Selective Detoxification of Murine Toxin from *Yersinia pestis*. Reaction of Heavy Metals with Essential Sulfhydryl and Tryptophan Residues†

Thomas C. Montie* and Diane B. Montie

ABSTRACT: Plague murine toxin in the native form is sensitive to high levels of metal sulhydryl reagents. Subunits (mol wt 12,000 or 24,000) formed from toxin A after treatment with 0.5% sodium dodecyl sulfate were detoxified by approximately a 1:1 mole ratio of Ag^+ or Hg^{2+} to sulfhydryl. Mild perturbation of toxin A with 0.05% sodium dodecyl sulfate rendered toxin susceptible to metallic ions at the same low ratios. Fluorescein mercuric acetate inactivated toxin A in 0.05% sodium dodecyl sulfate at mole ratios of 5–10 times reagent to SH. Tris-Cl buffer protected toxin against heavy metal inactivation. Evidence for specific reaction of metallic reagents with SH in toxin A included: (1) isolation of a ^{203}Hg–cysteine complex; (2) quenching of fluorescence emission of the SH reagent fluorescein mercuric acetate; (3) competition of Ag^+ for fluorescein mercuric acetate binding; (4) quenching of tryptophan fluorescence emission resulting from mercaptide bond formation. The lack of a general conformational change caused by mercaptide formation was indicated by the absence of a fluorescence shift in contrast to the shift in the fluorescence maximum induced by guanidine hydrochloride. Mercuric ions also interacted with tryptophan residues in toxin A in 0.05% sodium dodecyl sulfate as demonstrated by distinctive difference spectrum profiles (A_{max} 298 nm) comparable to profiles obtained by reaction of Hg^{2+} with tryptophan alone. The evidence strongly indicates the importance of SH groups in an essential site required for toxicity. Results further suggest an association of a tryptophan residue with the sulfhydryl site. Inactivation of subunits or polypeptide chains containing a single tryptophan or cysteine unit per mol wt 12,000 suggest that this is the minimum unit required for toxicity.

Two proteins toxic for mice and rats have been isolated both from culture lysates and directly from avirulent cells of *Yersinia (Pasteurella) pestis*, the plague organism. These proteins have been designated toxin A (mol wt 240,000) and toxin B (mol wt 120,000) (Montie *et al.*, 1966b). The toxins are complex, containing a number of small subunits with molecular weights of approximately 24,000; each subunit contains two polypeptide chains of mol wt 12,000 (Montie and Montie, 1971). Based on amino acid analysis, minimum molecular weight calculations, and the high level of repeating sequences observed in peptide fingerprint analysis, it is consistent to assume the presence of both one cysteine and one tryptophan residue per polypeptide chain in toxin A (Montie and Montie, 1971).

Previous studies using toxins purified by Sephadex column separations showed that the toxicity could be modified by addition of rather high levels of sulfhydryl inhibitors, particularly silver ions (Montie *et al.*, 1966a). It has also been demonstrated that tryptophan analogs selectively inhibit synthesis of toxic protein in growing cells of *Y. pestis* (Montie *et al.*, 1964). These findings led us to investigate further the possible importance of sulfhydryl groups and tryptophan in the toxic activity of these proteins. The results reported below support the concept of an essential role for sulfhydryl groups in an "active-site mechanism" and further implicate tryptophan residues in such a mechanism.

Materials and Methods

Cultural Conditions and Toxin Isolation and Assay. Cells of *Y. pestis*, avirulent strain Tjiwidej, were grown at room temperature in flask cultures on an enzymatic casein hydrolysate–glucose–salts medium. After 7-days incubation toxin is released during autolysis of the cells. Toxin was isolated following removal of cellular debris by centrifugation. Crude preparations containing both toxins A and B were isolated employing ammonium sulfate fractionation between 35 and 70% saturation (Englesburg and Levy, 1954). The concentrated crude toxin was further purified primarily by separation on a Büchler preparative gel electrophoresis as described previously (Montie and Montie, 1969), or by separation on Sephadex G-100 or G-200 columns (Montie *et al.*, 1966b). Toxin A obtained by the preparative gel method had a consistently higher specific toxic activity than toxin B. Therefore toxin A was employed in most of the experiments reported here unless otherwise stated. Toxin purity was assayed by acrylamide gel electrophoresis at pH 9.0. Biological activity was determined by intraperitoneal injection of toxin into 14–22-g mice. For each experiment mice only varying within 4 g were chosen for assay. Two inbred mouse strains (C-57 B1, and C_3H) were used for most of the assays since assay with the inbred mice proved more sensitive and reproducible than assay with the Swiss albino mice. Toxin LD_{50} values were determined from maximal and minimal lethal doses and expressed in terms of micrograms of Lowry protein (Oyama and Eagle, 1956) employing bovine serum albumin (Pentex) as the standard. Toxin samples were treated with inhibitor for a minimum of 30 min before injection.

Chemicals. Sodium dodecyl sulfate was obtained from Matheson, Coleman & Bell and recrystallized by dissolving it in 95% ethanol and precipitating with acetone. Isotopic mercuric chloride ($^{203}HgCl_2$, specific activity 1.01 Ci/0.01

† From the Department of Microbiology, University of Tennessee, Knoxville, Tennessee. *Received June 4, 1973.* This research was supported by the National Science Foundation Grant GB-12893 and a University of Tennessee Biomedical Sciences support grant (FR-7088).

mol) was obtained from Amersham-Searle Co. Radioactivity was measured using a Packard Tri-Carb, Model 3375, liquid scintillation counter.

Fluorescence Quenching Assay. Fluorescence quenching spectra of tryptophan were obtained by measuring excitation at 290 nm using a spectrofluorometer designed by Dr. J. Churchich (Churchich, 1967). The sample in a 1-cm cuvette was illuminated by monochromatic light obtained by passing the output of a xenon lamp through a Bausch and Lomb monochromator. An EMI phototube (62565) was used as the fluorescence detector.

Fluorescein Mercuric Acetate Assay for Sulfhydryls. The method of Heitz and Anderson (1968) modified from Karush *et al.* (1964) was used to determine the reaction of fluorescein mercuric acetate with sulfhydryls. Quenching of fluorescein mercuric acetate was measured by excitation of solutions at 495 nm and detection of emission at 525 nm. Measurements were made using an Aminco Bowman spectrofluorometer with a xenon mercury lamp, Pacific photometric recording photometer, Model 15.

