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"IN VIVO" (^{35}S)METHIONINE INTERACTION WITH RAT LIVER tRNA

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As part of a study to characterize the methionine role in tumorigenesis, we report that methionine sulfur interacts with rat liver tRNA "in vivo". (^{35}S)radioactivity remained associated to the nucleic acid after a number of treatments, including tRNA deacylation. Similar data were obtained after administration of (methyl-^{3}H)methionine, while no comparable tRNA labelling was detected when the aminoacid labelled in the aliphatic chain was given. Hplc analysis of (^{35}S)tRNA enzymic hydrolysate showed two unidentified UV-absorbing radioactive peaks. NMR spectra of these two peaks did not reveal any thiomethyl group.

An earlier report from this laboratory indicated an "in vivo" interaction between methionine and DNA involving the sulfur of the aminoacid (1). Two radioactive peaks were detected and partially characterized by hplc of (^{35}S)DNA enzymic hydrolysate. Thus, given the well known crucial role of methionine in oncogenesis and the possible mechanisms herein involved (2), it was decided to extend this study to tRNA which is the main target of bioalkylation by methionine (3).

MATERIALS AND METHODS

L-(methyl-^{3}H)methionine (200 mCi/mmol) and (L-^{35}S)methionine (1115 or 1198 Ci/mmole) were from New England Nuclear, Boston, MA.; L-[2(n)-^{3}H]methionine (9.5 Ci/mmol) and L-(2-^{14}C)methionine (58 mCi/mmol) were from Amersham, UK. Oligo-dT and DE52-cellulose were from Pharmacia and Whatman respectively. Enzymes were from Boehringer, Mannheim. Ribonucleosides were from Sigma.

Wistar male albino rats (180-200 g) were given radioactive methionine plus/or unlabelled aminoacid by intraperitoneal injection and then killed three hours later.

Rat livers were homogenized in 0.075 M NaCl/0.025 N EDTA, pH 7.6/0.1 % Triton X-100 and DNA was isolated and purified from nuclear pellet as described (1). Total RNA was extracted with phenol from supernatant fraction and treated with 0.25 mg/ml proteinase K in 1 M NaCl/0.01 M Tris-HCl, pH 7.8,/0.05 % SDS, 4 h, 37°C, followed by a second protease treatment with 1 mg/ml pronase

Abbreviations: cytidine, Cyd; uridine, Urd; guanosine, Guo; inosine, Ino; intraperitoneal, i.p.

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in the same buffer. The RNA was fractionated through an oligo-dT cellulose column in order to isolate mRNA (4). The first large UV-absorbing peak from oligo-dT chromatography was ethanol-precipitated and fractionated again on a DE52-cellulose column according to Brunngraber (5), in order to separate tRNA. The tRNA was deacylated by incubation in 1.8 M Tris-HCl, pH 8.0, at 37°C for 90 min (6) or in 0.1 M Tris-HCl, pH 9.5, at 37°C for 1 h (7). Nucleic acids were ethanol precipitated, dissolved in 10 mM NaCl and assayed for (^{35}S)radioactivity in Maxifluor by liquid scintillation counting in a LS 7800 Beckman.

25 $OD_{260\ nm}$ (^{35}S)tRNA in 1 ml 0.05 M Tris, pH 8.0/0.01 M NaCl/0.01 M EDTA was enzymically hydrolyzed to nucleosides using RNases (0.5 mg pancreatic and 100 units T1, both pre-treated at 80°C) plus alkaline phosphatase (5 units) at 37°C for 16 h. Phosphatase was pre-heated at 95°C for 10 min in 0.05 M Tris-HCl pH 8.0 to inactivate adenosine deaminase. Then reaction mixture was adjusted to pH 6.0, nucleases P1 (40 units) and S1 (10 units) plus wheat germ acid phosphatase (3 units) were added and incubation prolonged for further 12 h. The incubation reaction mixture was heated at 100°C for 10 min and then centrifuged to remove the denatured enzyme proteins.

