DNA Cross-Linking by Intermediates in the Mitomycin Activation Cascade†

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ABSTRACT: We have assayed the cross-linking of oligonucleotides containing repeated mitomycin-reactive CpG sites in order to assess the factors that enhance activation of the carbamoyl function at C_{10}, yielding efficient mitomycin cross-linking. Drugs studied include mitomycin C (MC), *N*-methylmitomycin A (NMA), and the aziridinomitosene of NMA (MS). Drugs were reduced both by catalytic hydrogenation and by dithionite. We find that cross-linking by fully reduced NMA can be increased severalfold by addition of either excess dithionite reductant or the oxidant $FeCl_3$. Enhancement by $FeCl_3$ is not seen with MC or MS, but excess dithionite increases cross-linking by all three compounds. We explain the action of Fe^{3+} by postulating production of the semiquinone of the monoadduct of mitomycin reacted at the C_1-position; according to this mechanism, departure of the carbamate from C_{10} is more efficient for the semiquinone than for the hydroquinone. However, our results imply that the hydroquinone can also function as a cross-linking agent. Excess dithionite, beyond that required for stoichiometric reduction, activates the carbamate 2–3-fold for cross-linking. We find that the fully reduced leucoaziridinomitosene is highly unstable in solution, yet it produces efficient cross-linking. Hence, this compound is highly reactive in DNA alkylation and a good candidate for the role of primary alkylating agent.

The mitomycins, potent antibacterial and anticancer drugs, have long been recognized as bioreductive alkylating agents. Extensively studied since their discovery in 1956 (Hata et al., 1956), the mechanism of their reaction with DNA remains fertile ground for investigation. Early hypotheses advanced by Iyer and Szybalski (1964) and Moore (1977) suggested that a two-electron reduction of mitomycin to a hydroquinone would lead to localization of electron density on nitrogen, triggering the loss of methanol and resulting in increased electrophilicity of centers C_1 and C_{10} (Scheme I). This mechanism explains mitomycin's requirement for reductive alkylation and its DNA cross-linking capability. However, since that time, evidence has accumulated suggesting that the mitomycin semiquinone radical is sufficiently activated to trigger the events that lead to the formation of an alkylating agent. Cross-linking could thus potentially be increased by maximizing the hypothetical mitomycin semiquinone concentration (Tomasz et al., 1974, 1987; Lown et al., 1978). Evidence for the presence of a semiquinone intermediate in the course of reduction of mitomycins has been reported (Patrick et al., 1964). More recently, inquiries into the chemical reactivity of mitomycin analogues and proposed intermediates of the activation cascade by Andrews et al. (1986), Kohn et al. (1987), and Egbertson and Danishefsky (1987) demonstrate that one-electron reduction is sufficient to activate mitomycins for elimination of the angular substituent. In addition, the mitosenyl semiquinone appears to be a better alkylating agent for small nucleophiles than is the mitosenyl hydroquinone (Egbertson & Danishefsky, 1987).

The experiments of Tomasz and collaborators (Tomasz et al., 1987, 1988) show clearly that the major impediment to the cross-linking reaction is activation of the carbamate (C_{10}) as a leaving group; most monoadducts are attached through reaction at the aziridine function (C_1). In the experiments reported here, we focus our attention on the cross-linking process, using a gel electrophoresis assay that conveniently distinguishes cross-linked oligonucleotides (Teng et al., 1989). We have explored the conditions that maximize cross-linking by mitomycins and aziridinomitosene (Danishefsky & Egbertson, 1986). Starting from fully hydrogen-reduced *N*-methylmitomycin A (NMA),[1] the yield of cross-linking can be improved severalfold by adding the oxidizing agent Fe^{3+}. Unexpectedly, a similar increase is achieved by exposure of the reduced system to the reducing agent sodium dithionite. The action of Fe^{3+} is specific to NMA and not seen with mitomycin C or aziridinomitosene. We discuss these and other results in terms of the role of the mitomycin semiquinone, and the ability of dithionite to activate the carbamate function.

