# A Requirement for β-Mercaptopyruvate in the *in Vitro* Thiolation of Transfer Ribonucleic Acid*

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ABSTRACT: The sulfurtransferase enzyme system which catalyzes the *in vitro* transfer of sulfur from cysteine to the thionucleotides in *Escherichia coli* soluble ribonucleic acid (sRNA) has previously been found to require pyridoxal phosphate. The pyridoxal phosphate is now shown to be required only for the generation of the α-keto acid analog of cysteine, β-mercaptopyruvate, which is active as a cofactor in the over-all reaction: [$^{35}$S]cysteine + sRNA (ATP, $Mg^{2+}$, β-mercaptopyruvate, and sulfurtransferases) → [$^{35}$S]thionucleotides in sRNA. Preincubation of a system containing [$^{35}$S]-cysteine, adenosine triphosphate (ATP), $MgCl_2$, pyridoxal phosphate, and enzyme for 60 min, followed by the addition of acceptor sRNA and a further 30-min incubation, is shown to result in the fixation of a greatly increased amount of sulfur in the sRNA as compared with a control lacking the preincubation. The activating material generated during this preincubation step was isolated on a cation-exchange column and identified as β-mercaptopyruvate by chromatography of its 2,4-dinitrophenylhydrazone. Thus, cysteine (pyridoxal-$PO_4$, $Mg^{2+}$, and enzyme) → β-mercaptopyruvate. β-Mercaptopyruvate was found to be present at the level of 0.5–1% in the commercial [$^{35}$S]cysteine. Removal of this contaminant from the labeled cysteine results in the complete inactivity of that cysteine as a sulfur donor without the preincubation step. The sulfur from β-mercaptopyruvate does not serve as the source of sulfur for the thionucleotides, since $^{35}$S-labeled β-mercaptopyruvate in the presence of unlabeled cysteine in the assay system does not result in the incorporation of radioactivity into the sRNA, while the converse labeling situation does. The formation of an addition compound containing cysteine and β-mercaptopyruvate is postulated as an intermediate in thionucleotide synthesis.

The transfer of sulfur from cysteine to the thionucleotides in *Escherichia coli* sRNA has been shown to occur *in vitro* (Hayward and Weiss, 1966; Lipsett and Peterkofsky, 1966), and one of the reported systems requires the addition of pyridoxal phosphate for maximal activity (Lipsett and Peterkofsky, 1966). The present work demonstrates absolute dependence of sulfur incorporation upon β-mercaptopyruvic acid, the keto acid derivative of cysteine. In the presence of β-mercaptopyruvate, the pyridoxal phosphate requirement is abolished. The sulfur incorporated into the sRNA originates from cysteine, not from the β-mercaptopyruvate, even though activity is shown to depend upon the presence of both components.

## Experimental Methods

*Isolation of β-Mercaptopyruvic Acid Fractions.* Separations were performed on a small cation-exchange column of Bio-Rad AG 50W-X2 ($H^+$ form) by the method of Katz and Comb (1963). The β-mercaptopyruvic acid was collected in 10 ml of 0.05 N HCl eluate and evaporated to dryness from water solution several times to remove the HCl. Isolation of this compound from commercial cystine preparations was carried out after reduction of the cystine with a tenfold excess of 2-mercaptoethylamine (Calbiochem), which was retained on the column. Recovery of β-mercaptopyruvic acid from preincubation mixtures was carried out as follows. The mixture was acidified to 0.05 N HCl, chilled, and centrifuged to remove the protein precipitate, and the supernatant fluid was chromatographed as above. Neither pyridoxal phosphate nor ATP[1] present in the preincubation mixtures was released from the column in the 0.05 N HCl eluate.

*Cysteine Purification.* Cysteine was freed of traces of β-mercaptopyruvate on a similar cation-exchange column. The reduction of disulfides was performed with 2-mercaptoethanol, which was washed off the column with water before the cysteine was eluted. The use of $KBH_4$ to perform the preliminary cystine reduction gave identical results in enzymatic experiments utilizing the cysteine so purified. In either case, the cysteine was recovered from the column by elution with 3 N HCl.

It is possible that the β-mercaptopyruvate represents a product of irradiation of cysteine. Marked generation of β-mercaptopyruvate has often been noted in solutions of [$^{35}$S]cysteine stored frozen for a few weeks.

