N²-Guanine specific transfer RNA methyltransferase I from rat liver and leukemic rat spleen*

Jan Kraus** and Matthys Staehelin

Biological Laboratories, Pharmaceutical Department, CIBA-GEIGY Ltd.,
Basel, Switzerland

Received 9 August 1974

ABSTRACT

An enzyme was purified from rat liver and leukemic rat spleen which methylates guanosine residues in tRNA to N²-methylguanosine. By sequence analysis of bulk *E. coli* tRNA methylated with crude extracts it was shown that the enzyme is responsible for about 50% of total m²G formed *in vitro*. The extent of methylation of a number of homogenous tRNA species was measured using the purified enzyme from both sources. Among tested *E. coli* tRNAs only tRNA^{Arg}, tRNA^{Phe}, and tRNA^{Val} yielded significantly more m²G than the bulk tRNA. The K_m for tRNA^{Arg} in the methylation reaction with enzymes from either tissue was 7.8×10^{-7} M as compared to the value 1×10^{-5} M obtained for the bulk tRNA. In a pancreatic RNase digest of bulk tRNA as well as of pure tRNA^{Arg}, tRNA^{Phe}, and tRNA^{Val}, A-m²G-Cp was found to be the only sequence methylated. Thus, the mammalian methyltransferase specifically recognizes the guanylate residue at position 10 from the 5'-end contained in a sequence (s⁴)U-A-G-Cp. Furthermore, there is no change between the enzyme from normal liver and leukemic spleen in the affinity for tRNA, the methylating capacity, and tRNA site and sequence recognition specificity.

INTRODUCTION

In 1962, Fleissner and Borek¹ discovered that tRNA is methylated after the formation of the polynucleotide by a group of enzymes, called methylases, which use S-adenosylmethionine as the methyl donor. The methylases are species specific², base and ring position specific³, tRNA site specific, and nucleotide sequence specific⁴⁻⁶. Enhanced tRNA methylase activity (2-10 fold increase compared with the normal tissue) was observed in crude extracts of 30 different neoplastic tissues⁷. Further information on tRNA methylation may be obtained from recent reviews⁸⁻¹².

1-adenine and N²-guanine methylation of *E. coli* tRNA with purified enzymes from both normal and leukemic rat tissue has been studied in our laboratory.

1-methyladenosine occurs in many eukaryotic tRNAs next to the common G-T-M-C-Rp sequence^{***}. It is present in close to one residue per chain in mammalian tRNA¹³, but is absent from *E. coli* tRNA¹⁴. An enzyme methylating *in vitro* the same adenylate residue in tRNA from *E. coli* and yeast has been purified and described previously^{4,13,15,16}.

Many tRNA species from eukaryotic cells contain methylated N²-guanosine derivatives¹⁷⁻²⁰ which are absent from *E. coli* tRNA^{21,22}. These derivatives occur at two sites in tRNAs from eukaryotic cells, namely N²-methylguanosine as the first base paired nucleotide in the stem of the DHU loop (site I), in general the 10th nucleotide from the 5'-end in a sequence U-R-m²G-Yp and N²-dimethylguanosine in an unpaired interstem region between the DHU arm and the anticodon arm (site II) most often in a sequence G-C-m²G-Yp. Recently two m²G residues were detected in tRNA^{Met} from mammalian cells^{23,24}. The second m²G residue was located at the site II, where m²G occurs in other tRNAs.

In known prokaryotic tRNA sequences neither m²G nor m₂²G occur, however, the corresponding unmodified guanylate residues are in most cases found in the same sequences at the same sites.

We have studied an enzyme capable of methylating guanylate residues at the N²-position and characterized the site and sequence specificity of the enzymes both from the normal and the leukemic rat tissue. In this paper the purification of N²-guanine methyltransferase I, which we believe specifically methylates site I, will be described. In the second paper it will be shown that another enzyme is specific for N²-guanine methylation at site II. This enzyme gives rise to N²-methylguanosine as well as to N²-dimethylguanosine.

MATERIALS AND METHODS

tRNAs: *E. coli* tRNA (strain B) was purchased from Mann Research Laboratories. Rat liver tRNA was prepared as described by Rogg, *et al.*²⁶. tRNA from brewer's yeast was isolated according to the method of Monier, *et al.*²⁷. tRNA^{Ser} (rat liver) with a purity of over 80% was prepared as described by Müller, *et al.*²⁸. tRNA^{fMet}, tRNA^{Glu}, tRNA^{Phe} (*E. coli* K12 M0) and tRNA^{Arg}, tRNA^{Val} (*E. coli* K12 M07) were generous gifts of Dr. A.D. Kelmers, Oak Ridge National Laboratory, Tenn., U.S.A.²⁹ tRNA^{Asp} (yeast) was kindly provided by Prof. Dirheimer, Strasbourg. The following tRNA's

(*E. coli* B) tRNA₁^{Leu}, tRNA₂^{Leu}, tRNA₃^{Leu}, tRNA₄^{Leu}, tRNA₁^{Ser}, tRNA₃^{Ser} and tRNA^{Tyr} were top-peak fractions of a partition column on which partly purified *E. coli* tRNAs were fractionated (Fig. 8 of Wehrli and Staehelin).³⁰ The iso-accepting tRNAs are numbered according to their peak elution positions on the partition column. The most polar isoacceptors tRNA₃^{Leu} and tRNA₄^{Leu} were probably identical to the two known sequenced species responding to codons of CU series.

S-Adenosyl-L-[Me-¹⁴C]Methionine: ¹⁴C-methyl labelled S-adenosyl-L-methionine (55 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, England. Non-labelled S-adenosyl-L-methionine chloride was obtained from Mann Research Laboratories.

Animals: Adult rats of Sprague-Dawley (CFE, albino) strain were killed and the livers were excised. Leukemic spleens were removed from the rats of the Fischer RL strain, killed 9 days after inoculation with Dunning R 3323 leukemia¹³.

Tissue was either used immediately after decapitation or frozen in liquid nitrogen and stored at -20°C in tightly closed plastic bags. Either fresh or frozen tissues were used for the preparation of the enzyme, since rapid freezing and storage of rat organs at -20°C did not quantitatively affect the methylating activity of the high-speed supernatant.