Materials and Methods

A 22 base pair oligonucleotide of sequence ATAAAT-$(CG)_5$TAAATA and its complement were synthesized on an Applied Biosystems DNA synthesizer and purified by Waters HPLC. They were labeled with [γ-^{32}P]ATP and T4 DNA kinase and hybridized after labeling. Calf thymus DNA was purchased from Sigma.

Mitomycin C (from Bristol Pharmaceutical Co.) and *N*-methylmitomycin A and its aziridinomitosene were dissolved in dimethyl sulfoxide and stored at −20 °C as 15 mM solutions. Drug solutions were used within 1 month.

Sodium dithionite, from Fisher Scientific, was dissolved in the appropriate volume of deaerated water and used within 5 min after preparation. Ferric chloride was obtained from Baker. Gel loading buffer (5×) contained 0.15% Bromophenol Blue, 0.15% xylene cyanol, 25% glycerol, 5 mM EDTA, and 50 mM sodium hydroxide.

A 10 mM sodium phosphate (pH 4.5 or 7.5) solution, containing the labeled oligomer (~10000 cpm), 1.5 mM calf thymus DNA, and 1.5 mM mitomycin was deaerated by flushing with nitrogen for at least 1 min. The reducing agent sodium dithionite was then rapidly added, and the sample, tightly capped, was left in an ice bath. The reaction was stopped after 1 h with 5 μL of gel loading dye. In some experiments, the DNA was added last to the fully reduced

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[1] Abbreviations: MC, mitomycin C; NMA, *N*-methylmitomycin A; MS, aziridonomitosene (Danishefsky & Egbertson, 1986).

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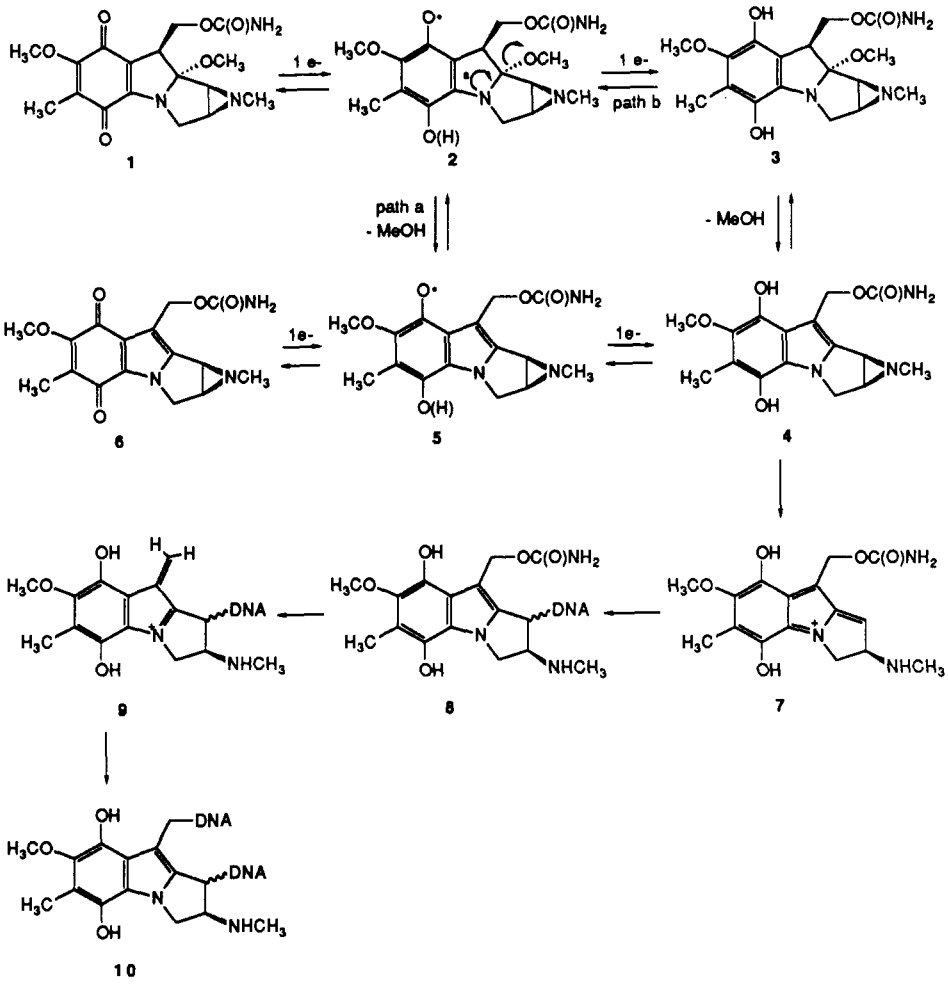
Scheme I: Mechanism of Mitomycin–DNA Interaction[a]



