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Transfer Ribonucleic Acid Sulfurtransferase Isolated from Rat Cerebral Hemispheres†

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ABSTRACT: A tRNA sulfurtransferase system has been isolated from rat cerebral hemispheres which is capable of transferring the labeled sulfur from [^{35}S]β-mercaptopyruvate to tRNA. While such enzymes have been reported in bacteria, none has been harvested previously from mammals. In addition to the enzyme and the sulfur donor, the transsulfuration reaction has a strict requirement for ATP, magnesium ion, and tRNA as sulfur acceptor. Of all the RNA species tested, only tRNA can accept sulfur; rRNA, mRNA, and synthetic ribohomopolymers lack this capacity. Two findings indicate that the sulfur is transferred onto the tRNA molecule by the brain enzyme: (1) on Sephadex G-100 chromatography, the ^{35}S label elutes with tRNA; (2) the labeled enzymatic product is sensitive to RNase treatment. Hydrolysis of the *in vitro* labeled [^{35}S]tRNA followed by DEAE-cellulose chromatography or electrophoresis reveals the formation of several thionucleotides, none of which is 4-thioUMP, the major thionucleotide synthesized by *Escherichia coli*. Fractionation of the subcellular components of rat cerebral hemispheres indicates that the sulfurtransferase activity is present predominantly in the soluble portion of the cytoplasm.

Thionucleotides are among the minor components of bacterial, yeast, and mammalian tRNAs (Carbon *et al.*, 1965, 1968; Lipsett, 1965; Baczynskyj *et al.*, 1968; Burrows *et al.*, 1968; Eliceiri, 1970). The usual role postulated for these minor constituents is that they regulate the secondary structure and consequently the functioning of tRNA. Earlier work suggested that the amino acid accepting ability of certain species of tRNA is controlled by thionucleotides; the latter must remain in the reduced form for the tRNA molecules to function as amino acid acceptors (Carbon *et al.*, 1965; Goehler and Doi, 1968). More recent studies have revealed the presence of thionucleotides in the anticodon region of certain tRNAs of *Escherichia coli*, yeast, and rat liver, which suggests that they are crucial to precise codon recognition by tRNA (Ohashi *et al.*, 1970; Yoshida *et al.*, 1971; Kimura-Harada *et al.*, 1971; Nishimura, 1972). Because of the central role played by tRNA in protein synthesis, the manner by which these unusual nucleotides come to be present in tRNA molecules presents a challenging riddle. Two basic mechanisms may be visualized for the biosynthesis of minor nucleotides in a tRNA molecule: insertion during nucleotide polymerization, or modification of nucleotides after polymerization. Investigations in bacterial systems by others and ourselves have indicated that the sulfur of thionucleotides originates from biochemical alteration occurring after polynucleotide assembly, through enzymatic transfer of the sulfur moiety of cysteine or β-mercaptopyruvate to tRNA (Hayward and Weiss, 1966; Lipsett and Peterkofsky, 1966; Wong *et al.*, 1970). The sulfur in *E. coli* tRNA has been shown to be derived from cysteine. Soluble extracts of *E. coli* capable of catalyzing such reactions were first described by

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Hayward and Weiss (1966) and by Lipsett and Peterkofsky (1966). Alkaline hydrolysis and chromatography of the *in vitro* thiolated tRNA indicated that the major product was 4-thioUMP, which was known to occur naturally in the tRNA of *E. coli* (Lipsett, 1965). More recently, we have isolated another *in vitro* sulfurtransferase system from *Bacillus subtilis* which, in contrast to the *E. coli* system, does not produce 4-thioUMP as the major product, but other thiopyrimidine nucleotides that are not yet identified (Wong *et al.*, 1970). The *B. subtilis* enzyme is capable of transferring the sulfur from either cysteine or β-mercaptopyruvate to tRNA. Of the two sulfur donors, β-mercaptopyruvate is by far the more efficient and exhibits a K_m 200–300 times smaller than the K_m for cysteine. Further, there are indications that when cysteine functions as the sulfur donor, it is first converted to β-mercaptopyruvate as an intermediate (Wong *et al.*, 1970).

While tRNA sulfurtransferases have been isolated from two bacterial systems, no such enzyme has been reported in mammals. Yet the existence of thiolated tRNA in mammalian species (Carbon *et al.*, 1965; Eliceiri, 1970; Kimura-Harada *et al.*, 1971) implies that such enzymes must exist in mammals. Recently, we have isolated a tRNA sulfurtransferase system from rat cerebral hemispheres which is capable of thiolating tRNA *in vitro* by catalyzing the transfer of sulfur from β-mercaptopyruvate to tRNA. It is the purpose of this report to describe the isolation of this mammalian tRNA sulfurtransferase and the study of some of its properties.

Materials and Methods

Enzyme Preparation. Male Buffalo rats 35–36 days old were used in the experiments, and all procedures were carried out at 0–4°. For each enzyme preparation, 15 rats were killed by decapitation. The cerebral hemispheres from every animal were excised rapidly, weighed, and transferred to a glass homogenizer equipped with a Teflon plunger. Chilled 0.14 M KCl–0.02 M Tris (pH 7.5) was then added in the amount of 10 ml/g of brain tissue. Homogenization was carried out by 3 up-and-down strokes at 4000 rpm. Afterward the pooled homogenate was centrifuged twice at 1600*g* for 10 min and once at 33,000*g* for 20 min. The supernatant was centrifuged again at 160,000*g* (49,000 rpm) for 120 min in a Spinco Type 50 titanium rotor. The resulting clear 160,000*g* supernatant was removed. A small portion of it was stored at −75° until assay; the remainder was subjected to ammonium sulfate fractionation. The proteins precipitating between 40 and 70% saturation with ammonium sulfate were collected by centrifugation at 33,000*g* for 20 min, resuspended in 15 ml of 0.02 M Tris (pH 7.5), and either stored at −75° until use or subjected to CM-cellulose treatment as follows.

