MODIFICATION OF TRANSFER RIBONUCLEIC ACID BY AN ALKYLATING FRAGMENT FROM S-(1,2-DICHLOROVINYL)-L-CYSTEINE

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SUMMARY: An alkylating fragment derived by enzymatic cleavage of [^{35}S]-(1,2-dichlorovinyl)-L-cysteine reacted, apparently covalently, with RNA isolated from E. coli, and from livers of the bovine calf, rat and rabbit. Transfer RNA was much more susceptible to alkylation than ribosomal RNA as revealed by gel filtration technique, and measurement of [^{35}S] substitution into nucleotides. Unfractionated E. coli tRNA modified by such reaction accepted most amino acids to the same extent as control tRNA, although about 40% less acceptance was observed for L-histidine, L-serine and L-tyrosine. Study of ribosomal binding, however, indicated an impairment of codon-anticodon interaction between synthetic polynucleotide messengers and amino acyl substituted, alkylated tRNA.

INTRODUCTION: Administration of a single dose (2.5-3 mg/kg) of S-(1,2-dichlorovinyl)-L-cysteine (DCVC) can induce fatal aplastic anemia in the bovine calf (1). An enzyme from calf liver cleaves DCVC and produces an alkylating fragment (AF)(2). This fragment reacts in vitro with DNA (3) and chromatin (4),bringing about a profound change in the physical and biological properties of these macromolecules. Model compounds like synthetic polynucleotides (5) and synthetic basic polyamino acids and their DNA complexes (6) also react with the fragment. With these modified, alkylated products the complementary interaction between polynucleotide strands and the interaction between DNA and polyamino acids were severely impaired. Analyses of (AF)-substituted DNA and polynucleotides by sequential enzymatic hydrolysis revealed that the (AF)-moiety linked two adjacent bases of the same chain (3,5).

The alkylating fragment from DCVC may be the ultimate toxicant. It also presumably reacts with DNA in vivo since DNA isolated from hemopoietic

tissues of calves treated with DCVC showed similar abnormal physical (7) and biological (8,9) characteristics as DNA treated with (AF) in vitro (3). Although DNA may be a prime target for (AF), it is considered important to study what effect (AF) has on other biological components with which it also reacts. In this paper observations are presented on (AF) interaction with tRNA and rRNA, and the resulting change in the biological function of modified tRNA.

EXPERIMENTAL: Total RNA from livers of calf, rat and rabbit was purified according to the procedure of Kruh et al. (10). This RNA was fractionated into tRNA and rRNA using 1 M NaCl (11). Each RNA fraction was subsequently purified by gel filtration through Sephadex G-100 (11) using 0.02 M tris-Cl, pH 7.5 containing 4 x 10^{-3} M $MgCl_2$. Unfractionated tRNA from E. coli K12, stripped of amino acids was purchased from General Biochemicals. [^{14}C] amino acids (sp. act. 106-460 mci/mmole) were from Schwartz/Mann. A mixture of amino acyl-tRNA synthetases as prepared by the method of Kelmers et al. (12), 70S ribosomes purified from E. coli RNase I, and synthetic polynucleotide messengers were obtained from Miles Laboratories.

Varying amounts of different RNA fractions were allowed to react in 0.5 ml final volume with 5 µmoles of [^{35}S] DCVC (7.5 x 10^4 CPM/µmole)(3), 50 µmoles of tris-Cl, pH 7.5 and lyase (sp. act. 9.5 units/ml)(2) for 30 min at 37°. Other variables are indicated for each experiment. To stop a reaction the mixture was cooled and one-tenth volume of 20% potassium acetate solution (pH 5.5) was added followed by two volumes of ice cold absolute ethanol. After the mixture was kept at -20° for 2 h the precipitate was collected by centrifugation and washed three times with ice cold absolute ethanol. RNA was then extracted with two 1 ml portions of 0.02 M tris-Cl, pH 7.5 containing 4 x 10^{-3} M $MgCl_2$, centrifuging each time to separate denatured lyase. Aliquots were used to measure absorbance, phosphorus (13) and radioactivity (3), or further fractionated into tRNA and rRNA by means of 1 M NaCl and/or gel filtration. When tRNA was to be used for amino-acylation assay, the above reaction mixture was increased suitably and the isolated tRNA was dissolved to a concentration of 40 A_{260} units/ml (about 2 mg).

