Base Sequence Selectivity in the Alkylation of DNA by 1,3-Dialkyl-3-acyltriazenes

Marilyn B. Kroeger Smith,*[,†] Lisa A. Taneyhill,[†,‡] Christopher J. Michejda,[†] and Richard H. Smith, Jr.[†,‡]

Molecular Aspects of Drug Design Section, Macromolecular Structure Laboratory, ABL−Basic Research Program, NCI−Frederick Cancer Research and Development Center, Frederick, Maryland 21702, and Department of Chemistry, Western Maryland College, Westminster, Maryland 21157

Received April 28, 1995[⊗]

The base sequence selectivity of DNA alkylation for a series of structurally related 1,3-dialkyl-3-acyltriazenes was examined with calf thymus DNA or polymers containing the sequences GGG, CGC, TGT, and AGA. The reaction products at the *N*7 and the O^6 positions of guanine were identified, quantitated, and then correlated with the decomposition rates of the triazenes, 1-(2-chloroethyl)-3-methyl-3-carbethoxy- (CMC), 1-(2-chloroethyl)-3-methyl-3-acetyl- (CMA), 1-(2-hydroxyethyl)-3-methyl-3-carbethoxy- (HMC), 1-(2-hydroxyethyl)-3-methyl-3-acetyl- (HMA), and 1,3-dimethyl-3-acetyl- (DMA). The results of these studies revealed that DNA sequences with runs of purines were more reactive toward alkylation by all of the triazenes tested, irrespective of whether the alkylation was measured by *N*7, O^6, or total guanine adducts. Within this generalization, the (hydroxyethyl)triazenes showed a preference for the AGA sequence, while the (chloroethyl)triazenes favored the GGG sequence. The structure of the 3-acyl group of the triazene also played a role in the extent of alkylation of a particular sequence of DNA. Both the (chloroethyl)- and the (hydroxyethyl)triazenes produced higher alkylation product yields for the 3-carbethoxytriazenes as compared with the 3-acetyl derivatives for most of the sequences examined. These overall patterns correlated well with the order of decomposition of the triazenes at 37 °C: HMC > DMA > HMA > CMC > CMA. This study has demonstrated how varying the structure of 1,3-dialkyl-3-acyltriazenes can modulate DNA alkylation, a finding which may be important in the design of new triazene antitumor agents.

Introduction

Several 1-(2-chloroethyl)-3-methyl-3-acyltriazenes have been shown to possess chemotherapeutic activity against a variety of transplanted tumors in mice (*1*). The postulated mechanism of action of these compounds at acidic or neutral pH (Figure 1) involves *N*2−*N*3 heterolysis, leading to production of an alkanediazonium ion. This intermediate can, in turn, either chloroethylate or hydroxyethylate DNA (*2*), depending on the structure of the acyltriazene. At basic pH, or in the presence of a hydrolytic enzyme such as esterase, deacylation becomes the predominant pathway. Acyl group removal produces two tautomeric dialkyltriazenes, which can then decompose to give either a chloroethylating or hydroxyethylating species, or a methylating agent (*2*). Depending on the triazene structure, varying ratios of methylation to chloroethylation or hydroxyethylation were observed in the reaction of the triazene with calf thymus DNA in the presence of esterase (*2*).

While the (hydroxyethyl)guanine adducts are thought to have little importance for antitumor activity, they may contribute to the carcinogenic potential of the title triazenes, as has been postulated for the 1-(2-chloroethyl)-1-nitrosoureas (*3, 4*). Chloroethylation of DNA, on the other hand, probably leads to the production of lethal interstrand cross-links (*5, 6*), which may be responsible for chemotherapeutic activity. Such cross-links are thought to occur by initial attack on the O^6 position of guanine, followed by intramolecular rearrangement involving the N^1 position of guanine. This then allows a $-CH_2CH_2-$ bridge to form between the N^1 of guanine and the N^3 position of a cytosine on the contralateral strand (*7*). It is possible that variations in the chemotherapeutic activity observed with a series of compounds may be linked to differences in this cross-linking ability.

In an effort to understand the mechanism of chemotherapeutic action of acyltriazenes, we examined the reaction of several compounds, including 1-(2-chloroethyl)-3-methyl-3-carbethoxytriazene (CMC)[1] and 1-(2-chloroethyl)-3-acetyl-3-methyltriazene (CMA), as well as their 2-hydroxyethyl analogs HMC and HMA and 1,3-dimethyl-3-acetyltriazene (DMA), with calf thymus DNA and with synthetic polymers of defined sequence. Experiments were undertaken to investigate whether differences in triazene structure would alter the observed initial DNA alkylation patterns. This type of information would be important in the design of chloroethylating agents that could modify a particular position on the various DNA bases, select for a particular sequence in the DNA, or lead to interstrand cross-links.

[†] ABL−Basic Research Program, NCI-FCRDC.
[‡] Western Maryland College.
[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1995.

[1] Abbreviations: CMC, 1-(2-chloroethyl)-3-methyl-3-carbethoxytriazene; CMA, 1-(2-chloroethyl)-3-methyl-3-acetyltriazene; HMC, 1-(2-hydroxyethyl)-3-methyl-3-carbethoxytriazene; HMA, 1-(2-hydroxyethyl)-3-methyl-3-acetyltriazene; DMA, 1,3-dimethyl-3-acetyltriazene; HPLC, high-pressure liquid chromatography; 7-MeG, 7-methylguanine; 7-HOEtG, *N*7-(hydroxyethyl)guanine; 7-ClEtG, *N*7-(chloroethyl)guanine; O^6-MeG, O^6-methylguanine; O^6-HOEtG, O^6-(hydroxyethyl)guanine.

