Studies of the Ethylation of Rat Liver Transfer Ribonucleic Acid after Administration of L-Ethionine

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1. The ethylated nucleosides present in tRNA isolated from the livers of rats treated with 0.5 g of L-ethionine/kg body wt. were investigated. Evidence that this tRNA contained *N*²-ethylguanine, *N*²*N*²-diethylguanine, *N*²-ethyl-*N*²-methylguanine, 7-ethylguanine, two ethylated pyrimidines and ethylated ribose groups was obtained. 2. Ethylation of bacterial tRNA was catalysed by extracts containing tRNA methylases prepared from rat liver by using *S*-adenosyl-L-ethionine as an ethyl donor, but the rate of ethylation was 20 times less than the rate of methylation with *S*-adenosyl-L-methionine as a methyl donor. 3. The principal product of such ethylation *in vitro* was *N*²-ethylguanine and traces of the other ethylated guanines and pyrimidines found in tRNA isolated from rats treated with ethionine *in vivo* were also found. 1-Ethyladenine was not formed, although 1-methyladenine is a major product of methylation of bacterial tRNA by these extracts, and 1-ethyladenine was not present in the rat liver tRNA isolated from ethionine-treated animals. 4. After injection of actinomycin D (15 mg/kg body wt.) or L-methionine (1.0 g/kg body wt.) before the ethionine, ethylation of tRNA was diminished by about 80% but not completely abolished. Administration of 1-aminocyclopentanecarboxylic acid (2.5 g/kg body wt.) to inhibit the formation of *S*-adenosyl-L-ethionine inhibited ethylation of tRNA by 44%. 5. These results suggest that not all of the ethylation of tRNA that occurs in the livers of rats treated with ethionine is mediated by the action of tRNA methylases acting with *S*-adenosyl-L-ethionine as a substrate, but that this pathway does occur and accounts for a major part of the observed ethylation. 6. The results are discussed with reference to ethionine-induced hepatocarcinogenesis.

Administration of L-ethionine to rats leads to the ethylation of proteins and nucleic acids in the liver (Farber & Magee, 1960; Stekol *et al.*, 1960; Natori, 1963; Stekol, 1965; Farber *et al.*, 1967*a,b*; Rosen, 1968; Ortwerth & Novelli, 1969; Friedman *et al.*, 1969; Swann *et al.*, 1971). Such ethylation of one or all of these macromolecules may be relevant to the induction of hepatomas by prolonged feeding of ethionine (Farber, 1963), as many carcinogens have been found to interact with proteins and nucleic acids within the tissues in which they produce tumours (Magee & Barnes, 1967; Miller, 1970).

Ethionine is readily converted into *S*-adenosyl-L-ethionine in the liver (Farber, 1963, 1967; Stekol, 1965; Smith & Salmon, 1965; Shull *et al.*, 1966). The ethylation of liver nucleic acids after ethionine administration could therefore be produced by *S*-adenosyl-L-ethionine substituting for *S*-adenosyl-L-methionine in the reactions catalysed by nucleic acid methylases (Borek & Srinivasan, 1966; Starr & Sells, 1969). A consequence of this mechanism would be that ethyl substituents should be found only at those sites of nucleic acids that are normally methylated. As tRNA is the most extensively methylated species of RNA

(Starr & Sells, 1969), the observation that tRNA was the most extensively ethylated nucleic acid species after ethionine administration (Natori, 1963; Stekol, 1965; Farber *et al.*, 1967*b*; Ortwerth & Novelli, 1969) is consistent with this hypothesis. However, direct experimental evidence on this question is conflicting. Ethylation of tRNA *in vitro* catalysed by cell-free extracts with *S*-adenosyl-L-ethionine as an ethyl donor has been reported to occur in some experiments (Hancock, 1968), but not to any significant extent in others (Borek & Srinivasan, 1965; Peterkofsky, 1965; Rodeh *et al.*, 1967; Ortwerth & Novelli, 1969). This question is of interest in studies of the mechanism of carcinogenesis, as other carcinogens such as diethylnitrosamine and *N*-ethyl-*N*-nitrosourea (Magee & Barnes, 1967; Druckrey *et al.*, 1967) also produce ethylation of nucleic acids and proteins (Swann & Magee, 1968, 1970, 1971), but these compounds would not be expected to give rise to *S*-adenosyl-L-ethionine within the cell, and diethylnitrosamine did not produce the preferential ethylation of tRNA obtained with ethionine (Swann & Magee, 1970, 1971).

The present paper describes further studies of the

ethylation of rat liver RNA after ethionine administration *in vivo*, and the transfer of ethyl groups from *S*-adenosyl-L-ethionine to tRNA *in vitro* in the presence of an extract prepared from rat liver. The products of such ethylations have been separated and tentatively identified. The results are discussed in relation to the mechanism of ethylation of nucleic acids in the livers of rats treated with ethionine.

