Pseudouridine Deficiency in Transfer Ribonucleic Acids from *Escherichia coli* Treated with 2-Thiouracil†

Steve Gurchinoff and Ivan I. Kaiser*

ABSTRACT: Some of the properties of transfer ribonucleic acid from *Escherichia coli* B grown in the presence of 2-thiouracil were examined. These transfer ribonucleic acids were shown to be nearly 50% deficient in pseudouridine. The major nucleotide content was essentially unchanged, as were the levels of dihydrouridine, ribothymidine, and 4-thiouridine. No evidence was found for the incorporation of 2-thiouracil. Cosedimentation on sucrose gradients and cochromatography on Sephadex with normal *E. coli* transfer ribonucleic acids did not reveal any differences. A slight alteration in the thermal denaturation profile was noted in the absence of Mg^{2+}, although this difference disappeared when Mg^{2+} was added. The extent of acceptance for each amino acid examined was about as great or greater than that of the control tRNA, with the exceptions of histidine and phenylalanine which were lower. The pseudouridine deficiency is probably the result of 2-thiouracil or one of its metabolites interfering with the pseudouridine-forming enzyme.

The pyrimidine analog, 2-thiouracil, was first used medicinally in the treatment of hyperthyroidism in the 1940's. Since that time, it has been mainly used as a tool to study cellular metabolism in a variety of systems. In bacteria its reported effects have been somewhat inconsistent. When added to cultures of *Escherichia coli*, for example, Strandskov and Wyss (1945) reported complete inhibition of growth, whereas other workers noted little or no effect (Amos *et al.*, 1958; Kerbiriou and Hervé, 1972). These inconsistencies probably result from growth under different conditions, such as the concentration of potassium ion in the medium (Amos *et al.*, 1958). The most extensive incorporation of the analog into the RNA of a bacterial system was in *Bacillus megaterium*, to the extent of about 5 mol % (Hamers, 1956). In *E. coli* Hamers and Hamers-Casterman (1961) reported SHUra[1] replaced 1–2% of the uracil residues (0.2–0.4 mol %) in RNA. Other workers, including ourselves, have not detected any SHUra incorporation into *E. coli* RNA (Cardeilhac, 1966; Goodrich and Cardeilhac, 1970; Bauernfeind and Grümmer, 1968). This inability to be extensively incorporated into RNA is apparently *not* the result of its inability to be converted to the triphosphate form (Amos *et al.*, 1958; Lindsay *et al.*, 1972; Yu *et al.*, 1973).

Reports have appeared in the literature which suggest that abnormal proteins are synthesized by *E. coli* in the presence of SHUra (Hamers and Hamers-Casterman, 1961; Kerbiriou and Hervé, 1972). These findings, particularly those of Kerbiriou and Hervé, strongly indicate that incorporation of SHUra or other modifications of m- or tRNA does occur in the presence of the analog.

Our findings suggest that if SHUra incorporation does occur, it must be slight. On the other hand, there is a definite deficiency of pseudouridine in the tRNA from 2-thiouracil-treated *E. coli*. This deficiency may be sufficient to alter tRNA function and lead to abnormal protein biosynthesis. With the exception of the pseudouridine deficiency and some anomalies in amino acid acceptance, sU-tRNA was similar to normal in most other properties examined.

Materials and Methods

Growth of Escherichia coli B. Agar slants of *E. coli* B were kindly provided by Dr. P. D. Bear. Overnight cultures of cells (500 ml of media + 1% glucose) were inoculated into 14-l. fermentor vessels (New Brunswick Magnaferm Fermentor) containing 9 l. of the minimal salts media of Davis and Mingioli (1950) along with 500 ml of 20% glucose. Cell growth was continued under vigorous aeration (16,000 cm^3/min) at 37° and growth was followed by measuring turbidity at 660 nm in a Coleman Junior II spectrophotometer using 10 × 75 mm cuvets. Additions of SHUra were made during early exponential growth and incubation was continued for three hours. Uridine-2-^{14}C, when used, was added 5 min after addition of the analog. Growth was terminated by cooling the fermentor and harvesting in a Sharples centrifuge. Viable cell numbers were determined by removing aliquots of the growing cells, making serial dilutions, and then plating the cells on trypticase soy agar plates. The plates were incubated at 37° for 6–8 hr and then counted.

Cells grown in the presence of uridine-2-^{14}C were harvested in a B-20 IEC centrifuge with an 872 rotor at 12,000 rpm for 5 min at 0°. The cell paste was washed in 0.01 M Tris-HCl (pH 7.4)–0.0001 M $MgCl_2$, centrifuged at 10,000*g* in the B-20 (870 rotor) for 15 min at 0°, and stored at −20° until used. Normal cells without the addition of the analog were grown in a similar manner.

