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# FORMATION OF THE THIOL ADDUCTS OF 4′-(9-ACRIDINYLAMINO)METHANESULFON-*m*-ANISIDIDE AND THEIR BINDING TO DEOXYRIBONUCLEIC ACID

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**Abstract**—We investigated the interactions of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (mAMSA) with thiol-containing compounds and the potential binding of the thiolytic adducts to DNA. All thiols tested (glutathione, cysteine, coenzyme A, 2-mercaptoethanol and lactate dehydrogenase) formed adducts with mAMSA as evidenced by changes in the absorption spectrum of mAMSA and induction of fluorescence. Spectral changes induced by the thiols were different, suggesting that each thiol induced specific changes in the electronic structure of the acridine nucleus. Treatment of glutathione with *p*-chloromercuribenzoate eliminated the absorption spectral changes and induction of fluorescence, indicating that the reduced-thiol group is involved. In high ionic strength buffer, addition of calf thymus DNA induced fluorescence-quenching of both the mAMSA–glutathione and mAMSA–cysteine adducts without spectral shift. Viscometric studies showed that mAMSA and mAMSA–glutathione intercalated into DNA and produced similar increases in the length of linear DNA.

4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (mAMSA)† is a 9-aminoacridine derivative that has antitumor activity in animals and man [1, 2]. It is currently in phase II–III clinical evaluation. The precise mechanism of cytotoxicity of mAMSA is not well understood, but its strong DNA intercalating properties [3] suggest that DNA may be one of the drug target sites. mAMSA induces single-strand breaks, double-strand breaks, and DNA protein cross-links in L1210 leukemia cells [4]. We have also shown that mAMSA is capable of degrading isolated plasmid DNA in the presence of cupric ion [Cu(II)] [5–7]. Furthermore, it has been suggested that mammalian DNA topoisomerase II is a possible target for mAMSA [8, 9]. mAMSA stimulates the formation of a topoisomerase II–DNA complex which may lead to DNA breakage [10].

mAMSA interacts with small molecular weight thiols or thiol-proteins resulting in the formation of mAMSA–thiol adducts [11, 12]. Adducts are produced as a result of nucleophilic attack at the C-9 position of mAMSA by thiols [12]. Since the mAMSA–thiol interaction is prominent and rapid [11], we have studied adduct formation in more detail.

There are three categories of thiol-containing compounds: (1) small molecular weight thiols, such as lipoate, coenzyme A, glutathione, and amino acids and related compounds (cysteine, homocysteine, 2-thiohistidine, ergothioneine and thioglycolate); (2) nonenzyme proteins, such as membrane proteins; and (3) enzymes of a variety of types. As these thiol-containing compounds are essential for the normal functioning of cells [13], interactions of mAMSA with thiols may affect cellular activities, which may contribute to the cytotoxic effects of mAMSA. Our recent studies showed that mAMSA interacts with membrane-localized thiol-containing proteins, resulting in the formation of fluorescent mAMSA membrane protein adducts [14]. Moreover, pharmacokinetic studies have shown that mAMSA is taken up rapidly by the liver, and glutathione-9-thioether of mAMSA has been identified as the major biliary metabolite [15]. Consequently, the formation of mAMSA–thiol adducts is one probable mechanism for intracellular detoxification and *in vivo* metabolism of mAMSA. Since there appears to be a close relationship between the binding of mAMSA to DNA and its antitumor potency [16], we have also examined the possible interaction of the adducts with DNA.

In the present studies, we demonstrate that mAMSA interacts with several small molecular weight thiols such as glutathione, cysteine, coenzyme A and 2-mercaptoethanol and also with one thiol-containing enzyme, lactate dehydrogenase. Both the mAMSA–glutathione and mAMSA–cysteine adducts bind to calf thymus DNA.

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† Abbreviations: CYS, cysteine; EB, ethidium bromide; GSH, glutathione; [$^3$H]GSH, L-[glycine-2-$^3$H]glutathione; LDH, lactate dehydrogenase; mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; MCE, 2-mercaptoethanol; and SH, reduced-thiol group.

## MATERIALS AND METHODS

*Chemicals and reagents.* mAMSA was supplied by Bristol Laboratories, Syracuse, NY. mAMSA solutions were prepared in dimethyl sulfoxide (1 mg/ml) and diluted with phosphate buffer (pH 7.4) as specified, to attain a final dimethyl sulfoxide concentration of 2–3%. Coenzyme A, cysteine, dimethyl

sulfoxide, ethidium bromide, glutathione, 2-mercaptoethanol, lactate dehydrogenase, *p*-chloromercuribenzoate, and sodium phosphate were obtained from the Sigma Chemical Co., St. Louis, MO. Ethyl acetate was purchased from the J. T. Baker Chemical Co., Phillipsburg, NJ. Sephadex G10 was obtained from Pharmacia Fine Chemicals, Piscataway, NJ. L-[Glycine-2-$^3$H]glutathione (sp. act. 1.8 Ci/mmole) was obtained from New England Nuclear, Boston, MA.

