DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards

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ABSTRACT

Nitrogen mustards alkylate DNA primarily at the N^7 position of guanine. Using an approach analogous to that of the Maxam-Gilbert procedure for DNA sequence analysis, we have examined the relative frequencies of alkylation for a number of nitrogen mustards at different guanine-N7 sites on a DNA fragment of known sequence. Most nitrogen mustards were found to have similar patterns of alkylation, with the sites of greatest alkylation being runs of contiguous guanines, and relatively weak alkylation at isolated guanines. Uracil mustard and quinacrine mustard, however, were found to have uniquely enhanced reaction with at least some 5'-PyGCC-3' and 5'-GT-3' sequences, respectively. In addition, quinacrine mustard showed a greater reaction at runs of contiguous guanines than did other nitrogen mustards, whereas uracil mustard showed little preference for these sequences. A comparison of the sequence-dependent variations of molecular electrostatic potential at the N^7-position of guanine with the sequence dependent variations of alkylation intensity for mechlorethamine and L-phenylalanine mustard showed a good correlation in some regions of the DNA, but not others. It is concluded that electrostatic interactions may contribute strongly to the reaction rates of cationic compounds such as the reactive aziridinium species of nitrogen mustards, but that other sequence selectivities can be introduced in different nitrogen mustard derivatives.

INTRODUCTION

Mechlorethamine (bis(2-chloroethyl)methylamine, nitrogen mustard, HN2) was the first clinically effective anticancer drug to be discovered (1). After more than 30 years of intensive drug development efforts, the nitrogen mustard derivatives L-phenylalanine mustard, cyclophosphamide, and chlorambucil are still among the most useful clinical agents (2). These compounds are known to alkylate DNA preferentially at guanine-N7 positions (3,4). Antitumor activity and high potency cell killing require the presence of two chloroethyl groups per nitrogen mustard molecule, probably because the effective DNA lesions are crosslinks (4-7).Crosslinks through bifunctional alkylation of guanine-N7 positions in a right-handed B form DNA helix can arise either by reaction with two adjacent guanines in the

same DNA strand or with guanines in opposite strands in the sequence:

5'-GC-3' .

3'-CG-5'

It is not known how such DNA lesions would selectively kill certain tumor cells, but it possibly could involve selective reactions with particular GC-rich regions in the cell genome.

Sequence selective reactions with DNA has been observed in the case of several compounds, including bleomycin (8-10), N-acetoxy-N2-acetylaminofluorene (11), mitomycin C (12), benzo(a)pyrene (13), aflatoxins (14,15), cis-dichlorodiammine platinum(II) (16) and chloroethylnitrosoureas (17). The relative extents of guanine-N7 addition reactions at various guanines can in principle be determined by an application of the rapid Maxam and Gilbert chemical method of DNA sequence determination (8,18). Grunberg and Haseltine (19) showed that this method can be applied to nitrogen mustards. In a 92 base pair fragment of human alpha DNA, data were obtained suggestive of sequence selective reactions of nitrogen mustard (HN2), as well as of differences between nitrogen mustard derivatives; however these results were not definitive and were not stated as firm conclusions.

The present investigation aims to determine the nature and degree of sequence selectivity of guanine-N7 alkylation of isolated DNA by several nitrogen mustards and to determine whether the sequence selectivity can be modified by structural alteration of the nitrogen mustard molecule.

MATERIALS AND METHODS

Mechlorethamine (bis-2-chloroethylmethylamine hydrochloride; HN2) was donated by Merck, Sharp and Dohme Research Lab. L-phenylalanine mustard, uracil mustard (NSC 34462), chlorambucil, phosphoramide mustard (NSC 26271), spiromustine (NSC 172112) and mustamine (NSC 364989) were obtained through the Developmental Therapeutics Program, National Cancer Institute. The following reagents were obtained from commercial sources: quinacrine mustard and triethanolamine, Fluka Chemical Corp.; dimethylsulfate (99.9%), Aldrich Chemical Company; piperidine, Fisher; T4 polynucleotide kinase and pBR322 DNA, Pharmacia P-L Biochemicals; Eco RI and Bam HI, New England Biolab; Sal I, International Biotechnologies Inc.; Hind III and ultrapure urea, Bethesda Research Laboratories; [gamma-^{32}P]ATP (7000 Ci/mmole), New England Nuclear.

Preparation of End-labeled DNA Fragments

The 3741 base pair Hind III to Sal 1 fragment of pBR322 was labeled at the 5' end of the Hind III site with T4 polynucleotide kinase as described by Maxam and Gilbert (18). The 276 base pair Bam HI to Sal I fragment 5' labeled at the Bam HI site was prepared similarly. Isolation of the fragments was by preparative electrophoresis on 0.8% agarose gels.