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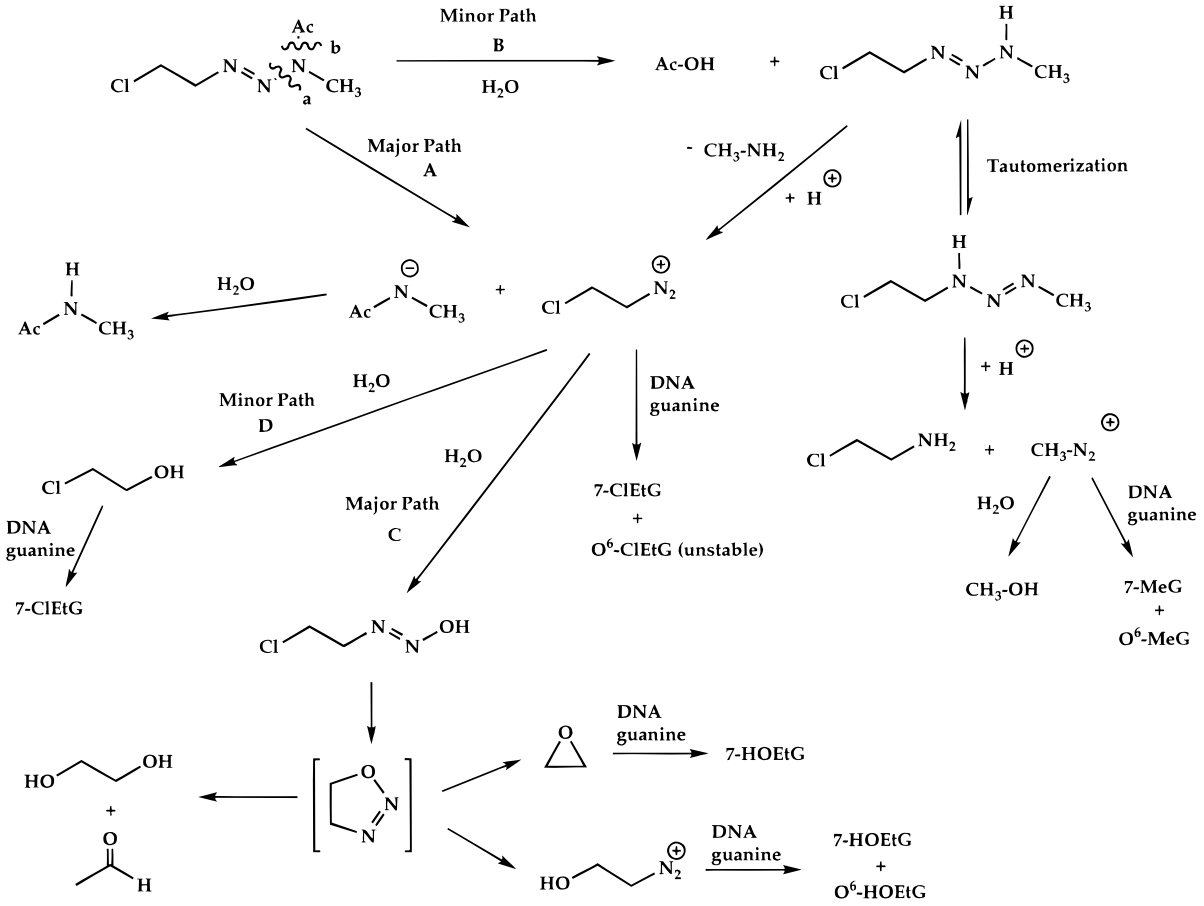


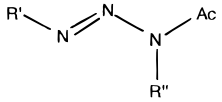
Figure 1. Decomposition pathways of 1-(2-chloroethyl)-3-acyl-3-methyltriazenes.

Materials and Methods

Caution! *Alkyltriazenes are potent biological alkylating agents, mutagens, and carcinogens. All experiments should be carried out in filter-equipped hoods, and proper clothing, especially gloves, should be worn in order to minimize exposure.*

Polydeoxyribonucleotides and Other Materials. Calf thymus DNA was purchased from Sigma Chemical Co. (St. Louis, MO). The synthetic DNA homopolymers poly(dG)·poly(dC) containing the triplet sequence GGG, the alternating polymers poly(dG-dC)·poly(dG-dC) containing the sequence CGC, poly(dA-dC)·poly(dG-dT) containing the sequence TGT, and poly(dA-dG)·poly(dC-dT) containing the sequence AGA were purchased from LBK/Pharmacia (Piscataway, NJ). The acyltriazenes HMC, HMA, CMC, CMA, and DMA were prepared, purified, and characterized according to the procedure of Smith et al. (*8*, *9*) and were >99% pure (NMR). The triazenes were dissolved in Burdick and Jackson dimethyl sulfoxide (VWR Scientific, West Chester, PA) immediately prior to use. Standard alkylated bases were either purchased (7-methylguanine, Sigma Chemical Co.) or synthesized in our laboratory (*10*), except for 7-(chloroethyl)guanine, which was a generous gift of Dr. David Ludlum, University of Massachusetts Medical School, Worcester, MA.

