

STUDIES ON THE FLUORESCENCE LABELING OF HUMAN RED BLOOD CELL MEMBRANE GHOSTS WITH 4'-(9-ACRIDINYLAMINO)METHANESULFON-*m*-ANISIDIDE

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Abstract—4'-(9-Acridinylamino)methanesulfon-*m*-anisidide (mAMSA) interacts with red cell membranes, resulting in the formation of fluorescent protein adducts. The mAMSA-membrane protein adducts exhibited an emission fluorescence maximum at 445 nm, with two shoulders at approximately 425 and 470 nm. The major labeled proteins were identified as spectrins 1 and 2 and bands 3, 4.1, 4.2 and 5. The fluorescence intensity increased with increasing mAMSA concentrations (0.03 to 1.5 mM), time (15–120 min), and temperature of the reaction. Results from sodium dodecyl sulfate gel electrophoresis show that mAMSA caused no detectable change in the molecular weight of membrane proteins. This indicates that mAMSA is a monofunctional, noncrosslinking agent. Other acridine analogs, 9-aminoacridine and acridine, did not fluorescently label membrane proteins, suggesting that the presence of the acridine nucleus is not sufficient for labeling. Addition of 2-mercaptoethanol to the mAMSA-membrane reaction mixtures reversed the fluorescence labeling. Furthermore, pretreatment of membrane proteins with *N*-ethylmaleimide or iodoacetamide prevented the formation of fluorescent mAMSA-membrane protein adducts. These data suggest that mAMSA interacts with sulfhydryl groups of the membrane proteins. When the membrane sulfhydryl groups were assayed by labeling with *N*-[ethyl-2-³H]ethylmaleimide, it was shown that the accessible membrane sulfhydryl groups were reduced after the mAMSA treatment. The above results suggest that mAMSA covalently binds to the sulfhydryl groups in the red cell membrane, with the production of fluorescent mAMSA-protein adducts.

4'-(9-Acridinylamino)methanesulfon-*m*-anisidide (mAMSA)[†] is a 9-aminoacridine derivative developed by Cain and Atwell [1]. It is currently in phase II-III clinical evaluation. Clinical studies suggest that mAMSA is active in acute leukemia [2, 3]. The precise mechanism of cytotoxicity of mAMSA is not well understood. mAMSA intercalates into DNA [4], and it has been suggested that the mAMSA-DNA interaction may correlate with the antitumor activity [5]. mAMSA induces DNA single-strand breaks, double-strand breaks and DNA protein crosslinks in L1210 leukemia cells [6]. Recent studies from our laboratory have shown that mAMSA, in the presence of the Cu(II) ion, induces breakage of the plasmid superhelical form I DNA [7, 8]. Although evidence supports the concept that the mAMSA-DNA interactions are important in its cytotoxic effects, other mechanisms of action, such as the interaction with cell membranes, cannot be excluded.

The antitumor antibiotic adriamycin, which intercalates into DNA, has been shown to interact with the cell membrane [9, 10] and affect the membrane properties, such as the concanavalin A-mediated cell agglutination [9] and the ion transport [11]. Another DNA intercalating agent, ellipticine, may interact with acidic phospholipids of the membranes [12]. Furthermore, it has been suggested that the diacridines, which were synthesized and developed as antitumor drugs because of their strong DNA intercalating properties [13, 14], are cytotoxic because of their interactions with cell membranes [15]. By using a spin-labeling technique [16], it has been shown that AMSA, an mAMSA analog, interacts strongly with membrane proteins and may induce protein conformational changes. Therefore, the cell membrane may be a common target for the DNA intercalating antitumor drugs.

The interaction of mAMSA with proteins has been studied. It was shown [17] that the nonfluorescent mAMSA reacts with the sulfhydryl containing plasma proteins to form highly fluorescent mAMSA-protein adducts. Adducts are produced as a result of nucleophilic attack at the C-9 position of mAMSA by the sulfhydryl groups of plasma proteins (see Fig. 1). mAMSA also interacts with red blood cells [17]. However, no study on the interaction of mAMSA with membrane proteins has been reported. Since the membrane sulfhydryl groups are essential for the normal functioning of the red blood cell, changes in the membrane sulfhydryl content may affect the cell structural integrity and the enzymatic and transport

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[†] Abbreviations: AMSA, 4'-(9-acridinylamino)-methanesulfonamide; mAMSA, 4'-(9-acridinylamino)-methanesulfon-*m*-anisidide; NEM, *N*-ethylmaleimide; [³H]NEM, *N*-[ethyl-2-³H]ethylmaleimide; SDS, sodium dodecyl sulfate; and SH, reduced-sulfhydryl group.

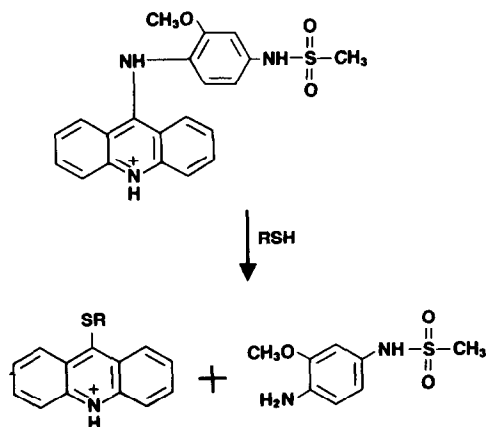


Fig. 1. Reactions of mAMSA with sulphydryl-containing compounds.