Experimental

Materials

L-[Me-¹⁴C]Methionine (50mCi/mmol), L-[Et-1-¹⁴C]ethionine (54mCi/mmol) and *S*-adenosyl-L-[Me-¹⁴C]methionine (54mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. L-[Et-1-³H]Ethionine (1.63mCi/mmol) and L-[Et-1-¹⁴C]ethionine (2.4mCi/mmol) were obtained from the New England Nuclear Corp., Boston, Mass., U.S.A. Labelled *S*-adenosyl-L-ethionine and *S*-adenosyl-L-methionine were prepared from the radioactive amino acids, purified and stored as previously described (Pegg & Williams-Ashman, 1969, 1970). The commercially obtained *S*-adenosyl-L-[Me-¹⁴C]methionine was also subjected to this purification procedure. L-Methionine, L-ethionine, ATP, 2-thioxanthine, adenosine, guanosine, cytidine, uridine and actinomycin D were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Ethylamine, diethylamine and tRNA isolated from *Escherichia coli* K12 were purchased from British Drug Houses Ltd., Poole, Dorset, U.K. *N*-Methylethylamine was obtained from Eastman-Kodak Ltd., Kirkby, Liverpool, U.K., and 1-aminocyclopentane-carboxylic acid from Ralph N. Emanuel Ltd., Wembley, Middx., U.K.

Preparation and assay of rat liver extracts containing tRNA methylases

Male Wistar albino rats (weighing 140–160g) bred in this Institute were killed by a blow on the head and the livers were rapidly removed, weighed and placed in ice-cold 0.25 M-sucrose–0.1 mM-EDTA–1 mM-2-mercaptoethanol–10 mM-tris–HCl solution, pH 7.5. All subsequent operations were performed at 0–4°C. The livers were homogenized in 3 vol. of the medium described above by using ten strokes of a rotating Teflon pestle in a glass homogenizer. The homogenate was centrifuged at 10000g for 10 min in an MSE High-Speed 18 centrifuge. The supernatant fraction was removed and centrifuged at 120000g for 2 h in a Spinco 50Ti rotor. The supernatant fraction from this centrifugation was treated with (NH₄)₂SO₄ as follows. To every 25 ml of ultracentrifuged supernatant fraction, 8.5 ml of a saturated solution of (NH₄)₂SO₄ (adjusted to pH 7.0 with

1 M-NH₃ and maintained at 2°C) was added. The mixture was stirred for 30 min and the precipitate that formed was removed by centrifugation at 10000g for 10 min. Additional saturated (NH₄)₂SO₄ solution (13 ml for every 25 ml of the original ultracentrifuged supernatant fraction) was then added to the supernatant, and after stirring for 30 min the precipitate that formed was collected by centrifugation at 10000g for 10 min. This precipitate was dissolved in 10 mM-tris–HCl–1 mM-2-mercaptoethanol–0.5 mM-EDTA solution, pH 7.8, to give a protein concentration of about 20 mg/ml, and this solution was dialysed against 4 litres of the same medium for 18 h. After dialysis the solution was stored at –20°C until required. The tRNA methylase activity was slowly lost at a rate of about 10% per week with storage under these conditions.

The assay of tRNA methylase activity was carried out as previously described (Pegg, 1971a). Bacterial tRNA that had been ethylated *in vitro* by the action of liver enzymes was re-isolated from the medium as described for methylated tRNA (Pegg, 1971a) and was then analysed as described below.

Preparation and analysis of ethylated rat liver tRNA

A group of five male rats (weighing about 200g) were each given 5 ml of water containing 100 mg of L-[Et-1-³H]ethionine (1.63 mCi/mmol) by intraperitoneal injection. The rats were then starved for 18 h before death. The livers were rapidly removed and RNA was isolated by the procedure of Swann & Magee (1968). The RNA was dissolved in water, an equal volume of 1.0 M-tris–HCl buffer, pH 8.5, was added and the solution incubated at 25°C for 30 min to deacylate any bound L-[Et-1-³H]ethionine. The pH was then adjusted to 5.0 by the addition of 1.0 M-acetic acid; 3 vol. of ethanol was then added and the solution left overnight at –20°C. The RNA was collected by centrifugation at 5000g for 15 min and dissolved in 5 mM-MgCl₂–10 mM-tris–HCl buffer, pH 7.5. This solution was applied to a column (25 cm × 2 cm diam.) of DEAE-cellulose that had previously been equilibrated with the 5 mM-MgCl₂–10 mM-tris–HCl buffer, pH 7.5. The column was washed with 250 ml of 0.3 M-NaCl–5 mM-MgCl₂–10 mM-tris–HCl solution, pH 7.5, at a flow rate of 40 ml/h, and the eluate discarded. The tRNA was then eluted with 100 ml of 0.8 M-NaCl–5 mM-MgCl₂–10 mM-tris–HCl solution, pH 7.5. This RNA was precipitated by the addition of 20 ml of 20% (w/v) sodium acetate (adjusted to pH 5 with acetic acid), followed by 3 vol. of ethanol. After storage overnight at –20°C, the RNA was collected by centrifugation at 5000g for 15 min, washed with ethanol and then with ether, dried in air and stored at –20°C.

Analysis of the ethylated components present in rat

liver tRNA after the administration of L-[Et-1-³H]-ethionine *in vivo*, and in bacterial tRNA after incubation with S-adenosyl-L-[Et-¹⁴C]ethionine and rat liver enzymes *in vitro*, was carried out after hydrolysis of the tRNA either by heating at 100°C for 30 min in 1.0M-HCl to yield free purine and pyrimidine nucleotides or by heating at 100°C for 90 min in 11.6M-HClO₄, which produces free purine and pyrimidine bases with subsequent degradation of the ribose moieties. The hydrolysates were then analysed by chromatography on paper and on columns of Dowex 50 (H⁺ form) as described below.

