Sulfur-Deficient Transfer Ribonucleic Acid. The Natural Substrate for Ribonucleic Acid Sulfurtransferase from *Escherichia coli**

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ABSTRACT: Sulfur-deficient tRNA, isolated after cysteine starvation of *Escherichia coli* HfrC, RC^{rel}, met⁻, cys⁻, λ, was used as the substrate for the crude mixture of *E. coli* RNA sulfurtransferases. This tRNA accepted nearly four times more sulfur *in vitro* than normal tRNA. Sulfur incorporation was inversely proportional to the 4-thiouridine content. The optimal concentrations of all of the required cofactors and substrates, ATP, Mg^{2+}, cysteine, and tRNA were determined. The apparent K_m for cysteine was 2.6×10^{-4} M and that for sulfur-deficient tRNA, 2.7×10^{-6} M. Chromatography of digests of *in vitro* thiolated [^{35}S]sulfur-deficient tRNA on DEAE-cellulose or Dowex (formate) demonstrated qualitative similarity to digests of *in vivo* labeled [^{35}S]tRNA. There were quantitative differences in the two patterns. Hence, 2-thiocytidine appeared to be the major thionucleotide formed during *in vitro* thiolation, while the major *in vivo* product was 4-thiouridine. This difference may be due to the thionucleotide content of sulfur-deficient tRNA, or to loss of activity of the 4-thiouridine sulfurtransferase in the enzyme preparation.

Transfer ribonucleic acid (tRNA) from *Escherichia coli* has been shown to contain four thionucleotides: s^4U[1] (Lipsett, 1965), s^2C, mnm^5s^2U (Carbon *et al.*, 1968), and ms^2i^6A (Burrows *et al.*, 1968). *In vivo* studies showed that the sulfur portion of these bases was derived from cysteine (Peterkofsky and Lipsett, 1965). Shortly thereafter, two laboratories described RNA sulfurtransferase (RNA thiolase) activity in cell-free extracts of *E. coli* (Lipsett and Peterkofsky, 1966; Hayward and Weiss, 1966). In these early studies normal tRNA was used as the substrate for sulfur acceptance. An enzyme system which catalyzes this transfer of sulfur was recently purified from both *Bacillus subtilis* and from *E. coli* (Wong *et al.*, 1970; Abrell *et al.*, 1971). In both these investigations, normal tRNA was used as the primary substrate despite its presumably being fully thiolated. In the second study cited, an experiment was done with a sulfur-poor tRNA obtained through chloramphenicol treatment of a strain auxotrophic for cysteine, but no differences in sulfur acceptance were seen between this and the normal tRNA.

The presumed natural substrate for the sulfurtransferase is a tRNA chain devoid of sulfur. The sulfur atoms are transferred from cysteine to this chain as one of a series of modifications to the newly synthesized tRNA molecule. Despite this, most previous investigations used normal tRNA as the sulfur acceptor. An exception was the use of sulfur-poor tRNA isolated from a cysteine auxotroph after chloramphenicol treatment as a substrate for the s^4U-specific sulfurtransferase (Abrell *et al.*, 1971). Despite its deficiency in s^4U this tRNA was equivalent to normal tRNA in sulfur acceptance. Perhaps a better substrate would be sulfur-deficient tRNA, which was isolated from a cysteine-requiring, RC^{rel} strain of *E. coli* (Harris *et al.*, 1969). This organism, called the C6 strain, produced tRNA deficient in thionucleotides when deprived of cysteine. This tRNA had a higher sulfur-accepting ability *in vivo* as compared to normal tRNA. We now report the use of sulfur-deficient tRNA, a mixture of normal and underthiolated tRNA populations, as the substrate for the mixture of RNA sulfurtransferases from *E. coli*.

Materials and Methods

L-[^{35}S]Cystine (106–120 mCi/mmole) and L-[^{14}C]cysteine (324 mCi/mmole) were obtained from Amersham Searle, Des Plaines, Ill., the dimer being converted to the monomer by a 20-fold excess of 2-mercaptoethanol before use. [^{35}S]Na_2SO_4 (590 mCi/mmole) was purchased from New England Nuclear Corp., Boston, Mass. L-Cysteine, CTAB, and pyridoxal phosphate were products of Sigma Chemical Co., St. Louis, Mo. *E. coli* B tRNA was obtained from Schwarz BioResearch, Orangeburg, N. J. Chromatographic materials were purchased from the following sources: DEAE-cellulose from Eastman Organic Chemicals, Rochester, N. Y., AG-1-X8 (Dowex 1) from Bio-Rad Laboratories, Richmond, Calif., and Dowex 50 (H^+ form) from J. T. Baker Co., Phillipsburg, N. J.

