PURIFICATION AND CHARACTERIZATION OF A TOXIC PROTEIN OF CLOSTRIDIUM BOTULINUM

TYPE E, STRAIN IWANAI

by

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B.Sc., Panjab University, 1959

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

M.Sc.

in the Department of

BACTERIOLOGY AND IMMUNOLOGY

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Abstract

Clostridium botulinum type E, strain Iwanai, was grown in a dialysate apparatus, modified from the original apparatus of Vinet and Fredette (1951), using GPB1 medium with 0.5 percent dextrose and 1.0% sodium thioglycollate, for five days at 30° C. The toxin was precipitated from the cell-free toxic filtrate with 0.60 saturated ammonium sulfate at 4° C overnight. The toxic precipitate was obtained by centrifugation at 4,500 R.P.M. at 0° C for 45 minutes and dissolved in 0.01 M sodium acetate buffer at pH 5.5. The preparation was dialysed for 24 hours against the same buffer in versene-treated dialysis sacs and the insoluble material was removed by centrifugation. This preparation was then applied to ion-exchange columns. DEAE (Selectacel) cellulose, suspended and kept in 2 M NaCl at 4° C, was packed into columns under gravity flow at room temperature, washed with 1N HCl and equilibrated with 0.01 M sodium acetate buffer at pH 4.5. The toxin, eluted by the same buffer, reprecipitated and rechromatographed, was further analysed in the ultracentrifuge and with electrophoresis. Toxicity of the pure preparation was found to be 7.5 x 10^6 MLD/mg N, the sedimentation coefficient, $S_{20}^W = 1.70$, and the molecular weight 18,600. Partial amino acid analysis on this preparation was also carried out.
ACKNOWLEDGMENTS

I take this opportunity to thank Dr. Julia Gerwing, under whose direct supervision this work was carried out. I have profited a great deal from her able guidance and knowledge. I am indebted to her also for allowing me to work on the problem of the purification of a toxic protein produced by Clostridium botulinum type E which has helped me to understand the problems of protein purification in general and ion exchange chromatography in particular.

I thank Dr. C.E. Dolman for placing his confidence in my research capabilities and making it possible for me to pursue this course of research.

I am grateful to Dr. M. E. Reichmann for his assistance and advice given regarding the biophysical studies carried out during the past two years.

I appreciate the continuous help given to me by Mr. Angus Macaulay.
Work has been done previously on various botulinus toxic proteins in order to purify and further characterize these materials. High molecular weights for type A, B, and D have been reported before but no attempts had been successful in obtaining botulinus type E in pure form. (Sakaguchi and Sakaguchi, 1959; Sakaguchi et al, 1963; Gerwing et al 1962; Gordon et al 1957). This toxin is highly lethal for man and its toxicity is potentiated by various proteolytic enzymes including trypsin (Dolman 1953, 1957; Sakaguchi and Tohyama, 1955; Duff et al 1956). In order to study these activities, a thorough investigation of the molecular size and character of this material, is necessary. This report has covered various analytical techniques in protein purification and characterization. A toxic preparation has been obtained which appears to be homogeneous by both ultracentrifugal and electrophoretic analysis.
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CHAPTER I

Analytical Methods in Protein Chemistry

The synthetic production of urea from an inorganic source (ammonium cyanate) by Wöhler in 1828, was the "beginning of the end" of an era infested with superstition and misinterpretation of scientific logic. A little more than a century later another important stage in the development of molecular biology was marked by the isolation and crystallization of the Tobacco Mosaic Virus by Stanley. During this period a number of organic compounds were isolated and their biological activity studied. Two decades were yet to pass before techniques were developed sufficiently to allow the structural studies on macromolecules of biological origin. In 1945 Sanger reported the complete amino acid sequence of the insulin molecule. Watson and Crick followed him by proposing the molecular structure of genetic material (DNA and RNA). The major advances made in the study of molecular biology during the past thirty years are a direct reflection on the techniques made available to the researcher during this time. The following pages deal briefly with the principles involved in the isolation and characterization of proteins.
(a) **Structure of Proteins**

Proteins are giant molecules with great diversity and complexity in structure; however, the basic structure of all proteins is the same in that they consist of long chains of amino acids. There are twenty L-amino acids, of common occurrence. The amino acids, with their dipolar ion groups form the \(-\text{NH-CH-CO-}\) group with the elimination of water during polymerization. The twenty amino acids have a different R-group or side chain which is specific for each amino acid. A peptide linkage of all or some of these combinations imparts to polypeptides their specific activity. There may be one or more polypeptide chains which may in turn consist of several thousand amino acid residues, that comprise a protein. The amino acids of the polypeptide are arranged in a genetically determined definite sequence which comprises the primary structure of proteins.

Long chain polymers, with their apparent tendency for a screw symmetry, tend to be dependent on their side chains for establishing stability. No configuration is stable unless it allows every imino-group to be hydrogen-bonded to a carbonyl belonging to the same chain or to a neighboring one. There are various ways in which hydrogen bonding occurs in polypeptide chains. The simplest is a planar zig-zag chain which occurs in synthetic polyamide (nylon). The other types of structures with hydrogen bonds occur within the same
chain and are designated as either alpha- or beta-helical. The X-ray diffraction photographs of crystalline proteins indicate that their structures are highly organized and specific, so that nearly every one of the thousands of atoms which constitute a protein molecule occupies a definite position. It must be concluded that polypeptide chains, no matter what their secondary structure may be, must somehow be folded to produce the compact and very nearly round molecules which comprise their tertiary structure.

(b) Isolation

The term "isolation" applied to proteins implies the obtaining of preparations with high specific activity per quantitative unit. Schwimmer and Pardee (1953) mentioned the object of protein "isolation" is to remove as completely as possible all proteins except that particular component in which resides the desired activity. Neilands and Stumpf (1958) suggest that "isolation" should be reserved for those rare instances where an investigator has tried and failed to demonstrate the chemical contamination of his preparation. The term "isolation" used here implies the preparation of soluble concentrated proteins, which does not necessarily indicate that it contains a single component.

Isolation methods significantly influence the final results as the integrity of the biological product can be maintained only with a consistent yield of that product under optimal conditions.
As there is detailed information available regarding isolation methods in the literature, this problem will be dealt with briefly in this report. The choice of source in the protein isolation is very important. Sources differ widely in the amounts of protein they contain; and also one source may contain undesirable material which is absent in the other. Also the source should be available in such amounts that a large quantity of the protein is obtained. In the case of bacteria, the cells are grown under optimal conditions for the production of the desired protein.

The protein from the source is either extracted by a solvent or precipitated by "common ion effect". A number of isolation methods are used: 1) Sedimentation, 2) Extraction, 3) Salt Fractionation, and 4) Solvent Fractionation.

(c) **Protein Solubility and Precipitation**

Proteins are soluble in pH's away from their iso-electric points but denaturation tends to occur. The presence of neutral salts at high concentration, absence of water, pH close to iso-electric point, processing and storage at low temperatures, the presence of lipids (in the case of lipoproteins), carbohydrates (especially in the case of mucoproteins), metal ions (metallo-proteins and metal-activated enzymes), enzyme substrates for preserving enzymes, toluene, etc., decrease the denaturation of proteins. Proteins can be obtained in the soluble form with the least denaturation at a pH away from the iso-electric point in the presence of
adequate amounts of neutral salts. The same protein can be precipitated near the iso-electric point. Temperature, pH and the electrolyte in which the protein is suspended will determine the solubility of that protein. Denaturation, precipitation and solubility of proteins are dealt with in detail by Alexander & Block (1960) and Fox and Foster (1957).

(d) Protein Purification

Separating a single protein species which satisfies the prevalent criteria of purity is called protein purification. There are a number of chemical methods used in protein purification which will not be elaborated on here, e.g., fractionation by organic solvents like ethanol, ether, acetone, methanol, dioxane and others; and the use of heavy metal ions like Zn, Pb, Hg, Fe, Cu, etc. Processes important in research include fractionations by acid and salts. Care must be taken in acid precipitations to provide enough neutral salts before precipitation to cut down denaturation. Precipitation by neutral salts is the safest way of fractionation.

1. Repeated Precipitation. The most commonly used salt for precipitation is \((\text{NH}_4)_2\text{SO}_4\). It is soluble in aqueous solution and consequently is very effective as a precipitating agent. Sodium sulphate has also been used chiefly because it does not introduce nitrogen into the protein samples. Repeated precipitation with \((\text{NH}_4)_2\text{SO}_4\) at various levels of concentration may yield a certain level of purity. Alexander and Block (1960)
describe a modification of the older methods of salting out proteins which is differential salting in. The proteins are precipitated as a mixture and the precipitate is extracted by salt solutions of gradually decreasing strength. They also mention the modification of the "salting out" method by Cohn. Instead of adding the salt solution directly to the protein sample, the protein sample is put into a dialysing sac which in turn is placed in the salt solution. The advantage of this method is that sudden changes in the composition of the protein solution are avoided, with a consequent reduction of the co-precipitation of protein fractions.

2. **Adsorption Chromatography.** This method includes two distinct processes: a) adsorbent is put in the sample of protein to which the protein adsorbs and is then extracted by either simply the addition of a buffer or by dialysis of the adsorbed material against the required buffer. This method has been successful for quite a long time. Common adsorbents used are Alumina-gel, Benzoic Acid, Calcium Phosphate gel, hydroxides, sulphides and celluloses, etc.

b) When adsorbent is packed into a column the procedure is called adsorption column chromatography. After packing, the protein sample is applied to the column and then the adsorbed protein is eluted by stepwise elution gradient. Tiselius et al (1956) describe in detail the preparation of chromatographic columns and introduced calcium hydroxylapatite as an adsorbent. Other adsorbents commonly used are: Hydrated Ca$_3$(PO$_4$)$_2$ (Swingle and Tiselius,
Silica gel, (Shepard and Tiselius, 1949), Diatomaceous earths, (Martin and Porter, 1951, and Porter and Press 1957) alumina (Zechmeister et al, 1938) and starch (Simonart and Chow, 1951). These methods have been quite successful in the separation of various proteins but there is no single reference available where the workers claimed that their protein preparation was pure.

Care must be taken in choosing the molar concentration, pH and temperature of the eluting buffer. Strongly adsorbed proteins may require buffers of ionic strengths which in turn cause their denaturation.

3. Ion Exchange Column Chromatography. The chromatographic column is packed with ion exchange materials which are available in a number of distinct varieties. Proteins being macromolecular in size, require a large amount of surface area for effective adsorption. The mesh size of the material is very important. There are two types of ion exchange materials available: resins and cellulosics:

**Resins:**

IR-50 (XE 64) - Hirs et al (1953) 200-400 mesh.


Dowex 50 (NH\textsubscript{4})\textsuperscript{+} - Sober et al 1949 200-400 mesh

Dowex 2 (Cl\textsuperscript{-}) - Bowman (1955) 200-400 mesh.

Amberlite IR-4B (Acetate\textsuperscript{-}) Waley (1957)

A Cation Exchange Resin on Celite Feitelson & Partridge (1956)
Celluloses


DEAE Cellulose Peterson & Sober (1956) Anion exchanger.

Ecteola Cellulose Peterson & Sober (1956).

Sulphomethyl Cellulose Porath (1957).

TEAE Cellulose Porath (1957).

