Mechanism of Deoxyribonucleic Acid Breakage Induced by 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide and Copper: Role for Cuprous Ion and Oxygen Free Radicals†

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ABSTRACT: 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (mAMSA) interacts with Cu(II) ion, as indicated by changes in the mAMSA absorption spectrum induced by Cu(II). The spectral changes are due to the oxidation of mAMSA by Cu(II), resulting in an oxidized mAMSA product and Cu(I). Two lines of evidence for the oxidation of mAMSA are as follows: (1) The spectral changes induced by manganese oxide, an oxidizing agent, were similar to those induced by Cu(II), and (2) the Cu(II)-induced spectral changes were reversed by a reducing agent, NADPH. Thin-layer chromatographic studies showed the oxidized mAMSA product to be N^1-methylsulfonyl-N^4-(9-acridinyl)-3-methoxy-2,5-cyclohexadiene-1,4-diimine (mAQDI). The involvement of Cu(I) in the reaction was demonstrated by the use of two Cu(I)-specific chelating agents, neocuproine and bathocuproine. Neocuproine or bathocuproine chelated the Cu(I) ions in the mixture, producing Cu(neocuproine)$_2^+$ complex or Cu(bathocuproine)$_2^+$ complex. The stoichiometry of mAMSA–Cu(II) interactions was determined by titrating the mAMSA–Cu(II) mixtures with bathocuproine. Job plots of the absorbance at 480 nm showed a clear end point at a Cu(II)/mAMSA ratio of 1.5/1, indicating that 1.5 equiv of Cu(II) reacts with 1 equiv of mAMSA to produce 1.5 equiv of Cu(I). Cu(I) plays an important role in the mAMSA–Cu(II)-induced DNA breakage, since in the presence of neocuproine the DNA breakage is inhibited. Up to 200 μM, Cu(I) by itself is virtually ineffective, in contrast to the mixture of mAMSA and Cu(II). This suggests that mAMSA, aside from reducing Cu(II) to Cu(I), may play a role in mediating DNA breakage. The DNA breakage was reduced in partially anaerobic conditions, indicating the involvement of molecular oxygen. Catalase and 4,5-dihydroxy-1,2-benzenedisulfonate inhibited the DNA breakage completely, indicating that hydrogen peroxide and superoxide radicals mediate DNA breakage. 1,3-Diazabicyclo[2.2.2]octane partially inhibited the breakage, suggesting that singlet oxygen is involved. DNA breakage was not inhibited by potassium iodide or mannitol, indicating that hydroxyl radicals are not involved. The available evidence supports a mechanism indicating the formation of a DNA·mAMSA·Cu(II) ternary complex and the subsequent oxidation–reduction of the complex to form DNA·mAQDI·Cu(I). The oxidation of the complexed Cu(I) may result in reduction of oxygen to oxygen free radicals which induce DNA breaks.

4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (mAMSA)[1] has been shown to have antitumor activity in animals and man (Cain & Atwell, 1974; Cain et al., 1974; Wilson et al., 1973; Issell, 1980; Legha et al., 1980; Benjamin et al., 1980; Rivera et al., 1980). Because of the promising antitumor activity of mAMSA and its potential clinical usefulness, biochemical studies have been initiated to define the mechanism of action of the drug. The mechanism by which the drug exhibits its antineoplastic activities is not understood, but its strong DNA-intercalating properties suggest that the drug receptor site may be DNA (Waring, 1976). We have shown that mAMSA is capable of degrading pDPT275 plasmid DNA in the presence of Cu(II) ion (Wong et al., 1984), whereas its noncytotoxic isomer, oAMSA, is less effective. This suggests that the drug–Cu(II)-induced DNA-breakage activity may correlate with their cytotoxic activities. The realization that a metal ion [Cu(II)] may play a central role in the mechanism of mAMSA-induced DNA breakage and that Cu(II) interacts with mAMSA and induces characteristic spectral changes has stimulated our interest in studying the nature of mAMSA–Cu(II) interactions and their relationship to their DNA-breaking activities.

A number of antitumor drugs, e.g., bleomycin (Sausville et al., 1976) and adriamycin (Someya & Tanaka, 1979), and 1,10-phenanthroline (Sigma et al., 1979) are able to form coordination complexes with transition metal ions. The metal interactions with bleomycin and 1,10-phenanthroline have been studied extensively. Both drugs bind to and break DNA (Haidle, 1971; Strong & Crooke, 1978; Huang et al., 1980, 1981; Mirabelli et al., 1979, 1980; Marshall et al., 1981). Their DNA-degrading reaction exhibits an oxygen requirement (Sausville et al., 1976, 1978a,b; Sigman et al., 1979) and can be terminated by metal chelating agents such as EDTA. This indicates that both the metal ions and molecular oxygen are involved in the DNA breakage (Sausville et al., 1976; Que et al., 1980). The available evidence supports the concept that bleomycin exhibits its DNA-cutting activity through the

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[1] Abbreviations: bathocuproine, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline; DABCO, 1,3-diazabicyclo[2.2.2]octane; EDTA, ethylenediaminetetraacetic acid; EB, ethidium bromide; KI, potassium iodide; mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; mAQDI, N^1-methylsulfonyl-N^4-(9-acridinyl)-3-methoxy-2,5-cyclohexadiene-1,4-diimine; $O_2^{\cdot-}$, superoxide free radicals; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; SOD, superoxide dismutase; Tiron, 4,5-dihydroxy-1,2-benzenedisulfonic acid disodium salt; neocuproine, 2,9-dimethyl-1,10-phenanthroline; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; Me_2SO, dimethyl sulfoxide.