Alkylation Reaction

Labeled DNA was incubated with alkylating agent in a buffer of 1 mM EDTA, 25 mM triethanolamine HCl, pH 7.2, in a total volume of 50 µl. After incubation at 20°C for 60 minutes, 50 µl of an ice-cold solution

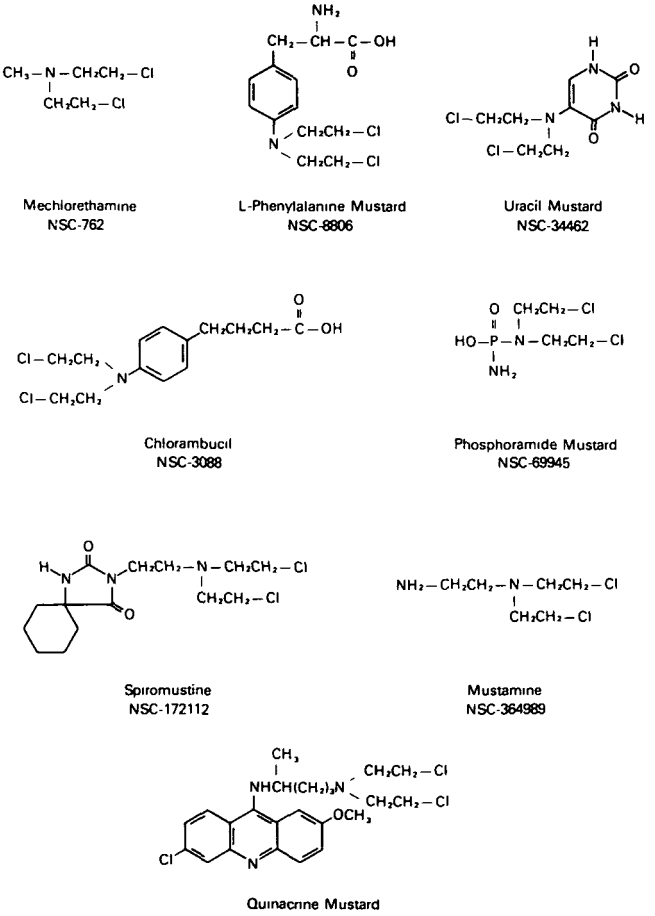


Figure 1. Structures of the nitrogen mustards used in this study.

containng 0.6 M sodium acetate, 20 mM EDTA, and 100 μg/ml tRNA was added and the DNA recovered by precipitation with three volumes of ethanol. After resuspending the pellet in 0.3 M sodium acetate, 1 mM EDTA, the DNA was ethanol precipitated again and the pellet washed with cold ethanol prior to vacuum drying.

Breaks at sites of N7-guanine alkylation were created by resuspending the salt-free DNA pellet in freshly diluted 1 M piperdine and incubating at 90°C for 20 minutes (18). Creation of breaks at alkylation sites is complete under these conditions; further incubation results in degradation of control DNA (unpublished data). After lyophilisation the radioactivity in each sample was determined by Cerenkov counting and the samples resuspended in loading buffer (18) to give 15,000 cpm/μl. Samples were heated at 90°C for 1 minute, and then chilled in an ice-bath before loading onto the gel.

Polyacrylamide Gel Electrophoresis

Electrophoresis of the DNA fragments was on 0.4 mm x 90 cm x 20 cm 6% polyacrylamide gels containing 7 M urea and a Tris-boric acid-EDTA buffer system (18). 2 μl samples were loaded and run for 3 hours at approximately 3600 volts. Following autoradiography of the dried gel, relative band intensities were determined by microdensitometry using a Beckman DU-8 scanning spectrophotometer with gel scanning accessory. The extent of alkylation for any dose of drug was determined by comparing the integrated area of the band corresponding to the full length fragment for the treated sample with that for an untreated sample and using the absolute value of the natural logarithm of that ratio to give the average number of breaks per molecule (13).

RESULTS

N7-guanine alkyl adducts render the imidazole ring of guanine susceptible to ring opening at elevated pH (20). Treatment with the secondary

Figure 2. The 3741 bp Hind III - Sal I fragment of plasmid pBR322 DNA, labeled at 5' end of the Hind III site, was reacted with the indicated compounds, precipitated and electrophoresed as described in Materials and Methods. Lane a: no drug; lane b: 250 μM phosphoramide mustard (phosphoramide mustard is a reactive metabolite of cyclophosphamide); lane c: 250 μM chlorambucil; lane d: 0.1 μM mustamine; lane e: 10 μM uracil mustard; lane f: 20 μM mechlorethamine; lane g: 50 μM L-phenylalanine mustard; lane h: 0.05 μM quinacrine mustard; lane i: 5 μM spiromustine; lane j: 500 μM dimethyl sulfate; lane k: A+G reaction (depurination with formic acid). Arrows indicate sites of preferential alkylation with mustamine (lane d).