The source and properties of the C6 mutant used here have been described (Harris *et al.*, 1969). *E. coli* Q13, deficient in RNase 1, was a gift of Dr. E. Schuytema.

tRNA Preparation. Normal tRNA was prepared by the method of von Ehrenstein (1967) from log-phase cells of strain C6 grown on fully supplemented medium. This tRNA has a $A_{335}:A_{260}$ absorbance ratio of 0.017, and this ratio was constant for various preparations. Sulfur-deficient tRNA was isolated by the same method from C6 cells starved 6 hr for cysteine. The $A_{335}:A_{260}$ values for these preparations

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[1] The abbreviations used are: s^4U, 4-thiouridine; s^2C, 2-thiocytidine; mnm^5s^2U, 5-methylaminomethyl-2-thiouridine; ms^2i^6A, 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine; ATP, adenosine 5′-triphosphate; CTAB, cetyltrimethylammonium bromide. The 2′- and 3′-nucleotides are indicated by a MP suffix; RNase, ribonuclease. RC^{rel} refers to relaxed control of RNA synthesis as exhibited during amino acid starvation.

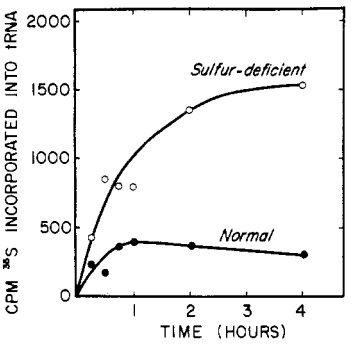

FIGURE 1: Kinetics of ^{35}S incorporation into normal (closed circles) and sulfur-deficient (open circles) tRNA *in vitro*. [^{35}S]Cysteine was added to the standard assay mixture at a specific activity of 20 × 10^6 cpm/μmole. The tRNA preparations were added at equal concentrations and the incubation time at 37° was varied as shown.

ranged between 0.007 and 0.009. *In vivo* labeled [^{35}S]tRNA was prepared from late-log cells of strain C6 grown in complete medium supplemented with 0.5 mCi/l. of [^{35}S]-cysteine.

Enzyme Preparation. The crude RNA sulfurtransferase mixture was prepared from *E. coli* Q13 by a modification of the method of Hayward and Weiss (1966). The 105,000*g* supernatant solution was further purified by application to a DEAE-cellulose column. The activity was adsorbed, and some proteins were removed by washing the column with 0.02 M Tris-HCl (pH 7.5), containing 0.002 M $MgCl_2$ and 0.01 M 2-mercaptoethanol. The enzyme was then eluted with 0.7 M potassium phosphate buffer (pH 7.5). The fractions highest in A_{280}:A_{260} absorbance were pooled and solid ammonium sulfate was added to 80% saturation (25°). The precipitate which formed was collected by centrifugation and dissolved in 0.01 M Tris-HCl (pH 7.5) with 0.001 M 2-mercaptoethanol present. This solution was dialyzed for 3 hr against three changes of the same buffer and stored frozen at −20°.

