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Sites of Methylation of Purified Transfer Ribonucleic Acid Preparations by Enzymes from Normal Tissues and from Tumours Induced by Dimethylnitrosamine and 1,2-Dimethylhydrazine

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1. The sites within the tRNA sequence of nucleosides methylated by the action of enzymes from mouse colon, rat kidney and tumours of these tissues acting on $tRNA^{Asp}$ from yeast and on $tRNA_2^{Glu}$, $tRNA^{fMet}$ and $tRNA_1^{Val}$ from *Escherichia coli* were determined. 2. The same sites in a particular tRNA were methylated by all of these extracts. Thus $tRNA_2^{Glu}$ was methylated at the cytidine residue at position 48 and the adenosine residue at position 58 from the 5'-end of the molecule; $tRNA^{Asp}$ was methylated at the guanosine residue at position 26 from the 5'-end of the molecule; $tRNA^{fMet}$ was methylated at the guanosine residues 9 and 27, the cytidine residue 49 and the adenosine residue 59 from the 5'-end; $tRNA_1^{Val}$ was methylated at the guanosine residue 10, the cytidine residue 48 and the adenosine residue 58 from the 5'-end. 3. All of these sites within the clover leaf structure of the tRNA sequence are occupied by a methylated nucleoside in some tRNA species of known sequence. It is concluded that methylation of tRNA from micro-organisms by enzymes from mammalian tissues *in vitro* probably does accurately represent the specificity of these enzymes *in vivo*. However, there was no evidence that the tumour extracts, which had considerably greater tRNA methylase activity than the normal tissues, had methylases with altered specificity capable of methylating sites not methylated in the normal tissues.

Enzymes present in mammalian tissues are known to be able to catalyse the methylation of RNA from yeast and bacteria, and the observations from many laboratories that these enzymes have greater activity in neoplastic tissues have led to suggestions that these tissues might contain tRNA methylases with specificities different from the enzymes present in normal tissues (Srinivasan & Borek, 1964; Borek, 1971; Borek & Kerr, 1972). Although there have been many studies of the activities of these enzymes in methylating unfractionated tRNA (Starr & Sells, 1969; Kerr & Borek, 1972; Borek & Kerr, 1972) only a few investigations of the sites at which methylated nucleosides are produced within the tRNA nucleotide sequence have been published (Baguley & Staehelin, 1968*a*,*b*, 1969; Baguley *et al.*, 1970; Kuchino & Nishimura, 1970; Kuchino *et al.*, 1971, 1972; Pegg, 1972*a*; Venkstern & Shershneva, 1972; Shershneva *et al.*, 1973*a*,*b*), and these papers have been confined to studies of the specificity of tRNA methylases from rat liver and spleen and tumours of these tissues. In the present paper I describe the investigation of the sites of methylation of $tRNA_2^{Glu}$, $tRNA_1^{Val}$, $tRNA^{fMet}$ of *Escherichia coli* and $tRNA^{Asp}$ of yeast by enzymes from mouse colon and colon tumours induced by 1,2-dimethylhydrazine and from rat kidney and kidney tumours induced by dimethylnitrosamine. Previous studies have shown that these tissue extracts catalyse the formation of N^2-methylguanosine when $tRNA^{Asp}$, $tRNA^{fMet}$ and $tRNA_1^{Val}$ were substrates for methylation, 1-methyladenosine and 5-methylcytidine when $tRNA^{fMet}$, $tRNA_2^{Glu}$ and $tRNA_1^{Val}$ were substrates and 1-methylguanosine when $tRNA^{fMet}$ was a substrate (Pegg, 1972*b*; Pegg & Hawks, 1974). A preliminary report of some of this work has been published (Pegg, 1972*c*).

Experimental

Methylation of tRNA

Purified tRNA species (purity greater than 90% based on ability to be converted into the appropriate aminoacyl-tRNA), *S*-adenosyl-L-[*Me*-^{14}C]methionine (55mCi/mmol) and extracts from mouse colon and colon tumours were obtained as previously described (Pegg & Hawks, 1974). Tumours of the rat kidney were induced by the administration of a single dose of dimethylnitrosamine to rats being fed a diet deficient in protein (McLean & Magee, 1970). Extracts from these tumours and normal rat kidneys containing tRNA methylase activity were prepared as previously described (Pegg, 1972*b*). Methylation of tRNA was carried out in a volume of 0.4ml containing 3nmol of tRNA, about 6mg of protein from the colon

and kidney extracts, 0.3M-ammonium acetate, 0.1M-Tris–HCl buffer, pH8.3, 0.05mM-EDTA, 0.1mM-dithiothreitol and 0.05mM-*S*-adenosyl-L-[*Me*-^{14}C]-methionine (55mCi/mmol). After incubation at 37°C for 15min the reaction was stopped by the addition of 1ml of phenol (previously saturated with water at room temperature). About 2mg of yeast tRNA in 1ml of water was then added and the tRNA isolated, washed and dried (Baguley & Staehelin, 1969). The dried tRNA was dissolved in 0.5ml of 50mM-Tris–HCl, pH7.5. Portions of the solution were used for the determination of radioactivity in the methylated nucleosides present (Pegg & Hawks, 1974) and the remainder was used for sequence analysis after degradation with ribonucleases.

Enzymic degradation of tRNA

Pancreatic ribonuclease A, ribonuclease T_1, snake venom phosphodiesterase and *E. coli* alkaline phosphatase were obtained from Sigma (London) Chemical Company, London S.W.6, U.K., and Worthington Biochemical Corp., Freehold, N.J., U.S.A. Guanyloribonuclease from *Actinomyces aureoverticillatus* (Tatarskaya *et al.*, 1967) was a generous gift from Dr. T. Venkstern, Institute of Molecular Biology, Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R. The conditions used for enzymic degradation of tRNA and oligonucleotide fragments were as described by Gangloff *et al.* (1970, 1972*a*) and Tatarskaya *et al.* (1967).