*Determination of drug concentration.* The concentrations of mAMSA and ethidium bromide were determined spectrophotometrically. For mAMSA, a molar extinction coefficient of $1.20 \times 10^4\ cm^{-1}\ M^{-1}$ at 434 nm (in water) was used [17]. A molar extinction coefficient of $12{,}400\ cm^{-1}\ M^{-1}$ at 325 nm was used for ethidium bromide [18].

*Preparation of the mAMSA thiol adducts.* mAMSA (60 $\mu$M) was incubated with 2 mM glutathione (GSH), 2 mM cysteine (CYS), 2-mercaptoethanol (MCE) or coenzyme A, and 100 units of lactate dehydrogenase (LDH), in 134 mM phosphate buffer (pH 7.4) at 37° for 24 hr. In some studies, mAMSA (60 $\mu$M) was incubated with various concentrations of GSH (0.1, 2 or 10 mM). Formation of the mAMSA–thiol adducts was monitored by both spectrophotometric and fluorometric measurements.

*Removal of unreacted mAMSA from mAMSA–glutathione and mAMSA–cysteine adduct preparations.* The unreacted mAMSA was separated from the thiol adduct by extracting the incubation mixture with ethyl acetate (five times, v/v = 1/2). Virtually all free mAMSA was extracted into the ethyl acetate layer, whereas thiol adducts remained in the aqueous phase [15]. The aqueous phase was lyophilized and redissolved in distilled water. The samples were desalted by being passed through a Sephadex G10 column (8 cm × 2.2 cm, 1 × i.d.) and eluted with distilled water. Fractions (1.65 ml/fraction) were collected with a LKB 2070 UltroRac II Fraction Collector, which was connected to a UVI Cord S UV Monitor (at 280 nm) and a 2210 Recorder. Elution profiles of the mAMSA–GSH and mAMSA–CYS adducts were obtained. Fractions were appropriately combined, and their absorption spectra were measured.

*Quantitation of mAMSA in the mAMSA–glutathione and mAMSA–cysteine adducts.* The procedure for using free mAMSA as the standard solution was similar to that as described by Gormley and Cysyk [19]. Aliquots (0.5 ml) of the mAMSA–GSH or mAMSA–CYS adducts were added to 0.5 ml of 2 N NaOH and incubated at 75° for 24 hr. After cooling to room temperature, 5 ml of benzene was added, and the tubes were shaken vigorously for 1 min and centrifuged. The organic phase was transferred to a clean test tube, and the extraction procedure was repeated twice with 5 ml of fresh benzene. The organic phases from the two extractions were then combined. After evaporation of the benzene under a gentle flow of air, 1 ml of 0.2 N NaOH was added to each tube and the tubes were shaken vigorously for 30 sec. The fluorescence of the samples was measured with an Aminco SPF-500 Recording Spectrofluorometer, with an excitation of 266 nm and emission of 470 nm.

*Spectrophotometric measurements.* The absorption spectra were measured with an automatic recording Hitachi model 110 Spectrophotometer in 1-ml and 1-cm quartz cuvettes. For these measurements, the mAMSA–thiol incubation mixtures (60 $\mu$M mAMSA, 0.1 ml) were diluted to 1 ml with 134 mM phosphate buffer, pH 7.4. For mAMSA–DNA binding studies, increasing amounts of calf thymus DNA were added to 7.5 $\mu$M mAMSA in 1 ml of 134 mM phosphate buffer, pH 7.4, and the absorbance changes at 435 nm were recorded.

*Fluorometric measurements.* Fluorescence measurements were performed in 1-ml and 1-cm quartz cuvettes with an Aminco SPF-500 Spectrofluorometer equipped with an X-Y Recorder. Aliquots of 25 $\mu$l of each mAMSA–thiol mixture were added to 1 ml of 134 mM phosphate buffer, pH 7.4. Samples were excited at 387 nm, and uncorrected fluorescence spectra were reported. For assays of DNA-induced fluorescence quenching of the mAMSA–GSH and mAMSA–CYS adducts, increasing amounts of calf thymus DNA were added to 10.5 $\mu$M mAMSA–GSH adduct or 11.5 $\mu$M mAMSA–CYS adduct in 134 mM phosphate buffer, pH 7.4. The fluorescence changes were recorded. The fluorescence was stable throughout the DNA titrations. The intrinsic fluorescence of the DNA or mAMSA was less than 1.0% of that of the mAMSA adduct.

*Viscometric studies of the DNA binding of mAMSA–glutathione adduct.* Viscosity was measured essentially as described [20, 21]. Increasing amounts of ethidium bromide, mAMSA or mAMSA–GSH adduct (after ethyl acetate extractions) were added to a fixed amount of calf thymus DNA solution (200 $\mu$g/ml) in 134 mM phosphate buffer, pH 7.4, in a Type 75 Cannon–Ubbelohde Viscometer thermostated at 25° with a Cannon constant temperature bath. The flow time was measured in triplicate with a model 221 Wescan Automatic Viscosity Timer to within ±0.03 sec.

