Autocatalytic Quinone Methide Formation from Mitomycin *c*†

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ABSTRACT: Mitomycin *c* in the presence of NADPH and brewers' yeast NADPH: (acceptor) oxidoreductase (Old Yellow enzyme, EC 1.6.99.1) is transformed, at pH 8.0 and with anaerobicity, to two major mitosene products (the *cis*- and *trans*-1-hydroxy-2,7-diaminomitosenes; respective yields of 45 and 30%). These arise by covalent trapping by solvent of a quinone methide intermediate, obtained by rearrangement of the mitomycin *c* hydroquinone. At lower pH (6.5), the major product of this reaction is 2,7-diaminomitosene, which arises by covalent trapping of the quinone methide by H^+. In the former instance the quinone methide acts as an electrophile and in the latter as a nucleophile. A detailed kinetic analysis indicates that the role of the NADPH and Old Yellow enzyme is to initiate an autocatalytic reaction, propagated by mitomycin *c* reduction by the mitosene hydroquinones (arising by the electrophilic pathway). The evidence supporting this conclusion is (1) the formation of oxidized mitosene products, under the rigorously anaerobic reaction circumstance, (2) the nonstoichiometric participation of NADPH, (3) a dependence of the velocity on the total mitomycin *c* concentration, (4) the pH dependence of the reaction, and (5) the accurate simulation of the overall kinetic course with a mathematical model of the autocatalytic pathway. These observations indicate that the autocatalytic pathway may be dominant during in vitro mitomycin *c* anaerobic reductive activation by other reducing agents and that (as with anthracycline reductive activation) oxidation of the mitosene hydroquinone obtained from nucleophile addition to the quinone methide may be a necessary event for the formation of *stable* covalent adducts in vivo.

The neoplastic cell is eradicated only with great difficulty. Toward this goal chemotherapy has achieved success in select instances, through careful administration of appropriate chemical agents. Yet despite much effort the number of successful antitumor antibiotics remains small, and for many of these the mechanistic basis of their antitumor efficacy remains unknown. One possible exception is mitomycin *c* (**1**) (Lown, 1979, 1982; Franck, 1979; Remers, 1979, 1980). Mitomycin *c* is an alkylating agent that is unmasked in vivo (Moore & Czerniak, 1981). It contains two latent functionalities, a carbinolamine and an aziridine, which under appropriate circumstances are transformed into electrophilic species. The presumed target of this drug in vivo is DNA, and the molecular structure of the resulting covalent adducts has yielded to extensive study (Hashimoto et al., 1980, 1983, 1984; Tomasz & Lipman, 1979, 1981; Tomasz et al., 1983, 1984). Appropriately, considerable attention has therefore been focused on the mechanism of adduct formation, and adduct formation is now recognized to occur under two different circumstances. One is acid activation (Cheng & Remers, 1977; Tomasz & Lipman, 1979), and the other is enzyme-mediated anaerobic reduction of the mitomycin *c* quinone (Iyer & Szybalski, 1964; Hornemann et al., 1976, 1983; Bean & Kohn, 1983; Tomasz & Lipman, 1981; Pan et al., 1984; Keyes et al., 1984). Evidence for the relevance of the latter pathway to the in vivo activity of mitomycin *c* has been provided by Sartorelli and colleagues, who have observed a selective toxicity of mitomycin *c* to hypoxic tumor cells [Kennedy et al., 1980; Keyes et al., 1984, 1985; see also Rauth et al. (1983)]. Thus, the chemistry associated with the reductive activation has relevance and has proven—perhaps not surprisingly—to exhibit rich complexity.

The principle uncertainties in the reductive activation process rest amid the classic puzzle of mechanistic organic chemistry, the understanding of how intermediates partition. An outline of the present situation for mitomycin *c* is given in Scheme I. Reduction of the quinone to the hydroquinone **2** renders the carbinolamine more labile, allowing the loss of methanol with the formation of the electrophilic imine. Although this imine may be trapped by exceptional nucleophiles (Hornemann et al., 1976), more commonly intramolecular tautomerization to the indole proceeds rapidly. This new intermediate however is also not stable, and aziridine ring opening ensues, leading to a quinone methide presumed to have structure **3**. Evidence for this quinone methide stems from its ambivalent character, where both electrophilic and nucleophilic characters are expressed in the reactions leading to the ultimate products. Three major products are obtained. At neutral pH in vitro the quinone methide's primary fate (pathway a, Scheme I) is a reaction as an electrophile toward water as a nucleophile, leading to the formation of the diastereomeric *cis*- and *trans*-1-hydroxy-2,7-diaminomitosenes (**6** and **7**). This stereoisomer formation argues against any alternative mechanism, not involving the quinone methide. Direct aziridine cleavage at the hydroquinone level should have given only a single product stereoisomer. Under slightly more acidic conditions, mitomycin reductive activation gives in addition to **6** and **7** the 2,7-diaminomitosene (**5**), arising from a nucleophilic reaction (pathway b) with a solvent-derived proton (Tomasz & Lipman, 1981; Kohn & Zein, 1983). Apart from the ambivalent quinone methide behavior, an additional distinction between these two pathways is that the electrophilic reaction of the quinone methide yields its products at the

