Mammalian tRNA sulfurtransferase: properties of the enzyme in rat liver

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ABSTRACT

Transfer RNA sulfurtransferase activity was detected in 105,000 x g supernatant preparations from rat liver and several other rat tissues. Sulfur is transferred from [^{35}S] cysteine to tRNA in a reaction which also requires ATP, Mg^{2+} , and supernatant protein. While [^{35}S] β -mercaptopyruvate appeared to be a substrate for this enzyme, the reaction product was sensitive to deacylation and the reaction was inhibited by [^{32}S] cysteine. Of the various nucleic acids tested, only tRNAs were effective sulfur acceptors, with rat liver tRNA being the poorest substrate. The [^{35}S] reaction product was sensitive to ribonuclease, cochromatographed with tRNA on methylated-albumin kieselguhr columns, and was converted to nucleotide material after alkaline hydrolysis. DEAE-cellulose chromatography of the neutralized [^{35}S] nucleotide digest revealed a single thionucleotide peak. These studies demonstrate that tRNA sulfurtransferase is present in various rat tissues, and that the requirements of the liver enzyme are similar to those of bacterial enzymes.

INTRODUCTION

Thionucleotides have been identified as minor components of tRNA from bacteria (3-6), yeast (7), plants (8), and mammalian tissues (9), and are also present in bacteriophage tRNA (10). Despite the universal occurrence of these modified bases, little is known of the enzymes involved in their synthesis.

Several reports of RNA sulfurtransferase in bacteria have appeared (11-14), but only the $s^4\text{U}$:sulfurtransferase (2) of Escherichia coli has been purified (14). The latter enzyme has been fractionated into two components, one which requires ATP, a divalent metal ion, tRNA and a sulfhydryl compound, and the other requires cysteine as sulfur donor and the product of the first enzyme. The enzymes of Bacillus subtilis have been studied (13), and differ from the enzymes of E. coli in the sulfur donor utilized and the thionucleotides formed in vitro (11,12). It was also found that the B. subtilis sulfurtransferases were more efficient with β -mercaptopyruvate than cysteine as substrate, while the E. coli enzyme was not active with the former sulfur donor. The reason for these differences is not understood at present.

To date, only one thionucleotide has been identified in mammalian tRNA, 5-methyl-2-thiouridine, which was isolated from tRNA^{lys} and tRNA^{glu} of rat liver (9). This thiobase is located in the first position of the anticodon in these tRNAs. The enzyme responsible for the synthesis of this thiobase has not yet been isolated, but a few studies of mammalian RNA sulfurtransferase(s) have appeared. Enzyme activity has been reported in preparations from rat brain (15), and in rat liver and Morris hepatomas (16). In these studies β -mercaptopyruvate was used as the sulfur donor and yeast tRNA as the acceptor molecule. Recently, we reported tRNA sulfurtransferase activity in rat liver and dye-induced rat hepatomas (17), in which cysteine served as the sulfur donor. We now report a more detailed analysis of the enzyme present in rat liver, and present evidence that cysteine, and not β -mercaptopyruvate, is the sulfur donor for this reaction system.

MATERIALS AND METHODS

Male, adult, albino Wistar strain rats were obtained from Hilltop Breeders, Scottdale, PA. ATP, polyuridylic acid, polyadenylic acid, cetyltrimethylammonium bromide, calf liver tRNA, salmon sperm DNA, and pancreatic ribonuclease were purchased from Sigma Chemical Co., St. Louis, MO. *E. coli* B tRNA was obtained from Schwarz Bioresearch, Orangeburg, N.Y. L-[³⁵S] cystine was purchased from New England Nuclear, Boston, MA., at specific activities of 550 to 22,000 mc/mmol (see below). All other chemicals were of the highest quality commercially available.

Enzyme Preparation

Tissues were excised as quickly as possible and placed immediately in ice-cold 50 mM Tris-HCl Buffer, pH 7.6, with 50 mM MgCl₂, 25 mM KCl, and 0.25 M sucrose (Buffer A). Cerebral cortices were dissected from the whole brains, and testes were decapsulated before homogenization; these operations and all subsequent ones being carried out at 4°. The tissues were minced and homogenized in four volumes of Buffer A using 10 up and down strokes with a glass-teflon homogenizer. Cell supernatant solutions were prepared by centrifugation for 90 min at 105,000 x g as previously described (17) and dialyzed two h against two changes of Buffer A minus sucrose. Protein was measured by the method of Lowry *et al.* (18) using bovine serum albumin as standard. The enzyme was assayed immediately after dialysis as a loss of activity occurred upon storage (see below).