Purification of N²-guanine methyltransferase I: An enzyme catalyzing specifically the formation of N²-methylguanine was prepared from rat liver and rat leukemic spleen using a modified procedure described earlier¹³. All operations were carried out at 0-3°C. The tissue (50 g of livers, 27 g of leukemic spleen) was cut into small pieces and homogenized in four volumes of cold homogenization medium containing 0.25 M sucrose, 10 mM MgCl₂, 1 mM dithiothreitol, 10 mM Tris-Cl pH 8.0 using a glass teflon motor driven homogenizer. The extract was centrifuged at 30000 x g for 10 min and the supernatant fluid was centrifuged at 105000 x g for 60 min. The high-speed supernatant from this centrifugation was passed through a funnel containing a small plug of glass wool in order to remove the upper lipid layer. The enzyme precipitated from the high-speed supernatant at pH 5 was prepared by the procedure introduced by Kerr.³¹ The methylating activity precipitated at pH 5 was further purified on a DEAE-cellulose column (Whatman DE-52, 2x18 cm) which had been equilibrated with buffer A (50 mM Tris-Cl, pH 8.0, containing 0.1 mM EDTA and 0.1 mM dithiothreitol). The redissolved pH 5 precipitate from rat liver (512 mg) or leukemic

spleen (354 mg) was applied to the column which was then washed with 120 ml of buffer A. Elution was carried out with a linear gradient of 0-0.3 M sodium chloride in buffer A (400 ml) at a flow rate of 1.5 ml/min. Fractions (10.5 ml) were collected, kept on ice, the absorbance at 280 nm measured and 0.2 ml aliquots were assayed for tRNA methylating activity. The column fractions of highest methylating activity were pooled and the enzyme precipitated by slowly adding solid ammonium sulphate to 60% saturation under stirring. The precipitate was collected by centrifugation and dissolved in a small volume of buffer B (10 mM Tris-Cl, pH 8.0; 0.1 mM EDTA; 0.1 mM dithiothreitol) and dialyzed for 4 hours against the same buffer. One part of the enzyme was stored and the other part used immediately for further purification.

Sephadex G-200 (Pharmacia, Sweden) was packed in a column (2 x 58 cm) and equilibrated with buffer A for 2 days. The column was first calibrated with Blue dextran 200 (m.w. $\sim 2 \times 10^6$) (Pharmacia, Sweden) to determine the void volume of the gel and then with bovine serum albumin (BSA: m.w. 6.9×10^4) (Fluka, Switzerland) as a molecular weight marker both at concentrations 2 mg/ml in 2.5 ml samples.

The enzyme pool from the DEAE cellulose was applied to the column and eluted with buffer A. The fractions containing enzyme activity were pooled and solid ammonium sulphate was added to 60% saturation. The precipitates were collected by centrifugation, and dissolved in small volumes of buffer B and dialyzed for 4 hours against buffer B (1 liter) with one intermediate change of buffer.

The specific activity, base specificity and methylating capacity of the extract were determined after each purification step. Unfractionated E. coli tRNA was used as the comparative standard substrate. The protein concentration of the enzymes was estimated by the method of Lowry, et al.³²

Storage of enzymes: All dialyzed enzyme preparations were made 1 mM in dithiothreitol and stored in small volumes in sealed glass ampoules under liquid nitrogen. The content of one ampoule was not thawed more than twice. No loss of activity was observed during storage lasting for 2 years.

Assay of tRNA-methyltransferase activity: If not otherwise stated, the following assay conditions were used: Each assay tube (0.3 ml) contained 0.4 M ammonium acetate pH 9.25, 10 μ M [CH_3 - ^{14}C]S-adenosylmethionine (sp. activity 5×10^4 cpm/nmole), 1 mM dithiothreitol, 5-20 μ g E. coli tRNA, and 0.1-0.6 mg of enzyme protein. The final pH of the assay mixture

was 8.7 due to the addition of labelled SAM which had been stored in diluted H_2SO_4 . The incubation was carried out at 37° for 3 hours. After the incubation the assay tubes were chilled, 1 ml of cold 2 N HCl was added and the tubes kept on ice. After 10 min. the precipitates were collected on filter disks (Whatman Glass Fibre Paper GF/C, 2.4 cm), washed with a total of 20 ml of 0.01 N HCl, and dried at 80° . The filters were placed in toluene containing 0.6% Butyl-PBD (Scintillator CIBA-GEIGY Ltd.) and counted.

Analysis of enzymic digest of ^{14}C -methylated tRNA by thin layer chromatography and electrophoresis: 10-20 μg bulk tRNA or 5-10 μg of individual tRNA was methylated, 60 μg carrier rat liver tRNA was added and tRNA was isolated from the reaction mixture⁴.

All tRNA samples and tRNA fragments were desalted³³ prior to enzymatic digestion and thin layer chromatography or electrophoresis (this procedure also removed [^{14}C -methyl]S-adenosylmethionine degradation products). 0.1-2.0 A_{260} units of desalted tRNA or oligonucleotides were digested in 0.2 ml using either T_1 -ribonuclease, pancreatic ribonuclease, snake venom diesterase, alkaline phosphatase or for the methylated nucleoside analysis a combination of snake venom diesterase and alkaline phosphatase. The digestion conditions have been described.³⁴

Since thin layer chromatography gives a much better separation of N^6 -methyladenosine than of 1-methyladenosine from other methylated nucleosides, the digest was heated in a sealed glass ampoule in presence of 2M ammonia for 20 min. at 100°C to convert all 1-methyladenosine present to N^6 -methyladenosine by the Dimroth rearrangement.^{35,36}

The following combinations of solvents for thin layer chromatography were used: First dimension - solvent system A: Isobutyric acid - 0.5 M NH_3 - 0.1 M EDTA (100:60:1.6, by vol.)³⁷. Second dimension - solvent system B: 2-propanol-water-15 M NH_4OH (105:30:15, by vol.)³⁸ and solvent system C: Tertiary butanol - 0.08 M formic acid - isoamylalcohol (50:50:2, by vol.)³⁹.

