precipitated by ethanol, collected by centrifugation, and finally dissolved in 0.5–1.0 mL of water. Storage was at −20 °C.

In some experiments where supernatants were desired, the cells were first broken by passing the resuspended washed cells [in 0.01 M Tris-HCl (pH 7.5)–0.01 M magnesium acetate, containing RNase-free DNase (5 μg/mL)] through a chilled, French pressure cell (15 000 psi). After low-speed centrifugation, the supernatant was either treated with phenol and the tRNAs extracted, essentially as described above for whole cells, or centrifuged for an additional 3–4 h at 150000*g* before extraction of the supernatant with phenol.

Transfer RNA Hydrolysis and Base Analysis for Experiments 1 and *2*. The tRNA in experiments 1 and 2 was digested to the nucleotide level by using RNase T_2 and pancreatic RNase (RNase A) as described (Brownlee, 1972). The digest mixture contained 5 A_{260} units of tRNA, 2 units/mL of RNase T_2, 0.05 mg/mL RNase A, and 0.05 M ammonium acetate (pH 4.5). The digestion was carried out for 6–8 h at 37 °C with a total volume of between 0.1 and 0.6 mL. About 1.0 A_{260} unit (<50 000 cpm) of the digestion mixture was spotted on a cellulose thin-layer plate and developed in two dimensions (Nishimura, 1972). The radioactive nucleotide spots were located by autoradiography on X-ray film, cut from the plates, and quantitated without elution by liquid scintillation counting. The mole % of each nucleotide was calculated by dividing the cpm obtained for the nucleotide by the total number of counts for all the nucleotides and multiplying by 100.

Experiment 3 Protocol. In experiment 3, cells were grown with different amounts of Ura and FUra present (see Table I). [^{14}C]-5-Fluorouracil (44.2 nCi/mL of culture media) was also added to each flask as a sensitive method for determining the extent of analogue incorporation. RNAs were phenol extracted from the low-speed centrifugation (30000*g*) supernatants as described earlier. Large amounts of high molecular weight ribosomal RNAs were present in these extracts as expected. Pooled tRNAs were recovered from Sephacryl S-200 columns and hydrolyzed to the nucleoside level either enzymatically or by alkali followed by alkaline phosphatase treatment (Kaiser, 1972). Except for H_6^5Urd and s^4Urd, the natural nucleoside levels in these digests were quantitated by high-pressure liquid chromatography on columns of Aminex A-5 in 20 mM $(NH_4)_2CO_3$ buffer (pH 9.2) at flow rates of 0.4 mL/min. Other details were as described (Kaiser & Young, 1975). The H_6^5Urd contents in experiment 3 were determined by the change in absorbance of intact tRNAs at 235 nm in 0.1 M KOH at room temperature (Molinar et al., 1968). Control tRNA had a dihydrouridine content of 1.99 mol %, assuming a tRNA concentration of 1 mg/mL has an $A_{260} = 24$ for a 1-cm path-length cell in 0.01 M Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 0.15 M KCl and the average weight of a micromole of nucleotide is 340 μg (Hedgcoth & Jacobson, 1968). A value of 9.2×10^3 was used for the molar extinction coefficient of dihydrouridine at 235 nm, pH 13 (Molinar et al., 1968). H_6^5Urd contents were also determined by the colorimetric assay of Hunninghake & Grisolia (1966), as modified by Jacobson & Hedgcoth (1970). The s^4Urd content of tRNAs was determined on concentrated samples with A_{260} values ranging from 12 to 42. The $A_{340nm}/A_{260nm} \times 100$ ratio of 2.03 found for normal tRNA was assumed to equal 0.875 mol % of s^4Urd (Kaiser, 1977). The mole percentages of FUrd and FCyd present in these samples were based on the specific activity of the [^{14}C]FUra in the original culture media, the specific activities of the ^{14}C-containing tRNAs, and assuming 1 A_{260} unit of intact tRNA in the above Tris–$MgCl_2$–KCl buffer contains 128.7 nmol of nucleotides.

Modified Nucleoside Formation in Preformed tRNAs. *E. coli* cultures were grown as described, with $^{32}PO_4^{2-}$ added 2 min after the addition of FUra and Ura at a final concentration of 20 and 10 μg/mL, respectively. After 2 h, the cells were harvested. One-half of these were frozen and maintained at −70 °C; the other one-half was washed in fresh media containing 50 μg/mL rifampicin, repelleted, and resuspended in fresh media containing 50 μg/mL rifampicin with no $^{32}PO_4^{2-}$, Ura, or FUra added. These resuspended cells were then incubated at 37 °C with shaking for 2 h, harvested, and stored at −70 °C. Control cultures containing only Ura (final concentration of 30 μg/mL) were carried out in parallel. Transfer RNAs were isolated from each batch and the nucleotide content—based on ^{32}P radioactivity—was determined.