Authentic ethylated bases for use as marker substances for this chromatography were synthesized. N²-Substituted guanines were prepared by the method of Elion *et al.* (1956). 7-Ethylguanine, 1-ethylguanine, 1-ethyladenine, 3-ethylcytosine and 3-ethyluracil were prepared by appropriate modification of published methods for preparation of the methylated bases (Jones & Robins, 1963; Broom *et al.*, 1963; Brookes & Lawley, 1962). 3-Ethyladenine and N⁶-ethyladenine were generous gifts from Dr. L. Rosen, Oak Ridge National Laboratory, Oak Ridge, Tenn., U.S.A.

Procedures for chromatography on paper and on columns of Dowex 50 were as described by Swann *et al.* (1971). The solvents used for paper chromatography were (1) butan-1-ol–conc. NH₃ (sp. gr. 0.88)–water (86:5:9, by vol.), (2) propan-2-ol–conc. HCl–water (65:18:17, by vol.), (3) butan-1-ol–water (17:3, v/v) and (4) methanol–conc. HCl–water (7:2:1, by vol.). Chromatograms in solvents 1 and 2 (Rosen, 1968) were run for 18 or 34 h according to the fractions being separated, and where appropriate the solvent was allowed to drip from the end of the paper. Distances moved by the ethylated purines relative to that moved by adenine, in solvents (1) and (2) respectively, were similar to those found by Rosen (1968), as follows: 7-ethylguanine (1.1, 1.3), N²-ethylguanine (1.6, 1.7), N²N²-diethylguanine (2.3, 2.1), 1-ethylguanine (1.3, 1.2), N²-ethyl-N²-methyl guanine (1.6, 1.6), 1-ethyladenine (1.7, 1.3), 3-ethyladenine (1.0, 1.7) and 6-ethyladenine (2.8, 1.1).

Determination of ethylation of RNA and concentrations of S-adenosyl-L-methionine, S-adenosyl-L-ethionine and S-adenosyl-L-homocysteine

Male rats (120 g) were injected intraperitoneally with actinomycin D (0.5 mg/ml in 0.15M-NaCl), L-methionine (30 mg/ml in water) or 1-aminocyclopentanecarboxylic acid (75 mg/ml in water). Control animals were injected with a similar volume of 0.15M-NaCl. Then 15 min later 0.3 ml of a solution of 21 mM-L-[Et-1-¹⁴C]ethionine (2.4 mCi/mmol) in 0.15M-NaCl was administered by intraperitoneal injection. The animals were killed 165 min after the second injection and the livers removed. Approx.

one-third of the liver was homogenized in 15 ml of ice-cold 0.5M-HClO₄, the homogenate centrifuged at 15000g for 30 min at 2°C and the supernatant from this centrifugation used for analysis as described below. The remaining two-thirds of the liver was used for the preparation of tRNA as described above. The dried RNA was dissolved in 1 ml of 0.2M-NaOH. A portion (0.5 ml) of this solution was used for the determination of radioactivity. Another portion of the solution was diluted to an appropriate volume with 0.2M-NaOH and the E₂₆₀ was measured. The ethylation of RNA after the administration of L-[Et-1-¹⁴C]ethionine was expressed as c.p.m. incorporated into RNA/mg of RNA isolated, assuming that 1 mg of RNA dissolved in 1 ml of 0.2M-NaOH has an E₂₆₀ of 24 in a 1 cm light-path cell.

One-half of the supernatant fraction obtained from centrifugation of the liver homogenate in 0.5M-HClO₄ was applied to a column (10 cm × 1 cm diam.) of Dowex 50 (X2; H⁺ form; 100–200 mesh), which had previously been equilibrated with 1M-HCl. The column was then washed with 200 ml of 1M-HCl at a flow rate of 150 ml/h. S-Adenosyl-L-methionine, S-adenosyl-L-ethionine and S-adenosyl-L-homocysteine remained bound to the resin and were then eluted with 50 ml of 4M-HCl. The E₂₅₆ of this solution was measured and the total content of the three nucleosides calculated by assuming that the molar extinction coefficient for each was 15000 litre·mol⁻¹·cm⁻¹. This approximation is correct within 10% (Schlenk & DePalma, 1957; Stekol, 1965; Salvatore *et al.*, 1968). The S-adenosyl-L-ethionine content of the solution was determined by measurement of the radioactivity present in the solution. The other half of the supernatant fraction was treated with alkali to degrade the sulphonium compounds, and the S-adenosyl-L-homocysteine present was isolated by chromatography on Dowex 50 as described by Salvatore *et al.* (1968). The S-adenosyl-L-methionine content of the liver extract was then calculated by subtraction of the S-adenosyl-L-ethionine and S-adenosyl-L-homocysteine values from the total. This procedure gave values for the S-adenosyl-L-methionine content of normal rat liver of 0.05 μmol/g wet wt. and for S-adenosyl-L-homocysteine of 0.07 μmol/g wet wt., which are in reasonable agreement with previously published results (Baldessarini & Kopin, 1966; Salvatore *et al.*, 1968), obtained by methods that are considerably more precise but less convenient, for assays in the presence of S-adenosyl-L-ethionine.

Determination of radioactivity

The preparation of samples for radioactivity measurements, assay of radioactivity and corrections for quenching were as previously described (Pegg, 1971a; Swann *et al.*, 1971). Samples dissolved in

0.2M-NaOH were counted for radioactivity after 0.5ml of the solution had been added to 15ml of Triton X-100-0.6% (w/v) 2,5-diphenyloxazole in toluene (1:5, by vol.).