Difference Spectra Measurements of Mercury–Tryptophan Reactions. Measurements of ultraviolet difference spectra were carried out using a Perkin-Elmer, Coleman Model 124, double-beam spectrophotometer equipped with a Beckman Model 1005, 10-in. linear–log recorder.

Results

Heavy-Metal Inactivation Related to Toxin Structural State. Previous studies with toxin obtained by Sephadex chromatography demonstrated that toxin was suspectible to sulfhydryl reagents (Montie *et al.*, 1966a). These studies also demonstrated that the most potent SH reagent found, silver ion, required an excess of from 40–60 to 1 (metal to SH) to achieve inactivation of isolated toxin. In general agreement with the above results experiments showed that more highly purified, "preparative-gel" toxin A (designated native toxin A) also became susceptible only at high ratios of metal to SH group (Table I). Almost no inactivation occurred at a level of 4-fold mercury equiv or 20–40-fold silver ion to SH. Some inhibition was noted at levels of 20:1 or 40:1 with mercury; the LD_{50} in the mercury-treated preparations increased from 1.0 to 2.5 or >4 μg of protein, respectively. In contrast were results using a dissociated polymeric intermediate from toxin A that had been separated from native polymer by preparative acrylamide gel electrophoresis. The increased mobility of this material in analytical acrylamide gels indicated a smaller size than native toxin confirmed by experiments using Sephadex filtration (Montie *et al.*, 1966a). It was shown that these preparations were almost totally inactivated by 20- or 40-fold excess of Ag^+ (Table I).

We had demonstrated previously that toxin dissociated in sodium dodecyl sulfate (0.5–1.0%) formed highly toxic 1.7S subunits (Montie *et al.*, 1968). It was suspected that the subunit preparations, because of their disaggregated condition, might be more sensitive to inhibition by metallic ions. Toxin was dissolved in 1% sodium dodecyl sulfate at 37° for 3 hr followed by dialysis against 0.1% sodium dodecyl sulfate. Final concentration in the dialysis bag is approximately equivalent to 0.5% sodium dodecyl sulfate (Montie and Montie, 1971). It was found that toxin dissociated in sodium dodecyl sulfate (0.5–1.0%) was characterized by increased susceptibility to metal SH reagents. Addition of approximately 1.5- to 3-fold equiv of Ag^+ to SH groups caused at least a 7-fold loss in toxicity (Table I). Even at levels below 1:1 Ag^+ to SH inactivation was observed. This subunit material, however, showed only slight inhibition with iodoacetate, iodoacetamide, *N*-ethylmaleimide, and dithiobis(nitrobenzoic acid). Some detoxification by the latter inhibitors had been obtained previously using polymeric toxin obtained by Sephadex filtration. In order to obtain even partial inhibition with these reagents, however, excess quantities (40–60-fold) were required which exceeded levels needed for metal inactivation of native toxin (Montie *et al.*, 1966a).

TABLE I: Inactivation of Native Toxin and Dissociated Toxin with Silver and Mercury Ions.

Expt	Sample[a]	Mole of Metal:Mole of SH	LD_{50} (μg of Protein)
1	Toxin A (native)[b]		<4
	Toxin A plus Ag^+	20:1	<4
		40:1	<4
2	Toxin A (native)[b]		1
	Toxin A plus Hg^{2+}	4:1	1
		20:1	2.5
		40:1[d]	>4
3	Toxin A (acrylamide) column "subunit"[c]		<4
	Toxin A plus Ag^+	20:1	6
		40:1	>24
4	Toxin A (acrylamide) column "subunit"[c]		<2
	Toxin plus Hg^{2+}	20:1	<4
		40:1	8–24
5	Toxin A + B[e] subunits in sodium dodecyl sulfate (0.5–1%)		3
	Toxin A + B plus Ag^+	0.31:1	5–10
		1.50:1	>20
		3.25:1	>10

[a] Toxin was dissolved in sodium phosphate buffer (0.01 M, pH 7.1). [b] Toxin obtained by preparative gel acrylamide electrophoresis. [c] Partial dissociation products of toxin A (see text). [d] Some of this material was precipitated at the high mercury concentration. [e] Toxin A and B mixture obtained by Sephadex column filtration.

Attempts to reverse SH-inhibited 1.7S subunits using excess glutathione (0.125 M) were unsuccessful. This was the case even when time of exposure to silver was limited to ten minutes in an effort to avoid nonspecific denaturation. These data suggested that the bound sodium dodecyl sulfate (equivalent to approximately one-half the toxin weight) created a barrier which prevented penetration of organic inhibitors, or organic reducing reagents.

In order to avoid either denaturation of toxin by sodium dodecyl sulfate or to avoid excess binding of sodium dodecyl sulfate to protein, but still perturb toxin, preparations of toxin A were treated with reduced concentrations of sodium dodecyl sulfate (0.05%). This procedure sufficiently altered toxin A, without inactivation, so that low concentrations of metals reacted with toxin (Table II). Results obtained following titration with mercury ions showed that mole ratios of metal to SH of 1.3:1 caused loss of two-thirds of the total toxicity of the preparation. Further addition of mercury ions to a