A second type of in vivo modification experiment was carried out which was similar to the one just described, except that no rifampicin was added during the second incubation. [^{14}C]Ura (80 nCi/mL of culture) was used to label the pyrimidines in place of $^{32}PO_4^{2-}$, and [^{3}H]FUra (116 nCi/mL of culture) was added to determine the extent of FUra incorporation. Final concentrations of both Ura and FUra were 15 μg/mL. Cytidine was also added (final concentration 10 μg/mL) in an effort to keep labeling of this nucleoside to a minimum. One-half of the initial cell harvests of both normal and FUra-treated cells were washed once in medium A containing 30 μg of Ura/mL and resuspended in the same medium. Growth was continued for 1 h at 37 °C in the *absence* of rifampicin and without additional label added. Enzymatically generated nucleotides from the isolated tRNAs were separated by TLC and located by autoradiography, and the labeled spots were cut out and quantitated by scintillation counting. Mole % values for Ψrd, rThd, and H_6^5Urd were based on the amount of label associated with the Urd derived nucleosides relative to the amount of activity found in these

three nucleosides *plus* Urd. This total was assumed to be 22.2 mol %. FUrd content was based on the ^3H-associated radioactivity of the intact tRNAs.

$NaDodSO_4$–Sephacryl S-200 Gel Chromatography and $NaDodSO_4$–Acrylamide Gels. All $NaDodSO_4$–Sephacryl S-200 gel chromatography was carried out at room temperature on 1 × 115 cm columns in a buffer consisting of 0.01 M Tris-HCl (pH 7.4), 0.01 M $MgCl_2$, 0.15 M NaCl, and 0.2% $NaDodSO_4$. Absorbance of each fraction (1.8 mL) was measured and 0.2 mL removed for determination of radioactivity in 3 mL of Biofluor by liquid scintillation counting.

$NaDodSO_4$–acrylamide gradient gels from 6 to 12% acrylamide were prepared and handled as described by Owens & Haley (1976).

Materials

Materials used and their suppliers were as follows: uracil (Mann Research); 5-fluorouracil (PCR); $H_3{}^{32}PO_4$ (Amersham); [^{14}C]uracil (Schwarz/Mann); 5-[^{14}C]fluorouracil (Schwarz/Mann); [^{3}H]-5-fluorouracil (Amersham); Sephadex G-75 and Sephacryl S-200 (Pharmacia); T_2 ribonuclease (Calbiochem); ribonuclease A (Worthington and Sigma); cellulose thin-layer plates (Eastman); X-ray film (Kodak X-Omat R); Aminex A-5 (Bio-Rad); rifampicin (Sigma); and Bifluor (New England Nuclear). Other chemicals used were either reagent grade or the best available.

Results

Bacterial Growth. Untreated *E. coli* B cells grew in a normal fashion exhibiting an exponential growth pattern when monitored by either absorbance at 660 nm or viable cell numbers determined by platings on tripticase agar plates. When *E. coli* cells were grown to an A_{660} of about 0.2 (5×10^8 cells/mL) and treated with 10 μg/mL FUra (*plus* 20 μg/mL Ura), the cells grew linearly instead of exponentially (data not shown). This dosage of FUra did not cause an exponential killing of the cells such as that reported by Beck & Howlett (1977), in a *Salmonella typhimurium* mutant grown under conditions where the biosynthesis of pyrimidine nucleosides is growth-rate limiting. Under the conditions described above, FUra treatment did not manifest any bacterial effects up to about 4 h of growth in the presence of the analogue.

Effect of Fluorouracil Incorporation on Modified Pyrimidines. All experiments were carried out with *E. coli* B cells grown for similar periods of time (generally 3 h) following the addition of FUra to the cultures in an early exponential phase of growth. The extent of FUra incorporation could be decreased significantly by the addition of Ura to the culture medium. As seen in Table I, small amounts of exogenous Ura compete very effectively in lowering FUra incorporation into mature tRNA molecules. Under our conditions, where the total exogenous pyrimidine base added to the culture media was about 30 μg/mL, we observed wide variation in the mole % of FUra incorporation between experiments. This is indicated in Table I and in other experimental results not shown. The reasons for the variations are unclear.

Despite these variations, the effects of FUra incorporation on the modified pyrimidines found in the tRNAs were consistent. In all cases shown, the mole percentage of the nucleoside is plotted vs. the FUrd content of the tRNA. Figure 1 illustrates the exponential decrease of rThd, Ψrd, and H_6^5Urd with increasing amounts of FUrd present in the tRNAs. Ribosylthymidine content (Figure 1A) was shown to drop off very rapidly at low levels of FUra incorporation. In experiments 1 and 2, a 65% decrease was noted in FUra-tRNAs