Sulfurtransferase Assay

Enzyme activity was measured using a modification of previously reported procedures (12,17). The standard assay mixture contained per 0.5 ml:25 μ moles

Tris-HCl, pH 8.0; 2.5 μ moles $MgCl_2$; 2.0 μ moles ATP at pH 7.0; 2.0 μ moles 2-mercaptoethanol; 0.4 mg *Escherichia coli* B tRNA; 5 nmoles [^{35}S] cysteine (200 μ Ci/ μ mole), and 0.1 mg of dialyzed supernatant protein. Incubations were carried out at 37°; and the reaction was terminated by the addition of 1.5 ml of 20 mM cysteine, 0.5 mg of *E. coli* B tRNA as carrier, and 0.05 ml of a 5% cetyltrimethylammonium bromide solution. The RNA precipitate was obtained by centrifugation, dissolved and precipitated by the addition of ethanol, deacylated to remove cysteinyl-tRNA, extracted with phenol, reprecipitated with ethanol, and filtered on Millipore polyvinyl chloride filters. The filters were washed, dried, and counted by the scintillation method. The above assay procedure has been described in detail elsewhere (17).

Isolation of [^{35}S] tRNA

[^{35}S] tRNA was recovered from the standard reaction mixture by the addition of 0.5 ml of 20 mM cysteine, 1.0 ml of redistilled phenol (88%), and 1.0 mg *E. coli* B tRNA as carrier. The mixture was shaken at 4° for 10 min, and the aqueous layer obtained after centrifugation at 17,000 x g for 20 min. The tRNA was precipitated by the addition 0.1 volume of 20% potassium acetate, pH 5.4, and two volumes of 95% ethanol. After at least one hour at -20°, the tRNA was collected by centrifugation as above as dissolved in 2 M Tris-HCl buffer, pH 8.0. After two hours at 37° the tRNA solution was dialyzed overnight against two changes of deionized water (1000 ml H_2O per ml sample).

Analysis of [^{35}S] tRNA

Labeled tRNA was analyzed on columns of methylated-albumin kieselguhr (MAK) by the method of Mandell and Hershey (19). The RNA sample was diluted to 50 μ g/ml in 0.1 M NaCl, 0.05 M sodium phosphate buffer, pH 6.7, and applied to a 2.5 x 30 cm MAK column. After a 50 ml wash with the same buffer, the column was eluted with a 240 linear gradient of NaCl, ranging from 0.1 to 1.6 M in NaCl in 0.05 sodium phosphate buffer, pH 6.7, at a flow rate of 1 ml per min. Fractions of three ml were collected, adjusted to 5% with TCA, filtered on Millipore SS filters, washed with cold 5% TCA and dried. The filters were counted by the scintillation method (20).

For nucleotide analysis, tRNA was dissolved in 0.3 M KOH (2 ml per mg tRNA), and incubated for 18 hours at 37°. The digest was neutralized, applied to a DEAE-cellulose column, and chromatographed as previously described (17).

Preparation of Sulfur Donors

L-[^{35}S] cysteine was prepared from the dimer by treatment with 0.05 M mercaptoethanol or 0.012 M dithiothreitol, and analyzed by descending paper chromatography using isopropanol:water:HCl (65:18.4:16.6). Radioactivity

present in 1 cm strips of the chromatogram was determined by the scintillation method. Unlabeled amino acids were detected with a ninhydrin spray reagent (1% in n-butanol), applied after the chromatogram was neutralized by repeated application of a saturated NaHCO_3 solution. β -Mercaptopyruvate was prepared by the method of Abrell *et al.* (14) and chromatographed in the above system. Unlabeled β -mercaptopyruvate was kindly provided by Dr. E. Kun, and this compound was located on chromatograms using 0.05 M I_2 -1.5% sodium azide spray reagent.