*Studies on the DNA binding of glutathione.* To test the binding of GSH to DNA, [$^3$H]GSH (2.7 nmoles; 5 $\mu$Ci) was incubated with 60 $\mu$g of calf thymus DNA in 1 ml of 134 mM phosphate buffer, pH 7.4, at 37° for 5 min. The sample mixture was then applied to a Sephadex G50 column (17 × 0.8 cm, 1 × i.d.) which had been equilibrated with 134 mM phosphate buffer, pH 7.4. The sample was eluted with the same phosphate buffer, and 0.8-ml fractions were collected. Absorbance (260 nm) of the fractions was measured for the detection of DNA. A 0.5-ml aliquot of each fraction was used in the measurements of radioactivity.

## RESULTS

*Changes in absorption spectrum of mAMSA on reacting with thiols.* Upon reaction with mAMSA for 24 hr, four of the five thiols tested (GSH, CYS, coenzyme A and MCE) induced marked changes and one (LDH) induced a small change in the absorption spectrum of mAMSA (Fig. 1, A to E). In general, all thiols induced a reduction of the spectral intensity. However, the characteristics of the spectral changes induced by each thiol were different. Coenzyme A (Fig. 1C) induced a pattern of multiple peaks, with

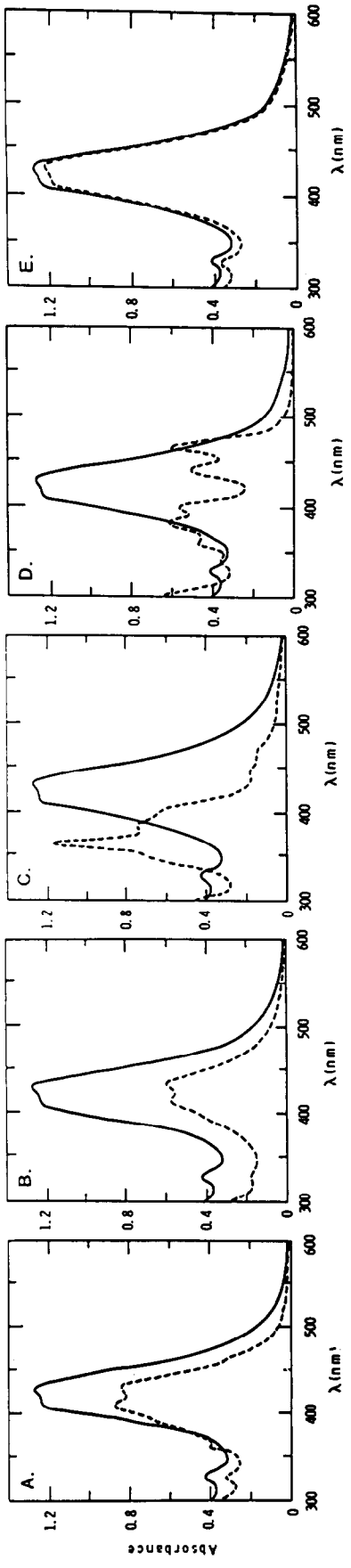


Fig. 1. Absorption spectral changes of mAMSA (60 μM) after incubation with (A) glutathione (2 mM); (B) cysteine (2 mM); (C) coenzyme A; (D) 2-mercaptoethanol; and (E) lactate dehydrogenase (100 units) in 134 mM phosphate buffer, pH 7.4, at 37° for 24 hr. Spectra were recorded at 0 time (——) and after 24 hr (– – –) of incubation.

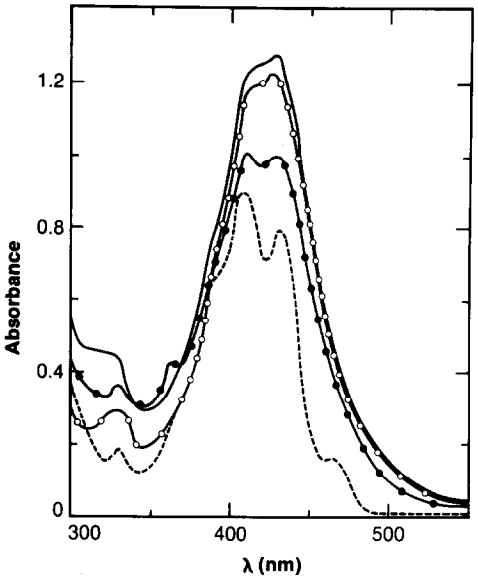


Fig. 2. Effects of various concentrations of glutathione on mAMSA spectral changes. mAMSA (60 μM) was incubated alone (——) or with 0.1 mM (—○—), 2 mM (—●—) or 10 mM (– – –) glutathione in 134 mM phosphate buffer, pH 7.4, at 37° for 24 hr.

intense peaks localized within the range of 330 to 410 nm. 2-Mercaptoethanol (Fig. 1D) induced a pattern of multiple, small peaks. Thus, all thiols tested reacted with mAMSA, and each thiol induced specific changes in the acridine nucleus, which is the source of the mAMSA absorption spectrum.

