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Reductive Activation of Mitomycin C†

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ABSTRACT: Mitomycin C, an antitumor antibiotic, is known to require reductive activation in order to function as an alkylating agent. In this work reduction has been carried out by using radiolytically produced formate radicals that reduce mitomycin C to its semiquinone in a clean rapid one-electron reaction. The ultimate products of the reduction are *cis*- and *trans*-2,7-diamino-1-hydroxymitosene (B_1 and B_2) and 2,7-diaminomitosene (C). The yields of these compounds were found to be the same when the rate of reduction was varied by 11 orders of magnitude. At pH 7, one mitosene molecule is formed for every two formate radicals, while at pH 9.1, about eight mitosene molecules are formed per formate radical. The ratio of (B_1 + B_2)/C is <0.4 at pH 5.7, 1.0 at pH 7, and >3.5 at pH 9.1. Observations have been made of changes in optical absorption due to the formation of the semiquinone and hydroquinone of both mitomycin C itself and 2,7-diamino-1-hydroxymitosene (B). The direct conversion of the semiquinone form of mitomycin C into the semiquinone of B proceeds slowly, if at all. The semiquinone form of B will rapidly reduce mitomycin C ($k = 7.2 \times 10^8$ M^{-1} s^{-1}). The hydroquinone of mitomycin C undergoes changes resulting in the formation of B and C. The yields of B and C depend on pH. The kinetic data fit a scheme in which the mitomycin C hydroquinone has a pK_a of 5.1, with its protonated form changing to C by a concerted rearrangement with $k = 1.2$ s^{-1}, its deprotonated form changing to the hydroquinone of B with $k = 0.015$ s^{-1}, and the hydroquinone of B reducing mitomycin C with $k = 5$ M^{-1} s^{-1}. The 2,7-diamino-1-hydroxymitosenes are thus formed in a chain reaction with the formation of 2,7-diaminomitosene as a chain termination step. It is suggested that increased cell toxicity at lower pH could be due to the precursor of 2,7-diaminomitosene being an effective alkylating agent.

Mitomycin C, the clinically important antibiotic antitumor drug isolated from *Streptomyces caespitous*, is believed to function by inhibiting DNA replication. This inhibition is brought about by covalent binding to cellular DNA, followed by cross-linking between the complementary strands, thus preventing replication (Tomasz et al., 1985; Iyer & Szybalski, 1963, 1964). It is generally accepted that mitomycin C must be activated by reduction before binding can occur. Activation can be brought about in a variety of systems, e.g., in rat liver microsomes (Tomasz & Lipman, 1981), in hypoxic tumor cells in vivo and in vitro (Keyes et al., 1985), by purified reducing enzymes (Pan et al., 1984; Peterson & Fisher, 1986), and by chemical reduction (Tomasz et al., 1986; Tomasz & Lipman, 1981; Lown et al., 1976).

Recently, the structure of the mitomycin C–nucleotide adduct has been proposed to be formed by covalent linkages at the N^2, N^7, or O^6 positions of guanine and also the N^6 of adenine (Tomasz et al., 1985, 1986, 1987; Hashimoto et al., 1984; Zein & Kohn, 1987; Pan et al., 1986). Some of these adducts has been isolated and characterized. The most recent work (Tomasz et al., 1987) used proton magnetic resonance, differential Fourier transform infrared spectroscopy, circular dichroism, mass spectrometry, and computer-constructed molecular modeling to show conclusively that the major covalent link occurs at the N^2 position of guanine. Activation results in both mono- and bifunctional adducts, i.e., N^2-guanosyl-1-mitosene and N^2,$N^{2'}$-bisguanosyl-1,10-mitosene. Reduction by sodium dithionite leads to almost exclusive bifunctional alkylation, whereas use of the reducing enzyme systems or H_2/PtO_2 in vitro results in >90% monofunctionality. The same bisadduct was formed when DNA was isolated from the liver of rats injected with mitomycin C. Computer simulations have shown that the drug molecule fits neatly into the minor groove for B-DNA without appreciable disruption of the DNA structure.

The increasing clinical importance of the drug and its analogues has brought about a recent upsurge in the study of the mechanisms of bioactivation of mitomycin C as well as other antitumor quinone drugs [e.g., Pan et al. (1984), Peterson and Fisher (1986), Bachur et al. (1978), Land et al. (1985), and Butler and Hoey (1987)]. It was originally believed that the activation consists of a two-electron reduction of mitomycin C to the hydroquinone that results in the molecular rearrangements required to produce reactive centers at carbon 1 and carbon 10. Using reduced nicotinamide adenine dinucleotide phosphate (NADPH) and Old Yellow enzyme to initiate the reduction of mitomycin C, a recent study (Peterson & Fisher, 1986) has also put forward a mechanism of activation involving the hydroquinone route. However, another

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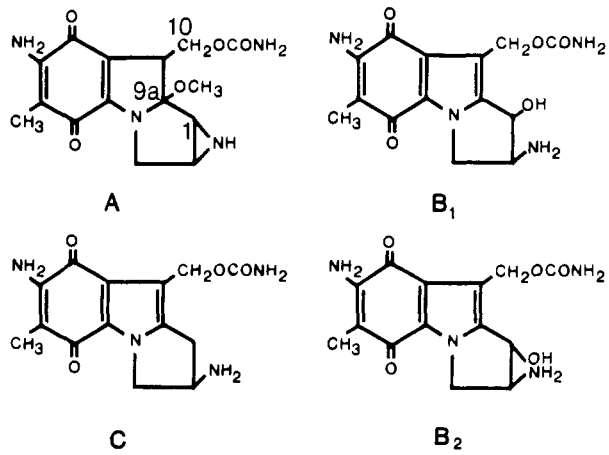


FIGURE 1: Structure of mitomycin C (A) and the acid hydrolysis products, *cis*- and *trans*-2,7-diamino-1-hydroxymitosene (B_1 and B_2) and 2,7-diaminomitosene (C).

