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## Ribonucleic Acid Sulfurtransferase from *Bacillus subtilis* W168. Sulfuration with $\beta$ -Mercaptopyruvate and Properties of the Enzyme System\*

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**ABSTRACT:** A ribonucleic acid (RNA) sulfurtransferase activity has been isolated from *Bacillus subtilis* W168 and purified some 150-fold; the procedures for enzyme isolation and purification are described. In this system, both cysteine and  $\beta$ -mercaptopyruvate function as sulfur donors; however,  $\beta$ -mercaptopyruvate is far more efficient in this capacity and exhibits a  $K_m$  200–300 times smaller than the  $K_m$  for cysteine. Studies on the properties of the partially purified enzyme

show that sulfurtransferase activity has an absolute requirement for adenosine triphosphate (other nucleotides are inactive), a divalent metal ion, and transfer RNA as the sulfur acceptor. Although other cellular species of RNA and synthetic ribohomopolymers do not accept sulfur in this system, denatured deoxyribonucleic acid prepared from various sources appears to function as a good sulfur acceptor in the *in vitro* reaction.

Several reports from this and another laboratory have described the catalytic transfer of cysteine sulfur to tRNA by cell-free bacterial extracts (Hayward and Weiss, 1966; Hayward *et al.*, 1966; Lipsett and Peterkofsky, 1966; Lipsett *et al.*, 1967). This activity, designated as RNA sulfurtransferase, requires enzyme, magnesium ion, ATP, cysteine as the sulfur source, and tRNA as the sulfur acceptor. A survey of several different microorganisms indicated that crude extracts from *Bacillus subtilis* W168 contained a relatively high level of sulfurtransferase activity and that partial purification of this enzyme activity was possible.

While studying the effects of various sulfur-containing

compounds on this enzyme activity *in vitro*, it was observed that the transfer of radioactive sulfur from [<sup>35</sup>S]cysteine to tRNA was markedly suppressed in the presence of  $\beta$ -mercaptopyruvate. Kinetic data indicated that this thiol keto acid behaved like a competitive inhibitor of the sulfurtransferase reaction; further studies with [<sup>35</sup>S] $\beta$ -mercaptopyruvate indicated that it was a very efficient sulfur donor for tRNA sulfuration, more so than with cysteine. In this communication, we wish to describe the preparation of the sulfurtransferase enzyme from *B. subtilis* W168 and its properties and to examine the nature of the sulfuration activity when either cysteine or  $\beta$ -mercaptopyruvate serves as the sulfur precursor.

### Methods

*B. subtilis* W168 was purchased from Grain Processing Corp., Muscatine, Iowa, and stored at  $-85^\circ$ . Extracts prepared from cells that had been frozen for 1 year or more still maintained most of their activity.

[<sup>35</sup>S]Cystine (crystalline) was purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y., and converted into [<sup>35</sup>S]cysteine by the following treatment. The labeled compound was dissolved in 0.02 M mercaptoethanol, adjusted

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to contain 0.05 N HCl, and adsorbed onto a  $1 \times 5$  cm column of Dowex 50( $H^+$ ), previously equilibrated with 0.05 N HCl. The column was washed discretely with 20 ml each of 0.05 N, 0.50 N, and 3 N HCl, respectively. Radioactive cysteine was eluted with 3 N HCl; these fractions were combined and dried *in vacuo* over pellets of sodium hydroxide. The [ $^{35}S$ ]-cysteine was dissolved in  $H_2O$ , adjusted to an appropriate concentration, and used directly in the experiments described here.

[ $^{35}S$ ] $\beta$ -Mercaptopyruvate (as the crystalline ammonium salt) and [ $^{14}C$ ]cystine were purchased from New England Nuclear Corp., Boston, Mass. [ $^{35}S$ ] $\beta$ -Mercaptopyruvate was dissolved in water and used directly. [ $^{14}C$ ]Cystine was purified and converted to [ $^{14}C$ ]cysteine by the procedure described for [ $^{35}S$ ]cystine.

Crystalline nonradioactive ammonium  $\beta$ -mercaptopyruvate was a generous gift from Dr. Ernest Kun (Kun, 1957) and was recrystallized once before its use. 2- and 3-mercaptopyruvic acids were gifts from Evans Chemetics, Inc., New York, N. Y.

**Enzyme Preparation.** SPINCO SUPERNATANT. All operations were conducted at 0–4°. Frozen cells (80 g) were suspended at 1 g/5 ml in a solution containing 0.02 M Tris (pH 7.5) and 0.002 M  $MgCl_2$ . This suspension was subjected to sonic oscillation with a Branson Sonifier and then centrifuged for 20 min at 28,000g. The supernatant was centrifuged at 30,000 rpm for 3 hr in a Spinco No. 30 rotor, and the clear supernatant was collected with care to avoid agitation of the loosely packed material.

**Ammonium Sulfate Treatment (60–80 Fraction).** Solid ammonium sulfate was added to the Spinco supernatant (0.368 g/ml) over a 15-min period with stirring to 60% of saturation. Stirring was continued for another 15 min, and the suspension was subjected to centrifugation at 28,000g for 20 min. The supernatant was then adjusted to 80% saturation with ammonium sulfate by adding 0.132 g/ml; the precipitate was collected by centrifugation as above, and then resuspended in 20 ml of 0.02 M Tris (pH 7.5) and stored at –85° overnight.

**Calcium Phosphate Gel Treatment (A-enzyme).** The 60–80 fraction was thawed and diluted with 0.02 M Tris (pH 7.5) to give a protein concentration of 5 mg/ml (about 140 ml) as estimated by the following formula: mg of protein per ml =  $1.54A_{280} - 0.76A_{260}$  (Kalckar, 1947). An equal volume of 0.01 M potassium phosphate buffer (pH 7.5) was added with stirring, followed by the addition of a concentrated suspension of calcium phosphate gel (about 20–25 ml) in the proportion of 0.5 mg of solid gel per mg of protein. After 15 min of stirring, the mixture was centrifuged at 28,000g for 20 min and the gel pellet was collected. This pellet was carefully suspended in 100 ml of 0.2 M potassium phosphate buffer (pH 7.5), stirred for 15 min, and then centrifuged as above. The eluate was discarded, and the gel pellet was extracted with 30 ml of 0.4 M phosphate buffer in the same fashion. After centrifugation, the eluate was treated with CM-cellulose (18 mg of dry weight per ml of eluate) by batch-wise addition and mixing for 15 min, and subsequent filtration through a Buchner funnel with mild suction. The filtrate was dialyzed against 20 volumes of saturated ammonium sulfate for 2 hr; the precipitate was collected by centrifugation and then resuspended in 5 ml of 0.02 M Tris (pH 7.5). This preparation was designated as “A-enzyme” and

used for most of the experiments described in this report. It was stored at –85° in 1-ml portions.

**Standard Assay System.** The reaction mixture, per 0.50 ml, contained 50  $\mu$ moles of Tris (pH 8.2 or pH 7.8), 5  $\mu$ moles of  $MgCl_2$ , 1.5  $\mu$ moles of ATP, 2.5  $\mu$ moles of 2-mercaptoethanol, 0.10  $\mu$ mole of [ $^{35}S$ ]cysteine or [ $^{35}S$ ] $\beta$ -mercaptopyruvate ranging in specific activity from  $10 \times 10^6$  to  $70 \times 10^6$  cpm per  $\mu$ mole, 1 mg of yeast tRNA, and varying amounts of enzyme. After incubation the reaction was stopped by the addition of 0.05 ml of 0.5 M EDTA (pH 7.5), 3 ml of cold  $H_2O$ , and 0.10 ml of cetyltrimethylammonium bromide (50 mg/ml); the solution was thoroughly mixed and allowed to stand in ice for 30 min, and the precipitate was collected by centrifugation. The pellet was dissolved in 0.50 ml of 2 M NaCl (at room temperature); 1 ml of 95% ethanol was added and the mixture was allowed to stand at –20° for 1 hr. The precipitate was collected by centrifugation, dissolved in 1 ml of 0.02 M Tris (pH 7.5), and then vigorously extracted by vortex mixing for 2 min with an equal volume of 90% phenol previously equilibrated with 0.1 M sodium phosphate buffer (pH 6.7). The aqueous phase was removed and RNA was precipitated by the addition of 0.15 ml of 20% potassium acetate (pH 5.4) and 2 volumes of ethanol. After standing at –20° for 1 hr, the RNA was sedimented by centrifugation and suspended in 0.50 ml of a solution containing 0.01 M NaCl and 0.01 M Tris (pH 7.5) and the entire sample was added to a scintillation mixture of Triton X100-toluene and counted in a Nuclear Chicago Mark I scintillation counter.