The following reference nucleosides were obtained from Sigma: N^2 -methylguanosine, N^2 -dimethylguanosine, 1-methylguanosine, 7-methylguanosine, 5-methylcytidine, 1-methyladenosine and N^6 -methyladenosine. The chromatographic mobilities of the methylated nucleosides and nucleotides in the above mentioned solvent systems as well as their R_{Up} in thin layer electrophoresis are indicated in Table I. Thin layer electrophoresis was carried out at pH 2.8 as described earlier.³⁴ The techniques for sample application

TABLE I

Chromatographic and Electrophoretic Mobilities of tRNA Major Components and Some of Their Methylated Derivatives

Symbol ^a	R _F in Solvents		R _{Ap} ^b in Solvents		R _{Up} ^c Electro-phoresis pH 2.8
	A	B	A	C	
A	0.80	0.48			
Ap	0.63	0.19	1.00	1.00	0.05
pA	0.58	0.07			
m ¹ A	0.75	0.47			
m ⁶ A	0.81	0.67			
G	0.55	0.25			
Gp	0.39	0.09	0.51	0.95	0.57
pG	0.32	0.02			
m ¹ G	0.61	0.44			
m ² G	0.64	0.33			
m ² G	0.74	0.41			
m ⁷ G	0.73	0.25 ^d			
U	0.58	0.43			
Up	0.43	0.13	0.61	1.05	1.00
pU	0.35	0.06			
C	0.70	0.35			
Cp	0.57	0.13	0.89	1.00	0.02
pC	0.50	0.06			
m ⁵ C	0.73	0.49			
m ⁵ Cp			0.93	0.91	-
m ² G-Cp			0.67	0.68	0.45
m ² G-Up			0.61	0.91	-
m ² G-Cp			0.75	0.70	0.43
A-m ² G-Cp			0.76	0.43	0.39
A-m ¹ A-G-Up			0.58	0.35	0.72
A-m ⁶ A-G-Up			0.45	0.54	-

^aThe nomenclature proposed by IUPAC-IUB Commission on Biochemical Nomenclature was used, see *Eur. J. Biochem.* **15**, 203-208 (1970).

^bMobilities relative to adenosine 3'-phosphate after developing twice in each solvent.

^cElectrophoretic mobility relative to uridine 3'-phosphate.

^dThis nucleoside undergoes ring scission in system B.

to the plate, for the location of the spots in UV light at low temperature, and the photographic recording of the results were previously described.^{33,40} In addition the radioactive spots were located by autoradiography of the plates (Agfa-Gevaert X-ray film, Structurix DW). The radioactive spots located by autoradiography and in the UV light were scraped off, the cellulose was extracted twice with 1 ml of water and removed by centrifugation. 0.5 ml aliquots were applied to filter disks, dried and the

radioactivity was estimated.

For further analysis in case of separated oligonucleotides the water was removed by rotatory evaporation and the residue further digested. The authenticity of identified nucleosides was ascertained by their spectra characteristics in acid and in alkali. The UV spectra were compared with those of reference substances and with those published.^{20,41}

Analysis of enzymic digest of ^{14}C -methylated tRNA by column chromatography: 10-20 μg of *E. coli* tRNA was methylated under above mentioned conditions in the presence of 20 μg rat liver tRNA.

Following the incubation, the content of 10 assay tubes was pooled and rat liver tRNA was added (10 mg). The tRNA was recovered from the mixture, digested with pancreatic ribonuclease and the digest was fractionated on a DEAE-cellulose column (1 x 50 cm).⁴

RESULTS

Properties of Unfractionated tRNA Methyltransferase Preparations.

In order to measure guanine specific methylation activity, tRNA samples methylated to the highest extent by enzyme preparations at different stages of purification were isolated from the incubation mixture, desalted and digested (Table II). The specific activities of the enzyme at each step were tested using an excess of tRNA, and the methylation extents were measured using limiting concentrations of tRNA (Table III). The results of nucleoside analysis (Table II) indicate that the N^2 -guanine methylated derivatives represented about 60-70 per cent of total newly formed methylated nucleosides in tRNA methylated both with the high-speed supernatant as well as with the pH 5 enzyme.

The methylation of *E. coli* tRNA with unfractionated rat liver extracts yielded under our conditions 3-4 times more m^2G than m_2^2G residues formed in tRNA chains. The relative yield of m_2^2G and m^1G residues was higher upon tRNA methylation with crude extract from leukemic spleen, while the amount of m^5C decreased.

The data in Table III show that the pH 5 precipitation increased markedly both the specific activity of the enzyme from liver and the extent of tRNA methylation. Although the specific activities of the preparations from leukemic spleens were higher than those of liver preparations, the methylation extent was in fact lower.

TABLE II

Products of tRNA Methylation with Fractionated
Methyltransferase Preparations*

Enzyme Source	Total Methylation Nucleosides					N ² -Guanine Methylation Nucleotides		
	m ¹ A	m ⁵ C	m ¹ G	m ² G	m ₂ ² G	m ² G-Yp	m ₂ ² G-Yp	R-m ² G-Yp
Rat Liver:								
105,000 x g supernatant	16.5	20.8	1.8	45	15.7	23	15.7	22
pH 5 enzyme	20.7	17.5	0.2	48.5	13	23	13	25.5
DEAE pool	51	0	0	49	0	0	0	49
Sephadex pool II	7	0	0	93	0	0	0	93
Rat Leukemic Spleen:								
105,000 x g supernatant	14	9	7.6	36.1	33.3	18.1	33.3	18
pH 5 enzyme	14.2	5.9	7.1	42.8	30	20.2	30	22.6
DEAE pool	32.3	4.4	5.3	55.8	2.2	6.1	2.2	49.7
Sephadex pool II	7	0	0	93	0	0	0	93

* Since m₂²G accounts for two radioactive methyl groups the results are expressed as % of total [¹⁴C-CH₃] nucleosides and nucleotides formed rather than % of total ¹⁴C incorporation.

Purification of N²-Guanine Methyltransferase I.

