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Comparative potencies of induction of point mutations and genetic duplications by the methylating agents methylazoxymethanol and dimethyl sulfate in bacteria

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Methylazoxymethanol (MAM) and dimethyl sulfate (DMS) are mutagens whose genetic effects can be ascribed to the methylation of DNA. While both methylate the N7 position of guanine heavily, only MAM strongly methylates the O^6 position of guanine. We evaluated the relative effectiveness and specificity of MAM and DMS in bacterial assays for the induction of point mutations and the formation of chromosomal duplications by genetic recombination. *Salmonella typhimurium* strain TS1121 was used to measure the formation of genetic duplications on the basis of the *aroC321* allele and mutations by reversion of the *hisG46* allele. Specific base pair substitutions and frameshift mutations were measured in a reversion assay based on *lacZ* alleles of *Escherichia coli*. The results show MAM to be the more potent mutagen and DMS the stronger recombinagen in the *Salmonella* assay. In the *lacZ* assay DMS induced several classes of base pair substitutions (GC→AT transitions, GC→TA transversions and AT→TA transversions), as well as lower frequencies of +1, –1 and –2 frameshift mutations. The activity of MAM as a base pair substitution mutagen was more specific than that of DMS, inducing only GC→AT transitions. It also induced +G, –G, –A and –CG frameshift mutations, though more weakly than it induced GC→AT transitions. Long known as a base pair substitution mutagen, the induction of frameshifts by MAM was unexpected. The results show that both DMS and MAM are effective inducers of base pair substitutions and modest inducers of frameshifts and that DMS exhibits a broader spectrum of mutagenic activity than does MAM.

Introduction

Methylazoxymethanol (MAM) and dimethyl sulfate (DMS) are monofunctional alkylating agents that methylate DNA. DMS almost exclusively alkylates nitrogen sites in the DNA bases, most strongly affecting the N7 position of guanine, followed by the N3 of adenine (Hoffmann, 1980; Loeb and Preston, 1986). In contrast, MAM distributes methyl groups more broadly, including the O^6 position of guanine (Kumari *et al*., 1985). The ratio of O^6-methylguanine to *N*7-methylguanine has been estimated as 0.003–0.004 for DMS (Zielenska *et al*., 1989; Vogel,E.W. and Nivard, 1994) and 0.188 for MAM (Kumari *et al*., 1985). O^6-alkylguanine tends to mispair when DNA replicates, causing GC→AT transitions (Vogel,E.W. and Nivard, 1994; Seo *et al*., 2000). In contrast, *N*7-methylguanine does not mispair, but it has indirect genetic consequences through secondary lesions such as abasic sites (Loeb and Preston, 1986; Zielenska *et al*., 1989; Laval *et al*., 1990; Goodman *et al*., 1993; Vogel,E.W. and Nivard, 1994; Glaab *et al*., 1999). The structures of MAM and DMS are shown in Figure 1.

Besides being useful as model compounds for studying mechanisms of mutagenesis, MAM and DMS are of interest as environmental mutagens and carcinogens. MAM is a mammalian metabolite of the colon carcinogen 1,2-dimethylhydrazine (Wolter and Frank, 1982; Kumari *et al*., 1985; Nelson *et al*., 1996). It also occurs in cycad plants in the form of its glucoside cycasin (Morgan and Hoffmann, 1983). Exposure may occur when cycads are used as foods with inadequate processing to remove MAM glycosides (Hoffmann and Morgan, 1984; Kisby *et al*., 1999). When cycasin is cleaved by the β-glucosidase activity of gut bacteria (Morgan and Hoffmann, 1983) or specific mammalian tissues, especially brain (Kisby *et al*., 1999), free MAM is released and can exert its toxicological effects. MAM is carcinogenic (Morgan and Hoffmann, 1983; Zeilmaker *et al*., 1991) and it is genotoxic in diverse assays (Morgan and Hoffmann, 1983; Hoffmann and Morgan, 1984). It has been reported to induce DNA strand breakage (Kumari *et al*., 1985), base pair substitutions and SOS induction in bacteria, gene mutations and mitotic recombination in yeast, and germ cell mutations in *Drosophila*. In mammalian systems it has been shown to induce gene mutations, sister chromatid exchange and unscheduled DNA synthesis in cultured cells and chromosome aberrations both *in vitro* and *in vivo* (Morgan and Hoffmann, 1983; Hoffmann and Morgan, 1984). The literature on MAM contains inconsistencies, however, such that positive and negative results are sometimes reported for the same genetic end point or assay (Morgan and Hoffmann, 1983).

DMS is used as a methylating agent in organic chemistry and as a model compound in genetic and toxicological studies (Hoffmann, 1980; International Agency for Research on Cancer, 1999). It is carcinogenic in laboratory animals and probably in humans (International Agency for Research on Cancer, 1999). Its genetic effects include the induction of DNA damage, mutations, genetic duplications and phage induction in bacteria; mutations in fungi, plants, *Drosophila*, fish and cultured mammalian cells; and cytogenetic alterations in plants, fish and mammalian cells (Hoffmann, 1980; Hoffmann *et al*., 1988; International Agency for Research on Cancer, 1999).

