Structure-Activity Relations of (2-Chloroethyl)nitrosoureas. 2. Kinetic Evidence of a Novel Mechanism for the Cytotoxically Important DNA Cross-Linking Reactions of (2-Chloroethyl)nitrosoureas[1]

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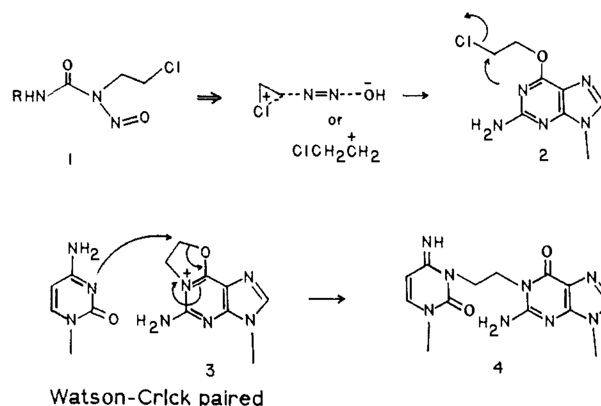
Abstract: The anticancer agents 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and 2-chloroethyl (methylsulfonyl)methanesulfonate (SoSo) should form the same initial 2-chloroethyl (ClEt) adduct at O^6-guanine (O^6-dG) in DNA, which is thought to be the first intermediate in a sequence that leads to lethal DNA interstrand cross-links, and should therefore have the same mechanism of cross-link formation. We determined rate constants for the in vitro cross-linking reactions of calf-thymus DNA treated with BCNU and SoSo (pH 7.2, 37 and 50 °C). For both drugs, cross-linking of DNA is first-order and probably reflects a rate-limiting rearrangement of initial adducts that form cross-links in subsequent fast steps. Values of k_{obsd} are different for the two drugs, however, with cross-linking for BCNU ca. 6-fold faster than for SoSo. On the basis of our kinetic data and other results, we propose two alternative mechanisms. SoSo may form ClEt-O^6-dG in duplexed B-DNA and cross-link by a mechanism that incorporates part of the standard model; after the helix opens, cyclization of the initial adduct to the putative ethanoguanine intermediate may occur, and cross-links may form by a displacement reaction during reclosure of the duplex. BCNU cross-linking may involve cyclization of a tetrahedral intermediate formed by addition of the imidourea form of CENUs to O^6-dG$_1$ in a 5'-dG$_1$dG$_2$dN$_3$-3' codon, where dN is any other base. In this mechanism, the direct precursor to DNA interstrand cross-links and (2-hydroxyethyl)-O^6-dG products is (2-(hydroxyazo)ethyl)-O^6-dG formed by a reaction between the cyclized tetrahedral intermediate and a major groove nucleophile on the adjacent dG. It is probable that the latter step is rate-limiting and that cross-link formation is not related to opening of duplex B-DNA.

(2-Chloroethyl)-1-nitrosoureas (CENUs)[2] are widely used for the treatment of solid systemic and brain tumors; clinically, the most effective of these agents is 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU).[3] It is generally accepted that CENUs kill cells by cross-linking the strands of duplex DNA[4] by a multistep mechanism usually formulated as shown in Scheme I:[5] Parent CENU hydrolyzes to reactive carbocations or to their precursors that alkylate DNA by either electrophilic addition to ("RCH_2^+") or direct displacement by ($RCH_2N_2^+$, RCH_2N=NOH) DNA nucleophiles.[6] The initial lesion is thought to be the (2-chloroethyl)-O^6-guanine (ClEt-O^6-dG) adduct **2** that cyclizes to the reactive N_1,O^6-ethano-dG **3**, which in turn reacts with N$_3$ of the Watson-Crick paired cytosine on the opposite strand to form the interstrand cross-linked base pair **4**.[7] The ClEt-N$_7$-dG adduct is thought to lead to the bis-N$_7$-diguanylethyl intrastrand cross-link.[8] Excision of **2** by the repair protein O^6-alkylguanine DNA alkyltransferase (GATase) in malignant tissue is thought to account for therapy-limiting resistance to CENUs.[9,10]

One factor limiting the clinical use of CENUs is myelotoxicity that may be caused by carbamoylation reactions of CENUs.[11] 2-Chloroethyl (methylsulfonyl)methanesulfonate (SoSo), an "S$_N$2" reagent that does not carbamoylate DNA or proteins, was identified in a search for chloroethylating agents that should give the adduct **2** and should cross-link DNA through the reactions predicted by the standard model.[12] SoSo is an effective cytotoxic agent against tumor cells in vitro,[13,14] but soon after in vitro testing of SoSo began, it became apparent that the mechanisms of action of BCNU and SoSo might be different.[15,16] Some of our work on the effects of heat[17] and X-rays[18] on cell kill in combination protocols with CENUs and the apparently anomolous behavior of CENUs with different structures on repair processes[19] suggested that mechanisms other than the standard model might be involved. Attempts to rationalize the solution chemistry of CENUs with

Scheme I



DNA reactions led to a study of solvent isotope effects on CENU hydrolysis,[1] computer graphics modeling studies,[20] an extensive

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(2) The abbreviations used are: CENUs, 1-(2-chloroethyl)-1-nitrosoureas; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; dG, deoxyguanine; ClEt, (2-chloroethyl) group; HOEt, (2-hydroxyethyl) group; GATase, O^6-alkylguanine DNA alkyltransferase; SoSo, 2-chloroethyl (methylsulfonyl)methanesulfonate; PNU, 1-*n*-propyl-1-nitrosourea; ANUs, alkylnitrosoureas; CT-DNA, calf-thymus DNA; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; HN2, bis(2-chloroethyl)methylamine; CNU, 1-(2-chloroethyl)-1-nitrosourea; dMCNU, 1-(2-chloroethyl)-3,3-dimethyl-1-nitrosourea.

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(9) Brent, T. P. *Cancer Res.* **1984**, *44*, 1887-1892 and references cited therein.

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† Dedicated to Professor Richard A. Sneen on the occasion of his retirement.

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review and reconsideration of published results for CENU chemistry,[21] and the studies reported here. On the basis of these results and the recently published work of other groups, we believe it is possible to provide a critique of the standard model; the more important arguments are summarized below.

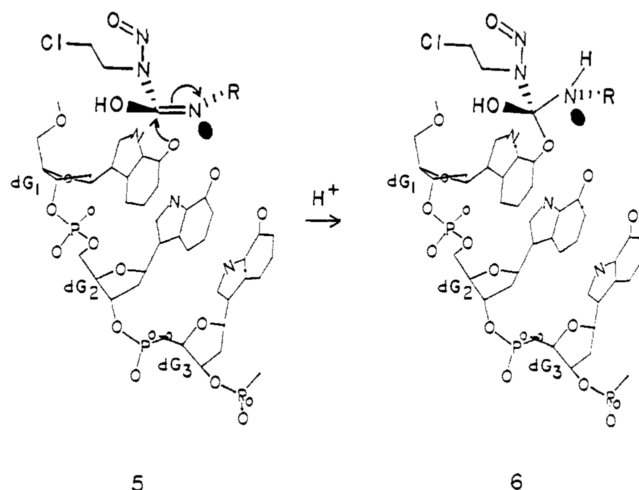
Critique of the Standard Model

Kinetics of Alkylation of DNA. Arguments against DNA alkylation by carbocations or their precursors were made elsewhere[22] and will not be reviewed in detail here. In the standard model, the rate-limiting step should be the rate of hydrolysis ($k_{alk} \gg k_{hydrol}$) and the kinetics should be second-order (or pseudo-first-order). Nonetheless, the rates of alkylation of DNA[23] or RNA[24] by alkylnitrosoureas (ANUs) are not the same as the rate of hydrolysis of the nitrosourea used, and the alkylation of DNA by propylnitrosourea (PNU) is strictly first-order,[23] which is consistent with rate-limiting intramolecular alkylation through an intermediate covalently bound to the surface of DNA but not with carbocation addition or bimolecular displacement reactions with carbocation precursors.