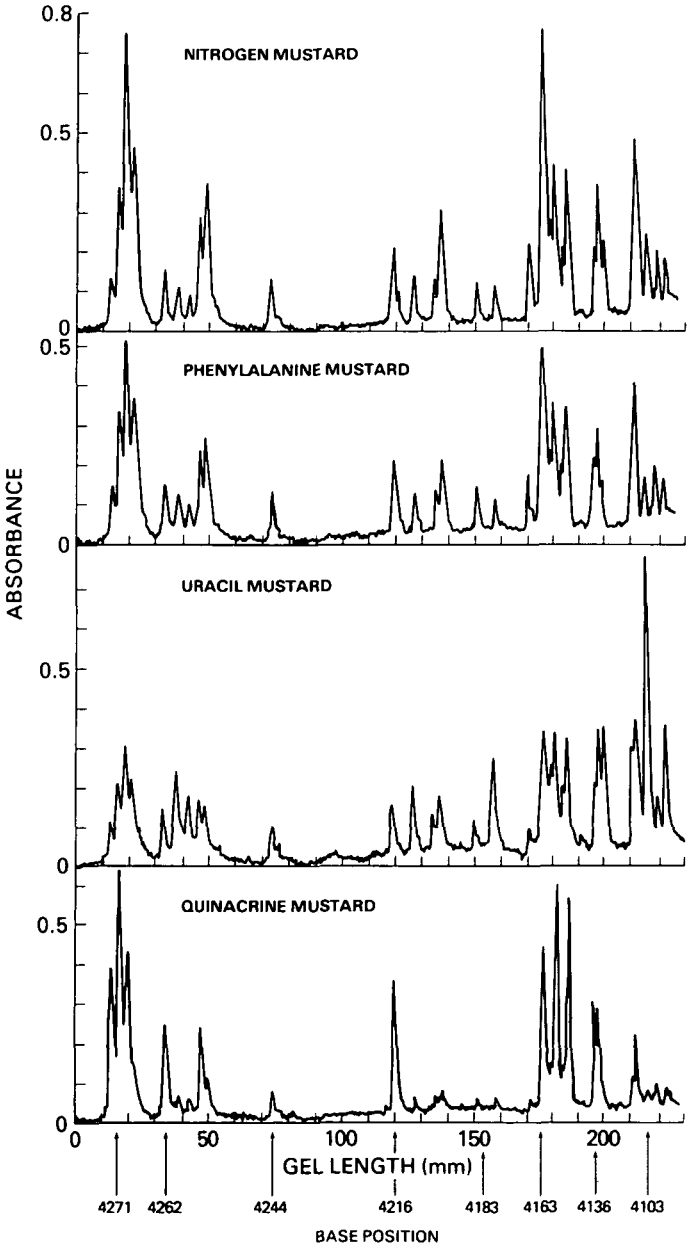
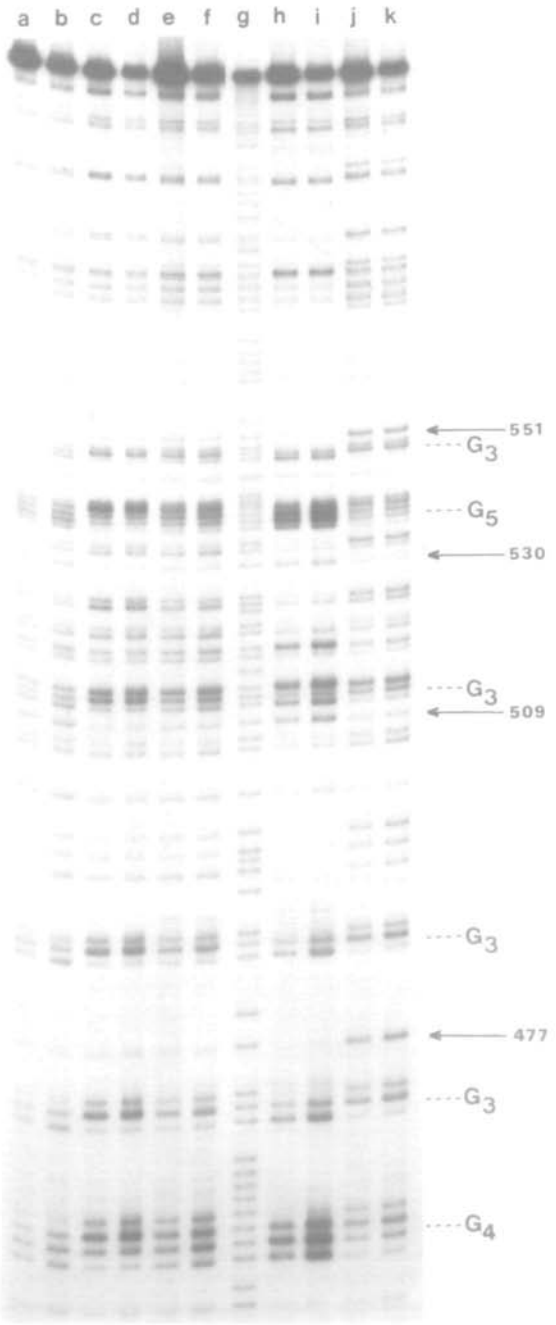