Preparation of tRNA. tRNA was prepared by direct phenol–sodium dodecyl sulfate extraction. The cell paste was resuspended in the Tris-HCl buffer mentioned above plus sodium dodecyl sulfate and bentonite to final concentrations of 0.5% and 1–2 mg/ml, respectively. An equal volume of 88% redistilled phenol was then added, and the mixture was placed on a wrist shaker for 20 min at room temperature, cooled, and centrifuged at 0° and 10,000*g* for 15 min. The

† From the Division of Biochemistry and Department of Chemistry University of Wyoming, Laramie, Wyoming 82071. *Received November 27, 1972.* Address correspondence to the Division of Biochemistry. This work was supported in part by Grant No. T-546 from the American Cancer Society. Journal Paper No. 565 of the Wyoming Agricultural Experiment Station.

[1] Abbreviations used are: SHUra, 2-thiouracil; sU-tRNA, tRNA isolated from bacteria grown in the presence of SHUra; A_{260} unit, a unit of material which in a volume of 1 ml will have an absorbance of one at 260 nm when measured in a cell of 1-cm path length.

aqueous supernatant was then aspirated and treated with phenol again. The first phenol layer was extracted with buffer, cooled, and centrifuged as above. The supernatants were combined and the RNA was precipitated with two volumes of cold 95% EtOH at −20°.

The RNA was further purified by the method of Zubay (1966). The final isopropyl alcohol precipitate was suspended in 1.8 M Tris-HCl (pH 8.0 at 37°), incubated at 37° for 90 min to strip tRNA of amino acids (Sarin and Zamecnik, 1964), and passed over long columns (0.9 × 230 cm) of Sephadex G-75 equilibrated with a buffer composed of 0.01 M potassium cacodylate (pH 7.0), 0.15 M KCl, 0.0005 M NaEDTA (standard buffer), and 0.005 M $MgCl_2$. The peak A_{260}-absorbing material was pooled and precipitated with two volumes of cold 95% EtOH at −20° overnight.

Sephadex Chromatography. Cochromatography of normal and ^{14}C-labeled sU-tRNA was carried out at room temperature on columns (0.9 × 230 cm) of Sephadex G-75 equilibrated with standard buffer *plus* Mg^{2+}. The absorbance of each fraction (3 ml) was determined and 2-ml aliquots of the peak fractions were added to vials containing 10 ml of a toluene–Triton 76 scintillation mixture (Noll, 1969) plus 4 drops of formic acid. The samples were counted in a Nuclear-Chicago Unilux IIA scintillation counter.

Ultraviolet Absorbance–Temperature Measurements. A Beckman-Gilford spectrophotometer was adapted for these studies essentially as described by Mandel and Marmur (1968). tRNA samples were diluted with standard buffer, *plus* or *minus* 0.005 M $MgCl_2$, to give starting absorbances between 1.5 and 1.8 A_{260} units. The samples were equilibrated at 25° for at least 30 min before raising the temperature in 5° increments. The temperatures were stabilized at each increment until no further increase in absorbance could be detected, before recording the reading. Absorbances were corrected for dilution as a result of solvent expansion at higher temperatures.

Sucrose Gradients. Linear sucrose gradients (5–20%) were prepared as described by Britten and Roberts (1960) in standard buffer $\pm Mg^{2+}$. Normal tRNA and ^{14}C-labeled sU-tRNA in a ratio of 6 to 1 were layered onto the gradients (0.20-ml total volume in standard buffer $\pm Mg^{2+}$). Centrifugation was carried out in the IEC B-60 ultracentrifuge at 41,000 rpm (195,000*g* average) using the SB-283 rotor for 24 hr at 20°. The tubes were punctured and 30-drop aliquots (approximately 0.75 ml) were collected manually. Each tube was diluted with 0.5 ml of the appropriate buffer and absorbances were determined with the Gilford 240. One milliliter of each tube was then added to 15 ml of toluene–Triton 76 scintillation mixture and counted in a Packard Tri-Carb scintillation spectrometer.

Hydrolysis of tRNA. tRNA was hydrolyzed to the nucleotide or nucleoside level as previously described by Kaiser (1972).

Analyses of Nucleotides and Nucleosides. The four major nucleotides were separated by the paper chromatographic method of Lane (1963) and their relative amounts determined as described (Kaiser, 1969).

Pseudouridylic acid content, based on absorbance measurements, was determined from chromatograms run on Whatman No. 1 in a solvent consisting of 700 ml of *tert*-butyl alcohol, 132 ml of 20.2% HCl, and H_2O to 1 l. (Smith and Markham, 1950). These values are reported relative to uridylic acid (see Table II), because of the poor separation of the other nucleotides in this solvent. The molar extinction coefficient used for pseudouridylic acid was 8.4×10^3 at pH 7.0 (Davis and Allen, 1957).

