CHROMATOGRAPHIC PURIFICATION AND MOLECULAR DIMENSIONS OF TYPE E CLOSTRIDIUM BOTULINUM TOXIN

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ABSTRACT

CHROMATOGRAPHIC PURIFICATION AND MOLECULAR DIMENSIONS OF TYPE E *CLOSTRIDIUM BOTULINUM* TOXIN

By

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The objective of this study was to clarify some of the discrepancies relative to the homogeneity, aggregation, and molecular weight of type E botulism toxins.

The *Clostridium botulinum* type E Vancouver Herring strain used for toxin production was grown in a physiological saline solution in which a dialysis sac filled with culture medium was suspended. Cultures were incubated for seven days at 30 C. The extracellular toxin was separated by removing the bacterial cells by centrifugation, and purified on DEAE cellulose colums at pH 6.0 following precipitation with ammonium sulfate at 60% saturation, and dialysis.

The toxic material was fractionated on DEAE cellulose columns into toxic and non-toxic components represented by separate peaks. Disc electrophoresis of the toxic fraction on pH 4.3 gels showed three bands, two of

which were not toxic. Passage of the toxic fraction through a Sephadex G-200 column resulted in two fractions with equal toxicity. Both fractions were eluted after cytochrome c, therefore the molecular weights were less than 12,200. Final purification of the toxic fraction was carried out on CM-Sephadex C-50. The toxic fraction was retarded by the gel and released when 0.5 M NaCl was applied to the column. Disc electrophoresis of the toxic fraction showed only one band indicating that the purified toxin was homogeneous with respect to the number of components present. The toxic fraction was eluted in one peak, after cytochrome c, on a calibrated Sephadex G-200 column. Therefore, the estimated molecular weight of the purified toxin was less than 12,200.

A test for hemagglutinating activity of the toxic fractions gave negative results, while nonpurified type A and B toxins used as controls were positive.

A possible aggregation effect of toxin with ribonuclease was observed when toxin purified on DEAE cellulose was treated with the enzyme. The preparation was separated into three peak fractions on a calibrated Sephadex G-200 column. An additional highly toxic compound of large molecular weight (approximately 240,000) was obtained in addition to the two small molecular weight fractions. However, the large molecular compound was unstable and disappeared after 3 to 4 days of storage at 4 C.

The adsorption or coupling of the toxin to blue dextran, to red blood cells and to bacterial cells was observed when purified toxin mixed with those large molecular weight compounds on Sephadex G-200 columns eluted with them in the void volume.

A possible aggregation phenomenon of the toxic protein was apparent at pH 7.0 and pH 9.1. Toxic material purified on DEAE cellulose columns eluted on Sephadex G-200 columns throughout the entire fractionation range of the G-200 gel. The toxin probably existed in the forms of aggregates of different sizes.

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TABLE OF CONTENTS

LIST OF FIGURES

Figure Page

LIST OF TABLES

Table Page

INTRODUCTION

Botulism has been known as a disease for over a century, but its association with food poisoning was not established until Van Ermengem described and isolated the causative organism in the 1890's. Since then a large number of cases have been recorded and many publications have been written on the subject throughout the world, indicating the high level of interest the disease has aroused.

Most of the reported cases implicated underprocessed home-prepared foods as the causative agents and Government agencies continue to emphasize the importance of safe thermal processes in food preservation.

The safe processing of commercial products is also a concern, since the presence of toxin in these products could affect very large numbers of consumers.

Human botulism is caused by the microorganisms of Clostridium botulinum types A, B, or E, however, it should be noted that the other types C, D, and F are also involved on rare occasions.

The microorganisms are Gram-positive, spore forming rods, motile by means of peritrichous flagella. They

produce powerful neurotoxins which upon ingestion or injection may cause death. Death usually results from respiratory paralysis, since the toxins act on the acetylcholine release mechanism. The full toxicity of type E toxin becomes available only after treatment with trypsin.

Type A and B toxins also possess hemagglutinating properties which can be retained without retention of toxicity. The hemagglutinating activity of type E toxin has not been demonstrated and relatively few data are available for type E toxin.

Numerous attempts have been made to purify these toxins and to determine their chemical composition or molecular structures. The purification procedures yielded products of reported high and uniform potency with widely different molecular weights, and questionable homogeneity.

Purification of the toxin formed by type E Clostridium botulinum has been aarried out by several techniques. Gordon et al. (1957) precipitated the toxin with ethanol and used ionized cellulose columns for purification. Gerwing et al. (1964) used ammonium sulfate to precipitate the toxin, further purified the concentrated dialysate on DEAE cellulose columns, and calculated a molecular weight of 18,600 (S_{20W} = 1.70S). Sakaguchi and Sakaguchi (1967) digested the "precursor" toxin first with ribonuclease and then used CM-Sephadex and Sephadex G-200 columns for purification and to estimate molecular weight.

Their results, in contrast to those of Gerwing et al., indicated a uniform molecular weight of 200,000 or more.

Due to the above discrepancies, attempts were made to clarify some of the questions relative to the homogeneity, aggregation, and molecular weight of the type E toxins.

LITERATURE REVIEW

Toxin Isolation and Purification

The isolation and purification of Clostridium botulinum toxins have involved a variety of techniques such as precipitation with acid, shaking with chloroform, precipitation with ethanol, high speed centrifugation, extraction with salt solutions, precipitation and crystallization from salt solutions, and recently chromatography on cellulose compounds.

One of the earliest isolation procedures, which was developed by Brieger and Kempner (1987), used zinc chloride to precipitate type B C.botulinum toxin. Sommer, Sommer and Meyer (1926) described a gentler method in which type A toxin was adsorbed to aluminum hydroxide and then eluted with ammonium phosphate. Snipe and Sommer (1928) used an acid precipitation procedure for concentrating the toxin, and thus provided a basis from which purification procedures have proceeded. None of these early methods produced highly purified toxins. The potency of these products was little more than one-thousandth of the toxicity reported by Lamanna, Eklund, and McElroy (1946), and Lamanna, McElroy and Eklund (1946) who isolated type A

toxin in crystalline form with a specific activity of 2.2 x 10^8 minimum lethal dose (MLD) per mg N. These workers used a procedure of precipitation with acid, followed by shaking with chloroform and final crystallization from an ammonium sulfate solution. Abrams, Kegeles and Hottle (1946) also isolated type A toxin in crystalline form by precipitating the toxin with sodium sulfate instead of shaking with chloroform. Duff et al. (1957) reported an improved method for isolating type A toxin that used acid precipitation at pH 3.5 with 3 N sulfuric acid, extraction of the toxin in 0.075 M calcium chloride at pH 6.5, a second acid precipitation at pH 3.7 with 1 N hydrochloric acid, a subsequent precipitation with 15 % alcohol at -5 C, and final crystallization from 0.9 M ammonium sulfate solution.

Cardella et al. (1958) precipitated type C toxin from the culture with 25 % ethanol, extracted the toxin from this precipitate with 0.05 M calcium chloride at pH 5.0, and reprecipitated the toxin with 15 % ethanol at -5 C.

Cardella et al. (1960) also purified type D toxin by slightly modifying the procedure used for type C toxin. The type D toxin after the initial ethanol precipitation was extracted with 0.075 M calcium chloride at pH 6.5 and reprecipitated with 10 % ethanol at -5 C.

Gordon et al. (1957) purified crude type E toxins by precipitating with 25 % ethanol in the cold, then extracted the toxin from the precipitate with 0.075 M calcium chloride solution at pH 6.0, and reprecipitated with 25 % ethanol at -5 C. Fiock, Yarinsky and Duff (1961) described the production, purification and conversion to toxoid of type E toxin treated with trypsin. They treated the culture with 0.1 % trypsin (pH 6.0) for 2 hr at 37 C. Purification was accomplished by precipitation with ammonium sulfate at 60 % saturation, extraction with calcium chloride, and reprecipitation with ethanol in the cold. The purified toxin was then converted to toxoid by incubation with formalin adsorbed on aluminum phosphate. Gerwing, Dolman and Arnott (1961) prepared highly purified type E toxin by modifying the method described by Gordon et al. (1957). They added 95 % ethanol to toxic filtrates to produce a final concentration of 35 % ethanol. After centrifugation the precipitate was resuspended in 0.05 M sodium acetate buffer (pH 6.0) and further purified on DEAE cellulose columns. Gerwing et al. (1964) further modified this purification procedure by using ammonium sulfate as an initial precipitating agent instead of ethanol. Sakaguchi and Sakaguchi (1961) and Sakaguchi, Sakaguchi and Imai (1964) extracted type E toxin from bacterial cells with 1 M acetate buffer at pH 6.0. The toxin was precipitated from the extract with ammonium sulfate to

obtain 50 % saturation, centrifuged, and the precipitate redissolved in 0.05 M acetate buffer. This precipitation procedure was repeated three times, and was followed by chromatography on Sephadex G-25, digestion, with ribonuclease and further purification on CM - Sephadex C-50.

Toxin Potency, Homogeneity, and Molecular Size

The purification procedures described yielded products of high and uniform potency. The specific activity for type A toxin ranged from 2.2×10^8 to 2.7×10^8 MLD per mg N. For type B toxin the range of specific activities was from 1.25×10^8 to 2.6×10^8 MLD per mg N. The data for type E toxin are less constant. Sakaguchi and Sakaguchi (1961) reported a potency of 1.7×10^5 MLD per mg N, while Gerwing, Dolman and Arnott (1961, 1962), and Gerwing *et al.* (1964) obtained a potency of 6.0×10^5 and 7.5×10^6 MLD per mg N, when ammonium sulfate was substituted for ethanol as a precipitating agent. However, a considerable variation exists in the reported molecular weights.

Lamanna, Eklund and McElroy (1946), Lamanna, McElroy and Eklund (1946) and Putnam, Lamanna and Sharp (1946) determined a molecular weight of 900,000 to 1,000,000 for type A toxin. On the basis of cysteine and cystine contents the minimum molecular weight of crystalline toxin was calculated as 45,000 (Buehler, Schantz, and

Lamanna, 1947). The high molecular weight toxin concept was supported by Duff et al. (1957) who obtained a value of S_{20W} = 14.5, implying that the molecular weight is indeed very large.

Gerwing, Dolman and Bains (1965) reported the isolation of a toxic moiety of low molecular weight for type A. The toxic substance was shown to be homogeneous using electrophoresis and ultracentrifugation and a calculated molecular weight of 12,200 was obtained.

The homogeneity of type A toxin obtained in crystalline form by Lamanna, McElroy and Eklund (1946) remained unquestioned for 20 years. However, observations made by Lamanna (1948) showed that the crystalline toxin also contained a hemagglutinin which could be dissociated from the toxin with a two to three-fold increase in specific activity (Lamanna and Lowenthal, 1951; Lowenthal and Lamanna, 1951). Wagman and Bateman (1951, 1953) and Wagman (1954) later found the molecular size and shape of purified crystalline type A toxin was dependent upon the pH and salt concentration of aqueous buffers used as solvents. They isolated toxic fragments formed at alkaline pH that had a molecular weight about 70,000.

Heckley, Hildebrand and Lamanna (1960) showed that toxin appearing in the lymph of orally poisoned rats had a significantly lower sedimentation constant than that obtained with undissociated crystalline toxin, which had a

molecular weight of 900,000. Similar results were reported on the toxin appearing in the plasma and lymph of rabbits injected intravenously by Hildebrand, Lamanna and Heckly (1961).

Schantz and Lauffer (1962) studied the diffusion rate of type A toxin and estimated the molecular weight between 10,000 and 20,000. Wagman (1963) found that purified type A toxin irreversibly dissociated at pH 9.2 with little loss in toxicity and formed a principal and a minor component with sedimentation constants of 7 S and 13.7 S. No reaggregation of the 7 S toxin was observed upon readjustment of the pH to 3.8. Therefore, he suggested that these products are components of large aggregates. The diffusibility of significant quantities of these toxic fragments through cellulose dialysing membranes was observed in several experiments but not in others.

Boroff and DasGupta (1964, 1966), DasGupta, Boroff and Rothstein (1966), and DasGupta and Boroff (1968) questioned the homogeneity of the crystalline toxin and they demonstrated that chromatographically the toxin can be resolved into a toxic (α) and into a hemagglutinin (β) fraction. The toxic fraction had a molecular weight of 150,000 at the physiological pH range of 7.0 to 7.4. The hemagglutinin appeared to exist in three forms of aggregation (β_1, β_2 and β_3) with molecular weights of β_1 = 290,000, β_2 = 500,000 and β_3 = 900,000. Boroff et al. (1966)

analyzed crystalline type A toxin in the ultracentrifuge for homogeneity and the α fraction was analyzed for molecular weight. The crystalline whole toxin resolved into two components with S values of approximately 7 S and 13.5 S. The α fraction was homogeneous and a molecular weight of 128,000 was determined.

A molecular weight of 60,000 was reported by Lamanna and Glassman (1947) for type D toxin using membrane diffusion techniques. Duff et al. (1957) found purified B toxin was essentially homogeneous when examined in the analytical ultracentrifuge. The toxin consisted of a main component of 14.9 S and a diffusely sedimenting minor component with an S value of 10.9. These results are similar to those reported by Wagman and Bateman (1951). They determined by sedimentation and diffusion measurements that the molecular weight of the major component in a similar preparation of type B toxin was about 500,000. Gerwing et al. (1966) showed that purified type B toxin was monophoretic and monodisperse in the ultracentrifuge and on the basis of the biophysical studies and amino acid analysis a molecular weight between 9,000 and 10,000 was calculated.