Enzyme Assay. The sulfurtransferase assay was a modification of that used by Hayward and Weiss (1966). The reaction mixture, per 1.0 ml, contained 61 μmoles of Tris-HCl (pH 8.5), 1.4 μmoles of ATP (adjusted to pH 7), 2.2 μmoles of $MgCl_2$, 6.7 μmoles of 2-mercaptoethanol, 0.027 μmole of pyridoxal phosphate, 340 μg of normal or sulfur-deficient tRNA, 0.23 μmole of [^{35}S]cysteine ranging in specific activity from 12 × 10^6 to 22 × 10^6 cpm per μmole, and 2.6 mg of enzyme. After incubation at 37° the reaction was stopped by the addition of 3 ml of 3.33 mM cysteine, 0.1 ml of 1% tRNA as carrier, and 0.1 ml of 0.5% CTAB. After 1 hr at 0° the precipitated material was collected by centrifugation, dissolved in 0.5 ml of 2.0 M NaCl–0.005 M Tris-HCl (pH 8.0), and then two volumes of 95% ethanol at −20° was added. After at least 1 hr at −20° the precipitate was collected and suspended in 0.5 ml of 0.005 M NaCl–0.5 M Tris-HCl (pH 10.0). This mixture was incubated for 45 min at 37° and extracted once with 88% phenol. The phases were separated by centrifugation and 0.2 ml of the aqueous phase was added to 0.02 ml of 20% potassium acetate (pH 5.4) and then 0.4 ml of 95% ethanol at −20° was added. This mixture was kept at −20° for at least one hour and was then filtered through Gelman VM6 membrane filters (0.45 μ). The filters were washed three times with 3 ml of ethanol–1.0 mM Tris-HCl (pH 7.2) (2:1, v/v), three times with 3 ml of ethanol–ether (2:1, v/v), and twice with the same volume of ether. The dried filters were then counted as previously described (Harris *et al.*, 1969). Under maximal conditions the counting efficiency for ^{35}S was 74%.

Isolation of Thiolated tRNA. The reaction mixture for large-scale sulfurtransferase reactions, using 100 A_{260} units of tRNA, was the same as the standard assay system, scaled up, and optimal levels of all required components were used (see below). [^{35}S]Cysteine was added at a specific activity of 13 × 10^6 to 33 × 10^6 cpm per μmole. After incubation for 90 min at 37° the reaction was terminated by the addition of an equal volume of 88% phenol. After extraction tRNA was precipitated from the aqueous phase as described above. The tRNA was dissolved in 2.0 M Tris-HCl (pH 8.0) and incubated 1 hr at 37° to hydrolyze esterified cysteine. Then 350 μmoles of 2-mercaptoethanol was added to reduce mixed disulfides between [^{35}S]cysteine and tRNA. Finally, the tRNA was precipitated, dissolved in distilled water, and dialyzed 3 hr in the cold against three changes of distilled water. All tRNA solutions were stored frozen at −20°.

Chromatography of tRNA Digests. tRNA was hydrolyzed by 0.3 M KOH for 18 hr at 37°. The digest was carefully adjusted to pH 8 using Dowex 50 (H^+) before chromatography. DEAE-cellulose chromatography was carried out by the method of Lipsett (1965), and a s^4UMP was prepared from a digest of *E. coli* B tRNA using this technique. The method of Carbon (1965) was used for Dowex 1 formate chromatography, and was used to isolate s^2CMP from *in vivo* labeled [^{35}S]tRNA. The absorbance of each fraction was measured and a sample from each was applied to Whatman No. 3MM disks (2.4 cm), which were counted by the scintillation method after they were dry. The urea present in pooled fractions from DEAE-cellulose runs was removed by the method of Rushizky and Sober (1962), and the nucleotides were concentrated by lyophilization.

Paper chromatography of the nucleotide fractions from the column runs was performed according to the method of Carbon *et al.* (1968). Spots were located by their uv absorption and the chromatogram was cut into strips and counted on a Tri-Carb strip counter Model 7201. The counting efficiency for ^{35}S was about 25%.

Analytical Measurements. Absorption of tRNA solutions was measured using either a Zeiss PMQ II or Beckman DU spectrometer. Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Results

Since the tRNA which accumulated in the C6 strain of *E. coli* during cysteine starvation was a good sulfur acceptor *in vivo* (Harris *et al.*, 1969), the sulfur acceptance *in vitro* was measured. The results shown in Figure 1 indicate that sulfur-deficient tRNA accepted between three and four times more sulfur than did the normal tRNA. Incubation for 17 hr resulted in loss of ^{35}S from the RNA fraction, perhaps due to the action of endogenous nuclease. Because this tRNA was a new substrate for the sulfurtransferase system a reevaluation of the requirements for sulfur transfer was necessary. This was done by measuring the effect of the omission of various components of the complete system. The results, shown in Table I, demonstrate that absolute requirements exist for ATP and Mg^{2+}, but not for pyridoxal phosphate. Incorporation in the absence of sulfur-deficient tRNA was probably due to endogenous tRNA in the enzyme preparation. When enzyme concentrations higher than those reported in the table were employed, the differences between the blank and experimental values increased. The addition of pancreatic