Pseudouridine and ribothymidine content, based on radioactivity, were determined by two-dimensional thin-layer chromatography of the nucleoside digests of uridine-^{14}C-labeled normal and sU-tRNAs (Hedgcoth and Jacobson, 1968). After locating the appropriate spots under ultraviolet light, they were marked and scraped into vials containing 10-ml of toluene–Triton 76 scintillation mix and counted.

The 4-thiouridylic acid content was determined by comparing the A_{336}/A_{260} ratio of normal and sU-tRNA in neutral buffer (Lipsett, 1965). tRNA samples at a concentration of *ca.* 1 mg/ml were used for the scan from 300 to 380 nm.

The dihydrouridine assays were carried out as previously described (Kaiser, 1971).

Aminoacylation of tRNA. tRNA was aminoacylated by the filter paper disk method essentially as described by Rubin *et al.* (1967). Each reaction mixture contained the following components at the final indicated concentrations: potassium glycylglycinate (pH 8.0 at 24° (0.1 M)), NaATP (0.002 M), magnesium acetate (0.02 M), ammonium chloride (0.005 M), 2-mercaptoethanol (0.01 M), ^{14}C-labeled amino acid (700–3000 pmol), tRNA (0.06–0.4 A_{260} unit), and aminoacyl synthetases (49 μg) (prepared from late-logarithmic phase *E. coli* Q-13 cells according to Muench and Berg (1966), in a final volume of 0.25 ml. When glycine-^{14}C was used in the aminoacylation, the potassium glycylglycinate buffer was replaced with Tris-HCl (pH 8.0 at 37°, 0.1 M). Following incubation at 37° for 20 min, a 100-μl aliquot was withdrawn and pipetted onto 2.2-cm Whatman No. 3 paper disks and washed (Rubin *et al.*, 1967). After drying, the disks were counted in 10 ml of toluene scintillation fluid (4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 l. of toluene) in either a Packard Tri-Carb or Nuclear-Chicago Unilux II scintillation counter at an efficiency of either 60 or 72%, respectively. The aminoacylation values reported in Table III represent the average value of at least three different tRNA concentrations run in duplicate. Blanks not containing tRNA were processed with the samples and the appropriate corrections were made. Under the reaction conditions reported here, incorporation was linearly dependent upon the tRNA concentration. Label precipitated by cold trichloroacetic acid could be solubilized by heating in 7% trichloroacetic acid at 90° for 10–15 min and by incubating in 1.8 M Tris-HCl (pH 8) at 37° for 90 min (Sarin and Zamecnik, 1964).

Materials. Ultra Pure Tris, 2-thiouracil, ribonuclease-free sucrose (density grade, Ultra Pure), and *E. coli* B tRNA were purchased from Schwarz BioResearch and Schwarz/Mann. The enzymes ribonuclease, venom phosphodiesterase, and bacterial alkaline phosphatase were purchased from Worthington Biochemical. Pseudouridine and dihydrouridine were obtained from the Sigma Chemical Co. Dr. Paul T. Wigler (Memorial Research Center, University of Tennessee) generously donated the authentic 2-thiouridine. Uridine-2-^{14}C (50 Ci/mol) was from International Chemical and Nuclear Corp. Radioactive amino acids (^{14}C labeled with specific activities of 25–270 Ci/mol) were purchased from International Chemical and Nuclear Corp., Nuclear-Chicago, and Schwarz BioResearch.

Phenol was distilled *in vacuo* and stored in tightly stoppered flasks at 4°. All other chemicals and reagents were analytical or reagent grade.

Results

Bacterial Growth. The effect of SHUra on the growth rate of *E. coli* B may be seen in Figure 1. Addition of the analog is

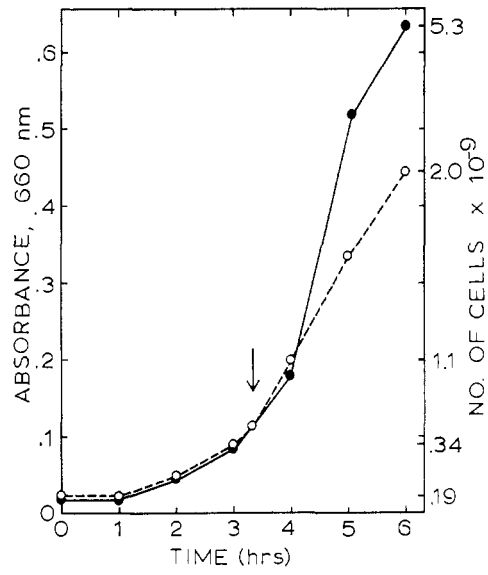

FIGURE 1: Growth curves of *E. coli* B. The arrow indicates the addition of 2-thiouracil to a final concentration of 500 μg/ml: (●) normal; (○) SHUra treated.

indicated by the arrow. Between 4 and 6 hr (termination) the SHUra-treated cells have undergone one doubling while the normal cells have doubled more than two times. Cell counts showed that the number of viable, SHUra-treated cells (2×10^9) represented only about 40% of the number of viable normal cells (5.3×10^9) at the time of harvesting (6 hr). The absorbance at 660 nm of the analog-treated cells (0.445) is 70% of that of the normal cells at termination of growth.