[a] Structures are as follows: **1**, *N*-metylmitomycin A (NMA); **2**, mitomycin semiquinone; **3**, leucomitomycin; **4**, leucoaziridinomitosene; **5**, mitosenyl semiquinone; **6**, mitosene; **7**, **9**, quinone methides; **8**, monofunctionally alkylated leucomitosene; **10**, bifunctionally alkylated leucomitosene.

mitomycin. When ferric chloride was used, it was dissolved in deaerated water and added at different times after reducing the drug in the presence of DNA. pH changes due to addition of $FeCl_3$ were less than 0.2 unit. The final volume of the samples was always 20 μL, since smaller volumes do not allow good deaeration. Small variations in this procedure are reported under Results.

Reactions with the hydroquinone of NMA were performed in a dry box under continuous flushing of nitrogen. The mixture of labeled DNA, buffer, and water was first deaerated in a nitrogen atmosphere; then the drug was added and finally the reducing or oxidizing agent. The mitomycin, as a 15 mM solution in DMSO, had been previously reduced by flushing with hydrogen in the presence of the catalyst Pd/C for ~20 s, always working under nitrogen. It had been filtered through a cotton plug, tightly capped, and used within 1 h.

The samples were denatured by heating at 90 °C for 3 min, loaded on a 20% denaturing polyacrylamide gel, TBE buffer, and electrophoresed at 60 W for 20 min. The gel was autoradiographed, and the bands corresponding to the cross-linked and un-cross-linked DNA were cut out from the gel and counted in a Packard 1500 TRI-CARB liquid scintillation counter. Quantitation of the results was based on the fraction of total DNA counts found in the cross-linked band.

Results

Previous studies in our laboratory (Teng et al., 1989) have shown that mitomycins cross-link oligomers containing several repeated CpG dinucleotides better than oligomers having isolated CpG sites. Hence, the sequence chosen for our assay (with five successive CpG sites) is an efficient cross-linking target for mitomycins and allows good quantitation of the results. Figure 1 illustrates the gel electrophoresis assay used to detect and quantitate the products. As shown, cross-linked oligonucleotides run much more slowly than non-cross-linked strands, whereas monoadducts have little influence on electrophoretic mobility (unpublished results).

As reducing agent, we used sodium dithionite because other reductants, such as ascorbic acid or sodium borohydride, did not give enough cross-linked product to allow a good quantitation of the results. Experiments were performed in phosphate buffer both at pH 4.5 and at pH 7.5: in acidic conditions, the amount of cross-link increases significantly, as expected, due to the consumption of protons during the reaction (Iyer & Szybalski, 1964). Attention was concentrated on the neutral pH value, which is closer to physiological conditions. The concentration of phosphate buffer used was sufficient to maintain the desired pH value during the reaction between sodium dithionite and mitomycin.

