Further Investigation of the Increased Transfer Ribonucleic Acid Methylase Activity in Tumours of the Mouse Colon

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1. Extracts prepared from tumours of the mouse colon induced by 1,2-dimethylhydrazine were considerably more active in catalysing the methylation of tRNA than were extracts from normal colon. The enhanced activity was observed when both unfractionated 'methyl-deficient' tRNA and purified tRNA preparations from yeast and bacteria were used as substrates for methylation. 2. The methylated bases produced in these reactions were identified. There were no differences between the products of the reaction catalysed by extracts of tumour and normal colon. 3. The increased activity of tRNA methylases was not due to the presence in the extracts of stimulatory or inhibitory molecules of low molecular weight such as polyamines or *S*-adenosylhomocysteine. 4. Other enzymes concerned with tRNA metabolism (RNA polymerase, ATP-tRNA adenylyltransferase, aminoacyl-tRNA ligases) were also increased in activity in the tumour tissue. 5. The extent of methylation of a limiting amount of tRNA was greater when tumour extracts were compared with controls, but in no case was it possible to achieve a stoichiometric methylation of the purified tRNA preparations used as substrates, and the tumour extracts were not able to methylate tRNA obtained from normal mouse colon. We conclude that the tumours contained greater activities of tRNA methylases but that there was no evidence for changes in the specificity of these enzymes during neoplastic growth.

The possibility that changes in the cellular content of tRNA methylases may play an important role in the development and growth of tumours (Srinivasan & Borek, 1964, 1966; Craddock, 1970) has been supported by numerous reports of increases in the activity of these enzymes in extracts prepared from malignant cells and of the content of methylated bases in tRNA prepared from tumours (summarized by Borek, 1971; Borek & Kerr, 1972). Recently, however, these reports have been contested because it has been shown that methylation of tRNA catalysed by crude tissue extracts *in vitro* is profoundly influenced by the concentration in the assay medium of cations such as polyamines (Leboy, 1970, 1971; Pegg, 1971*a*) and by other compounds such as nicotinamide, glycine and *S*-adenosylhomocysteine (Pegg, 1971*b*; Kerr, 1972; Murai *et al.*, 1972; Gross & Wildenhauer, 1972; Kuchino & Endo, 1972). Also it has been reported that no differences could be found in the content of methylated bases of some tumours and appropriate control tissues (Randerath *et al.*, 1971; Klagsbrun, 1972). Further, in many of the comparisons of tRNA methylases from normal and neoplastic cells the control tissue was not really representative of the cells from which the tumour was derived, and since organ-specific differences of tRNA methylase activity have been reported (Kerr & Borek, 1972; Leboy & Piester, 1973) the

relevance of the changes in tRNA methylase activity to the neoplastic state is not clear.

Most of the studies of tRNA methylases of mammalian cells have been carried out with bacterial tRNA as a substrate for methylation because it is not at present possible to obtain tRNA from mammalian cells that has not already been methylated *in vivo*. tRNA from *Escherichia coli* is methylated to a lower extent than that from eukaryotes (Staehelin, 1971) and can be further methylated *in vitro* by extracts from mammalian cells (Starr & Sells, 1969; Kerr & Borek, 1972), although 'undermethylated' tRNA obtained from a methionine-requiring mutant 'grown' in the absence of the required amino acid is a better substrate. It is now well established that extracts derived from a range of tissues contain a number of enzymes (at least nine) capable of producing a range of methylated bases when acting on such bacterial tRNA (Rodeh *et al.*, 1967; Baguley & Staehelin, 1968*a,b*; Starr & Sells, 1969; Kuchino & Nishimura, 1970; Leboy, 1971; Staehelin, 1971; Pegg, 1971*a*, 1972*a*; Kuchino *et al.*, 1971, 1972; Liao *et al.*, 1972; Kerr & Borek, 1972). The increased tRNA methylase activity found in studies of the rate and extent of methylation of bacterial tRNA by crude extracts from tumours could be due to a general increase in the activity of all of these enzymes, or to a selective increase in some of

them, or to the appearance of enzymes in the tumours capable of methylating sites in the tRNA not recognized by the control extracts. However, the complexity of the reaction, owing to the heterogeneity of the substrate, has prevented detailed analysis. Also, in such a system it is most unlikely that assay conditions employed can be optimal for all of the enzymes contributing to the methylation of the tRNA.

Tumours of the mouse colon produced by the administration of 1,2-dimethylhydrazine (Druckrey *et al.*, 1967; Wiebecke *et al.*, 1969) have a greater tRNA methylase activity than has normal colon. Such tumours, which form multiple nodules within the colon, can be readily dissected from the normal tissue and provide a useful system for the study of biochemical changes occurring during carcinogenesis and subsequent tumour growth (Pegg & Hawks, 1971). The present paper describes further studies of tRNA methylases and other enzymes concerned with tRNA metabolism in mouse colon and colonic tumours. The tRNA methylases present in these tissues have been studied with purified tRNA preparations from *E. coli* and from yeast as substrates for methylation. Several purified tRNA species were substrates for methylases present in these extracts. Each produced a characteristic range of methylated bases, which was much simpler than that found when unfractionated 'methyl-deficient' tRNA from *E. coli* was utilized as a substrate. No evidence that the colon tumours contained tRNA methylases with a specificity different from those of normal colon was found.