The 15 ml of resuspended 40–70% ammonium sulfate precipitate was diluted fivefold with 0.02 M Tris (pH 7.5) and allowed to pass by gravity through a pad of CM-cellulose (20 mg of dry cellulose/mg of protein) in a coarse sintered-glass funnel. The filtrate was dialyzed against 20 volumes of saturated ammonium sulfate for 2 hr. The protein precipitate was collected by centrifugation at 33,000*g* for 20 min, resuspended in 15 ml of 0.02 M Tris (pH 7.5), and stored at −75°. This preparation was designated as the CM-cellulose enzyme.

Assay of Sulfurtransferase Activity. The standard assay mixture was 0.5 ml in volume and contained 5 nmol of ammonium [^{35}S]β-mercaptopyruvate, 0.5 mg of yeast tRNA, varying amounts of an enzyme fraction, 50 μmol of Tris (pH 7.4), 1 μmol of ATP, 3 μmol of $MgCl_2$, and 1 μmol of β-mercaptoethanol. The standard control consisted of the above mixture with 25 μmol of EDTA added. This control was based on the fact that the reaction had an absolute requirement for Mg^{2+} ion; in the presence of EDTA, which bound Mg^{2+} ion, the reaction could not take place.

The assay mixture was incubated at 37° for 20 min. At the end of this period, the reaction was terminated by adding 0.05 ml of 0.5 M EDTA (pH 7.5). This was followed by the addition of 3 ml of ice-cold 0.05 M β-mercaptoethanol and 0.1 ml of a 50-mg/ml solution of cetyltrimethylammonium bromide. The precipitate formed after standing for 30 min in ice was collected by centrifugation and dissolved in 0.5 ml of 2 M NaCl; then 1 ml of ethanol was added. After 1 hr at −20°, the precipitate was sedimented by centrifugation, resuspended in 1 ml of 0.02 M Tris (pH 8.2), and extracted for 2 min by vigorous Vortex mixing with 1 ml of phenol preequilibrated with 0.1 M sodium phosphate (pH 6.7). After the extraction, the aqueous phase, which contained RNA, was recovered. To it was added 0.1 ml of 2 M KOAc (pH 5.4) and 2 ml of ethanol. After 1 hr at −20°, the RNA precipitate was harvested by centrifugation, resuspended in 1 ml of 0.01 M NaCl–0.01 M Tris (pH 8.2), and transferred quantitatively to a glass counting vial. Twenty milliliters of a toluene–Triton X100 scintillation fluid (Patterson and Green, 1965) was then added, and the mixture was counted in a Nuclear-Chicago Mark II liquid scintillation spectrometer.

Definition of Enzyme Unit. A unit of enzyme is defined as that amount of protein which catalyzes the transfer of 1 pmol of ^{35}S from [^{35}S]β-mercaptopyruvate to yeast tRNA per 0.5 ml of standard assay mixture in 20-min incubation at 37°, under the conditions described above.

Radioactive and Nonradioactive Substrates. Ammonium [^{35}S]β-mercaptopyruvate ranging from 150 to 650 Ci per mol in specific activity was purchased from New England Nuclear Corp., Boston, Mass. The compound was dissolved in water and used directly. Unused portions were stored at −20°. Authentic nonradioactive ammonium β-mercaptopyruvate was a generous gift from Dr. Ernest Kun (Kun, 1957).

Preparation of Nucleic Acids for Use as ^{35}S Acceptors. Yeast tRNA was purchased from Schwarz-Mann and served as the sulfur acceptor for most of the experiments described here. Prior to use, it was extracted three times with phenol preequilibrated with 0.1 M sodium phosphate (pH 6.7), precipitated with ethanol, resuspended in water, and dialyzed overnight against several changes of water. *E. coli* B tRNA was also obtained from Schwarz-Mann and purified in the same manner. Rat liver tRNA and rabbit liver tRNA were purchased from General Biochemicals, extracted with phenol, precipitated with ethanol, and purified with Sephadex G-100 chromatography. Synthetic ribohomopolymers (poly(U), poly(C), poly(A), and poly(G)) were procured from Miles Laboratory, Inc. and used without further purification. rRNA from *E. coli* B was prepared from isolated ribosomes by phenol extraction (Gierer and Schramm, 1956). The material which precipitated with 1 M NaCl at 4° was used as rRNA after extensive dialysis against water. mRNA from the bacteriophage MS2 was also obtained by phenol extraction.

Isolation of [^{35}S]tRNA for Analysis. For all studies on product analysis, the homologous rat liver tRNA was used as the sulfur acceptor. The [^{35}S]tRNA was prepared and isolated as follows. A 3-ml standard reaction mixture, containing 3 mg of rat liver tRNA and approximately 180 units of CM-cellulose enzyme, was incubated at 37° for 20 min, after which the reaction was stopped by the addition of EDTA and chilling in ice as usual. The reaction mixture was then extracted three times with an equal volume of phenol preequilibrated with 0.1 M sodium phosphate (pH 6.7), and precipitated twice with 0.1

TABLE I: Partial Purification of Sulfurtransferase from Rat Cerebral Hemispheres.[a]

Fraction	Vol (ml)	A_{280}/A_{260}	Total Units	Protein Concn (mg/ml)	Sp Act. (Units/mg of Protein)
160,000g supernatant	122	0.63	19,520	3.12	51.3
40–70% $(NH_4)_2SO_4$ precipitate	15	1.37	13,380	8.36	106.7
CM-cellulose enzyme	15	1.47	7,080	7.17	65.8

[a] Procedures for enzyme preparation and the definition of an enzyme unit are given under Materials and Methods. Protein concentrations were determined by the method of Lowry *et al.* (1951). Values for total units and specific activity were calculated from ^{35}S incorporation data obtained with standard assays of enzyme activity for the various fractions shown.

volume of 2 M KOAc (pH 5.4) and two volumes of ethanol. The final pellet of [^{35}S]tRNA was resuspended in 0.5 ml of 0.05 M NH_4HCO_3 (pH 8.6)–0.0014 M β-mercaptoethanol, and chromatographed on a 90 × 1 cm column of Sephadex G-25, using the same buffer for elution. The excluded [^{35}S]tRNA was recovered by precipitation with KOAc and ethanol, then resuspended in water or other solutions for further use.