However, the homogeneity of the purified B toxin was seriously questioned by Boroff, DasGupta and Fleck (1968). They examined the toxin and separated it into two components, a toxic and a non-toxic component. The

molecular weight of the toxic component was 100,000 or greater and resembled the component isolated from the crystalline toxin of type A. Recently Gerwing, Morrell and Nitz (1968) demonstrated that the toxin was synthesized in the later stage of logarithmic growth and was released into the supernatant fluid of the culture during lysis of the cells. Sedimentation studies of the intracellular toxin showed one fraction with low molecular weight (S = 2.5) and low specific activity, and one fraction with high molecular weight (S = 15.3) and with specific activity similar to that isolated from culture lysates. The high molecular weight toxin was composed of an aggregate of small sub-units.

The presents of two toxic units was also shown in type A toxin by Hauschild and Hilsheimer (1968). They found two equally toxic units with molecular weights of 400,000 to 450,000 and 150,000 to 200,000.

Only two estimations of the molecular weight of type E C. botulinum toxin are available. Sakaguchi, Sakaguchi and Imai (1964) reported that purified type E toxin did not penetrate Sephadex G-200 gel, therefore, the molecular weight must be 200,000 or larger. Ultracentrifugal studies on purified type E toxin done by Fiock, Yarinsky and Duff (1961) showed that the partially purified fraction produced by their procedure was not homogeneous. Two components with calculated S values of 12.5

and 4.7 were observed. The more quickly sedimenting boundary represented the major component. Gerwing, Dolman and Arnott (1962) also analysed a highly purified preparation of type E botulinum toxin ultracentrifugally. They obtained sedimentation constants of 5.6 for the toxic component and 1.1 for the non-toxic component. Gerwing et al. (1964) later reported that a highly purified type E toxin was electrophoretically and ultracentrifugally homogeneous and possessed a calculated molecular weight of 18,600. Recalculation of the molecular weight by Gerwing, Dolman and Ko (1965) based on the analysis of amino acid residues showed that the molecular weight of the pure toxin was around 14,000 to 16,000 or roughly 20 % less than the figure based on ultracentrifugal analysis.

Kitamura, Sakaguchi and Sakaguchi (1967) found the purified material below pH 6.0 homogeneous with S_{20W} = 12.3. However, this material above pH 7.0 resolved into two fractions, a toxic and a non-toxic fraction each with S values of 7.3

Composition and Active Site of the Toxin

Buehler, Schantz and Lamanna (1947) studied the amino acid composition of type A toxin by microbiological assay. They found that the total number of amino acid residues were 7754 and that cysteine was the limiting amino acid. Analysis showed a total sulfur content of 0.436 %

and was accounted for in the methionine, cysteine and half-cystine content. The nitrogen content of the toxin was 16.2 % which was slightly higher than the 14.2 % nitrogen in the toxin reported by Abrams, Kegeles and Hottle (1946).

Lamanna (1959) defined the toxins as simple globular proteins composed exclusively of amino acids. He described type A crystalline toxin as a white odorless protein with "unknown taste" and with an isoelectric point about pH 5.6.

Stefayne et al. (1964) using chromatographic techniques for amino acid determination of the toxin found agreement with Buehler's report for 14 amino acids. Some values for tyrosine, phenylalanine, tryptophan, serine and glycine were different.

Schantz and Spero (1957) studied the active site of the toxin by reacting the toxin with ketene. They found that 98 % of the toxicity was lost when 19 % of the amino groups had reacted. Nitrous acid also deactivated the toxin rapidly. Spero and Schantz (1957) interpreted this as a deamination effect. Weil et al. (1957) found a loss of toxicity due to photo-oxidation when methylene blue was present. Boroff and Fitzgerald (1958) and Boroff (1959) reported that when toxin is treated with alkali, with certain iron slats, or with antisera to the toxin, there is a decrease in flourescence which correlates with the resulting decrease in toxicity. They suggested that the structure

responsible for flourescence and toxicity is the same or related.

Schantz, Stefanye and Spero (1960) refuted this opinion. They inactivated the toxin with urea without the parallel reduction in flourescence. Spero (1958) reported that the loss of toxicity at alkaline pH is caused by the ionization of the ε-amino groups of lysine. Toxin dissolved in buffered solutions at a temperature of 15 C and pH up to 10.58 was very stable for 3 hr, but 50 % of the toxicity was lost at pH 10.97 within minutes, and at pH 11.2 all toxicity was lost in 1 to 2 minutes.

Coleman and Meyer (1922) observed that viable type A spores heated at 80 C for 30 min still produced toxin. Grecz and Lin (1967) made a distinction between heat sensitive vegetative toxin and heat resistant spore toxin. The vegetative toxin was inactivated by temperatures as low as 70 C within 15 seconds, while spore toxin was heat resistant and was not destroyed by heating for 10 min at 80 C.

To date heat resistant spore toxin has been found in type A but not with types B and E (Kempe and Graikoski 1962, and Schneider, Grecz and Anellis 1962). Type B and E toxins released in the medium were denatured by heating for 10 min at 80 C.

Cartwright and Lauffer (1958) observed that at pH 6.9 toxin was detoxified in minutes at 50 C, while at 40 C the toxin was stable for more than 1 hour.

Boroff and DasGupta (1964) reacted type A toxin with 2-hydroxy-5-nitro-benzylbromide (HNBB) and contended that inactivation was due to tryptophan modification, since HNBB reacts specifically with tryptophan. Actually HNBB reacts with cysteine also (Koshland, Harkhanis and Latham, 1964).

Gerwing et al. (1966a, 1966b) found 17 amino acids and approximately 85 amino acid residues in type B toxin. Arginine was identified as the single N-terminal amino acid residue. They concluded that cysteine played a critical role in toxicity since toxicity was rapidly lost when cysteine residues reacted with para-chloromercuricbenzoate (PCMB), or N-(4 dimethyl-amino-3,5-dinitrophenyl)-maleimide (DDPM). They found no tryptophan in type B toxin.

Gerwing, Dolman and Ko (1965) reported the loss of 18 of the amino acid residues present in the original protein when type E toxin was activated by trypsin. The N-terminal amino acid residue was glycine in the non-activated toxin and arginine in the trypsin treated toxin.

The sequence data for type A, B and E botulinum toxins were published by Gerwing, Mitchell and Van Alstyne (1967). They concluded that the cysteine residue, in combination with other amino acid residues which may be close

to it in terms of protein tertiary structure, is involved in the toxic activity of these proteins.

Due to the above discrepancies, the need for additional studies is apparent.

MATERIALS AND METHODS

Microorganism. Clostridium botulinum type E Vancouver Herring (VH) strain was used for toxin production. The strain was obtained from Dr. C. F. Schmidt, Continental Can Company, Chicago, Illinois.

Culture medium. A medium consisting of 5.0 % trypticase (B.B.L.), 0.5 % peptone (Difco), 1.0 % yeast extract (Difco), 0.2 % sucrose, and 0.02 % sodium thioglycollate at pH 7.2 (TPSY) was used. This medium ahd previously been found to yield excellent growth, sporulation and toxin production by type E strains of C. botulinum.

The organisms were grown and toxin was produced by a modified version of the sac method of Vinet and Fredette (1951). Two- or 3-liter Erlenmeyer flasks were filled with physiological saline solution and a dialysis sac filled with medium was suspended in each flask. The ratio of the medium to saline solution was approximately 1:4. An inoculum of 15 ml of a 12 to 16 hr actively growing culture was introduced into the saline solution and incubated at 30 C for 7 days. Toxin assays and protein determinations were carried out daily.

Toxicity assays. White mice weighing 15 to 20 g were injected intraperitoneally with 0.5, 0.2, and 0.1 ml of serially diluted toxic preparations. Samples were digested with 1.0 % trypsin (Difco, 1:250) in an equal volume of 0.05 M sodium phosphate buffer at pH 6.0. Further dilutions were made in the same phosphate buffer. Two mice were injected per dilution. The highest dilution at which the mice died within 96 hr was used for mouse lethal dose (MLD) calculations.

Analytical methods. Procedures used to obtain protein concentrations varied according to the accuracy that was required.

The spectrophotometric method (Warburg and Christian, 1942) was used for a rapid estimation of protein concentration of the fractions. Protein was determined spectrophotometrically by measuring the absorption of light at wavelengths of 280 and 260 nanometers, with a correction for the nucleic acid content from the data given by Warburg and Christian (1942) for known mixtures of crystalline enolase and yeast nucleic acid (Merck). The determination was carried out in silica cells (d = 1.0 cm). One aliquot of the protein solution was suitably diluted with water or buffer to a volume of 3.0 ml and the absorbance was read on a Beckman DBG spectrophotometer (Beckman Instruments, Fullerton, California) at 280 and 260 nm

against a blank containing the solvent. The protein or nucleic acid concentration was then obtained from the nomograph.

The Biuret reaction (Gornall et al., 1949) was applied to determine protein concentration in the partially purified toxic supernatants.

The method of Lowry et al. (1951) was used to estimate the protein concentration in the purified fractions.

Total nitrogen determinations were carried out by the micro-Kjeldahl method (Kabat and Mayer, 1948) on the purified concentrated toxins used for ultracentrifugal work.

Purification procedures. Purification procedures were modified from the procedures of Gerwing et al. (1946). A schematic flow sheet of the procedures used in these studies is presented in Figure 1. The bacterial cells were separated from the toxin by centrifugation at 5,000 x g for 20 min at 4 C. The toxic supernatnat was then filtered through a Millipore membrane filter with a mean pore size of 0.45 μm, and the toxin was precipitated overnight at 4 C with crystalline ammonium sulfate $[(NH_4)_2SO_4]$ at 60 % saturation. The precipitate was then collected by centrifugation for 20 min at 10,000 x g at 4 C, dissolved in 0.05 M sodium phosphate buffer at pH 6.0, and dialysed overnight at 4 C. Any insoluble material present after

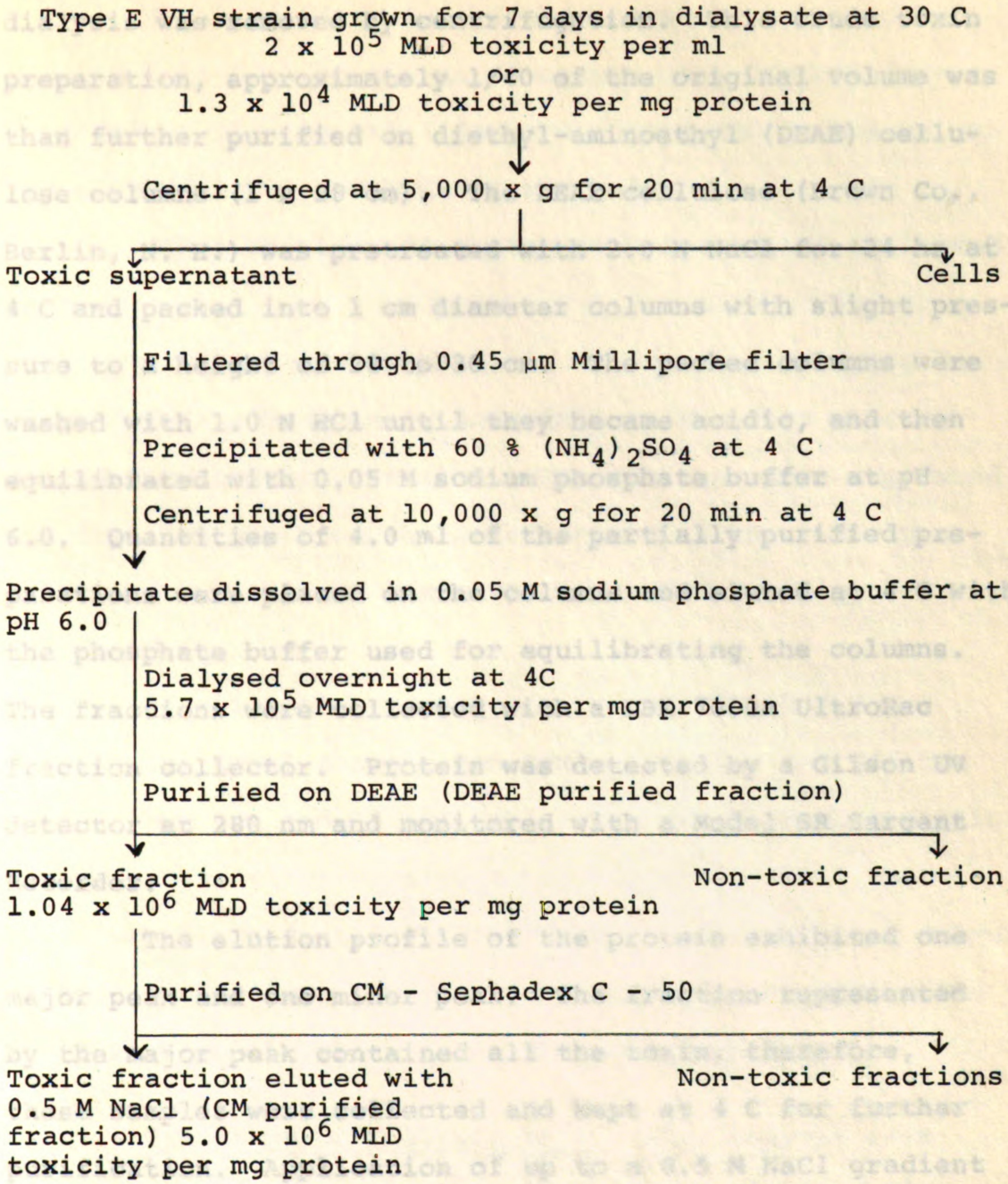


Figure 1. Schematic diagram of the purification procedure of type E Clostridium botulinum toxin

dialysis was removed by centrifugation. This crude toxin preparation, approximately 1/40 of the original volume was than further purified on diethyl-aminoethyl (DEAE) cellulose columns (1 x 28 cm). The DEAE cellulose (Brown Co., Berlin, N. H.) was pretreated with 2.0 N NaCl for 24 hr at 4 C and packed into 1 cm diameter columns with slight pressure to a height of 28 to 30 cm. The packed columns were washed with 1.0 N HCl until they became acidic, and then equilibrated with 0.05 M sodium phosphate buffer at pH 6.0. Quantities of 4.0 ml of the partially purified preparations were placed on the columns and eluted at 4 C with the phosphate buffer used for equilibrating the columns. The fractions were collected with a LBK 7000A UltroRac fraction collector. Protein was detected by a Gilson UV detector at 280 nm and monitored with a Model SR Sargent Recorder.