Separation of tRNA digestion products was carried out on a Perkin Elmer liquid chromatograph equipped with an LC-100 column oven, an LC-55B spectrophotometric detector with a 254nm filter and a Shimadzu C-R64 Chromatopac. The column was a 5 µm RP-18 Lichrosorb prefitted with a 7 µm guard column (both from Merck) and isocratically eluted with 0.005 M ammonium formate, pH 4.8/0.5 % methanol, at 37°C and at a flow rate of 1 ml/min. The four main ribonucleosides were identified by using authentic standards; some of the minor modified ribonucleosides were identified analyzing in the UV (8) the relative fractions. Hplc fractions were then assayed for radioactivity as previously described. Ultraviolet spectra were obtained with a Beckman DU-8 spectrophotometer.

For NMR spectroscopy, samples were dissolved in $^{2}H_2O$ and analyzed with a Bruker AM-300 spectrometer. Chemical shifts were given in ppm using the solvent signal ($^{2}H_2O$ = 4.8 ppm) as reference.

RESULTS AND DISCUSSION

Both tRNA and DNA from rat liver became labelled after the animals had been given (^{35}S)methionine by i.p. injection, while no radioactivity was found in association to poly (A^+)RNA or rRNA. As shown in Table 1, the highest extent of labelling was found in tRNA, the radioactivity ratio of RNA to DNA being 13.3.

Table 1 "In vivo" labelling of rat liver nucleic acids by (^{35}S)methionine. Rats were injected i.p. with the radioactive amino acid (1.6 mCi, 25 mg/kg b.w.) and nucleic acids isolated and analyzed for ^{35}S-cpm as described under Methods. Data are reported as mean value ± S.D. Number of experiments in parentheses.

Nucleic acid	pmol (^{35}S)/O.D. $_{260\ nm}$	
DNA	2.6 ± 0.3	(3)
tRNA	34.5 ± 3.1	(5)
mRNA	0	(5)
rRNA	0	(5)

Table 2 Stability to purification procedure of (^{35}S)radioactivity bound to tRNA following "in vivo" exposure to sulfur-labelled amino acid. Rats were injected with (^{35}S) methionine (2.2 mCi, 10 mg/kg b.w.) and RNA extracted and analyzed for radioactivity. Data are reported as the means of 5 experiments ± S.D.

Treatment	pmol sulfur/OD_{260}
Phenol extraction	8.6 ± 1.8
Proteinase K	5.4 ± 1.3
Pronase	4.9 ± 2.1
DE-52 chromatography	9.3 ± 1.6
Deacylation	5.7 ± 1.8

Table 2 shows the stability of (^{35}S)binding to tRNA during the purification procedure. Not even deacylation treatment removed radioactivity, thus indicating that the binding of the label to tRNA was not due to the formation of methionyl-tRNA.

To further control the specificity of this interaction, tRNA labelling was analyzed after administration to the rats of methionine which was isotopically labelled in the methyl group, in the sulfur or in the aliphatic chain. Table 3 illustrates that, as expected, administration of (^{3}H-methyl)-methionine labelled the nucleic acid: the extent of interaction was higher than that found when (^{35}S)methionine was given. Practically no labelling of tRNA occurred after the administration of methionine labelled in the aliphatic chain.

In order to investigate the nature of the products formed in (^{35}S)methionine-tRNA interaction, (^{35}S)tRNA was enzymatically hydrolyzed and analyzed by hplc. A representative chromatogram is reported in Fig. 1. Reversed-phase separation of enzymic digest of radioactive tRNA showed only two radioactive peaks: the first was fast eluted by the aqueous buffer while the second could only

Table 3 tRNA labelling by methionine labelled in the methyl group, in the sulfur or in the aliphatic chain. Rats received 10 mg amino acid/kg b.w. plus: 5.5 mCi (^{3}H-methyl)- or 2.2 mCi (^{35}S)- or 2.8 mCi [2(n)-^{3}H]- or 1.3 mCi L-(2-^{14}C)-methionine*. Data represent the means from experiments shown in parentheses ± S.D.