NMR Decomposition and Product Studies. The extent of decomposition of the various acyltriazenes (Figure 2) after 48 h at 37 °C was measured in 0.05 M sodium phosphate buffer with 0.25 M NaCl plus 0.0025 M fumaric acid in D_2O adjusted to pH 7.5 with NaOD. Buffer was added to a weighed amount of triazene, sealed in glass ampules, and incubated for 0, 24, or 48 h. The initial triazene concentration was 0.0100 M. At the end of the reaction time, an aliquot of the reaction solution was removed and analyzed by ^{1}H NMR on an XL-200 Varian spectrometer. Assignment of the NMR peaks arising from the decomposition of the triazenes was made by comparison with authentic samples of starting materials and products and confirmed by coincidence of peaks upon addition of authentic material. Quantities were determined by comparative integration of the product peaks and confirmed by use of the vinyl signal from the fumaric acid as an internal standard. Rates of decomposition were calculated from the amount of starting material remaining after 24 h (HMC) or 48 h (DMA, HMA, CMC, and CMA) according to the equation: $\ln(A_0/A) = kT$.



R'	R''	Ac	Acyltriazene
CH_3	CH_3	$COCH_3$	DMA
HOEt	CH_3	$COCH_3$	HMA
HOEt	CH_3	CO_2Et	HMC
ClEt	CH_3	$COCH_3$	CMA
ClEt	CH_3	CO_2Et	CMC

Figure 2. Structures and abbreviations of 1,3-dialkyl-2-acyltriazenes.

The conversion of the acyltriazenes CMC, CMA, HMC, and HMA to methanol was measured in 0.02 M sodium phosphate buffer with 0.01 M NaCl plus 0.05 M maleic acid in D_2O adjusted to pH 7.4 with NaOD (nominal pH 6.96). Buffer was added to a weighed amount of triazene, sealed in ampules, and incubated for 90 h at 70 °C. The initial triazene concentration in each reaction was 0.02 M. In addition, the production of methanol was also assayed from the decomposition of CMA in the presence of calf thymus DNA (final triazene concentration 0.005 M). Assignment of the NMR peaks arising from the decomposition of the triazenes was made as described above, using maleic acid as the internal standard.

Alkylation Assay. The alkylation of calf thymus DNA or the synthetic polymers (0.52 mg/mL in 20 mM sodium phos-

phate buffer, 10 mM NaCl, pH 7.0) by the acyltriazenes was accomplished at 37 °C. The reaction was initiated by the addition of 10 μL of a 0.5 M solution of the triazene in DMSO (5 mM final concentration) to 990 μL of the calf thymus DNA or polymer solution in buffer. The reaction was mixed on a Vortex mixer and incubated for 48 h. The reactions were stopped by the addition of 3 mL of cold absolute ethanol, and the DNA was pelleted by centrifugation (15 min at 5000*g*). The pellet was washed first with cold 2% sodium acetate in ethanol and allowed to sit in this solution for 15 min. The solvent was then decanted off and replaced by ethanol/ether (1:1) for 5 min and finally by ether. After the ether was decanted, the DNA pellets were dried in a vacuum desiccator and stored at −20 °C until hydrolysis.

The stability of the 7-(chloroethyl)- and 7-(hydroxyethyl)-guanine adducts to depurination was measured by reacting a 50 μL aliquot of CMC or CMA with 4950 μL of calf thymus DNA or poly(dG)·poly(dC) (0.52 mg/mL). The alkylation of the guanine was allowed to proceed at 37 °C for 48 (CMC) or 90 h (CMA). At the end of this time, the DNA was isolated by precipation with 15 mL of ethanol and was then washed and dried as described above. The dried DNA was redissolved in 5 mL of buffer, and 1 mL aliquots were removed at time points between 0 and 48 h. The aliquots were precipitated, dried, and stored as described above until hydrolysis.

Analysis of DNA Adducts. Hydrolysis and chromatography of the DNA adducts (7-MeG, 7-HOEtG, 7-ClEtG, O^6-MeG, and O^6-HOEtG) were carried out as previously described (*10*). Quantitation of the adducts was accomplished by comparison of the areas of the HPLC chromatographic peaks to a standard curve, which was remeasured each day. Results are reported as mmol of alkylated base/mol of guanine on at least triplicate experiments.

Polymer Decomposition Kinetics. The kinetics of triazene disappearance in the presence and absence of the poly(dG-dC)·poly(dG-dC) and the poly(dG)·poly(dC) polymers were measured in the following manner. Reactions consisted of 990 μL of buffer (pH 7.0) or synthetic polymer (0.52 mg/mL) dissolved in the buffer and 10 μL of a 0.5 M DMSO solution of either CMC or CMA (final concentration 5 mM). In place of some of the buffer, EDTA was added to various reaction mixtures to give a final concentration of 2.3 mM. The reactions were allowed to run for times up to 119 h, with 50 μL aliquots withdrawn at times of 0, 3, 7, 24, 30, 48, 57, 72, 95, and 119 h. The aliquots were assayed immediately by HPLC using a reverse phase C_{18} column at a flow rate of 1 mL/min with an isocratic solvent mixture of water/methanol (3:1). The triazene peak was monitored at 234 nm and was integrated by a computer program. The rate constants reported are the average of at least two determinations.

Results

NMR Studies. The approximate rates of triazene decomposition, as determined from the amount of triazene remaining after 48 h at 37 °C, are shown in Table 1. The relative order of decomposition of the acyltriazenes at 37 °C was found to be the same as that seen at 70 °C (HMC > DMA > HMA > CMC > CMA) (*11*). The ratios of the rates at each temperature were comparable except for CMA, whose rate of decomposition at 37 °C was roughly half of that predicted by assuming roughly parallel temperature dependence of the rates.