The elution profile of the protein exhibited one major peak and one minor peak. The fraction represented by the major peak contained all the toxin, therefore, these samples were collected and kept at 4 C for further purification. Application of up to a 0.5 M NaCl gradient on the columns did not elute more toxin.

The approximate protein content of the major fraction was determined by the spectrophotometric method. The nucleic acid content was also calculated from the adsorbance at 280/260 nm.

Final purification of the toxin was achieved by placing 1 to 2 ml of the major sample on a CM-Sephadex C-50 column (1 x 28 cm). Toxin was retarded on the column and eluted with 0.5 M NaCl in the buffer.

Other final purification techniques also evaluated were:

a. Disc electrophoresis was carried out in gels at pH 4.3 as subsequently described in the disc electrophoresis methodology section. The proteins were separated and then the parts of the gels containing the toxic protein were cut out and toxin was extracted overnight as described.

b. Disc electrophoresis in gels at pH 4.3 was carried out until the two non-toxic proteins ran off the gels. Then the end of the tubes were capped with dialysis membranes and electrophoresis was continued until the toxic protein migrated off the gels. The toxin was driven in this manner into the few drops of buffer occupying the space between the gel and the dialysis membrane.

Treatment of dialysis sacs. All dialysis sacs used in the experiments were boiled for 5 min in 0.01 M ethylenediaminetetra-acetic acid (EDTA) solution at pH 7.0 (Gerwing et al., 1964) to prevent the inactivation of toxin by surface-active agents. The bags were washed in distilled water after they were boiled.

Determination of sedimentation coefficients. A Beckman-Spinco model E analytical centrifuge equipped with a synthetic boundary cell was used for determining the sedimentation coefficient. Determinations were made at 20 C for 40 min at 59,780 rpm using protein concentrations of 3.85 mg and 6.12 mg protein per ml of 0.05 M sodium phosphate buffer at pH 6.0.

Concentration of toxin. The toxic preparation was concentrated by evaporation for the centrifugal studies. Dialysis bags were filled with DEAE purified toxic fractions and were suspended in front of a fan at 4 C. Evaporation was completed when approximately 90 % of the liquid was lost. The concentrates were dialyzed overnight against 0.05 M phosphate buffer at pH 6.0 and the nitrogen content was determined by micro-Kjeldahl determination. Protein concentrations were calculated by using the equation protein = N x 6.25.

Disc electrophoresis. Disc electrophoresis of the toxic fractions was performed in pH 4.3, 7.0 % acrylamide gel, using pH 5.0 β-alanine-acetic acid buffer, and in pH 8.9, 7.0 % acrylamide gel using pH 8.3 tris-glycine buffer with a modification of the David (1964) technique. The concentrations of stock solutions and preparations of working solutions were different from those of Davis and are given below. A sample of 50 to 100 mg of protein was

placed on the gel and electrophoresis was carried out at 4 C with a current of 3 m. Amp. per tube, applied to an analytical temperature regulated disc electrophoresis apparatus (Polyanalyst, Buchler Instruments, Fort Lee, N.J.). The current was obtained from a power supply regulated with a Heathkit variable voltage regulator, Model PS-3. (Heath Co., Benton Harbor, Mich.). Two tubes per sample were used for electrophoresis: one was stained to detect the bands while the other one was used for toxin assay. Portions of the unstained gel corresponding to the bands on the stained gel were cut out, macerated, and extracted overnight at 4 C in physiological saline phosphate buffer at pH 6.0 (1 to 2 ratio), then trypsinized and assayed for toxin.

Stock solutions for positively and negatively charged proteins were prepared and mixed as listed in the appendix.

Test for hemagglutinating activity. The rapid test of DasGupta and Boroff (1968) was used in which a drop of human red blood cells in suspension was mixed with a drop of partially purified toxin on a microscopic slide. Agglutination was observed with or without the aid of a microscope.

Quantitative estimation of hemagglutination was done by adding 0.5 ml of a 2.0 % suspension of human red

blood cells in physiological saline to 0.5 ml of serially diluted purified toxin (1 to 10 then 2 fold dilutions). The mixtures were incubated at 37 C for 60 min and then at 4 C overnight. Reactions were considered as positive in which all cells were clumped at the bottom of the tube and resuspended as distinct granules, flakes, or flocculent masses in an otherwise clear fluid. The most dilute sample which caused cell clumping was recorded.

Estimation of molecular size. The size of the toxic fractions was estimated by gel filtration (Andrews 1965). Columns were prepared as follows: to Sephadex G-200 gel (water regain 20 ± 2 g/g) distilled water was added and the gel was allowed to swell on a boiling water bath for 5 hr. The hydrated gel was deaerated under vacuum and the columns (1 x 28 cm) were filled at 4 C and equilibrated with 0.05 M sodium phosphate buffer at pH 6.0. The void volume was determined with blue dextran 2,000 (M.W. 2×10^6). Blue dextran (0.2 g) was dissolved in 25 ml of distilled H_2O and 0.05 to 0.1 ml was applied on the column. The column was calibrated with hemoglobin (M.W. 6.7×10^4), cytochrome c (M.W. 1.22×10^4), and aldolase (M.W. 1.4 to 1.5×10^5).

Solutions of 4 mg/ml of hemoglobin, 5 mg/ml of cytochrome c and 4 mg/ml of aldolase were prepared and 0.05 to 0.1 ml of each were applied on the column. The elution

volumes (V_e) of each of these compounds were carefully measured and plotted versus their molecular weights on semi-log paper. A straight line relationship was obtained. The molecular weights of the unknown toxic proteins were determined by obtaining their elution volumes on the column and then determining their molecular weights from the standard plot.

The sources of proteins used were as follows: human hemoglobin, (lot No. T-3131, Mann Research Laboratories, N.Y., N.Y.); aldolase, (rabbit muscle, lot No. U-114, Mann Res. Lab., N.Y., N.Y.); cytochrome c, (equine heart, Type III, lot No. 87B-7131, Sigma Chemical Co., St. Louis, Missouri). Blue Dextran 2,000 and Sephadex G-200 were obtained from Pharmacia Chemical Company, Uppsala, Sweden.

Preparation of buffers. Phosphate buffers were prepared by titrating 0.05 M Na_2HPO_4 with 0.05 M NaH_2PO_4 to the required pH. The Tris-HCl buffers were prepared by titrating 0.05 M Tris with 0.05 M HCl to the required pH.

RESULTS AND DISCUSSION

Toxin Production

The modified dialysis sac procedure was used for growth and toxin production of type E *Clostridium botulinum*. The procedure was modified in that the saline containing the inoculum was placed outside and the medium was placed inside the sac.

The original sac method was used successfully for toxin production by Gerwing, Dolman and Arnott (1961). They obtained toxic filtrates with 3.2×10^3 MLD activity per mg N, or 2.0×10^4 MLD activity per mg protein. The toxic filtrate produced by our modification had a slightly lower activity of 1.3×10^4 MLD per mg protein, however, our method did not require the construction of an apparatus. We harvested the toxin after 7 days of incubation while they separated their toxin from the cells after 5 days of incubation. We found a difference in toxin production among the type E strains. The Kalamazoo strain reached a peak of toxin production after 4 to 5 days of incubation. Due to the differences in methods, in number of days of incubation and in strains used for toxin production, the

method to obtain maximum toxin production must be determined for each strain.

The sac technique has several advantages over the usual bottle or flask technique. The bacteria are grown and toxin is produced in a dialysate, therefore, the produced toxin has fewer impurities than the toxin produced in bottle or flask cultures. The toxins produced in the saline are free from nondialysable constituents in the medium, and the large amount of impurities in the medium do not interfere with the purification procedures. The nutrients constantly diffuse into the saline and provide a renewed supply of nutritive substances for growth. Some of the dialysable metabolic products which might restrain growth are removed. Thus much heavier growth could be obtained.

The Erlenmeyer flasks were capped with aluminum foil which allowed the gas produced to escape and eliminated the danger of a flask explosion by seating of the screw cap.

The medium in the sacs was used at 1, 2, or 4-fold concentrations. Best results were obtained when the 2 fold concentration of TPSY medium was used. There were fewer numbers of bacteria produced in normal TPSY medium than when a 2 fold concentration was used. The 4 fold concentration was unsatisfactory because the highly concentrated medium enhanced diffusion of water into the bags and constant leakage occurred at the top of the sacs. Thus a

route for bacterial contamination was opened and a constant loss of growth medium was also encountered. Therefore, the medium at 2 fold concentration was used for toxin production. Two milliliters of sample were removed daily for toxin and protein assays. The amount of toxin produced in the cultures steadily increased during the first 7 days of incubation (Table 1). A slight reduction in the amount of

Table 1.--Toxin production of the VH strain in "dialyzed" TPSY medium

Days of incubation at 32 C	Toxin in dialysate in MDL/ml
1	4×10^2
2	2×10^3
3	2×10^4
4	4×10^4
5	4×10^4
6	2×10^5
7	4×10^5
8	2×10^5
9	1×10^5

toxin produced was noticed on the 8th and 9th days of incubation, therefore, toxin was separated from the cells on the 7th day of incubation by centrifugation at 5,000 x g at 4 C. The activity of the toxin was measured in MLD rather than in LD_{50}. The purpose of the toxin assay was to find the time when the cultures contained the maximum amount of toxin and for this purpose determination of MLD

was quite satisfactory. Considerably fewer mice were required for the MLD titration than the LD_{50}, and the labor involved was also greatly reduced.

The appearance of the cultures was followed by observing a representative sample of the cultures daily with the aid of the microscope fitted with phase contrast optics. The cultures consisted of mostly vegetative cells and very few spores on the first and second day of incubation. About 40 % sporulation and a great number of actively growing vegatative cells were observed on the third day. The number of growing vegetative cells decreased sharply on the fifth and sixth days of incubation, and the number of free spores also greatly increased. Cell disintegration and accumulation of cell debris was observed from the third day on. Gerwing, Morrell and Nitz (1968) studied the synthesis and nature of toxin of type B C. botulinum in growing cells and demonstrated that the toxin was synthesized in the latter stage of logarithmic growth and was released into the supernatant fluid during lysis of the cells.

Toxin Isolation and Purification

A sufficient number of flasks were prepared so that approximately 14 liters of toxic supernatant was obtained and the toxic material was precipitated as indicated in Figure 1, with 60 % saturation of crystalline ammonium sulfate $(NH_4)_2SO_4$. The toxic precipitate appeared as a

whitish flocculent material which settled out when left overnight at 4 C. The precipitate was sedimented by centrifugation and the sedimented toxin material was dissolved in 0.05 M sodium phosphate buffer at pH 6.0. The toxic material was water soluble and dissolved very easily in the pH 4.5 to 7.0 buffers used in our studies.

The partially purified material, approximately 1/20 to 1/40 of the original culture volume, contained 5.7 x 10^5 MLD toxicity per mg protein and was not ready for further purification of DEAE cellulose columns. The toxic material was dialyzed overnight at 4 C against the phosphate buffer to remove all traces of $(NH_4)_2SO_4$. Most of the purification was done at 4 C to prevent a loss in toxin activity. The dialysate was assayed for toxicity to determine if any toxin diffused through the sac. Ordinary dialysing sacs will permit materials with molecular weights below 30,000 to pass through. Throughout the course of our studies toxin in the dialysate was never observed after precipitation with $(NH_4)_2SO_4$. The medium in the dialysis sac suspended in the saline solution in which the cultures were growing also never contained any toxicity. Therefore, the toxic material produced by the bacteria and the active material precipitated by $(NH_4)_2SO_4$ were probably large enough not to pass through the membrane.

The partially purified toxic material separated on DEAE cellulose columns into a major fraction and a minor

fraction (Figure 2). The major peak samples were highly toxic and contained 1.0 x 10^6 MLD toxicity per mg protein. The specific activity of this purified material was approximately 50 % higher than that prepared by $(NH_4)_2SO_4$ precipitation. The smaller fraction was only slightly toxic and contained 1 x 10^3 MLD toxicity per mg protein. Since the small fraction followed the major fraction fairly closely, probably some overlapping occurred that caused the negligible activity of this fraction.

