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Nonenzymatic methylation of DNA by the intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction

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Incubation of DNA with S-adenosyl-L-methionine (SAM) in neutral aqueous solution leads to base modification, with formation of small amounts of 7-methylguanine and 3-methyladenine. The products have been identified by high performance liquid chromatography of DNA hydrolysates and by the selective release of free 3-methyladenine from SAM-treated DNA by a specific DNA glycosylase. We conclude that SAM acts as a weak DNA-alkylating agent. Several control experiments, including extensive purification of [^{3}H-methyl]SAM preparations and elimination of the alkylating activity by pretreatment of SAM with a phage T3-induced SAM cleaving enzyme, have been performed to determine that the activity observed was due to SAM itself and not to a contaminating substance. We estimate that SAM, at an intracellular concentration of 4 x 10^{-5} M, causes DNA alkylation at a level similar to that expected from continuous exposure of cells to 2 x 10^{-8} M methyl methanesulphonate. This ability of SAM to act as a methyl donor in a nonenzymatic reaction could result in a background of mutagenesis and carcinogenesis. The data provide an explanation for the apparently universal occurrence of multiple DNA repair enzymes specific for methylation damage.

Key words: alkylating agents/DNA repair/ethionine/3-methyladenine/SAM

Introduction

Exposure of DNA to simple methylating agents such as methyl methanesulphonate (MMS) and dimethyl sulphate (DMS) results in the alkylation of ring nitrogens of purine residues, with 7-methylguanine and 3-methyladenine occurring as the most abundant lesions. Trace amounts of O-alkylated bases such as O^6-methylguanine are also formed, although these latter adducts are generated more efficiently by alkylating agents which react by an S_N1 (unimolecular nucleophilic substitution) mechanism, for example, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (Lawley and Shah, 1972). Several different DNA repair enzymes, which specifically recognize these various forms of methylation damage, have been found to be widely or universally distributed among different organisms (Lindahl, 1982). It would appear that living cells are as well equipped to deal with DNA damage caused by methylation (Strauss *et al.*, 1975; Lawley and Brookes, 1968) as they are to deal with u.v. radiation damage and spontaneous hydrolytic lesions.

The reasons for the development of such effective repair mechanisms for alkylated DNA have been far from clear. N-Nitroso compounds may occur as common environmental mutagens (Fine *et al.*, 1977), and one possibility is that cells are often exposed to such agents and have responded to this challenge during evolution. However, metabolic activation of nitrosamines to mutagenic derivatives does not usually occur in bacteria, which nevertheless are proficient in repair of DNA alkylation damage. Another possibility is that normal intracellular methyl group donors could occasionally react with (and damage) DNA by a nonenzymatic mechanism. In this respect, the methyl group of S-adenosylmethionine (SAM) is highly reactive due to the positive charge on the sulphur and can be donated to a large number of methyl group acceptors by various methyltransferases; methylammonium compounds such as N^5-methyltetrahydrofolic acid have, by comparison, much lower transfer potential (Coward, 1977). We now show that incubation of purified DNA with [^{3}H-methyl]SAM of high specific radioactivity leads to detectable methylation of the DNA.

Results

Products of DNA methylation with [^{3}H]SAM

When a neutral aqueous solution of DNA was made 1.2 x 10^{-5} M in [^{3}H]SAM, followed directly by ethanol precipitation of the DNA, washing, and subsequent dialysis of redissolved material, ~0.001% of the radioactivity remained associated with the DNA. Analysis of an acid hydrolysate by high performance liquid chromatography (h.p.l.c.), employing a strong cation exchange column (Partisil 10 SCX), showed that the radioactive material was only comprised of SAM and degradation products of SAM, which apparently had adsorbed to the DNA. That is, the degradation products included an unidentified peak at 13.5 min, as well as peaks eluting early from the column; material with such chromatographic properties was also detected in commercial [^{3}H]SAM preparations not exposed to DNA. No radioactive material was found at the elution positions of 7-methylguanine (8 min) and 3-methyladenine (20 min).