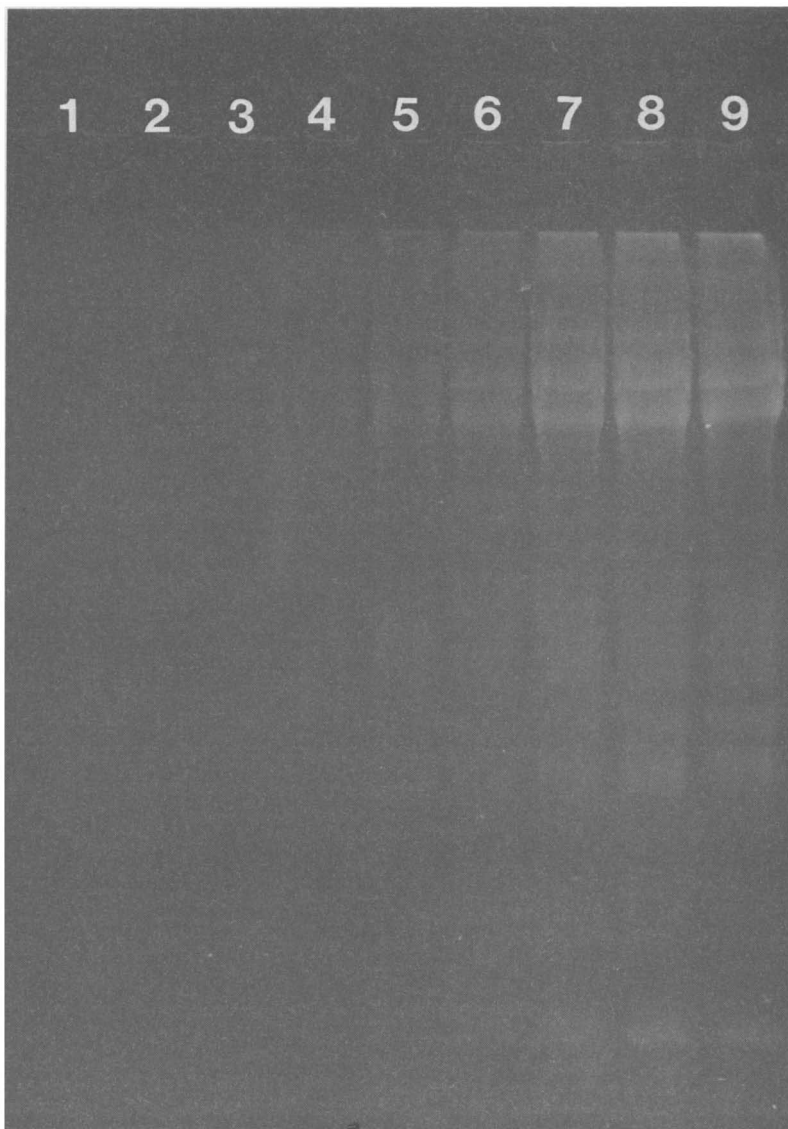
functions of the cell membrane [18]. Therefore, the interactions between mAMSA and membrane sulphydryl proteins may have biological relevance.

In the present studies, we demonstrate that mAMSA may interact with human red cell membrane ghosts, resulting in the formation of fluorescent mAMSA-protein adducts. These studies suggest that, in addition to DNA, the cell membrane may be a target for mAMSA and that the cytotoxic effects may be mediated in part via interactions with sulphydryl groups in the key membrane proteins.

MATERIALS AND METHODS

Materials. mAMSA base (NSC 249992) was supplied by Bristol Laboratories, Syracuse, NY. mAMSA solution was prepared by dissolving the drug in dimethyl sulfoxide (30 mg/ml), and diluting it with distilled water to the desired concentration.

(A)