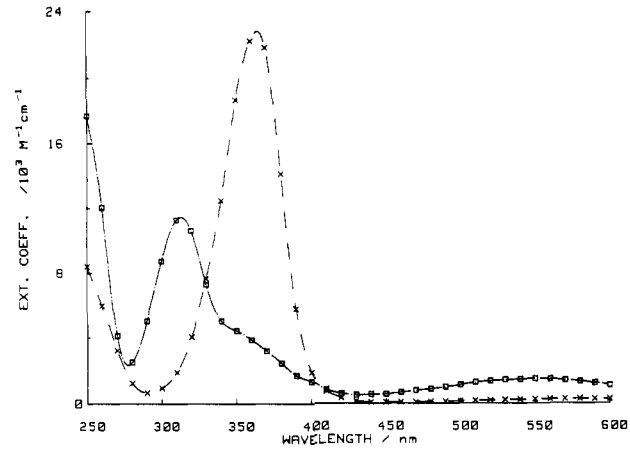


FIGURE 2: Absorption spectra of mitomycin C (A) (×) and mitosenes B_1, B_2, and C (identical spectra) (□).

more widely held view (Pan et al., 1982, 1984; Andrews et al., 1986; Stinson, 1986; Kohn et al., 1987; Egbertson & Danishefsky, 1987) is that the drug is not activated via the two-electron route but rather by a one-electron pathway in which the semiquinone radical undergoes the changes intramolecularly.

Pulse radiolysis has been used in our laboratory to investigate a variety of biochemical intermediates produced by one-electron reactions including the semiquinones of ubiquinone (Land & Swallow, 1970), NAD^+ (Farrington et al., 1980), and adriamycin (Land et al., 1985). In the present work, this technique has been used to study the intermediates in the activation of mitomycin C. Our results imply a mechanism of activation involving the hydroquinone route. Kinetic and spectroscopic information about intermediate steps in the reaction is provided.

Experimental Procedures

Materials. Mitomycin C was obtained from Kyowa Hakka Kogyo Co. Ltd. Bovine milk xanthine oxidase (EC 1.2.3.2) was from Sigma. All other reagents were of the highest grades available from BDH. The *cis*- and *trans*-2,7-diamino-1-hydroxymitosenes were prepared according to literature methods (Stevens et al., 1964) and purified by high-performance liquid chromatography (HPLC). 2,7-Diaminomitosene was prepared enzymically at pH 5 (Pan et al., 1984; Tomasz & Lipman, 1981). The absorption spectra of mitomycin C and *cis*-diamino-1-hydroxymitosene are shown in Figure 2. The spectra of *cis*- and *trans*-2,7-diamino-1-hydroxymitosene are very similar to that of 2,7-diaminomitosene.

Methods. The HPLC apparatus consisted of a Gilson 301 pump and a custom-built mixer and gradient controller. The column was a Techsil 10 ODS (25 × 1.0 cm). Analytical separations were effected by using a linear gradient from 100% system I (10 mM potassium phosphate at pH 7.0) to 100% system II [methanol/10 mM potassium phosphate at pH 7.0 (1:1 v/v)] in 25 min at a flow rate of 4.5 mL/min. A Hewlett-Packard 8451A diode array spectrophotometer served as detector, and absorptions were measured at 310 nm in a 70-μL flow cell.

A Varian TEM Ltd. ^{60}Co source (nominal activity, 8.5×10^{13} Bq) was used for the steady-state irradiations. Pulse radiolysis experiments were conducted at the Paterson Institute linear accelerator facility (Keene, 1964). The pulse lengths were less than 0.1 μs. The optical detection system consisted of a xenon lamp, a Kratos monochromator, and an EMI 9558QA photomultiplier. Capillary cells (3-mm internal diameter) had optical path lengths of 2.5 cm except where stated. Optical difference spectra were normalized to a dose of 10 Gy and 2.5-cm optical length. The signals from the photomultiplier were recorded on a Tektronix 7612D programmable digitizer and analyzed by a Hewlett-Packard 9836s computer.