Separation of ribonuclease digests of tRNA by column chromatography

Digests of tRNA produced by the action of ribonuclease A were separated by chromatography on DEAE-cellulose (Whatman DE 52) in the presence of 7M-urea (Tomlinson & Tener, 1962; Tener, 1967). The column (60cm×1cm diam.) was equilibrated with 7M-urea–20mM-Tris–HCl, pH7.5, and the digest applied in this buffer solution. After washing with 50ml of the starting buffer, the eluate being discarded, the column was eluted with a linear gradient of 0.1–0.45M-NaCl in the starting buffer at a flow rate of 40ml/h. Fractions (5ml) were collected and the E_{260} was measured. Digests of tRNA produced by ribonuclease T_1 were separated by chromatography on columns (80cm×1cm diam.) of DEAE-Sephadex A-25 (purchased from Pharmacia, Uppsala, Sweden) as described by Rushizky *et al.* (1964) and Tener (1967). In this case, after application of the sample in 7M-urea–20mM-Tris–HCl, pH7.5, and washing as described above the column was eluted with 400ml of a linear gradient of 0.14–0.4M-NaCl in the starting buffer and then with a further 100ml of 1.0M-NaCl in the starting buffer. The flow rate of the column was maintained at 20–30ml/h and fractions of 5ml were collected.

A sample from each fraction of the column eluates was assayed for radioactivity as described below and those samples forming a peak of radioactivity were pooled, freed of urea and salt (Baguley & Staehelin, 1969; Gangloff *et al.*, 1972*a*) and concentrated by evaporation at reduced pressure and room temperature. A sample was used for the identification of the labelled methylated bases present and the remainder was used for further degradation by nucleases, as well as electrophoresis or chromatography.

Other methods

Mild treatment with alkali to degrade 7-methylguanosine and to convert 1-methyladenosine into N^6-methyladenosine was done as described by Wintermeyer & Zachau (1970) by incubation in 0.1M-Tris–HCl, pH9.5 at 50°C. After degradation with alkaline phosphatase and snake venom phosphodiesterase to convert oligonucleotides into nucleotides and a nucleoside from the 5′-end of the molecule, the products were separated by chromatography on DEAE-cellulose paper with 0.05M-ammonium formate, pH3.0 (Furlong, 1967). Nucleosides run with the solvent front in this system whereas nucleotides are retarded. Electrophoresis was carried out as described by Barrell (1971) and Gangloff *et al.* (1970) at a voltage gradient of 50V/cm. Radioactivity was assayed by mixing 1ml of aqueous solutions with 15ml of Triton X-100–0.6% 2,5-diphenyloxazole in toluene (1:2, v/v) and counting in a liquid-scintillation counter (Packard Tri-Carb 3320). The efficiency was about 60% for ^{14}C and was determined by the addition of an internal standard. Samples on paper were counted for radioactivity directly in the presence of 10ml of 0.6% 2,5-diphenyloxazole in toluene at an efficiency of 55% for ^{14}C.

Results

Amounts of radioactivity incorporated into tRNA

Table 1 shows the amounts of radioactivity incorporated into methylated nucleosides after the incubation of each of the tRNA species used in this study with 0.3M-ammonium acetate, *S*-adenosyl-L-[*Me*-^{14}C]methionine and extracts from colon, kidney and tumours derived from these tissues. The methylated nucleosides produced by the colon extracts were as described in the preceding paper (Pegg & Hawks, 1974). The kidney extracts produced the same products although the relative amounts of each nucleoside were somewhat different. These results differ from those previously obtained when methylation was carried out in the presence of spermidine rather than ammonium acetate (Pegg, 1972*b*) in the finding

Table 1. *Methylation of tRNA species by enzymes from rat kidney, mouse colon and tumours of these tissues indicating size of the oligonucleotide fragments containing the methylated products after digestion of the tRNA by ribonucleases A and T_1*

$tRNA_2^{Glu}$, $tRNA^{fMet}$, $tRNA^{Asp}$ and $tRNA_1^{Val}$ were methylated by extracts from the tissues shown and radioactivity present in each of the methylated nucleosides formed was determined as described in the Experimental section. After digestion of the labelled tRNA species with ribonucleases A or T_1 or both, the size of the oligonucleotide fragment containing each of the methylated bases was determined by chromatography on DEAE-cellulose or DEAE-Sephadex columns as described in Fig. 1. >6 indicates an oligonucleotide containing more than 6 nucleosides and a + sign after the number indicates that the oligonucleotide containing the labelled methylated nucleoside was eluted from the column preceding the peak of isopliths containing the number of nucleosides shown.

		10^{-3} × Radioactivity present after methylation with extract shown (c.p.m.)				Number of nucleoside residues in oligonucleotide containing labelled products after digestion with ribonuclease shown		
Substrate	Products	Colon	Colon tumour	Kidney	Kidney tumour	T_1	A	A+T_1
$tRNA_2^{Glu}$	1-Methyladenosine	9.1	48.0	3.3	8.9	>6	3+	2+
	5-Methylcytidine	13.6	58.5	11.5	25.4	6	3	3
$tRNA^{fMet}$	1-Methyladenosine	3.9	18.7	2.5	5.9	>6	3+	3+
	N^2-Methylguanosine	5.1	17.1	2.4	5.5	4	2	1
	1-Methylguanosine	2.6	11.9	3.6	8.7	2+ and 3	5	1+
	5-Methylcytidine	2.2	9.0	0.8	2.7	3+	1	1
$tRNA^{Asp}$	N^2-Methylguanosine	7.6	23.3	3.5	7.5	4	4	1
$tRNA_1^{Val}$	1-Methyladenosine	6.2	27.9	2.8	7.3	5+	2+	1+
	5-Methylcytidine	5.5	31.0	1.5	5.0	3+	1	1
	N^2-Methylguanosine	7.6	20.6	4.2	9.3	5	3	2

that 5-methylcytidine was formed when $tRNA^{fMet}$ was a substrate for methylation.