Cochromatography. A mixture of normal tRNA and ^{14}C-labeled sU-tRNA in a ratio of 18 to 1 was passed over long columns of Sephadex G-75 and gave the profile shown in Figure 2. The ^{14}C-labeled sU-tRNA elutes in essentially the same position as the normal tRNA.

Melting Temperatures and Thermal Denaturation. Ultraviolet absorbance–temperature profiles for normal and sU-tRNA may be seen in Figure 3A,B. The corrected absorbance at each temperature divided by the initial absorbance at 25° (A_t/A_{25}) was plotted *vs.* the temperature of the solution (Mandel and Marmur, 1968). In the presence of Mg^{2+}, normal and sU-tRNAs have very similar denaturation profiles and T_m's of

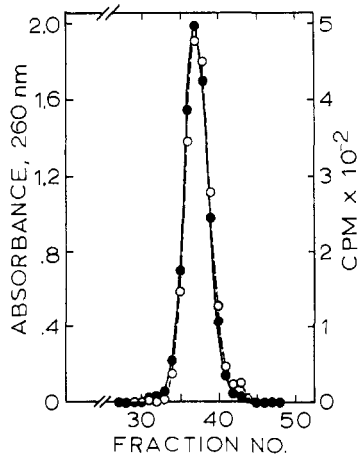

FIGURE 2: Cochromatography of normal and ^{14}C-labeled sU-tRNAs on Sephadex G-75 in standard buffer *plus* Mg^{2+}: (●) absorbance; (○) radioactivity.

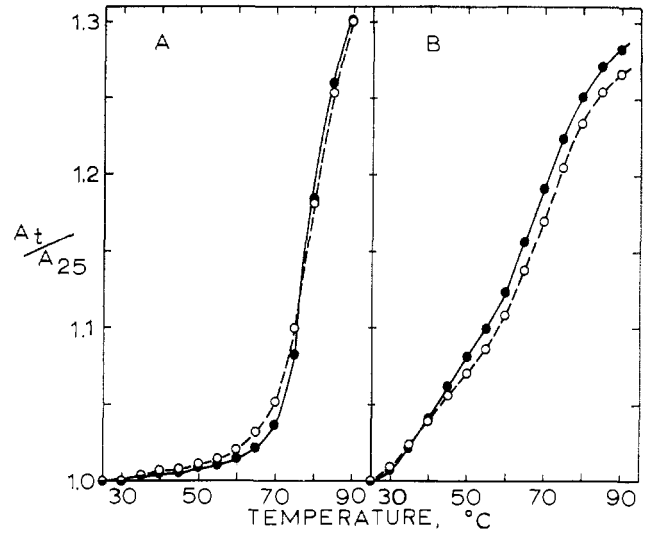

FIGURE 3: Ultraviolet absorbance–temperature profile of normal and sU-tRNA: (A) standard buffer *plus* Mg^{2+}; (B) standard buffer; (●) normal tRNA; (○) sU-tRNA.

78.5 and 78.0°, respectively (Figure 3A). Both samples had a hyperchromicity of slightly more than 26%.

In the absence of Mg^{2+}, the profiles are also similar, although the sU-tRNA exhibits slightly less hyperchromicity than normal tRNA, as shown in Figure 3B (24.2% *vs.* 22.6%). The T_m values for normal and sU-tRNA were 62.5 and 64.5°, respectively.

Nucleotide and Nucleoside Composition of Normal and sU-tRNA. The major nucleotide composition of normal and sU-tRNA is shown in Table I. The three tRNAs have essentially the same composition.

The pseudouridine and ribothymidine content of the tRNAs is shown in Table II. Determinations based on both spectrophotometric and radioactivity measurements showed about a 50% decrease in the pseudouridine content of sU-tRNA relative to normal material. The ribothymidine content was essentially unchanged.

The content of 4-thiouridine was estimated by looking at A_{336}/A_{260} ratios. Absorbance scans of normal and sU-tRNA, in 0.05 M Tris-HCl (pH 7.2) buffer, gave $A_{336}/A_{260} \times 100$ values of 1.96% for normal and 1.39% for sU-tRNA. This represents 71% of the normal value. Lipsett (1965) and others have shown that normal tRNA has an absorbance at 336 nm of 1.5–2.0% of that at 260 nm.