On incubation of the DNA/[^{3}H]SAM mixture for 4 h at 37°C, the amount of radioactive material associated with DNA increased ~3-fold. This could be partly accounted for by adsorption of a larger proportion of [^{3}H]SAM and SAM degradation products to the DNA, but in addition two new peaks of radioactivity were found in the hydrolysate. These were eluted in the positions expected for 7-methylguanine and 3-methyladenine (Figure 1). The identities of the two compounds were confirmed by paper chromatography. The relative proportions of methylated purines were those found in DNA exposed to simple alkylating agents such as MMS. Thus, when the GC-rich DNA from *Micrococcus luteus* was employed as methylation target, the 3-methyladenine peak was ~10% the size of the 7-methylguanine peak (Figure 1B), while in calf thymus DNA hydrolysates, the former peak was ~21% of the latter (see Figure 3A). No radioactive material eluting at the position of N^6-methyladenine (14.5 min) was detected. Moreover, purification of the [^{3}H]SAM-treated DNA by neutral CsCl density gradient centrifugation prior to base analysis did not cause a detectable decrease (<15%) in

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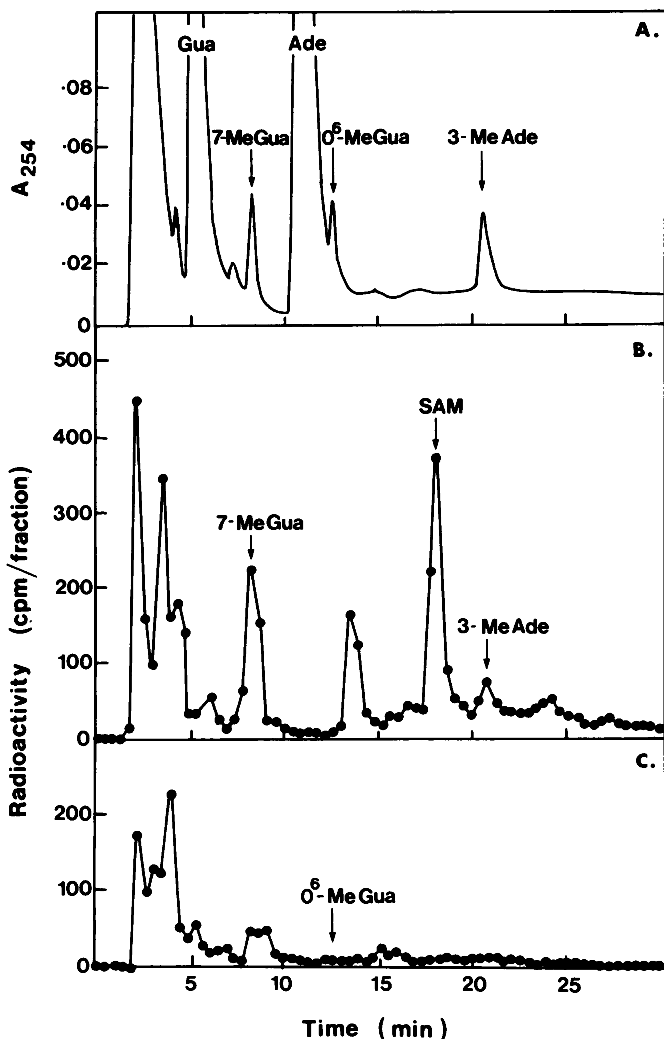

Fig. 1. Methylation products in DNA exposed to SAM. *M. luteus* DNA (0.25 mg) was incubated with 100 μCi [^{3}H]SAM for 4 h at 37°C, precipitated with ethanol, hydrolyzed in 0.1 M HCl, and analyzed by h.p.l.c. (for details, see Materials and methods). **Frame A**, absorbance at 254 nm of the hydrolysate, which had been supplemented before chromatography with reference compounds 7-methylguanine (7-MeGua), O^6-methylguanine (O^6-MeGua), and 3-methyladenine (3-MeAde). Pyrimidine nucleotides elute early from the column. **Frame B**, the radioactivity in the fractions collected. SAM degradation products are seen at 2–5 min and at 13.5 min. **Frame C**, a separate experiment to search for radioactive O^6-methylguanine. 1 mg *M. luteus* DNA was incubated with 400 μCi [^{3}H]SAM as above, and DNA isolated following hydrolysis at 80°C and neutral pH to remove N^3- and N^7-methylated purines. The purine residues remaining in DNA were then released by hydrolysis in 0.1 M HCl and analyzed by h.p.l.c.

the amount of 7-methylguanine found in DNA, showing that the methylated purine was present in DNA rather than in contaminating RNA or other material. These results indicate that SAM can act as a weak DNA methylating agent in neutral aqueous solution.