Assay of amino acid acceptor activity of tRNA was performed following the method of Rubin et al. (14). The reaction mixture (80 µl) contained 8 µmoles of tris-Cl, pH 7.5, 1.6 µmoles of magnesium acetate, 1.6 µmoles of potassium chloride, 0.4 µmole of ATP, 0.8 µmole of 2-mercaptoethanol, 1 A_{260} unit of either control or (AF)-tRNA, 0.1 µci of ^{14}C-amino acids (0.217-0.943 nmole) and 0.6 mg of synthetase concentrate. The mixture was incubated at 30° for 10 min. Aliquots (20 µl) were applied to Whatman No. 3MM filter paper discs (24 mm) and the discs were washed (15), dried and the radio-activity was counted with a liquid scintillation spectrometer.

To prepare [^{14}C] amino acyl-tRNA for ribosomal binding study, the above reaction mixture was scaled up ten-fold. The charged tRNA was reisolated and purified as described by Fink et al. (16). Ribosomal binding was measured according to the method of Nirenberg and Leder (17). Each reaction mixture (50 µl) contained 5 µmoles of tris-Cl, pH 7.2, 2.5 µmoles of ammonium chloride, 0.5 µmoles of magnesium chloride, 2 A_{260} units of ribosomes, 0.2 A_{260} unit of polynucleotide messenger as indicated in the table and 3 pmoles of [^{14}C] amino acyl-tRNA (amino acid equivalent). The incubation was at 23° for 20 min. Each specimen was processed for analysis as described (17).

RESULTS: When [^{35}S] DCVC was reacted with lyase in presence of purified calf liver total RNA, [^{35}S](AF) became firmly attached to RNA. The binding was pronounced with saturating levels of RNA. With high concentrations of RNA the binding was linear to the amount of lyase present as shown in Fig. 1.

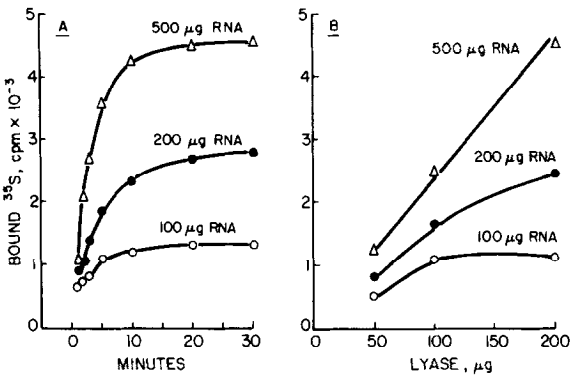
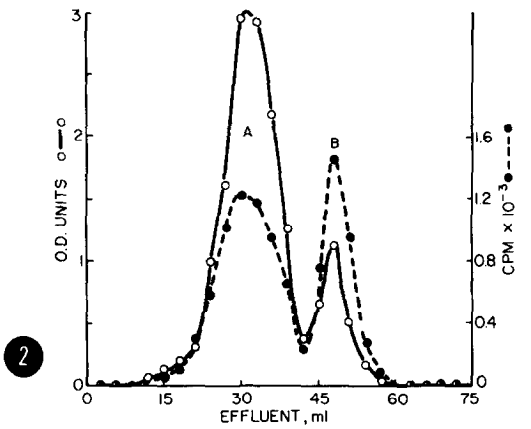


Fig. 1: Incorporation of [^{35}S] into calf liver total RNA. Different amounts of RNA were incubated with [^{35}S] DCVC for (A) various time periods and (B) with different enzyme concentration as described in the text.

After fractionation into tRNA and rRNA by the NaCl method, the higher specific activity was in tRNA. Thus in a typical experiment total RNA after [^{35}S](AF) reaction had 9,270 CPM/mg. After NaCl fractionation and purification, the specific activity in tRNA was 28,800 CPM/mg while in rRNA it was 7,500 CPM/mg. Fractionation of [^{35}S](AF)-bound total RNA by gel filtration on Sephadex G-100 also showed higher specific radioactivity in tRNA fractions than in the RNA of high molecular weight (Fig. 2). When each component of RNA was reacted separately with [^{35}S] DCVC and lyase, nucleotide modification was observed to the extent of about 5% in rRNA but about 15% in tRNA (Fig. 3). Substitution pattern was similar as observed with RNA fractions from other sources as shown in Table I.