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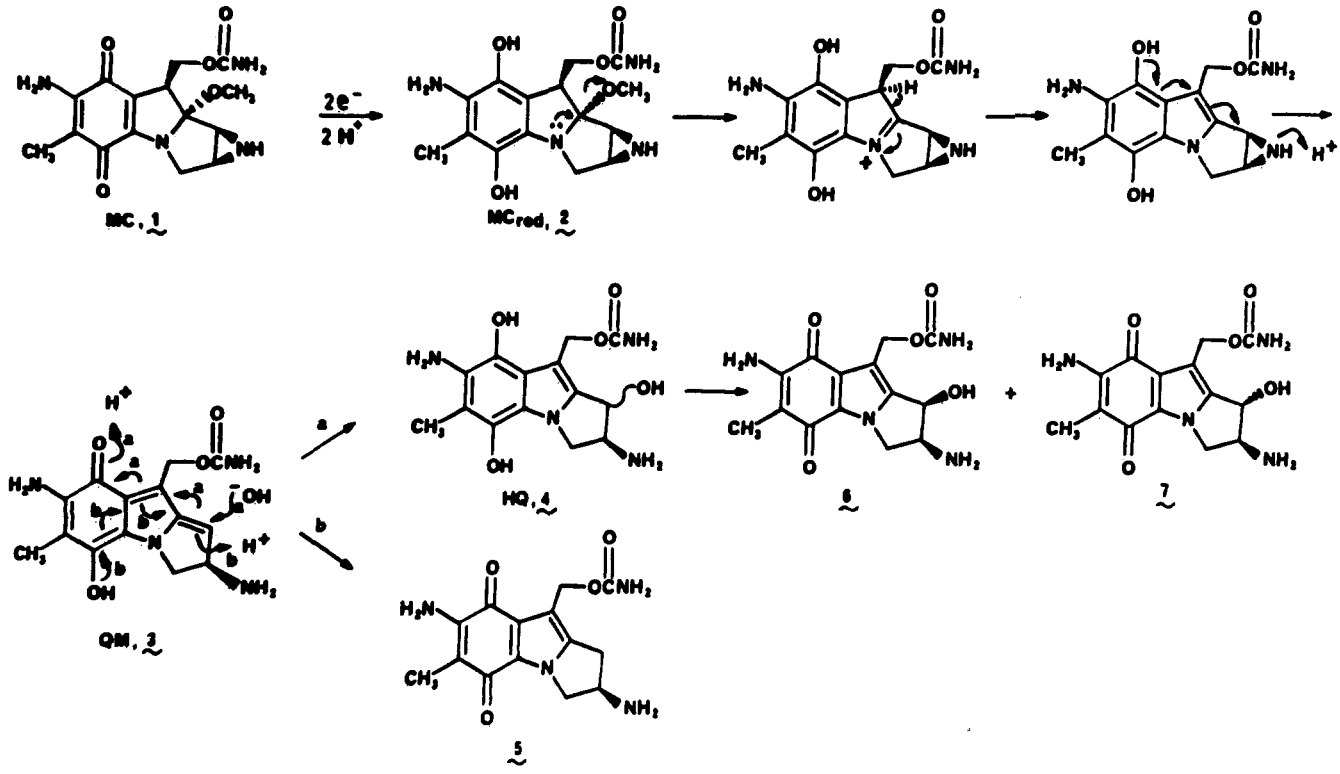
Scheme I



hydroquinone oxidation level (pathway a) while nucleophilic reaction yields its product at the quinone oxidation level (pathway b).

The central focus in the understanding of the anaerobic reductive activation of mitomycin *c* is the partitioning of this quinone methide between its nucleophilic and electrophilic character. An identical circumstance exists in the behavior of quinone methides from reductive activation of the anthracyclines (Kleyer & Koch, 1984; Kleyer et al., 1984; Ramakrishnan & Fisher, 1983, 1986; Fisher et al., 1985) and likely characterizes the behavior of quinone methides in general (Skibo, 1986). With the anthracyclines, nucleophile addition to the quinone methide formed upon their reductive activation likewise provides a hydroquinone adduct. Adduct formation is however reversible, and the evidence suggests that re-formation of the quinone methide, by ejection of the newly acquired nucleophile as a leaving group, may indeed be the favored direction. For the anthracyclines, *stable* adduct formation demands oxidation of the hydroquinone adduct to the quinone oxidation level. This is most simply accomplished by quinone/hydroquinone comproportionation, and the overall reaction becomes autocatalytic in character. Whether a similar situation exists for the mitomycin quinone methide has not been previously established; nevertheless, there can be little doubt that oxidation of the mitosene hydroquinone derived from nucleophile addition to the quinone methide **3** will be a stabilizing event. Indeed, it appears that in many of the previous studies of nucleophile trapping by **3** the resulting adducts were initially generated at the *quinone* oxidation level. This apparently peculiar circumstances has been noted upon only by Tomasz and Lipman (1981), who correctly noted that "the hydroquinone forms of the products are probably reoxidized to quinone during isolation and/or by intermolecular electron transfers between various quinones, semiquinones, and hydroquinones present during the reaction." The full implications of these electron transfers are now addressed. In this study, the anaerobic reductive activation of mitomycin *c* has been observed under an unusually simple set of experimental conditions. This has allowed the essential features of the quinone methide partitioning to be described for the first time, and establishes an autocatalytic basis for mitomycin *c* reductive activation to its quinone methide.

Experimental Procedures

Materials. Mitomycin *c*, as the clinical form Mutamycin, is the generous gift of Dr. W. T. Bradner of the Bristol-Myers Co. NADPH, glucose oxidase, and catalase are obtained from Sigma. Old Yellow enzyme [NADPH: (acceptor) oxidoreductase, EC 1.6.99.1] is isolated as described by Abramovitz and Massey (1976). After purification, this enzyme possesses an absorbance ratio at 278 to 462 nm of 10.7, indicating near homogeneity. The V with O_2 as an acceptor at 30 °C is 0.7 s^{-1} both in 0.10 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 8.0 buffer and in 0.05 M [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane hydrochloride (Bistris-HCl) pH 6.5 buffer.

The mitosene products, which are necessary as chromatographic standards, are obtained by known chemical transformations of mitomycin *c*. The *cis*- and *trans*-1-acetoxy-2-acetamido-7-aminomitosenes are obtained by acid hydrolysis and acetylation (Stevens et al., 1964) and purified by preparative liquid chromatography (see below). 2(*R*)-Acetamido-7-aminomitosene is obtained by mitomycin *c* reduction under slightly acidic conditions (Tomasz & Lipman, 1981), acetylation, and liquid chromatographic purification. 1a-*N*-Acetylmitomycin *c* is obtained by direct acetylation of mitomycin *c* (Kinoshita et al., 1971). The identity of each is established by comparison of the observed physical properties with the literature values. The reported absolute configurations are based on those for mitomycin *c* (Shirahata & Hirayama, 1983).

trans-1(R)-Acetoxy-2(S)-acetamido-7-aminomitosene: ^1H NMR [300 MHz, $(CD_3)_2SO$] δ 8.50 (d, 1 H, N*H*Ac, J = 6.7 Hz, D_2O exchangeable), 6.63 (s, 2 H, NH_2, D_2O exchangeable), 6.38 (s, 2 H, OCONH_2, D_2O exchangeable), 5.89 (d,

