Protein Toxins of *Pasteurella pestis*. Subunit Composition and Acid Binding*

Thomas C. Montie and Diane B. Montie

ABSTRACT: The subunit structure of two mouse toxic proteins was investigated. Toxins A (240,000 molecular weight) and B (120,000 molecular weight) contain basic subunits of approximately 24,000 molecular weight determined after dissociation in SDS and assay by sodium dodecyl sulfate–gel electrophoresis and gel filtration. This value is in agreement with an $S^0_{20,w}$ of 1.7 S and the small number of peptides obtained for each toxin. Smaller polypeptide chains (12,000–14,000) are attained by dissociation with sodium dodecyl sulfate or citric acid and separation at low protein concentrations. Tryptic fingerprints showed a total of 14–16 peptides with 7 peptides alike or the same in toxins A and B. The toxins are dissociated with citric or acetic acid (pH 2.0–4.0). The rate and extent of dissociation with acetic acid is enhanced by the addition of chelating agents.

Citric acid remains bound to tryptic peptides following digestion and separation by electrophoresis and chromatography. EDTA does not bind to toxin in acid, indicating metals are completely removed, but with citric acid a complex may be formed through a metal bridge. These investigations together with previous studies showing differences in tryptophan composition and polypeptide chains in acid gel electrophoresis indicate that toxin B contains five or six subunits (toxin A 10 or 12 subunits) with each subunit containing two unlike 12,000 molecular weight chains, one chain common to both toxins A and B.

Two proteins toxic for mice and rats are produced by an avirulent strain of the plague bacillus *Pasteurella pestis*. Molecular weights of toxins A and B have been estimated to be 240,000 and 120,000, respectively (Montie *et al.*, 1966). Preliminary investigations indicated that both toxins contain the same size subunits after dissociation with sodium dodecyl sulfate (SDS).[1] These subunit preparations retained a large percentage of toxic activity initially present in the "native polymer" (Montie *et al.*, 1968). More recently, the toxins were dissociated in a phenol–acetic acid–urea electrophoretic system. These profiles showed two polypeptide chains present in each toxin with one chain common to both proteins (Montie and Montie, 1969).

Our overall investigations have centered on the comparative structure of these proteins in an effort to: (1) identify parallel properties that may explain their selective toxicity for mice and rats and (2) aid in the understanding of macromolecular interaction with specific host receptors. Data are presented in this report supporting the concept that the toxins are composed of basic subunits with each subunit containing two 12,000 molecular weight chains. The degree of homology between toxins A and B is compared by tryptic peptide mapping. In addition, a rather unique interaction of toxin with citric acid is reported suggesting a possible role of metals in toxin structure. The possible relationship of toxin binding to toxic interaction in the host is discussed.

Experimental Section

Isolation of Toxin. Toxins A and B were isolated using an ammonium sulfate fractionation followed by separation on Sephadex G-100 or G-200 columns (Montie *et al.*, 1966) or by a Büchler preparative gel electrophoresis apparatus as described previously (Montie and Montie, 1969). Toxin purity was assayed by disc electrophoresis at pH 9, and biological activity was determined by intraperitoneal injection of 16–18-g Swiss albino mice in the manner described previously (Montie *et al.*, 1966). Toxin LD_{50} values were from 1 to 4 μg of protein for toxin A and 2 to 8 μg for toxin B.

Ultracentrifugation Studies. Sedimentation velocity experiments were performed at 20° and 59,780 rpm in a Spinco Model E ultracentrifuge equipped with a schlieren optical system. A conventional 12-mm cell with an aluminum centerpiece was used. The samples were dissolved in SDS (Matheson Coleman & Bell, 95% pure recrystallized from ethanol) and sodium phosphate buffer (0.01 M, pH 7.1).

Molecular Weight Determination by Gel Electrophoresis. The molecular weight of toxin components was estimated by a modified method of Shapiro *et al.* (1967), using the following standard proteins: pepsin and trypsin (twice crystallized, Worthington Biochemicals), cytochrome *c* (from horse heart, Type III, Sigma Chemical Co.), bovine serum albumin (Pentex, twice crystallized), and ribonuclease A (Worthington twice crystallized). Samples were examined on cylindrical gels (Canalco) or using a slab technique with pulsing voltage (Ortec Co., Oak Ridge, Tenn.). Bands were stained with Amido Black or coomassie blue. Protein resistant to staining as a result of excess SDS in the gel was first fixed with 50% trichloroacetic acid overnight and then stained with coomassie blue in 20% trichloroacetic acid (Lenard, 1970).

Molecular Weight Determination by Gel Filtration. Toxin was dissociated either by dissolving it in 1 or 2 ml of 1.0% SDS for 17–24 hr or by pretreatment (37°, 3 hr) in 1.0% SDS followed by 17-hr dialysis against 0.1% SDS as described for SDS–gel electrophoresis (Shapiro *et al.*, 1967). Molecular weight determinations were carried out as described by Andrews (1965) on Sephadex G-100 or G-200 columns (1.5 × 90 cm). Columns were equilibrated with 0.1 M sodium phos-

* From the Department of Microbiology, University of Tennessee, Knoxville, Tennessee. *Received December 28, 1970.* This research is currently supported by a National Science Foundation grant (GB-12893), University of Tennessee Biomedical Grant FR-7088, and was previously supported in part by Public Health Service Grant 5 R01 AI-03866-08 from the National Institute of Allergy and Infectious Disease.

[1] Abbreviations used are: SDS, sodium dodecyl sulfate; V_0, void volume; V_e, elution volume.

phate buffer (pH 7.1). Blue Dextran was incubated and applied in SDS with standard protein to determine V_0. To avoid binding, Blue Dextran was not mixed with cytochrome *c* or toxin samples, but marker dye was run before and after passage of these proteins.