DEAE-cellulose Chromatography of Alkaline Hydrolysate of [^{35}S]tRNA. Approximately 3 mg of [^{35}S]tRNA, obtained from the Sephadex G-25 chromatography described above, was hydrolyzed in 0.5 ml of 0.3 M KOH at 37° for 18 hr. The hydrolysate was diluted with water to about 20 ml and neutralized with Dowex 50 (H^+ form) to pH 10. The resin was then removed promptly by filtration, and the filtrate was further neutralized to pH 8.6 with dilute HCl. About 8 A_{320} units of nonradioactive 4-thio-2′(3′)-UMP was then added as marker and the entire mixture was adjusted to 0.01 M NH_4HCO_3 (pH 8.6) in a volume of 100 ml. This solution was applied to a 30 × 1 cm DEAE-cellulose column preequilibrated with the same buffer. The column was then washed with 60 ml of 0.02 M NH_4HCO_3 (pH 8.6), and eluted with a linear gradient consisting of 180 ml of 0.05 M NH_4HCO_3 (pH 8.6) in 7 M urea in the mixing chamber and 180 ml of 0.25 M NH_4HCO_3 (pH 8.6) in 7 M urea in the reservoir, as described by Lipsett (1965). Fractions (4 ml each) were collected, and their A_{260}, A_{280}, A_{320}, and radioactivity were determined.

Paper Electrophoresis of Nucleotides Derived from [^{35}S]tRNA. The [^{35}S]tRNA obtained from Sephadex chromatography was suspended in 0.01 M NH_4HCO_3 (pH 8.6) and treated with RNase (50 μg of pancreatic RNase and 10 μg of T_1 RNase/0.2 ml of reaction) by incubating at 37° for 2 hr. Afterward one-half of the RNase-treated material was incubated with crystalline *E. coli* alkaline phosphatase (29 μg/0.1 ml of reaction) at 37° for 1 hr. The RNase digests, with and without alkaline phosphatase treatment, were adjusted to contain 0.05 M NH_4HCO_3 (pH 8.6) and 0.0014 M β-mercaptoethanol. That amount of each digest sufficient to give 6000–8000 cpm was spotted on Whatman No. 3MM paper and electrophoresed in the same buffer at 400 V for 7 hr in the cold. Afterward the paper was air-dried, cut into small strips, placed in glass vials, and counted with 0.5 ml of water and 10 ml of scintillation fluid.

Isolation of Subcellular Fractions from Rat Cerebral Hemispheres and Determination of Their Sulfurtransferase Activity. To determine the distribution of tRNA sulfurtransferase activity in the various subcellular fractions, the cerebral hemispheres derived from 10 male Buffalo rats (35–36 days old) were homogenized in 0.14 M KCl–0.02 M Tris (pH 7.5) in the amount of 10 ml/g of tissue as described under Enzyme Preparation. The homogenate was then subjected to differential centrifugations according to the method of Ivanov *et al.* (1967), with the exception that the sucrose–$CaCl_2$ buffer in the reported procedure was replaced by 0.14 M KCl–0.02 M Tris (pH 7.5) in all steps. The nuclear fraction obtained from the above method, which was contaminated with cell debris and unbroken cells, was further purified by centrifugation in 2.05 M sucrose–0.02 M Tris (pH 7.5)–0.001 M $MgCl_2$ for 2 hr at 25,000 rpm in a Spinco SW 25.1 rotor.

Afterward all particulate fractions were leached with 0.02 M Tris (pH 7.5) at 0° for 40–60 min, then centrifuged at 33,000g for 20 min. The supernatants from these leached particulate fractions as well as the supernatant from the whole homogenate were assayed for their sulfurtransferase activity with the standard assay mixture, from which the total activity of each subcellular fraction was calculated.

Miscellaneous Materials. Male Buffalo rats were purchased from Simonsen Laboratories, Inc., Gilroy, Calif. Nonradioactive 4-thio-2′(3′)-UMP was isolated from the alkaline hydrolysate of *E. coli* B tRNA by the method of Lipsett (1965). 4-Thiouridine was obtained from Sigma Chemical Co. Pancreatic RNase, pancreatic DNase, and *E. coli* alkaline phosphatase were procured from Worthington Biochemical Corp. T_1 RNase was obtained from Calbiochem. Hexokinase (Type III yeast) was purchased from Sigma Chemical Co.

Results

Enzyme Purification. As outlined in Table I, sulfurtransferase activity was demonstrated in the 160,000g supernatant obtained from the whole homogenate of rat cerebral hemispheres. A 40–70% ammonium sulfate fractionation of the supernatant led to a twofold increase in specific activity with retention of 65–75% of the original activity. But more importantly, the ammonium sulfate fractionation led to a stable enzyme preparation which retained its activity for several months when stored at −75°. For this reason, we have found it convenient to use the 40–70% ammonium sulfate precipitate as the enzyme source for most of the studies described in this report.

Treatment of the resuspended 40–70% ammonium sulfate precipitate with CM-cellulose resulted in a loss of enzyme units as well as a decrease in specific activity (Table I). Nonetheless, the treatment was effective in removing RNase activity from the enzyme preparation, so that the [^{35}S]tRNA produced was comparatively intact. For this reason, the CM-cellulose enzyme was used mainly for the preparation of [^{35}S]tRNA for product analysis (see Materials and Methods).