When a nitrous oxide saturated solution of sodium formate (100 mM), phosphate buffer (10 mM, pH 7.0), and quinone (30 μM–2.0 mM) is irradiated, the following reactions are initiated:

$$H_2O \rightarrow e_{aq}^-, {}^\bullet OH, {}^\bullet H, H_2O_2, H_2 \quad (1)$$

$$e_{aq}^- + N_2O \rightarrow {}^\bullet OH + N_2 + OH^- \quad (2)$$

$${}^\bullet OH({}^\bullet H) + HCO_2^- \rightarrow H_2O(H_2) + CO_2^{\bullet -} \quad (3)$$

$$CO_2^{\bullet -} + Q \rightarrow CO_2 + Q^{\bullet -} \quad (4)$$

The quinone, Q, and the semiquinone, $Q^{\bullet -}$, may have a number of protonated forms. The concentration of $CO_2^{\bullet -}$ radicals produced by the pulse was less than 10% that of the quinone unless otherwise stated. The phosphate concentration was maintained at 10 mM to avoid the formation of mitosene phosphates (Tomasz & Lipman, 1981; Pan et al., 1984). The temperature was 21 ± 2 °C throughout. Solutions containing more than 100 μM mitomycin C were freed from sodium chloride (240 mg/10 mg of mitomycin C in the commercial preparation) by dissolving the material in acetone, centrifuging to remove the sodium chloride, and then vacuum distilling to remove the acetone.

Results

Formation of Products. The reduction of mitomycin C (1.5 mM) by hypoxanthine/xanthine oxidase (40 μL, 0.1 unit) at pH 7 was observed by the change in absorption spectrum in the range 420–700 nm. Reduction products were analyzed by HPLC. The results were essentially the same as those previously reported (Pan et al., 1984) except that mitosene phosphates were not found at the low phosphate concentration employed and that since the reduction was stopped when only 10% of the original concentration of mitomycin C is converted, the enzyme reduces only mitomycin C and not the subsequent metabolites. Consequently, only three main products were observed. The positions of the peaks of the HPLC profiles and the absorption spectra of the species at these peaks were identical with those of authentic *cis*- and *trans*-2,7-diamino-1-hydroxymitosenes (B_1 and B_2, respectively) and 2,7-diaminomitosene (C). Reduction by $CO_2^{\bullet -}$ radicals produced by a pulse of radiation (150 Gy) gave the same products in

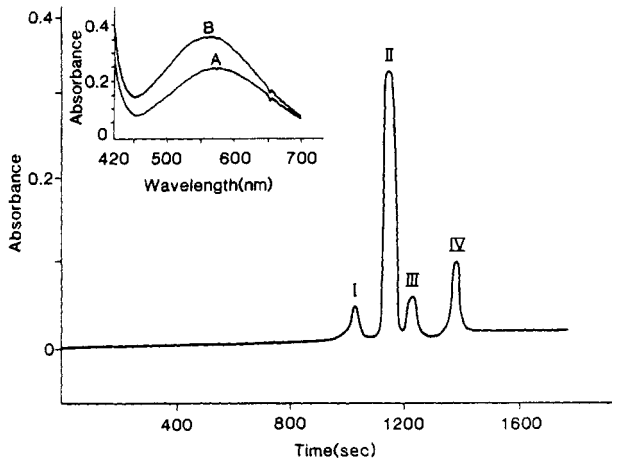


FIGURE 3: HPLC analysis of the products from the reduction of mitomycin C by $CO_2^{\bullet-}$ radicals. The HPLC profiles, recorded at 310 nm, were ascribed as I = B_2, II = mitomycin C, III = B_1, and IV = C. The irradiated solution consisted of N_2O-saturated mitomycin C (1.0 mM) containing sodium formate (0.1 M) and sodium phosphate (0.01 M, pH 7.0). (Inset) Optical absorption of the solution before (A) and after (B) irradiation.

the same proportion (Figure 3).

To ascertain whether the intramolecular changes occur through the semiquinone or the hydroquinone, the radiation dose rate was varied. At the lowest dose rates, it is known that mutual reaction of the semiquinone radicals would be diminished in rate, giving the best possible conditions for any intramolecular process to occur. At the highest dose rates, the radicals would be more likely to disproportionate to the hydroquinone before they could decompose. At pH 7 the same semiquantitative yield of mitosenes was obtained when equal concentrations of $CO_2^{\bullet-}$ radicals were produced at rates of production varying by 11 orders of magnitude, i.e., from 0.32 nM s^{-1} (^{60}Co irradiation) to 30 M s^{-1} (linear accelerator irradiation). On the other hand, the concentrations of mitosenes formed were found to depend strongly on pH as had been found previously using the xanthine oxidase system (Pan et al., 1984). By use of γ-irradiation to produce $CO_2^{\bullet-}$, the ratio of $(B_1 + B_2)/C$ was found to be <0.4 at pH 5.7, 1.0 at pH 7, and >3.5 at pH 9.1. The stoichiometry at pH 5.7 corresponded approximately to the formation of one mitosene molecule ($B_1 + B_2 + C$) for every two $CO_2^{\bullet-}$ radicals introduced into the solution. Mitosenes were formed in higher yields at pH 7, while at pH 9.1 more than eight mitosene molecules were formed per two $CO_2^{\bullet-}$. It was not practical to examine solutions below pH 5 or above pH 9 because of hydrolysis of mitomycin C (Pan et al., 1984; Stevens et al., 1964).

Reaction Kinetics. Pulse radiolysis was used to produce the semiquinone radicals of mitomycin C. After a few tens of microseconds, the net result of delivering the pulse will be to convert some parent into semiquinone. The difference spectrum [OD(semiquinone) − OD(quinone)] produced 88 μs after the pulse at pH 7 is shown in Figure 4. Since the parent has little absorption in the visible region, it is clear that the peak at 510 nm is entirely due to formation of the semiquinone radical.