RESULTS

Sulfur Donor

There is a discrepancy in the literature concerning the sulfur donor for the mammalian tRNA sulfurtransferases, as both cysteine (17) and β -mercaptopyruvate (15,16) have been used. Before testing these compounds as substrates for the enzymes in rat liver and brain, we felt it important to analyze these sulfur donor preparations to assure their identity and purity. A typical analysis of $[^{35}\text{S}]$ cysteine is shown in Figure 1A. A small amount of residual cystine remained after mercaptoethanol treatment, and this component increased on storage. With dithiothreitol reduction, overnight treatment at 25° resulted in complete conversion of cystine to cysteine, but longer contact resulted in the appearance of new sulfur-containing peaks. For these reasons, fresh cysteine was prepared for each experiment. As is seen in Figure 1A, commercial $[^{35}\text{S}]$ cysteine is contaminated with from 2-5% β -mercaptopyruvate, as previously reported (21). However, cysteine was not detected in β -mercaptopyruvate preparations (Figure 1B).

Fig. 1. Thin layer chromatography of $[^{35}\text{S}]$ cysteine (A) and $[^{35}\text{S}]$ β -mercaptopyruvate (B). Approximately 1 μCi of $[^{35}\text{S}]$ cysteine and 0.5 μCi of $[^{35}\text{S}]$ β -mercaptopyruvate were chromatographed as described, the chromatogram cut into strips, and counted. The markers show the positions of the unlabeled standards on the chromatogram.

Table I shows the results of experiments comparing cysteine and β -mercaptopyruvate as substrates for tRNA sulfurtransferase preparations from rat liver and cerebral cortex. The assay procedure used here included a step to hydrolyze cysteinyl-tRNA, while previous studies with β -mercaptopyruvate were carried out without deacylation (13,15,16). The data show that deacylation lowered the amount of sulfur incorporated into tRNA when cysteine was used as the substrate, and after an additional hour of deacylation no further reduction in sulfur incorporation was noted. Quite unexpectedly, sulfur incorporation into tRNA with mercaptopyruvate as sulfur donor was reduced to background levels by deacylation. The experiments with the cerebral cortex enzyme were carried out under our assay conditions, but a similar result was obtained with partially purified brain enzyme and assay procedures reported by Wong *et. al.* (15). Therefore, the results with β -mercaptopyruvate observed here were not peculiar to our reaction conditions or enzyme preparation.

Table I: Cysteine and β -Mercaptopyruvate
as Sulfur Donors^a

<u>Substrate</u> <u>Before Deacylation</u>	<u>pmoles Sulfur Incorporated^b</u>	
	<u>Liver Enzyme</u>	<u>Brain Enzyme</u>
Cysteine	20.70 $\pm$ 2.8 (3)	22.60 $\pm$ 7.1 (6)
Mercaptopyruvate	2.75 $\pm$ 1.25(4)	6.83 $\pm$ 2.6 (4)
<u>After Deacylation</u>		
Cysteine	2.30 $\pm$ 0.05(3)	1.35 $\pm$ 0.3 (3)
Mercaptopyruvate	0.25 $\pm$ 0.25(5)	0.11 $\pm$ 0.11(5)

^aThe standard mixture was used in this experiment, with 1.0 μ Ci [³⁵S] cysteine and [³⁵S] β -mercaptopyruvate (specific activity = 275.5 mCi/mmol) being present. The samples were either processed as described in Methods (after deacylation) or the deacylation step (17) was omitted from the assay procedures.

^bThe data are given as averages $\pm$ the S.E.M., with the number of determinations for each condition being given in parenthesis. These results were taken in part from a M.S. thesis of William St.Clair, West Virginia University (1976), the experiments being carried out in this laboratory.

It is unlikely that sulfur incorporation from β -mercaptopyruvate (before deacylation) is due to cysteine contaminants (see Figure 1), and we could not detect any cysteine in β -mercaptopyruvate assay mixtures during the course of the reaction. In one experiment, we observed a 93% reduction in sulfur incorporation into tRNA with [³⁵S] β -mercaptopyruvate as substrate when a

15-fold molar excess of [32 S] cysteine was added to the assay medium. We conclude that cysteine is a better substrate than β -mercaptopyruvate, and that results obtained with the latter substrate (15,16) should be reevaluated.

Characteristics of the System

The above findings prompted an evaluation of the reaction system with the true sulfur donor. The incorporation of sulfur from cysteine to the tRNA molecule is lowered when certain of the components of the standard reaction mixture are omitted (Table II). It is seen that the reaction requires the presence of tRNA, ATP, magnesium ion, and cysteine. Omission of tRNA lowers incorporation to a finite level. This value has been subtracted in subsequent

Table II: Liver Sulfurtransferase Requirements

Reaction Mixture	pMoles [35 S] Incorporated ^a into tRNA
Complete	1.17
Complete (Zero Time)	0.41
No Enzyme	0.37
Heated Enzyme	0.37
No tRNA	0.54
No ATP	0.50
No MgCl ₂	0.22
Pancreatic RNase added ^b	0.37
[32 S] Cysteine added ^c	0.58

^aThe standard reaction mixture was used here, with 1.0 μ Ci of [35 S] cysteine (Specific activity = 275.5) being added. The data are averages of two separate determinations.

