ENZYMATIC THIOLATION OF E. COLI SRNA

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Transfer RNA (sRNA[1]), which contains several unusual constituents such as pseudouridine,[2] methylated bases,[3, 4] dihydrouridine,[5] and inosine,[6] has recently been found to contain still another class of minor components, the thionucleotides. These include 4-thiouridine[7] and a 2-thiopyrimidine,[8] although there are more members of this class as yet unidentified.[9] In the case of the methylated bases, the methyl groups have been found to arise as additions to the four common bases in the polynucleotide chain of an sRNA precursor.[10] The present work indicates that the sulfur moieties may also be introduced at the macromolecular level.

Studies with growing cells using the isotope dilution technique have shown that cysteine or cystine, of all the common sulfur intermediates tested, are the most direct donors of the sulfur atom of thionucleotides.[11] Evidence is presented here that in a cell-free system, S^{35}-cystine can donate its sulfur atom to *E. coli* sRNA to form S^{35}-labeled thionucleotides. Some cofactor and acceptor requirements of the reaction are outlined. In addition, data are presented to show that the acceptor pyrimidine of sRNA which results in the formation of 4-thioUMP is a uracil unit.

Materials and Methods.—Yeast and *E. coli* B sRNA were obtained from General Biochemicals, Inc., and further purified by phenol extraction and dialysis. Rabbit liver sRNA was prepared by the method of Cantoni *et al.*[12] C^{14}-cytidine, uniformly labeled, was obtained from Schwarz Bio-Research, S^{35}-cystine from Amersham and from Schwarz BioResearch, and S^{35}-H_2SO_4 from New England Nuclear Corp. Alcohol-resistant membrane filters (Metricel type GA-6) were obtained from Gelman Instrument Company, Ann Arbor, Michigan.

The sulfur-poor sRNA was prepared using mid-log phase cells of *E. coli* 58-161 (met^-), a methionine auxotroph showing "relaxed control" of nucleic acid synthesis. The cells were grown in a minimal medium[13] containing 2×10^{-5} *M* sulfate and 4.2×10^{-5} *M* methionine. The sRNA was prepared by the method of Fleissner and Borek,[10] and showed an absorbance at 335 mμ equal to 0.76 for each 100 A_{260} units, as compared to a normal level of 1.5–1.7 for *E. coli* B.

Ribosomal RNA was prepared from a sample of ribosomes from *E. coli* K12S obtained from Dr. A. Weissbach. The ribosomal pellet from a 105,000 $\times$ *g* centrifugation was washed with water to dissociate polysomes and remove any last traces of sRNA before recentrifuging at 105,000 $\times$ *g* for 3 hr. The pellet was taken up in 0.01 *M* Tris buffer, pH 7.4, and shaken with an equal volume of 90% phenol. The aqueous supernatant was combined with a buffer washing of the phenol layer and precipitated with 2 vol of cold ethanol. The RNA precipitate was dialyzed overnight against distilled water. A spectrum of the material gave no evidence for an absorption peak at 335 mμ.

The cell-free enzyme extract used for the *in vitro* incorporation studies was the S-100 fraction prepared from *E. coli* B according to the method of Nirenberg,[14] and kindly provided by Dr. J. D. Capra. It was stored frozen in small tubes in liquid nitrogen until used.

Paper electrophoresis was carried out with the apparatus of Markham and Smith[15] using a potential of 20 v/cm. Single- and double-channel counting was performed in a Packard liquid scintillation spectrometer, using 10 ml Bray's solution[16] as a solvent.

Results.—The pyrimidine precursor of 4-thioUMP in sRNA: To determine whether 4-thioUMP arises from the substitution of sulfur in the 4 position of uracil or of cytosine in the sRNA molecule, sRNA was prepared from *E. coli* B grown under conditions where the uridine and cytidine components of the nucleic acids

were unequally labeled with C^{14}. The specific activity in the 4-thioUMP isolated from this same sRNA indicated which of the pyrimidine nucleotides had served as the sulfur acceptor.