Results

Administration of 500mg of L-[Et-1-³H]ethionine (1.63mCi/mmol)/kg body wt. to male rats 18h before death led to considerable incorporation of radioactivity into RNA. The total RNA isolated from the livers of these rats had a specific radioactivity of 1277d.p.m./mg, and the specific radioactivity of tRNA, prepared from this RNA by fractionation on columns of DEAE-cellulose, was 10545d.p.m./mg. It is therefore apparent that tRNA was much more extensively ethylated than rRNA, which forms most of the total extracted RNA. This finding is in agreement with previous reports in which much lower doses of ethionine were used (Natori, 1963; Stekol, 1965; Farber *et al.*, 1967b; Ortwerth & Novelli, 1969). The ethylation detected in the present experiments corresponds to about 1 mol of ethyl groups/120mol of tRNA, so that even with the very large dose of ethionine administered only a small number of tRNA molecules within the liver were ethylated.

After hydrolysis of a sample of the ethylated tRNA by heating at 100°C in 1M-HCl for 30min, and chromatography of the products on Dowex 50 (H⁺ form) with a gradient of 1-3M-HCl, four major fractions containing radioactivity were obtained (fractions I-IV, Fig. 1). Fraction I (35% of the total radioactivity) was eluted from the column at the beginning of the gradient in the region occupied by pyrimidine nucleotides. Fractions II, III and IV, which represented 14, 30 and 14% of the total radioactivity respectively, were eluted from the column after guanine but before adenine. Most of the radioactivity in fraction IV was found to correspond to 7-ethylguanine both by the position of elution from the Dowex column and by paper chromatography (solvents 1 and 2). Similarly most of the radioactivity in fraction 3 was found to correspond to N²-ethylguanine (solvents 1-3). 3-Ethyladenine and 1-ethylguanine were also eluted from the Dowex column in positions overlapping fraction III, but none of the radioactivity present in this fraction corresponded to the former on paper chromatography and only a very small peak of radioactivity corresponding to 1-ethylguanine in solvent 1 was found. Fraction II was resolved into two portions on paper chromatography in solvent 2. The faster-moving component corresponded to N²N²-diethylguanine and the slower-moving component corresponded to N²-ethyl-N²-methylguanine in solvents 1 and 2. Fraction I was evaporated to dryness and heated at 100°C for 60min in 11.6M-HClO₄ to produce free pyrimidine bases, which were then separated by chromatography on

Dowex 50 (H⁺ form) and on paper (solvents 2 and 3). Two radioactive components were separated, one corresponding to a cytosine derivative and one to a uracil derivative. These compounds were not positively identified owing to the absence of known standards, but they did not correspond to 3-ethyluracil and 3-ethylcytosine, and by comparison with the properties of methyl derivatives the ethylated pyrimidines could be 5-ethylcytosine and 5-ethyluracil. A substantial portion of the radioactivity from fraction I (23% of the total labelling of the tRNA) was lost after the treatment with HClO₄. As the fractions from the Dowex column were evaporated to dryness in a stream of warm air before their radioactivity was counted, and the samples applied to chromatography paper were also dried in air before and after chromatography, it is likely that this radioactive material was converted into a volatile product by hydrolysis in 11.6M-HClO₄. This procedure is known to hydrolyse 2'-O-alkyl groups from nucleosides (Baskin & Dekker, 1967) and it is therefore probable that the radioactivity made volatile by HClO₄ treatment comes from ethylation of the ribose moieties of the tRNA. [It has been reported that 3-methyluracil is destroyed by heating with 60% HClO₄ (Iwanami & Brown, 1968). However, at least 80% of the 3-ethyluracil could be recovered unchanged after treatment with HClO₄ under the conditions used to hydrolyse the pyrimidine nucleotide fraction in the present experiments, and therefore the degradation of this

Fig. 1. Ion-exchange chromatography of hydrolysed tRNA isolated from livers of rats treated with L-[Et-1-³H]ethionine

The dose of ethionine (sp. radioactivity 1.63mCi/mmol) was 500mg/kg body wt. About 1.5mg of tRNA was heated for 60min in 1 ml of 1M-HCl before chromatography on Dowex 50 (X12; H⁺ form) with an exponential 1-3M-HCl gradient. ▲, E₂₆₀^{1cm} ●, radioactivity. Py, Pyrimidine nucleotides; G, guanine; A, adenine; radioactive peaks are numbered I to IV as described in the text.

Table 1. *Ethylated bases present in rat liver tRNA after ethionine administration in vivo and in bacterial tRNA after incubation with S-adenosyl-L-ethionine and rat liver extracts in vitro*

About 1.5mg of tRNA (5040c.p.m.) isolated from rats treated with 500mg of L-[Et-1-³H]ethionine (1.63mCi/mmol)/kg body wt. was analysed as described in the text (Expt. A). Results of Expt. B show similar analysis of 1 mg of tRNA (2500c.p.m.) re-isolated after incubation with rat liver extracts as described in Table 2. N.D., not detected.