Sizes of oligonucleotide fragments containing methylated nucleosides

After digestion of the labelled tRNA species with either ribonuclease A or ribonuclease T_1 the size of the oligonucleotide fragments which contained the methylated nucleosides was determined by chromatography on DEAE-cellulose or DEAE-Sephadex columns and is indicated in Table 1. It was found that the sites of methylation were the same for all the four tissue extracts studied, as described in detail below.

An example of the chromatography of oligonucleotide fragments is shown in Fig. 1, which gives the results obtained for $tRNA_2^{Glu}$ methylated by colon extracts. After digestion with ribonuclease A, radioactivity due to labelled 5-methylcytidine was present in a trinucleotide. Radioactivity due to 1-methyladenosine was present in an oligonucleotide which was eluted before the trinucleotide (Figs. 1*a* and 1*c*). 1-Methyladenosine has a positive charge at pH7.5 and oligonucleosides containing this nucleoside are eluted from columns of DEAE-cellulose at positions preceding the peak due to oligonucleotides one residue shorter than they actually are (Tener, 1967; Baguley & Staehelin, 1969; Dirheimer *et al.*, 1972). Therefore the labelled 1-methyladenosine present in $tRNA_2^{Glu}$ is present in a tetranucleotide after digestion with ribonuclease A. This conclusion was confirmed by very mild treatment of the oligonucleotide with alkali. Such treatment converts 1-methyladenosine into N^6-methyladenosine (Lawley & Brookes, 1963), after which the oligonucleotide behaves as a tetranucleotide on chromatography on DEAE-cellulose.

The chromatography of ribonuclease T_1 digests of methylated $tRNA_2^{Glu}$ on DEAE-Sephadex is shown in Figs. 1(*b*) and 1(*d*). It was not possible to determine accurately the size of oligonucleotides greater than six nucleotides long and the column was therefore eluted with a linear gradient of NaCl until the hexanucleotide isopliths were obtained and then with 1 M-NaCl to elute the larger fragments as a single peak. Radioactivity due to 5-methylcytidine was present in a hexanucleotide and that due to 1-methyladenosine was present in an oligonucleotide larger than this (Figs. 1*b* and 1*d*).

The other tRNA species were treated in a similar manner and the sizes of the oligonucleotides containing each of the methylated nucleosides are shown in Table 1.

Positions of methylations in individual tRNA species

The oligonucleotide fragments containing methylated nucleosides were further analysed by standard procedures (Barrell, 1971; Dirheimer *et al.*, 1972). This analysis combined with the knowledge of the complete sequences of the tRNA species enabled the sites of methylation to be determined. A summary of these results is shown in Table 2. The analysis of

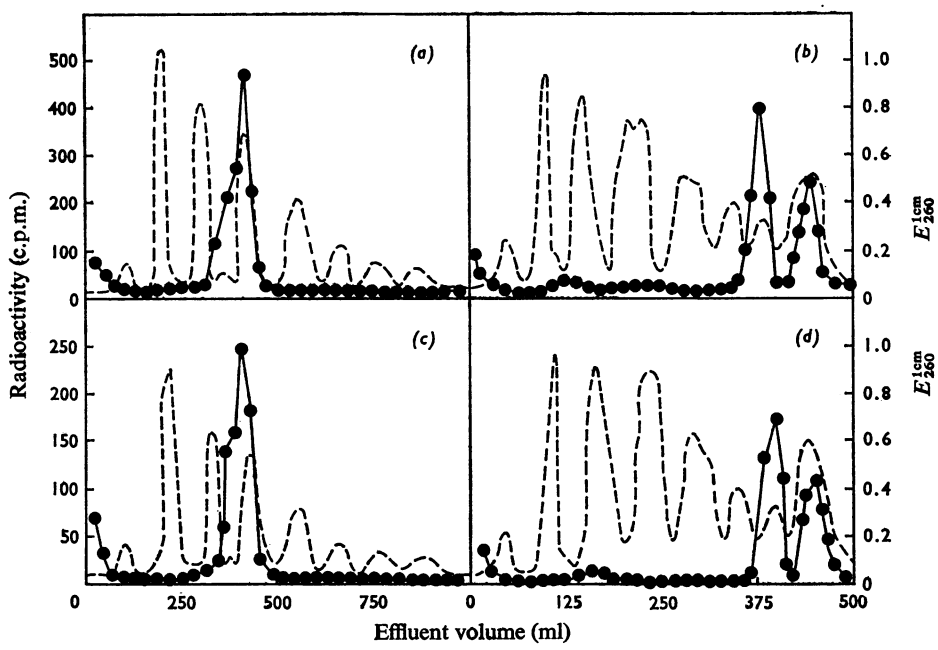


Fig. 1. *Fractionation of ribonuclease A and ribonuclease T_1 digests of ^{14}C-methyl labelled $tRNA_2^{Glu}$ on DEAE-cellulose or DEAE-Sephadex columns*

$tRNA_2^{Glu}$ was methylated by incubation with enzymes from normal colon (*a* and *b*) or colon tumours (*c* and *d*), combined with carrier tRNA, digested with ribonuclease A (*a* and *c*) or ribonuclease T_1 (*b* and *d*) and fractionated as described in the text. Radioactivity present in 1 ml samples from the column fractions (●) and the E_{260} (----) were measured.

the sites of formation of N^2-methylguanosine in $tRNA_2^{Glu}$, $tRNA^{Asp}$ and $tRNA^{fMet}$, of 1-methyladenosine in $tRNA_2^{Glu}$ and $tRNA_1^{Val}$ and of 5-methylcytidine in $tRNA_2^{Glu}$ were straightforward. Additional details of the other determinations are given below.

