

PURIFICATION OF INTRACELLULAR AND
EXTRACELLULAR TOXINS FROM FOUR STRAINS OF
CLOSTRIDIUM BOTULINUM TYPE E

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This is to certify that the
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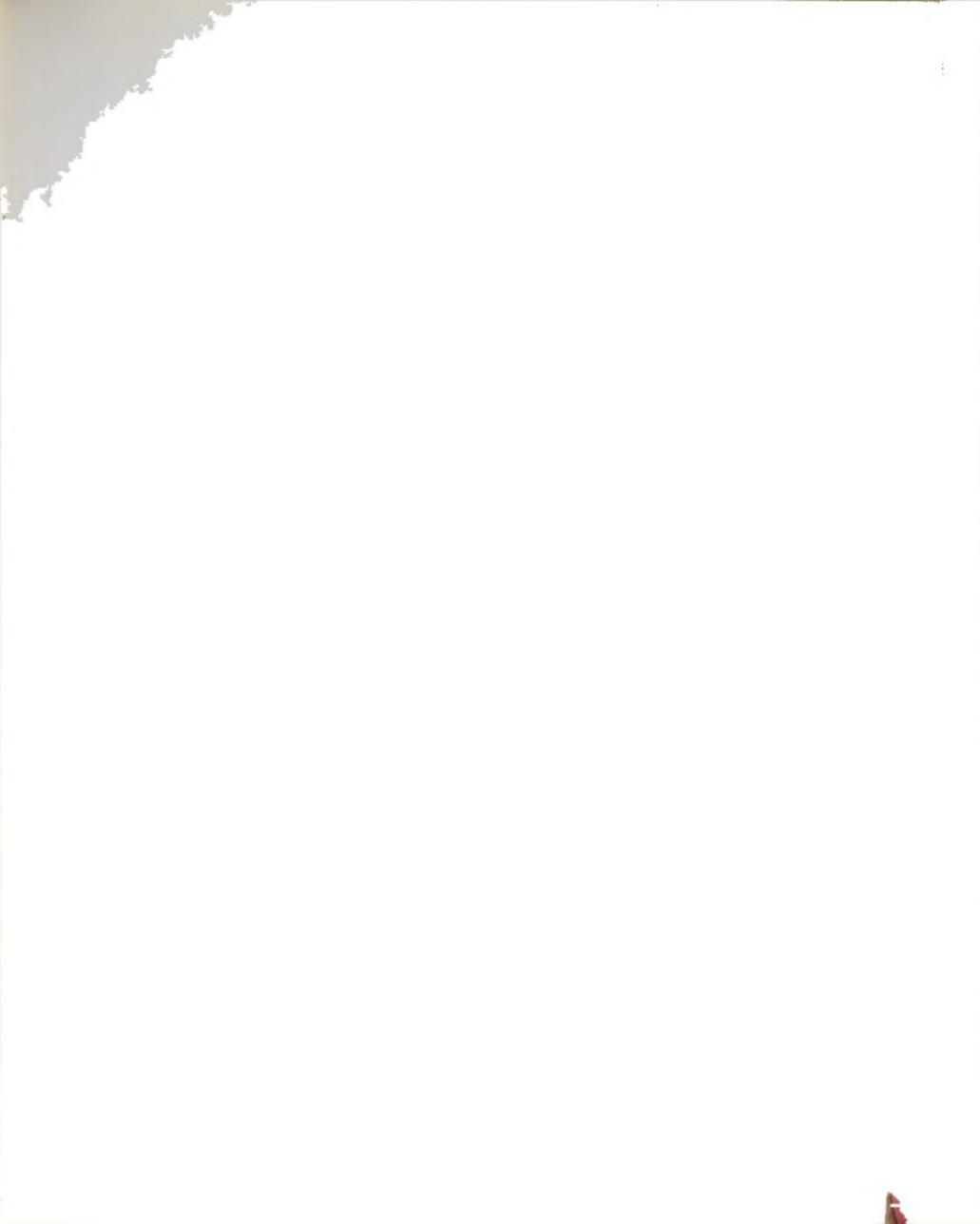
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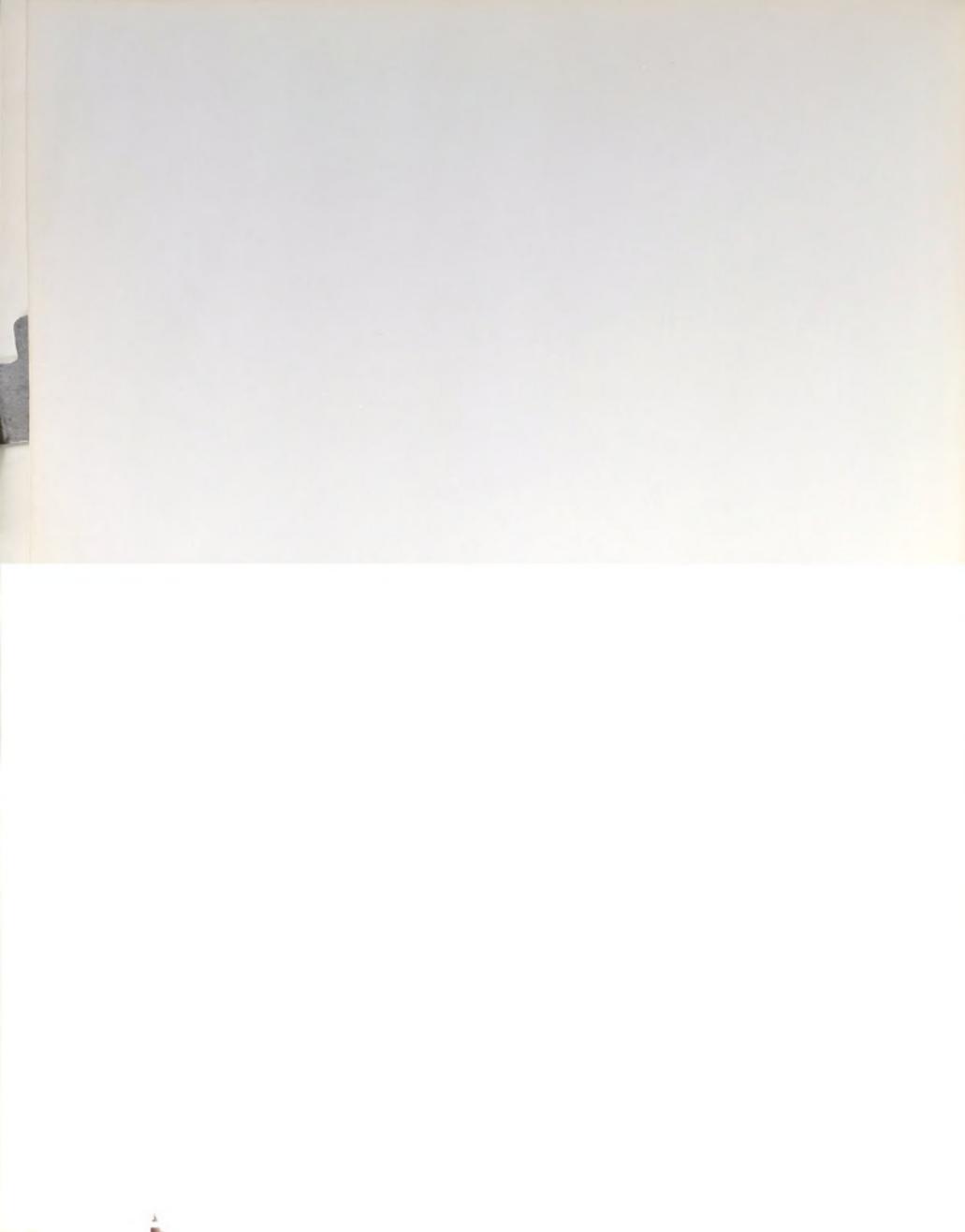
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ABSTRACT

PURIFICATION OF INTRACELLULAR AND EXTRACELLULAR TOXINS FROM FOUR STRAINS OF CLOSTRIDIUM BOTULINUM TYPE E

By

Merlin Dennis Breen

The toxin from type E *Clostridium botulinum* has been reported to have a molecular weight from 5,000 to 250,000. The conditions used to produce and purify the toxin have varied and this may account for the differences reported in molecular weight.

Four different cellular strains of C. botulinum (Kalamazoo, VH, Hazen and Iwanai) were grown to produce extracellular toxin. All four strains were grown in the same medium using the dialysis sac culture method at 32 C for 7 days. The cells were removed by centrifugation and the supernatant and fluid was precipitated by adding ammonium sulfate to 60% of saturation. The toxic precipitate was dissolved in 0.05 M sodium phosphate buffer, pH 6.0, and concentrated by ultrafiltration. The toxin was eluted through a column of Diethyl aminoethyl cellulose and applied to a CM Sephadex column. The toxin adsorbed onto the CM Sephadex column and was recovered with the application of a 0-0.5 M NaCl linear gradient.



For experiments on intracellular toxin, the Kalamazoo strain of type E C. botulinum was grown for 3 days at 30 C. The cells were removed by centrifugation, washed twice and extracted twice with 0.2 M phosphate buffer at pH 6.0. The toxin in this cellular extract was precipitated by addition of ammonium sulfate to 50% of saturation. The precipitate was recovered by centrifugation and dissolved in 0.02 M acetate buffer, pH 6.0. This partially purified toxin absorbed on a CM Sephadex column and was recovered with a 0-0.5 M NaCl linear gradient.

Both the extracellular and intracellular toxin preparations contained 2 main protein components as demonstrated by electrophoresis in a β -alanine-acetate acid buffer system at pH 4.3 in polyacrylamide gels. The slowest moving band (of lesser intensity) was indicated to be the toxin. The molecular weight of the proteins was determined using a sodium dodecyl sulfate polyacrylamide gel electrophoresis system. Under these conditions, the previously mentioned toxin band dissociated into two bands. The molecular weight of the lighter component was 111,000-120,000. The heavier component, believed to be the toxic component, had a molecular weight of 125,000-142,000.

The molecular weight of C. botulinum type E toxin does not appear to depend upon the cellular strain, the purification procedure, or the source (intracellular or extracellular). From the results of this investigation, the toxin is presumed to have a molecular weight of 125,000 to 142,000; toxin of low molecular weight was not detected.

The first part of the report discusses the background and objectives of the study. It highlights the importance of understanding the factors that influence the performance of the system under investigation. The second part of the report presents the methodology used for data collection and analysis. This includes a detailed description of the experimental setup and the statistical methods employed to analyze the results. The third part of the report discusses the findings of the study, which show that the system performs well under most conditions, but there are some areas for improvement. Finally, the report concludes with a summary of the key findings and recommendations for future research.

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A THESIS

Submitted to
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G82792

To my wife and son



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LITERATURE REVIEW

Introduction. Botulism has occurred for centuries, most commonly from consumption of contaminated sausage or of low-acid, preserved vegetables. The causative agent was demonstrated by Van Ermengen in the 1890's to be a soluble toxic substance, not the bacterium itself (Dolman, 1964). He also isolated and described Clostridium botulinum, the anerobic spore-forming bacillus which produced the toxin. Further, he demonstrated the disease in laboratory animals by injecting a cell-free filtrate of the culture. The toxicity was destroyed in 5 minutes at the temperature of boiling water or in one hour at 70 C.

The toxic component, as mentioned above, is very heat labile when compared to the spores of C. botulinum. The toxin is also labile to ultraviolet light, air, and alkalinity. It is relatively stable under low pH conditions, during precipitation by alcohol or ammonium sulfate, dialysis and mild proteolysis (Schantz, 1964).

The molecular action of the toxin has been difficult to clearly elucidate. It is known that the peripheral nerves are most affected as compared to the central nervous system. The toxin interferes with the release of acetylcholine at neuromuscular junctions (Whaler, 1967). Lamanna and Hart (1967) reported the amount of toxin which constitutes a lethal dose is independent of body weight for mice but related to body weight in rats. The onset of symptoms includes nausea, vomiting, weakness, dizziness and lassitude (Rogers et al., 1964). The optic



nerves are usually affected resulting in double vision and blurred vision. Dryness of the mouth, difficulty in speech and in breathing are the other primary symptoms. Death usually results due to respiratory failure.

The concentration and purity of toxin can be measured by gel-diffusion and by the techniques commonly used to measure the concentration and purity of proteins. But assay by injecting mice and observing death is the most sensitive and specific assay known. Intraperitoneal (IP) injections are the simplest, requiring the least skill to properly complete. A more difficult, but quicker method is intravenously (IV) injections. While IP injections are simple, dilutions must be made over a specific range and the mice must be observed for 48 hours. IV injections require just a few hours observation, with the time-to-death related to the concentration of the toxin, giving a rapid measurement of toxin concentration (Kitamura et al., 1968).

Early Studies of C. botulinum. Early studies of C. botulinum toxin purifications were conducted for the purpose of producing antisera to the toxin. Kemper in 1897 and Leuchs in 1910 were the first to prepare antisera, while Snipe and Sommer in 1928 precipitated toxin by adjusting the pH to 3.5 (cited in Cooper, 1964). Various methods have been used for purifying C. botulinum toxin including isoelectric precipitation, shaking with chloroform, ethanol precipitation, chromatography in modified cellulose and crystallization from salt solutions. Precipitation with salts is now quite common, particularly with ammonium sulfate.