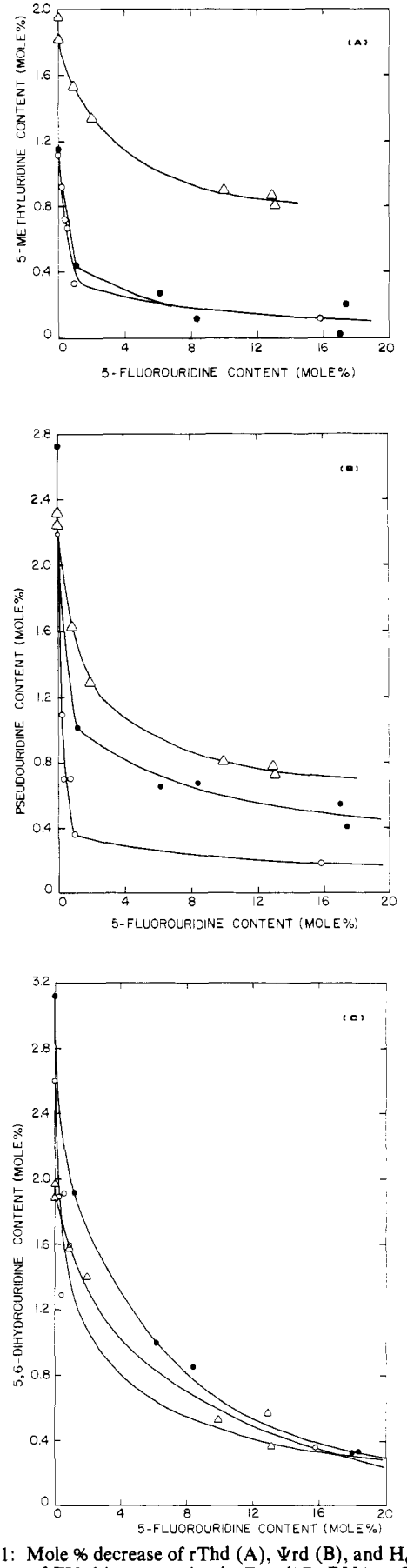

FIGURE 1: Mole % decrease of rThd (A), Ψrd (B), and H_6^5Urd (C) vs. mole % of FUrd incorporation in *E. coli* B tRNAs. Conditions for cell growth and mole % determinations are described under Methods. (Experiment 1) O—O; (experiment 2) ●—●; (experiment 3) Δ—Δ.

Table I: Effect of Different Ura/FUra Ratios in the Growth Media on the Mole % of FUra Incorporation into *E. coli* tRNAs[a]

experiment 1			experiment 2			experiment 3		
Ura (μg/mL)	FUra (μg/mL)	mol % FUra	Ura (μg/mL)	FUra (μg/mL)	mol % FUra	Ura (μg/mL)	FUra (μg/mL)	mol % FUra
30	0	0.0	30	0	0.0	31.5	0	0.0
25	5	0.20	22	8	1.07	31.5	0.43	0.008
22	8	0.34	15	15	6.13	15.7	16.1	0.901
15	15	0.54	6	24	8.35	9.4	22.5	1.93
10	20	0.91	2	28	17.00	3.1	28.7	9.90
0	30	15.80	0	30	17.40	1.3	30.7	13.11
						0	31.6	12.94

[a] Mole % of FUra for the different experiments was determined as described under Methods.

containing about 1 mol % of FUrd. Experiment 3 also shows an exponential decrease, although the numbers are less dramatic than those observed in the other cases. These differences are largely the result of the experimental analyses used. In experiments 1 and 2, the nucleoside compositions were based on the ^{32}P content of the nucleotides separated by TLC (see Methods section). The $[^{32}P]PO_4^{2-}$ was added to the culture of growing *E. coli after* the addition of the FUra, so that normal tRNAs present before FUra addition would not be labeled and consequently not contribute to the label found in the nucleotides. Nucleoside analyses in experiment 3 were also carried out on a mixture of tRNAs which contained both the normal tRNAs present *before* FUra addition and the FUra-containing tRNAs. In the methodology used, however, no differentiation could be made between the nucleosides present *before* and *after* addition of the analogue. As such, the modified pyrimidines present *before* FUra addition tend to elevate the numbers somewhat relative to the ^{32}P results. The same reasoning holds for the Ψrd data presented in Figure 1B, for experiment 3. In addition, there appears to be a constant interfering component eluting from the Aminex A-5 column coincident with the rThd that elevates the base line by about 0.75 mol %. While this changes the quantitative aspects of the rThd measurements, they are still qualitatively similar to those observed in the first two experiments.

Ψrd and H_6^5Urd contents of FUra-containing tRNAs were also shown to decrease more rapidly than expected, assuming a random replacement of Urd by FUrd (Figures 1B and 1C). Both of these modified pyrimidines appear quite sensitive to low levels of FUra incorporation. It has been assumed until recently that the decrease in the modified nucleosides derived from Urd and modified at the 5 position in FUra-containing tRNA results from direct impairment of the modifying enzyme by the fluorine atom of the FUra that has replaced the Ura destined to be modified. This is clearly an insufficient explanation of our results unless there is a preferential incorporation of FUra residues into those Ura positions destined to be modified, as proposed for rThd some years ago (Baliga et al., 1969). We currently have two pieces of evidence that argue *against* the preferential incorporation theory. First, at low levels of FUra incorporation, the sum of the decreases of Ψrd, rThd, and H_6^5Urd *exceeds* the extent of FUrd incorporation. That is, in tRNAs containing up to about 4 mol % FUrd, the decreases noted in just these three modified pyrimidines exceed the FUrd content. This is illustrated in Figure 2. Secondly, at low levels of FUrd incorporation, the Urd contents of FUra-tRNAs consistently show an initial increase, followed by a linear decrease with increasing FUrd content (Figure 3). If FUra was being preferentially incorporated into those Urd positions destined to be modified, no initial increase in Urd should be seen. Lu et al. (1976) and Tseng et al. (1978) have also noted that the decreases in the