Sucrose Gradient Analysis. Sucrose gradients (4.5 ml) were formed from 5 to 20% in KCl (0.05 M) and stored overnight at 4°. Toxin or standard proteins were dissolved in 0.1 M citric acid and 0.05 M KCl and placed at 37° for 4 hr. Samples were centrifuged for 17 hr at 39,000 rpm in a Spinco SW50 rotor. Tubes were fractionated by drop counting and protein peaks determined by optical density readings at 230 and 280 mμ.

Amino-Terminal Amino Acid Analysis. Amino-terminal amino acids were determined by the fluorodinitrobenzene method as described by Chaykin (1966). Approximately 10 mg of DNP-toxin was placed in a loosely capped tube and heated in an autoclave from 105 to 108°, 4–5 psi, for 5 hr. The final concentrated ether extracts were spotted on silica gel thin-layer chromatograms (Eastman Kodak, 6060, impregnated with fluorescent indicator). The first dimension was run in the upper layer of a two-phase solvent consisting of toluene–pyridine–2-chloroethanol–0.8 N ammonia solution (100:30:60:60, v/v), and the second dimension was run in chloroform–benzyl alcohol–glacial acetic acid, 70:30:3, v/v) (Pataki, 1968).

Trypsin Digestion and Peptide Mapping of the Toxins. Toxin (18 mg/10 ml) was dissolved in 8 M urea and 1 M NaCl and incubated for 1–2 hr at room temperature. The solution was exhaustively dialyzed against 0.1 M NH_4HCO_3 (pH 7.8) at 4°. Trypsin, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated (Worthington), at a ratio of 1:40, trypsin: protein was added to the dialyzed material, digested at room temperature for 6 hr, and lyophilized. The digest (0.5–0.7 mg) was dissolved in 10–18 μl of electrophoresis buffer (pyridine–acetic acid–water (10:1:100, v/v) containing 0.05 μmole of Clelands reagent (dithiothreitol) and spotted on prewet cellulose thin-layer chromatograms (Eastman Kodak, 6065, with fluorescent indicator). A Gelman electrophoresis apparatus (3270-50) was used and separation achieved at 220 V and 10 mA for 3 hr at 4°. Butanol–propionic acid–water (3:2:1, v/v) solvent was employed for chromatography in the second dimension.

Results

Sedimentation Values of Subunits in Sodium Dodecyl Sulfate. Previous experiments showed that toxins A or B treated for 3 hr at 37° followed by dialysis against 0.1% SDS for 16 hr (standard conditions) gave toxic material (LD_{50}, 50 to 100% of LD_{50} of starting material) that sedimented in a single peak $S_{20,w}$ = 1.4 S (Montie *et al.*, 1968). Reports in the literature have indicated that SDS alone in concentrations of 0.5% or above will peak in the ultracentrifuge, *s* value = slightly below 1.0*S*. The SDS peak may coalesce with small molecular weight macromolecules and distort the later *s* value (Di Sabato and Kaplan, 1961; Rudback and Milner, 1968). We have calculated by dry weight measurements that 0.5 to 1% SDS remains in the dialysis bag with toxin following treatment under standard conditions. To avoid possible inaccuracies resulting from excess accumulation of SDS in the dialysis bag, toxin A was submitted to 1% SDS and further diluted with 0.1% SDS after dialysis. The *s* values were calculated for three different dilutions and extrapolated to zero concentration. A value of 1.7 $s^0_{20,w}$ was obtained. Since no SDS peak can be observed in the ultracentrifuge at concentrations of 0.1% SDS and protein concentration effects were minimized the 1.7S figure should represent an accurate value. In confirmation, a value of 1.7 S was also obtained from a preparation of toxin dissolved in 1.0% SDS at room temperature followed by removal of excess SDS by cold precipitation.

TABLE I: Subunits Obtained from Toxins A and B.

Method	Av Value[a] B	A
1. Acrylamide gel sodium dodecyl sulfate electrophoresis[b]	24,000	24,000 30,000
2. Sephadex gel filtration sodium dodecyl sulfate dissociation	13,000 22,000	11,500 24,000
3. Sucrose gradient citric acid dissociation	<14,400	

[a] Basic subunits are approximately 24,000 and polypeptide chains are considered to have a value of approximately 12,000. Values are for the major peaks obtained. [b] Values ±10%.

Experiments reported in a preliminary report indicated that exposure of toxin B to 0.1% SDS for 30 min at 27° resulted in subunits of 2.5 S and the remaining approximately 50% of the sample sedimented at 7.6 S (Montie *et al.*, 1968). The time of these treatments was extended to 5 and 20 hr to determine if further depolymerization would occur. The results were identical with those found at the shorter time periods. We concluded that toxin in 0.1% SDS could not be depolymerized to the 1.7S form found after higher concentrations of SDS are initially used.

Molecular Weight Determination on Acrylamide Gels and Gel Filtration. Molecular weight studies were carried out using the SDS acrylamide gel electrophoresis technique of Shapiro *et al.* (1967). Subunit bands were obtained at approximately 24,000 molecular weight with toxin A or B (Table I, Figure 1). When the SDS concentration in the gel was increased to 0.5%, more consistent isolation of fully dissociated subunit was achieved.

In some runs toxin A gave a band of somewhat higher molecular weight, but we believe this is caused by incomplete dissociation. This interpretation is supported by the fact that ribonuclease, for example, ran as a dimeric protein in 0.1% SDS, but when the concentration was increased to 0.5% SDS conversion into the monomer resulted. Attempts to raise the concentration of SDS to 1.0% in the gels introduced problems in the electrophoretic procedure and in the staining technique (see Lenard, 1970).