Duff et al. (1956) used a purification procedure which included precipitation with acid at pH 3.5 followed by extraction with 0.075 M CaCl_2 , acid precipitation, precipitation with 15% ethanol at -5°C and crystallization with 0.9 M ammonium sulfate. Gerwing et al. (1961) precipitated the toxin with ammonium sulfate and then further purified the toxin by chromatography on a Diethylaminoethyl cellulose column (DEAE cellulose).

The definition of the active site of the toxin of Clostridium botulinum has been a difficult problem to resolve. Boroff et al. (1967) reported that the toxin is photoxidized in the presence of methylene blue. They attributed this toxicity loss to either tryptophan or methionine. The rate of destruction of tryptophan by photoxidation closely followed the photoxidation destruction of toxin. Iodacetate, which affects methionine almost exclusively, did not affect toxicity; 2-hydroxy, 5-nitrobenzyl bromide (HNBB) also inactivated the toxin. This chemical affects only tryptophan and cysteine. Gerwing et al. (1966) however maintained that cysteine residues are involved in the active site. They did not find tryptophan by their analysis of the toxin. One of their investigations studied disulfide bond reduction as it affects detoxification of the toxin; they reported detoxification closely follows the reduction of disulfide. Schantz and Spero (1951) found little loss in toxicity after treatment with ketene and thus discounted the role of cysteine in the active site of toxin. Gerwing et al. (1967) compared amino acid sequences near the cysteine residue of types A, B and E toxin and found very little difference in this region.



Studies of Type E Toxin. Investigations on Type E. C.

botulinum toxin were intensified after outbreaks of botulism due to ingestion of smoked fish occurred in 1963 in US. The toxin, from Type E, normally associated with marine items, required activation to reach full toxicity (Duff et al., 1956 b). Treatment with a proteolytic enzyme, usually trypsin, produces about a 100X increase in toxicity. Thus the difference between type E toxin and other types was thought to be significant; but it appears the proteolytic strains of Types A and B are self-activated by proteolytic enzymes produced by the vegetative cell (Das Gupta, 1971).

Two general methods have been used to produce purified type E toxins. The first method, used by Gerwing et al. (1961), utilized culture systems in dialysis sacs. The culture phase, 0.85% NaCl, in dialysis sacs was surrounded by the culture medium of beef infusion fortified with 1.0% peptone, 0.5% NaCl, 0.2% Na_2HPO_4 , 0.1% sodium thioglycolate and 2.0% glucose. The dialysis sac culture method was first described by Vinet and Fredette (1951). The culture medium was changed daily and the culture was incubated at 30 C for 5 days. The cells were removed by centrifugation. The supernatant fluid was filtered through a Seitz filter and the filtrate was used as the crude toxin preparation. Emodi (1969) used a modification of the dialysis sac culture system, using the following medium: 5% tripticase, 0.5% peptone, 1.0% yeast extract, 0.2% sucrose and 0.1% sodium thioglycolate (TPSY) at pH 7.2, inside of the sacs at 2X strength. The culture was inoculated outside the sac into physiological saline. These flasks were incubated at 32 C for



7 days. The culture phase was centrifuged to remove the cells and the supernatant fluid containing the toxin was retained for further purification.

Alternately, Kitamura et al. (1968) grew cells in deep broth culture with a medium consisting of 1% glucose, 0.5% yeast extract, 2.0% peptone and 0.25% sodium thioglycolate at a pH of 6.3. After growth for 4 days at 30 C, the cells were removed by centrifugation, washed twice and extracted twice with 0.2 M phosphate buffer at pH 6.0. These extracts were used in further toxin purification procedures.

Purification of the crude toxins has also been attempted using various methods. Gerwing et al. (1961) precipitated toxin by adding 95% ethanol to a final concentration of 35% by volume (-15 C). This precipitate was dissolved in pH 7.0 acetate buffer and applied to a DEAE cellulose column. The toxin was eluted using 0.0-0.5 M sodium acetate buffer at pH 6.5. A toxicity of $3.6 - 7.2 \times 10^5$ MLD/mg N was obtained. Gerwing et al. (1964) also precipitated the toxin by the addition of dry ammonium sulfate to 60% of saturation and eluted the toxin through DEAE cellulose columns with 0.01 M acetate buffer at pH 4.5. These modifications increased the toxicity to 7.5×10^6 MLD/mg N.

Kitamura et al. (1968) purified a cellular extract by precipitating the toxin by the addition of dry ammonium sulfate to 50% of saturation. The precipitate was dissolved and chromatographed on Sephadex C-50 (CM-Sephadex). The percolate, containing the toxin was subjected to a RNase treatment and then adsorbed on the CM-Sephadex column and eluted from the column with NaCl at approximately 0.07 M NaCl contained in acetate buffer.



The toxin was concentrated by a second precipitation with ammonium sulfate at 50% of saturation and eluted through G-200 Sephadex columns. The filtrate had a specific toxicity of 4.8×10^7 LD₅₀/mg N.

Molecular Weight. The molecular weight of type E C. botulinum toxin has been studied by several research groups. Since toxins from all C. botulinum types have similar pharmacological reaction, the toxins would be expected to have similar size, shape, amino acid composition and similar action on their host.

The type A toxin, which has been the most frequently studied, was reported by Buehler et al. (1947) to have a minimum molecular weight of 45,000, to be composed only of amino acids and to contain 16.2% Nitrogen. Putman et al. (1946) reported that type A toxin had a molecular weight of 900,000. However, Boroff et al. (1968) reported that type A toxin had a molecular weight of 740,000 and could be disassociated into two components, a toxic component of 150,000 and a nontoxic component of 500,000. Wageman (1954) reported that the toxin molecule would disassociate under certain conditions of pH and ionic strength to give smaller units whose molecular weights ranged from 40,000 to 100,000. Lamanna and Lowenthal (1951) demonstrated that the two components, one toxic and one possessing hemagglutinating activity, could be separated by utilizing DEAE Sephadex. Meyer and Lamanna (1959) reported that the molecular weight of the toxic component was 150,000.

The reported molecular weight of type B toxin has also been inconsistent. Toxin purified on DEAE Sephadex was reported to have



a mol. wt. greater than 100,000 (Boroff et al., 1968), while an unchromatographed toxin was found to have a molecular weight similar to type A toxin or about 700-900,000 (Duff et al., 1957).

The molecular weight reported for type E toxin varies from 5000 to 300,000. Emodi and Lechowich (1969) reported finding two toxic components with molecular weights of 5,000 and 7,000 from the VH strain. Dolman and Gerwing (1967) reported a molecular weight of 14-18,000 for type E toxin with molecular weights of Types A and B toxins of the same magnitude. These two studies on type E toxin employed elution through DEAE cellulose as the primary purification step.

Kitamura et al. (1968) reported a molecular weight of about 250,000 for type E toxin. The toxin consisted of two main dissociable components, of which one was toxic and the other which was not. Both had molecular weights of 150,000.

Recently DasGupta and Sugiyama (1972) found that toxins of types A, B and E all contained a toxic component with a molecular weight of about 150,000. Their determination of molecular weight was based on electrophoretic patterns in polyacrylamide gels containing sodium dodecyl sulfate.

The activation phenomenon of type E toxin is also a factor in determining the molecular weight. Sakaguchi et al. (1964) reported the activation did not affect the molecular weight, while Gerwing et al. (1965) reported the activation tended to fragment the molecule, resulting in a 20% reduction in molecular weight. DasGupta and Sugiyama (1972) reported that with types A and B toxin

or type E toxin after activation, the 150,000 molecular weight component, under reducing conditions, splits into a 100,000 and a 50,000 molecular weight component. This suggests that the activation of type E toxin is due to the breakage of one peptide bond.

The discrepancy found between molecular weights reported by Dolman and Gerwing (1967) and the other reports was suggested by Gerwing et al. (1965) to be due to the repeated ammonium sulfate precipitations used by Sakaguchi et al. (1964) in their purification procedure. Recently Helmsch and Sugiyama (1972) reported that DEAE cellulose and CM-cellulose chromatography at pH 6.0 reduced the molecular weight of the toxin from 250,000 to 150,000. This reduction in molecular weight may explain why the toxin found by Gerwing et al. (1964) is reduced in size, but does not account for the contrast between molecular weight of 16,000 and 150,000.

Other factors which contribute to this discrepancy other than chromatographic differences could be: the pH of the growth medium, the type of buffer used in purification, the strain of type E used and whether the toxin is intracellular or extracellular.

The pH of the growth medium was suggested by Kitamura et al. (1968) to be very important. They reported that the toxin is broken down rapidly at pH 7.0. Thus the toxin grown by Dolman et al. (1964) at a high pH could have been fragmented by the pH and further fragmented by a small amount of proteinases present in the culture.

Emodi (1969) also used a culture medium with an initial pH of 7.2 compared to an initial pH of 6.3 for Kitamura et al. (1968). Kitamura et al. (1968) also used the Hazen strain of type E C. botulinum while Emodi (1969) used VH strain and Gerwing et al. (1961) used the Iwanai strain.

The greatest difference in molecular weight appears between the extracellular toxin isolated by Gerwing et al. (1961) which was purified by DEAE cellulose, and the toxin studied by Kitamura et al. (1968) which was extracted from bacterial cells and purified using CM-Sephadex chromatography.

In the examination of specific toxicity, the highest specific toxicity for type E toxin was 1.2×10^8 LD₅₀/mg N. This was reported by Heinsch and Sugiyama (1972) for a toxin which had a molecular weight of 150,000. Gerwing et al. (1965) appear to have a less pure preparation with a specific activity of 2.3×10^7 MLD/mg N. A specific activity of 4.8×10^7 LD₅₀/mg N was reported for type E toxin by Sakaguchi and Sakaguchi (1967). The study of type E toxin by Emodi and Lechowich (1967) did not include a report of the final specific activity.

The above mentioned discrepancies in molecular weight need to be resolved. This investigation was designed to control the variables involved in purification and to determine the actual molecular weight of the toxin from four different strains of type C. botulinum.

METHODS AND MATERIALS

Microorganisms. Clostridium botulinum type E was used in this investigation. The four strains used, Vancouver Herring (VH), Kalamazoo, Iwanai and Hazen were obtained from the Department of Food Science and Human Nutrition, Michigan State University.

Inoculum preparation. Stock cultures (tubes of culture frozen after 24 hours of growth) were inoculated into a deep culture tube containing cooked meat medium (Difco) and incubated at 32 C. After growth was evident as determined by turbidity and gas production, one ml of the culture was transferred to a second tube containing 15 ml of cooked meat medium and the tubes were incubated for 24 hours at 32 C. After a third transfer in cooked meat medium the culture was transferred to a tube of medium consisting of 5% trypticase (BBL, Cockville, Maryland), 0.5% peptone (Difco, Detroit, Michigan), 0.2% sucrose, 1.0% yeast extract (Difco) and 0.1% sodium thioglycolate (TPSY) adjusted to pH 6.3. After the three successive transfers in the TPSY medium, five ml of the culture was inoculated into a dialyzed sac culture system in a 200-ml Erlenmeyer flask and the flask was incubated for 24 hours at 32 C.

Toxin Production. Unless otherwise noted, the TPSY medium described in the previous section was used throughout this investigation for toxin production. Extracellular toxin was produced in the dialysis sac culture system first described by Vinet and



Fredette (1951) as modified by Emodi (1969). A dialyzing sac containing double-strength TPSY medium was suspended in 0.85% saline in a 2-liter flask. Thirty ml of a 24-hour TPSY culture was inoculated into the saline portion of each of 4 flasks, and the flasks were incubated at 32 C for 7 days. All dialyzing membranes (both for toxin production and toxin dialysis) were soaked in distilled water for 15 minutes followed by boiling with 0.05 M ethylenediaminetetracetate (EDTA) at pH 7.0 for 15 minutes. The membranes were then rinsed in distilled water and stored in water at 4 C until used.

Cells for intracellular toxin production were cultured in a Fermacell 40-liter Fermentor (New Brunswick Scientific Co.). Approximately 10^9 washed spores of the Kalamazoo strain, contained in 100 ml of 0.05 M phosphate buffer at pH 6.0, were inoculated into 40 liters of TPSY medium and incubated at 30 C for 80 hours without agitation.

Toxin Assays. Prior to determining their toxicities, the samples were incubated with equal volumes of 0.5% trypsin (Difco) in 0.05 M phosphate buffer, pH 6.0 for 1 hour at 37 C in order to activate any toxin which might be present. Portions of "activated" samples were injected intraperitoneally into Swiss-Webster female mice (15-20 g body weight) to determine the toxicity of the samples. The mice were observed for 48 hours for symptoms typical in botulism death.