Products of CENU Treatment of DNA. If carbocations were intermediates in a common mechanism for hydrolysis and DNA alkylation, product ratios should be the same for both processes, and DNA should be alkylated randomly with regard to base sequence. Hydrolysis of CENUs (pH 7–7.4, 37 °C) yields chloroethanol (the equivalent of chloroethylated DNA products) and acetaldehyde (the equivalent of hydroxyethylated DNA products) in a ratio of ca. 2:1,[25-28] but in DNA treated with CENUs, HOEt-N_7- and HOEt-O^6-dG predominate over ClEt-N_7 by ratios of ca. 2:1.[29] ClEt-N_7-dG is stable to hydrolysis and is not the precursor to HOEt-N_7-dG;[30] it is assumed that the HOEt-O^6-dG arises by hydrolysis of **3**. Product reversal suggests that either the mechanisms for hydrolysis and DNA alkylation are entirely different or the less important hydrolysis pathway (hydroxyethylated products) becomes dominant in DNA reactions.

Bimolecular reaction of the "S_N2" reagents dimethyl sulfate and SoSo with DNA nucleophiles gives random distributions of products at N_7-dG.[31] In contrast, CENUs and ANUs alkylate DNA regioselectively in contiguous runs of guanines in vitro

Chart I



(pBR-322 DNA,[31] oligomers[32]), in bacteria (*Escherichia coli* genomic DNA[33]), and in situ (HA-*ras*-1 oncogenes in rodents[34]), a selectivity that would not be expected for carbocations or precursors. It is significant that dG_1 is not alkylated either in vitro or in situ,[31,33,34] and that ^{14}C-labeled RN_3 groups of CENUs or ANUs are covalently bound to DNA,[35] albumin,[36] polylysine,[37,38] and histone.[38] Regioselectivity is consistent with an intermediate covalently bound to DNA at dG_1 in a $dG_1dG_2dN_3$ codon (dN = any other base).[1,39] In the reaction with albumin, the presence of ^{14}C-labeled covalent products from groups on both N_1 and N_3 suggests that the whole molecule of parent drug is involved in the reaction.[1] One of us (T.P.B.)[19] has found differential GATase repair phenomena for DNA treated with CENUs with different R groups on N_3, and Seidenfeld[41] has found recently that CENUs that may form both adducts simultaneously on DNA or protein cause different cellular effects than CENUs that can either carbamoylate or alkylate, but not both.

The rate-limiting step in CENU hydrolysis at near neutral pH[1] is formation of a tetrahedral intermediate[42] by addition of water to the imido urea derived from parent CENU. Lown and Chauhan[43] reported that 2-(alkylimido)-3-nitrosooxazolidines,

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(39) Reaction of the tetrahedral intermediate may not be restricted to addition at O^6-dG_1; indeed, we suspect that the stability of RN_3-DNA adducts is more compatable with formation of ureas rather than carbamates, which suggests that N^6-dA may be a site of initial reaction. Pegg and Dolan[32,40] have evidence that the presence of 5′-dAdG-3′ pairs increases the methylnitrosourea (MNU) alkylation at dG on the same strand of oligomers. The simplest oligomer they studied has no contiguous 5′-dGdG-3′ or 5′-dAdG-3′ pairs on the same strand, but has the complementary strand doublet (5′-dGdA-3′)-(3′-dTdG-5′). Because our computer modeling studies suggest that adducts at O^6-dG can react readily with bases on the opposite strand, tetrahedral intermediates formed at N^6-dA could react in a similar manner, and regioselective reactions could occur on the same strand ("cis-strand" alkylation) and across the major groove ("trans-strand" alkylations). In fact, assigning relative amounts of Me-O^6-dG adducts (pmol/μmol oligomer) to various cis- and trans-strand dAdG pairs in the Pegg–Dolan oligomers allows calculation of expected amounts of alkylation, which agree fairly well with the experimentally determined values for five oligomers. This tantalizing hint of regioselective cis-strand alkylation is being explored.
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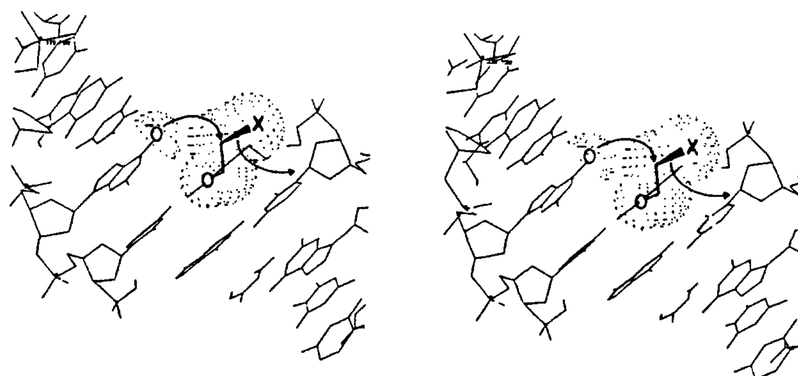

Figure 1. Stereodiagram (cross-eyed) that shows the most favorable geometry for displacement by an opposite strand nucleophile on a 2-X-Et-O^6-dG adduct in the major groove of B-DNA.

which rapidly hydrolyze in aqueous buffers to acetaldehyde, ethylene glycol, and carbamates, alkylate DNA at rates and extents similar to those found for CENUs. These results and the apparent necessity of a precursor lesion covalently bound to DNA for regioselective alkylations suggested that the initial step in DNA alkylation for both ANUs[22] and CENUs[1] may be formation of a tetrahedral intermediate by addition of the imidourea at O^6-dG_1 (Chart I). One of the possible reactions for 6 is direct displacement on the ClEt group to form 2 and/or ClEtN_7-dG_2.

Despite the centrality of 2 to the standard model, it has never been isolated from DNA treated with CENUs, and only recently has the synthesis of the purine analogue of ClEt-O^6-guanine and its cyclization to N_1,O^6-ethanoguanine, the intermediate equivalent to 3 in DNA, been reported.[44,45] The primary product of hydrolysis is HOEt-N_1-guanine, which by the standard model should be the primary product of treatment of guanosine with BCNU. Nonetheless, treatment of guanosine with BCNU under physiologic conditions yields HOEt-N_7-dG as the primary product;[46] generation HOEt-N_1-dG was not reported, but it may be present in minor amounts.[47] These results are inconsistent with the intermediacy of ethanoguanine in CENU–DNA cross-linking reactions, but may be rationalized as an *inter*molecular displacement of N_7-guanosine on an imidourea–guanine adduct (models show that the *intra*molecular displacement is not probable).

Computer Modeling Studies. As a part of studies on the effect of X-irradiation on CENU cross-linking,[18,20] we modeled possible reactions of (2-X-ethyl)-O^6-dG adducts (where X is a leaving group) using the UCSF MIDAS computer graphics program.[48] Our results show that the geometries for reaction of this adduct in duplex B-form oligo$(dG\text{-}dC)_5$ are not compatible with the standard model. Cyclization of 2 to 3 in duplex B-DNA is not possible because the Watson–Crick paired cytosine on the opposite strand blocks the XEt-O^6-dG from assuming the appropriate geometry. In the most sterically favorable configuration for reaction with any base nucleophile, CH_2X is above and astride the major groove and in a position to undergo facile displacement by a nucleophile on the complementary base Watson–Crick paired to the adjacent (5′–3′) base in the alkylated strand. (The stereodiagram shown in Figure 1 is for $dG_1[X\text{-}Et\text{-}O^6]dG_2dC_3$ and the attacking nucleophile is O^6-dG'_3 on the opposite strand.) If 3 could form in normal duplex DNA, the angles necessary for displacement on 3 by the Watson–Crick paired dC on the opposite strand are too severe to allow reaction. (As discussed below, perturbation of the helix may allow this reaction to occur at other sites.)

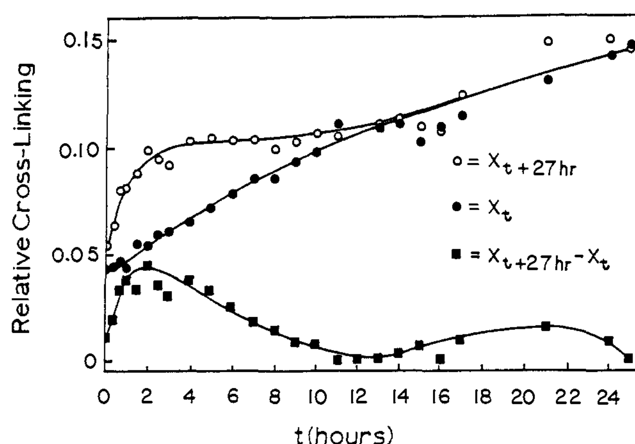

Figure 2. Typical plot for relative cross-linking of CT-DNA treated with SoSo at 37 °C. The three plots shown are discussed in the text.