In the first set of experiments, different ratios of reductant/drug were used for a given concentration of mitomycin (NMA, MS, or MC), in order to determine the dependence of the percentage of cross-linked DNA on the amount of reducing agent. The results of several gels are plotted in Figure 2 for NMA. A saturation phenomenon is clearly observed, with a plateau beyond a 3-fold molar excess of dithionite, yielding maximum cross-linking of about 35%. It is known

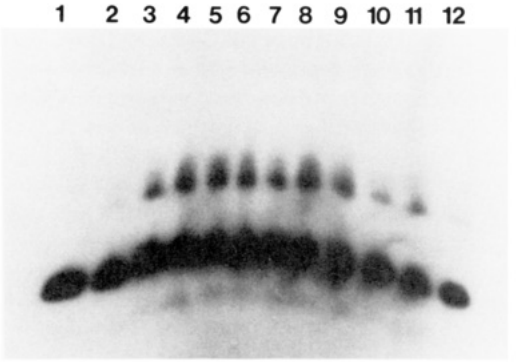


FIGURE 1: Twenty percent denaturing gel used to assay the effect on the cross-linking reaction of adding ferric chloride to mitomycin reduced by dithionite (1:1:1 drug:dithionite:$FeCl_3$ molar ratio). The first lane on the left contains control DNA, not treated with mitomycin; the easy reannealing of the sequence used can cause some apparent cross-link (upper band), the amount of which was subtracted from the other samples in quantitative studies. Successive lanes (2–12) show the effect of adding ferric chloride at increasing times after the reductant: 0, 1, 5, 10, 20, 30, and 45 min; 1, 2, 4, and 8 h. Maximal enhancement is observed after about 5 min, followed by a broad plateau implying an intermediate that is stable for several hours. The preparation of the samples is reported under Materials and Methods.

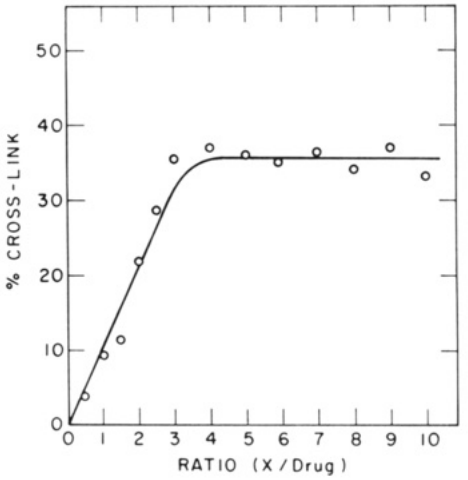


FIGURE 2: Effect of the molar ratio X/drug, where X = $Na_2S_2O_4$, on the percentage of cross-link. The concentration of the drug was 1.5 mM. The amount of cross-linked oligonucleotide is ~10% when a stoichiometric ratio of reducing agent is used, very close to the value found for hydrogen-reduced NMA. (Electrophoretic mobilities of hydrogen- and dithionite-reduced cross-linked products were indistinguishable.) The experimental data clearly define a saturation curve, with the plateau occurring beyond a molar ratio of ~3.

that sodium dithionite undergoes different parallel reactions, is very unstable, and decomposes rapidly in the presence of oxygen. Therefore, the reduction reaction might occur with partial efficiency (~30%), thus explaining the requirement for excess dithionite. However, results presented below for NMA indicate that dithionite contributes to the cross-linking reaction beyond the requirement for stoichiometric reduction.

In a second set of experiments, we measured the effect of adding ferric chloride to the reaction mixture of DNA and reduced mitomycin. Addition of this oxidizing agent within approximately an hour after sodium dithionite significantly enhances the percentage of cross-linked DNA (Figure 1). This effect is found only for NMA; ferric chloride does not affect the yield of the reaction when the drug used is MC or MS. (The source of this contrast may be the difference in the reduction potentials for the three compounds: −0.4 V for MC and MS in comparison to −0.2 V for NMA.) Since sodium dithionite provides two electrons, while ferric chloride can