In an attempt to study the formation of O^6-methylguanine in DNA, the DNA incubated with [^{3}H]SAM was first heated at neutral pH to remove purines methylated at the N^3 and N^7 positions, then subjected to acid hydrolysis to liberate any O^6-methylguanine formed (Frei *et al.*, 1978). No O^6-methylguanine was found. While a 7-methylguanine peak containing 1800 c.p.m. was obtained in the neutral hydrolysate, <10 c.p.m. of O^6-methylguanine were present in the acid hydrolysate (Figure 1C). The data show that the amount of

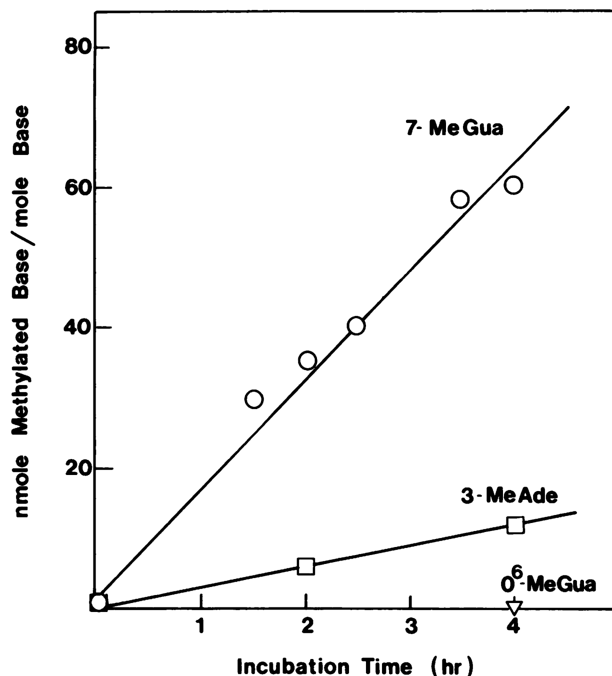

Fig. 2. Kinetics of formation of 7-methylguanine and 3-methyladenine in DNA incubated with 1.2 x 10^{-5} M SAM in 0.15 M cacodylate buffer, pH 7.0. Data were obtained by a combination of h.p.l.c. analysis, as shown in Figure 1, and paper chromatography of DNA hydrolysates. Yields are expressed as 7-methylguanine/unmodified guanine residue in DNA, and similarly, 3-methyladenine/unmodified adenine residue in DNA.

O^6-methylguanine generated was $<1\%$ of that of 7-methylguanine. These results are consistent with a DNA alkylation pattern of SAM resembling that of MMS and DMS (Lawley and Shah, 1972). In contrast, if SAM had acted by the same mechanism as the methylating agents MNNG and N-methyl-N-nitrosourea, similar amounts of O^6-methylguanine and 3-methyladenine should have been formed, and this would have been detected.

Properties of the reaction

The methylation of DNA by SAM, as revealed by the generation of 7-methylguanine and 3-methyladenine residues in DNA, occurred linearly as a function of time (Figure 2). SAM is susceptible to hydrolytic degradation at neutral and alkaline pH, and the decomposition of the [^{3}H]SAM in the standard reaction mixture was tested by h.p.l.c. analysis. At the end of the 4-h incubation period, only 86% of the radioactive material remained as SAM. Thus, it was impractical to prolong incubations further, since the effective concentration of SAM would gradually decrease, and in addition part of the 3-methyladenine present in DNA would be released by hydrolysis. The formation of 7-methylguanine and 3-methyladenine in DNA was not suppressed to a detectable extent by the inclusion of Tris (10 mM), 2-mercaptoethanol (5 mM), or ethanol (2%) in reaction mixtures. While cacodylate buffer was routinely employed in these experiments because of the non-reactivity of the buffer ions with alkylating agents (Uhlenhopp and Krasna, 1971), the SAM-dependent methylation would also be expected to occur under other ionic conditions.