E. coli B was cultured at 37°C in 3 liters of an inorganic salts medium[13] containing 3 per cent glucose and supplemented with P^{32}-phosphate (0.4 mc/liter). When the A_{650} reached 0.49, 40 mg of uridine, 2 mg of cytidine, and 3.33 μc of C^{14}-cytidine (uniformly labeled) were added to each liter of culture. Aerobic incubation was continued for an additional hour. The bacteria were collected by centrifugation in a refrigerated centrifuge and washed once by resuspension in water. Using the method of Fleissner and Borek,[10] 504 A_{260} units of sRNA were obtained from the 6.55 gm wet weight of cells. This sRNA was digested 18 hr in 0.3 *N* KOH at 37°C, neutralized with solid Dowex-50-H^+ form, and fractionated on a DEAE-cellulose column, using an elution scheme similar to that in Figure 1. The crude column fractions of CMP and UMP were purified by paper electrophoresis in 0.05 *M* formate, pH 3.5, and the 4-thioUMP was isolated by electrophoresis in 0.05 *M* NH_4HCO_3, pH 8.6. Table 1 shows the C^{14}/P^{32} ratios of the starting preparation of sRNA and of samples of CMP, UMP, and 4-thioUMP after DEAE fractionation (crude nucleotide fractions) and after elution from the electrophoretic strips (purified fractions).

Comparison of the specific activity (ratio C^{14}/P^{32}) of the 4-thioUMP with that of CMP and UMP in the purified fractions clearly indicates a correspondence in values between the thionucleotide and UMP. Thus, it is apparent that in the case of 4-thioUMP, the sulfur transfer reaction involves the replacement of an oxygen rather than an amino group from the pyrimidine moiety, as would be the case if the pyrimidine precursor were cytosine. While we were not able in this experiment to isolate a measurable quantity of the 2-thiopyrimidine reported by Carbon *et al.*,[8] the biosynthesis of this nucleotide through a sulfur substitution in the 2 position of either uracil or cytosine in the RNA would also involve replacement of an oxygen.

Synthesis of thionucleotides in a cell-free system: Previous studies with whole cells[11] indicated that cysteine was the source of the sulfur atom of the thionucleotides in *E. coli* sRNA. On the basis of these observations, extracts of *E. coli* were tested for the capacity to incorporate S^{35} from S^{35}-cystine into sRNA. Table 2 shows the incorporation of sulfur into the sRNA in the presence of a dialyzed cell extract, ATP, $MgCl_2$, pyridoxal phosphate, and S^{35}-cystine. The presence of each of these components is required for full activity. In addition to a certain amount of radio-

TABLE 1

THE PRECURSOR OF THE PYRIMIDINE MOIETY OF 4-THIOUMP

Fraction	Corrected (cpm) C^{14}	Corrected (cpm) P^{32}	C^{14}/P^{32}
C^{14}-P^{32} RNA	5449	3991	1.37
C^{14}-P^{32} RNA, alkali-digested	2032	1781	1.14
Crude CMP	774	299	2.58
Purified CMP	1023	313	3.26
Crude UMP	1132	876	1.29
Purified UMP	158	207	0.76
Crude 4-thioUMP	36	51	0.72
Purified 4-thioUMP	32	47	0.67

The fractions were prepared as noted in the text. The samples were dissolved in a total of 1 ml water and mixed with 10 ml Bray's scintillation solvent[16] for counting. All samples were counted on the same day to avoid complications arising from the short half life of P^{32}.

TABLE 2

REQUIREMENTS FOR INCORPORATION OF RADIOACTIVITY FROM S^{35}-CYSTINE INTO sRNA

Component omitted	Cpm incorporated	% Inhibition
None	10,139	—
None (zero time)	1,439	—
ATP	3,038	70
$MgCl_2$	4,225	58
Pyridoxal phosphate	2,141	79
Enzyme	2,307	77
RNA	4,831	53

Incubation mixtures contained, in a volume of 0.2 ml, in μmoles: Tris-chloride, pH 7.4, 20; ATP, 2; $MgCl_2$, 2; S^{34}-cystine, 0.2 (2.5×10^6 cpm/μmole); pyridoxal phosphate, 0.002; *E. coli* sRNA, 10 A_{260} units; cell extract, 0.03 ml. After incubation for 60 min at 37°C, the mixtures were diluted to 1.0 ml and then shaken with 1.0 ml 88% phenol. The phenol phase was re-extracted with another 1.0-ml aliquot of water. The combined aqueous fractions were acidified to 5% TCA, and the precipitated sRNA was collected on Millipore filter disks. Radioactivity was determined by dissolving the Millipore membrane in 10 ml of Bray's[16] scintillation solution.

activity which appears in an unincubated zero-time blank, and which may represent material occluded in the TCA precipitate, there is also an appreciable amount of radioactivity which appears in mixtures incubated without enzyme and in mixtures incubated without sRNA, over and above that in the unincubated blank. The nature of the bound radioactivity in these cases is under investigation.