These findings suggest that the standard model does not adequately explain the DNA cross-linking reactions of CENUs. Because SoSo may well cross-link by some form of the standard model and because possible differences between CENU and SoSo cross-linking might be reflected kinetically, we measured the rate of formation of precursor lesions and the time course for cross-linking of calf-thymus DNA (CT-DNA) treated with BCNU and SoSo at 37 and 50 °C, pH 7.2, under various conditions. Rate constants for cross-linking calculated from these data are different for the two agents. On the basis of an analysis of our kinetic data and other results, we propose a modified version of the standard model for SoSo cross-linking and a novel mechanism for CENU cross-linking of DNA that does not involve the adduct 2.

Results

The percent cross-linking vs time (x_t) was measured by using the ethidium bromide intercalation assay,[9,19,49] which depends on the difference in DNA-bound fluorescence before and after cross-linking occurs, either in the continuous presence of drug or after treatment for a given period followed by removal of drug by precipitating DNA (pulse treatment). For continuous treatment protocols, t_0 was the time of addition of drug; for pulse treatment protocols, t_0 was the time incubation in the absence of drug was begun. Values were Poisson normalized to cross-links/molecule (X_t) using the equation $X_t = -\ln(1 - x_t)$. Values of X_t are either the average of two determinations for one experiment or the average of single determinations for several experiments and were generally within ±10%. Slopes of all the plots

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Table I. Rate Constants for Cross-Linking of Calf-Thymus DNA

drug	X_∞[a]	$X_\infty^{\text{K-S}}$	$10^3 k_{obsd}$, min^{-1}	r	$10^3 k_{\text{K-S}}$,[b] min^{-1}	τ, h	r
			37 °C, Continuous Drug Treatment				
10 mM BCNU	0.59	0.61	2.5	0.97	2.6	5	1.0
20 mM SoSo	0.18[c]	0.18	1.0	0.99	1.2	24	0.96
			37 °C, Drug Absent				
10 mM BCNU	0.48	0.50	2.6	0.98	2.5	2	0.95
20 mM SoSo		0.15	0.77	0.95	0.88	8	0.82
100 mM SoSo[d]		0.26	0.4	0.99	0.3	6	0.99
			50 °C, Continuous Drug Treatment				
10 mM BCNU	0.59	0.63	13	0.98	11	0.5	0.99
30 mM BCNU	0.60		13	0.99	14	1	0.97
20 mM SoSo		0.21	2.4	0.95	4.3	4	0.87
40 mM SoSo		0.31	2.6	0.98	2.7	4	0.99
			50 °C, Drug Absent				
10 mM BCNU	0.61	0.63	13	0.99	12	2	0.98
20 mM SoSo		0.23	3.2	0.98	3.6	2	0.95
50 mM SoSo[d]		0.20	2.2	0.95	2.4	4	0.96

[a] For BCNU, X_∞ was the experimentally determined value; for SoSo, X_∞ was estimated from Kezdy-Swinbourne plots. [b] Calculated from slopes of Kezdy-Swinbourne plots. [c] At 24 h. [d] From ref 53.

were calculated by linear regression.

Rates of Precursor Formation. The relative number of precursor lesions at any time t that may form cross-links was determined by measuring X_t and $X_{t+t'}$, where t' is a period of additional incubation of a portion of the sample used to determine X_t, typically 24–27 h. Plots of $X_{t+t'} - X_t$, the number of potential precursor lesions, vs t gives a curve for the appearance and decay of precursor lesions. A typical plot of $X_{t+t'} - X_t$ (squares) is shown in Figure 2 for treatment of CT-DNA with 20 mM SoSo at 37 °C; similar plots were obtained for 20 mM SoSo at 50 °C and for BCNU at 37 and 50 °C (not shown). Pseudo-first-order rate constants for precursor lesion formation for SoSo and BCNU (10–20 mM drug used to treat 200 μg/mL DNA) were calculated from the forward portion of curves using the equation $\ln [(X_\infty - X_t)/X_\infty] = -kt$, with X_∞ taken as the maximum of the curve.

Rate constants are approximately the same for 10 mM BCNU (0.034 min^{-1}, 37 °C; 0.067 min^{-1}, 50 °C) and 20 mM SoSo (0.029 min^{-1}, 37 °C; 0.065 min^{-1}, 50 °C), and these concentrations were used as the standards. For BCNU, k_{obsd} for precursor formation is ca. 3-fold greater than the hydrolysis rate constant at the same pH (14×10^{-3} min^{-1}).[5a,28] The value for the rate constant for hydrolysis of SoSo has not been reported, but alkylation of DNA with SoSo is complete within 4 h.[15] Datum points for the decay portions of the curves are too scattered to yield useful rate constants. For instance, the correlation coefficient for k_{decay} for SoSo, 37 °C, is 0.56.

Kinetics of Cross-Linking. Plots of X_t vs t (not shown) were obtained for both drugs at 37 and 50 °C. Maximum cross-linking for 10 mM BCNU was typically 0.6 cross-link/molecule, while values for 20 mM SoSo were significantly lower (ca. 0.15 cross-link/molecule). Rate constants for the overall reaction leading to cross-linking were calculated from these plots with the equation $\ln [(X_\infty - X_t)/X_\infty] = -k_{obsd}t$. Rate plots for these data are shown in Figure 3; values from these plots and from plots not shown are listed in Table I. Details for each drug are discussed separately.

SoSo. Plots of X_t vs t for treatment of CT-DNA with 20 mM SoSo are different than those for BCNU and are different for the two treatment protocols. For the continuous-treatment protocol, X_t for SoSo continues to increase slowly (for up to 49 h; see Figure 2, circles). After the initial decay, the curve for potential precursors rises and decays a second time, with a maximum at ca. 18 h (Figure 2, squares). Gibson et al.[14] have seen similar behavior in cells. We suspect that this small, late increase in new precursor lesions may reflect reaction and cross-linking of a hydrolysis product of SoSo, possibly a cyclic derivative related to the cyclo-SoSo compounds being studied by Gibson et al.[50] In

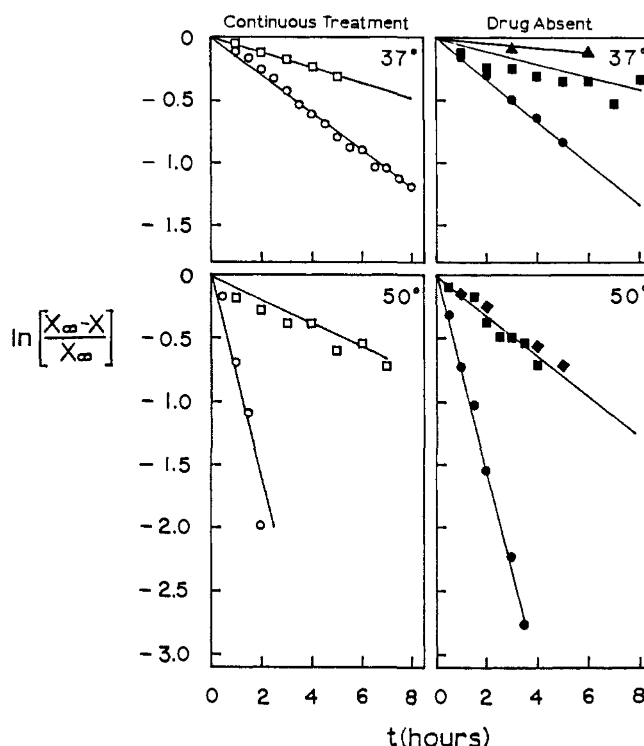


Figure 3. Rate plots for cross-linking of CT-DNA treated with 10 mM BCNU (circles) or 20 mM SoSo (squares) either in the continuous presence or in the absence of drug. For SoSo, the plot for 100 mM drug (triangles) at 37 °C and for 50 mM drug (diamonds) at 50 °C were calculated from data in ref 53.

our experiments, this behavior does not occur if the drug is removed after an initial pulse treatment. In the drug-absent protocol, X_t increases initially, declines slightly, and then either plateaus or increases slightly. Because of this variable behavior, it is not possible to obtain values of X_∞ directly from plots of X_t vs t for either protocol. Therefore, X_∞ was estimated by using the Kezdy-Swinbourne graphic method,[51] which has the advantage of not being particularly sensitive to slower secondary reactions; points from the initial portions of the curves were used. (As a check on the accuracy of the Kezdy-Swinbourne method in this instance, values of X_∞ for BCNU were also estimated graphically and found to be within ±5% of the experimental values.)