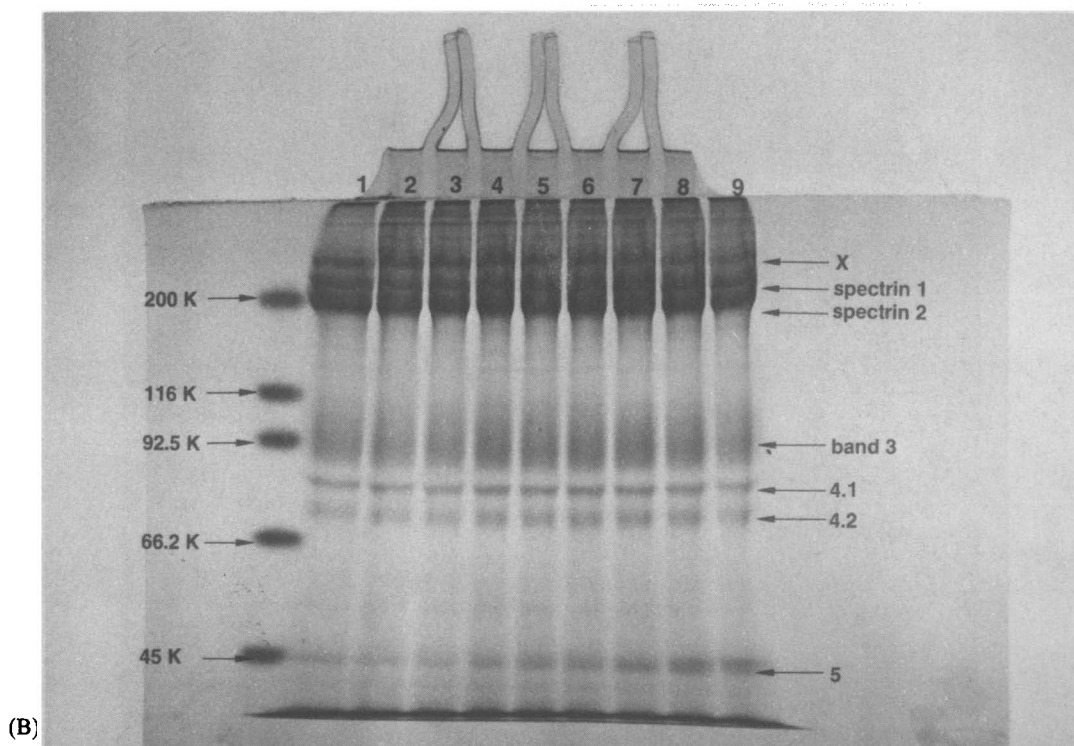


Fig. 2. (A) Fluorescence-labeled membrane proteins obtained from human red blood cell ghosts after treatments with various concentrations of mAMSA. Lane 1, ghost preparation (3.5 mg/ml protein concentration, suspended in 5 mM phosphate buffer, pH 8) treated with 2 μ l of distilled water; lane 2, 1.3% dimethyl sulfoxide; lanes 3-9, 0.03, 0.06, 0.13, 0.25, 0.5, 1.0 and 1.5 mM mAMSA respectively. Drug treatments were performed at 37° for 2 hr. The membrane proteins (17.5 μ g total protein) were separated by using sodium dodecyl sulfate (0.1%)/acrylamide (7.5%) slab gel electrophoresis. The gel was fixed in methanol-acetic acid-water (40:10:50, by vol.). The fluorescent mAMSA-labeled protein bands were visualized by irradiating the gel with u.v. light. (B) The Coomassie blue protein stain of the same gel shown in Fig. 2A. A standard protein mixture was used to mark molecular weight ranges, including myosin (200 K), β -galactosidase (116 K), phosphorylase B (92.5 K), bovine serum albumin (66.2 K) and ovalbumin (45 K).

The final concentration of dimethyl sulfoxide in the membrane ghost reaction mixture was less than 2%. 9-Aminoacridine, acridine, bovine muscle actin, dimethyl sulfoxide, Na₂EDTA, glycerol, glutathione, hydrogen peroxide (30%), iodoacetamide, 2-mercaptoethanol and trichloroacetic acid were obtained from the Sigma Chemical Co., St. Louis, MO. Acrylamide, bis(*N,N*-methylene-bis-acrylamide), sodium dodecyl sulfate (SDS), glycine, *N,N,N',N'*-tetramethylethylenediamine (TEMED), ammonium persulfate, Coomassie blue R-250 and bromophenol blue were obtained from Bio-Rad Laboratories, Richmond, CA. *N*-Ethylmaleimide was obtained from Calbiochem-Behring, San Diego, CA. Phosphate-buffered saline (Dulbecco "A" tablet) was obtained from KC Biological, Inc., Lenexa, KS. *N*-[ethyl-2-³H]Ethylmaleimide (sp. act. 55.8 Ci/mmole) and Econofluor were obtained from the New England Nuclear Corp., Boston, MA. Beckman Ready-Solve HP/b scintillation fluid was obtained from Beckman Instruments, Inc., Fullerton, CA.

Preparation of unsealed human red blood cell membrane ghosts. Fresh human blood (Group O⁺), anticoagulated with sodium citrate, was obtained from normal individuals with informed consent. Blood

was kept at 4° overnight before use. The buffy coat was removed after centrifugation (2300 g, 10 min). Red cells were washed twice with phosphate-buffered saline, pH 7.4. Unsealed ghosts were obtained from osmotic cell lysis as described by Dodge *et al.* [19]. Hemolysis was initiated by rapidly and thoroughly mixing the packed red cells with 40 vol. of ice-cold 5 mM phosphate buffer, pH 8. The membrane ghosts were pelleted by centrifugation at 23,000 g for 10 min. Ghosts were washed three times, each with 5 vol. of 5 mM phosphate buffer, pH 8. The ghosts obtained were creamy white and morphologically intact. They were stored at -20° until use. The method of Lowry *et al.* [20], calibrated with bovine serum albumin and modified by the addition of SDS, was used to determine protein concentration.

Treatment of membrane ghosts with drugs. Membrane ghosts were suspended in 5 mM phosphate buffer, pH 8 (3-4 mg/ml protein concentration). Aliquots of 150 μ l of the ghost suspension were incubated with different concentrations of mAMSA (0.03 to 1.5 mM), at 37° for various time intervals (15 min-2 hr). When the effects of reducing agents were studied, additional treatments (1 mM 2-mercapto-