Without necessarily implying the state of protonation of the radicals or the hydroquinone, the dismutation of the semiquinone may be represented by

$$2AH^{\bullet} \rightarrow AH_2 + A \qquad (5)$$

The rate constant for reactions 5 at pH 7.0 was determined by giving various doses of radiation (producing concentrations

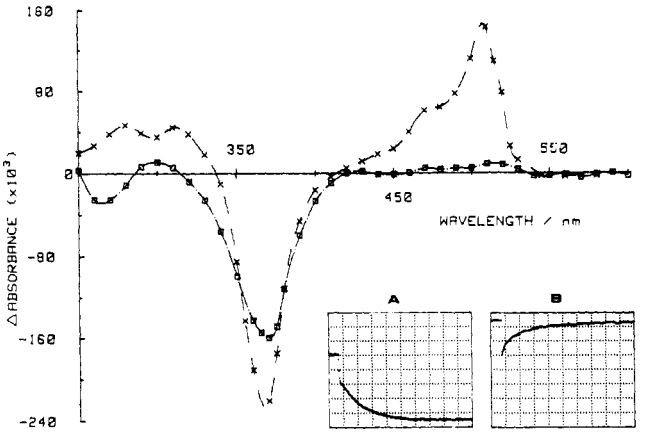


FIGURE 4: Optical difference spectra produced by the reduction of mitomycin C (30 μM) by $CO_2^{\bullet-}$. Solution conditions are given under Experimental Procedures: (×) 88 μs after pulse; (□) 92 ms after pulse. (Inset A) Time scale = 15 μs/division; sensitivity = 100 mV/division. (Inset B) Time scale = 10 ms/division; sensitivity = 100 mV/division. The wavelength observed was 510 nm in each case.

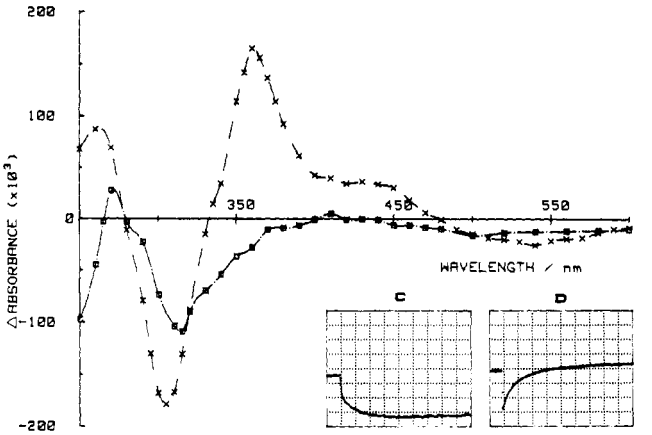


FIGURE 5: Optical difference spectra produced by the reduction of metabolite B (30 μM) by $CO_2^{\bullet-}$: (×) 45 μs after pulse; (□) 8.92 ms after pulse. (Inset C) Time scale = 10 μs/division; sensitivity = 100 mV/division. (Inset D) Time scale = 1 ms/division; sensitivity = 100 mV/division. The wavelength observed was 360 nm in each case.

of $CO_2^{\bullet-}$ from 5 to 25 μM) to solutions of mitomycin C (50–250 μM). The rate constant for the decay at this pH was found to be $2k = (5.1 \pm 1) \times 10^7$ M^{-1} s^{-1}, independent of dose and mitomycin C concentration. After reaction 5 is essentially complete, the net result is that some parent has been converted to hydroquinone. The difference between the spectrum of the hydroquinone and that of the parent, measured at 92 ms after the pulse, is also shown in Figure 4. Typical traces showing semiquinone formation and dismutation are shown as insets in Figure 4.

Admission of oxygen between 0.1 and 1 s after the formation of the hydroquinone (pH 7) caused the absorption spectrum to revert to that of the unirradiated solution. This shows that the semiquinone had not undergone any rearrangement before dismuting and that the rate constant for any conversion of the hydroquinone into C or the hydroquinone of B_1 and/or B_2 must be less than 1 s^{-1}.

A pulse radiolysis study was undertaken for the individual *cis*- and *trans*-2,7-diamino-1-hydroxymitosenes (B_1 and B_2). Numerous experiments showed no difference between the isomers in the kinetics and spectra obtained. The difference in spectrum obtained by conversion of B into its semiquinone ($BH^{\bullet}$) at pH 7 is shown in Figure 5. $BH^{\bullet}$ has a peak at 370 nm and is evidently quite different from $AH^{\bullet}$ (Figure 4). This difference emphasizes that the semiquinone radicals of mito-

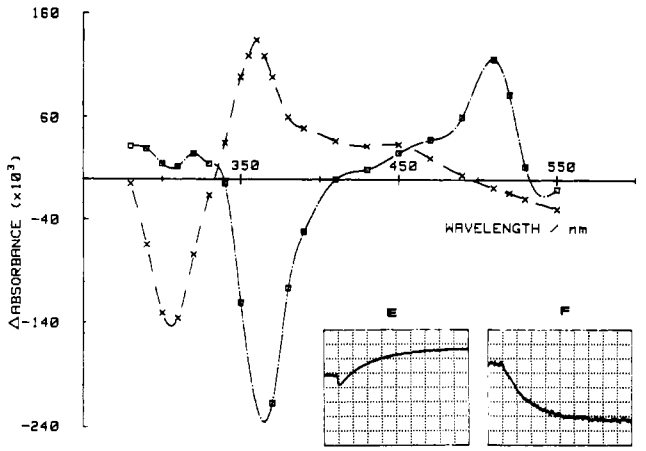


FIGURE 6: Optical difference spectra produced by the reduction of metabolite B (200 μM) by $CO_2^{\bullet-}$ in the presence of mitomycin C (20 μM): (×) 4.5 μs after pulse; (□) 225 μs after pulse. (Inset E) Wavelength = 370 nm; time scale = 25 μs/division; sensitivity = 200 mV/division. (Inset F) Wavelength = 510 nm; time scale = 25 μs/division; sensitivity = 50 mV/division.