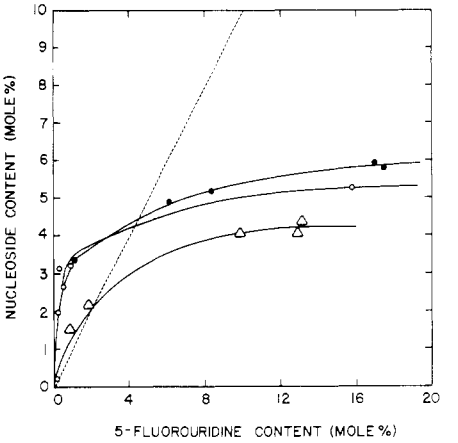

FIGURE 2: Total mole % *decrease* of rThd, Ψrd, and H_6^5Urd with increasing FUrd incorporation into *E. coli* B tRNAs. The dashed line represents FUrd incorporation. The curves represent data from three separate experiments with the symbols as defined in Figure 1.

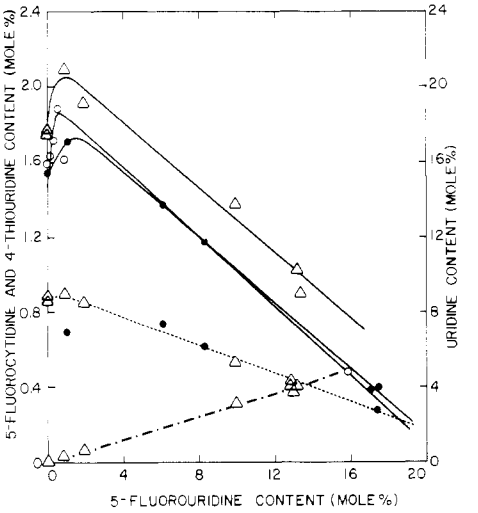

FIGURE 3: Effects of FUrd incorporation on the mole % of Urd, s^4Urd, and 5-fluorocytidine (FCyd) in *E. coli* B tRNAs. Different symbols reflect data from different experiments as indicated in Figure 1. Urd contents (—); s^4Urd (- - -); and FCyd (—·—). Content of s^4Urd was not determined in experiment 1 and FCyd content was not determined in experiments 1 and 2.

modified Urd derivatives (Ψrd, rThd, and H_6^5Urd) are greater than the amount of FUrd incorporated at low levels in tRNAs from tissues and tumors of mice administered either FUra or FUrd.

The major sulfur-containing modified nucleoside found in *E. coli* tRNAs, s^4Urd, is not affected by FUra in the same manner as rThd, Ψrd, and H_6^5Urd. Instead of showing an exponential decrease with increasing FUrd incorporation, s^4Urd decreases linearly (Figure 3). This suggests that the thiolase responsible for s^4Urd formation can utilize FUra-

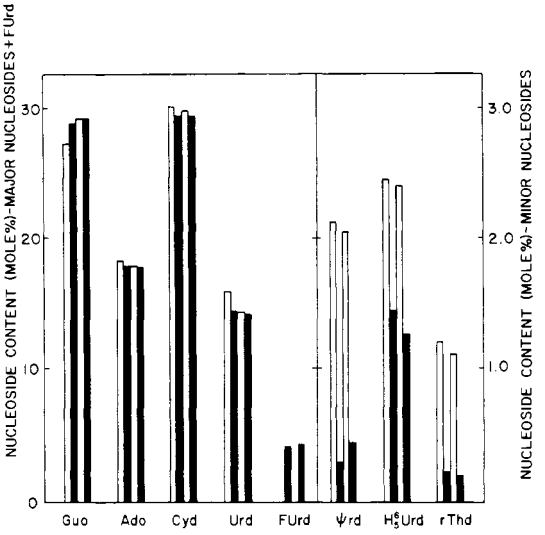

FIGURE 4: In vivo modification of preformed FUra-containing tRNAs in *E. coli*. Cells grown in the presence of FUra were harvested, washed, and reincubated in the absence of FUra, but in medium containing rifampicin. The open bars (□) represent nucleoside contents of control tRNAs isolated from cultures grown with no FUra added. The closed bars (■) represent nucleoside contents of FUra-containing tRNAs isolated from cells treated with FUra. See Methods for details. The first two bars from the left in each set of four represent tRNAs from the first harvest of cells (*minus* and *plus* FUra, respectively), whereas the second two bars correspond to normal and FUra-containing tRNAs isolated *after* reincubation in the presence of rifampicin.

containing tRNA as a substrate. If a FUrd residue has replaced the Urd destined to be thiolated, however, no stable thiolation occurs. This is in keeping with earlier results from this laboratory, which found no evidence for the formation of 4-thio-5-fluorouridine in *E. coli* tRNA (Kaiser, 1969, 1972).