TABLE I: Requirements for Sulfur Transfer from [^{35}S]Cysteine to Sulfur-Deficient tRNA.[a]

Component Omitted	Cpm in RNA Fraction
None	551
None (zero time)	318
Enzyme	329
Sulfur-deficient tRNA	378
ATP	341
$MgCl_2$	395
$MgCl_2$, EDTA added	297
Pyridoxal phosphate	568
None, RNase added	319
[^{35}S]Cysteine, [^{14}C]cysteine added[b]	250

[a] The reaction mixture was as described except that 0.45 mg of enzyme protein was used. The specific activity of [^{35}S]cysteine was 22.8 $\times$ 10^6 cpm/μmole. Where indicated, the following additions were made: EDTA, 2.5 μmoles; and pancreatic RNase, 10 μg. Incubation was at 37° for 60 min. [b] [^{35}S]Cysteine was replaced by [^{14}C]cysteine at the same specific activity.

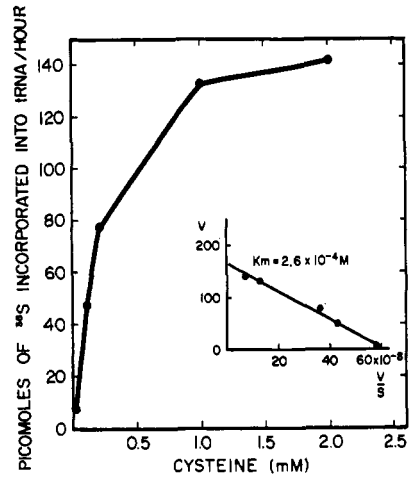

FIGURE 3: Cysteine-dependent incorporation of sulfur into sulfur-deficient tRNA. [^{35}S]Cysteine (1.3 μCi of a 120 μCi/μmole solution) was added to the standard mixture and [^{32}S]cysteine was added to give the desired concentration. The data are corrected for differences in specific activity and for incorporation in the absence of added tRNA. Incubation was for 2 hr at 37°. Inset: Augustinnsson plot of data (Augustinnsson, 1948). Velocity is expressed in pmoles of ^{35}S incorporated per hr and substrate concentration as molarity $\times$ 10^{-3}.

RNase lowered the incorporation to the level of the zero time sample. Finally, the low level of incorporation when [^{14}C]-cysteine was substituted for [^{35}S]cysteine shows that the carbon chain was not transferred to the tRNA molecule. The reaction was dependent on added enzyme as shown in Figure 2. Sulfur incorporation increases linearly up to about 1.0 mg of enzyme after which the sulfur transfer becomes limited by tRNA rather than by added enzyme fraction.

The results shown in Table I prompted us to investigate the effect of varying the concentrations of the required components. The concentrations of $MgCl_2$ and ATP used in the standard assay system were close to being optimal. This was not the case for cysteine. Figure 3 shows that the optimal cysteine level is 1.0 mM, about four times higher than used in the standard system. When the cysteine concentration was raised to this level we found increased incorporation into normal tRNA as well. Preliminary experiments showed that the difference in incorporation between normal and sulfur-deficient tRNA was less at the higher cysteine concentration. This might suggest that nonspecific incorporation occurred at the higher cysteine concentration.

The data obtained in these studies on the effects of varying substrate concentrations can also be analyzed to determine the apparent Michaelis–Menten constants. Since these data are not based on genuine initial velocities, these apparent constants will probably be greater than the true composite constants. The value found for cysteine was 2.6 $\times$ 10^{-4} M (Figure 3) and that for sulfur-deficient tRNA was 2.7 $\times$ 10^{-6} M (Figure 4). An analysis of the data obtained on the thiolation of tRNA over a 90-min period showed that under these

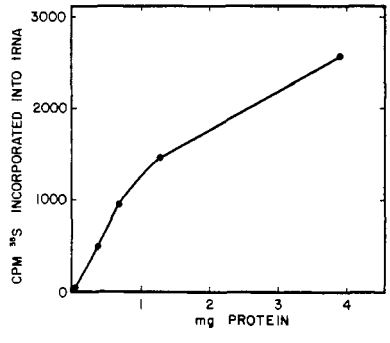

FIGURE 2: Incorporation of sulfur into tRNA as a function of enzyme concentration. Sulfur-deficient tRNA was incubated for 2 hr at 37° in the presence of various amounts of enzyme. The data were corrected for incorporation observed in the absence of added tRNA. The specific activity of [^{35}S]cysteine was 16 $\times$ 10^6 cpm/μmole.