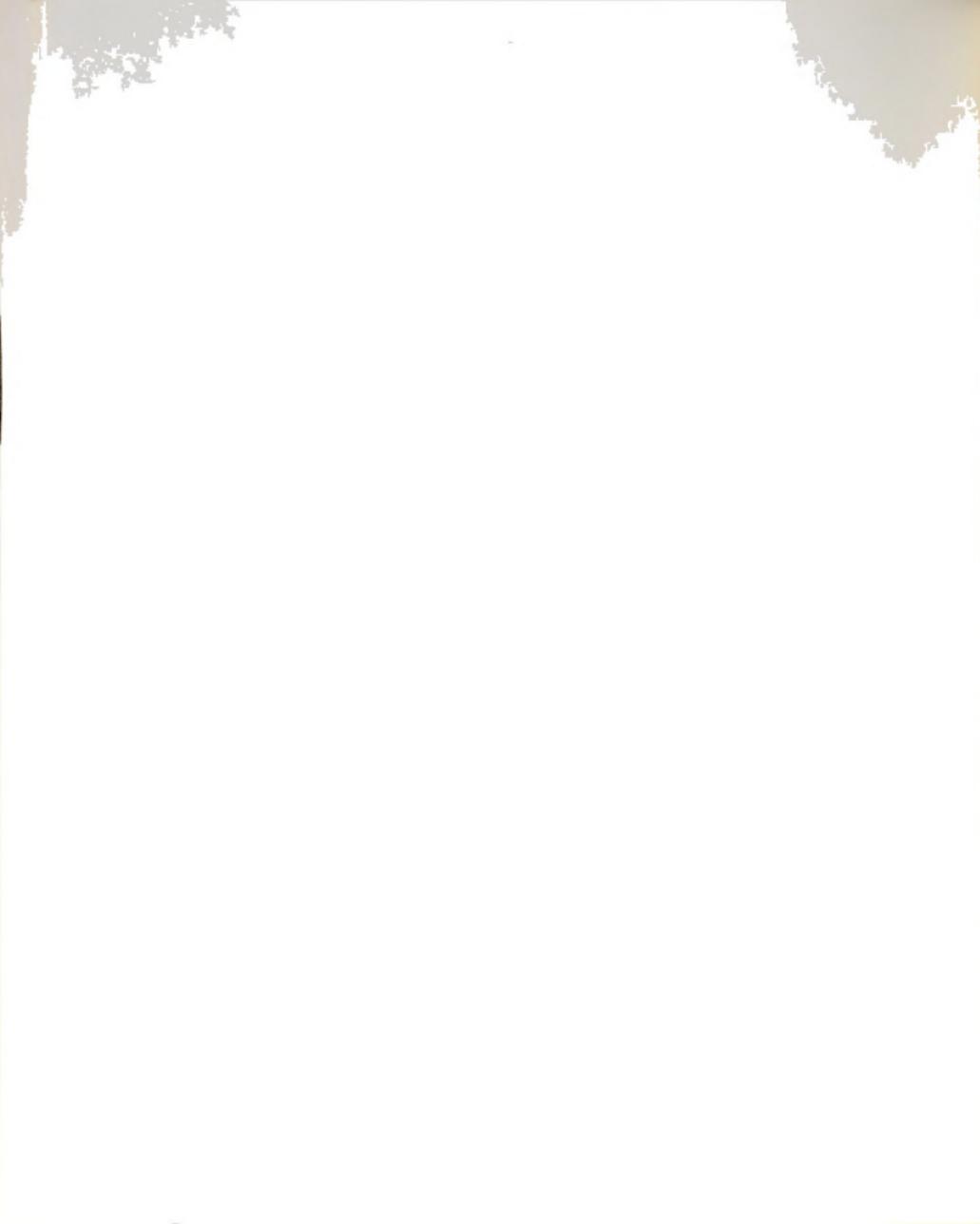
Estimates of the toxicity of samples were determined by injection of serial dilutions of the samples into mice. The highest dilution producing death within 48 hours was used to estimate the toxicity of the sample.

The mouse lethal dose (MLD) per ml of sample was determined by duplicate injections of 0.1, 0.25 and 0.5 ml of 1:10 serial dilutions of samples into separate mice. The MLD/ml was designated as the reciprocal of the highest dilution of the sample which produced death in both mice.

The 50% lethal dose (LD_{50}) per ml of sample was calculated using the method of Reed and Meunch (1938). Four mice were injected per dilution over a range of proportionately equal intervals. This range included dilutions which killed all four mice and dilutions which killed none of the four mice. The dilution which would have killed 50% of the mice was calculated and the reciprocal of that dilution was designated as the LD_{50} /ml for that sample.

Protein Determination. The method of Lowry *et al.* (1951) was used for all protein determinations. Bovine serum albumin (BSA) was used as a standard reference. Duplicate tubes containing dilutions of the standards and unknowns were used in each analysis. Absorption at 660 nm was measured using a Beckman DB-G Spectrophotometer and the average value for each sample was used to calculate the protein concentration. Nitrogen content of the samples was calculated assuming that the toxin contained 16.2% nitrogen.

Purification of Extracellular toxin (Figure 1). After 7 days of incubation, the culture phase of the dialysis sac system was centrifuged ($27,000 \times g$; 85 ml/min.) with a Sorvall RC-2 refrigerated centrifuge equipped with a KSB-R continuous centrifuge system. The supernatant fluid was retained and cooled to 4 C.



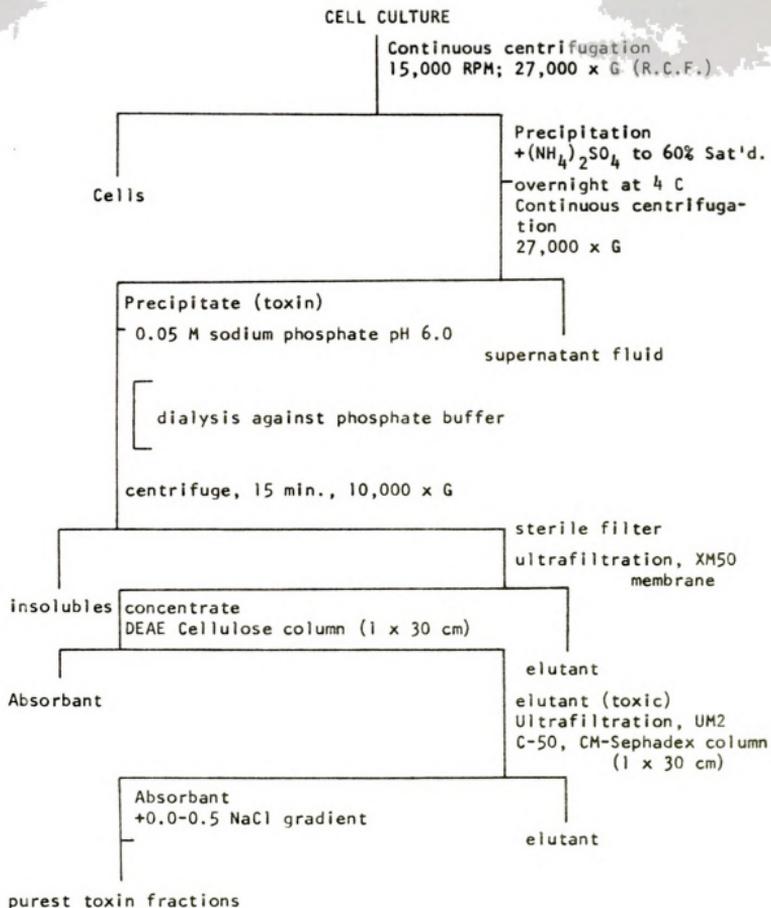


Figure 1. The procedure used for purification of extracellular type *E. C. botulinum* toxin from the culture phase of a dialysis sac culture system.

The toxin was precipitated from the supernatant fluid by the addition of dry ammonium sulfate to 60% of saturation (423.6 g per 1000 g). After stirring overnight, the precipitate was removed by continuous centrifugation at 27,000 x \underline{g} (approximately 85 ml/min). The liquid phase was discarded and the toxic precipitate was dissolved in 0.05 M phosphate buffer at pH 6.0 and dialyzed overnight against 3 changes of the same buffer. The toxic solution was centrifuged at 15,000 x \underline{g} for 15 minutes, filtered through a 0.45 μ m-pore-size Millipore filter and stored at 4 C.

Diethylaminoethyl (DEAE) cellulose (Cellex D, Bio Rad Laboratories, Richmond, California) was suspended in 0.1 M HCl, stirred and centrifuged. The DEAE cellulose was then rinsed in 0.1 M NaOH followed by a rinse in 0.1 M HCl and suspended in 0.05 M phosphate buffer at pH 6.0. The DEAE cellulose was cooled to 4 C, poured into a 1 x 30 cm column, allowed to settle, and packed with the aid of slight N₂ gas pressure. After eluting the column with 0.05 M phosphate buffer at pH 6.0 for 24 hours the sample was applied. For repeated use, the column was subjected to elution with 0.1 M HCl followed by elution with 0.05 M phosphate buffer at pH 6.0 until the pH of the elutant returned to 6.0.

Crude toxin was concentrated approximately 12-fold using a model 52 Amicon ultrafiltration unit with an XM-50 membrane. Four ml of the concentrated crude toxin was layered on a 1 x 28 cm DEAE cellulose column and eluted using 0.05 M phosphate buffer at pH 6.0 containing 0.5% NaN₃. (The toxin was eluted from the column

with the sample front.) The toxic fractions were pooled and concentrated by ultrafiltration using a UM-2 membrane (Amicon).

C-50 Sephadex (Carboxymethyl-Sephadex, Pharmacia, Uppsala, Sweden) was hydrated by soaking in 0.05 M phosphate buffer at pH 6.0 overnight at room temperature. The C-50 was decanted and mixed with additional buffer, stored for two additional days at room temperature, cooled to 4 C and poured into a 1 x 30 cm column. After a column height of approximately 10 cm had formed, the column was allowed to elute and pack overnight using 0.05 M phosphate buffer at pH 6.0.

The toxin which had eluted from the DEAE cellulose column was applied to the C-50 Sephadex column (1 x 28 cm). The toxin adsorbed onto the column and after the column had been thoroughly eluted, the adsorbed protein was recovered by using a 0-0.5 M NaCl linear gradient in phosphate buffer (0.05 M, pH 6.0). The toxic fractions were desalted and concentrated by ultrafiltration prior to being subjected to electrophoresis.

Purification of Intracellular Toxin (Figure 2). Forty liters of TPSY culture from the fermentor were centrifuged by continuous centrifugation at $15,000 \times g$ (approximately 85 ml/min). The cells were washed twice with 0.05 M sodium acetate buffer at pH 5.0 and suspended in 0.2 M phosphate buffer at pH 6.0 (the extraction buffer). The extraction of toxin from the cells was carried out at 37 C for 2 hours and then at 4 C overnight. This cell suspension was centrifuged and the supernatant fluid was saved. The cells were re-suspended in 0.2 M phosphate buffer at pH 6.0 and extracted at 37 C for 2 hours and at 4 C overnight. The cells were removed

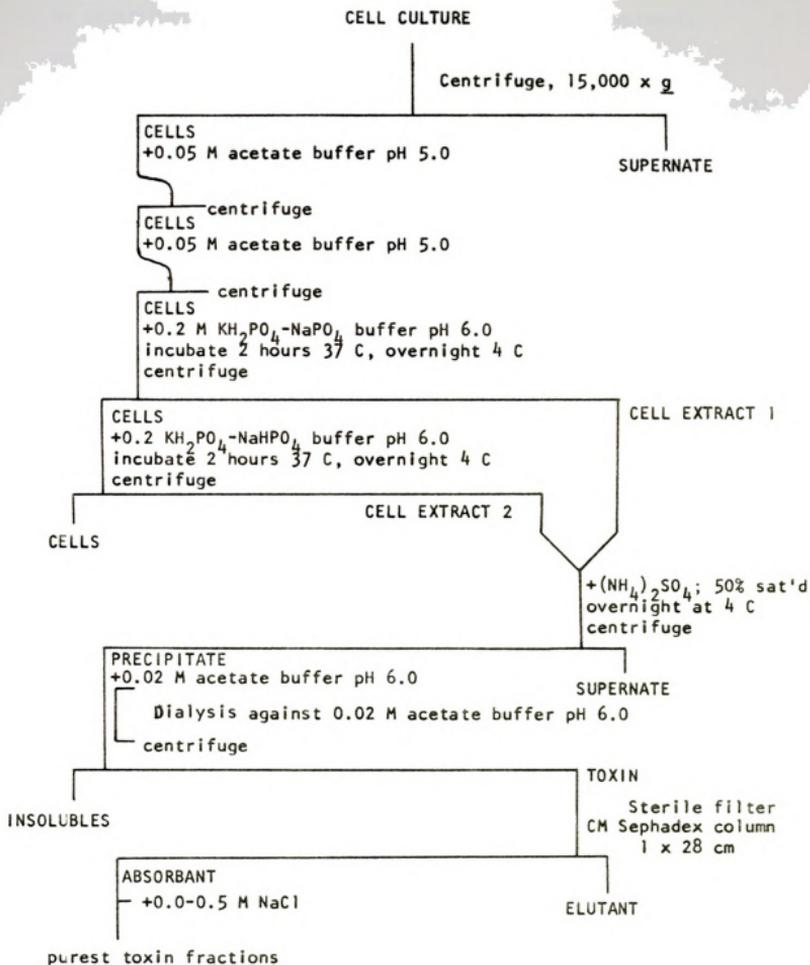


Figure 2. The procedure used to extract and purify intracellular toxin from 3-day old cells of Kalamazoo strain of type E *C. botulinum*.

by centrifugation and discarded and the two cellular extracts were combined.

