Clostridium botulinum Type E Toxin: Effect of pH and Method of Purification on Molecular Weight

HENRY S. SACKS and SCOTT V. COVERT

Department of Microbiology and Institute of Comparative and Human Toxicology, Albany Medical College of Union University, Albany, New York 12208

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The toxin of Clostridium botulinum type E was isolated from intact cells and from toxic culture filtrates by column chromatography at three pH values. 4.5, 5.3, and 6.0. At pH 6.0 and 5.3, the isolated toxin was in a form with a molecular weight (MW) of 86,000. This toxin was homogeneous on polyacrylamide gel electrophoresis and gel filtration and had an optical density ratio, 280 nm/260 nm, greater than 2.0. It did not dissociate at higher pH levels, but was dissociated into nontoxic components of approximately 12,000 MW when reduced and alkylated in the presence of 6 M guanidine hydrochloride. At pH 4.5, smaller amounts of an impure toxic moiety with a MW of 12,000 were found. After storage for 6 months, the 86,000-MW moiety had lost 60% of its lethality. Gel filtration revealed that the bulk of the toxicity was associated with a component having a MW of 150,000. Toxic components with MW of 12,000 and over 200,000 were also found. The toxin appears to polymerize or aggregate when in a pure form, so that most, if not all, of the MW previously reported for the toxin may belong to different polymers of a monomer with a MW of 12,000 or less. Treatment of the 86,000-MW toxin with trypsin resulted in an 18- to 128-fold increase in lethality, but no detectable change in MW.

Estimates for the molecular weights (MW) of toxic proteins isolated from Clostridium botulinum type E have ranged from 5,000 (3) to near 900,000 (16). Most attempts to isolate the toxic moiety have involved the use of selective precipitation, gel filtration, and ion-exchange chromatography, but there have been important differences in the techniques employed. Gerwing et al. (5, 6) purified toxic proteins from cell-fee culture filtrates by gel filtration and ion-exchange chromatography of solutions buffered at pH 4.5. They estimated the MW of their preparation to be 12,000 to 18,000. Emodi and Lechowich (3) precipitated toxin from the culture medium with ammonium sulfate after first spinning out the cells. Gel filtration on Sephadex G-200 suggested that the toxic moieties had MW of 5,000 and 9,000. Kitamura (10) has conducted ultracentrifugal analyses of a toxin prepared by the method of Emodi and Lechowich and found evidence for a high-molecular-weight (HMW) toxin in the preparation. These methods have been classified as preparations of released toxin (10).

An alternative approach has been to extract retained botulinum toxin from whole cells, using 1 M sodium acetate or 0.2 M phosphate buffer (12). Gel filtration and ion-exchange chromatography at pH 6.0 yielded a product with a MW estimated by ultracentrifugation to be 350,000 (13). This toxin was homogeneous at pH 6.0, but at pH 8.0 it could be separated into toxic and nontoxic components, labeled E6 and E3, respectively, each with a MW of 150,000.

The present study is an attempt to clarify the roles of pH and of released or retained origin in determining molecular size of the toxin obtained, and to study further the dissociation of the toxin.

MATERIALS AND METHODS

Organisms and culture methods. Samples of C. botulinum type E, VH strain, (obtained from E. J. Schantz, Fort Detrick, Frederick, Md.) were stored in a dry-ice chest. Organisms were grown in a liquid medium containing 1.5% Casitone (Difco Laboratories, Detroit, Mich.), 0.25% yeast extract (Baltimore Biological Laboratory, Cockeysville, Md.), 1% glucose, 0.05% CaCl2, 0.1% K2HPO4, and 0.37% cysteine hydrochloride. The bacteria were incubated at 30°C in flasks containing 2 to 8 liters of medium.