Figure 3. Densitometric scans of the guanine N7-alkylation pattern produced by nitrogen mustard (mechlorethamine), L-phenylalanine mustard, uracil mustard, and quinacrine mustard. Scans correspond to Figure 2, lanes f, g, e, and h, respectively.

amine piperidine converts these modified base sites into strand breaks (18). If the DNA is labeled only at one end of one strand, and is of known sequence, the lengths of the labeled fragments produced after alkylation and subsequent alkaline piperidine treatment indicate the position of the original alkylation (18). Fragments differing in size by only one nucleotide can be resolved on high resolution DNA sequencing gels, and the intensity of the autoradiographic image of each band gives an indication of the amount of alkylation at that site. Using this approach we have examined the sequence selectivity of guanine -N7 alkylation of several nitrogen mustard derivatives (figure 1). A 3741 base pair fragment of pBR322 DNA was reacted with these drugs, treated with piperidine, and electrophoresed (figure 2). In the region of the gel where they can be resolved, the bands produced corresponded to positions of guanines. For each of the drugs the intensity varied greatly from band to band. Furthermore, the relative intensities of some bands was markedly influenced by the non-alkylating moiety of the drug. Compared to the parent compound mechlorethamine (lane f), phosphoramide mustard (lane b), chloambucil (lane c), L-phenylalanine mustard (lane g), and spiromustine (lane i) showed similar patterns of alkylation intensities. In general these agents showed strong alkylation at the two runs of three contiguous guanines (positions 4028-4030 and 4040-4042), whereas isolated guanines were alkylated relatively weakly. In contrast to the other mustards, uracil mustard (lane e) had greatly enhanced reaction with the guanine in the 5'-TGCC-3' sequence at base position 4103. Quinacrine mustard (lane h) had yet again a different pattern with an enhanced reaction at the isolated guanines in 5'-AGT-3' sequences at base positions 4151 and 4157, and in a 5'-TGT-3' sequence at base position 4216. In a part of the sequence not clearly resolved by the gel, mustamine (lane d) showed two sites (indicated by the arrows) of enhanced reaction not observed with the other compounds. Differences between uracil mustard, quinacrine mustard, and nitrogen mustard and phenylalanine mustard can be clearly seen in densitometric scans (figure 3) of portions of the corresponding lanes of figure 2. The relative reaction intensities in relation to the base sequence are schematically summarized in Figure 6a.

It is pertinent to note that the doses for each drug used for these experiments were chosen so as to give a comparable extent of alkylation (approximately 1 break per 500 bases, see Materials and Methods). Also,



the differences observed were not markedly dependent on the solvent condition of the reaction, e.g. 0.1 M and 1.0 M Na^+, 10 mM Mg^{2+}, 20% ethanol (data not shown).

Given the observed preference of these agents for the two $(G)_3$ sequences, we examined the alkylation of the 276 base pair Bam H1-Sal I fragment of pBR322 DNA, a sequence which was not contained in the fragment examined in figure 2 but which has several occurences of three or more contiguous guanines (Figure 4). As can be seen from figure 4, and the corresponding microdensitometric scans in figure 5, mechlorethamine (lanes c and d), L-phenylalanine mustard (lanes e and f), and quinacrine mustard (lanes h and i) reacted strongly with these runs of guanines. From the microdensitometric analysis the average intensity of guanines within the runs of contiguous guanines was determined (Table 1). There seems to be a pattern of overall increasing reaction with increasing guanine number in such sequences by mechlorethamine, L-phenylalanine mustard and particularly quinacrine mustard. In contrast, uracil mustard showed little preferential reaction with these sequences. All drugs however showed a lower overall reaction for the single $(G)_5$ sequence (5'-CCGGGGGAC-3') than of the single $(G)_4$ sequence (5'-ATGGGGAA-3').

There seem to be some differences between mechlorethamine, L-phenylalanine mustard and quinacrine mustard in their relative reaction with individual bases in runs of contiguous guanines (Figure 4). This can be seen more clearly in the corresponding microdensitometric scans in Figure 5 (e.g. compare the reaction with the guanines in the $(G)_4$ sequence at positions 461-464, and the $(G)_3$ sequences at positions 471-473, 485-487, and 511-513).