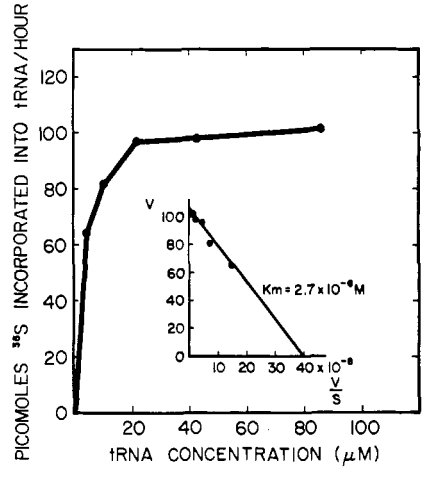

FIGURE 4: Incorporation of sulfur into tRNA as a function of the concentration of sulfur-deficient tRNA. Various amounts of tRNA were incubated for 2 hr in the standard assay mixture. The specific activity of [^{35}S]cysteine was 16 $\times$ 10^6 cpm/μmole. The data were corrected for incorporation in the absence of added tRNA. Inset: the data are plotted by the Augustinnson method. Velocity is expressed as pmoles of ^{35}S per hr and the substrate concentration as molarity $\times$ 10^{-6}.

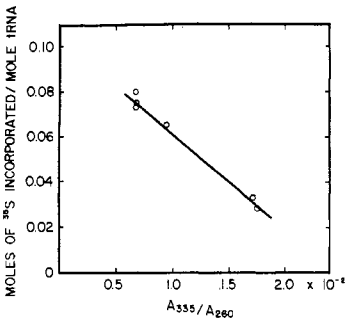

FIGURE 5: The sulfur acceptance of tRNA preparations with different $A_{335}:A_{260}$ values (s^4U content). The reaction mixture was as described except that the cysteine concentration was 1.0 mM (specific activity of 2.2×10^6 cpm/μmole). The reaction mixture was incubated for 90 min at 37°. The molecular weight of tRNA was assumed to be 28,000.

conditions the sulfur transfer was inversely proportional to the orginial concentration of one of the thionucleotides, s^4U (Figure 5). While these data represent neither true initial velocities nor extent of saturation, they do imply a correlation between the total sulfur uptake and the degree of deficiency of one of the thionucleotides.

Characterization of the Reaction Products. To investigate the nature and amount of all thionucleotides formed, digests

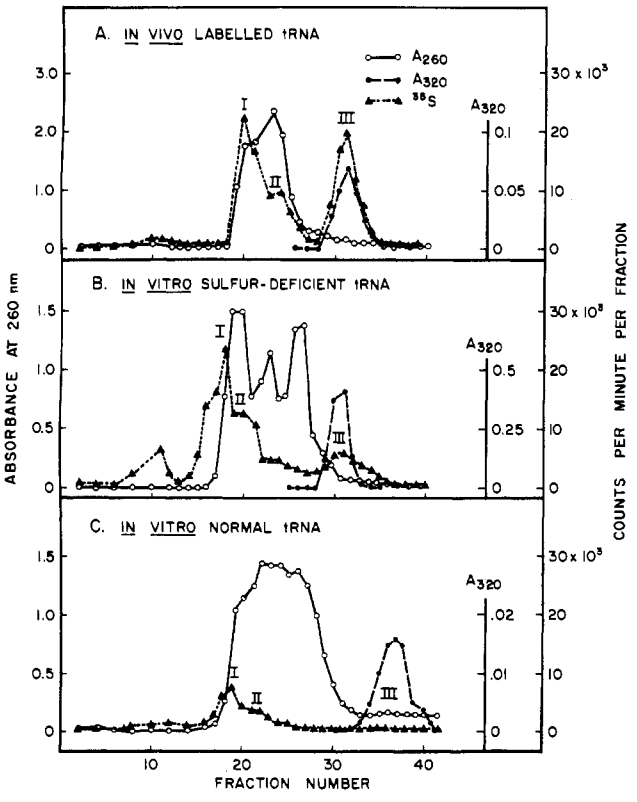

FIGURE 6: DEAE-cellulose chromatography of neutralized digests of various tRNA preparations. Fifty A_{260} units of each tRNA was applied in each case, with various amounts of s^4UMP added as marker. Nucleotides were eluted by a linear gradient of NH_4HCO_3 (pH 8.6) in 7 M urea. The gradient was from 0.05 to 0.25 M in NH_4HCO_3. The volume of each fraction was 3.2 ml. (A) *In vivo* labeled [^{35}S]tRNA isolated from *E. coli* C6. (B) *In vitro* labeled [^{35}S]sulfur-deficient tRNA. (C) *In vitro* ^{35}S-labeled normal tRNA.