*Assay System.* The complete assay system is de-

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[1] Abbreviations used: ATP, adenosine triphosphate; TCA, trichloroacetic acid; UMP, uridine monophosphate.

TABLE I: Effect of Preincubation upon Sulfur Transfer into sRNA.

| System | Incorporated Radioactivity (cpm) Total | Net[a] |
|---|---|---|
| Complete, no preincubation | 14,610 | 8,680 |
| Complete, preincubated | 17,090 | 14,935 |
| Preincubated without | | |
| Enzyme | 13,085 | 10,930 |
| Substituted boiled enzyme | 17,110 | 10,780 |
| ATP | 19,685 | 17,535 |
| $MgCl_2$ | 11,585 | 9,430 |
| Pyridoxal phosphate | 11,770 | 9,615 |

[a] Corrected as described in Experimental Methods. The basic assay system included, in a volume of 0.25 ml: 20 μmoles of Tris·Cl, pH 7.8; 2 μmoles of ATP, 2 μmoles of $MgCl_2$, 2 mμmoles of pyridoxal phosphate, 10 $A_{260}$ *E. coli* sRNA, 30 mμmoles of [$^{35}$S]cystine (sp act. $5 \times 10^7$ cpm/μmole), and 0.05 ml of enzyme solution (Lipsett and Peterkofsky, 1966). Each value represents the average of three samples. Preincubation, where included, was carried out for 60 min at 37° in the absence of sRNA. The assay step was incubated for 30 min at 37° after the addition of sRNA and any other missing ingredients. At the end of the assay, the mixture was precipitated with 2 ml of 5% TCA and treated as described in Experimental Methods.

TABLE II: Nonincorporation of Radioactivity into sRNA from Preincubation Product or Cysteine Contaminant.

| Addition | $^{35}$S Incorporation (cpm) Total | Control (enzyme omitted) | Control (sRNA omitted) | Net[a] |
|---|---|---|---|---|
| | Experiment I | | | |
| Purified[b] [$^{35}$S]-cysteine | 507 | 33 | 425 | 49 |
| Preincubation product[b] | 334 | 116 | 176 | 42 |
| Preincubation product + [$^{32}$S]-cysteine | 33 | 27 | 29 | (−23) |
| Preincubation product + [$^{35}$S]-cysteine | 1629 | 150 | 553 | 926 |
| | Experiment II | | | |
| Purified [$^{35}$S]cysteine | 1157 | 64 | 1096 | (−3) |
| Cysteine contaminant[b] + [$^{32}$S]-cysteine | 147 | 27 | 60 | 60 |
| Cysteine contaminant + purified [$^{35}$S]cysteine | 1865 | 195 | 1060 | 610 |
| β-Mercaptopyruvate + purified [$^{35}$S]cysteine | 1945 | 60 | 955 | 730 |

[a] Corrected as described in Experimental Methods. [b] Purification of [$^{35}$S]cysteine, the preincubation product, and the cysteine contaminant is described in Experimental Methods. The assays were carried out as described for the basic assay system in Table I. The assay mixtures contained, where shown, 0.5 mμmole of [$^{35}$S]cysteine (30,000 cpm), 0.1 mμmole of cysteine contaminant (5000 cpm), 0.1 mμmole of preincubation product (5000 cpm), 0.1 mμmole of β-mercaptopyruvate (Kun, 1957), and 0.5 (expt I) or 10 mμmole (expt II) of unlabeled cysteine.

scribed in Table I. The reaction was terminated by the addition of 2 ml of 5% TCA. The mixture was filtered through a cellulose nitrate filter, which was washed with additional TCA and counted in 10 ml of Bray's (1960) solution in a scintillation counter. Nonspecific uptake of labeled cysteine into both protein and sRNA during the incubation (Hayward and Weiss, 1966; Lipsett and Peterkofsky, 1966) necessitates a correction. This correction was determined by incubating one sample without enzyme, another without sRNA, adding back the missing ingredients just before TCA precipitation, and subtracting the fixed counts in those samples from the values determined for the complete system. This method may lead to overcorrection, as evidenced by occasional negative incorporation values, but the routine determination of a more accurate correction became too cumbersome.