Data for SoSo are less reliable than data for BCNU because of the inherent inaccuracy of measurements of X_t at low levels

(50) Gibson, N. W.; Hartley, J. A.; Kohn, K. W. *Cancer Res.* **1986**, *46*, 1679–1683.

(51) Espenson, J. H. *Chemical Kinetics and Reaction Mechanisms*; McGraw-Hill: New York, 1981; pp 24–30.

of cross-linking.[52] One of us (T.P.B.)[53] has published plots of X_t vs t for treatment of CT-DNA with 50 mM (50 °C) and 100 mM (37 °C) SoSo that have higher values of X_t than those determined in this study. At 37 °C, k_{obsd} for treatment with 20, 40, and 100 mM SoSo decreases with concentration; we believe this represents increased accuracy at higher treatment concentrations and not a decrease in k_{obsd}, which would be overestimated at the lower values of X_t. Our best value for k_{obsd} at 37 °C is the value for 100 mM SoSo ($k_{obsd} = 0.4 \times 10^{-3}$ min^{-1}). The same tendency is seen in k_{obsd} for treatment with 20, 40, and 50 mM SoSo at 50 °C; our best value for k_{obsd} at 50 °C is 2.2×10^{-3} min^{-1}. SoSo cross-linking is not pseudo-first-order.

Because of these variations, values of the thermodynamic quantities for SoSo are much less reliable than those for BCNU. For our best values for k_{obsd}, we calculate $\Delta H^{\ddagger} = 31.5$ kcal/mol and $\Delta S^{\ddagger} = +20.5$ eu. For the less reliable values of k_{obsd}, calculated values are within the same range ($\Delta H^{\ddagger} = 25.3$ kcal/mol, $\Delta S^{\ddagger} = +15.0$ eu).

BCNU. Plots of X_t vs t for the continuous-treatment and drug-absent protocols are distinctly different. In the drug-absent protocol, X_t reaches a maximum value, plateaus, and remains at that value for 2–4 h. Similar plots for cross-linking of intracellular DNA in several cell lines have been reported.[54] In the continuous presence of BCNU, however, plots of X_t vs t peak and then decay. This "loss" of cross-links may reflect degradation of the DNA because of single strand breaks that lead to loss of fluorescence in the assay and therefore may not represent a true degradation of cross-links. This loss of X_t does not affect the value of k_{obsd} for cross-linking, however (Table I).

Infinity values were taken as either the maxima (continuous treatment) or the plateau values (drug absent) in plots of X_t vs t. Values of k_{obsd} for either protocol are essentially the same ($2.5–2.6 \times 10^{-3}$ min^{-1} at 37 °C and 13×10^{-3} min^{-1} at 50 °C, Table I). Moreover, k_{obsd} for treatment with 10, 20, and 30 mM BCNU at 50 °C are the same (continuous treatment, not shown) and cross-linking is not pseudo-first-order. The calculated value of $\Delta H^{\ddagger}$ is 25.3 kcal/mol, and the value of $\Delta S^{\ddagger}$ is +2.3 eu.

Lown et al.[55] measured X_t for λ-DNA treated with 5 mM BCNU and CCNU at pH 7, 37 and 50 °C, and found that the curves for the two CENUs were the same at each temperature (invariant with the RN_3 group). Rate constants for λ-DNA calculated from these data are 2-fold faster than those for CT-DNA ($k_{obsd} = 3.5 \times 10^{-3}$ min^{-1} at 37 °C, 27×10^{-3} min^{-1} at 50 °C), which may reflect differences in the structures of the DNAs.

Data for intracellular cross-linking of DNA in L-1210[54a] and 9L[54b] tumor cells treated with BCNU at 37 °C, pH 7–7.4, are available from which the rate constants for cross-linking may be estimated. For the first 3–4 h—cross-linking is complete in 6 h—k_{obsd} for both cell lines is essentially the same as k_{obsd} for CT-DNA at 37 °C. For L-1210 cells, k_{obsd} is the same for treatment with 16, 32, 50, and 100 μM BCNU.[54a] Intracellular cross-linking is not a pseudo-first-order process.

Bis(2-chloroethyl)methylamine [Nitrogen Mustard (HN2)]. The rate of cross-linking of CT-DNA with HN2 (0.2 mM, 37 °C, pH 7, continuous treatment) was determined as described for BCNU and SoSo. The cross-linking reaction is fast ($k_{obsd} = 50 \times 10^{-3}$ min^{-1}) and X_t declines rapidly after the initial phase of the reaction. The same behavior is found for HN2 cross-linking of L-1210 cells,[54a] and the rate of cross-linking in this cell line ($45–50 \times 10^{-3}$ min^{-1}) is essentially the same as the rate for CT-DNA. Moreover, cross-linking rate constants are the same as the overall k_{obsd} for hydrolysis of parent compound[56] ($k_{obsd} = 45–50 \times 10^{-3}$ min^{-1}). Thus both the initial alkylation and subsequent cross-linking involve rate-limiting rearrangement to aziridines.

Discussion

The mechanisms of cross-linking for BCNU and SoSo are different and probably involve different initial adducts. Strictly first-order cross-linking suggests further that either rearrangement of initial adducts on DNA or subsequent reactions of reactive products are rate-limiting. Our results for HN2 confirm that this general sequence is possible for DNA cross-linking reactions and can be used to rationalize the mechanisms of cross-linking for SoSo and BCNU.

SoSo–DNA Cross-Linking. The major product of reaction of SoSo with CT-DNA is ClEt-N_7-dG (ca. 95%),[15] which cannot form *inter*stand cross-links. Minor products have been seen on HPLC, but they have not been isolated and identified despite major efforts by several groups.[57,58] Because there are few alternatives and because the low levels of interstrand cross-linking are similar to results of Alexander et al.[13] and Gibson et al.[14] for the intracellular reactions, which suggest that a minor product leads to interstrand cross-links, we assume as a working hypothesis that the initial precursor lesion for SoSo is **2**. (If the cross-linking precursor is the β-ethyl sulfonate ester formed by displacement of chloride, the arguments made below would be the same.)

In the absence of cyclization to **3**, the most obvious mechanism for cross-linking is direct displacement by an opposite strand nucleophile (Figure 1, X = Cl). (Our modeling studies show that displacement on the methylenes of a possible three-membered O^6-dG oxonium ion is not possible in the major groove.[20]) While the rate constant and enthalpy of activation are within acceptable ranges for this reaction, the entropy of activation is not. Therefore, it is doubtful that SoSo cross-linking is a simple displacement reaction.

The second possibility is the sequence of reactions shown in Scheme II. It is clear that the rate of cyclization (k_2, Scheme II) to **3** is not rate-limiting because k_{obsd} for cyclization of the purine analogue of **2** is 0.04 min^{-1} at 37 °C, pH 7,[44] and is 10^2 faster than the rate of cross-linking. Moreover, the rate-limiting cyclization reaction should have a high negative $\Delta S^{\ddagger}$, while our calculated values for $\Delta S^{\ddagger}$ is +10–20 eu. Results of our computer modeling studies discussed above suggest that cyclization of **2** to **3** can occur only if there is local disruption of the DNA helix. This is known to occur "spontaneously", with normal "breathing" of DNA ($k_{cl} > k_{op}$ and $k_{cl}' = k_{cl}$) estimated to uncoil up to 9–10 base pairs;[59a] alkylation in the major groove may cause sufficient disruption that "breathing" is more facile. Thus, if the distance between uncoiled strands is sufficient, the geometric restrictions are removed and cyclization of **2** to **3** might occur. (In the MIDAS structures, the ClEt-O^6-dG group fits into the interstrand space at a displacement of 7 Å between strands.[20]) Cross-links could then form between **3** and an opposite strand base nucleophile during closure to duplex DNA ($k_4 > k_2$). For the open/close equilibrium for ribooligonucleotides, ΔS° has been estimated to be on the order of +20 eu,[59b] and in reactions with "loose" transition states involving significant changes in geometries, such as the denaturation of proteins,[60a] ΔS° is often approximately $\Delta S^{\ddagger}$.[60b] This suggests that entropic factors control SoSo cross-linking. This mechanism incorporates the essential elements of the standard model for CENU cross-linking after formation of the initial lesion[61] and predicts that SoSo cross-linking occurs

(52) Lown, J. W.; Joshua, A. V.; McLaughlin, L. W. *J. Med. Chem.* **1980**, *23*, 798–805. Lown et al. found a slow rate of cross-linking at 37 °C for λ-DNA treated with 2-chloroethyl trifluoromethanesulfonate, which should form DNA products similar to those formed by SoSo.