Product	Expt. A (tRNA ethylated <i>in vivo</i>)		Expt. B (tRNA ethylated <i>in vitro</i>)	
	Radioactivity present (c.p.m.)	% of total	Radioactivity present (c.p.m.)	% of total
Ethyluracil	264	5	138	6
Ethylcytosine	295	6	120	5
N ² -Ethylguanine	1249	25	1692	68
N ² N ² -Diethylguanine	355	7	103	4
7-Ethylguanine	679	13	120	5
N ² -Ethyl-N ² -methylguanine	395	8	N.D.	—
1-Ethylguanine	123	2	N.D.	—
1-Ethyladenine	N.D.	—	N.D.	—
6-Ethyladenine	N.D.	—	N.D.	—
Ethylated ribose*	1159	23	N.D.	—

* Radioactivity present in fraction 1 from the Dowex column and rendered volatile by heating in 11.6M-HClO₄ for 90 min at 100°C.

pyrimidine could not be responsible for the loss of radioactivity from the pyrimidine nucleotide fraction of the ethylated tRNA. It is known that the exact ratio of HClO₄ to nucleic acid is critical in preventing degradation of pyrimidine bases during hydrolysis of nucleic acids (Wyatt, 1952), so the difference in stability between 3-methyluracil in the experiments of Iwanami & Brown (1968) and 3-ethyluracil in the present study could be due to differences in the conditions employed for HClO₄ treatment rather than to differences in the stability of these compounds towards HClO₄.]

A summary of the ethylated products found in rat liver tRNA after ethionine administration is shown in Table 1 (Expt. A). The identification of these ethylated products was based solely on the results of chromatography on Dowex 50 (H⁺ form) and on paper with at least two solvent systems and is not completely satisfactory for the positive characterization of these products, but the small amount of material obtained even after treatment of the animals with very large doses of ethionine makes isolation and rigorous characterization of this material very difficult. The identification described in Table 1 should therefore be regarded as tentative, but the comparisons between the compounds found in tRNA after ethionine administration *in vivo* and the ethylated products formed by rat liver enzymes *in vitro* as described in detail below should be more reliable.

An extract prepared from rat liver homogenates as

described above catalyses the incorporation of methyl groups from S-adenosyl-L-methionine into tRNA of *E. coli* K12 (Pegg, 1971a). All of the tRNA methylase activity of the liver that could be detected under the assay conditions used in these experiments was present in this extract. When S-adenosyl-L-ethionine was substituted for S-adenosyl-L-methionine in the assay medium, radioactivity was incorporated into tRNA, but the rate of incorporation of ethyl groups was much less than that of methyl groups (Table 2). The difference between the rates of methylation and ethylation could not be decreased by increasing the concentration of S-adenosyl-L-[Et-1-¹⁴C]ethionine present in the assay medium, as a maximal rate of reaction was obtained in the presence of 40 μM-S-adenosyl-L-ethionine.

The incorporation of label from S-adenosyl-L-[Et-1-¹⁴C]ethionine into tRNA was not due to the presence of contaminating L-[Et-1-¹⁴C]ethionine and the consequent formation of ethionyl-tRNA by an aminoacyl-tRNA synthetase, as ATP did not stimulate the reaction and a large excess of unlabelled ethionine or methionine did not inhibit the incorporation (Table 2). In contrast, a large excess of unlabelled S-adenosyl-L-methionine did completely inhibit the ethylation of tRNA.

To investigate the nature of the products formed by the ethylation of bacterial tRNA by liver enzyme(s) *in vitro* the reaction mixture was scaled up by a factor of 20. The tRNA was re-isolated after incubation at

Table 2. *Ethylation and methylation of bacterial tRNA catalysed by liver extracts*

The assay medium contained 20 μ mol of tris-HCl buffer, pH 8.6, 1.0 μ mol of spermidine, 0.2 mg of tRNA (*E. coli* K12), 0.8 mg of liver protein and either 0.01 μ mol of *S*-adenosyl-L-[Me- 14 C]methionine (60000 c.p.m./nmol) or 0.02 μ mol of *S*-adenosyl-L-[Et-1- 14 C]ethionine (70000 c.p.m./nmol) in a total volume of 0.2 ml.

Addition to assay medium	Incubation time (min)	Ethyl groups incorporated into tRNA in the presence of <i>S</i> -adenosyl-L-ethionine (nmol)	Methyl groups incorporated into tRNA in the presence of <i>S</i> -adenosyl-L-methionine (nmol)
None	15	0.006	0.181
None	30	0.010	0.327
None	45	0.014	0.465
None	60	0.019	0.595
L-Methionine (20 μ mol)	30	0.009	0.310
L-Ethionine (20 μ mol)	30	0.011	0.342
ATP (10 μ mol) + MgCl ₂ (2.5 μ mol)	30	0.008	0.283
<i>S</i> -Adenosyl-L-methionine (10 μ mol)	30	<0.001	—