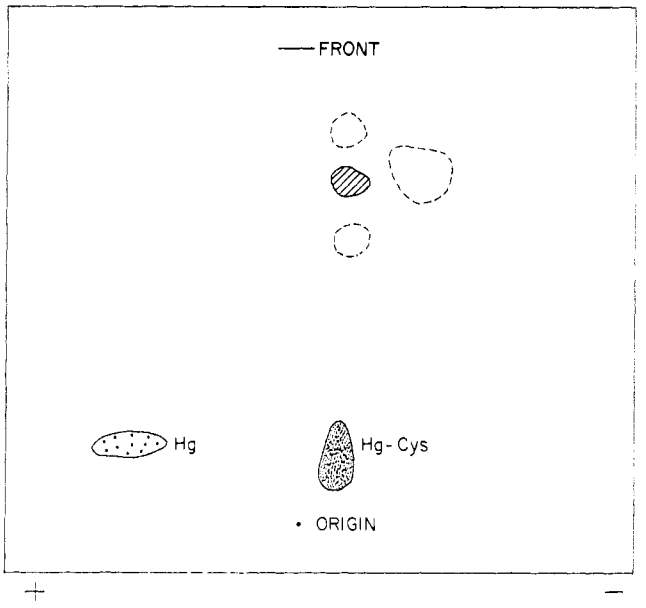

FIGURE 1: Isolation of mercury-labeled cysteine from Pronase digests of toxin A. Toxin A (7.3 mg of protein) was dissolved in 1.0 ml of 0.05% sodium dodecyl sulfate (pH 7.3). Radioactive mercury chloride (25 μCi) was reacted for 3 hr with toxin at a mole ratio of Hg^{2+}:SH of 4:1. The reaction mixture was dialyzed and further denatured with 8 M urea and 1 M sodium chloride, dialyzed, and treated with trypsin as described previously (Montie and Montie, 1971). The tryptic digest (1.2 mg) was concentrated by lyophilization and treated with 0.05 mg of Pronase at 37° for 3.5 hr and then treated for 2 hr at 25°. The preparation was concentrated by lyophilization, and the sample was subjected to fingerprinting on 20 × 20 cm cellulose preformed sheets (Eastman Kodak no. 6065). Electrophoresis (400 V and 20 mA for 1.5 hr) was performed using a Shandon electrophoresis apparatus in the first dimension in pyridine–acetic acid–water (10:1:100, v/v). Butanol–proprionic acid–water (3:2:1, v/v) solvent was employed for ascending chromatography in the second dimension for 3 hr. Spots were developed with ninhydrin. The chromatogram was then placed on Kodak No-Screen X-Ray Film and exposed for 1 week prior to development. Designated radioactive spots were as follows: Hg–Cys, mercury–cysteine complex; Hg, free mercury; striped spot, weakly labeled, suspected mercury–tryptophan complex; dashed spots, weakly labeled spots, probably small tryptophan-containing peptides.

level of 10:1 increased the LD_{50} tenfold. Toxin A was inhibited by a number of silver salts comparable to inhibition with mercury (Table II). These data indicated that the anionic component was not important in the detoxification mechanism by cationic metals.

TABLE II: Inactivation of Toxin A in 0.05% Sodium Dodecyl Sulfate with Silver and Mercury Ions.

Sample[a]	Mole of Metal:Mole of SH	LD_{50} (μg of Protein)
Toxin A		0.3–0.4
Toxin A + mercuric chloride	1.3:1	1.0
	2:1	1.5
	4:1	1.5
	10:1	3.0
Toxin A + silver nitrate	2:1	0.75
Toxin A + silver acetate	2:1	0.75
Toxin A + silver sulfate	4:1	1.5

[a] Toxin A (5 mg) was dissolved in 10 ml of 0.001 M sodium phosphate (pH 7.1) and sodium dodecyl sulfate (0.05%). Samples were incubated in the dark and then injected.

TABLE III: Inactivation of Toxin A in 0.05% Sodium Dodecyl Sulfate with Fluorescein Mercuric Acetate (FMA).

Expt	Sample	Mole of Metal: Mole of SH)	Toxin (μg Injected)	Mice Died/ Mice Injected	LD_{50} (μg of Protein)
1[a]	Toxin		0.4	0/6	0.7
			1.0	6/6	
	Toxin + FMA	1:1	1.0	5/6	<1.0
		2:1	1.0	5/6	<1.0
		4:1	1.0	3/6	1.0
			2.0	6/6	
2[b]	Toxin		2.0	4/6	2
	Toxin + FMA	10:1	2.0	0/6	>4 to <20
			4.0	0/6	
			20.0	6/6	

[a] Toxin A (0.4 mg) was dissolved in 4.0 ml of sodium pyrophosphate (0.01 M, pH 7.5) and sodium dodecyl sulfate (0.05%). FMA was dissolved in sodium pyrophosphate (0.1 M, pH 8.0). [b] Toxin A (1.2 mg) was dissolved in 3.2 ml of 0.01 M sodium phosphate (pH 7.1) and sodium dodecyl sulfate (0.05%). FMA was dissolved in sodium pyrophosphate (0.1 M, pH 8.0).

Fluorescein mercuric acetate, another sulfhydryl reagent, was also found to be a potent inhibitor of toxicity. Unlike the metal ions, however, fluorescein mercuric acetate showed little to no inhibition of the toxin in 0.5–1.0% sodium dodecyl sulfate, but it was effective in 0.05% sodium dodecyl sulfate (Table III). This result supported our previous concept that high concentration of protein-bound sodium dodecyl sulfate form a barrier which inhibits penetration by certain larger, and perhaps negatively charged organic SH reagents. Even at low sodium dodecyl sulfate concentrations (0.05%), toxin was less sensitive to fluorescein mercuric acetate than to the metals. The precise degree of inhibition at low concentration of fluorescein mercuric acetate was difficult to establish accurately. Results in Table III showed that at mole ratios of 1:1 or 4:1 fluorescein mercuric acetate to SH, inactivation increased the LD_{50} merely by 0.3 μg of protein. Addition of tenfold fluorescein mercuric acetate, however, clearly demonstrated a loss of most of the toxic activity.

The sodium dodecyl sulfate concentration was decreased to 0.05%, and preparations were tested for reversal of metal inhibition in the presence of high concentrations of reducing agents. These attempts were unsuccessful even though metal concentration, time of toxin exposure, and buffer conditions were varied in an effort to achieve optimum conditions for toxin reactivation. In some experiments EDTA was added to a reducing environment without any enhancement of reactivation. These results suggest that low concentrations of sodium dodecyl sulfate (0.05%) surround the active site.

Influence of Buffer Conditions on Toxin Inactivation. Because of the increased solubility of metallic salts and their respective toxin complexes in Tris buffer (pH 7.5–8.0), we tested Tris-Cl as the standard inactivation medium. However, toxin in Tris (pH 8.0) and 0.05% sodium dodecyl sulfate treated with silver ions was much less susceptible to inactivation when compared to toxin in sodium phosphate buffer

TABLE IV: Relationship of Buffer to Toxin Susceptibility to Silver Ions.