The tRNA methylating activities present in the pH enzyme both from rat liver and leukemic spleen were fractionated on a DEAE cellulose column. The bulk of the proteins was not bound to the DEAE-cellulose and had been washed out before the salt gradient was started. tRNA methylating activity was then eluted at approximately 0.12-0.18 M sodium chloride concentrations. The fractions containing the highest activity were pooled and the methylating activity was recovered from the column eluate by ammonium sulphate precipitation and dialysis. The DEAE enzyme pool from both columns methylated tRNA to the same extent, namely 6 nmoles methyl group/mg tRNA. When the tRNA methylated with the enzyme from normal liver was analyzed for radioactive nucleosides, equal amounts of 1-methyladenosine and N²-methyl-guanosine were found. No N²-dimethylguanosine was detected. In the tRNA

methyalted with the DEAE pool enzyme from the leukemic spleen 56% of the radioactive nucleosides were represented by N²-methylguanosine and 32% by 1-methyladenosine with only traces of others. Also in this case only 2.2% of N²-dimethylguanosine was found (Table II). It is noteworthy that the relative activity of 1-adenine methyltransferase in the DEAE pool enzyme is higher than in the high-speed supernatant. This may indicate that either some other N²-guanine methylating activity was removed or that the activity present was partly inactivated. The first possibility appeared to be true.²⁵ The DEAE pools were highly enriched in 1-adenine methyltransferase and N²-guanine methyltransferase (Table II). In order to separate these two activities the DEAE eluates were applied to a column of Sephadex G-200.

Figure 1 shows that the methylating activity was distinctly separated into two peaks. The methylating activities from both the normal and the leukemic tissue were eluted with the same elution volumes. The fractions corresponding to the first and second peak were pooled (as evident in the figure) and the methylating activities recovered by ammonium sulphate precipitation and dialysis. The nucleoside analysis of the methylated tRNA samples showed that 96% of the methylation by Sephadex pool I from both columns was due to 1-adenine methyltransferase and 93% of the methylation by Sephadex pool II in both cases due to N²-guanine methyltransferase.

The elution positions of 1-adenine methyltransferase and N²-guanine methyltransferase on Sephadex G-200 column were significantly different thus indicating a considerable difference in their molecular weights. The elution volumes of 1-adenine methyltransferase were in both cases identical to the elution volumes of Blue dextran (m.w. $\sim 2 \times 10^6$). These data would suggest that the molecular weight of 1-adenine methyltransferase is higher than 8×10^5 which is the exclusion molecular weight limit for Sephadex G-200 gel. The elution volume of N²-guanine methyltransferase was in both cases slightly higher than that of bovine serum albumin ($\sim 7 \times 10^4$). By comparison of the results with the molecular weight calibration curve⁴³ for Sephadex G-200, we could make a rough estimate of the molecular weight of N²-guanine methyltransferase to be in the order of 1×10^5 .

Fig. 1. Fractionation of DEAE cellulose-purified enzymes from rat liver (A), and leukemic rat spleen (B) on a Sephadex G-200 column. The DEAE pool from liver (26.5 mg) and leukemic spleen (43 mg) was applied to the column. Volumes of the samples were 2.3 ml. The buffer A (900 ml) was placed in a Mariotte flask and the column was eluted (150 ml) under an operating pressure of 18 cm at a flow rate of 2.35 ml/10 min. The fractions (2.35 ml) were collected, the absorbance at 280 nm measured and 0.1 ml aliquots were assayed for tRNA methylating activity for 1 hour at 37° using 100 μ g of tRNA. Note the different scale for (B). The arrows indicate the elution volumes of Blue dextran 2000 and of bovine serum albumin.

TABLE III
Purification of N²-Guanine Methyltransferase I from Rat Liver (L) and Leukemic Rat Spleen (LS)

	Protein (mg)		Activity (U)*		Sp. Activity (U/mg)		Methylation Extent	
	L	LS	L	LS	L	LS	L	LS
Total methylating activity:							nmols CH ₃ /mg tRNA	
105,000 x g supernatant	3840	2360	461	897	0.12	0.38	2.8	2.7
pH 5 precipitate	512	366	261	307	0.52	0.84	11.0	5.4
DEAE pool	78.5	87	62.8	141	0.88	1.62	6.0	6.0
N ² -guanine methylating activity:**							nmols A-m ² G-Cp/mg tRNA	
105,000 x g supernatant	3840	2360	101.4	161.5	0.026	0.068	0.62	0.49
pH 5 precipitate	512	366	66.5	76.9	0.13	0.21	2.8	1.22
DEAE pool	78.5	87	31.4	70.5	0.44	0.81	2.9	3.0
Sephadex pool II***	4(12)	6.9(13.8)	5.4(16.2)	30(60)	1.34	4.4	3.0	4.0

*One unit of enzyme (U) catalyzed the incorporation of 1 nmole of methyl group into tRNA in 1 hour.

**The approximate percentage of N²-guanine methyltransferase I has been calculated as 22% and 18% respectively, for the supernatant enzyme from liver and leukemic spleen, 25% and 23%, respectively, for the pH 5 enzyme, and 50% for the DEAE pools.

***One third of the DEAE enzyme pool from liver and one half of the DEAE enzyme pool from leukemic spleen were applied to the Sephadex G-200 column. The numbers within parentheses correspond to entire volumes of DEAE enzyme pools.

Oligonucleotide Analysis of Bulk E. coli tRNA Methylated with N²-Guanine Methyltransferase.

The nucleoside analysis of methylated tRNA has shown that the enzyme pool II from the Sephadex column was a methyltransferase with a high N²-guanine specificity. To decide which oligonucleotide sequence was methylated, the methylated tRNA was subjected to digestion with pancreatic RNase. Figure 2 shows that almost all the radioactivity (85%) present in the pancreatic RNase digest was found in one peak in the trinucleotide region with some radioactivity (15%) in the second peak in the tetranucleotide region. The fractions corresponding to the first and second radioactive peaks were pooled. After evaporation of the volatile buffer, the trinucleotides from the first peak were separated by thin layer electrophoresis at pH 2.8. Sequence analysis of the dephosphorylated radioactive trinucleotide by snake venom phosphodiesterase after dephosphorylation by alkaline phosphatase showed that the trinucleotide had the structure A-m²G-Cp. The sequence of the radioactive tetranucleotide from peak 2 could not be completely elucidated, because of the limited amount of radioactive material, but all radioactivity was associated with N⁶-methyladenosine 5'-phosphate.

tRNA samples methylated with Sephadex enzyme pool II from normal as well as from leukemic tissue were digested with pancreatic RNase. The radioautographs of the oligonucleotides separated by thin layer chromatography were identical indicating that both enzymes methylated only guanylate residues in the A-G-C sequence. N²-guanine methylation in crude enzyme preparation can also be due to another N²-guanine methyltransferase (see paper 2), which methylates sites liberated mainly as dinucleotides in pancreatic RNase digest. To study to which extent N²-guanine methylation in crude extracts was due either of the N²-guanine methyltransferase sequence studies were also carried out.