As shown in Fig. 2, the GSH-induced mAMSA spectral changes increased with increasing concentrations of GSH. GSH (0.1 mM) induced a small decrease in the mAMSA absorption spectrum, whereas 2 mM induced a more significant reduction (a 20% decrease of $A_{435}$). The spectrum obtained at 10 mM GSH was similar to that of the purified mAMSA–GSH adduct (see Fig. 6A), indicating that a high percentage of mAMSA had reacted with GSH to form the mAMSA–GSH adduct.

*Fluorescence properties of the mAMSA–thiol adducts.* mAMSA does not exhibit detectable fluorescence [22]. However, thiolysis results in the cleavage of the anisidide side chain and the subsequent formation of the fluorescent mAMSA–thiol adducts [11]. Figure 3 shows the emission spectra (excitation at 387 nm) of the mAMSA–thiol adducts formed after a 24-hr incubation with GSH (A), CYS (B), coenzyme A (C), MCE (D) or LDH (E). The fluorescence characteristics of the CYS and LDH adducts are very similar, with a doublet at 435 and 458 nm, and a shoulder around 485 nm. The fluorescence characteristics of the coenzyme A and MCE adducts are similar, with a doublet at 420 and 442 nm, and a shoulder around 470 nm. Qualitatively, the fluorescence spectra of these two pairs of thiol adducts are very similar, except that those of coenzyme A and MCE show a 20 nm shift to shorter wavelengths. The spectrum of the GSH adduct has a prominent peak at 452 nm and two shoulders at around 424 and 475 nm. Thus, each adduct displays a unique fluorescence spectrum.

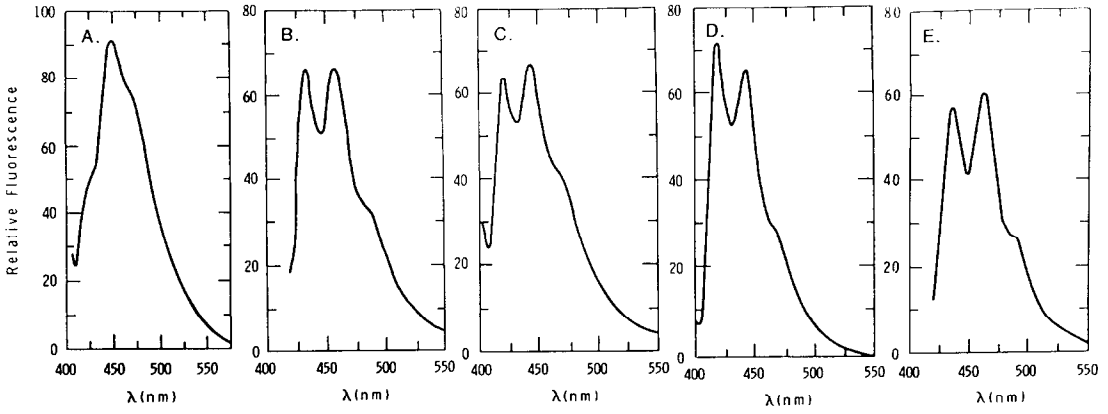


Fig. 3. Induction of fluorescent product from the interaction of mAMSA (60 μM) with (A) glutathione (2 mM), (B) cysteine (2 mM), (C) coenzyme A, (D) 2-mercaptoethanol, and (E) lactate dehydrogenase in 134 mM phosphate buffer, pH 7.4, at 37°. Fluorescence spectrum (ex. 387 nm) was recorded after 24 hr of incubation. The incubating mixture was diluted 40-fold with 134 mM phosphate buffer before measurements.

*Involvement of the reduced-sulfhydryl group in adduct formation.* To confirm that the reduced sulfhydryl group is involved in adduct formation, 1 mM GSH was incubated with 2 mM *p*-chloromercuribenzoate, a sulfhydryl specific oxidizing agent, in 134 mM phosphate buffer, pH 7.4, at 37° for 1 hr. mAMSA was then added to the incubation mixture (final concentration 120 μM), and the sample mixture was further incubated for 24 hr at 37°. There was no change in the mAMSA absorption spectrum, nor an appearance of fluorescence (data not shown), suggesting that the SH group is necessary in the adduct formation.

*Spectral properties of the purified mAMSA–GSH and mAMSA–CYS adducts.* Figure 4 shows the absorption spectrum of mAMSA–GSH adduct after removal of unreacted mAMSA by repeated extraction with ethyl acetate. The adduct shows a major peak at 405 nm, a minor peak at 430 nm and two shoulders at approximately 360 and 390 nm. The absorption changes include reduction in absorption intensity (as compared to mAMSA), an increase in peak number, and a shift to shorter wavelength. The fluorescence characteristics of the mAMSA–GSH adduct before (Fig. 3A) or after (data not shown) removal of unreacted mAMSA were similar; both exhibited a peak at 425 nm and two shoulders around 424 and 475 nm. Because mAMSA does not exhibit detectable fluorescence, its presence does not affect the fluorescence properties of the adduct.