The preparation of the above resins and cellulosics has been reviewed by Alexander and Block (1960) and for detailed information, reference may be made to the individual authors. The grain size of the resin, technique of packing the column, ionic strength and pH of the buffers and the temperature are the important factors which influence the ion exchange chromatography of proteins. The mechanism of ion exchange involved in the case of the chromatography of amino acids is well understood. Proteins on the other hand are large molecules and it is difficult to visualise the mechanism. Polyvalency of proteins influences the separation as does the iso-electric point, the dimensions and molecular weight of the protein. Cytochrome-C with an iso-electric point around pH 9.1 (Hirs et al, 1953) was purified on IRC-50 while proteins with iso-electric points on the acidic side may not yield the same results. It is also a well known fact that proteins, with their iso-electric points on the basic side are more easily separated than those with iso-electric points on the acidic side. Boardman and Partridge (1955) noted that the physical factors involved in the bonding
of proteins at low pH's are hydrogen bonds and the Wander-Waal's forces, most probably due to removal of a layer of electrostatically bound water. Detailed physical characteristics and chromatographic behavior of proteins is reviewed by Turba (1960) and Zittle (1953).

Ion exchange cellulosics have been quite useful in separating various serum proteins. (Sober & Peterson 1956, 1961), and various other proteins -- (Zimmerman et al, 1962, Aposhrian and Kornberg, 1962, Garen et al 1960, Shukuya 1960, Maxwell 1960, Snoswell 1959, Crawford and Yanofsky 1959, Levin 1958 and Malmstrom 1957). The references given here deal with purification of proteins of bacterial origin and some viruses. Our experience in this laboratory has shown that DEAE cellulose (Selectacel) has been the best and most convenient ion exchange material for our purposes. The toxic proteins produced in this laboratory were not even adsorbed on Dowex-50 while polysterene showed only partial adsorption.

It is sometimes advisable to use more than one ion exchange material in a stepwise manner. There is evidence in the literature that this approach has been successful in several instances (Zimmerman et al, 1962, Aposhrian & Kornberg 1962).

Materials used for the purification of proteins of bacterial origin other than cellulose are as follows:
Neilands and Stumpf (1958) believe that ion exchange chromatography is essentially an electrophoretic separation in which the resin serves as one electrode and gravity as the other. It may yet develop into a valuable tool as chemical industry places more new resins in the hands of protein chemists (Colowick & Kaplan 1955).

4. **Zone Electrophoresis**. A large number of communications have appeared during recent years dealing with the electromigration of proteins, peptides and amino-acids in a stabilizing media. If the stabilizing medium used is paper, the technique is called paper zone electrophoresis.

The major parts of a simple, low voltage, electrophoresis apparatus are two electrodes, one positive and one negative; a filter paper which is dipped into the buffer present in buffer troughs built on either side and a glass cover to cut down the evaporation of buffer. Levels of buffer on each side are the same. The sample is applied, with great care not to scratch the paper, with a smooth-tipped capillary or capillary tube. The line of application must be at least 7-8 mm. clear of the both edges. Concentration of the protein sample should be 2-10% and must not exceed 0.005/ml/cm. The choice of buffer is dependent on the iso-electric point of the protein sample to be separated. A potential of 5-10V/cm is applied across the strip; high
potentials generate more heat and reproducible results are difficult to obtain. A known protein sample should be tested before applying the unknown sample. The proteins are detected by staining after heating the strip at 110°C for 20 minutes. Some of the available dyes used are Amido-Black 10B, lissamine green, ninhydrin and bromophenol blue. Zones can be marked on the strip by the absorption in ultra-violet light prior to staining and the zones can be cut and eluted for quantitative purposes.

The main physical factors affecting migration rates on paper are the type and concentration of buffer, control of evaporation, electro-endosmosis, and the electric field. The application of paper zone electrophoresis is limited and cannot be successfully used in quantitative research. This technique is a valuable tool in the initial qualitative work.

Zone electrophoresis in stabilizing media such as granular starch, powdered cellulose or resin beads offers considerable advantages over paper zone electrophoresis, which are:

1. Large amounts of proteins can be separated during one run and are available for further analytical work.
2. The evaporation problem is not as acute, and
3. Some of the media are suitable for the determination of mobilities and iso-electric points. The apparatus used in most laboratories is the same as that originally devised by
Kunkel and Tiselius (1951) for paper zone electrophoresis. A significant development was made by Smithies in 1955 with the introduction of starch gel as a stabilizing media in zone electrophoresis. A combination of electrophoresis in agar gel with serological precipitation called immuno-electrophoresis was developed by Graham and Williams (1955) in which, after a normal electrophoretic run anti-serum is applied lengthwise on one side. The antiserum diffuses through the stabilizing media and gives a specific precipitation reaction.

Separations are also possible on a micro-scale using cellulose acetate (Kohn, 1957) which is an ideal medium for immuno-electrophoresis (Consden & Kohn, 1959).

Zone electrophoresis in vertical columns has been technically developed by Haglund and Tiselius (1950), Porath (1954), and Flodin and Kupke (1956), the obvious advantage being the collection of fractions as in Column chromatography.

For the detailed study of these techniques, reference must be made to the original authors and valuable information is also given by Bailey (1962). There are few references available where these techniques are exploited for the purification of proteins of bacterial origin.

The specific activity of a protein/unit can be determined either with the help of ultraviolet absorption at the suitable wave length or by calculating the amount of nitrogen/unit protein. The nitrogen content of a protein can best be obtained from samples free of carbohydrates and

E) Criteria of Purity

The characteristic of a pure protein is that it gives one boundary under ultracentrifugation and electrophoresis. These criteria of purity are a "must" in contemporary techniques apart from other determinant characteristics.

(1) Crystallization: This was considered to be a criterion of purity in the late 1920's and reports were made even more recently where (Lamanna, 1946) emphasis was laid on crystallization. However, several crystalline proteins have been shown to be quite impure; these include Type "A" botulinus toxin (Wagman & Bateman 1951, Wagman, 1954), heart lactic dehydrogenase, ribonuclease, lysozyme and Sumner's urease (c.f. Neilands and Stumpf 1958). Therefore crystallinity in itself is an extremely unreliable index of purity. Also crystals formed under certain conditions may differ entirely in configuration from those formed under another set of conditions. Isomorphous proteins may be present in the same crystals, indicating false visual purity (Fox and Foster 1957). Baronwski (c.f. Schwimmer and Pardee, 1953) has shown that crystallinity is certainly an indication of a certain level of purity and crystalline proteins are usually not denatured.

(2) Biological criteria. Determination of the specific biological activity per unit may be used as one of the criteria of purity. Pure proteins show much higher biological activity per unit than crude preparations.
(3) The chemical structure of a preparation must be consistent at all times with the existing properties of the pure component.

(4) Purified protein will in most cases give a single peak under ion-exchange or adsorption column chromatography. Changes in pH, ionic strength as well as the buffer are essentially tried in order to confirm the purity of the sample.

(5) Paper, starch and immuno-zone electrophoretic techniques indicate a very high level of purity and one or all must be tried depending on the sample. The choice of buffers is very important in these techniques.

(6) **Ultracentrifugal analysis.** Protein preparations are analysed with the ultracentrifuge for establishing purity and for calculating the sedimentation coefficient. This technique was developed in 1922 when Svedverg started exploring the field. Beckman-Spinco is the supplier of all ultracentrifuges in North America.

Ultracentrifugation includes two major techniques; the sedimentation velocity and the sedimentation equilibrium method. The former method is used most commonly for the determination of purity, while with the latter method the diffusion coefficient can be calculated.

The sedimentation velocity method is based on spinning a protein preparation at a very high speed in an ultracentrifuge. During this process, the heavier solutes leave the solvent and accumulate towards the periphery of the cell.
This migration of the solute particles leaves a region in the cell containing only solvent particles and a zone where the concentration of the solute is uniform. There is also a third place in addition to the above where the concentration varies with distance from the axis of rotation. This is called the boundary. The sedimentation velocity method, in brief, is based on observations, by optical methods, of the movement of the boundary which in turn is a measure of the movement of the solute molecules in the plateau region. The rate at which the solute particles move is a function both of the molecular weight of the solute and of the functional resistance which other molecules or particles experience as they move through the solvent. Purity of the preparation can be indicated if one uniform boundary is shown after an adequate length of time. Faulty observations may be made if the preparation used was of high concentration and no analysis was made at dilute levels. This method is not the ultimate technique in the determination of purity as a single boundary may be shown with different proteins having the same sedimentation coefficient; a detailed review has been given by Schachman (1957).

(7) **Diffusion Electrophoresis.** Diffusion is the movement of one solute into another when the latter is of lower concentration than the former. The diffusion coefficient is the rate of diffusion from one solute to the other in a given period of time under a driving force. Diffusion electrophoresis is an apparatus by which the diffusion coefficient of a protein is determined. Molecular weight can then be calculated
from the sedimentation studies and diffusion coefficient. The Tiselius electrophoresis cell, with only slight modifications, is the ideal cell. Schachman (1957) has covered the working of this cell and its modifications, etc. with great detail and lucidity. The results of diffusion electrophoresis are also used as a criterion of purity. Calculations for molecular weight determinations as well as other diffusion techniques have been reviewed by Alexander and Block (1961).

The Tiselius electrophoresis cell is most frequently used for determining homogeneity and electrophoretic mobility of protein preparations. The material is applied to the cell and subjected to an electric current. The different components form concentration gradients under these conditions which can be observed with Schlieren optics. Different components separate according to their mobilities under the conditions of the run. In the case of a homogeneous preparation only one moving peak will be observed.

F. Amino Acid Analysis

Preparations with 13.5% or more nitrogen content are considered as pure protein preparations and amino acid analysis may be carried on these preparations. (Alexander and Block 1961 Vol. 2) which in no case means that there is only one protein species present. Amino acid analysis of preparations containing more than one component is a waste of time.
The first step in the amino-acid analysis is the hydrolysis of proteins. It is brought about with addition of acids such as hydrochloric, sulphuric, hydriodic, formic and others; the detailed procedure for these acids is given by Alexander & Block (1961, Vol.2). Column chromatography of the hydrolysed protein by the methods of Moore & Stein (1954) will give the researcher both qualitative and quantitative results regarding the amino-acid constituents and ratios for the protein being studied (Tristram, 1963). Under these conditions, an empirical formula for the purified protein may be calculated.
CHAPTER II
Review Literature on Botulinus Proteins

"There is an old Dutch word for chemistry, 'scheikunde', which literally means the 'art of separation'. Indeed, separation methods form the basis of chemistry, and the definition of a pure chemical substance ultimately depends on separative operations". (Tiselius 1961; cf. Heftman 1961). "Scheikunde" or the "art of separation" also forms the basis for biochemical research.

During the past two decades, there has been considerable interest in the problems associated with determining the specific activity and chemical structure of certain deadly proteins of bacterial origin, and efforts have been made to obtain these toxins in the pure state. One group of such substances are the exotoxins produced by types, A, B, C (alpha and beta), D, and E, of Clostridium botulinum. The toxins, for the experimental work, were either obtained from filtrates or extracted from whole bacterial cells. There were different methods used for the production and purification of various types of botulinus toxins which are discussed in detail as follows:

(a) Clostridium Botulinum Type A

The purification of this toxin was attempted as far back as 1906 but significant contributions were not made until 1926 when Sommer et al used colloidal Al (OH)₃,
to which the toxin is strongly adsorbed. They tried to elute the adsorbed toxin with weak acetic acid (molarity not specified) at pH 3.8 and ammonia (pH 8.4). This was unsuccessful but thirty to fifty percent of the adsorbed toxin was finally recovered after treatment with a 0.5% solution of \((\text{NH}_4)_2\text{HPO}_4\) + 0.1% glycerol at 28° C for five hours. The toxin was freed of salts by dialysis and evaporated to dryness at 40° C. This dried toxic preparation was soluble in water. A drop in toxicity was observed during dialysis and the final yield was 0.15 to 1.8%.