Expt	Sample[a]	Mole of Metal:Mole of SH	Toxin (μg Injected)	Mice Died/Mice Injected	LD_{50} (μg of Protein)
1	Toxin in Tris (pH 8.0)		2.5	6/6	<2.5
	Toxin in phosphate (pH 7.1)		2.5	2/6	2.5
	Subunits in Tris		5.0	6/6	<5.0
	Subunits in phosphate		5.0	0/6	10.0
			10.0	2/5	
	Subunits in Tris + Ag^+	3:1	2.5	3/6	2.5–5.0
			5.0	4/6	
	Subunits in phosphate + Ag^+	3:1	10.0	0/5	>10.0
2	Toxin in Tris (0.001 M, pH 7.5) and SDS[b] (0.05%)		0.25	0/6	0.5
			0.5	2/6	
	Toxin in Tris (0.001 M, pH 7.5) and SDS (0.05%) + Ag^+	4:1	0.5	3/6	0.5
			1.0	6/6	
	Toxin in SDS (0.05%) + Ag^+ + 30 min + Tris (0.85 M, pH 7.5)	4:1	0.5	6/6	<0.5
	Toxin in SDS (0.05%) + Ag^+ + 30 min + glutathione (0.125 M) and Tris (0.85 M) (pH 7.5)	4:1	0.5	2/6	0.5
			1.0	6/6	

[a] Expt 1: toxin or subunit (1.7 S) preparation used was a mixture of toxin A + B from Sephadex columns; buffer concentrations were 0.01 M. Expt 2: native toxin A preparations from preparative acrylamide gel electrophoresis were used. [b] SDS = sodium dodecyl sulfate.

(pH 7.1) and sodium dodecyl sulfate (0.05%) treated with silver ions (Table IV). Little to no change in LD_{50} of the silver-treated subunit preparation was noted in Tris buffer. Also, it was observed that untreated toxin generally retained or showed increased activity in Tris (LD_{50} < 2.5 μg) compared to toxin in sodium phosphate (LD_{50} = 2.5 μg). Experiments performed with Tris adjusted to pH 7.5 (Table IV) suggested that the protective effect was related to the nature of Tris buffer itself, rather than an activity related to pH. In Table IV (expt 2) results showed that 0.001 M Tris and 0.05% sodium dodecyl sulfate not only protected toxin against silver, but also, at higher concentrations of Tris (0.85 M) and 0.05% sodium dodecyl sulfate, the specific toxic activity was increased from 0.5 μg to well below 0.5 μg. Inclusion of reduced glutathione in the high Tris concentration sample did not enhance activity.

Reaction of Toxin with ^{203}Hg; Isolation of the Cysteine Complex. Although mercury and silver ions are considered effective SH reactants (Webb, 1966) direct proof of their reaction with toxin sulfhydryls was sought. Toxin was treated with $^{203}HgCl_2$ and submitted to protease digestion in an effort to obtain a labeled complex. Extensive dialysis was performed against urea or guanidine hydrochloride and finally against sodium bicarbonate (Montie and Montie, 1971). The dialyzed protein was lyophilized and then resuspended for trypsin and then Pronase digestion. It was shown that the ^{203}Hg label on tryptic peptides could be removed by addition of dithiothreitol.

The labeled amino acid digest was subjected to two-dimensional electrophoresis–chromatography as described previously (Montie and Montie, 1971). A large radioactive spot was observed near the origin (Figure 1). The location of this spot corresponded to the mobility of standard Hg–cysteine. Moreover, this spot was never visible in peptide digests labeled with mercury. These results indicated that Hg^{2+} was attached to cysteine residues following addition of metal to toxin in 0.05% sodium dodecyl sulfate. Several weakly radioactive spots were also observed in or near the neutral electrophoretic zone. These spots showed rapid mobility in the chromatographic solvent. The location of one of these spots (R_F 0.58) suggests that it is a mercury–tryptophan complex in agreement with data reported below. Three spots were designated as peptides containing tryptophan since these spots are coincident with peptides from tryptic digests of toxin prelabeled with [^{14}C]tryptophan. These spots probably represent small peptides because they are found in Pronase digests. They may also represent peptides containing cysteine.

Fluorescence Quenching of Fluorescein Mercuric Acetate by Toxin. Fluorescein mercuric acetate has been used to titrate sulfhydryl groups specifically (Karush *et al.*, 1964). Reaction of sulfhydryls results in quenching of fluorescein mercuric acetate emission at 525 nm. It was noted that reaction of fluorescein mercuric acetate with toxin was time dependent. Maximum quenching occurred after approximately 30 min. Toxin was titrated in tubes containing a constant concentration of toxin followed by incubation with increasing amounts of fluorescein mercuric acetate for 3 hr to ensure maximum reaction. A constant ratio of fluorescein mercuric acetate to toxin where maximum quenching of fluorescein mercuric acetate occurred is indicative of the total number of sulfhydryl groups according to Karush *et al.* (1964) and Heitz and Anderson (1968).

The data in Figure 2 show that the fluorescein mercuric acetate fluorescence was quenched in the presence of toxin. In these experiments toxin was kept in excess, and fluorescein mercuric acetate was allowed to react for 2–3 hr to achieve