Experimental

S-Adenosyl-L-[Me-¹⁴C]methionine (55 mCi/mmol), L-[Me-¹⁴C]methionine (50 mCi/mmol), L-[U-¹⁴C]-lysine (306 mCi/mmol), L-[U-¹⁴C]leucine (312 mCi/mmol), [5-³H]UTP (1 Ci/mmol) and [8-¹⁴C]ATP (34 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. ATP, GTP, UTP, CTP, dithiothreitol and methylated bases used as markers were obtained from the Sigma (London) Chemical Co., London S.W.6, U.K., and Cyclo Chemical Corp., Los Angeles, Calif., U.S.A. 1,2-Dimethylhydrazine was a gift from Dr. R. Preussmann, Krebsforschungszentrum, Heidelberg, Germany. α -Amanitin was a gift from Dr. Th. Wieland, Max-Planck-Institut für Medizinische Forschung, Heidelberg, Germany. tRNA_{3^{ser}} from *E. coli* B was a gift from Dr. S. Nishimura, National Cancer Centre Research Institute, Tokyo, Japan. tRNA^{fMet} and tRNA_{2^{Glu}} from *E. coli* K-12 MO7 (Hancer *et al.*, 1969; Weeren *et al.*, 1970) were obtained from Dr. A. Kelmers, Oak Ridge National Laboratory, Oak Ridge, Tenn., U.S.A. Purified yeast tRNA species (Gillam *et al.*, 1968;

Simsek & Rajbhandary, 1972; Weissenbach *et al.*, 1972) were gifts from Dr. I. Gillam, Department of Biochemistry, University of British Columbia, Vancouver, Canada. tRNA_{1^{Val}} from *E. coli* M.R.E. 600 was purified starting from the preparation purchased as tRNA^{Val} from Boehringer, Mannheim, Germany, by the procedures of Nishimura (1971).

Preparation of tissue extracts

Tumours of the descending colon were induced in N.M.R.I. mice bred in the Courtauld Institute by 24 weekly injections of 1,2-dimethylhydrazine (Wiebecke *et al.*, 1969; Pegg & Hawks, 1971; Hawks, 1972). Tumour tissue or corresponding segments of normal colon (Hawks *et al.*, 1972; Pegg & Hawks, 1971) was removed from the animals as rapidly as possible after cervical dislocation and used for the preparation of the tissue extracts. All further operations were performed as near to 0°C as possible.

Nuclei were isolated by homogenization of the tissue in 5 vol. of 0.25 M-sucrose–5 mM-MgCl₂–50 mM-Tris–HCl, pH 7.5, containing 1% (w/v) *N*-acetyl-L-cysteine (Hawks, 1972). The homogenate was filtered through cheesecloth and centrifuged at 600g for 10 min at 2°C. The pellet was resuspended in 2 M-sucrose–5 mM-MgCl₂–25 mM-KCl–50 mM-Tris–HCl, pH 7.5, layered over 10 ml of the same medium and centrifuged at 64000g (*r*_{av} 9.1 cm) for 1 h at 2°C. The resulting pellet was suspended in 25 mM-KCl–50 mM-Tris–HCl (pH 7.5)–20% (v/v) glycerol and used for RNA polymerase assays and the determination of DNA.

Supernatant protein extracts for the assay of ATP-tRNA adenyltransferase, aminoacyl-tRNA ligases and tRNA methylases were obtained by homogenization of the tissue with 5 vol. of 10 mM-Tris–HCl (pH 7.5)–1 mM-dithiothreitol–0.1 mM-EDTA. The homogenate was centrifuged for 4 h at 165000g (*r*_{av} 5.9 cm) in the type 50Ti rotor of the Spinco model L2(50) centrifuge at 2°C. The supernatant fraction from this centrifugation was used for the assay of aminoacyl-tRNA ligases and ATP-tRNA adenyltransferase after dialysis overnight against 4 litres of the buffer used for homogenization.

For the assay of tRNA methylases the supernatant fluid was further fractionated by the addition of 4 ml of saturated (NH₄)₂SO₄ solution (saturated at 4°C and adjusted to pH 7.0 with conc. NH₃) for every 15 ml of solution. After stirring for 20 min at 0°C the precipitate was removed by centrifugation at 15000g for 15 min and a further 20 ml of saturated (NH₄)₂SO₄ solution was added. After stirring for 20 min at 0°C the precipitate was collected by centrifugation at 15000g for 15 min and dissolved in 10 mM-Tris–HCl (pH 7.8)–1 mM-dithiothreitol–1 mM-EDTA at a protein concentration of 25–30 mg/ml. The solution was dialysed against 4 litres of the same medium over-

night and stored frozen at -30°C for up to 1 week before use. No loss of tRNA methylase activity occurred under storage and more than 95% of the tRNA methylase activity of the colon extracts was present in this fraction. A similar proportion of the protein present in homogenates from normal colon and from colon tumours was present in the final supernatant fraction used for the assay of tRNA methylases.

Enzyme assays

tRNA methylase activity was measured by determination of the incorporation of labelled methyl groups from *S*-adenosyl-L-[*Me*- ^{14}C]methionine into tRNA (Pegg, 1971a, 1972a). The assay medium contained various cations as shown in the relevant tables, up to 0.2 mg of protein from the colon extracts, 15 μmol of Tris-HCl, pH 8.5, 500 pmol of the tRNA preparations (where the tRNA concentration was not known exactly it was assumed that 1650 pmol had $E_{260}^{1\text{cm}}$ 1), 10 nmol of *S*-adenosyl-L-[*Me*- ^{14}C]methionine, 5 nmol of EDTA and 5 nmol of dithiothreitol in a total volume of 0.15 ml. The rate of methylation of tRNA was proportional to the time of incubation for at least 30 min and was proportional to the protein added under these conditions.

The extent of methylation of small amounts of tRNA in the presence of an excess of the tissue extracts was measured in a similar manner, but in this case the assay medium contained 20 nmol of *S*-adenosyl-L-[*Me*- ^{14}C]methionine, 20 μmol of Tris-HCl, pH 8.5, 20 pmol of tRNA, 3–4 mg of protein, 60 μmol of ammonium acetate, 150 nmol of EDTA and 150 nmol of dithiothreitol in a total volume of 0.2 ml. Incubation for 45 min at 37°C produced a maximal extent of methylation of tRNA under these conditions, and addition of extra *S*-adenosyl-L-[*Me*- ^{14}C]methionine, or extra protein or further incubation, did not increase the incorporation of methyl groups into tRNA. Controls in which no tRNA was added were included in all assays and the incorporation of methyl groups into protein was subtracted from the total incorporation into material insoluble in trichloroacetic acid (Pegg, 1971a). In experiments where the rate of methylation of tRNA by a small amount of protein was measured this incorporation into protein was less than 5% of the methylation of tRNA, but in the assays of extent of methylation of small amounts of tRNA, radioactivity incorporated into protein was up to 45% of the total radioactivity. All of the latter assays were carried out in triplicate for each protein extract with and without added tRNA, and the results agreed within 5% in all cases.