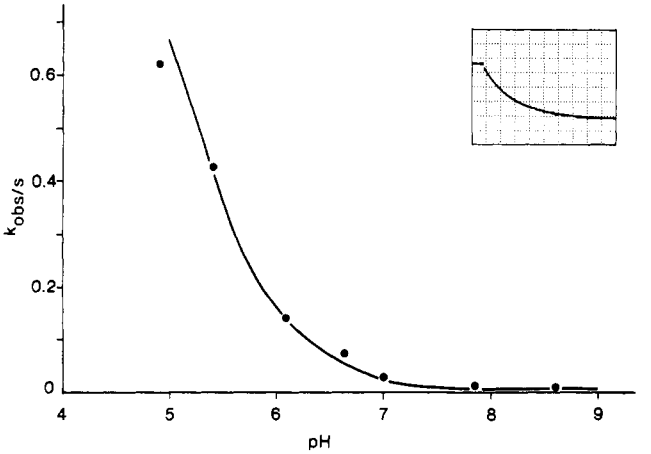


FIGURE 7: pH dependence of the decay of the hydroquinone of mitomycin C (30 μM). The radiation dose was equivalent to [$CO_2^{\bullet-}$] 200 μM. (Inset) Wavelength = 310 nm; time scale = 1 s/division; sensitivity = 500 mV/division; pH 5.72. The curve is calculated from eq 11.

mycin C have not rearranged during our experiments. The rate constant for the decay of the semiquinone radicals, represented formally as

$$2BH^{\bullet} \rightarrow BH_2 + B \qquad (6)$$

was found to be $2k = (4.2 \pm 0.8) \times 10^8$ M^{-1} s^{-1} (pH 7), independent of dose and hydroxymitosene concentration. The difference between the spectrum of the hydroquinone of hydroxymitosene and that of the parent is also shown in Figure 5. Comparison with the corresponding difference spectra for mitomycin C (Figure 4) shows some dissimilarities in the region below 400 nm. Insets in Figure 5 show the rates of formation and dismutation of the semiquinone radicals.

When single pulses were given to solutions at pH 7 containing high concentrations of hydroxymitosene (200 μM) and low concentrations of mitomycin C (20 and 50 μM) it was observed that the semiquinone radicals of the hydroxymitosene can efficiently reduce mitomycin C.

$$BH^{\bullet} + A \rightarrow B + AH^{\bullet} \qquad (7)$$

$$k = (7.2 \pm 0.7) \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$$

Changes in absorbance produced by the conversion of B into $BH^{\bullet}$ (4.5 μs) and by the subsequent reaction 7 (225 μs) are shown in Figure 6. Typical traces are shown in the insets. Reaction 7 is consistent with the one-electron-reduction potentials of mitomycin C, which has been measured as −310 mV (Butler & Hoey, 1986), and hydroxymitosene, −420 mV (Butler and Hoey, unpublished experiments).

The hydroquinone of mitomycin C was observed to undergo further reactions over a period of tens of seconds after the pulse (see inset to Figure 7). The absorption changes over these time scales were consistent with the formation of mitosene quinones as found after enzymic reduction. The reactions of the hydroquinone of mitomycin C in the presence of minimal amounts of mitomycin C itself were studied by producing the hydroquinone with single large pulses of radiation ([$CO_2^{\bullet-}$] = 200 μM) delivered to N_2O-saturated solutions of mitomycin C (30 μM), sodium formate (0.1 M), and phosphate buffer (10 mM) at pH values in the range 4.9–8.6. At pH 5.2, where the reaction was relatively fast, the absorption spectrum of the pulsed solution in the region 270–570 nm was found to change steadily over about 10 s from that of the hydroquinone to that of the products, without any observable intermediate. The same first-order kinetics were seen at every wavelength. A typical example of the kinetics is shown in the inset of Figure 7. In order to study the pH dependence of the reaction, observations were made over periods of as much as 100 s at 310 nm, where it can be seen from Figure 4 that the hydroquinone of mitomycin C has little absorption. At this wavelength, the mitosenes have an extinction coefficient of 11 300 M^{-1} cm^{-1}, and it can be calculated from Figure 5 that the hydroquinone of B has an extinction coefficient of ~5000 M^{-1} cm^{-1}. Absorptions at 310 nm were found to build up after the pulse in a first-order manner at a rate that depended on pH as shown in Figure 7. This pH dependence can be explained by the following scheme, in which the hydroquinone changes directly to product, the protonated (AH_3^+) form of the hydroquinone yielding C while the unprotonated (AH_2) form yields BH_2.

$$AH_3^+ \rightleftharpoons AH_2 + H^+ \qquad (8)$$

$$AH_3^+ \rightarrow C \qquad (9)$$

$$AH_2 \rightarrow BH_2 \qquad (10)$$

The pK_a of the hydroquinone (eq 8, equilibrium constant K_8) and the rate constants for reactions 9 and 10 were derived from a least-squares analysis of the results in Figure 7 by using the formula

$$k_{obsd} = (k_9[H^+] + k_{10}K_8)/(K_8 + [H^+]) \qquad (11)$$

The values found were $pK_a = 5.1 + 0.15$, $k_9 = 1.2 \pm 0.2$ s^{-1}, and $k_{10} = 0.015 \pm 0.005$ s^{-1}. The solid line in Figure 7 is calculated from these values.