In this study we measured the relative effectiveness and specificity of MAM and DMS in assays for genetic duplications in *Salmonella typhimurium* and point mutations in *Escherichia coli*. The *aroC321* assay in *Salmonella* detects a large genetic duplication that forms by homologous recombination (Hoffmann *et al*., 1983, 1985, 1989; Hoffmann, 1992). Like other *aroC* mutants, *aroC321* strains are deficient in chorismate

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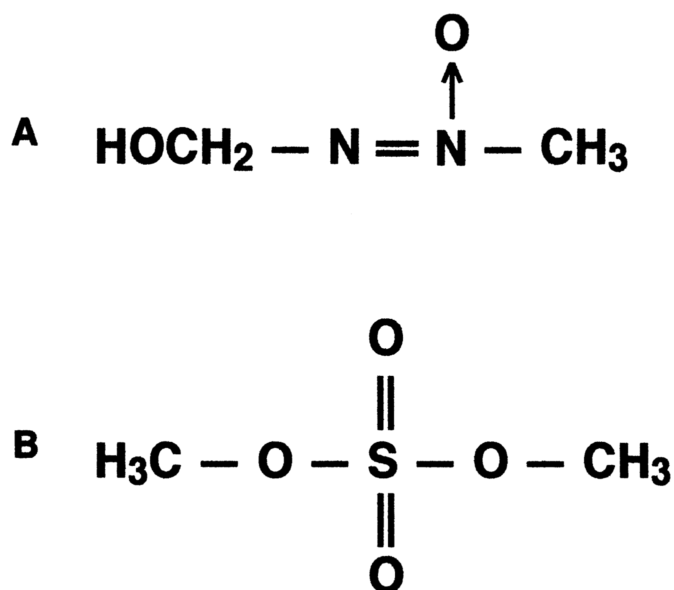

Fig. 1. Structures of the methylating agents MAM (**A**) and DMS (**B**).

synthase. They require phenylalanine, tyrosine and tryptophan. The *aroC321* allele is an unusual leaky mutation, in that *aroC321* strains give rise to genetically unstable tryptophan-independent (Trp^+) derivatives that still require phenylalanine and tyrosine. The unstable Trp^+ revertants, which arise at a high spontaneous frequency ($>10^{-4}$) and give rise to many Trp^- segregants, contain two copies of the leaky *aroC321* allele, along with a surrounding region that includes ~30% of the chromosome. Since the duplication forms by $recA^+$-dependent recombination (Hoffmann *et al*., 1985), its induction can be considered a recombinagenic effect. Strain TS1121, which contains the *aroC321* and *hisG46* alleles, permits the simultaneous measurement of recombinagenic effects and base pair substitution mutations by selecting for Trp^+ and His^+ revertants, respectively.

The *lacZ* reversion assay in *E.coli*, developed by Cupples and colleagues, detects Lac^+ revertants that result from specific base pair substitutions in strains CC101–CC106 (Cupples and Miller, 1989; Josephy, 2000) or frameshift mutations in strains CC107–CC111 (Cupples *et al*., 1990; Josephy, 2000). Each strain carries a specific *lacZ* mutation on an F′ episome and reverts by a single mutational mechanism. The pattern of reversion in the 11 strains therefore provides a simple measure of the spectrum of mutations induced.

Our results in the *aroC321* and *lacZ* assays are interpreted with respect to the relative potency of mutagenic and recombinagenic effects, methylation of oxygen and nitrogen sites in DNA, S_N1 and S_N2 mechanisms of methylation, mispairing and non-coding lesions in DNA, and the relationship of specific revertibility to forward mutation spectra.

Materials and methods

Chemicals

MAM (methylazoxymethanol acetate, CAS no. 592-62-1) was purchased from Sigma Chemical Co. (St Louis, MO). DMS (CAS no. 77-78-1) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Since they are unstable in water, aqueous solutions were prepared immediately before treatment.

Bacterial strains

Salmonella typhimurium strain TS1121 (*aroC321 hisG46*) was used to measure genetic duplications on the basis of *aroC321* and reversion of *hisG46* (Hoffmann *et al*., 1985). The *lacZ* reversion assay was conducted in *E.coli strains* CC101–CC111, obtained from Dr Claire Cupples (Concordia University, Montreal, Canada) (Cupples and Miller, 1989; Cupples *et al*., 1990; Josephy, 2000).

Media

Salmonella typhimurium strain TS1121 was grown in Difco Nutrient Broth from single colonies on Difco Nutrient Agar. Cell densities and survival were measured on Vogel–Bonner Medium E (Vogel,H.J. and Bonner, 1956) containing 1.5% Difco Bacto Agar and 2% D-glucose (Maron and Ames, 1983), supplemented with 0.2 mM L-phenylalanine, 0.2 mM L-tyrosine, 0.2 mM L-tryptophan and 0.5 mM L-histidine. Bacteria were plated in 2.5 ml of molten top agar (45°C) on the same medium lacking histidine to select for His^+ revertants. Top agar was 0.6% Difco Bacto Agar containing a trace (0.036 mM) of L-histidine and 0.5% NaCl. Dilutions were spread on medium lacking tryptophan to select for Trp^+ duplicants. *Escherichia coli* strains CC101–CC111 were picked from single colonies on LB medium (Sambrook *et al*., 1989) and cultured in Vogel–Bonner Medium E containing 2% glucose and 15 μM thiamine. The selection medium for quantifying Lac^+ revertants was Vogel–Bonner Medium E containing 0.2% α-lactose as sole carbon source.