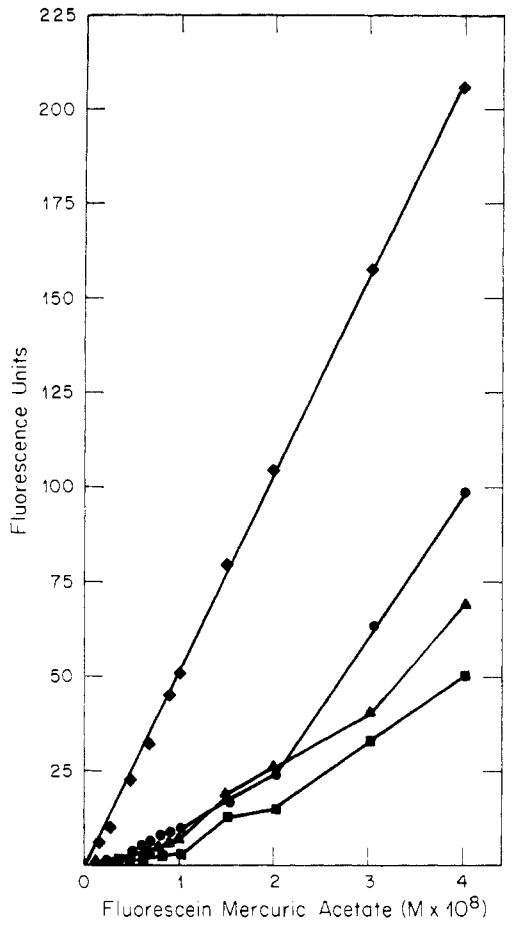

FIGURE 2: Fluorescence quenching of fluorescein mercuric acetate by toxin. Toxin A (1.8 mg of protein) was dissolved in 1 ml of 0.05% sodium dodecyl sulfate in sodium pyrophosphate buffer (0.1 M, pH 8.0). Toxin was diluted in water and 0.05% sodium dodecyl sulfate giving a final molarity of 0.001 M sodium pyrophosphate in a total volume of 3.0-ml solutions containing: no toxin (♦), 7.5 μg of toxin (●), 15.0 μg of toxin (▲), 30 μg of toxin (■). Reaction mixtures were incubated for 3 hr at 25° and readings were recorded at 525 nm.

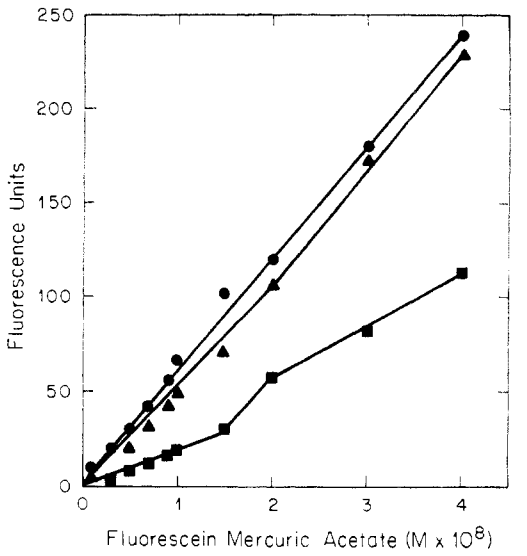

FIGURE 3: Silver ion interference of fluorescein mercuric acetate interaction with toxin SH. Toxin A (212 μg of protein) was dissolved in 1.0 ml of 0.05% sodium dodecyl sulfate in sodium pyrophosphate (0.1 M, pH 7.5), and then reacted with silver sulfate (8:1 mole ratio of Ag^+:SH) for 1.5 hr. The reaction mixture was then diluted to 40.0 ml with 0.05% sodium dodecyl sulfate. Fluorescein mercuric acetate was added to separate 3.0-ml aliquots of the reaction mixtures containing 15 μg of toxin (final pyrophosphate buffer was 0.001 M). The reaction mixtures were incubated 2 hr at 25°, and readings recorded at 525 nm: fluorescein mercuric acetate alone (●), toxin + fluorescein mercuric acetate (■), toxin + silver sulfate + fluorescein mercuric acetate (▲).

maximum quenching. No precise stoichiometric relationship was evident as described for yeast alcohol dehydrogenase by Heitz and Anderson (1968). The lowest ratios of toxin SH to fluorescein mercuric acetate were 5-, 10-, and 20-fold at the terminal titration point (4×10^{-8} M fluorescein mercuric acetate) for the 7.5-, 15-, and 30-μg toxin curves, respectively. If lower ratios of toxin to fluorescein mercuric acetate were used, or if excess fluorescein mercuric acetate was titrated against toxin, the method lacked sensitivity, and the results were inconclusive. Since only one cysteine residue has been identified per 12,000 mol wt unit of toxin, it is understandable that large amounts of toxin were required to obtain measurable amounts of quenching. For comparison purposes cysteine also was titrated with fluorescein mercuric acetate. No time dependence was observed for the latter reaction. The maximum quenching obtained was only approximately 40% of the control fluorescein mercuric acetate sample even at concentrations of a 200-fold excess cysteine to fluorescein mercuric acetate (4×10^{-8} M). A 200-fold excess of toxin to fluorescein mercuric acetate gave 80% quenching. Apparently toxin protein contains additional quenching properties probably due to interaction of bound fluorescein mercuric acetate with adjacent side chains. Both requirement for excess fluorescein mercuric acetate, and also the increase in time dependence for this reaction compared to immediate quenching of tryptophan observed with heavy metals, underline the difficulty of penetration into sulfhydryl sites by fluorescein mercuric acetate.

Using a similar approach, evidence was obtained showing that fluorescein mercuric acetate and silver ions were reacting at the same sites in toxin molecules. The experiment described in Figure 3 was similar to the one outlined in Figure 2 except that some toxin samples were pretreated with silver ions (8:1, Ag^+:SH) for 90 min followed by fluorescein mercuric acetate treatment. The lack of any appreciable fluorescence quench in the samples pretreated with silver indicated that silver ions were effectively competing with fluorescein mercuric acetate for sulfhydryl sites.

Tryptophan Fluorescence Quench in Toxin by Silver or Mercury Ions. The reaction of silver or mercury ions with sulfhydryls was assayed by titrating the fluorescence quenching of tryptophan residues of toxin A in 0.05% sodium dodecyl sulfate. Quenching may be the result of the formation of mercaptide bonds which interfere with tryptophan fluorescence and induce quenching by intersystem crossing (Steiner, 1971). This quenching may also indicate proximity of a tryptophan residue to a free sulfhydryl. As we increased the silver ion level from a ratio of 2:1 Ag^+:SH to 4:1, we observed a decrease in the tryptophan fluorescence emission spectrum, and almost complete quench was observed at 8:1 (Figure 4A). The abolition of tryptophan emission, with a maximum at 337 nm, coincided with a loss of toxicity. Tyrosine fluorescence contributed an insignificant amount to total fluorescence emission.