(53) Brent, T. P.; Lestrud, S. O.; Smith, D. G.; Remack, J. S. *Cancer Res.* **1987**, *47*, 3384–3387.

(54) (a) Ewig, R. A. G.; Kohn, K. W. *Cancer Res.* **1977**, *37*, 2114–2122. (b) Tofilon, P. J.; Williams, M. E.; Deen, D. F. *Radiat. Res.* **1984**, *99*, 165–174.

(55) Lown, J. W.; McLaughlin, L. W.; Chang, Y.-M. *Bioorg. Chem.* **1978**, *7*, 97–110. Values for rate constants were calculated from these data as described in the text.

(56) Cohen, B.; van Artsdale, E. R.; Harris, J. *J. Am. Chem. Soc.* **1952**, *74*, 1875–1878. Rates were calculated from plots of [Cl⁻] vs time reported by Rauen, H. M.; Reisch, A. *Arzneim.-Forsch.* **1964**, *14*, 752–757.

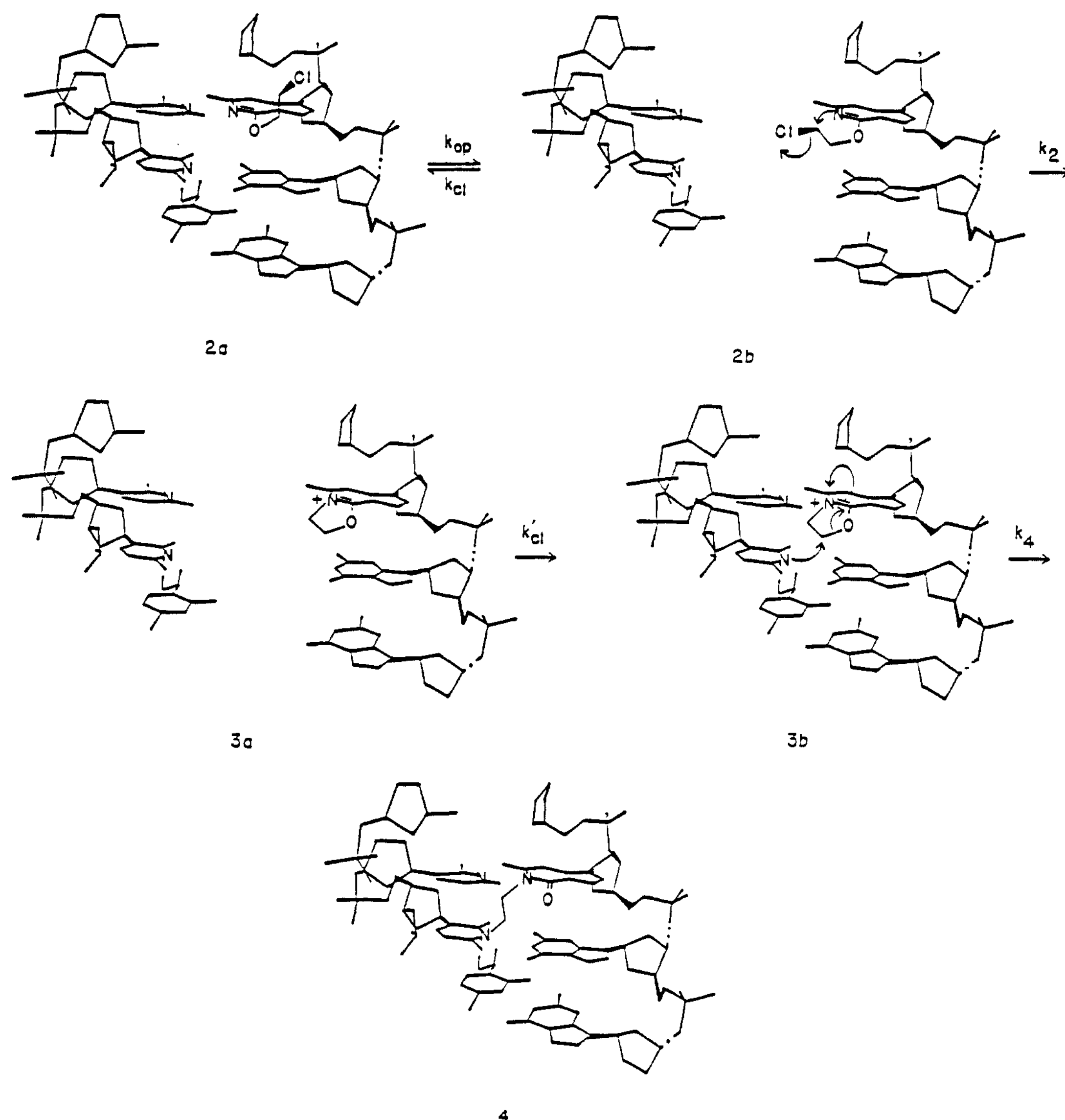
(57) Struck, R. F., personal communication, 1987.

(58) Gibson, N. W., personal communications, 1987, 1988.

(59) (a) von Hipple, P. H.; Wong, K.-Y. *J. Mol. Biol.* **1971**, *61*, 587–613. (b) Mandal, C.; Kallenbach, N. R.; Englander, S. W. *Ibid.* **1979**, *135*, 391–411. Although, see: Gueron, M.; Kochoyan, M.; Leroy, J.-L., *Nature (London)* **1987**, *328*, 89–92.

(60) See: (a) Maskill, H. *The Physical Basis of Organic Chemistry*; Oxford: London, 1985; p 255. (b) Moore, W. J. *Physical Chemistry*; Prentice-Hall: Englewood Cliffs, 1965; p 299.

Scheme II



regiospecifically, but in the opposite direction (5′–3′ on the opposite strand) than CENU cross-linking.

BCNU–DNA Cross-Linking. A mechanism for BCNU cross-linking must take into account the initial formation of a stable precursor lesion, first-order kinetics, regioselective alkylation, and a reversal of DNA product distribution compared with hydrolysis products. The rate of precursor formation and the stability of the initial lesion may be explained by addition of the imidourea of CENUs to O^6-dG to yield a tetrahedral intermediate.[1,22] [1-(2-Chloroethyl)-3,3-dimethyl-1-nitrosourea (dMCNU), which cannot form an imidourea, does not alkylate or cross-link DNA;[55] DNA treated with BCNU at pH 5, where little or no imidourea would be formed, is not alkylated.[31]] Protonation of the anion on N_3 by solvent water should occur primarily from above the major groove ("exo" protonation); because rotation about the N_3–C_2 bond is restricted by the proximity of the RN_3 group to the surface of DNA (Figure 4), the exo protonated tetrahedral intermediate cannot form proper gauche rotomers for antiperiplanar collapse by the Deslongchamps rules,[42b,62] which may account for the unusual stability of the lesion and the reversal of products in DNA alkylation.

The tetrahedral intermediate may react with nucleophiles of adjacent bases on the same strand in two ways; either sequence would account both for the first-order kinetics—a bimolecular, intramolecular reaction—and for the regioselectivity. First, ClEt-O^6-dG and ClEt-N_7-dG could form by displacement on the methylene α to the nitrosated N_1. ClEt-O^6-dG so formed could then undergo the sequence proposed for SoSo (Scheme II), but the faster rate for BCNU cross-linking compared to SoSo and the absence of HOEt-N_1-dG among the products of CT-DNA treated with CENUs with different alkyl groups on N_3[29] suggest that the ClEt-O^6-dG adduct is involved in only a minor way in this sequence.[61] The stability of ClEt-N_7-dG to hydrolysis[30] suggests that HOEt-N_7-dG arises from another species.