^b10 μ g of pancreatic ribonuclease (Sigma) was added to the standard reaction mixture.

^c8.4 nmoles of [32 S] cysteine was added to the standard reaction mixture.

experiments so that the data reflect incorporation into added tRNA. Sulfur incorporation was sensitive to pancreatic ribonuclease treatment, and lowered by dilution of the isotope with [32 S] cysteine. Considerable incorporation was observed in the absence of either tRNA or enzyme, and other experiments showed that similar blank values were seen when both tRNA and enzyme were omitted. When higher cysteine levels were used (see Figure 3A) net incorporation of sulfur into tRNA was increased relative to the blank values, which remained relatively constant. Hence, the blank incorporation appears to be non-specific and did not interfere with later experiments.

The time course of the reaction is shown in Figure 2A where it is seen that the reaction is nearly complete by 10 minutes at a protein level of 100

μg . Sulfur incorporation was proportional to protein concentration up to nearly 200 μg of protein per assay mixture (Figure 2B). Amounts of protein above 500 μg resulted in a decrease in sulfur incorporation into tRNA.

The reaction is pH dependent, having a pH optimum of between 7.5 and 8.0 (Figure 2C). Our previous experiments were carried out at pH 8.5 (17), the optimal pH of the bacterial system (12,20). This would cause a reduction in activity of about 20% for the assays carried out at the higher pH. In other experiments the reaction was found to be temperature dependent, with the rate of sulfur transfer at 10° being 1/3 that at 37°.

Figure 2A. Time course of the tRNA sulfurtransferase reaction. The standard mixture used here contained 10 μM [³⁵S] cysteine and 100 μg of supernatant protein. Samples were incubated for the indicated times and processed as described in Methods. The data are averages of two separate experiments.

Figure 2B. Effect of the protein level on sulfur transfer to tRNA. Various amounts of supernatant protein were added as shown, the data being averages of two separate experiments.

Figure 2C. Effect of pH on sulfurtransferase activity. The standard assay system was used, except that the reaction mixture was buffered with 25 μmoles of Tris-HCl at the indicated pH values. Each point is an average of two separate determinations.

The rate of sulfur transfer to tRNA was dependent on the cysteine concentration as shown in Figure 3A. The data do not show simple saturation kinetics, suggesting that more than one reaction may be taking place. In fact, sulfur incorporation increased at a slower rate between 100 and 400 μ M cysteine. Because more than one enzymatic event may be occurring here it will be necessary to purify the enzyme before meaningful kinetic experiments can be performed.

Figure 3B shows the dependence of the reaction on tRNA, where saturation occurred at 1 mg of added *E. coli* B tRNA. Hence, most of our experiments were carried at a sub-saturating tRNA concentration. These data illustrate one of the major problems in measuring this enzyme, namely the low molar sulfur incorporation into tRNA. At saturation, there is approximately 1 mole of sulfur incorporated per 12,000 moles of tRNA, possibly due to a low transfer efficiency into but a few of the tRNA isoacceptors in this tRNA mixture. The use of a thionucleotide-free tRNA isoacceptor would greatly facilitate future work on tRNA sulfurtransferases (see below).

Sulfur Acceptor

The ability of various nucleic acids to serve as sulfur acceptors in the sulfurtransferase system was tested. Table III shows that only tRNA served as an acceptor with no incorporation observed using rRNA, DNA, poly U or poly A.

Figure 3A. Effect of cysteine concentration on sulfur transfer to tRNA. Various amounts of unlabeled cysteine was added to 2.0 μ Ci of [³⁵S] cysteine in the standard reaction mixture. The data were corrected for the resulting differences in specific activity, and are a composite of several separate experiments.

Figure 3B. Effect of the tRNA level on the sulfurtransferase reaction. Various amounts of *E. coli* B tRNA were added to the standard reaction mixture. The data are averages of several separate experiments.