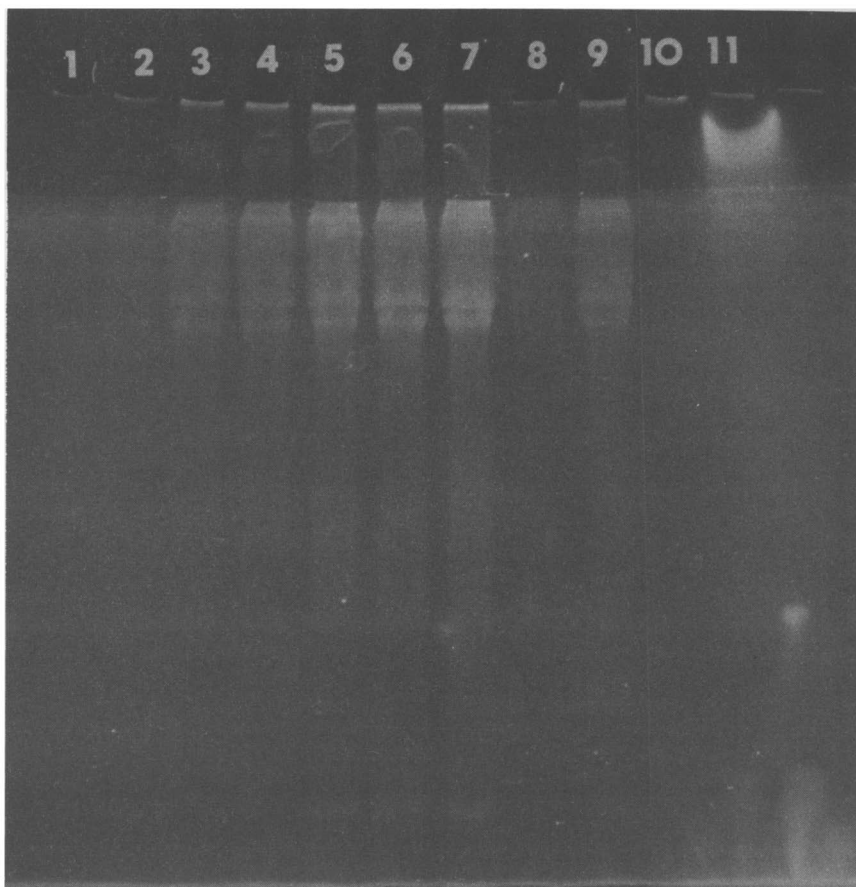


Fig. 3. Time and temperature dependencies of the mAMSA labeling of membrane proteins. Lane 1, untreated ghosts; lanes 2–7, treatments (37°) with 1 mM mAMSA for 0 min, 15 min, 30 min, 60 min, 90 min, and 120 min, respectively; lanes 8 and 9, treatments with mAMSA (1 mM, 2 hr) at 4° and 20° , respectively; lanes 10 and 11, treatments (37° , 2 hr) with 1 mM 9-aminoacridine and acridine, respectively.

ethanol or 5 mM glutathione) were included (37° for 30 min) after the mAMSA–ghost incubations. In some experiments, ghosts were pretreated with *N*-ethylmaleimide (5 mM) or iodoacetamide (5 mM) at 37° for 30 min, prior to the addition of mAMSA. Stock solutions of *N*-ethylmaleimide (250 mM) and iodoacetamide (750 mM) were prepared by dissolving the compounds in distilled water and used within 15 min.

Electrophoretic analysis of cell membrane proteins. After drug treatment, 50 μ l of termination buffer (SDS, 20%, w/v; EDTA, 2 mM, glycerol, 40%, w/v; Tris, 250 mM, pH 6.8) was added into the ghost suspension. The samples were boiled for 5 min and subjected to SDS (0.1%), acrylamide (7.5%) slab gel electrophoresis, as described by Laemmli [21]. After electrophoresis, the gels were fixed in methanol–acetic acid–water, 40:10:50 (by vol.). The fluorescent mAMSA-labeled protein bands were visualized by irradiating the gels with a transilluminator (Ultraviolet Products, Inc.). Permanent records were obtained by photographing the gels with a Polaroid CU-5 Land Camera, equipped with a No. 8 Kodak Wratten gelatin filter (Eastman Kodak Co., Rochester, NY) and type 665 Land Film. The negative films of gel were used for densitometric

scannings. Densitometric scanning of the gels or gel negative pictures were performed with a Beckman DU-8 UV/VIS Spectrophotometer, equipped with a slab gel scan compuset module (Beckman Instruments).

Labeling of membrane proteins with *N*-[ethyl-2- 3 H]ethylmaleimide. The sulfhydryl content of proteins was assayed by labeling with *N*-[ethyl-2- 3 H]ethylmaleimide (3 H]-NEM). A 100- μ l ghost suspension [control or 1 mM mAMSA-treated (2 hr at 37°)] was incubated with 25 μ l of *N*-ethylmaleimide mixture which contained 1 mM nonradioactive NEM and 0.448 nmole [3 H]NEM (55.8 Ci/mmmole). The reaction mixtures were incubated at 37° for 30 min. Under these conditions, all the free sulfhydryl groups would be labeled with NEM [22]. The membrane proteins were then subjected to electrophoresis, as described above. Gels were stained with Coomassie blue, destained, and sliced according to the staining profile. To a 1–2 mm gel slice, 0.5 ml hydrogen peroxide (30%) was added. The gel slice was incubated at 60° until dissolved, and 15 ml of Beckman Ready-Solve HP/b was then added. Radioactivities were determined with a Beckman LS 7800 Scintillation Counter (Beckman Instruments, Inc.). The counting efficiency was 55–60%.

