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Cellular Reactions of O^6-Methylguanine, a Product of some Alkylating Carcinogens

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Cultures of a purine-requiring mutant of Chinese hamster ovary cells (CHO-104b), randomly bred hamster embryo cells, or *Escherichia coli* B_{s-1} were treated with non-toxic doses of ^{3}H-labelled O^6-methylguanine. DNA and RNA were isolated and subjected to enzymic digestion to nucleosides at pH8. The products of digestion were analysed by ion-exchange chromatography on columns of Dowex 50 (NH_4^+ form) at pH8.9. No ^{3}H-labelled O^6-methylguanosine was detected in nucleic acid digests. ^{3}H-labelled O^6-methylguanine was O-demethylated yielding [^{3}H]guanine in CHO-104b cells. Radioactivity in nucleic acid digests was associated with thymidine, guanosine, deoxyguanosine and an unidentified early-eluting product. Reports of similar unidentified products from nucleic acids labelled with various agents are discussed.

Methylation of the O-6 of guanine in nucleic acids by alkylating nitroso compounds (dimethylnitrosamine, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, or *N*-methyl-*N*-nitrosourea) has been observed in bacteriophage (Lawley *et al.*, 1971), bacteria (Lawley & Orr, 1970), mammalian cells (Lawley & Thatcher, 1970), and in whole animals (Frei, 1971; O'Connor *et al.*, 1972, 1973). Loveless (1969) suggested that the resulting 2-amino-6-methoxypurine (O^6-methylguanine), could be a promutational group, and thus account for part of the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. Because O^6-methylguanine lacks a proton at N-1 it could imitate the coding properties of adenine, and induce transition mutations during subsequent replication cycles.

However, the nitroso alkylating agents give rise to several other base modifications in addition to O^6-methylguanine, and it is difficult to assign biological effects to any single reaction. In an attempt to develop a system for studying the effects of methylation of the O-6 of guanine alone, we investigated the incorporation of the base O^6-methylguanine by bacteria and mammalian cells. To promote incorporation, a purine-requiring mutant (Taylor *et al.*, 1971) was tested.

A precedent for the incorporation of an exogenous modified purine was provided by studies of 6-thioguanine. The incorporation of this base into DNA of ascites or mammary tumours was correlated to carcinostasis (LePage, 1963), and more recently LePage (1971) demonstrated that over one-half of the guanine in bone marrow could be replaced by 6-thioguanine. As with O^6-methylation, the presence of a thio group at position 6 makes it unlikely that N-1 will contribute a proton during base-pairing. Evidence that 6-thioguanine may in fact have coding properties similar to those of adenine was provided by Price & Timson (1971). These authors observed that the antimitotic effect of 6-thioguanine on human lymphocytes could be competitively inhibited by adenine, but not guanine or hypoxanthine.

However, in the present work no O^6-methylguanosine or deoxyguanosine could be detected in RNA or DNA after incubation of cells with the ^{3}H-labelled base. O^6-Methylguanine was taken up by cells and a small proportion of the base was O-demethylated to guanine. Radioactivity in the nucleic acids could be accounted for primarily by labelled guanosine, thymidine, deoxyguanosine and an unidentified product.

Materials and Methods

2-Amino-6-methoxypurine (O^6-methylguanine) was prepared by the method of Balsiger & Montgomery (1960). A sample (approx. 1g) was recrystallized by the authors, and was ^{3}H-labelled by The Radiochemical Centre, Amersham, Bucks., U.K., by a catalytic ^{3}H-exchange procedure, and further purified as described below; radioactivity was present in both the methyl group and in the purine ring. [^{14}C]Formate (sodium salt) was also obtained from The Radiochemical Centre.

Deoxyribonuclease I (EC 3.1.4.5) from bovine pancreas, alkaline phosphatase (EC 3.1.3.1) type III from *Escherichia coli*, and phosphodiesterase (EC 3.1.3.1) type II from *Crotalus adamanteus* venom were

obtained from Sigma Chemical Co., London S.W.6, U.K. Ribonuclease A, (EC 2.7.7.16) from Worthington Biochemical Corp., Freehold, N.J., U.S.A., was the 'lyophilized, phosphate-free' grade.