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formation of a complex of DNA·bleomycin·chelated Fe(II)·molecular oxygen. Oxidation of the bound Fe(II) ion occurs in this complex with concomitant reduction of oxygen to produce oxygen free radicals (Povirk et al., 1979; Huang et al., 1980; Suguira, 1980). Studies on the DNA cleavage induced by 1,10-phenanthroline in the presence of a reducing agent suggest that this drug complexes with Cu(II) and the $(1,10\text{-phenanthroline})_2Cu^+$ complex may bind to DNA. Hydroxyl free radicals are generated in the vicinity of susceptible bonds, resulting in DNA breakage (Que et al., 1980).

In the present studies, we demonstrate that (1) during the course of mAMSA–Cu(II) interaction, Cu(I) and mAQDI (an oxidized mAMSA product) are produced, (2) partial removal of molecular oxygen reduces the DNA breakage induced by mAMSA and Cu(II), and (3) singlet oxygen, hydrogen peroxide, and superoxide free radicals may be involved in DNA cutting. These results suggest that Cu(I), mAMSA, and oxygen free radicals may play a role in DNA breakage. Therefore, the DNA breakage induced by mAMSA and Cu(II) can be compared to that induced by bleomycin or 1,10-phenanthroline. mAMSA may be one of a class of molecules that induces DNA breakage in vitro by facilitating the redox reaction of a metal ion close to a site where damage to DNA may readily occur.

Materials and Methods

Materials

mAMSA was supplied by Bristol Laboratories, Syracuse, NY. The mAMSA solution was prepared by dissolving the drug powder in dimethyl sulfoxide (3 mg/mL) and diluting with distilled water to 1 mg/mL. The final concentration of dimethyl sulfoxide in the DNA reaction mixture was 2–3%. pDPT275 plasmid is a second step copy number mutant derived from R100(NR1) and the pDPT275 DNA was isolated according to the procedure of Clewell & Helinski (1970). Bathocuproinedisulfonate, catalase, $CuSO_4 \cdot 5H_2O$, dimethyl sulfoxide, Na_2EDTA, ethidium bromide, KI, mannitol, NADPH, neocuproine hydrochloride, Tiron, and Tris were obtained from Sigma Chemical Co., St. Louis, MO. Agarose-ME was obtained from Bethesda Research Laboratories, Gaithersburg, MD. Active grade manganese(IV) oxide was obtained from Aldrich Chemcial Co., Milwaukee, WI. Bovine erythrocyte superoxide dismutase was from Miles Laboratories, Elkhart, IN. CuCl and silica gel IB2F plates (20 × 20 cm, 0.1 mm) were purchased from J. T. Baker Chemical Co., Philipsburg, NJ.

Methods

Gel electrophoretic and spectrophotometric assays have been described in the preceding paper (Wong et al., 1984).

Reaction of mAMSA with pDPT275 DNA. The reaction mixture (60 μL) contained 10 mM Tris-HCl (pH 7.5), 0.74 μg of pDPT275 DNA, and varying amounts of mAMSA and Cu(II), as described in the preceding paper (Wong et al., 1984). In some studies, various amounts of neocuproine or different free radical scavengers were added into the Cu(II)-containing buffer solutions before adding the mAMSA and DNA. To perform studies under lower oxygen partial pressure, nitrogen was bubbled through the assay mixtures containing the complete system [mAMSA + Cu(II) + DNA in Tris buffer] for 1.5 min. The test tubes (Eppendorf microtubes) were then filled with nitrogen and quickly sealed with stoppers. The Tris buffer and $CuSO_4$ solution were purged with nitrogen for 15 min before use.

Thin-Layer Chromatography. Analytical silica gel IB2-F plates (20 × 20 cm, 0.1 mm thickness) were used for TLC studies. Aliquots of 10 μL of the mAMSA–Cu(II) mixture (300 μM/600 μM) or mAMSA–manganese oxide mixture (300 μM/0.01%) were incubated for 30 min and spotted. The plates were developed with 1-butanol plus acetic acid plus water, 4:1:1. After chromatography, the separated migration bands were scraped, extracted with absolute methanol (0.5 mL), and centrifuged in a microfuge for 2 min to remove insoluble residue. An aliquot of 300 μL of the clear supernatant was then mixed with 700 μL of Tris-HCl buffer (10 mM, pH 7.5) and subjected to spectrophotometric measurement.