The cellular extract was subjected to precipitation by the addition of dry ammonium sulfate to 50% of saturation (380 g per 1000 ml). After stirring overnight, the precipitate was recovered by centrifugation, dissolved in 60 ml of 0.02 M sodium acetate buffer at pH 6.0 and dialyzed overnight against the same buffer. The dialyzed extract was centrifuged and the supernatant fluid was filtered through a 0.45 μ m-pore-size Millipore filter. This filtrate containing the crude intracellular toxin was stored at 4 C.

A C-50 Sephadex (CM-Sephadex) column was prepared as previously described except that 0.02 M acetate buffer at pH 6.0 was used in place of the phosphate buffer. The crude intracellular toxin was concentrated approximately 12-fold and 4 ml of the concentrated toxin was layered onto a 1 x 28 cm C-50 Sephadex column. The toxin was adsorbed onto the column and was removed by the application of a 0-0.5 M NaCl linear gradient contained in 0.02 M sodium acetate buffer at pH 6.0. The fractions containing each absorbance peak were pooled for each peak and concentrated and desalted by ultrafiltration using a UM-2 membrane (Amicon).

G-200 Sephadex columns were prepared as previously described for CM-Sephadex. Standard materials used to calibrate the column included Blue Dextran 2000 (Pharmacia Fine Chemicals Inc.), human hemoglobin (Mann Research Laboratories) and lysozyme (Nutritional Biochemical Corp.).

Electrophoresis. Acid Gel electrophoresis: Disc gel electrophoresis was used to determine the purity of the samples. The gels contained 7% acrylamide and KOH-acetic acid buffer at pH 4.3. The buffer used in the buffer tanks was .035 M β -alanine-acetic acid buffer at pH 5.0 as described by Emodi (1969). Acrylamide, Prep/cryl grade, N, N' methylenebisacrylamide (bis) and N, N, N', N' Tetramethylethylenediamine (Temed) were obtained from Canalco Industries Corporation and ammonium persulfate from E-C Apparatus Corp. The gels were polymerized with light and installed in a Poly-analyst temperature-regulated disc electrophoresis apparatus (E-C Apparatus Corp.). The buffers were pre-chilled to 4 C and the cooling jacket was cooled by tap water (18 C). Twenty to one hundred microliters of sample were carefully layered onto each gel. A direct current of $1\frac{1}{2}$ milliampere (ma) per gel was applied for 45 minutes and then was raised to 3 ma/gel. After $3\frac{1}{2}$ hours the gels were stained with Buffalo Black (Allied Chemical) or Coomassie Brilliant Blue R (Sigma Chemical Co.) (dissolved in 125 ml of H₂O, 125 ml methanol and 25 ml of glacial acetic acid/g of dye) for 2-12 hours. The gels were destained in 7% acetic acid with the aid of direct current for 20 minutes and then allowed to stand overnight in 7% acetic acid.

Sodium Dodecyl Sulfate Electrophoresis: The method of sodium dodecyl sulfate (SDS) electrophoresis as described by Weber and Osborn (1969) was used in this investigation. Their dialysis buffer [0.01 M sodium phosphate, 1.0% SDS (95% grade), and 0.01% mercaptoethanol at pH 7.0] was prepared double-strength and combined



with samples on a 1:1 basis and incubated at 37 C for two hours before being applied to the gels.

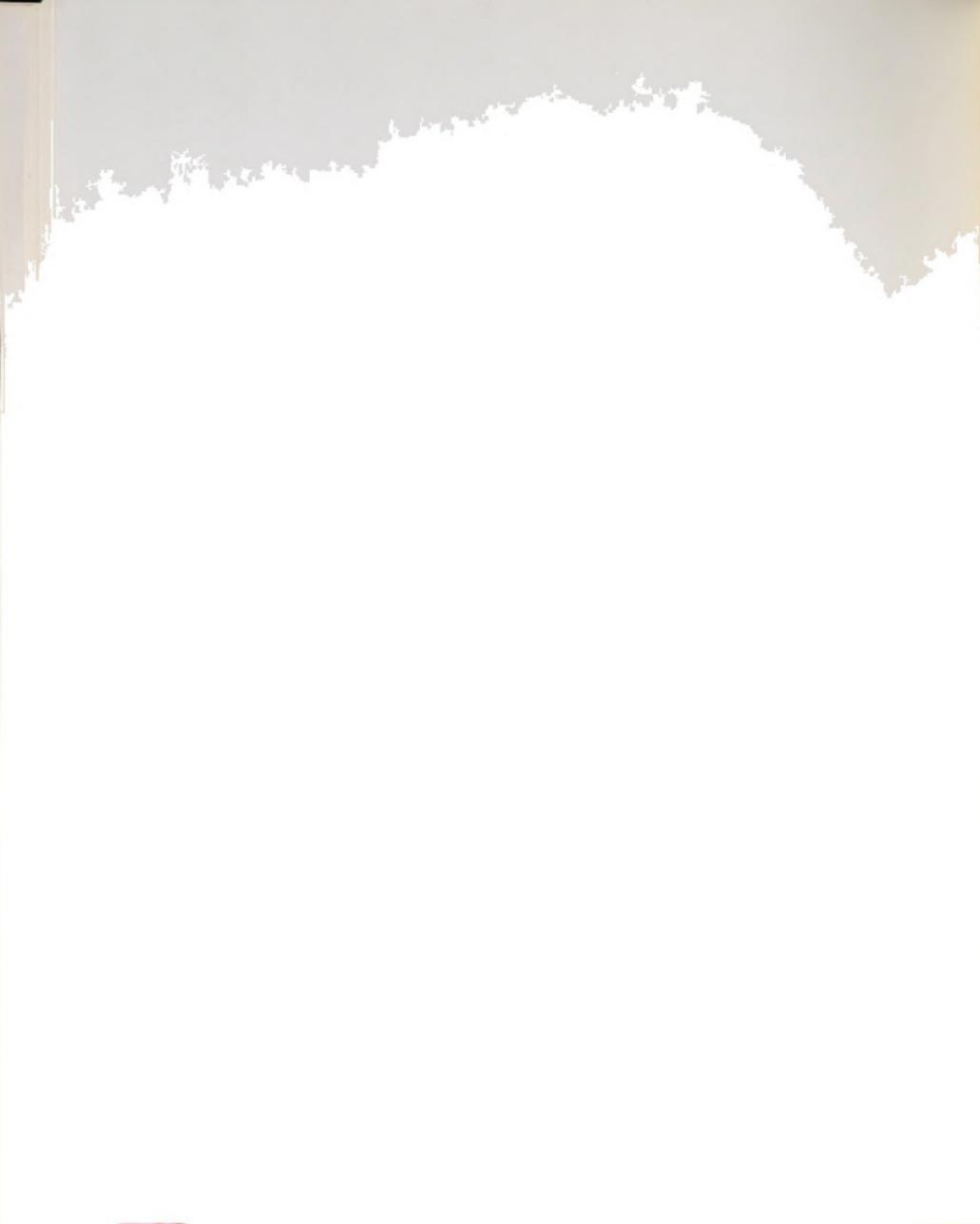
The gels, which contained 10% acrylamide and 0.03% bis, were held at room temperature during electrophoresis. Five μ l of mercaptoethanol were layered onto each gel prior to application of 50-100 μ l of the incubated sample. A current of 8-10 ma/gel was used for 4-6 hours. The buffer in the buffer tanks was diluted to $\frac{1}{2}$ of that used by Weber and Osborn (1969). The gels were removed and stained with Coomassie Brilliant Blue R followed by destaining as described above.

Proteins of known molecular weight were applied with and without the unknown samples. These proteins were: Phosphorylase a, 94,000 mol. wt. (Sigma); Catalase, 60,000 mol. wt.; Lysozyme, 14,300 mol wt. and BSA, 68,000 mol. wt. (all from Nutritional Biochemical Corp.). The electrophoretic mobility of the proteins was plotted on a linear scale vs their molecular weight on a log scale. The standard curve was then used to determine the molecular weight of the protein bands which resulted from electrophoresis of the toxic samples.

RESULTS AND DISCUSSION

Growth Conditions. Media used for production of type E C. botulinum have included TPSY used by Emodi (1969), and fortified beef extract used by Gerwing et al. (1961). The pH of the medium must be favorable for toxin production, but must not affect the stability of the toxin. The pH of media used for toxin production has ranged from 7.2 (Emodi, 1969) to pH 6.3 (Kitamura et al., 1968). Figure 3 depicts the change in pH during the growth of type E C. botulinum in deep culture tubes (15 ml screw-cap test tubes). Since Kitamura et al. (1967) reported that toxin began to dissociate near pH 7.0, this pH level should be avoided during the latter stages of growth when toxin is released into the medium. Both cultures exhibited a deep in pH during the first 24 hours, thus both media had pH values below 7.0 when toxin would be released from the cells.

The next step was to determine the effect of pH on toxin production. Two strains (Hazen and VH) were grown in tubes of TPSY, each at two different pH values, 6.3 and 7.2. After 6 days of growth at 32 C the cultures were centrifuged and the toxicity of the supernatant fluid was determined. The results are listed in Table 1.



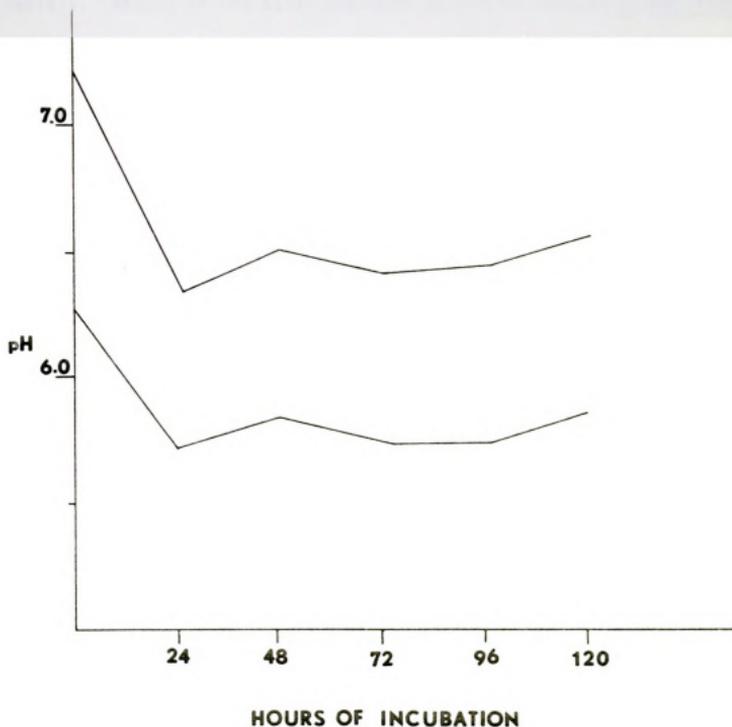


Figure 3. The pH of cultures during growth of type *E. coli* botulinum (Hazen strain) contained in TPSY medium (initial pH values of 7.2 and 6.3) at 32 C in screw cap test tubes.

Table 1. Assays of the toxin produced by two strains of C. botulinum type E in TPSY medium incubated 5 days at 32 C.

<u>Strain</u>	<u>pH</u>	<u>Fate of mice injected with the sample dilution indicated</u>	
		<u>death</u>	<u>survival</u>
VH	6.3	1:100,000	not assayed
Hazen	6.3	1:100,000	not assayed
VH	7.2	1:4,000	1:10,000
Hazen	7.2	1:10,000	1:100,000

Since TPSY at pH 6.3 produced higher yields of toxin for both strains, this pH was used in all subsequent experiments for toxin production.

Experiments were conducted to maximize toxin production using the dialysis sac culture method. Emodi (1969) reported that double-strength medium should be used inside of the sacs for optimal production of toxin. The ratio of 1:4, medium to saline, used by him was originally employed for toxin production. This combination produced toxin levels too low to use as starting material for toxin purification. Ratios of 2:1 and 1:1, saline to culture medium, were tested to determine if these ratios would produce higher yields of toxin. For these experiments, Kalamazoo and Hazen strains were grown for six days at 32 C. The results are shown in Table 2.