*Studies on the mAMSA–GSH thiol adducts by column chromatography.* Figure 5 shows the chromatogram of the mAMSA–GSH reaction mixture (after ethyl acetate extractions) eluted from a Sephadex G10 column. It exhibits a major elution peak at fraction number 23, and two shoulders at approximately fractions 15 and 30, indicating the presence of at least three different types of mAMSA–GSH reaction products. The absorption spectra of some

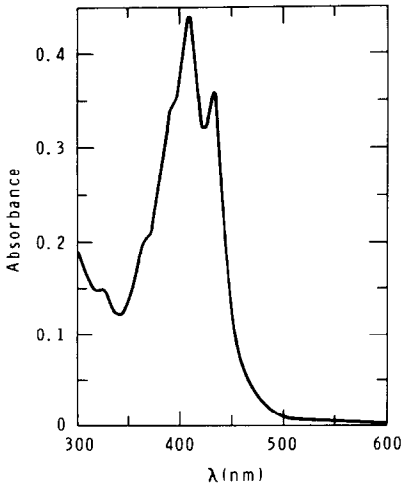


Fig. 4. Absorption spectrum of the ethyl acetate extracted mAMSA-GSH adduct. mAMSA (60 μM) was incubated with glutathione (2 mM) at 37° for 24 hr. The incubation mixture (2 ml) was subjected to ethyl acetate extractions (five times, v/v = 1/2). The aqueous phase was lyophilized and redissolved in distilled water (2 ml), and the absorption spectrum was then recorded.

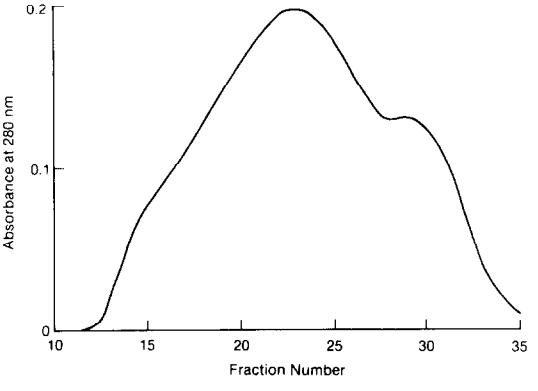


Fig. 5. Column chromatogram of the mAMSA–GSH adducts. The mAMSA–GSH incubation mixture was extracted with ethyl acetate (five times, v/v = 1/2). It was then applied on a Sephadex G10 column (8 cm × 2.2 cm, 1 × i.d.), which was eluted with distilled water. The elution profile was obtained by monitoring the absorbance at 280 nm of the eluted fractions (1.65 ml/fraction).

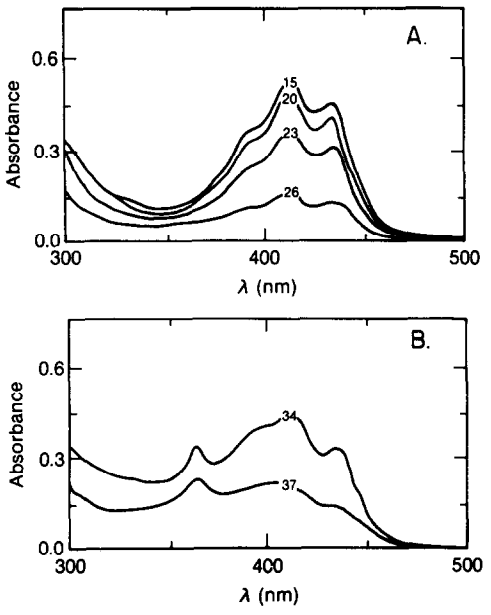


Fig. 6, A and B. Absorption spectra of several of the eluted fractions that were obtained by passing the mAMSA–GSH adducts through a Sephadex G10 column. The fraction numbers were as indicated.

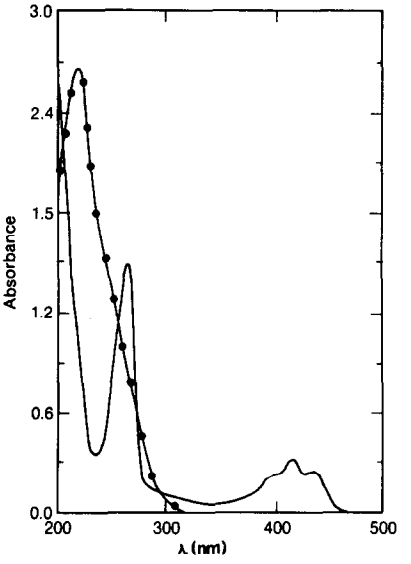


Fig. 7. Absorption spectra of two of the eluted fractions (fractions 19 and 36) that were obtained by passing the mAMSA–CYS adducts through a Sephadex G10 column. Key: (——) fraction 19; (—●—) fraction 36.

of the fractions were measured. Fractions 15, 20, 23 and 26 (Fig. 6A) show similar spectral characteristics (a major peak at 405 nm, a minor peak at 430 nm, and a shoulder at 390 nm), which are different from those of fractions 34 and 37 (Fig. 6B; absorption peaks at 435, 410 and 360 nm).