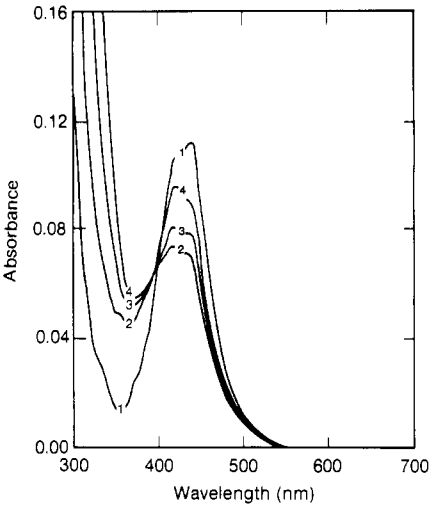

FIGURE 1: Effect of NADPH on the absorption spectrum of mAMSA + Cu(II). mAMSA (10 μM) was incubated with Cu(II) (100 μM) in 1 mL of Tris-HCl buffer (10 mM, pH 7.5). Absorption spectra were recorded at 0 time (1) and at 3 h (2) of incubation. Then 0.3 (3) or 0.5 mM (4) NADPH was added to the reaction mixture which had been treated with Cu(II) for 3 h. Spectra were obtained 25 min after the addition of NADPH.

Stoichiometric Titration of Cu(I) Production. The concentrations of Cu(I) produced in the mAMSA–Cu(II) reaction mixtures were determined by titrating with bathocuproine as follows: mAMSA (5 or 10 μM in 10 mM Tris-HCl buffer, pH 7.5) was mixed with varying amounts of Cu(II), and 10 μL of 10 mM stock bathocuproine aqueous solutions was then added to attain a final bathocuproine concentration of 100 μM. The samples were subjected to spectrophotometric measurements, as described previously (Wong et al., 1984). The absorbance at 480 nm was recorded.

Results

Effects of NADPH on the Cu(II)-Induced mAMSA Spectral Changes. We have demonstrated that mAMSA interacts with Cu(II) by using absorption spectroscopy (Wong et al., 1984). Addition of Cu(II) ion resulted in a decrease in the absorption above 400 nm and a simultaneous increase below 400 nm. In the present studies, the chemical nature of these absorption spectral changes has been explored. Figure 1 shows that when mAMSA (10 μM) was incubated with 100 μM Cu(II) for 3 h, characteristic mAMSA spectral changes similar to those reported previously were obtained (Wong et al., 1984). Subsequent addition of 0.3 mM NADPH induced an immediate slight increase in absorption at 435 and 420 nm (A_{435} and A_{420}), accompanied by a decrease in A_{330} (not shown). The spectral changes increased gradually with increasing time of incubation. Maximal spectral changes occurred at 25 min after the addition of NADPH (Figure 1). No further spectral change could be obtained with extended periods of incubation (up to 2 h). The NADPH-restored spectrum resembled that of untreated mAMSA. When 0.5

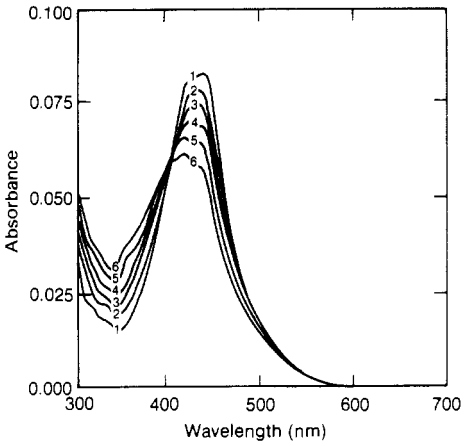

FIGURE 2: Interaction of mAMSA with manganese oxide. mAMSA concentration was 10 μM in 1 mL of 10 mM Tris-HCl buffer, pH 7.5. Manganese oxide was added to attain a final concentration of 0.8 mg/mL of H_2O. Absorption spectra were recorded at different time periods after the addition of manganese oxide: 0 (1), 10 (2), 25 (3), 60 (4), 120 (5), and 180 (6).

mM NADPH was used, a greater restoration was obtained. The spectrum was thus more like that of mAMSA.

Oxidation of mAMSA by Manganese Oxide. Figure 2 shows that when an oxidizing agent, manganese oxide (0.8 mg/mL H_2O), was incubated with mAMSA (10 μM), there was a decrease in the absorption above 395–400 nm and a simultaneous increase below 395–400 nm. An isosbestic point at approximately 395 nm was obtained. The absorption spectral changes increased with time. The characteristics of the spectral changes induced by manganese oxide are similar to those induced by Cu(II) (Wong et al., 1984).

Analysis of the mAMSA–Cu(II) Interaction Products by Thin-Layer Chromatography. Thin-layer chromatography (not shown) of the mAMSA–Cu(II) reaction mixtures showed a yellow band (band 1; R_f 0.63) and a brown band (band 2; R_f 0.52). Band 1 has the same R_f value as that of mAMSA. Incubation of mAMSA with manganese oxide gave two migration bands that have R_f values (0.63 and 0.52) equivalent to the two bands obtained from the mAMSA–Cu(II) reaction mixture (not shown). The absorption spectrum of band 1 (Figure 3A) was similar to that of mAMSA (a major peak at 435 nm and a shoulder at approximately 420 nm), suggesting that band 1 may be unreacted mAMSA. Band 2, produced from reactions with either Cu(II) or manganese oxide, exhibits four absorption peaks (λ_{max} = 400, 377, 300, and 255 nm) (Figure 3B).