Under the experimental conditions used for Figure 7, the absorption changes observed at pH 9 were small and produced very slowly. However, larger and more rapid changes were observed at this pH when relatively high concentrations of mitomycin C remained after reduction. Typical traces at 550 nm, where mitomycin C and its hydroquinone have little absorption but the mitosenes have a broad peak, are shown in Figure 8. These observations were attributed to reaction of the hydroquinone form of 2,7-diamino-1-hydroxymitosene (BH_2, from reaction 10) with the excess of mitomycin C (A):

$$BH_2 + A \rightarrow B + AH_2 \qquad (12)$$

The mitomycin C hydroquinone, which will be little protonated at this pH, then reacts according to reaction 10, thus producing a chain reaction. The chain is terminated by reaction 9 via reaction 8. Reaction 12 would also occur at lower pH values but would tend to be masked by reaction 9 The

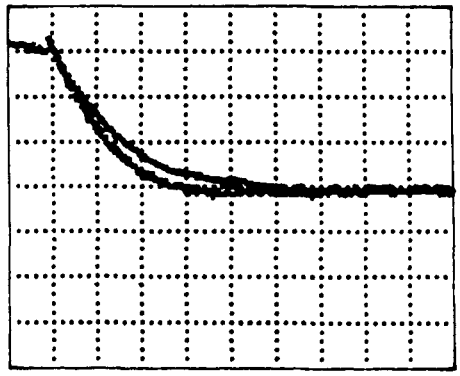


FIGURE 8: Typical traces for the production of mitosenes at high mitomycin C concentration. The radiation dose was equivalent to $[CO_2^{\bullet-}]$ 30 μM. (Upper trace) Wavelength = 550 nm; time scale = 35 s/division; sensitivity = 200 mV/division; pH 9.0; [mitomycin C] = 0.5 mM. (Lower trace) As above but [mitomycin C] = 2.0 mM.

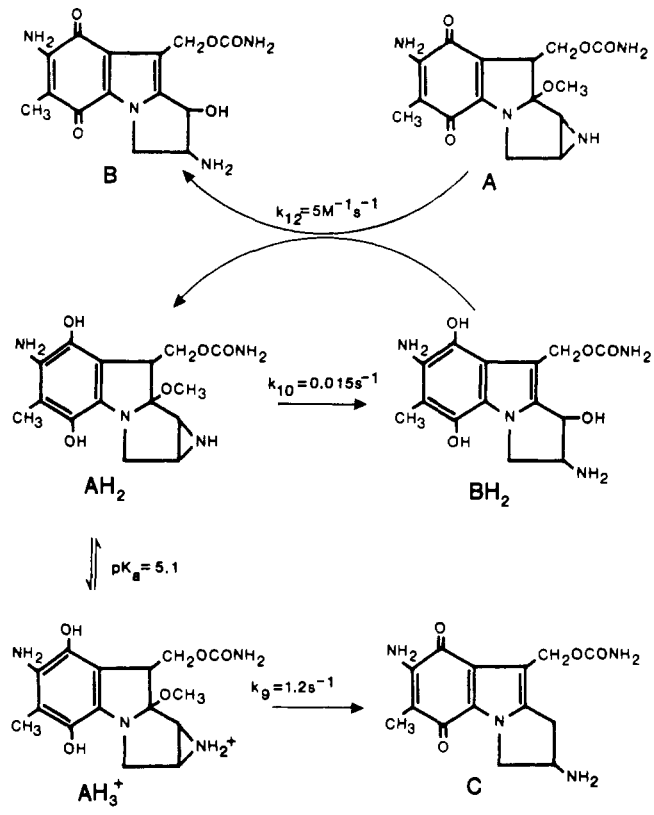
rate constant for reaction 12 was obtained by a computer-simulated Runge–Kutta–Merson method. This involved estimating the variations in the 550-nm absorption with time for different concentrations of mitomycin C (0.2–2.5 mM) at pH 9, using the estimated pK_a of reaction 8, the rate constants for reactions 9 and 10, and selected values for the rate of reaction 12. A good correspondence between the observed and calculated rate constants was obtained with $k_{12} = 5 \pm 2$ M^{-1} s^{-1}.