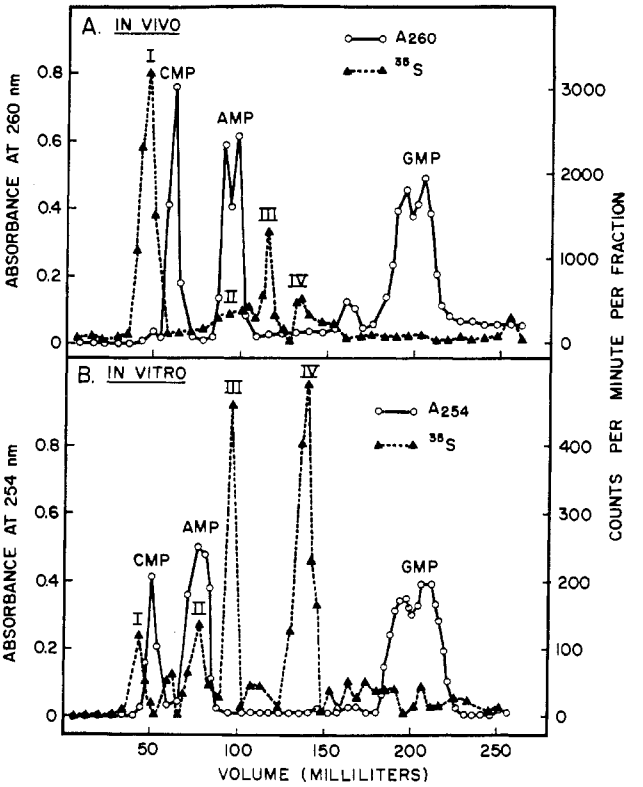

FIGURE 7: Dowex 1 formate chromatography of neutralized digests of *in vivo* and *in vitro* labeled [^{35}S]tRNA. Fifty A_{260} units of each tRNA was applied and the nucleotides were eluted using a formic acid gradient according to the method of Carbon (1965). Absorption at 260 nm was measured in the Zeiss PMQII spectrometer and absorption at 254 nm was taken from an ISCO UA-2 ultraviolet analyzer recording. (A) *In vivo* labeled [^{35}S]tRNA isolated from *E. coli* C6. (B) *In vitro* labeled [^{35}S]sulfur-deficient RNA.

of *in vitro* and *in vivo* labeled [^{35}S]tRNA were chromatographed on DEAE-cellulose according to the method of Lipsett (1965). This technique successfully separated s^4UMP from the other nucleotides as shown in Figure 6A for *in vivo* labeled [^{35}S]tRNA. Equal amounts of incorporation were seen in the s^4UMP (peak III) and mononucleotide regions (peaks I and II). Figure 6B,C shows that *in vitro* less incorporation was seen in the s^4UMP region than in the other parts of the chromatogram. In these studies the possibility of disulfide bond formation is not ruled out so that peaks I and II may be artifically high. Sulfur-deficient tRNA, thiolated *in vitro*, accepted more sulfur in both the s^4UMP and earlier eluting peaks when compared to the acceptance by normal tRNA.

To identify the other thionucleotides formed digests of *in vivo* and *in vitro* labeled [^{35}S]tRNA were chromatographed on Dowex formate (Carbon, 1965). Figure 7 shows that the pattern of sulfur incorporation was similar for these two tRNA preparations, and agreed well with previously reported results (Carbon *et al.*, 1965). According to their identification, peak I was mnm^5s^2UMP and peak III was s^2CMP (Carbon *et al.*, 1968). Labeled cysteine, when chromatographed with unlabeled tRNA, yielded a peak in the region of peak IV of Figure 7. Peak IV appears to be a major product of the reaction. However, since Dowex chromatography of the [^{35}S]tRNA digest results in the loss of 95% of the applied counts, this peak could really represent a much smaller fraction of incorporated sulfur. If these losses were similar in the different experiments, then the *in vitro* system generated comparatively

more s^2CMP than mnm^5s^2UMP whereas the opposite was found for the *in vivo* studies.