Properties of the Transsulfuration Reaction. The general characteristics of the transsulfuration reaction catalyzed by the 40–70% ammonium sulfate fraction and with [^{35}S]β-mercaptopyruvate as the substrate are summarized in Table II. Radioactivity is incorporated into tRNA when [^{35}S]β-mercaptopyruvate is incubated in a complete reaction mixture with the enzyme. ^{35}S incorporation requires the addition of enzyme; en-

TABLE II: Requirements of Transsulfuration Reaction with β-Mercaptopyruvate as Sulfur Donor and tRNA as Acceptor.[a]

Reaction Mixture	^{35}S Incorp into tRNA (pmol)
Complete	24.4
No enzyme	1.4
Heated enzyme	1.0
No ATP	1.6
No $MgCl_2$	1.3
Add EDTA	1.1
No tRNA	1.0
Add RNase	1.3
Add DNase	23.4
Add nonradioactive β-mercaptopyruvate (1-fold)	13.2
Add nonradioactive β-mercaptopyruvate (10-fold)	2.9

[a] The complete reaction mixture was the same as the standard assay system described under Materials and Methods and contained 5 nmol of [^{35}S]β-mercaptopyruvate (1.84×10^5 cpm/nmol) as the radioactive substrate and 24.4 units of the 40–70% $(NH_4)_2SO_4$ fraction as enzyme. Where indicated, one of the following was also present: (1) 24.4 units of heated (5 min at 100°) 40–70% $(NH_4)_2SO_4$ fraction in place of the same amount of unheated enzyme, (2) 25 μmol of EDTA, (3) 50 μg of pancreatic RNase and 10 μg of T_1 RNase, (4) 50 μg of pancreatic DNase, and (5) 5 or 50 nmol of nonradioactive β-mercaptopyruvate. Incubation was for 20 min at 37°.

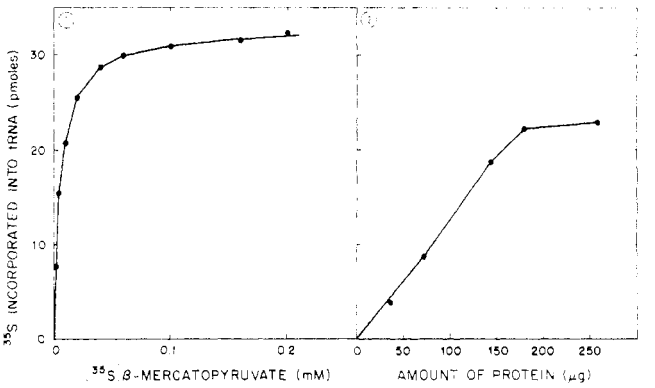

FIGURE 1: ^{35}S incorporation into tRNA as a function of the concentration of the sulfur donor, [^{35}S]β-mercaptopyruvate. The reaction mixture, containing 20.8 units of 40–70% $(NH_4)_2SO_4$ fraction, was the same as the standard assay system described under Materials and Methods, except that the concentration of [^{35}S]β-mercaptopyruvate (2.37×10^5 cpm/nmol) was varied as indicated above. Incubation was for 20 min at 37°.

FIGURE 2: Effect of varying enzyme concentration on ^{35}S incorporation into tRNA. The reaction mixture, containing 5 nmol of [^{35}S]β-mercaptopyruvate (2.28×10^5 cpm/nmol), was the same as the standard assay system described under Materials and Methods, except that the concentration of the 40–70% $(NH_4)_2SO_4$ fraction (in μg of protein/0.5 ml of reaction mixture) was varied as indicated above. Incubation was for 20 min at 37°.

zyme that has been heated to 100° for 5 min or more is inactive. ^{35}S incorporation also requires the addition of ATP, Mg^{2+} ion, and tRNA to the reaction system. RNase completely abolishes the formation of labeled product, while DNase has no such effect, indicating that the ^{35}S is transferred to RNA.

When authentic, nonradioactive β-mercaptopyruvate is added to the reaction mixture in amounts one- or tenfold that of [^{35}S]β-mercaptopyruvate, the decrease in percentage of ^{35}S incorporation into tRNA reflects almost exactly the dilution of the radioactive substrate. This finding indicates that [^{35}S]β-mercaptopyruvate, and not some radioactive contaminant, is the actual sulfur donor.

The extent of transsulfuration is dependent on the concentration of the sulfur donor, [^{35}S]β-mercaptopyruvate (Figure 1); it is also dependent on the concentration of the enzyme, the 40–70% ammonium sulfate fraction (Figure 2), and on the concentration of the sulfur acceptor, yeast tRNA (Figure 3).

The ability of various types of RNA to serve as ^{35}S acceptors

TABLE III: The Ability of Various RNAs to Serve as Acceptors in the Transsulfuration Reaction.[a]

Type of RNA Used as Acceptor	^{35}S Incorp into RNA (pmol)
None	0.8
Rat liver tRNA	22.7
Rabbit liver tRNA	18.8
Yeast tRNA	22.3
E. coli B tRNA	24.1
E. coli B rRNA	0.3
MS2 RNA	0.4
Poly(U)	0.1
Poly(C)	0.2
Poly(A)	0.2
Poly(G)	0.5

[a] The complete reaction mixture, containing 5 nmol of [^{35}S]β-mercaptopyruvate (2.13×10^5 cpm/nmol) and 22.3 units of 40–70% $(NH_4)_2SO_4$ fraction, was the same as the standard assay system described under Materials and Methods. Where indicated, 0.5 mg each of the various RNAs shown replaced yeast tRNA. Incubation was for 20 min at 37°.

TABLE IV: Specificity of Nucleotide Requirement for Sulfurtransferase Activity.[a]

Nucleotide Present in Reaction Mixture	^{35}S Incorp into tRNA (pmol)
None	1.4
ATP	23.0
GTP	1.1
UTP	0.8
CTP	1.0
ADP	16.0
ADP + hexokinase + glucose	1.0
ATP + hexokinase	22.8
ATP + glucose	23.1
AMP	1.0

[a] The complete reaction mixture, containing 5 nmol of [^{35}S]β-mercaptopyruvate (2.11×10^5 cpm/nmol) and 23.0 units of 40–70% $(NH_4)_2SO_4$ fraction, was the same as the standard assay system described under Materials and Methods. Where indicated, ATP was omitted or replaced by GTP, UTP, CTP, ADP, or AMP (1.0 μmol each). In three instances, 20 units of hexokinase (52.8 μg) and/or 20 μmol of glucose was added. Incubation was for 20 min at 37°.