Compared to its reaction with other isolated guanines quinacrine mustard (lanes h and i) shows particularly strong alkylation with the

Figure 4. The 276 bp Bam HI - Sal I fragment of plasmid pBR322 DNA, labeled at the 5' end of the Bam HI site, was reacted with the indicated agents and prepared for electrophoresis as described in Materials and Methods. Lane a: 0.5 mM dimethyl sulfate; lane b: 1 mM dimethyl sulfate; lane c: 20 μM mechlorethamine; lane d: 40 μM mechlorethamine; lane e: 50 μM L-phenylalanine mustard; lane f: 100 μM L-phenylalanine mustard; lane g: A+G reaction (depurination with formic acid); lane h: 0.05 μM quinacrine mustard; lane i: 0.1 μM quinacrine mustard; lane j: 10 μM uracil mustard; lane k: 20 μM uracil mustard. Numbered arrows indicate the base position in pBR322, and the positions and lengths of runs of guanines are indicated by dotted lines.

TABLE 1. Average intensity of guanine N7-alkylation in runs of 2-5 guanines relative to the average intensity of a single isolated guanine.

Drug	Average Alkylation Intensity per Guanine				
	$(G)^a$	$(G)_2{}^b$	$(G)_3{}^c$	$(G)_4{}^d$	$(G)_5{}^d$
Nitrogen Mustard	1.0 (0.46-1.62)	1.87 (1.41-2.75)	3.60 (1.33-4.86)	6.43	2.77
Phenylalanine Mustard	1.0 (0.73-1.27)	1.59 (1.03-1.90)	2.53 (1.32-3.64)	5.14	2.76
Quinacrine Mustard	1.0 (0.28-3.87)	1.58 (0.83-3.65)	3.95 (1.76-6.46)	11.05	5.28
Quinacrine Mustard[e]	1.0 (0.50-2.04)	2.83 (1.48-6.51)	7.06 (3.14-11.54)	19.7	9.44
Uracil mustard	1.0 (0.38-2.24)	1.16 (0.98-1.25)	1.72 (0.92-2.23)	1.98	1.22
Uracil Mustard[f]	1.0 (0.52-1.61)	1.55 (1.28-1.68)	2.32 (1.24-3.17)	2.66	1.64
Dimethylsulphate	1.0 (0.51-1.16)	1.16 (0.82-1.62)	1.5 (0.94-1.84)	2.17	1.49

[a] Average intensity (with range) of all isolated guanines from position 450-550 unless otherwise stated.
[b] Mean and range of five occurrences within the sequence.
[c] Mean and range of four occurrences within the sequence.
[d] Single occurrence within the sequence.
[e] Excluding the two prefered sites (5'-PyGT-3') at positions 509 and 529.
[f] Excluding the two prefered sites (5'-PyGCC-3') at positions 477 and 550.

two occurances of 5'-CGT-3' sequences within the fragmemt at positions 509 and 530 as indicated in figure 4. Uracil mustard showed enhanced reaction with the two occurances of 5'-CGCC-3' sequences at positions 477 and 551. The results are summarised and compared to the sequences examined in figure 6. In particular, quinacrine mustard showed a preference for runs of contiguous guanines that was greater than that observed for other mustards, and, compared with other isolated guanines a uniquely enhanced reaction with several occurences of 5'-GT-3' (eg. at base positions 4263, 4216, 4157, 4151, 4134, 4136, and 509). Uracil mustard reacted preferentially at three 5'-PyGCC-3' sites (base positions 4216, 477, and 551). A more detailed analysis covering a broader range of sequences is in progress.

