THE ORIGIN OF THE SULFUR IN s-RNA

Alan Peterkofsky and Marie N. Lipsett

National Institute of Dental Research and National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland

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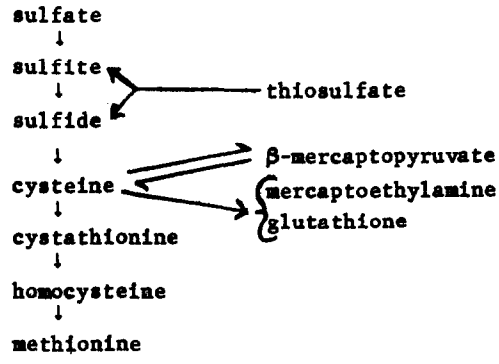
The recent identification of thionucleotides as normal constituents of E. coli s-RNA (Carbon et al., 1965; Lipsett, 1965 a) raises the question of the pathway of biosynthesis of these compounds. Current evidence suggests that sulfur metabolism in enteric bacteria may be represented by the following scheme (Leinweber and Monty, 1963):



This paper presents data indicating that the sulfur which eventually appears in the s-RNA thionucleotides arises more directly from cysteine than from any other of the above listed compounds.

Cultures of E. coli B were grown to late log phase in glucose-salts medium (Vogel and Bonner, 1956) containing S^{35}-labelled inorganic sulfate, 2×10^{-4}M, as the sulfur source. Isotope dilution studies were done by supplying unlabelled sulfur-containing compounds in parallel cultures, usually in tenfold molar excess over the inorganic sulfate. The s-RNA was isolated from the

washed cells by the method of Fleissner and Borek (1962). After treatment with phenol and precipitation of the s-RNA with ethanol, the precipitate was taken up in 0.1 M glycine buffer, pH 10.3, and incubated at 37° for 1 hour to remove any bound amino acids (Zubay, 1962). Radioactivity in the s-RNA preparations was determined by counting samples in a dioxane counting fluid (Bray, 1960) in a liquid scintillation spectrometer.

Some parameter was needed to indicate that the total sulfur content of the various s-RNA preparations was within normal limits if the S^{35}-labelling was to be meaningful. For this purpose, we took advantage of the fact that the absorption peak of 4-thiouridylic acid (4-TU)[1], the major thionucleotide present in this s-RNA (Lipsett , 1965 a, b) occurs at 335 mμ. Hence the concentration of this nucleotide may be determined directly from the spectrum of the intact s-RNA at neutral pH.

None of the isotope dilution studies altered the 4-TU content of the s-RNA signficantly. The absorptions at 335 mμ of the s-RNA preparations examined in this study were within the normal range of 1.28-I.81 per cent of that at 260 mμ.

More than half of the total nucleotide sulfur is present as 4-TU, as is shown in the following experiment. An alkaline hydrolysate of the s-RNA obtained from cultures grown in S^{35}-sulfate was fractionated on a urea-DEAE-cellulose column (Lipsett, 1965 a). At least 55% of the total radioactivity could be accounted for in the 4-TU peak. This represents a lower limit for the nucleotide, since any 4-TU disulfide would not have eluted in this position[2]. The isotope dilution experiments to be reported deal with changes in specific activity of 90-95%. Therefore, it is reasonable to conclude that the results reported here probably apply to the biosynthesis of all the thionucleotides, certainly to the biosynthesis of 4-TU. Actual isolation and determination of the specific activity of the 4-TU component was not carried out routinely.

[1] Abbreviations used are: 4-TU, 4-thiouridylic acid; DEAE-cellulose, diethylaminoethyl cellulose.

Table I shows that the addition of either cystine or cysteine decreased the amount of S^{35} from inorganic sulfate appearing in the s-RNA by

TABLE I

Inorganic S^{35}-sulfate as a Thionucleotide Precursor

Cells were grown in glucose-salts medium containing 2×10^{-4} M S^{35}-sulfate, specific activity 7.85×10^5 cpm/μmole. Yields of s-RNA per gram of wet cells were similar within each experiment.