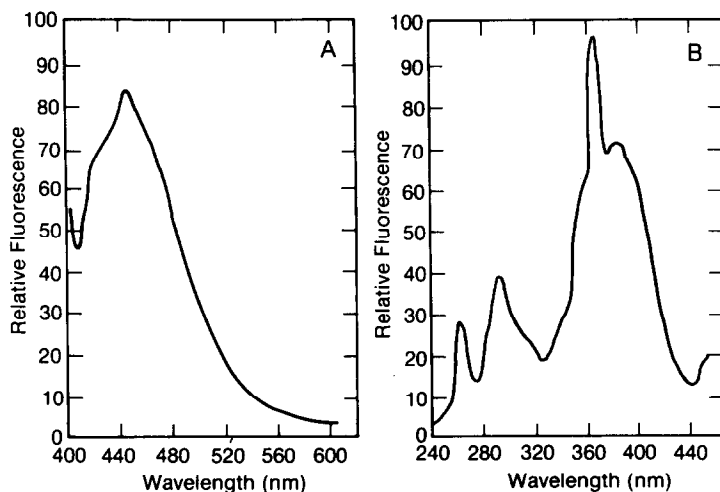


Fig. 4. (A) Fluorescence emission spectrum (excited at 387 nm) of the mAMSA-labeled membrane ghosts. The ghost preparation (3.5 mg/ml protein concentration) was treated with 0.03 mM mAMSA at 37°. Fluorescence spectrum was recorded after 2 hr of incubation. (B) Fluorescence excitation spectrum (emission at 450 nm) of the mAMSA-labeled membrane ghosts.

Determination of the sulfhydryl content of actin. Lyophilized bovine muscle actin was dissolved in 5 mM phosphate buffer, pH 8, to a final concentration of 3 μ M (0.14 mg/ml). Aliquots of the actin solution (100 μ l) were treated with various concentrations of mAMSA (0.3, 1, 3 and 10 μ M) at 37° for 1 hr. A 5- μ l mixture containing 1 pmole [3 H]-NEM (55.8 Ci/mmol) and 2 nmoles nonradioactive NEM was then added. The reaction mixtures were incubated at 37° for 1 hr. The actin was then precipitated by adding 2 ml of ice-cold trichloroacetic acid (10%) and vacuum filtered using Whatman GF/C filters. The filters were washed three times (3 ml each) with ice-cold trichloroacetic acid (5%) and dried. The amount of radioactivity incorporated into actin was determined by liquid scintillation spectrometry with the addition of 10 ml Econofluor.

Fluorometric measurements. Fluorescence measurements were performed in a 1-ml, 1-cm quartz cuvette with an Aminco SPF-500 Ratio Spectrofluorometer, equipped with a Hewlett-Packard 9815 A/S Calculator and an HP 7225A Graphic Plotter. For fluorescence measurements, 90 μ l ghost samples were diluted to 0.9 ml with 5 mM phosphate buffer, pH 8. Uncorrected fluorescence measurements are reported.

RESULTS

Reactions of mAMSA with human red blood cell membrane ghosts. Human red blood cell membrane ghosts were labeled by mAMSA to yield fluorescent mAMSA-membrane protein adducts. The generic reaction is shown in Fig. 1. The adducts are stable with time. The gels may be stored in fixative for several days prior to u.v. photography without any loss in fluorescence intensity.

Labeling of red blood cell membrane ghosts resulted in several major protein bands that were detected by their fluorescence on sodium dodecyl sulfate polyacrylamide gels (Fig. 2A). The same gel was also stained with Coomassie blue protein stain

(Fig. 2B). The major labeled proteins were identified as spectrins 1 and 2 and bands 3, 4.1, 4.2 and 5 according to the nomenclature of Fairbanks *et al.* [23]. An extra band, band x, was obtained from our control ghost samples which were incubated at 37° for 2 hr. However, fresh ghosts or ghosts treated with a reducing agent (e.g. 2-mercaptoethanol) did not give band x. This may indicate that band x is a crosslinked product (of some unidentified membrane proteins) which is formed during the 2-hr incubation.

As shown in Fig. 2A, the fluorescence intensity of most of the protein bands increased with increasing mAMSA concentrations (0.03 to 1.5 mM, lanes 3–9). Densitometric scanning results of the gel lanes in Fig. 2B (Coomassie blue-stained protein gels) showed that there was no apparent change in the protein patterns induced by dimethyl sulfoxide (1.3%) or mAMSA treatment (1 mM) (data not shown).

Figure 3 shows that, in the presence of a constant concentration of mAMSA (1 mM), increasing the time of incubation of mAMSA with the ghosts (0, 15, 30, 60, 90 and 120 min) resulted in increased fluorescent adduct production (lanes 2–7). Maximum adduct production was obtained at approximately 90–120 min of incubation. The extent of adduct production also increased with increasing incubation temperature. When the mAMSA-labeling reaction (1 mM mAMSA, 2 hr) was performed at 4° (Fig. 3, lane 8), very few, if any, adducts were detected. However, at higher temperatures (20° and 37°), there was an increase in fluorescent adduct formation (lanes 9 and 7, respectively). Other acridine analogs, 9-aminoacridine and acridine, were tested for their abilities to fluorescently label membrane proteins. The fluorescence obtained in lane 11 was due to the fluorescence of free acridine. Neither drug labeled the membrane proteins (Fig. 3, lanes 10 and 11), suggesting that the presence of the acridine moiety was not sufficient for labeling.