It has been demonstrated that in vitro incubation of manganese oxide with mASMA yields the quinone–imine analogue, mAQDI, which is the putative oxidation product of mAMSA (Shoemaker et al., 1982; Gaudich & Przybylski, 1983). The UV spectrum of mAQDI (λ_{max} = 377, 300, and 255 nm) (Gaudich & Przybylski, 1983) was very similar to that of band 2 produced from either mAMSA–Cu(II) or mAMSA–manganese oxide interactions. Thus, the band 2 product may be mAQDI. In other experiments (data not shown) 200 μM mAMSA was incubated with 100 μM Cu(II) for 2 h before the addition of 67 μM NADPH. The mixture was incubated for another 25 min. TLC studies of the mixture (not shown) showed that only one band, which had the same R_f value (0.63) as that of mAMSA, was obtained. These results and spectral studies shown in Figure 1 suggest that regeneration of mAMSA by the reduction of mAQDI with NADPH.

Production of Cu(I) from mAMSA–Cu(II) Interactions. We have used two Cu(I)-specific chelating agents to determine

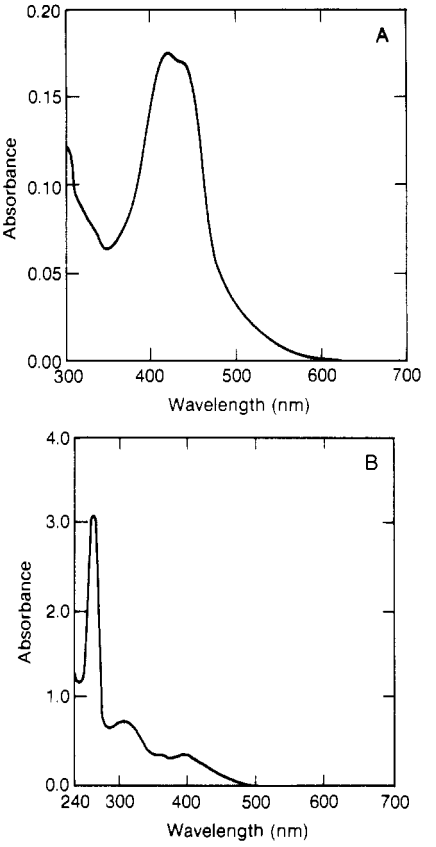

FIGURE 3: (A) Absorption spectrum for band 1 obtained from thin-layer chromatography of mAMSA–Cu(II) interaction. Sample was prepared as described under Materials and Methods. (B) Absorption spectrum for band 2 obtained from thin-layer chromatography of mAMSA–Cu(II) interaction. Sample was prepared as described under Materials and Methods.

whether the mAMSA–Cu(II) interactions resulted in the reduction of Cu(II) to Cu(I). The two agents employed are neocuproine and bathocuproine. Neocuproine complexes with Cu(I) to form the Cu(neocuproine)$_2^+$ complex which has an absorption peak at 450 nm (Nebesar, 1961). Bathocuproine, on the other hand, forms an intense orange complex with Cu(I) which absorbs maximally at 480 nm (Joselow & Dawson, 1951).

As can be seen in Figure 4, Cu(II) did not react with either chelating agent. However, when Cu(I) was added to the neocuproine- or bathocuproine-containing solutions, absorption peaks at 450 and 480 nm were obtained, respectively. Addition of neocuproine to the mAMSA–Cu(II) mixture gave the Cu(I)-specific reaction product which had an absorption maximum at 450 nm. With bathocuproine, a reaction product which had an absorption peak at 480 nm was obtained. This suggests that Cu(I) may be produced during mAMSA–Cu(II) interaction. No spectral changes was obtained as mAMSA was incubated with either neocuproine or bathocuproine.

Stoichiometry of the mAMSA–Cu(II) Interactions. To determine the amount of Cu(I) produced in the reaction mixture, bathocuproine was employed. The Cu(bathocuproine)$_2^+$ complex (ϵ_m = 13 500) (Joselow & Dawson, 1951) absorbs maximally at a wavelength (λ_{max} = 480 nm) that is separated from the absorption of the yellow mAMSA (λ_{max} = 435 nm) so that their absorption spectra do not overlap significantly.

Figure 5 shows the job plots of the absorbance at 480 nm vs. the mAMSA/Cu(II) ratio. When mAMSA was mixed with increasing concentrations of Cu(II), there was an increase in Cu(I) production, as evidenced by an increase in A_{480} after