Effect of Fluorouracil Incorporation on Major Nucleosides and Modified Purines. We detected no change in the content of the major nucleotides, except for uridylic acid, in any of the FUra-containing tRNAs (data not shown). In addition, there were no decreases in any of the modified purine nucleosides examined, which included 2-methyladenosine, 6-methyladenosine, 7-methylguanosine, and nucleoside Q. There were some fluctuations in the contents of the nucleosides, but no pattern was apparent. These findings are in agreement with those of others who have examined tRNAs from *E. coli* treated with FUra for modified purine content (Baliga et al., 1969; Lowrie & Bergquist, 1968).

Attempts to Further Modify FUra-tRNAs in Vivo. As a first step in getting some insight into the nature of the inhibition of rThd, Ψrd, and H_6^5Urd synthesis in FUra-tRNAs, we examined the ability of preformed, undermodified FUra-tRNAs to be modified in vivo in the absence of FUra. *E. coli* B cultures were grown for 2 h in the presence of a mixture of FUra and Ura at final concentrations of 20 and 10 μg/mL, respectively. The cells were harvested and washed, and one-half of the culture was resuspended in media lacking FUra, but containing rifampicin—to inhibit any new tRNA synthesis. The second incubation was continued for 2 h with additional rifampicin (50 μg/mL) added after 1 h (see Methods for details). Separate experiments showed that at the concentrations of rifampicin used over 90% of all de novo RNA synthesis was inhibited. Results of the nucleotide analyses of the FUra-containing and control tRNAs grown in the absence and presence of rifampicin are shown in Figure 4. In keeping with the earlier results, the Ψrd, rThd, and H_6^5Urd levels of FUra-tRNAs are reduced dramatically, to levels of 14, 19, and 59% of control values, respectively. These values remained essentially unchanged after an additional 2 h of incubation in the absence of FUra, but in the presence of rifampicin to prevent de novo RNA synthesis. There were also no changes noted in the contents of adenosine, guanosine, and cytidine. Urd showed a slight decrease, but less than could be accounted for from the extent of FUra incorporation (4.3 mol %). Inspection of Figure 3, however, indicates that the Urd content is decreasing at 4 mol % of FUrd and is similar to control tRNA in its Urd composition. This experiment was repeated under conditions where the extent of FUra incorporation into the FUra-tRNAs was 0.5 mol % (data not shown). Subsequent removal of the FUra and continued incubation in the presence of rifampicin gave essentially the results shown in Figure 4. That is, there was no indication that in vivo modification of preexisting FUra-tRNAs was occurring.

The inability of undermodified FUra-RNAs to undergo additional modification in vivo is not the result of dead cells. It also is not caused by the inability of undermodified, mature FUra-tRNAs to be modified—at least partially—since we (I. I. Kaiser and R. L. Hancock, 1972, unpublished data; D. A. Frendewey and I. I. Kaiser, 1978, unpublished data) and others (Baliga et al., 1969) have shown *E. coli* FUra-tRNAs capable of accepting methyl groups in vitro with heterologous and homologous methylases. It has also been shown that mature tRNAs that are methyl- and pseudouridine deficient can serve as substrates for ribosylthymidine synthetase and pseudouridine synthetase in vitro (see Shugart, 1978, and references therein; Cortese et al., 1974; Kwong et al., 1977). It is not yet known whether dihydrouridine synthetase can function on mature-sized tRNAs or whether it requires the precursor form. These observations, and those of Tseng et al. (1978) who found reduced activity of ribosylthymidine synthetase in extracts of mammalian cells treated with FUra, suggest that FUra or one of its metabolic products may become tightly bound to the modifying enzymes and inhibit their activity.

If the modifying enzymes are inhibited by FUra or one of its metabolic products in essentially an irreversible manner, then additional enzyme synthesis may be required for modification of preformed FUra-tRNAs. This would presumably require additional modifying enzyme mRNA to be both transcribed and translated, which would not occur in the presence of rifampicin. In in vivo modification experiments where no rifampicin was added, additional RNA synthesis did occur as evidenced by the increase in total tRNA isolated from the second harvest, a decrease in its specific activity, and a decrease in the FUrd content (see Figure 5). This RNA synthesis had only a slight effect on the observed modification of FUra-tRNAs (Figure 5). The increases seen in Ψrd and H_6^5Urd after reincubation in the absence of rifampicin were insignificant. Ribosylthymidine did show a slight increase (0.24 mol %), although it was certainly less than a quantitative conversion.