aroC321 assay and histidine reversion in Salmonella

Two kinds of chemical exposure were used: treatment of non-growing cells in buffer and treatment in growth medium. In both cases, nutrient broth cultures of strain TS1121 were grown overnight to stationary phase in a shaker at 37°C. To treat non-growing cells, a fresh culture was centrifuged and resuspended in 67 mM phosphate buffer, pH 7. Approximately 1.2×10^9 cells in 300 μl were distributed to Eppendorf tubes containing 300 μl of freshly prepared solutions of MAM or DMS in the same buffer. The bacteria were treated in a shaker for 90 min at 37°C, centrifuged, resuspended in buffer lacking the mutagen and plated on media selective for His^+ revertants and Trp^+ duplicants. In the reversion assays, 2×10^8 cells were plated at all dosages. In duplication assays, plating densities were adjusted between 1×10^5 and 6×10^5 cells/plate to correct for anticipated toxicity so as to have approximately equal numbers of viable cells per plate in all cases. Further dilutions were plated on fully supplemented minimal medium to determine survival. To treat growing cells, 10 μl (~2×10^7 cells) of a fresh culture was inoculated into 600 μl of nutrient broth containing MAM or DMS. The bacteria were grown for 16 h in a shaker at 37°C and plated on the same media as for treatments in buffer. Plating was in triplicate and colonies were counted after 44 h at 37°C. Revertant and recombinant frequencies are reported as means with standard errors.

lacZ reversion assay in E.coli

Strains CC101–CC111 derived from single colony isolates were subcultured (10 μl in 5.5 ml) in Vogel–Bonner Medium E containing 2% D-glucose and 15 μM thiamine, grown for 16 h at 37°C, centrifuged and suspended in 50 mM phosphate buffer, pH 7, at 4×10^9 cells/ml. Treatments were initiated by mixing bacteria 1:1 with fresh solutions of MAM or DMS in the same buffer. After 2 h in a shaker at 37°C, treatments were terminated by the addition of 10 ml of 0.9% NaCl, centrifugation and resuspension in saline. Bacteria were surface plated in triplicate or quadruplicate on minimal lactose medium to select for Lac^+ revertants.

Results

The genetic activity of MAM in *S.typhimurium* strain TS1121 is shown in Table I. MAM causes dose-dependent increases in the frequencies of Trp^+ and His^+ colonies, indicating the induction of genetic duplications and base pair substitution mutations, respectively. Table II shows the recombinagenic and mutagenic effects of DMS in the same assay. Like MAM, DMS causes dose-dependent increases in the frequencies of both homologous recombination and point mutations.

Both compounds exhibit dose-dependent toxicity in strain TS1121, but DMS is toxic at lower concentrations than MAM. The difference in toxicity impedes a comparison of the two compounds at equal concentrations in a short-term treatment in buffer. While the mutagenic effect of DMS occurs at a lower absolute concentration, it is weaker than that of MAM when compared at comparable levels of toxicity. Moreover, a comparison at similar concentrations in growing bacteria (e.g. 2 mM) suggests that MAM is the more effective mutagen and DMS the more effective recombinagen. Even when compared at similar toxicities, MAM does not surpass DMS in recombi-

Table I. Induction of genetic duplications and base pair substitutions by MAM in *S.typhimurium* strain TS1121 (*aroC321 hisG46*)

MAM (mM)	Survival (%)	Relative cell density (%)	His^+ revertants per plate	Trp^+ recombinants per plate	Revertants per 10^8 cells	Recombinants per 10^4 cells
(A) Treatment in phosphate buffer (pH 7, 90 min, 37°C)						
0	100		0.3	53.0	0.1 ± 0.1	3.7 ± 0.18
8	98		82.7	58.7	29.7 ± 3.7	4.2 ± 0.17
16	94		1238.7	95.3	463.9 ± 21.0	7.2 ± 0.24
32	93		4581.0	294.0	1735.2 ± 112.4	16.7 ± 0.78
64	82		11274.0	499.0	4840.1 ± 427.8	21.4 ± 0.54
(B) Treatment in growth medium (16 h, 37°C)						
0		100	0.3	38.3	0.1 ± 0.1	1.3 ± 0.4
2		98	10393.3	224.3	1853 ± 353	8.0 ± 1.0
4		87	13884.0	488.0	2788 ± 302	14.7 ± 1.0
8		71	9987.7	653.3	2460 ± 370	16.1 ± 1.6
16		52	13804.3	1246.3	4648 ± 779	27.6 ± 4.1
32		20	12746.0	734.7	11181 ± 809	21.4 ± 1.5

Table II. Induction of genetic duplications and base pair substitutions by DMS in *S.typhimurium* strain TS1121 (*aroC321 hisG46*)

DMS (mM)	Survival (%)	Relative cell density (%)	His^+ revertants per plate	Trp^+ recombinants per plate	Revertants per 10^8 cells	Recombinants per 10^4 cells
(A) Treatment in phosphate buffer (pH 7, 90 min, 37°C)						
0	100		0.3	60.0	0.1 ± 0.1	4.1 ± 0.3
0.5	95		143.0	103.7	51.8 ± 3.0	7.5 ± 1.0
1	43		376.7	180.0	301.3 ± 81.9	14.4 ± 0.7
2	4		102.0	98.3	879.3 ± 86.3	28.1 ± 3.8
(B) Treatment in growth medium (16 h, 37°C)						
0		100	0.0	79.0	0.0 ± 0.0	2.8 ± 0.2
0.5		94	78.3	181.3	14.6 ± 2.5	6.8 ± 0.5
1		74	627.0	302.0	148.6 ± 8.7	14.3 ± 0.5
2		57	1220.0	663.0	375.4 ± 1.3	30.7 ± 1.6
4		57	1300.5	784.5	400.2 ± 6.3	24.1 ± 1.2

nagenicity. The comparison of mutagenic and recombinagenic potencies is clearest when calculated as slopes of induced recombinational events per induced mutation: 36–57 for MAM and 262–705 for DMS. Thus, the *aroC321* assay shows DMS to be highly recombinagenic relative to its mutagenic activity, whereas MAM is a more potent mutagen than a recombinagen.