In pancreatic RNase digests of tRNA methylated with either high-speed supernatant or pH 5 enzyme radioactive N²-methylguanosine was detected in about equal amounts in the dinucleotide and trinucleotide sequences (Table II). Thus only half of the N²-monomethylguanosine is due to methylation by each N²-guanine methyltransferase and only 18-22% of the methylated activity of unfractionated methyltransferase preparations is due to N²-guanine methylation of A-G-C sequences at site I.

Defining N²-guanine methyltransferase I as that activity utilizing

Fig. 2. DEAE-cellulose chromatography of a pancreatic RNase digest of *E. coli* tRNA previously methylated with N²-guanine methyltransferase I. tRNA was methylated to an extent of 3 nmoles methyl group/mg tRNA with one of the earlier preparation of the liver enzyme (about 86% pure with respect to other methylating activities). The tRNA was digested and m¹A which possesses a positive charge was converted to m⁶A by heating the digest at pH 8.6 for 45 minutes at 100°C.⁴ Fractionation of oligonucleotides was achieved at pH 8.6 by a linear gradient of triethylammonium bicarbonate 0.04-0.35 M. The fractions (5.7 ml) were collected, the absorbance at 260 nm measured (—), aliquots of the fractions were dried in scintillation vials and the radioactivity counted (.....).

the sequence Y-A-G-C as a substrate, a summary of its purification can be presented (Table III).

The yield of N^2 -guanine methyltransferase from rat liver was 0.32 enzyme units/g tissue with a recovery of 16% and a degree of purification of 57 times. The yield of N^2 -guanine methyltransferase from rat leukemic spleen was 2.22 enzyme units/g tissue with a recovery of 37% and a degree of purification of 65 times. The N^2 -guanine methyltransferase I activity was about three times increased in purified enzyme preparations from leukemic spleen. However, the enzymes from the leukemic tissue did not methylate *E. coli* tRNA to a greater extent.

Methylation of Individual tRNAs with N^2 -Guanine Methyltransferase I.

The pool of maximal tRNA methylating activity from DEAE cellulose column eluate and the N^2 -guanine methyltransferase I (Sephadex pool II) were used to methylate individual tRNAs from various sources and the maximal methylation extents were estimated. In cases when relatively high levels of methylation were reached the analysis of methylated nucleosides was performed. The results are summarized in Table IV. It can be seen that different tRNAs varied considerably in their ability to accept methyl groups. No methylation of tRNA₁^{Ser} from rat liver with N^2 -guanine methyltransferase I from either source was observed. Bulk yeast tRNA was a relatively poor substrate for the DEAE pool enzyme consisting of equal amounts of l-adenine methyltransferase and N^2 -guanine methyltransferase I. The best acceptor of methyl groups from the DEAE enzyme pool among the individual *E. coli* tRNAs used was tRNA₂^{Glu}. Also tRNA₂^{Leu}, tRNA₃^{Leu}, tRNA₁^{Ser}, tRNA₃^{Ser}, tRNA^{Tyr} proved to be better substrates for the DEAE pool enzyme than the bulk tRNA (Table IV.d). However, when either the analysis of methylated nucleosides was carried out (Table IV.c) or the results compared with those of separated N^2 -guanine methyltransferase I (Table IV.a,b) it was concluded that in all these cases the increased methylation was due to l-adenine methylation. Among the tRNAs tested only tRNA^{Phe}, tRNA^{Val} and mainly tRNA^{Arg} appeared to be substrates for N^2 -guanine methyltransferase I. N^2 -guanine methyltransferase I from both sources methylated tRNA^{Phe} and tRNA^{Val} to an extent of approximately 0.15 mole methyl group/mole tRNA. Liver and leukemic spleen enzyme methylated tRNA^{Arg} to an extent of 0.5 and 0.7 methyl group per tRNA molecule, respectively. The real values of methyl incorporation are probably higher since the tRNA substrates used were only about 70% pure.²⁹

TABLE IV

 N^2 -Guanine Methylation and Sequences of Individual tRNAs

tRNA	Site I Nucleotides 8-13	Other Y-A-G-C Sequences	Extent of Methylation mole CH_3 /mole tRNA			
			a	b	c	d
<u>E. coli</u> : bulk			0.10	0.12	0.09	0.18
Leu I						0.09
Leu II				<0.01		0.21
III	U-G-G-Y-G-G	+	<0.01		<0.01	0.18
Ser I				0.02		0.36
III				0.01		0.27
Glu II	U-C-G-U-C- U	-			0.02	0.42
fMet	sU-G-G-A-G-C	+			0	0.12
Tyr	sU-sU-C-C-C-G	-			0	0.21
Phe	sU-A-G-C-U-C	-	0.15	0.15		0.27
Val	sU-A-G-C-U-C	+	0.16	0.12		
Arg	sU-A-G-C-U-C	+	0.52	0.74		
<u>Yeast</u> : bulk				<0.01		0.02
Asp	U-A-G-U-U- U	-				0.03
<u>Rat Liver</u> : bulk			<0.01	<0.01		<0.01
Ser I	U-G-G-C-acC-G	-	0	0		<0.01

- a) Rat liver N^2 -guanine methyltransferase I.
b) Leukemic spleen N^2 -methyltransferase I.
c) DEAE-enzyme; N^2 -guanine methylation.
d) DEAE-enzyme; total methylation.

A comparison of the results obtained with enzymes from both the normal tissue and the leukemic tissue revealed no significant difference in their tRNA methylating capacities. The only difference that was observed was with respect to tRNA^{Arg} methylation extent, however, when the dependence of ^{14}C -methyl incorporation into tRNA^{Arg} on amount of protein in the assay mixture was measured (Fig. 3), saturation of the substrate with the enzyme from normal tissue, was not reached (curve 1). The specific activity of the normal enzyme was significantly lower than that of the leukemic enzyme (about 3 x, Table III), however, the almost linear course of curve 1 allowed us to conclude that the methylation extent would have gone up further at high enzyme concentrations and possibly reached the plateau values of tRNA^{Arg} methylation with the leukemic enzyme.