Sommer and Snipe (1928) investigated the adsorptive properties of type A toxin further on colloidal alumina, and elucidated the role of the pH factor in this process. The toxic product obtained gave a positive Biuret test, a slight Molisch test and contained a small amount of gelatinase. These workers (Snipe and Sommer 1928) later showed that the toxin was precipitated by lowering the pH of the toxic filtrate. The optimum pH for precipitation was found to be 3.0 and gave a yield of 28.5%. Further attempts to purify the toxin by repeated dissolution at pH 7.0 and reprecipitation at pH 4.4 resulted in the loss of most of the toxic activity.

Sommer et al (1928) attributed the loss of toxicity during dialysis to a very small extent to diffusion. They noted that a large amount was irreversibly inactivated, for which no explanation was given. The maximum stability of
the toxin was also reported to be between pH 4.0 and pH 5.0.

Sommer (1936) obtained preparations of high toxicity by using repeated acid precipitations with N/10 HCl between pH 3.5 and 4.0 and with subsequent dissolution in sodium acetate buffer (molarity not specified). The preparations contained lethal doses of $4.10^{-9}$ gm = $2.10^{-7}$ gm per Kg mouse. The yield of the first precipitate was 50% but decreased after further reprecipitations. He also noted that the toxin was gradually destroyed at room temperature and that the presence of trivalent ions enhances this effect.

Stockinger and Ackerman (1941) found that toxic filtrates were stable between pH 1.5 and pH 8.5 and carried out fractional precipitations of toxic filtrates at pH 4.0 and 1.5. They found that the bulk of the toxin was precipitated at the later pH. The concentrated material was treated with "takadiastase" to remove carbohydrate-and phosphate-containing material (amounting to 10 - 20%), and preliminary analyses showed that the purified toxin is a simple, unconjugated protein of the albumin class and contained less than 1 percent nitrogen and four percent sulfur. It contained 10,000 to 20,000 MLD/mg toxin. They also noted the significance of disulfide bonds and observed that the destruction of these bonds brought about the loss of toxicity.

Abrams and co-workers (1946) reported that type A toxin was stable at room temperature between pH 1.0 and 6.0 (the maximum being between pH 4.0 - 5.0) but that the toxin was rapidly destroyed at pH's above 7.0. They used
Sommer's (1936) acid precipitation technique and adjusted the toxin filtrates to pH 3.5. The precipitated toxin then was thrice extracted in 1/4 the original volume of 1% sodium acetate solution at pH 6.5; and the three extractions were pooled and the insoluble material discarded. The toxin was precipitated again at pH 3.5; washed twice in distilled water; redissolved in 1/4 the original volume of 1% sodium acetate solution, then precipitated with 10, 20, 40 and 50 per cent ethanol; and further precipitated with saturated Na₂SO₄ at pH 6.5 at room temperature. The material obtained was soluble in distilled water and fractional precipitation at 0.18 and 0.4 saturated Na₂SO₄ was carried out. The toxic fraction was contained in the 0.4 saturated Na₂SO₄ preparation, had a toxicity of 60 x 10⁶ mouse LD/mg N., and had an isoelectric point at pH 5.6. This toxin gave a positive test for carbohydrates and phosphorus. The toxin was electrophoretically homogeneous. The authors note that the "homogeneous" fraction had fewer mouse LD/mg N than did certain impure fractions. Another toxic fraction with toxicity of 220 x 10⁶ MLD/mg N was obtained by using a slightly different procedure. The buffer used in this case was 1% sodium phosphate buffer at pH 6.8. The rest of the procedure was essentially the same as given before and the recovery rate was 34 per cent. This component was twice crystallized in 0.10 to 0.30 saturated (NH₄)₂SO₄ at 4°C.
This toxic fraction did not satisfy the criteria of purity used by Abrams et al which were: 1) maximum toxicity per mg nitrogen; 2) electrophoretic homogeneity and 3) a single ultraviolet absorption band at 278 m\(\mu\).

The procedure for purification developed by Lamanna et al (1946) varied to a certain extent from the technique used by Abrams et al (1946). The toxin was precipitated at pH 3.5 with 2 N HCl, the supernatant siphoned off, and the precipitate washed in distilled water. The washed precipitate was resuspended in water, to which 1 M NaCl and 0.075 M sodium acetate solutions were added and the pH adjusted to 6.5. The precipitate was discarded and 1/6 to 1/10 volume C.P. chloroform was added and shaken under CO\(_2\) for five minutes at pH 5.8 and 6.1. The gel and the excess chloroform were discarded and the toxin present in the aqueous gel was precipitated with 20% saturated (NH\(_4\))\(_2\) SO\(_4\). The precipitate was obtained by lowering the pH to 5.0 by the addition of HCl. The toxic precipitate was resuspended in 1 M NaCl + 0.075 M sodium acetate solution at pH 6.5. To this suspension, 0.9 gm (NH\(_4\))\(_2\) SO\(_4\) /ml toxin was added and a coloured precipitate formed which was discarded. The supernatant was allowed to stand at 4° C overnight; the toxic crystals which formed had a nitrogen content of 14.3%. The yield was 15 to 20% and the toxicity was 4.5 x 10\(^9\) mouse LD 50/ mg N. The pure toxin had the properties of a protein and fourteen amino acids were identified as well as phosphate and sulfur.
Kegeles (1946) calculated the diffusion constant for the crystalline toxin (Abrams et al 1946 and Lamanna et al 1946) and recorded the viscosity measurements. The molecular weight of the toxin was reported to be 1,130,000. (N.B. Unhomonogeneity was observed electrophoretically after these calculations.).

Putnam et al (1946) studied the electrophoretic, sedimentation and diffusion characteristics of Lamanna's (1946) crystalline toxin in 0.1 M sodium acetate buffer at pH 4.38. They noted that the boundary spread was greater than that attributable to diffusion alone. The molecular weight of the material sedimenting with a coefficient of $S_{20,w} = 17.30$ was calculated as 900,000. The molecules were assumed to be ellipsoids and an estimate that there were $2.1 \times 10^7$ molecules per Mouse LD$_{50}$ was made.

Buehler et al (1947) studied the elemental and amino acid composition of Lamanna et al (1946) toxin and identified nineteen amino acids. The minimum possible molecular weight was reported to be 44,944 and an empirical formula was given. The presence of phosphorus was felt to be an impurity (possibly the residue of nucleo-proteins). The criteria of purity used were ultracentrifugation, electrophoretic and serological behavior.

Putnam and his associates used the chloroform shaking method in 1948 to purify the toxic material. They obtained photographs of the electrophoretic migration of the
crystalline toxin and calculated the diffusion coefficient. The sedimentation coefficient was reported to be $S_{20} W = 17.35$ and the molecular weight 900,000. Boundary spreading indicated the polydisperse character of the toxin molecule. The immunological homogeneity was also studied and indicated.

Lamanna and Doak (1948) gave a full report on the serological behavior of Lamanna's (Lamanna et al 1946) crystalline toxin. They claimed that the antitoxin response in Rabbit and Horse was complete and that the toxin was a single antigenic component.

Carl Lamanna (1948) worked on the hemagglutination property of the crystalline toxin. The amorphous as well as the crystalline toxins reacted with the red blood cells of chickens, guinea-pigs, rabbits, sheep, and man, although the toxin itself was not adsorbed onto the red blood cells. The activity of hemagglutination and toxicity was decreased by treatment with formaldehyde.

Lamanna and Lowenthal (1951) further studied the haemagglutinating and the toxic activities and a material was separated which caused the hemagglutinating activity. The type specific antiserum neutralized both the activities while type non-specific antiserum did not. The toxic activity was, however, more sensitive to heat while the hemagglutinating activity was sensitive to an acid pH and was neutralized by type B antitoxin. Oudin-plate technique
also demonstrated the separability of the two properties when type A antitoxin was used. The multiplicity of type A crystalline toxin conflicted with the apparent homogeneity of material shown by ultracentrifugation and electrophoresis. In the light of this later data, they hypothesized the formation of a stable complex of the two substances with the crystallization technique applied. This was later confirmed by Lowenthal and Lamanna (1953).

Lamanna and Aragon (1956) reported the isolation of a protein component from the crystalline toxin which was responsible for the hemagglutinating activity.

The peculiarity of the high molecular weight of type A crystalline toxin was noted by Wagman and Bateman (1951). They showed that the sedimentation constants varied with the concentration of the preparation used, and that by raising the pH, dissociation of the large molecules took place and a slowly sedimenting component having considerable toxic activity with no haemagglutinating activity could be isolated. Wagman in 1954 reported the sedimentation coefficient of this active component to be $S_{20}^W = 6.55$ and the molecular weight 70,000. He kept the toxin in 0.05 M acetate buffer of pH 3.8 at $5^\circ C$. He prepared the crystalline toxin by using Abrams (1946) method and estimated the iso-electric point to be pH 5.5. The crystalline toxin was dialyzed at pH 6.5 to 8.0 with phosphate buffers of ionic strengths 0.13 and 0.5. (higher ionic strength buffers gave better separation).
With this treatment a product was obtained which had a sedimentation coefficient of $S_{20}^W = 6.55$ and contained fifteen percent of the total toxic activity. This component polymerised at pH 3.8 and a heavy component was formed which was obtained with phosphate buffer of ionic strength 1.0 (Wagman 1954). The low molecular weight material had two to three times the specific activity of the large complex. The heavy component was similar to the original component and he considered the possibility that toxin molecules were aggregated with non-toxic material.

Halliwell (1954) extracted the toxin from lysed cells and precipitated it in acid citrate buffer containing 0.18 M sodium citrate ($H_2$) and 0.02 M sodium citrate ($Na_2$) at pH 4.0. Further precipitation was done with ammonium sulphate. The samples contained $70 \times 10^6$ M LD/mg N. However, further batches were prepared by this method with toxicities comparable to those obtained by Lamanna et al. (1946). Both the toxins were crystalline and homogeneous electrophoretically. The two preparations differed greatly in stability; the low activity material being readily inactivated in 2 minutes in 3.5 N HCl at 16.2°C. Both the toxins were stable in buffers of pH 5.0 to 6.5 at 0°C. The low titre material was rapidly destroyed at 30°C at pH 7.0 while the high titre material was stable under these conditions for 26 hours.
A high state of purity was obtained by Duff et al. (1952, 1957) by acid precipitation at pH 3.5. The precipitate was washed with distilled water and the toxic component was extracted from the precipitate with 0.075 M CaCl₂ and reprecipitated at pH 3.7. The precipitate was again dissolved in phosphate buffer at pH 6.8. Further precipitation was done with fifteen percent ethanol in the cold. This preparation was crystallized by precipitation with (NH₄)₂SO₄ and had a specific activity of 269 x 10⁶ mouse intraperitoneal LD₅₀/mg N. This toxin appeared to be homogeneous in the analytical ultracentrifuge.

Since 1957, no further data have come to light regarding the purification and characterization of botulinus type A toxin. The accumulated information documented above strongly indicates that the criteria for purity at our disposal today have not been satisfied regarding this material.