Table III shows the spectrum of reversion induced by MAM in the *lacZ* reversion assay. MAM is a potent inducer of GC→AT transitions in CC102, but it elicited no response in any of the other base pair substitution strains. It also induced +G, –G, –A and –CG mutations in the frameshift tester strains. The frameshift mutagenicity of MAM is weaker than its induction of transitions. Table IV shows the spectrum of reversion induced by DMS under the same conditions. Statistically significant, dose-dependent increases in revertant frequency were observed in strain CC102, which detects GC→AT transitions, strain CC104, which detects GC→TA transversions, and strain CC105, which detects AT→TA transversions. Thus, DMS exhibited a broader spectrum of mutagenic activity than MAM in the *lacZ* reversion assay. The responses to DMS in the transversion strains were weaker than those in strain CC102. DMS was also mutagenic in four of the five strains that revert by frameshift mutations: +G frameshifts in CC107, –G frameshifts in CC108, –CG frameshifts in CC109 and –A frameshifts in CC111. On the basis of numbers of induced revertants at a given dosage or fold increase over numbers of spontaneous revertants, the frameshift mutagenicity is small relative to the prominent induction of base pair substitutions.

Discussion

Differences among alkylating agents in mutagenicity and carcinogenicity have long been ascribed to the relative S_N1 and S_N2 character of the reaction mechanisms (Osterman-Golkar *et al*., 1970; Lawley, 1974; Hoffmann, 1980; Vogel,E. and Natarajan, 1982; Zielenska *et al*., 1989; Elespuru *et al*., 1991; Glaab *et al*., 1999). Agents with a high Swain–Scott substrate constant (Swain and Scott, 1953), such as DMS (Vogel,E.W. and Nivard, 1994), have been described as typical S_N2 alkylating agents that selectively methylate highly nucleophilic sites, such as base nitrogens in DNA (Hoffmann, 1980; Zielenska *et al*., 1989). In contrast, agents such as *N*-methyl-*N*-nitrosourea (MNU) that have a lower Swain–Scott constant (Vogel,E.W. and Nivard, 1994) and alkylate oxygen sites have been described as having more S_N1 character (Zielenska *et al*., 1989). Loechler (1994) has argued persuasively that interpreting the mutagenicity of alkylating agents with respect to S_N2 and S_N1 mechanisms is incorrect. His reasoning is supported on several grounds, notably including evidence that all the commonly discussed alkylating agents act through S_N2 mechanisms and that the Swain–Scott principle does not properly describe the distribution of alkyl groups in DNA. With highly reactive alkylating agents such as *N*-ethyl-*N*-nitrosourea, oxygen sites are disproportionately affected relative to what one might expect on the basis of their relative nucleophilicity. Loechler suggests that 'high oxyphilic' versus 'low oxyphilic' would be better descriptors than S_N1 and S_N2 for the reactions of alkylating agents with DNA.

Table III. Mutagenicity of MAM in the *lacZ* reversion assay in *E.coli*

Strain[a]	Reversion mechanism[a]	MAM (mM)	Revertants per plate (± SEM)[b]
CC102	GC→AT	0	0.75 ± 0.25
		8	10.00 ± 0.71[c]
		16	64.75 ± 3.47[d]
		32	274.50 ± 18.31[d]
		64	535.25 ± 11.69[d]
		128	674.00 ± 3.49[d]
CC107	+1 (+G)	0	10.00 ± 0.41
		8	17.25 ± 1.38[e]
		16	25.75 ± 0.63[d]
		32	51.75 ± 2.56[d]
		64	127.25 ± 1.93[d]
		128	145.50 ± 2.33[d]
CC108	–1 (–G)	0	3.25 ± 0.48
		8	2.00 ± 0.71[c]
		16	5.75 ± 1.38[c]
		32	11.50 ± 0.50[e]
		64	38.75 ± 3.07[d]
		128	15.25 ± 3.07[d]
CC109	–2 (–CG)	0	49.00 ± 3.24
		8	51.00 ± 3.54[c]
		16	48.00 ± 3.14[c]
		32	62.75 ± 5.69[c]
		64	118.75 ± 6.90[d]
		128	120.75 ± 3.57[d]
CC111	–1 (–A)	0	9.00 ± 0.71
		8	8.50 ± 0.96[c]
		16	11.75 ± 0.85[c]
		32	14.00 ± 0.71[d]
		64	19.25 ± 1.31[d]
		128	8.00 ± 0.71[c]

[a]There were no dose-related or statistically significant increases in numbers of revertants per plate in strains CC101 (AT→CG), CC103 (GC→CG), CC104 (GC→TA), CC105 (AT→TA), CC106 (AT→GC) and CC110 (+A).
[b]Analysis of variance with a Dunnett multiple comparisons test.
[c]NS, non-significant.
[d]$P < 0.01$.
[e]$P < 0.05$.