Similar results were found with mercury ions with respect to quenching of tryptophan fluorescence (Figure 4B). This indicated a parallel mode of action for these metal ions. Increased binding of sulfhydryl, as assayed here, correlated

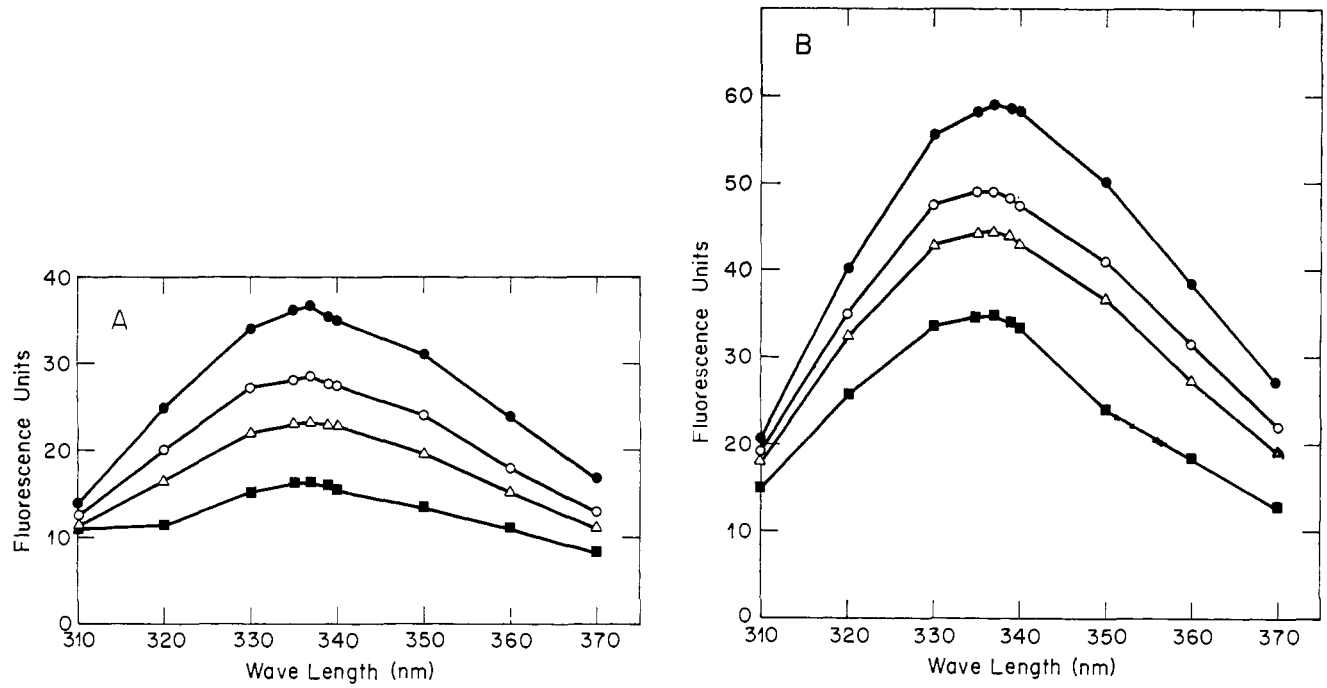

FIGURE 4: Quenching of tryptophan fluorescence. (A) By silver ions: toxin A (228 μg of protein) was dissolved in 0.05% sodium dodecyl sulfate in sodium phosphate (pH 7.1). Silver sulfate was added sequentially and changes in the emission spectrum at excitation wavelength of 290 nm were recorded after each addition: no addition (●), 2:1 Ag^+:SH (○), 4:1 (△), 8:1 (■). The control toxin sample at 0.6 μg killed 6/6 mice injected. Treated sample (4:1) at 1.0 μg killed 0/6 mice injected. (B) By mercury ions: toxin A (228 μg) was dissolved in 0.05% sodium dodecyl sulfate in sodium phosphate buffer (0.01 M, pH 7.3). Mercuric chloride was added sequentially and spectrum changes at excitation wavelength of 290 nm were recorded after each addition: no addition (●), 1:1 Hg^{2+}:SH (○), 2:1 (△), 4:1 (■). The control sample at 0.06 μg killed 6/6 mice injected. Treated sample (4:1) at 0.6 μg killed 4/6 mice injected.

with inactivation of toxin. Native toxin A in buffer alone did not show fluorescence quenching in the presence of low levels of metal. This lack of response correlated with resistance to inactivation of biological activity.

In contrast to the effects of mercury or silver ions, when a more general denaturant, guanidine hydrochloride (6 M), was employed, toxin fluorescence was quenched coincident with a shift in tryptophan maximum from 337 to 350 nm. We concluded that a conformational change occurred in guanidine hydrochloride solutions, but that such major structural changes did not occur following inactivation of toxin in the presence of 0.05% sodium dodecyl sulfate with low concentrations of silver or mercury ions.

Detection of a Mercury–Tryptophan Complex in Toxin. Ramachandran and Witkop (1964) and more recently Chen (1971) reported results indicating that mercuric ions can form a 1:1 complex with free tryptophan. Complex formation does not occur with silver ions. The mercury–tryptophan complex is readily distinguished by difference spectra showing a maximum at 297 nm and a valley at 288 nm. It was found that toxin A treated with low concentrations of mercury ions showed evidence for formation of complex (Figure 5). At a ratio of Hg^{2+} to tryptophan of 2.5:1 a reaction was readily detectable. At levels of 10:1 maximum amount of the tryptophan–toxin reaction was observed. The spectrum for mercury which was reacted with free tryptophan was almost identical with the toxin profiles confirming that mercury interacted with tryptophan in the toxin.

Free tryptophan was titrated with mercury ions by using difference spectrum measurements at 297 nm and varying the ratio of mercury to tryptophan as the tryptophan concentration was increased. The linear plot which resulted showed that the 1:1 reaction of mercury with tryptophan is independent of the mole ratio of mercury to tryptophan, provided mercury is not limiting.