The products from the decomposition of the various triazenes (see Figure 1), measured by NMR, were the same as determined previously (*11*). The *N*1 products were either methanol (DMA), ethylene glycol and acetaldehyde (HMC, HMA), or 2-chloroethanol, ethylene glycol, and acetaldehyde (CMC, CMA). The *N*3 product was *N*-methylacetamide from the acetyltriazenes and ethyl *N*-methylcarbamate from the carbethoxytriazenes.

Table 1. Rates of Decomposition of 1,3-Dialkyl-3-acyltriazenes in Phosphate Buffer[a] at pH 7.5

acyltriazene	k_{obs}[b] (s^{-1}) at 70 °C	k_{obs}[c] (s^{-1}) at 37 °C
HMC	2.22×10^{-3}	3.3×10^{-5}
DMA	7.16×10^{-4}	1.3×10^{-5}
HMA	4.53×10^{-4}	7.8×10^{-6}
CMC	2.46×10^{-4}	3.2×10^{-6}
CMA	4.46×10^{-5}	2.5×10^{-7}

[a] Buffer concentration 0.05 M; ionic strength of 0.25 M held constant with added $NaClO_4$. [b] The rate constants are an average of at least two independent runs varying by no more than ±3%; triazene initial concentration 3.0×10^{-5} M; data taken from ref 11. [c] The rate constants were calculated from the amount of acyltriazene decomposition in 48 h (see Materials and Methods); triazene initial concentration 0.0100 M.

The appearance of methanol as a product following incubation of various triazenes other than DMA for 90 h at 70 °C in sealed ampules was reinvestigated by NMR. The spectra showed that when all of the triazene had decomposed, the maximum amount of methanol produced was 3.4% from HMA and 1.5% from CMA, while both HMC and CMC showed no detectable peak for methanol. Incubation experiments with CMA in the presence of DNA at 37 °C showed no increase in the levels of methanol, showing that the presence of DNA did not change the spectrum of decomposition products.

Alkylation Studies. The analysis of synthetic DNA polymer or calf thymus DNA alkylation products by HPLC coupled with fluorescence detection, following alkylation by CMC, CMA, HMC, HMA, or DMA, revealed the formation of the adducts 7-MeG, 7-HOEtG, 7-ClEtG, O^6-MeG, and/or O^6-HOEtG, depending on the triazene investigated. The results of these studies are summarized in Table 2. Since poly(dG)·poly(dC) and poly(dG-dC)·poly(dG-dC) contain 50% guanine, while poly(dA-dC)·poly(dG-dT) and poly(dA-dG)·poly(dC-dT) contain 25% guanine and calf thymus DNA contains 21.7% guanine (*12*), the yields have been "normalized" by dividing the actual millimoles of alkylated guanine detected by the mole percentage of guanine present in the polymer sequence. The data reported in this manner allow for direct comparison of the susceptibility of any particular sequence in the polymer to preferential alkylation of DNA guanine upon exposure to a given triazene for 48 h at 37 °C.

As expected, the composition of the polymer sequences was found to be important in the alkylating pattern of the triazenes. For any or all of the 7-alkyl guanine adducts from each triazene, it can be seen that DNA sequences which have runs of purines were alkylated to a greater extent than are sequences which alternate purines and pyrimidines. However, within the framework of this generalization, the structure of the triazene was also found to be a determinant in the observed sequence selectivities. Both of the (hydroxyethyl)triazenes examined hydroxyethylated calf thymus DNA to a greater extent than the other triazenes. For the 7-HOEtG adduct from HMC, the sequence of reactivity for the polymers was in the order: AGA > GGG > CGC ≫ TGT, while for HMA the order was AGA > GGG ≫ CGC > TGT. In addition, both (hydroxyethyl)triazenes formed an unknown 7-alkylguanine product (data not shown), as has been reported previously both by us (*2*) and others (*13*). Both of the (chloroethyl)triazenes chloroethylated and/or hydroxyethylated the GGG polymer best, followed by calf thymus DNA ≫ AGA > TGT > CGC, depending

Table 2. Alkylation of DNA Polymers by Various Acyltriazenes

			mmol of alkylated base/mol of guanine				
agent	polymer	N	7-MeG	7-HOEtG	7-ClEtG	O6-MeG	O6-HOEtG
HMC	poly(dG)·poly(dC)	GGG	0.08	9.92		0.16	5.45
	poly(dG-dC)·poly(dG-dC)	CGC	nd[a]	8.99		0.12	5.89
	poly(dA-dC)·poly(dG-dT)	TGT	nd	1.53		0.05	2.19
	poly(dA-dG)·poly(dC-dT)	AGA	0.67	13.08		0.32	9.69
	ct DNA	NGN	4.27	12.52		0.26	8.05
CMC	poly(dG)·poly(dC)	GGG	nd	18.30	9.99	0.12	0.10
	poly(dG-dC)·poly(dG-dC)	CGC	0.18	0.41	2.42	0.04	0.30
	poly(dA-dC)·poly(dG-dT)	TGT	0.47	0.53	0.94	nd	0.61
	poly(dA-dG)·poly(dC-dT)	AGA	0.72	1.57	7.71	nd	0.90
	ct DNA	NGN	2.09	5.27	9.29	nd	0.44
HMA	poly(dG)·poly(dC)	GGG	1.32	7.50		1.09	4.56
	poly(dG-dC)·poly(dG-dC)	CGC	0.39	3.83		0.79	3.30
	poly(dA-dC)·poly(dG-dT)	TGT	nd	3.22		0.43	0.56
	poly(dA-dG)·poly(dC-dT)	AGA	1.24	11.52		1.54	4.99
	ct DNA	NGN	1.81	22.95		1.92	4.60
CMA	poly(dG)·poly(dC)	GGG	1.08	3.81	2.56	0.30	0.05
	poly(dG-dC)·poly(dG-dC)	CGC	0.56	0.10	0.14	0.28	0.11
	poly(dA-dC)·poly(dG-dT)	TGT	1.12	0.21	1.19	0.11	0.54
	poly(dA-dG)·poly(dC-dT)	AGA	1.53	0.38	nd	0.31	0.68
	ct DNA	NGN	2.56	1.01	2.07	0.42	0.22
DMA	poly(dG)·poly(dC)	GGG	87.40			30.92	
	poly(dG-dC)·poly(dG-dC)	CGC	33.41			17.31	
	poly(dA-dC)·poly(dG-dT)	TGT	34.52			16.52	
	poly(dA-dG)·poly(dC-dT)	AGA	177.89			63.23	
	ct DNA	NGN	167.16			77.68	