Table IV. Mutagenicity of DMS in the *lacZ* reversion assay in *E.coli*

Strain[a]	Reversion mechanism[a]	DMS (mM)	Revertants per plate (± SEM)[b]
CC102	GC→AT	0.0	0.33 ± 0.33
		0.125	4.00 ± 1.53[c]
		0.25	47.00 ± 3.79[d]
		0.5	226.33 ± 1.45[d]
		1.0	479.67 ± 4.33[d]
		2.0	455.33 ± 12.17[d]
CC104	GC→TA	0.0	1.00 ± 0.41
		0.125	2.75 ± 0.48[c]
		0.25	4.50 ± 0.87[c]
		0.5	8.00 ± 1.29[e]
		1.0	15.00 ± 1.47[d]
		2.0	38.00 ± 2.61[d]
CC105	AT→TA	0.0	2.00 ± 0.71
		0.125	2.75 ± 0.75[c]
		0.25	3.00 ± 0.71[c]
		0.5	8.75 ± 0.85[d]
		1.0	14.50 ± 1.55[d]
		2.0	20.25 ± 1.31[d]
CC107	+1 (+G)	0.0	15.50 ± 1.55
		0.125	23.75 ± 1.03[e]
		0.25	40.50 ± 1.26[d]
		0.5	73.00 ± 2.27[d]
		1.0	74.25 ± 3.07[d]
		2.0	10.50 ± 1.55[c]
CC108	–1 (–G)	0.0	9.50 ± 0.87
		0.125	11.25 ± 0.75[c]
		0.25	10.00 ± 0.41[c]
		0.5	39.75 ± 3.71[d]
		1.0	65.25 ± 3.75[d]
		2.0	62.75 ± 4.44[d]
CC109	–2 (–CG)	0.0	66.00 ± 2.68
		0.125	63.75 ± 1.11[c]
		0.25	106.00 ± 4.14[d]
		0.5	142.75 ± 6.17[d]
		1.0	166.75 ± 4.01[d]
		2.0	196.50 ± 7.71[d]
CC111	–1 (–A)	0.0	7.25 ± 0.48
		0.125	10.50 ± 0.87[c]
		0.25	17.75 ± 1.49[d]
		0.5	26.25 ± 2.29[d]
		1.0	33.50 ± 3.59[d]
		2.0	27.25 ± 1.75[d]

[a]There were no dose-related or statistically significant increases in numbers of revertants per plate in strains CC101 (AT→CG), CC103 (GC→CG), CC106 (AT→GC) and CC110 (+A).
[b]Analysis of variance with a Dunnett multiple comparisons test.
[c]NS, non-significant.
[d]$P < 0.01$.
[e]$P < 0.05$.

Differences between MAM and DMS in recombinagenic and mutagenic potencies and in mutation spectra can be explained by their patterns of alkylation of DNA. Following the terminology of Loechler (1994), MAM is a highly oxyphilic mutagen whereas DMS is not. The difference is reflected in the O^6/N7 ratio of alkylation: ~0.188 for MAM (Kumari *et al*., 1985) and 0.003–0.004 for DMS (Zielenska *et al*., 1989; Vogel,E.W. and Nivard, 1994). O^6-methylguanine tends to mispair with thymine, causing GC→AT transitions (Seo *et al*., 2000). Unlike the mispairing bases O^6-alkylguanine and O^4-alkylthymine (Richardson *et al*., 1987; Zielenska *et al*., 1989; Seo *et al*., 2000), other alkylated bases exert their genetic effects indirectly through secondary, non-coding lesions. *N*7-methylguanine and *N*3-methyladenine tend to undergo depurination, which produces abasic sites (Loeb and Preston, 1986; Laval *et al*., 1990; Vogel,E.W. and Nivard, 1994; Glaab *et al*., 1999), and N7 methylation triggers ring-opening, producing formamidopyrimidine derivatives (Laval *et al*., 1990; Glaab *et al*., 1999). Abasic sites hinder replication (Goodman *et al*., 1994) and they give rise to base pair substitutions (Goodman *et al*., 1993, 1994; Glaab *et al*., 1999), including transitions (Glaab *et al*., 1999) and especially GC→TA transversions (Loeb and Preston, 1986; Laval *et al*., 1990; Strauss, 1991). Mutagens also stimulate inducible responses that process DNA damage, sometimes giving rise to mutations in doing so. The mutagenic consequences are well known for some of these responses, such as the SOS system, but less so for others. DMS has been shown to induce UVM (Humayun, 1998), an inducible response designated 'UV modulation of mutagenesis' that is independent of the SOS and adaptive responses and processes non-coding lesions into mutations (Wang *et al*., 1995).

The large increases that we observed in the frequency of His$^+$ revertants at non-toxic concentrations in the *Salmonella* assay (Table I) confirm earlier studies showing that MAM is a potent mutagen (Morgan and Hoffmann, 1983). The specificity of its base pair substitution mutagenesis for strain CC102 in the *lacZ* assay (Table III) is consistent with the finding that 23 of 24 forward mutations recovered in the *lacI* gene after treatment of *E.coli* with MAM acetate in a mouse host-mediated assay were GC→AT transitions (Zeilmaker *et al*., 1991). Our finding that MAM induces several classes of

frameshift mutations is unexpected. Most assays of MAM for frameshift mutagenesis have been negative (Morgan and Hoffmann, 1983), though there is an isolated positive result with mammalian metabolic activation in an Ames assay in *Salmonella* strain TA1538 (Simmon, 1979).