The purine-requiring cell line CHO-104b as described by Taylor *et al.* (1971) was obtained from Dr. Milton W. Taylor, University of Indiana, Bloomington, Ind., U.S.A. Hamster embryo cells were prepared from randomly bred hamsters as described by Lawley & Thatcher (1970). *E. coli* B_{s-1} were cultured as described by Lawley & Orr (1970).

For CHO-104b cell culture Eagle's minimal essential medium containing 100i.u. of penicillin G/ml and 10i.u. of streptomycin/ml was supplemented with 7% (w/v) foetal calf serum, 35μg of L-proline/ml, 13μg of L-asparagine/ml and 4μg of hypoxanthine/ml. This supplemented medium is termed PAX medium and medium supplemented with serum, proline and asparagine is termed PA medium. For plating experiments the concentration of foetal calf serum was increased to 14% (w/v).

Cells were detached from the surface with trypsin in sodium phosphate buffer (0.25%, w/v), supplemented with 100i.u. of penicillin G/ml and 10i.u. of streptomycin sulphate/ml.

Purification of ^{3}H-labelled O^6-methylguanine

Crude ^{3}H-labelled O^6-methylguanine (50mg, nominally 2.04mCi/mg) was dissolved in 50ml of hot methanol. Silica gel (25g) was added, and the slurry was dried in a desiccator. The dry silica mixture was added to the top of a silica gel column (60cm × 2cm) and the ^{3}H-labelled O^6-methylguanine was eluted with chloroform–methanol (6:1, v/v); 20 fractions (each 25ml) were collected and one drop of each was assayed for radioactivity. Fractions 6–13 were pooled and evaporated to dryness. The specific radioactivity of the product was 1.05mCi/mg. Although still slightly yellow, the product gave the predicted u.v. absorption value on the basis of the value for the authentic compound of $\epsilon = 7.9 \times 10^3$ litre·mol^{-1}·cm^{-1} at 280nm (Balsiger & Montgomery, 1960), and contained no radioactive contaminants after chromatography.

Treatment of cells

Eight Thompson bottles were inoculated with 2×10^7 cells in 150ml of PAX medium, and incubated at 37°C for 48h. To 'starve' cultures, the medium was removed and replaced with 100ml of purine-free PA medium for 36h.

For harvest, two Thompson bottles were handled at a time. The medium from each was decanted into a 250ml centrifuge jar. The cell layers were rinsed with phosphate-buffered saline, pH7.4 (Dulbecco & Vogt, 1954) and detached with 25ml of trypsin-containing solution. After suspension with a pipette the cells were returned into the 'conditioned' medium, and kept at 37°C until cells from all eight bottles were suspended. The cells were then centrifuged at about 4*g* for 5min, and pooled in 40ml of PA medium in a 50ml spinner flask. A portion (0.1ml) was diluted into the PAX plating medium and set aside for cell count and colony assay. The total yield from eight Thompson bottles was about 6×10^8 cells.

The ^{3}H-labelled O^6-methylguanine was dissolved in 0.5ml of 0.1M-HCl and rinsed into the cell suspension with 10ml of purine-free medium. Two portions (0.1ml) were removed for assay of radioactivity. After the desired incubation period (up to 30min) cell suspension (0.2ml) was added to 0.2ml of ice-cold trichloroacetic acid (10%, w/v); two portions (0.1ml) of cell suspension were removed for ^{3}H radioactivity assay and 0.1ml of cell suspension was added to 9.9ml of PAX plating medium for eventual dilution, cell count and colony assay.

The reaction was stopped by pipetting 12.5ml of cell suspension into each of four centrifuge jars containing 190ml of ice-cold phosphate-buffered saline. The cells were pelleted at about 4*g* for 5min, and the diluted medium was collected for ^{3}H radioactivity assay and chromatography. The cells were pooled into two batches, each with 13.2ml of 0.01M-Tris–HCl, pH8.0, and lysed with 1.4ml of sodium dodecyl sarcosinate (6%, w/v). Nucleic acids were isolated at once, or the cell lysate was frozen.