The yield of 7-methylguanine formed in DNA was directly proportional to the DNA concentration of the reaction mixture. (The same appeared to be true for 3-methyladenine, but since this compound was formed in smaller amounts, its

presence was difficult to quantitate at low DNA concentrations). A 5-fold difference in generation of 7-methylguanine was observed on changing the DNA concentration from 0.2 to 1 mg/ml. Addition of non-radioactive SAM (4×10^{-5} M) to the standard reaction mixture (containing 1.2×10^{-5} M [^{3}H]SAM) did not reduce the amount of radioactive material recovered as 7-methylguanine in DNA. Thus, the total amount of 7-methylguanine formed appeared to be proportional to the total SAM concentration in the reaction mixture. This would be expected, since only a very minor proportion of the potentially reactive guanine residues in DNA were methylated in these experiments.

Treatment of methylated DNA with 3-methyladenine-DNA glycosylase

Relatively small amounts of radioactive 3-methyladenine were detected in the DNA hydrolysates by h.p.l.c., and an additional means of identification of this product seemed desirable. This is particularly so, since 3-methyladenine in DNA is an important inactivating lesion for cells exposed to methylating agents (Karran *et al.*, 1980). A DNA repair enzyme, *Escherichia coli* 3-methyladenine-DNA glycosylase I, catalyses the release of free 3-methyladenine from methylated DNA, while 7-methylguanine and several other methylation products are not liberated (Riazuddin and Lindahl, 1978). Consequently, on exposure of SAM-treated DNA to this enzyme, any 3-methyladenine generated should be selectively removed. The results of such an experiment are shown in Figure 3. The 3-methyladenine was, in fact, no longer present in hydrolysates of enzyme-treated DNA, while 7-methylguanine remained in the DNA. Duplicate samples showed identical results within experimental error, and the data confirm that formation of 3-methyladenine in DNA can occur as a consequence of incubation with SAM.

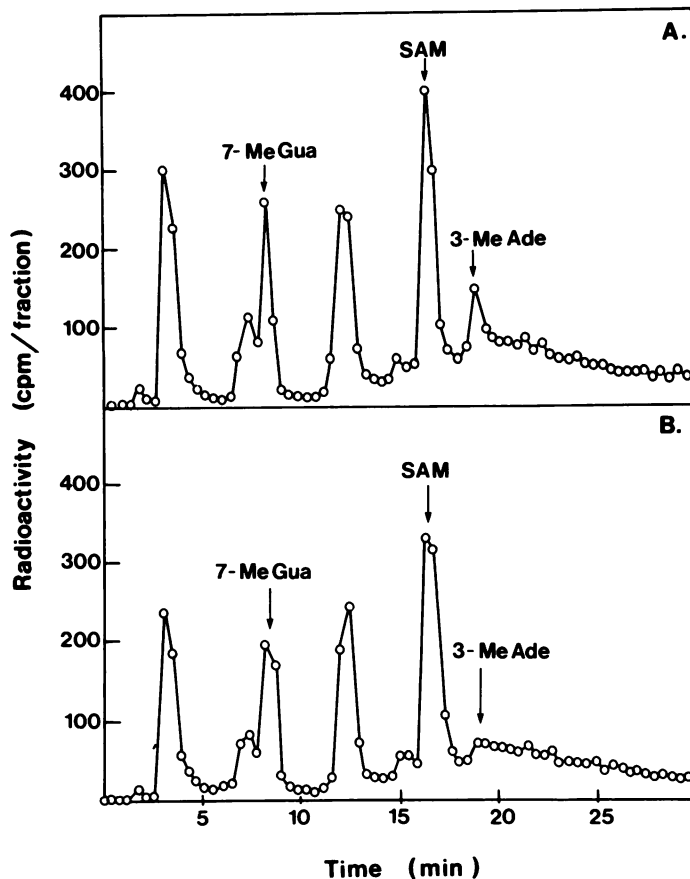

Fig. 3. Selective removal of 3-methyladenine from [^{3}H]SAM-treated DNA by a DNA glycosylase. Calf thymus DNA (0.5 mg), previously incubated with 200 μCi [^{3}H]SAM, was divided into two equal aliquots. One of these was incubated without the reagent enzyme, while the other was incubated with 3-methyladenine-DNA glycosylase. **Frame A**, the h.p.l.c. profile of the hydrolysate of the control sample. **Frame B**, analysis by h.p.l.c. of the material remaining in DNA after treatment with 3-methyladenine-DNA glycosylase.