Discussion

Results of these studies using purified plague murine toxin have underlined the importance of free sulfhydryl groups for biological activity in this protein. Toxin A of high specific toxic activity purified by preparative gel electrophoresis was inhibited by silver or mercury ions only at relatively high metal concentrations. A similar response was obtained with toxin A or B isolated by separation on Sephadex columns (Montie *et al.*, 1966a). These results have emphasized that the majority of sulfhydryl groups are not readily accessible in the native protein. These groups become more available to sulfhydryl reagents after toxin is dissociated to subunits or when the protein is slightly perturbed with weak detergent concentrations. Previous data (Montie *et al.*, 1966b; Montie and Montie, 1971) indicating that toxin A contains identical subunits with important single sulfhydryl and tryptophan residues per 12,000 mol wt unit are reinforced in these studies which demonstrate nearly stoichiometric reactions between SH and low concentrations of silver or mercury ions. At low concentrations fluorescein mercuric acetate, a demonstrated sulfhydryl reagent, inhibited biological activity. Interaction of the metal SH reagents with toxin A, as documented by fluorescence quenching, was correlated closely with loss in toxicity. Detection of partial toxicity loss at ratios of metal to SH below one indicated that a 1:1 reaction is occurring.

The significance of the structural state of toxin with respect to detoxification by SH reagents is also worth emphasizing. Thus, subunit material which is partially dissociated during purification exhibits a somewhat enhanced sensitivity to in-

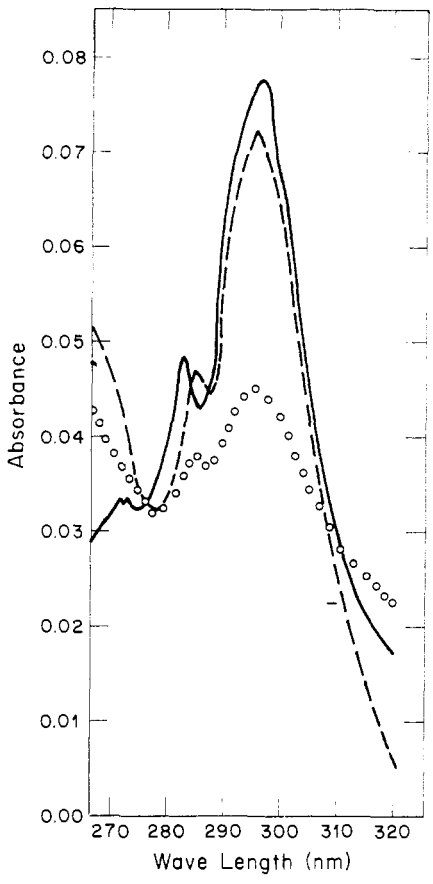

FIGURE 5: Interaction of mercury ions with free tryptophan and tryptophan in toxin. Tryptophan was dissolved in 0.05% sodium dodecyl sulfate in 0.01 M sodium phosphate (pH 7.2). Mercuric chloride was added at a mole ratio of 4:1 (—) Hg^{2+}:tryptophan, and difference spectra were recorded between tryptophan and tryptophan plus mercury. Toxin A (440 μg of protein) was dissolved in 0.05% sodium dodecyl sulfate in 0.01 M sodium phosphate (pH 7.2). Mercuric chloride was added at a mole ratio of ions to tryptophan in toxin of 2.5:1 (○) and 10:1 (- -), and difference spectra were recorded after 2 min.

activation by these reagents when compared to the parent polymer material. The dissociated material thus seems to represent a conformational form intermediate between native polymer and sodium dodecyl sulfate perturbed material as judged by response to SH inhibitors.

Although sulfhydryl groups are not readily accessible in native toxin molecules, addition of sodium dodecyl sulfate, which does not affect toxicity, in some way enhances interaction of inhibitor with SH. This absence of any deleterious action of the detergent itself, together with the observation that tryptophan fluorescence in sodium dodecyl sulfate is identical with the fluorescence spectrum of untreated toxin, strongly indicates that little or no change in native structure has occurred in sodium dodecyl sulfate. These findings are in agreement with results of Reynolds and Tanford (1970), who demonstrated that sodium dodecyl sulfate does not reduce proteins to random coils, but in fact facilitates retention of a high degree of order. Moreover, Jirgensons and Capetillo (1970) noted that sodium dodecyl sulfate orders to a degree the structure of previously unordered peptide chains of certain nonhelical proteins. The sodium dodecyl sulfate action appears in contrast to the denaturing action of guanidine hydrochloride as indicated by the shift in the fluorescence spectrum with the latter compound.

Slight perturbation with 0.05% sodium dodecyl sulfate resulting in SH susceptibility may indicate that the hydrophobic environment near the metal ion sensitive site is important in protection of SH. In support of this concept Seibles (1969) concluded from experiments using ^{35}S-labeled sodium dodecyl sulfate that at low concentrations binding of sodium dodecyl sulfate to β-lactoglobulin occurred in small discrete regions involving a histidine residue. This interpretation of selectivity of action of sodium dodecyl sulfate seems more acceptable than the concept of sodium dodecyl sulfate altering coiled protein structure, thus eliminating the protection of SH by steric hindrance and exposing the SH residue.

It is interesting that apparently Tris also created a favorable environment for toxicity. The observation that Tris enhances toxin activity cannot be readily explained except to state that this buffer contributes in a more general way toward maintenance of toxin conformation. Additional protection by Tris against metals probably involved formation of stable complexes between Tris and silver ions as reported by Cecil and McPhee (1959).

Inactivation of toxin with concentrations of sulfhydryl reagent equivalent to thiol indicates that primarily sulfhydryl groups are reacting. The following evidence further supports this conclusion. (1) A radioactive spot coinciding with known Hg–cysteine was identified in proteolytic digests following reaction of $^{203}Hg^{2+}$ with toxin. (2) Fluorescein mercuric acetate, a specific SH reagent, is quenched following reaction with toxin. This reaction is blocked by pretreatment with silver ions. (3) Silver and mercury ions form mercaptides which induce quenching of tryptophan fluorescence emission.