Estimation of subunit size after treatment with SDS was carried out using Sephadex G-100 and G-200 columns. Some investigations have indicated that SDS disturbs the molecular sieving capacity of Sephadex (Rosenberg and Guidotti, 1968). Experiments conducted in our laboratories confirmed that a series of proteins of a wide range of molecular weights are voided in Sephadex G-50 or G-75 columns equilibrated with 0.1% SDS. Rudman *et al.* (1968) showed that aberrations in the sieving properties of Sephadex were avoided by removing SDS from column and eluent, by lengthening the column, and by reducing the volume and percentage SDS in the sample. Utilizing the latter technique, toxins A and B or standard proteins were treated with 1 or 2 ml of 1% SDS, reacted for 24 hr at room temperature and passed through G-100 or G-200 columns (1.5 × 90 cm). A major peak was obtained with a

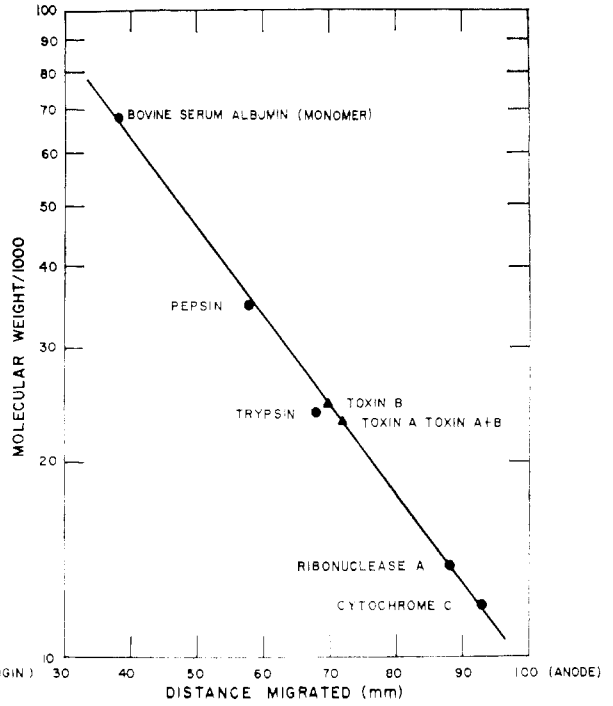

FIGURE 1: Molecular weight determination by acrylamide gel electrophoresis in SDS. Toxins and standards were added at 340 μg/gel and submitted to electrophoresis at 3 mA/gel for 16 hr. The gels contained 10% acrylamide and 0.5% SDS. Gels were fixed and stained as described in Experimental Section.

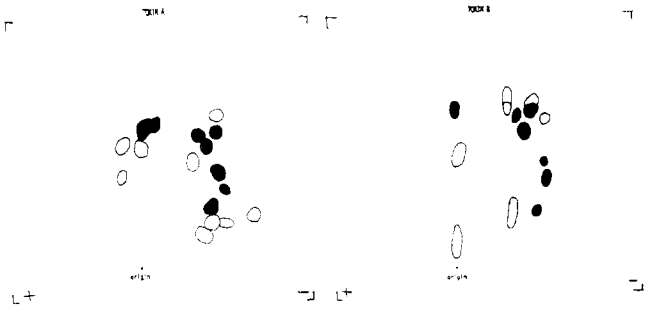

FIGURE 2: Comparative fingerprints of tryptic digests of toxins A and B. Protein was digested and peptides separated as described in Experimental Section. Chromatography (second dimension) was in butanol–propionic acid–water (3:2:1, v/v). Peptides were identified with ninhydrin reagent. Shaded spots indicate peptides common to both toxins A and B; unshaded spots are unlike peptides.

molecular weight of 12,000–14,000 with a minor peak at approximately 22,000 (Table I). In some experiments where higher concentrations of toxins and/or shorter columns (60 cm) were used, a major peak measuring approximately 24,000 was obtained. Apparently dilution of protein is necessary for dissociation to the 13,000 molecular weight species.

Peptide Maps of Toxins A and B. Toxin A or B was subjected to further analysis after denaturation and hydrolysis with trypsin. Toxin was denatured with glacial acetic acid or by treatment with 8 M urea. Using either denaturation technique resulted in peptide maps of from 12 to 16 peptides when using either of two fingerprinting techniques on thin-layer cellulose or paper. Routinely, thin-layer electrophoresis followed by chromatography was the technique utilized. The lack of any ninhydrin-positive material at the origin indicated that complete tryptic digestion had occurred. Toxin A gave 16 peptides and toxin B 14 peptides (Figure 2). Seven peptides were very similar or the same in both toxins. Therefore, toxin A contains 9 different peptides and toxin B contains 7. Confirmation of the peptide distribution was verified by using a second solvent system for chromatography, isoamyl alcohol–propionic acid–pyridine–H_2O (35:2:35:27, v/v). These results are consistent with a basic subunit molecular weight in the area of 24,000 based on approximately 70 arginine and lysine residues per 120,000 molecular weight (Montie *et al.*, 1966).

The frequency of unlike peptides between A and B was somewhat surprising in view of the similarity in amino acid content (Montie *et al.*, 1966). However, the recent demonstration of two unlike polypeptide chains in A and B would explain these results (Montie and Montie, 1969).

Some difficulty was initially encountered in identifying peptides because of amino acids present in the maps (not pictured). Almost all of the amino acids remained in the neutral zone during electrophoresis. Amino acids were differentiated from peptides by using standards of [^{14}C]amino acids, by color reaction with collidine in ninhydrin. Following tryptic digestion, amino acids were removed from the major portion of the peptide fraction by passing this material through Sephadex G-25 fine columns (1.5 × 90 cm) containing acetic acid (0.2 N). The peptides obtained by passing the hydrolysate through Sephadex G-10, G-15, and G-25 columns were estimated to be from 1500 to 2500 molecular weight.

Amino-Terminal Residues. Application of the fluorodinitrobenzene technique was followed by separation of DNP derivatives on silica gel, thin-layer chromatograms. Results showed alanine to be the predominant N-terminal amino acid in both toxins A and B. The per cent yield obtained of alanine, of the theoretical yield, was 17–20 or 34–40% depending on an assumption of 12 or 10, 6 or 5 polypeptide chains, respectively. These results support the concept that toxins A and B contain a common polypeptide chain.