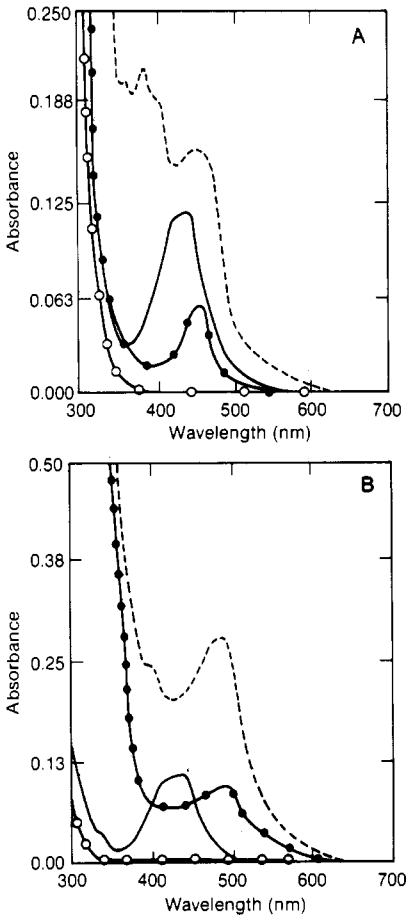

FIGURE 4: (A) Detection of mAMSA-induced Cu(I) production by neocuproine. The concentration of neocuproine used was 100 μM. (○) Neocuproine + Cu(II) (100 μM); (●) neocuproine + Cu(I) (7 μM); (—) mAMSA (10 μM) + neocuproine; (---) mAMSA (10 μM) + Cu(II) + neocuproine. Neocuproine was added immediately after the mixing of mAMSA and Cu(II). (B) Detection of mAMSA-induced Cu(I) production by bathocuproine. The concentration of bathocuproine used was 100 μM. (○) Bathocuproine + Cu(II) (100 μM); (●) bathocuproine + Cu(I) (7.5 μM); (—) mAMSA (10 μM) + bathocuproine; (---) mAMSA (10 μM) + Cu(II) + bathocuproine. Bathocuproine was added immediately after the mixing of Cu(II) and mAMSA.

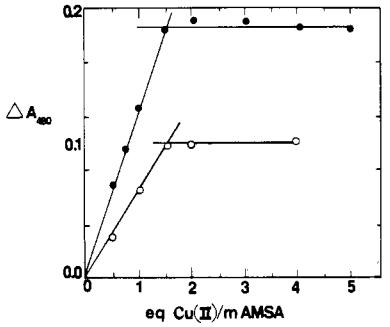

FIGURE 5: Stoichiometry of the mAMSA–Cu(II) interactions. (○) A titration of 5 μM mAMSA; (●) 10 μM mAMSA. The absorbance difference at 480 nm (ΔA_{480}) of the samples with or without Cu(II) addition is plotted vs. the equivalents of Cu(II) added per equivalent of mAMSA. The x-axis value at the intersection of the two lines is taken to represent the ratio of Cu(II) to mAMSA. The value obtained here in both titrations are 1.5 or 1.5 equiv of Cu(II) to 1 equiv of mAMSA.

titrating with bathocuproine. The reduction in A_{480} due to the addition of Cu(II) to mAMSA is negligible when compared to the increase in A_{480} after addition of bathocuproine. In the presence of 5 μM mAMSA, a maximum increase in A_{480} was

Table I: Production of Cu(I) from mAMSA–Cu(II) Interactions Calculated from Spectroscopic Data[a]

Cu(II) added (μM)	A_{480}	ΔA_{480}[b]	Cu(I)[c] (μM)
	(A) 5 μM mAMSA		
0	0.0152		
2.5	0.0522	0.0370	2.74
5.0	0.0787	0.0635	4.70
7.5	0.1105	0.0953	7.06
10.0	0.1141	0.0989	7.33
20.0	0.1181	0.1029	7.62
	(B) 10 μM mAMSA		
0	0.0275		
5.0	0.0954	0.0679	5.03
7.5	0.1227	0.0954	7.05
10.0	0.1531	0.1256	9.30
15.0	0.2112	0.1837	13.61
20.0	0.2171	0.1896	14.00
30.0	0.2175	0.1900	14.07
40.0	0.2132	0.1857	13.76
50.0	0.2102	0.1827	13.53

[a] Assays were carried out as described under Materials and Methods. [b] ΔA_{480} was obtained from A_{480} of the samples minus A_{480} of mAMSA with no Cu(II) addition. [c] Concentrations of Cu(I) were calculated by using the equation $A = \epsilon cl$, in which ϵ = 13 500 and l = 1 cm.

obtained at 7.5 μM Cu(II), which is equal to an mAMSA/Cu(II) ratio of 1.5/1. There was little further increase in A_{480} at Cu(II) concentration up to 20 μM. With 10 μM mAMSA, an end point of titration was achieved at 15 μM Cu(II), which is also equal to an mAMSA/Cu(II) ratio of 1.5/1. Thus, in the presence of either 5 or 10 μM, a Cu(II)–mAMSA stoichiometry of 1.5:1 was obtained.

From the absorbance data (A_{480}) shown in Figure 5, the amounts of Cu(I) produced were calculated and shown in Table I. The data suggest that addition of 1 equiv of Cu(II) produced 1 equiv of Cu(I) at concentrations below the equilibrium levels.