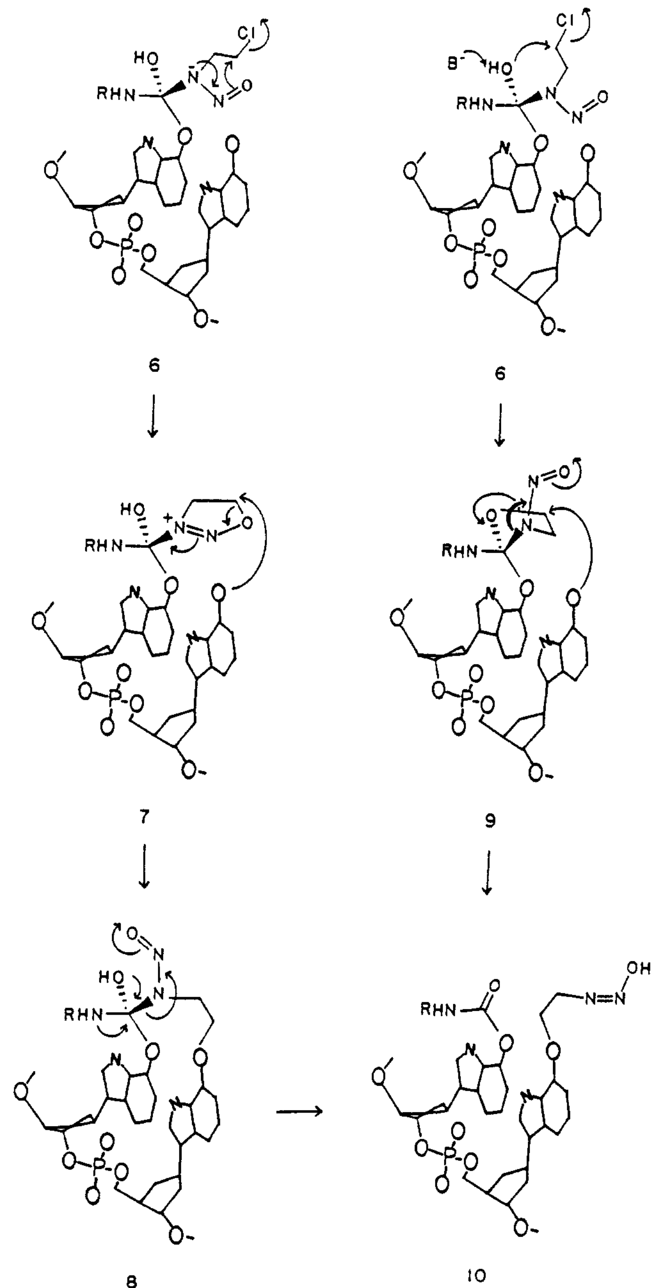
Second, products may be formed after rearrangement of the tetrahedral intermediate. Displacement by nucleophiles of adjacent bases on the rearranged tetrahedral intermediate may form a reactive alkyl adduct such as the diazoic acid **10** (Scheme III). A priori, **10** can hydrolyze to the HOEt-DNA adducts, react with amino groups on proteins to give DNA–protein cross-linked products,[4] or react with base nucleophiles on the same or opposite strands to give cross-linked base pairs.

Two probable rearrangements are shown in Scheme III. The "anchimeric" pathway (**6** → **8**) is analogous to the process first proposed by Chaterjii et al.[63] for CENU hydrolysis and is

(61) Small amounts of ClEt-O^6-dG adducts may form in DNA treated with CENUs and may react by the sequence shown in Scheme II. Hydrolysis of the ethano intermediate in the open helix (**3a**, Scheme II) may be the source of HOEt-N_1-dG in CT-DNA treated with 1-(2-chloroethyl)-1-nitrosourea (CNU) [Bodell, W. J.; Tokuda, K.; Ludlum, D. B. *Proc. Am. Assoc. Cancer Res.* **1987**, *28*, 285 (Abstract 1129)]. The ca. 2-fold difference[29] in HOEt-N_7-dG/HOET-O^6-dG for BCNU and other CENUs[15-19] and CNU[30] treatment of CT-DNA suggest that CNU (N_3 not alkylated) is not a good model compound for intracellular DNA reactions.

(62) Deslongchamps, P. *Tetrahedron* **1975**, *31*, 2463–2490.

Scheme III



equivalent to the cyclization of [β-(tosyloxy)ethyl]nitrosamines reported by Michejda et al.[64] (Other similar displacement[65] or addition reactions[66] of nitroso groups have been proposed.) The sequence **6** → **9** is equivalent to the cyclization of (β-halo-ethyl)ureas[67] and of 3-(2-chloroethyl)urea adducts formed by treating lysine or polylysine with BCNU.[37] As shown, regioselective displacement on either cyclic structure by an adjacent O^6-dG leads to **10**. There is evidence from the hydrolysis chemistry to differentiate between these pathways.

The products of hydrolysis of BCNU at pH 7.0–7.4 are drastically different from the products at pH 5.0; under slightly acidic conditions, hydroxyethylated products far outweigh chloroethylated products,[29] similar to the reversal found for DNA alkylation products. Brundrett[26] has argued that acetaldehyde arises by the anchimeric mechanism at pH 5.0 (A_1 in pathway A, Scheme IV). Neither BCNU nor dMCNU can form an imidourea at this acidic pH. Thus the anchimeric cyclization to **12** is a probable first step in the reaction. The elimination pathway proposed for BCNU (**12** to **14**, R″ = ClEt) is not possible for dMCNU (**11**, R′ = R″ = Me), which nonetheless yields acetaldehyde and ethylene glycol as the major products of hydrolysis at pH 7.4 (32% and 13%, respectively). This suggests an alternative pathway. The intermediate **12** should be reactive[64] and may hydrolyze to **16** that in turn cyclizes to the tetrahedral intermediate **17**. Concerted hydrolysis of **17** yields the *Z*- and *E*-diazoic acids[68] that collapse to acetaldehyde and ethylene glycol, respectively.[69] There is ample evidence for the addition of a β-hydroxyethyl group to the carbonyl of a nitrosourea, albeit from N_3 and not N_1.[70] Nonetheless, the ratio of acetaldehyde/ethylene glycol for hydrolysis of dMCNU, 1,3-bis(2-hydroxyethyl)-1-nitrosourea, and 1-(2-hydroxyethyl)-3-cyclohexyl-1-nitrosourea at pH 7–7.4, 37 °C, are the same.

At near neutral pH, products arise from the tetrahedral intermediate **20**. Antiperiplanar collapse of the rotomer **22** gives the (*E*)-2-chloroethyl-1-diazoic acid **23** that hydrolyzes to chloroethanol (pathway B_2, Scheme IV).[42b] It is commonly assumed that acetaldehyde arises from CH_3CHCl^+ that forms by a 1,2 hydride shift of either $^+CH_2CH_2Cl$ or the nitrogen-separated ion pair that may precede the formation of the "bare" primary carbocation. If this were true, the cation should partion proportionally to products; the fact that CNU gives acetaldehyde and chloroethanol in the ratio of 15:85 while other CENUs give ratios of ca. 12:25 suggests that a common intermediate is not involved.

The source of acetaldehyde at near neutral pH may be an intermediate analogous to **17**. (Lown and Chauhan found that only ca. 10% of acetaldehyde—or 1.5–2% of the total products—arises by an anchimeric mechanism.[71]) As shown in pathway B_1, Scheme IV, buffer-catalyzed cyclization[72] of the tetrahedral intermediate **20** may occur, and hydrolysis of **21** may lead to acetaldehyde.[73] Indeed, hydration of 2-(alkylimido)-3-nitrosooxazolidines would give the intermediate **21**; the products

(63) Chaterjii, D. C.; Greene, R. F.; Gallelli, J. F. *J. Pharm. Sci.* **1978**, *67*, 1527–1532.

(64) (a) Michejda, C. J.; Koepke, S. R. *J. Am. Chem. Soc.* **1978**, *100*, 1959–1960. (b) Koepke, S. R.; Kupper, R.; Michejda, C. J. *J. Org. Chem.* **1979**, *44*, 2718–2722. (c) Michejda, C. J.; Kroeger-Koepke, M. B.; Koepke, S. R.; Krupper, R. J. In N-nitrosamines; Anselme, J.-P., Ed.; ACS Symposium Series 101; American Chemical Society: Washington, DC, 1979; pp 77–89.

(65) Padwa, A.; Cimiluca, P.; Eastman, D. *J. Org. Chem.* **1972**, *37*, 805–812.

(66) Kirmse, W.; Engelmann, A.; Hesse, J. *J. Am. Chem. Soc.* **1972**, *95*, 625–626.