37°C for 60 min, hydrolysed in 1 M-HCl at 100°C for 30 min and the products of hydrolysis were separated by chromatography on paper and Dowex 50 (H⁺ form) columns (Fig. 2), as described above for tRNA ethylated *in vivo*. The major product of the reaction was identified as *N*²-ethylguanine (68% of the total radioactivity). Other ethylated purines found were 7-ethylguanine (5%) and *N*²*N*²-diethylguanine (4%). The remaining label was found in the pyrimidine nucleotide fraction, and after hydrolysis with 11.6 M-HClO₄ two labelled products, with the same chromatographic properties as the ethylated pyrimidines found after administration of ethionine *in vivo*, were isolated. However, in this case, virtually none of the radioactivity in the pyrimidine nucleotide fraction was rendered volatile after treatment with HClO₄, and hence no ethylation of the ribose moiety of the bacterial tRNA had occurred. A summary of the ethylated products produced by the action of liver enzymes acting on tRNA from *E. coli* K12 is shown in Table 1, Expt. B. To obtain direct experimental evidence as to whether the ethylation of RNA *in vivo* after ethionine administration depends on enzymes acting with *S*-adenosyl-L-ethionine as an ethyl donor, the effects of compounds known to affect the synthesis of *S*-adenosyl-L-ethionine or RNA on the ethylation of RNA was studied. Table 3 shows the ethylation of RNA and the tissue concentrations of *S*-adenosyl-L-ethionine and *S*-adenosyl-L-methionine in control animals, rats treated with actinomycin D to stop RNA synthesis, rats treated with a large dose of L-methionine to raise the intracellular concentration of *S*-adenosyl-L-methionine and inhibit the formation of *S*-adenosyl-L-ethionine, and rats injected with 1-aminocyclopentanecarboxylic acid,

Fig. 2. Ion-exchange chromatography of hydrolysed tRNA from *E. coli* K12 re-isolated after incubation with *S*-adenosyl-L-[Et-1- 14 C]ethionine and rat liver extract as described in Table 2

Hydrolysis and chromatography were carried out as described in Fig. 1. Δ , E_{260}^{cm} $\bullet$, radioactivity.

which inhibits ATP-L-methionine *S*-adenosyltransferase (Lombardini *et al.*, 1970). The results of these experiments (Table 3) show that 165 min after the administration of 6.3 μ mol of L-[Et-1- 14 C]ethionine to male rats weighing 120 g the concentration of *S*-adenosyl-L-ethionine in the liver was 0.045 μ mol/g wet wt. of tissue and was approximately equal to the concentration of *S*-adenosyl-L-methionine. The RNA of the liver was ethylated, with a specific radioactivity of 240 c.p.m./mg. It should be emphasized that the method for the measurement of the *S*-adenosyl-L-methionine content in these experiments is dependent

Table 3. *Effects of actinomycin D, 1-aminocyclopentanecarboxylic acid and methionine on concentrations of S-adenosyl-L-ethionine, S-adenosyl-L-methionine and S-adenosyl-L-homocysteine, and on ethylation of tRNA in rat liver*

Treatment of animals was as described in the text. Results are expressed as the means of the number of determinations shown in parentheses $\pm$ S.D.

Treatment of animals	Ethylation of tRNA (c.p.m./mg)	S-Adenosyl-L-methionine (nmol/g of liver)	S-Adenosyl-L-ethionine (nmol/g of liver)	S-Adenosyl-L-homocysteine (nmol/g of liver)
Control (6)	240 $\pm$ 31	47 $\pm$ 8	45 $\pm$ 5	74 $\pm$ 11
Actinomycin D (15 mg/kg)	37 $\pm$ 13	80 $\pm$ 19	81 $\pm$ 21	40 $\pm$ 19
1-Aminocyclopentanecarboxylic acid (2.5 g/kg)	135 $\pm$ 42	27 $\pm$ 9	9 $\pm$ 3	71 $\pm$ 24
L-Methionine (1.0 g/kg)	48 $\pm$ 17	345 $\pm$ 95	3 $\pm$ 1	180 $\pm$ 50

on complete recovery of this nucleoside and S-adenosyl-L-homocysteine from the liver extracts and on the determination of the S-adenosyl-L-homocysteine content. Although the results on control animals were consistent with other published values and the results were consistent within a particular test group of animals, the tissue concentrations of S-adenosyl-L-methionine should be regarded only as reasonable approximations.

1-Aminocyclopentanecarboxylic acid (2.5 g/kg body wt.) was administered to a group of rats 15 min before the ethionine. As this compound has been reported to inhibit S-adenosyl-L-methionine formation by the rat liver enzyme (Lombardini *et al.*, 1970), it was hoped that this dose of 1-aminocyclopentanecarboxylic acid (the maximum tolerated by the animals) would prevent the formation of S-adenosyl-L-ethionine in the liver. However, although inhibition of the synthesis of S-adenosyl-L-ethionine did occur, the concentration was decreased only to about 20% of the control value. There was a considerably smaller decrease in the S-adenosyl-L-methionine content of the liver, to 55% of the control value. Lombardini & Talalay (1971) have noted that administration of 1-aminocyclopentanecarboxylic acid can actually lead to a rise in the concentration of S-adenosyl-L-methionine in rat liver under some conditions, but the mechanism by which such changes in the content of liver sulphonium compounds are produced is unclear. The ethylation of RNA in the liver was decreased by the treatment with 1-aminocyclopentanecarboxylic acid from 240 c.p.m./mg to 135 c.p.m./mg. This decrease could be due to the increase in the ratio of S-adenosyl-L-methionine to S-adenosyl-L-ethionine, from about 1 in control animals to 3 in animals treated with 1-aminocyclopentanecarboxylic acid.

When 1 g of L-methionine/kg body wt. was administered to rats 15 min before the ethionine, the content of S-adenosyl-L-ethionine in the liver was decreased to only 7% of the control value, whereas the S-adenosyl-L-methionine content was increased by 700%.