DISCUSSION

Although radiolytic reduction of mitomycin C takes place by an unambiguous one-electron route, our results show that the loss of methanol and opening of the aziridine ring do not take place until after the semiquinone radicals have dismuted to yield the hydroquinone. It is true that the ring aromatization that promotes the changes could in principle take place at the semiquinone stage (Pan et al., 1984), but our experiments indicate that the aromatization is too slow to take place before the radicals disappear. When the reduction is carried out enzymically, the rate-determining step will be the addition of the first electron; rapid dismutation ($2k = 5.1 \times 10^7$ M^{-1} s^{-1}) will lead to linear enzyme kinetics that are first order with respect to mitomycin C concentration but still consistent with activation via the hydroquinone. Another argument in favor of changes occurring at the semiquinone stage is that electrochemical reduction of mitomycin C in dimethyl sulfoxide, a nonphysiologically relevant solvent, to the radical anion resulted in the formation of about nine products when water was added. These products resembled those that could be formed from extensive enzyme reduction. In contrast, reduction anaerobically to the dianion resulted in the formation of only two products, one which was not formed by the enzyme (Andrews et al., 1986). However, no mitomycin C remained in the solutions that had been reduced to the dianion, indicating such a high degree of reduction that the initial products would have been further reduced, leading to increasingly degraded mitosenes. Thus, the products obtained on reduction to the radical anion differed from those on reduction to the dianion because of excessive reduction in the latter case. Had the degree of reduction been the same, the radical anion and the dianion would have given the same products.

Our mechanism for the formation of products B and C from the hydroquinone of mitomycin C is summarized in Scheme I. It is central to our scheme that the aziridine ring of the hydroquinone protonates with p$K_a \approx 5.1$. The pK_a of the aziridine ring in mitomycin C itself has been measured as 3.2

Scheme I



(Stevens et al., 1964) and 2.8 (McClelland & Lam, 1985), which represents the lowest pK_a recorded for an aziridine (Dermer et al., 1969). The low pK_a has been attributed to the electron-withdrawing groups present in mitomycin C and also to the aziridine nitrogen being situated above the plane of the ring structure where it could be influenced by the neighboring carbamate moiety at carbon 10 and by the more distant electron-poor quinone system. When the quinone is reduced to the hydroquinone, the electron deficiency is removed. Thus, it is reasonable that the pK_a of the aziridine ring should increase to 5.1. It is the AH_3^+ form that is kinetically active. Our experiments provide no evidence for a quinone methide as an intermediate between AH_3^+ and C (Tomasz & Lipman, 1981; Peterson & Fisher, 1986). If such an intermediate is formed, its rate of disappearance must be faster than its rate of formation.

The chain reaction represented by reactions 10 and 12 is similar to the autocatalytic pathway proposed in a recent study in which Old Yellow enzyme and NADPH had been used to effect the reduction (Peterson & Fisher, 1986). Considering the difference in the experiments, the rate constants for the bimolecular step, 5 ± 2 M^{-1} s^{-1} (this work) and 12 M^{-1} s^{-1} (Peterson & Fisher, 1986), are in good agreement. The internal oxidation–reduction of the mitomycin C hydroquinone to 2,7-diaminomitosene (reaction 9) has been suggested previously (Tomasz & Lipman, 1981) and, indeed, the pH dependence of the formation and yield of this reaction as found from catalyic hydrogenation are consistent with Scheme I.

A recent publication (Egbertson & Danishefsky, 1987) has shown that the products from the reduction of *N*-methylmitomycin A, by dithionite in aqueous pyridine, are formed in greater than stoichiometric yields. These results can be explained by the chain reaction of a hydroquinone with the *N*-methylmitomycin A quinone.

Although Scheme I represents the activation of mitomycin C coming about through the hydroquinone, reaction 12 could,

in principle, go via the semiquinone:

$$BH_2 + B \rightleftharpoons 2BH^{\bullet} \quad (13)$$

$$2BH^{\bullet} + 2A \rightarrow 2B + 2AH^{\bullet} \quad (14)$$

$$2AH^{\bullet} \rightleftharpoons AH_2 + A \quad (15)$$

$$\text{sum: } BH_2 + A \rightarrow B + AH_2 \quad (12)$$

Reactions 13, 14, and 15 are essentially similar to reactions 6, 7, and 5, respectively. The high rate constant for reactions 5 and 6 and the independence of the rate constants on quinone concentrations show that the equilibria represented by (15) and (13) are strongly in favor of dismutation. However, corresponding equilibria have been demonstrated for adriamycin (Land et al., 1985). The driving force for the overall reaction [(12)] is the fast rate of reaction 7 or 14 and the fast dismutation of $AH^{\bullet}$. The overall slowness of the reaction could be due to the direction of the equilibrium [(13)].

Biological Consequences. The cytotoxicity of mitomycin C, which is brought about as a consequence of bioreduction, must depend on the available oxygen and on the pH of the environment. We have previously demonstrated that the semiquinone radicals of mitomycin C react rapidly, $k = (2.2 \pm 0.2) \times 10^8\ M^{-1}\ s^{-1}$ (Butler et al., 1985), with oxygen to form the superoxide radical, which can lead to a variety of forms of damage to biological components (Gutteridge et al., 1985, and references cited therein). It is therefore possible that some of the killing of aerobic cells by mitomycin C could be due to superoxide formation. Furthermore, in view of the high rates of reaction of the semiquinone and hydroquinone of mitomycin C with oxygen and the slowness of reductive activation, we propose that activation of mitomycin C would only occur to any great extent when redox cycling of the drug and oxygen had significantly reduced the oxygen tension.

One of the main conclusions from the work is that activation of mitomycin C comes about from the hydroquinone. However, at physiological pH, hydroquinone can readily be formed from the dismutation of semiquinones in the absence of oxygen. Therefore, previous attempts to specifically generate one-electron reduction of mitomycin C in vitro (Pan et al., 1984; Tomasz & Lipman, 1981) or to inhibit two-electron reduction in cell cultures (Keyes et al., 1984, 1985) should be reevaluated in light of the interrelationship between semiquinones and hydroquinones.