In large-scale incorporation studies, the purification procedure described below completely removes the nonenzymatically fixed label from the RNA. In the TCA-precipitation assay, however, the blank corrections which must be made for the protein and RNA components are very vexing. It is possible that the cysteine is being held in mixed disulfide linkages with either protein sulfur or thionucleotide sulfur. Similar blank values are found when [$^{14}$C]cysteine is used, without a corresponding net increase in fixed radioactivity in the complete system. As purification of the enzyme system proceeds (J. S. Norton and M. N. Lipsett, unpublished work), a reduction in the required enzyme protein has correspondingly reduced the blank correction to less than 10% of the total fixed radioactivity.

## Results

Table I indicates that preincubation in the presence of all components except the acceptor sRNA is stimulatory to the incorporation system. The addition of the

TABLE III: Effect of Cysteine Metabolites upon Incorporation of $^{35}S$ from Cysteine into sRNA.

| Addition | $^{35}S$ Incorporation (cpm) | | | |
|---|---|---|---|---|
| | Total | Control (enzyme omitted) | Control (sRNA omitted) | Net[a] |
| | Experiment I | | | |
| None | 20,430 | 1300 | 13,760 | 5,370 |
| Homocysteine | 7,940 | 1300 | 6,745 | (−105) |
| β-Mercaptopyruvate | 32,750 | 1625 | 14,895 | 16,230 |
| β-Mercaptoethylamine (cysteamine) | 9,960 | 935 | 9,560 | (−435) |
| Cysteic acid | 16,180 | 1745 | 7,660 | 6,775 |
| Cysteinesulfinic acid | 17,080 | 2185 | 9,490 | 5,405 |
| S-Methylcysteine | 13,030 | 1005 | 11,945 | 80 |
| Sodium sulfate | 21,340 | 1470 | 14,370 | 5,500 |
| | Experiment II | | | |
| None | 5,510 | 490 | 3,860 | 1,160 |
| β-Mercaptopyruvate | 33,420 | 720 | 19,120 | 13,580 |
| β-Hydroxypyruvate | 3,640 | 385 | 3,760 | (−505) |
| Pyruvate | 6,020 | 465 | 3,470 | 2,085 |
| Serine | 7,010 | 700 | 3,440 | 2,870 |
| $Na_2S_2O_3$ | 4,385 | 490 | 3,035 | 860 |

[a] Corrected as described in Experimental Methods. The incorporation assays were carried out as described for the basic assay system in Table I. The assay mixtures in expt I contained 30 mμmoles of unpurified [$^{35}S$]cystine ($1.8 \times 10^6$ cpm) and in expt II, 12 mμmoles of unpurified [$^{35}S$]cystine ($7 \times 10^5$ cpm) along with 50 mμmoles of unlabeled cysteine.

acceptor sRNA after 60-min preincubation, followed by an additional 30-min incubation, results in the fixation of much more sulfur than is observed in a comparable 30-min assay without the preincubation. The effect is dependent upon the presence in the preincubation medium of pyridoxal phosphate, $Mg^{2+}$, and enzyme, but not ATP.

The products of a preincubation mixture were acidified and separated on a cation-exchange column, as described under Experimental Methods. Cysteine is retained on such a column, along with any other aminated materials which are charged at this pH. A substantial amount of radioactivity which was initially present as cystine was now in a form that was not retained on the column. This material was freed of HCl, concentrated, and tested in an assay system lacking pyridoxal phosphate. In these assays, commercial [$^{35}S$]cystine (Schwarz) was first freed of a labeled contaminant (comprising between 0.5 and 1% of the total radioactivity) as described above. Table II indicates that neither the preincubation product nor the purified [$^{35}S$]cysteine is active by itself, but the two components together result in the fixation of a substantial amount of radioactivity into sRNA. The addition of the $^{35}S$-labeled preincubation product or of the cystine contaminant (shown below to be identical) together with unlabeled cystine do not result in the incorporation of radioactivity into the sRNA. Therefore, these materials seem to facilitate the transfer of sulfur from cysteine, but are themselves not sulfur donors.

Since the generation of this activating material in the preincubation step is dependent upon the presence of cysteine and pyridoxal phosphate in that step, and since the active contaminant isolated from cystine is also sulfur labeled, it seemed probable that the material was formed by a simple alteration of cysteine. A wide variety of unlabeled compounds which could conceivably arise as metabolic products from cysteine was tested. Table III shows that of all the materials tested, β-mercaptopyruvic acid is the only one which stimulates the incorporation system. The addition of this compound in concentrations one-half that of the cysteine is able to bring about the incorporation of sizeable amounts of label into the sRNA. While both pyruvate and serine had some stimulatory activity, they were both less than 20% as active as β-mercaptopyruvate.