Purification and enzymatic cleavage of [^{3}H]SAM

Since the amount of SAM-induced DNA methylation in the experiments was low, it was imperative to exclude contamination by trace amounts of methylating agent in the [^{3}H]SAM samples employed. However, the amount of DNA methylation caused by SAM was very similar (less than a 2-fold difference) with several batches of unpurified [^{3}H]SAM obtained from two different suppliers. We have, nevertheless, employed [^{3}H]SAM preparations purified by two ion-exchange chromatography steps in most experiments, although recent batches of [^{3}H]SAM (for example, lot 19 from the Radiochemical Centre) have shown the same DNA-alkylating activity (within experimental error) before and after column purification. As estimated by h.p.l.c. analysis, the chromatography steps employed for SAM purification reduced the level of contaminating material in the [^{3}H]SAM preparations at least 10-fold. It is noteworthy that [^{3}H]SAM is prepared by enzymatic condensation of [^{3}H]methionine with ATP, and not by chemical synthesis involving a methylating agent.

If the DNA-alkylating activity were due to SAM itself, it should be possible to abolish the reaction by specific enzymatic cleavage of SAM prior to incubation with DNA. A suitable enzyme for such an experiment is induced by phage T3 in *E. coli* (Gefter *et al.*, 1966); this enzyme, present in large amounts in extracts of phage-infected cells, cleaves SAM to thiomethyladenosine and homoserine. Treatment of [^{3}H]SAM with a partly-purified preparation of T3 SAMase caused the conversion of 94% of the radioactive material to thiomethyladenosine, as determined by h.p.l.c., while a control [^{3}H]SAM preparation incubated under the same conditions (with bovine serum albumin instead of SAMase) remained intact. Treatment of DNA with these two preparations showed that most of the methylating activity of the SAM preparation treated with SAMase had been destroyed. The apparent amount of 7-methylguanine recovered in a hydrolysate of DNA treated with SAMase-cleaved [^{3}H]SAM was 25% that of the control (both samples contained the same amount of DNA). Since only 6% of the SAM remained after SAMase treatment, as determined by h.p.l.c., the value of 25% 7-methylguanine remaining appears high. One explanation is that a trace of radioactive thiomethyladenosine (which has chromatographic properties similar to 7-methylguanine) was adsorbed to DNA, causing an overestimation of the 7-methylguanine in the hydrolysate. The experiment serves to illustrate that at least 80% of the DNA-methylating activity of the [^{3}H]SAM preparation may be ascribed to SAM itself, rather than to an unknown contaminating alkylating agent.

Radiochemistry

During storage of [^{3}H]SAM solutions of high specific radioactivity, radiolytic products that could have methylating activity accumulate and could thus account for the results obtained. Moreover, a direct radiochemical reaction is

theoretically possible, in view of the large amounts of radioactive SAM employed in the reaction mixtures and the low yield of DNA methylation products obtained. These possibilities were tested by exposing a [^{3}H]SAM/DNA mixture to external X-irradiation equivalent to ~10 times the dose that would result from internal ^{3}H-irradiation during a standard incubation, and also by checking a heavily X-irradiated [^{3}H]SAM sample as methyl donor. (The dose from internal ^{3}H disintegrations in stock solutions of [^{3}H]SAM was estimated to be 3 Gy/day, or 300 rad/day, while ~0.1 Gy, or 10 rad, would be the dose in a [^{3}H]SAM/DNA mixture during a 4-h incubation). The yield of methylated products in DNA was not detectably altered by the external radiation treatments, precluding a significant contribution from a radiochemical reaction.

Discussion

Nonenzymatic methylation of DNA with SAM has not been described previously, although Paik *et al.* (1975) found that SAM can cause carboxyl methylation of proteins at glutamic acid and aspartic acid residues. The mixing of DNA and SAM in solution to obtain methylation of DNA base residues is a very simple experiment, and it may be questioned why this reaction has not been discovered earlier. SAM is a very weak methylating agent, so its induction of DNA damage is difficult to detect without access both to relatively large amounts of radioactive SAM preparations of high specific activity and the product resolution offered by h.p.l.c. analysis of DNA hydrolysates. It should be emphasized that, whereas large amounts of radioactive material were consumed in these experiments, the finite SAM concentration in the reaction mixtures employed (1.2×10^{-5} M) is 3- to 4-fold lower than the average intracellular SAM concentration in various types of cells and tissues (Salvatore *et al.*, 1971). SAM is a reactive high-energy compound, highly susceptible to nucleophilic attack, so its weak but significant intrinsic ability to alkylate DNA is not surprising.