Fig. 3. Dependence of ^{14}C -methyl incorporation into tRNA^{Arg} on the amount of protein in the assay. Assay tubes contained $2\text{ }\mu\text{g}$ of tRNA^{Arg} and various amounts of enzyme from normal liver (curve 1) or from leukemic spleen (curve 2). Incubation was carried out at 37° for 3 hours.

Oligonucleotide Analysis of tRNA^{Arg} , tRNA^{Phe} , and tRNA^{Val} Methylated with N^2 -Guanine Methyltransferase I.

A number of individual tRNAs from *E. coli* were tested as substrates for N^2 -guanine methyltransferase I. Among tested tRNAs only tRNA^{Arg} , tRNA^{Phe} , and tRNA^{Val} were found to act as substrates for the enzyme, since the nucleoside analysis of these *in vitro* methylated tRNAs yielded radioactive N^2 -methylguanosine. Further characterization of the enzyme involved determination of the oligonucleotide methylated in these tRNAs and the location of this oligonucleotide within the tRNA since the primary sequences of tRNA^{Arg} , tRNA^{Phe} , and tRNA^{Val} are known.

tRNA^{Arg} , tRNA^{Phe} , and tRNA^{Val} were methylated with both enzyme preparations and subjected to digestion with pancreatic RNase. The mixture of oligonucleotides was in each case separated on thin layer plate in two dimensions and the radioactive spot was located by autoradiography. Figure 4 depicts the separation of digestion products of tRNA^{Arg} previously methylated with N^2 -guanine methyltransferase I from the leukemic tissue. Very similar results were obtained when other five digestion mixtures were separated.

The dephosphorylated oligonucleotide was digested with snake venom diesterase. All the radioactivity was associated with N^2 -methylguanosine 5'-phosphate, and in addition the nonradioactive cytidine 5'-phosphate and adenosine were detected. Therefore in all three tRNAs methylated with either enzyme preparation the structure of the radioactive

oligonucleotide released by pancreatic RNase was A-m²G-Cp.

To study the kinetics of methylation, the time course was studied both under the conditions of limiting tRNA as well as with excess amounts of tRNA. When tRNA in the methylation reaction was not significantly rate limiting the time course was almost linear within 3 hours of incubation at 37° (curve 1 in Figure 5). Curve 2 illustrates the methylation of tRNA under the conditions of limiting tRNA. Under such conditions saturation was approached at the level of 4 nmoles/mg tRNA. This experiment indicates that the enzyme is active for several hours at 37° and that the tRNA was still capable of accepting methyl groups after the long exposure to the enzyme at 37° and it indicates further that prolonged incubation may be required to ensure full methylation of the tRNA substrate, whereas shorter incubation times could be used to measure kinetic parameters. Agris, et al.⁴⁴, indeed methylated tRNA samples for up to

^{14}C -Methyl incorp.
nmoles CH_3 /mg tRNA

Fig. 5. Time course of methylation. Assay tubes contained 0.6 mg enzyme protein and either 50 μg (curve 1) or 10 μg of total *E. coli* tRNA (curve 2). Results were corrected for enzyme blank values (10% of total counts).

9 hours at 37° in order to obtain stoichiometrical molar ratios of methyl incorporation.

Using bulk *E. coli* tRNA and tRNA^{Arg} as substrates, the affinity for tRNA of N^2 -guanine methyltransferase I from both normal liver and leukemic spleen was measured (Fig. 6). It was discovered that the enzyme from

Fig. 6. Lineweaver-Burk plot for methylation at 37° of bulk *E. coli* tRNA (A) and tRNA^{Arg} (B) with N^2 -guanine methyltransferase I from rat liver (o—o), and from leukemic rat spleen (●—●). Assay tubes contained 10–160 μg of bulk tRNA or 5–40 μg of tRNA^{Arg} and either 100 μg of liver enzyme or 17 μg (A), 68 μg (B) of leukemic spleen enzyme and were incubated for 1 hour.

leukemic tissue had identical affinity for tRNA in the methylation reaction as had the enzyme from normal tissue. The K_m for bulk tRNA, and tRNA^{Arg} were 10 μ M and 0.78 μ M, respectively. Assuming that the methyl-accepting species in *E. coli* comprised about 10% of the total tRNA used, with respect to the results on methylation extent of bulk tRNA (Table IV), the K_m for bulk tRNA was 1 μ M.

Figure 7 shows a comparison of methylation kinetics of tRNA^{Phe}, tRNA^{Val}, and tRNA^{Arg}. It is obvious from the picture that the affinity of N²-guanine methyltransferase I for tRNAs specific for phenylalanine and valine was much lower under our experimental conditions, than was the affinity for tRNA^{Arg}. The precise K_m values for these tRNAs were not measured, however, from the experiment shown, it can be concluded that the kinetic parameters of methylation of tRNA^{Phe} and tRNA^{Val} lay between the values obtained for tRNA^{Arg} and total *E. coli* tRNA, namely between 0.78 μ M and 10 μ M, respectively.

Fig. 7. The kinetics of methylation of individual tRNAs from *E. coli* with N²-guanine methyltransferase I from leukemic rat spleen. Assay tubes contained either 68 μ g of enzyme and various amounts of tRNA^{Arg} (o—o) and tRNA^{Phe} (o---o) or 34 μ g of enzyme and various amounts of tRNA^{Val} (o....o). The incubation was carried out at 37° for 1 hour.

DISCUSSION

The unfractionated tRNA methylating activities present in the high-

speed supernatant and in the pH 5 precipitate were examined. It was demonstrated that the tRNA methylating activity of pH 5 precipitate from normal liver and the extent of tRNA methylation were about 4 times higher than those of the high-speed supernatant (Table III). These findings corroborate the results of Kerr^{31,45} who demonstrated the presence of a non-dialyzable natural inhibitor of protein character consisting of a high and a low molecular component, in adult organ extracts. The inhibitor was separated from the methylases by pH 5 precipitation of the high-speed supernatant. Kerr, *et al.*⁴⁶ have also suggested that the high molecular weight inhibitor was absent in several tumor extracts which may account for the much lower increase in the specific activity of the pH 5 enzyme from leukemic spleen.