RNA polymerase assays were carried out by using the isolated nuclei as a source of enzyme and DNA template. The assay medium contained, in a volume

of 0.2 ml, 20 μmol of Tris-HCl, pH 8.0, 0.2 μmol of dithiothreitol, 0.2 μmol each of ATP, GTP, CTP, 1 μCi of 5-[^3H]UTP, 3 μmol of KCl, 5% (v/v) glycerol, about 0.1 mg of nuclear DNA and either 3 mM-MnCl₂+0.3 M-(NH₄)₂SO₄ or 5 mM-MgCl₂ and 1 μg of α -amanitin/ml. After incubation at 37°C portions (0.1 ml) were removed and radioactivity incorporated into RNA was measured (Smuckler & Hadjiolov, 1972). Activity was proportional to the time of incubation for up to 20 min at 37°C and to the amount of nuclei added up to 0.2 mg of DNA. RNA polymerase activity of both normal colon and colon tumours was inhibited by more than 90% by 50 μg of actinomycin D/ml and activity in the presence of 3 mM-MnCl₂-0.3 M-(NH₄)₂SO₄ was inhibited by more than 80% by 1 μg of α -amanitin/ml.

ATP-tRNA adenylyltransferase activity was determined by measurement of the incorporation of [^3H]-ATP into rat liver tRNA that had previously been incubated with rat liver enzymes to remove the terminal adenosine (Littauer & Daniel, 1966). The assay medium contained 8 μmol of Tris-HCl, pH 8.8, 0.5 mg of rat liver tRNA, 0.8 μmol of MgCl₂, 0.8 μmol of phosphoenolpyruvate, 2 μg of pyruvate kinase, 0.14 μCi of [^3H]-ATP (35.4 mCi/mmol) and 0.5–1.0 mg of protein from the colon extracts in a total volume of 0.2 ml. Incorporation was proportional to the time of incubation at 37°C for about 20 min and was proportional to the protein concentration present up to 1 mg.

Aminoacyl-tRNA ligase activity was determined by measurement of the incorporation of ^{14}C -labelled amino acids into aminoacyl-tRNA (Makman, 1966). The assay medium contained 1 mg of yeast tRNA, 4 μmol of potassium phosphate buffer, pH 7.2, 0.4 μmol ATP, 0.8 μmol of MgCl₂, 0.1 μmol of dithiothreitol, 0.01 μmol of CTP, about 0.2–0.3 mg of protein from the colon extracts and either 0.5 μCi of L-[*Me*- ^{14}C]methionine (50 mCi/mmol), 0.625 μCi of L-[U- ^{14}C]lysine (306 mCi/mmol), or 0.625 μCi of L-[U- ^{14}C]leucine (312 mCi/mmol) in a total volume of 0.2 ml. Activity was proportional to the amount of colon protein added up to 0.5 mg of protein and was proportional to the time of incubation at 37°C for up to 20 min.

Analysis of methylated bases produced in vitro

The tRNA was incubated in a total volume of 0.4 ml containing 100 mM-Tris-HCl, pH 7.8, 40 μM -*S*-adenosyl-L-[*Me*- ^{14}C]methionine (55 mCi/mmol), about 10 mg of protein, 1 nmol of the tRNA and the cation concentration shown for 15 min at 37°C . The reaction was then stopped by adding an equal volume of phenol (saturated with water) followed by 0.2 ml of a solution of yeast tRNA (10 mg/ml in water). The tRNA was then isolated and washed to remove any bound *S*-adenosyl-L-[*Me*- ^{14}C]methionine (Pegg,

1971a). The dried tRNA was hydrolysed to free bases by heating with 0.05 ml of 70% (w/v) HClO_4 at 100°C for 90 min (Wyatt, 1952). The hydrolysate was cooled, 2 ml of water was added and the black residue removed by centrifugation. Then 2 ml of 0.1 M-HCl containing marker methylated bases (about 0.5 mg of each) was added and the combined solution applied to a column (10 cm $\times$ 1 cm) of Dowex 50W (X12; H^+ form; 200–400 mesh) equilibrated with 1 M-HCl. The column was eluted with an exponential gradient of 1–3 M-HCl starting with 400 ml of 1 M-HCl in the mixing flask. About 180 fractions of 5 ml each were collected at a flow rate of 25–40 ml/h. 5-Methyluracil was found in fractions 4–8, 5-methylcytosine in fractions 42–52, N^2N^2 -dimethylguanine was present in fractions 68–76, N^2 -methylguanine in fractions 74–82, 1-methylguanine in fractions 82–92, 7-methylguanine in fractions 94–110 and 1-methyladenine in fractions 136–158. The methylated guanine derivatives were further separated by paper chromatography (Iwanami & Brown, 1968). The radioactivity present in the tubes corresponding to the other methylated products coincided with the authentic marker base on chromatography on paper in at least two solvents (Pegg, 1971a; Iwanami & Brown, 1968). All of the radioactivity applied to the column was present in these products, except for a small amount that was

not retained by the column. This was produced from the breakdown of *S*-adenosylmethionine during the hydrolysis, as it was also present in control tubes that had no tRNA present during incubation. Radioactivity was not found in 3-methyluracil (present in fractions 6–10), 3-methylcytosine (52–60) or 6-methyladenosine (170–190). 3-Methyluracil has been reported to be destroyed by heating in conc. HClO_4 (Iwanami & Brown, 1968), but under the conditions used to degrade the tRNA in the present experiments at least 90% of added 3-methyluracil was recovered, and the absence of this pyrimidine from the methylated bases, found to be formed *in vitro*, was not due to degradation during hydrolysis of the tRNA.