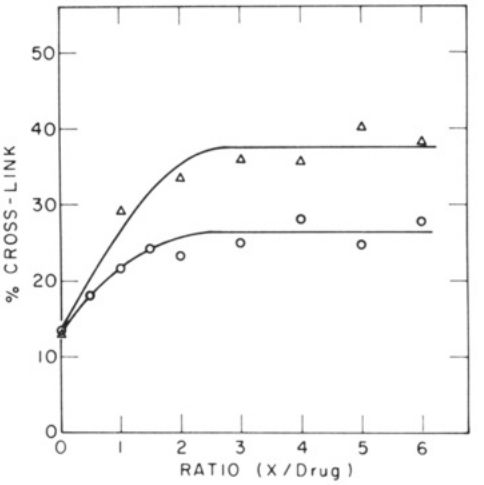


FIGURE 3: Percentage of cross-linked product upon addition of X moles of ferric chloride (△) or sodium dithionite (○) per mole of hydrogen-reduced NMA. The reducing agent enhances the reaction yield by 2-fold, and Fe^{3+} by about 3-fold. Again, the amount of cross-linked DNA does not increase after a ratio of 2 to 3.

accept only one electron, reductant:oxidant:drug ratios of 1:1:1, 1:2:1, and 2:2:1, were investigated, with very similar results in all three cases. When sodium dithionite is present at a concentration 3 or 4 times larger than mitomycin, ferric chloride does not seem to affect the amount of cross-linked product. Furthermore, increasing the time delay in the addition of the oxidizing agent eliminates the enhancement; adding ferric chloride after several hours or more does not improve the yield of the cross-linking reaction. Finally, addition of ferric chloride to the oligonucleotide and unreduced NMA does not give any cross-linked DNA. These results suggest that the fully reduced mitomycin is not the only cross-linking species, nor is it the strongest.

To shed further light on the reaction mechanism, a series of experiments was performed using hydrogen-reduced NMA in a nitrogen atmosphere. The drug was used within 1 h after its reduction. An NMR spectrum, taken 6 h after reducing the drug, still showed the presence of at least 98% of the fully reduced compound, confirming the absence of degradation products. Either sodium dithionite or ferric chloride, or both, was added to the mixture of drug (1.5 mM) and DNA. In Figure 3, percent cross-link is plotted as a function of the ratio of reductant or oxidant to drug. Again, a saturation phenomenon is observed. An interesting feature is that the addition of sodium dithionite enhances the amount of the cross-linked DNA beyond the level seen with the hydrogen-reduced compound; however, this effect is less than that observed upon addition of ferric chloride.

We reasoned that a possible explanation for the dithionite effect could be the presence of some unreduced NMA (2–5%) that could react with the reducing agent. To test this hypothesis, sodium dithionite (final concentration 3.0 mM) was added to samples in which the drug concentration ranged between 0.075 and 1.5 mM. If a very small amount of reduced mitomycin were responsible for the 2-fold increase in cross-linking found in the previous experiment, we should obtain a dramatic cross-linking response at low drug concentration, followed by saturation. In fact, the relation between percent cross-link and drug concentration is linear from 0.1 mM NMA (2% cross-linking) to 1.5 mM NMA (28% cross-linking), and no saturation is observed.

Some samples containing hydrogen-reduced NMA were treated first with sodium dithionite and then, after 5 min, with ferric chloride, or vice versa, but the addition of the second

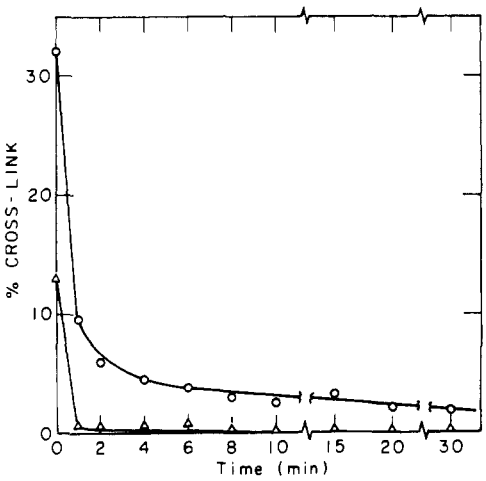


FIGURE 4: Effect of time delay in the addition of labeled oligonucleotide to fully dithionite-reduced NMA (○) and MS (△). The lability of the active cross-linking species when the drug used is MS is proven by the almost total lack of cross-linking capability within the first minute after reduction. NMA, reduced with 2 equiv of sodium dithionite, yields an alkylating agent whose concentration decreases on a time scale of several minutes. Similar results are obtained with catalytically hydrogenated drugs. Note that the decay of reactivity of free reduced NMA shown here is much faster than the decay of the species that reacts with $FeCl_3$ to enhance cross-linking (Figure 1).