Purification of retained toxin from CEX. The general scheme for purification of retained toxin is given in Fig. 1. The cells were harvested by continuous centrifugation at room temperature in a DeLaval Gyrotester (The DeLaval Separator Company, Poughkeepsie, N.Y.). The cells were washed twice by
suspension of C. botulinum in distilled water and centrifuging them in a Sorvall RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) with a GSA rotor at 10,000 rpm (13,600 × g) for 15 min. The cells were extracted with a 0.05-culture volume of 1.0 M acetate buffer (pH 6.0) at 4°C for 24 h. The extract was precipitated by adding ammonium sulfate (special enzyme grade, Mann Research Laboratories, New York, N.Y.) to 70% saturation (470 g/liter). The precipitate was allowed to form for 24 h at 4°C and was then collected by centrifugation in a Sorvall RC2-B centrifuge at 13,600 × g for 15 min. The precipitate was suspended in 0.1 volume of 0.05 M acetate buffer (pH 6.0). Insoluble material was removed by centrifugation.

The CEX was desalted, and low-molecular-weight (LMW) contaminants were removed by gel filtration with Sephadex G-50 columns (2.5 by 40 cm). Flow was regulated at 2 ml/cm²/h with a Holter bilateral roller pump (The Holter Company, Bridgeport, Pa.). Samples (5 ml) of the outflow from the columns were collected in a Gilson model VL linear fractionator (Gilson Medical Electronics, Middleton, Wisc.). Elution with 0.05 M acetate buffer (pH 6.0) was followed by optical density (OD) measurements at 260 and 280 nm in a Beckman model DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

The contents of the tubes containing the highest toxic activity concentrations were pooled. The pooled toxin solution was applied to columns (2.5 by 15 cm) packed with Whatman DE-52. The toxin was eluted by a stepwise gradient of NaCl in the same acetate buffer. NaCl was added in 0.1-M increments to a final concentration of 1.0 M NaCl.

Eluates with the highest toxicity were pooled. The toxin was precipitated by the addition of ammonium sulfate to 70% saturation (470 g/liter). The precipitate was allowed to form for 24 h at 4°C and was then collected by centrifugation in a Sorvall RC2-B centrifuge with SE-12 rotor at 10,000 rpm (10,300 × g) for 15 min. The precipitate was suspended in 0.1 volume of acetate buffer (0.05 N, pH 6.0), and the insoluble material was removed by a second centrifugation.

The final product was obtained by gel filtration on columns (2.5 by 97.5 cm) of Sephadex G-200. The toxin was filtered through sterile membrane filters (Millipore Corp.) and stored at 4°C. Samples were removed periodically to determine toxin stability.

Purification was carried out by the same general procedure, but by substituting the following buffers for 0.05 N acetate (pH 6.0): 0.05 N acetate (pH 4.5) and 0.05 N acetate (pH 5.3). One series was run with 0.025 N acetate buffer of pH 4.5, which also contained 0.05 M guanidine hydrochloride (ultrapure, Mann Research Laboratories). Also, for one series, the ammonium sulfate precipitations were omitted. Instead, the CEX was concentrated by adding Sephadex G-25 (coarse; 20 g/100 ml). The Sephadex was allowed to swell for 1 h. The concentrated CEX was recovered by vacuum filtration through a Buchner funnel lined with 400-mesh nylon netting. Sephadex G-25 (coarse) was added again, and the procedure was repeated. In this series, smaller columns (1 by 30 cm) of Sephadex G-200 were used.

Purification of released toxin from culture filtrate. The procedures for purification of toxin from the culture filtrate were similar to that just described for CEX, except that the starting material was the supernatant remaining after centrifugation of the cells. Purifications from the culture filtrate were carried out at pH values of 4.5, 5.3, and 6.0. The purifications at pH 4.5 and 6.0 were done in the presence of 0.05 N guanidine hydrochloride within the buffers.

Trypsinization. Samples of toxin were incubated with bovine pancreatic trypsin (EC 3.4.4.4; recrystallized two times, Sigma Chemical Co., St. Louis, Mo.) in a final concentration of 0.01% for 2 to 6 h in a 37°C water bath. Action of the enzyme was halted by the addition of soybean trypsin inhibitor type 1-S (Sigma, crystallized two times), or by adjustment of the pH to 4.0.