Of the various tRNAs tested, sulfur-deficient tRNA from the C6 mutant of *E. coli* showed the highest sulfur acceptance, but not much higher than observed for *E. coli* B tRNA. This suggests an increased acceptance by only a fraction of the isoacceptors in this tRNA preparation, which contains one-half the

Table III: Various Nucleic Acids as Sulfur Acceptors

Nucleic Acid	pMoles Sulfur ^a Incorporated
None	0.72 (8)
<i>E. coli</i> B tRNA	1.86 (4)
Sulfur-deficient tRNA ^b	2.31 (3)
Rat Liver tRNA	0.78 (3)
Rat Brain tRNA	1.74 (2)
Calf Liver tRNA	1.17 (4)
Rat Liver rRNA	0.79 (3)
Salmon Sperm DNA	0.64 (4)
Polyuridylic Acid	0.70 (2)
Polyadenylic Acid	0.41 (2)

^aThe values are averages with the number of determinations being given in parenthesis.

^bThe sources of the commercial nucleic acids are given in Methods. Rat liver and brain tRNA were isolated by phenol extraction of whole tissue, and has been previously described (30). Rat liver rRNA was isolated from the microsomal fraction by phenol extraction and ethanol precipitation. Sulfur-deficient tRNA was isolated from *E. coli* C6 as previously described (24). The standard assay mixture was used here which contained 0.2 mg of each nucleic acid, 100 µg of protein, and 1.0 µCi [³⁵S] cysteine (200 mCi/mmmole).

Table IV: Tissue Distribution of RNA Sulfurtransferase in the Rat

Tissue ^a	Specific Activity ^b (units/mg)
Liver	10.2
Brain	6.0
Testes	10.0
Lung	9.9
Kidney	7.8
Muscle	16.1
Heart	11.6
Adrenals	13.6

^aEach tissue was removed from the rat as quickly as possible, frozen, and stored at -60°. Frozen tissues were thawed slightly, sliced, minced, then homogenized and processed as described for liver. Muscle was taken from the hind limbs, and testes were decapsulated before freezing.

^bA unit of enzyme activity is that amount of supernatant protein which catalyzes the incorporation of 1.0 pmole of [³⁵S] into tRNA in 10 min at 37° under the standard assay conditions. The data are averages of two separate experiments, each tissue being assayed in triplicate.

normal thionucleotide content (20). These data further suggest that the sites available for thiolation with liver enzymes are different than those recognized by the *E. coli* enzymes. Incorporation into brain tRNA was higher than seen with *E. coli* tRNA. This may mean that tRNA sulfurtransferases are tissue specific, as was true for the tRNA methyltransferases (22). Finally, the low incorporation seen with liver tRNA is consistent with the idea that tRNAs are not further modified by homologous enzymes.

Tissue Distribution

We observed tRNA sulfurtransferase activity in both rat liver and cerebral cortex (Table I), and reported the presence of the enzyme in rat hepatomas as well (17). Table IV provides evidence that tRNA sulfurtransferase activity is also present in several other rat tissues. A 2- to 3-fold activity range was noted, with the lowest activity being found in brain and the highest in muscle.

Characterization of the Reaction Product

To prove that the sulfur incorporated in the assay method was actually into tRNA, the reaction product was isolated from the standard reaction mixture and chromatographed on a MAK column. Figure 4 shows that sulfur was incorporated into tRNA, but that the majority of [35 S] was found in an oligonucleotide peak eluting with the void volume. When the isolation was carried out with bentonite present, incorporation into the latter fraction was lowered, suggesting that some breakdown of tRNA occurred during isolation. Incorporation into this low molecular weight material was not investigated further.

Figure 4. Methylation-kieselguhr chromatography of the [35 S] reaction product. [35 S] tRNA was isolated from the standard reaction mixture as described in Methods. Twenty μ Ci of [35 S] cysteine (specific activity - 275.5) was used to label RNA. The position of the tRNA peak was verified by separate chromatography of *E. coli* B tRNA.

