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1,2,5,6-Diepoxyhexane and 1,2,7,8-Diepoxyoctane Cross-Link Duplex DNA at 5′-GNC Sequences

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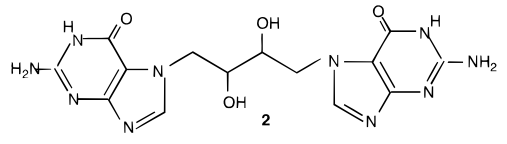
The carcinogenicity of epoxide compounds has been attributed to covalent binding to DNA. Whereas monoepoxides form only monoadducts, diepoxides can form both monoadducts and interstrand cross-links. The latter are believed to be the more significant cytotoxic lesions as diepoxides are frequently more carcinogenic and mutagenic than their monoepoxide analogues. We therefore examined the relative DNA interstrand cross-linking capabilities of several diepoxides with respect to chain length, molecular flexibility, reported carcinogenic potential, and DNA sequences targeted. Using denaturing polyacrylamide gel electrophoresis, we found that 1,2,5,6-diepoxyhexane and 1,2,7,8-diepoxyoctane share the 5′-GNC target sequence previously found for 1,2,3,4-diepoxybutane [Millard, J. T., and White, M. M. (1993) *Biochemistry* **32**, 2120−2124] and that the efficiency of cross-linking this sequence may reflect carcinogenicity. 1,2,5,6-Diepoxycyclooctane, the biologically inactive rigid analogue of 1,2,5,6-diepoxyhexane, was found to be a poor cross-linker of all DNA sequences examined. Moreover, increasing the diepoxyalkane chain length did not result in enhanced cross-linking ability.

Introduction

Butadiene (C_4H_6) is an important monomer in the production of synthetic rubber, nylon, and other polymers (*1*). The National Institute for Occupational Safety and Health has estimated that 65 000 workers are exposed to this compound annually (*2*). Butadiene is also a component of cigarette smoke (*3*), plastics, and gasoline (*4*). Upon inhalation and absorption, butadiene is metabolized in the liver to its mono- and diepoxides (Scheme 1; *5*−*7*). The metabolites 3,4-epoxy-1-butene and 1,2,3,4-diepoxybutane (DEB,[1] **1**) alkylate DNA, producing point mutations, deletions, and cancer (*8*−*10*). Indeed, epidemiological studies indicate that excess mortality from lymphatic and hematopoietic cancers occurs among workers exposed to butadiene (*11*−*13*). Other epoxides are found as pesticides, sterilizing agents, mycotoxins, mammalian metabolic products, and air pollutants (*9, 14, 15*). Because of the prevalence of potentially hazardous epoxides surrounding living organisms, we are studying their biochemical mode of action.

Diepoxides were first postulated to act as biological cross-linking agents in 1951 (*8*). Later, Brookes and Lawley proposed that DEB binds to the N7 position of guanine, similarly to nitrogen mustards, the first clinically utilized antitumor drugs (*16*). They further suggested that such bifunctional alkylating agents must span at least 8 Å to cross-link the minimal N7-to-N7 distance, contained at the duplex sequence 5′-GC (*17*). Despite the isolation of conjugate **2** from DEB-treated DNA hydrosylates (*18*), the alkyl chain of DEB can stretch only 4 Å, leaving some doubt about its capacity for interstrand cross-linking (*19, 9*). However, DNA demonstrates considerably greater conformational flexibility than previously anticipated: nitrogen mustard (*20*−*22*) and DEB (*23*) both cross-link duplex DNA through distal guanines at the sequence 5′-GNC (where N is any base) in preference to either 5′-GC or 5′-CG sequences. Despite the different ultimate biological effects of the carcinogenic DEB and the chemotherapeutic nitrogen mustard, they share the same genomic target. Other molecular factors must therefore be involved in determining the consequences of DNA cross-linkers *in vivo*.