Methionine labeled in the:	pmol/$OD_{260\ nm}$	
methyl group	9.12 ± 2.7	(3)
sulfur	5.74 ± 1.8	(5)
aliphatic chain	0.95 ± 0.4	(3)
	0.33*	(1)

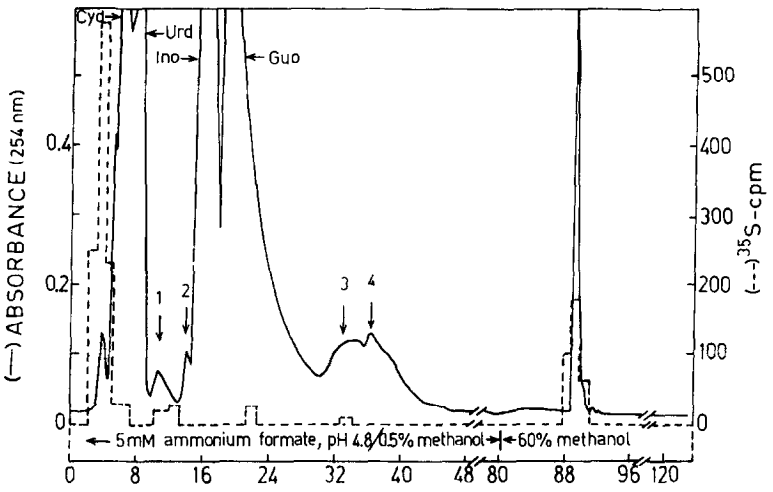

Fig. 1 - Reversed-phase separation of the enzymic hydrolysate of radioactive tRNA isolated from rat liver 3 h after i.p. injection of (^{35}S)methionine (1.6 mCi, 25 mg/kg b.w.). The arrows indicate minor modified ribonucleosides: 1. 5-methyluridine ; 2. 1-methylinosine; 3. N^2-methylguanosine; 4. $N^{2,2}$-dimethylguanosine.

be eluted by 60% methanol. No radioactivity was found in the four main ribonucleosides or in the minor modified ones. It has to be noted that the majority of the adenosine was found as inosine, notwithstanding the pre-heating treatment of alkaline phosphatase.

Under these conditions, (^{35}S)methionine (about 15,000 cpm) eluted at 8 min (Fig. 2).

The UV behaviour of the two radioactive peaks obtained from enzymic digest of (^{35}S)tRNA is reported in Fig. 3. The buffer-

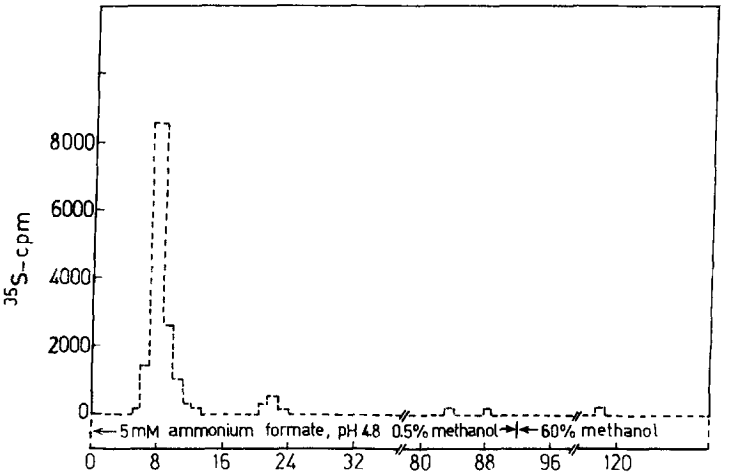

Fig. 2 - Reversed-phase chromatography of (^{35}S)methionine.