The identity of the three fractions found to stimulate the uptake of sulfur from purified cysteine into sRNA (*i.e.*, chemically synthesized β-mercaptopyruvate, the contaminant found in commercial [$^{35}S$]cystine, and the product produced during incubation of cysteine with enzyme and pyridoxal phosphate) was established in the following manner. All three materials were converted into 2,4-dinitrophenylhydrazones by the procedure of Kun and Garcia-Hernandez (1957). For this purpose, the two $^{35}S$-labeled fractions were first mixed with 1 mg of unlabeled β-mercaptopyruvate

TABLE IV: Cofactor Requirements in β-Mercaptopyruvate-Supplemented System.

| Component Omitted | $^{35}S$ Incorporation (cpm) | | | |
|---|---|---|---|---|
| | Total | Control (enzyme omitted) | Control (sRNA omitted) | Net[a] |
| | Experiment I | | | |
| None | 7279 | 1499 | 2715 | 3065 |
| β-Mercaptopyruvate | 2194 | 1602 | 2175 | (−1683) |
| ATP | 5821 | 1135 | 3236 | 1450 |
| $MgCl_2$ | 7882 | 1065 | 2997 | 3820 |
| Pyridoxal phosphate | 6657 | 1239 | 2392 | 3026 |
| | Experiment II | | | |
| None[b] | 3130 | 834 | 153 | 2143 |
| β-Mercaptopyruvate | 1010 | 300 | 445 | 265 |
| ATP and β-Mercaptopyruvate | 935 | 300 | 460 | 175 |
| Pyridoxal phosphate and β-mercaptopyruvate | 775 | 300 | 450 | 15 |

[a] Corrected as described under Experimental Methods. [b] Assay conditions for this one sample were identical with the complete system in expt I, with 0.5 mμmole of purified [$^{35}S$]cysteine and 1 mμmole of β-mercaptopyruvate. The incorporation assays were carried out as described in Table I for the basic assay system. The incubations in expt I included 1 mμmole of β-mercaptopyruvate and 0.5 mμmole of purified [$^{35}S$]cysteine (30,000 cpm). In expt II, unpurified [$^{35}S$]cysteine was used (0.5 mμmole, 30,000 cpm) and the β-mercaptopyruvate was omitted where noted.

carrier. The 2,4-dinitrophenylhydrazine mixtures were extracted with ethyl acetate after 5-days' reaction time, and the solutions were dried with sodium sulfate and concentrated. Paper chromatography was carried out in 83% ethanol–water (Kun and Garcia-Hernandez, 1957) and the resulting chromatograms were scanned in a strip scanner. The known disulfide of the 2,4-dinitrophenylhydrazone of β-mercaptopyruvic acid has an $R_F$ in this system of 0.18, and the sulfur-labeled dinitrophenylhydrazones from the cystine contaminant and from the preincubation product were both found to have $R_F$ values of 0.17 and to travel together with the known carrier spot, which was visible. Further, when the labeled spots were eluted and reduced with 2-mercaptoethanol, the sulfur-labeled material was again found to cochromatograph in this system with the reduced form of known β-mercaptopyruvic acid 2,4-dinitrophenylhydrazone, this time with an $R_F$ of 0.63.

A study was made of the effect of the addition of β-mercaptopyruvate on the cofactor requirements of the system. In a system containing purified [$^{35}S$]-cysteine, as indicated in Table IV, the 30-min incorporation is now shown to be independent of pyridoxal phosphate, and completely dependent upon added mercaptopyruvate. No dependence upon added magnesium is seen, but the system has not been rigorously purified from divalent cations. Only partial dependence upon added ATP is now apparent.

To verify the conclusion that the label introduced into the sRNA in the assay system in the presence of purified [$^{35}S$]cysteine and β-mercaptopyruvate is indeed present as thionucleotide sulfur, an incubation was set up on a large scale using 2 μM purified [$^{35}S$]cysteine (sp act. 24 × $10^6$ cpm/μmole), 20 mμM β-mercaptopyruvate, 80 mM Tris·Cl (ph 7.8), 8 mM ATP, 8 mM $MgCl_2$, and, in each ml of reaction mixture, 40 $A_{260}$ *E. coli* sRNA and 0.6 ml of enzyme solution. After incubation for 30 min at 37°, the reaction was stopped by the addition of an equal volume of 88% phenol. The phenol layer was washed once with water, and the sRNA in the combined washings and aqueous layer was precipitated with ethanol. The precipitate was redissolved in 0.1 M glycine buffer (pH 10.3) and incubated at 37° for 1 hr to remove cysteine held in aminoacyl linkage. At the end of this time, 140 μmoles of 2-mercaptoethanol was added to reduce away any possible cysteine held in mixed disulfide linkage on the sRNA. The material was precipitated with ethanol and exhaustively dialyzed against running distilled water.