5-Methylcytidine was found, in ribonuclease T_1 digests of $tRNA^{fMet}$ and $tRNA_1^{Val}$, to be present in an oligonucleotide which was eluted from the DEAE-Sephadex columns just preceding the main peak of trinucleotides. This observation suggested that the oligonucleotide might contain a nucleoside which has a strong positive charge at pH7.5. Both of these tRNA species contain 7-methylguanosine, which does have such a charge, in a sequence G-m^{7}G*-U-C-Gp. This sequence yields the tetranucleotide m^{7}G-U-C-Gp on digestion with ribonuclease T_1, and this product is known to be eluted from DEAE-Sephadex at neutral pH values in the same position as the labelled product containing 5-methylcytidine (Seno *et al.*, 1969; Harada *et al.*, 1969). 7-Methylguanosine is very easily degraded in alkali, yielding 2,4-diamino-6-hydroxy-5-methylformamidopyrimidine (Lawley & Brookes, 1963). Such treatment at pH9.5, as described in the Experimental section, changed the electrophoretic mobility of the fragment produced by T_1 ribonuclease treatment (which then migrated faster towards the anode) and led to a change in the behaviour of the fragment on DEAE-Sephadex. After the treatment with alkali the fragment was eluted as a tetranucleotide. Under all conditions tested the behaviour of the oligonucleotides containing 5-methylcytidine from $tRNA^{Val}$ and $tRNA^{fMet}$ were identical.

1-Methylguanosine was present in a pentanucleotide in ribonuclease A digests of $tRNA^{fMet}$. 1-Methylguanosine was present at the 5'-end of the fragment. Digestion of methylated $tRNA^{fMet}$ with the guanyloribonuclease from *A. aureoverticillatus* [which is somewhat better than ribonuclease T_1 at attacking linkages to methylated guanosine residues (Tatarskaya *et al.*, 1967)] produced a dinucleotide of the form N-m^{1}G-cyclic-p. This information indicates that 1-methylguanosine is present in a sequence G-Y-m^{1}G-R-R-R-Yp, and there are two possible

* Abbreviations: m^{7}G,7-methylguanosine; m^{5}C,5-methylcytidine; m^{1}G, 1-methylguanosine; m^{2}G, N^2-methylguanosine; m^{1}A, 1-methyladenosine; s^4U, 4-thiouridine; R, unspecified purine nucleoside; Y, unspecified pyrimidine nucleoside; N, unspecified nucleoside that is not guanosine.

Table 2. *Summary of the analysis of oligonucleotide fragments produced by ribonucleases A and T_1 acting on tRNA species methylated in vitro by mammalian enzymes*

The table shows the methylated nucleoside formed with the indicated tRNA species acting as a substrate for methylases from kidney and colon. The sequences found to contain the methylated nucleoside after digestion with ribonuclease A to T_1, the sequence in the tRNA which could give rise to these products and the number (from the 5′ end) of the nucleoside methylated are also shown.

tRNA substrate	Product of methylation	Product from ribonuclease A digestion	Product from ribonuclease T_1 digestion	Probable form of sequence	Position in tRNA
$tRNA_2^{Glu}$	5-Methylcytidine	A-A-m^{5}Cp	[N,(Y-A-A-m^{5}C)]Gp	G-U-A-A-m^{5}C-A-Gp	48
$tRNA_2^{Glu}$	1-Methyladenosine	G-m^{1}A-A-Yp	>6 nucleotides	C-G-m^{1}A-A-U-C-C-C-C-U-A-Gp	58
$tRNA^{Asp}$	N^2-Methylguanosine	m^{2}G-R-R-Yp	N-N-Y-m^{2}Gp	G-A-A-U-m^{2}G-G-G-Cp	26
$tRNA^{fMet}$	N^2-Methylguanosine	m^{2}G-Yp	N-N-Y-m^{2}Gp	G-C-U-C-m^{2}G-Up	27
$tRNA^{fMet}$	5-Methylcytidine	m^{5}Cp	m^{7}G-U-m^{5}C-Gp	G-m^{7}G-U-m^{5}C-Gp	49
$tRNA^{fMet}$	1-Methyladenosine	A-m^{1}A-A-Yp	>6 nucleotides	G-T-U-C-A-m^{1}A-A-U-C-Gp	59
$tRNA^{fMet}$	1-Methylguanosine	m^{1}G-R-R-R-Yp	Y-m^{1}G-cyclic-p	s^{4}U-m^{1}G-G-A-G-Cp	9
$tRNA_1^{Val}$	N^2-Methylguanosine	A-m^{2}G-Yp	N-N-Y-A-m^{2}Gp	G-A-U-s^{4}U-A-m^{2}G-Cp	10
$tRNA_1^{Val}$	5-Methylcytidine	m^{5}Cp	m^{7}G-U-m^{5}C-Gp	G-m^{7}G-U-m^{5}C-Gp	48
$tRNA_1^{Val}$	1-Methyladenosine	G-m^{1}A-Yp	m^{1}A-Y-N-N-N-Gp	C-G-m^{1}A-U-C-C-C-Gp	58