Confirmatory evidence for the presence of different N terminals between the toxins has not been obtained although DNP-serine and DNP-glycine were found associated with toxins A and B. The yields of serine and glycine were approximately 8 and 3%, respectively, of the theoretical based on 10 polypeptide chains/120,000 molecular weight. The amount of glycine obtained varied between preparations presumably because glycine-DNP is rather unstable to acid (Elmore, 1968). Confirmation of the glycine N terminal was obtained by limiting hydrolysis time from 30 to 60 min.

Toxins A and B preparations also revealed the presence of trace amounts of aspartic acid, glutamic acid, threonine, and cystine. The dicarboxylic amino acids were not eliminated by sample pretreatment in 8 M urea and 1 M NaCl followed by dialysis, or by washing with 10% trichloroacetic acid.

Interaction of Toxin with Organic Acids. Toxin A and toxin B in solution are denatured as the pH is lowered from neutrality to below 5.5. Concomitantly lethal activity for mice is lost. We attempted to depolymerize toxin by rapidly lowering the pH to 2.0–3.0 thus avoiding isoelectric precipitation at pH 4.7–5.0. Toxin was treated with citric acid (0.1 M, pH 2.2) for 1 hr at 37° or for 24 hr at 25–27°. The solution was passed through a Sephadex G-50 column containing 0.001 M potassium phosphate buffer. Two peaks emerged, one at the void with a pH of 7 and the second at approximately 1.5 void volumes with a pH of 2.8–3.8. The majority of protein was contained in the second peak when the temperature was raised

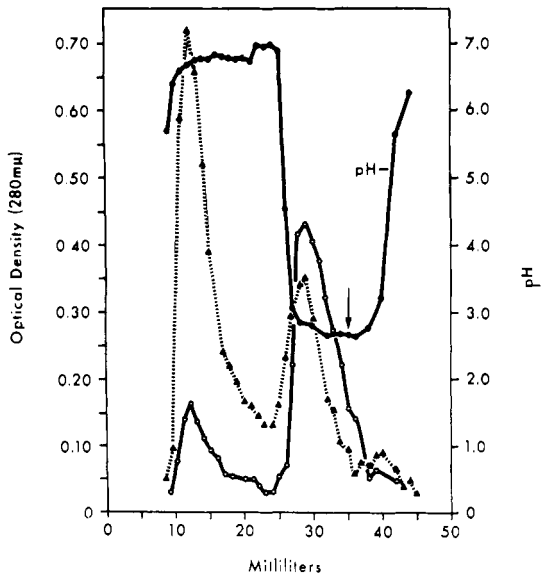

FIGURE 3: Separation of citric acid dissociated polypeptide by gel filtration. A mixture of toxins A and B were dissociated in citric acid (0.1 M) at 37° for 1 hr (▲) or for 24 hr (○). Separation was achieved in Sephadex G-50 columns equilibrated with potassium phosphate buffer (0.001 M, pH 7.0). The pH plot (●) was obtained from data from the 1-hr sample fractions. Arrow designates the V_e for citric acid alone run under the same column conditions.

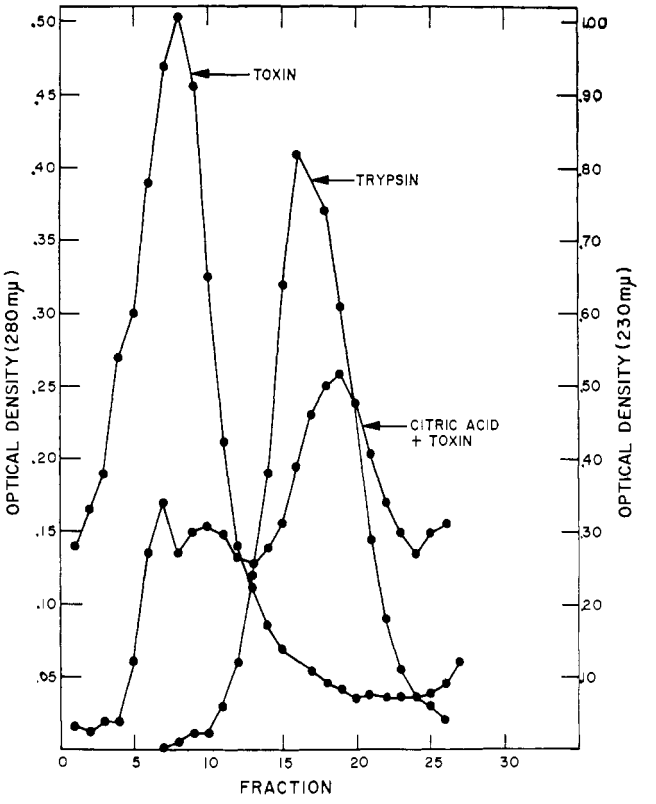

FIGURE 4: Sucrose gradient profiles of toxin following dissociation in citric acid. A mixture of toxins A and B (4.4 mg) was dissolved in 0.5 ml of citric acid (0.1 M) and centrifuged before running on gradient (citric acid and toxin). Toxin (A plus B) standard, 3.2 mg, was dissolved in 0.5 ml of 0.002 M sodium phosphate. Trypsin standard (4.0 mg) was dissolved in 0.5 ml of citric acid. Samples were incubated at 37° for 7 hr. Samples (0.5 ml) were layered on 2–20% gradients and centrifuged for 17.5 hr. Total volume of each gradient was 5.3 ml. Fractions of 0.2 ml were collected from the bottom of the gradient (fraction 1). Fractions were diluted to 1.0 ml and read at 280 mμ (toxin and trypsin standards). Citric acid plus toxin 230-mμ readings are plotted. Profiles were identical with 280-mμ readings (not shown).

to 37° for 24 hr (Figure 3). These results show that depolymerization is time and temperature dependent.