[a] nd = adduct not detected.

Table 3. DNA Alkylation Ratios

	7-HOEtG + 7-ClEtG/7MeG		7-HOEtG/7-ClEtG		7-HOEtG/7-MeG				O^6/7-HOEtG				O^6/7-MeG				
DNA polymer	CMC	CMA	CMC	CMA	CMC	CMA	HMC	HMA	CMC	CMA	HMC	HMA	CMC	CMA	HMC	HMA	DMA
poly(dG)·poly(dC)	nd	5.9	1.8	1.5	25.92	3.5	123	5.7	0.01	0.01	0.55	0.61	0.17	0.28	2.0	0.82	0.35
poly(dG-dC)·poly(dG-dC)	15.3	0.4	0.17	0.7	2.2	0.18	nd	9.7	0.73	1.1	0.66	0.86	0.2	0.5	nd	2	0.52
poly(dA-dC)·poly(dG-dT)	3.1	1.3	0.56	0.18	1.1	0.24	nd	nd	1.2	2.5	1.4	0.17	nd	0.1	nd	nd	0.48
poly(dA-dG)·poly(dC-dT)	13	0.25	0.2	nd	2.2	0.25	19.5	9.3	0.57	1.8	0.7	0.43	nd	0.2	0.5	1.2	0.36
ct DNA	7	1.2	0.57	0.49	2.5	0.4	2.9	12.7	0.08	0.22	0.64	0.2	nd	0.17	0.06	1.1	0.46

on the triazene. However, they all methylated calf thymus DNA preferentially to the other sequences, with the exception of DMA, which methylated the AGA polymer equally well. Thus, the one triazene which is a pure methylating agent did not follow the pattern of the other acetyltriazenes.

Polymers with runs of purines are also preferentially alkylated in the O^6-position of guanine, as compared with those containing runs of alternating pyrimidines and purines. For all of the triazenes, the O^6-HOEtG adduct was formed to the greatest extent in the AGA sequences; however, the second best DNA for the formation of this product from the (hydroxyethyl)triazenes was calf thymus DNA. The (chloroethyl)triazenes surprisingly alkylated the TGT polymer second, followed by calf thymus DNA. For O^6-MeG, calf thymus DNA is alkylated to the greatest extent by all of the compounds except for HMC, where AGA was methylated slightly better than calf thymus DNA. Thus, for O^6-methylation, the three acetyltriazenes followed the same pattern, while the carbethoxytriazenes showed a different selectivity.

As can be seen from the Table 2, the acyltriazene which was the best overall alkylating agent varied with the sequence examined. DMA alkylated DNA to a greater extent than any other triazene in all sequences. The order of the rate of decomposition of the triazenes (see above) paralleled total 7-alkylation only in the GCG or AGA polymers. For calf thymus DNA, the order was HMA > HMC ≥ CMC > CMA, while for poly(dG)·poly(dC), the order was CMC ≥ HMC > HMA ≥ CMA. For poly(dA-dC)·poly(dG-dT) the pattern was again different with HMA > CMA > CMC > HMC. The rates of decomposition of the triazenes were also reflected in the alkylation by all of the triazenes on the O^6-position of guanine. For calf thymus DNA and the polymers, the order of alkylation followed the order of triazene decomposition.

The ratios of the adducts (Table 3) varied with both the sequence of the polymer as well as the structure of the triazene. The (hydroxyethyl)triazenes showed higher 7-HOEt/MeG ratios than do the (chloroethyl)triazenes, indicative of the fact that the former compounds can only hydroxyethylate DNA. The ratio of 7-ClEt/7-MeG or 7-HOEt/7-MeG for CMC was always greater or equal to that for CMA in all the sequences examined. These ratios reflect the experimental alkylation levels for CMA relative to CMC (Table 2), which showed an elevation in the amount of 7-MeG in all sequences examined. This slight shift in adduct preference (i.e., elevation in the methylation level at the expense of chloroethylation or hydroxylation by CMA) is not attributable to the 3-acetyl group since HMA and HMC clearly do not show the same shift in the 7-HOEt/MeG ratio. For the CMC and CMA, the ratio of 7-HOEt/ClEtG was either 9 or 2 times higher in poly(dG)·poly(dC) as compared to poly(dG-dC)·poly(dG-dC). However, within the context of each GC se-

Table 4. Rates of Depurination of 7-Alkylguanine Adducts

	k_{obs} (s^{-1})	
	7-ClEtG	7-HOEtG
CMC/ct DNA	3.6×10^{-6}	3.2×10^{-6}
CMC/GGG polymer	6.3×10^{-7}	
CMA/ct DNA	1.8×10^{-6}	6.4×10^{-6}
CMA/GGG polymer	2.5×10^{-6}	

quence, both triazenes gave similar numbers for this ratio, showing that the enhanced apparent reactivity toward runs of guanines was not due to a preference for chloroethylation over hydroxyethylation.