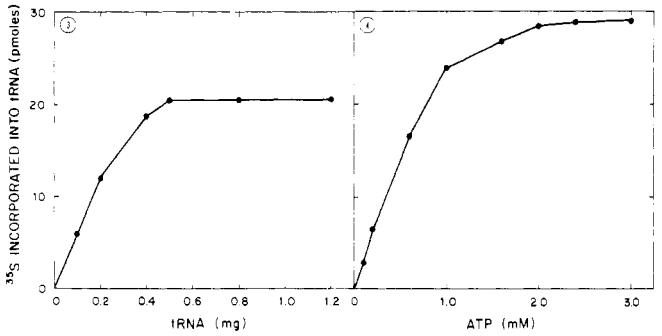

FIGURE 3: ^{35}S incorporation as a function of the concentration of the sulfur acceptor, yeast tRNA. The reaction mixture, containing 5 nmol of [^{35}S]β-mercaptopyruvate (2.48 × 10^5 cpm/nmol) and 20.5 units of 40–70% $(NH_4)_2SO_4$ fraction, was the same as the standard assay system described under Materials and Methods, except that the tRNA concentration was varied as indicated above. Incubation was for 20 min at 37°.

FIGURE 4: ^{35}S incorporation into tRNA as a function of ATP concentration. The reaction mixture, containing 5 nmol of [^{35}S]β-mercaptopyruvate (1.49 × 10^5 cpm/nmol) and 28.7 units of 40–70% $(NH_4)_2SO_4$ fraction, was the same as the standard assay system described under Materials and Methods, except that the concentration of ATP was varied as indicated above. Incubation was for 20 min at 37°.

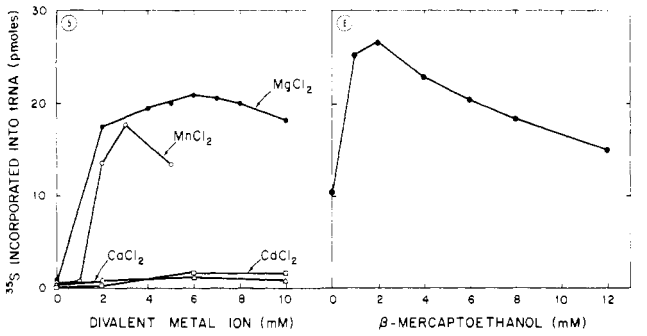

FIGURE 5: Effect of divalent metal ion concentration on sulfurtransferase activity. The reaction mixture, containing 5 nmol of [^{35}S]β-mercaptopyruvate (2.48 × 10^5 cpm/nmol) and 21.0 units of 40–70% $(NH_4)_2SO_4$ fraction, was the same as the standard assay system described under Materials and Methods, except that $MgCl_2$, $MnCl_2$, $CaCl_2$, or $CdCl_2$ was used at varying concentrations. Incubation was for 20 min at 37°.

FIGURE 6: Effect of β-mercaptoethanol concentration on sulfurtransferase activity. The reaction mixture, containing 5 nmol of [^{35}S]β-mercaptopyruvate (1.61 × 10^5 cpm/nmol) and 26.6 units of 40–70% $(NH_4)_2SO_4$ fraction, was the same as the standard assay system described under Materials and Methods, except that the concentration of β-mercaptoethanol was varied as indicated above. Incubation was for 20 min at 37°.

is shown in Table III. All tRNAs tested, whether from rat liver, rabbit liver, *E. coli* B, or yeast, can serve as ^{35}S acceptors. Because yeast tRNA has a reasonably high ^{35}S-accepting capacity among the tRNAs tested, gives rise to the same thionucleotides as the homologous rat liver tRNA with our sulfurtransferase system, and is readily available commercially, we have found it convenient to use yeast tRNA in all the standard assays. Table III also shows that rRNA from *E. coli* B, mRNA from the phage MS2, and synthetic ribohomopolymers such as poly(U), poly(C), poly(A), and poly(G) are ineffective as ^{35}S acceptors. These findings indicate that among the different kinds of RNA, only tRNA can accept sulfur.

The dependence of sulfurtransferase activity on ATP concentration is shown in Figure 4. The ability of ATP to function as the activating nucleotide in the transsulfuration reaction is highly specific, since ATP cannot be replaced by other nucleotides such as GTP, UTP, or CTP (Table IV). The ^{35}S transfer obtained with ADP is apparently due to the formation of ATP by the following reaction: 2ADP $\rightleftarrows$ ATP + AMP. This reaction is catalyzed by adenylate kinase, with which the 40–70% ammonium sulfate fraction appears to be contaminated. This speculation is confirmed when the addition of hexokinase and glucose to the reaction system completely abolishes the ^{35}S transfer. Hexokinase or glucose individually has no effect on the sulfurtransferase activity (Table IV). AMP is totally ineffective as the activating nucleotide.

The transsulfuration reaction also requires activation by a divalent metal ion. Either $MgCl_2$ or $MnCl_2$ may fulfill this role (Figure 5). Of the two, $MgCl_2$ is the more effective. Maximal sulfur transfer occurs at a concentration of 6 mM for magnesium ion and 3 mM for manganese ion. Higher concentrations lead to suppression of ^{35}S incorporation into tRNA. Other divalent metal ions such as calcium and cadmium ions are incapable of activating the transsulfuration reaction (Figure 5).

Optimal formation of [^{35}S]tRNA requires the presence of some reducing agent such as β-mercaptoethanol. In the absence of this thiol, [^{35}S]tRNA formation is about one-half to one-third of the maximum (Figure 6). Above a certain concentration, β-mercaptoethanol has an inhibitory effect on the reaction. The stimulatory effect of this thiol is probably due in part to its ability to maintain the substrate, [^{35}S]β-mercaptopyruvate, in the reduced form.