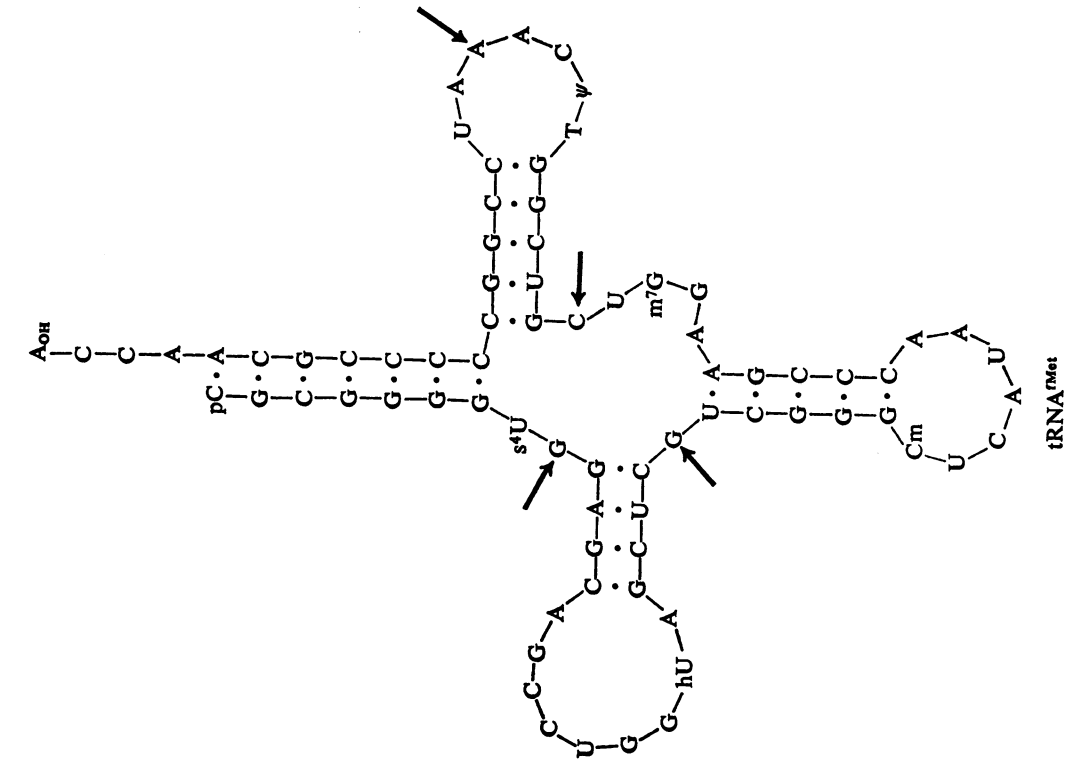
sequences in $tRNA^{fMet}$ which could be methylated to form this product. These are the sequences G-C-G-G-G-G-s^{4}Up and G-2^{4}U-G-G-A-G-Cp. The labelled pentanucleotide obtained by digestion with pancreatic ribonuclease moved, on electrophoresis at pH3, at a slower speed (0.6 times) than did Up. This suggests that the methylated sequence is m^{1}G-G-A-G-Cp, since it is known that G-G-G-G-Up moves faster than Up and that G-G-A-G-Cp moves more slowly than Up at this pH (Barrell, 1971). Therefore the site of formation of 1-methylguanosine is probably the residue at position 9 from the 5′-end of $tRNA^{fMet}$.

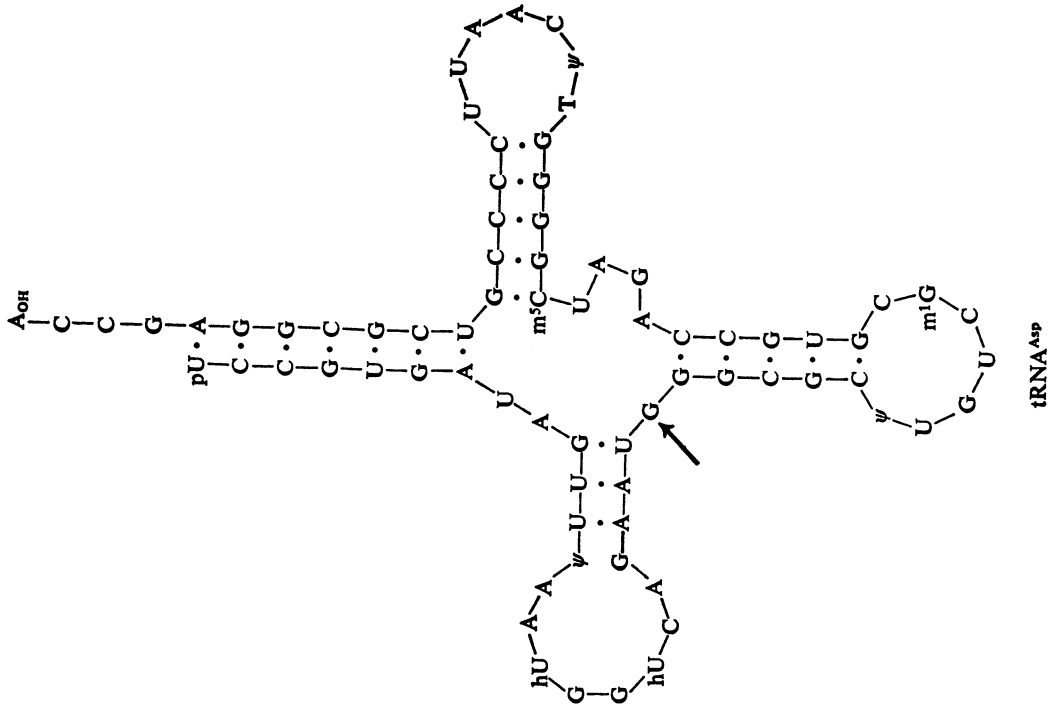
The tetranucleotide containing 1-methyladenosine produced by ribonuclease A digestion of $tRNA^{fMet}$ had the sequence (m^{1}A,A$_2$)Yp. The position of the 1-methyladenosine in this sequence was shown, by the following procedure, to be the second nucleoside from the 5′-end. Treatment of the fragment with alkali under conditions that converted 1-methyladenosine into 6-methyladenosine was followed by digestion with alkaline phosphatase followed by digestion with snake venom phosphodiesterase. This revealed that 6-methyladenosine was not present at the 5′-terminus. Partial digestion of the oligonucleotide with ribonuclease T_2 gave rise to a number of products, including a trinucleotide which could be shown to have 6-methyladenosine at its 5′-terminus as described above. This trinucleotide must therefore be of the form m^{6}A-A-Yp.