Spectroscopic studies. Panels A and B of Fig. 4 show the emission spectrum (excited at 387 nm) and

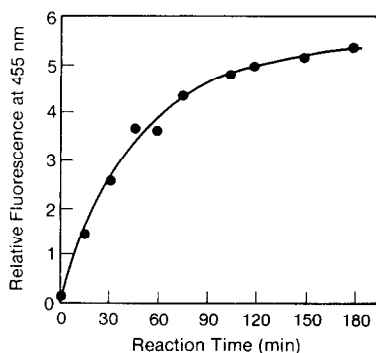


Fig. 5. Increase in the production of fluorescent mAMSA-membrane protein adducts with increasing labeling time. Ghost preparations (3.5 mg/ml protein) were incubated with 0.03 mM mAMSA at 37°. Relative fluorescence of the ghost sample emission at 455 nm was measured at different times of incubation.

the excitation spectrum (emission at 450 nm) of the mAMSA-membrane protein adducts. When excited at 387 nm, the fluorescence spectrum showed a peak at 445 nm, and two shoulders around 425 and 470 nm.

With an emission at 450 nm, the excitation spectrum showed a major peak at 365 nm, 3 minor peaks at 260, 290 and 387 nm, and a shoulder around 350 nm. Both mAMSA and the membrane ghost preparation showed only weak fluorescence. The relative fluorescence (emission at 455 nm) increased with increasing incubation time (Fig. 5). The fluorescence spectrum, however, did not vary as a function of reaction time.

Effects of 2-mercaptoethanol and glutathione on the mAMSA-labeling reaction. As shown in Fig. 6, there was a significant diminution of fluorescent labeling as a result of post-treatment with 2-mercaptoethanol (1 mM) after the mAMSA-labeling reaction (lane 4). With a higher concentration of 2-mercaptoethanol (5 mM), the fluorescence labeling was completely abolished (data not shown). However, addition of glutathione (5 mM) did not reverse the labeling reaction (lane 6). Neither 2-mercaptoethanol (lane 3) nor glutathione (lane 5), when used alone, resulted in fluorescent products.

Effects of n-ethylmaleimide and iodoacetamide pretreatment on the mAMSA-labeling reaction. N-Ethylmaleimide and iodoacetamide are monofunctional

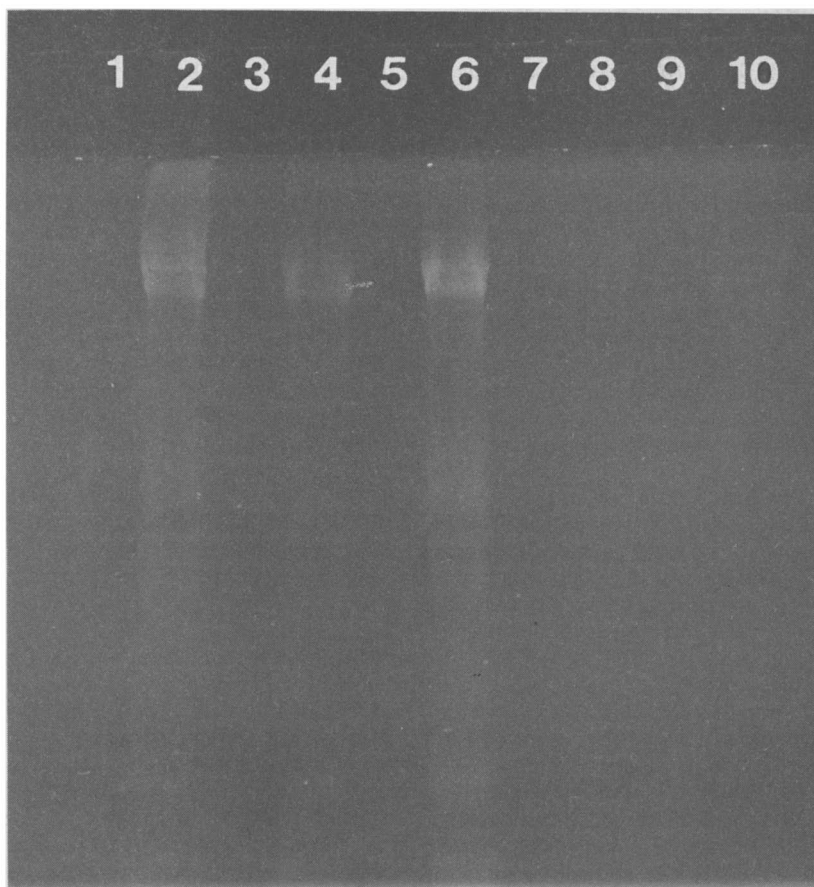


Fig. 6. Effects of several sulfhydryl reacting agents on the mAMSA labeling of membrane proteins. The concentrations of drugs used were: mAMSA and 2-mercaptoethanol, 1 mM; glutathione, NEM and iodoacetamide, 5 mM. The mAMSA treatments were 2 hr, whereas for other drugs they were 30 min. Samples were prepared as described under Materials and Methods. Lane 1: ghost preparation incubated at 37° for 2 hr; lane 2: treatment with mAMSA; lane 3: 2-mercaptoethanol; lane 4: mAMSA, then 2-mercaptoethanol; lane 5: glutathione; lane 6: mAMSA, then glutathione; lane 7: NEM; lane 8: NEM, then mAMSA; lane 9: iodoacetamide; and lane 10: iodoacetamide, then mAMSA.