Model experiments on nonenzymatic transmethylation from simple methylsulfonium compounds to various nucleophiles have established that the reaction occurs by an S_N2 mechanism (Coward and Sweet, 1971). The present data on methylation of DNA by SAM agree with such a mechanism, as judged from the spectrum of products observed. In contrast, an S_N1 reagent should have generated detectable amounts of the highly mutagenic lesion O^6-methylguanine. Lawley and Shah (1972) have shown that the difference between alkylating agents reacting with DNA by an S_N1 or S_N2 mechanism is quantitative rather than qualitative, in that typical S_N2 agents such as MMS and DMS also cause the formation of O^6-methylguanine in DNA, albeit at a 20- to 50-fold lower level than that observed with S_N1 agents. Extrapolating to the present case, it seems likely that SAM would also generate O^6-methylguanine in DNA, but at a level considerably lower than that of 3-methyladenine. The high cost of [^{3}H]SAM has so far precluded an experimental verification.

MMS reacts in the same manner and to the same extent with DNA either *in vivo* or *in vitro* (Strauss *et al.*, 1975), and the same would be expected to be true for SAM. The DNA in eukaryotic cells is unlikely to be protected from SAM by compartmentalization, since SAM must be present in the nucleus to serve as the cofactor for DNA methylases. In comparison with MMS (Lawley and Shah, 1972; Strauss *et al.*, 1975), the present data suggest that SAM is 1000–3000 times weaker as a DNA-alkylating agent. Thus, we estimate that at an intracellular SAM concentration of 4×10^{-5} M, the amount of nonenzymatic DNA methylation would be comparable to that expected from continuous exposure of cells to 2×10^{-8} M MMS. This is a dose several orders of magnitude lower than that employed in conventional mutagenicity experiments with bacteria or human cells (Hoppe *et al.*, 1978). A fluctuation test designed to determine small increases over the spontaneous mutation rate (Green *et al.*, 1976) can readily detect, as mutagenic in *E. coli*, MMS concentrations as low as 4×10^{-6} M, the MMS-induced mutations occurring both by error-prone repair and by direct miscoding. We conclude that nonenzymatic methylation of DNA by SAM would be expected to contribute to the background mutation rate, but it would only account for a minor part in most systems. The possibility that metabolic processes within cells themselves play a major role in spontaneous mutagenesis has recently been emphasised by Sargentini and Smith (1981).

Assuming that SAM acts on DNA in a similar fashion *in vivo* and *in vitro*, 4×10^{-5} M SAM would be expected to generate 4000 7-methylguanine, 600 3-methyladenine, and 10–30 O^6-methylguanine residues in the DNA of a mammalian cell during a 24-h period. In addition, several other minor products should be formed, such as 3-methylguanine. The lack of a strong mutagenic response to this continuous challenge within a cell may be ascribed to the efficiency with which repair of alkylation damage to DNA occurs. In the absence of such repair, the background mutation rate due to unavoidable SAM-induced methylation would appear to be unacceptably high. This argument provides a reason for the apparently universal distribution of effective DNA repair enzymes that specifically remove methylated bases (Lindahl, 1982). It is, moreover, an intriguing possibility that other reactive donor compounds in cells also modify DNA by nonenzymatic routes to a low extent, and that separate repair enzymes exist to deal with such damage. For example, acetyl-CoA can acetylate histones in a nonenzymatic reaction (Paik *et al.*, 1970); it is not known, however, if similar acetylation of DNA base residues can occur.