Comparison of the O^6/7-MeG or O^6/7-HOEtG ratios (Table 3) between different triazenes reveals some interesting trends. The (hydroxyethyl)triazenes, in general, and HMA, in particular, had higher O^6/7-MeG ratios, i.e., larger amounts of O^6 methylation, than did DMA, which, in turn, was the same or higher than those of the (chloroethyl)triazenes. It therefore appears that the *N*1-alkyl group on the triazene is the important determinant in the partitioning of methylation between the O^6- and 7-positions on guanine. The conclusions that can be drawn from the O^6/7-HOEtG ratios are less clear. In general, the major differences in this ratio for the (chloroethyl)triazenes are seen when comparing the calf thymus DNA or the poly(dG)·poly(dC) sequences to the others; in the former two cases, the ratio is much lower. This ratio in the CGC, TGT, and AGA sequences for CMA was approximately double that for CMC. Except for the poly(dG)·poly(dC) sequence, the (chloroethyl)triazenes had ratios which were the same or higher than the (hydroxyethyl)triazenes. The presence of a carbethoxy group in the triazene changed the ratio only for the poly(dG)·poly(dG) and the calf thymus DNA sequences (where the HMC ratio was quite a bit higher than it was for CMC); in the other sequences the ratio was identical for both triazenes.

The stability of the 7-HOEtG and 7-ClEtG adducts was measured after incubation of the DNA polymers in phosphate buffer following previous reaction with either CMC or CMA. As can be seen from the rates of decomposition of the adducts in Table 4, the 7-HOEtG and the 7-ClEtG adducts produced from both triazenes in the calf thymus DNA reactions decomposed, presumably by depurination. However, only 7-ClEtG depurinated from reactions with the GGG polymer. For CMC, the rate of 7-ClEtG depurination was faster if this adduct was produced from calf thymus DNA reactions than if it arose from reactions with GGG; however, the reverse was true with the 7-ClEtG adduct from CMA. The 7-HOEtG adduct from calf thymus DNA reactions with CMA depurinated faster than the same adduct from CMC.

Triazene Decomposition Studies in the Presence of Polymer. The rates of decomposition of CMC or CMA in phosphate buffer alone or in the presence of synthetic DNA polymer in buffer at 37 °C were analyzed by HPLC measurement of the disappearance of substrate (data not shown). The rates of decomposition in buffer alone are essentially the same as those determined by NMR analysis, making some allowance for the differences in the methods. The presence of nucleic acid polymer did not substantially alter these rates within experimental error. For example, the rate of decomposition of CMC in the presence of poly(dG)·poly(dC) was 2.04×10^{-6} s^{-1} by HPLC analysis, as compared with 3.15×10^{-6} s^{-1} determined from the NMR study. The results also revealed that the rates of decomposition of the two triazenes, in either the presence or absence of the chelating agent EDTA, were not significantly different irrespective of the polymer. Therefore, the polymers themselves are not acting as catalysts in triazene decomposition.

Discussion

The fact that DNA base sequence can affect patterns of alkylation by various agents; including nitrosoureas and 1-aryltriazenes, has been previously documented. For example, even the simple ethyl- and methylnitrosoureas show such selectivity, with the methyl analog exhibiting more reactivity toward sequences which are composed exclusively of A-T base pairs, while the ethyl analogs demonstrate a preference for consecutive G-C bases (*14*). Various (chloroethyl)nitrosoureas have been shown to favor alkylation at runs of guanines rather than at random base sequences of DNA, although the degree of selectivity was found to vary with both the position alkylated and the nature of the electrophilic species (chloroethyl or hydroxyethyl) (*12*, *15*, *16*). Several 1-aryl-3-alkyltriazenes and (alkyltriazenyl)imidazoles were also examined for their ability to selectively alkylate a fragment of pBR322 DNA (*17*). The monomethyl and monochloroethyl triazenes alkylated guanines extensively at the 7-position of guanine with a preference for runs of contiguous guanines, similar to but not as striking as that observed for the (chloroethyl)nitrosoureas.

The results of the present study reveal that, like the nitrosoureas, acyltriazenes exhibit definite preferences in the alkylation of various DNA polymer sequences, with the selectvity varying with the structure of the triazene. The selective alkylation effects are not likely to be due to DNA conformational changes during the course of the reaction, since it has been previously reported that the double-stranded polymers used in our study prefer the B-DNA conformation under the reaction conditions employed (*16*). All of our reactions were carried out for the same length of time in an attempt to eliminate any variables except for the differential rates of triazene decomposition. Further, the rate of disappearance of the triazene was shown to be independent of the presence of DNA. Since one can eliminate the above possibilities as contributing factors in the observed sequence selectivity, we must then consider the structural differences between the triazenes as the pertinent factors in their observed selective alkylation of DNA.