These (AF)-substituted RNA specimens were highly resistant to enzymatic hydrolysis, even more so than (AF)-modified DNA (3). Thus combined actions of pancreatic RNase, snake venom phosphodiesterase, and E. coli alkaline



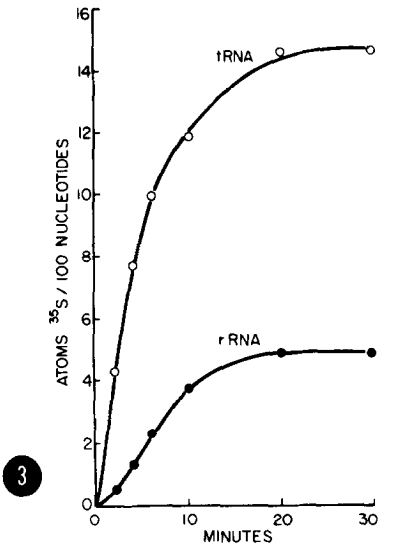


Fig. 2: Fractionation of [^{35}S](AF)-RNA on Sephadex G-100. One mg of calf liver total RNA was reacted with [^{35}S] DCVC and lyase and fractionated after reisolation as described in the text. Peak A represents high mol. wt. RNA, peak B represents tRNA.

Fig. 3: Extent of nucleotide substitution in calf liver tRNA and rRNA with [^{35}S](AF). Reaction mixture is same as described in the text except: 0.2 mg of lyase, 0.6 μmole of tRNA or 2 μmoles of rRNA (nucleotides).

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phosphatase under various conditions did not release any smaller fragment suitable for analysis. Substituted RNA was also resistant to hydrolysis in 0.3 N NaOH at 37° up to 1 h. Prolonged hydrolysis completely volatilized the radioactivity. The nature of interaction of the alkylating fragment with RNA has therefore not been ascertained.

Since E. coli tRNA showed maximum substitution (Table I), and since all the other E. coli components were readily available, this modified tRNA was tested for its capacity to accept amino acids. The assays were conducted with limiting tRNA concentration in the presence of an excess of amino acyl-tRNA synthetase. The amino acid acceptance capacities of normal tRNA and (AF)-substituted tRNA are shown in Table II. The activity of several tRNAs did not change due to (AF)-substitution. In fact the only changes noted were

TABLE I

Incorporation of [^{35}S](AF) *in vitro* in RNA[1]

Source of RNA	Atom [^{35}S]/100 Nucleotides	
	tRNA	rRNA
Bovine calf liver	14.64	4.87
Rabbit liver	12.38	3.47
Rat liver	11.30	2.82
E. coli K12	18.80	-

1. Reaction mixture as described in the text: 0.2 mg of lyase, 0.5-0.7 μmole of tRNA or 1-2 μmoles of rRNA (nucleotides).

TABLE II

Amino acid acceptance capacity of tRNA[1]

L-Amino acids	Acceptance, pmoles/A_{260} unit	
	Control tRNA	(AF)-tRNA
Alanine	45.3 ± 3.1	52.1 ± 4.2
Arginine	67.5 ± 3.6	69.0 ± 6.0
Aspartic acid	38.7 ± 1.6	39.8 ± 3.2
Glycine	29.4 ± 1.4	28.3 ± 2.3
Histidine	13.1 ± 1.1	8.6 ± 0.6
Isoleucine	44.3 ± 3.7	41.2 ± 3.2
Leucine	114.7 ± 8.5	102.6 ± 7.9
Lysine	39.5 ± 2.2	30.2 ± 2.8
Methionine	83.1 ± 5.3	79.1 ± 6.1
Phenylalanine	37.2 ± 2.6	33.6 ± 3.0
Proline	28.6 ± 2.1	27.2 ± 1.2
Serine	23.4 ± 2.3	16.1 ± 1.4
Threonine	38.2 ± 3.6	41.1 ± 3.9
Tyrosine	39.4 ± 3.1	23.5 ± 2.6
Valine	70.3 ± 5.2	72.8 ± 6.9

1. Reaction conditions as described in the text. Each value represents mean ± standard deviation calculated from four separate experiments.

for three tRNA species- histidine, serine and tyrosine, which were inhibited to the extent of about 40%. Activity of tRNA treated with either lyase or DCVC alone was not different from that of control untreated specimen when tested with different amino acids.