Methylating agents acting by an S_N2 mechanism, that is, MMS and DMS, are carcinogens (Clapp *et al.*, 1968; Swann and Magee, 1969; Kleihues *et al.*, 1972; Druckrey *et al.*, 1970). Consequently, SAM should also be a weak carcinogen, and perturbation of SAM metabolism might lead to an increased cancer frequency. Treatment of rats with the hepatotoxic and carcinogenic agents hydrazine and carbon tetrachloride results in the formation of small amounts of methylated guanine residues in the DNA of the liver, with the methyl groups originating from methionine. It has been proposed that this carcinogenic DNA methylation might be caused by SAM (Becker *et al.*, 1981). A particularly interesting case is found with the methionine analogue, ethionine. Prolonged feeding of rats with the latter causes liver cancer in virtually all animals so exposed (Farber, 1963). Large amounts of S-adenosyl-L-ethionine have been found to accumulate in the liver under these conditions, and 7-ethylguanine has been detected in liver DNA from such animals (Swann *et al.*, 1971). Ethionine, after a delay, also induces DNA repair in rat liver, tentatively ascribed as a consequence of the accumulation of S-adenosyl-L-ethionine which might react nonenzymatically with DNA (Craddock and Henderson, 1978).

The sites of base alkylation by SAM and MMS differ from

those employed in transmethylation reactions catalyzed by DNA methylases. In the latter, two extremely poor methyl acceptor sites are known to be used, i.e., the 5-position of cytosine and the N^6-position of adenine. The resulting methylated bases serve as biological signals, and are not recognized by the multiple DNA repair enzymes that may have evolved in response to the nonenzymatic methylation of DNA by SAM.

Materials and methods

Purification of [^{3}H]SAM

Several different batches of [^{3}H]SAM (sp. act. 13 – 15 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, and from the New England Nuclear Corp. These were further purified by anion-exchange chromatography on Dowex 1, followed by cation-exchange chromatography either on Dowex 50 (Shapiro and Ehninger, 1966) or on sulfopropyl-Sephadex (Glazer and Peale, 1978). In a typical experiment, the [^{3}H]SAM (1 mCi, obtained in dilute H_2SO_4 containing 10% ethanol) was neutralized with potassium cacodylate and applied to a column (0.5 x 1 cm) of Dowex 1-X8 (200 – 400 mesh), equilibrated with HCO_3^-. The [^{3}H]SAM was eluted with 1.6 ml 0.01 M NaCl. This solution was made 0.1 M with respect to NaCl and applied to a column (0.5 x 1 cm) of Dowex 50W-X8 (200 – 400 mesh), equilibrated with Na^+. The column was washed with 3 ml 0.1 M NaCl, and the [^{3}H]SAM was eluted with 0.8 ml 6 M HCl. The eluted material was evaporated to dryness under reduced pressure, dissolved in 2 ml 0.01 M HCl, and applied to a second Dowex 50W column, equilibrated with 0.6 M HCl. The column was washed with 3 ml 0.6 M HCl, and the [^{3}H]SAM eluted with 0.8 ml 6 M HCl. The final yield was ~50%. This radioactive material was evaporated to dryness as above, redissolved in 0.01 M HCl and stored at –80°C.

In some preparations, SP-Sephadex C-25 (Pharmacia Ltd.) was employed instead of Dowex 50. In such cases, the [^{3}H]SAM eluted from Dowex 1 was made 0.05 M with respect to HCl and applied to a column (0.5 x 2 cm) of SP-Sephadex, equilibrated with 0.01 M HCl. The column was washed with 1 ml 0.15 M HCl, and the [^{3}H]SAM eluted as a broad peak with 0.5 M HCl. The most active fractions were pooled, evaporated to dryness and used directly.

Treatment of [^{3}H]SAM with SAMase

SAMase was partly purified from bacteriophage T3-infected *E. coli* (Gefter *et al.*, 1966). The wild-type phage and the *E. coli* host strain B834 were obtained from F.W. Studier, Brookhaven National Laboratory. For an enzyme preparation, exponentially growing bacteria in 500 ml broth were infected with phage T3 at high multiplicity. Incubation of the culture at 37°C was continued for 10 min, with vigorous aeration, followed by chilling to 0°C, collection of the cells by centrifugation, washing with ice-cold 0.05 M potassium phosphate (pH 7.1), and freezing for storage at –80°C. The cell pellet (0.15 g) was suspended in 0.8 ml 0.02 M Tris-HCl (pH 7.8), 1 mM dithiothreitol. After disruption of the cells by sonication, glycerol was added to a final concentration of 5%, and debris removed by centrifugation. The crude cell extract, which contained 25 mg protrein and 750 units SAMase, was partly purified on a column (1 x 5 cm) of DEAE-cellulose equilibrated with 0.02 M Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, 5% glycerol. In contrast to most of the proteins in the extract, SAMase was not retarded on the column, and a 25-fold purification was achieved. The preparation was stored frozen at –80°C and remained stable under those conditions. Enzyme activity was estimated according to Gefter *et al.* (1966), and SAMase assays were performed by incubating protein samples with [^{3}H]SAM followed by chromatography of the reaction products on small Dowex 50 columns.