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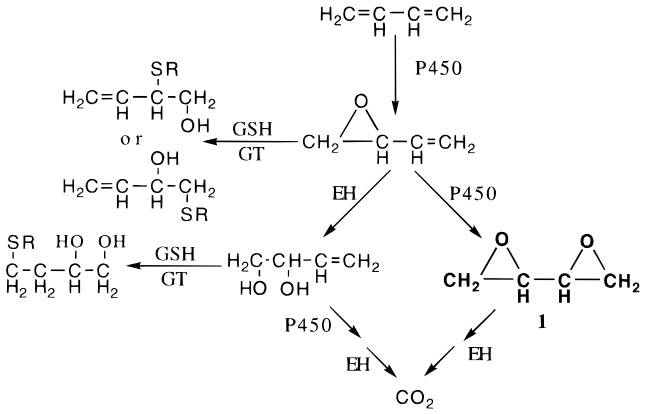
Scheme 1. Metabolism of 1,3-Butadiene to Diepoxybutane and Other Compounds in the Liver by Cytochrome P450 (P450), Glutathione (GSH), Glutathione *S*-Transferase (GT), and Epoxide Hydrolase (EH)



With the goal of finding a correlation to biological activity, we examined the relative cross-linking efficiencies of several diepoxides with respect to alkyl chain length, molecular flexibility, reported carcinogenicity, and DNA sequences targeted. We used denaturing polyacrylamide gel electrophoresis to verify the cross-linking capability of the putative cross-linkers 1,2,5,6-diepoxyhexane (DEH, **3**) and 1,2,7,8-diepoxyoctane (DEO, **4**) as well as to demonstrate the poor cross-linking capability

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[1] Abbreviations: DEB, 1,2,3,4-diepoxybutane; DEH, 1,2,5,6-diepoxyhexane; DEO, 1,2,7,8-diepoxyoctane; DECO, 1,2,5,6-diepoxycyclooctane; PAGE, polyacrylamide gel electrophoresis.

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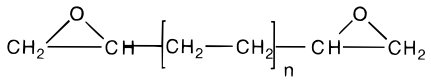
Table 1. DNA Duplexes Used in These Studies

nucleotide sequence	descriptor
$^{5'}$AACCTATTGGGCGGGATTA$^{3'}$ $^{3'}$GATAACCCGCCCTAATTAA$_{5'}$	GGGCGGG
$^{5'}$AATATATTGCAATAT$^{3'}$ $^{3'}$TATAACGTTATATAA$_{5'}$	TGCA
$^{5'}$AATATATTCGAATAT$^{3'}$ $^{3'}$TATAAGCTTATATAA$_{5'}$	TCGA
$^{5'}$AATATATGGCCATAT$^{3'}$ $^{3'}$TATACCGGTATATAA$_{5'}$	GGCC
$^{5'}$AATATATGATCATAT$^{3'}$ $^{3'}$TATACTAGTATATAA$_{5'}$	GATC

of 1,2,5,6-diepoxycyclooctane (DECO, **5**). Our results suggest that cross-linking efficiency may be related to the carcinogenic potential of these agents.



n=1; **3**
n=2; **4**

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Experimental Procedures

Preparation of Radiolabeled DNA Duplexes. Oligonucleotides shown in Table 1 were purchased from Operon Technologies, Inc. (Alameda, CA), and purified through denaturing polyacrylamide gel electrophoresis (PAGE) as described previously (*23*). Radioisotopic labeling of 3′-ends was with [α-^{32}P]dATP/Klenow fragment; 5′-ends, with [γ-^{32}P]ATP/T4 polynucleotide kinase under standard conditions followed by ethanol precipitation with sodium acetate (*24*).

Reaction with Diepoxides. Radiolabeled duplex DNA (0.5 OD_{260}) was incubated in 0.3 mM sodium acetate (pH 5) and 250 mM diepoxide (100 μL total volume) for 2 h at 37 °C. Identical products, although in lower yield, were found when the reaction medium was Tris-EDTA buffer (pH 7.2). Epoxide reactions have been reported to be acid-catalyzed (*25*). The slow reaction rate of diepoxides relative to other cross-linkers, such as nitrogen mustards, necessitated the high concentrations used (*26*). Incubation was followed by ethanol precipitation and lyophilization. The resulting pellet was dissolved in 100 μL of purified water and a 5 μL aliquot removed for Cerenkov counting to determine total cpm in the reaction. The remaining sample was lyophilized for denaturing PAGE.