1 H, H-1, J = 2.2 Hz), 5.04 (d, 1 H, H-10, J = 12.4 Hz), 4.94 (d, 1 H, H-10, J = 12.4 Hz), 4.69 (m, 1 H, H-2), 4.47 (dd, 1 H, H-3, J = 13.2 and 6.7 Hz), 4.06 (dd, 1 H, H-3, J = 13.2 and 2.2 Hz), 2.02 (s, 3 H, NHCOCH_3), 1.81 (s, 3 H, OAc), 1.75 (s, 3 H, 6-CH_3). Irradiation at 4.6 collapses the 8.50 and 5.89 doublets and removes the vicinal couplings to the C-3 hydrogens.

cis-1(S)-Acetoxy-2(S)-acetamido-7-aminomitosene: ^{1}H NMR [300 MHz; $(CD_3)_2SO$] δ 8.33 (d, 1, NHAc, J = 8.2 Hz, D_2O exchangeable), 6.63 (s, 2 H, H_2, D_2O exchangeable), 6.36 (s, 2 H, OCONH_2, D_2O exchangeable), 6.03 (d, 1 H, H-1, J = 5.8 Hz), 5.00 (m, 2 H, H-10, non-first-order doublets), 5.00 (m, 1 H, H-2), 4.51 (dd, 1 H, H-3, J = 12.4 and 8.1 Hz), 3.87 (dd, 1 H-3, J = 12.4 and 8.4 Hz), 2.03 (s, 3 H, NHAc), 1.84 (s, 3 H, OAc), 1.75 (s, 3 H, 6-CH_3). Irradiation at 5.0 collapses the 8.33 and 6.03 doublets and removes the vicinal couplings to the C-3 hydrogens.

2(R)-Acetamido-7-aminomitosene: ^{1}H NMR [300 MHz, $(CD_3)_2SO$] δ 8.42 (d, 1 H, NHAc, J = 6.6 Hz), 6.50 (br s, 4 H, 7-NH_2, -$CONH_2$), 5.00 (s, 2 H, H-10), 4.77 (m, 1 H, H-2), 4.33 (dd, 1 H, H-3, J = 12.9 and 7.2 Hz), 3.93 (dd, 1 H, H-3, J = 12.8 and 4.2 Hz), 3.11 (dd, 1 H, H-1, J = 16.7 and 7.9 Hz), 2.67 (dd, 1 H, H-1, J = 16.6 and 4.2 Hz), 1.80 (s, 3 H, NHAc), 1.72 (s, 3 H, 6-CH_3).

1a-N-Acetylmitomycin c: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.46 (br s, 2 H, OCONH_2), 5.10 (s, 2 H, 7-NH_2), 4.84 (dd, 1 H, H-10, J = 11.0, 4.8 Hz), 4.44 (d, 1 H, H-3, J = 13.3 Hz), 4.04 (t, 1 H, H-10, J = 10.9 Hz), 3.65 (dd, 1 H, H-9, J = 10.9 and 4.7 Hz), 3.51 (dd, 1 H, H-3, J = 10.9 and 1.4 Hz), 3.48 (d, 1 H, H-1, J = 4.3 Hz), 3.24 (dd, 1 H, H-2, J = 4.2 and 1.3 Hz), 3.18 (s, 3 H, 9a-OCH_3), 2.09 (s, 3 H, NAc), 1.76 (s, 3 H, 6-CH_3).

Liquid chromatography is done on a Beckman dual 110A pump system using a variable-wavelength Hitachi spectrophotometer for peak detection and a Hewlett-Packard 3390A integrator for peak analysis. The wavelength used for mitosene observation is 310 nm; reported yields are not extinction coefficient corrected. Analytical separations are accomplished on a Rainin Microscorb C18 column (0.46 × 25 cm) and preparative separations on a Du Pont Zorbax C18 column (2.1 × 25 cm). An isocratic elution with 60:40 (v/v) H_2O–CH_3OH is used for both, and comparison is made to the above standards. Representative k' values as follows: 3.5, *N*-acetylmitomycin *c*; 4.0, 2-acetamido-7-aminomitosene; 4.4, *cis*-1-acetoxy-2-acetamido-7-aminomitosene; 6.0, *trans*-1-acetoxy-2-acetamido-7-aminomitosene.

Recovery and Isolation of Mitosene Products. At the completion of the enzymatic reaction, the solution is exposed to the atmosphere and centrifuged to collect all precipitated mitosenes. The supernatant is passed through a small DEAE column (0.5 × 3.0 cm) to remove the enzyme, NADP(H), and the minor mitosene product (see below). The effluent is added to the precipitate, and the mitosenes brought to dryness in vacuo. These are dissolved in a small volume of dry pyridine, and one-third volume of acetic anhydride is added. After 1 h, the solution is taken to an oil by evaporation in vacuo, and then toluene is added and evaporated, to assist solvent removal. The residue is dissolved in a minimal amount of CH_3OH, diluted with water, and absorbed to a small C18 column (Rainin Spice cartridge). After a thorough washing with water, the mitosene mixture is removed with CH_3OH, diluted appropriately with water, and analyzed on the liquid chromatograph.

Recovery of Old Yellow Enzyme following Mitomycin Activation. An anaerobic solution of mitomycin *c* (2.4 mM), NADPH (2.4 mM), and Old Yellow enzyme (8.1 μM), having a total volume of 7.0 mL of 0.10 M Tris-HCl pH 8.0 buffer, is reacted at 30 °C for 1 h. [General procedures for these anaerobic reactions are given in Fisher et al. (1985).] The reaction is complete at this time. It is opened to the atmosphere and centrifuged, and the supernatent is removed to an Amicon centrifugation cone. The enzyme in this supernatent is concentrated and further washed with several small portions of water before final concentration. The enzyme is applied to a G-25 gel filtration column (1.7 × 20 cm) and eluted with 0.05 M Tris-HCl pH 8.0 buffer. Old Yellow enzyme is recovered from this column as a brown band, possessing an absorption spectrum strongly suggestive of covalent modification by mitosene moieties (Fisher & Olsen, 1982). Within experimental error the catalytic activity of this modified enzyme for O_2 reduction by NADPH is identical with that for the native enzyme.