Table 1. Reduction of the [^3H]NEM labeling of membrane protein by treatment with mAMSA*

Band number	Percent reduction of radioactivity
Spectrin 1	26.0 \pm 4.3
Spectrin 2	27.0 \pm 10.2
Band 3	43.0 \pm 5.0
Band 4.1	30.8 \pm 12.1
Band 4.2	26.2 \pm 15.5
Band 5	28.7 \pm 5.3
Total	28.1 \pm 4.8

* Ghosts were treated with 1 mM mAMSA at 37° for 2 hr (3.5 mg/ml protein concentration). The mAMSA-non-reacting sulfhydryl groups were then labeled with *N*-[ethyl-2- ^3H]ethylmaleimide. Peptides were separated by gel electrophoresis, and radioactivity of defined bands was determined. Values are expressed as mean \pm S.E., $N = 4$.

sulfhydryl alkylating agents [24]. When the ghosts were treated (30 min, 37°) with *N*-ethylmaleimide (5 mM) or iodoacetamide (5 mM) (Fig. 6, lanes 8 and 10, respectively) and then labeled with mAMSA, a minimal amount of fluorescence was associated with the membrane proteins. Fluorometric measurements showed that, after pretreatment with NEM or iodoacetamide, only 5–10% of the fluorescence intensities were obtained from the mAMSA–ghosts reaction mixture as compared to that of ghosts that were not pretreated. Neither NEM nor iodoacetamide produced fluorescent adducts with membrane proteins. Furthermore, neither NEM nor iodoacetamide reacted with mAMSA, as demonstrated by thin-layer chromatography (data not shown). Consequently, since the reactions with NEM and iodoacetamide are irreversible and completed within 30 min [22], and neither reacted with mAMSA, the reduction in labeling was not due to a reaction of unreacted mAMSA with the alkylators.

Effects of mAMSA on the distribution of membrane sulfhydryl proteins. Table 1 shows that the membrane protein SH groups were reduced after mAMSA

treatment. In these experiments, the membrane SH groups were labeled by pretreating the ghosts with mAMSA (1 mM) at 37° for 2 hr. That such treatment provided maximum mAMSA–adduct formation has been shown in Fig. 5. The SH groups that had not reacted with mAMSA were then labeled with [^3H]NEM. After electrophoresis of the membrane proteins, the gel lanes were sliced and the total radioactivities in the gel lanes and also of individual bands were determined. mAMSA treatment resulted in a 28% (± 4.8 , $N = 4$) decrease in total [^3H]NEM labeling (Table 1), as indicated by the difference in radioactivities between controls and mAMSA-treated samples. This suggests that the total number of SH groups accessible to NEM was reduced after the mAMSA treatment. [^3H]NEM labeling was also reduced in individual proteins, with band 3 exhibiting the highest reduction ($43 \pm 5.0\%$, $N = 4$). That these results were not due to interactions of free mAMSA with [^3H]NEM is further supported by the fact that, under the conditions of the incubation, no reaction between mAMSA and NEM was observed. Furthermore, in experiments in which the membranes were dialyzed after mAMSA treatment against an mAMSA-free medium, no differences in inhibition were observed.

Reactions of mAMSA with actin. To better quantitate the reaction of mAMSA with sulfhydryl groups in proteins, its reaction with actin was studied. Bovine muscle actin consists of a single polypeptide chain which is rich in sulfhydryl groups. Treatment of actin with mAMSA reduced the number of protein sulfhydryl groups. This was demonstrated by a decrease in the labeling with [^3H]NEM. Figure 7 shows that, with increasing concentration of mAMSA (0.3 to 10 μM), there was a gradual decrease in the actin sulfhydryl content. A maximal decrease was obtained at 3–10 μM mAMSA. Furthermore, fluorometric measurements showed that increasing concentrations of mAMSA resulted in concomitant increases in fluorescence intensities of the mAMSA–actin reaction mixtures. The increase plateaued at 3–10 μM mAMSA. Therefore, in the

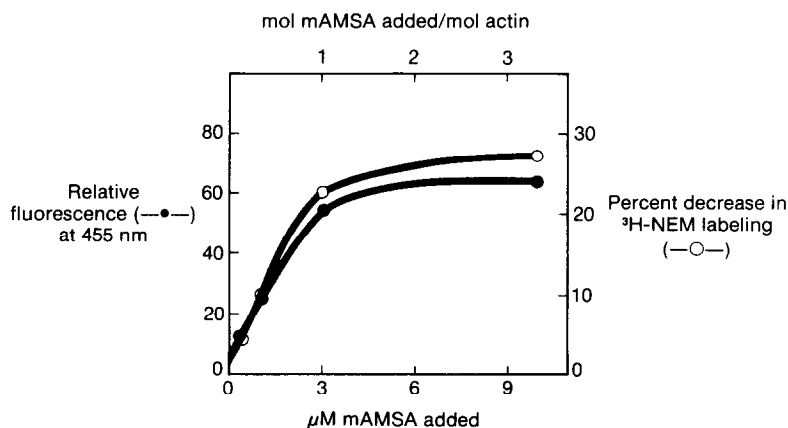


Fig. 7. Parallelism of the increase in fluorescence intensity of mAMSA-labeled actin and the percent decrease of [^3H]NEM labeling as a function of the molar ratio of mAMSA and actin. Actin solutions (3 μM) were incubated with mAMSA at 37° for 2 hr before subjected to fluorometric measurements. Determination of the actin sulfhydryl content was described in Materials and Methods. Key: (●) relative fluorescence; and (○) percent decrease in [^3H]NEM labeling.

range of 0.3 to 10 μ M mAMSA, the increase in fluorescence intensity of mAMSA-actin paralleled a decrease in the sulfhydryl content of actin.