**Enzyme Unit.** A unit of enzyme activity is defined as the amount of protein which catalyzes the incorporation of 1  $\mu$ mole of sulfur from  $\beta$ -mercaptopyruvate into yeast tRNA in 20 min at 37° under the standard assay conditions described above.

**Nucleic Acid Preparations.** Yeast tRNA was purchased from Schwarz BioResearch and served as the sulfur acceptor for most of the work described here. Prior to its use, it was extracted with 90% phenol several times, precipitated with ethanol, and dialyzed overnight with several changes of water. *B. subtilis* tRNA and *Escherichia coli* tRNA were purchased from Schwarz BioResearch, rat liver tRNA from General Biochemicals, synthetic ribohomopolymers (poly U, poly C, and poly A) from Miles Laboratory, Inc.; these preparations were used without further purification. *E. coli* rRNA was prepared from isolated ribosomes by the phenol extraction procedure (Gierer and Schramm, 1956). The material that precipitated with 1 M NaCl at 4° was used as rRNA after extensive dialysis against water. MS2 RNA and T2 and T4 DNA were isolated from their respective phages by phenol extraction. DNA was prepared from *Micrococcus lysodeikticus* and rat liver by the procedure of Marmur (1961). Calf thymus and salmon sperm DNA were purchased from Sigma Biochemicals. All of the DNA preparations were further treated with RNase (DNase free) and pronase (DNase free), extracted several times with 90% phenol, and dialyzed against 0.01 M NaCl–0.01 M Tris (pH 7.5). Denatured DNA was prepared by heating in a boiling water bath for 10 min followed by rapid quenching in an ice bath.

**Alkaline Hydrolysis and DEAE-Cellulose Chromatography.** Sulfur-labeled RNA was hydrolyzed in 1 ml of 0.3 N KOH for 18 hr at 37°. The hydrolysate was diluted with water to about 20 ml and triturated with Dowex 50( $H^+$ ) to pH 10,

TABLE I: Purification of Sulfurtransferase from *B. subtilis* W168.<sup>a</sup>

Fraction	Volume (ml)	Protein Concentration (mg/ml)	$A_{280}/A_{260}$	Total Units	Specific Activity (units/mg of protein)	Ratio of Specific Activities of $\beta$ -MP: Cysteine
Spinco Supernatant	374	12.0	0.58	5130 (2510)	1.12 (0.56)	2.0
60–80% $(\text{NH}_4)_2\text{SO}_4$ Precipitate	20	60.0	0.61	4400 (2240)	3.67 (1.87)	2.0
A-Enzyme	5	0.675	0.62	550 (165)	163 (49)	3.3

<sup>a</sup> Enzyme purification and the definition of an enzyme unit are described under Methods. Protein concentrations were determined by the method of Lowry *et al.* (1951). Values for total units and specific activity were calculated from <sup>35</sup>S-incorporation data obtained from standard enzyme assays for the various fractions shown; values outside parentheses were obtained with [<sup>35</sup>S] $\beta$ -mercaptopyruvate ( $\beta$ -MP) and those inside parentheses with [<sup>35</sup>S]cysteine.

the resin was removed by filtration, and the filtrate was further neutralized to pH 8.6 with dilute HCl. Nonradioactive 4-thio(2',3')UMP was then added as marker and the entire mixture was adjusted to contain 0.01 M  $\text{NH}_4\text{HCO}_3$  (pH 8.6) in a volume of 100 ml. This mixture was applied to a  $1.8 \times$

15 cm column of DEAE-cellulose previously equilibrated with the same buffer, washed with 100 ml of 0.02 M  $\text{NH}_4\text{HCO}_3$  (pH 8.6), and eluted with a 360-ml linear gradient of the same buffer (0.05 M to 0.24 M) containing 7 M urea as described by Lipsett (1965). Fractions of 4.5 ml each were collected and examined for their absorbance at 260, 280, and 320 m $\mu$  and their radioactivity.

**Paper Electrophoresis.** Radioactive RNA was hydrolyzed with alkali as described above. The hydrolysate was neutralized to pH 7 with a predetermined amount of 0.5 M citric acid and then adjusted to contain 0.0014 M 2-mercaptoethanol. Alternatively, the [<sup>35</sup>S]tRNA was hydrolyzed with RNase (50  $\mu\text{g}$  of pancreatic and 10  $\mu\text{g}$  of T1/0.36 ml of reaction) by incubation for 1 hr at 37°. A portion of the RNase-treated material was incubated with crystalline *E. coli* alkaline phosphatase (27  $\mu\text{g}$ /0.2 ml of reaction) for 1 hr at 37°. The RNase digests, with or without alkaline phosphatase treatment, were adjusted to contain 0.0014 M 2-mercaptoethanol. The hydrolyzed materials were electrophoresed on Whatman No. 3MM paper in a buffer containing 0.05 M  $\text{NH}_4\text{HCO}_3$  (pH 8.6)–0.0014 M 2-mercaptoethanol for 6 to 9 hr at 400 V in the cold. The paper was then air dried, cut into small strips, and placed in vials for scintillation counting.

**Isolation of [<sup>35</sup>S]tRNA for Analysis.** Synthetic [<sup>35</sup>S]tRNA was isolated from an *in vitro* sulfurtransferase reaction mixture by extracting 3 times with phenol (after the addition of EDTA and chilling to 0°) and then precipitated twice with 2 volumes of ethanol and 0.1 volume of 20% KOAc (pH 5.4). The radioactive tRNA was resuspended in 0.5 ml of 0.05 M  $\text{NH}_4\text{HCO}_3$  (pH 8.6)–0.0014 M 2-mercaptoethanol and chromatographed on a  $90 \times 1$  cm column of Sephadex G-25 using the same buffer for elution. The excluded [<sup>35</sup>S]-tRNA was concentrated by precipitation with ethanol, centrifugation, and resuspension in water.

Radioactive tRNA was isolated from late log *E. coli* B cells grown on a minimal medium containing  $\text{Na}_2^{35}\text{SO}_4$  as previously described (Hsu *et al.*, 1967). A similar procedure was used for the growth and preparation of [<sup>35</sup>S]tRNA from *B. subtilis* W168.

TABLE II: Requirements of Sulfurtransferase Reaction with [<sup>35</sup>S]Cysteine as the Sulfur Donor.<sup>a</sup>

Reaction Mixture	Radioactive Substrate	<sup>35</sup> S Incorporated into tRNA (m $\mu$ moles)
Complete	[ <sup>35</sup> S]cysteine	1.62
Omit enzyme	[ <sup>35</sup> S]cysteine	0.02
Heated enzyme	[ <sup>35</sup> S]cysteine	0.02
Omit ATP	[ <sup>35</sup> S]cysteine	0.01
Omit $\text{MgCl}_2$	[ <sup>35</sup> S]cysteine	0.01
Omit tRNA	[ <sup>35</sup> S]cysteine	0.01
Add RNase	[ <sup>35</sup> S]cysteine	0.01
Add DNase	[ <sup>35</sup> S]cysteine	1.62
Add nonradioactive cysteine (20 $\times$ excess)	[ <sup>35</sup> S]cysteine	0.09
Add nonradioactive cysteine (100 $\times$ excess)	[ <sup>35</sup> S]cysteine	0.03
Complete	[ <sup>14</sup> C]cysteine	0.01

<sup>a</sup> Reaction mixture was as described for the standard assay system under Methods and contained either [<sup>35</sup>S]cysteine ( $17.8 \times 10^6$  cpm/ $\mu\text{mole}$ ) or [<sup>14</sup>C]cysteine ( $21.1 \times 10^6$  cpm/ $\mu\text{mole}$ ) as the radioactive substrate and 3.2 units of the 60–80%  $(\text{NH}_4)_2\text{SO}_4$  fraction. Where indicated, the following were also present: 2  $\mu\text{moles}$  and 10  $\mu\text{moles}$  of nonradioactive cysteine, 50  $\mu\text{g}$  of pancreatic RNase or DNase, and 3.2 units of enzyme heated for 5 min in a boiling water bath. Incubation was for 20 min at 37°.