RNA isolated from the livers of these rats was ethylated, but the specific radioactivity of the RNA was 48 c.p.m./mg, which is only 20% of the control value. Although S-adenosyl-L-ethionine was detectable in the livers of these animals, and it therefore cannot be ruled out that ethylation of RNA was mediated through this nucleoside, a more probable explanation would be that, as postulated by Ortwerth & Novelli (1969), another pathway for the ethylation of nucleic acids does exist, and that this pathway is responsible for 20% of the total ethylation of RNA.

Administration of 15 mg of actinomycin D/kg body wt. to rats 30 min before the labelled ethionine decreased the specific radioactivity of the ethylated tRNA in the liver from 240 c.p.m./mg to 37 c.p.m./mg. Thus even this very large dose of actinomycin D did not prevent the ethylation of RNA entirely, and the ethylation in the presence of actinomycin D was similar in magnitude to that found when large doses of methionine were administered. It is noteworthy that there was a significant increase in both S-adenosyl-L-ethionine and S-adenosyl-L-methionine in the livers of rats treated with actinomycin D. The reason for this increase is unclear, but may be related to inhibition of the synthesis of either acceptors of alkyl groups or enzymes catalysing the transfer of these groups.

Discussion

The ethylated bases found in rat liver tRNA after the administration of a large dose of L-ethionine (500 mg/kg body wt.) in the present study are in reasonable agreement with those found by Rosen (1968), who used a much smaller dose of L-ethionine. This result is compatible with the hypothesis that the formation of ethylated bases in the livers of rats treated with ethionine is mediated through the action of tRNA methylases, as all the ethylated products

found are known to have methylated equivalents in tRNA isolated from normal rats (Craddock, 1969; Pegg, 1971a; Starr & Sells, 1969). However, there are marked differences in the relative proportions of alkylated bases when the ethylated products are compared with the normal methylated bases. 1-Methyladenine and 1-methylguanine are major components of the complement of methylated bases in tRNA of rat liver (Dunn, 1959; Smith & Dunn, 1959; Baguley & Staehelin, 1968; Craddock, 1969; Pegg, 1971a), but, as reported by Rosen (1968), the ethyl analogues are virtually absent from tRNA of ethionine-treated animals.

It was found in the present work that a crudely fractionated extract containing tRNA methylases prepared from rat liver did catalyse the transfer of ethyl groups from *S*-adenosyl-L-ethionine to bacterial tRNA. The principal ethylated product formed *in vitro* under the incubation conditions used in these experiments was *N*²-ethylguanine, although traces of *N*²*N*²-diethylguanine, 7-ethylguanine and ethylated pyrimidines were also produced. As rat liver tRNA devoid of methyl groups is not at present available to act as a substrate in the reaction catalysed by liver tRNA methylases, it is necessary to use tRNA from a different organism, which contains some sites for methylation recognizable by liver enzymes in assays of liver tRNA methylases (Borek & Srinivasan, 1966; Starr & Sells, 1969). The substrate used in the present experiments was tRNA from *E. coli* K12, which had been fully methylated *in vivo* by the bacterial enzymes. Although it is possible to obtain tRNA from a methionine auxotroph of *E. coli* which had been starved of methionine for some hours before harvesting the cells and this tRNA is to some extent undermethylated by the bacterial methylases (Borek & Srinivasan, 1966), there is only a small increase in the rate of methylation by rat liver enzymes of this tRNA compared with the rate of methylation of normal bacterial tRNA (Leboy, 1970; Pegg, 1971a). This result indicates that the majority of sites for methylation recognized by rat liver enzymes within these preparations are present in both the undermethylated and the normal tRNA. This finding is supported by work in which a number of purified tRNA preparations from yeast and *E. coli* (grown under normal conditions) have been shown to be methylated by enzymes from mammalian cells and the nucleotide sequences surrounding the methylated bases elucidated (Baguley *et al.*, 1970; Kuchino & Nishimura, 1970; Pegg, 1972). It is possible, however, that the liver extracts do contain tRNA methylases that were not detectable by the assay method used, and that additional enzymes catalysing the ethylation of tRNA may be present. However, with *S*-adenosyl-L-methionine as a substrate for the methylation of bacterial tRNA by this liver extract, 1-methyladenine was found to be a major product of the reaction,

representing about 30% of the total incorporation of methyl groups (Pegg, 1971a). It is therefore apparent that the enzyme(s) forming 1-methyladenine in rat liver, which are detected by this assay method, are unable to utilize *S*-adenosyl-L-ethionine as a substrate to form 1-ethyladenine. This result is in agreement with the absence of this compound from the ethylated purines found after extraction of tRNA from animals treated with L-ethionine *in vivo*. A similar argument applies to the absence of 1-ethylguanine from tRNA ethylated *in vitro* and *in vivo*, but as 1-methylguanine is only a minor product of the methylation of bacterial tRNA by rat liver enzymes *in vitro* (Pegg, 1971a), the absence of the ethyl analogue of this base from tRNA ethylated *in vitro* is not really conclusive. It is noteworthy that in the presence of sufficiently high concentrations of *S*-adenosyl-L-ethionine the transfer of methyl groups from *S*-adenosyl-L-methionine to tRNA by rat liver enzymes was completely inhibited (Pegg, 1971b), and therefore it is possible that tRNA may be produced in the livers of ethionine-treated animals which is deficient in 1-methyladenine but does not have the methyl group replaced by an ethyl moiety.