The techniques employed by most of the workers involved in this problem were based on the initial precipitation of toxic matter with subsequent repeated precipitations obtained either by lowering the pH or "salting out". A significant departure from these methods was introduced by Lamanna (1946) with his "chloroform shaking process", the advantage of which is doubtful. An unduly high significance has been placed on the obtaining of the toxic component in a crystalline form. Crystallization of material indicates merely that one species of crystal has formed in a given preparation and it is abundantly clear that two or more distinct molecular
structures may be involved in the formation of such a crystal (Neilands and Stumpf, 1958).

The initial biophysical studies carried out on crystalline type A toxin indicated inhomogeneity, both in the ultracentrifuge (Putnam et al. 1946), wherein it was noted that the boundary spread was greater than could be attributed to ordinary diffusion, and during electrophoresis (Kegeles, et al., 1946). Later data (Wagman and Bateman 1951, 1953, and Wagman, 1954) showed that the crystalline material could be dissociated and a smaller toxic entity isolated. Lamanna himself (Lowenthal & Lamanna 1951) later postulated the formation of a stable complex during crystallization.

In the light of general knowledge today, the assumption that a protein molecule with the remarkable biological activity of Type A toxin has a molecular weight of about a million appears unrealistic; and one can deduce with some certainty that this protein has not, to date, been purified to a degree wherein its properties may be satisfactorily studied.

(b) *Clostridium botulinum* Type B

Lamanna and Glassman (1947) developed a technique for the production and purification of Type B toxin. The 'OKRA' strain of Type B was grown at 34°C for fourteen days and the culture was brought to pH 4.0 with 2 N HCl. The culture was allowed to stand at room temperature and a
precipitate formed which gave a 90% recovery of the original toxicity of the whole culture. The supernatant was discarded and the precipitate was suspended in 1/40 of the original volume in distilled water. The pH was brought to 2.0 with 2 N HCl. The precipitate was discarded and the filtrate contained 80% of the original toxic activity. This clear filtrate was brought to pH 4.0 with 2 N NaOH and a flocculent precipitate was obtained (the authors noted that the recovery rate was more consistent and higher at pH 5.0) which was then resuspended in 1/4 of the original volume in 0.5 M NaCl at pH 2.0 for 10 to 15 minutes. The precipitate was recovered by centrifugation and resuspended in 1/4 original volume of acidified distilled water at pH 2.0. The toxin was reprecipitated by bringing the pH to between 5.0 and 5.5. The final recovery rate was 50%. The purified toxin was greyish yellow when solid and yellow brown in solution. It gave a positive biuret test and was negative for carbohydrates. The maximum absorption was at 277 nm and the amino nitrogen was 15.5%. The molecular weight determination was carried out by calculating the diffusion coefficient at pH 2.0 and was reported to be 60,000 (the electrophoretic analysis showed that pH 4.5 was on the acid side of the isoelectric point). The toxin was insoluble between pH 4.0 and 7.0 and the presence of salts decreased its solubility in water. Electrophoresis which was carried out between pH 1.8 and 3.8 gave a single boundary, and the specific toxicity was 5.9 x 10^9 mouse LD 50/mg N. Lability of the material appeared to be increased after purification.
Wagman and Bateman (1951) studied the behaviour of Lamanna and Glassman's purified (1947) type B toxin at pH 3.0 in the analytical ultracentrifuge and reported the molecular weight as 500,000. They isolated another slow moving component with sedimentation coefficient $S_{20\ W} = 10.7$ which represented about 30% of the total toxic material.

Duff and co-workers (1954) showed that the toxin could be extracted from acid precipitates with CaCl$_2$ at pH 1.5 to 6.5, the greater recovery being between pH 1.5 to 2.5. This material was not soluble above pH 4.5. Recovery of this toxin was extremely low at pH 5.5 to pH 6.5 but the component obtained after further purification was soluble between pH 5.5 and 6.5. Purification was completed by precipitating with acid and then with ethanol in the cold.

Millonig in 1956 used the "OKRA" strain and produced toxic filtrates with the dry casein method. A precipitate was obtained with (NH$_4$)$_2$SO$_4$ to a final saturation of 0.6 at pH 7.0. Final purification was obtained in 1 M NaCl at pH 6.0 with 0.3 saturated (NH$_4$)$_2$SO$_4$.

Duff et al (1957) grew the toxin in trypticase yeast extract, cystine hydrochloride and glucose. The cultures contained $2.0 \times 10^6$ mouse intraperitoneal LD$_{50}$/ml or greater. The precipitate formed by acidification at pH 4.5 was washed with distilled water and the toxin was extracted from it with 0.05 M calcium chloride solution at pH 6.0 between 30° C and 35° C. Further precipitation was done at pH 3.7 and the toxin was dissolved in 0.1 M phosphate
buffer at pH 5.5 or 0.1 M phosphate buffer at pH 6.8. This material gave a specific activity of $262 \times 10^6$ mouse LD$_{50}$/mg N and when examined in the analytical ultracentrifuge, two components were obtained; one main component with $S_W^{20}$ of 14.9, and a diffusely sedimenting component with $S_W^{20}$ of 10.9 at pH 3.0.

The method used by Lamanna and Glassman (1947) for the purification of botulinus type B toxin is essentially based on repeated precipitations at various pH's. It is indicated in the results that a large amount of toxicity was lost after each precipitation and subsequent suspension. Since proteins show minimum solubility near the isoelectric pH, the fractionation around that pH is valid (Neilands and Stumpf 1958) but denaturation may occur if a pH beyond the isoelectric point is used (Alexander and Block 1958). Also, the lack of neutral salts in the solution aids in denaturation. Wagman and Bateman (1951) showed that there were two components in the purified toxin of Lamanna and Glassman (1947) and were successful in separating them.

Duff et al (1954, 1957) made no significant contributions towards purification of botulinus type B toxin as: 1) they used essentially the same technique as Lamanna and Glassman (1947), and 2) Specific toxicity/mgN was much lower than Lamanna and Glassman's (1947) purified toxins.
It is quite clear that botulinus type B toxin obtained by these workers was unhomogeneous and satisfactory tests for determining the purity were not carried out.

(c) Clostridium Botulinum Type C

Sterne and Wentzel (1950) developed an apparatus for their production of toxins in carboys with inserted cellophane bags in which the organisms were grown. They used unconcentrated corn-steep liquor (3.5% of total solids) at pH 7.2 with 1% NaCl as media. The toxicity obtained was $3.0 \times 10^6$ MLD/mg.N. No further attempts were made at this time to purify the product.

Boroff et al (1952) extracted the toxin from 48 hour cells, washed three times with distilled water, grown on media composed of peptic digest of liver and beef muscle enriched with 1% glucose, at pH 7.0, with 1 M NaCl and 0.1 M Sodium citrate solutions kept for 6 days in the cold. The supernatant was dialyzed against distilled water for 48 hours and a white precipitate appeared. Both the precipitate and the supernatant were toxic. The supernatant and the precipitate were treated separately in the following ways:

The supernatant was brought to pH 6.0 with 6N HCl and the precipitate obtained was of feeble toxicity and was discarded. The pH of the supernatant was further decreased to 4.0 and a

+ The culture on which Boroff et al (1952) did their work was mistakenly understood to be of type D but was later proved to be of type C.
toxic precipitate was recovered with partial solubility at pH 2.0. The toxin was reprecipitated at pH 4.7 (pH change was brought about by the addition of 1 N NaOH). The precipitate was washed in phosphate buffer at pH 4.5 and redissolved at pH 5.7. The pH was brought to 7.2 and the material dialyzed against 0.85% salt solution. The toxicity of the final product was $1.4 \times 10^7$ mouse LD$_{50}$/mg N.

The precipitate was treated with 0.1 M sodium citrate solution and 1 M NaCl. The toxic extract was dialysed for two days at 0°C against distilled water. This toxin went into solution at pH 9.0 and was completely detoxified but was insoluble between pH 4.0 and 7.0. Both the toxins were unstable at 40°C but stable at 4°C. It was noted that heat at 60°C applied for 30 minutes completely detoxified the toxins.

The type C strain, isolated by Robinson and Theiler in 1928, was grown in media composed of 4% proteose peptone, 2.0% pancreatic digest of casein (N-Z amine type B, Sheffield, Norwich, N.Y.), 0.2% yeast extract (BBL) and 1.0% glucose, by Cardella et al in 1958. Four litres of media in carboys were inoculated with 10% of its volume of a 24 hour inoculum and incubated at 33°C for five days. The filtrate was treated with 95% ethanol to a final concentration of 25% for 18 to 24 hours at 5°C, after which the supernatant was discarded. The precipitate was diluted to 1/8 culture volume with distilled water and the volume was further diluted to 1/4 culture volume with 1 M CaCl$_2$.
(34)

(final concentration of CaCl₂ was 0.5M). The pH was adjusted to 5.0, stirred for 1 hour at room temperature and was centrifuged at 4°C. The pH of the supernatant was further increased to 6.0 with 1 N NaOH, and a precipitate was obtained with 50% ethanol, added to a final concentration of 15% and allowed to stand at -5°C for 18-24 hours. The precipitate was dissolved in 0.4 M succinate buffer at pH 5.0 and the solution clarified by centrifugation at 4°C. These workers indicated that ethanol precipitation from the cultures at pH 5.5 to 6.0 was complete and the recovery was much higher than the acid precipitation method in which recovery was low. They also tried precipitation with 40% saturated (NH₄)₂SO₄ at room temperature overnight. The precipitate was obtained after centrifugation at 4,000 RPM at 4°C and resuspended in water to 1/4 culture volume. The rest of the procedure was the same as used with ethanol precipitates.

Boroff and co-workers used the acid precipitation method to purify type C botulinus toxin. None of the workers characterized this toxin and also failed to clarify whether this type belonged to C₂ or Cβ of botulinus toxins. Thus it is very difficult to correlate the results obtained by various workers in this field. Lack of sufficient neutral salts, precipitation of toxin at pH's beyond the iso-electric point and treatment of this toxin protein with concentrated ionized solutions (Block and Alexander 1960) could have added to significant denaturation. This botulinus toxin has not been purified and the results obtained indicate no helpful knowledge for further work.
(d) Clostridium botulinum Type D

Sterne and Wentzel (1950) used media composed of unconcentrated corn-steep liquor (3.5% of total solids) with 0.5% glycerol and 0.1% sodium chloride, for the mass production of type D botulinus toxin. They developed a technique for the production of toxins in carboys with inserted cellophane sacs and obtained toxicities up to $130 \times 10^6$ mouse LD/mg N. Purification was tried with 0.40 saturated $(\text{NH}_4)_2\text{SO}_4$ at pH 5.8 and a precipitate was obtained which was redissolved (solution not specified) and reprecipitated with 25-30% saturated $(\text{NH}_4)_2\text{SO}_4$. The preparation was electrophoretically homogeneous and a molecular weight of 1,000,000 was reported. The purified toxin gave a specific toxicity of $4 \times 10^{12}$MLD/mg N.

Wentzel et al (1950) reported that this preparation turned out to be polydisperse when the diffusion experiment was performed.