Since the enzyme system used here might be expected to have cysteine-sRNA synthetase activity, the reisolated sRNA after incubation was treated to remove any amino acids bound through aminoacylation. Mild alkaline treatment (30 min at 37°C, pH 10) did not dissociate any radioactivity from the acid-precipitable portion of the mixture. Furthermore, when 1-C^{14}-cystine was substituted for S^{35}-cystine, there was no appreciable incorporation above a zero-time control. These data suggest that cystine is reacting with the sRNA to donate its sulfur atom but not its complete carbon chain. The sulfur incorporation into sRNA in this system is time-dependent over periods up to 90 min.

In order to determine whether thionucleotides were actually formed in the *in vitro* incubation mixtures, a column chromatographic comparison was made of the alkaline digests of (*a*) sRNA isolated from *E. coli* cultured in medium containing S^{35}-sulfate[11] (*in vivo* labeled sRNA) and of (*b*) sRNA reisolated after incubation with S^{35}-cystine and enzyme extract under the conditions outlined in Table 2 (*in vitro* labeled sRNA). The S^{35}-labeled digests were fractionated on a DEAE-cellulose column as shown in Figure 1.

The elution profile of mononucleotides derived from *in vivo* labeled sRNA (Fig. 1*A*) shows four clearly defined radioactive regions in this case, although peak *III* is a cystine contaminant rather than nucleotide material. The first peak corresponds to the thiopyrimidine described by Carbon *et al.*[8] The second major radioactive fraction (*II*) is a third thionucleotide, which has the acid-lability properties of a purine nucleotide.[9] Absolute chemical identification is not yet available. The remaining radioactive peak, *IV*, which elutes just before pGp, is 4-thioUMP.

Column chromatography of the mononucleotides from *in vitro* labeled sRNA (Fig. 1*B*) indicates roughly the same pattern of radioactivity. To clarify the identification, the column fractions containing the various sulfur-labeled compounds were concentrated and separated by paper electrophoresis in 0.05 *M* NH_4HCO_3, pH 8.6. The mobilities of the thionucleotide portions of peaks *I*, *II*, and *IV* cor-

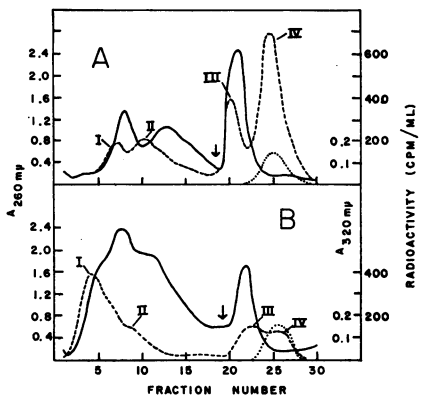

FIG. 1.—DEAE-column chromatography of S^{35}-labeled nucleotides. To neutralized KOH digests of sRNA in 0.015 *M* NH_4HCO_3, pH 8.6, was added 2 A_{330} units of carrier 4-thioUMP. The digests were applied to DEAE-columns (1.2 × 7.8 cm) equilibrated with 0.01 *M* NH_4HCO_3, pH 8.6. Elution was carried out with 0.025 *M* NH_4HCO_3 until the 280/260 ratio of the eluate indicated that most of the CMP, UMP, and AMP had been eluted. At the *arrow*, the eluting fluid was changed to 0.05 *M* NH_4HCO_3, pH 8.6, in 7 *M* urea. Elution was continued until the absorption at 320 mμ had returned to the base line, indicating that GMP and the 4-thioUMP peak were eluted. Fractions of 1.2 ml were collected, and 1-ml samples of each fraction were counted. In the case of the urea-containing samples, 0.2 ml were counted with 0.2 ml ethanol in 10 ml Bray's solution. The *solid line* indicates A_{260}, the *dotted line* A_{320}, and the *dashed line* the radioactivity measurements. Curve *A:* a digest of sRNA prepared from *E. coli* grown in S^{35}-sulfate medium, 50 A_{260} units containing 25,000 cpm S^{35}. Curve *B:* a digest of sRNA labeled by *in vitro* incubation in S^{35}-cystine. For details, see text. The total sample contained 92 A_{260} units, with 20,000 cpm as S^{35}.

responded to those of the three thionucleotides described above, i.e., 9.6, 13.3, and 15.2 cm/hr, respectively. Peak *III* and a portion of peak *I* migrated as nonphosphorylated and probably nonnucleotide material, with mobilities of 0–1 cm/hr. Thus, chromatographic and electrophoretic evidence indicates that thionucleotides are being synthesized on sRNA in the cell-free system.