Examination of FUra-Treated Cells for tRNA Precursor Accumulation. Most of our experiments have involved extracting FUra-treated *E. coli* cells directly with phenol or first breaking the cells and carrying out the extraction on the supernatant, following low-speed centrifugation. Under both procedures, we have seen no evidence for the accumulation of higher molecular weight tRNA precursor material in FUra-treated cells, although contaminating RNAs make the interpretation difficult. To overcome this problem, we have ruptured cells grown in the presence of [^{14}C]FUra and subjected the recovered supernatant from low-speed centrifugation to high-speed centrifugation (150000*g* for 4 h). Most

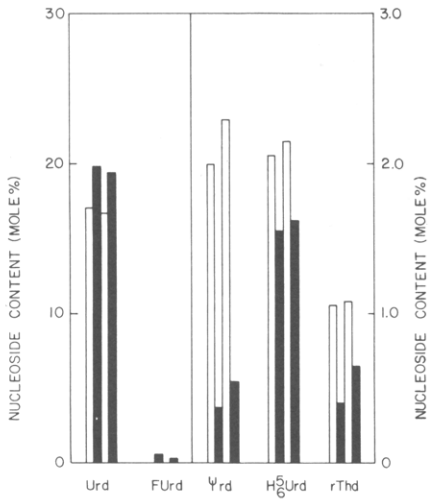

FIGURE 5: In vivo modification of preformed FUra-containing tRNAs in *E. coli* in the absence of rifampicin. Conditions and symbols are as described under Methods section and Figure 4. The second two bars in each set of four correspond to normal and FUra-containing tRNAs isolated after reincubation in the *absence* of rifampicin.

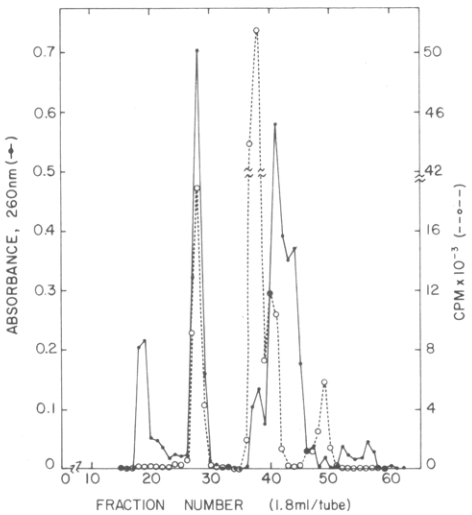

FIGURE 6: Sephacryl S-200–NaDodSO$_4$ chromatography of S-150 supernatant from *E. coli* B treated with [^{14}C]FUra. Approximately 1.68×10^6 cpm of radioactivity (620 μg of protein) was applied to the column and chromatography was carried out as described in the Methods section. Recovery of radioactivity was greater than 90%. The FUra-containing tRNAs eluting between fractions 26 and 30 were shown in a separate experiment to contain 8.4 mol % FUrd. The cpm values correspond to 0.2-mL fraction aliquots.

contaminating [^{14}C]FUra-containing RNAs should be pelleted, leaving primarily mature and higher molecular weight precursor tRNAs in the supernatant. When this S-150 supernatant was made 0.2% in NaDodSO$_4$ and passed directly over a Sephacryl S-200–NaDodSO$_4$ column, no evidence of precursor tRNA was seen (Figure 6). To better examine the tRNA region, the S-150 supernatant was electrophoresed on 6–12% gradient acrylamide gels (Figure 7, slot C). In this figure, the [^{14}C]FUra-tRNA pattern is compared with [^{14}C]FUra-tRNAs and [^{14}C]Ura-tRNAs isolated by phenol treatment of supernatants following low-speed centrifugation of extracts from [^{14}C]FUra- and [^{14}C]Ura-treated *E. coli* cells and recovered from Sephacryl S-200 columns. There are no apparent qualitative changes between the labeled tRNA bands in the absence or presence of S-150 proteins. Compare, e.g., [^{14}C]FUra-tRNA bands electrophoresed in the presence of (slot C) and absence (slot D) of S-150 proteins and [^{14}C]-Ura-tRNA bands in their presence (slot A) and absence (slot

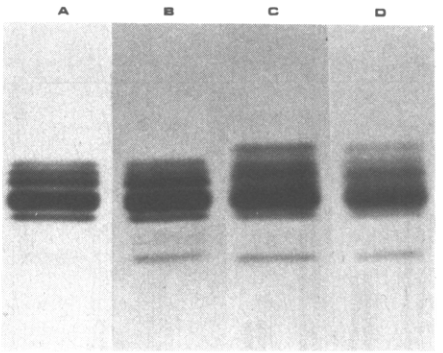

FIGURE 7: Autoradiographs of 6–12% NaDodSO$_4$–acrylamide gels showing [^{14}C]Ura- and [^{14}C]FUra-labeled tRNAs. Slots A and C correspond to patterns obtained by running S-150 supernatants from normal and FUra-containing *E. coli*, respectively, after having been grown in the presence of the radioactive base. Slots B and D represent phenol extracted normal and FUra-containing tRNAs isolated from the same cells and recovered from Sephadex G-75 columns (see Methods). Migration is from top to bottom.