Other methods

Protein was measured by the method of Lowry *et al.* (1951) with dry crystalline bovine serum albumin as a standard. Radioactivity of samples on paper discs was determined as described by Pegg (1971a). Radioactivity of samples from the Dowex-column eluates was assayed after evaporation of the samples as described by Swann & Magee (1968). DNA was determined by the method of Burton (1968) with calf thymus DNA as a standard. 'Methyl-deficient' tRNA from *E. coli* K12W6 was prepared as

Table 1. Rates of methylation of tRNA species by colon extracts

tRNA methylase activity was measured in the presence of the cations shown, about 150 μg of protein, 500 pmol of tRNA, 100 mM-Tris-HCl, pH 8.5, and 0.067 mM-*S*-adenosyl-[$\text{Me-}^{14}\text{C}$]methionine in a total volume of 0.15 ml. Results are calculated as pmol of methyl groups incorporated into tRNA/mg of protein per 10 min of incubation at 37°C . N.D., not determined.

tRNA substrate	Colon extract	Rate of methylation in the presence of cation shown			
		5 mM-Mg $^{2+}$	25 mM-Putrescine	0.3 M-NH $_4^+$	2 mM-Spermidine
Unfractionated (<i>E. coli</i>)	Normal	11	69	65	47
	Tumour	28	284	250	206
Unfractionated (rat liver)	Normal	<1	<1	<1	<1
	Tumour	<1	<1	<1	<1
Unfractionated (mouse colon)	Normal	<1	<1	<1	<1
	Tumour	<1	<1	<1	<1
tRNA $^{\text{tMet}}$ (<i>E. coli</i>)	Normal	4	20	29	38
	Tumour	14	104	119	182
tRNA $^{\text{Glu}}$ (<i>E. coli</i>)	Normal	<1	32	48	34
	Tumour	1	116	224	182
tRNA $^{\text{Val}}$ (<i>E. coli</i>)	Normal	16	N.D.	41	47
	Tumour	35	N.D.	167	180
tRNA $^{\text{Ser}}$ (<i>E. coli</i>)	Normal	6	36	26	47
	Tumour	12	144	106	200
tRNA $^{\text{Asp}}$ (yeast)	Normal	15	8	16	10
	Tumour	41	23	49	34
tRNA $^{\text{Phe}}$ (yeast)	Normal	<1	1	3	1
	Tumour	<1	2	7	3
tRNA $^{\text{Met}}$ (yeast)	Normal	<1	1	1	1
	Tumour	<1	1	2	2
tRNA $^{\text{Asn}}$ (yeast)	Normal	<1	1	1	1
	Tumour	<1	2	2	2

described by Fleissner & Borek (1966). Rat liver and mouse colon tRNA were isolated by the method of Brunngraber (1962).

Results

As previously reported (Pegg & Hawks, 1971), the transfer of methyl groups from *S*-adenosyl-L-methionine to unfractionated 'methyl-deficient' bacterial tRNA by extracts derived from colon tumours proceeded at a rate 2–4 times faster than that catalysed by extracts from normal colon (Table 1). The dialysed soluble protein extracts from mouse colon used in these experiments as a source of tRNA methylases required the addition of a cation as well as *S*-adenosyl-methionine to catalyse the methylation of tRNA. The results given in Table 1 show that the enhanced rate of methylation by tumour extracts was seen whether the methylation of tRNA was stimulated by Mg^{2+} , NH_4^+ , putrescine or spermidine, although the latter three compounds were much more effective cofactors than $MgCl_2$. Stimulation of the methylation of tRNA by NH_4^+ or polyamines to a much greater extent than by Mg^{2+} has previously been reported for tRNA methylases of rodent liver and kidney (Rodeh *et al.*, 1967; Kaye & Leboy, 1968; Leboy, 1970, 1971; Pegg, 1971a).

The colon extracts were also able to methylate highly purified (greater than 90% homogeneous) $tRNA^{Met}$, $tRNA_2^{Glu}$, $tRNA_3^{Ser}$ and $tRNA_1^{Val}$ from *E. coli* and $tRNA^{Asp}$ from yeast (Table 1), but yeast $tRNA^{Phe}$ was a very poor substrate, and yeast $tRNA_1^{Met}$ and $tRNA_2^{Asp}$ and unfractionated tRNA from mouse colon and rat liver were not methylated under the assay conditions tested. In all cases where methylation did occur, the tissue extracts from tumours produced a greater rate of methylation than those from normal tissues, but in no case did the tumour extract methylate tRNA when the normal tissue did not.

It is clear from Table 1 that the cation requirement for a maximal rate of methylation to take place is dependent on the tRNA added as substrate but not on the source of the tissue extract. Thus $tRNA_2^{Glu}$ is inactive as a substrate for methylation by extracts from normal colon or from tumours when 5mM- $MgCl_2$ was added but was highly active when 0.3M-ammonium acetate was added. $tRNA^{Asp}$ in contrast was almost as active a substrate for methylation by the colon extracts in the presence of Mg^{2+} as when polyamines or NH_4^+ was added. The other purified tRNA species from *E. coli* were methylated in the presence of Mg^{2+} but were more active substrates when polyamines or NH_4^+ were present. These observations are consistent with the hypothesis that the colon extracts contain a number of enzymes producing different methylated bases at different sites within the tRNA molecule. These

enzymes have differing requirements for cations and tRNA substrates, so that the purified tRNA preparations are each methylated by only a limited number of the methylases present in the colon extracts.