(67) Kreling, M.-E.; McKay, A. F. *Can. J. Chem.* **1959**, *37*, 504–505.

(68) (*E*)- and (*Z*)-alkyldiazotates and presumably the alkyldiazoic acids have strikingly different stabilities. See ref 6 and references cited therein.

(69) This is formally equivalent to the deaminative hemipinacol rearrangement. For nitrous acid deamination of simple β-hydroxyamines, see: Saavedra, J. E. *J. Org. Chem.* **1981**, *46*, 2610–2614. For solvent isotope studies on the origin of the carbonyl oxygen, see: Günther, B.-R.; Kirmse, W. *Liebigs Ann. Chem.* **1980**, 518–532. For an argument that diazotates or diazonium ions and not free cations are involved, see: Chērest, M.; Felkin, H.; Sicher, J.; Šipoš, F.; Tichý, M. *J. Chem. Soc.* **1965**, 2513–2521. For carbonyl compounds produced from non "vibrationally excited" intermediates, see: Newman, M. S.; Edwards, W. M. *J. Am. Chem. Soc.* **1953**, *76*, 1840–1845. Note that hydrolysis of *threo*- and *erythro*-1,3-bis(3-chloro-2-butyl)-1-nitrosourea at pH 7.4, 37 °C produces isobutyraldehyde and butanone as the major products, but in different amounts, perhaps by sterically controlled anchimeric cyclization and subsequent collapse [Brundrett, R. B.; Colvin, M. *J. Org. Chem.* **1977**, *42*, 3538–3541.]

(70) See: Morikawa, T.; Tsujihara, K.; Takeda, M.; Arai, Y. *Chem. Pharm. Bull.* **1983**, *31*, 1646–1651 and preceding papers in this series.

(71) Lown, J. W.; Chauhan, S. M. S. *J. Org. Chem.* **1982**, *47*, 851–856.

(72) Chaterjii et al.[63] found that increasing the concentration of phosphate buffer had no effect on the rate of hydrolysis of chlorozotocin (CHLZ), 1,3 bis(2-fluoroethyl)-1-nitrosourea (BFNU), or BCNU at pH 7, 37 °C, but had a dramatic effect on the production of either chloride or fluoride, which is proportional to the production of acetaldehyde. Plots of log (X_M/X_0) vs the concentration of buffer, where X_0 is the percentage of halide produced by hydrolysis in 0.01 M phosphate and X_M is the percentage for 0.1–0.4 M phosphate, are linear for CHLZ and BFNU and the slopes are parallel. (Only two points are available for BCNU; the slope of the two point line is essentially the same as the lines for CHLZ and BFNU.) Edie plots for these data (plots of $X_M - X_0$ vs $(X_M - X_0)/[PO_4]$) are also linear and have the same slopes (see: Pollack, S. J.; Jacobs, J. W.; Schultz, P. G. *Science (Washington, D.C.)* **1986**, *234*, 1570–1573). Moreover, Brønsted-like plots of the log of the relative yield of chloroethanol and acetaldehyde (equivalent to log k_{rel}) vs pK_A are linear for hydrolysis of CCNU at pH 7, 37 °C in various buffers.

(73) Note that this sequence differs substantially from the mechanism suggested in Scheme III (right) of ref 1. There are intriguing (but incomplete) data for the 2-bromoethyl compounds that suggest the reaction may be more complex [Yoshida, K.; Yano, K. *Bull. Chem. Soc. Jpn.* **1983**, *56*, 1557–1558].

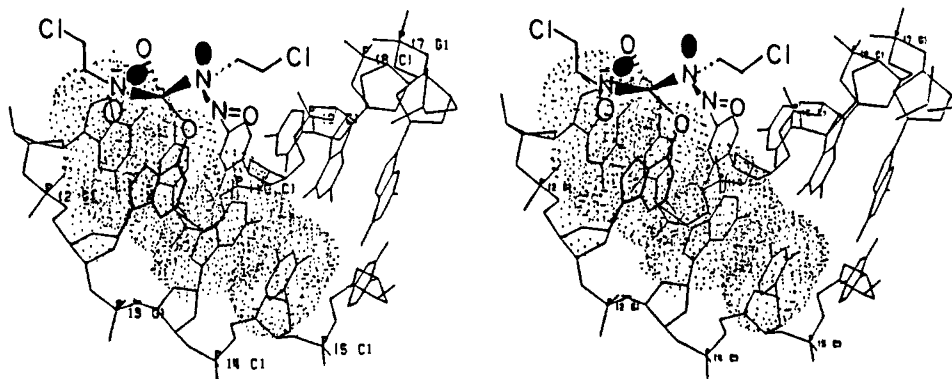


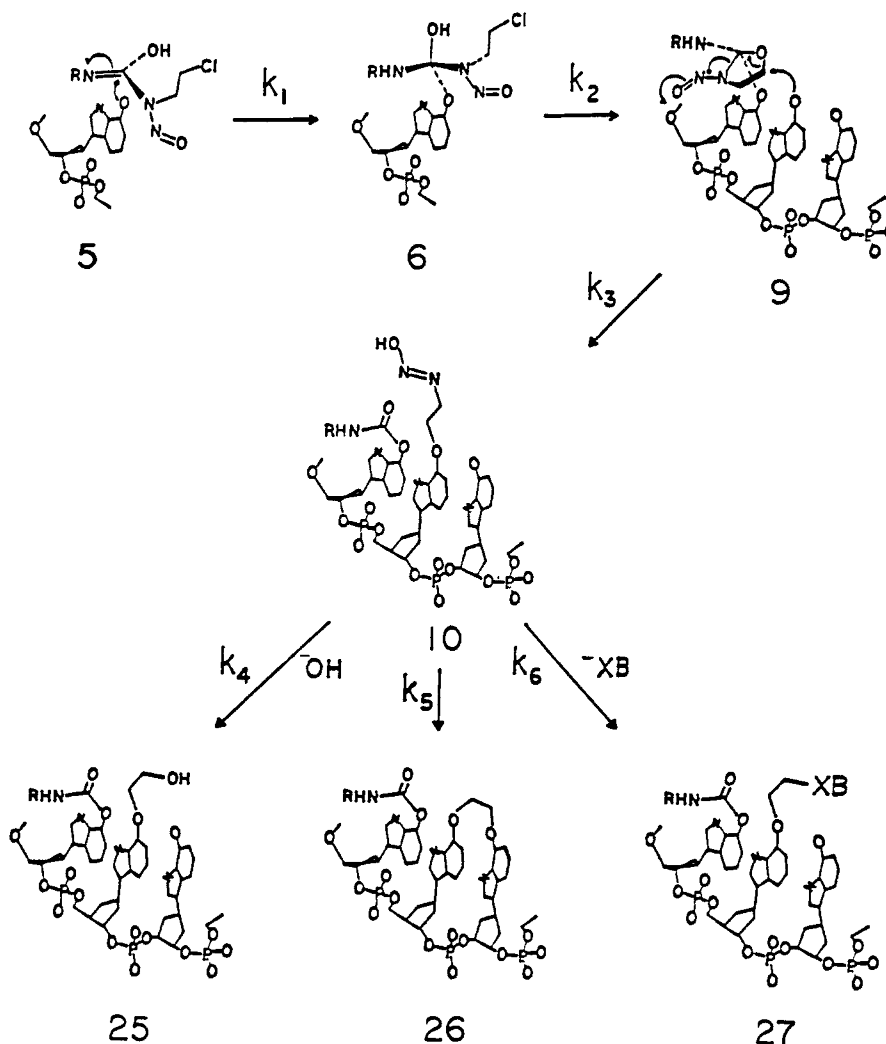
Figure 4. Stereodiagram (cross-eyed) for the tetrahedral intermediate formed by addition of the imidourea of BCNU to O^6-dG. Rotation is restricted and protonation should occur from above the major groove.

Scheme IV

of hydrolysis of these reactive compounds are acetaldehyde and ethylene glycol.[43]

We have suggested above that the stability of the DNA precursor lesion is the result of the inability of the tetrahedral intermediate to form the proper gauche rotomers for antiperiplanar collapse. Because the anchimeric sequence in pathway A, Scheme III, ultimately depends on the ability of **8** to undergo antiperiplanar collapse, it is more likely that the cyclization to **9** occurs; a displacement gives the diazoic acid **10**, the subsequent reactions of which nicely account for the product reversal in DNA reactions. Thus while CENU hydrolysis is a kinetically controlled process, DNA reactions are thermodynamically controlled to the extent

Scheme V



that steric factors suppress the more favorable hydrolysis pathway.