The dihydrouridine content of normal and sU-tRNA was

TABLE I: Base Composition of Commercial, Normal, and sU-tRNAs.[a]

Nucleotide	Commercial tRNA (n = 3)	Normal tRNA (n = 9)	sU-tRNA (n = 12)
A	21.43 ± 0.08	20.58 ± 0.34	20.37 ± 0.58
G	32.09 ± 0.22	31.84 ± 1.16	31.91 ± 1.68
U	17.03 ± 0.26	15.49 ± 0.55	16.58 ± 0.89
C	29.46 ± 0.05	32.09 ± 0.57	31.15 ± 0.92

[a] The nucleotides were separated by paper chromatography (Lane, 1963) as described in Methods. Expressed as mole per cent plus and/or minus standard deviation.

TABLE II: Pseudouridine and Ribothymidine Content of Normal and sU-tRNAs.[a]

	$[\psi/(\psi + U)] \times 100 \pm SD$	
Pseudouridine	Normal tRNA	sU-tRNA
Uridine-^{14}C	13.79 ± 0.06	7.64 ± 0.00
Absorbance	13.49 ± 0.27	6.94 ± 0.67
Absorbance		11.09[b]
Absorbance		7.99[c]
Ribothymidine	$[rT/(rT + U)] \times 100 \pm SD$	
Uridine-^{14}C	7.64 ± 0.07	6.78 ± 0.00

[a] Analyses were carried out on either unlabeled or uridine-^{14}C-labeled normal and sU-tRNAs as described in Methods. [b,c] Values for tRNA extracted from cells grown in the presence of 50 and 1000 μg per ml of 2-thiouracil, respectively.

compared by two different procedures. Method A (Molinaro *et al.*, 1968) showed the dihydrouridine content of normal tRNA to be 2.44 mol % and that of sU-tRNA to be 1.78 mol % (73% of normal). Method B (Jacobson and Hedgcoth, 1970) showed the dihydrouridine content for normal and sU-tRNA to be 2.66 and 2.43 mol % (92% of normal), respectively.

Aminoacylation of Normal and sU-tRNA. Table III shows that tRNA from cells exposed to SHUra for 3 hr accepted all of the 17 amino acids examined. The extent of acceptance for each amino acid was about as great or greater than that of the control tRNA, with the exceptions of histidine and phenylalanine which were lower. The glutamic acid amino acid acceptance activities of both batches of sU-tRNA were elevated over the normal material, with batch II also showing high charging values for five other amino acids (Gln, Gly, Lys, Met, and Val).

The time course of the charging reactions of normal and sU-tRNA (batch II) for lysine and glycine were identical under our reaction conditions. Addition of either more synthetase or tRNA to the reaction mixture, after 20 min of incubation, did not appreciably alter the picomoles of glycine or lysine bound per A_{260} unit of tRNA.

The extent of aminoacylation of the tRNAs seems high, particularly the batch II sU-tRNAs. Even though only seventeen amino acids were examined, the extent of charging was 100%. Possible reasons for these high values will be considered in the Discussion.

TABLE III: Aminoacylation of Normal and sU-tRNAs (Per Cent tRNA).[a]

		sU-tRNA	
	Normal tRNA	Batch I	Batch II
Alanine	3.61	2.85	3.12
Arginine	8.06	6.84	8.39
Asparagine	5.38	5.13	5.03
Aspartic acid	4.53	5.12	5.13
Glutamic acid	2.40	4.19	3.58
Glutamine	2.99	3.58	4.38
Glycine	7.91	9.37	10.38
Histidine	3.06	1.57	2.13
Isoleucine	5.38	4.80	5.19
Leucine	7.52	7.56	8.06
Lysine	5.30	5.52	6.97
Methionine	5.31	5.60	7.32
Phenylalanine	4.15	3.87	2.94
Proline	3.15	3.20	3.54
Serine	4.36	5.25	5.21
Threonine	6.81	5.83	7.28
Valine	8.73	9.08	11.31

[a] tRNA concentrations were determined by measuring the absorbance at 260 nm using the extinction $E^{0.1\%}_{1\,cm} = 23$ (in standard buffer *plus* Mg^{2+}), and assuming an average molecular weight of 27,000. This gave 1610 pmol/A_{260} unit.

Discussion

Growth inhibition of *E. coli* B by 2-thiouracil (500 μg/ml) has been shown to be approximately 60% after 3 hr at 37° (Figure 1). The extent of inhibition varies with the growth medium and the strain of organism used but appears to be at least partially dependent upon the potassium ion concentration. Amos *et al.* (1958) showed, for example, that for complete inhibition of *E. coli* K12 in medium containing 50 mequiv of potassium/l., 800 μg/ml of SHUra was required; with one-tenth as much potassium, only 20 μg of SHUra was needed. The medium used to grow *E. coli* B in this report contained 102 mequiv of potassium/l.