For the mAMSA–CYS reaction mixture, two major elution peaks (at fractions 19 and 36) were resolved (data not shown). The absorption spectrum (Fig. 7) of fraction 19 exhibits two major peaks at 410 and 260 nm, a minor peak at 435 nm and a shoulder at 395 nm, which is a characteristic of the acridine chromophore. Fraction 36, however, shows a major peak at 220 nm and a shoulder at 250 nm, with no absorption in the visible region (Fig. 7). This suggests that fraction 19 consists of the mAMSA–CYS adduct, whereas fraction 36 may consist of the unreacted free cysteine and/or the mAMSA anilino tail which is displaced after reacting with cysteine.

*Quantitation of the mAMSA–thiol adducts.* Alkaline hydrolysis of mAMSA or its thiol adduct results in the cleavage of anisidide side chain and the formation of the highly fluorescent 9(OH)-acridone [23]. Employing free mAMSA as a standard, the fluorescence of the hydrolytic product can be measured to quantitate the amount of acridine moiety present in the mAMSA–thiol adducts. In agreement with previous reports [19], such an assay (data not shown) was sensitive, and the fluorescence–mAMSA concentration relationship was linear to a concentration of 2.5 μM mAMSA.

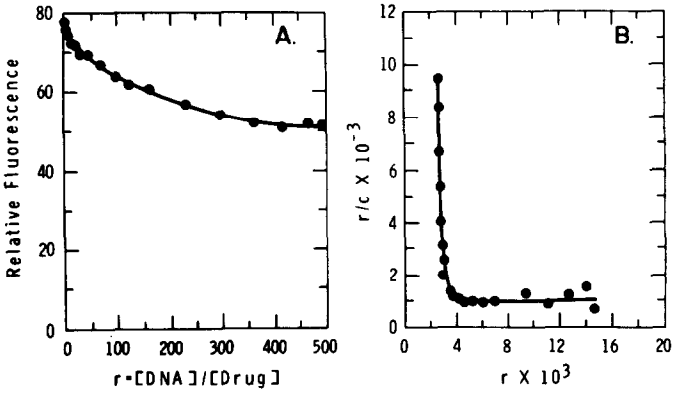


Fig. 8. (A) Quenching of fluorescence of the mAMSA–GSH adducts by increasing concentrations of calf thymus DNA. Increasing concentrations of DNA were titrated against 10.5 μM mAMSA–GSH adducts in 134 mm phosphate buffer, pH 7.4; (B) Scatchard analysis of the quenching effects of DNA on the fluorescence of the mAMSA–GSH adducts as shown in panel A. The parameters $r$ (moles of ligand bound per nucleotide), $c$ (moles per liter of free ligand), and $r/c$ were calculated by the method of Scatchard [24].

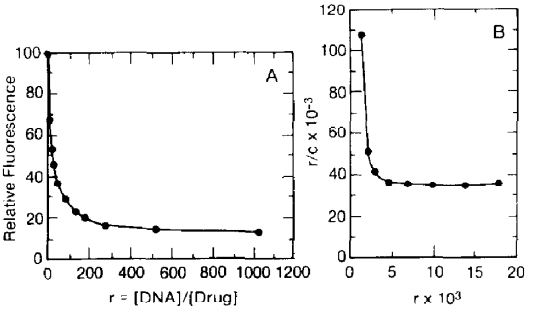


Fig. 9. (A) Quenching of fluorescence of the mAMSA–CYS adducts by increasing concentrations of calf thymus DNA. Increasing concentrations of DNA were titrated against 11.5 μM mAMSA–CYS adducts in 134 mM phosphate buffer, pH 7.4. (B) Scatchard analysis of the quenching effects of DNA on the fluorescence of the mAMSA–CYS adducts as shown in panel A.

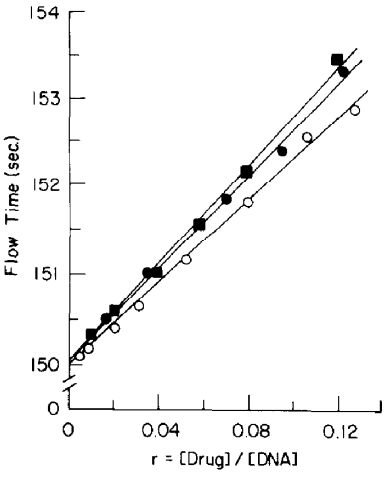


Fig. 11. Viscometric titrations of the effects of EB (—■—), mAMSA (—●—) and mAMSA–GSH adducts (—○—) on calf thymus DNA in 134 nM phosphate buffer, pH 7.4, at 25°. Viscosity expressed by flow time was plotted with increasing input drug/DNA ratios for each drug. The concentration of the DNA was 200 μg/ml.

Assuming that such a linear relationship is also valid for the mAMSA–thiol adducts, we calculated the concentration (in terms of acridine moiety) of the GSH and CYS adducts (after ethyl acetate extractions) to be 12 and 15 μM respectively, when 60 μM mAMSA was reacted with a 2 mM concentration of each thiol. Thus, recoveries of 20 ± 4% for mAMSA–GSH and 25 ± 5% for mAMSA–CYS were obtained.