The analysis of the methylated bases produced *in vitro* by the colon extracts acting on heterologous tRNA is also consistent with this hypothesis. As shown in Table 2, seven labelled methylated bases were found in the tRNA isolated from incubation with the normal colon extracts; the same bases in approximately the same proportions were found when tRNA methylated by extracts from tumours was analysed. The major products of methylation were 1-methyladenine, N^2 -methylguanine, 1-methylguanine and 5-methylcytosine, and the minor products of the reactions were N^2N^2 -dimethylguanine, 7-methylguanine and 5-methyluracil. $tRNA^{Met}$ was a substrate for the formation of only four of these bases, i.e. 1-methyladenine, N^2 -methylguanine, 1-methylguanine and 5-methylcytosine. $tRNA_3^{Ser}$ was also a substrate for the formation of four methylated bases, i.e. 1-methyladenine, 5-methylcytosine, N^2 -methylguanine and N^2N^2 -dimethylguanine. $tRNA_1^{Val}$ was a substrate for the formation of only three methylated bases, 1-methyladenine, 5-methylcytosine and N^2 -methylguanine. $tRNA_2^{Glu}$ was methylated to give only two products, 1-methyladenine and 5-methylcytosine (the small amount of 1-methylguanine also found was probably due to the presence of contaminating tRNA species in the $tRNA_2^{Glu}$, as the amount formed was very small and increased in proportion to the other products when the tRNA concentration was increased). $tRNA^{Asp}$ was a substrate for the formation of only one base, N^2 -methylguanine. As shown in Table 2 the colon tumours produced the same spectrum of methylated bases as the normal colon extracts with each of the tRNA substrates tested; the proportions of the bases produced were approximately the same in both cases.

The experiments described above indicate that tRNA methylases are more active in the colon tumours than in the normal colon. They do not indicate any change in specificity in the tRNA methylases of colon tumours permitting the methylation of sites in the tRNA substrates not methylated by the enzymes in normal tissue. It has been suggested that the extent of methylation of a limiting amount of tRNA by an excess of the tissue extract is indicative of the capacity of the extracts to methylate sites in the tRNA, and that any changes in the extent of methylation when extracts from tumours are compared with extracts from normal tissue provide evidence for such a change in specificity of tRNA methylases (Srinivasan & Borek, 1964, 1966; Craddock, 1970; Borek, 1971; Borek & Kerr, 1972). The extent of methylation of a limiting amount of bacterial tRNA by an excess of enzymes from mouse colon extracts was therefore investigated by studying

Table 2. Methylated bases produced by colon extracts methylating various tRNA species

The methylated bases were separated as described in the Experimental section from tRNA methylated *in vitro* by colon extracts as shown. Methylation of unfractionated tRNA was carried out in the presence of 25 mM-putrescine, tRNA^{Met}, tRNA^{Val} and tRNA^{Glu} in the presence of 2 mM-spermidine, tRNA^{Glu} in the presence of 0.3 M-NH₄⁺ and tRNA^{Asp} in the presence of 0.2 M-NH₄⁺. Results are expressed as the percentage of the total radioactivity incorporated into tRNA present in each methylated base. At least 5000 c.p.m. were present in each tRNA preparation analysed.

Methylated product	% of radioactivity in each methylated base											
	Unfractionated tRNA		tRNA ^{Met}		tRNA ^{Glu}		tRNA ^{Ser}		tRNA ^{Asp}		tRNA ^{Val}	
	Normal	Tumour	Normal	Tumour	Normal	Tumour	Normal	Tumour	Normal	Tumour	Normal	Tumour
1-Methyladenine	29	37	28	33	38	45	17	21	1	—	32	35
N ² -Methylguanine	17	13	37	30	—	—	29	18	98	99	40	39
N ² N ² -Dimethylguanine	5	5	—	—	—	—	4	3	—	—	—	—
1-Methylguanine	19	12	19	21	3	1	—	—	—	—	—	—
7-Methylguanine	2	2	—	—	—	—	—	—	—	—	—	—
5-Methylcytosine	20	22	16	16	59	55	50	58	1	1	28	26
5-Methyluracil	8	10	—	—	—	—	—	—	—	—	—	—

the incorporation of methyl groups into 20 pmol of tRNA as a function of the amount of protein present in the extract and the time of incubation. Prolonged incubation beyond 1 h at 37°C led to a decrease in the total methylated tRNA that could be detected (possibly because of the presence of nucleases in the extracts), and therefore the time of incubation was limited to 45 min. After 45 min of incubation at 37°C the maximum extent of methylation of tRNA occurred in the presence of 3–4 mg of protein and with 0.3 M-ammonium acetate (Fig. 1). When larger amounts of protein were present the rate of methylation of tRNA was inhibited (Fig. 1). This inhibition was not caused by the breakdown of the *S*-adenosylmethionine added as a methyl donor by other methyl transferases in the colon extracts, because it could not be prevented by the addition of extra *S*-adenosyl-[Me-¹⁴C]methionine either at the start of the incubation or after 45 min of incubation at 37°C. The greatest extent of methylation of the added bacterial tRNA catalysed by extracts from colon tumours amounted to 0.8–0.9 mol of methyl groups incorporated/mol of tRNA and was significantly greater than the maximal extent of methylation of the tRNA by extracts from normal colon, which amounted to only 0.4–0.5 mol of methyl groups/mol of tRNA. This greater extent of methylation of a limited amount of bacterial tRNA by the tumour extracts is similar to that reported for many other tumours (Borek, 1971; Borek & Kerr, 1972). However, because the substrate was unfractionated tRNA, containing many different molecules with different sites capable of accepting methyl groups, no information on the stoichiometry of methylation of particular sites can be obtained from such studies.

Fig. 1. Effect of protein concentration on methylation of 'methyl-deficient' tRNA from *E. coli*

'Methyl-deficient' tRNA from *E. coli* (20 pmol) was methylated in the presence of 0.3 M-ammonium acetate. The amount of protein from normal colon extracts (○) or extract from colon tumours (●) is shown; incubation was for 45 min at 37°C.