N²-guanine methyltransferase I had a considerably lower molecular weight ($\sim 1 \times 10^5$) than 1-adenine methyltransferase ($> 8 \times 10^5$), as judged from their elution volumes on Sephadex G-200. It is difficult to imagine that 1-adenine methyltransferase has really a molecular weight of over 800,000 when compared to other enzymes, such as aminoacyl-tRNA synthetases with a molecular weight in a range 50,000-300,000⁴⁷ and RNA-polymerase having a molecular weight of about 500,000. One possible explanation is that tRNA methyltransferases from rat liver were found *in vitro* in large complexes with other tRNA modifying enzymes, tRNA, aminoacyl-tRNA synthetases and lipids.⁴⁷ Agris, *et al.*⁴⁴ separated the activity for the formation of 1-methyladenine from HeLa-cells into more peaks of activity. It was suggested that the enzyme occurred as a multiprotein complex and the apparent multiple enzymes for the formation of 1-methyladenine were merely complexes in the various stages of dissociation. In agreement with that we found that the N²-guanine methyltransferase I, although well separated from the "high" molecular 1-adenine methyltransferase, was always contaminated (5-20%) by some "low" molecular 1-adenine methylating activity.

The availability of some tRNAs with known sequences allowed us to study the site and sequence specificity of N²-guanine methyltransferase I. The only tRNAs which acted as substrates for this enzyme were tRNA^{Arg}, tRNA^{Phe}, and tRNA^{Val} all containing U-A-G-Cp sequence at site I. The oligonucleotide analysis of these *in vitro* methylated tRNAs has demonstrated that the labeled m²G was indeed present in a sequence A-m²G-Cp. This is in close agreement with the result of oligonucleotide analysis of bulk tRNA methylated with N²-guanine methyltransferase I from normal and

leukemic tissue. The only oligonucleotide containing ^{14}C -labeled m^2G was identified as $\text{A-m}^2\text{G-Cp}$. This trinucleotide, prior to methylation and digestion, had to be a part of a sequence Y-A-G-C- which occurs only once in tRNA^{Phe} and twice in tRNA^{Val} and tRNA^{Arg} . The only identical position in the clover-leaf model where this sequence is present in all 3 tRNAs is the position between the amino acid arm and the dihydrouridine arm. Thus the site of N^2 -guanine methylation with the target guanosine being 10 nucleotides far from the 5'-end is identical in vitro to the site I of in vivo N^2 -guanine methylation. The sequence (S^4) U-A-G-C- which was recognized in vitro is present as a $\text{U-A-m}^2\text{G-C-}$ sequence at site I in 5 eukaryotic tRNAs specific for tyrosine, lysine and phenylalanine. It was also reported that m^2G was located in this position in *E. coli* $\text{tRNA}_1^{\text{Val}}$, tRNA^{Phe} and $\text{tRNA}_1^{\text{Ala}}$ methylated in vitro with purified enzyme from liver of rat⁴⁸ and of rabbit⁴⁹.

The presence of an unmodified guanylate residue in position 10 is not the only decisive factor for recognition of this site by the enzyme. It was demonstrated that the guanylate at site I in yeast tRNA^{ASP} and rat $\text{tRNA}_1^{\text{Ser}}$ occurring in sequences U-A-G*Up and U-G-G*-Cp , respectively, was not methylated in vitro. No methylation of the guanylate residue at site I in a sequence U-G-G*-A-G-Cp in $\text{tRNA}_f^{\text{Met}}$ and in a sequence U-C-G*-Up in $\text{tRNA}_2^{\text{Glu}}$ was detected, nor did the enzyme recognize the guanylate at N^2 -guanine methylation site II in $\text{tRNA}_f^{\text{Met}}$ and yeast tRNA^{ASP} . Also no guanosine methylation of three tRNA^{Leu} species and of two tRNA^{Ser} species was observed. Nor is the sequence U-A-G-C itself the recognition site for the enzyme, since this sequence is present in other regions of $\text{tRNA}_f^{\text{Met}}$ which was not methylated. Apparently the purine in position 9 must be adenine, since the sequence $\text{G-m}^2\text{G-Yp}$ was not detected in digests of methylated bulk tRNA. In many eukaryotic tRNAs m^2G follows the m^1G in position 9. Since m^1G does not occur in *E. coli* tRNAs at this position we could not test whether the enzyme would recognize a $\text{m}^1\text{G-G-C}$ sequence.

It is still unexplained why tRNA^{Arg} was methylated to almost 5 x higher extent than tRNA^{Phe} and tRNA^{Val} . The sequence $\text{s}^4\text{U-A-G*-C-U-C-A-G}$ between positions 8 and 15 is identical in these tRNAs, they all consist of 76 nucleotides and have a short extra arm. One possible explanation may be the fact that while in tRNA^{Phe} and tRNA^{Val} the target G is firmly base-paired with C, the G in tRNA^{Arg} is "paired" with an U and may be better accessible to the enzyme. It seems that one of the factors necessary for the in vitro recognition of site I in *E. coli* tRNA by a

mammalian enzyme is the sequence A-G-C, perhaps as a part of a longer sequence in terms of a primary structure, but the recognition of guanylate residue at site I may also depend on other parts of the primary sequence, on the presence of other minor bases or on the conformation of the sequence within the molecule.

The specific activity of N²-guanine methyltransferase I was about three times increased in the enzyme preparation from the leukemic spleen. However, the elution volume of this enzyme from Sephadex G-200, its tRNA methylating capacity, tRNA sequence specificity, and affinity for *E. coli* bulk tRNA and tRNA^{Arg} were identical to the enzyme purified from normal liver. These findings are in close agreement with the previous results from our laboratory¹³ on the substrate specificity of l-adenine methylase from the same two tissues. Also others^{50,51} have shown that the methylases from cancerous tissues do not possess an altered specificity towards the tRNA. Randerath⁵², too, found no changes in the minor base composition of tumour tRNA as compared to RNA from normal tissue.

FOOTNOTES

* A preliminary report has been given at the Symposium of the Societe de chimie biologique, Strasbourg (1971).

** Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520, U.S.A.

*** Two m¹A residues were reported⁴² in tRNA^{Phe} from rabbit liver, the second residue was found in the DHU arm.

REFERENCES

1. Fleissner, E. and Borek, E. (1962) Proc. Natl. Acad. Sci. U.S. **48**, 1199-1203.
2. Srinivasan, P.R. and Borek, E. (1964) Biochemistry **3**, 616-619.
3. Hurwitz, J., Gold, M. and Anders, M. (1964) J. Biol. Chem. **239**, 3462-3473.
4. Baguley, B.C. and Staehelin, M. (1968) Biochemistry **7**, 45-50.
5. Kuchino, Y. and Nishimura, S. (1970) Biochem. Biophys. Res. Commun. **40**, 306-313.
6. Johnson, L., Hayashi, H. and Söll, D. (1970) Biochemistry **9**, 2823-2831.
7. Borek, E. (1971) Cancer Res. **31**, 596-597.
8. Starr, J.L. and Sells, B.H. (1969) Physiological Reviews **49**, 623-669.
9. Craddock, V.M. (1970) Nature **228**, 1264-1268.
10. Söll, D. (1971) Science **173**, 293-299.

11. Kerr, S.J. and Borek, E. (1972) in Advances in Enzymology (Meister, A., ed.) Vol. 36, pp. 1-27, Interscience Publishers, New York.
12. Gauss, D.H., von der Haar, F., Maelicke, A. and Cramer, F. (1971) in Annual Review of Biochemistry (Snell, E.E., ed.), Vol. 40, pp. 1045-1078, Annual Reviews Inc., Palo Alto, California.
13. Baguley, B.C. and Staehelin, M. (1968) Eur. J. Biochem. **6**, 1-7.
14. Dunn, D.B. (1961) Biochim. Biophys. Acta **46**, 198-200.
15. Baguley, B.C., Wehrli, W. and Staehelin, M. (1970) Biochemistry **9**, 1645-1649.
16. Baguley, B.C. and Staehelin, M. (1969) Biochemistry **8**, 257-262.
17. Dunn, D.B. (1959) Biochim. Biophys. Acta **34**, 286-288.
18. Smith, J.D. and Dunn, D.B. (1959) Biochim. Biophys. Acta, **31**, 573-575.
19. Dunn, D.B. and Flack, I.H. (1967) Abstr. 4th Fed. Eur. Biochem. Soc., p. 82.
20. Smith, J.D. and Dunn, D.B. (1959) Biochem. J. **72**, 294-301.
21. Dubin, D.T. and Gülnalp, A. (1967) Biochim. Biophys. Acta **134**, 106-123.
22. Nichols, J.L. and Lane, B.G. (1968) Can. J. Biochem. **46**, 1487-1495.
23. Simsek, M., RajBhandary, U.L., Boissard, M. and Petrisant, G. (1974) Nature **247**, 518-520.
24. Piper, P.W. and Clark, B.F.C. (1974) Nature **247**, 516-518.
25. Kraus, J. and Staehelin, M. (1974) Nucl. Acids Res. **1**, 1479-1496
26. Rogg, H., Wehrli, W. and Staehelin, M. (1969) Biochim. Biophys. Acta **195**, 13-15.
27. Monier, R., Stephenson, M.L. and Zamecnik, P.F. (1960) Biochim. Biophys. Acta **43**, 1-8.
28. Müller, P., Wehrli, W. and Staehelin, M. (1971) Biochemistry **10**, 1885-1890.
29. Weeren, H.O., Ryon, A.D., Heatherly, D.E. and Kelmers, A.D. (1970) Biotechnol. Bioeng. **12**, 889-912.
30. Wehrli, W. and Staehelin, M. (1971) Biochemistry **10**, 1878-1885.
31. Kerr, S.J. (1970) Biochemistry **9**, 690-695.
32. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. **193**, 265-275.
33. Rogg, H., Wehrli, W. and Staehelin, M. (1971) in Methods in Enzymology (Grossman, L. and Moldave, K., eds.) Vol. XX, Part C, pp. 118-125, Academic Press, New York.
34. Rogg, H. and Staehelin, M. (1971) Eur. J. Biochem. **21**, 235-242.
35. Brooks, P. and Lawley, P.D. (1960) J. Chem. Soc. 539-545.
36. Macon, J.B. and Wolfenden, R. (1968) Biochemistry **7**, 3453-3458.
37. Krutilina, A.I., Venkstern, T.V. and Bayev, A.A. (1964) Biochemistry USSR **29**, 288-292.
38. Hacker, B. and Mandel, L.R. (1969) Biochim. Biophys. Acta **190**, 38-51.
39. Bergquist, P.L. and Scott, J.F. (1964) Biochim. Biophys. Acta **87**, 199-211.

40. Rogg, H. and Staehelin, M. (1969) Biochim. Biophys. Acta **195**, 16-22.
41. Venkstern, T.V. and Bayev, A.A. (1968) Spectra of Nucleic Acid Compounds, IFI/PLENUM, New York.
42. Keith, G., Picaud, F., Weissenbach, J., Ebel, J.P., Petrissant, G. and Dirheimer, G. (1973) FEBS Letters **31**,
43. Andrews, P. (1965) Biochem. J. **96**, 595-606.
44. Agris, P.F., Spremulli, L.L. and Brown, G.M. (1974) Arch. Biochem. Biophys. **162**, 38-47.
45. Kerr, S.J. (1971) Proc. Natl. Acad. Sci. U.S. **68**, 406-410.
46. Kerr, S.J., Sharma, O.K. and Borek, E. (1971) Cancer Res. **31**, 633-636.
47. Söll, D. and Schimmel, P.R. (1974) in The Enzymes (Boyer, P., ed.) Vol. 10, pp. 490-538, Academic Press, New York.
48. Kuchino, Y. and Nishimura, S. (1970) Biochem. Biophys. Res. Commun. **40**, 306-313.
49. Roe, B., Michael, M. and Dudock, B. (1973) Nature New Biol. **246**, 135-138.
50. Pegg, A.E. (1972) Biochim. Biophys. Acta **262**, 283-289.
51. Kuchino, Y., Endo, H. and Nishimura, S. (1972) Cancer Res. **32**, 1243-1250.
52. Randerath, K. (1971) Cancer Res. **31**, 658-661.