Kinetics and Mathematical Modeling. The kinetics are obtained on a Cary 219 spectrophotometer equipped with a Varian-Apple II digital interface. The software used for data acquisition and modeling is written in ths laboratory. All kinetics are obtained under rigorously anaerobic conditions. A representative kinetics experiment is described. A stock mitomycin *c* solution is obtained by reconstitution of a Mutamycin vial (containing 20 mg of mitomycin *c* and 40 mg of mannitol) with 20 mL of anaerobic buffer and the resulting solution transferred to a Schlenk tube under positive N_2 pressure. Transfer of a 3.0-mL portion of this solution to an anaerobic 1.0-cm path-length cell is made by gas-tight syringe. This cell contains 3.0 mg of solid NADPH and has been previously flushed with N_2. Old Yellow enzyme (200 μL of a 0.15 mM solution) is made anaerobic by equilibration with a moist N_2 gas stream and transferred by syringe to the cell to initiate the reaction. To ensure that anaerobicity is maintained, all buffers contain 30 mM glucose, 2.3 units mL^{-1} glucose oxidase, and 6.5 units mL^{-1} catalase, and all reactions are kept under positive N_2 pressure. Mitosene formation is monitored by the increase in absorbance at 550 nm. For several experiments with higher mitomycin *c* concentrations, a 5.0-mm path-length cell is used.

The values reported in Table I are obtained by numerical solution by Euler approximation methods (Kreyzig, 1979). The program, written to operate on an Apple IIe computer, calculates as a function of time the concentrations of mitomycin *c*, the 1-hydroxy-2,7-diaminomitosene diastereomers, 2,7-diaminomitosene, and the reduced species. The reduced species are treated as a single entity, as there is no way to quantitatively distinguish among the mitomycin *c* hydroquinone, the quinone methide, the mitosene hydroquinones, and the other reduced species shown in Scheme I. The rate constants are varied empirically until an optimal visual fit to the kinetic data and product ratios is obtained. (It is assumed that the extinction coefficient of the reduced species at 550 nm is nil.) An extinction coefficient for the mitosenes at 550 nm is estimated to be 1050 M^{-1} cm^{-1} with reference to the mitosene band at 310 nm (ϵ = 11 100 M^{-1} cm^{-1}; Tomasz & Lipman, 1979). The extinction coefficient for mitomycin *c* at 363 nm is 21 800 M^{-1} cm^{-1}, from which ϵ_{550nm} = 250 M^{-1} cm^{-1} is determined.

Results

In the absence of an enzyme catalyst, the bimolecular rate constant for NADPH oxidation by mitomycin *c* is immeasurably small. When, however, brewers' yeast NADPH:(acceptor) dehydrogenase (Old Yellow enzyme) is added to an anaerobic solution of these two reagents and the reaction

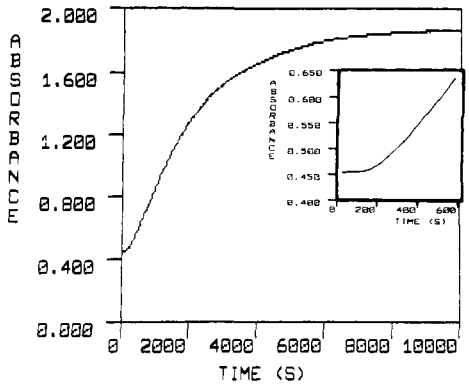


FIGURE 1: Representative progress curve at 550 nm for mitosene formation. The reaction is set up as described under Experimental Procedures, with final reagent concentrations of 1.8 mM NADPH, 1.8 mM mitomycin *c*, and 4.8 μM Old Yellow enzyme in anaerobic 0.10 M Tris-HCl pH 8.0 buffer, 30 °C. Some mitosene product precipitation from the solution occurs during the course of the reductive activation and contributes to the final absorbance. The insert is a magnification of the lag phase seen at the beginning of the reaction.

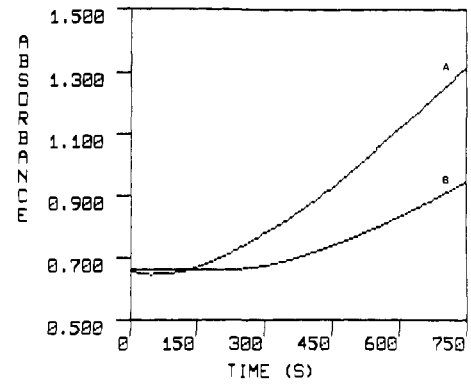


FIGURE 2: Effect of Old Yellow enzyme concentration on progress curve at 550 nm for mitosene formation. The final concentrations are 0.9 mM NADPH, 2.6 mM mitomycin *c*, and 7.8 μM (curve A) and 4.0 μM (curve B) Old Yellow enzyme in anaerobic 0.10 M Tris-HCl pH 8.0 buffer, 30 °C.

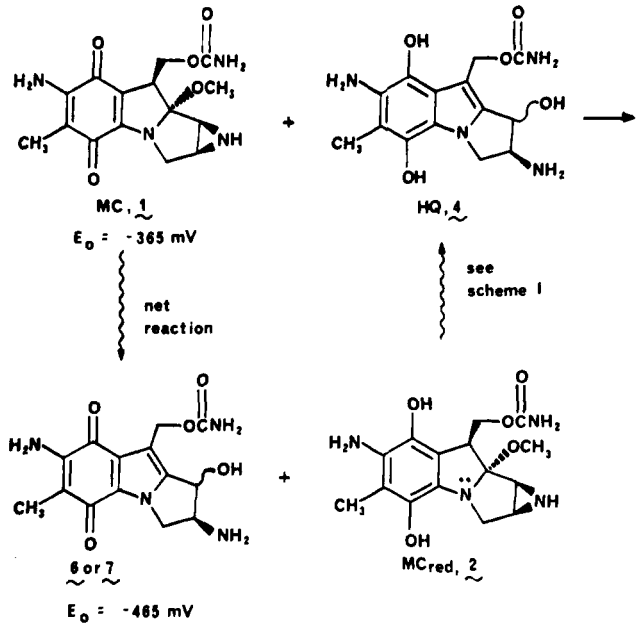
is monitored at 550 nm for mitosene formation, an absorption increase is observed (Figure 1). This indicates reductive activation of the mitomycin *c* (leading to the formation of mitosene products), and examination of the reaction at that point where the 550-nm absorbance increase has ceased indicates virtually complete consumption of the mitomycin *c*. The nature of the reaction is not, however, straightforward, and the precise role that the enzyme plays as the catalyst of this transformation is addressed by the following experiments.