Nucleoside analyses of the tRNA isolated from SHUra-treated *E. coli* cells revealed nearly a 50% reduction in the pseudouridine content, when compared with normal tRNA. tRNA from yeast (*Saccharomyces cerevisiae*) grown in the presence of SHUra also showed a reduced pseudouridine content, with no change in the amount of dihydrouridine (S. Gurchinoff and I. I. Kaiser, unpublished data). The major nucleosides along with the modified pyrimidines, ribothymidine, 4-thiouridine, and 5,6-dihydrouridine, were present in essentially normal amounts. During these analyses, no evidence for the incorporation of 2-thiouracil into sU-tRNA was found. A number of the solvent systems used to separate the hydrolysis products of sU-tRNA were capable of separating authentic 2-thiouridine from the other nucleosides. Amounts present in the order of 1 mol % should have been detected.

When the normal and sU-tRNAs were cochromatographed on Sephadex G-75 and cosedimented on sucrose gradients (not shown) in the presence and absence of Mg^{2+}, no significant differences were noted. These findings suggest that the overall molecular dimensions of both tRNAs are similar. There was a slight difference in the thermal denaturation profile of normal and sU-tRNA in the absence, but not in the presence, of Mg^{2+}. This difference may reflect the deficiency of pseudouridine residues in the hydrogen-bonded regions of the sU-tRNAs.

Earlier reports suggested that SHUra was incorporated into the RNA of tobacco mosaic virus (Jeener and Rosseels, 1953) and *Bacillus megaterium* (Hamers, 1956) in place of uracil to the extent of about 5 mol %. In *E. coli* the incorporation into RNA appears to be much less extensive, even though pathways leading to the formation of 2-thiouridine triphosphate presumably exist (Amos *et al.*, 1958; Lindsay *et al.*, 1972; Yu *et al.*, 1973). Hamers and Hamers-Casterman (1961) reported thiouracil replaced 1–2% of the uracil residues in *E. coli* RNA. This would represent about 0.2–0.4 mol % of the total nucleotide content in the RNA and might not have been detected by our methods. Other workers have reported that

SHUra *is not* incorporated into *E. coli* RNA (Cardeilhac, 1966; Goodrich and Cardeilhac, 1970; Bauernfeind and Grümmer, 1968).

Preliminary work on the functional activities of sU-tRNAs indicate that they are active in accepting amino acids. The reasons for the differences in accepting activity of the samples shown in Table III are not clear, however. They may merely reflect either changes in the relative amounts of the types of tRNAs which are formed, as a result of altered cellular metabolism caused by high concentrations of SHUra, or the selective extraction of tRNAs from the cells. Misacylation cannot be ruled out at the present time, although it seems unlikely since these reactions were carried out with a mixture of tRNAs and synthetases (see Yarus, 1972).

The high percentage of charging may be partially accounted for by asparagine and glutamine deamidases present in the synthetase preparation. Conversion of either or both of these labeled monoamido amino acids to their corresponding acid form during the charging reaction, would yield abnormally high aminoacylation values for these two amino acids. Another possible source of high counts might have been the presence of residual amounts of "other" labeled amino acids in the ^{14}C-labeled amino acid samples, since the other 19 unlabeled amino acids were not added. It should be pointed out, however, that isotope dilution experiments showed the expected decrease in incorporating activity with the concomitant decrease in specific activity for the amino acids examined (Asp, Gly, Met, and Pro).

The biosynthesis of abnormal β-galactosidase (Hamers and Hamers-Casterman, 1961) and aspartate transcarbamylase (Kerbiriou and Hervé, 1972) in *E. coli* grown in the presence of SHUra has been attributed to the incorporation of the analog into the m- and/or tRNA. Although this remains a possibility, the deficiency of pseudouridine in sU-tRNA may also allow abnormal tRNA function at some stage during protein biosynthesis. Recent work with *E. coli* mutants indicate that single base changes in tRNA molecules in places other than the anticodon can result in dramatic functional changes (Anderson and Smith, 1972).

Work by Singer *et al.* (1972) indicates that the *his T* class of mutant cells from *Salmonella typhimurium* lack the enzyme activity which converts uridine to pseudouridine in the anticodon region of tRNA. Lack of this modification is not essential to the life of the cell and in the case of $tRNA^{His}$ isolated from a *his T* mutant shows normal charging and the same affinity for histidyl-tRNA synthetase as normal $tRNA^{His}$ (Brenner and Ames, 1972). The pseudouridine modifications are, however, apparently necessary for the $tRNA^{His}$ to be competent in repression and strongly suggest that they play a regulatory role in $tRNA^{His}$ function (Singer *et al.*, 1972).