B). There are, however, differences between the normal and FUra-containing tRNA patterns (compare slots B and D). The additional, slow-moving band present in the analogue-containing tRNA samples may represent a small accumulation of monomeric tRNA precursors. If so, they are not separated from other mature tRNAs on Sephacryl S-200 columns since the tRNAs run in slot D were recovered from symmetrically shaped tRNA peaks from the columns (data not shown), similar to that seen in Figure 6. In any case, it is clear that massive accumulations of precursor tRNAs do not occur in *E. coli* cells treated with FUra and that our nucleoside analyses have been carried out on mature-sized molecules. An earlier report did indicate that FUrd interferes with tRNA maturation in *Drosophila* cells (Vogrin, 1976).

In working with the S-150 supernatants, we observed that the majority (74%) of the radioactivity from [^{14}C]Ura-labeled cells was acid precipitable. Sephacryl S-200–NaDodSO$_4$ chromatography confirmed this, in that 86% of the total recovered cpm was eluted in the void volume and tRNA peak (data not shown). In contrast, only 16% of the [^{14}C]FUra-labeled material could be acid precipitated. As may be seen in Figure 6, the majority (80%) of the recovered [^{14}C]FUra label elutes after the tRNA peak. The nature of this low molecular weight radioactive material is currently under investigation.

Discussion

Substitution of FUrd for Urd residues destined to be subsequently modified at the 5 position during the maturation process physically prevents the modification. In addition, there appears to be a second type of indirect inhibition present which preferentially inhibits the formation of three modified pyrimidines derived from Urd, namely, Ψrd, rThd, and H_6^5Urd. The content of all three modified nucleosides is much lower than predicted from a random substitution of Urd by FUrd. It is also clear that there *is not* a preferential incorporation of FUrd into those Urd residues destined to be modified since the decrease in just Ψrd, rThd, and H_6^5Urd exceeds FUrd content at low levels (<4 mol %) of incorporation. Further, there is a concomitant increase in Urd concentration at low levels of FUrd incorporation, which should not be observed if FUra were preferentially incorporated for Urd residues destined to be modified.

Not all modified nucleosides derived from Urd are affected the same way. The content of the sulfur-containing nucleoside, s^{4}Urd, is inversely proportional to FUrd content, indicating

that FUrd substitution for Urd *is* random and that the FUrd is not an acceptable substrate for the thiolase, which is in keeping with earlier results from this laboratory (Kaiser, 1969, 1972).

The specific effects observed only with Ψrd, rThd, and H_6^5Urd indicate that the enzymes responsible for the formation of these modified pyrimidines are being specifically inhibited. Attempts to modify tRNAs in vivo that contain low levels of FUrd, but that are grossly deficient in Ψrd and rThd and partially deficient in H_6^5Urd, were unsuccessful when cells were incubated in either the presence or absence of rifampicin (see Figures 4 and 5). They further suggest that sufficient residual inhibitor is retained by the cells to prevent any newly formed modifying enzyme from effectively acting on modified-nucleoside deficient, preformed FUrd-containing tRNAs. FUra-tRNAs containing low levels of FUrd can be methylated in vitro as shown by Baliga et al. (1969) and others (I. I. Kaiser and R. L. Hancock, 1972, unpublished data; D. A. Frendewey and I. I. Kaiser, 1978, unpublished data), indicating that they are still suitable substrates and are not so grossly altered structurally to be unrecognized by the modifying enzymes. *E. coli* purine base tRNA modifying enzymes do not appear affected by FUra incorporation—even when incorporation levels are high (Baliga et al., 1969; Lowrie & Bergquist, 1968; D. A. Frendewey and I. I. Kaiser, unpublished data). Tseng et al. (1978) also noted reduced ribosylthymidine synthetase activity in mouse liver and mammary tumor cells after FUra treatment. This reduced activity persisted for long periods after treatment and was unaffected by dialysis. The inability to modify the FUra-tRNAs *was not* the result of nonviable cells.