Discussion

Fig. 2 shows the sequences of $tRNA^{Asp}$ (Gangloff *et al.*, 1972*b*), $tRNA^{fMet}$ (Dube *et al.*, 1969), $tRNA_2^{Glu}$ (Ohashi *et al.*, 1972) and $tRNA_1^{Val}$ (Yaniv & Barrell, 1969). The arrows show the nucleosides attacked by tRNA methylases of colon and kidney. The structure of $tRNA_2^{Glu}$ shown is that reported by Ohashi *et al.* (1972), which differs from that found by Munninger & Chang (1972) in that the latter authors found an additional unidentified nucleoside in the anticodon loop. The positions for the cytidine and adenosine residues found to be methylated by mammalian enzymes, as given in Table 2, must therefore be increased by 1 if the latter structure is correct.

It was not possible to characterize rigorously oligonucleotide fragments containing methylated bases introduced by the action of mammalian enzymes *in vitro*, because only a small amount of material was present in the labelled oligonucleotides and suitable unlabelled methylated oligonucleotides to act as markers were not available. The procedures by which the sites of methylation were determined therefore depend entirely on the specificity of ribonucleases A and T_1 and on the determination, by chromatography on DEAE-cellulose, of the size of the oligonucleotides produced by the action of these enzymes. This approach has obvious limitations, but the results obtained do appear to be in close agreement with the known sites of occurrence of methylated nucleosides in tRNA. At least 40 tRNA sequences are now known and the methylated nucleosides are confined to only a few sites in the tRNA structure (Staehelin, 1971; Dirheimer *et al.*, 1972). Thus, 1-methyladenosine, when present in tRNA species, has always been found as the second nucleoside after the sequence T-ψ-Cp in the loop usually referred to as the 'TψC loop'. [Recently $tRNA_1^{Met}$ has been shown not to contain the sequence