The isolation of $tRNA^{Gly}_{IA\text{ and }B}$ from *Staphylococcus epidermidis* is also of interest (Stewart *et al.*, 1971; Roberts, 1972). These tRNAs are completely lacking all minor bases with the exception of 4-thiouridine (Roberts, 1972). They accept glycine (by the same glycine tRNA synthetase that aminoacylates the three other isoaccepting species found in *S. epidermidis*) and participate in peptidoglycan synthesis, but not in protein synthesis (Stewart *et al.*, 1971). The replacement of the common sequence GTψC by the sequence GUGC might explain this change. Additional support for the involvement of this sequence in protein elongation comes from the primary sequence studies of $tRNA_f^{Met}$ from eukaryotic initiator tRNAs (Simsek and RajBhandary, 1972; RajBhandary *et al.*, 1973; Piper and Clark, 1973). Since methionyl-$tRNA_f^{Met}$ in the cytoplasm of eukaryotes does not need to be formylated to initiate protein biosynthesis, it has been proposed that the absence of the GTψC sequence may prevent this tRNA from inserting methionine internally in a protein (Simsek and RajBhandary, 1972). These findings suggest that sU-tRNA, deficient in pseudouridine, may function abnormally in protein biosynthesis.

Formation of pseudouridine, as well as other modified nucleosides, in tRNA occurs after the polymeric chain is formed (Söll, 1971). The inhibition of pseudouridine formation in cells grown in the presence of SHUra probably results from an inhibition of the pseudouridine-forming enzyme by 2-thiouracil or one of its metabolites. This inhibition of pseudouridine formation by 2-thiouracil at the macromolecular level also appears to have its counterpart at the mononucleotide level. In *Agrobacterium tumefaciens* the enzyme pseudouridylate synthetase catalyzes the formation of pseudouridylic acid from uracil and ribose 5-phosphate (Suzuki and Hochster, 1966). *In vitro* studies with a partially purified enzyme preparation from this organism indicate that 2-thiouracil is an effective inhibitor of this enzyme (Y.-C. Liu and C. Hedgcoth, 1972, personal communication). When present at ten times the concentration of uracil, the inhibition is 100%. Heinrikson and Goldwasser (1964) also found an interaction between 2-thiouracil and pseudouridylate synthetase isolated from the protozoan *Tetrahymena pyriformis*. These workers reported that 2-thiouracil could serve as a substrate in place of uracil for the enzyme, although with somewhat less efficiency, and be converted to the nucleotide, presumably 5-ribosyl-2-thiouracil 5′-monophosphate.

There are a number of questions that remain to be answered about this pseudouridine-deficient tRNA. Work is in progress to determine the ability of cells to recover their capacity for pseudouridine biosynthesis after removal of SHUra, the location of the missing pseudouridines, and the effect the deficiency has on other tRNA functions.

References

Amos, H., Vollmayer, E., and Korn, M. (1958), *Arch. Biochem. Biophys. 77*, 236.

Anderson, K. W., and Smith, J. D. (1972), *J. Mol. Biol. 69*, 349.

Bauernfeind, A., and Grümmer, G. (1968), *Z. Naturforsch. B 23*, 564.

Brenner, M., and Ames, B. N. (1972), *J. Biol. Chem. 247*, 1080.

Britten, R. J., and Roberts, R. B. (1960), *Science 131*, 32.

Cardeilhac, P. (1966), *Proc. Okla. Acad. Sci. 47*, 422.

Davis, B. D., and Mingioli, E. S. (1950), *J. Bacteriol. 60*, 17.

Davis, F. F., and Allen, F. W. (1957), *J. Biol. Chem. 227*, 907.

Goodrich, M. E., and Cardeilhac, P. (1970), *Biochim. Biophys. Acta 222*, 621.

Hamers, R. (1956), *Biochim. Biophys. Acta 21*, 170.

Hamers, R., and Hamers-Casterman, C. (1961), *J. Mol. Biol. 3*, 166.

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

Heinrikson, R. L., and Goldwasser, E. (1964), *J. Biol. Chem. 239*, 1177.

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

Jeener, R., and Rosseels, J. (1953), *Biochim. Biophys. Acta 11*, 438.

Kaiser, I. I. (1969), *Biochemistry 8*, 231.

Kaiser, I. I. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 17*, 249.