Inhibition of DNA Breakage by Neocuproine. To examine whether the Cu(I) produced during the mAMSA–Cu(II) interaction is essential for the DNA breakage, a Cu(I)-specific chelating agent, neocuproine, was added to the mAMSA–Cu(II)–DNA incubation mixture. Neocuproine forms a stable complex with Cu(I) in aqueous solution, and we have confirmed (data not shown) that the complex does not break DNA (Que et al., 1980). The inhibition of DNA breakage by neocuproine was examined by using three different ratios of mAMSA and Cu(II) and varying the amount of neocuproine. When increasing concentrations of neocuproine were added, there was a progressive decrease in the production of form II DNA accompanied by an increase in the retention of form I DNA. Results in Figure 6 show that the percent inhibition of form II production is a function of the ratio of [neocuproine]/[Cu(II)]. The percent inhibition of form II production was calculated as $(A - B)/A \times 100$ (A and B are the percentages of form II obtained without and with the presence of neocuproine, respectively). The percent inhibition reached a plateau at a [neocuproine]/[Cu(II)] of 1.5 to 2, regardless of the ratio of mAMSA and Cu(II) used in the mixture. The [neocuproine]/[Cu(II)] stoichiometry obtained agrees with the reported neocuproine–Cu(I) complex formation in which two neocuproine molecules chelate one copper ion. These data suggest that neocuproine may inhibit the mAMSA–Cu(II)-induced DNA breakage by complexing with Cu(I).

DNA Breakage Induced by CuCl. The effectiveness of DNA cutting induced by mAMSA–Cu(II) or by Cu(I) (from

Table II: DNA Breakage Induced by mAMSA–Cu(II) or by Exogenous Cu(I)

mAMSA + 100 μM Cu(II)		Cu(I) (from CuCl)	
mAMSA added[a] (μM)	Cu(I) production[b] (μM)	added[c] (μM)	% of form II DNA
	Part A		
0	0		8.2
5	7.5		12.7
10	15		14.9
20	30		19.2
30	45		25.5
42	63		32.1
50	75		28.0
100	100		37.1
	Part B		
		10	11.0
		20	14.0
		40	11.1
		80	11.6
		200	10.2

[a] The incubation mixture contains 100 μM Cu(II) and 0.74 μg of pDPT275 DNA with various concentrations of mAMSA added in 10 mM Tris-HCl buffer (pH 7.5). [b] Concentrations of Cu(I) were determined by titrating the mAMSA–Cu(II) mixtures with 100 μM bathocuproine as described under Materials and Methods. [c] The incubation mixture contains 0.74 μg of pDPT275 DNA with various concentrations of CuCl added in 10 mM Tris-HCl buffer (pH 7.5).

CuCl) was compared. Table II shows that in the presence of 100 μM mAMSA and 100 μM Cu(II), which produces 100 μM Cu(I) (as determined by titrations with bathocuproine), a maximum of 25% DNA breakage was obtained. However, when Cu(I) was added, at concentrations up to 200 μM it induced a much less extent of DNA breakage (3%).

Effects of Partial Anaerobic Condition and Free Radical Scavengers on DNA Breakage. The mAMSA–Cu(II)-induced DNA breakage was studied under a reduced oxygen partial pressure. Also, the effectiveness of several free radical scavengers or inhibitors of DNA breakage was studied. DABCO is a singlet oxygen scavenger (Ouannes & Wilson, 1968). Both Tiron and SOD eliminate superoxide free radicals (Greenstock & Miller, 1975; McCord & Fridovich, 1969). Catalase eliminates hydrogen peroxide. Both KI and mannitol remove hydroxyl radicals. As shown in Table III, DNA cleavage was reduced by approximately 50% under a reduced oxygen partial pressure. This suggests that molecular oxygen is involved in DNA breakage. DABCO partially (44%) inhibited the DNA breakage, indicating that singlet oxygen may be involved. Catalase (100 μg/mL) inhibited the DNA breakage almost completely (82%), suggesting the requirement of hydrogen peroxide in the mAMSA–Cu(II)-induced DNA breakage. Since DNA breakage was not affected by boiled catalase, the inhibition by native enzyme may be due to the catalytic activity

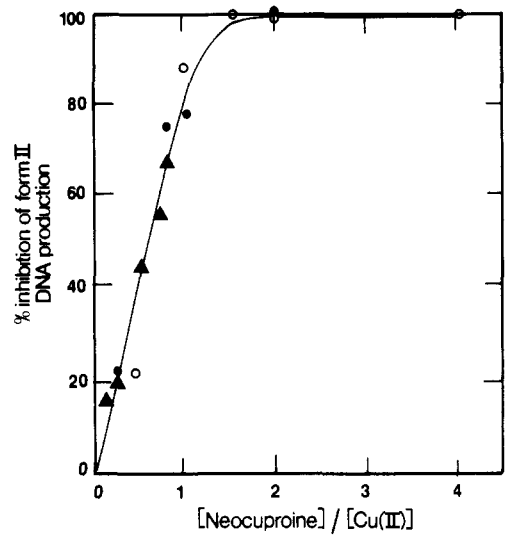

FIGURE 6: Inhibition of mAMSA–Cu(II)-induced DNA breakage by neocuproine. DNA was treated with different concentrations of mAMSA and Cu(II). (○) [mAMSA]/[Cu(II)] = 120 μM/60 μM, (●) 60 μM/120 μM, and (▲) 90 μM/60 μM.

(removal of hydrogen peroxide) rather than physical binding to DNA or mAMSA.