The separation of the toxin of type E strain Iwanai into two components using DEAE cellulose was reported by Gerwing et al. (1964). They obtained a highly toxic fraction containing 7.5 x 10^6 MLD per mg N, and a non-toxic fraction. Our results are in agreement with theirs, except they obtained a higher specific activity of the toxin. This might be explained by the difference in buffer systems used. Sakaguchi, Sakaguchi and Imai (1964) pointed out that the toxin was approximately 5-fold less active when pH 7.5 phosphate buffer was used for elution than when acetate buffer at pH 6.0 was used. Other variants such as strain differences should also be taken into account.

The principle involved in DEAE cellulose purification is that the toxic material elutes frontally while most of the other components are strongly adsorbed to the acidified DEAE cellulose and are released when the pH is altered or when the molarity of the eluent is changed.

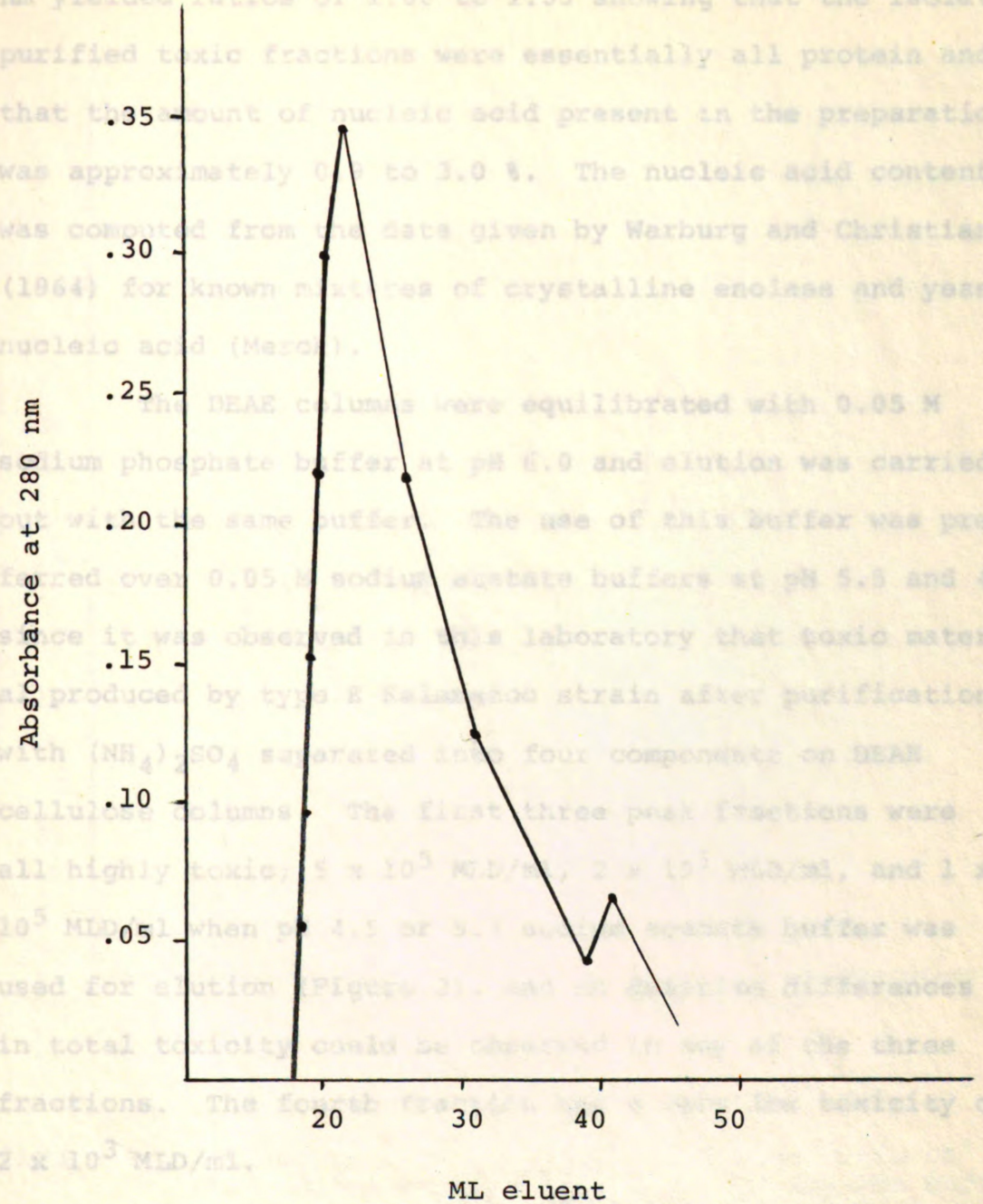


Figure 2. Elution patterns of Type E VH toxin on DEAE cellulose with 0.05 M sodium phosphate buffer, pH 6.0. The major component contains the toxin

Absorbance of the major peak fractions at 280/260 nm yielded ratios of 1.06 to 1.35 showing that the isolated, purified toxic fractions were essentially all protein and that the amount of nucleic acid present in the preparation was approximately 0.9 to 3.0 %. The nucleic acid content was computed from the data given by Warburg and Christian (1964) for known mixtures of crystalline enolase and yeast nucleic acid (Merck).

The DEAE columns were equilibrated with 0.05 M sodium phosphate buffer at pH 6.0 and elution was carried out with the same buffer. The use of this buffer was preferred over 0.05 M sodium acetate buffers at pH 5.5 and 4.5 since it was observed in this laboratory that toxic material produced by type E Kalamazoo strain after purification with $(NH_4)_2SO_4$ separated into four components on DEAE cellulose columns. The first three peak fractions were all highly toxic; 5×10^5 MLD/ml, 2×10^5 MLD/ml, and 1×10^5 MLD/ml when pH 4.5 or 5.5 sodium acetate buffer was used for elution (Figure 3), and no definite differences in total toxicity could be observed in any of the three fractions. The fourth fraction had a very low toxicity of 2×10^3 MLD/ml.

Toxic activity of 7×10^4 MLD/ml was obtained only in the third peak fraction when the same toxic material was eluted with pH 6.0 phosphate buffer (Figure 4). The other three fractions contained no activity. Although the

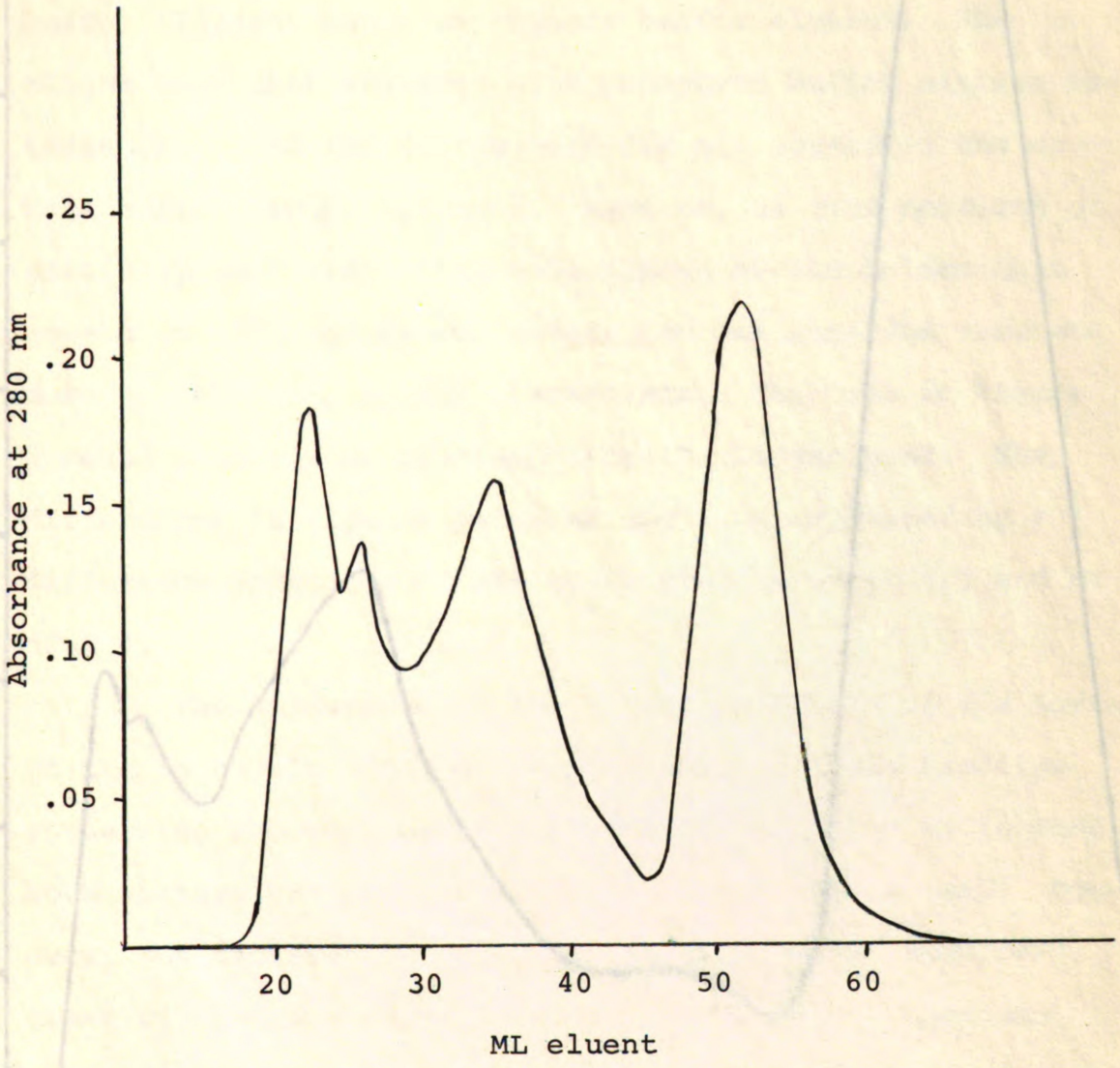


Figure 3. Elution of 2 ml of type E Kalamazoo toxin on DEAE cellulose with 0.05 M sodium acetate buffer, pH 4.5. The first three components contain the majority of the toxicity

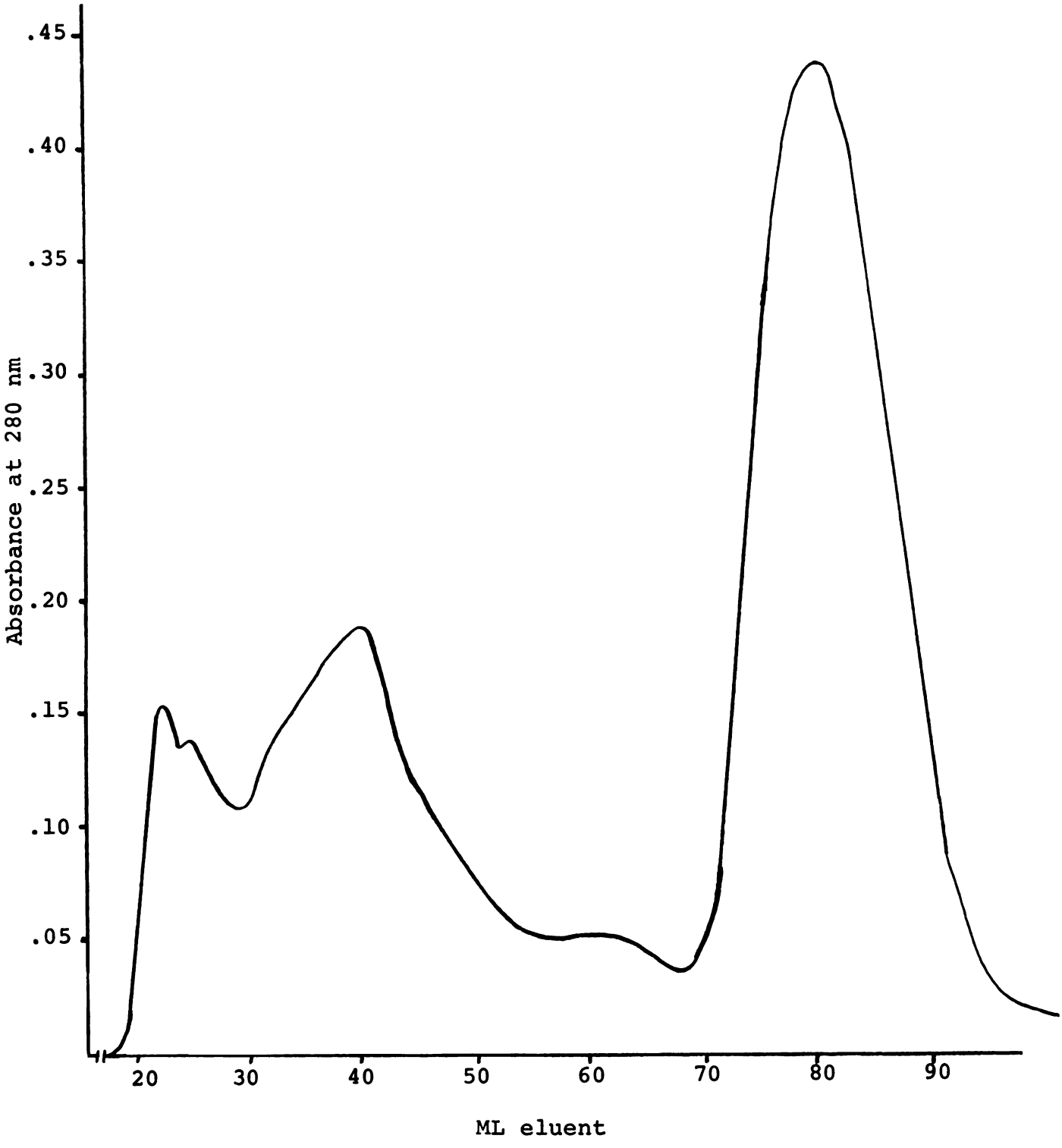


Figure 4. Elution of 2 ml of type E Kalamazoo toxin on DEAE cellulose with 0.05 M sodium phosphate buffer, pH 6.0. The third component contains the toxin

number of peaks were the same with both buffers, the heights and areas of the peaks were different. The non-toxic fraction produced a much larger peak with phosphate buffer elution than with acetate buffer elution. The slight hump that appeared with phosphate buffer elution between the third and fourth peak did not appear in the acetate buffer elution pattern. However, as more portions of partially purified toxins were placed on the column this non-toxic compound became larger and was regarded more and more and the hump became discernible. The hump in Figure 3 could probably be overlapped by the larger peak. The differences in elution patterns might be explained by a difference in charge of the toxic protein at pH 4.5 and at pH 6.0.