*Interaction of the mAMSA–thiol adducts with DNA by fluorescence quenching assay.* Calf thymus DNA induced a quenching effect on the fluorescence of the mAMSA–GSH and mAMSA–CYS adducts. In 134 mM phosphate buffer, the DNA-induced fluorescence quenching occurred throughout the entire spectra of the GSH adduct and CYS adduct (data not shown). At a GSH adduct/DNA concentration ratio of 500, a maximum reduction of 36% in fluorescence was noted (Fig. 8a). For the CYS adduct, a maximum reduction of 86% fluorescence was observed at an adduct/DNA concentration ratio of 500 (Fig. 9A). These results indicated that both the GSH and CYS adducts interacted with DNA molecules in 134 mM phosphate buffer. At this high ionic strength, ionic interactions are minimal. Since mAMSA interacts with DNA primarily by intercalation of its acridine nucleus into base pairs [3], it is likely that the adducts may intercalate into DNA by the same mechanism.

The binding data shown in Figs. 8A and 9A were analyzed by the Scatchard [24] method, as previously described [25, 26]. Figures 8B and 9B show the Scatchard plots in which $r_b/c$ was plotted against $r_b$, according to $r_b c = Kn - Kr_b$. In this equation, $r_b$ is the number of moles of ligand bound per nucleotide phosphate; $c$ is the molar concentration of the free drug; $K$ is the apparent association constant; and $n$ is the number of drug binding sites per nucleotide phosphate. As shown in Figs. 8B and 9B, hyperbolic curves were obtained. It is therefore difficult to obtain meaningful binding parameters ($K$ and $n$ values) by using the Scatchard method.

Although to obtain more meaningful binding par-

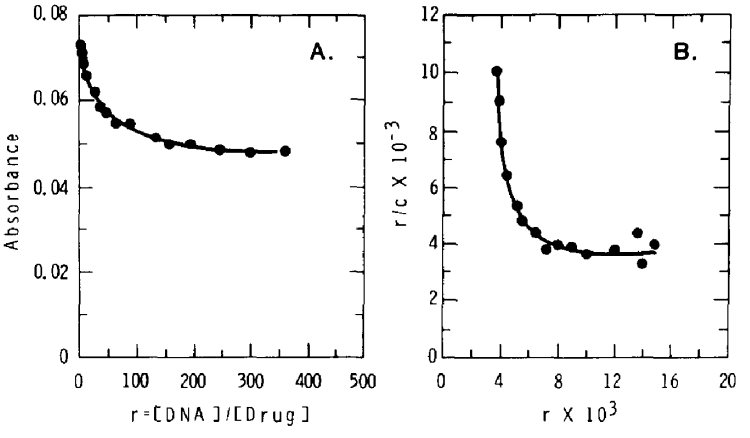


Fig. 10. (A) Quenching of mAMSA absorbance at 435 nm by increasing concentrations of calf thymus DNA. Increasing concentrations of DNA were titrated against 7.5 μM mAMSA in 134 mM phosphate buffer, pH 7.4. (B) Scatchard analysis of the quenching effects of DNA on the mAMSA absorbance shown in panel A.

ameters an analysis procedure according to McGhee and von Hippel [27] may be necessary, an approximate affinity constant can be obtained from the Scatchard plot using data points between $r = 3.5 \times 10^{-3}$ and $4.0 \times 10^{-3}$. Calculated this way, the mAMSA–GSH adduct gives a $K$ value of $1.00 \times 10^5\ M^{-1}$ and an $n$ value of 0.005. The $n$ value is equivalent to the binding of one molecule of mAMSA–GSH adduct per one hundred DNA base pairs. For the mAMSA–CYS adduct, the $K$ value is $1.73 \times 10^6\ M^{-1}$ and the $n$ value is 0.039, which is equivalent to the binding of one molecule of mAMSA–CYS adduct per thirteen DNA base pairs.

*Quenching of mAMSA absorption by DNA.* The addition of calf thymus DNA induced a quenching effect on the absorption of mAMSA. Figure 10A shows that, in 134 mM phosphate buffer, a maximum decrease of 35% in absorbance at 435 nm was obtained at a [mAMSA]/[DNA] ratio of 1/400. When the binding data were analyzed using the Scatchard method, a hyperbolic curve was obtained (Fig. 10B). If the data points between $r = 5.5 \times 10^{-3}$ and $6.0 \times 10^{-3}$ are considered, the $K$ value of mAMSA to DNA is $0.9 \times 10^6\ M^{-1}$, with $n$ equal to 0.012, which is equivalent to the binding of one molecule of mAMSA per eighty-three base pairs.