The molecular weight indicated by these workers is very high and the possibility exists that their molecular species was unhomogeneous and represents toxic molecules aggregated with non-toxic material. There are not enough data given to further analyse the procedure. The method is based on "salting out" with $(\text{NH}_4)_2\text{SO}_4$ which is quite a gentle treatment and decreases the possibility of denaturation (Neilands & Stumpf 1958).
(e) **Clostridium botulinum** Type E

Gordon *et al* (1957) obtained whole culture filtrates by growing a type E culture in 2.0% proteose peptone, 2.0% yeast extract (BBL) and 1.0% dextrin at 30° C for 7 days. The precipitate was obtained from the whole culture by the addition of 95% ethanol to a final concentration of 25% which also contained 1% Bentonite at -7° C. The precipitate was brought up to 1/6 of the culture volume with water and stirred at room temperature for one hour. The preparation was further diluted to 1/4 culture volume with water, and 1 M CaCl$_2$ was added to a final concentration of 0.075 M at pH 6.0. The precipitate obtained after stirring for 2 hours at room temperature was discarded. The supernatant was treated with ethanol in the previously described manner and the precipitate was dissolved in 0.08 M phosphate buffer at pH 6.0. Final precipitation was carried out with ethanol and the precipitate was dissolved in 0.02 M succinate buffer at pH 5.5. The preparation thus obtained was believed to be 410 times more pure than the original whole culture with a toxicity of 19,000,000 LD$_{50}$/mg N.

Sakaguchi and Sakaguchi (1959) worked on 48-hour, three times distilled water washed cells which were grown on media composed of beef liver infusion broth, brain heart infusion broth (Difco), V.F. broth, (peptic digest of beef and liver) and 0.5% glucose. The washed cells were stirred at 37° C overnight in molar acetate buffer at pH 6.0. The extract was treated with (NH$_4$)$_2$SO$_4$ to a final concentration of 50-60%
saturation and dialyzed against water. The supernatant was lyophilized and purification was attempted with starch electrophoresis using acetate buffer at pH 6.0. The toxic fraction isolated by this method contained a considerable amount of ribonucleic acid and had a toxicity of $6 \times 10^4$ to $9 \times 10^5$ MLD/mgN.

Gerwing et al (1961, 1962) exploited the ion exchange chromatographic technique for the purification of ethanol precipitated toxins from cultures grown in cellophane sacs. The precipitate was dissolved in 0.05 M sodium acetate at pH 6.0 in 1/20 of the original culture volume. Ten millilitres of this toxic preparation were then applied to columns packed with DEAE (Selectacel) cellulose pretreated with 2 M sodium acetate solution at $4^\circ$ C for 24 hours, washed with distilled water and then three times with 95% ethanol, dried, and suspended in distilled water to make a homogeneous particle size slurry. The toxin was eluted with ascending salt concentration starting from distilled water to 0.5M sodium acetate at a constant pH of 6.5. The toxin recovered was dialysed, lyophilized, resuspended in 0.05 M sodium acetate at pH 6.0 and rechromatographed in the same way. The authors report that a ten-fold purification can be obtained by using this method. The toxicity of the purified preparation was $5.8 \times 10^5$ MLD/mg N. The ultracentrifugal study of this toxin showed two components with sedimentation constant $S_{20W} = 5.6$ and 1.1, the toxic component being present in the more rapidly moving boundary.
Gordon and coworkers (1957) claimed that their toxic protein obtained after repeated ethanol precipitations was 410 times more pure than the crude product which in no way satisfied any criteria of purity.

Sakaguchi and Sakaguchi (1959) also failed to obtain any satisfactory results and the methods used for obtaining the toxins from cultures was more difficult, messy and less reliable than the method used by Gerwing et al (1961, 1962). Gerwing et al (1961, 1962) for the first time used ion exchange chromatography for the purification of ethanol-precipitated toxin which was a significant divorce from the methods used before. They obtained a partially purified toxin component. Their treatment of chromatographic material before usage did not exploit the exchange potential of the resin which led to poor resolution in the eluted fractions.

In summary, various methods have been applied to purify the A, B, C, D & E toxic proteins from the crude solutions from 1926 to date. Mainly, acid precipitation, "salting out" and more recently ion exchange chromatography were the basic techniques. The purified toxic proteins obtained by various workers had not satisfied the criteria of purity and were essentially unhomogeneous.
CHAPTER III

Preliminary Experiments

The major work involved throughout this research project centered on the solution to problems concerning the purification of the toxic protein produced by *Clostridium botulinum* type E. The two immediate techniques to be developed were for the mass production of this toxic protein with consistently high yields and the concentration of toxin from the toxic filtrates, maintaining the toxic material in a soluble and biologically active form. The final stages of the project were concerned with the separation of a single protein species imparting the toxic activity. The work of Gerwing et al. (1961, 1962) was used as a guide at the start and the available information was exploited to find the best and easiest method of solving these problems.

The preliminary experiments performed imparted valuable information and formed the basis of the final successful technique.

(a) Mass Production of Toxin

Cultures were grown in tubes of GPBl broth for 24 hours at 37°C and were inoculated to a dialysate column (Fig. 1, Appendix 1) using GPBl broth with 1.0% sodium thioglycollate and 2.0% dextrose (Appendix II). A major problem of this technique was the risk of contamination of the media during the incubation period which contributed to the production of low toxicity filtrates. A modified apparatus was developed (Fig. 2, Appendix III) which was easy to handle, less prone to
contaminate the media and gave continuously high titre toxic filtrates.

The dialysate cultures were harvested after 80 to 90 hours growth at 30° C. It was realized later that if growth was allowed to continue for 120 hours at 30° C in the dialysate columns using GPB1 media with 1% sodium thioglycollate and 0.5% dextrose, better and more consistent yields were obtained. This was previously shown by Dolman, C.E. (unpublished data). It was also observed that the activity of the dialysate toxins was decreased due to the cellophane sacs. (Sommer et al. (1926), also observed this type of detoxification in the case of Clostridium botulinum type A). It was noted that the cellophane sacs if used after boiling in 0.1M Versene adjusted to pH 7.0 and washing several times with distilled water, had little effect on the toxin production and dialysates of consistently high toxic titres were obtained.

(b) Purification of the Concentrated Toxin

The procedure of Gerwing et al. (1961, 1962) was tried with ethanol precipitated toxin which was suspended in 0.05 M sodium acetate solution at pH 6.5, for purification (Fig. 3). This procedure was tried with various alterations such as the flow rate, column length and diameter ratio and variations in ionic strengths of eluting buffers. Although none of these experiments were completely successful in obtaining the toxin in the pure state, important data was obtained in some cases. These data will be given to demonstrate the development of the final and successful technique.
**FIG. 3** Chromatography of Botulinus Type E, ethanol precipitated toxin on a column packed with DEAE pretreated with 2 M sodium acetate solution. The graph shows the elution pattern of the preparation.
Procedure A: The column was packed with DEAE (Selectacel) Cellulose by the general method described by Gerwing et al. (1961) except that the DEAE cellulose was ionized in 2 M NaCl overnight, after which the column was washed with distilled water. Ten ml of the ethanol precipitated toxin was applied and elution was carried out with distilled water. A large amount of non-toxic material was eluted with the distilled water (Figure 4) although the toxin remained on the column. The inferences drawn from these results were: 1) there is a water-soluble non-toxic component in the toxic preparations, and 2) these components are not adsorbed onto the column under the given conditions.

Procedure B: In this procedure, the DEAE (Selectacel) Cellulose was treated with 2 M NaCl solution overnight at 4°C before packing. The columns were packed under slight positive pressure. Ten ml of the ethanol precipitated toxin was applied to the column and stepwise elution was carried out with 180 ml of distilled water and 1 M sodium acetate solution at pH 6.5 respectively (Figure 5).

Further experiments were done to study the resolution and separation of various components, using eluting agents of constant molarity but different pH's (Fig. 6). The pH values of the effluents were also studied (Fig. 7).

The last experiment in this series was done by using Dowex as the ion exchange material (Fig. 8).
Separation of a nontoxic and water soluble material from the toxic preparation of botulinus type E with chromatography on DEAE, 2 M NaCl pretreated columns. Elution was carried out with distilled water.
FIG. 5. Stepwise elution gradient of botulinus type E ethanol precipitated toxin on DEAE cellulose (selectacel) columns, pretreated with 2 M NaCl at 4°C, with distilled H₂O and 1 M Sodium Acetate solution at pH 6.5
Stepwise elution gradient of botulinus type E, ethanol precipitated toxin, using 1 M sodium acetate solution at pH 6.5, pH 4.5 and pH 7.0 respectively, water-soluble non-toxic material is previously removed with distilled H₂O.

(DEAE - cellulose in 2 M NaCl is used in the column)
Stepwise elution gradient of botulinus type E, ethanol precipitated toxin (1 M Sodium Acetate solution at pH 6.5 and pH 4.5 is used as eluent. The column is packed with DEAE cellulose, pretreated with 2 M NaCl at 4°C.)
Chromatograph of botulinus type E, ethanol precipitated toxin on a column packed with Dowex pretreated with 2 M NaCl. Elution is carried out with distilled H2O and 1 M Sodium Acetate solutions (pH 6.5 and pH 4.5 respectively).
Procedure C: At this stage, the washing of the packed columns with 1N HCl prior to equilibration, was introduced. It was felt that this treatment was necessary to release all the extraneous material sticking onto the column after the previous run.

The column size chosen in this case was 14 mm x 725 mm. DEAE (Selectacel) Cellulose was treated with 2 M NaCl overnight at 4°C and columns were packed under slight positive pressure.

The columns were washed with 1N HCl and then with distilled water till the pH of the effluent was at 5.5. Ten ml of the ethanol precipitated toxin (suspended in 0.05 M sodium acetate solution at pH 6.5) was then applied to the column. Stepwise elution was carried out with the following solutions (applied in the given order).

1. Distilled water 225 ml.
2. 0.05 M sodium acetate solution at pH 6.5 675 ml.
3. 0.10 M "  "  "  "  " 200 ml.
4. 0.20 M "  "  "  "  " 285 ml.
5. 0.40 M "  "  "  "  " 365 ml.
6. 0.60 M "  "  "  "  " 200 ml.

The column was run at 4°C with an average flow rate of 10-15 ml/hr and 5.0 ml fractions were collected. (Figures 9 and 10). Fractions 139-147 contained the toxic activity and were pooled. The biological activity of this fraction was then calculated as \( 5.0 \times 10^6 \text{ MLD/mgN} \). This toxin gave a sedimentation coefficient of \( S_{20} = 1.7 \) and appeared to be a single component. The
Fig. 9. Chromatography of botulinus type E, ethanol precipitated toxin on DEAE cellulose column, pretreated with 2M NaCl. The column is washed with 1N HCl and distilled water after packing, and stepwise elution was carried out with distilled H₂O and 0.05 M and 0.1 M sodium acetate solutions at pH 6.5 respectively.
Fig. 10. Schlieren photograph of ultracentrifugal behaviour of botulinus type E, ethanol precipitated and purified toxin.
material, however, appeared to be polydisperse because spreading of the boundary was more rapid than could be accounted for purely by ordinary diffusion.

(c) The preparation of Soluble Concentrated Toxin

The toxic filtrates were treated with 95% ethanol to a final concentration of 35% at -15° C overnight. The precipitate obtained after centrifugation was suspended in 0.05 M sodium acetate solution at pH 6.5. This suspension always contained a large amount of insoluble toxic material. This problem was investigated by suspending the toxic ethanol precipitate in distilled water and then centrifuged at 0° C at 15,000 RPM for 30 minutes. Two fractions were obtained after centrifugation, i.e. the non-toxic supernatant and the toxic residue. The toxic residue was resuspended in distilled water to get rid of all the non-toxic water-soluble components. Attempts were then made to obtain this residue in a completely soluble form by suspending the residue in various buffers and salt concentrations (Procedure A, Table 1). As it appeared that at least 50% of the toxin was irreversibly insoluble under the given conditions, further attempts to concentrate the toxin were carried out using (NH)\textsubscript{4}SO\textsubscript{4} as the initial precipitating agent. (Procedure B, C, D, Table 2). Under these conditions, a high titre soluble concentrated toxic preparation was obtained. The recovery rate was 75-80%.
Schematic procedures for the preparation of high titre soluble concentrated toxin.