Thymidylate synthetase catalyzes the reductive methylation of 2′-deoxyuridylate to thymidylate by using the coenzyme 5,10-methylenetetrahydrofolate as the one-carbon donor. Cells treated with FUra generate 5-fluoro-5′-deoxyuridylate, which can serve as a quasi-substrate for thymidylate synthetase leading to the formation of a stable, inactive 5-fluoro-2′-deoxyuridylate:methylenetetrahydrofolate:thymidylate synthetase covalent complex (Santi & McHenry, 1972). Whether or not the modifying enzymes responsible for Ψrd and rThd—and to a lesser extent H_6^5Urd formation—are all specifically inhibited in FUra-treated cells by a similar mechanism is presently a matter of speculation. Our results suggest that the mechanisms of formation of the three modified nucleosides do have a common feature, although we have found no evidence for a covalent interaction between highly substituted radioactive FUra-tRNA and crude supernatant extracts of *E. coli* known to have ribosylthymidine and pseudouridine synthetase activity (I. I. Kaiser, unpublished data). While the ribosylthymidine synthetase from *E. coli* uses *S*-adenosylmethionine as the one-carbon donor, the same enzyme from *Streptococcus faecalis* uses 5,10-methylenetetrahydrofolate in the formation of rThd in its tRNAs (Delk et al., 1976). Any covalent bond formed between the *S. faecalis* ribosylthymidine synthetase and FUra residues in FUra-tRNAs would be expected to be stabilized by this coenzyme if the reaction were similar to that found with 5-fluoro-2′-deoxyuridylate and thymidylate synthetase. Yet, in vitro experiments which use a purified ribosylthymidine synthetase from *S. faecalis* and FUra-containing tRNAs from *E. coli* gave no indication of potent inhibition (A. S. Delk, unpublished data). We are in the process of examining FUra-treated *E. coli* cells for a low molecular weight FUra metabolite that inhibits these modifying enzymes.

We found no evidence for the accumulation of precursor tRNAs in FUra-treated *E. coli* cells, indicating that the prior formation of Ψrd, rThd, and H_6^5Urd is not essential for proper processing. Transfer RNA bands of normal and FUra-containing material do, however, exhibit quantitative differences on acrylamide gels (Figure 7). Free 5S rRNA was largely absent from high-speed supernatants from analogue-treated cells, which is in keeping with an earlier report (Johnson et al., 1969).

References

Baliga, B. S., Hendler, S., & Srinivasan, P. R. (1969) *Biochim. Biophys. Acta 186*, 25.

Beck, C. F., & Howlett, G. J. (1977) *J. Mol. Biol. 111*, 1.

Brownlee, G. G. (1972) *Determination of Sequences in RNA*, p 254, American Elsevier, New York.

Cortese, R., Kammen, H. O, Spengler, S. J., & Ames, B. N. (1974) *J. Biol. Chem. 249*, 1103.

Delk, A. S., Romeo, J. M., Nagle, D. P., & Rabinowitz, J. C. (1976) *J. Biol. Chem. 251*, 7649.

Frendewey, D. A., & Kaiser, I. I. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1733.

Giege, R., Heinrick, J., Weil, J. H., & Ebel, J. P. (1969) *Biochim. Biophys. Acta 174*, 43.

Hedgcoth, C., & Jacobson, M. (1968) *Anal. Biochem. 25*, 55.

Heidelberger, C. (1965) *Prog. Nucleic Acid Res. Mol. Biol. 4*, 1.

Horowitz, J., & Chargaff, E. (1959) *Nature (London) 184*, 1213.

Horowitz, J., Ou, C. N., Ishaq, M., Ofengand, J., & Bierbaum, J. (1974) *J. Mol. Biol. 88*, 301.

Hunninghake, D., & Grisolia, S. (1966) *Anal. Biochem. 16*, 200.

Jacobson, M., & Hedgcoth, C. (1970) *Anal. Biochem. 34*, 459.

Johnson, J. L., Yamamoto, K. R., Weislogel, P. O., & Horowitz, J. (1969) *Biochemistry 8*, 1901.

Kaiser, I. I. (1969) *Biochim. Biophys. Acta 182*, 449.

Kaiser, I. I. (1971) *FEBS Lett. 17*, 249.

Kaiser, I. I. (1972) *J. Mol. Biol. 71*, 339.

Kaiser, I. I. (1977) *Arch. Biochem. Biophys. 183*, 421.

Kaiser, I. I., & Young, P. A. (1975) *J. Chromatogr. 111*, 242.

Kaiser, I. I., Jacobson, M., & Hedgcoth, C. (1969) *J. Biol. Chem. 244*, 6707.

Kwong, L. K., Moore, V. G., & Kaiser, I. I. (1977) *J. Biol. Chem. 252*, 6310.

Lowrie, R. J., & Bergquist, P. L. (1968) *Biochemistry 7*, 1761.

Lu, L. W., Chiang, G. H., Tseng, W.-C., & Randerath, K. (1976) *Biochem. Biophys. Res. Commun. 73*, 1075.

Molinar, M., Sheiner, L. B., Neelson, F. A., & Cantoni, G. L. (1968) *J. Biol. Chem. 243*, 1277.

Nishimura, S. (1972) *Prog. Nucl. Acid Res. Mol. Biol. 12*, 49.

Owens, J. R., & Haley, B. E. (1976) *J. Supramol. Struct. 5*, 91.

Santi, D. V., & McHenry, C. S. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1855.

Sarin, P. S., & Zamecnik, P. C. (1964) *Biochim. Biophys. Acta 91*, 653.

Shugart, L. (1978) *Biochemistry 17*, 1068.

Tseng, W.-C., Medina, D., & Randerath, K. (1978) *Cancer Res. 38*, 1250.

Vogrin, V. G. (1976) *Genetics 83*, s80.