DISCUSSION

In the present studies, we demonstrate that mAMSA interacted with red cell membrane proteins to produce fluorescent adducts. The red blood cell membrane was chosen as the system for investigation because: (1) membrane ghosts can be isolated by simple hypotonic lysis; (2) the red cell membrane is a well characterized system; (3) the red cell carries large numbers of reduced sulfhydryl groups (2×10^6 /cell) on its membrane proteins [25], and (4) it has been demonstrated that AMSA, an mAMSA analog, may react with red blood cell membrane proteins [16].

The following observations demonstrate that mAMSA reacts with the sulfhydryl groups of membrane proteins: (1) post-treatment of mAMSA-ghost preparation with 2-mercaptoethanol reversed the fluorescent labeling of membrane proteins by mAMSA; and (2) pretreatment of membrane proteins with *N*-ethylmaleimide or iodoacetamide prevented the mAMSA labeling. A characteristic in the sulfhydryl-disulfide oxidation reaction is that it can readily be reversed by sulfhydryl-containing reducing agents [26, 27]. It has been demonstrated that 2-mercaptoethanol can reverse the (1,10-phenanthroline)₂Cu⁺-induced disulfide crosslinking of membrane proteins [27]. Our studies demonstrate that 2-mercaptoethanol reversed the fluorescence labeling of membrane proteins with mAMSA (Fig. 6). This suggests that mAMSA labeling of membrane proteins may involve oxidation of sulfhydryl groups. Dithiothreitol, another sulfhydryl-containing reducing agent, also reversed the mAMSA labeling (unpublished observation). However, glutathione, which serves as a reductant of membrane protein disulfide in intact red blood cells [28], was unable to reverse the mAMSA labeling of isolated membrane ghosts (Fig. 6). This is probably because glutathione, *in vitro*, is not so potent a reducing agent as 2-mercaptoethanol or dithiothreitol.

mAMSA does not induce intermolecular protein crosslinking between the major red blood cell membrane proteins, as indicated from the data that labeling by mAMSA produced no apparent change on the Coomassie blue stained gel pattern (Fig. 2B). However, the possibility that mAMSA may induce the association of polypeptides into oligomers, which cannot be distinguished by using denaturing SDS gel electrophoresis, cannot be excluded.

It has been demonstrated by fluorometric measurements that three fluorescent acridine analogs, 9-aminoacridine, 9-amino-3-chloro-7-methoxyacridine and quinacrine, interact with membrane, resulting in fluorescence quenching of the compounds [29]. These interactions are mediated through hydrogen bonding and/or ionic interaction between the drugs and membrane components. Our results (Fig. 3) suggest that, when subjected to electrophoresis, the 9-aminoacridine and acridine-membrane protein complexes may dissociate because these types of

interactions are not sufficient to withstand an electric current. Therefore, no fluorescent band could be obtained by gel electrophoresis when 9-aminoacridine or acridine was used to label red cell membrane.

The mAMSA-membrane protein adducts exhibited fluorescent spectra (Fig. 4, A and B) that were similar to those of the mAMSA-plasma protein adducts [17], but different from those of 9-aminoacridine and acridine. Since mAMSA and the membrane ghost preparation show only weak fluorescence, the measurements of the increase in relative fluorescence of mAMSA-ghosts mixture can be used to monitor adduct formation. The data obtained from fluorometric measurements (Fig. 5) agree with the results obtained from densitometric scanning (data not shown) of the fluorescent gels (Fig. 3). Studies on the mAMSA labeling of actin showed that there was a close correlation between the extent of the decrease in NEM-reacting sulfhydryl groups in actin and the extent of fluorescent mAMSA-actin adduct formation (Fig. 7). This indicates that the sulfhydryl groups of actin are the sites of fluorescent mAMSA labeling.

Figure 5 shows that there was an increase in adduct production with increasing incubation time, reaching a plateau at approximately 90–120 min. Moreover, addition of mAMSA at the plateau state did not increase fluorescence (data not shown). This saturation of mAMSA labeling is probably because the remaining sulfhydryl groups are inaccessible to mAMSA. The rate of mAMSA labeling is relatively slow as compared to the reactions of *N*-ethylmaleimide and iodoacetamide. Both compounds saturate the accessible membrane sulfhydryl proteins within 1 hr [22]. As demonstrated by the [³H]NEM assay, mAMSA did not react extensively with membrane proteins under our conditions. It reacted with only 28% of the total sulfhydryl groups (Table 1). This suggests that not all membrane sulfhydryl groups are accessible to mAMSA.

All the labeled proteins (spectrins 1 and 2, bands 3, 4.1, 4.2 and 5) play an important role in the red blood cell membrane function. Spectrins 1 and 2 and bands 4.1 and 5 constitute the cytoskeleton of the red cell [30, 31]. A change in the state of the cytoskeleton produced by drug-membrane interaction may affect the shape and deformability of the red cell as well as the lateral distribution of membrane proteins [32]. Band 3 is the anion channel protein which serves to transport a variety of inorganic and organic anions across the lipid bilayer [33]. Band 5 is identified as actin [34]. Consequently, the effects of mAMSA on inorganic and organic ion transport should be characterized. Furthermore, it is possible that the labeling of sulfhydryl proteins may take place not only in red blood cells, but also in other cell types, such as the lymphocytes and tumor cells, since these cells carry large numbers of sulfhydryl groups on their cell surface [35].

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