Assay for the degree of dissociation was routinely carried out on columns equilibrated with low molarity buffers (0.001–0.002 M) at neutral pH. When columns were equilibrated with an excess hydrogen ion concentration (>0.002 M acetic acid) this resulted in aggregation and voiding of the total toxin sample. Columns were equilibrated with 0.1 M KCl to avoid possible adsorption of toxin to dextran under the weak buffer conditions. Under the latter conditions dissociated protein was obtained.

Attempts to measure the size of the dissociated protein on Sephadex were not successful. The V_e of toxin polypeptide relative to standard proteins showed a wide variation depending on the pore size Sephadex employed. Rate of movement through Sephadex was not merely a function of size.

Stronger evidence indicating that isolation of dissociated polypeptide was occurring on columns was obtained using sucrose gradients (Table I). Dissociated toxin protein sedimented at a slower rate than trypsin (23,800) (Figure 4) or lysozyme (14,400). Sedimentation studies with the Model E ultracentrifuge proved unsuccessful since the high concentration of protein required resulted in reaggregation of the polypeptide material.

In an effort to understand the mechanism involved in dissociation in citric acid, toxins A and B were treated with other acids. Toxin dissolved in acetic acid (0.1 M) for 22–24 hr at 37° resulted in only a partial conversion into polypeptide units. The indication that chelation with metals may be an important factor in dissociation with citric acid was strengthened by the complete dissociation in acetic acid after addition of 10^{-3} M EDTA. Both *o*-phenanthroline or 8-hydroxyquinoline substituted for EDTA in enhancing toxin dissociation. *o*-Phenanthroline immediately turned red in the presence of acetic acid and toxin and gave slight color at neutral pH. A less intense reaction of toxin with 8-hydroxyquinoline in acid was also noted. Attempts to substitute hydrochloric or boric acids for the organic acids were unsuccessful since the toxins were precipitated by these acids. EDTA addition did not enhance the solubility of toxin in boric acid. Toxin remained soluble in phosphoric acid (0.01 M) when incubated for 24 hr (37°). Results from Sephadex filtration indicated dissociation in phosphoric acid.

The effectiveness of citric acid as a toxin depolymerizing agent compared with other acids suggested some specific interaction occurred between citric acid and toxin molecules. Undissociated protein eluting at the void volume from Sephadex G-50 columns gave a pH near neutrality. Dissociated protein fractions from the same columns gave pH values slightly below 3.0 (Figure 3). The low pH obtained was attributed to citric acid bound to the polypeptide unit. On the other hand, trypsin and cytochrome *c* were found not to bind citric acid. Association of citric acid with toxin polypeptide unit was confirmed using [^{14}C]citric acid. Radioactivity coincident with polypeptide units in G-50 profiles was demonstrated. Dissociated protein peak fractions were combined and passed through a second G-50 column. Radioactivity coincided directly with the polypeptide peak. Protein from this peak was concentrated, neutralized with ammonium hydroxide, and treated with trypsin. Tryptic peptides were passed through a

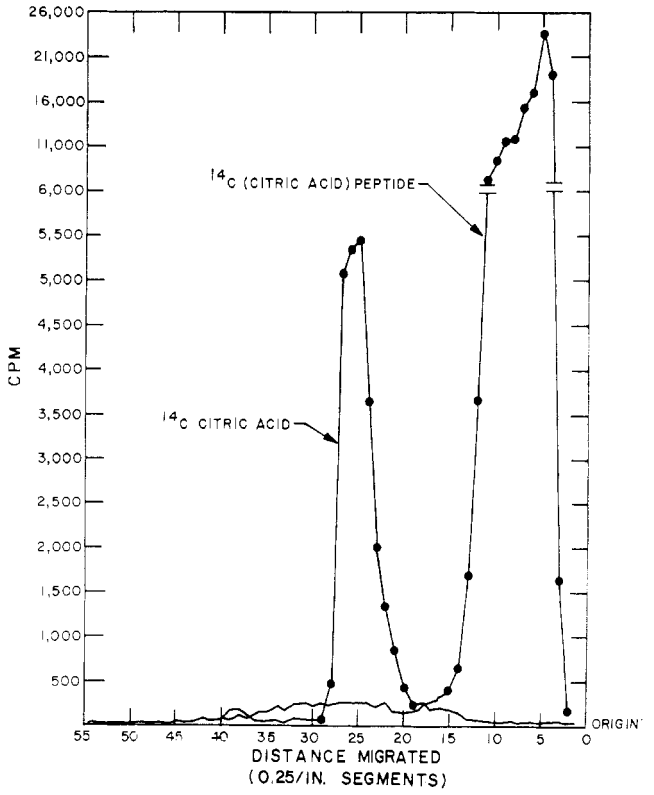

FIGURE 5: Chromatography of citric acid–toxin peptide complex. Toxin B (5.5 mg) was dissolved in 2 ml of citric acid (0.1 M) containing 0.2 ml of [^{14}C]citric acid ([1,5-^{14}C]citric acid, specific activity 1.84 mCi/mmole, International Nuclear and Chemical Corp.), and incubated for 24 hr (37°). The sample was clarified by centrifugation and passed through a sequence of two Sephadex G-50 columns equilibrated in 0.001 M potassium phosphate (pH 7.0). The retarded peaks containing [^{14}C]citric acid–protein complex were combined and lyophilized. The sample was adjusted to pH 8.0 with ammonium hydroxide and treated with trypsin at room temperature overnight. This sample was passed through a Sephadex G-25 fine column equilibrated with 0.1 M acetic acid. The peptide peak fractions were pooled and lyophilized. The sample was chromatographed in butanol–acetic acid–water (4:1:1, v/v). on Whatman No. 3MM paper for 16 hr and peptides located with ninhydrin. [^{14}C]Citric acid was used as a marker. The dried chromatogram was divided into 0.25-in. segments and each segment was counted in a Packard liquid scintillation counter.