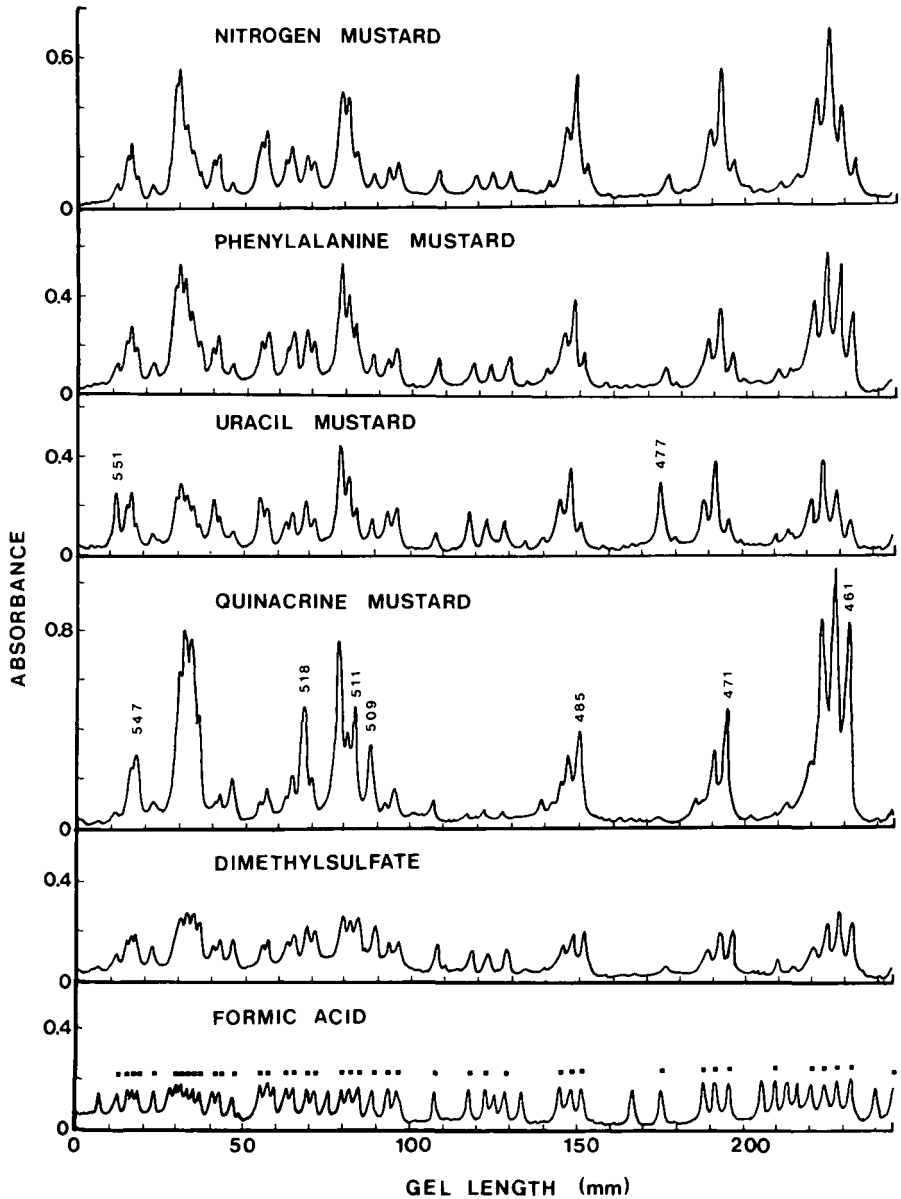
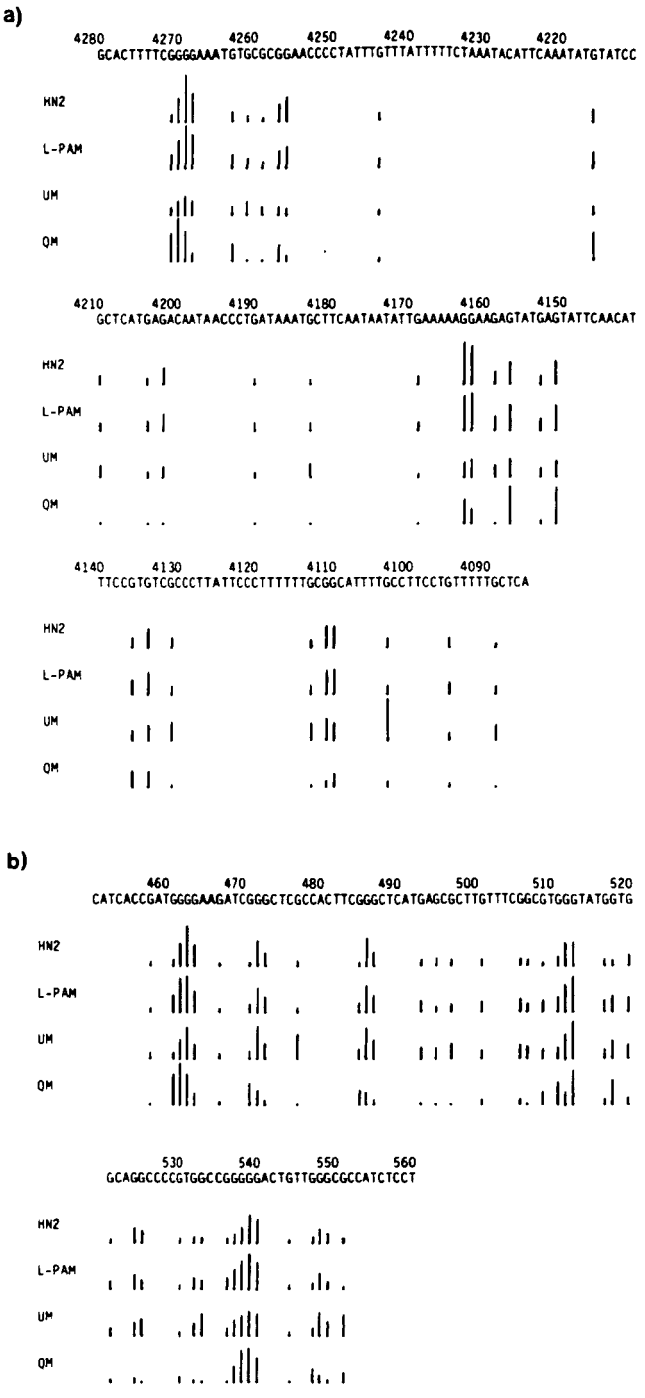