TABLE III: Effect of Different Sulfur-Containing Compounds on Cysteine-Sulfur Transfer to tRNA.<sup>a</sup>

Reaction Mixture	<sup>35</sup> S Incorporated into tRNA (mμmole)
Complete plus EDTA	0.02
Complete	0.67
Add glutathione	0.64
Add methionine	0.69
Add homocysteine	0.48
Add β-mercaptopyruvate	0.06
Add thiosuccinate	0.48
Add cysteamine	0.54
Add cysteic acid	0.56

<sup>a</sup> Reaction mixture, containing 0.1 μmole of [<sup>35</sup>S]cysteine ( $15 \times 10^6$  cpm/μmole) and 2.1 units of A-enzyme, was as described for the standard assay system under Methods. Each of the sulfur-containing compounds indicated above was added in the amount of 1 μmole. Incubation was for 20 min at 37°.

**Preparation of DNPH<sup>1</sup> Derivatives.** Formation of 2,4-dinitrophenylhydrazine derivatives and their chromatography on paper were carried out essentially as described by Kun and Garcia-Hernandez (1957).

#### Experimental Section

A partial purification (some 150-fold) of the sulfurtransferase activity in extracts from *B. subtilis* W168 can be effected by the procedure described under Methods and summarized in Table I. When the individual fractions were assayed with [<sup>35</sup>S]β-mercaptopyruvate the total number of enzyme units and the specific activities were at least twice those obtained with [<sup>35</sup>S]cysteine. The material absorbing at 260 mμ contained in the partially purified fractions can be largely removed by passage over various anion exchangers (i.e., DEAE-cellulose), but such procedures result in considerable loss of enzyme units with little increase in specific activity, and in addition to which the sulfurtransferase activity becomes quite unstable. In consequence, we have found it convenient to use the "A-enzyme" for the studies described in this report since this preparation is stable for several months when stored at -85°, with only slight losses of activity.

In Table II it is shown that the transfer of cysteine sulfur to an RNA acceptor requires the absolute participation of enzyme, ATP, magnesium ion, and tRNA. That tRNA is the sulfur acceptor in this system is suggested by the absence of <sup>35</sup>S product when RNase, but not DNase, is included in the incubation mixture. The substitution of [<sup>14</sup>C]cysteine for [<sup>35</sup>S]cysteine also results in no labeled

<sup>1</sup> Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966) are: CTAB, cetyltrimethylammonium bromide; DNPH, 2,4-dinitrophenylhydrazine.

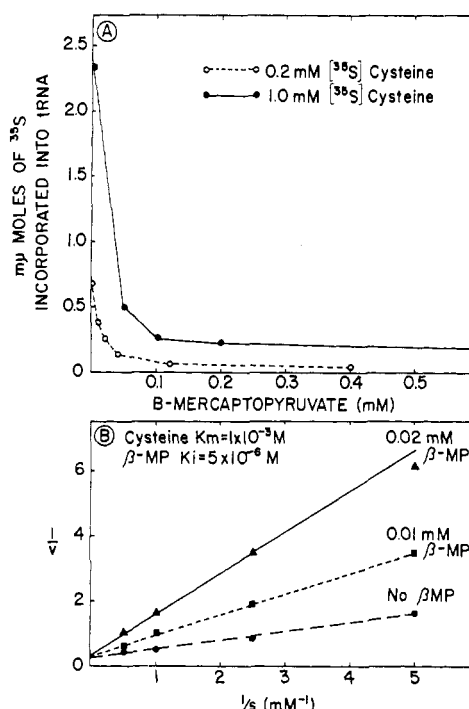


FIGURE 1: Effect of β-mercaptopyruvate (β-MP) on sulfuration when [<sup>35</sup>S]cysteine is used as the labeled sulfur donor. In A, the complete reaction mixture, containing either 0.2 or 1.0 mM [<sup>35</sup>S]cysteine ( $15 \times 10^6$  cpm/μmole) and various concentrations of non-radioactive β-mercaptopyruvate as shown above, was the same as the standard assay system described under Methods. Each reaction contained 2.0 units of A-enzyme and incubation was for 20 min at 37°. In B, the conditions for reactions were as in A except that the [<sup>35</sup>S]cysteine concentration was varied from 0.2 to 2 mM either in the absence or presence of 0.01 and 0.02 mM β-mercaptopyruvate. Values for 1/v and 1/s represent the reciprocal quantities of the mμmoles of <sup>35</sup>S incorporated into tRNA and the concentration of [<sup>35</sup>S]cysteine used.

product, indicating that only the sulfur moiety of cysteine, and not cysteine carbon, is transferred to tRNA in this reaction. When nonradioactive cysteine is added to the system, a reduction in labeled product formation occurs which corresponds very closely to the expected dilution for the labeled substrate. Although these results indicate that [<sup>35</sup>S]cysteine functions as the source of sulfur in this system, they do not necessarily imply that cysteine is the "immediate" sulfur precursor.

During the course of one experiment directed toward answering this question, it was observed that the presence of β-mercaptopyruvate in the sulfurtransferase system resulted in a striking reduction of tRNA labeling whereas other sulfur-containing compounds had no marked effect (Table III). Further studies revealed that the reduction of [<sup>35</sup>S]tRNA formation was dependent upon the concentration of β-mercaptopyruvate and that this apparent "inhibitory" response was elicited by extremely small molar quantities of the thiol keto acid relative to the [<sup>35</sup>S]cysteine concentration (Figure 1A). Reciprocal plots of 1/v vs. 1/s, in the absence and presence of β-mercaptopyruvate (at two levels), suggested that the thiol keto acid functioned as a competitive inhibitor of cysteine in the sulfurtransferase reaction (Figure 1B). From these data, the K<sub>i</sub> for β-mercaptopyruvate ( $5 \times 10^{-6}$

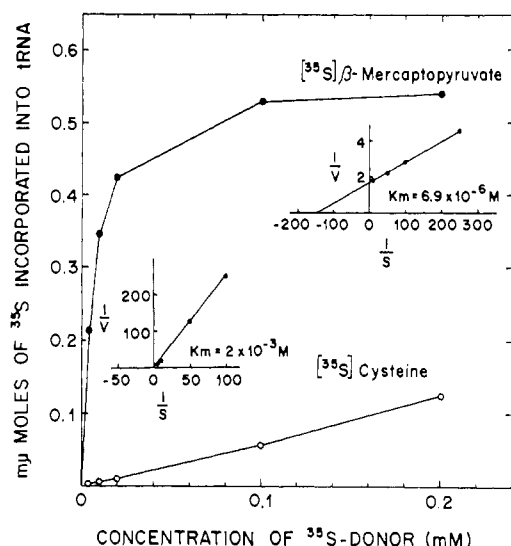


FIGURE 2: Sulfurtransferase activity as a function of the concentration of  $\beta$ -mercaptopyruvate and cysteine. The reaction mixture, containing 0.54 unit of A-enzyme, was similar to the standard assay system described under Methods except that both  $[^{35}\text{S}]\beta$ -mercaptopyruvate ( $60 \times 10^6$  cpm/ $\mu$ mole) and  $[^{35}\text{S}]$ cysteine ( $35 \times 10^6$  cpm/ $\mu$ mole) were used separately as the labeled substrate, and their concentrations were varied as indicated above. Incubation was for 20 min at  $37^\circ$ .