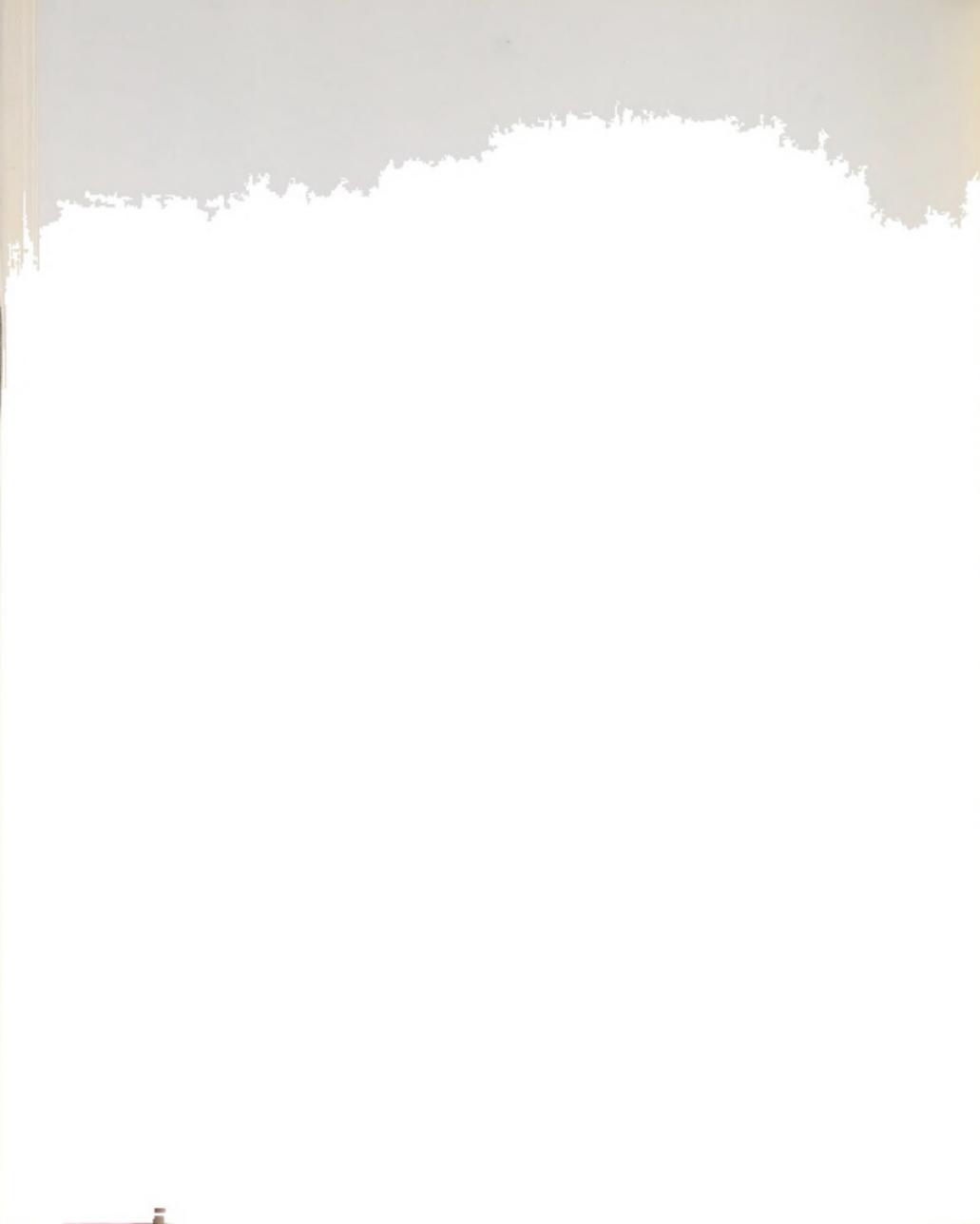
Although there was a slight problem of overflow of the culture phase from the flasks due to gas production during growth, the increase in total toxin yields prompted the adoption of a culture system containing equal volumes of saline and double strength medium.

Concentration Steps. Concentration of toxin was necessary prior to purification by column chromatography. Ultrafiltration equipment now available allows concentration and dialysis of proteins much more rapidly than by per-evaporation or concentration by polyethylene glycol (PEG). Additionally, ultrafiltration membranes are available in various pore sizes and can be chosen to optimize the rapid movement of solvent with retention of molecules of a specific molecular size. An Amicon model 52 ultrafiltration cell was used



Table 2. Assays of the toxin produced by two strains of C. botulinum type E in a dialysis sac culture at two different concentrations of Tpsy medium at pH 6.3.

<u>Strain</u>	<u>Saline:Medium</u>	Fate of mice injected with the sample dilution indicated	
		<u>death</u>	<u>survival</u>
Kalamazoo	2:1	1:2,000	1:10,000
Kalamazoo	1:1	1:20,000	1:40,000
Hazen	2:1	1:1,000	1:2,000
Hazen	1:1	1:20,000	1:40,000



in this investigation. The ultrafiltration membrane used to concentrate toxins (XM-50) was reported by Amicon to retain greater than 95% of aldolase (142,000 mol. wt.), 90% of human serum albumin (67,000 mol. wt.) and 80% of ovalbumin (45,000 mol. wt.). This membrane was used to concentrate 63 ml of crude toxin to 5.4 ml. There was no apparent loss of toxin during this 12-fold concentration step.

Toxins which have been chromatographed on DEAE cellulose have been reported to have molecular weights ranging from 5,000 to 18,000 (Emodi and Lechowich, 1969; Gerwing et al., 1967). Heimsch and Sugiyama (1972) reported that toxin chromatographed on DEAE cellulose on CM cellulose had a molecular weight of 150,000 as compared to 250,000 for the same sample before chromatography. The UM-2 ultrafiltration membrane (Amicon) was used to concentrate eluted and adsorbed fractions from the DEAE cellulose and the C-50 Sephadex columns. Since this ultrafiltration membrane was designed to retain 95% of all molecules having a molecular weight greater than 2,000, all toxin sizes previously reported would not be lost during concentration.

Purification Methods. Each of four strains, Kalamazoo, Iwanai, Hazen and VH, were grown using the dialysis sac culture method. After centrifuging the culture phase, the toxin was precipitated from the supernatant fluid by the addition of dry ammonium sulfate to 60% of saturation, the precipitate was removed by centrifugation at 27,000 x g and the precipitate was dissolved in 0.05 M phosphate buffer at pH 6.0. After dialysis against the same buffer, 140 ml of crude toxin was obtained from the original 3-4 liters of culture.



When 2-4 ml of crude toxin was layered on a 2 x 80 cm G-200 Sephadex column one major toxic peak was eluted (Figure 4). The elution volume for the toxic fraction was found within a narrow volume for all cellular strains used in this investigation. Calibration of the column with lysozyme, hemoglobin and blue dextran permitted the plotting of a standard curve of elution volume vs molecular weight. After chromatography of the crude toxin, the toxic fractions were detected at elution volumes which corresponded to molecular weights ranging from 148,000 to 168,000 on the standard curve (Figure 5).

When larger amounts of crude toxin were applied to the column, toxin was detected in fractions representing a wide range of elution volumes, without a sharp peak of toxicity. Since measurement of the absorbance at 280 nm yielded four distinct absorbance peaks, the toxin was not being eluted as a distinct protein species. This may have resulted from inherent binding of toxin to non-toxic protein or to the column, or possibly from applying too much protein to the column. Due to the problems encountered, this procedure was not used as a primary purification step.

Primary purification was accomplished by elution through DEAE cellulose followed by adsorption on C-50 Sephadex (Emodi, 1969). A sample of approximately 60 ml of crude toxin was concentrated to 4 ml and applied to a 1 x 28 cm DEAE cellulose column using 0.05 M sodium phosphate buffer at pH 6.0. Figure 6 represents the absorption profile obtained when the DEAE cellulose column elutant was monitored at 280 nm. A large peak, which appeared early, contained



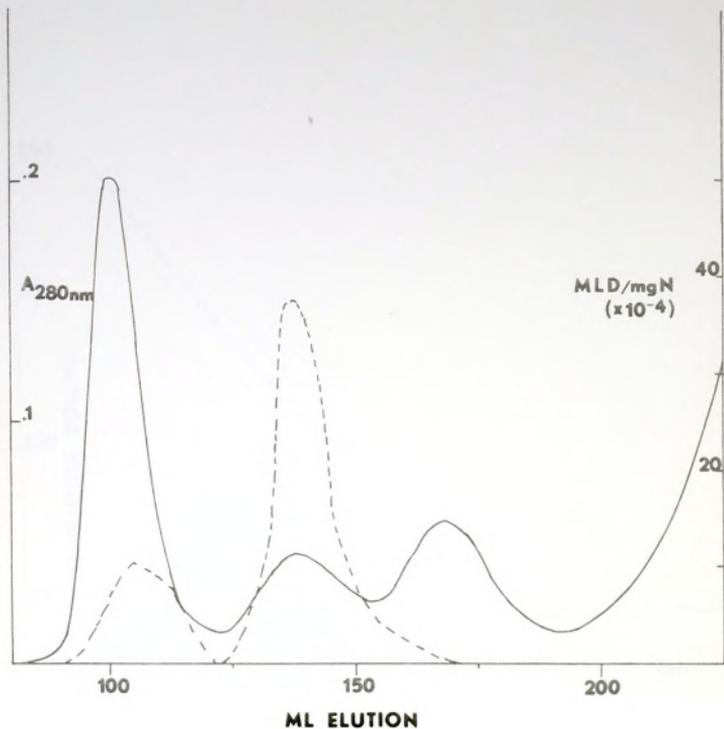


Figure 4. Absorbance at 280 nm (solid line) and toxicity in MLD/mg N (broken line) of elutant from a 2 x 80 cm G-200 Sephadex column after application of one ml of crude toxin from the Iwanai strain to type E C. botulinum.



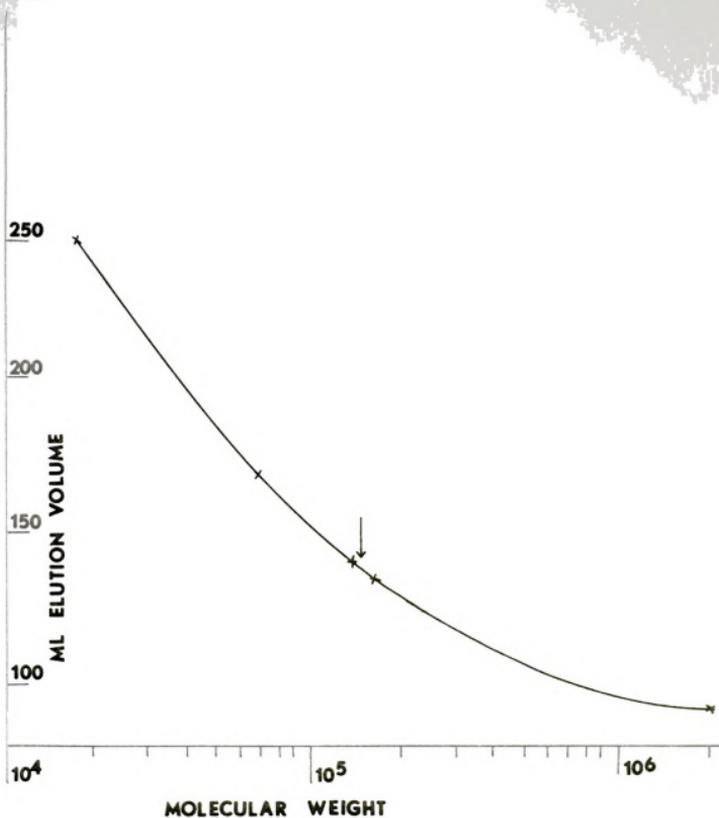


Figure 5. The standard curve used for assigning molecular weights of proteins as determined by applying the proteins to a 2 x 80 cm column of G-200 Sephadex and measuring the volume required to elute the proteins. Extracellular toxin eluted as indicated by the arrow.

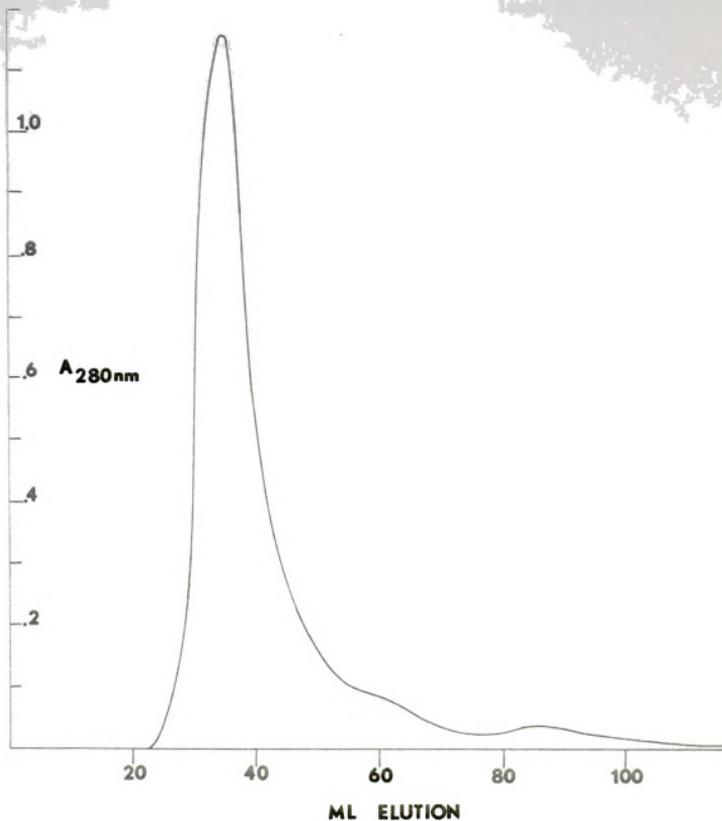


Figure 6. Absorbance at 280 nm of elutant from a 1 x 28 cm column of DEAE cellulose after application of four ml of concentrated type E *C. botulinum* toxin followed by elution with 0.05 M sodium phosphate buffer at pH 6.0 (the toxin eluted with the major absorption peak).

most of the protein and toxin. This peak was followed by a small shoulder or trailing peak which contained little toxin.

The toxic fractions were pooled, concentrated using ultrafiltration with an Amicon UM-2 membrane and applied to a C-50 Sephadex column. A large frontal peak eluted first, followed by low absorption (Figure 7). The large peak usually included a small amount of toxin, but most of the toxin adsorbed to the column. The adsorbed toxin could be eluted from the column by the addition of 0.5 M NaCl to the phosphate buffer. Use of a linear salt gradient from 0.0-0.5 M NaCl produced a spreading of the absorbant peak containing the toxin resulting in a leading shoulder on the main absorbant peak (Figure 8).