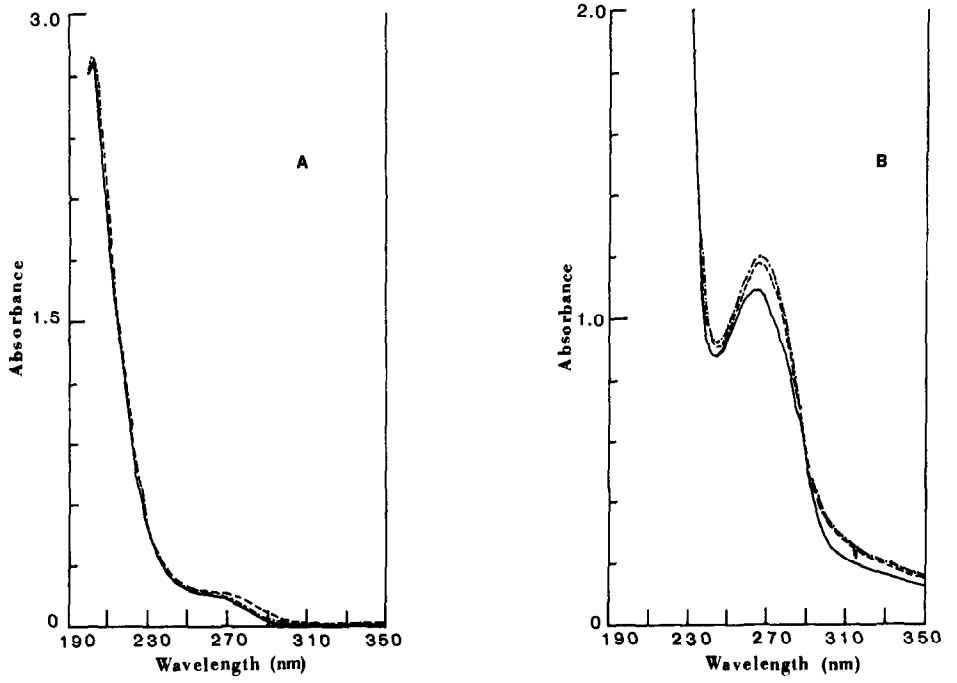

Fig. 3 - UV-spectra of buffer-eluted (A) and methanol-eluted (B) radioactive peak isolated from (^{35}S)tRNA enzymic digest.

eluted peak was characterized by far UV absorbance (fig. 3A), while the methanol-eluted peak showed additional absorbance at about 260 nm (fig. 3B). Neither appeared to correspond to any of the known sulfur-nucleosides reported in the literature (3,8).

Fig. 4 illustrates the NMR spectra of the two peaks obtained by hplc of the enzymic digest of the tRNA isolated from animals given 25 mg unlabelled methionine per kg b. w. Spectral lines could not be assigned by employing available ribonucleosides as models: however, there is a marked diversity between the two compounds. In particular, the buffer-eluted peak presented a sharp signal at 1.9 ppm (fig. 4A), which is characteristic of the methyl group. The signal corresponding to the thiomethyl group (2.2 ppm), which should have been indicative of methionine presence or of a thiomethylated compound (9), was absent in both.

Taken together, these data indicate that methionine sulfur binds "in vivo" to tRNA producing a characteristic labelling pattern and specific radioactive compounds. Actually the radioactive products are not yet identified and, likewise, the reaction mechanism of the interaction is still to be elucidated, nor we can actually exclude an indirect rather than direct kind