The first curious feature of this reaction is the overall shape of the progress curve at 550 nm. The curve has a sigmoidal appearance, showing a lag phase (approximately inversely proportional in duration to the enzyme concentration) and catalytic phase (approximately directly proportional). One obvious explanation to account for the lag phase is the presence of residual oxygen. Oxygen will quickly oxidize the mitomycin *c* hydroquinone (**2**), before methoxide expulsion and conversion to mitosene products can occur (Danishefsky & Ciufolini, 1984; Bachur et al., 1979). Since Old Yellow enzyme catalyzes O_2 reduction by NADPH, the lag phase could then result from catalytic turnover of residual oxygen prior to the anaerobicity absolutely necessary for mitomycin *c* reductive activation. This explanation is, however, easily excluded as a possibility. When rigorous precautions to exclude O_2 are made, the lag phase remains. Furthermore, the zero-order reduction of O_2 by NADPH as catalyzed by Old Yellow enzyme occurs in a period shorter than the observed lag phase. When the aerobic activity of Old Yellow enzyme is measured with an oxygen electrode, the velocity for O_2 reduction catalyzed by this enzyme is completely unaffected by the presence of mitomycin *c*. Hence, mitomycin *c* is not a competitive inhibitor of O_2 reduction, nor does it augment O_2 reduction. Molecular oxygen is simply the preferred oxidant of the two on a kinetic basis. [From the midpoint two-electron potential for Old Yellow enzyme reduction of −472 mV vs. SCE (Stewart & Massey, 1985), the reduction of both oxygen and mitomycin *c* (midpoint two-electron potential of −368 mV vs. SCE (Rao et al., 1977) by this enzyme is thermodynamically favorable.] As the duration of the lag phase is inversely related to the enzyme concentration (Figure 2), it is clearly related to the role the enzyme plays as a catalyst of this reaction but not to the presence of O_2 or to the ability of the enzyme to reduce O_2. An explanation of the lag phase is provided by attention to the nature of the reaction being catalyzed.

The second curious feature of the kinetic progress curves is that a 550-nm absorbance increase is observed at all. The 550-nm absorbance rise indicates that mitosenes are being produced at the quinone oxidation level, since mitomycin and mitosene hydroquinones are yellow (Tomasz, 1976; Danishefsky & Ciufolini, 1984; D. M. Peterson, unpublished data). Indeed, the best estimate from the final 550-nm absorbance is that greater than 90% of the mitomycin has been converted to oxidized mitosene products. As should then be expected, exposure of the reaction at its conclusion to O_2 results in no detectable color change, as would be seen for O_2-dependent hydroquinone oxidation. Product analysis indicates that mitosene products are indeed formed. Four major oxidized mitosene products are recovered. The least abundant of these (approximately 5% of the total) absorbs to DEAE-cellulose and gives other indications of being an NADPH–mitosene adduct (see below), whose formation has previously been suggested under similar circumstances (Tomasz & Lipman, 1981). The three remaining products are 2,7-diaminomitosene (**5**) (approximately 8%), *trans*-1-hydroxy-2,7-diaminomitosene (**7**) (30%), and *cis*-1-hydroxy-2,7-diaminomitosene (**6**) (45%). (Completing the product mixture is a number of minor as yet unidentified mitosenes.) An accounting for the origin of the major products, **6** and **7**, follows from the known chemistry concerning mitomycin *c* reductive activation (Scheme I). Sequential loss of methoxide and tautomerization of the enzyme-generated hydroquinone **2** provides the quinone methide **3**, which undergoes nucleophilic attack by solvent to produce the diastereomeric 1-hydroxy-2,7-diaminomitosene hydroquinones. Yet these accumulate during the course of the reaction at the quinone oxidation level; clearly then there must be a subsequent redox event to accomplish this. The obvious solution to this circumstance is that mitomycin *c* itself operates as the oxidant (Scheme II). Not only are the mitosene hydroquinones robustly capable of this reduction (their midpoint potential is fully 100 mV more negative than that of mitomycin *c*; Rao et al., 1977), but quinone/hydroquinone comproportionation is invariably rapid, and further, once mitomycin *c* is reduced it is placed on the pathway to the mitosene hydroquinone (Scheme I), which will continue the chain reaction. Hence, there exists the basis for an autocatalytic reaction.

This assertion is experimentally verifiable. If autocatalytic, the anaerobic reductive activation of mitomycin *c* by Old Yellow enzyme must be (1) nonstoichiometric with respect to NADPH, (2) dependent on Old Yellow enzyme for initiation, but then proceed substantially independent of the enzyme during the autocatalytic phase (greater than zero order in mitomycin *c*), and (3) pH-dependent. This latter conclusion

Scheme II



follows from the appearance of the 2,7-diaminomitosene as a product. This has been previously observed as a product of mitomycin *c* reductive activation and is known to account for a significantly greater portion of the mitosene products at lower pH (<7; Tomasz & Lipman, 1981; Pan et al., 1984). Since it arises as a consequence of a nucleophilic reaction of the quinone methide with H^+, with the transformation of the quinone methide to the mitosene quinone oxidation level, its formation represents a chain-terminating event. Where this is an appreciable product, the autocatalytic reaction will be less efficient. The outcomes of the experiments testing these three predictions are given in turn.

Under otherwise identical conditions (anaerobic 0.10 M Tris-HCl pH 8.0 buffer, 7.8 μM Old Yellow enzyme), the kinetics of mitosene formation at 550 nm from a 2.0 mM mitomycin *c* solution are observed in the presence of 0.25, 0.33, 0.66, and 1.0 equiv (with respect to mitomycin *c*) of NADPH. Within experimental error, the length of the initial lag phase, the overall forward velocity, the extent of mitomycin *c* consumption, and the final mitosene product distribution are identical for each. (The yield of the least mitosene product, tentatively assigned as an NADPH–mitosene adduct, is, however, proportional to the initial NADPH concentration.) The principal rate and product-determining events do not therefore require a stoichiometric quantity of NADPH.