Cell number in the samples set aside for colony assay was then determined with a haemocytometer and Coulter counter. The cells were diluted serially, and 100 cells were plated in 10ml of PAX plating medium. After 1h at 0°C, the sample in trichloroacetic acid was spun at 4*g*, and the supernatant was chromatographed on Whatman 3MM paper in propan-2-ol – aq. NH_3 (sp.gr. 0.88) – water (7:1:2, by vol.).

The manipulation of the cells during treatment did not induce cell lysis, as estimated by cell counts at various time-intervals. However, if the cells were kept in phosphate-buffered saline instead of 'conditioned' medium, some cell lysis did occur.

For the longer incubation period of 3h, the cells were treated while attached to the surface, and were subsequently harvested with trypsin. Although this procedure avoided the 'artificial' suspension of cells before treatment, it required extensive amounts of labelled precursor. In addition, treatment before suspension made it impossible to stop the reaction abruptly. Any conditions which would permit trypsin to function would also permit cellular metabolism.

For treatment of CHO-104b cells with [^{14}C]-formate, eight Thompson bottles were seeded, grown in complete PAX medium, and suspended in purine-

free medium immediately before treatment, as described above. [^{14}C]Formate (0.133μmol/ml of cell suspension, 10.8mCi/mmol) was added as an aqueous solution, and portions of the suspension were diluted in ice-cold phosphate-buffered saline after 10 and 30min. The cells were suspended in 20ml of *p*-aminosalicylic acid (sodium salt, 6%, w/v), and were lysed with one-tenth vol. of sodium dodecyl sulphate (10%, w/v).

Separation of DNA and RNA

CsCl (19.2mg) was added to 15.0ml of cell lysate and dissolved. The refractive index was checked and adjusted to a refractive index, *n* 1.400, sp.gr. 1.706. The lysates were transferred to 25ml centrifuge tubes, and were balanced, topped with liquid paraffin, capped, and balanced again. The lysates were centrifuged in a Ti 8×25 rotor, MSE 65 centrifuge at 35000rev./min (*i.e.* 107000*g*, $r_{av.}$ 8cm) for 65–70 h at temperature setting 3, about 14°C (except where otherwise indicated). The density of the CsCl gradient ranged from 1.83 to 1.63. About 22 fractions were collected with a Perpex peristaltic pump (LKB Instruments, Croydon, Surrey, U.K.) connected to a capillary tube inserted into the bottom of the gradient. Fractions were timed (30s) since the DNA band was too viscous to form discrete drops. A sample (0.05ml in 10ml of Triton X-100 phosphor) of each fraction was assayed for radioactivity with a Packard Tri-Carb 375 or 3375 liquid-scintillation counter. A second sample (0.05ml in 4.0ml of water) was assayed for u.v. absorption with a Unicam SP.800 spectrophotometer. The viscous fractions (11–14) were pooled, diluted 1:10 with water, and the DNA was precipitated with one-tenth vol. of cetyltrimethylammonium bromide solution (1%, w/v). The DNA was picked out and rinsed twice with sodium acetate (2%, w/v) in aq. ethanol (70%, v/v), and dried in ethanol and ether. The total yield was about 6mg of DNA.

Two RNA fractions were obtained. First the pellet from the CsCl gradient was dissolved in 25ml of water. Secondly, fractions containing high u.v. absorption (1–9) were pooled and diluted to 100ml with water. RNA was precipitated from each with 2vol. of ethanol and collected by centrifugation at 4*g* for 10min. The RNA was washed twice with each of aq. sodium acetate in ethanol, dry ethanol and ether.

Nucleic acids from hamster embryo cells, *E. coli* B_{s-1}, and CHO-104b cells treated with [^{14}C]formate were obtained by the modified procedure of Kirby (Kirby, 1957; Lawley & Thatcher, 1970). Protein was extracted with 1vol. of phenol reagent (containing 500g of phenol, 62ml of *m*-cresol, 62ml of water and 0.62g of 8-hydroxyquinoline); DNA and RNA were selectively precipitated with 1.5vol. of 2-ethoxyethanol and 2vol. of ethanol respectively.