The most important result of the present study in that regard was that DNA sequences containing runs of purines (this includes calf thymus DNA) are the most reactive toward alkylation by all of the triazenes tested, whether measured by *N*7, O^6, or total guanine adducts. Within that generalization, (hydroxyethyl)triazenes showed a preference for the AGA sequence, while (chloroethyl)triazenes favored GGG. The high levels of alkylation observed in calf thymus DNA for all of the adducts, as compared with the levels of the comparable adducts in the other polymers, had also been observed by Briscoe et al. (*11*, *15*). They noted that, following alkylation by 1,3-bis(2-chloroethyl)-1-nitrosourea, the 7-HOEtG level resulting from averaging data obtained separately using four synthetic polymers was the same as the experimental value detected in calf thymus DNA. This finding was also obtained in the DNA alkylation data by choloreth-

yltriazenes in the present study. In the case of the hydoxyethyltriazenes, however, calf thymus DNA was alkylated to a greater extent than any of the synthetic polymers; the reason for this finding is unknown, but is clearly reflective of the structural difference in the *N*1-alkyl group.

The basis for the preferential alkylation in purine runs is most likely the result of a complex mixture of competing factors. It has been suggested (*12*) that one reason for this may be that guanines in runs of guanines are the most electronegative of the sequences tested (GGG ≫ AGA > TGT > CGC) (*17*). In their study of (chloroethyl)nitrosoureas, which showed the same high selectivity for alkylation in runs of guanines as we observed, Briscoe et al. (*12*) found that this electronegativity hypothesis correlated well for the 7-HOEt adduct from various (chloroethyl)nitrosoureas and moderately well for the 7-ClEtG adduct. Examination of the levels of the 7-(hydroxyethyl)guanine adduct from our studies reveals that the (chloroethyl)triazenes alkylate between 10−12 times more in the GGG sequence than they do in the AGA sequence. However, the 7-ClEtG levels are only slightly elevated in the GGG sequence, as compared to AGA. Therefore, the electronegativity of a sequence of guanines is most likely only one contributing factor in total 7-alkylation.

A second important factor may involve steric effects by neighboring bases. This, along with the decreased nucleophilicity, probably accounts for the lower alkylation of TGT by the triazenes, where the 5-methyl group of thymine may hinder the accessibility of the *N*7 of the guanine (*18*). However, steric considerations should not be involved in the lower amount of hydroxyethylation observed in the AGA sequence as compared to the GGG sequence, since both the *N*7 and O^6 positions in GGG and AGA should be equally open to attack. The reason for the preference for the AGA sequences by the (hydroxyethyl)triazenes is therefore unknown.

A third factor which may influence the observed elevated alkylation yields in runs of purines may involve the stability of the various adducts themselves. The (chloroethyl)triazenes yielded more 7-HOEtG in the GGG polymer than in calf thymus DNA, yet approximately equivalent amounts of 7-ClEtG are produced in both polymers. A clue to understanding this result is provided by the data on depurination of the 7-ClEt and 7-HOEtG adducts formed from the triazenes in the presence of calf thymus DNA or poly(dG)·poly(dC) (see Table 4). While both adducts were slowly lost through depurination from the calf thymus DNA reaction, only the the 7-ClEtG adduct was lost following reaction with the GGG polymer. The 7-HOEtG adduct from the GGG reaction, by comparison, is relatively stable. Thus the observed higher yield of 7-HOEtG from GGG, as compared with that from calf thymus DNA, may simply be the result of the partial decomposition of this adduct from calf thymus DNA during the 48 h reaction period.

Comparing the two class of triazenes, structural differences that affect the pathway of decomposition were also seen to play a significant role in the noted patterns of alkylation. The results of the studies clearly showed that DMA, a substance which produces exclusively methanediazonium ion, was the most effective alkylating agent of all of the acyltriazenes tested. This presumably is simply a result of the high susceptibility of DNA towards methylating agents, as compared with other types of alkylating agents and the fact that the only alkylation products possible from DMA are the result of methylation.

A second variable examined was whether the alkylating triazene bears an *N*1 hydroxyethyl or chloroethyl group. Inspection of the yields of *N*7-HOEtG and O^6-HOEtG in Table 2 reveals that, with almost all of the polymers, the levels for the (hydroxyethyl)triazenes are higher than those for the (chloroethyl)triazenes. The observed enhancement is not a simple rate effect; HMC decomposed about 10 times faster in buffer at neutral pH than did CMC. However, the level of O^6-HOEtG produced by HMC in the GGG homopolymer was about 54-fold higher than that produced by CMC. The HMC/CMC ratio for this same adduct in calf thymus DNA was about 19. The same type of comparison holds for HMA and CMA.

Figure 1 shows the mechanisms by which (hydroxyethyl)- and (chloroethyl)- triazenes decompose to alkylating agents, resulting in the formation of O^6- and *N*7-HOEtG. Given that a portion of the alkylating agents produced by (chloroethyl)triazenes lead to (chloroethyl)-guanines, it is reasonable to expect that (hydroxyethyl)-triazenes, purely hydroxyethylating agents, would give higher overall yields of (hydroxyethyl)guanines.