reagent in both cases does not affect the reaction yield over a relatively wide range of reductant:oxidant:drug ratios (data not shown), suggesting that the fundamental steps of the reaction occur in the first few minutes. To shed further light on this point, we added at different times the labeled oligonucleotides to NMA and MS previously reduced with 2 equiv of sodium dithionite. A large, but not complete, decrease in cross-linking yield is evident after the first minute when the drug is NMA (Figure 4). In contrast, it is clear that reduced MS loses its reactivity immediately after reduction. A parallel experiment performed by reducing MS with hydrogen and Pd/C gave exactly the same result. This confirms the results of Egbertson and Danishefsky (1987), who found that reduced MS is unstable and decays rapidly after reduction. These results show that the reduced or leucoaziridinomitosene must react rapidly with DNA in order to produce good cross-linking yields during its short-lived presence in solution.

The requirement for excess dithionite, and the increased cross-linking yield when dithionite is added to the catalytically hydrogenated drug, suggests that sodium dithionite, or its decomposition products, reacts in some additional way besides simply reducing mitomycin to the hydroquinone. An attempt was made to identify which compounds are present when NMA or MS undergoes catalytic and/or chemical (dithionite) reduction, using thin-layer chromatography. The experiment was conducted under nitrogen, using two different eluants: 20:80 acetone/ethyl acetate and 15:85 methanol/chloroform. The samples were (a) hydrogen-reduced NMA and MA (i) without (ii) with 1 equiv, and (iii) with 3 equiv of sodium dithionite and (b) NMA and MS (i) with 1 equiv and (ii) with 3 equiv of sodium dithionite, all in phosphate buffer, pH 7.5, and 10–20% DMSO (the same conditions as used for the samples assayed by electrophoresis). Every sample was run on TLC under a nitrogen atmosphere immediately after the 15 min after the addition of the reducing agent. The vials were then opened to the air for at least 20 min and reanalyzed by TLC. Surprisingly, reoxidation due to the oxygen present in the air is very slow, and the brown-orange color characteristic of the reoxidized compound appears only at the surface after several minutes. Unfortunately, there is no way to stain these compounds, and an evaluation of their presence is based only on their color, sometimes leading to uncertain results. These experiments exclude the presence of large amounts of MS upon reduction of NMA and confirm that no changes seem to occur after the first minutes. Even after reoxidation, no new spots are visible.

Previous experiments had shown that bubbling hydrogen in a solution of MS in pyridine, in the presence of Pd/C as a catalyst, for ~20 s yields the leucoaziridinomitosene (structure **4** in Scheme I); if continued for ~3–5 min, the ene–pyrrole is produced (Egbertson & Danishefsky, 1987). The reduction state of this compound is exactly the same as that of the hydroquinone. The ene–pyrrole is apparently a very good alkylating agent, since it can easily delocalize the electrons received upon interaction with nucleophiles into the quinone ring.

In order to determine whether this is the real cross-linking agent, the following experiments were performed: working in a nitrogen atmosphere, and dissolving MS both in pyridine and in DMSO, to exclude any solvent effects, some samples were treated with hydrogen for ~4 min, some others for ~20 s, but then sodium dithionite was added (2.5-fold molar excess over drug). An NMR spectrum was taken of all the samples, indicating ~80% of ene–pyrrole. The labeled oligonucleotide was added to the same samples, immediately after the reaction and a few minutes later, but the gel assay showed the complete absence of any cross-linked product. Thus, even though sodium dithionite seems to assist the formation of ene–pyrrole, especially when the solvent used as pyridine, we cannot consider the ene–pyrrole to be the primary cross-linking agent. This would be expected since this species could only function as a monoalkylating agent.