	Addition*	s-RNA Specific Activity cpm/OD_{260}	Isotope Dilution %
A.	None	1380	
	DL-homocystine	1090	21
	DL-homocysteine	1210	12
	L-cystine	8	99
	L-cysteine	14	99
	DL-methionine	1390	(-1)
B.	None	1740	
	L-cysteine (2×10^{-5}M)	1310	25
	(2×10^{-4}M)	4	99.8
	(2×10^{-3}M)	7	99.6
	D-cysteine	12	99.3
	Mercaptoethylamine	1570	10
	$Na_2S_2O_3$ (1×10^{-3}M)	298	83

*All additions were made at a concentration of 2×10^{-3}M, except where otherwise n

[2]Electrophoresis of our alkaline digests (pH 3.5 formate) revealed four additional S^{35}-labelled nucleotide regions in addition to 4-TU. It is likely that at least one of these activities represents the disulfide of 4-TU, whether natural or artefactual. A second is most probably the thionucleotide reported by Carbon et al. (1965).

about 99%. Isotope dilution is obtained with both D and L isomers of cysteine. These amino acids have been shown to repress the pathway leading from sulfate to cysteine (Ellis et al., 1964) which doubtless explains the extreme isotope dilution observed even when cysteine is added at a level equimolar to inorganic sulfate. The slight amount of isotope dilution observed with homocysteine and homocystine may be ascribed to a limited ability of these compounds to enter the cysteine pool. Similar experiments indicated that methionine and mercaptoethylamine were without effect on labelling of s-RNA in this

system. Added inorganic thiosulfate reduced the specific activity of the s-RNA by roughly the amount expected from the dilution of the labelled sulfate through a pool of thiosulfate tenfold larger.

TABLE II

Short-term Incorporation of S^{35}-sulfate

Cells were grown in a glucose-salts medium containing 2×10^{-4} M unlabelled sulfate until the OD_{650} reached 0.14. At this time, carrier-free sulfate was added (Expt. A, 3.7×10^7 cpm S^{35}-sulfate/liter, Expt. B, 1.9×10^7 cpm/liter) along with the unlabelled sulfur compounds in the concentrations noted. After about two hours' further growth, or when the OD_{650} reached 1.0, the cells were harvested and the s-RNA isolated as usual.

	Addition	s-RNA Specific Activity	Isotope Dilution
		cpm/OD_{260}	%
A.	None	383	
	Cysteine (2×10^{-4}M)	11	97
B.	None	132	
	$Na_2S_2O_3$ (1×10^{-3}M)	9	93
	Na_2S (2×10^{-3}M)	4	97

Since both thiosulfate and sulfide are unstable in solution for long periods, short-term labelling experiments were performed with these compounds. Table II presents the results of experiments in which the S^{35}-sulfate and a tenfold excess of unlabelled thiosulfate or sulfide, or an equimolar amount of unlabelled cysteine, were added to cultures already entering the log phase of growth. Examination of the s-RNA from cells harvested after 2-3 hours' growth under these conditions indicated that all three unlabelled compounds were capable of diluting the amount of label found in the s-RNA.

In order to decide whether thiosulfate, sulfide, or cysteine was the more direct precursor of the thionucleotides, cells were grown in S^{35}-labelled cystine. Supplementation with unlabelled sulfate, thiosulfate, or homocysteine, using up to tenfold molar excess, failed to dilute the specific activity of the s-RNA (Table III). Likewise, neither Na_2S nor cystathionine showed a

preferential sulfur incorporation over cysteine into s-RNA. The limited dilution by glutathione probably is due to a partial degradation of the tripeptide to cysteine.

The addition of a tenfold molar excess of β-mercaptopyruvate, prepared by the method of Kun (1957), depressed the specific activity of the s-RNA to 26% of the control value when S^{35}-cystine was the sulfur source (Table III). It was not clear whether this isotope dilution arose because mercaptopyruvate underwent a transamination reaction to form cysteine, thus diluting the isotope,

TABLE III

S^{35}-L-Cystine as a Thionucleotide Precursor

Expt. A - The cells were grown in glucose-salts medium containing 2×10^{-4} M S^{35}-L-cystine (Amersham), sp. act. 5×10^4 cpm/μmole. Growth was somewhat slower in the homocysteine media, but the yields of s-RNA per gram of wet cells were similar in all cases.