Polyacrylamide gel electrophoresis was carried out by modification of the Hjerten method (9). Electrophoresis was done at pH 8.0 in 0.05 tris(hydroxymethyl)aminomethane-phosphate buffer and at pH 4.0 in 0.07 N glycine-acetic acid. Samples were diluted with 30% sucrose solution to give protein concentrations of approximately 0.5 mg/ml and were layered.

Fig. 1. Flow diagram for the purification of C. botulinum type E toxin. This scheme was used with minor modifications throughout the range of conditions employed.
over gels in Pyrex tubes (5 by 70 mm). A loading current of 2 mA per tube was applied for 15 min and then increased to 5 mA per tube for the remainder of the run (2 to 3 h). The gels were stained with 1% Buffalo black in 7% acetic acid.

**Mouse assay.** Toxicity determinations were made on toxin samples diluted with a 0.85% NaCl solution containing 0.1% gelatin. Lethality was determined by intraperitoneal injections of 0.5-ml serial twofold dilutions into female white mice (17- to 20-g) using two to six mice per dilution. The mean lethal dose (LD₅₀) was calculated by the Reed and Muench method (14).

**Characterization of the toxin molecule.** MW of toxin preparations were determined by gel filtration on a column (2.5 by 100 cm) of Sephadex G-200 (1). The eluting buffer was 0.05 N K₂HPO₄·KH₂PO₄, pH 6.0. The column was calibrated with the following proteins: lysozyme (Sigma, grade I), MW = 14,300; bovine pancreatic trypsin (Sigma, crystallized two times), MW = 22,000; egg albumin (Sigma, grade V), MW = 45,000; bovine albumin (Sigma, fraction V), MW = 68,000; bovine gamma globulin (Mann Research Lab., fraction II), MW = 156,000; and bovine thyroglobulin (Sigma, type I), MW = 600,000. The MW quoted above are commonly accepted literature values.

Each protein (10 to 20 mg) was dissolved in 2 ml of eluting buffer and applied to the column. Flow was regulated at 2 ml per cm² per hr by a Holter bilateral roller pump. Samples (2 ml) were collected in a Gilson fractionator. Elution of protein was monitored by measuring OD at 280 nm with a Beckman model DB spectrophotometer. Each protein was run at least four times.

Gel filtration in the presence of guanidine hydrochloride was carried out by using a modification of the method of Fish et al. (4). Protein samples were dissolved in 0.5 ml of 6 M guanidine hydrochloride, pH 6.5, (ultrapure, Mann Research Laboratories) and applied to columns (1 by 30 cm) of Sephadex G-200. They were eluted with the same solvent. Reduced and alkylated samples were prepared by dissolution in 0.5 ml of 6 M guanidine hydrochloride–0.1 M 2-mercaptoethanol (pH 8.6) for 4 h and then by adding iodoacetic acid to 0.25 M and lowering the pH to 6.5 before applying the samples to the column.

**RESULTS**

**Preparation of retained toxin at pH 6.0.** After 96 h of culture, approximately 90% of the recoverable toxin was intracellular and extractable with sodium acetate solution. The toxic material was concentrated by ammonium sulfate precipitation rather than by lyophilization, because we found that the latter procedure greatly reduced the lethality of the preparation.

Gel filtration of the concentrated CEX is shown in Fig. 2. The principal peak at the void volume (Vᵥ) of the column contained the toxin. However, it also contained a large percentage of ribonucleic acid (RNA), as shown by the fact that the extinction at 280 nm is less than that at 260 nm, and by a positive Bial reaction for pentose. The RNA was separated from the toxin by ion-exchange chromatography (Fig. 3). The first peak contained up to 160,000 LD₅₀/ml. This is the only peak for which the extinction at 280 nm exceeds that at 260 nm. The latter peaks, which contained the RNA, all had less than 8,000 LD₅₀/ml. Only those samples with OD₂₆₀/OD₂₈₀ ratios greater than 1.9 were found to be free of RNA by Bial reaction.