Discussion

Mitomycins act in a variety of ways in biological systems; we have directed our attention to their ability to cross-link the strands of DNA, a process with dramatic consequences for the cell. It is known that these drugs undergo an enzymatic reduction in order to express their biological activity, and this reaction leads to many different intermediates that can account for the complexity of the mechanism of cross-linking. Using sodium dithionite and ferric chloride as reducing and oxidizing agents, respectively, we tried to characterize the compound or compounds proximally responsible for this action.

A main generalization to be drawn from our experiments is the finding that both the oxidant Fe^{3+} and the reductant dithionite increase by severalfold the cross-linking yield produced by catalytically hydrogenated NMA. We also showed that the oxidizing agent enhances the percentage of cross-link when added to the mixture of DNA and chemically reduced drug. We interpret the ferric chloride effect, which is specific for NMA among the drugs tested, in terms of production of mitomycin semiquinone. According to our data, at least two major classes of intermediates capable of cross-linking DNA are present: first, one-electron reduction products, the mitomycin or aziridinomitosene semiquinones (structures **2** or **5** in Scheme I); second, a fully reduced drug, the leucomitomycin (structure **3**) or the leucoaziridinomitosene (structure **4**) which occurs upon further reaction with sodium dithionite. Treating NMA with sodium dithionite should lead to structures **2**, **3**, **4**, and **5** in Scheme I, according to path a or b.

Our experiments show that the amount of cross-linked DNA increases upon addition of ferric chloride to fully reduced NMA, structure **3**, which has the potential for converting by loss of the methoxy group to compound **4**. Since both our

results and the work of Egbertson and Danishefsky (1986) show that the leucoaziridinomitosene **4** is very short-lived, it is not a plausible candidate for the subsequent Fe^{3+} oxidation process, the reactant for which is found to be present many minutes after the reduction (Figure 1). On the other hand, leucomitomycin and its monofunctional adduct with DNA are expected to be stable, and are therefore likely targets for reoxidation by Fe^{3+}. This reaction yields one or both of the corresponding semiquinones. Since the response to ferric chloride decays more slowly than does the DNA reactivity of reduced NMA (compare Figures 1 and 4), we conclude that the probable target for ferric chloride oxidation is the leucomitomycin monoadduct with DNA (structure **8** in Scheme I). Oxidation of **8** yields its semiquinone and thereby activates the carbamate as leaving group for the cross-linking reaction. The semiquinone of **8** also plays a key role in the cross-linking reaction mechanism proposed by Tomasz et al. (1988). We argue against some nonoxidative role of Fe^{3+} in activating the carbamate on the basis of the failure to observe enhancement effects in the case of the two other drugs (MS and MC), which have significantly altered reduction potentials.

We believe that formation of the mitomycin semiquinone activates departure of the carbamate group; the semiquinone is therefore a better cross-linking agent than the hydroquinone. However, our results do not support obligatory participation of the semiquinone: the increased cross-linked yield upon dithionite treatment of reduced NMA is highly unlikely to result from back-oxidation to the semiquinone. We conclude that both hydroquinone and semiquinone are capable of cross-linking DNA, with some advantage in reactivity of the C_{10}-position enjoyed by the latter.

The kinetic differences observed between MA and NMA can be understood on the basis of the kinetics of elimination of the methoxy group that distinguishes NMA from MS. According to Egbertson and Danishefsky (1987), loss of the alcohol group occurs spontaneously from the mitomycin semiquinone (structure **2** in Scheme I) and possibly from the leucomitomycin (structure **3** in Scheme I), as proposed by Hoey et al., (1988), the driving force being the formation of pyrrole. This fact gives an explanation for the different rates of decay of reactivity shown by the reduced forms of NMA and MS. According to Scheme I, leucoaziridinomitosene is formed almost immediately from MS upon reduction with sodium dithionite, and if DNA is not already present, it is rapidly consumed in reactions with water or other nucleophiles. On the other hand, in the case of NMA, both the mitomycin semiquinone and the leucomitomycin are stable prior to the obligatory loss of methanol, which is a slow step compared to the alkylation reaction. This is why even several minutes after NMA reduction there is still measurable cross-linking activity, whereas cross-linking by MS requires that DNA be present during reduction.