Superoxide dismutase (SOD) catalyzes the reaction $2O_2^{-}\cdot + 2H^+ \rightarrow O_2 + H_2O_2$, and it has been used extensively as an indicator of the involvement of superoxide free radicals ($O_2^{-}\cdot$) in a variety of reactions (McCord & Fridovich, 1969). Results of our studies show that SOD, either alone or in the presence of Cu(II), induces DNA breakage. There was a 5–10% increase in the form II DNA production when SOD or SOD plus Cu(II) was added to the system. Neither SOD nor boiled SOD had any inhibitory effect on the mAMSA–Cu(II)-induced DNA breakage. However, the breakage was blocked (81%) by Tiron, which is another superoxide scavenger. The discrepancy between the effectiveness of SOD and that of Tiron may be due to the intrinsic DNA-cutting activity of SOD. Neither KI nor mannitol, at 50 mM, affected the mAMSA–Cu(II)-induced DNA breakage, indicating that hydroxyl radicals may not be formed or, if formed, are not freely diffusable and detected by the OH· traps.

Discussion

Previous studies employing spectrophotometry, fluorometry and agarose gel electrophoresis of isolated plasmid DNA (Wong et al., 1984) suggested that mAMSA interacted with Cu(II) and induced DNA breakage. In the present studies, several important features of the mechanism involved in DNA breakage have been revealed: (1) mAMSA was oxidized by Cu(II), resulting in the formation of mAQDI (an oxidized mAMSA product) and Cu(I); (2) DNA breakage was mediated by Cu(I), but direct addition of Cu(I) was less effective; (3) molecular oxygen was required for efficient DNA break-

Table III: Percentage Inhibition of Form II DNA Production after Treatment of mAMSA and Cu(II) in the Presence of Scavengers or under Partial Anaerobic Condition[a]

scavengers or anaerobic condition[b]	% inhibition of form II DNA production	scavengers or anaerobic condition[b]	% inhibition of form II DNA production
KI	7.6	boiled catalase	10.0
mannitol	4.4	SOD	0
DABCO	44.0	boiled SOD	0
Tiron	81.3	partial anaerobic condition	51.5
catalase	82.2		

[a] Data were obtained from scanning of the negative film of the agarose gel (data not shown). [b] The concentrations used were the following: KI, 50 mM; mannitol, 50 mM; DABCO, 50 mM; Tiron, 50 mM; catalase, 100 μg/mL; SOD, 83 μg/mL.

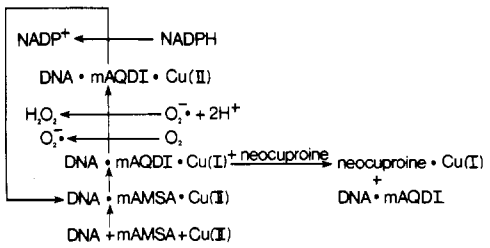

FIGURE 7: Proposed model for the degradation of DNA by mAMSA and Cu(II). mAMSA intercalates with DNA, and Cu(II) interacts with mAMSA. A DNA·mAMSA·Cu(II) ternary complex may be formed. A redox reaction of mAMSA and Cu(II) in the complex may occur, with the formation of a DNA·mAQDI·Cu(I) complex. The complex may act as a catalyst for Cu(I) to Cu(II) oxidation, generating oxygen free radicals which may break DNA.

age; (4) hydrogen peroxide, superoxide free radicals, and singlet oxygen appeared to be involved in the DNA breakage whereas hydroxyl radicals were not.

On the basis of our observations, a reaction mechanism is proposed (Figure 7). mAMSA intercalates with DNA and Cu(II) interacts with mAMSA. A ternary complex, DNA·mAMSA·Cu(II), may then be formed. A redox reaction of mAMSA and Cu(II) in the ternary complex [DNA·mAMSA·Cu(II)] may occur, with the formation of a DNA·mAQDI·Cu(I) complex. This ternary complex may act as a catalyst for the Cu(I) to Cu(II) oxidation, which reduces molecular oxygen to generate a variety of reduced oxygen species. These reduced species may then induce DNA breaks. Additional evidence for the production of reduced oxygen (superoxide) derives from experiments (data not shown) employing the method of Fridovich (1970) in which cytochrome *c* was reduced by the superoxide produced by the mAMSA–Cu(II) interaction.

Potentiometric studies (Loach, 1976) indicate that the aminoacridines undergo a two-electron reversible oxidation, with an E_0' of −0.360 mV at pH 7. This is certainly accessible to the oxidation by Cu(II), which has an E_0' of 0.158 mV (pH 7). Accordingly, the redox reaction between aminoacridines and Cu(II) is thermodynamically favorable. In the present studies we have demonstrated that mAMSA, being an 9-aminoacridine derivative, is also oxidized by Cu(II). The following is evidence for the oxidation of mAMSA: (1) mAMSA spectral changes induced by Cu(II) could be reversed by adding a reducing agent, NADPH. Thin-layer chromatography studies confirmed that addition of NADPH to the mAMSA–Cu(II) incubation mixture regenerated the mAMSA. (2) When an oxidizing agent, manganese oxide, was incubated with mAMSA, an absorption spectral change similar to that produced by mAMSA and Cu(II) was obtained. Thin-layer chromatographic studies show that the oxidized mAMSA product may be mAQDI. This agrees with the clear isosbestic point (395 nm) obtained from the spectrophotometric studies suggesting that mAMSA is converted to a single species.