Kaiser, I. I. (1972), *J. Mol. Biol. 71*, 339.
Kerbiriou, D., and Hervé, G. (1972), *J. Mol. Biol. 64*, 379.
Lane, B. G. (1963), *Biochim. Biophys. Acta 72*, 110.
Lindsay, R. H., Tillery, C. R., and Wong, Yu M. Y. (1972), *Arch. Biochem. Biophys. 148*, 466.
Lipsett, M. N. (1965), *Biochem. Biophys. Res. Commun. 20*, 224.
Mandel, M., and Marmur, J. (1968), *Methods Enzymol. 12B*, 195.
Molinaro, M., Sheiner, L. B., Neelon, F. A., and Cantoni, G. L. (1968), *J. Biol. Chem. 243*, 1277.
Muench, K. H., and Berg, P. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row, p 375.
Noll, H. (1969), *in* Techniques in Protein Biosynthesis, Vol. 2, Campbell, P. N., and Sargent, J. R., Ed., New York, N. Y., Academic, p 101.
Piper, P. W., and Clark, B. F. C. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 30*, 265.
RajBhandary, U. L., Simsek, M., Ziegenmeyer, J., Heckman, J., Petrissant, G., and Ghosh, H. P. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 585 Abstr.
Roberts, R. J. (1972), *Nature (London), New Biol. 237*, 44.
Rubin, I. B., Kelmers, A. D., and Goldstein, G. (1967), *Anal. Biochem. 20*, 533.
Sarin, P. S., and Zamecnik, P. C. (1964), *Biochim. Biophys. Acta 91*, 653.
Simsek, M., and RajBhandary, U. L. (1972), *Biochem. Biophys. Res. Commun. 49*, 508.
Singer, C. E., Smith, G. R., Cortese, R., and Ames, B. N. (1972), *Nature (London), New Biol. 238*, 72.
Smith, J. D., and Markham, R. (1950), *Biochem. J. 46*, 509.
Söll, D. (1971), *Science 173*, 293.
Stewart, T. S., Roberts, R. J., and Strominger, J. L. (1971), *Nature (London) 230*, 36.
Strandskov, F. B., and Wyss, O. (1945), *J. Bacteriol. 50*, 237.
Suzuki, T., and Hochster, R. M. (1966), *Can. J. Biochem. 44*, 259.
Yarus, M. (1972), *Nature (London), New Biol. 239*, 106.
Yu, M. W., Sedlak, J., and Lindsay, R. H. (1973), *Arch. Biochem. Biophys. 155*, 111.
Zubay, G. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davies, D. R., Ed., New York, N. Y., Harper & Row, p 455.

A Molecular Weight Determination of the 16S Ribosomal Ribonucleic Acid from *Escherichia coli*†

Juan P. Ortega‡ and Walter E. Hill*,§

ABSTRACT: Sedimentation equilibrium experiments coupled with an accurate determination of the partial specific volume ($\bar{v}$) were used to obtain a mol wt of 6.4×10^5 for 16S ribosomal RNA from *Escherichia coli*. This value is significantly greater than those previously reported, which were obtained through a combination of sedimentation velocity, viscosity, and light scattering experiments or by chemical methods. This new value for the molecular weight of 16S rRNA implies that there are 260,000 daltons of protein present on the 30S ribosomal subunit and about 1900 nucleotides present in the 16S rRNA strand. It also gives clear evidence that the 30S ribosomal subunit is heterogeneous with respect to its protein complement.

The molecular weight of the 16S ribosomal RNA (rRNA) was first determined by Kurland (1960) using combined sedimentation velocity, viscosity, and light scattering measurements. Later Stanley and Bock (1965) obtained a similar molecular weight using sedimentation velocity and viscosity measurements. Midgley (1965) also obtained the molecular weight of this macromolecule by calculating a value based on the stoichiometry of periodate-oxidized RNA with [*carbonyl*-^{14}C]isonicotinic acid hydrazide. As a result of these studies, the molecular weight of 16S rRNA has been given as 5.5–5.6 $\times 10^5$, with a precision of about $\pm 15\%$.

However, due to the recent studies by Kurland *et al.* (1969), Voynow and Kurland (1971), and Traut *et al.* (1969), showing the possible heterogeneity of the 30S ribosomal protein complement, as well as the meaningful work of Fellner *et al.* (1972) in sequencing the 16S rRNA, it has become apparent that there is a critical need for a more refined determination of the molecular weight of the 16S rRNA. This study was made for this purpose.

Materials and Methods

Escherichia coli strain MRE 600 (RNase I$^-$) supplied by Dr. James Young, University of Wisconsin, was grown in glucose and minimal salts media, harvested in the middle of the logarithmic growth phase, and stored at $-76°$ until needed.

Preparation of 30S Ribosomal Subunits. The 30S subunits were isolated using the method of Hill *et al.* (1969b) except

† From the Department of Chemistry, University of Montana, Missoula, Montana 59801. *Received March 29, 1973.* This research was supported in part by grants from the National Science Foundation (GB17497) and from the U. S. Public Health Service (GM 17436).

* Address correspondence to this author at the University of Montana.

‡ Fellow of the Consejo Nacional de Investigaciones Científicas y Technológicas (CONICIT) de la República de Venezuela.

§ Public Health Service Career Development Awardee (3 K04 GM19692).