M) was found to be 200 times smaller than the  $K_m$  for cysteine ( $1 \times 10^{-3}$  M), indicating a much stronger affinity of the enzyme for  $\beta$ -mercaptopyruvate. Although the thiol keto acid analog of cysteine appeared to function as a competitive inhibitor,

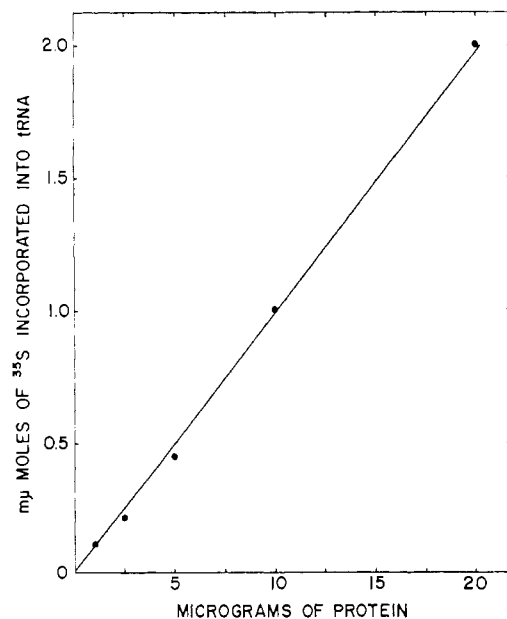


FIGURE 3: Effect of varying enzyme concentration on the sulfuration of tRNA. The reaction mixture, containing 0.10  $\mu$ mole of  $[^{35}\text{S}]\beta$ -mercaptopyruvate ( $68.5 \times 10^6$  cpm/ $\mu$ mole), was as described for the standard assay system under Methods except that the A-enzyme protein concentration was varied as shown above. Incubation was for 20 min at  $37^\circ$ .

TABLE IV: Effect of Different Sulfur-Containing Compounds on  $[^{35}\text{S}]$ tRNA Formation with  $[^{35}\text{S}]\beta$ -Mercaptopyruvate as Substrate.<sup>a</sup>

Reaction System	<sup>35</sup> S Incorporated into tRNA ( $\mu$ mole)
Complete	0.35
Replace tRNA with rRNA	0.02
Add $\beta$ -mercaptopyruvate (non-radioactive, 10 $\times$ )	0.04
Add cysteine	0.32
Add 2-mercaptopropionic acid	0.30
Add 3-mercaptopropionic acid	0.31
Add cysteamine	0.37
Add cysteic acid	0.31
Add cystathionine	0.40
Add methionine	0.34
Add glutathione	0.33
Add thiosuccinic acid	0.40

<sup>a</sup> Complete system, containing 0.10  $\mu$ mole of  $[^{35}\text{S}]\beta$ -mercaptopyruvate ( $57.2 \times 10^6$  cpm/ $\mu$ mole) and 0.35 unit of A-enzyme, was as described under Methods. Where indicated, 1 mg of *E. coli* rRNA replaced yeast tRNA and each of the sulfur-containing compounds indicated was added in the amount of 1  $\mu$ mole. Incubation was for 20 min at  $37^\circ$ .

an alternative explanation of the above results could not be excluded, namely, that in this system  $\beta$ -mercaptopyruvate could also serve as a sulfur precursor for tRNA sulfuration.

When  $[^{35}\text{S}]\beta$ -mercaptopyruvate replaced labeled cysteine in the *in vitro* sulfurtransferase system, it was found to be an excellent sulfur donor for the sulfuration of tRNA. Figure 2 shows the formation of  $[^{35}\text{S}]$ tRNA as a function of the concentration of the two  $^{35}\text{S}$  substrates when assayed separately, and it is immediately apparent that  $\beta$ -mercaptopyruvate is far more efficient for tRNA sulfuration than cysteine at the low concentrations of substrate examined. Reciprocal plots of  $1/v$  vs.  $1/s$  indicate that the  $K_m$  for  $\beta$ -mercaptopyruvate ( $6.9 \times 10^{-6}$  M) is about 300 times smaller than the  $K_m$  for cysteine ( $2 \times 10^{-3}$  M), in close agreement with our previous data.

Table IV shows that when  $[^{35}\text{S}]\beta$ -mercaptopyruvate is used as the sulfur donor in the *in vitro* system, only the addition of nonradioactive  $\beta$ -mercaptopyruvate effectively reduces the extent of tRNA labeling to a level that roughly approximates the dilution of the labeled substrate. The addition of nonradioactive cysteine, as well as other closely related sulfur-containing compounds, has essentially little effect on the extent of  $[^{35}\text{S}]$ tRNA formation. The data of Table IV also allow one to conclude that the purity of the  $[^{35}\text{S}]\beta$ -mercaptopyruvate is fairly high, 90% or better, which is in agreement with the following findings. When 0.134 mg of the commercial ammonium  $[^{35}\text{S}]\beta$ -mercaptopyruvate ( $50.4 \times 10^6$  cpm) was added to 51.3 mg of nonradioactive crystalline ammonium  $\beta$ -mercaptopyruvate, giving an esti-

TABLE V: Requirements of Sulfurtransferase Reaction with  $\beta$ -Mercaptopyruvate as the Sulfur Donor.<sup>a</sup>

Reaction Mixture	<sup>35</sup> S Incorporated into tRNA (mμmole)
Complete	0.97
Omit enzyme	0.04
Omit ATP	0.05
Omit MgCl <sub>2</sub>	0.03
Omit tRNA	0.02
Add RNase	0.04
Replace tRNA with rRNA	0.02

<sup>a</sup> Complete reaction mixture, containing 0.1 μmole of [<sup>35</sup>S]β-mercaptopyrivate ( $68.5 \times 10^6$  cpm/μmole) and 0.97 units of A-enzyme, was the same as the standard assay system described under Methods. Where indicated, 20 μg of pancreatic RNase was added, and 1 mg of *E. coli* rRNA replaced yeast tRNA. Incubation was for 20 min at 37°.

mated specific activity of  $0.981 \times 10^6$  cpm/mg, the specific activities after three successive recrystallizations were  $0.966 \times 10^6$ ,  $0.892 \times 10^6$ , and  $0.923 \times 10^6$  cpm/mg, respectively.

**Properties of the Sulfurtransferase System.** The *in vitro* properties of the *B. subtilis* sulfurtransferase enzyme ascertained with [<sup>35</sup>S]β-mercaptopyrivate appear to be very similar to those found with [<sup>35</sup>S]cysteine. It may be seen in Table V that as with cysteine the transfer of labeled sulfur from β-mercaptopyrivate to tRNA shows an absolute requirement for enzyme, ATP, metal ion, and tRNA; the

TABLE VI: Nucleotide Specificity for Sulfurtransferase Activity.<sup>a</sup>

Nucleotide Present in Reaction Mixture	<sup>35</sup> S Incorporated into tRNA (mμmole)
None	0.05
ATP	0.41
UTP	0.07
CTP	0.07
GTP	0.08
ADP	0.12
ADP + hexokinase and glucose	0.07
AMP	0.06

<sup>a</sup> Complete reaction mixture, containing 0.10 μmole of [<sup>35</sup>S]β-mercaptopyrivate ( $63.5 \times 10^6$  cpm/μmole) and 0.41 unit of A-enzyme, was the same as for the standard assay system described under Methods. Where indicated, UTP, CTP, GTP, ADP, or AMP (1.5 μmoles each) replaced ATP, and 0.1 mg of hexokinase plus 15 μmoles of glucose were added to the reaction system. Incubation was for 20 min at 37°.