Table 3. Extent of methylation of tRNA preparations by colon extracts

The extent of methylation of tRNA in the presence of excess protein from the colon extracts was measured as described in the Experimental section. The reaction was carried out in the presence of the concentration of the cation shown and 15 pmol of all the tRNA species, except tRNA^{Asp} where 20 pmol were added.

tRNA	Cation and concn.	Methylation of tRNA (mol of methyl groups incorporated/mol of tRNA)	
		Normal colon	Tumour of colon
Unfractionated	25 mM-Putrescine	0.5	0.9
fMet	1.5 mM-Spermidine	0.8	1.3
Glu ₂	0.3 M-NH ₄ ⁺	0.5	1.1
Ser ₃	1.5 mM-Spermidine	0.8	1.4
Asp	2 mM-Mg ²⁺	0.3	0.6

Table 3 shows similar results for the extent of methylation of purified tRNA preparations by colon extracts under the conditions described above. In all cases the extent of methylation produced by the tumour extracts was greater than that found when extracts from normal colon were used, but the extent of methylation was in all cases much less than that expected for a stoichiometric formation of one molecule of each of the methylated bases known to be produced in the methylation reaction (from Table 2) per molecule of added tRNA. Thus, tRNA^{Asp} that was methylated with the formation of only one methylated base, N²-methylguanine, was alkylated to only 30% of the expected value by normal colon extracts and to 60% of the expected value by the tumour extracts. Similarly, as tRNA^{Glu} was methylated with the formation of two bases, 5-methylcytosine and 1-methyladenine, the theoretical maximal incorporation of methyl groups would be 2 mol/mol and the observed values amount to only 25% and 55% of this respectively. The possibility that the production of a methylated product at one site within the molecule prevents methylation from occurring at other sites cannot be ruled out for the other bacterial tRNA species tested. However, as values greater than 1 mol of methyl groups/mol of tRNA were achieved this possibility appears unlikely, and again methylation by extracts from normal colon amounted to only 20–30% of the expected value and to 30–60% when tumour extracts were used as a source of enzyme. It is clear therefore from these experiments that the extent of methylation of a limiting amount of tRNA by these crude tissue extracts cannot be regarded as a true indication of the capacity of the tissue to recognize sites for methylation within the tRNA added as substrate.

Table 4. Activity of RNA polymerase, ATP-tRNA adenylyltransferase and aminoacyl-tRNA ligases in colon extracts

Assays were carried out as described in the Experimental section. RNA polymerase activity is given as c.p.m. incorporated/0.1 mg of DNA per 10 min of incubation at 37°C. ATP-tRNA adenylyltransferase activity is expressed as pmol of ATP incorporated/mg of protein per 10 min of incubation at 37°C. Activity of aminoacyl-tRNA ligases is given as pmol of the amino acid incorporated/mg of protein per 15 min incubation at 37°C. Results are shown ± s.d. for the number of estimations shown.

Activity assayed	Activity measured with the tissue extract shown	
	Normal colon	Colon tumour
RNA polymerase in presence of Mg ²⁺ /α-amanitin	1250 ± 219 (5)	3792 ± 614 (5)
RNA polymerase in presence of Mn ²⁺ /(NH ₄) ₂ SO ₄	2652 ± 413 (5)	9486 ± 1083 (5)
ATP-tRNA adenylyltransferase	54.4 ± 15.2 (5)	158.6 ± 43.5 (8)
L-Methionine-tRNA ligase	26.3 ± 5.2 (5)	48.8 ± 14.2 (5)
L-Leucine-tRNA ligase	13.6 ± 3.1 (5)	31.8 ± 11.4 (5)
L-Lysine-tRNA ligase	19.4 ± 4.3 (5)	36.5 ± 8.1 (5)

The possibility that the increased tRNA methylase activity found in the colon tumours was accompanied by a general increase in the activity of other enzymes concerned in RNA metabolism was investigated by measuring the activity of RNA polymerases, ATP-tRNA adenylyltransferase and aminoacyl-tRNA ligases. Table 4 shows the activities of nuclear RNA polymerases of nuclei isolated from normal and cancerous colon. Both the nuclear RNA polymerase sensitive to α-amanitin and stimulated by (NH₄)₂SO₄ and the nucleolar RNA polymerase, which is insensitive to α-amanitin (Roeder & Rutter, 1969; Chambon *et al.*, 1970; Jacob *et al.*, 1970), were 3–4-fold more active in nuclei from colon tumours.

Table 4 shows that the activity of the soluble enzyme responsible for the addition of the terminal adenosine moiety of tRNA species, ATP-tRNA adenylyltransferase, was also considerably more active in supernatant extracts prepared from the colon tumours than in supernatant extracts prepared from normal colon. The activities of L-methionine-tRNA ligase, L-lysine-tRNA ligase and L-leucine-tRNA ligase in the colon extracts were also measured (Table 4). Again the activities of these enzymes were all increased severalfold when supernatant fractions from colon tumours were compared with fractions from normal colon. (The substrate used as an acceptor of amino acids in these experiments was yeast tRNA.

This may not be recognized correctly by all the aminoacyl-tRNA synthetases in colon. However, the activities were measured under conditions where the amount of amino acid attached to the tRNA was proportional to the amount of protein added and independent of the amount of tRNA present, so satisfactory comparisons can be made between the two extracts, although the absolute activities may not be the same as those that would have been obtained had homologous tRNA been used.)

Discussion

Experiments in which tRNA methylase activity of tumour extracts have been compared with that of normal tissues (Craddock, 1970; Borek, 1971; Borek & Kerr, 1972) can be criticized on several grounds. (a) The tumour may have been derived from a single cell type within the control tissue and extracts prepared from the entire control tissue may not have a tRNA methylase activity typical of these particular cells. (b) Methylation *in vitro* may be influenced by the concentration of compounds such as polyamines, Mg^{2+} or *S*-adenosylhomocysteine in the tissues being compared and apparent changes in tRNA methylase activity may be due to differences in the amounts of these compounds in the two tissues. (c) The experiments have been carried out in an artificial system in which tRNA from one species (usually bacterial) has been methylated by a crude protein fraction containing a number of methylases from another species. In many cases the assays have been carried out under conditions far removed from those giving an optimal rate of methylation *in vitro* (Kaye & Leboy, 1968; Rodeh *et al.*, 1967; Baguley & Staehelin, 1968a; Pegg, 1971a).