Like previous studies, our results (Tables II and IV) indicate that DMS is a base pair substitution mutagen. The modest frameshift mutagenicity of DMS is also compatible with earlier studies, in that DMS has given both weakly positive and negative results in assays for frameshift mutations (Hoffmann, 1980). A forward mutation spectrum determined for DMS in the *lacI* gene in *E.coli* (Zielenska *et al*., 1989) showed a predominance of base pair substitutions (111 of 121 mutations). They were primarily GC→AT transitions (90/111), followed in frequency by GC→TA transversions (14/111) and a few AT→GC transitions (3/111) and AT→TA transversions (4/111). The forward mutation spectrum is therefore similar to our results in the *lacZ* reversion assay, in which DMS induced predominantly GC→AT transitions with lesser induction of GC→TA and AT→TA transversions (Table IV). In contrast to DMS, the oxyphilic methylating agent MNU induced exclusively GC→AT transitions (39/39) in the *gpt* gene of *E.coli* (Richardson *et al*., 1987), a spectrum compatible with our finding that GC→AT was the only base pair substitution induced by the oxyphilic MAM in the *lacZ* reversion assay.

Abasic sites resulting from depurination of *N*7-methylguanine and *N*3-methyladenine (Loeb and Preston, 1986) can explain the induction by DMS of GC→TA and AT→TA transversions, respectively, as adenine is preferentially inserted opposite abasic sites (Loeb and Preston, 1986; Strauss, 1991). The induction of GC→AT transitions by DMS is harder to explain and is apt to involve multiple mechanisms. While there is relatively little O^6-methylguanine produced, DMS is an effective alkylator, and a small fraction of the methyl groups are on this position (Zielenska *et al*., 1989; Vogel,E.W. and Nivard, 1994). These infrequent but highly mutagenic lesions probably contribute to the GC→AT transitions. Transitions may also stem from the major alkylation products through depurination, as bases other than adenine are sometimes inserted opposite abasic sites (Loeb and Preston, 1986; Strauss, 1991). In a study of depurination in *E.coli*, thymine was inserted half as frequently as adenine opposite abasic sites resulting from the loss of guanine by depurination, making it the second most frequently inserted base (Kunkel, 1984). Thus, the yield of GC→AT transitions following DMS treatment may reflect an accumulation from mispairing of rare O^6-methylguanine residues, insertion of thymine opposite sites of depurination and, perhaps, other less well characterized mechanisms.

A conceptual question about the use of specific reversion assays is whether they provide information comparable to more laborious forward mutation assays or whether other influences, most notably effects of neighboring bases and the general sequence context, are so prominent that reversion assays cannot provide a realistic sense of the mutation spectrum. Sequences may differ in specificity because the efficiency of bypass of abasic sites and of insertion of bases is influenced by neighboring bases (Goodman *et al*., 1994). However, in the case of MAM and DMS in the *lacZ* reversion assay (Tables III and IV), the results for base pair substitutions are compatible with published forward mutation spectra (Zielenska *et al*., 1989; Zeilmaker *et al*., 1991). The similarity suggests that neighboring base influences are relatively small or that *lacZ* strain CC102 fortuitously mimics the most sensitive sequence in forward mutation assays (Zielenska *et al*., 1989; Goodman *et al*., 1994) in having a purine residue (G in CC102) 5′ to the target base.

A coherent view of the base pair substitution mutagenicity of methylating agents emerges by combining the results for MAM and DMS in the *lacZ* reversion assay with data reported by Ohta *et al*. (2000) for methyl methanesulfonate (MMS), *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) and MNU in the same assay. MMS resembles DMS in methylating nitrogen sites almost exclusively, whereas MNU and MNNG, like MAM, also effectively methylate oxygen sites (Vogel,E.W. and Nivard, 1994). The base pair substitutions induced by MNU and MNNG were 97 and 96% GC→AT transitions, respectively (Ohta *et al*., 2000). In contrast, only 56% of the mutations induced by MMS were GC→AT transitions, and there was a substantial increase in transversions: 22% AT→TA and 16% GC→TA (Ohta *et al*., 2000). Thus, both data sets indicate that oxyphilic alkylators (e.g. MAM) are more specific for GC→AT transitions than non-oxyphilic alkylators. The greater proportion of transversions among the mutations induced by MMS in the study of Ohta *et al*. (2000) than by DMS in our study may be ascribable to their using *uvrA* pKM101 strains, in that the *mucAB* genes carried by pKM101 enhance the induction of transversions more strongly than transitions (Watanabe *et al*., 1994).

By using strains CC107–CC111, we observed that MAM and DMS induce frameshift mutations, though more weakly than they induce base pair substitutions. The modest induction of frameshifts may be ascribable to these agents stimulating or stabilizing slipped mispairing (Streisinger *et al*., 1966; Hoffmann and Fuchs, 1997) in the repetitive target sites of the *lacZ* frameshift strains. It is also possible that the frameshift mutagenesis occurs indirectly through the saturation of mismatch repair, as proposed by Cupples *et al*. (1990) for ethyl methanesulfonate and MNNG in the *lacZ* reversion assay.