In Scheme V the various steps are combined and the total mechanism for CENU alkylation and cross-linking is shown. The reactive intermediate **10** may either hydolyze to **25**, form the as-yet-unidentified intrastrand product **26**, or regioselectively form interstrand cross-links (**27**) by reaction with an opposite strand nucleophile (see Figure 1). We believe that it is possible to define the rate-limiting step in this sequence. The value of k_1 is reported here and is fast compared with the rate of cross-linking; we assume that k_{4-6} would be fast as well because hydrolysis or ethanolysis of alkyldiazotates is essentially instantaneous.[74] Thus the rate-limiting step may be either k_2 or k_3. We have paid particular attention to the various hydrolysis mechanisms because k_{obsd} for hydrolysis of CCNU and BCNU[75] at ca. pH 5.5, 37 °C (2.2 and 1.8×10^{-3} min^{-1}, respectively) and dMCNU[26] at pH 7.4, 37 °C (2.7×10^{-3} min^{-1}), all of which give acetaldehyde and ethylene glycol as the major products and only minor amounts of chloroethanol at slightly acidic pHs, are essentially the same as k_{obsd} for BCNU cross-linking of DNA at the same pH and temperature ($2.5-2.6 \times 10^{-3}$ min^{-1}). The value and sign of $\Delta S^{\ddagger} = +2.0$ eu is consistent with the displacement reaction to form **10** and not with the cyclization to form **9**. Moreover, in preliminary experiments we found that BCNU cross-linking of poly(dGdA)·poly(dCdT), pH 7, 50 °C, is 2-fold slower than the rate of cross-linking of CT-DNA; cross-linking would occur after formation of HON=NEt-N^6-dA by displacement on the cyclized tetrahedral intermediate, a reaction that should be slower than displacement by O^6-dG.[76] Some of the products predicted by this mechanism have not been reported, but not all of the products have been isolated and identified.[58]

Conclusions

We are confident that all results for BCNU are consistent with the tetrahedral intermediate mechanism, but we recognize that it may represent a crude approximation to the events that take place on the surface of DNA; aspects of the mechanism are under active investigation. We are encouraged, however, that the initial rate constants for BCNU cross-linking of DNA at 37 °C in L-1210 and 9L cells are essentially the same as the rates of cross-linking of CT-DNA reported here and are strictly first-order for L-1210 cells. We are less confident in the mechanism for SoSo because neither the minor alkylation products nor the structures of cross-linked base pairs have been identified. A precise description of the mechanism must await these results. Nonetheless, the mechanism in Scheme II may be a useful guide to design other experiments.

Intermediates and products suggested by these mechanisms may be important for therepy-limiting intracellular repair processes. One of us (T.P.B.) has shown that 4-fold more GATase is needed to repair SoSo precursor lesions than is needed to repair BCNU precursor lesions.[53] Moreover, BCNU precursor lesions react with GATase to form covalent products, the structures of which have not yet been defined.[77] Excision repair of cross-linked base pairs

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(76) We also found that HN2 and SoSo cross-link CT-DNA and poly(dGdA)poly(dCdT) at exactly the same rates at 50 °C, which adds further proof that BCNU and SoSo cross-link by different mechanisms.

may be therapy-limiting. Our mechanisms suggest that SoSo and BCNU produce cross-linked base pairs with different regioselectivities, the structures of which may account for differential repair phenomena.

There is evidence, however, that DNA cross-linking may not be a primary cytotoxic mechanism in some, often viral-transformed, cell lines. Gibson in particular has found that alkylation of BE cell DNA with MeSoSo, and less so with EtSoSo, causes almost as much cell kill as treatment with the chloroethyl compound.[58] In these and possibly in other cell lines, regioselective alkylation may be an important cytotoxic mechanism or may be responsible for other events. Seidenfeld's results for induction of heat shock enzymes by CENU treatment of BE cells may be related to proximity formation of carbamoyl (or urea) and alkyl adducts in cells.[41]

It is clear that the cytotoxic mechanisms of these and other anticancer drug are complex. SoSo and BCNU are two of the structurally *simple* compounds that are used clinically for the treatment of human neoplasia. Despite a great deal of often elegant work, we are only now beginning to have a vague understanding of mechanisms of action of these and other anticancer drugs and of mechanisms of DNA alkylation that lead to mutagenic and carcinogenic transformations in situ.

Experimental Section

Determination of the Percent Cross-Linking of CT-DNA. For DNA treatment, BCNU (10–20 mM) and HN2 (0.2 mM) were dissolved in absolute ethanol and SoSo (20–40 mM) was dissolved in dimethyl sulfoxide. CT-DNA (200 μg/mL in 50 mM potassium phosphate buffer, pH 7.2) was either treated continuously (no drug removal) or pulse treated with the drugs for up to 1 h (drug-absent protocol). In the latter, unreacted drug was removed by precipitating DNA with 2.5 volumes of cold (−20 °C) ethanol, washing the precipitate with ice-cold 80% ethanol, and redissolving precipitate in fresh warm buffer, which was incubated at either 37 ± 0.2 or 50 ± 0.2 °C in a thermostated water bath.

The percent cross-linking was determined by using the method of Morgan and Paetican.[49] Briefly, about 10 μg of treated DNA in buffer was added to 3 mL of a solution of ethidium bromide (1 μg/mL), 0.4 mM EDTA, and 20 mM potassium phosphate (pH 11.8). Fluorescence was measured in 1-cm^2 cuvettes at ambient temperature in a Perkin-Elmer LS-F fluorescence spectrophotometer using 525-nm excitation and 600-nm emission wavelengths. The solution was heated to 100 °C for 5 min. After the solution was cooled rapidly in ice water, the fluorescence was measured again. The fraction of the original fluorescence retained is the fraction of DNA cross-linked.

Kezdy-Swinbourne Method.[51] Values of X_∞ were determined from plots of X_t vs $X_{t+\tau}$ where τ is an interval between datum points generally chosen to equal 1.5–2 half-lives. Slopes were calculated by linear regression for various values of τ, and the slope with the best correlation coefficient was used. In general, larger values of τ give more accurate estimates of X_∞, and we chose the largest value of τ consistent with good plots. Values of τ on the order of 24–27 h are available from the precursor formation data. X_∞ was estimated graphically by constructing lines with the first datum point and the calculated slope; the intersection of this line with the 45° line is X_∞. For small or large values of τ, k_{obsd} calculated with either the slopes of Kezdy-Swinbourne plots or the estimated X_∞ values were generally within ±10%, but deviations of as much as 20% were found at the lower temperature and lower concentrations used. Rate constants can be calculated directly from slopes of Kezdy-Swinbourne plots with the equation $k_{K-S} = (\ln \text{slope})/\tau$; values are listed in Table I.

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Registry No. BCNU, 154-93-8; SoSo, 88343-72-0.

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Hydrolysis of a Peptide Bond in Neutral Water

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Abstract: A radioassay for measuring amide bond hydrolysis at neutral pH and room temperature is presented. A peptide labeled at the terminal amino acid is attached to a polyacrylamide resin. The derivatized resin is incubated in buffer, and the release of radiolabel into bulk solution is monitored as a function of time. These data are used to calculate a rate constant for the process leading to radiolabel release. Control experiments indicate that this process is amide bond hydrolysis. The rate of hydrolysis of resin-PhePhePheGly at neutral pH and room temperature was found to be 3×10^{-9} s^{-1}, which corresponds to a half-life of approximately 7 years.

Simple hydrolysis of an unactivated amide has never been observed in pure water. What is known about the rate of amide hydrolysis under neutral conditions has been determined either by inference from the properties of more reactive functionalities, such as esters or amides activated by conjugation, or by extrapolation of results from amide hydrolyses studied under more vigorous conditions. Perhaps the mildest conditions reported for hydrolysis of an unactivated amide are those used by Meriwether and Westheimer.[1] They established the rate of glycinamide hydrolysis to be 2×10^{-6} s^{-1} at pH 9.3 and 65 °C.

Our interest in the hydrolysis of amides stems from ongoing work here which is directed toward the construction of a semisynthetic peptidase for the selective hydrolysis of certain C-terminal peptides. The design of artificial enzymes has been the focus

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