This RNA fraction was hydrolyzed with 0.3 N KOH (18 hr, 37°), neutralized, and separated on a DEAE-cellulose column at pH 8.6, as shown in Figure 1. Over 95% of the radioactivity applied to the column was recovered in the three thionucleotide peaks shown in the figure, and in this experiment approximately one-half of this was in the peak coinciding with the 320-mμ absorption peak of 4-thio-UMP. This fraction was pooled, desalted on a small DEAE-cellulose column, and lyophilized, and the material was electrophoresed in 0.05 M $NH_4HCO_3$ buffer (pH 8.6, 100 v, 1.5 hr) to separate any contaminating materials (Lipsett and Peterkofsky, 1966). Strip scanning of the paper electrophoretogram showed that the area of radioactivity corresponded to the area of ultraviolet absorption

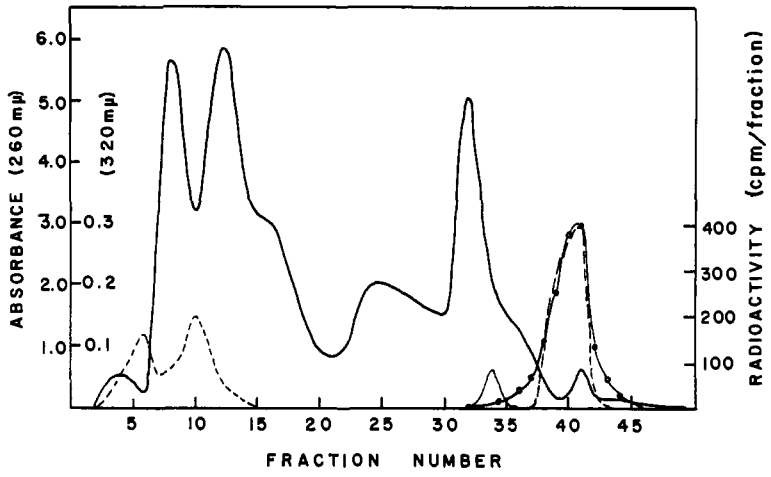


FIGURE 1: DEAE-chromatography of alkali-digested $^{35}S$-labeled sRNA product. The alkaline digest was prepared as described in the text, neutralized, and applied to a DEAE-column (1.4 × 13 cm) equilibrated with 0.01 M $NH_4HCO_3$, pH 8.6. The material was eluted with 0.03 M $NH_4HCO_3$ through fraction 30, at which point the eluting buffer was changed to 0.05 M $NH_4HCO_3$ in 7 M urea. Fractions of 5.8 ml were collected, and 1-ml aliquots were counted in Bray's (1960) scintillation fluid in a liquid scintillation counter. (———) Absorbance at 260 mμ. (●—●—●) Absorbance at 320 mμ. (- - - -) Radioactivity.

of the 4-thio-UMP, with a mobility of 15.2 cm/hr.

A similar incubation was carried out using the same system with the omission of β-mercaptopyruvate. In this case, the recovered sRNA was totally devoid of radioactivity when isolated by the above procedure. A third incubation carried out without β-mercaptopyruvate, but supplemented with 8 μM pyridoxal phosphate to activate the generation of β-mercaptopyruvate *in vitro*, resulted in the fixation of only about half as much radioactivity in the isolated sRNA as was found in the β-mercaptopyruvate-supplemented system. (This is not surprising, since the incubation time was only 30 min, and it is shown in Table III that the 30-min incorporation in a pyridoxal phosphate containing system is markedly stimulated by additional β-mercaptopyruvate. The generation of β-mercaptopyruvate in this system is very inefficient.) The DEAE-cellulose chromatography pattern on this sRNA was identical in shape with that in Figure 1 and to the previously published pattern (Lipsett and Peterkofsky, 1966).