tRNA^Met


tRNA^Asp

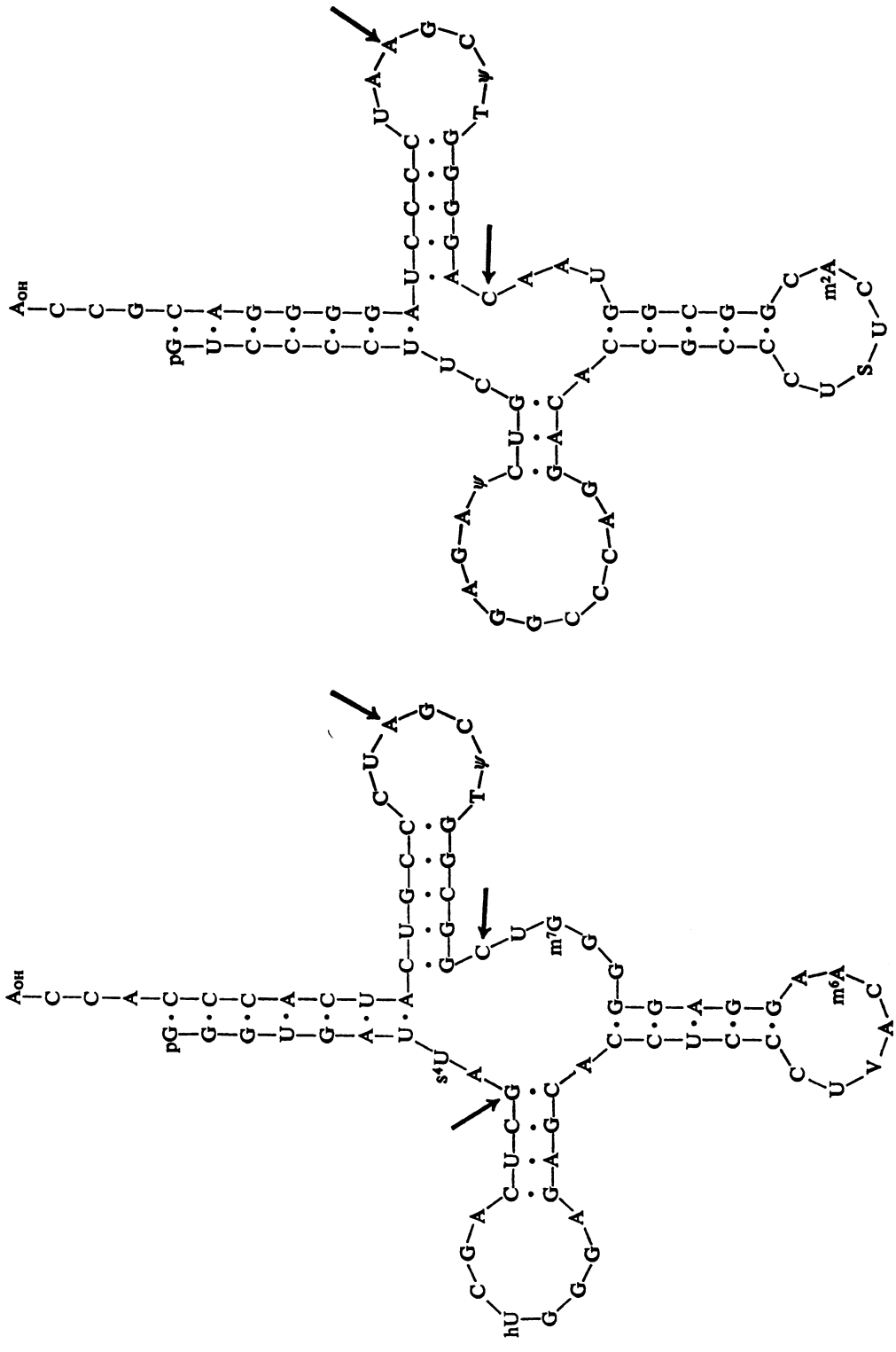


Fig. 2. *Nucleotide sequences of tRNA^{Asp}, tRNA^{fMet}, tRNA$_1^{Val}$ and tRNA$_2^{Glu}$, indicating sites attacked by tRNA methylases of kidney, colon and tumours of these tissues*

Sites found to be methylated are indicated by arrows.

T-ψ-Cp, which is common to all other tRNA species involved in protein synthesis, but it does have 1-methyladenosine in the same position as in other tRNA species, namely as the fifth residue in this loop (Simsek & Rajbhandary, 1972).] *E. coli* $tRNA^{fMet}$, $tRNA_2^{Glu}$ and $tRNA_1^{Val}$ were methylated by the extracts from kidney and colon to produce 1-methyladenosine at this site, although the sequences A-m^1A-A-Up, G-m^1A-A-Up and G-m^1A-Up respectively were different in each case. This finding is in agreement with other reports of the production of 1-methyladenosine at this site or in these sequences when unfractionated yeast or *E. coli* tRNA (Baguley & Staehelin, 1968*b*, 1969; Staehelin, 1971), yeast $tRNA^{Ser}$ (Baguley *et al.*, 1970) or *E. coli* $tRNA^{fMet}$ (Kuchino *et al.*, 1971; Spremulli *et al.*, 1972; Shershneva *et al.*, 1973*b*) were methylated by enzymes from rat liver or HeLa cells.

5-Methylcytidine has been found in three positions in tRNA sequences. It occurs in the stem of the anticodon arm in yeast $tRNA^{Phe}$, but in all other tRNA species in which it has been found, 5-methylcytidine is present as the first nucleoside in the stem of the 'TψC loop' and/or as the residue before this, thereby forming the last nucleoside of the 'extra arm' region. 5-Methylcytidine was formed at the latter site in the *E. coli* tRNA species studied in this paper (Fig. 2) in the sequence A-A-m^5C-Ap in $tRNA_2^{Glu}$ and the sequence m^7G-U-m^5C-Gp in $tRNA_1^{Val}$ and $tRNA^{fMet}$. Although the sequence attacked was the same, $tRNA_1^{Val}$ was a better substrate for methylation at this site than $tRNA^{fMet}$ under the conditions examined. No satisfactory explanation for this finding is available. Although according to Dube *et al.* (1969) 25% of $tRNA^{fMet}$ molecules have the sequence A-U-C-Gp instead of m^7G-U-C-Gp, at least 80% of the $tRNA^{fMet}$ molecules in the preparation used in the present experiments had 7-methylguanosine (A. E. Pegg, unpublished work) and the difference cannot be explained solely by such a small amount of tRNA with a different sequence. 5-Methylcytidine has been reported to be formed by rat liver enzymes acting on $tRNA^{fMet}$ and yeast $tRNA^{Val}$ (Shershneva *et al.*, 1973*a*,*b*; Venkstern & Shershneva, 1972) at the same position as found here for the action of enzymes from colon and kidney.

N^2-Methylguanosine is present, in at least ten tRNA species from yeast, as the first residue in the stem of the dihydrouridine-containing loop, and it was in this position that $tRNA_1^{Val}$ was methylated in the sequence A-m^2G-Cp. N^2N^2-Dimethylguanosine is found in many yeast tRNA species at the residue between the stem of the 'dihydrouridine loop' and the stem of the anti-codon loop'. This position was the site of formation of N^2-methylguanosine in $tRNA^{fMet}$ and $tRNA^{Asp}$ (Fig. 2). It is not possible at present to say whether this methylation is the first stage of the formation of the dimethyl derivative and that the second step does not take place under the conditions used *in vitro* or whether N^2-methylguanosine is really a normal product of methylation of tRNA by mammalian enzymes at this site. Rat liver enzymes have also been shown to catalyse the formation of N^2-methylguanosine in the sequence C-m^2G-Up in $tRNA^{fMet}$ and in the sequence U-m^2G-Gp in $tRNA^{Asp}$ (Kuchino & Nichimura, 1970; Pegg, 1972*a*).

1-Methylguanosine is present adjacent to the anticodon in $tRNA^{Asp}$, but in many other tRNA species it is found as the nucleoside between the stem of the 'amino acid arm' and the 'dihydrouridine arm', which was the site of formation of this product by colon and kidney enzymes acting on $tRNA^{fMet}$.