Purification of Cross-Linked DNA through Denaturing PAGE. Cross-linked samples were dissolved in 5 M aqueous urea/xylene cyanole and loaded onto a 20% polyacrylamide gel (19:1 acrylamide/bis(acrylamide), 50% urea, 0.35 mm thick, 41 × 37 cm), run on a Hoefer Poker Face sequencer at 65 W. Autoradiography was used to visualize single-stranded and cross-linked DNA, of roughly half the mobility of the corresponding single strands. The major cross-linked band was excised from the gel and Cerenkov counted. For poorly cross-linked duplexes, the region of the gel where centrally cross-linked product would be expected was located by comparison to a mitomycin C cross-linked DNA standard (*23, 27, 28*). The percentage of cross-linked DNA was determined from the ratio of cpm in the gel slices to the total cpm in the reaction. DEB, DEH, DEO, and DECO reactions were all run simultaneously in duplicate with the same DNA sequence on each gel to minimize variability between samples. Multiple trials were performed and standard deviations calculated as reported.

Table 2. Interatomic Distances between Epoxide Groups (from ref 19)

compd	greatest separation (Å)
diepoxybutane	4.0
diepoxycyclooctane	4.3
diepoxyhexane	6.6
diepoxyoctane	8.9

DNA Cleavage Reactions. The DNA GGGCGGG (Table 1) was 3′- or 5′-end radiolabeled on the top strand. Gel-purified cross-linked DNA or monoalkylated DNA (an aliquot of a cross-linking reaction used without further purification) was heated at 90 °C in aqueous piperidine to cleave at sites of N7 alkylation (*29*). The resulting fragments were purified to single-base resolution through 25% denaturing PAGE run as above at 65 °C. Gels were dried (Hoefer Drygel Sr.) onto Whatman 3MM paper, and autoradiograms were obtained through overnight exposure. Densitometry (Hoefer GS-300) data were smoothed, plotted, and integrated (Hoefer Scientific GS370 Densitometry Program, Version 2.0) to obtain plots of cleavage intensity for each nucleotide. Bands were assigned through reference to Maxam−Gilbert G-lanes (*29*). Total monoalkylation efficiency was calculated by subtracting the intensity of the peak corresponding to the remaining single strands from the sum of the intensities of all the peaks.

Synthesis of Diepoxides. We used the epoxidation method of Paul and Tchelitcheff (*30*) to synthesize 1,2,5,6-diepoxyhexane. A solution of 1,2,5,6-hexadiene (4.7 g, 57 mmol) and *m*-chloroperoxybenzoic acid (33.9 g, 196 mmol) in 150 mL of dichloromethane was mixed at 4 °C for 15 h. Following vacuum filtration, a dilute NaOH solution was added to neutralize the acid byproduct of the reaction. The organic phase was separated, refiltered, and dried with anhydrous sodium sulfate. The residue was run through a silica gel column with a 3:7 ether/hexane solvent system, and three fractions were collected. IR analysis showed the desired 1,2,5,6-diepoxyhexane to be in fraction three. Purity was 99% as monitored by GC/MS, neglecting response factors. Synthesis of 1,2,4,5-diepoxypentane was unsuccessful by this method, as well as through epoxidation with magnesium monoperoxyphthalate hexahydrate (*31*), so this compound could not be used in our studies. 1,2,3,4-Diepoxybutane, 1,2,7,8-diepoxyoctane, and 1,2,5,6-diepoxycyclooctane were all purchased from Aldrich (Milwaukee, WI).

Results

Diepoxide reactivity toward DNA was monitored according to length of the carbon chain (Table 2). We compared interstrand cross-linking by DEB, DEH, and DEO. Moreover, we considered conformational flexibility by including the rigid diepoxycycloalkane DECO. We also examined the monofunctional compound ethylene oxide (CH_2CH_2O) in addition to these bifunctional agents although diepoxides are frequently more carcinogenic and mutagenic than their monoepoxide analogues (*19, 9*). For example, the mutagenicity of 3,4-epoxy-1-butene is much lower than that of DEB (*10, 32*). Moreover, DEB has been found to be some 30 times more effective, per primary alkylation, at generating mutations than ethylene oxide, despite similar reaction rates (*9*).