The potent mutagenicity of MAM in the *Salmonella* assay (Table I) and its induction of GC→AT transitions in the *lacZ* assay (Table III) are probably ascribable to O^6 methylation, whereas the broader spectrum of base pair substitutions induced by DMS (Table IV) undoubtedly encompasses mechanisms more indirect than mispairing, including the processing of apurinic sites. The strong recombinagenicity of DMS (Table II) suggests greater dependence on absolute amounts of DNA damage, including depurinating lesions, than on mispairing lesions. N7 methylation, while less mutagenic than O^6 methylation, is probably effective in stimulating the recombinational change that leads to genetic duplications.

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References

Cupples,C.G. and Miller,J.H. (1989) A set of *lacZ* mutations in *Escherichia coli* that allow rapid detection of each of the six base substitutions. *Proc. Natl Acad. Sci. USA*, **86**, 5345–5349.

Cupples,C.G., Cabrera,M., Cruz,C. and Miller,J.H. (1990) A set of *lacZ* mutations in *Escherichia coli* that allow rapid detection of specific frameshift mutations. *Genetics*, **125**, 275–280.

Elespuru,R.K., Stupar,L.L. and Gordon,J.A. (1991) Discrimination of mutagenic intermediates derived from alkylating agents by mutational patterns generated in *Escherichia coli*. *Carcinogenesis*, **12**, 1161–1167.

Glaab,W.R., Tindall,K.R. and Skopek,T.R. (1999) Specificity of mutations induced by methyl methanesulfonate in mismatch repair-deficient human cancer cell lines. *Mutat. Res.*, **427**, 67–78.

Goodman,M.F., Creighton,S., Bloom,L.B. and Petruska,J. (1993) Biochemical basis of DNA replication fidelity. *Crit. Rev. Biochem. Mol. Biol.*, **28**, 83–126.

Goodman,M.F., Cai,H., Bloom,L.B. and Eritja,R. (1994) Nucleotide insertion and primer extension at abasic template sites in different sequence contexts. *Ann. N. Y. Acad. Sci.*, **726**, 132–142.

Hoffmann,G.R. (1980) Genetic effects of dimethyl sulfate, diethyl sulfate, and related compounds. *Mutat. Res.*, **75**, 63–129.

Hoffmann,G.R. (1992) Bacterial assays for recombinagens. *Mutat. Res.*, **284**, 125–146.

Hoffmann,G.R. and Fuchs,R.P.P. (1997) Mechanisms of frameshift mutations: insight from aromatic amines. *Chem. Res. Toxicol.*, **10**, 347–359.

Hoffmann,G.R. and Morgan,R.W. (1984) Putative mutagens and carcinogens in foods: V. Cycad azoxyglycosides. *Environ. Mutagen.*, **6**, 103–116.

Hoffmann,G.R., Walkowicz,M.J., Mason,J.M. and Atkins,J.F. (1983) Genetic instability associated with the *aroC321* allele in *Salmonella typhimurium* involves genetic duplication. *Mol. Gen. Genet.*, **190**, 183–188.

Hoffmann,G.R., Catuogno,L.S., Linnane,J.F. and Parente,L.A. (1985) Effects of DNA-repair processes on the induction of genetic duplications in bacteria by ultraviolet light. *Mutat. Res.*, **151**, 25–33.

Hoffmann,G.R., Boyle,J.F. and Freemer,C.S. (1988) Induction of genetic duplications in *Salmonella typhimurium* by dialkyl sulfates. *Environ. Mol. Mutagen.*, **11**, 545–551.

Hoffmann,G.R., Freemer,C.S. and Parente,L.A. (1989) Induction of genetic duplications and frameshift mutations in *Salmonella typhimurium* by acridines and acridine mustards: dependence on covalent binding of the mutagen to DNA. *Mol. Gen. Genet.*, **218**, 377–383.

Humayun,M.Z. (1998) SOS and Mayday: multiple inducible mutagenic pathways in *Escherichia coli*. *Mol. Microbiol.*, **30**, 905–910.

International Agency for Research on Cancer (1999) Dimethyl sulfate. *IARC Monogr. Eval. Carcinog. Risk Chem. Hum.*, **71**, 575–588.

Josephy,P.D. (2000) The *Escherichia coli lacZ* reversion mutagenicity assay. *Mutat. Res.*, **455**, 71–80.

Kisby,G.E., Kabel,H., Hugon,J. and Spencer,P. (1999) Damage and repair of nerve cell DNA in toxic stress. *Drug Metab. Rev.*, **31**, 589–618.

Kumari,M.L., Kamat,P.L., D'Ambrosio,S.M., Witiak,D.T. and Milo,G.E. (1985) A comparative study of dimethylhydrazine regio-isomers and the methylazoxymethanol metabolite of 1,1- and 1,2-dimethylhydrazine in relation to transformation in human fibroblasts. *Cancer Lett.*, **29**, 265–275.

Kunkel,T.A. (1984) Mutational specificity of depurination. *Proc. Natl Acad. Sci. USA*, **81**, 1494–1498.

Laval,J., Boiteux,S. and O'Connor,T.R. (1990) Physiological properties and repair of apurinic/apyrimidinic sites and imidazole ring-opened guanines in DNA. *Mutat. Res.*, **233**, 73–79.

Lawley,P.D. (1974) Some chemical aspects of dose-response relationships in alkylation mutagenesis. *Mutat. Res.*, **23**, 283–295.