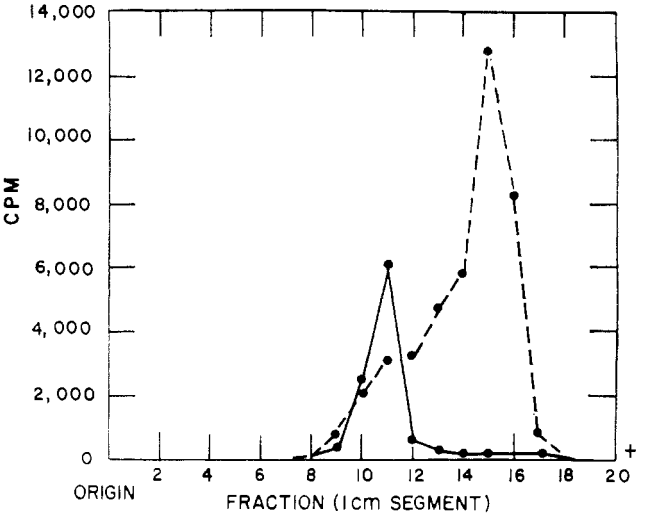

FIGURE 6: Electrophoresis of citric acid–toxin peptide complex. Toxin B (5.0 mg) was dissolved in 1.8 ml of citric acid (0.1 M) and treated with labeled citric acid as described (Figure 5). The sample was passed through a Sephadex G-50 column equilibrated with (0.001 M) sodium phosphate (pH 7.1). Retarded peak fractions were combined, lyophilized, and adjusted to pH 8.0 with ammonium hydroxide. The sample was treated with 0.05 mg of trypsin for 8 hr. The sample was spotted on paper (Whatman No. 3MM) and submitted to high-voltage electrophoresis for 2 hr at 7° on a Savant High-voltage flat-plate electrophoresis apparatus. Separation was achieved at 2000 V and 50 mA in pyridine–acetic acid–water (25:1:225, v/v). Dried paper was cut into segments and assayed by counting in a Packard liquid scintillation counter. [^{14}C]Citric acid–peptide complex (--), [^{14}C]citric acid marker (—).

Sephadex G-25 (fine) column. Results showed all the radioactivity associated with a single major peptide peak. Labeled citric acid bound to tryptic peptides was additionally demonstrated by paper chromatography (Figure 5) and paper electrophoresis (Figure 6). Citric acid was associated with one and possibly two peptides containing strong negative charges. In contrast, preparations of toxin denatured with acetic acid and treated in the same manner did not give rise to unusual peptides with negative charges. The binding of citric acid, but not acetic acid was sufficient to change the net charge on these peptides.

It was previously noted that citric acid polypeptide units were retarded more than ribonuclease or cytochrome *c* on Sephadex G-50 and G-25 in weak buffer eluents. Retardation was not a consequence of dextran acting as a specific adsorbent because similar results were obtained with Bio-Gel P-20 columns. The unusually small size obtained suggested polypeptide units were not being filtered strictly on the basis of their Stokes radii. To further examine this question we passed [^{14}C]citric acid–toxin complex through a Sephadex G-10 column equilibrated with 0.1 M KCl and weak potassium phosphate (pH 7.0). Under these conditions ribonuclease is completely voided. Toxin polypeptide bound approximately 20% of the citric acid. The complex eluted at twice the void volume and was completely separated from unbound acid (2.5 V_0). Because of the extensive retardation of polypeptide units in G-10 columns together with the variability in apparent molecular size on all columns tested, we concluded that the rate of filtration of acid-dissociated toxin molecules through macromolecular pores is regulated to some extent by the acid-bound component.

Experiments were carried out to test the hypothesis that EDTA binds to toxin polypeptide unit, in the presence of acetic acid, mimicking the citric acid action. Toxin B treated with [^{14}C]EDTA and acetic acid (0.1 M) was passed through a Sephadex G-10 column. Labeled EDTA showed no binding and emerged at the void completely separated from retarded polypeptide unit. Polypeptide fractions had a pH of 2.8 indicating acetic acid was bound.

Toxin Resistance to Alkaline Solutions. The effect of alkaline pH in initiating dissociation was tested using sodium glycinate buffer (0.085 M, pH 10.2). Toxin B was incubated at 37° for 21 hr. In another experiment EDTA (0.05 M) was added to the glycinate with incubation at 27° for 52 hr. These solutions in separate assays were passed through a Sephadex G-50 column equilibrated with glycinate (0.043 M, pH 10.0). No retarded peaks were evident in either case. These results demonstrate the necessity of an acid environment for toxin dissociation.

Discussion

A comparison of the components of toxin A (240,000 molecular weight) and toxin B (120,000 molecular weight) has sub-

stantiated an earlier report that both toxins are complex proteins containing the same size subunits (Montie *et al.*, 1968). The toxins are depolymerized with 1.0% sodium dodecyl sulfate from 7.8S native molecules to biologically active molecules of 1.7 S. The latter value may be somewhat low since toxin in 0.1% SDS gives a value of 2.4 S for the slow peak suggesting it is free from aberrations introduced by an excess of SDS. In this regard Chang and Snell (1969) found that histidine decarboxylase (190,000 with ten subunits) gave a single peak of 1.9 S in 1% SDS and a value of 2.5 S for a slow peak and 8.8 S for the fast peak (native) protein in 0.1% SDS. The toxins in SDS (0.5 to 1.0%) are disaggregated to basic subunits of approximately 24,000 molecular weight as determined by SDS–acrylamide gel electrophoresis. Results from fingerprint analysis of tryptic peptides are consistent with a molecular weight for a basic subunit of 24,000 based on the number of arginine and lysine residues. It is worth noting that size estimates for the tryptic peptides of 1500–2500 correspond to a predicted number of 14 to 16 actually obtained. The evidence indicates that toxin may be dissociated further to 12,000 molecular weight chains. Polypeptide, mol wt 12,000, was isolated under dilute conditions when toxin was dissociated with 1.0% SDS for 16–24 hr at room temperature and assayed in Sephadex columns. Evidence for a small polypeptide <14,400 also was indicated by sucrose gradient centrifugation after depolymerization with citric acid. A value of 12,000 would agree with a minimum molecular weight of 12,000 based on 1 cysteine residue/chain (Montie *et al.*, 1966). The question remains whether the biologically active 1.7S material represents 12,000 chains or the 24,000 basic subunits.