The first step in a cross-linking event is the formation of a monoalkylated product. We tested the importance of the initial alkylation step for a variety of epoxides on a ^{32}P end-labeled DNA with a core sequence of 5′-d(GGGCGGG). Because cross-linking efficiency is low, we used the distribution of total alkylation to approximate monoalkylation frequency. Following reaction with epoxide and subsequent piperidine treatment (to cleave at N7-alkylated guanines), we purified the resulting fragments to single-base resolution through 25% denaturing PAGE and assigned bands via a Maxam−

Table 3. Monoalkylation by Epoxides: Percent Total Alkylation of the GGGCGGG Duplex (See Table 1) and Average Relative Percentages of Monoadduct Formation as a Function of Nucleotide Position[a]

	% cleavage at						
agent	G1	G2	G3	G4	G5	G6	% total alkylation
ethylene oxide	20	16	17	17	15	15	ND[b]
DEB	22	18	13	17	19	11	37
DEH	21	18	13	19	16	13	ND
DEO	20	16	15	17	16	16	15
DECO	16	17	20	14	17	16	12

[a] Numbering is from 5′-3′. Piperidine cleavage products of both 5′- and 3′-end-labeled DNA were averaged. [b] Not determined.

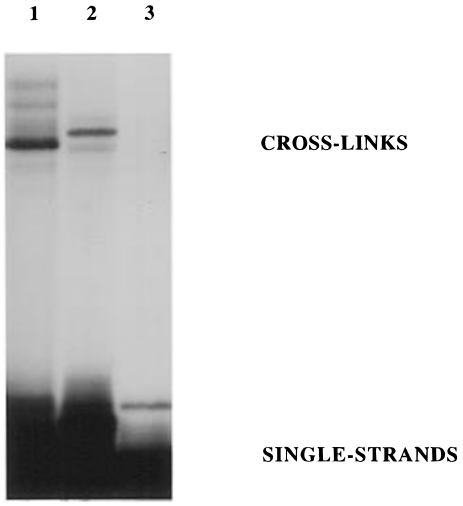

Figure 1. Denaturing PAGE analysis of radiolabeled DNA GGCC (see Table 1) incubated for 2 h with 250 mM DEB (lane 1), 250 mM DEO (lane 2), and 0 mM diepoxide in 0.3 M NaOAc (pH 5) (lane 3).

Gilbert G sequencing reaction. Relative cleavage intensities at each position correspond to alkylation frequency. The average monoalkylation frequency is fairly uniform among dG residues in this duplex for all epoxides tested (Table 3). Any interstrand cross-linking preference exhibited by these epoxides must therefore occur at the second step of cross-link formation: the conversion of monoadducts to cross-links. This agrees with our previous results for DEB (*23*). However, the relative efficiencies for monoalkylation differed for the diepoxides tested, with DEB > DEO > DECO.

We then incubated several duplex DNA sequences with DEH, DEO, and DECO to verify their cross-linking capabilities. Oligonucleotides with different guanine-containing four-base cores (TGCA, TCGA, GGCC, and GATC; see Table 1 for sequences) were exposed to diepoxides and analyzed by 20% denaturing PAGE. These reactions were extremely heat-sensitive, so samples were loaded directly onto gels without heat denaturing. In addition to recovering chiefly single strands and presumably monoadducts, less mobile products were also formed in these reactions (Figure 1). These somewhat diffuse bands of reduced mobility are indicative of agents with some flexibility in their sequence specificity, such as the pyrrolizidine alkyloids (*33*). The major product was quantified in order to distinguish between the relative cross-linking efficiencies of the variable core sequences in the duplexes studied. A mitomycin C centrally cross-linked reference was used to pinpoint the appropriate area of the gel for poorly cross-linked duplexes (*23, 28*).

Table 4. Average Cross-Linking Efficiencies of DNA's with Sequences Shown in Table 1[a]

	core sequence			
agent	TGCA[b]	TCGA[b]	GGCC	GATC
DEB	0.08 ± 0.02	0.09 ± 0.01	0.98 ± 0.37	0.86 ± 0.33
DEH	0.09 ± 0.04	0.02 ± 0.00	0.34 ± 0.16	0.08 ± 0.02
DEO	0.05 ± 0.05	0.07 ± 0.06	1.08 ± 0.50	0.30 ± 0.11
DECO			0.016 ± 0.004	0.014 ± 0.003

[a] Values indicate percent interstrand cross-linked product relative to total starting material (see Experimental Procedures). [b] From Cerenkov counting of gel region where centrally cross-linked material is expected.