Loeb,L.A. and Preston,B.D. (1986) Mutagenesis by apurinic/apyrimidinic sites. *Annu. Rev. Genet.*, **20**, 201–230.

Loechler,E.L. (1994) A violation of the Swain-Scott principle, and *not* S_N1 versus S_N2 reaction mechanisms, explains why carcinogenic alkylating agents can form different proportions of adducts at oxygen versus nitrogen in DNA. *Chem. Res. Toxicol.*, **7**, 277–280.

Maron,D.M. and Ames,B.N. (1983) Revised methods for the Salmonella mutagenicity test. *Mutat. Res.*, **113**, 173–215.

Morgan,R.W. and Hoffmann,G.R. (1983) Cycasin and its mutagenic metabolites. *Mutat. Res.*, **114**, 19–58.

Nelson,R.L., Abcarian,H., Nelson,T.M., Misumi,A., Kako,H., Rizk,S. and Sky-Peck,H. (1996) The effect of dietary selenium deficiency on acute colorectal mucosal nucleotoxicity induced by several carcinogens in the rodent. *Am. J. Surg.*, **172**, 85–88.

Ohta,T., Watanabe-Akanuma,M. and Yamagata,H. (2000) A comparison of mutation spectra detected by the *Escherichia coli* Lac^+ reversion assay and the *Salmonella typhimurium* His^+ reversion assay. *Mutagenesis*, **15**, 317–323.

Osterman-Golkar,S., Ehrenberg,L. and Wachtmeister,C.A. (1970) Reaction kinetics and biological action in barley of mono-functional methanesulfonic esters. *Radiat. Bot.*, **10**, 303–327.

Richardson,K.K., Richardson,F.C., Crosby,R.M., Swenberg,J.A. and Skopek,T.R. (1987) DNA base changes and alkylation following *in vivo* exposure of *Escherichia coli* to N-methyl-N-nitrosourea or N-ethyl-N-nitrosourea. *Proc. Natl Acad. Sci. USA*, **84**, 344–348.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Seo,K.-Y., Jelinsky,S.A. and Loechler,E.L. (2000) Factors that influence the mutagenic patterns of DNA adducts from chemical carcinogens. *Mutat. Res.*, **463**, 215–246.

Simmon,V.F. (1979) *In vitro* mutagenicity assays of chemical carcinogens and related compounds with *Salmonella typhimurium*. *J. Natl Cancer Inst.*, **62**, 893–899.

Strauss,B.S. (1991) The 'A rule' of mutagen specificity: a consequence of DNA polymerase bypass of non-instructional lesions? *BioEssays*, **13**, 79–84.

Streisinger,G., Okada,Y., Emrich,J., Newton,J., Tsugita,A., Terzaghi,E. and Inouye,M. (1966) Frameshift mutations and the genetic code. *Cold Spring Harbor Symp. Quant. Biol.*, **31**, 77–84.

Swain,C.G. and Scott,C.B. (1953) Quantitative correlation of relative rates. Comparison of hydroxide ion with other nucleophilic reagents toward alkyl halides, esters, epoxides, and acyl halides. *J. Am. Chem. Soc.*, **75**, 141–147.

Vogel,E. and Natarajan,A.T. (1982) The relation between reaction kinetics and mutagenic action of monofunctional alkylating agents in higher eukaryotic systems: interspecies comparisons. In deSerres,F.J. and Hollaender,A. (eds), *Chemical Mutagens: Principles and Methods for Their Detection.* Plenum Press, New York, NY, Vol. 7, pp. 295–336.

Vogel,E.W. and Nivard,M.J.M. (1994) The subtlety of alkylating agents in reactions with biological macromolecules. *Mutat. Res.*, **305**, 13–32.

Vogel,H.J. and Bonner,D.M. (1956) Acetylornithinase of *E.coli*: a partial purification and some properties. *J. Biol. Chem.*, **218**, 97–106.

Wang,G., Palejwala,V.A., Dunman,P.M., Aviv,D.H., Murphy,H.S., Rahman,M.S. and Humayun,M.Z. (1995) Alkylating agents induce UVM, a *recA*-independent inducible mutagenic phenomenon in *Escherichia coli*. *Genetics*, **141**, 813–823.

Watanabe,M., Nohmi,T. and Ohta,T. (1994) Effects of the *umuDC*, *mucAB* and *samAB* operons on the mutational specificity of chemical mutagenesis in *Escherichia coli*: II. Base substitution mutagenesis. *Mutat. Res.*, **314**, 39–49.

Wolter,S. and Frank,N. (1982) Metabolism of 1,2-dimethylhydrazine in isolated perfused rat liver. *Chem.-Biol. Interact.*, **42**, 335–344.

Zeilmaker,M.J., Horsfall,M.J., van Helten,J.B.M., Glickman,B.W. and Mohn,G.R. (1991) Mutational specificities of environmental carcinogens in the *lacI* gene of *Escherichia coli* H. V: DNA sequence analysis of mutations in bacteria recovered from the liver of Swiss mice exposed to 1,2-dimethylhydrazine, azoxymethane, and methylazoxymethanolacetate. *Mol. Carcinog.*, **4**, 180–188.

Zielenska,M., Horsfall,M.J. and Glickman,B.W. (1989) The dissimilar mutational consequences of S_N1 and S_N2 DNA alkylation pathways: clues from the mutational specificity of dimethylsulphate in the *lacI* gene of *Escherichia coli*. *Mutagenesis*, **4**, 230–234.

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