The rate of the oxidized mitosene formation is approximately proportional to the initial mitomycin *c* concentration (Figure 3). This dependence may be explained either by a high K_M for a simple enzymatic reaction or by the autocatalytic pathway. The first possibility, of a high K_M, is completely inconsistent with the observation of a lag phase (when substrate is maximal and there is no product, the velocity should be greatest) and is excluded on this basis. (This exclusion is substantiated by the ability to reproduce this initial lag phase with a chemical reductant, sodium dithionite.) In contrast, the reaction rate in the autocatalytic process would have such a dependence on the total mitomycin *c* concentration.

As previously discussed, the change in products accompanying a pH change to 6.5 should profoundly limit the autocatalytic process. The catalytic activity for Old Yellow enzyme reduction of O_2 by NADPH is not pH dependent; it is reasonable to assume that this is likewise true for mitomycin *c*

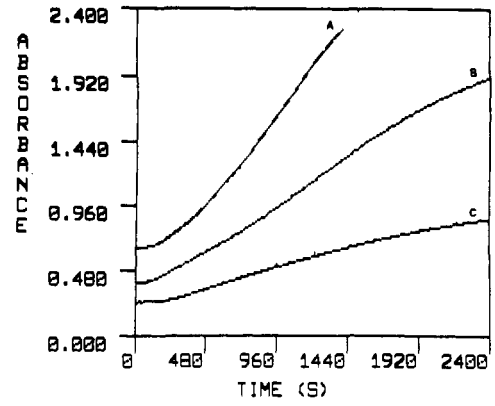


FIGURE 3: Effect of mitomycin *c* on progress curve at 550 nm for mitosene formation. The final concentrations are 0.9 mM NADPH, 7.8 μM Old Yellow enzyme, and 2.6 mM (curve A), 1.6 mM (curve B), and 1.0 mM (curve C) mitomycin *c* in anaerobic 0.10 M Tris-HCl pH 8.0 buffer, 30 °C.

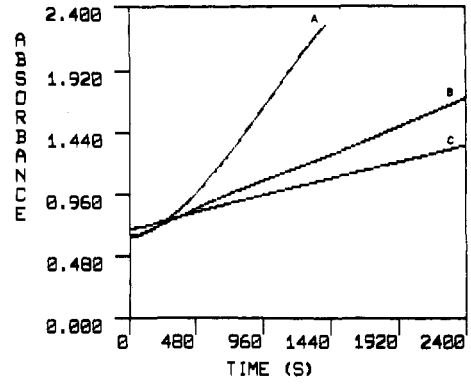


FIGURE 4: Effect of pH on progress curve at 550 nm for mitosene formation. The final concentrations are as follows: (curve A) 0.9 μM NADPH, 2.6 mM mitomycin *c*, and 7.8 μM Old Yellow enzyme in anaerobic 0.10 M Tris-HCl pH 8.0 buffer; (curve B) 2.6 mM NADPH, 2.6 mM mitomycin *c*, and 13.2 μM Old Yellow enzyme in anaerobic 0.02 M Bistris-HCl pH 6.5 buffer; (curve C) 2.6 mM NADPH, 2.8 mM mitomycin *c*, and 6.9 μM Old Yellow enzyme in anaerobic 0.02 M Bistris-HCl pH 6.5 buffer.

reduction. A comparison of the kinetic course for anaerobic mitomycin *c* reduction at pH 6.5 and 8.0 is given in Figure 4. The rate of mitosene formation at the lower pH is slower, and there is much less indication of an initial lag. This is consistent with a less efficient autocatalytic process, as a consequence of the competitive and irreversible trapping of the quinone methide by H^+. Product analysis of the lower pH reaction supports this belief. After 1.5 h, approximately 75% of the mitomycin *c* is consumed and has provided the 2,7-diaminomitosene as the major product (56% as determined chromatographically) and the hydroxymitosenes **6** (16%) and **7** (12%) as minor products. (The remaining products are the presumed NADPH adduct and other unknowns.) The mitosene arising from the chain termination indeed corresponds to the major reaction product.

In order to verify the conclusion that the Old Yellow enzyme catalyzed reduction of mitomycin *c* is the initiation step of a faster autocatalytic reaction, a mathematical simulation of the progress curves at 550 nm for mitosene formation and of the product compositions was undertaken. The enzymatic initiation is assumed to follow saturation kinetics. Since the nature of the "reduced" intermediates and their precise interrelationship may only be surmised, it is further assumed that the autocatalytic (chain propogating) and nucleophilic (chain terminating) pathways have velocities directly proportional to the reduced intermediates produced by the enzymatic reaction.

Scheme III[a]

$$-\frac{d[1]}{dt} = \frac{V[1]}{K_M + [1]} + k_{autocatalytic}[1][1_{red}]$$

$$\frac{k[5]}{dt} = k_{H^+}[H^+][1_{red}]$$

$$\frac{d[6+7]}{dt} = k_{autocatalytic}[1][1_{red}]$$

$$\frac{d[1_{red}]}{dt} = \frac{V[1]}{K_M + [1]} - k_{H^+}[H^+][1_{red}]$$

$$A_{550nm} = 250\ M^{-1}[1] + 1050\ M^{-1}[5 + 6 + 7]$$

[a] The conversion of the mitomycin *c* hydroquinone to the quinone methide is assumed rapid. The term $[1_{red}]$ is a composite of the mitosene hydroquinones and the quinone methide.

Table I: Comparison of Observed and Simulated Product Distributions (%) for Mitomycin *c* Reductive Activation[a]

	1	5	6 + 7
pH 8.0, 1 h, 7.8 μM OYE, 2.6 mM MC			
calcd	3	7	90
obsd	4	8	88
pH 6.5, 1.5 h, 13.2 μM OYE, 2.6 mM MC			
calcd	29	37	34
obsd	24	42	34

[a] The observed values are normalized to exclude the small quantities of unknown mitosenes and to include the presumed NADP adduct under **6 + 7** (as this adduct also arises by nucleophilic addition). Hence, the "observed" values given here differ slightly from the actual observed values, given in the text.