Three separate fractions from a spreading absorbant peak from a C-50 Sephadex column, such as that shown in Figure 8, were assayed for toxicity, analyzed for protein content and applied to a pH 4.3 polyacrylamide gel electrophoresis system. These fractions were found to have different toxicities and different protein contents. Figure 9 illustrates the fractions of the absorbant peak and the electrophoretic patterns obtained from the three fractions which were assayed. Fraction 47 contained 3 distinct bands. The band with the lowest mobility was labelled B_1 , the next lowest, B_2 , and the band with the highest mobility, B_3 . Fraction 45 contained 2 significant bands, equivalent to B_1 and B_3 , and Fraction 50 revealed only one band, B_3 .

The protein concentrations and specific toxicities of these fractions are shown in Table 3.

Since fraction 45 had a higher specific toxicity than either fraction 47 or 50, band B_3 which was the most intense band in both

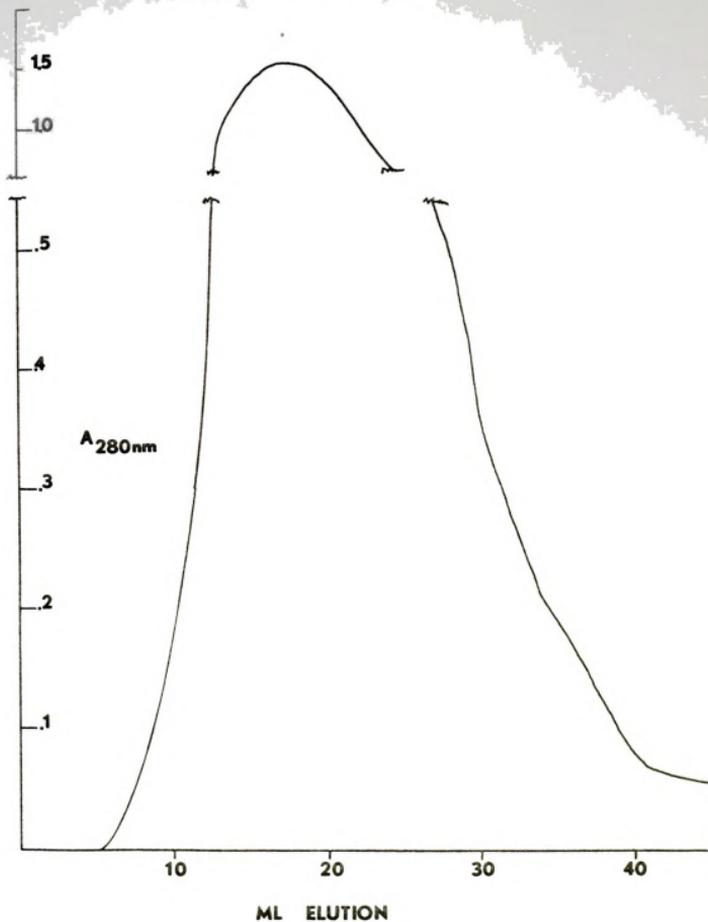


Figure 7. A sample of extracellular toxin, after elution through a column of DEAE cellulose, was applied to a 1 x 28 cm C-50 Sephadex column and eluted with 0.05 M sodium phosphate and monitored at 280 nm (a low level of toxin was present in the absorbance peak).

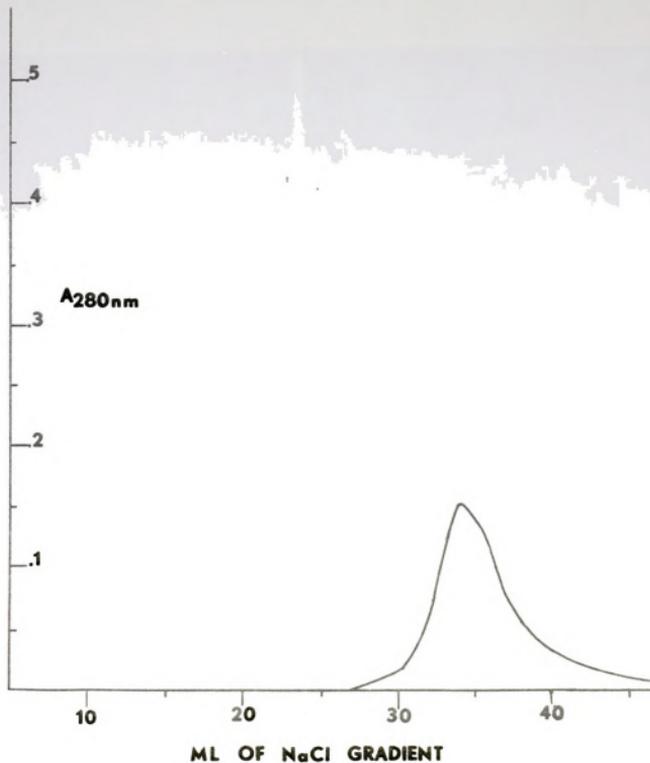


Figure 8. Adsorbed toxin, eluted through a column of DEAE cellulose and adsorbed onto a 1 x 28 cm column of C-50 Sephadex was eluted with a 0-0.5 M NaCl gradient in 0.05 M sodium phosphate buffer at pH 6.0. (The toxin eluted before the 280 nm absorbant peak.)

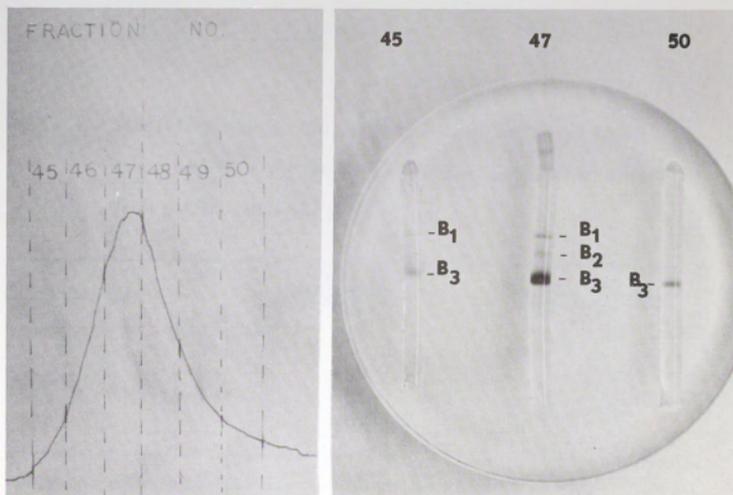


Figure 9. The fractions collected during the elution of the extracellular toxin adsorbed to a C-50 Sephadex column with a 0-0.5 M NaCl linear gradient in 0.05 M sodium phosphate buffer at pH 6.0, and the protein bands from three of the fractions after electrophoresis in 7% polyacrylamide gel at pH 4.3 and stained with Buffalo Black stain.

Table 3. Specific toxicity and protein concentration of three fractions from the adsorbed extracellular toxin eluted from a C-50 Sephadex column.

<u>Fraction No.</u>	<u>ug protein/ml</u>	<u>MLD/mg protein*</u>
45	67	3.0×10^6
47	618	1.6×10^5
50	223	$<4.5 \times 10^5$

*Only one mouse was used for each dilution instead of two.



fractions 47 and 50 did not represent the toxin. Band B₁ was presumably the toxin band because its presence and intensity were directly related to toxicity.

All of the fractions in the spreading absorbant peak from the C-50 Sephadex column were pooled, desalted and concentrated (ultrafiltration with UM-2 membrane). The resulting sample was applied to a pH 4.3 polyacrylamide gel electrophoresis system. The resulting electrophoretic pattern contained bands B₁ and B₃. Band B₂ was found in addition to B₁ and B₃ only for the sample of Iwanai toxin and that sample contained more total protein than the other samples.

Intracellular Toxin. The cellular extract of the toxin was partially purified by ammonium sulfate precipitation and dialyzed according to the method described by Kitamura et al. (1968) except that TPSY medium at pH 6.3 and the Kalamazoo strain were used. The extract was concentrated by ultrafiltration (XM-50 membrane) and layered onto a C-50 Sephadex column (1 x 28 cm) using 0.02 M acetate buffer at pH 6.0. The absorbance of the elutant from the column was monitored at 280 nm (Figure 10). Assay of the elutant demonstrated toxicity only in fractions from absorbance maximum and those fractions had low toxicity. This contracts with the results of Kitamura et al. (1968) who reported that toxin was eluted through a C-50 Sephadex column under similar conditions. Since this toxic sample was prepared according to their methods, other procedures in preparation of the column or toxin not reported by Kitamura et al. (1968) may have affected the results they obtained. The only difference in production of toxin between their experiment and this study (other than those

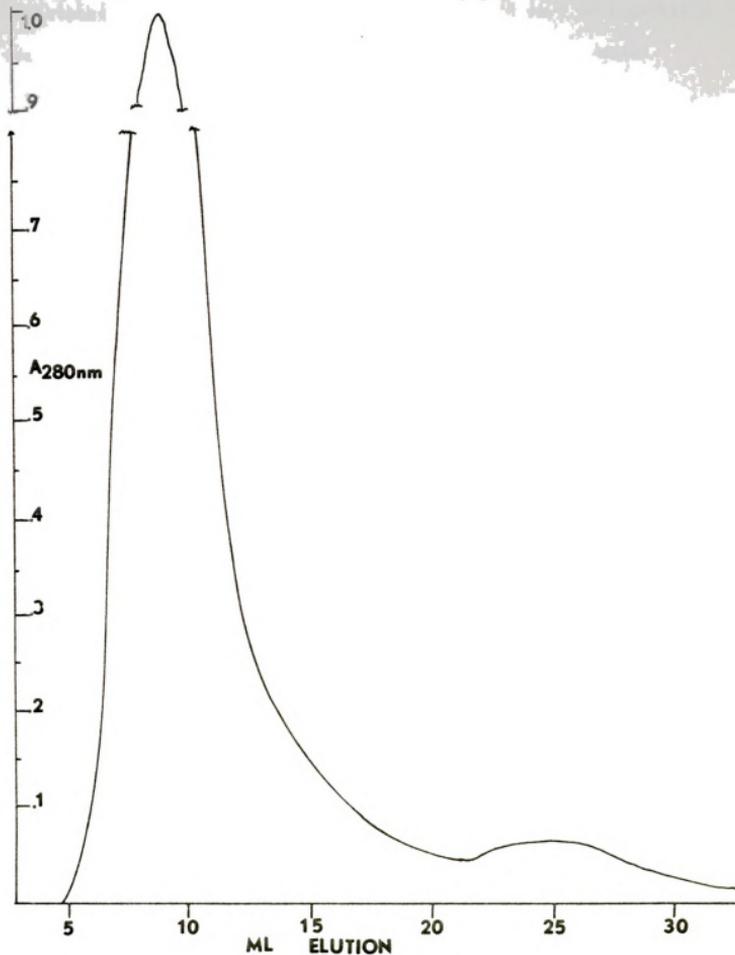


Figure 10. Absorbance at 280 nm of the elutant from a 1 x 28 cm column of C-50 Sephadex after application of a concentrated cellular extract followed by elution with 0.02 M sodium acetate buffer at pH 6.0. The absorption peak contained low levels of toxin.



mentioned above) was that they used cells after 4 days of incubation as compared to 3 days of incubation in this study. This would not be expected to account for the observed differences in toxin characteristics.

A linear salt gradient of 0.0-0.5 M NaCl contained in 80 ml of buffer was applied to the column and the absorption of the eluant was measured at 280 nm (Figure 11). Four areas of absorption are noted: a low, early peak, a leading shoulder, a main peak and a late peak. Toxin assays of fractions from these 4 areas (identified as F_1 , F_2 , F_3 and F_4 on Figure 11) revealed that only F_2 and F_3 contained significant amounts of toxin. F_2 contained 400,000 MLD/ml, which amounts to a specific toxicity of 3.9×10^6 MLD/mg of protein or 2.4×10^7 MLD/mg N. F_3 had a specific toxicity of 1.7×10^6 MLD/mg of protein or 1.0×10^7 MLD/mg N.