[^{3}H]SAM (0.3 mCi in 0.3 ml 7 mM Tris-HCl, pH 7.5) was incubated with 2.5 units SAMase (3.4 μg protein) for 30 min at 37°C. An additional 2.5 units SAMase were added, and the incubation continued for 30 min. The reaction mixture was then made 0.02 M with HCl and stored at –80°C. As a control, a separate aliquot of [^{3}H]SAM was incubated with bovine serum albumin instead of SAMase under identical conditions.

Methylation of DNA with [^{3}H]SAM

M. luteus DNA was prepared essentially according to Marmur (1961), with the inclusion of proteinase K and phenol treatment steps, and further purification of the DNA by CsCl density gradient centrifugation. Calf thymus DNA was purchased from Worthington.

Reaction mixtures (0.55 ml) contained 0.1 – 0.5 mg DNA and 1.2×10^{-5} M [^{3}H]SAM (100 μCi) in 0.15 M potassium cacodylate, 1 mM EDTA, pH 7.0. After 4 h at 37°C, the solution was chilled, and the DNA precipitated with two volumes cold ethanol, washed with 75% ethanol, and redissolved in 0.5 ml 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. The DNA was dialyzed overnight against 2 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, and then for 6 h against the same buffer without NaCl.

Treatment of methylated DNA with 3-methyladenine-DNA glycosylase

3-Methyladenine was enzymatically removed from methylated DNA by treatment with *E. coli* 3-methyladenine-DNA glycosylase I. Fraction V of the enzyme preparation described previously (Riazuddin and Lindahl, 1978) was employed. Calf thymus DNA (0.25 mg), methylated with [^{3}H]SAM, was incubated with 5 microunits 3-methyladenine-DNA glycosylase I in 1 ml of 0.07 M Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM 2-mercaptoethanol for 30 min at 37°C. A control sample of the methylated DNA was incubated in the same fashion, but with no enzyme added. Reactions were terminated by ethanol precipitation of the DNA.

Analysis of methylation products

Methylated DNA preparations were ethanol-precipitated and hydrolyzed in 0.1 M HCl at 70°C for 40 min to release the purine residues. Hydrolysates were analyzed by h.p.l.c. In some experiments, the DNA was first hydrolyzed in 0.1 M NaCl, 0.01 M potassium phosphate, 1 mM trisodium citrate (pH 7.4) at 80°C for 16 h. This treatment selectively liberates N^7- and N^3-methylated purines while O^6-methylguanine residues are retained in the DNA (Frei *et al.*, 1978; Karran *et al.*, 1979). The DNA was then precipitated with 0.1 M HCl, washed with 75% ethanol, hydrolyzed in 0.1 M HCl at 70°C, and the hydrolysate analyzed as above.

Chromatography of DNA hydrolysates, supplemented with appropriate reference compounds, was carried out on a Hewlett Packard 1084 B liquid chromatograph system equipped with a Whatman Partisil 10 SCX column (0.46 x 25 cm). The column was at 40°C during separations. The buffer system described by Frei *et al.* (1978) was modified to improve the separation of 3-methyladenine and SAM. Buffer A contained 0.02 M ammonium formate, pH 4.5, and buffer B 0.3 M ammonium formate, pH 4.5. Elution was performed at a flow rate of 1 ml/min, with buffer A for 5 min, a linear gradient of buffer A to B from 5 to 15 min, and buffer B from 15 to 30 min. Fractions were collected for radioactivity measurements by liquid scintillation counting.

The identities of the methylated purines were confirmed by paper chromatography in isopropanol/concentrated NH_3/H_2O (7:1:2) and in methanol/ethanol/concentrated HCl/H_2O (50:25:6:19). Reference compounds were localized under u.v. light, and each lane was cut transversely into 1-cm pieces, which were analyzed for radioactivity.

O^6-Methylguanine was synthesized according to Balsiger and Montgomery (1960). 7-Methylguanine, N^6-methyladenine and SAM were obtained from Sigma, and 3-methyladenine from Fluka AG.

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