Scheme III lists the relevant equations, where a Euler approximation is then used for the integrations (chosen time constant of 1 s). In Figure 5 are superimposed actual progress curves and the optimal fits generated by this simulation. The best values obtained for the following variables, by the iterative fit to the actual progress curves and product distributions, are as follows: V, mitomycin *c* reduction by Old Yellow enzyme at saturating NADPH, 0.033 s^{-1}; K_M, mitomycin, 2 mM; $k_{autocatalytic}$, 12 M^{-1} s^{-1}; k_{H^+}, quinone methide protonation, 7 × 10^4 M^{-1} s^{-1}. Table I indicates the agreement between the observed product distributions and the predicted values from this simulation. Although we can provide no assurances that the above values constitute a unique solution, all are reasonable, and the overall fidelity provides reassurance that the interpretation of the mechanism for mitosene formation as an autocatalytic process is correct.

Regardless of the kinetic course of the reaction (autocatalytic or stoichiometric), the pivotal intermediate is the quinone methide **3**. Two experiments and observations indicate that this intermediate is identical with the quinone methide studied previously. Its ambivalent character, leading to a now characteristic product mixture (Tomasz & Lipman, 1981), is the best evidence to this point. The alkylating ability of the quinone methide formed in this enzymatic reaction is further

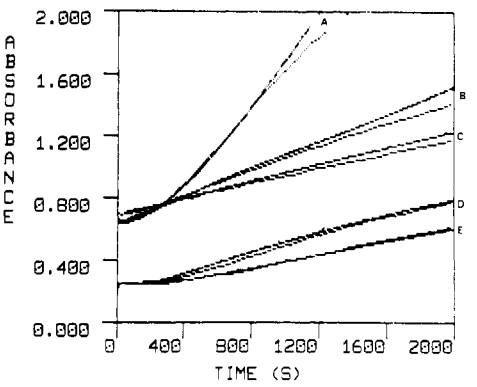


FIGURE 5: Mathematical model of autocatalytic reaction. The equations in Scheme III are integrated numerically to generate theoretical curves that are superimposed on actual data. The kinetic constants used to obtain the theoretical curves are V_{max} = 0.033 s^{-1}, K_M = 2mM, $k_{autocatalytic}$ = 12 M^{-1} s^{-1}, and K_{H^+} = 7 × 10^4 M^{-1} s^{-1}. The enzyme, hydronium ion, and mitomycin *c* concentrations determine the shapes of the theoretical curves. The final concentrations are as follows: (A) 2.6 mM mitomycin *c* and 7.8 μM Old Yellow enzyme, pH 8.0; (B) 2.6 mM mitomycin *c* and 13.2 μM Old Yellow enzyme, pH 6.5; (C) 2.8 mM mitomycin *c* and 6.9 μM Old Yellow enzyme, pH 6.5; (D) 1.0 mM mitomycin *c* and 7.8 μM Old Yellow enzyme, pH 8.0; (E) 1.0 mM mitomycin *c* and 4.0 μM Old Yellow enzyme, pH 8.0. The agreement between product ratios of curves A and B between actual and theoretical data is shown in Table I.

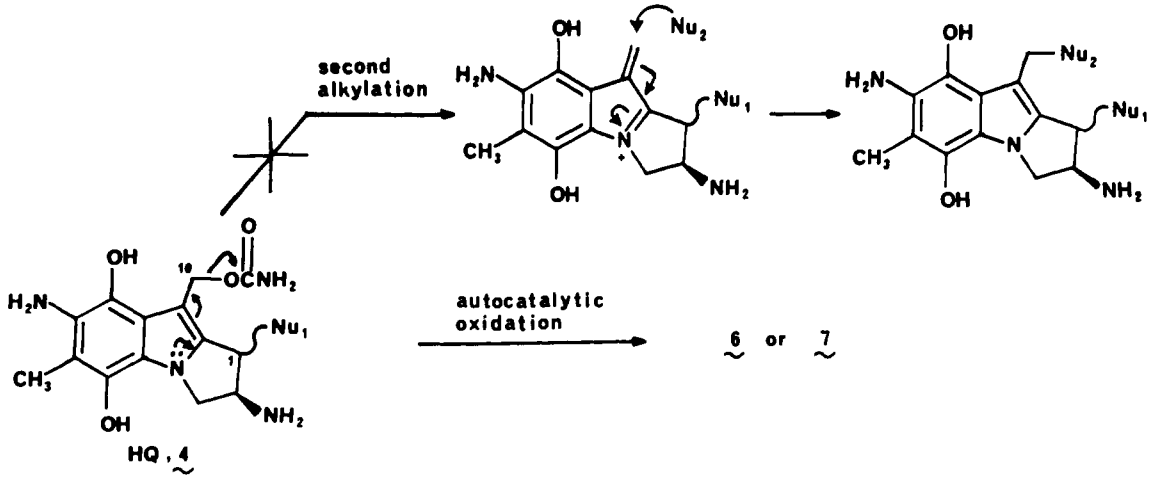
attested by the reproducible isolation of a product in the presence of NADPH, which shows the purple color of a mitosene and which absorbs to anion exchange resin. Although a complete structural proof has not been undertaken, several circumstantial observations suggest this product arises from quinone methide alkylation of NADPH. The yield of the adduct (while never large) is approximately proportional to the initial NADPH concentration, and the absorption spectrum contains a 260-nm absorption in addition to the 310- and 550-nm mitosene peaks. These characteristics are virtually identical with those of an adduct first suggested as being formed by Tomasz and Lipman (1981).

Last, Old Yellow enzyme itself gives evidence of having undergone quinone methide alkylation. At the completion of the reductive activation, Old Yellow enzyme may be recovered without loss of catalytic activity. Yet upon purification this enzyme is isolated as a brown—not yellow—protein. The absorption spectrum of the modified protein is consistent with covalent modification by the quinone methide, as it contains additional absorption bands, superimposed upon those of the flavin chromophore, near 310 and 550 nm (as previously discussed: Fisher & Olsen, 1982). The difference spectrum between Old Yellow enzyme subjected to mitomycin *c* activation and pure enzyme has peaks resembling a mitosene spectrum (250, 310, and 550 nm). An estimate of the number of covalent adducts per protein is difficult due to uncertainty concerning the extinction coefficients in the difference spectrum, variability within the experiment, and apparent hydrolytic instability of these adducts on the enzyme after their formation. The best estimate is that between one and six adducts are formed between the quinone methide and amino acid nucleophiles.