A model of toxin structure consistent with current data can be discussed. Preliminary electron micrographs of toxin B (120,000) show a structure containing either 5 or 6 subunits in a planar-radial arrangement typical of a number of polymeric proteins so far examined (Schnebli *et al.*, 1970; Boeker *et al.*, 1969). A subunit of 24,000 supports the concept of a pentagonal arrangement. However, currently the molecular weight estimates are not sufficiently accurate to distinguish between the possibility of 5 or 6 subunits per toxin B molecule. Toxin A (240,000) probably possesses a double layer of subunits as found in arginine decarboxylase containing 10 subunits (Boeker *et al.*, 1969) or glutamine synthetase containing 12 subunits (Valentine *et al.*, 1968).

It is proposed from previous electrophoretic separations of polypeptides under acid conditions that each toxin contains one unlike chain (Montie and Montie, 1969). Toxin A subunits contain chains designated nos. 1 and 2 and toxin B contains 2 and 3 chains. Tryptophan analysis has revealed that toxin B contains approximately 30% less tryptophan than toxin A (Montie *et al.*, 1966). The calculated number of tryptophan residues is 7 residues/120,000 g of toxin B and 10.9 residues/120,000 g of toxin A. In view of the error inherent in measuring tryptophan in proteins these values could represent 10–12 residues for toxin A and 5–7 residues per toxin B. Therefore, we propose that toxin A contains homogeneous subunits with each 12,000 chain (no. 1 or 2) containing a single tryptophan residue. Toxin B probably contains the same general arrangement except for the important difference that each subunit contains a no. 2 and a 3 chain with a single tryptophan in no. 2 as in toxin A, but no tryptophan in chain 3. A tryptophan-deficient chain in toxin B would account for the decreased tryptophan values. The presence of 5 or 6 homologous subunits would satisfy the requirements for intramolecular binding and an orderly geometrical arrangement with heterologous bonding (Monod *et al.*, 1965).

The identical tryptic peptides found in both toxins consequently would represent the components of polypeptide 2 containing alanine as the N-terminal amino acid and the heterologous peptides represent those from polypeptides 1 (toxin A) and 3 (toxin B). We would further suggest that chain 2 is the polypeptide responsible for toxicity since it is found in both toxic proteins. Experiments are being designed to test this hypothesis.

Dissociation of toxin molecules with organic acids is a complex reaction not completely understood. Data from sucrose gradients indicate that small molecular weight protein is formed after acid treatment. Dissociation with citric acid is related to its attachment to protein and an apparent chelation action. The interaction of toxin with organic acids is somewhat unique since other proteins tested did not exhibit acid binding. The presence of bound free amino acids detected during peptide and N-terminal analysis may relate to the intrinsic property of toxin protein to bind certain organic acids.

Several mechanisms are involved for both binding of organic acids to polypeptides and the resulting dissociation. Some recent model studies using nuclear magnetic resonance spectroscopy are pertinent to the binding problem. Hanlon *et al.* (1963) showed, using trifluoroacetic acid and a model amide, that the principle action between the two was the complete protonation of the amide donated from the trifluoroacetic acid anion. However, Stewart *et al.* (1967) using poly-L-alanine as a model found very little proton donation from trifluoroacetic acid. They proposed that the principal acid–polymer interaction is hydrogen bonding without complete proton transfer with the proton remaining primarily bonded to the acid. With an increase in acid concentration intramolecular hydrogen bonding in the peptide is suppressed in favor of hydrogen bonding between trifluoroacetic acid and polypeptide. These authors further concluded that this action is the driving force converting polypeptide from the helical into the random coil form. A complex of organic acid hydrogen bonded to the carboxyl groups of the toxin as found by Stewart *et al.* (1967) for a model system could explain the loose binding of acetic and citric acid to toxin protein. Consequently, acid binding would disrupt the intrachain hydrogen bonding of the native protein resulting in toxin dissociation. Hydrogen bonding in solution would be favored by intermediate acid concentrations (pH 3.0), where 50% of the citrate would still be ionized. It is conceivable that some dissociation by acid can be attributed to larger intramolecular repulsive forces which develop as the carboxyl groups (pK = 4) on the protein are titrated below pH 4 (Stellwagen and Schachman, 1962; Kauzman, 1954).

The synergistic action of chelating agents with acetic acid supports the view that citric acid functions through both hydrogen-bonding and chelation mechanisms. Acetic acid binding is reversed by neutralization indicating that only weak hydrogen bonds and ionic systems are involved. A plausible explanation for these results is that metals are participants in intersubunit binding. Citric acid may substitute for a polypeptide forming a stable acid–metal–polypeptide bridge. The data suggest that metal is extracted completely from toxin by EDTA since EDTA is not bound to protein. This conclusion is further supported by the observations that both *o*-phenanthroline and 8-hydroxyquinoline gave positive tests for metallic ion. The *o*-phenanthroline reaction is a qualitative test for ferrous iron. The level of hydrogen ion may influence the extent of metal complexing also decreasing it in the case of high acidity where lower concentrations of the ionized complexing forms exist. Therefore, raising the hydrogen concentra-

tion in the Sephadex column would tend to reverse both the chelation and hydrogen-bonding effects explaining the voiding of protein in these experiments. In addition, subunits may reaggregate when they become fully denatured by excessive acidification.