The use of tRNA molecules from one species as substrates for the study of tRNA methylases from another does therefore yield results that are in agreement with known tRNA structures, but it must be remembered that it is not known at what stage during the maturation of the tRNA precursor molecule in mammalian tissues that methylation occurs (Burdon, 1971; Nishimura, 1972). It is not surprising that *E. coli* tRNA species are good substrates for the formation of 1-methyladenosine, N^2-methylguanosine and 5-methylcytidine by mammalian enzymes, as *E. coli* tRNA contains very little, if any, of these methylated bases (Nishimura, 1972), but for studies of the formation of ribothymidine, 3-methylcytidine, 7-methylguanosine and methylated sugars in tRNA, tRNA species from *E. coli* and yeast do not appear to be suitable substrates. Thus some means of obtaining unmethylated tRNA from mammalian cells is most important for further studies of mammalian tRNA methylases. The use of homogeneous tRNA preparations as substrates for tRNA methylases, as in the present work, is essential for studies of the activities of tRNA methylases. The presence of a large excess of tRNA molecules that are not substrates for a particular methylase may actually be inhibitory to the methylation of a tRNA that is a substrate, as the rate of formation of 1-methylguanosine is actually greater when purified $tRNA^{fMet}$ is employed as substrate than when a preparation of unfractionated tRNA containing a similar amount of $tRNA^{fMet}$ is used (Pegg, 1972*b*). Further purification of the extracts containing tRNA methylases is also necessary to determine whether one enzyme is responsible for the formation of a particular methylated base at the same site in a variety of tRNA sequences, as described above, or whether there is one enzyme for each sequence. It is clear that the structure of the tRNA as a whole is important in determining whether methylation occurs at a particular nucleotide sequence, because nucleotide sequences similar to those methylated are found in other regions of the tRNA structure but are not attacked. Studies of the methylation of large fragments of tRNA and reconstituted tRNA molecules by liver tRNA methylases are in agreement with this

interpretation (Kuchino *et al.*, 1971; Shershneva *et al.*, 1971, 1973*a*).

Conversely the sequence surrounding the nucleoside that may be methylated may also be important in determining whether methylation can occur. $tRNA^{fMet}$, $tRNA_2^{Glu}$ and $tRNA^{Asp}$ have a guanosine residue at the same position as that in $tRNA_1^{Val}$ which was found to be methylated by the colon and kidney enzymes. Similarly, $tRNA^{Asp}$ has an adenosine residue in the same position as that methylated in $tRNA^{fMet}$, $tRNA_2^{Glu}$ and $tRNA_1^{Val}$. However, these nucleosides were not attacked under the conditions used. Some caution is necessary in interpreting negative results, because the access of the tRNA methylase to the site that could be attacked might be hindered in some way by the structure of the tRNA. Methylation might occur under different incubation conditions that alter the structure of the tRNA. Kuchino *et al.* (1971) reported that in the presence of Mg^{2+}, methylation of $tRNA^{fMet}$ to give 1-methyladenosine occurred only when fragments of the molecule were used as substrates rather than the intact $tRNA^{fMet}$, which was found to be a substrate for the formation of 1-methyladenosine under different assay conditions (Leboy & Piester, 1973; Spremulli *et al.*, 1972; Nau *et al.*, 1972; Shershneva *et al.*, 1973*b*). In the presence of Mg^{2+} alone the methylation of tRNA by crude extracts from mammalian tissues produces mainly methylated guanosine derivatives, but addition of polyamines or NH_4^+ allows the formation of 1-methyladenosine and 5-methylcytidine (Leboy, 1971; Pegg, 1972*a*,*b*; Leboy & Piester, 1973). These latter products are formed in the 'TψC loop' and the 'extra arm' region, which may be oriented in the tertiary structure of the tRNA (Levitt, 1969; Cramer, 1971; Kim *et al.*, 1973) such that methylation occurs only when the tRNA structure is altered.

Finally, although extracts from colon and kidney tumours have greater activities of tRNA methylases than do the normal tissues, the specificities of these enzymes towards nucleotide sequences in six tRNA species of known sequence [the four described in the present study and $tRNA_1^{Met}$ and $tRNA_2^{Arg}$, which were not attacked (Pegg & Hawks, 1974] are similar and there is therefore no evidence to support proposals that methylations due to tRNA methylases might exert a controlling influence in differentiation and neoplastic growth (Srinivasan & Borek, 1964, 1966; Craddock, 1970; Borek, 1971; Borek & Kerr, 1972; Kerr & Borek, 1972). Similar conclusions have been reached by Baguley & Staehelin (1968*b*) and by Kuchino *et al.* (1972). It is possible that studies with a wider range of purified tRNA species might reveal differences in tRNA methylase specificity in different tissues. Also variations in the ability to modify the residues adjacent to the anti-codon, which are frequently modified either by methylation but usually more extensively (Nishimura, 1972), may be important in cellular control mechanisms. However, at present, methods for the study of the enzymes responsible for the formation of these products are limited by the lack of suitable substrates. There is evidence for a role of such modifications in controlling the activity of the tRNA in protein synthesis (Gefter & Bikoff, 1971; Nishimura, 1972), but the function of the other methylated bases in tRNA [and it is these bases that are formed in the assay system used *in vitro* in the studies of tRNA methylases cited as evidence for changes in tRNA methylase activity in neoplastic growth (Craddock, 1970; Borek, 1971; Borek & Kerr, 1972)] is at present unknown. One possibility might be that they increase the stability of the active configuration of tRNA *in vivo* both by protecting against nuclease action and by aiding in the formation of intramolecular hydrogen bonds. If this were the case it might be expected that tissues with a high rate of growth and nucleic acid synthesis might have elevated tRNA methylase activities.

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