Digestion of DNA and RNA

DNA (2–3mg) was dissolved in 1.0ml of 0.01M-sodium acetate at 37°C. DNAase* (0.2ml, 0.5mg/ml) was added, and the solution was incubated for 1h. The pH was adjusted with 0.1M-Tris–HCl, pH8.0. Snake venom phosphodiesterase (0.03 unit in 0.1ml) and bacterial alkaline phosphatase (3.6 units in 15μl) were added. After 16h at 37°C the digestion was checked by t.l.c. (Polygram Cel 300UV) in propan-2-ol–aq. NH_3 (sp.gr. 0.88)–water (7:1:2, by vol.). RNA (3–4mg) was dissolved in 1.0ml of water and treated with 0.1ml (0.1mg/ml) of RNAase for 1h. The pH was adjusted to 8.0 and the RNA was digested to nucleosides as described for DNA.

Column chromatography

The nucleoside digests were chromatographed on Dowex 50 (NH_4^+ form), less than 400 mesh, at pH8.9, with 0.3M-ammonium formate, as described by Lawley & Shah (1972). Fractions (99 drops, 6.0ml) were collected and counted for radioactivity in 10ml of Triton X-100 phosphor in a Packard Tri-Carb liquid-scintillation counter. The efficiency of detection of ^{3}H radioactivity was 21%. Radioactivity in all tabulated peaks was at least ten times background values. The procedures for purification, digestion and chromatography did not degrade ^{3}H-labelled O^6-methylguanine (Lawley & Shah, 1972).

Results

The modified purine 6-amino-2-methoxypurine (O^6-methylguanine) proved relatively non-toxic to mammalian cells. In a colony-forming assay, the continuous presence of 50μg of O^6-methylguanine/ml of culture medium decreased plating efficiency of CHO-104b cells from 80 to 72%. Increase of the dose to 100μg/ml caused a further decrease of plating efficiency to 40%. In the experiments reported here, doses of ^{3}H-labelled O^6-methylguanine in the range 50–100μg/ml were used, but the exposure time was limited to short periods. Samples of each labelled cell suspension before and after treatment were diluted and plated for colony assay. In no case was the plating efficiency after treatment less than 90% of that observed before treatment. The metabolic activities detected were therefore associated with healthy cells.

The percentages of radioactivity in the major labelled products of DNA, from cells treated in actively growing cell cultures with ^{3}H-labelled O^6-methylguanine, digested enzymically to nucleosides, and separated on Dowex 50 (NH_4^+ form), are shown in Table 1. Undigested material, eluted near the origin,

* Abbreviations: deoxyribonuclease, DNAase; ribonuclease, RNAase.

Table 1. *Distribution of radioactivity in the products of digestion of DNA from cells incubated with ^{3}H-labelled O^6-methylguanine*

Details of the treatment of cells, separation of DNA, digestion of DNA to nucleosides, and column chromatography of the digest on Dowex 50 (NH_4^+ form) pH 8.9, are given in the Materials and Methods section. 'Starved' cells were incubated in purine-free medium for 36h before treatment.

Cell Type ...	CHO-104b				CHO-104b (starved)		Hamster embryo			*E. coli* B_{s-1}
Dose (μg of ^{3}H-labelled O^6-methylguanine/ml)	105	83.3	83.3	51.6	45.7	102	102	100	100	100	160
Incubation time (min)	5	5	10	15	180	30	30	180	180	20	180
Extent of incorporation ($10^{-2}\times$d.p.m./mg of DNA)	30	16	—	114	6700	128	210	—	95000	—	190000
% of ^{3}H in fractions 9–10 R_{dA}* 28 (unidentified)	8.4	10.4	8.4	8.0	10.6	8.7	9.1	15.2	6.5	9.5	6.5
% of ^{3}H as dT (R_{dA} 39)	17.8	10.3	11.3	23.0	10.7	5.4	3.8	2.8	4.5	2.0	—
% of ^{3}H as G (R_{dA} 70)	12.8	27.2†	36.7†	11.0	15.0	63.4‡	78.9‡	59.2	58	56	60
% of ^{3}H as dG+dA (R_{dA} 100)	46.9	37.1	36.7	54.0	63.7	16.2	7.4	22.4	24	23	32

* R_{dA} denotes (peak fraction no. of elution of product/fraction no. of elution of deoxyadenosine)$\times$100.
† CsCl gradient centrifuged at 56000*g*, 25°C.
‡ CsCl gradient centrifuged at 56000*g*, 50°C.