Kitamura et al. (11) found that their toxic eluate at pH 6.0 from DE-52 was homogeneous on electrophoresis at pH 6.0, but had two components at pH 8.0. They used ion-exchange chromatography at pH 8.0 to separate these components. We were unable to confirm this finding: polyacrylamide gel electrophoresis at pH 8.0 showed that the central portion of our pH 6.0 eluate from DE-52 contained only one component (Fig. 4). The material was also homogeneous on ion-exchange chromatography at pH 8.0.

The toxin was then chromatographed on a column of Sephadex G-200 to obtain the final

![Fig. 2. Gel filtration of concentrated CEX on a Sephadex G-50 column (2.5 by 40 cm) at pH 6.0.](http://aem.asm.org/)

![Fig. 3. Chromatography of CEX on a Whatman DE-52 column (2.5 by 15 cm) at pH 6.0 with stepwise addition of NaCl. (A, B, and C) Points where samples were taken for electrophoresis. Only the first peak contained significant toxicity.](http://aem.asm.org/)
product (Fig. 5). There was one major peak at approximately $1.8 \times V_0$. The leading and trailing edges of this peak were not symmetrical, indicating that they may have contained more than one component. The central area of the peak closely approximated a normal curve and contained the toxin in a highly purified form (Fig. 6). There was also a small nontoxic fraction that was eluted considerably later. Rechromatography of the toxin on Sephadex G-200 gave a single symmetrical peak.

**Preparation of retained toxin at pH 4.5.** Lowering the pH from 6.0 to 4.5 produced a considerable change in the behavior of the toxic material on Sephadex G-50 (Fig. 7). The first peak, at $V_0$, was smaller and less toxic, and the OD at 280 nm exceeded that at 260 nm. Whereas at pH 6.0 there had been a small peak at around $2.3 \times V_0$, there was a much larger, more toxic fraction at this point. The fractions collected at this point were turbid. Upon standing overnight at 4 C, a precipitate formed. This was collected by centrifugation and was suspended in phosphate buffer (pH 6.0). It contained low levels of toxicity (2,000 LD$_{50}$/ml to 4,000 LD$_{50}$/ml); the toxicity of the supernatant fluid remained high (200,000 LD$_{50}$/ml).

This fluid was next chromatographed on Whatman DE-52. A toxic fraction eluted frontally; increasing the ionic strength of the eluant buffer by adding NaCl resulting in the elution of a large nontoxic fraction with OD$_{280}$/OD$_{260}$ less

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**Fig. 4.** Polyacrylamide gel electrophoresis of a pH 6.0 eluate from Whatman DE-52 at elution volumes of 65 (A), 75 (B), and 88 (C) ml.

**Fig. 5.** Final purification of toxin from CEX on a Sephadex G-200 column (2.5 by 97.5 cm) at pH 6.0. (D, E, and F) Points where samples were taken for electrophoresis. OD at 280 nm (—); OD at 260 nm (—–).

**Fig. 6.** Polyacrylamide gel electrophoresis of eluate from Sephadex G-200 at elution volumes of 200 (D), 270 (E), and 350 ml (F).
than unity. The fractions from the toxic peak were pooled, precipitated with ammonium sulfate, dissolved in pH 4.5 acetate buffer, and chromatographed on Sephadex G-200. The toxin eluted in a single, nearly symmetrical peak at 1.8 × Vₐ, just as it had in the purification at pH 6.0. That is, after chromatography on DE-52 and precipitation with ammonium sulfate, the apparent MW of the toxic material, as measured by Sephadex gel filtration at pH 4.5, had changed from approximately 20,000 to approximately 80,000.