The quantitative aspects of the β-mercaptopyruvate stimulation of sulfur transfer are explored in Table V. The relation between the β-mercaptopyruvate:cysteine ratio and the incorporation of sulfur label indicates that β-mercaptopyruvate is not required in stoichiometric amounts for maximal transfer. For example, while maximal incorporation is shown to occur at a molar ratio of 0.06, the incorporation drops to only about 65% of maximum when the mercaptopyruvate concentration is reduced to 0.01 that of cysteine. At this low level (corresponding to 0.005 mμmole of β-mercaptopyruvate), the net number of micromoles of $^{35}S$ fixed into sRNA is 0.036, or about seven times the amount of mercaptopyruvate present. Other parameters have also been explored in this system. If the mercaptopyruvate:cysteine ratio rises above 0.1, there is a decrease in sulfur fixation which becomes progressively more pronounced as the mercaptopyruvate concentration rises. At a fixed level of mercaptopyruvate (0.05 mμmole), the amount of acceptor sRNA is not limiting, since a fivefold increase in its concentration did not affect the net sulfur fixation. Alteration of the

TABLE V: Effect of β-Mercaptopyruvate Concentration on the Incorporation of Sulfur from Cysteine into sRNA.

| β-Mercaptopyruvate (mμmoles) | Ratio (mercaptopyruvate:cysteine) | $^{35}S$ Incorporation (cpm) Total | Net[a] |
|---|---|---|---|
| None | 0 | 1223 | (−520) |
| 0.005 | 0.01 | 3380 | 1827 |
| 0.01 | 0.02 | 3653 | 1976 |
| 0.02 | 0.04 | 4213 | 2605 |
| 0.03 | 0.06 | 4127 | 2705 |
| 0.05 | 0.10 | 3756 | 2115 |

[a] The correction was determined as in Experimental Methods. These values represent an average of two determinations. The incorporation assays were carried out as described for the basic assay system in Table I. Pyridoxal phosphate was omitted from the assay mixture. β-Mercaptopyruvate was included as the ammonium salt (Kun, 1957) where noted. Each sample contained 0.5 mμmole of purified [$^{35}S$]cysteine (30,000 cpm).

enzyme concentration to one-half or two times the standard amount produced roughly proportional changes in the 30-min incorporation. Doubling the amount of [$^{35}$S]cysteine in the system produced a decrease (less than 20%) in the amount of sulfur fixation. All of these observations indicate that the amount of enzyme is probably the only limiting factor in our assay system.

## Discussion

The addition of β-mercaptopyruvate in catalytic amounts in the reaction mixture has eliminated the need for pyridoxal phosphate, but not for ATP. The data in Table II showing the requirements for the production of β-mercaptopyruvate indicate that ATP is not necessary for this first reaction. However, it must be borne in mind that the molar quantities of β-mercaptopyruvate produced during the 60-min preincubation are far less than the molar quantities of pyridoxal phosphate present. Under these circumstances, a need for ATP in order to regenerate pyridoxal phosphate might not become apparent.

It is unlikely that β-mercaptopyruvate is producing its effect by way of a sulfhydryl protection of the thiolating enzymes themselves, since other sulfhydryl compounds tested produced no such effect, even at levels far above that at which the maximal β-mercaptopyruvate stimulation is seen. In addition, the loss of a pyridoxal phosphate requirement in β-mercaptopyruvate-fortified systems is hard to explain in terms of a protective action of the added sulfhydryl grouping on the enzymatic system.

One possible mechanism by which β-mercaptopyruvate could act to facilitate the transfer of cysteine sulfur in the incorporation pathway to thionucleotides is through the formation of an addition compound containing β-mercaptopyruvate and cysteine. Such a complex could be free or enzyme bound, and in the final steps of the actual sulfur transfer it is likely that the carbon chain of the cysteine moiety is separated from the cysteine sulfur. Incorporation experiments using [$^{14}$C]cysteine (labeled uniformly or in the carboxyl group) have indicated that there is no $^{14}$C either in the sRNA isolated after incubation in the complete enzyme system (Hayward and Weiss, 1966; Lipsett and Peterkofsky, 1966) or in the TCA-precipitable material after the usual background corrections (see Methods). Whether further experimentation will support the hypothesis of such an addition compound remains to be seen, but any postulated mechanism for this sulfurtransferase must provide a role for β-mercaptopyruvate.

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