Discussion

Sulfur-deficient tRNA was a better sulfur acceptor in the *in vitro* sulfurtransferase system when compared to normal tRNA, presumably containing a full complement of nucleotides. The maximum incorporation was about 1 mole of sulfur into all thionucleotides per 12–14 moles of tRNA. This is considerably higher than reported by Abrell *et al.* (1971) for the s^4U sulfurtransferase purified from *E. coli* namely, 1 mole of sulfur into s^4U per 100 moles of normal tRNA. In addition to their studies of s^4U sulfurtransferase activity with normal tRNA, these investigators reported the use of a sulfur-poor tRNA which was obtained from a cysteine-requiring strain of *E. coli* following chloramphenicol treatment and cysteine deprivation. Although based on the 335:260 absorbancy ratio this tRNA had a low s^4U content, its sulfur acceptance was no greater than that observed for normal, or for Baker's yeast tRNA. Sulfur-deficient tRNA, as isolated from the C6 mutant (Harris *et al.*, 1969), may be closer to the natural, biological substrate for the group of sulfurtransferase enzymes and judging from the amount of sulfur acceptance appears to be clearly different from sulfur-poor tRNA.

The requirements for sulfur transfer were similar to those reported by Hayward and Weiss (1966), but differed somewhat from those cited by Abrell *et al.* (1971). An absolute requirement was shown for ATP, Mg^{2+}, tRNA, and enzyme. No requirement was observed for pyridoxal phosphate, and preincubation of the system was without effect (results not shown). Hence, a definite involvement of β-mercaptopyruvate in sulfur transfer as previously reported was not observed (Lipsett *et al.*, 1967; Wong *et al.*, 1970). This compound was not tested directly, however. Under our conditions incorporation was linear with enzyme concentrations up to 1.0 mg of protein/tube, but increased incorporation was seen at higher levels. The apparent composite Michaelis constant for cysteine was 2.6×10^{-4} M, and for sulfur-deficient tRNA, 2.7×10^{-6} M. The apparent K_m for cysteine is similar to that reported by Wong *et al.* (1970) for *B. subtilis*, but is greater than the K_m reported for the s^4U sulfurtransferase from *E. coli* (Abrell *et al.*, 1971). The value reported here, and the requirements of the system, refer to overall sulfur incorporation due to all of the site-specific sulfurtransferases. Further, the data may differ because sulfur-deficient tRNA is a different substrate than those used by others.

Earlier investigations have discussed the possibility of sulfur exchange being interpreted as sulfur transfer (Hayward *et al.*, 1966; Abrell *et al.*, 1971). This possibility is less likely in our experiments since the sulfur-deficient tRNA was a far better acceptor than the normal sulfur-containing molecule. While referring only to s^4U, the data shown in Figure 5 also buttress the contention that the results shown in this paper reflect sulfur transfer rather than sulfur exchange.

To determine which thionucleotides were formed during the *in vitro* reaction digests of the [^{35}S]sulfur-deficient tRNA reaction product were chromatographed on DEAE-cellulose and Dowex 1 formate. The chromatographic patterns were compared to those for digests of *in vivo* labeled [^{35}S]tRNA, and also to previously reported patterns (Lipsett, 1965; Carbon *et al.*, 1965, 1968). We determined that three of the four thionucleotides in *E. coli* tRNA were labeled in our system, s^4-UMP, mm^5s^2UMP, and s^2CMP. Sulfur incorporation into sulfur-deficient tRNA was greater at the nucleotide level compared to normal tRNA. However, due to the instability of thionucleotides during chromatography we were unable to estimate the exact amounts formed *in vitro*.

The relative proportions of the thionucleotides formed *in vivo* differed from those formed *in vitro*. *In vivo*, s^4UMP amounted to one-half of the total sulfur incorporated into tRNA (Figure 6A), and mnm^5s^2UMP was the major nucleotide recovered from Dowex chromatography. This was not the case for *in vitro* labeled sulfur-deficient tRNA, where s^4UMP was found in a lesser amount than the other thionucleotides (Figure 6B). Of these, s^2CMP was found in the greatest abundance (Figure 7B). These differences could be due to loss of site-specific enzymes during preparation, incomplete cofactor supplementation, or to the thionucleotide content of sulfur-deficient tRNA.