What is surprising is the range of variation in the O^6-HOEtG/7-HOEtG ratio seen with different triazenes and polymers. Clearly, this is a result of a complex interaction of several factors. As has already been mentioned, the varying stability of the same adduct (e.g., *N*7-HOEtG) in different polymers will skew the apparent yields and ratios of the products. Further, it can be seen from Figure 1 that (chloroethyl)triazenes lead to (hydroxyethane)guanines by two different pathways, C and D. One of these, C, leads to the same species, the hydroxyethanediazonium ion, which is presumably the sole alkylating agent derived from (hydroxyethyl)triazenes. This would lead to the expectation that the O^6-HOEtG/*N*7-HOEtG ratio from both types of triazenes would be identical. However, the presence of alternative pathways by which (chloroethyl)triazenes can produce (hydroxyethyl)guanines through different alkylating agents (e.g., chloroethanol, ethylene oxide, and oxadiazoline) (*6*) would be expected to give different O^6-HOEtG/*N*7-HOEtG ratios. Because some of these alternative pathways have been reported to give exclusively *N*7-HOEtG, it is even conceivable that in some cases a (chloroethyl)triazene could give a higher yield of this adduct than does the corresponding (hydroxyethyl)triazene.

A third factor important in the extent of alkylation of a given type of DNA was the structure of the triazene acyl group. For most DNA sequences, 3-carbethoxytriazenes gave higher yields of all types of adducts when compared with 3-acetyltriazenes. This holds true for both the (hydroxyethyl)- and (chloroethyl)triazenes. These overall patterns tend to mirror and may be explained by the rates of decompostion of the triazenes at 37 °C in phosphate buffer: HMC > HMA > CMC > CMA. The differences in yields are roughly of the same order as the differences in the rates of triazene decomposition.

Comparison of the ratios of 7-HOEtG + 7-ClEtG/7-MeG for CMA vs CMC (see Table 3) shows that an elevation of methylation occurred from CMA at the expense of hydroxyethylation and chloroethylation (i.e., lower ratios for CMA in all polymer sequences). On an absolute percent yield basis, a 0.0018% yield of 7-MeG was produced from CMC, while this number was 0.01% for CMA (5.5 times greater). Under the conditions of the

experiment, increased deacylation of CMA (pathway B, Figure 1) would be necessary to account for this additional methylation. Deacylation of CMA would produce 2-(chloroethyl)-3-methyltriazene as the first product. This, in turn, has been shown preferentially to give the methanediazonium ion. In order to further investigate the increased production of 7-methylguanine from CMA, we attempted to trap the methanediazonium ion as methanol by incubation of this triazene at 37 °C in a sealed ampule in buffer, both with and without DNA present. The results from the NMR spectrometry showed some (1.5%) methanol in reaction mixtures without DNA and no methanol when DNA was present, presumably because the methanediazonium ion was scavenged by the DNA. No more than a trace of methanol could be found in a similar experiment from CMC. Thus it appears that CMA gives a higher yield of the methanediazonium ion than does CMC. While this yield is small, the high alkylation efficiency of this species accounts for what might otherwise seen as unusually low 7-HOETG + 7-ClEtG/7MeG ratio for CMA. We further showed that the presence of different DNA polymers had no effect on the rate of triazene decomposition, as compared with the decomposition of the same triazenes in buffer alone. However, the presence of the polymer did not increase the rate.

One of the interesting, but still unexplained, findings of the aforementioned alkylation study using the pBR322 fragment (*17*) was that the nonalkylating portion of a triazene could influence the ultimate alkylation spectrum. In that study, two different (chloroethyl)triazenes produced different patterns of alkylation, suggesting that they did not act through a common intermediate (chloroethyl diazohydroxide), as had been previously postulated was the case in the alkylation of DNA by the (chloroethyl)nitrosoureas (*20*−*24*). In our study we have observed that the ratio of product from CMC to CMA for any given sequence paralleled the ratio (13) of the rate of decomposition of the two compounds, suggesting the same decomposition pathway was operative for both compounds. This is not the case, however, for the (hydroxyethyl)triazenes. HMA alkylated the 7-position of guanine from 0.5 to 2.3 times as much as did HMC, depending on the sequence. HMA, however, decomposes at a rate which is 0.2 times that of HMC. Moreover, in all of the sequences examined except calf thymus DNA, the amount of methylation from HMA vs HMC appeared to be elevated at the expense of hydroxyethylation (see ratios in Table 3). This correlates with the small amount of methanol detected from HMA, but not HMC, in the NMR experiments. The fact that HMA also showed this increased amount of methylation means that the presence of the acetyl group alone may possibly be a sufficient condition for deacylation to occur. However, the exact reason(s) for an acetyl vs a carbethoxy acyl group on the triazene affecting the balance of decomposition via different pathways awaits further experimentation.

DNA modifications by acyltriazenes are subject to multiple factors. One of these is the structure of the acyl group, which appears to offer considerable potential as a means of modulating the activity of acyltriazenes. The acyl group exerts its major influence through its electronic characteristics, which serve to modulate the rate and mechanism by which acyltriazenes decompose into reactive products. In the extreme, the acyl group can change the fundamental chemistry of the acyltriazenes, as had been observed in the case of 1-(2-chloroethyl)-3-methyl-3-(*N*-methylcarbamoyl)triazene (*25*). In addition, the acyl group also appears to influence DNA alkylation by a more subtle mechanism, which is as yet undefined. In any case, the modulation of alkylation by the presence of specific groups in the acyl position of the triazene has important implications in the design of new, more potent chemotherapeutic alkylating agents.

Acknowledgment. This research was sponsored by the National Cancer Institute, DHHS, under Contract NO1-CO-46000 with ABL. R.H.S. was supported in part by the National Science Foundation (CHE-9215925). The authors wish to thank Mr. John Klose for the NMR spectral determinations and Dr. Grzegorz Czerwinski for his assistance in the interpretation of the NMR data. The contents of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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TX9500742