The fractions representing the peak areas around F_2 and F_3 were separately pooled, desalted and concentrated. Upon electrophoresis, the sample representing peak F_2 contained band B_1 and a weak band B_3 with three faster moving bands which were less intense and less distinct. The sample representing the peak F_3 contained band B_1 and B_3 with band B_3 more intense than in the sample representing peak F_2 . Bands B_1 and B_3 were present in all of the samples which were analyzed by pH 4.3 polyacrylamide gel electrophoresis. However, the highest specific toxicities were obtained when band B_3 was the weakest in intensity. This again supports the theory that B_1 represents a single toxin band with B_3 the major protein contaminant. Attempts to recover toxin from



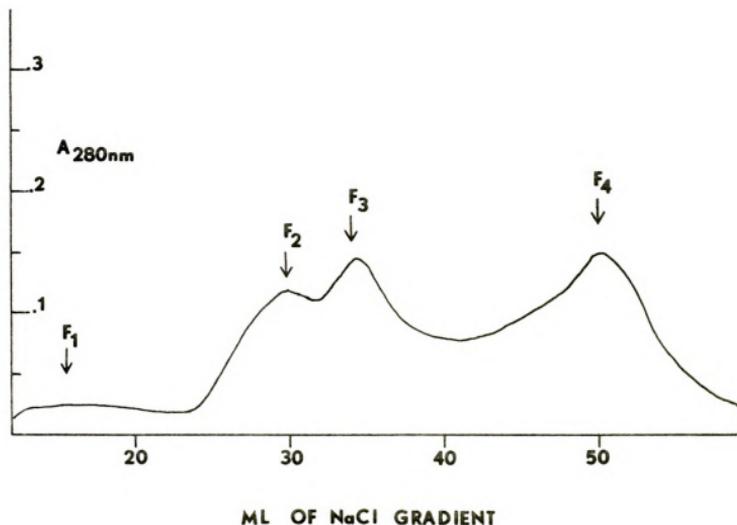


Figure 11. Absorbance at 280 nm of elutant from a 1 x 28 cm column of C-50 Sephadex after application of a sample of concentrated cellular extract, elution with 0.02 M sodium acetate buffer at pH 6.0 followed by elution with a 0-0.5 M NaCl linear gradient in 80 ml of the same buffer (fractions from peaks F₂ and F₃ contained the highest specific toxicities).



the gels after electrophoresis, including the extraction method described by Emodi (1969), have been unsuccessful even when 100,000 MLD's of toxin were applied to the gels.

A second portion of the cellular extract was concentrated and dialyzed against 0.05 M phosphate buffer at pH 6.0. Utilizing the procedure for purification of extracellular toxin, the concentrate was applied to a DEAE cellulose column and the absorbance of the eluant was measured at 280 nm (Figure 12). The main difference in the absorbance at 280 nm of the DEAE cellulose elutants of samples of extracellular and intracellular toxins was the larger quantity of protein present in the samples of extracellular toxin. Only the first major peak contained a significant amount of toxin. The fractions from the major peak were pooled, concentrated and applied to a C-50 Sephadex column (1 x 28 cm) and eluted with 0.05 M phosphate buffer at pH 6.0. Adsorbed protein was released with the use of a 0-0.5 M NaCl linear gradient contained in 80 ml of 0.05 M phosphate buffer at pH 6.0 (Figure 13). Peak F_1 of Figure 13 represents the fraction containing the highest toxicity measured. This result is similar to that obtained for intracellular toxin which was adsorbed onto C-50 Sephadex without previous DEAE cellulose chromatography. The total toxin recovered in this experiment utilizing 0.05 M phosphate buffer at pH 6.0 was lower than that recovered in the previous experiment utilizing acetate buffer. However, the apparent loss of toxin is probably due to the numerous additional steps conducted in this experiment.



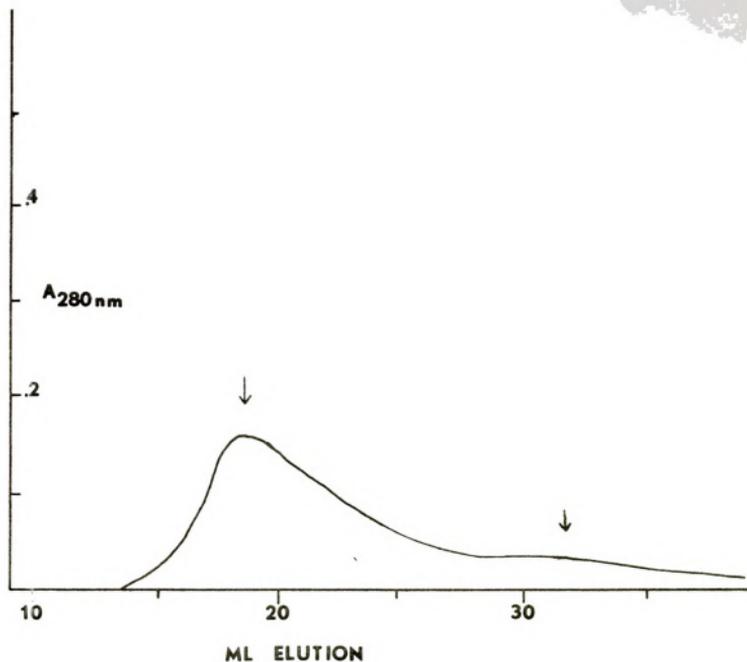


Figure 12. Absorbance at 280 nm of elutant from a 1 x 28 cm DEAE cellulose column after application of four ml of concentrated cellular extract followed by elution with 0.05 M sodium phosphate buffer at pH 6.0. (The toxin eluted with the absorption peak.)



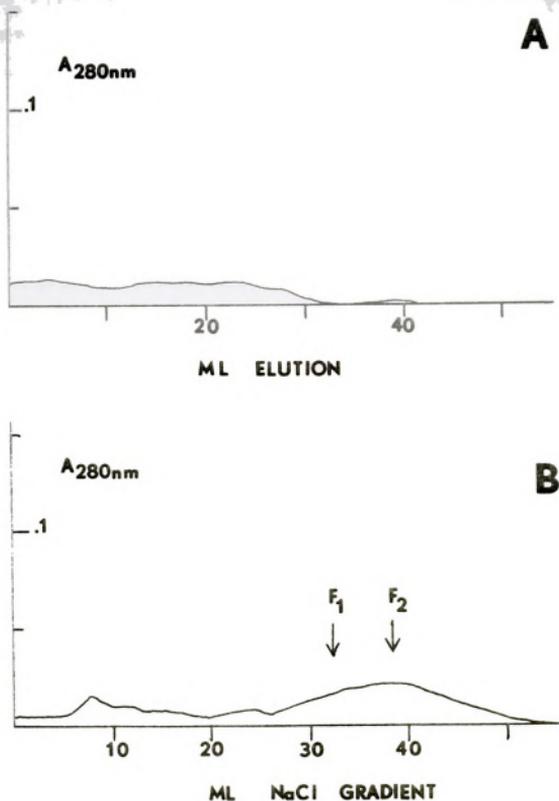


Figure 13. A-Absorbance at 280 nm of cellular extract from the Kalamazoo strain of type E *C. botulinum* (previously eluted through a column of DEAE cellulose) after application onto a 1 x 28 cm C-50 Sephadex column and eluted with 0.05 M sodium phosphate buffer at pH 6.0. B-Absorbance at 280 nm during the removal of the adsorbed toxin from the C-50 Sephadex column (described in part A) after application of a 0-0.5 M NaCl linear gradient in 0.05 M sodium phosphate buffer at pH 6.0. (Fractions collected at F_1 and F_2 contained toxin with F_1 having the highest specific toxicity.)



Molecular Weight Determination. The electrophoretic system described by Weber and Osborn (1969) which included β -mercaptoethanol and sodium dodecyl sulfate (SDS) was used to determine the molecular weight of the toxin purified in this study. The only modification of their method was to utilize the buffer in the buffer tanks at one-half of the strength they utilized. This system masks the charges of the proteins with SDS. Thus, the relative electrophoretic mobility of proteins in SDS is not due to the natural charge of the protein, but is determined by characteristics such as size and shape. Therefore, this system can be used for molecular weight determinations based on the relative mobility of protein molecules. A standard curve is developed by applying proteins of known molecular weight and plotting mobility on a linear scale vs molecular weight on a log scale. Proteins of known and unknown molecular weight are applied to the same and neighboring electrophoresis gels and the molecular weight of the "unknowns" can be determined from the standard curve.

When a sample containing toxin was applied to this SDS electrophoretic system, the protein bands moved very little in 4 hours. The single band B_1 , considered to be the toxin in the acid electrophoresis system, was replaced by two slightly separated bands in the SDS electrophoresis system. These two bands were separated further by reducing the cross-linking factor (bis) in the polyacrylamide gel by 1/2 (Figure 14).

Table 4 shows the results of the molecular weights of the bands of various toxic preparations purified in this investigation. Figure 15 shows the standard curve used in determining the molecular weights of the bands. The main limitation in assigning molecular

The first part of the document is a letter from the Secretary of the State to the Governor, dated the 10th of the month. It contains a report on the state of the treasury and the public debt. The Secretary states that the treasury is in a state of comparative health, and that the public debt is being gradually reduced. He also mentions the progress of the public works and the state of the agriculture and commerce.

The second part of the document is a report from the Board of Directors of the Bank of the State, dated the 15th of the month. It contains a statement of the assets and liabilities of the bank, and a report on the management of the bank during the year. The Board states that the bank has been successful in its operations, and that its assets are in a state of security.

The third part of the document is a report from the Board of Directors of the Bank of the City, dated the 20th of the month. It contains a statement of the assets and liabilities of the bank, and a report on the management of the bank during the year. The Board states that the bank has been successful in its operations, and that its assets are in a state of security.

The fourth part of the document is a report from the Board of Directors of the Bank of the County, dated the 25th of the month. It contains a statement of the assets and liabilities of the bank, and a report on the management of the bank during the year. The Board states that the bank has been successful in its operations, and that its assets are in a state of security.

Table 3. Molecular weights of protein bands observed when sodium dodecyl sulfate polyacrylamide gels were run at pH 4.3 with 5000 volt.

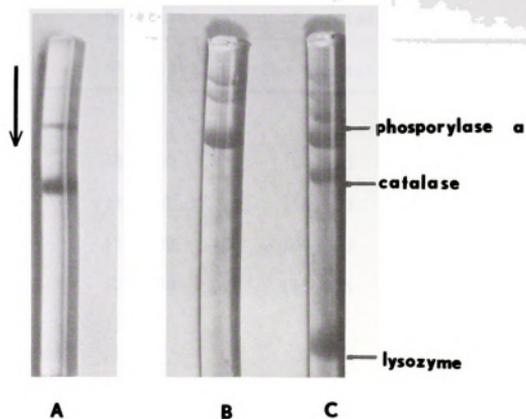


Figure 14. The gels resulting when a toxin sample was applied to a pH 4.3 polyacrylamide gel electrophoresis system (A), a toxin sample was applied to a SDS polyacrylamide gel electrophoresis system (B) and when a toxin sample and proteins of known molecular weight were applied to a SDS polyacrylamide gel electrophoresis system (C).



Table 4. Molecular weight of protein bands obtained after electrophoresis of various toxic samples in SDS polyacrylamide gel (10%, with 0.14% bis).

Sample	Molecular weight of protein bands		
	Slowest	2nd slowest	3rd slowest
<u>Extracellular</u>			
Kalamazoo	142,000	120,000	83,000
Iwanai	134,000	118,000	78,000
VH	127,000	109,000	80,000
Hazen	125,000	111,000	81,000
<u>Intracellular</u>			
C-50 Sephadex (Acetate)			
early fractions	137,000	120,000	--
main peak	135,000	119,000	85,000
DEAE Cellulose, C-50 Sephadex (Phosphate buffer)			
early fractions	137,000	122,000	--



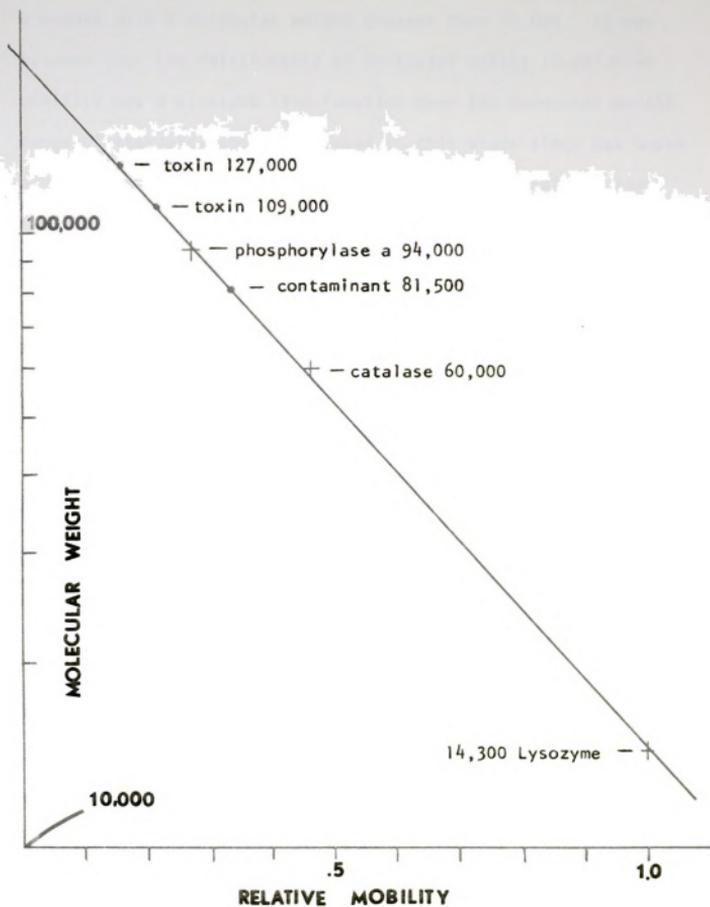


Figure 15. The standard curve of the molecular weights of proteins vs their relative mobility in a SDS gel electrophoresis system. The molecular weights of proteins was determined by measuring their mobility and comparing their relative mobility to the relative mobility of lysozyme, catalase and phosphorylase a, using the mobility of lysozyme as 1.0.



weights to the bands representing toxin was the lack of a protein standard with a molecular weight greater than 94,000. It was assumed that the relationship of molecular weight to relative mobility was a straight line function over the molecular weight range of standards and toxins used in this study since Das Gupta and Sugiyama (1972) reported that they found a straight line function for this system using proteins up to a molecular weight of 200,000.