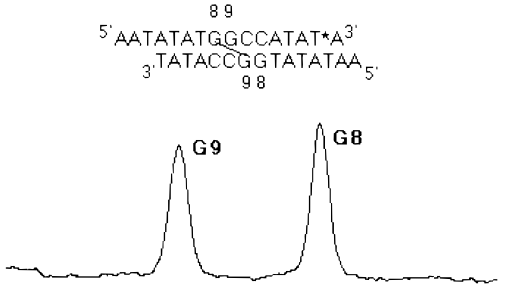

Figure 2. Densitometric analysis of piperidine cleavage products of DEO-cross-linked DNA (3′-end radiolabel: * = ^{32}P). Lettering denotes the residue cleaved. Cleavage bands of equal intensities indicated linkage between G8 and G9 on opposite strands, or cross-linking at the sequence 5′-d(GNC) as shown.

Oligonucleotides containing central 5′-TGCA and 5′-TCGA sequences were cross-linked extremely poorly, with yields of less than 0.1%, for all diepoxides tested (Table 4). This agrees with previous results for DEB, which also demonstrates poor reactivity for cross-linking 5′-GC and 5′-CG sequences (*23*).

The oligonucleotide containing the core 5′-GGCC showed substantial reactivity (Table 4). This DNA is cross-linked through distal guanines at the sequence 5′-GNC by DEB (*23*). We verified this connectivity for DEO and DEH through isolation and subsequent piperidine cleavage of the major cross-linked band. The electrophoretic mobilities of the resulting radiolabeled fragments corresponded to those of a Maxam−Gilbert G reaction. Equal intensities of the piperidine cleavage bands for both guanines confirmed that N7 alkylation occurred at the sequence 5′-GNC in this duplex (Figure 2).

The duplex containing 5′-GATC, corresponding to reaction of distal guanines at the sequence 5′-GNNC, also showed significant cross-linking by DEB and DEO (Table 4). Complete piperidine cleavage of the major cross-linked band verified the guanine N7 connectivity. DEH (∼0.1%) and DECO (∼0.01%) both showed extremely poor cross-linking of the 5′-GNNC sequence.

All the agents studied demonstrated greater cross-linking of the duplex containing the sequence 5′-GNC than those containing 5′-GNNC, 5′-GC, or 5′-CG. However, DEO and DEB showed substantial reactivity toward the 5′-GNNC sequence as well. To verify the core preference and control for the experimental variability involved in comparing distinct duplexes, we presented all of these sequences in a single duplex with the core 5′-d(GGGCGGG). DEB- and DEO-cross-linked DNA's were piperidine cleaved, and the resulting products were analyzed through single-base resolution denaturing PAGE. Integration of the densitometry data produced relative percentages of the cleavage products, which were averaged for both 5′- and 3′-radiolabeled duplex DNA (Table 5). Relative product abundance followed the order G2 >

Table 5. Average Relative Percentages of Cross-Linking by Diepoxybutane and Diepoxyoctane as a Function of Nucleotide Position in the Duplex GGGCGGG (See Table 1)[a]

	% cleavage at					
agent	G1	G2	G3	G4	G5	G6
DEB	23	44	9	8	7	9
DEO	24	31	14	9	9	14

[a] Numbering is from 5′ to 3′ end. Piperidine cleavage products of both 5′- and 3′-end-labeled DNA were averaged.

G1 > G3, G4, G5, G6, indicating that both diepoxides again showed preferential cross-linking of the 5′-GNC sequence. The next most favored site for cross-linking was at 5′-GNNC. The other sequences, 5′-GC, 5′-CG, 5′-CGG, and 5′-CGGG, were all poorly cross-linked. Thus, despite its significantly longer chain length, DEO was confirmed to share the sequence preference of DEB. However, DEO showed only a 1.5-fold preference for cross-linking 5′-GNC relative to 5′-GNNC in this duplex, reduced from the 2-fold preference demonstrated by DEB. Radiolabeling of the bottom strand verified linkage to the single central G; virtually complete piperidine fragmentation in all cases verified that cross-linking was in fact from guanine N7-to-N7 positions.