*Viscometric studies on the interactions of mAMSA–GSH with DNA.* Viscometric studies were performed to determine if mAMSA–GSH intercalates into DNA. Figure 11 shows the viscometric changes, expressed as flow time in seconds, of sheared calf thymus DNA treated with ethidium bromide, mAMSA or mAMSA–GSH adduct in 134 mM phosphate buffer, pH 7.4. The two DNA intercalators, ethidium bromide and mAMSA, exhibited characteristic increases in DNA viscosity with increasing drug concentrations. The mAMSA–GSH adduct induced viscosity changes in DNA to an extent similar to that induced by mAMSA. Thus, the mAMSA–GSH adduct may intercalate with DNA, inducing DNA conformation distortions to an extent comparable to those induced by mAMSA.

*Interactions of free GSH with DNA.* When the incubation mixture of calf thymus DNA and [$^3$H]GSH was separated on a Sephadex G50 column, the high molecular weight DNA was obtained in the first five fractions (0.80 ml/fraction), whereas [$^3$H]GSH was eluted between fractions 6 and 10. There was virtually no coelution between the DNA and [$^3$H]GSH, indicating their lack of interaction. These results suggest that the binding of the mAMSA–GSH adduct to DNA is mediated by its acridine nucleus, rather than by the GSH side chain.

## DISCUSSION

By employing spectrophotometric and fluorometric methods, we have studied the thiolysis reaction of several small molecular weight thiols, including glutathione, cysteine, coenzyme A and 2-mercaptoethanol, and a thiol-containing protein, lactate dehydrogenase, with mAMSA. In these studies, a 2 mM concentration of the nonprotein thiols was used because most cell types contain 2–5 mM of intracellular glutathione, which is the dominant nonprotein thiol inside the cell [28]. Cysteine and other thiols are present in trace amounts (1–10 $\mu$M or less) in cells [29]. Thus, the *in vitro* reactions of mAMSA and thiols reported here were performed under conditions (37°, 2 mM thiol) analogous to the intracellular environment, allowing for the comparative study of the relative reactivities of thiols with mAMSA in the *in vitro* and *in vivo* systems.

All thiols tested interact with mAMSA, as evident from the changes in the absorption spectrum of mAMSA and the inductions of fluorescence spectra. Since each thiol induced characteristic changes in the mAMSA absorption spectrum and generated specific fluorescence spectra, this may suggest that each thiol induces unique changes in the electronic state of the acridine nucleus of the mAMSA molecule. *p*-Chloromercuribenzoate is an effective, thiol-specific reacting agent. Pretreating GSH with *p*-chloromercuribenzoate prevented the GSH-induced changes in the mAMSA absorption and fluorescence characteristics. This suggests that (1) the interactions between thiols and mAMSA require the presence of reduced sulfhydryl groups; and (2) the absorption spectral changes and the appearance of the fluorescence result from the thiolysis reaction.

As revealed from chromatographic analysis, the thiolytic products formed by mAMSA and GSH are heterogeneous. These thiolytic products may be produced by substitution of GSH into various sites of mAMSA. It has been reported that at least two types of mAMSA–GSH adducts can be identified. They are the mAMSA–GSH conjugate in which the thioether linkage occurs at the 5-position of the anilino ring, and the GSH-9-thioether of acridine [30–33]. Details of the methods for separation and identification of the mAMSA–GSH adducts have been reported by Shoemaker *et al.* [31, 33] and Przybylski *et al.* [30, 32]. Although at present no structural studies have been performed to show the chemical nature of our mAMSA–GSH adducts, studies on the absorption and fluorescence spectra of the eluting fractions suggest that all the reaction products contain the acridine chromophore.

Since the cytotoxic effects of mAMSA may be related to its ability to interact with DNA [3, 34], it is of interest to study whether the thiolytic adducts also interact with DNA. In this report, by using fluorescence quenching and viscometric assays, we have shown that both the mAMSA–GSH and mAMSA–CYS adducts interact with DNA. The mAMSA–GSH adduct induces an increase in DNA contour length to an extent similar to that induced by the two intercalating agents, mAMSA and EB. However, recent studies have shown that the mAMSA-5′-GSH adduct is not cytotoxic against L1210 cells [33]. Since GSH cannot permeate or be transported by the red blood cell membranes [35], it is likely that the loss of cytotoxicity of the mAMSA-5′-GSH is due to its inability to enter cells.

Once entering the cells, mAMSA may form specific adducts with small molecular weight thiols, and these adducts may produce specific biological effects. Our observations suggest that the adducts formed from each type of thiol exhibit unique changes in the electronic state of the acridine nucleus. Thus, at least some types of adducts interact with DNA and they may interact with DNA with

different characteristics. As the acridine moiety of the adduct intercalates into two adjacent DNA base pairs, the presence of new functional groups may provide additional interactions between mAMSA and DNA. It has been reported that the binding affinity of mAMSA to DNA could be increased by the introduction of a lysyl residue at its side chain [35]. Therefore, functional groups such as GSH, CYS or lysyl residue may form hydrogen bondings, hydrophobic or ionic interactions with DNA, which may be important in the cytotoxic effects of mAMSA. Conversely, the interaction of mAMSA with other thiol-containing compounds, perhaps higher molecular weight thiols, may reduce DNA binding.

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