was not included in the calculations. All DNA preparations, whether separated on CsCl gradients or by the modified phenol method of Kirby (1957) followed by chromatography on Sephadex G-200, contained significant amounts of labelled guanosine. Further purification to remove the residual RNA was not undertaken since the ribo- and deoxyribo-nucleosides were well separated and readily identified. The major labelled deoxyribonucleoside was deoxyguanosine; deoxyadenosine, which was only partially separated from deoxyguanosine, contained less than one-sixtieth of the label in deoxyguanosine. Minor labelled products included thymidine, probably labelled in the methyl group, and an unidentified product, eluted just before uridine in fractions 9–10, R_{dA} 28 (for definition of R_{dA} see Table 1). Traces of radioactivity were detected in the elution position of deoxycytidine, but this was not in a well-defined peak, and could have arisen from 'tailing' of the guanosine. No incorporation of unchanged O^6-methylguanine was observed.

The same pattern of labelled products was obtained from CHO-104b cells treated under normal growth conditions; from CHO-104b cells treated after 36h in purine-free medium; from hamster embryo cells; or from *E. coli* B_{s-1}.

The extent of labelling of nucleic acid was approximately proportional to dose and to time of exposure to ^{3}H-labelled O^6-methylguanine.

The distribution of radioactivity in products of enzymic digestion of RNA to nucleosides is shown in Table 2. The major radioactive product was guanosine. Only small amounts of radioactivity were associated with adenosine. As with DNA, an unidentified product was consistently detected in fractions 9–10, R_A 31 (for definition of R_A see Table 2). The absence of any ^{3}H-labelled O^6-methylguanosine, even after short incubation times, suggested that the unknown product did not arise through degradation of ^{3}H-labelled O^6-methylguanosine after its incorporation into nucleic acid. In addition, it is unlikely that this product was adsorbed low-molecular-weight material. The absence of any trace of the base ^{3}H-labelled O^6-methylguanine indicated the low-molecular-weight compounds had been efficiently removed. Nonetheless, chromatography of ^{3}H-labelled O^6-methylguanine in propan-1-ol–aq. NH_3 (sp.gr. 0.88) (7:1, v/v) gave rise to a degradation product at R_F 0.88. After elution into water, this spot had the same R_A value on Dowex 50 (NH_4^+ form) as the unknown labelled product from the nucleic acid digests. The unknown product was not xanthine, which was eluted earlier.

None of the labelled products detected in DNA or RNA were derived from trace contaminants in the original ^{3}H-labelled O^6-methylguanine, which gave only a single radioactive peak in the position of O^6-methylguanine after column chromatography. However, several radioactive peaks were detected after

Table 2. *Distribution of radioactivity in the products of digestion of RNA from cells incubated in ^{3}H-labelled O^6-methylguanine*

Details of the treatment of cells, separation of RNA, digestion of RNA to nucleosides, and column chromatography of the digest on Dowex 50 (NH_4^+ form) pH 8.9, are given in the Materials and Methods section. 'Starved' cells were incubated in purine-free medium for 36h before treatment.

Cell Type ...	CHO-104b					CHO-104b (starved)			*E. coli* B_{s-1}
Dose (μg of ^{3}H-labelled O^6-methylguanine/ml)	104	104	83.3	51.6	45.7	0.3	102	102	160
Incubation time (min)	5	10	10	15	180	15	30	30	180
Extent of incorporation ($10^{-2}\times$d.p.m./mg of RNA)	30	—	95	210	4100	220	1100	1500	380000
% of ^{3}H in fractions 9–10, R_A* 31 (unidentified)	9.2	6.7	7.2	11.1	8.0	5.8	6.2	7.1	3
% of ^{3}H as guanosine (R_A 86)+adenosine (R_A 100)	82.6	86.5	90.8	82.3	90.0	89.5	93.0	89.9	84

* R_A denotes (peak fraction no. of elution of product/fraction no. of elution of adenosine)$\times$100.

chromatography on Dowex 50 (NH_4^+ form) at pH 8.9 of the low-molecular-weight fraction of the cell lysate (top fraction of the CsCl gradient, under the lipoprotein pad). Major peaks occurred at the elution positions of O^6-methylguanine and guanine. Minor unidentified peaks were observed early in the elution profile.