In a parallel experiment, tRNA was isolated and hydrolyzed to the nucleotide level using 0.3 M KOH. After neutralization, the digest was chromatographed on DEAE-cellulose by the method of Lipsett (3). Figure 5 shows that a single peak of 35 S was detected which eluted with the first peak of nucleotide material. A similar result was obtained when the tRNA fraction on MAK was

Figure 5. DEAE-cellulose chromatography of an alkaline digest of $[^{35}\text{S}]$ tRNA. Dialyzed $[^{35}\text{S}]$ tRNA was isolated and hydrolyzed to nucleotides with 0.3 M KOH as described in Methods. After neutralization of the digest with Dowex-50(H^+), the sample was applied to a 1 x 30 cm DEAE-cellulose column equilibrated with 0.01 M NH_4HCO_3 , pH 8.6. The column was washed with 60 ml of the same buffer and a 360 ml linear gradient of NH_4CHO_3 , from 0.05 M to 2.5 M in 7 M urea, was applied. Fractions of 3.2 ml were collected, and 1 ml aliquots were removed for counting with 10 ml of ACS scintillation cocktail (Amersham-Searle, DesPlaines, IL).

hydrolyzed and chromatographed on DEAE-cellulose. Previous data from this laboratory showed three apparent thionucleotides formed in vitro by the liver enzymes (17). The difference can be attributed to a change in the isolation procedure used here in which the reaction was stopped by the addition of 20 mM cysteine and deacylation was increased from one to two hours. Further, the $[^{35}\text{S}]$ tRNA samples analyzed here were extensively dialyzed against distilled H_2O before KOH hydrolysis, a procedure not used in the previous experiments. Hence, the other two peaks were possibly due to contaminants present in the $[^{35}\text{S}]$ cysteine used, or the result of incomplete deacylation of tRNA. Finally, chromatography of an unlabeled tRNA hydrolysate with $[^{35}\text{S}]$ cysteine showed radioactivity in this region, but not coincident with the proposed thionucleotide.

DISCUSSION

This report is the first detailed analysis of tRNA sulfurtransferase in rat liver cell supernatants. Enzyme activity was also detected in supernatants from rat cerebral cortex, kidney, lung, testes, muscle, and adrenal glands. The reaction catalyzed is the transfer of sulfur from cysteine to acceptor sites in tRNA. The required components of the reaction are enzyme, tRNA, Mg^{2+} , ATP, and cysteine. In this regard, the requirements are similar to those reported previously for *E. coli* sulfurtransferases (11,12,14).

Purification of liver tRNA sulfurtransferase was hindered by the instability of the enzyme on storage, even when sulfhydryl agents were present. Overnight storage at -60° resulted in a 50% loss of activity. The enzyme did

not precipitate at pH 5, and this pH inactivated the enzyme in the supernatant. DEAE-cellulose fractionation was unsuccessful as all activity was lost during chromatography. Finally, although the activity was fractionated in a 40-60% $(\text{NH}_4)_2\text{SO}_4$ pellet, there was a 2-fold loss in specific activity over the original supernatant. Recent experiments show that greater than 50% of the enzyme is found in the pellet fraction when rat liver supernatants are centrifuged at $105,000 \times g$ for an additional 18 hours. This procedure is currently being tried as a first step in the purification scheme, with hopes that the enzyme will become stable at some stage of purification.

There are several significant differences between this study and those of Wong *et. al.* which deserve mention. First, the sulfur donor used here was cysteine, while β -mercaptopyruvate was used in the studies with rat brain (15), and with liver and hepatoma sulfurtransferases (16). We have shown here that while sulfur incorporation from the latter substrate was demonstrable with liver and brain preparations, that chemical deacylation lowered the ^{35}S in tRNA to blank levels. Further, addition of unlabeled cysteine to [^{35}S] β -mercaptopyruvate assays inhibited incorporation into tRNA. While this might suggest that β -mercaptopyruvate was converted to cysteine and subsequently acylated to tRNA, we were unable to detect such a conversion in reaction mixtures. It is possible that β -mercaptopyruvate was directly acylated to tRNA, but we have no evidence of this. Next, yeast tRNA served as sulfur acceptor in the studies of Wong *et. al.* (15,16), while *E. coli* B tRNA was used here. While unlikely that yeast [^{35}S] tRNA labeled by β -mercaptopyruvate would be insensitive to deacylation, it is possible that adduct formation between this sulfur donor and a minor base unique to yeast tRNA had occurred. Finally, the pH optimum of 7.4 reported for the brain enzyme (15) differs from the value reported here.