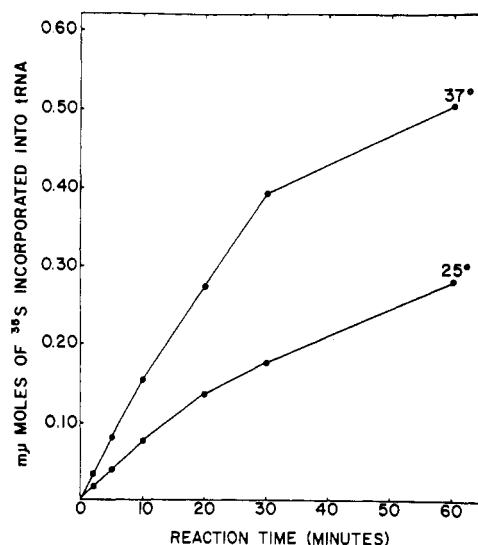


FIGURE 4: Effect of varying time and temperature on the sulfuration of tRNA. The reaction mixture, containing 0.10 μmole of [<sup>35</sup>S]β-mercaptopyrivate ( $68 \times 10^6$  cpm/μmole) and 0.28 unit of A-enzyme, was as described for the standard assay system under Methods. Reaction time and temperature of incubation were varied as shown above.

presence of RNase or substitution of rRNA for tRNA leads to base-line quantities of radioactivity incorporated into the nucleic acid fraction.

Figure 3 shows that in this system [<sup>35</sup>S]tRNA formation is dependent upon enzyme and there is a direct and linear relationship between the amount of sulfur transferred to tRNA and the amount of enzyme added to the reaction mixture. The rate of sulfur transfer at 37° remains fairly constant for the first 30 min of reaction and then changes to a slower rate over the next half hour (Figure 4). At 25°, the rate of tRNA sulfuration over the first 30 min is roughly

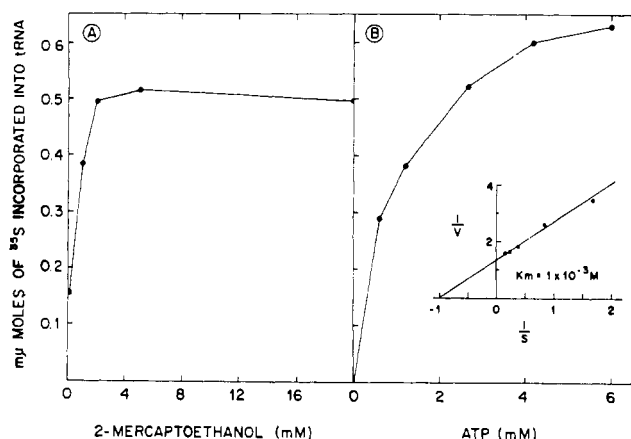


FIGURE 5: Effect of varying concentrations of 2-mercaptoethanol and ATP on the sulfuration of tRNA. The reaction mixture, containing 0.10 μmole of [<sup>35</sup>S]β-mercaptopyrivate ( $66 \times 10^6$  cpm/μmole) and 0.5 unit of A-enzyme, was as described for the standard assay system except that the concentration of 2-mercaptoethanol and ATP was varied in A and B, respectively, as indicated above. Incubation was for 20 min at 37°.

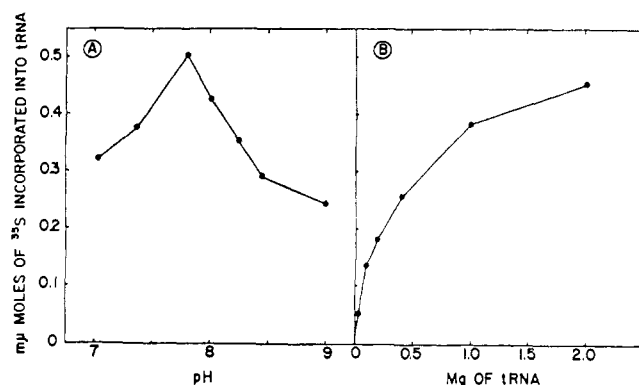


FIGURE 6: Sulfurtransferase activity as a function of pH and tRNA concentration. The reaction mixture, containing 0.10  $\mu$ mole of [<sup>35</sup>S] $\beta$ -mercaptopyruvate ( $62 \times 10^6$  cpm/ $\mu$ mole) and 0.4 unit of A-enzyme, was as described for the standard assay system under Methods except that the hydrogen ion and tRNA concentrations were varied in A and B, respectively, as indicated above. Incubation was for 20 min at 37°.

half of that observed at 37°, indicating the temperature-dependent nature of the sulfurtransferase activity.

Optimal transfer of sulfur from  $\beta$ -mercaptopyruvate to tRNA requires the presence of some reducing agent such as 2-mercaptoethanol; in the absence of this reagent [<sup>35</sup>S]tRNA formation is about one-third of maximum (Figure 5A). The relationship between sulfurtransferase activity and ATP concentration is shown in Figure 5B. Reciprocal plot of the incorporation data indicates that the  $K_m$  for ATP is approximately  $1 \times 10^{-3}$  M. The data of Table VI indicate that the nucleotide requirement for this enzyme system can only be fulfilled by ATP; UTP, CTP, GTP, ADP, and AMP are inactive. The slight sulfuration of tRNA that is seen with ADP disappears when hexokinase and glucose are also

TABLE VII: Various RNAs as Acceptors in the Sulfurtransferase System.<sup>a</sup>

Type of RNA Used as Acceptor	<sup>35</sup> S Incorporated into RNA (mμmole)
None	0.06
Yeast tRNA	0.35
<i>B. subtilis</i> tRNA	0.30
<i>E. coli</i> tRNA	0.30
Rat liver tRNA	0.29
<i>E. coli</i> rRNA	0.04
MS2 RNA	0.07
Poly C	0.07
Poly A	0.06
Poly U	0.04

<sup>a</sup> Complete reaction mixture, containing 0.10  $\mu$ mole of [<sup>35</sup>S] $\beta$ -mercaptopyruvate ( $51.5 \times 10^6$  cpm/ $\mu$ mole) and 0.35 units of A-enzyme, was the same as the standard assay system described under Methods. Where indicated, 1 mg each of the different RNAs shown above replaced yeast tRNA. Incubation was for 20 min at 37°.

TABLE VIII: DNA as Acceptor for Sulfur in the Transferase System.<sup>a</sup>

Type of Nucleic Acid	Amount of Nucleic Acid ( $\mu$ g)	<sup>35</sup> S Incorporated into Nucleic Acid (mμmole)
<i>E. coli</i> rRNA	1000	0.04
Yeast tRNA	1000	0.32
Salmon DNA (native)	410	0.05
Salmon DNA (denatured)	410	0.41
Salmon DNA (denatured) plus DNase	410	0.06
Salmon DNA (denatured) plus RNase	410	0.35
Calf thymus DNA (native)	405	0.05
Calf thymus DNA (denatured)	405	0.35
T4 DNA (native)	254	0.05
T4 DNA (denatured)	254	0.17
T2 DNA (denatured)	204	0.12
<i>M. lysodeikticus</i> DNA (denatured)	410	0.11
Rat liver DNA (denatured)	410	0.13

<sup>a</sup> Complete system, containing 0.10  $\mu$ mole of [<sup>35</sup>S] $\beta$ -mercaptopyruvate and 0.32 unit of A-enzyme, was the same as the standard assay system described under Methods. Where indicated, *E. coli* rRNA and various DNA preparations (native or denatured) replaced yeast tRNA in the amounts shown above. Incubation was for 20 min at 37°. The assay was modified as follows: the reaction was stopped with EDTA (as before), chilled in ice, and allowed to stand for 10 min in the cold after the addition of 0.10 ml of 1 N NaOH. The mixture was neutralized with 0.10 ml of 1 N HCl; 3 ml of water was added followed by precipitation of the nucleic acid with CTAB. From this point on, the assay was the same as before.

present, suggesting that adenylate kinase is a contaminant of the sulfurtransferase preparation. Hexokinase and glucose by themselves do not affect the sulfurtransferase reaction (not shown here).