The difference in the elution patterns of the toxin of the VH strain produced similar toxin elution profiles containing two components only one of which was toxic when both acetate and phosphate elution buffers were used. However, the decision to use phosphate buffer was made because of the need for a standard purification procedure which could be applicable to other type E strains. Therefore, all subsequent initial purifications on DEAE were carried out with phosphate buffer at pH 6.0.

Toxin eluted with phosphate buffer showed about 5-fold less toxicity than toxin eluted with sodium acetate buffer. This phenomenon was also observed by Sakaguchi,

Sakaguchi and Imai (1964) who found that potencies of toxins eluted by pH 7.5 phosphate buffer were lower than those obtained with acetate buffer at pH 6.0. This may reflect a lower degree of stability at the higher pH, or sodium acetate buffer may keep the toxin stable for a longer time than phosphate buffer.

Application of a NaCl gradient on the columns up to 0.5 M concentration did not elute more toxic material. Therefore, the toxic fractions were pooled and kept for further purification. A second precipitation with $(NH_4)_2SO_4$ and purification of the toxic fractions on DEAE was also investigated. However, this additional procedure did not increase the degree of purification. Disc electrophoresis of the fractions indicated three bands in both fractions. Both fractions produced the same pattern of one dense and two slight bands. Therefore, this second purification treatment was not repeated. Disc electrophoresis of the fractions will be discussed in detail later.

Final purification of the toxic fraction was carried out on CM-Sephadex C-50 gel. This ion exchange gel retarded the toxin when eluted with phosphate buffer at pH 6.0. The toxin strongly adsorbed to the acidified gel, while some of the other non-toxic proteins were eluted with the buffer. Toxin was released from the gel in one small fraction when 0.5 M NaCl was applied to the column (Figure 5). Toxin assay showed that this toxic fraction

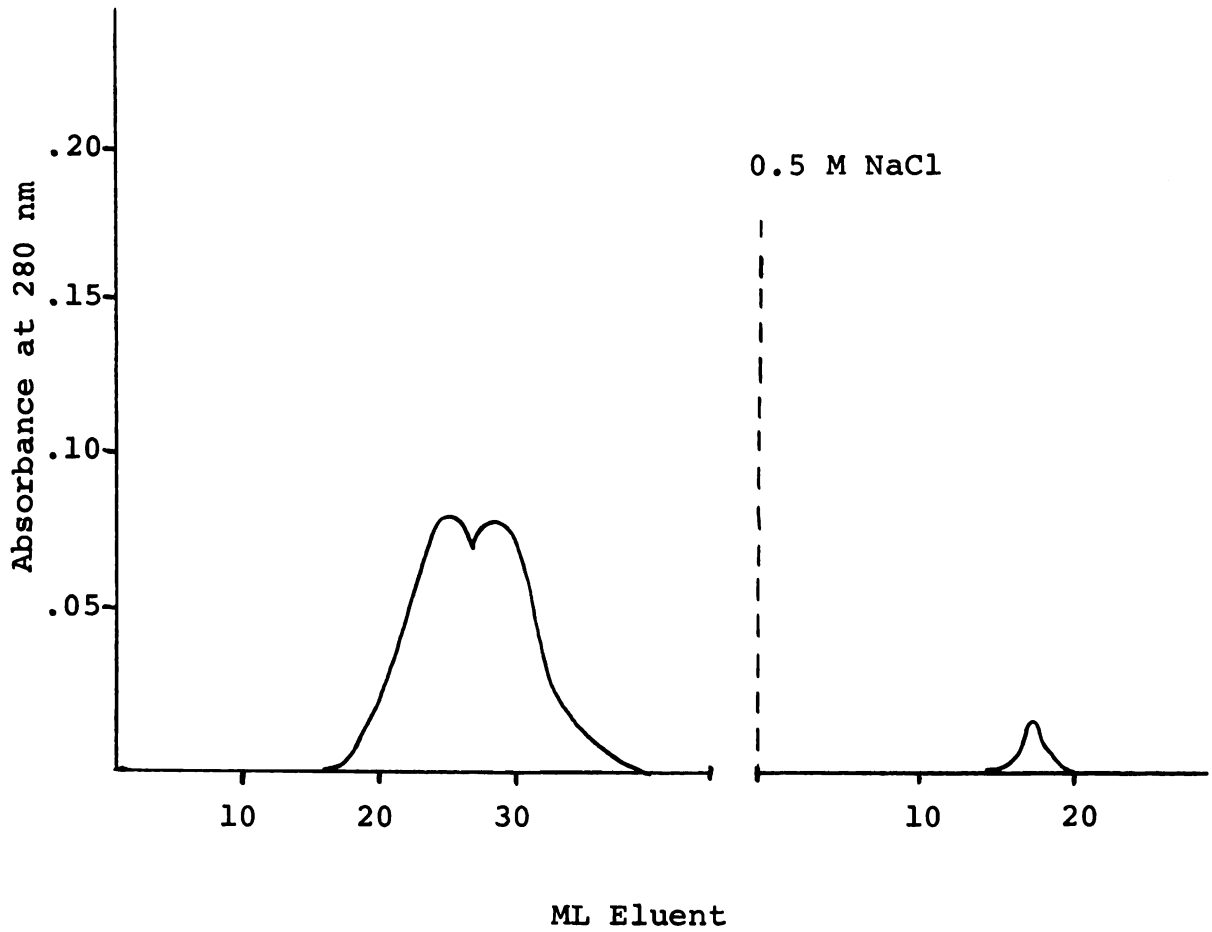


Figure 5. Elution of 1.5 ml of DEAE purified type E VH toxin on CM-Sephadex C-50. The peak eluting after NaCl application contains the toxic component

contained approximately 5 x 10^6 to 1 x 10^7 MLD toxicity per mg protein, which was a give to tenfold increase in activity when compared to the activity obtained after DEAE chromatography. A continuous increase in activity of the toxin was demonstrated throughout our purification procedures. Our final preparation contained a toxic activity of 5 x 10^6 to 1 x 10^7 MLD per mg protein. Gerwing et al. (1964) with similar procedures obtained 7.5 x 10^6 MLD toxicity per mg N, or 4.7 x 10^7 MLD toxicity per mg protein, while Kitamura, Sakaguchi and Sakaguchi (1967) with a different procedure obtained 5 to 10 x 10^7 MLD toxicity per mg N or 3.1 to 6.2 x 10^8 MLD toxicity per mg protein. The latter workers obtained the highest activity, but their starting material and purification procedures were different. They extracted the toxin from the bacterial cells with phosphate buffer and purified only the extracellular toxin. Their purification procedure of the toxin involved RN-ase digestion, CM-Sephadex purification and Sephadex G-200 gel filtration. They used acetate buffer at pH 6.0 while Gerwing et al. (1964) used acetate buffers at pH 4.5 and 5.5 and we used phosphate buffer at pH 6.0 throughout the purification procedures.

Kitamura, Sakaguchi and Sakaguchi (1967) used the Hazen strain for toxin production. Our attempt to grow this strain in TPSY medium or in the peptone-glucose-yeast

extract medium used by them was unsuccessful. This strain grew very poorly in both media and produced little toxin.

The CM-Sephadex C-50 column when packed and equilibrated with phosphate buffer at pH 6.0 had a height of approximately 28 cm. CM-Sephadex gels are supplied in their sodium forms. They are strongly hydrophilic and swell in water and salt solutions. Application of NaCl to the column shrunk the gel to a height of 18 to 20 cm. Because of this excessive shrinking and swelling property it was hard to work with this gel since it was difficult to obtain a constant flow rate. Therefore, other final purification procedures were also investigated.

Disc gel electrophoresis purification procedures use the principle that proteins are separated because they possess a different net charge at different pH and the rate of migration of the molecules in an electric field depends upon the net charge. The first or simpler procedure involved the separation of the proteins by disc gel electrophoresis, followed by extraction of the toxic protein from the gel. The portions of the gels containing the toxin were cut out and extracted overnight in a 1:2 mixture of physiological saline and phosphate buffer at pH 6.0. A highly purified toxin preparation could be obtained in this manner since the toxin readily diffuses from the gel into buffered saline. Some loss of protein could occur with this method since the overnight extraction may not

extract all the protein. The use of a longer extraction time, such as two or more days, may result in the loss of samples through microbial contamination.

The second method employed a procedure that utilized the difference in rate of migration of the proteins in the gel. The non-toxic proteins pass through the gel first, then the tube is removed and capped with a dialysis membrane and the slowest moving toxic protein is captured in the few drops of buffer occupying the space between the gel and the dialysis membrane. The dialysis membrane is placed on the end of the tubes after all non-toxic proteins have migrated into the buffer system. This method worked well but through leakage around the membrane the loss of protein is possible, and some care is required in placing the dialysis membranes on the end of the tubes without enclosing an air bubble. The membrane material must be a type that would not allow smaller molecules to diffuse through, therefore, cellulose sausage casing was used. It was observed during our experimentation that purified toxin diffused through ordinary dialysis bags. Since dialysis bag materials do not carry any specification as to the retention of molecular sizes, the diffusion of smaller colecular compounds through the membranes could be established only on a trial and error basis. Cellulose sausage casing will retain materials with molecular weights above 4,000.

The diffusion of purified toxin through the dialysis membranes used in most of our toxin production was observed upon several occasions. This indicated that these purified molecules were smaller than those present in toxic cultures or those obtained after precipitation with $(NH_4)_2SO_4$. It is possible that the toxin in its original form exists as a large molecule, or as an aggregate, or it may combine with some large non-toxic protein. Chromatography on DEAE cellulose may fractionate the protein into smaller peptide units, or it may disaggregate the large aggregates into their original small units.

Homogeneity of the Toxin

The toxin fraction purified on DEAE cellulose appeared to be homogeneous with respect to molecular size when examined by ultracentrifugation (Figure 6). One slowly moving component was observed and a sedimentation coefficient of $S_{obs} = 0.6$ was calculated. However, at the beginning of the centrifugation two small components were also observed which later diffused. This may have been due to the presence of very small amounts of impurities or the presence of some aggregated materials. But since microscopic appearance of the protein solutions showed no impurities or aggregated materials, it was possible that small amounts of other proteins were also present in the preparation. Therefore, the partially purified preparations,

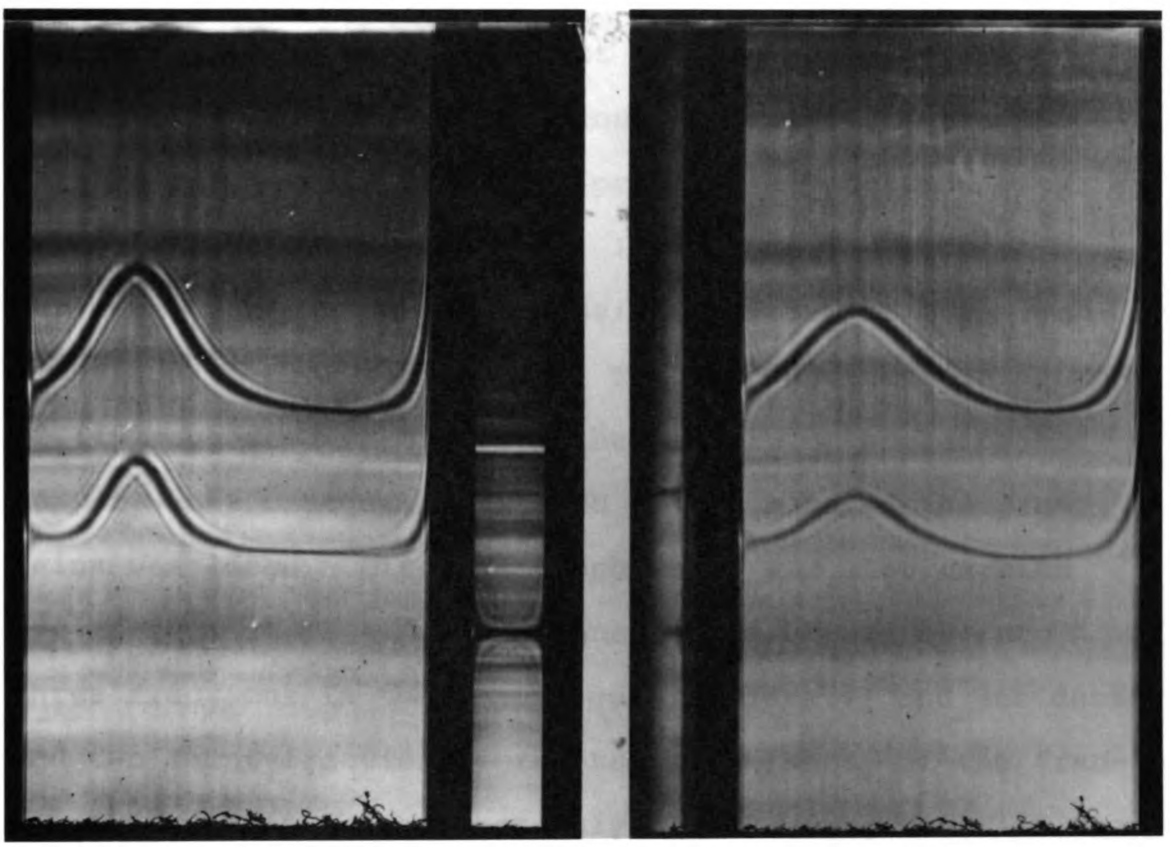


Figure 6. Ultracentrifuge photographs of DEAE purified type E VH toxin at 59780 rpm, 0 min and 32 min after reaching full speed, in 0.05 M sodium phosphate buffer, pH 6.0