Figure 5. Densitometric scans of the guanine-N7 alkylation pattern produced by nitrogen mustard (mechlorethamine), L-phenylalanine mustard, uracil mustard, quinacrine mustard, dimethylsulfate, and formic acid. Scans correspond to Figure 4, lanes d, f, k, i, b, and g respectively. Numbers above peaks indicate the base position in pBR322, and filled boxes in the formic acid scan indicate the guanine positions.



DISCUSSION

The effects of nucleotide sequence context on the covalent reaction of many different compounds have been described and recently reviewed (21). We have examined the effects of sequence context on the reaction of a group of compounds having a common reactive species, the chloroethylaziridinium group (22). We have found that the non-alkylating moiety to which the chloroethylaziridium group is attached can strongly influence the sequence selectivity of covalent binding to guanine N7 positions.

Muench et. al. (15) considered two possible explanations for the sequence specificity of aflatoxin: 1) guanines in different sequence contexts have inherently different reactivities, or 2) the reactive chemical has specific non-covalent interactions with the DNA double helix that vary with sequence and lead to differences in the subsequent covalent interactions with those sequences. They conclude that the latter explanation is consistent with their observation that the reaction of aflatoxin with single stranded DNA is weak and not sequence specific, and that non-reactive analogs of aflatoxin compete for reactive sites in DNA. The difference between the sequence specific reaction of quinacrine mustard and uracil mustard with that of other nitrogen mustards observed in the present study strongly suggests that non-covalent interactions are occurring between the non-alkylating moieties of these drugs and DNA prior to covalent reaction.

On the other hand all the nitrogen mustard derivatives we have studied so far react with guanines in a sequence dependent fashion. Almost all (with the possible exception of uracil mustard) show an enhanced reaction with guanines flanked by other guanines as opposed to reaction with isolated guanines. Grunberg and Haseltine (19) had noted enhanced reactivity of mechlorethamine and phosphoramide mustard with pairs of guanines in a segment of alpha DNA, a reiterated sequence in the human genome. Our recent experiments indicate that the reaction of alkyldiazohydroxides with guanine-N7 positions also is greatly enhanced in guanines flanked by other guanines (17). Similarly aflatoxin showed the highest level of reaction at GG and GGG sequences (15).

Figure 6. Summary of alkylation intensities for mechlorethamine, L-phenylalanine mustard, uracil mustard and quinacrine mustard compared with the corresponding sequence. Alkylation intensities at a given sequence position on each fragment were determined as peak height relative to the highest peak of alkylation for that compound on that fragment.