Cross-linking in EMT6 tumor cells by mitomycin C has shown to be strongly pH dependent and increases on going from pH 7.5 to 5.7 (Kennedy et al., 1985). The results presented here would therefore suggest that the precursor could be the protonated hydroquinone and that competition could exist between the production of 2,7-diaminomitosenes and the DNA adduct. A similar conclusion can be reached by considering the work wherein it has been shown that one of the main products from reduction of mitomycin C by catalytic hydrogenation is the 2,7-diaminomitosene (Tomasz & Lipman, 1981). This effect has been attributed either to changes in enzyme activity or to acid-catalyzed decomposition of mitomycin C. However, the results presented here would suggest that the precursor to cross-linking could be the protonated hydroquinone, which is strongly favored at acid pH. It could therefore be proposed that the carbon-1 position of the hydroquinone could react with the N^2 position of guanine if produced in close proximity to DNA or alternatively with a solvent proton to produce C. The structures of the known covalent cross-link adducts between mitomycin C and DNA would not discount this proposal (Tomasz et al., 1987).

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Registry No. B_1, 54911-19-2; B_2, 54911-18-1; C, 78598-43-3; AH_2, 92056-69-4; BH_2, 102633-17-0; AH_3^+, 113033-35-5; mitomycin C, 50-07-7.

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Characterization and Chemical Properties of Phosphoribosylamine, an Unstable Intermediate in the de Novo Purine Biosynthetic Pathway†

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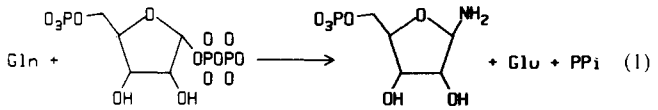
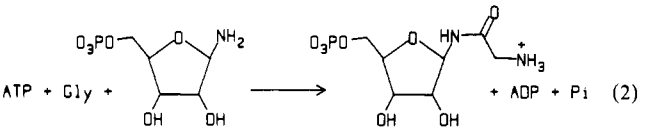
ABSTRACT: Incubation of [1-^{13}C]-5-phosphoribosyl pyrophosphate ([1-^{13}C]PRPP) and glutamine with PRPP amidotransferase results in rapid production and disappearance of two new resonances at 89.3 and 85.9 ppm. These resonances coincide with two of the products produced upon incubation of [1-^{13}C]ribose 5-phosphate with NH_3. Extensive NMR studies (^{15}N and ^{1}H–^{13}C chemical shift correlation spectra) have allowed assignment of these resonances to β- and α-phosphoribosylamine. These studies represent the first spectral observations of this chemically reactive intermediate. The rate of interconversion of α- to β-phosphoribosylamine as a function of pH has been determined by saturation and inversion-transfer NMR methods. The rate of formation of 5-phosphoribosylamine (PRA) from ribose 5-phosphate and NH_3 and its rate of decomposition as a function of pH have been determined with a glycinamide ribonucleotide synthetase trapping system fashioned after earlier studies of Nierlich and Magasanik [Nierlich, D. P., & Magasanik, B. (1965) *J. Biol. Chem. 240*, 366]. Phosphoribosylamine has a $t_{1/2}$ = 38 s at 37 °C and pH 7.5. The pH-independent equilibrium constant for ribose 5-phosphate and NH_3 with phosphoribosylamine has been established, 2.5 M^{-1}, by use of these rate constants as well as by NMR methods. This equilibrium constant and the rates of nonenzymatic interconversion of α- and β-PRA provide essential background for studying the mechanism of glycinamide ribonucleotide synthetase and investigating the possibility of channeling phosphoribosylamine between this enzyme and the first enzyme in the purine pathway.

The first committed step in the purine biosynthetic pathway involves the conversion of 5-phosphoribosyl pyrophosphate (PRPP)[1] and glutamine to 5-phosphoribosylamine (PRA), glutamate, and pyrophosphate by PRPP amidotransferase (eq 1). Although PRA is widely accepted to be the product of



(1)

this amidotransferase, it has never been directly isolated and characterized, presumably due to its chemical instability. The evidence which strongly suggests its existence is based on the trapping of PRA, produced both enzymatically and chemically from the reaction of ribose 5-phosphate (R-5-P) and NH_3, by GAR synthetase. This trapping involves the second enzyme in the purine pathway, GAR synthetase, and its substrates, glycine and ATP (Goldthwait, 1956) (eq 2). This reaction



(2)

produces glycinamide ribonucleotide (GAR), which is stable, is isolable, and has been well characterized (Hartman et al., 1956; Peabody et al., 1956; Chettur & Benkovic, 1977). In addition to the fact that PRA had only been characterized indirectly at the time these studies were undertaken, the "quantitative" stability of PRA was also controversial. In 1956, Goldthwait, measuring NH_3 release as a criterion for PRA decomposition, concluded that between pH 7 and pH 8 only

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[1] Abbreviations: PRPP, 5-phosphoribosyl pyrophosphate; PRA, 5-phosphoribosylamine; GAR, glycinamide ribonucleotide; R-5-P, ribose 5-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; FID, free induction decay.

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