Procedure A (unsuccessful)

Toxic Filtrate
(dialysate toxin centrifuged and filtered)

+ 

95% ethanol to a final concentration of 35% at -15°C overnight.

Centrifuged at 0°C (15,000 RPM, 30")

Precipitate (Toxic) → Suspended in dist. H₂O (Non toxic discard)

Centrifuged at 0°C (15,000 RPM, 30")

Residue (Toxic) → Supernatant (Non toxic discard)

Resuspended in distilled H₂O and centrifuged at 0°C. Discarded the non toxic supernatant and resuspended in 0.01 M sodium acetate buffer at pH 5.5.

Centrifuged at 0°C.

Residue (Toxic) → Resuspended in 0.5 M Sodium Acetate Buffer at pH 5.5. (Low toxicity)

Centrifuged at 0°C (15,000 RPM, 30")

Residue (Toxic) → Supernatant (Toxic)

Suspended in 0.5 M NaCl solution.
Table 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>MLD/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxic filtrate</td>
<td>3,000</td>
</tr>
<tr>
<td>Ethanol precipitate suspended in distilled water</td>
<td>30,000</td>
</tr>
<tr>
<td>ethanol supernatant</td>
<td>30</td>
</tr>
<tr>
<td>Water insoluble residue of ethanol precipitate</td>
<td>20,000</td>
</tr>
<tr>
<td>Water soluble portion of ethanol precipitate</td>
<td>30</td>
</tr>
<tr>
<td>Second water insoluble residue suspended in 0.01 M sodium acetate buffer at pH 5.5.</td>
<td>10,000</td>
</tr>
<tr>
<td>Second water soluble portion of ethanol precipitate</td>
<td>30</td>
</tr>
<tr>
<td>0.01 M sodium acetate buffer at pH 5.5. soluble portion</td>
<td>100</td>
</tr>
<tr>
<td>0.01 M sodium acetate buffer at pH 5.5. insoluble portion suspended in 0.5 M sodium acetate buffer at pH 5.5.</td>
<td>3,000 to 10,000</td>
</tr>
<tr>
<td>0.5 M sodium acetate buffer at pH 5.5. soluble portion</td>
<td>2,000</td>
</tr>
<tr>
<td>0.5 M sodium acetate buffer at pH 5.5 in soluble portion suspended in 0.5 M NaCl solution</td>
<td>2,000</td>
</tr>
<tr>
<td>Preparation</td>
<td>MLD/ ml</td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Toxic filtrate</td>
<td>1,000</td>
</tr>
<tr>
<td>33% saturated (NH₄)₂SO₄ ppt. suspended in 0.01 M sodium acetate buffer at pH 5.5.</td>
<td>1,000</td>
</tr>
<tr>
<td>Supernatant</td>
<td>100</td>
</tr>
<tr>
<td>50% saturated (NH₄)₂SO₄ precipitate suspended in 0.01 M acetate buffer at pH 5.5.</td>
<td>3,000</td>
</tr>
<tr>
<td>Supernatant</td>
<td>100</td>
</tr>
<tr>
<td>60% saturated (NH₄)₂SO₄ precipitate suspended in 0.01 M sodium acetate buffer at pH 5.5.</td>
<td>10,000 to 20,000</td>
</tr>
<tr>
<td>Supernatant</td>
<td>30</td>
</tr>
</tbody>
</table>
Procedure B (unsuccessful)

**Toxic Filtrate**

\[ \text{Saturated } (\text{NH}_4)_2\text{SO}_4 \] to a final concentration of 33% saturation precipitated overnight at 4°C.

Centrifuged at 4,500 RPM for 45 minutes at 0°C.

\[ \text{Precipitate} \rightarrow \text{Supernatant} \]

(Toxic) (Toxic)

Procedure C (unsuccessful)

**Toxic Filtrate**

\[ (\text{NH}_4)_2\text{SO}_4 \] to a final concentration of 50% saturation kept in fridge overnight.

Centrifuged at 4,500 RPM for 45 minutes at 0°C.

\[ \text{Precipitate} \rightarrow \text{Supernatant} \]

(Toxic) (Toxic)

Procedure D (successful)

**Toxic Filtrate**

\[ (\text{NH}_4)_2\text{SO}_4 \] to a final concentration of 60% saturation kept in fridge overnight.

\[ \text{Precipitate} \rightarrow \text{Supernatant} \]

(Toxic) (Non-Toxic)

Suspended in 0.01 M Sodium Acetate Buffer at pH 5.5.

Centrifuged at 4,500 RPM for 45 minutes at 0°C.

\[ \text{Residue} \rightarrow \text{Supernatant} \]

(Non-Toxic) (Toxic)
Finally, after obtaining the high titre concentrated soluble toxin, attempts were made to purify this material by using the following method.

DEAE (Selectacel) Cellulose pretreated with 2 M NaCl solution overnight at 4°C was packed into columns (column size 14 mm. x 725 mm) and was washed with 1 N HCl. Ten ml of the (NH$_4$)$_2$SO$_4$ precipitated toxin (dissolved in 0.01 M sodium acetate buffer at pH 5.5.) was applied to the column, equilibrated with the same buffer. Elution gradient was then tried with 0.01 M sodium acetate buffer at pH 5.5. and 1M NaCl. Sodium chloride provided a steady increase in molarity (from 0. to 0.5 M) in a constant background of buffer at pH 5.5. The elution was carried out at room temperature as well as the packing of the column, washing and equilibration (Fig. 11a, 11b). The encouraging results observed here were further investigated and formed a basis for the successful experimental work. An analogous run with columns equilibrated in pH 4.5 buffer rather than pH 5.5. was carried out. The results (Fig. 12) showed much better resolution of toxic material and subsequent runs were made using buffer at pH 4.5 for both equilibration and elution.
Fig. 11a. Chromatography of botulinus, type E, (NH₄)₂SO₄ precipitated toxin on a DEAE cellulose column.
FIG. 12. Chromatography of botulinus, type E, \((\text{NH}_4)_2\text{SO}_4\) precipitated toxin on a DEAE cellulose column.
CHAPTER IV
Materials and Methods

The strain of *Clostridium botulinum* type E used in this work was isolated by Nakamura et al. (1956) from an outbreak of fish-borne botulism in Hokkaido, Japan. This strain was toxigenic, non-proteolytic, gas producing and named as the Iwanai strain of *Clostridium botulinum*, type E. This strain gave a consistent toxicity of 1,000 - 3,000 MLD/ml when grown in culture tubes of GPB1 medium. (Appendix II).

The strain, maintained as a pure culture in GPB1 medium, was obtained through the courtesy of Connaught Laboratories, Western Division, University of British Columbia, and the stock cultures were periodically checked on Brain Heart Infusion agar plates (Appendix IV).

Tubes containing 15 ml of GPB1 containing 1% thiglycollate and 2% dextrose added aseptically as 50% dextrose were used for the maintenance of stock cultures as well as for the growth of the seed cultures. The cultures were grown at 37°C for 24 hours.

GPB1 media with 1% thiglycollate (DIFCO) was used in large volume toxin production. Fifty percent dextrose was added to the GPB1 media after autoclaving to a final concentration of 0.5%.
(a) Toxin Production
Large volume toxin production involved the use of a dialysis apparatus (Vinet and Fredette 1951) modified in this laboratory in order to cut down bacterial contamination of media from the apparatus as well as from outside (Appendix I, Fig. 1). The cultures were grown in the cellophane sacs (Appendix III, Fig. 2) for five days at 30° C with daily changes of media. The seed culture of 30 ml in two GPBl tubes was incubated at 37° C for 18-24 hours prior to inoculation.

(b) Toxin Precipitation
The Seitz filtered dialysate toxins were treated with saturated \((\text{NH}_4)_2\text{SO}_4\) to a final concentration of 60% saturation for 10-16 hours at 4° C. The toxic precipitate, obtained after centrifugation of the filtrate at 4,500 RPM for 45 minutes at 0° C. was suspended in 0.01M sodium acetate buffer at pH 5.5., and the volume brought to approximately 1/20 the original filtrate volume. This material was dialysed against 0.01 M sodium acetate buffer at pH 5.5. overnight. The insoluble non-toxic material was removed by centrifugation.

(c) Toxin Purification
DEAE (Selectacel) cellulose synthesized and described by Peterson et al (1956) was used as the ion exchange material. This material was suspended in 2.0 M NaCl solution (10 gms of the cellulose per litre of 2 M NaCl solution) and kept at 4° C. Columns of varying lengths with diameter to length ratio of 1/20 were packed under gravity flow at room temperature (Appendix V). The columns were then washed with 1 N HCl and
equilibrated with 0.01M sodium acetate buffer at pH 4.5 at room temperature. Five ml of the concentrated toxin were then applied to the column and eluted frontally with 0.01 M sodium acetate buffer at pH 4.5 at room temperature. Fractions of 5 ml were collected on an automatic fraction collector model V-10 (Gilson Medical Electronics).

(d) **Treatment of Dialysis Paper**

The dialysis paper used in the production of toxins as well as in dialysis was pretreated with boiling 0.1 M Versene adjusted to pH 7.0 for five minutes and washed several times in distilled water.

(e) **Assay Technique**

Serial decimal dilutions of the toxin were made in 0.85% of saline solution depending on the expected toxicity and inoculated intraperitoneally into mice of weight between 15-25 gms with the use of #26 needles. Rough titrations were carried out by using one mouse per dose while five mice per dose were used for an accurate assay. The smallest dose which killed the animal in 48 hours was calculated as the MLD/ml.

(f) **Analytical Techniques**

Various fractions collected during the frontal elution were analysed for 280 m\(\mu\) and 260 m\(\mu\) absorbing material on a Beckman Model DU spectrophotometer using a B.D.C.C. quartz curvette. Sodium acetate buffer of 0.01 molarity and pH 4.5 was used in the standard cell.
Total nitrogen was determined by using the semi-micro Kjeldahl technique (Kabat & Mayer 1948). Boiling chips used in this technique were coated with selenium which replaced copper-sulphate as the catalyst for the digestion process.

A Beckman/Spinco Model E analytical ultracentrifuge and a Spinco Model H electrophoresis diffusion apparatus were used for analysis of the material prepared in this work.

The amino-acid analysis of the purified component was carried out on an automatic amino-acid analyser built by Reichmann (Federal Agricultural Research Station, Vancouver, British Columbia) based on the Moore & Stein (1954, 1956) technique.

(g) Preparation of Buffer solution
Various buffer solutions used in this work were prepared by calculating components with Henderson-Hasselbalch equation (Appendix VI).
CHAPTER V
Results

(a) Degree of purification. The activity per mg.N of the various toxic preparations is summarized in table 3. It can be seen that a two thousand-fold increase in specific activity was obtained during purification.

Table 3.