The extent of ^{35}S incorporation into tRNA is dependent on the time and temperature of incubation (Figure 7). The transsulfuration reaction can take place over a fairly broad pH range, but maximal sulfur transfer occurs at pH 7.4 (Figure 8).

Nature of the Labeled Product of Transsulfuration. It has already been indicated that the product of the transsulfuration reaction behaves like RNA, judging from its sensitivity to RNase (Table II). When the labeled product is chromatographed on Sephadex G-100, almost all the radioactivity is excluded in a position coincident with tRNA; a small amount of ^{35}S appears much later in a region known to exclude ATP and other low molecular weight compounds (Figure 9A). When the ^{35}S-labeled product is treated with RNase prior to Sephadex chromatography, the radioactivity and ultraviolet-absorbing material are no longer seen in the position for elution of tRNA but are now excluded in the same region as the low molecular weight substances (Figure 9B).

When the *in vitro* labeled product is exposed to alkali under conditions that hydrolyze RNA and then chromatographed on

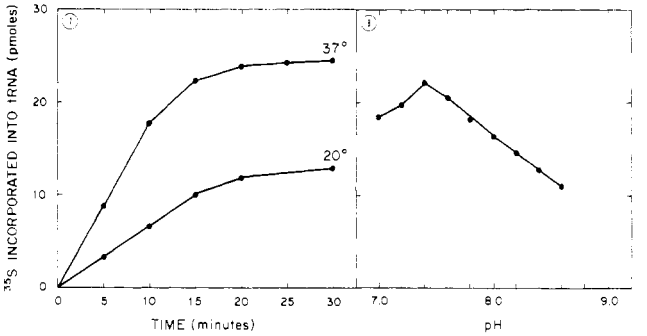

FIGURE 7: Effect of time and temperature of incubation on ^{35}S incorporation into tRNA. The reaction mixture, containing 5 nmol of [^{35}S]β-mercaptopyruvate (2.09 × 10^5 cpm/nmol) and 23.9 units of 40–70% $(NH_4)_2SO_4$ fraction, was the same as the standard assay system described under Materials and Methods. Time and temperature of incubation were varied as indicated above.

FIGURE 8: Effect of pH on sulfurtransferase activity. The reaction mixture, containing 5 nmol of [^{35}S]β-mercaptopyruvate (2.48 × 10^5 cpm/nmol) and 22.1 units of 40–70% $(NH_4)_2SO_4$ fraction, was the same as the standard assay system described under Materials and Methods, except that the pH of the mixture was varied (with Tris buffers) as indicated above. Incubation was for 20 min at 37°.

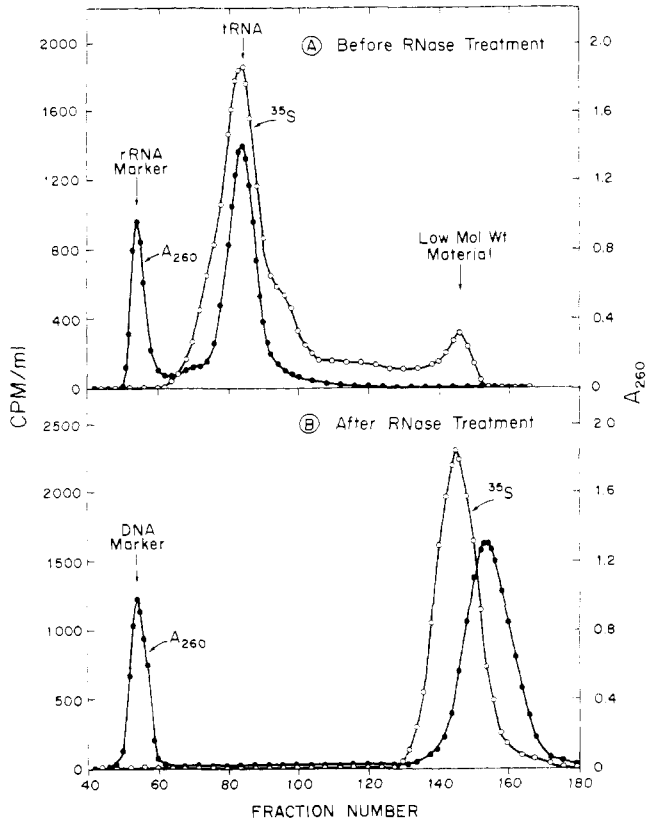

FIGURE 9: Sephadex G-100 chromatography of *in vitro* labeled rat liver [^{35}S]tRNA before and after RNase treatment. The [^{35}S]tRNA was prepared in a standard reaction mixture and isolated by phenol extraction, ethanol precipitation, and Sephadex G-25 chromatography to remove the vast amount of unreacted [^{35}S]β-mercaptopyruvate (6.00 $\times$ 10^5 cpm/nmol), as described under Materials and Methods. The [^{35}S]tRNA was then used in the following experiments: In (A), approximately 3 mg of [^{35}S]tRNA (114,000 cpm), mixed with 0.6 mg of marker *E. coli* B rRNA, was chromatographed on a 90 $\times$ 2.4 cm column of Sephadex G-100; elution was carried out with 0.05 M NH_4HCO_3 (pH 8.6)–0.0014 M β-mercaptoethanol, and 3-ml fractions were collected. In (B), approximately 3 mg of [^{35}S]tRNA (110,000 cpm) was incubated with 50 μg of pancreatic RNase and 10 μg of T_1 RNase in a volume of 0.5 ml for 2 hr at 37°; then 0.6 mg of heat-denatured calf thymus DNA was added as a marker, and the entire mixture was chromatographed on Sephadex G-100 as described for (A). (●) A_{260}; (○) ^{35}S.