Expt. B - The cells were grown to an OD_{650} of 0.18 in 2×10^{-4} M unlabelled cysteine. At this time, S^{35}-cystine (0.1 μmole, 10^7 cpm) was added to each liter, along with 2×10^{-3} M unlabelled sulfur compound as noted. Cells were harvested after about 2 hours' further growth, when the OD_{650} reached 1.0.

	Addition	s-RNA Specific Activity	Isotope Dilution
		cpm/OD_{260}	%
A.	None	83	
	$MgSO_4$ (2×10^{-4}M)	71	14
	$Na_2S_2O_3$ (1×10^{-4}M)	94	(-13)
	(1×10^{-3}M)	86	(-3)
	DL-homocysteine thiolactone (2×10^{-4}M)	83	0
	(2×10^{-3}M)	87	(-5)
B.	None	130	
	$Na_2S_2O_3$ (1×10^{-3}M)*	110	16
	Na_2S	121	7
	Glutathione	105	20
	Cystathionine	150	(-15)
	β-mercaptopyruvate	33	74

*The 4-TU of this s-RNA preparation had an absorption at 335 mμ of only 0.94% that at 260 mμ.

or whether the mercaptopyruvate was indeed serving as a sulfur donor for thionucleotides without passing through the cysteine pool. In order to distinguish between these two possibilities, E. coli were grown on S^{35}-cystine in the presence or absence of unlabelled mercaptopyruvate. One portion of each culture was used for the preparation of s-RNA, while another portion was used to prepare a crude protein fraction. It is clear from the data of Table IV that the extent of isotope dilution of the s-RNA due to the presence of mercaptopyruvate was essentially the same as the isotope dilution in the protein.

TABLE IV

The Effect of β-Mercaptopyruvate on S^{35} Incorporation from Cystine into s-RNA and into Protein

The cells were grown as in Expt. B of Table III. Preparation of the s-RNA was carried out as usual to the stage of the 100,000 x g supernatant. At this point, one third of the supernatant was used for s-RNA isolation. The remainder was precipitated with an equal volume of saturated $(NH_4)_2SO_4$, the precipitate redissolved and exhaustively dialyzed against distilled water. The yield of protein from the two samples, as determined by the method of Lowry et al. (1951), was essentially the same. Contamination with RNA was negligible, as evidenced by the fact that solutions containing 1 mg/ml protein had absorbancies of only 1.0-1.2 at 260 mμ.

Addition	s-RNA		Protein	
	Specific Activity	Isotope Dilution	Specific Activity	Isotope Dilution
	cpm/OD_{260}	%	cpm/mg	%
None	173		18,600	
β-mercaptopyruvate	56	67	4850	74

It seems most probable, therefore, that mercaptopyruvate added to the cell culture was partially converted to cysteine, thus creating a reduction in the specific activity of the cysteine pool which is reflected in the labelling of both thionucleotides and protein.

The stage at which sulfur is incorporated into the nucleotides of s-RNA is not known. It is difficult to see how thionucleotides can be formed at the nucleotide level without at the same time placing some awkward restrictions on the process of s-RNA replication. On the other hand, it is known that 2-thiouracil can easily be incorporated into nucleotides and thence into ribonucleic

acids (Hamers, 1956; Mandel et al., 1957). The extent of replacement of uracil in these cases is rather large, and there is no evidence that it is replaced in anything but a random manner. In the present case of s-RNA and 4-TU, where the occurrence of the thiopyrimidine is so low, and where it is known that it is not evenly distributed among the various amino acid acceptors (Doctor and Lipsett), the most attractive theory is that introduction of the sulfur from cysteine occurs on the polynucleotide chain of an s-RNA precursor, in a manner analogous to the formation of the methylated bases of s-RNA (Fleissner and Borek, 1962).

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