Gerwing et al. (8) have suggested that one possible reason for what they considered the aggregation of Sakaguchi toxin preparation is the use of repeated precipitations with ammonium sulfate. To evaluate this possibility, the purification at pH 4.5 was repeated, substituting concentration with Sephadex G-25 for the (NH₄)₂SO₄ precipitations. This led to a considerable reduction in concentration of toxin in all stages. The eluate from Sephadex G-50 again showed two toxic peaks. Both behaved similarly on DE-52, a toxic fraction eluting frontally but with an OD₂₈₀/OD₄₀₀ ratio reduced to 1.4 to 1.6. The eluate, from DE-52, of the first peak from the G-50, was concentrated again with Sephadex G-25 and passed through a Sephadex G-200 column (1 by 30 cm) at pH 4.5 (Fig. 8).

There were two toxic components in the eluate. The substances comprising both the major peak and a second smaller peak, with an OD₂₈₀/OD₄₀₀ ratio less than 1.0, were toxic, that corresponding to the earlier larger peak of OD being the most lethal. The toxin of the earlier peak contained 20,000 LD₅₀/ml, whereas the latter one contained 8,000 LD₅₀/ml.

The second G-50 peak, after concentration with Sephadex G-25, was passed through Sephadex G-200 at pH 4.5 and gave similar results: two toxic peaks with the first containing the bulk of the activity.

Samples of the second peak eluted from Sephadex G-50 were run in an analytical ultracentrifuge. Results indicated that this fraction contained two major components. A rapidly sedimenting moiety was found to contain all of the toxicity present in the sample. The rate of sedimentation for this component was consistent with a MW of approximately 90,000. The slower component, with a MW of about 20,000, was nontoxic. The final product (the eluate from Sephadex G-200) contained only the rapidly sedimenting toxic moiety.

To further reduce the possibility of aggregation of the toxin molecules, the eluting buffer was changed to 0.025 N acetate; guanidine hydrochloride was added to this buffer in a concentration of 0.05 M. Elution patterns obtained were indistinguishable from those shown in Fig. 7 and 8 where guanidine was not used.

Samples were also purified using 0.05 N acetate buffer at pH 5.3. This gave a small reduction in the OD₂₈₀ of the first peak extracted from Sephadex G-50, with a corresponding increase in the OD₄₀₀ of the last peak. However, there was no shift of toxicity from the first to the second peak, and no precipitate formed in the fractions collected from the second G-50 peak. Toxic samples from the first peak were pooled and chromatographed on Whatman DE-52 at pH 5.3 and then precipitated and chromatographed on Sephadex G-200. The final product obtained at pH 5.3 eluted from Sephadex G-200 at 1.8 × Vₐ, just as it had at the higher and lower pH values.

**Purification of released toxin from culture filtrate.** Chromatography on Sephadex G-50 at
pH 4.5 (Fig. 9) gave two toxic components, the first at the $V_o$ and the second considerably later. The first toxic peak contained 2.5 times the activity as measured in $LD_{50}$/ml, of the second peak, although the second peak gave higher OD readings.

Ion-exchange chromatography of each toxic peak on Whatman DE-52 at pH 4.5 resulted in the frontal elution of a toxic component with an $OD_{280}/OD_{260}$ ratio of 1.9 or greater. These toxic fractions were precipitated by the addition of 70% saturated ammonium sulfate and the dissolved precipitates were chromatographed on Sephadex G-200. In each case, the result was elution of a single toxic peak at $1.8 \times V_o$.

Similar purifications carried out at pH 5.3 and 6.0 resulted in nearly complete elimination of the lethality of the second eluate peak from Sephadex G-50.

The addition of 0.05 M guanidine hydrochloride to each of the eluting buffers used did not change the behavior of the toxin.