previously examined ultracentrifugally were tested by disc electrophoresis to determine if the preparation was homogeneous with respect to number of components present. Disc electrophoresis was carried out in 8.0 % acrylamide gels at first, but a decision to change to 7.0 % gels was made after it was observed that the proteins moved very little after 2 hr of electrophoresis. Electrophoresis carried out in gels at pH 8.9 produced a substance with low electrophoretic mobility. It was difficult to observe whether the material consisted of one or more fractions due to poor resolution. The low electrophoretic mobility suggested that either the protein or proteins had very few net charges at this pH or the size of the proteins was large. Disc electrophoresis was also carried out in gels at pH 4.3. The concentrate separated into three fractions in these acid gels (Figure 7) and one dense and two faint fractions were observed. Assay of the fractions for toxin showed that only the one fraction with the lowest electrophoretic mobility was toxic. Acid gel disc electrophoresis was performed also on unconcentrated DEAE purified fractions not used for ultracentrifugal studies and the same three materials resulted. A few milliliters of the preparation were treated with 4.0 and 8.0 M urea to ensure that three different proteins were present in the purified preparation. Urea causes an alteration in the conformation of proteins and competes with the hydrogen bond in the native protein and produces an unfolding. Disc

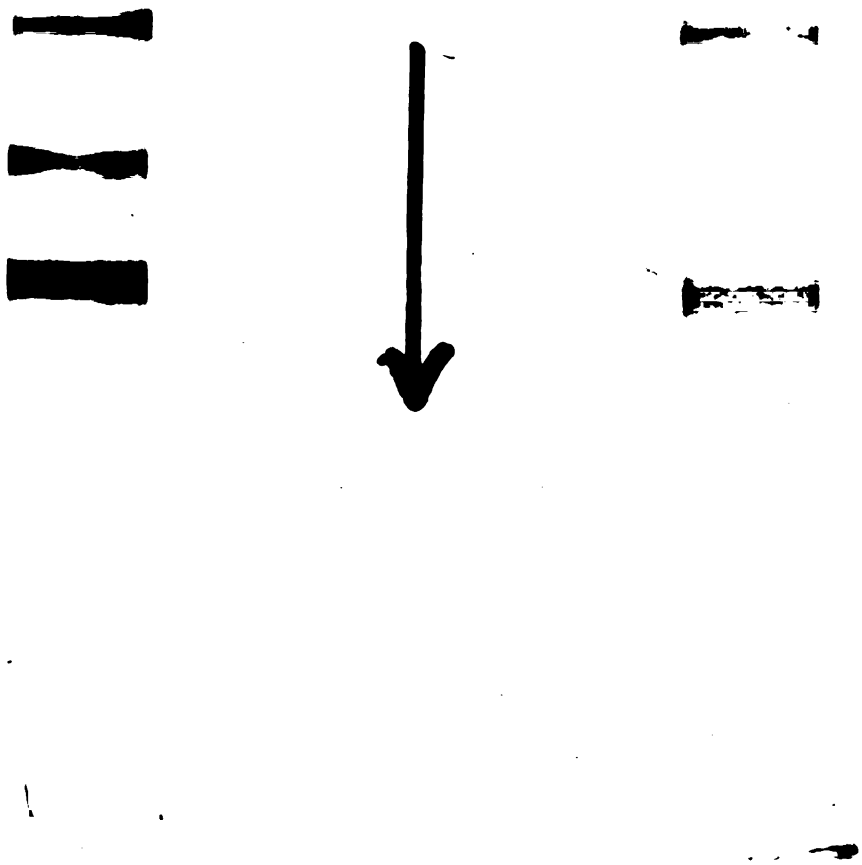


Figure 7. Replicate disc electrophoresis patterns of purified type E VH toxin on pH 4.3, 7.0 % acrylamide gel using pH 5.0 β alanine-acetic acid buffer and a current of 3 mAmp with the cathode at the bottom. The top band contains the toxin

electrophoresis carried out in the preparation treated with urea produced similar results to those observed without the urea treatment. One major and two minor fractions were observed. Since urea quickly detoxifies the toxin, the toxicity of the fraction with the lowest electrophoretic mobility was not established. It is possible that urea by unfolding the molecule also affects the toxic site or sites thereby causing the loss of activity. The loss of toxicity of type A toxin after 6 M urea treatment was previously reported by Schantz, Stefanye and Spero (1960).

Disc electrophoresis established that three different proteins were indeed present in the DEAE purified preparation, and that the preparation was not homogeneous with respect to the number of components present. Only one toxic protein, the one with the lowest electrophoretic mobility, was detected.

Although the shape and size of the protein molecule influence the absolute rate of electrophoretic migration, the major factor is the net charge. It is most likely that the toxin has the least number of net charges, but it may also be possible that it is a large molecule and moves more slowly through the gel than the other proteins.

The disc electrophoresis data thus suggest the possibility that when the molecular size and homogeneity of the preparation was determined with the analytical ultracentrifuge, the protein with the small sedimentation coefficient was not the toxic protein but represented instead

the major non-toxic protein producing the large dense band on the gel. The toxic protein could have been simply unobservable because it was present in very low concentration in the preparation. Since its exact proportion of toxin in the purified preparation is unknown, it is quite possible that even if samples with higher protein concentration would be analyzed centrifugally, the toxic protein would not be identifiable by an absorption peak. Gerwing et al. (1964) using similar purification techniques, analyzed the purified material with the ultracentrifuge using a protein concentration of 1 %, and observed the presence of a single major homogeneous component. A rapidly sedimenting component producing a small shoulder was also observed and accounted for as a small percentage of apparent impurities. The peak moved slowly with a sedimentation coefficient of S_{20W} = 1.70 S and our findings are in agreement with theirs in respect to indication of a small protein molecule. However, Kitamura, Sakaguchi and Sakaguchi (1967) with their different purification method obtained a purified toxic product which appeared to be homogeneous below pH 6.0 with an S_{20W} = 12.3 S. Their result was in good agreement with an S_{20W} of about 11.5 reported by Sakaguchi and Sakaguchi (1967) for a purified homogeneous toxic product.

It was also observed during our disc electrophoresis experiments that if the concentration of protein applied to the gels was decreased the two faint components

could not be observed and only a sharp band representing the major protein would be present. This proved that it is quite possible to fail to detect the toxin and to come to the conclusion that a homogeneous compound is present, even when the preparation is not homogeneous.

Very poor resolution was obtained when the basic gel was used for disc electrophoresis. It is possible that at basic pH the proteins carried very few net charges and they were close to their isoelectric points. The isoelectric point of type E toxin is not known yet. Crystalline type A toxin is reported to have an isoelectric point at pH 5.6 (Putnam, Lamanna and Sharp, 1946 and 1948).

The toxin purified on CM-Sephadex was also examined using disc electrophoresis. Electrophoresis was conducted using pH 4.3 gel, and only one component was obtained (Figure 8). This indicated that the preparation contained one protein with a very low electrophoretic mobility. Toxin assay of the gel showed that the protein was highly active and it traveled with the same mobility as the toxic fraction on the control. The DEAE cellulose partially purified toxin was used as a control. It would appear that with this final purification step the separation of the toxin molecule from other proteins was achieved, and a toxic product homogeneous with respect to the number of components was obtained.

A B

Figure 8. Disc electrophoresis patterns of type E VH toxin, B. before and A. after CM-Sephadex purification on pH 4.3, 7.0 % acrylamide gel with pH 5.0 β alanine-acetic acid buffer and a current of 3 mAmp

Homogeneity of the fractions purified on DEAE and on CM-Sephadex were also tested on Sephadex G-200 gel columns. Molecules larger than the largest pores of the hydrated Sephadex cannot penetrate the gel particles and, therefore pass through the bed in the liquid phase outside the particles and are thus eluted first.

Smaller molecules, however, penetrate the gel particles to a varying extent depending on their size and shape, and are eluted from a Sephadex bed in order of decreasing molecular size.

Therefore, Sephadex G-200 column chromatography should indicate whether a size difference exists among the proteins present in the material purified on DEAE cellulose and it would also give an estimate of the size of these proteins.

Rechromatography of the toxic components eluted from DEAE cellulose on a Sephadex G-200 column (1 x 28 cm) produced two fractions of equal toxicity (Figure 9). This indicated that the preparation was not homogeneous with respect to molecular size.

Toxin assay of the eluent occasionally showed that toxic material appeared in the eluent before any material absorbed at 280 nm could be detected with our apparatus. The protein content of these eluates was so low that the detector could not detect changes in absorbancy. Since this phenomenon could not always be observed, it is possible

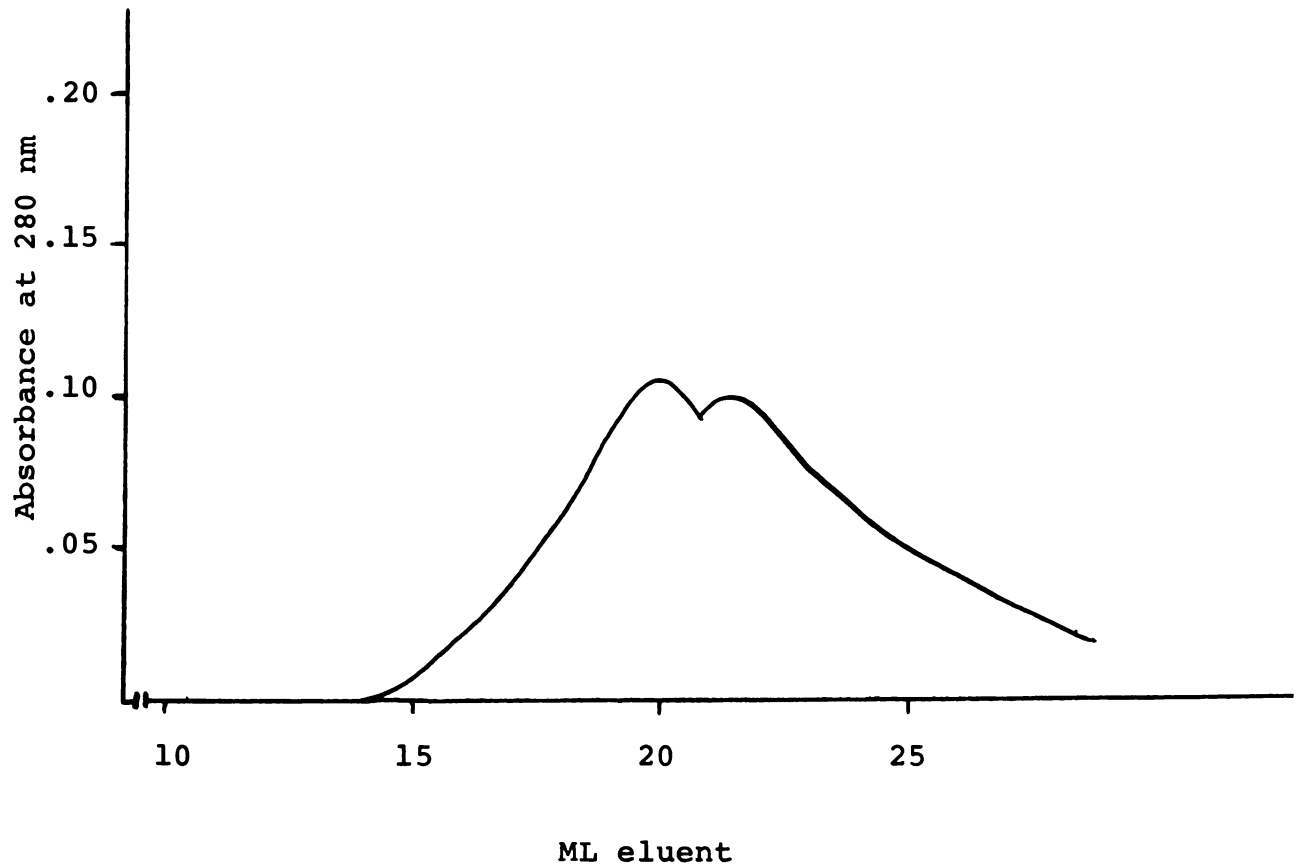


Figure 9. Elution of DEAE purified type E VH toxin on Sephadex G-200 column with 0.05 M sodium phosphate buffer at pH 6.0

that the toxin may aggregate or disaggregate with time into smaller or larger units depending on the treatment to which it is subjected. The low protein content of these eluates suggested that the toxin is a protein with very high toxic activity. Eluates were found containing 5×10^4 MLD activity per ml, while absorbance at 280 nm was practically zero.