Discussion

Goldacre et al. (*34*) first suggested almost fifty years ago that the cytotoxicity of bifunctional alkylating agents might be related to their DNA cross-linking reactions. Although attempts to correlate DNA binding with carcinogenicity have generally been successful, some metabolites bind DNA without being carcinogenic (*35*). Clearly, investigations of chemically similar agents with differing biological effects are needed to understand the basis of carcinogenesis. Previously, we compared the DNA cross-linking preferences of diepoxybutane to the chemically analogous nitrogen mustard mechlorethamine (*23*). We found that, despite their vastly different biological effects, these compounds target the same genomic site: 5′-GNC. Interstrand cross-linking sequence preferences alone therefore cannot explain their differing biological activities. In this study, we compared the DNA cross-linking efficiencies of a variety of diepoxides, also with differing biological consequences, to elucidate a molecular rationale for their resulting activities.

We first tested monoalkylation by epoxides to determine the relative importance of this step and the subsequent formation of cross-links. Monoalkylation can be sequence specific, such as for mitomycin C (*36, 37*), or sequence random. Many carcinogens, including nitrogen mustard, demonstrate a preference for runs of contiguous guanines, attributed to increased nucleophilicity (*38, 39*). However, epoxides do not seem to follow this pattern. The monoepoxide ethylene oxide and the diepoxides DEB, DEH, DEO, and DECO all showed fairly uniform alkylation patterns among the guanines in the sequence 5′-GGGCGGG. Any sequence preference for cross-linking must therefore arise at the step of monoadduct conversion to cross-link. Interestingly, there was a difference in relative monoalkylation abilities, with DEB yielding 2- to 3-fold more alkylated product than DEO and DECO.

The effect of the chain length of bifunctional alkylating agents was first pondered almost thirty years ago (*18*). Although diepoxybutane exhibits the biological properties of a DNA cross-linker, it contains a chain of only four carbon atoms, which is unlikely to span the putative 8 Å distance between reactive groups in B-DNA. Lawley and Brookes pose this dilemma, but do not resolve it (*19*). It has been reiterated in the literature that N7-to-N7 cross-linking between deoxyguanines is impossible for diepoxides shorter than at least seven carbon atoms (*40*). For example, the high frequency of multilocus deletions among DEO-induced *Neurospora crassa* mutants relative to diepoxypentane-induced mutants has been attributed to DNA cross-linking: diepoxypentane was considered unlikely to cross-link N7 of guanines because its alkyl chain can extend only 5.2 Å (*41*). We therefore conducted a systematic study on the effect of diepoxide alkyl chain length to resolve the question of their capacities for interstrand cross-linking. DNA's of defined sequence were used to determine possible sequence preferences for cross-linking, which has not been possible in previous studies demonstrating DEB cross-linking in whole cells (*42*−*44*).

We compared cross-linking efficiencies of diepoxybutane, diepoxyhexane, diepoxyoctane, and diepoxycyclooctane for several DNA sequences. Somewhat diffuse bands on denaturing polyacrylamide gels indicated that cross-links resulted from linkage of central dG residues and also, to a lesser extent, of other residues. Hyperreactivity with duplex termini has been noted for several cross-linking agents which do not possess a rigid sequence specificity, presumably because the reactivity of the terminal residues is higher than that of those in the duplex regions (*33*). Terminal cross-links were present to a larger extent in DNA's that were poorly cross-linked. The major product was quantified to determine the relative reactivities of the tested core sequences.

Quantifying the centrally dG-cross-linked material led to the following order of reactivity for all of the diepoxides tested: 5′-GGCC > 5′-GATC ≫ 5′-TGCA, 5′-TCGA. This is consistent with a shared preference for the sequence 5′-GNC, although other sequences are also cross-linked less efficiently. Therefore, despite their different chain lengths, DEB, DEH, DEO, and the nitrogen mustard mechlorethamine all target 5′-GNC preferentially.

The relative cross-linking efficiencies for the preferred 5′-GNC sequence followed the order DEB, DEO > DEH > DECO. Nitrogen mustard interstrand cross-linking efficiency (*45*) and sequence specificity (*46*) have been correlated with biological activity. It is difficult to make this comparison for diepoxides because of some discrepancies in the literature as to the relative carcinogenicities of these compounds. Mutagenicity has been correlated to carcinogenic activity (*10, 47, 48*), and several reports confirm both the carcinogenicity and mutagenicity of DEB (*9, 49*). For example, DEB is a moderately active carcinogen in rodents after skin application (*14, 50*), intraperitoneal injection (*51*), and subcutaneous injection (*52, 53*). DEH has been classified as a weak carcinogen upon subcutaneous injection in mice and rats (*52*) and skin painting in mice, yet it induced about twice as many cancers as DEO, although at twice the concentration (*54*). The mutagenicity of DEH is greater than that of DEO in *Salmonella typhimurium* (*55*). Furthermore, biological activity has been reported to decrease with chain length from the four- to the ten-carbon diepoxides (*56*−*58*). However, it has also been reported that DEB, DEH, and DEO have about the same carcinogenic effectiveness (*9*). It is clear that diepoxycyclooctane is inactive in mammalian systems (*14, 19, 54, 59*) as well as in the Ames