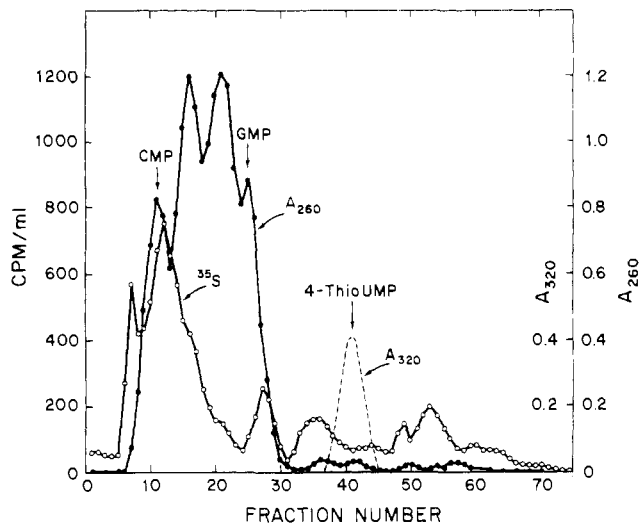

FIGURE 10: DEAE-cellulose chromatography of alkaline hydrolysate of *in vitro* labeled rat liver [^{35}S]tRNA: The sulfur donor, [^{35}S]β-mercaptopyruvate, had a specific activity of 5.88 $\times$ 10^5 cpm/nmol. The conditions for [^{35}S]tRNA preparation, isolation, alkaline hydrolysis, and DEAE-cellulose chromatography were as described under Materials and Methods. About 3 mg of [^{35}S]tRNA (108,000 cpm) was used in the preparation of the alkaline hydrolysate, and 8 A_{320} units of 4-thio-2′(3′)-UMP was added as marker. Recovery of the total radioactivity applied to the column was about 85%. (●) A_{260}; (--) A_{320}; (○) ^{35}S.

DEAE-cellulose, the bulk of the radioactivity distributes itself in several peaks in the region where the major nucleotides are normally found under the experimental conditions used (Figure 10). Little label is seen to elute with the 4-thio-2′(3′)-UMP that was added as a marker prior to chromatography. A small amount of labeled material elutes after 4-thio-2′(3′)-UMP; similar material has been observed by Eliceiri (1970) on DEAE-cellulose chromatography of alkaline hydrolysate derived from *in vivo* labeled [^{35}S]tRNA of mouse lymphoma cells.

Figure 11 shows the electrophoretic patterns of the hydrolysate of *in vitro* labeled [^{35}S]tRNA with and without additional alkaline phosphatase treatment. The hydrolysate contains a major product which migrates toward the anode at a slightly slower rate than 4-thio-2′(3′)-UMP (Figure 11A). Treatment with alkaline phosphatase retards significantly its rate of anodal migration, as would be expected if the ^{35}S is associated with a nucleotide, but again the dephosphorylated ^{35}S-product does not coincide with 4-thiouridine (Figure 11B). In addition to this major product, several minor, slower-moving ^{35}S-containing nucleotides also appear to be present (Figure 11A).

Distribution of Sulfurtransferase Activity in the Various Subcellular Fractions of Rat Cerebral Hemispheres. Assays performed with the leaching fluids obtained from the nuclear, mitochondrial, and microsomal fractions as well as the supernatant of the whole homogenate indicate that the bulk of the tRNA sulfurtransferase activity is present in the supernatant fraction (Table V). A minute quantity of the activity is also found in the leaching fluids from the mitochondrial and microsomal fractions. Without further purification by sucrose gradient, it is impossible to state whether such low levels of enzyme activities are the result of contamination of the mitochondrial and microsomal fractions by the cell sap or whether they represent true activities residing in these cell organelles. In view of the report of thionucleotides in the mitochondrial tRNA of chicken liver (Lalyre-G. and Titchener, 1971), however, these activities, though low, may be meaningful. The repeated failures to demonstrate tRNA sulfurtransferase activity in the nuclear fraction may be real or a consequence of the leaching procedure used, which may not be effective in extracting any tRNA sulfurtransferase activity that is potentially present in the nuclei.

Discussion

A sulfurtransferase system has been partially purified from the whole homogenate of rat cerebral hemispheres which is capable of transferring the sulfur from β-mercaptopyruvate to tRNA *in vitro*. In addition to the sulfur donor, enzyme, and tRNA as acceptor, the *in vitro* system requires ATP and magnesium ion for activation and a thiol such as β-mercaptoethanol for optimal sulfur transfer.

Among the different varieties of RNA, only tRNA is capable of accepting sulfur in this mammalian sulfurtransferase system. Both homologous and heterologous tRNAs are effective in this respect, but rRNA, mRNA, and synthetic ribohomopolymers are ineffective. This specificity implies that certain structural attributes peculiar to tRNA are requisite for the ability to accept sulfur.

Chromatography of the ^{35}S-labeled enzymatic product on Sephadex G-100 before and after RNase treatment indicates

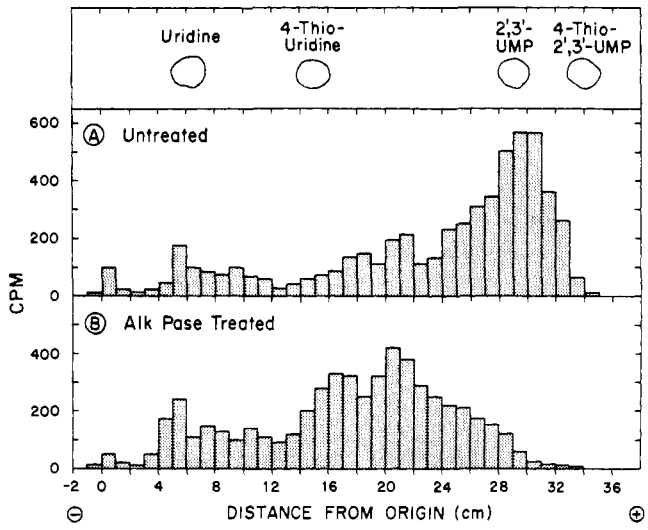

FIGURE 11: Electrophoresis of RNase digest of *in vitro* labeled rat liver [^{35}S]tRNA. The [^{35}S]β-mercaptopyruvate used as sulfur donor had a specific activity of 5.76×10^5 cpm/nmol. The conditions for [^{35}S]tRNA preparation, hydrolysis with RNase, treatment of the RNase digest with alkaline phosphatase, and electrophoresis were as described under Materials and Methods. In (A), the RNase digest was electrophoresed directly. In (B), the RNase digest was further treated with alkaline phosphatase before electrophoresis. Total cpm spotted was about 6000 in each instance. Nonradioactive markers including 4-thio-2'(3')-UMP, 2'(3')-UMP, 4-thiouridine, and uridine were electrophoresed simultaneously.