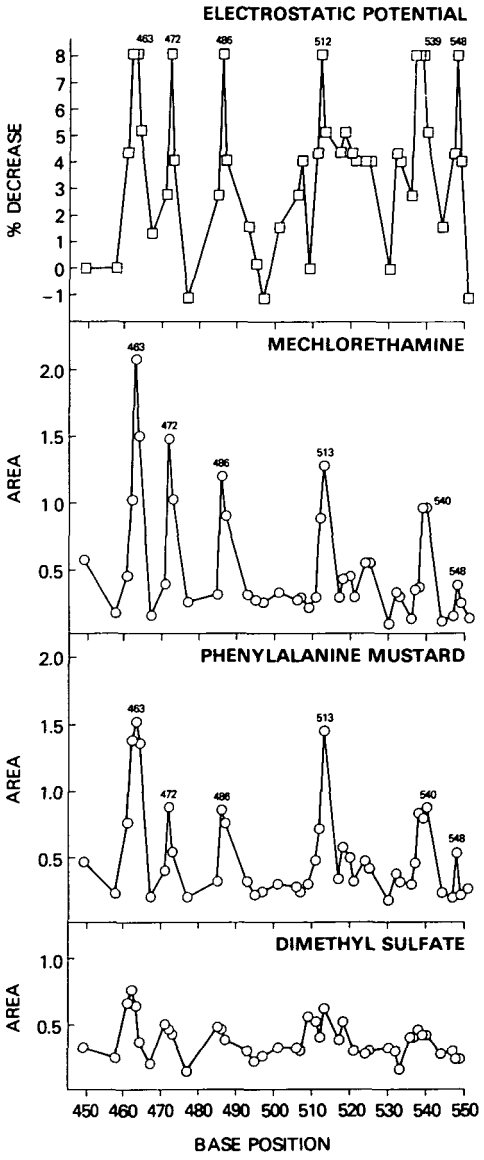


Figure 7. Upper panel: the deviation (% change) in the electrostatic potential at guanine N7 from -238.9 kcal/mol was calculated for each guanine from base 440 to base 550 in pBR322 using values reported by Pullman and Pullman (24). The numbers take into account the influence of only the immediate 5' and 3' neighboring bases. Lower three panels: plotted areas from the densitometric scans of figure 4 lanes f (L-phenylalanine mustard), d (nitrogen mustard, mechloethamine) and b (dimethyl sulfate). Numbers above peaks indicate the base position in pBR322 DNA.

What might explain the enhanced reactivity of such sequences? These sequences would be expected to have greater stacking interactions than those in a GT dinucleotide (23), and so would not be expected to have a more accessible N7 site. Hence accessibility of the N7 position does not seem to be a major factor. Another possibility is that the neighboring bases might alter the reactivity of the guanine N7 position via electrostatic interactions. A local increase in the electronegativity near the guanine N7 position would be expected to increase the affinity for a positively charged species such as the chloroethylaziridinium ion. Pullman and Pullman (24) have calculated the effects on the molecular electrostatic potential at the N7 site of guanines in duplex DNA produced by adjacent base pairs. Using these numbers we have plotted the sequence dependent variations in the electrostatic potential at the guanine N7 position for bases 440-550 of pBR322 (figure 7). The sequence dependent variations in alkylation for this same region were examined in figure 4. When the experimentally determined variations in alkylation intensities are compared with the calculated variations in electrostatic potential a distinct similarity in some regions can be seen; in fact, the regions of greatest agreement are sequences of contiguous guanines. The relationship is not a perfect one: peaks of intense alkylation by nitrogen mustard and phenylalanine mustard at positions 513 and 539 seem to be offset slightly from the peaks of electronegativity at positions 512 and 537, respectively. Other discrepancies can be seen in the figure. It should be noted that the calculations for variations in the electrostatic potential take into account only the contribution of the two bases surrounding the guanine position examined. However, the correlation is good enough to suggest that sequence dependent variation in electrostatic potential at the guanine-N7 position is a causal factor in determining the sequence specificity of many agents (especially cationic reactants) that attack this site. Alternatively, the variations in electrostatic potential may parallel some other feature of the DNA configuration that is a causal factor contributing to alkylating agent sequence specificity. (It is pertinent to note that the sequence specific variations in the electrostatic potential at other sites of guanine in duplex DNA may be different from those at the N7 position, and that the reactive sites of guanine are much less electronegative in single stranded DNA (24).)

Since the nitrogen mustard derivatives used in this study are bifunctional it may be anticipated that after one alkylating moiety

has reacted with a guanine in a DNA helix the other one can go on to react with a neighboring guanine in the same or complementary strand. Such reactions would thus be expected to enhance the reactivity of contiguous guanines in the same strand (sites of intra-strand crosslinks) or guanines in 5'-GC-3' sequences (sites of inter-strand crosslinks). One would predict that intra-strand crosslinking at a GG site would lead to an apparent enhancement of strand breakage at the guanine closest to the labeled end. In fact, when we compared the alkylation of one fragment of pBR322 labeled at its 5' end with alkylation of the same fragment labeled at its 3' end there were no evident differences in the patterns of strong and weak alkylation in contiguous guanines (data not shown). Thus it would appear that intra-strand crosslinks are not a major contributing element to the patterns of alkylation we have observed. A more detailed and quantitative comparision of 3' and 5' labeled fragments may reveal the actual extent of intra-strand crosslink formation at certain sites. As for inter-strand crosslinks, we actually observe that 5'-GC-3' sites are some of the least reactive sites for almost all of the nitrogen mustards, with the possible exception of uracil mustard. Thus our data suggest that the formation of inter-strand crosslinks by most nitrogen mustards may be limited by not only the low frequency of 5'-GC-3' sites but also by the weak alkylation reaction at these sites.

An important significance of our observations is that these alkylating agents may preferentially attack regions of the genome rich in contiguous guanines. Some transforming genes, including the c-H-ras oncogene, have such regions, which constitute regions of enhanced reactivity with some chemotherapeutic agents (unpublished results). Whether preferential reaction with such regions (control regions?) of these genes may partially explain their chemotherapeutic potential is an intriguing speculation. Further understanding of the mechanisms contributing to the sequence selectivities of such agents may lead to the rational design of drugs with markedly enhanced sequence preferences.

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