The most difficult feature of our results to explain is the reaction enhancement that results when excess dithionite is added to catalytically reduced NMA; the same enhancement is probably also responsible for the observed 3:1 ratio of dithionite to drug required for optimal activation. [As reported by Teng (1987), this ratio is independent of the concentration of added drug, eliminating any contribution from contaminating oxidants in the other reagents.] The site of action must be related to activation of the carbamate as leaving group, beyond the effect of simple reduction. This aspect of our work does not fit readily into the reaction scheme proposed by Tomasz et al. (1988), according to which the semiquinone drug monoadduct complexed at C_1 (the semiquinone of **8**) forms a cross-link or remains as monoadduct depending on the relative rates of reaction with DNA or competing nucleophiles, and on the rate of the additional competing reoxidation reaction. Full hydrogen reduction of the drug, and strict maintenance of reducing conditions as adhered to in our experiment, should render the reoxidation rate negligible. However, addition of dithionite improves cross-linking yields by roughly 2-fold under these conditions. If dithionite does not act by preventing reoxidation (and the ferric chloride effect indicates that some reoxidation is actually favorable), it must act in some other way. A possible mechanism involves ejection of the C_{9a} methoxide and formation of a dithionite or bisulfite adduct [cf. Hornemann et al. (1976)] in the leucomitomycin. The negative charge on the adduct could serve as an intramolecular base to facilitate E_2-like elimination of the carbamate. Coejection of the bisulfite leads to a compound of form **9**, which serves as the reactive agent for cross-linking. Additional studies will be required to shed further light on this issue.

References

Andrews, P. A., Pan, S.-S., & Bachur, N. R. (1986) *J. Am. Chem. Soc. 108*, 4158–4166.

Danishefsky, S. J., & Egbertson, M. (1986) *J. Am. Chem. Soc. 108*, 4648–4650.

Egbertson, M., & Danishefsky, S. J. (1987) *J. Am. Chem. Soc. 109*, 2204–2205.

Hata, T., Sano, Y., Sugawara, R., Matsume, A., Kanamori, K., Shima, T., & Hoshi, T. (1956) *J. Antibiot., Ser. A 9*, 141–146.

Hoey, B. M., Butler, J., & Swallow, J. (1988) *Biochemistry 27*, 2608–2614.

Hornemann, U., Ho, Y.-K., Mackey, J. K., Jr., & Srivastava, S. C. (1976) *J. Am. Chem. Soc. 98*, 7069–7074.

Iyer, V. N., & Szbalski, W. (1964) *Science 145*, 55–58.

Kohn, H., Zein, N., Lin, X. Q., Ding, J.-Q., & Kadish, K. M. (1987) *J. Am. Chem. Soc. 109*, 1833–1840.

Lown, J. W., Begleiter, A., Johnson, D., & Morgan, A. R. (1976) *Can. J. Biochem. 54*, 110–119.

Lown, J. W., Sim, S., & Chen, H.-H. (1978) *Can. J. Biochem. 56*, 1042–1047.

Moore, H. W. (1977) *Science 197*, 527–532.

Patrick, J. B., Williams, R. P., Meyers, W. E., Fulmor, W., Cosulich, D. B., Broschard, R. W., & Webb, J. S. (1964) *J. Am. Chem. Soc. 86*, 1889–1890.

Remers, W. A. (1979) in *The Chemistry of Antitumor Antibiotics*, Vol. 1, pp 221–276, Wiley, New York.

Teng, S. P., Woodson, S. A., & Crothers, D. M. (1989) *Biochemistry 28*, 3901–3907.

Tomasz, M., Mercado, C. M., Olson, J., & Chatterjie, N. (1974) *Biochemistry 13*, 4878–4887.

Tomasz, M., Lipman, R., Chowdary, D., Pawlak, J., Verdine, G., & Nakanishi, K. (1987) *Science 235*, 1204–1208.

Tomasz, M., Chawla, A. K., & Lipman, R. (1988) *Biochemistry 27*, 3182–3187.