<table>
<thead>
<tr>
<th>No.</th>
<th>Toxin Preparation</th>
<th>MLD/mgN</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Original Toxic Filtrate</td>
<td>$3.2 \times 10^3$</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>Ammonium sulfate precipitate, after dialysis and centrifugation</td>
<td>$4.9 \times 10^4$</td>
<td>80</td>
</tr>
<tr>
<td>3.</td>
<td>Pure preparation</td>
<td>$7.5 \times 10^6$</td>
<td>50</td>
</tr>
</tbody>
</table>

(b) Elution Chromatography. Ammonium sulfate precipitated toxin, dissolved in 0.01M sodium acetate buffer at pH5.5 and eluted with same buffer at pH4.5, came out frontally. After the passage of 100 ml. of the eluent, two peaks were obtained; the former being extremely toxic and the later peak non-toxic (Fig. 13). The major portion of the non-toxic material remained adsorbed onto the column and would be removed in one step with 1N HCl (Fig. 14) or more slowly using a gradient or stepwise elution. Chromatographed, reprecipitated toxin gave a single peak under the same set of conditions indicating a high level of purity. (Fig. 15) Final pure
Chromatography of botulinus type E, $(\text{NH}_4)_2\text{SO}_4$ precipitated toxin on a DEAE cellulose column, pretreated with 2M NaCl, washed with 1N HCl; equilibrated and eluted with 0.01 M sodium acetate buffer at pH 4.5.
Chromatography of botulinus type E (NH₄)₂SO₄ precipitated toxin (same conditions as in Fig. 13). This figure shows the elution of the adsorbed non-toxic material with 1N HCl from the column after elution of the toxin.
Fig. 15. Rechromatography of botulinus type E, chromatographed and reprecipitated toxin. The figure shows a single sharp peak.
preparation contained $7.5 \times 10^6$ MLD/mgN and the yield was 50%.

(c) **Ultra-Violet adsorption spectrum.** The purified toxin showed a UV absorption spectrum typical of most proteins with maximum absorption at 277 nm (Fig. 16). The absence of any subsidiary absorption at other wavelengths has indicated at least the lack of non-protein impurities.

(d) **Ultracentrifugal Analysis.** The ultracentrifugal analysis showed the presence of a single, apparently homogeneous component in an artificial boundary cell, run at 59780 RPM. Schlieren photographs were taken at sixteen minute intervals (Fig. 17). The sediment coefficient was calculated as $S_{20}^W = 1.70$. The shadow of a rapidly moving component was also observed which could indicate a low percentage of impurities or the presence of aggregated molecules.

(e) **Electrophoretic analysis.** The behaviour of the material was studied extensively at pH4.5 in a Spinco - Model H electrophoresis diffusion apparatus and showed the presence of a large single homogeneous component with a mobility of $+5.66 \times 10^{-5}$/cm$^2$/Vol. sec. (Fig. 18). The results also showed a small, immobile peak, which disappeared rapidly and was assumed to be the boundary.

(g) **Diffusion properties.** A Spinco Model H Electrophoresis Diffusion apparatus was used for diffusion properties and the diffusion coefficient was calculated at $4.8 \times 10^{-7}$ cm$^2$/sec. at $1^\circ$ C and $8.87 \times 10^{-7}$ cm$^2$/sec. at $30^\circ$ C over a period of 72 hours.
Fig. 16. Ultra-violet absorption spectrum of pure botulinus type E toxin.
Fig. 17. Ultracentrifugal analysis of botulinus type E, purified toxin. Schlieren photographs were taken at sixteen minute intervals. Ultracentrifuge was run at 59780 RPM.
Fig. 18. Electrophoretic behaviour of botulinus type E, purified toxin.
(71)

hours.

(g) **Molecular weight determination.** The molecular weight was calculated to be 18,600 with the following equation:

\[
M = \frac{RTS_{20}^O W}{D_{20} (1-VP)}
\]

- **M** = Molecular weight.
- **R** = Gas Constant
- **T** = Absolute temperature.
- **S_{20}^W** = Sedimentation coefficient at \(20^\circ C\).
- **V** = Partial specific volume.
- **P** = Solution Density.
- **D_{20}** = Diffusion coefficient at \(20^\circ C\).

(h) **Amino Acid Analysis.** The purified toxin was hydrolysed in vacuo in 6N HCl at \(106^\circ C\) for 18 hours. HCl was removed by freeze-drying and the amino-acid preparation was washed three times in distilled water and flash-evaported at \(42^\circ C\). These preparations were run on Moore and Stein (1954) columns in order to obtain the amino acid ratios. Tryptophane, always destroyed under these conditions, has not as yet been calculated. Methionine and cysteine, which must be calculated as methionine, sulfone and cysteic acid in order to give accurate results on the Moore & Stein (1954) columns, have not yet been evaluated although performic acid oxidations have been carried out on hydrolysates. Results for the remaining amino acids are shown in Table 4.
TABLE 4
The amino acid analysis of type E toxin.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Micromoles per ml.</th>
<th>mg/ml</th>
<th>Molecular ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.137</td>
<td>0.0176</td>
<td>9</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.0152</td>
<td>0.0021</td>
<td>1</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.041</td>
<td>0.0064</td>
<td>3</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.246</td>
<td>0.0283</td>
<td>16</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.072</td>
<td>0.0073</td>
<td>5</td>
</tr>
<tr>
<td>Serine</td>
<td>0.112</td>
<td>0.0098</td>
<td>8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.166</td>
<td>0.0214</td>
<td>11</td>
</tr>
<tr>
<td>Proline</td>
<td>0.095</td>
<td>0.0092</td>
<td>6</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.180</td>
<td>0.0106</td>
<td>12</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.093</td>
<td>0.0066</td>
<td>6</td>
</tr>
<tr>
<td>Valine</td>
<td>0.089</td>
<td>0.0088</td>
<td>6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.147</td>
<td>0.0166</td>
<td>10</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.144</td>
<td>0.0163</td>
<td>10</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.0517</td>
<td>0.0084</td>
<td>4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.074</td>
<td>0.0109</td>
<td>5</td>
</tr>
</tbody>
</table>
CHAPTER VI
Discussion

The basic aims of this research project, i.e., the purification and characterization of botulinus type E toxin, have been successfully accomplished. The problems encountered in the process were mainly the ones connected with the production, concentration and purification of this toxin.

The method of producing toxins in dialysis sacs introduced by Vinet and Fredette (1951) and modified in this laboratory (Appendix III, Fig. 2) was used, and consistent yields of 3,000 to 5,000 MLD/ml of toxic filtrates were obtained. Simplification of the basic technique has significantly cut down on media contamination which was one of the major hurdles in obtaining good yields of toxin.

The inactivation of botulinus toxins during dialysis was noted by Sommer et al (1926, 1928) working with Type A. No explanation was given at that time for this loss of activity. Decrease in toxicity of botulinus type E toxin was also noted during dialysis in this laboratory, and the assumption was made that the toxin was irreversibly destroyed by the surface active compounds present on the cellophane sacs. Pretreatment of the cellophane sacs with 0.1 M Versene at pH7.0 alleviated the negative effect of the surface active compounds with the result that little toxicity was lost during dialysis. It was confirmed that the Versene pretreated cellophane sacs used in the production of dialysate toxins appreciably stabilized the toxic yields.
The dialysate toxins are superior to those produced in flasks as the larger media components are not present. In purification studies, such as those undertaken here, it is imperative to start with filtrates containing as little as possible extraneous matter, particularly proteins. Indeed, the behaviour of flask toxins was markedly different, in ion-exchange chromatography, from dialysate toxins, under the same set of conditions. (Fig. 19). Gordon et al (1957) worked on the flask toxins which contributed to the variance of their results from the ones obtained here. The method used by Sakaguchi and Sakaguchi (1959, 1963, 1964) of extracting type E toxins from washed cells has possible disadvantages to the methods used here. These workers obviously have had considerable difficulty in obtaining their toxic components in a state free from nucleic acids and nuclear-proteins. It is possible that the toxin, before it is released by autolysis from the cells, is linked or aggregated in some way to such cell components.

The superiority of \((\text{NH}_4)_2\text{SO}_4\) over ethanol as a precipitating agent was noted. Ethanol precipitates were essentially insoluble in the various electrolytes tried, while \((\text{NH}_4)_2\text{SO}_4\) precipitates were easily soluble. Ethanol may bring about partial denaturation during precipitation resulting in aggregation. This type of denaturation may be attributed to the lack of adequate electrolytes and to pH's beyond the isoelectric point (Alexander and Block, 1960). Ammonium sulfate, on the other hand, caused no appreciable denaturation and
FIG. 19. Comparative chromatographic behaviour of dialysate and flask toxins of botulinus type E, precipitated and eluted under similar conditions.
aggregation. Sodium sulfate may also be used in precipitation; the advantage being that it does not impart any ammonia to the preparation.

The chromatographed toxic preparation contained 1,000 times more toxicity per mg. N than the crude toxic precipitates. The toxic protein was eluted frontally while the impurities remained adsorbed onto the column. (Fig. 14). Sodium acetate buffer solutions of 0.01 molarity and of pH's ranging from 3.8 to 4.8 successfully eluted the toxic protein. A number of other pH's were tried as well as elution gradients which did not yield the same results. (Figs. 11a, 11b, 12, 20). Diameter to length ratio of the column was found to be very important. It was also observed that the molarity of the buffer solutions used did influence the results. The pH at which precipitated toxins were resuspended was critical with respect to successful elution of pure toxin. Toxic preparations in buffers of pH's other than 5.5 when added to column, either did not give frontal elution or gave frontal peaks containing impurities.

DEAE (Selectacel) cellulose was used as an ion exchange material for chromatography of the concentrated toxin. This cellulose is an anion exchanger and 20-40% of the CH₂OH and/or OH groups on the cellulose molecule form an ether linkage when treated with dichloro-diethyl amino ethyl hydrochloride with the removal of the ions from the ethyl group (Appendix VIII).
Fig. 20. Chromatographic behavior of botulinus type E, (NH₄)₂SO₄ precipitated toxin.
This cellulose was suspended in 2M NaCl and kept at 4° C. (Fig. 21). Columns after packing were treated with 1N HCl till the pH was less than 0.01. This treatment removed excess Na⁺ and replaceable OH groups, imparting to the cellulose excess H⁺. Sodium acetate buffer during equilibration takes up H⁺ and Cl⁻ ions and the cellulose must be converted into the cellulose Cl⁻, CH₃COO⁻ form at pH 4.5, as the total removal of H⁺ and Cl⁻ ions would result in a basic pH. This combination of Cl⁻ and CH₃COO⁻ ions on the cellulose builds up appropriate conditions in sieving, adsorption and ion exchange, which allows the toxin to be frontally eluted while non-toxic material remains adsorbed on the column. This non-toxic material can be removed from the columns by treatment with 1N HCl (Fig. 14). Excess H⁺ decreases the adsorptive capacity of DEAE celluloses; this is why adsorption under pH 4.0 is due to Vander Waal's forces and hydrogen bonding. (Boardman and Partridge, 1955). This explains to a certain extent the inability of DEAE cellulose to give results below pH 3.8. It can be safely emphasized here that the number of Cl⁻ and CH₃COO⁻ ions present on this ion exchange material is important in the elution of type E botulinus toxin in the pure form. Elution patterns for various Clostridium botulinum type E strain toxins have shown similar behavior on DEAE (Selectacel) cellulose treated in this way.

A possible improvement to routine ion exchange chromatography for the purification of proteins is currently being developed in this laboratory. It is hoped that the principles of ion exchange and those of electrophoresis may be combined and exploited maximally (Appendix VII, Fig. 22).
Fig. 21. Changes in pH during the treatment of DEAE Cellulose (OH⁻ form) with 1M NaCl.
The criteria of purity used satisfy the contemporary requirements for the integrity of the purified preparation.

Crystallization was not applied, as this criterion is unreliable. Crystalline proteins do not necessarily contain one protein species as two protein species may be isomorphous and are constituents of one type of crystals. A different set of crystals may also be formed under another set of conditions.