In the presence of neocuproine, the mAMSA–Cu(II)-induced DNA breakage was inhibited. Data suggest that the Cu(I)–chelator inhibits the DNA breakage by complexing with Cu(I), which is essential for DNA breakage. However, we cannot rule out the possibility that neocuproine may bind to DNA directly and physically hinder the DNA breakage induced by mAMSA and Cu(II). The 200 μM added Cu(I) (from CuCl) is much less effective as compared to a mixture of mAMSA and Cu(II) which produces 100 μM of Cu(I). This suggests that mAMSA, aside from reducing Cu(II) to Cu(I), may play a role in mediating DNA breakage. It has been demonstrated that mAMSA binds to DNA by intercalation of the acridine chromophore between adjacent base pairs (Waring, 1976). As a result, the oxygen free radicals generated by the mAMSA–Cu(II) interaction are close to the DNA strand. Since hydrogen peroxide and superoxide radicals have a high reactivity, they may have a short lifetime in aqueous solution. It may be difficult for them to diffuse through a great distance from the site of generation. Hence, binding of mAMSA to DNA may reduce the diffusion distance from the site of oxygen radical generation to the targets in DNA and enhance the cutting efficiency. The mAMSA–Cu(II)-induced DNA breakage was reduced when a nonspecific intercalating agent, EB, was added to the DNA incubation mixture (data not shown), possibly by occupying intercalative sites essential for cleavage of DNA. Inasmuch as mAMSA is dissolved in Me_2SO, a possible artifact is that Me_2SO might induce aerobic oxidation of mAMSA. This is unlikely. Me_2SO (2–10%) did not induce spectral changes in mAMSA indicative of oxidation. Nor did Me_2SO and mAMSA in the absence of Cu(II) or the presence of other cations induce DNA breakage.

Biologically active metal ions appear to be rquired for several drugs to induce DNA cutting. Bleomycin and 1,10-phenanthroline are examples of drug–metal complexes that induce in vitro DNA breakage. The observations that Cu(II) interacts with mAMSA and that neocuproine can inhibit the DNA breakage suggest that mAMSA may interact with Cu(II) and possibly form a complex. mAMSA contains heteroatoms such as O, N, and S in its structure. It therefore may be able to form coordination complexes with copper ions.

The available evidence from studies of the induction of DNA breakage by mAMSA and Cu(II) is consistent with our proposed model. DNA cutting in the cell may very frequently be mediated by such events, and this mechanism may be a generally important process.

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Registry No. mAMSA, 51264-14-3; mAQDI, 87764-57-6; copper, 7440-50-8; hydrogen peroxide, 7722-84-1; superoxide radical, 11062-77-4; hydroxyl radical, 3352-57-6.

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7S RNA, Containing 5S Ribosomal RNA and the Termination Stem, Is a Specific Substrate for the Two RNA Processing Enzymes RNase III and RNase E†

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ABSTRACT: The 7S RNA, a precursor of 5S rRNA that contains 5S rRNA and the termination stem and loop, is a substrate for RNase E and is also a substrate for RNase III. The cleavage by RNase III is in the stem, 11 nucleotides downstream from the 3′ end of the mature 5S rRNA and 8 nucleotides downstream from the RNase E cleavage site. Near the cleaved nucleotides there are three base pairs that appear in the same relative positions in most known RNase III cleavage sites. The large product of the RNase III cleavage reaction, which is a 5S rRNA that contains 11 extra nucleotides at the 3′ end, is a substrate for RNase E. This suggests that the information for the 3′-end cleavage by RNase E resides mainly in the 5S rRNA itself. Using *rnc rne* strains, carrying the plasmid that leads to the accumulation of 7S RNA, we showed that the 7S RNA does not result from an RNase III cleavage but is apparently a proper transcription termination product.

The study of RNA processing enzymes is impaired to a large extent by the unavailability of large quantities of simple substrates. This is mainly due to the fact that the proper substrates are precursor RNAs that accumulate either in small quantities during the normal metabolism of RNA molecules or in larger quantities in appropriate mutants, blocked directly or indirectly in RNA processing reactions. This is also true for ribonuclease III (RNase III), the enzyme responsible for the primary processing of p16[1] and p23 rRNAs and for the cleavage of polycistronic mRNA precursors of some bacteriophages of *Escherichia coli* [for reviews, see Robertson (1982), Gegenheimer & Apirion (1981), and Pace (1984)]. These natural substrates of RNase III are several thousand nucleotides long, and obtaining them in significant quantities is a troublesome procedure.

We have been studying recently a precursor of 5S rRNA (designated 7S RNA) that accumulates in substantial quantities in the absence of functional RNase E in strains carrying a multicopy plasmid that contains an active 5S rRNA gene (Szeberényi & Apirion, 1983; Elford & Holmes, 1983; Szeberényi et al., 1983). The 7S RNA is only 165 nucleotides long and contains a perfect stem of 15 base pairs which could

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[1] Abbreviations: p16, p23, and p5 rRNAs, precursors to 16S, 23S, and 5S rRNAs, respectively; Na_2EDTA, ethylenediaminetetraacetic acid disodium salt; PEIC, poly(ethylenimine)–cellulose; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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