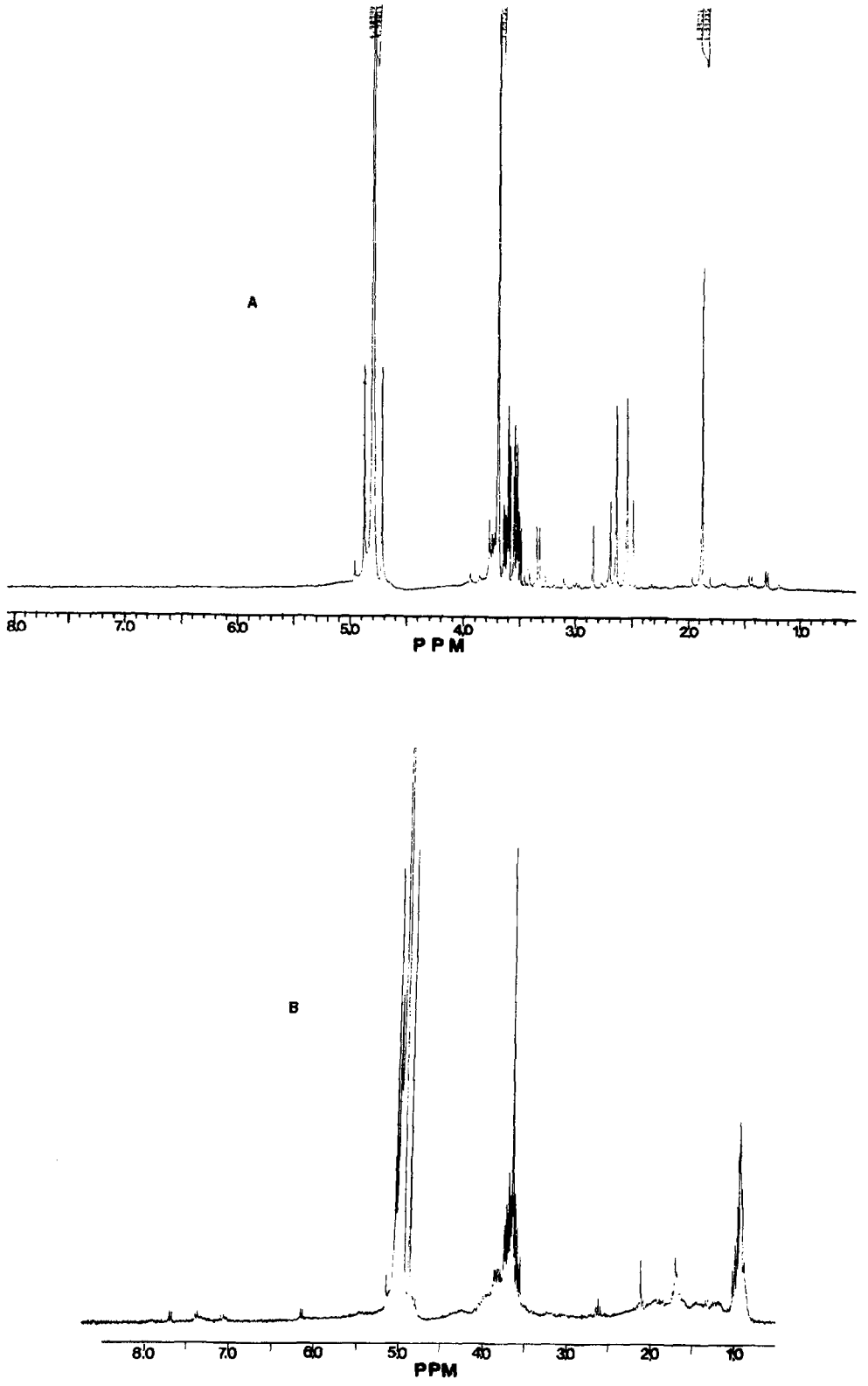

Fig. 4 - Proton magnetic resonance spectra of buffer-eluted (A) and methanol-eluted (B) peaks.

of reaction, i.e. mediated by other compound(s) which might be the true receptor(s) of the methionine sulfur. However it seems reasonable to explain this binding as due to a specific reaction and not to some experimental artifact. In fact, the double protein digestion should allow us to exclude protein contamination. Moreover, the logical possibility that (^{35}S)methionine-charged tRNA is still present in our tRNA preparations is not consistent with the data of Tables 2 and 3, or with NMR spectra. Thus, as well as the alkyl group and the aliphatic chain which are involved in bioalkylation reactions and in polyamine biosynthesis respectively, also methionine sulfur might play a metabolic role. Experiments are in progress to clarify the most important questions regarding the chemical nature and the reaction mechanism of this sulfur-tRNA association.

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