Discussion

A careful examination of the kinetics and products of an enzymatic, anaerobic reductive activation of mitomycin *c* has been made. This provides evidence in support of enzymatic initiation of an autocatalytic process, propagated by mitosene hydroquinone/mitomycin *c* quinone comproportionation. Under this chosen circumstance, the autocatalytic process accounts for the majority of the mitomycin *c* consumption *and*

Scheme IV



provides the means whereby stable C-1 adduct formation occurs. In these two respects a mechanistic equivalence between the mitomycin and anthracycline quinones is established. It is likely that an autocatalytic component for mitomycin *c* reductive activation has contributed significantly to previous model studies, as these frequently have employed millimolar mitomycin *c* concentrations; but it is much less certain whether the autocatalytic pathway is a significant contributor to the in vivo antineoplastic activity of this antibiotic. Nevertheless, it is apparent that future in vitro studies, attempting to mimic the mitomycin *c* reductive activation processes that occur in vivo, must be designed with attention to this possibility.

The elucidation of the autocatalytic pathway is made possible by the choice of Old Yellow enzyme as the catalyst to initiate and sustain the pathway. This enzyme has several attributes that simplify the overall kinetics and allow the autocatalytic pathway to become the predominant mode of activation. These include the apparent pH independence of its activity, its ability to withstand the harshness of quinone methide alkylation, and, most of all, its poor ability to reduce mitomycin *c* and complete inability to reduce mitosene quinones (which are notably poorer oxidants). As a result, the lag phase at pH 8 is well-defined, and the conversion to mitosene quinones proceeds in high yield (>90%). There is a reasonable likelihood that had an enzyme of stronger reducing ability been chosen—such as NADPH:cytochrome P-450 oxidoreductase—that these features would be less obvious.

Several important aspects of mitomycin *c* activation are not however resolved by this improvement in our knowledge. In particular, it is not possible to comment on the relative stability of the mitomycin *c* hydroquinone (**2**). Danishefsky and Ciufolini (1984) have suggested from their own studies on mitomycin reduction in pyridine that *general* circumstances may exist where these hydroquinones are modestly stable and slowly undergo loss of methoxide to the imine precursor of **3**. Previously, the loss of **2** was presumed quite rapid. While it is evident that mitomycin *c* hydroquinone is not accumulating under these autocatalytic circumstances, its *relative* stability remains unknown. As there is however no independent evidence that suggests that formation of **3** from the hydroquinone in aqueous solution is slow, we presume it to occur relatively fast. Possibly, a requirement for proton assistance to the CH_3O^- leaving group is fulfilled in water but not, obviously, in pyridine. The involvement of the other reduced intermediates is inferred only by the nature of the oxidized products formed. This is unfortunate since an understanding of the interrelationships among the reduced species, in particular that between the quinone methide and the hydroquinone, is paramount to establishing the connection between reductive alkylation and the antibiotic activity.

The inability to distinguish between the individual reduced species requires that the reduced compounds be considered as one entity in the mathematical model. The good agreement between theoretical and actual data suggests that the implicit assumptions are valid. The first assumption is that the transformation of **2** to the quinone methide is fast relative to electrophilic trapping and autocatalytic oxidation. This makes the process kinetically invisible in the reaction scheme. The second assumption is that the majority of reduced compounds are either in the quinone methide or hydroquinone form, and these are in rapid equilibrium. The resulting equilibrium constant is contained in the $k_{autocatalytic}$ and k_{H^+} rate constants. Although an equilibrium between the quinone methide and hydroquinone is not strongly substantiated, it may be important. As noted before, the stability of the mitosene adducts depends on the oxidation state of the quinone. A possible retro-Michael reaction involving the C-1 moiety on the hydroquinone regenerates the quinone methide, establishing an equilibrium between these species (reverse of pathway a, Scheme I). The possibility of nucleophile expulsion does not exist for the oxidized mitosenes. Hence, oxidation of the hydroquinone stabilizes adducts at C-1.

The collapse of hydroquinone adducts has important implications with respect to the lifetime of the quinone methide. It may be necessary for the alkylating agent to translate through solution before its "target" (presumably DNA) is reached. In solution, a large number of other nucleophiles including water are capable of C-1 adduct formation. If collapse of the hydroquinone were not possible, the alkylating agent would become irreversibly trapped (inactivated) by these "nontarget" nucleophiles; this would limit the lifetime of the quinone methide and thus its ability to translate through solution. This scenario, however, requires oxidation of the hydroquinone before stable adducts are formed. The autocatalytic reaction provides a requisite mechanism for this step.

Another interesting means for oxidizing mitosene hydroquinone adducts is recently reported by Zein and Kohn (1986) and has been previously observed (D. M. Peterson, unpublished results). The hydroquinone imine (Scheme IV) is ambivalent in nature (analogous to the quinone methide) and can be oxidized by a solvent-derived proton. This generates a C-10 methyl group, and the resulting mitosene is at the quinone oxidation state. Hence, this reaction also traps nucleophiles at C-1.

The observations concerning the outcome of the autocatalytic pathway may also identify the circumstances where double activation of mitomycin *c*, which is necessary for the formation of DNA cross-links, may occur. The evidence for an ability of mitomycin *c* to form interstrand DNA cross-links is strong (Iyer & Szybalski, 1963; 1964; Lown et al., 1976; Lown, 1982). This process is believed to involve the ejection of the carbamate moiety as a second leaving group, to form an electrophilic α,β-unsaturated imine. Substantiation of this notion has been provided by Hornemann and co-workers (Hornemann et al., 1979, 1983), who have shown substitution of the carbamate by xanthate, using dithionite reductive activation. Yet under the autocatalytic conditions no evidence of C-10 functionalization is seen. A possible explanation is as follows. It is most reasonable to view cleavage of the C-10 carbamate as a reaction requiring a hydroquinone oxidation state (Scheme IV). In the autocatalytic process, hydroquinone oxidation is fast, and it is most improbable that the hydroquinones accumulate. Thus, this oxidation may compete against carbamate loss and account for the absence of C-10 functionalization in in vitro mitomycin *c* reductive activation. It is anticipated, now that there exists cognizance of the autocatalytic process, that experimental design which directly addresses this issue will be straightforward.

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