test (*60*). Therefore, carcinogenic potential seems to follow the order DEB > DEH > DECO, with DEO somewhere in the middle. Cross-linking efficiency also follows this order, with the possible exception of DEO.

It is perhaps surprising that cross-linking efficiency does not increase with diepoxyalkane length. Therefore, it appears that chain length does not dictate the weak interstrand cross-linking capacity of DEB relative to nitrogen mustard, as previously postulated (*42*), and that other factors must be involved in mustard's enhanced efficiency.

Diepoxycyclooctane can be viewed as a diepoxyhexane molecule containing epoxide functions held rigid with respect to one another by an ethylene bridge. The chemical reactivity of these two compounds is similar (*19*). However, whereas DECO is completely inactive as a carcinogen, its open-chain flexible analogue DEH *is* somewhat carcinogenic (*52, 54*). Moreover, we found that DECO is a poor cross-linker relative to DEH. DECO cross-links the sequence 5′-GGCC about 5% as well as does DEH. Therefore, flexibility of reaction centers seems to be important for both carcinogenicity and DNA cross-linking.

To control for the inherent errors involved in cutting and quantifying gel bands, the cross-linking sequence preference of DEO was confirmed with a single DNA duplex containing a 5′-GGGCGGG core. The relative sequence reactivities in this duplex were about 3 GNC : 2 GNNC : 1 GC, CG (Table 5). The longer alkyl chain length of DEO appeared to reduce the absolute cross-linking specificity, as DEB showed a 2-fold preference for cross-linking GNC relative to GNNC and a 4-fold preference relative to GC and CG (Table 5 and ref 23). Alley et al. recently reported the reduced cross-linking specificity of 2,5-bis(1-azridinyl)-1,4-benzoquinone relative to mechlorethamine, also attributed to its longer tether length (*61, 62*). The slightly different relative reactivities in this duplex from those found when comparing the individual duplexes described above can be attributed to the impact of flanking bases as well as experimental error in cutting gel slices. Neighboring bases have been shown to have a significant impact on cross-linking by other agents [for example, mitomycin C (*37, 63*)], although the influence of flanking sequence is secondary to the absolute core sequence preference for cross-linking.

Considerable DNA distortion has been postulated to result from an N7-to-N7 cross-link at 5′-GNC by nitrogen mustard (*20, 21, 64*) and DEB (*23*). DNA bending upon mustard interstrand cross-linking has been experimentally verified (*65*). The need for a narrowed major groove may in fact dictate the preferred cross-linking site of these compounds: X-ray crystallographic analysis confirms the intrinsic bending of the sequence 5′-GGCC (*66*). It is likely that diepoxide cross-linking also contributes to net DNA bending, which may have significance *in vivo*. The underwinding and bending triggered by the antitumor agent cisplatin are believed to be responsible for binding of both its intrastrand and interstrand cross-links by high-mobility group proteins (*67, 68*). Furthermore, binding of such proteins could shield cross-linked lesions from repair and contribute to the mechanism of cytotoxic action (*69*). Therefore, the unique three-dimensional structures generated by diepoxides of varying chain lengths might result in differential repair processes *in vivo*, which could in turn mediate cytotoxicity at the cellular level. We hope to determine experimentally the geometry of the cross-linked adducts for these diepoxides to test this theory.

In summary, we have demonstrated that the putative cross-linkers DEH and DEO cross-link DNA through the N7 of distal deoxyguanines at the 5′-d(GNC) sequence, also the preferred site for DEB and mustard cross-linking. DECO was a poor cross-linker of all sequences tested. Cross-linking efficiencies, and not the sequences targeted, of these diepoxides correlate with their carcinogenicities. Moreover, increasing the alkyl chain length did not result in enhanced diepoxide cross-linking efficiency.

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