Figure 6 depicts the formation of sulfated tRNA as a function of the concentration of hydrogen ion and yeast tRNA. Formation of [<sup>35</sup>S]tRNA occurs over a broad pH range but optimum reaction is observed at a pH of about 7.8 (Figure 6A). The detection of sulfurtransferase activity is dependent upon the presence of an adequate sulfur acceptor. As shown in Figure 6B, this requirement can be satisfied with yeast tRNA, and a direct relationship exists between the amount of tRNA present and the extent of [<sup>35</sup>S]tRNA formation. Under the conditions used here, saturation of the system with yeast tRNA is somewhat greater than 2 mg/0.5 ml of reaction mixture. As far as other RNAs serving as sulfur acceptors in this system are concerned, our studies indicate that although a variety of different tRNAs can be sulfated, other species of RNA such as rRNA, MS2 RNA, and synthetic ribohomopolymers do not accept sulfur (Table VII). On the other hand, sulfur-accepting

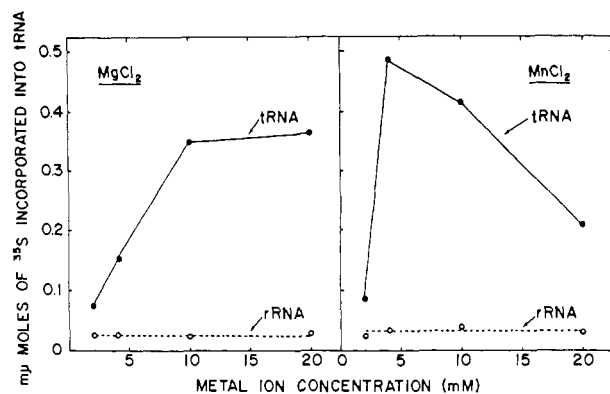


FIGURE 7: Effect of divalent metal ion concentration on sulfurtransferase activity. The reaction mixture, containing 0.10  $\mu$ mole of [ $^{35}$ S] $\beta$ -mercaptopyruvate ( $62 \times 10^6$  cpm/ $\mu$ mole) and 0.35 unit of A-enzyme, was as described for the standard assay system under Methods except that  $\text{MgCl}_2$  or  $\text{MnCl}_2$  were used at varying concentrations and either tRNA or rRNA was present during the reaction, as indicated above. Incubation was for 20 min at  $37^\circ$ .

activity is not limited to tRNA. Table VIII indicates that several different types of DNA, in their denatured but not in their native form, may also function as sulfur acceptors for sulfurtransferase activity. Denatured salmon sperm and calf thymus DNA seem to be better in this respect than denatured viral and bacterial DNA, and on a weight for weight basis they also appear to be much better acceptors than yeast tRNA. That DNA is sulfated in the *in vitro* reaction is suggested by the disappearance of labeled product in the presence of DNase but not RNase; it is still not possible, however, to exclude the possibility that some contaminant in the DNA preparation rather than the DNA itself is involved in the formation of the  $^{35}\text{S}$  product.

The sulfurtransferase enzyme requires activation by a divalent metal ion. Figure 7 shows that either  $\text{MgCl}_2$  or  $\text{MnCl}_2$  can activate the system. Optimum sulfur transfer occurs at a concentration of 10 mM for magnesium ion and about 4 mM for manganese ion; higher concentrations of manganese ion result in suppression of [ $^{35}\text{S}$ ]tRNA formation. Similar assays with other heavy metal ions such as  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Zn}^{2+}$  have led to misleading results because of the ready formation of insoluble metal-sulfur complexes which coprecipitate with RNA and cannot be completely removed by the assay procedure employed.

**Nature of the Labeled Reaction Product.** The sulfurtransferase  $^{35}\text{S}$  product appears to be RNA as indicated by its physicochemical properties, *i.e.*, precipitability with ethanol and CTAB, nondialyzability, alkaline instability under conditions which digest RNA, and sensitivity to the action of RNase. Some of these and other properties are illustrated by the following experiments. When enzymatically prepared labeled product was chromatographed on Sephadex G-100, almost all the radioactivity was excluded in a position coincident with tRNA; a small amount of  $^{35}\text{S}$  appeared much later in a region known to exclude ATP and other low-molecular-weight compounds (Figure 8A). When the  $^{35}\text{S}$ -labeled product was treated with RNase prior to Sephadex G-100 chromatography, then radioactivity and ultraviolet-absorbing material were no longer seen in the position for elution of tRNA but

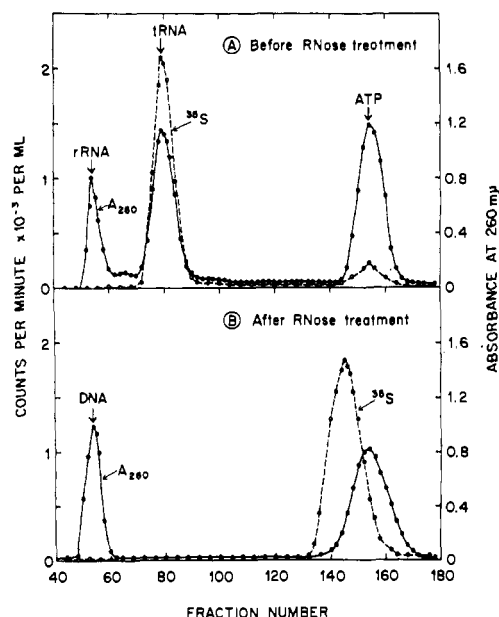


FIGURE 8: Sephadex G-100 chromatography of *in vitro* prepared [ $^{35}\text{S}$ ]tRNA before and after RNase treatment. Radioactive [ $^{35}\text{S}$ ]tRNA was prepared in a 2-ml standard sulfurtransferase system with [ $^{35}\text{S}$ ]cysteine as the sulfur donor and isolated from the reaction mixture by phenol extraction and ethanol precipitation as described under Methods. In A, approximately 1 mg of labeled tRNA (60,000 cpm) mixed with 0.4 mg of marker *E. coli* rRNA was chromatographed on a  $90 \times 2.4$  cm column of Sephadex G-100; the elution solvent was 0.05 M  $\text{NH}_4\text{CO}_3$  (pH 8.6)–0.0014 M 2-mercaptoethanol and 3-ml fractions were collected. In B, 0.8 mg of the labeled tRNA was exposed to pancreatic and T1 RNase for 1 hr at  $37^\circ$ ; 0.4 mg of denatured T4 DNA was added as a marker, and the entire mixture was chromatographed on Sephadex G-100 as described for A.

were now excluded in the same region as the low-molecular-weight substances (Figure 8B). The fact that the radioactivity is not exactly coincident with, but slightly precedes, the 260-m $\mu$  absorbing material may indicate incomplete enzymatic digestion; that the major  $^{35}\text{S}$  digestion products are oligonucleotides; or that after RNase treatment disulfide interactions occur between the digestion products, giving rise to larger radioactive fragments.

When the synthetic  $^{35}\text{S}$  product was exposed to alkali under conditions that hydrolyze RNA, and then chromatographed on DEAE-cellulose, the radioactivity distributed itself almost exclusively in the region where the major nucleotides are routinely found under the conditions used here; no label was seen to elute with the 4-thiouridylate which was added as a marker prior to chromatography (Figure 9A). Chromatography of a hydrolysate of [ $^{35}\text{S}$ ]tRNA isolated from *B. subtilis* W168 cells grown in a medium containing radioactive sulfate also shows that very little radioactivity elutes with marker 4-thiouridylate, most of the isotope being found with the bulk of the hydrolyzed 260-m $\mu$ -absorbing material (Figure 9B). With [ $^{35}\text{S}$ ]tRNA isolated from *E. coli* B cells, the distribution of radioactivity after alkaline hydrolysis and DEAE chromatography is just the reverse of the labeled profiles described above; the bulk of the isotope elutes with 4-thiouridylate, and relatively few counts are found associated with the 260-m $\mu$ -absorbing material (Figure 9C).