The effect of SH reagents in causing quenching of tryptophan fluorescence has been reported by a number of authors. Beers and Reich (1969) showed quenching of fluorescence in *Clostridium botulinum* toxin type B. Rogers (1969) demonstrated similar activity when experimenting with a series of sulfhydryl-containing dehydrogenase enzymes. These authors attributed fluorescence changes to an indirect effect of SH block in causing alteration in conformation of these proteins. Chloromercuribenzoate caused a slight shift in the fluorescence emission spectrum of native botulinum type B toxin suggesting a general structural alteration (Beers and Reich, 1969). In contrast the tryptophan emission spectrum of murine toxin is not shifted by addition of low levels of mercury or silver ions. Consequently, we explain quenching in murine toxin as due to an energy-transfer mechanism resulting from an overlap of the mercaptide-bond absorption with tryptophan fluorescence (Steiner, 1971). Chen (1971) explains the effects of mercury on fluorescence of egg ovalbumin in terms of an energy-transfer mechanism with a critical transfer distance of 17.0 Å.

A finding of particular interest was that mercury also may be interacting directly with the indole ring of tryptophan residues in toxin. The possibility of this type of reaction was indicated by detection of a specific difference spectrum (297-nm maximum). Such a reaction was first reported by Ramachandran and Witkop (1964). Chen (1971) studied the reaction with free tryptophan in detail. Additionally he offered the following data supporting this explanation for Hg^{2+} reaction in certain proteins: (1) the difference spectrum with a 297-nm maximum was absent in metal-treated staphylococcal nuclease which contained cysteine, but no tryptophan; (2) this spectrum was detected in a series of proteins and enzymes containing tryptophan; (3) formation of difference spectra was inhibited by competition with Tris or chloride groups indicating the interaction of Hg^{2+} with weak ligands rather than SH.

The importance of tryptophan in toxin has been earlier suggested by the observation that cell extracts are less toxic following incorporation of tryptophan analogs into *Y. pestis* cells (Montie *et al.*, 1964).

It is worthy of note that other toxic proteins contain essential tryptophan residues. Such residues are found in snake neurotoxins (Seto *et al.*, 1970), and also the botulinum toxin type A (Boroff and Dasgupta, 1966) or type B (Beers and Reich, 1969). Botulinum type B may also contain an essential sulfhydryl group possibly close to the tryptophan residue (Beers and Reich, 1969). Kandel and Collier (1972) have recently reported an important tryptophan in diptheria toxin.

It seems likely, from all the data, that both sulfhydryl and tryptophan are located in the same microenvironment that is associated with toxic activity in the protein. Montie *et al.* (1966b) have reported that there are single cysteine and tryptophan residues per 12,000 mol wt unit in toxin A. This finding would suggest the 12,000 mol wt unit is the minimum unit required for toxicity. Identification of such small toxic subunits dissociated with detergent has been documented (Montie and Montie, 1971). Recent studies concerned with toxin action *in vivo* have indicated that toxin has insulin-like effects correlated with its lethal activity (Wennerstrom and Montie, 1973). The importance of this finding relates to the interesting report by Lavis and Williams (1970) that a series of thiol compounds gave insulin-like effects when incubated with isolated fat cells of the rat. These insulin-like effects were related to the presence of sulfhydryl residues. These thiols at lower concentrations seemed to share with insulin a common pathway of action. The parallel between insulin, thiols, and toxin is currently being investigated.

Acknowledgment

The authors thank Dr. Jorge Churchich for the use of his spectrofluorometer. Also, we thank Dr. Richard Irwin for his helpful suggestions. The authors acknowledge the very able technical assistance of Ms. Carole Kennedy and Ms. Joan Freesh.

References

Beers, W. H., and Reich, E. (1969), *J. Biol. Chem. 244*, 4473.

Boroff, D. A., and Dasgupta, B. R. (1966), *Biochim. Biophys. Acta 117*, 289.

Cecil, R., and McPhee, J. R. (1959), *Advan. Protein Chem. 14*, 256.

Chen, R. F. (1971), *Arch. Biochem. Biophys. 142*, 552.

Churchich, J. E. (1967), *J. Biol. Chem. 242*, 4414.

Englesberg, E., and Levy, J. B. (1954), *J. Bacteriol. 68*, 57.

Heitz, J. R., and Anderson, B. R. (1968), *Arch. Biochem. Biophys. 127*, 637.

Jirgensons, B., and Capetillo, S. (1970), *Biochim. Biophys. Acta 214*, 1.

Kandel, J., and Collier, R. J. (1972), *Abstr. Annu. Meeting Amer. Soc. Microbiol., Minneapolis, Minn.*, 105.

Karush, F., Klinmar, N. R., and Marks, R. (1964), *Anal. Biochem. 9*, 100.

Lavis, V. R., and Williams, R. H. (1970), *J. Biol. Chem. 245*, 23.

Montie, T. C., and Montie, D. B. (1969), *J. Bacteriol. 100*, 535.

Montie, T. C., and Montie, D. B. (1971), *Biochemistry 10*, 2094.

Montie, T. C., Montie, D. B., and Ajl, S. J. (1964), *J. Exp. Med. 120*, 1201.

Montie, T. C., Montie, D. B., and Ajl, S. J. (1966a), *Arch. Biochem. Biophys. 114*, 123.

Montie, T. C., Montie, D. B., and Ajl, S. J. (1966b), *Biochim. Biophys. Acta 130*, 406.

Montie, T. C., Montie, D. B., Leon, S., Kennedy, C., and Ajl, S. J. (1968), *Biochem. Biophys. Res. Commun. 33*, 423.

Oyama, V. F., and Eagle, H. (1956), *Proc. Soc. Exp. Biol. Med. 91*, 305.

Ramachandran, L. K., and Witkop, B. (1964), *Biochemistry 3*, 1603.

Reynolds, J. A., and Tanford, C. (1970), *J. Biol. Chem. 245*, 5161.

Rogers, K. S. (1969), *Enzymologia 36*, 153.

Seibles, T. S. (1969), *Biochemistry 8*, 2949.

Seto, A., Sato, S., and Tamiya, N. (1970), *Biochim. Biophys. Acta 214*, 483.

Steiner, R. F. (1971), *Biochemistry 10*, 771.

Webb, J. L. (1966), Enzyme and Metabolic Inhibitors, Vol. III, New York, N. Y., Academic Press, p 818.

Wennerstrom, D. E., and Montie, T. C. (1973), *Abstr. Annu. Meeting Amer. Soc. Microbiol., Miami Beach, Fla.*, 109.