Specific activity is one important measure in determining the purity of toxins. The toxins prepared in this study (Table 5) had only about one-tenth of the specific activity that was reported for some preparation of C. botulinum type E toxin in the literature (Kitamura et al., 1968). In view of the presence of contaminating proteins, demonstrated in electrophoresis, this result would be expected. Samples containing less band B₃ (proportionately more band B₁) had higher specific activities. Band B₁ is indicated as the band representing the toxin.

Two research groups have reported a molecular weight for type E C. botulinum toxin below 20,000. Emodi and Lechowich (1969), reported two components of 5,000 and 7,000 molecular weight. Doiman and Gerwing (1967) reported a toxin of 14,000-18,000 molecular weight. Both reports described purification of extracellular toxin by chromatography with DEAE cellulose. Kitamura (1970) also used DEAE cellulose for purification of extracellular toxin, but found a molecular weight of 150,000 as determined by sucrose density gradient centrifugation. The toxin of 150,000 molecular weight agrees with that of Kitamura et al.



Table 5. Specific toxicity of various toxic samples.

Sample	toxicity/ml	ug protein/ml	LD ₅₀ /mgN
Extracellular			
Kalamazoo	1.87×10^5	394	2.9×10^6
Iwanai	9.39×10^5	1600	3.6×10^6
VH	5.60×10^3	20	1.7×10^6
Hazen	1.35×10^4	95	8.7×10^5
Intracellular			
C-50 (acetate buffer)			
early fractions	1.13×10^5	222	3.1×10^6
main peak	6.30×10^4	280	1.4×10^6
DEAE cellulose, C-50 Sephadex (Phosphate buffer)			
early fractions	2.24×10^4	30	4.6×10^6



(1968) who studied intracellular toxin. One difference in purification was that Selectacel grade (Brown Co.) of DEAE cellulose was used by Emodi and Lechowich (1969) and by Dolman and Gerwing (1967) whereas Kitamura (1971) used Cellex D (Bio Rad Laboratories) DEAE cellulose. Thus, the brand of DEAE cellulose used in the investigations could be responsible for the differences reported for the molecular weight of type E toxin.

Kitamura et al. (1967) reported that the toxic protein of 250,000 molecular weight could be dissociated into two components, each component with a molecular weight of about 150,000. Heimsch and Sugiyama (1972) applied toxin (250,000 molecular weight) to columns of DEAE cellulose and of CM cellulose. The toxin which was obtained after chromatography had a molecular weight of 150,000.

Das Gupta and Sugiyama (1972) reported that all types of C. botulinum produced a toxic component which had a molecular weight of approximately 150,000. If this toxic component from proteolytic strains of C. botulinum was treated with mercaptoethanol, a disulfide bond reducing agent, it would separate into two components, one with a molecular weight of approximately 50,000 and the other with a molecular weight of about 100,000. Toxin from type E strains demonstrated this characteristic only after activation by proteolytic enzymes.

The two protein bands which were separated only by SDS polyacrylamide gel electrophoresis in this investigation would thus be expected to represent the toxic and non-toxic proteins reported by Kitamura et al. (1967). They reported that both of these proteins have molecular weights of 150,000. Since the error in molecular



weight determinations using SDS polyacrylamide gel electrophoresis is about 10%, the results in this investigation for the larger component (molecular weight of 125,000-142,000) are essentially in agreement with the molecular weight of 150,000 (Kitamura et al, 1967; Heimsch and Sugiyama, 1972; and Das Gupta and Sugiyama, 1972).

The non-toxic component, which has not been studied by SDS electrophoresis could be represented by the smaller component (111,000-120,000 mol. wt.) found in this investigation. The smaller component migrated with the larger component in acid gel electrophoresis, in DEAE cellulose chromatography and CM Sephadex chromatography. The SDS gel electrophoresis (with disulfide bond-reducing conditions) could cause changes in the molecule of the smaller component which would result in an apparent molecular weight of 111,000-120,000.

Results obtained with both G-200 Sephadex gel filtration and SDS electrophoresis indicate the toxin from four cellular strains of type E C. botulinum used in this investigation, both intracellular and extracellular, had a molecular weight of about 150,000. Ultrafiltration with an Amicon XM-50 membrane without loss of toxin through the membrane also supports the fact that the toxin has a molecular weight greater than 50,000. The low molecular weight components previously observed by some investigators were not detected during this investigation.

SUMMARY AND CONCLUSIONS

Clostridium botulinum type E extracellular toxin was produced from each of four cellular strains: Kalamazoo, VH, Hazen and Iwanai. The toxin was produced by growing the proper strain by the dialysis sac culture method at 32 C for 7 days.

Intracellular type E C. botulinum toxin was produced by growth of the Kalamazoo strain for 3 days at 30 C. The cells were removed by centrifugation, washed twice, and extracted twice with 0.2 M phosphate buffer at pH 6.0. The cellular extracts were used for purification of intracellular toxin.

Both the extracellular and intracellular toxins were eluted through columns of diethylaminoethyl (DEAE) cellulose, adsorbed to columns of CM Sephadex, and eluted from the columns of C-50 Sephadex with a 0-0.5 M NaCl linear gradient. They each contained 2 main protein components as demonstrated by electrophoresis in polyacrylamide gels at pH 4.3. The molecular weight of the proteins was determined using a sodium dodecyl sulphate polyacrylamide gel electrophoresis system. Under these conditions, the band which contained the toxin dissociated into two bands. The molecular weight of the lighter component was 111,000-120,000. The heavier component, believed to be the toxic component, had a molecular weight of



125,000-142,000. In contrast to some previous work, toxic components with molecular weights of less than 20,000 were not detected in this investigation.



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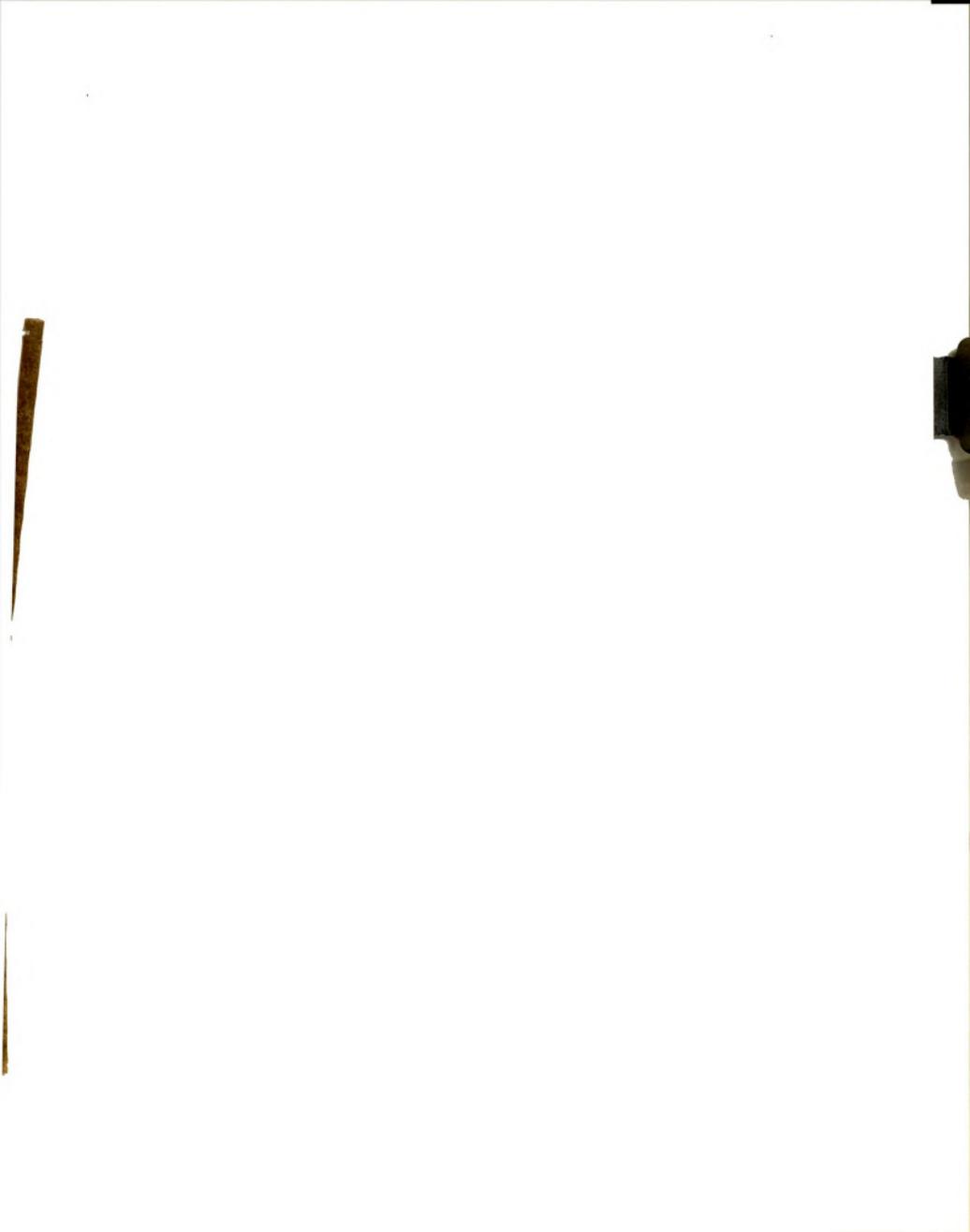
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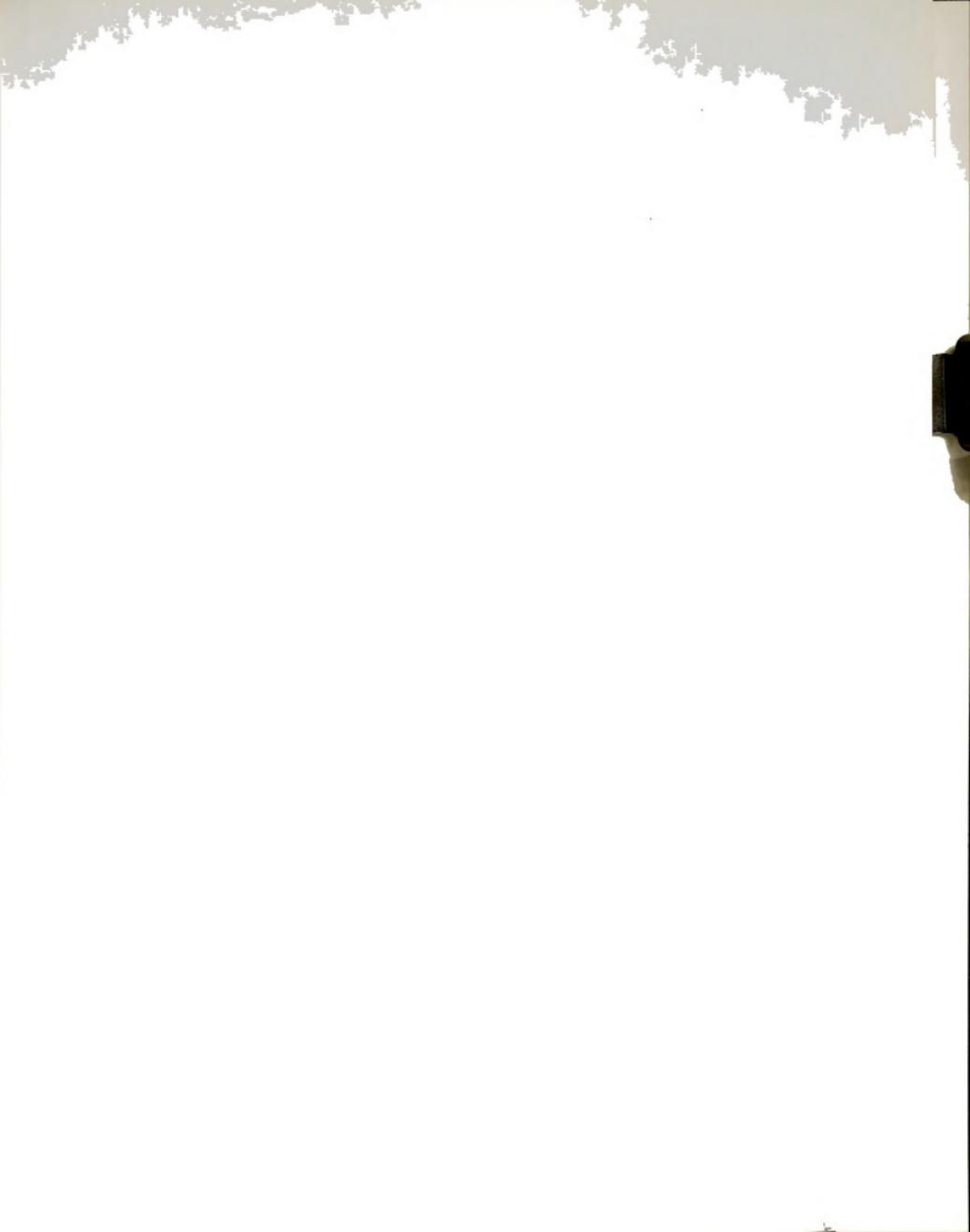
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