The results given in the present paper show clearly that colon tumours are more active in catalysing the methylation of tRNA than is normal mouse colon and the first two criticisms outlined above can be excluded from consideration. The results were obtained with extracts that had been dialysed to free them from Mg^{2+} , polyamines, *S*-adenosylhomocysteine, nicotinamide and glycine, which could affect the methylation (Leboy, 1970, 1971; Pegg, 1971a,b, 1972a; Kerr, 1972; Murai *et al.*, 1972; Gross & Wildenhauer, 1972; Kuchino & Endo, 1972). Changes in the amounts of these compounds, which are known to be present in altered amounts in some tumours (Williams-Ashman *et al.*, 1972; Tabor & Tabor, 1972), could not account for the differences in the rate of methylation that were observed. Also the colon tumours induced by 1,2-dimethylhydrazine are derived from the mucosal cells that form about 70% of the normal colon extracts, and therefore the increased activity of the tumours cannot be solely due to a change in the relative proportions of cells in the tissue (Pegg & Hawks, 1971; Hawks, 1972).

The third criticism cannot be excluded from the present results, as satisfactory methods for the quantitative separation of individual tRNA methylases from mammalian tissues have not yet been devised and it is not possible to obtain methyl-deficient tRNA from mammalian tissues to use as a substrate for methylation *in vitro*. [tRNA isolated from the livers of rats treated with ethionine may prove to be of value in this respect (Pegg, 1972b; Rajalakshmi, 1973).] It was therefore necessary to use bacterial and yeast tRNA species as substrates for methylation. The use of purified tRNA preparations has considerable advantages in these assays because these substrates resulted in a less complex pattern of methylation. The cations required for specific methylations to take place could be studied, the stoichiometry of methylation could be measured and the sites of methylation within the nucleotide sequence of the tRNA could be determined (Pegg, 1974).

Under all the conditions investigated there were no marked differences in the relative proportions of the methylated bases produced when extracts from normal colon were compared with extracts from colon tumours. No indications that the tumours contained methylases capable of attacking sites in tRNA that are not attacked by enzymes from normal colon were obtained. It is clear from the present results that the extent of methylation of a limiting amount of tRNA by an excess of the crude tissue extract could not be correlated with the ability of enzymes in the extract to recognize sites for methylation within the tRNA substrate. The reason why the preparations from colon tumours apparently catalyse a greater extent of methylation under these conditions than do preparations from normal colon is unclear. However, artifacts of no physiological importance might affect the extent of methylation of small amounts of tRNA by an excess of a crude tissue extract. For example, nucleases might degrade the tRNA, added as substrate, sufficiently to prevent methylation; other RNA present in the extracts, although not in itself a substrate for methylation, might bind to the methylases and prevent reaction; other proteins present in the extracts might bind to the tRNA added and prevent access of the methylases. Any of these factors combined with the enhanced activity of the methylases present in the tumour extracts might produce the observed increase in the extent of methylation without any change in the specificity of the methylases. This conclusion is supported by studies which showed that a greater extent of methylation of added tRNA was produced by purification of tRNA methylases from liver and spleen (Baguley & Staehelin, 1968a,b).

Several recent reviews of the known sequences of tRNA species have noted that methylated nucleosides occur at only a very limited number of sites within

the tRNA structure (Zachau, 1969; Staehelin, 1971; Dirheimer *et al.*, 1972). All of the tRNA preparations that were methylated by colon extracts contain an unmodified nucleoside that can be converted into the methylated derivatives which were produced *in vitro* at these particular sites within the tRNA structure. It is shown in the accompanying paper that methylation does indeed take place at these sites (Pegg, 1974). Conversely, tRNA₁^{Met} and tRNA₂^{Arg} (Simsek & Rajbhandary, 1972; Weissenbach *et al.*, 1972) do not have suitable residues for methylation at these positions and these tRNA species were not substrates for methylation by enzymes from normal colon or colon tumours.

These observations suggest that methylation *in vitro* of tRNA of micro-organisms by heterologous enzymes from mammalian cells may provide meaningful studies of the activities of these enzymes *in vivo* even though the substrate is unnatural. They do not provide any evidence to support the hypothesis that methylation of tRNA might play a regulatory role in cell growth because the activities of the different methylases were all increased to approximately the same extent in the colon tumours. It might be argued that further studies with a larger number of tRNA substrates are needed to provide evidence of such changes. However, the increase in tRNA methylase activity was of the same order as the increased activity of other enzymes involved in RNA metabolism and protein synthesis. Therefore it appears more likely that the enhanced activity of tRNA methylases in these tumours is simply part of a general increase commensurate with the greater growth rate.