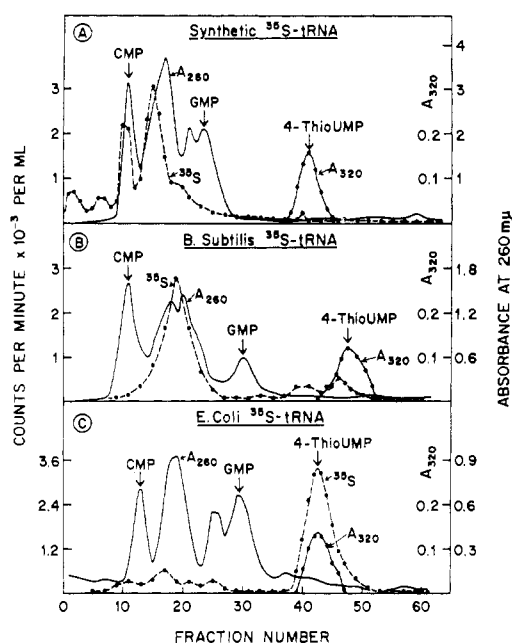


FIGURE 9: DEAE-cellulose chromatography of alkaline-hydrolyzed synthetic, *B. subtilis*, and *E. coli* [ $^{35}\text{S}$ ]tRNA. Conditions for [ $^{35}\text{S}$ ]tRNA preparation, alkaline hydrolysis, and DEAE chromatography were as described under Methods. Recovery of the total radioactivity applied to the column was 75–85%. (A) [ $^{35}\text{S}$ ]tRNA was prepared using the *B. subtilis* W168 sulfurtransferase enzyme; [ $^{35}\text{S}$ ]cysteine served as sulfur donor and yeast tRNA was the sulfur acceptor in a standard assay reaction. (B) [ $^{35}\text{S}$ ]tRNA was isolated from *B. subtilis* W168 cells grown in a medium containing  $\text{Na}_2^{35}\text{SO}_4$ . (C) [ $^{35}\text{S}$ ]tRNA was isolated from *E. coli* B cells grown in a medium containing [ $^{35}\text{S}$ ]Na $_2\text{SO}_4$ .

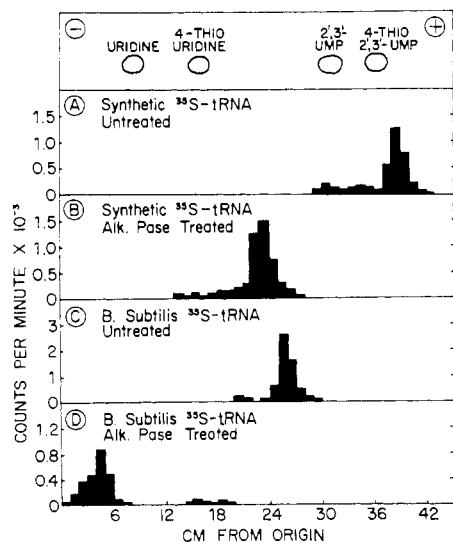


FIGURE 10: Electrophoresis of RNase-hydrolyzed synthetic and *B. subtilis* [ $^{35}\text{S}$ ]tRNA. Conditions for [ $^{35}\text{S}$ ]tRNA preparation, hydrolysis with RNase, and electrophoresis were as described under Methods. (A) Synthetic [ $^{35}\text{S}$ ]tRNA was prepared as in Figure 9A. (B) Same as in A except that the hydrolyzed product was further treated with alkaline phosphatase as reported under Methods. (C) The *B. subtilis* [ $^{35}\text{S}$ ]tRNA was prepared as in Figure 9B. (D) Same as in C except that the hydrolyzed product was further treated with alkaline phosphatase.

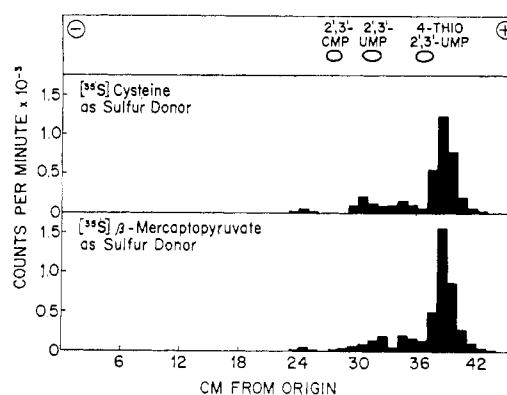


FIGURE 11: Comparison of electrophoretic properties of alkaline-hydrolyzed [ $^{35}\text{S}$ ]tRNA prepared either with [ $^{35}\text{S}$ ]cysteine or [ $^{35}\text{S}$ ]β-mercaptopyruvate. [ $^{35}\text{S}$ ]tRNA was prepared using the *B. subtilis* W168 sulfurtransferase A-enzyme, yeast tRNA, and either [ $^{35}\text{S}$ ]cysteine or [ $^{35}\text{S}$ ]β-mercaptopyruvate (as indicated above) in a standard assay reaction. The conditions for [ $^{35}\text{S}$ ]tRNA preparation, alkaline hydrolysis, and paper electrophoresis were as described under Methods.

Figure 10 shows the electrophoretic patterns of the alkaline hydrolysates of [ $^{35}\text{S}$ ]tRNA before and after alkaline phosphatase treatment; the [ $^{35}\text{S}$ ]tRNA was prepared either by the *in vitro* sulfurtransferase system or isolated from *B. subtilis* W168 cells. Under the conditions used, hydrolysis of the *in vitro* labeled [ $^{35}\text{S}$ ]tRNA gives rise to a major radioactive product which migrates toward the anode at a slightly faster rate than 4-thioUMP does (Figure 10A). Treatment with alkaline phosphatase significantly slows the rate of anodal migration, as would be expected if the  $^{35}\text{S}$  were associated with a nucleotide; however, the alkaline phosphatase treated radioactivity still migrates faster than 4-thio-uridine (Figure 10B). By comparison, after hydrolysis of [ $^{35}\text{S}$ ]tRNA from *B. subtilis* W168, the labeled sulfur-containing nucleotides migrate significantly more slowly than the synthetic hydrolyzed products (Figures 10C and 10D). The chromatographic and electrophoretic properties of the products formed after hydrolysis of the synthetic [ $^{35}\text{S}$ ]tRNA, and their behavior after exposure to alkaline phosphatase, suggest that they are nucleotides. It seems clear, however, that the sulfated nucleotides derived from *in vitro* prepared [ $^{35}\text{S}$ ]tRNA are different from the sulfur-containing nucleotides derived from *B. subtilis* [ $^{35}\text{S}$ ]tRNA.

The alkaline digest products of [ $^{35}\text{S}$ ]tRNA prepared enzymatically, either with radioactive cysteine or β-mercaptopyruvate, behave electrophoretically in an identical manner (Figure 11). It appears, therefore, that the sulfated tRNA product is the same with either thiol precursor, which supports the idea of a common enzymatic pathway for both substrates. It should be noted that the synthetic hydrolyzed [ $^{35}\text{S}$ ]tRNA products are electrophoretically similar when hydrolysis is carried out either with a mixture of RNase (pancreatic and T1) as in Figure 10, or with alkali as in Figure 11.

## Discussion

These studies show that a partially purified protein fraction from extracts of *B. subtilis* W168 catalyzes the transfer of

sulfur from either cysteine or  $\beta$ -mercaptopyruvate to a nucleic acid acceptor in the presence of ATP and a divalent metal ion. Although sulfurtransferase activity has previously been shown to utilize cysteine for the sulfuration of RNA *in vitro*, no such role has been described for  $\beta$ -mercaptopyruvate.

It is possible that the labeling of tRNA observed with [ $^{35}\text{S}$ ] $\beta$ -mercaptopyruvate might be due to some minor radioactive contaminant in the isotope preparation. Several points militate against this possibility although it cannot be absolutely excluded. (1) Various thiol-containing compounds closely related to the  $^{35}\text{S}$  substrate did not reduce the extent of [ $^{35}\text{S}$ ]tRNA formation when added to the reaction system; in contrast, nonradioactive crystalline  $\beta$ -mercaptopyruvate was quite effective in this regard. (2) Repeated recrystallization of a mixture of radioactive and unlabeled  $\beta$ -mercaptopyruvate showed no significant change in specific radioactivity. (3) The [ $^{35}\text{S}$ ] $\beta$ -mercaptopyruvate preparation reacts with 2,4-dinitrophenylhydrazine and exhibits a chromatographic pattern similar to the one obtained with the DNPH derivative of crystalline  $\beta$ -mercaptopyruvate.