Specificity for the sulfur-accepting molecule: Several types of RNA were examined for their ability to serve as sulfur acceptors in the system described above. The data are presented in Table 3. The best acceptor in this series is normal sRNA from *E. coli* B, which already contains a natural complement of 4-thiouridine as judged from its spectrum [I(*b*)].[17] To explore the possibility that sRNA which was deficient in thionucleotides might be a better acceptor in the thiolation reaction, such material was prepared. The 4-thioUMP-poor sRNA prepared from *E. coli* 58–161 (see *Methods*) contained about half of the normal complement of 4-thioUMP as measured spectrally, and yet this preparation of sRNA showed essentially the same rate of S^{35}-acceptor activity as the normal sRNA [I(*c*)].

It has already been indicated that uracil is the acceptor pyrimidine for the sulfur in 4-thioUMP synthesis. Since periodate oxidation of 4-thiouridine as the nucleoside or as a component of intact sRNA leads to removal of the sulfur and regeneration of uridine,[7] it was thought that such treatment of sRNA might expose additional sites for thiolation. Accordingly, *E. coli* sRNA was treated at room temperature with 0.0025 *M* sodium metaperiodate until the 335-mμ absorption peak had disappeared (about 3 hr), after which the material was precipitated with ethanol, dialyzed, and lyophilized. A test of the sulfur-accepting activity of one such preparation indicated that it was totally inactive [I(*d*)].

Ribosomal RNA free of sRNA was prepared from *E. coli* K12S as described under *Methods*. The spectrum of a concentrated solution of this material showed no absorption peak in the region of 335 mμ, indicating very little if any 4-thioUMP in the RNA preparation. This material appeared to be slightly active as an acceptor in our system. However, a further test of this apparent activity (expt. II, lines *e*, *f*) suggests that the incorporation observed is nonenzymatic. Yeast

TABLE 3

SUBSTRATE SPECIFICITY FOR INCORPORATION OF RADIOACTIVITY FROM S^{35}-CYSTINE INTO RNA

	RNA added	Incubation time (min)	Cpm incorporated	Cpm incorporated (enzyme omitted)	Cpm incorporated (corrected)*
I (*a*)	None	20	1430	—	—
(*b*)	*E. coli* sRNA	"	4330	670	2900
(*c*)	*E. coli* sRNA (sulfur-deficient)	"	4510	1100	3080
(*d*)	*E. coli* sRNA (periodate-oxidized)	"	1500	780	70
(*e*)	*E. coli* ribosomal RNA	"	1960	1160	530
(*f*)	Yeast sRNA	"	1450	330	20
(*g*)	Rabbit liver sRNA	"	900	280	0
II (*a*)	None	15	535	—	—
(*b*)	None	30	990	—	—
(*c*)	*E. coli* sRNA	15	3514	632	2979
(*d*)	*E. coli* sRNA	30	3974	1158	2984
(*e*)	*E. coli* ribosomal RNA	15	1370	930	835
(*f*)	*E. coli* ribosomal RNA	30	2050	1300	1060
(*g*)	Poly U	15	692	506	157
(*h*)	Poly U	30	592	606	0

Incubations were carried out under the conditions of Table 2. The incubation mixtures contained 0.01 μmole S^{35}-cystine (650,000 cpm). Where indicated, 10 A_{260} units of an RNA were included in the mixture. In experiment I, the reactions were terminated as described in Table 2. In experiment II, the reactions were terminated by shaking with phenol as described in Table 2, and the aqueous extracts were then treated with 0.1 vol of 20% potassium acetate and 2 vol of ethanol. The precipitated RNA was collected on alcohol-resistant filter membranes and counted with 10 ml Bray's solution.

* Cpm corrected for that incorporated in incubation mixtures not containing added RNA.

sRNA, which shows no 4-thioUMP absorption peak,[7] was inactive in this reaction. Rabbit liver sRNA, which also lacks the 4-thioUMP absorption peak, but which shows some of the behavior on iodine oxidation which is tentatively associated with disulfide formation,[8] was not an acceptor in our system. Polyuridylic acid was likewise inactive.

Discussion.—Since the biosynthesis of thionucleotides involves the transfer of sulfur from a cystine-derived donor to the purine or pyrimidine acceptor in the whole sRNA molecule, some thought must be given to the nature of the accepting bases. In particular, the origin of 4-thioUMP was important, since this base participates in the hydrogen-bonding scheme of sRNA.[17] It is assumed that the most likely bonding partner is adenosine. If the sulfur-accepting base were cytosine, sulfur transfer would accomplish the transformation of a potential G-C pair to an A-4-thioU pair. This possibility seemed important enough as a factor in the final secondary structure of sRNA to be worthy of investigation. As it turns out, actual determination of the pyrimidine sulfur acceptor indicates that it is uracil which is transformed into 4-thioU. Therefore, any alteration in base pairing occurring as a result of thionucleotide formation involves the changing of an A-U pair to an A-4-thioU pair, which may affect the stability of the secondary structure, but probably not the over-all shape of the molecule.