The thiolation of tRNA is envisioned as one of a series of modifications of some precursor molecule, such that the final product is a fully active molecule. This might suggest that thionucleotides are involved in determining the functions of the completed tRNA. Further, the role that a particular thionucleotide plays may depend upon its location in the tRNA chain (Abelson *et al.*, 1970). For example, s^4U is found at the 8th or 9th position from the 5′ end of the chain (Dube and Marker, 1969; Doctor *et al.*, 1969; Gefter and Russell, 1969; Barrell and Sanger, 1969; Nishimura *et al.*, 1969; Ninio *et al.*, 1969), yet has been shown to contribute differently to structure stabilization in these various species (Seno *et al.*, 1969). Other thionucleotides have been found in the anticodon region; ms^2i^6A as the first base next to the 3′ end of the anticodon (Doctor *et al.*, 1969; Gefter and Russell, 1969; Nishimura *et al.*, 1969), and mnm^5s^2U may be present in the anticodon itself (Ohashi *et al.*, 1970). At these positions thionucleotides may be involved in determining codon recognition or ribosome binding (Gefter and Russell, 1969).

We previously reported differences in amino acid acceptance between sulfur-deficient and normal tRNA (Harris *et al.*, 1969). Preliminary experiments now indicate that certain of the affected activities are sensitive to *in vitro* thiolation (Harris and Titchener, 1970). These findings are consistent with the previously reported work showing that aminoacylation can be altered by derivatization of thionucleotides (Reid, 1968; Carbon *et al.*, 1965; Carbon and David, 1968). Future studies using pure tRNA species and site-specific sulfurtransferases may be useful in elucidating the role of the individual thionucleotides in aminoacylation and ribosome binding.

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Template Activities of the ΦX-174 Replicative Allomorphic Deoxyribonucleic Acids*

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ABSTRACT: The template activity of the allomorphs of the replicative form (RF) DNA of ΦX-174 for mRNA synthesis by DNA-dependent RNA polymerase *in vitro* was investigated. When RFI DNA, which does not have a break on either strand, and RFII DNA, derived from RFI by introducing a single-strand break on either strand by DNase, were compared, RFI DNA had a significantly higher rate of transcription. The increased rate was due to the difference of the molecular configuration between these two allomorphs rather than the existence of the nick in RFII. RFI structure increased the stability of the initiation complex between RNA polymerase and the DNA. RNAs synthesized from either RF could be translated to ΦX-174-specific proteins *in vitro*.

The process of transcription of DNA molecules by DNA-dependent RNA polymerase *in vitro* is influenced by at least three factors: environmental conditions such as salt concentration; protein components which may or may not be a constituent of the enzyme; and the molecular structure of the template DNA. For example, the effects of mono- or divalent ions are well documented (Chamberlin and Berg, 1962; Maitra *et al.*, 1967; Richardson, 1969, 1970; Millette and Trotter, 1970). Also, the recent studies of protein factors which have specific roles during the transcription process have led to the discovery of σ (Burgess *et al.*, 1969), ρ (Roberts, 1969), and ψ (Travers *et al.*, 1970). Each has a distinct function during transcription.

The molecular structure of DNA affects the whole transcription scheme as shown in the case of the transcription of denatured DNA (Chamberlin and Berg, 1964a; Sinsheimer and Lawrence, 1964; Bassel *et al.*, 1964). In this case, the RNA product was isolated as an RNA–DNA hybrid until the ratio of template DNA to product RNA reached one. Then free RNA appeared in the reaction mixture. The number of 5′ termini of the product RNA is much greater than the number of RNA polymerase molecules even without adding ρ factor or at low-salt concentration indicating that RNA polymerase can terminate and reinitiate the reaction (Maitra *et al.*, 1967). On the contrary, when native DNA is used as a template under the low-salt condition RNA polymerase stays on the DNA and cannot reinitiate the synthesis of RNA (Bremer and Konrad, 1964). The importance of the configuration or molecular structure of double-stranded DNA for transcription *in vivo* was suggested in the T4 bacteriophage system, in which the DNA must be "competent" to allow

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