The sulfurtransferase data presented here are open to several interpretations. (1) Cysteine functions as a sulfur donor in the partially purified system because of prior conversion into its thiol keto acid analog,  $\beta$ -mercaptopyruvate. (2) Both  $\beta$ -mercaptopyruvate and cysteine function as substrates with the same enzyme. (3) Two separate sulfurtransferase enzymes exist, each utilizing a different thiol substrate. Although we cannot decide which one of these alternatives is correct, we have observed that when [ $^{35}\text{S}$ ]cysteine is used in the present enzyme system, a radioactive DNPH-reactive material can be isolated which behaves like the DNPH derivative of crystalline  $\beta$ -mercaptopyruvate in the chromatography system of Kun *et al.* (1956). This observation favors the interpretation that cysteine functions as a sulfur donor by conversion to  $\beta$ -mercaptopyruvate. However, regardless of which interpretation is correct, it appears from [ $^{35}\text{S}$ ]tRNA analysis that similar enzymatic products are formed when either labeled cysteine or  $\beta$ -mercaptopyruvate is utilized in the sulfurtransferase reaction.

Lipsett *et al.* (1967), employing a crude sulfurtransferase preparation from *E. coli*, observed that  $\beta$ -mercaptopyruvate exerts a marked stimulatory effect on cysteine-sulfur transfer to RNA but that  $\beta$ -mercaptopyruvate itself is inactive as a sulfur donor. These results, which are contradictory to the data reported here, might be partially reconcilable by the differences in source and purity of the enzymes employed by the two groups. However, when we assayed *E. coli* extracts for sulfurtransferase activity using [ $^{35}\text{S}$ ] $\beta$ -mercaptopyruvate, good incorporation into RNA was found; hence, the difference in results between the two groups probably is not due to enzyme source. When we used the procedure of Lipsett and coworkers (1967) to prepare [ $^{35}\text{S}$ ] $\beta$ -mercaptopyruvate (by the isolation of a small radioactive contaminant in [ $^{35}\text{S}$ ]cysteine), it was found to be inactive as a sulfur donor in the sulfurtransferase reaction as reported by the Lipsett group. Since our crystalline [ $^{35}\text{S}$ ] $\beta$ -mercaptopyruvate preparation was quite active as a sulfur donor under identical assay conditions, we conclude that the two preparations of [ $^{35}\text{S}$ ] $\beta$ -mercaptopyruvate must be different. Although the Lipsett group found that the presence of  $\beta$ -mercaptopyruvate was required for cysteine-sulfur transfer, they also noted that

with increased concentration of this thiol keto acid, cysteine-sulfur transfer into RNA becomes progressively inhibited, similar to the observations in this report. As discussed earlier, if cysteine functions as a sulfur donor by conversion into mercaptopyruvate then the observed inhibition would be due to dilution of the [ $^{35}\text{S}$ ] $\beta$ -mercaptopyruvate generated from [ $^{35}\text{S}$ ]cysteine during reaction. Their requirement for small amounts of  $\beta$ -mercaptopyruvate could reflect the need for some reducing agent similar to the 2-mercaptoethanol requirement in our work here (Figure 5A), as well as help in maintaining an effective concentration of the thiol keto acid during reaction. With respect to this latter point,  $\beta$ -mercaptopyruvate is fairly unstable when incubated at 37°, pH 8, losing 50% of its sulfur-donating ability within 30 min.

The presence of  $\beta$ -mercaptopyruvate in bacterial and mammalian cells has been described by several different groups (Tamiya, 1954; Kun *et al.*, 1956; Chatagner and Sauret-Ignazi, 1956). It has been suggested that this thiol keto acid arises by transamination of cysteine with an  $\alpha$ -keto acid (Meister *et al.*, 1954). If the *in vivo* formation of  $\beta$ -mercaptopyruvate occurs *via* transamination, then this enzymatic activity could regulate the extent of cellular tRNA sulfuration as well as other enzymes known to desulfurate mercaptopyruvate (Kun, 1969). The consequences of such regulatory mechanisms upon tRNA function, especially with respect to protein synthesis, cannot be presently evaluated since the role of sulfur-containing nucleotides in tRNA function has not been clarified.

So far, the only RNA species capable of serving as a sulfur acceptor in the sulfurtransferase reaction has been tRNA. Chromatography on Sephadex G-100, before and after RNase treatment, indicates that the radioactive sulfur is intimately associated with tRNA or its enzymatic breakdown products. After alkaline hydrolysis, the synthetic  $^{35}\text{S}$  products chromatograph on DEAE-cellulose and electrophorese in a manner expected for mononucleotide components. It is apparent, however, that the electrophoretic properties of the hydrolyzed radioactive products from the synthetic and naturally prepared [ $^{35}\text{S}$ ]tRNA are not the same. This could be accounted for if the sulfur-containing nucleotides of *B. subtilis* tRNA were further modified, *in vivo* (Carbon *et al.*, 1968; Burrows *et al.*, 1968; Hecht *et al.*, 1969). On the other hand, it is possible that the tRNA nucleotides sulfated *in vitro* are different from those sulfated *in vivo*. The synthetic and naturally sulfated tRNA appear to be similar to the extent that neither one shows a significant amount of  $^{35}\text{S}$  associated with 4-thiouridylic acid, which is the major sulfur nucleotide in *E. coli* tRNA (Lipsett, 1965). Also, using [ $^3\text{H}$ ]pyrimidine- or [ $^3\text{H}$ ]purine-labeled *E. coli* tRNA as sulfur acceptor in the *in vitro* reaction with the *B. subtilis* enzyme system, sulfuration occurs primarily on pyrimidine-containing nucleotides (>90%) which is similar to what we have found for the natural sulfur-containing nucleotides of tRNA isolated from *B. subtilis* W168 (data not shown here).

Contrary to the situation found for methylation of tRNA (Gold *et al.*, 1963), both homologous and heterologous tRNAs are good sulfur acceptors. This could be explained if different bases were sulfated *in vitro*; if sulfur functions were removed during tRNA isolation; or if the tRNA preparations contained pre-tRNA molecules (Bernhardt and Darnell, 1969) that were not fully thiolated. In view of the strict

specificity of sulfurtransferase for tRNA, it is interesting that denatured DNA was also sulfurated in the *in vitro* system. Whether the deoxynucleotides of DNA are actually sulfurated has not been rigorously characterized, but we have observed that DNA isolated from *E. coli* grown in a  $^{35}\text{S}$  medium contains isotope that bands with DNA upon equilibrium centrifugation in alkaline CsCl, is not removed by treatment with pronase, RNase, or strong alkali, and decays at a rate expected for  $^{35}\text{S}$ .

The exact mechanism by which sulfur is transferred from the donor substrate to the tRNA acceptor is unknown. The *in vitro* reaction is unaffected by the addition of pyridoxal phosphate or pyridoxal inhibitor. The participation of  $\beta$ -mercaptopyruvate as a sulfur donor in transsulfurase-catalyzed reactions is well known (Kun, 1969), and involves the transfer of sulfur from mercaptopyruvate to such acceptors as sulfite, cysteinesulfinate, cyanide, and certain SH compounds without any apparent activation of the sulfur donor or the sulfur acceptors. The specific requirement for ATP in the tRNA sulfurtransferase reaction is, therefore, unique and suggests the possible formation of an activated intermediate, either of the sulfur donor or of specific bases in tRNA. If an activated intermediate is formed, it is largely resistant to high concentrations of hydroxylamine (0.1 M) added to the reaction mixture, which does not alter the extent of [ $^{35}\text{S}$ ]tRNA formation. It seems unlikely, therefore, that ATP is involved in carboxyl activation as it is with amino acids to form aminoacyl-AMP. More detailed and extensive information is required for elucidating the role of ATP and the general mechanism of the tRNA sulfurtransferase action described in this report.

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