that the radioactive sulfur is an integral part of tRNA or its RNase digest. The electrophoretic properties of the ^{35}S-products formed after hydrolysis of the *in vitro* labeled rat liver tRNA, and their behavior after exposure to alkaline phosphatase, suggest that they are sulfur-containing nucleotides. DEAE-cellulose chromatography of the alkaline digest of *in vitro* labeled rat liver tRNA reveals the formation of several thionucleotides, none of which is 4-thioUMP, the major thionucleotide of *E. coli*. This observation is in accord with that of Lipsett (1965), who found no 320-nm-absorbing material in unfractionated rat liver tRNA. It is also in agreement with the report of Eliceiri (1970), who found no 4-thioUMP in the alkaline hydrolysate of *in vivo* labeled [^{35}S]tRNA isolated from mouse lymphoma cells.

The mechanism by which sulfur is transferred from β-mercaptopyruvate to the acceptor tRNA is not known. The specific requirement for ATP in the transsulfuration reaction suggests the formation of an activated intermediate, either of the sulfur donor or of specific bases in the acceptor tRNA. Kinetic data from the study of Abrell *et al.* (1971) with *E. coli* tRNA sulfurtransferase using cysteine as the sulfur donor indicate that ATP may be involved in activating tRNA, but the nature of the intermediate has not yet been elucidated, nor have attempts at demonstrating phosphate or pyrophosphate exchange with ATP been successful.

Fractionation of the subcellular components of rat cerebral hemispheres indicates that the tRNA sulfurtransferase activity is localized predominantly in the soluble portion of the cytoplasm. It is of interest that another group of enzymes involved in the biosynthesis of minor nucleotides, the tRNA methylases, are also localized predominantly in the cell supernatant (Burdon *et al.*, 1967; Culp and Brown, 1968).

Studies not presented here indicate that tRNA sulfurtransferase activity is also present in the soluble cytoplasm of rat liver and kidney. Other organs have not yet been investigated for their tRNA sulfurtransferase activities.

The reason for studying the tRNA sulfurtransferase in the brain is twofold. First, the great stability of the 40–70% ammonium sulfate precipitate obtained from the cell supernatant makes it convenient to use the brain enzyme for any protracted period of study. Second, there is some suggestion from recent reports that abnormal metabolism of β-mercaptopyruvate may be responsible for certain instances of genetically determined mental retardation (Ampola *et al.*, 1969; Crawhall *et al.*, 1969). Since β-mercaptopyruvate is a substrate for tRNA sulfurtransferase, studies of the factors controlling the action of this enzyme may have some bearing in understanding the manifestations of such a genetic abnormality.

TABLE V: Distribution of Sulfurtransferase Activity in Various Subcellular Fractions of Rat Cerebral Hemispheres.[a]

Subcellular Fraction	% Sulfurtransferase Act.
Nuclei	0.0 ± 0.0
Heavy mitochondria	0.5 ± 0.3
Light mitochondria	0.4 ± 0.1
Microsomes	0.6 ± 0.2
Supernatant	98.5 ± 0.4

[a] Preparation of the various subcellular fractions and assay for sulfurtransferase activity were as described under Materials and Methods. The above data represented the average ±1 standard deviation from three separate experiments on fractionation. For each experiment, 10 male Buffalo rats were used.

The function of thionucleotides in tRNA is not yet entirely elucidated. The studies of Carbon *et al.* (1965) and Goehler and Doi (1968) indicate that certain species of tRNA from rabbit liver, *E. coli*, and *B. subtilis* lose their ability to accept amino acids following oxidation with dilute iodine solutions. Because this inactivation is readily reversible by reduction of the iodine-exposed tRNAs with thiosulfate or glutathione, the phenomenon was interpreted as potentially due to disulfide-bond formation involving thionucleotides in the tRNA molecules (Carbon *et al.*, 1965).

More recent studies have unveiled the presence of 2-thiouridine derivatives in the first position of the anticodon of $tRNA^{Glu}$ from such diverse sources as yeast, *E. coli*, and rat liver (Yoshida *et al.*, 1970, 1971; Ohashi *et al.*, 1970; Kimura-Harada *et al.*, 1971). Further, there are indications that these strategically placed thionucleotides may play a role in precise codon recognition by tRNA through imposition of strict base-pairing between codon and anticodon and consequently prevention of wobbling. Sekiya *et al.* (1969) first drew attention to the fact that one species of glutamic acid accepting tRNA from yeast, $tRNA_3^{Glu}$, specifically recognized GAA from the two known code words for glutamic acid, GAA and GAG, in trinucleotide-stimulated ribosome binding assays. Such specificity in codon recognition was contrary to the prediction of Crick's wobble hypothesis (1966). The subsequent discovery of 2-thiouridine-5-acetic acid methyl ester in the first position of the anticodon of yeast $tRNA_3^{Glu}$ readily explained this specificity in codon recognition, since the 2-thiouridine derivative in the first position of the anticodon could pair only with A in the third position of the codon but hardly with G (Yoshida *et al.*, 1970, 1971). Parallel studies by Ohashi *et al.* (1970), Kimura-Harada *et al.* (1971), and Agris *et al.* (1973) with $tRNA^{Glu}$ from *E. coli* and rat liver further confirmed the correlation between the existence of a 2-thiouridine derivative in the first position of the anticodon and preferential recognition of A in the third position of the codon for glutamic acid.

Should thionucleotides be proven to play an indispensable

role in the functioning of tRNA, then the enzyme tRNA sulfurtransferase could play a potentially regulatory role in protein synthesis by controlling the extent of tRNA thiolation.

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