To determine whether the unknown product in fractions 9–10 could have arisen through metabolism utilizing the C_1-unit removed during O-demethylation, cells were incubated with [^{14}C]formate. Culture conditions were identical with those used for ^{3}H-labelled O^6-methylguanine treatment, without preliminary 'starving' in purine-free medium. Nucleic acids were isolated by the phenol method of Kirby (1957), and digested to nucleosides.

Chromatography of the RNA digest on Dowex 50 (NH_4^+ form) at pH 8.9 demonstrated four labelled peaks. One was coincident with adenosine with a shoulder in the guanosine region. Thus, although reportedly a purine-requiring line, CHO-104b cells could utilize formate in purine biosynthesis. However, the incorporation was more efficient in the biosynthesis of adenosine than guanosine.

Three additional peaks occurred in fractions 5, 10 and 14, coincident with undigested material, the unknown spot after incubation with ^{3}H-labelled O^6-methylguanine, and thymidine, respectively. Since thymidine was heavily labelled by formate this last may have come from DNA in the RNA fraction. The nature of the products in fractions 5 and 10 was not established.

Chromatography of the digest of [^{14}C]formate-labelled DNA revealed some label associated with the deoxyguanosine and deoxyadenosine peak, and a contaminant of labelled RNA. The thymidine peak was extensively labelled, but only a trace of radioactivity was associated with fraction 10.

Discussion

Incorporation of O^6-methylguanine into nucleic acid could not be detected in mammalian cells capable of normal purine biosynthesis, in a purine-requiring cell line, or in *E. coli* B_{s-1}. Although incorporation of trace amounts could not be absolutely disproven, the techniques were sufficiently sensitive to detect as little as 1 mol of O^6-methylguanine in 10^8mol of nucleotide. Thus, unlike the purine analogue 6-thioguanine, O^6-methylguanine was not accepted by the nucleic acid synthesizing systems.

The occurrence of labelled guanine in cytoplasm, DNA and RNA after treatment of actively growing cell cultures with ^{3}H-labelled O^6-methylguanine confirmed the presence of enzymic O-demethylase activity in all of the cell types studied.

O-Demethylase activity had previously been reported in several systems. Henderson & Mazel (1964)

demonstrated weak *O*-demethylase activity in mouse liver microsomes (microsomal fraction), with 2-amino-4-methoxytrimethylenepyrimidine as substrate. However, the activity seemed characteristic of the liver microsomes only, since it did not occur in Ehrlich-ascites-tumour cells. In the present study, *O*-demethylase activity, with 2-amino-6-methoxypurine as substrate, evidently occurred in mammalian embryonic or ovary cells, and in bacteria.

Baer & Drummond (1966) demonstrated demethylation of 6-methoxypurine ribonucleoside to inosine. In addition, the enzyme known as 'adenosine deaminase' was shown by Wolfenden (1966) to be capable of replacing N, S, or O–CH_3 with O at the N-6 position of adenosine or its derivatives. The biological importance of *O*-demethylase activity is not known.

O^6-Methylguanine was a product of the reaction of exogenous ^{14}C-labelled methylating agents with nucleic acids in bacteriophage, bacteria and mammalian cells. The decline in ^{14}C in the position of O^6-methylguanine after chromatography of DNA hydrolysates has been interpreted as enzymic excision of material containing this modified base (Lawley & Orr, 1970; O'Connor *et al.*, 1973). Since in the cited cases, the label was in the methyl group rather than in the purine ring, the decline of label in the O^6-methylguanine might also represent *O*-demethylation, perhaps carried out by deaminating enzymes. However, at the nucleic acid level, *O*-demethylation seems less likely than excision of nucleotides. Wolfenden *et al.* (1967), used adenylic acid oligonucleotides as substrates to demonstrate that deaminase activity decreased as chain length increased. Therefore, it would appear that demethylation of methylated purines might occur for low-molecular-weight precursors, but 'errors' in high-molecular-weight nucleic acids would be corrected by some excision mechanism. Once the modified nucleotides were excised, enzymes with *O*-demethylase activity would rapidly remove the methyl group at the O^6-position, and return the guanine for normal metabolism.