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References

- Baguley, B. C. & Staehelin, M. (1968a) *Biochemistry* **7**, 45–50
- Baguley, B. C. & Staehelin, M. (1968b) *Eur. J. Biochem.* **6**, 1–7
- Borek, E. (1971) *Cancer Res.* **31**, 596–597
- Borek, E. & Kerr, S. (1972) *Advan. Cancer Res.* **15**, 163–190
- Brunngraber, E. F. (1962) *Biochem. Biophys. Res. Commun.* **8**, 1–6
- Burton, K. (1968) *Methods Enzymol.* **12B**, 163–166
- Chambon, P., Gissinger, F., Mandel, J. L., Keding, C., Gnizdorski, M. & Mihalac, M. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 693–707
- Craddock, V. M. (1970) *Nature (London)* **228**, 1264–1268
- Dirheimer, G., Ebel, J. P., Bonnet, J., Gangloff, J., Keith, G., Krebs, B., Kuntzel, B., Roy, A., Weissenbach, J. & Werner, C. (1972) *Biochimie* **54**, 127–144
- Druckrey, H., Preussmann, R., Matzkies, F. & Ivankovic, S. (1967) *Naturwissenschaften* **54**, 285–286
- Fleissner, E. & Borek, E. (1966) *Procedures Nucl. Acid Res.* **1**, 461–465
- Gillam, I., Blew, D., Warrington, R. C., Tigerstrom, M. & Tener, G. M. (1968) *Biochemistry* **10**, 3459–3468
- Gross, H. J. & Wildenhauer, D. (1972) *Biochem. Biophys. Res. Commun.* **48**, 58–64
- Hancher, C. W., Phares, E. F., Novelli, G. D. & Kelmers, A. D. (1969) *Biotechnol. Bioeng.* **11**, 1055–1070
- Hawks, A. M. (1972) Ph.D. Thesis, University of London
- Hawks, A. M., Swann, P. F. & Magee, P. W. (1972) *Biochem. Pharmacol.* **21**, 432–435
- Iwanami, Y. & Brown, G. M. (1968) *Arch. Biochem. Biophys.* **124**, 472–482
- Jacob, S. T., Sajdel, F. M., Muecke, W. & Munro, H. N. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 681–691
- Kaye, A. M. & Leboy, P. S. (1968) *Biochim. Biophys. Acta* **157**, 289–302
- Kerr, S. J. (1972) *J. Biol. Chem.* **247**, 4248–4252
- Kerr, S. J. & Borek, E. (1972) *Advan. Enzymol. Relat. Areas Mol. Biol.* **36**, 1–27
- Klagsbrun, M. (1972) *J. Biol. Chem.* **247**, 7443–7451
- Kuchino, Y. & Endo, H. (1972) *J. Biochem. (Tokyo)* **71**, 719–723
- Kuchino, Y. & Nishimura, S. (1970) *Biochem. Biophys. Res. Commun.* **40**, 306–313
- Kuchino, Y., Seno, T. & Nishimura, S. (1971) *Biochem. Biophys. Res. Commun.* **43**, 476–483
- Kuchino, Y., Endo, H. & Nishimura, S. (1972) *Cancer Res.* **32**, 1243–1250
- Leboy, P. S. (1970) *Biochemistry* **7**, 1577–1584
- Leboy, P. S. (1971) *FEBS Lett.* **16**, 117–120
- Leboy, P. S. & Piester, P. (1973) in *Polyamines in Normal and Neoplastic Growth* (Russell, D. H., ed.), pp. 103–110, Raven Press, New York
- Liau, M. C., O'Rourke, C. M. & Hurlbert, R. B. (1972) *Biochemistry* **11**, 629–636
- Littauer, U. Z. & Daniel, V. (1966) *Procedures Nucl. Acid Res.* **1**, 353–361
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Makman, M. H. (1966) *Procedures Nucl. Acid Res.* **1**, 424–436
- Murai, J. T., Jenkinson, P., Halpern, R. M. & Smith, R. A. (1972) *Biochem. Biophys. Res. Commun.* **46**, 999–1003
- Nishimura, S. (1971) *Procedures Nucl. Acid Res.* **2**, 542–564
- Pegg, A. E. (1971a) *Biochim. Biophys. Acta* **232**, 630–642
- Pegg, A. E. (1971b) *FEBS Lett.* **16**, 13–17
- Pegg, A. E. (1972a) *Biochim. Biophys. Acta* **262**, 283–289
- Pegg, A. E. (1972b) *Biochem. J.* **128**, 59–68
- Pegg, A. E. (1974) *Biochem. J.* **137**, 239–248
- Pegg, A. E. & Hawks, A. M. (1971) *Biochem. J.* **122**, 121–123
- Rajalakshmi, S. (1973) *Proc. Amer. Ass. Cancer Res.* **14**, 153
- Randerath, K., Mackinnon, S. K. & Randerath, E. (1971) *FEBS Lett.* **15**, 81–84
- Rodeh, R., Feldman, M. & Littauer, U. Z. (1967) *Biochemistry* **6**, 451–460

- Roeder, R. G. & Rutter, W. J. (1969) *Nature (London)* **224**, 234–236
- Simsek, M. & Rajbhandary, U. L. (1972) *Biochem. Biophys. Res. Commun.* **49**, 508–515
- Smuckler, E. A. & Hadjiolov, A. A. (1972) *Biochem. J.* **129**, 153–166
- Srinivasan, P. R. & Borek, E. (1964) *Science* **145**, 548–553
- Srinivasan, P. R. & Borek, E. (1966) *Progr. Nucl. Acid Res. Mol. Biol.* **5**, 157–189
- Staehelin, M. (1971) *Experientia* **27**, 1–11
- Starr, J. L. & Sells, B. H. (1969) *Physiol. Rev.* **49**, 623–669
- Swann, P. F. & Magee, P. N. (1968) *Biochem. J.* **110**, 39–47
- Tabor, H. & Tabor, C. W. (1972) *Advan. Enzymol. Relat. Areas Mol. Biol.* **36**, 203–268
- Weeren, H. O., Ryon, A. O., Heatherly, D. E. & Kelmers, A. D. (1970) *Biotechnol. Bioeng.* **12**, 889–912
- Weissenbach, J., Martin, R. & Dirheimer, G. (1972) *FEBS Lett.* **28**, 353–355
- Wiebecke, B., Lohrs, U., Gimmy, J. & Eder, M. (1969) *Z. Gesamte Exp. Med.* **149**, 277–278
- Williams-Ashman, H. G., Coppoc, G. L. & Weber, G. (1972) *Cancer Res.* **32**, 1924–1932
- Wyatt, G. R. (1952) *J. Gen. Physiol.* **36**, 201–204
- Zachau, H. G. (1969) *Angew. Chem. Int. Ed. Engl.* **8**, 711–727