An attempt was made to carry out the purification without using precipitation with (NH$_4$)$_2$SO$_4$ but, due to the large volumes, the low concentrations of toxin, and the relative inefficiency of the Sephadex G-25 method of concentration, this attempt had to be abandoned. Instead, samples of culture filtrate were chromatographed at both pH 4.5 and 6.0 on Sephadex G-200 before treatment of any kind, other than removal of cells and cell debris (Fig. 10). At pH 6.0, the toxic activity was found in the peak at 290 ml ($1.8 \times V_o$). At pH 4.5, significant toxic activity was found at the $V_o$ of the Sephadex G-200, at $1.8 \times V_o$ (290 ml), and at $2.7 \times V_o$ (435 ml). The last peak, at pH 6.0, was nontoxic.

**Lethality.** The toxin preparations contained $6.9 \times 10^4$ $LD_{50}$/mg to $6.4 \times 10^4$ $LD_{50}$/mg N before trypsinization and $1.2 \times 10^4$ $LD_{50}$/mg to $8.0 \times 10^4$ $LD_{50}$/mg N after trypsinization.

**MW determination of purified toxin.** To eliminate variables introduced by variations in size and packing of columns, $K_{av} = (V_e - V_o)/(V_t - V_o)$, where $V_e$ is the elution volume of a given protein and $V_t$ is the total volume of the column, was plotted against the log of the known MW of the protein (Fig. 11). The method of least squares was used to calculate the equation of the straight line best fitting the values obtained for the calibrating proteins. Then, plotting the $K_{av}$ for the toxin of _C. botulinum_ type E on the same line at its value of 0.35, one finds on the abscissa the projected MW of 86,000 for the purified protein. The same result was obtained for both trypsinized and untrypsinized samples of toxin.

**Gel filtration in 6 M guanidine hydrochloride.** Treatment with 6 M guanidine hydrochloride resulted in a complete loss of toxic activity. The elution pattern of toxin in 6 M guanidine hydrochloride is the solid line of Fig. 12. There are at least four small peaks with $K_{av}$ between 0.33 and 0.60, and one large peak with a $K_{av}$ of 0.75.

Pretreatment of the toxin with 2-mercaptoethanol and iodoacetic acid eliminates the small peaks and gives a single peak with a $K_{av}$ of 0.75 (dashed line of Fig. 12). The shoulder on the right side of the peak corresponds to the elution volume of the 2-mercaptoethanol and iodoacetic acid when they are run alone. These data suggest that the toxin with a MW of 86,000 can be split into components of lower MW under conditions which usually do not break peptide bonds.

**Stability of the toxin.** A sample of purified retained toxin was stored in pH 6.0 phosphate buffer for up to 1 year at 4 C. During the time it lost more than 60% of its original lethality. The results of gel filtration of this sample on Sephadex G-200 are shown in Fig. 13. There was a major peak with a $K_{av}$ of 0.23 and a smaller peak with a $K_{av}$ of 0.74, plus a shoulder on the first peak at the $V_o$, and apparently components...
of several other sizes as well. Toxic activity was found to be present in the peaks corresponding to the $K_m$ values of 0.23 and 0.74 and the shoulder at the $V_p$. This suggests that the bulk of the toxin was present in a form with a $MW$ of 160,000, with smaller amounts of toxin with $MW$ of 12,000 and over 200,000.

**DISCUSSION**

At pH 6.0, a highly purified toxin could be prepared from the CEX of *C. botulinum* type E. The homogeneity of this preparation was demonstrated by the formation of a single symmetrical peak on Sephadex G-200, and by the presence of a single band on polyacrylamide gel electrophoresis at pH 4.0 and 8.0. The ratio of the OD at 280 nm to that at 260 nm was 2.0.

The same procedure carried out at pH 6.0 on the culture filtrate gave essentially similar results. The toxic eluate from Sephadex G-50 contained a yellow pigment which was separable by ion-exchange chromatography. Although the concentration of toxin was lower in the final preparations from culture fluid than in those from bacterial CEX, filtration through Sephadex G-200 indicated that the $MW$ of both preparations were 86,000. This confirms the finding of Kitamura (10), that the origin of the toxin is not a significant factor in the size of the final product isolated. The toxin released into the culture medium appears to be the same as that extracted from intact cells.