Although the rate of ethylation of tRNA by rat liver enzymes *in vitro* was an order of magnitude less than the rate of methylation catalysed by the same extracts, it is still likely that such enzymes are involved in ethylation of tRNA *in vivo*. There is normally no pool of unmethylated tRNA in rat liver (Borek & Srinivasan, 1966; Starr & Sells, 1969) and it is therefore probable that tRNA methylases are present in considerable excess of the amounts required to react at the same rate as that of RNA synthesis. After administration of ethionine to rats, *S*-adenosyl-L-ethionine is known to accumulate in the liver in concentrations greatly in excess of those of *S*-adenosyl-L-methionine (Farber *et al.*, 1964; Smith & Salmon, 1965; Shull *et al.*, 1966), and would therefore replace *S*-adenosyl-L-methionine in those reactions in which either nucleoside can take part.

The observations discussed in detail above therefore provide substantial evidence that ethylation of tRNA can be mediated through the action of tRNA methylases, but the possibility remains that some other means of ethylation of nucleic acids could also take place in animals treated with ethionine. Indeed, some evidence to support this hypothesis is available. Administration of actinomycin D in a dose sufficient to inhibit virtually all RNA synthesis (Merits, 1963) did not completely abolish the ethylation of tRNA (Table 3). As methylation (and presumably ethylation) by tRNA methylases occurs very shortly after the synthesis of the nucleic acid such treatment with actinomycin D would be expected to prevent ethylation completely by preventing the formation of any more substrate RNA to be ethylated. Also, treatment of animals with very large doses of methionine to

produce a large excess of *S*-adenosyl-L-methionine over the *S*-adenosyl-L-ethionine present in the liver would be expected to prevent ethylation by tRNA methylases. Such treatment did not completely inhibit incorporation of ethyl groups into tRNA, although this was markedly decreased (Table 3). Similar results and conclusions were reported by Ortwerth & Novelli (1969) and led these authors to suggest that ethylation of tRNA after ethionine administration was not mediated through enzymes acting with *S*-adenosyl-L-ethionine as an ethyl donor, but by the action of some other compound produced from ethionine (or by ethionine itself). The effect of 1-aminocyclopentanecarboxylic acid on the ethylation of nucleic acids after ethionine administration does not support this hypothesis. Administration of large doses of this compound decreased the concentration of *S*-adenosyl-L-ethionine in the liver and produced some inhibition of the ethylation of tRNA (Table 3), but considerable ethylation of tRNA still occurred. If all of the ethylation of tRNA was produced by the reaction of an intermediate generated from ethionine other than *S*-adenosyl-L-ethionine, the inhibition of the formation of this nucleoside by 1-aminocyclopentanecarboxylic acid might be expected to favour the formation of the other intermediate and hence to lead to a rise in ethylation of tRNA. [Ortwerth & Novelli (1969) have shown that a linear increase in ethylation of tRNA with the dose of ethionine employed takes place over a range that far exceeds that used in these experiments].

Other available evidence suggests that part of the ethylation of nucleic acids after treatment with ethionine may occur by a mechanism other than that mediated by tRNA methylases. After administration of large doses of ethionine to rats, 7-ethylguanine was found to be present in the liver DNA (Swann *et al.*, 1971). As 7-methylguanine is not a normal component of rat liver DNA (Shank & Magee, 1967; Swann & Magee, 1968; Craddock *et al.*, 1968), and no DNA methylase forming 7-methylguanine has yet been described, another mechanism by which 7-ethylguanine is formed must be postulated. It is known that *N*-7 of guanine is a preferred site for attack on nucleic acids by certain chemical alkylating agents (Lawley, 1966), and compounds of this type (or compounds converted into such alkylating agents within the cell) such as diethylnitrosamine or *N*-ethyl-*N*-nitrosourea have been shown to be potent carcinogens (Druckrey *et al.*, 1967; Magee & Barnes, 1967; Swann & Magee, 1970, 1971). If ethionine administration can lead to the production within the liver of such an ethylating agent, the alkylation produced by this intermediate may be of more importance in carcinogenesis by ethionine than the replacement of naturally occurring methyl groups in nucleic acids by ethyl groups as a consequence of nucleic acid methylases acting on *S*-adenosyl-L-ethionine, even if

the latter represents a much greater proportion of the total incorporation of ethyl groups into nucleic acids. Unfortunately, as 7-methylguanine is a normal component of tRNA (Dunn, 1959; Borek & Srinivasan, 1966; Starr & Sells, 1969; Craddock, 1969; Pegg, 1971*a*), it is not possible to determine whether 7-ethylguanine found in tRNA after ethionine treatment is produced by the action of tRNA methylases or by the action of such a hypothetical agent. It may be significant in this context that the ratio of *N*²-alkylguanine to 7-alkylguanine in tRNA isolated from animals treated with labelled methionine was 3.1 (Pegg, 1971*a*), whereas this ratio was only 1.8 in the ethionine-treated rats used in the present study.

The primary target molecule for carcinogens has not yet been positively identified, although many investigators have suggested that interaction with DNA may play a crucial role in carcinogenesis (Brookes, 1966; Lawley, 1966; Colburn & Boutwell, 1968). As the change produced by carcinogens must be inheritable, this hypothesis has obvious attractions, but Weinstein (1969) and Weinstein *et al.* (1971) have argued that changes induced in tRNA molecules could lead to alterations in gene expression producing tumours. Since ethionine interacts with both DNA (Swann *et al.*, 1971) and tRNA neither of these possibilities can definitely be excluded.

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