These studies have been directed toward elucidation of those unique properties responsible for toxicity in the mouse toxins. An important structural feature may lie in the property of the binding of small ligands. Metal binding may impart structural organization to the toxin giving it increased structural stability and may be important with respect to attachment in the bacterial cell. ATPase, for example, is held in bacterial membranes by Mg^{2+} bridges (Schnebli *et al.*, 1970). Binding competence may also relate to toxin attachment at specific receptor sites in the mouse or rat. The toxin readily binds to ribosomes (T. C. Montie, 1966, unpublished data) and alters mitochondrial membranes (Kadis *et al.*, 1966). A number of bacterial toxins show unique binding properties. The inherent quality of selective binding by many toxic proteins is reflected, for example, in the specificity of the Streptolysins O and S for certain membrane lipid substrates (Bernheimer, 1968), and for tetanus toxin with nervous tissue ganglioside (van Heyningen and Mellanby, 1968). Botulinium toxin is adsorbed rather selectively at the myoneural junction of muscle cells and nerve endings (Zacks *et al.*, 1962) and sarcolemma (Zacks and Scheff, 1968). It is interesting that Botulinium toxin and Pasteurella toxin show certain parallel characteristics. Both proteins are released as rather large polymers from the cell by autolysis. Both proteins are dissociated into small subunits in the presence of acid as assayed by Sephadex columns (Riesen, 1966) and by SDS (Beers and Reich, 1969). Both toxins contain important tryptophan residues (Boroff and Das Gupta, 1966; Montie *et al.*, 1966) and a small number of important SH groups (Beers and Reich, 1969; Montie *et al.*, 1966). Furthermore, both toxins contain a high mole percentage of hydrophobic amino acids (*Pasteurella pestis* toxin = 50% and Botulinum type B = 42%) and a high percentage of acidic amino acids. These properties, along with a low level of sulfhydryl amino acids are typical of membrane lipoproteins (Benson, 1968). We suspect these parallels may relate to a similar anatomical origin of both toxins in the bacterial cell surface which in turn relates to penetration and binding capacity in the host.

Acknowledgments

We thank Miss Charlotte Urbano and Miss Carol Kennedy for their able technical assistance in this work. We would also like to acknowledge the support of the Albert Einstein Medical Center, Philadelphia, Pa., where part of this work was carried out.

References

Andrews, P. (1965), *Biochem. J. 96*, 595.
Beers, W. H., and Reich, E. (1969), *J. Biol. Chem. 244*, 4473.
Benson, A. A. (1968), *in* Membrane Models and the Formation of Biological Membranes, Bolis, L., and Pethica, B. A., Ed., Amsterdam, North Holland Publishing Co., p 190.
Bernheimer, A. W. (1968), *Science 159*, 847.
Boeker, E. A., Fisher, E. H., and Snell, E. E. (1969), *J. Biol. Chem. 244*, 5239.
Boroff, D. A., and Das Gupta, B. R. (1966), *Biochim. Biophys. Acta 117*, 289.
Chang, G. W., and Snell, E. E. (1968), *Biochemistry 7*, 2012.
Chaykin, S. (1966), Biochemistry Laboratory Techniques, New York, N. Y., Wiley, p 56.
Di Sabato, G., and Kaplan, N. O. (1961), *J. Biol. Chem. 239*, 438.
Elmore, D. T. (1968), Peptides and Proteins, New York, N. Y., Cambridge University Press, p 27.
Hanlon, S., Russo, S. F., and Klotz, I. M. (1963), *J. Amer. Chem. Soc. 85*, 2024.
Kadis, S., Trenchard, A. V., and Ajl, S. J. (1966), *J. Biol. Chem. 241*, 5605.
Kauzman, W. (1954), *in* The Mechanism of Enzyme Action, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., Johns Hopkins Press, p 70.
Lenard, J. (1970), *Biochemistry 9*, 1129.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Montie, T. C., and Montie, D. B. (1969), *J. Bacteriol. 100*, 535.
Montie, T. C., Montie, D. B., and Ajl, S. J. (1966), *Biochim. Biophys. Acta 130*, 406.
Montie, T. C., Montie, D. B., Leon, S. A., Kennedy, C. A., and Ajl, S. J. (1968), *Biochem. Biophys. Res. Commun. 33*, 423.
Pataki, G. (1968), Techniques of Thin-Layer Chromatography in Amino Acid and Peptide Chemistry, Ann Arbor, Mich., Ann Arbor Science Publishers, Inc., p 133.
Riesen, W. H. (1966), *Proc. Conf. Botulinum Toxin.* Edgewood Arsenal (1965), Edgewood Arsenal Special Publication, EASP 100-1.
Rosenberg, S. A., and Guidotti, G. (1968), *J. Biol. Chem. 243*, 1985.
Rudback, J. A., and Milner, K. C. (1968), *Can. J. Microbiol. 14*, 1173.
Rudman, D., Garcia, L. A., Abell, L. L., and Akgun, S. (1968), *Biochemistry 7*, 3136.
Schnebli, H. P., Vatter, A. E., and Abrams, A. (1970), *J. Biol. Chem. 245*, 1122.
Shapiro, A. L., Viñuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.
Stellwagen, E., and Schachman, H. K. (1962), *Biochemistry 6*, 1056.
Stewart, W. E., Mandelkern, L., and Glick, R. E. (1967), *Biochemistry 6*, 143.
Valentine, R. C., Shapiro, B. M., and Stadtman, E. R. (1968), *Biochemistry 7*, 2143.
van Heyningen, W. E., and Mellanby, J. (1968), *J. Gen. Microbiol. 52*, 447.
Zacks, S. I., Metzger, J. F., Smith, C. W., and Blumberg, J. M. (1962), *J. Neuropathol. Exp. Neurol. 21*, 610.
Zacks, S. I., and Scheff, M. F. (1968), *Science 159*, 643.