When the purification of CEX was carried out at pH 4.5, the toxic activity was eluted from the Sephadex G-50 at two points. The major part of the toxic material was washed from the column quite late, which suggests that it had a LMW. Yet, further purification yielded a toxic molecule with a $MW$ of 86,000. Ultracentrifugal analysis suggested that the toxic component in the eluate from Sephadex G-50 also had a $MW$ close to this figure.

A possible explanation for this seeming disagreement is that at least a portion of the toxin in the crude preparation had a LMW, but that this form is not stable. This LMW toxin may be in equilibrium with a polymer or aggregate of a higher $MW$, the equilibrium being affected by other components of the mixture. If the gel filtration separated, or partially separated, the toxin from some substance that stabilized it in the form with a LMW, the result would be a shift to a form with a higher $MW$.

This hypothesis is supported by the behavior of the samples which were concentrated with Sephadex G-25 instead of (NH$_4$)$_2$SO$_4$. In these samples, the toxin with a LMW persisted throughout the purification procedure, albeit in low concentrations, so that a toxic fraction eluted from the final adsorption onto Sephadex G-200 in a volume suggesting a $MW$ of around 12,000. This held true whether the fraction passed through the ion-exchanger and Sephadex G-200 came originally from the first or the second toxic peak eluted from Sephadex G-50. Both these peaks were ultimately found to con-
sist of over 90% HMW toxin and a small amount of LMW toxins. The HMW toxin peak had an OD_{280}/OD_{200} ratio of 2.0, while the LMW toxin peak had a ratio of 1.3.

These results support Gerwing's contention that the use of (NH₄)_2SO₄ precipitation tends to cause aggregation of the toxin. The LMW toxin prepared by us had notable similarities to the Gerwing preparation, for which her group calculated a MW of 14,000 to 16,000. Our extrapolated value of 12,000 seems to be in reasonable agreement. Another similarity between the two preparations is the OD_{280}/OD_{200} ratio, but our results do not support Gerwing's finding that such a preparation is free of RNA. On the contrary, our results suggest that the presence of impurities may very well permit the toxin to exist in a LMW form. The trypsinized toxin prepared by Emodi and Lechowich (3) was found on gel filtration to exist in two LMW forms, 5,000 and 9,000, but their preparation was not homogeneous on electrophoresis. This may be taken as further evidence that the toxin exists in LMW forms only when in the presence of stabilizing impurities, although the digestion by RNase and the high OD_{280}/OD_{200} ratios Emodi and Lechowich obtained made it unlikely that the stabilizer was RNA.

Gerwing (5, 6) reported that the use of low concentrations of guanidine salts reduced the tendency of her preparation of LMW to form aggregates. We were unable to confirm this finding. The addition of 0.05 M guanidine hydrochloride to our buffers did not reduce the high yields of HMW toxin nor increase the yield of LMW toxin. Stronger guanidine hydrochloride (6 M) caused our HMW toxin to deaggregate, but, with this concentration of guanidine, the toxic activity was destroyed. Gel filtration of HMW toxin in 6 M guanidine hydrochloride yielded at least five peaks. The largest peak eluted in a volume which suggested a MW of around 12,000. The identity of this peak with our LMW toxin was possible, but could by no means be considered proven since the eluate was nontoxic. In addition, guanidine, by rupturing hydrogen bonds, reduces the protein molecule to a random coil, so that the elution behavior could be altered by a change in the shape of the molecule.

The fact that not all the toxin was reduced to components of approximately 12,000 MW suggests that factors other than hydrogen bonding play a part in the aggregation of the toxin. When samples of toxin in 6 M guanidine were treated with 2-mercaptoethanol and iodoacetic acid, a procedure which reduces and alkylates sulfhydryl bonds, the peaks associated with substances of a higher MW disappeared, and a single elution peak was obtained at a volume indicating a MW of around 12,000.