The fraction purified on CM-Sephadex showed the presence of only one component after passing through a Sephadex G-200 column. Toxin assay showed that the peak fraction was toxic. Therefore, this fraction on Sephadex G-200 proved to be homogeneous with respect to molecular size.

Estimation of Size

The size of the fraction purified on DEAE was first estimated using the ultracentrifuge. However, as was discussed previously, the small protein indicated by the sedimentation coefficient of $S_{obs} = 0.6$ may not have been the toxic protein. Furthermore, the disc electrophoresis data showed that the protein preparations used for ultracentrifugal studies were not homogeneous. Therefore, the data obtained with the ultracentrifuge should be accepted with reservation when the size of the toxic molecule is estimated.

Disc electrophoresis indicated that the toxic fraction in the preparation purified on DEAE was present in low concentration, and calibrated Sephadex G-200 columns (1 x 28 cm) were found more suitable than the ultracentrifugal studies for estimating molecular size.

Rechromatography of the toxic fractions eluted from DEAE cellulose on calibrated Sephadex G-200 columns produced two fractions of equal toxicity (Figure 9). Both peaks were eluted after cytochrome c (Figure 10), indicating that the molecular weights of the substances were less than 12,200. Elution of these fractions was carried out with phosphate buffer at pH 6.0. Fractions eluted with pH 7.0 phosphate buffer exhibited the same two peaks in their elution profile and the elution volumes were in close agreement with those obtained at pH 6.0. Evidently no change in size occurred with the change of pH.

The fraction purified on CM-Sephadex was then applied to a calibrated G-200 column and only one material eluted after cytochrome c (Figure 10). Therefore, the molecular weight of this toxic protein was less than 12,200.

Gerwing et al. (1964) obtained a toxic protein with a molecular weight of 18,600. Recalculation of the molecular weight by Gerwing, Dolman and Ko (1965) based on the amino acid residues showed that the molecular weight of their pure toxin was around 14,000 to 16,000 or roughly 20 % less than the figure based on ultracentrifugal

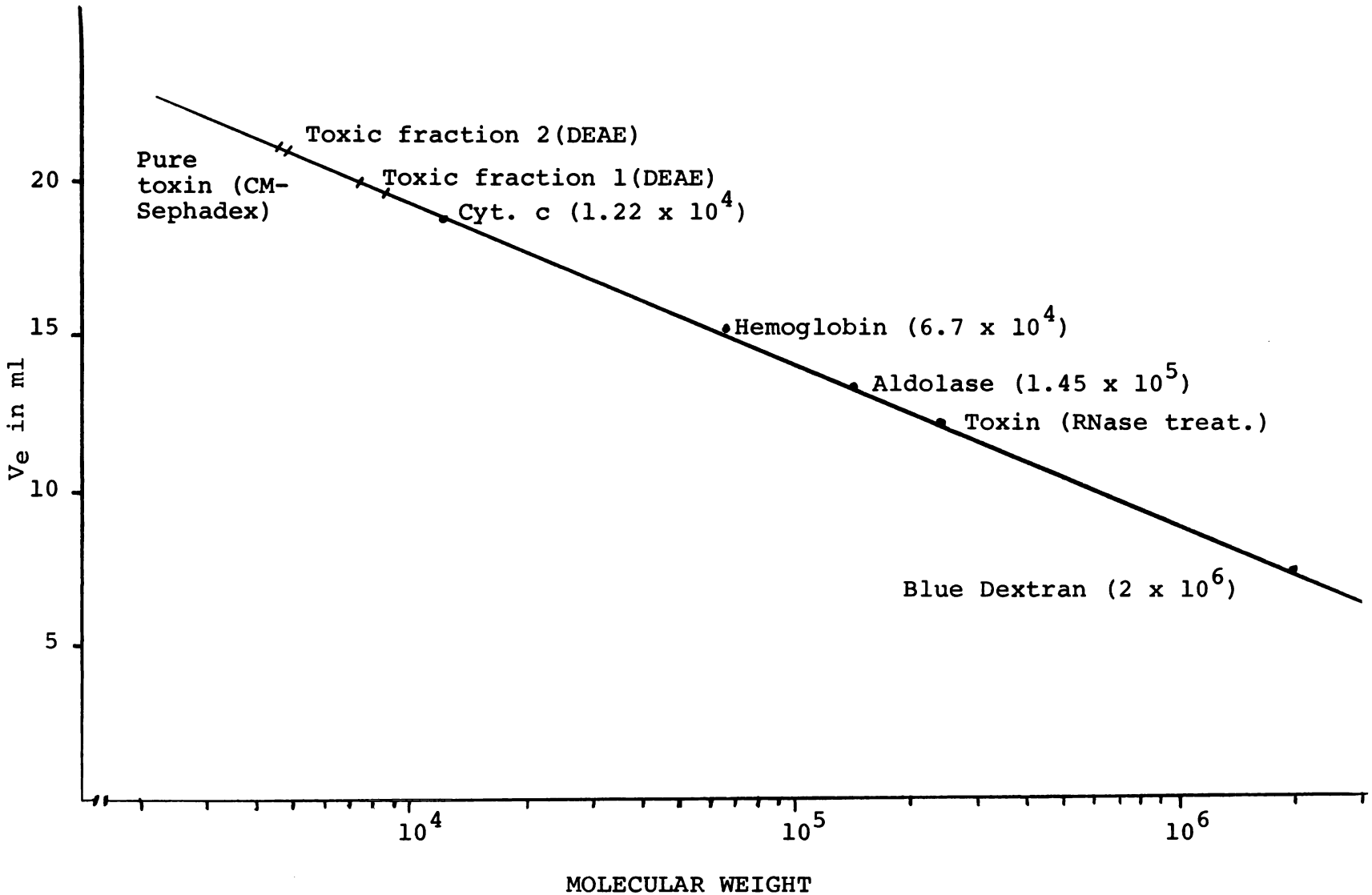


Figure 10. Molecular weights of toxic fractions as determined on a Sephadex G-200 column (1 x 28 cm). Proteins were eluted with 0.05 M sodium phosphate buffer pH 6.0. The molecular weights of proteins are stated within the parentheses

analysis. These two reports are in agreement with our finding that botulinum toxin has a small molecular weight.

A purified toxin of large molecular weight such as reported by Sakaguchi and Sakaguchi (1967) with S_{20W} = 11.5 S, and Kitamura, Sakaguchi and Sakaguchi (1967) with S_{20W} = 12.3 S, was never obtained in any of our studies.

The concept that type E C. botulinum toxin has a small molecular weight was supported by the fact that when fractions purified on DEAE were dialyzed against phosphate buffer at pH 6.0 the buffer became toxic, indicating that toxin molecules diffused through the dialysis membrane. The dialysis bags used were known to retain compounds with molecular weights of 30,000 or more. The toxic materials diffusing through the membrane must thus have had molecular weights less than 30,000.

Several times during our experimentation a possible aggregation phenomenon was observed. Toxin purified on DEAE cellulose and placed on DEAE cellulose columns at pH 7.0 eluted in a sharp frontal peak and when this toxic fraction was placed on a calibrated Sephadex G-200 column at pH 7.0 for size estimation, the toxin started to elute before cytochrome c and eluted constantly until the exclusion limit was reached. Since no peak was obtained, it is reasonable to assume that the toxin was in form of aggregates of different sizes and it is possible that at basic pH the toxin would aggregate into larger sizes. This

aggregation phenomenon was also investigated at pH 8.5 and pH 9.1.

The fraction purified on DEAE were rechromatographed on DEAE columns equilibrated with 0.05 M Tris-HCl buffers at pH 8.5 and pH 9.1 and eluted with the same buffers. No toxic material was eluted from the columns at pH 8.5. Application of a sodium chloride gradient up to 0.5 M concentration on the column also did not elute toxic material. Since the type E toxin is very unstable at this pH it was quite possible that by the time it was eluted it had lost all its activity, or that at this pH the toxic protein was very close to its isoelectric point and may have been insoluble. Studies using disc gel electrophoresis also showed that a toxic preparation in pH 8.9 gel moved very little, and thus these proteins at this pH probably had very few net charges. The toxic preparation chromatographed on DEAE cellulose columns at pH 9.1 separated into three peak fractions. Only the first peak fraction possessed toxic activity. When this toxic sample was placed on a Sephadex G-200 column, the toxic protein started to elute almost at the void volume and eluted continuously until the lower exclusion limit was reached. No real toxic fraction was observed, except for the two fractions which eluted after cytochrome c.

The continuous elution of toxic material within the exclusion limits of the gel may indicate that the protein

is in forms of aggregates of many sizes. DasGupta and Boroff (1968) separated type A toxin into α and β components on DEAE cellulose columns with Tris-HCl buffers at pH 8.0. Both components were large molecules with molecular weights of 150,000 and 500,000. But since type A toxin is stable at basic pH while type E is not, the determination of stable large molecular aggregates or small disaggregates of type E toxin cannot be performed with good scientific techniques until type E toxin can be stabilized at basic pH.

The adsorption of toxin to a large molecule was also observed when toxic fractions purified on DEAE were mixed with blue dextran. The toxin eluted with blue dextran in the void volume (Figure 11). The elution of type A toxin simultaneously with dextran was observed by Hauschild and Hilsheimer (1968). Whether dextran has an aggregating effect on the toxin, or the toxin simply adsorbs to dextran is not known. The toxin eluted with dextran was highly active, contained 2×10^5 MLD toxicity per ml while the two toxic fractions eluting after cytochrome contained 2×10^3 and 1×10^3 MLD of activity per ml, respectively. Since the protein content of our blue dextran fraction was very low and was not measurable with the protein determinations used in the course of these studies, it is reasonable to assume that the toxin is a highly active protein, and adsorbs on blue dextran in a pure form.

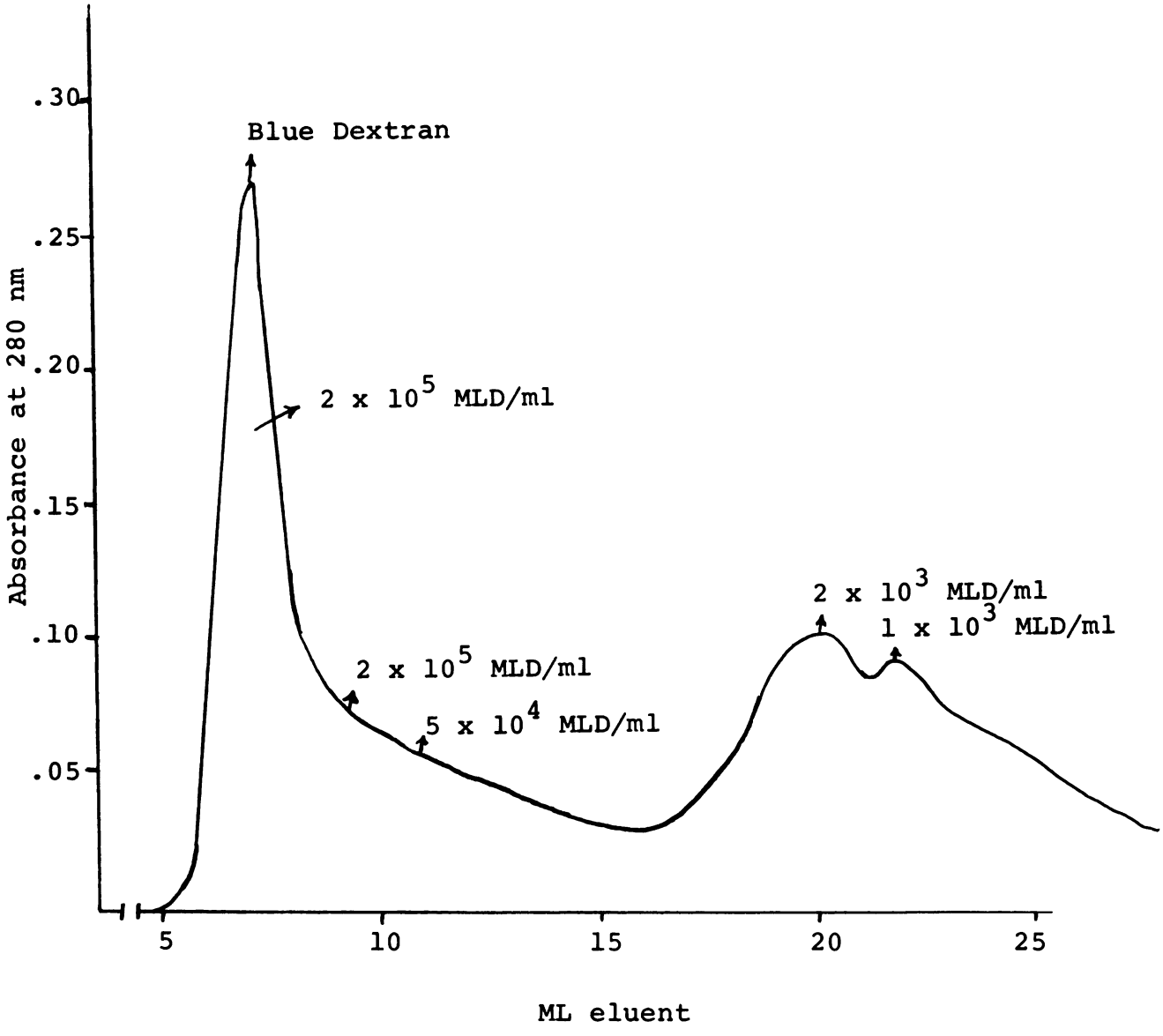


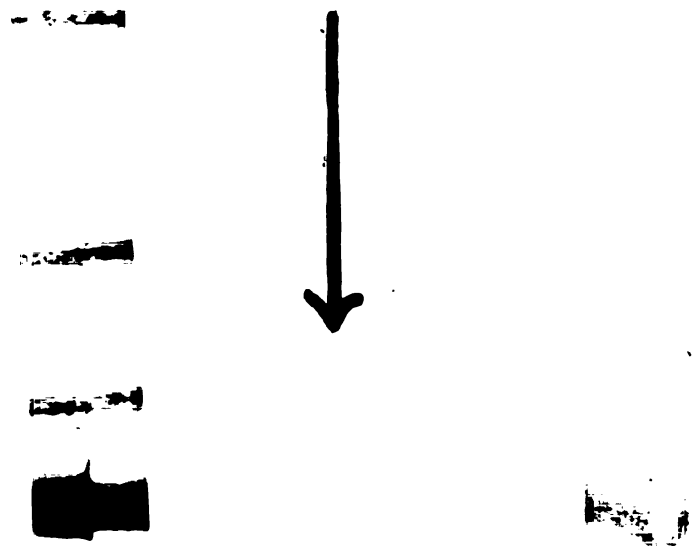
Figure 11. Elution of type E VH toxin mixed with blue dextran on Sephadex G-200 column with 0.05 M sodium phosphate buffer, pH 6.0