The pyridoxal phosphate requirement for sulfur transfer is not unexpected, since this coenzyme is commonly needed for reactions involving β-eliminations from amino acids. In our system this requirement is not absolute, but no rigorous attempt was made to free the enzyme preparation of pyridoxal beyond a thorough dialysis. It may be noted, however, that in an incorporation reaction which showed 35 per cent inhibition without added coenzyme, the presence of 4×10^{-3} *M* hydroxylamine, which is a pyridoxal inhibitor, increased the inhibition to 69 per cent.

Since sRNA which already contains the normally occurring amount of thionucleotides is an adequate substrate, and since our system does not incorporate sulfur into periodate-treated *E. coli* sRNA, which might be supposed to have additional unfilled acceptor sites, the possibility exists that the reaction described here is not a net synthesis of thionucleotides, but rather a partial reaction. A possible interpretation of these observations is that the normal pathway for thiolation of sRNA involves an initial activation of the sRNA acceptor site as a prerequisite for the actual sulfur attachment. Such an activating system may not be present in the enzyme preparation used here, either because of inactivation or depletion of some cofactor. The results reported here may represent exchange through a reversible final step in the over-all reaction leading to thionucleotide synthesis.

Summary.—In whole sRNA, the pyrimidine sulfur acceptor which yields 4-thiouridylic acid is shown to be uracil and not cytosine. Incubation of a dialyzed cell-free extract from *E. coli* with sRNA and S^{35}-cystine leads to labeling of the thionucleotides of the sRNA. The enzymatic reaction requires ATP, magnesium chloride, and pyridoxal phosphate. *E. coli* sRNA serves as a good sulfur acceptor substrate, while *E. coli* ribosomal RNA, periodate-treated *E. coli* sRNA, yeast or rabbit liver sRNA, and polyuridylic acid are inactive.

Elsewhere in this issue, another system is described which will also incorporate sulfur into sRNA.[18]

[1] Abbreviations used are: sRNA, transfer RNA; 4-thioUMP, the 2′(3′) phosphate ester of 4-thiouridylic acid; AMP, CMP, UMP, GMP, the 2′(3′) phosphate esters of adenosine, cytidine, uridine, and guanosine; TCA, trichloroacetic acid; DEAE-cellulose, diethylaminoethylcellulose.

[2] Cohn, W. E., *J. Biol. Chem.*, **235**, 1488 (1960).

[3] Dunn, D. B., J. D. Smith, and P. F. Spahr, *J. Mol. Biol.*, **2**, 113 (1960).

[4] Hall, R. H., *Biochem. Biophys. Res. Commun.*, **13**, 394 (1963).

[5] Madison, J. T., and R. W. Holley, *Biochem. Biophys. Res. Commun.*, **18**, 153 (1965).

[6] Holley, R. W., G. A. Everett, J. T. Madison, and A. Zamir, *J. Biol. Chem.*, **240**, 2122 (1965).

[7] Lipsett, M. N., *J. Biol. Chem.*, **240**, 3975 (1965).

[8] Carbon, J. A., L. Hung, and D. S. Jones, these PROCEEDINGS, **53**, 979 (1965).

[9] Lipsett, M. N., and A. Peterkofsky, unpublished observations.

[10] Fleissner, E., and E. Borek, these PROCEEDINGS, **48**, 1199 (1962).

[11] Peterkofsky, A., and M. N. Lipsett, *Biochem. Biophys. Res. Commun.*, **20**, 780 (1965).

[12] Cantoni, G. L., H. V. Gelboin, S. W. Luborsky, H. H. Richards, and M. F. Singer, *Biochim. Biophys. Acta*, **61**, 354 (1962).

[13] Vogel, H. J., and D. M. Bonner, *J. Biol. Chem.*, **218**, 97 (1956).

[14] Nirenberg, M. W., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1963), vol. 6, p. 17.

[15] Markham, R., and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

[16] Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).

[17] Lipsett, M. N., *Biochem. Biophys. Res. Commun.*, **20**, 224 (1965).

[18] Hayward, R. S., and S. B. Weiss, these PROCEEDINGS, **55**, 1161 (1966).