A report has appeared recently showing the dissociation of a trypsinized 150,000-MW toxin into components of 50,000 and 102,000 MW under reducing conditions (2). These components were not alkylated after reduction, however, and the possibility therefore remains that some recombination may have taken place. Both hydrogen bonding and covalent bonding appear, therefore, to be factors in the aggregation of the toxin. This may explain why preparing LMW toxin was so difficult, at least under the conditions studied.

Since these findings indicated that the MW of the toxin isolated was affected by conditions of the purification scheme, untreated samples of culture filtrate were chromatographed directly on Sephadex G-200 columns. These studies revealed toxic activity associated with fractions of at least three MW, 12,000, 86,000, and 200,000 or more. This last figure corresponds to toxic activity eluting in the Vₘ volume, so that only a lower limit can be stated. The heaviest fraction may correspond to either the 12S toxin assigned a MW of 350,000 by Kitamura et al. (11) or to the 14S toxic moiety prepared by Schantz and Spero (16).

Further evidence for the aggregation of the toxin is its behavior with age. When preparations of purified 86,000-MW toxin were stored for long periods, both their lethality and homogeneity gradually decreased. Gel filtration of a sample of toxin which had been stored for 6 months showed that the bulk of the preparation was in a form with a MW of 150,000; a toxic component with a MW of 12,000 was present in comparatively small amounts. It is also possible that a toxic form with a MW over 200,000 was present, but that it was separated incompletely from the 150,000-MW form.

We were unable to confirm the finding by Kitamura et al. (12) of an E₉ component, a nontoxic fraction with a MW of 150,000, which separated from their 12S toxin at pH 8.0. Our 86,000-MW toxin was homogeneous on electrophoresis at pH 4.0 and pH 8.0, and could not be resolved into two components by ion-exchange chromatography at pH 8.0. It is possible that E₉ is less strongly bound to an 86,000-MW toxin than to the larger form found by Kitamura et al. and that, therefore, it separated from the toxin at an early stage of the purification process.

The possibility that all of the type E toxins characterized thus far represent different degrees of polymerization of the same molecule is attractive. The form assigned a MW of 9,000 by
Emodi and Lechowich thus may be identical to the form to which we have tentatively assigned a MW of 12,000. The fact that Gerwing (7) arrived at a figure of 14,000 to 18,000 for her LMW preparation by ultracentrifugal analysis rather than by gel filtration would make the agreement among the three sets of figures reasonably good.

Although the present study found no evidence for an even smaller monomer, the possibility remains that the toxin with a MW of 5,000 found by Emodi and Lechowich is the monomer and the 9,000 to 16,000 form is a dimer or trimer of this.

This idea of variable polymerization is made more reasonable by the present finding of a previously unrecorded MW of 86,000 for a toxic moiety. This figure is suggestively close to one-half the value of 150,000 assigned by Sakaguchi et al. (15) to their E component, and even closer to one-fourth the MW of 350,000 they gave for their 12S protoxin. This dissociation of the 86,000 moiety into components of 12,000, which has been demonstrated, would complete the range.

Until a method is devised for obtaining reasonably pure toxin of LMW, the possibility cannot be ruled out that the toxin activity of the LMW preparations is due to the presence of a HMW toxin as a contaminant. A lethality of 10^4 LD\textsubscript{50}/mg N can be obtained from a preparation of pure LMW toxin, but this same figure can be obtained from a preparation containing 99% of a nontoxic LMW component and 1% HMW toxin with a lethality of 10^4 LD\textsubscript{50}/mg N. Under these circumstances, the HMW toxin might be very difficult to detect.

Our results support the finding of Sakaguchi et al. (15) that the MW of the toxin is unchanged by the action of the proteolytic enzyme trypsin. We found identical elution patterns from Sephadex G-200 for trypsinized and untrypsinized samples of our 86,000-MW toxin.

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