This adsorption or coupling phenomenon of the toxin to large molecular entities was also observed with red blood cells and with bacterial cells. Toxin preparations were mixed with red blood cells and eluted on a Sephadex G-200 column. The red blood cells eluted in the void volume and were highly toxic. Presumably, toxin was adsorbed to the cells. The two substances eluting after cytochrome c were also present but only carried slight toxic activity.

It was also noticed that the purified material became contaminated very easily and supported microbial growth. This contaminated toxic material was passed through a Sephadex G-200 column and a highly toxic material appeared in the void volume while the two fractions that subsequently eluted were less toxic. Contaminated toxic material filtered through a Millipore filter did not contain this toxic fraction at the void volume. The Millipore filter apparently removed the microbial cells, but should have permitted the toxin to pass through even if it was in an aggregated form separate from the cells. It is unlikely that bacteria growing in the toxic material would incorporate the toxic protein without any change, or without destroying its toxicity. The best probable explanation is that toxin is adsorbed to the cell walls.

Since a purified toxin was never found in the course of our studies, similar to the one (M.W. 200,000)

reported by Sakaguchi and Sakaguchi (1967) after treatment of their toxic material with RNase, the possibility of an aggregating effect of the enzyme was investigated. The toxic material purified on DEAE containing approximately 0.9 to 3.0 % of nucleic acid was treated with 10 μg/ml RNase at room temperature. The digestion was completed in about 2 hr. This mixture digested with RNase was now 100 % protein, as demonstrated by the absorbance at 280/260 nm. The preparation eluted as two fractions on a DEAE cellulose column with phosphate buffer at pH 6.0. Both fractions eluted before the NaCl gradient application. The gradient produced no additional absorption peaks. Assay for toxin showed that the first component contained more than 90 % of the toxin. Samples of the toxic and non-toxic fractions were placed on pH 4.3 disc gels and when electrophoresis was carried out as before, the toxic component yielded four fractions while the non-toxic component yielded two fractions (Figure 12) equivalent to the third and fourth fraction of the toxic sample. The third fraction of the non-toxic sample was extremely faint but was observable. Toxin assay of the components showed that the slowest moving material contained essentially all the toxin. This result correlates well with our previous findings. The toxic protein in disc gels at pH 4.3 always moved very slowly and always had the lowest electrophoretic mobility. The toxic component was also missing from the non-toxic fraction.



A B

Figure 12. Disc electrophoresis patterns of the toxic and non-toxic fractions eluted from DEAE cellulose after RNase treatment
A. Toxic fraction
B. Non-toxic fraction

Rechromatography on a Sephadex G-200 column of the preparation treated with RNase produced the usual two fractions with equal toxicity of 2 to 4 x 10^4 MLD per mg protein and a small fraction with an activity of about 1.3 x 10^5 MLD per mg protein. This fraction had an elution volume of 14.12 ml (Figure 10) or an estimated molecular weight of 240,000. However, this toxic material was not stable. Preparations kept at 4 C for 3 to 5 days or longer contained this fraction no longer. This may suggest that RNase had an aggregation effect on the toxin, but with time the toxin disaggregated to its smaller size.

Test for hemagglutinating activity of the sample purified on DEAE gave negative results, while unpurified type A and B toxins used as controls were found to contain hemagglutinin. DasGupta and Boroff (1968) separated type A toxin into a toxic and a non-toxic fraction on DEAE cellulose columns with Tris-HCl buffers at pH 8.0. The non-toxic fraction was a powerful hemagglutinin. The presence of hemagglutinin with type E toxin has not yet been demonstrated. Kitamura, Sakaguchi and Sakaguchi (1967) separated type E toxin to a toxic α and a non-toxic β fraction and tested the β fraction for hemagglutinating activity. No activity was detected. Since type E toxins differ from type A and B toxins in several properties, it is quite possible that the lack of demonstrable hemagglutinin is one more differentiating property. Conversely, type E toxin

has a hemagglutinin fraction, the activity of this component may be very weak without trypsin activation and can not be observed.

The purification procedure employed in our studies produced a highly purified type E toxin. Studies of the toxin should now be expanded to the toxins produced by the other type E strains as well as those of the other toxin types of C. botulinum. The scope of the experiments should be increased so that sufficient quantities of purified toxin can be obtained for chemical studies.

SUMMARY

The isolation, purification, homogeneity and molecular size of type E Clostridium botulinum toxin was studied.

The Vancouver Herring strain was used for toxin production. Cultures of this strain were grown in physiological saline and nutrients required for the microorganisms were supplied in dialysis bags filled with trypticase-peptone-yeast extract-sucrose (TPSY) medium suspended in the saline solution. The toxins produced by this method were free from nondialysable medium constituents. The maximum amount of toxin was produced by the sixth to seventh days of incubation. Cells were separated from the toxin by centrifugation and the toxin was precipitated from the supernatant with crystalline ammonium sulfate at 60 % saturation. The precipitate after dialysis against phosphate buffer was redissolved after centrifugation in 0.05 M phosphate buffer at pH 6.0. The activity of this partially purified toxic material was 5.7×10^5 MLD per mg protein. Further purification was accomplished by applying the toxic material to a DEAE cellulose column at pH 6.0, which resulted in a major toxic fraction with an activity of 1.0×10^6 MLD per mg protein and a minor slightly toxic fraction.

Absorbance of the toxic fraction at 280/260 nm yielded ratios of 1.06 to 1.35, indicating the presence in the purified material of approximately 0.9 to 3.0 % nucleic acid.

The purified fraction appeared homogeneous when examined by ultracentrifugation. One very slowly moving component was observed and a sedimentation coefficient of S_{obs} = 0.6 was calculated. Apparently the component observed with the centrifuge represented a very small molecule.

Disc electrophoresis studies of the protein preparation used for the ultracentrifugal experiments showed that the preparation consisted of three different entities. Material producing one dense and two slight bands were observed in pH 4.3 gels. Toxin assay indicated that the material that produced minor band with the lowest electrophoretic mobility contained the toxin. It is possible that the toxic protein is present in the preparation in a very minor proportion and that the peak produced with the ultracentrifuge represented the major portion of the protein fraction, while there was no evidence of the minor toxic protein because of its low concentration.

The purified toxin applied to a calibrated Sephadex G-200 column separated into two equally toxic fractions. Both fractions eluted after cytochrome c, suggesting that their molecular weights were less than 12,200.

The disc electrophoresis and the gel filtration experiments showed that the toxic material purified on DEAE was not homogeneous with respect to the number and size of components.

Final purification of the toxic fraction was done on CM-Sephadex C-50 columns at pH 6.0. This acidified gel retarded the toxin until the molarity of the eluant was changed by adding 0.5 M NaCl to the eluting buffer. The eluted toxic fraction now contained an activity of 5×10^6 MLD per mg protein and eluted as one absorption peak.

Disc electrophoresis studies of the doubly chromatographed toxin showed only one toxic component that produced only one band in the pH 4.3 gel.

The molecular weight of this toxic protein was estimated by the gel filtration technique. The toxin eluted as a one peak fraction after cytochrome c, indicating that it had a molecular weight of less than 12,200.

The disc electrophoresis and the gel filtration results suggested that the toxin purified on CM-Sephadex was not essentially homogeneous with respect to number of components and molecular size.

A possible aggregation phenomenon of the toxic protein was observed when elution on DEAE cellulose columns were carried out with buffers at pH 7.0 and 9.1. Toxic fractions purified on DEAE cellulose columns with these buffers and rechromatographed on Sephadex G-200 columns

eluted irregularly throughout the entire fractionation range of the G-200 gel. This may indicate that the toxin at basic pH existed in forms of aggregates of different sizes.

The adsorption or coupling of the toxin to blue dextran, to red blood cells, and to bacterial cells was demonstrated. These large molecular entities eluted in the void volume on Sephadex G-200 columns. Purified toxin mixed with these large molecular weight compounds eluted with them in the void volume. However, if bacterial cells and red blood cells were removed from the mixture by Millipore filtration no toxic material appeared at the void volume.

A possible aggregation effect of RNase was observed when toxin purified on DEAE was treated with the enzyme. The preparation treated with RNase eluted in a toxic and a non-toxic fraction on DEAE columns at pH 6.0. The toxic fraction separated into four bands upon electrophoresis in pH 4.3 gels, while the non-toxic fraction separated into two bands. The two slowest moving components, one of them representing the toxin, were absent in the non-toxic fraction.

Rechromatography of the preparation treated with RNase was performed on calibrated Sephadex G-200 columns for size estimation, and an additional highly toxic fraction as well as the two equally toxic fractions were

produced. This highly toxic fraction eluted before aldolase and its estimated molecular weight was about 240,000. It appears that RNase treatment aggregated the toxin into a large unstable molecular aggregate, which upon storage for 3 to 4 days at 4 C disappeared by probably again disaggregating into small units.

Tests for hemagglutinating activity of type E toxin were negative, while crude type A and B toxin preparations were found to possess hemagglutinating activity as previously reported.

The toxin of Clostridium botulinum type E Vancouver Herring strain was thus shown to be a low molecular weight protein of less than 12,000, based upon ammonium sulfate precipitation, DEAE cellulose and CM-Sephadex C-50 column chromatography. The aggregation of the toxin to large molecular weight substances was indicated and should be the subject of additional investigation.

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APPENDIX

APPENDIX

Stock Solutions for Negatively Charged Proteins

A. To 48 ml of 1 N HCl, 36.3 g of tris (hydroxymethyl) aminomethane (TRIS), 0.23 ml of N, N, N', N'-Tetramethylethylenediamine (TEMED) and H_2O were added to make 100 ml (pH 8.9 to 9.0).

B. To 48 ml of 1 N HCl, 5.98 g of TRIS, 0.46 ml of TEMED and H_2O were added to make 100 ml (pH 6.6 to 6.8).

C. To 60 g of acrylamide, 0.4 g of N, N'-Methylenebisacrylamide (BIS) and H_2O were added to make 135 ml.

D. To 10 g of acrylamide, 2.5 g of BIS and H_2O were added to make 100 ml.

E. To 4 mg of riboflavin, H_2O was added to make 100 ml.

F. Catalyst: To 0.14 g of ammonium persulfate, H_2O was added to make 100 ml (prepared fresh weekly).

G. Tracking Dye: 0.001 % bromphenol blue in H_2O.

H. Protein stain: To 250 ml H_2O, 250 ml methanol, 50 ml glacial acetic acid, and 2 g amido black were added.

I. Buffer: To 6.0 g of TRIS, 28.8 g of glycine and H_2O were added to make 1,000 ml (pH 8.3). A 1/10 dilution was used.

Stock Solutions for Positively Charged Proteins

AA. To 48 ml of 1 N KOH, 17.2 ml of acetic acid, 4.0 ml of TEMED and H_2O were added to make 100 ml (pH 4.3).

BB. To 48 ml of 1 N KOH, 2.89 ml of acetic acid, 0.46 ml of TEMED, and H_2O were added to make 100 ml (pH 6.7).

CC. Catalyst: To 0.28 g of ammonium persulfate H_2O was added to make 100 ml (prepared fresh weekly).

DD. Buffer: To 31.2 g of β-alanine, 8.0 ml of acetic acid and H_2O were added to make 1,000 ml (pH 5.0). A 1/10 dilution was used.

Working solutions were prepared as follows:

Lower gel; 7.0 % - 1 part of A or AA, 1.4 parts of C and 2.1 parts of H_2O

Lower gel; 8.0 % - 1 part of A or AA, 1.6 parts of C and 1.9 parts of H_2O were combined with catalyst in a 1:1 ratio.

Upper gel; 1 part of B or BB, 2 parts of D, 1 part of E and 2 parts of H_2O were mixed.