β -Mercaptopyruvate was initially thought to be a required component of the *E. coli* sulfurtransferase system (21), but on purification this requirement was lost (14). Later work demonstrated that β -mercaptopyruvate merely reversed an amino acid inactivation of the *E. coli* $s^4\text{U}$:sulfurtransferase, which requires pyridoxal-5'-phosphate as a cofactor (23). β -Mercaptopyruvate and other α -keto acids were equally effective in reversing this inactivation. We could not demonstrate a requirement for pyridoxal-5'-phosphate for the rat liver sulfurtransferase system, but this could be due to high levels of this cofactor in our crude preparations. The results reported here clearly demonstrate that cysteine is the substrate for the enzymes present in rat liver, and suggest that the results of experiments with β -mercaptopyruvate (15,16)

should be reevaluated. Because of this finding it was necessary to determine the characteristics of the enzyme in rat liver supernatants using the true sulfur donor.

Only tRNA appears to serve as a sulfur acceptor in the in vitro sulfur-transferase system. Rat liver tRNA was a poor acceptor, presumably because it has nearly all available sites filled by the liver enzymes. On the other hand, rat brain tRNA was among the better sulfur acceptors, suggesting that the sulfurtransferases may be tissue specific. The low sulfur incorporation with all tRNAs may necessitate the use of a specific isoacceptor lacking the thiobase in question. Interestingly, sulfur-deficient tRNA from the C6 mutant of E. coli (24) had the highest sulfur acceptance, presumably because it has additional sites available for thiolation. DNA, rRNA, and synthetic homopolymers were inactive as sulfur acceptors. Hence, the mammalian sulfur transferases specifically recognize tRNA and may require a definite structure for thiolation.

The product of the in vitro reaction was identified as tRNA by MAK column chromatography. Alkaline digests of this [³⁵S] tRNA were then chromatographed on DEAE-cellulose to see if sulfur was incorporated into nucleotide material. The results demonstrate a single thionucleotide peak which elutes with the first nucleotide material on DEAE-cellulose. The low amount of this thiobase obtained, along with the lack of an appropriate standard, prevents us from identifying it at this time. However, nucleotide digests of in vitro labeled tRNA from E. coli (11,14,20) and rat brain (15) also contain thionucleotides which chromatograph at this position on DEAE-cellulose. We are currently evaluating the isolated hepatocyte system (25) and certain cell lines maintained in culture to see if [³⁵S] tRNA can be isolated for use as an in vivo marker, and an aid to identification of the presumed in vitro labeled thionucleotide.

The function of thionucleotides in tRNA is not fully understood at present. Since the only thiobase identified in mammalian tRNA is 5-methyl-2-thiouridine (9), studies involving 2-thiouridines will be discussed here. It is known that 5-methyl-2-thiouridine is located in the "Wobble" position, or first anticodon position, in tRNA^{lys} and tRNA^{glu} from rat liver (9). Recently, Rudloff and Hilse isolated tRNA^{lys}, tRNA^{glu} and tRNA^{gln} from rabbit liver and reticulocytes, and demonstrated that they were inactivated for aminoacylation by iodine treatment (26), previously shown by Carbon et. al. to attack 2-thiouridine in mammalian tRNA (27). Iodine treatment lowered aminoacylation of only the tRNA isoacceptors which recognize codons ending with adenosine (26).

Studies of *E. coli* tRNA^{glu} have suggested that 2-thiouridine is responsible for selective recognition of codons ending with adenosine (28). That is, a tRNA with s²U in the first position of the anticodon will recognize XYA only, and not XY_G^A as would be predicted by the Wobble hypothesis. Taken together, these studies suggest two roles for s²U in tRNA: as part of the synthetase recognition site, and in the selective recognition of codons.

Because of the importance of s²U to tRNA function, a regulation of the sulfurtransferase in the cell appears necessary. Transfer RNA from rapidly growing cells is often undermodified (29), possibly due to changes in the activity of tRNA modification enzymes. If this occurred with sulfurtransferases, a change in codon recognition would follow, which in turn could alter the translational capacity of certain tRNA isoacceptors. In this regard, we observed no difference in tRNA sulfurtransferase levels in rat liver and dye-induced hepatomas (17). However, enzyme activity in various types of tumors will have to be measured before any definite conclusions about regulation of sulfurtransferase activity can be made. These experiments are currently in progress.

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2. Abbreviations used: s⁴U, 4-thiouridine; s²U, 2-thiouridine; poly U, polyuridylic acid; poly A, polyadenylic acid; sulfur-deficient tRNA, thionucleotide-deficient tRNA isolated after cysteine starvation of *Escherichia coli* HfrC, rel⁻, cys⁻, met⁻, λ.
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