An unknown radioactive compound was eluted in fractions 9–10, R_A 31, after chromatography of four widely differing preparations. Radioactivity occurred in fractions 9–10 of enzymic digests of DNA and RNA from first, mammalian cells, and secondly, bacteria, incubated with ^3H-labelled O^6-methylguanine. Thirdly, decomposition of ^3H-labelled O^6-methylguanine during chromatography in propan-2-ol–aq. NH_3 (sp.gr. 0.88) (7:1, v/v) resulted in a product which, after elution and column chromatography, appeared in fractions 9–10. Finally, radioactivity occurred in fractions 9–10 of the enzymic digest of RNA from cells incubated with [^{14}C]formate. However, only a small amount of ^{14}C occurred in fractions 9–10 of the corresponding DNA digest after incubation with [^{14}C]formate.

Radioactivity in fractions 9–10 of digests from ^3H-labelled O^6-methylguanine-labelled material seemed most likely to have arisen from (1) a decomposition product of the ring structure, or (2) subsequent metabolism of the methyl group. The rapid and extensive labelling of thymidine is evidence that the [^3H]-methyl group was available for metabolism. The absence of detectable ^3H-labelled O^6-methylguanine made postulate (1) unlikely. However, the demonstration of a degradation product of ^3H-labelled O^6-methylguanine, chromatographically similar to the material from nucleic acids, supported postulate (1).

The occurrence of products that were eluted early from Dowex 50 (H^+ or NH_4^+ form) has been reported for a variety of nucleic acid preparations. Schoental (1967) reported a fraction containing sugar moieties, and possibly some methylated products, after analysis of DNA treated with *N*-[^{14}C]methyl-*N*-nitrosourethane. Craddock (1971) reported an unknown product from DNA after treatment of rats with di[^{14}C]methylnitrosamine, and tentatively identified it as a formaldehyde metabolite. However, unlike the present observation with [^{14}C]formate as precursor, this product was found in greater abundance associated with DNA than with RNA.

O'Connor *et al.* (1973) reported an early-eluting product during column chromatography of DNA from rats treated with di[^{14}C]methylnitrosamine. This unidentified product was excised from DNA *in vivo* more slowly than was O^6-methylguanine. When [^{14}C]methyl methanesulphonate was used as alkylating agent, much less of the early-eluted product was observed (O'Connor *et al.*, 1972).

Lawley & Shah (1972) observed significant amounts of label eluted early in chromatographic analyses of RNA or polynucleotides, particularly poly(U), treated with *N*-[^{14}C]methyl-*N*-nitrosourea. They suggested that the early fractions might contain hydrolytic products from phosphotriesters. Less of the unknown fraction was obtained if di[^{14}C]methyl sulphate was substituted as alkylating agent.

Bannon & Verly (1972) obtained evidence consistent with the formation of phosphotriester groups after treatment of DNA with [^3H]ethyl methanesulphonate and [^3H]methyl methanesulphonate. The proportion of DNA alkylation accounted for by phosphate esters was about 15% for the ethyl derivative and 1% for the methyl derivative.

Since phosphotriesters from DNA are stable in neutral or mildly acid or alkaline conditions, unlike those from RNA (Brown *et al.*, 1955; Brown & Todd, 1955) it is reasonable to assume that products derived from hydrolysis of phosphotriesters, and which eluted early from Dowex 50 columns, would be more in evidence with RNA than with DNA. However, it remains unlikely that the methyl group of O^6-methylguanine could be transferred to phosphodiester groups by chemical reaction, and there is no evidence

for biosynthesis of methyl phosphotriester groups in normal nucleic acids (Brown & Todd, 1955).

In summary, therefore, whereas the present work cannot definitely eliminate or support any of the various suggestions as to the nature of the unidentified early-eluted products, it seems most reasonable that these derive from incorporation of label from the methyl group of O^6-methylguanine, or, less likely, from degradation of the purine moiety thereof. No assignment of structure of these products appears possible at present, and it remains uncertain whether they derive from impurities in the nucleic acid preparations.

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