TABLE III

Polynucleotide codon directed binding of amino acyl-tRNA to ribosomes[1]

Amino Acyl-tRNA	Polynucleotide Codon	Amino acyl-tRNA bound to ribosomes, pmoles		
		Control tRNA	(AF)-tRNA	% of Control
Arg-tRNA	CGU	2.43	1.73	71
Gly-tRNA	G	1.37	1.00	73
Leu-tRNA	CU	1.78	0.65	37
	ACU	1.31	0.60	46
Lys-tRNA	A	2.04	1.32	65
Met-tRNA	UG	0.60	0.16	27
Phe-tRNA	U	2.34	1.52	65
Pro-tRNA	C	1.70	0.98	58
Ser-tRNA	CU	1.83	0.97	53
	AGU	1.24	0.70	56
	CGU	0.77	0.43	56
Thr-tRNA	ACU	1.97	1.27	64
Val-tRNA	UG	0.59	0.29	50
	AGU	1.85	0.83	45
	CGU	1.88	0.95	50

1. Reaction conditions as given in the text. Each value is mean of duplicate experiments.

As shown in Table III, when the (AF)-tRNA was aminoacylated with different amino acids, all these tRNA species showed less binding to ribosomes in the presence of their respective polynucleotide codons. These values ranged from 27-73% of the binding of control amino acyl-tRNA. Inhibition was also observed in the case of a particular amino acyl-tRNA when a different but otherwise specific polynucleotide codon was used to stimulate ribosomal binding. The binding to ribosomes in the absence of polynucleotides was only about 10-20% of template directed binding for all control and (AF)-tRNA species.

DISCUSSION: Many alkylating agents, aromatic amines, and polycyclic hydrocarbons are known to react with RNA (18,19). Transfer RNA molecules are believed to be critical targets for such alkylations *in vivo* (11,19, 20,21), and they can also be modified *in vitro* by alkylating agents (16,22,

23,24). The extent of reaction varies with the alkylating agent used, but the capacity of these agents to react with tRNA is equal to or greater than that with DNA (19). The extent of reaction with tRNA as observed here using a toxic chemical having a different biological property is greater than that observed with native DNA (3) and synthetic double stranded polynucleotides (5). However, the extent of substitution in tRNA is much less than that found in denatured or nicked DNA (3) and in single stranded polydeoxyribonucleotides (5), but comparable to that observed in single stranded polyribonucleotides (5).

The (AF)-modified DNA shows abnormal physical characteristics, and is completely devoid of template activity (3). Single stranded DNA and polynucleotides after modification with (AF) show less complementary base interaction as studied by hybridization (25) and renaturation (5). Similar conclusions can be drawn in the case of (AF)-modified tRNA. The most significant change noticed is the inhibition of codon-anticodon interaction after introduction of (AF)-residues in tRNA molecules. Although a major function of tRNA is its amino acid acceptance capacity, this property is not lost for a majority of amino acids due to (AF)-alkylation. It is possible that (AF) preferentially reacts with those sites in histidine, serine and tyrosine tRNA species which are recognized by their respective synthetases. Alkylating agents which are potentially carcinogenic, on the other hand, can render tRNA inactive with respect to its capacity to accept many amino acids (16,20,22). From the present study it is apparent, however, that (AF) reacts with bases at or near the anticodon regions of most tRNA species, thereby significantly altering their codon response capability. Reaction with regions of tRNA recognized by specific binding sites on ribosomes is also a possibility. To obtain more information concerning this aspect, individual pure tRNA species should be used.

It has been established that DCVC exerts its toxic effect primarily at the replication and transcription levels (3,8,9). However the important

role of tRNA in cell regulation is well recognized, and there is evidence that certain aspects of this regulation and cell differentiation are normally exerted at the level of translation (20). The possibility that alteration in the translation apparatus of cells -especially in those where replication rate is low- as a molecular mechanism of chemical toxicity cannot, therefore be ignored. Such a mechanism is thought to be operative in chemical hepatic carcinogenesis (16,18,19,20). Although DCVC administration induces aplasia in the bone marrow with concommitant loss of circulating white cell population, gross pathological changes are also noticed in liver, kidney and other organs at the terminal stage of toxicity (1). DCVC also inhibits growth of E. coli, and produces change in morphology of the cells even when the density of the bacterial population during treatment is high (26). In replicating tissues, the toxic effect is perhaps due to alkylation of unprotected DNA with (AF) (7,8,9), whereas in resting tissues this reaction does not take place because DNA is protected as bound chromatin which seldom dissociates (4). In such tissues the presence of (AF)-residues released from DCVC may introduce changes in specific regions of tRNA, thereby altering interaction with activating enzymes, messenger RNAs or ribosomes. Therefore a derangement of translation process in the cells may have an important role in the pathology of DCVC toxicity.

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