Emphasis was laid on:

1) biological activity per unit N, 2) paper electrophoresis, 3) column-chromatography, 4) ultracentrifugal analysis and 5) diffusion electrophoresis, as the criteria for purity.

The purified toxic protein gave a typical Gaussian curve in the schlieren optical systems used, indicating the presence of a single major component in the electrophoretic and ultracentrifugal studies. An extremely small percentage of impurity was apparent which took the form of a rapidly sedimenting small shoulder which appeared sixteen minutes after full speed was attained in the ultracentrifuge. This was supposed to consist of aggregated toxic molecules and to be insignificant due to its low concentration in comparison to the total protein present. A small immobile peak having the behavior of a boundary, was seen in the electrophoretic analysis which did not
move when the sample was run at pH 4.5 and pH 5.5., and dis-appeared after about thirty minutes. If this immobile boundary peak had been due to a contaminant rather than to an interface reaction, one would have expected to have observed at least a degree of mobility at one of the pH's used for the investigation. Paper electrophoresis and ion exchange column chromatography indicated the presence of one single component in the toxic preparation.

Sakaguchi et al (1964) recently hypothesized the molecular weight of type E toxin to be larger than 200,000. They did not support the results reported by Gerwing et al (1962). These authors joined the galaxy of previous workers who reported very high molecular weights for other botulinus toxins (Kegeles, 1946; Putnam et al, 1946, 1948; Lamanna and Glassman, 1947; Wagman, 1954). The molecular weight determination of ethanol as well as ammonium sulfate precipitated toxic preparations was shown to be less than 20,000 (sedimentation coefficient for both the toxic preparations in the ultracentrifuge being 1.7) in this laboratory.

The data obtained in this work, combined with the critical analysis made of previous research done on the purification of botulinus toxins, indicate that the high molecular weight concept widely accepted for these substances, is probably erroneous.
SUMMARY

1. A method for the purification of the toxic protein produced by botulinus type E, strain Iwanai, has been developed and a preparation has been obtained which appears to be homogeneous under ultracentrifugal and electrophoretic analysis.

2. The sedimentation coefficient of the pure preparation is $S_{20}^0 = 1.7$ and the molecular weight is calculated to be 18,600.

3. Amino acid analysis of the preparation has been carried out, although values for tryptophane, methionine and cysteine are yet to be calculated.

4. Molecular structure of the DEAE (Selectacel) cellulose has been explained and an apparatus is included in the appendices which will be used for future purification work.

5. A modified dialysate apparatus has been built for the mass production of botulinus toxins.
Dialysis apparatus for the mass production of \textit{Clostridium botulinum} toxins was first reported by Vinet and Fredette in 1951.

The apparatus consisted of a pyrex tubing (6.4 x 120 cm.) with two ends made for #9 rubber stoppers. This tube had two outlets, one on either side approximately six inches from each end. The outlet on the lower end was connected to two (six litre) pyrex glass bottles through a T-tube. A loose cotton plug was placed in the upper outlet which served as a pressure release from the glass tubing. Cellophane tubing was inserted into the glass and fixed at either end with rubber stoppers. The saline and innoculum was introduced through T-tube connection in the lower stopper, the other outlet of which was used for sampling and harvesting. A piece of cotton wool plugged glass tubing was inserted through the upper stopper which allowed the escape of gas produced during bacterial growth.

The glass tube with cellophane sac, saline, and discard bottles with respective rubber tubing connections was sterilized for 1-1/2 hours under 15 pounds pressure. The media bottle was sterilized separately for 1 hour at 15 pounds pressure and 230°C. The apparatus was then set up in an upright position in the 30°C incubator. Eight hundred ml of the sterile saline was added to the cellophane sac and the media was pumped with negative pressure into the outside of the
cellophane sac. All the outlets were clamped. The medium was allowed to diffuse through the cellophane sac for overnight and then 40 ml. of a 24 hours' seed culture was inoculated through the saline bottle. Complete changes of medium were made every 24 hours and the cultures were harvested after 80 to 90 hours' incubation at 30°C.
Fig. 1. Dialysate apparatus for the mass production of botulinus, type E toxin.
This medium was used for tube cultures with added meat particles and for the mass production of dialysate and flask toxins.

Finely ground beef (round steak) was infused overnight in tap water at 4°C, and then at 65°C for 45 minutes in the top of a double boiler. Meat particles were taken off by straining through "cheese cloth" muslin and the filtrate was allowed to boil for 3 minutes. The infusion was then cooled to approximately 60°C and filtered through a whatman #1 filter paper in order to obtain fat and meat fibre free preparation. The volume was made up to the original with tap water and the peptone and salt constituents per litre were added as follows:

- NaCl 5 gms.
- Proteose peptone (DifCO) 10 gms.
- Na$_2$HPO$_4$ .12 H$_2$O 2 gms.
- Sodium thioglycollate 1 gm.

The pH was adjusted to 7.8 after dissolving the ingredients.

Fifteen ml. of the medium and 1 gm. of air dried meat particles were dispensed into 20 x 170 m.m. pyrex tubes, plugged with non-absorbent cotton, and sterilized in the autoclave for 30 minutes at 15 pounds pressure and 230°F. The medium for the production of dialysate toxins was dispensed in six litre quantities into 10 litre aspirator, plugged and autoclaved for 90 minutes at 15 pounds pressure and 230°F. Fifty percent
dextrose solution was sterilized at 230°F and 15 pounds pressure, and added to tube medium (2%) and bottle medium (.05%).
### APPENDIX III

**Dialysis apparatus for the mass production of botulinus toxins**

#### Table 5

**Parts of Dialysate Apparatus**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name</th>
<th>Description</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Glass tubings</td>
<td>(a) 5.6 x 90 cms; thick pyrex glass annealed, dialysate column.</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) 6.5 x 75 mms.</td>
<td>7</td>
</tr>
<tr>
<td>2.</td>
<td>Brass plates</td>
<td>(a) outer brass plates, 7.0 cms diameter with 4.45 cms. diameter empty space, 6.5 mms. thick, and two holes on either side (6.5 mm. diameter).</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) inner brass plates, rectangular (7.0 x 2.5 cms) with three holes (one hole in the centre and two on either side of 6.5 mm. diameter).</td>
<td>2</td>
</tr>
<tr>
<td>3.</td>
<td>Rubber corks</td>
<td>(a) outer rubber corks #7 (both have 4.45 cms diameter, hole in the centre. The cork used on upper side of the columns have two extra (6.5 mm. diameter) holes on either side of the central one. The cork used at the lower end have one hole (6.5 mm. diameter) on one side of the central one.</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) inner rubber corks #4 with holes in the centre (6.5 mm. diameter)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) rubber cork for the discard bottle #5 with two holes (6.5 mm. diameter) in the centre and 25 mms. apart.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d) rubber cork #2 with 6.5 mm. diameter hole in the centre.</td>
<td>1</td>
</tr>
<tr>
<td>4.</td>
<td>Pressure Rubber Tubings</td>
<td>(a) Six inches long for connecting media and toxin outlets</td>
<td>2</td>
</tr>
</tbody>
</table>

* Modified from the original of Vinet and Fredette (1951)
Table 5 (continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5. Aspirators</td>
<td>(a) Media bottle, 10 litres capacity</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(b) Media discard bottle, 8 litres capacity</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(c) Saline bottle, two litres capacity</td>
<td>1</td>
</tr>
<tr>
<td>(b) Two feet long for connecting media and saline inlets</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>6. Cellophane Sac</td>
<td>3-1/2 feet long, versene pretreated</td>
<td>1</td>
</tr>
<tr>
<td>7. Brass rods</td>
<td>3-3/4 feet long, 6 mm. diameter</td>
<td>2</td>
</tr>
<tr>
<td>8. Screws</td>
<td>(a) For screwing the inner plates</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>(b) For screwing the outer plates</td>
<td>4</td>
</tr>
<tr>
<td>9. Stopcocks</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

After the assembly, the dialysate apparatus (Fig.2) is autoclaved according to the specifications given in Table 6.

<table>
<thead>
<tr>
<th>Name</th>
<th>Temperature</th>
<th>Time in Minutes</th>
<th>Pressure in pounds/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assembled Dialysate Columns</td>
<td>230° F</td>
<td>120</td>
<td>15</td>
</tr>
<tr>
<td>Medium Bottle</td>
<td>230° F</td>
<td>90</td>
<td>15</td>
</tr>
<tr>
<td>Saline Bottle</td>
<td>230° F</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>Discard Bottle</td>
<td>230° F</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>50% Dextrose Solution</td>
<td>230° F</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

Various connections are made aseptically after autoclaving. Eight hundred ml of saline is then passed into the cellophane sac and the media is filled outside up to the inside
level of saline. After 18-24 hours, media is discarded and the dialysate is innoculated with 24 hours tube cultures; two tubes of which are added aseptically to the saline bottle and passed into the cellophane sac. The outside of the sac is again filled with medium which is changed after every 24 hours for the first three days while only a half change is made during the last two days of incubation. The toxin is harvested after incubation for five days at 30°C.
Fig. 2. Modified dialysate apparatus for the mass production of botulinus type E toxin.
**APPENDIX IV**

**Brain Heart Infusion Agar**

The dehydrated medium was rehydrated by dissolving 37 gms in 1,000 ml of distilled water. This medium with 1.5% agar was autoclaved for 15 minutes at 15 pounds pressure and 230° F. Eighteen ml. of the cold (45°C) medium was aseptically poured into each plate.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Ingredients/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf brains, infusion from</td>
<td>200 grams</td>
</tr>
<tr>
<td>Beef heart, infusion from</td>
<td>250 grams</td>
</tr>
<tr>
<td>Proteose Peptone, Difco</td>
<td>10 grams</td>
</tr>
<tr>
<td>Bacto-dextrose</td>
<td>2 grams</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5 grams</td>
</tr>
<tr>
<td>Disodium Phosphate</td>
<td>2.5 grams</td>
</tr>
</tbody>
</table>
APPENDIX V

Preparation of the ion exchange chromatographic column used for the elution of Clostridium botulinum type E, strain Iwanai toxin.

DEAE Cellulose (Selectacel) is obtained commercially in the OH form. Ten grams of this material was suspended in one litre of 1N HCl for about one hour at room temperature after which the HCl was removed by negative pressure. The material was washed three times in distilled water and a homogeneous suspension was then made in 1N NaOH. This process was repeated three times and the material was finally suspended in one litre of 2M NaCl and kept at 4°C.

The acid cleaned and distilled H₂O washed glass columns with sintered glass at their lower ends were partially filled with 2M NaCl solution to which the cellulose preparation was added, and the columns were packed by gravity flow the subsequent addition of cellulose and 2M NaCl.

The packed columns were then washed with 1N HCl till the pH dropped to 0.01. The columns were then ready for equilibrating and for further use, etc.
APPENDIX VI

Henderson-Hasselbalch Equation

\[ \text{pH} = \text{pKa} + (\log \frac{\text{salt}}{\text{acid}}) \]

where \( \text{pH} = \) hydrogen ion concentration.
\( \text{pKa} = \) ionization constant.
\( \text{salt} = \) salt concentration of the buffer
\( \text{acid} = \) acid concentration of the buffer

pKa values equals pH when the acid in solution is half neutralized. The pH is about one unit lower when 10% acid is neutralized and pH is one unit higher when 90% acid is neutralized. One can pick buffers which will maintain the pH relatively constant in the desired range and the choice is based on their pKa values.
APPENDIX VII

ELECTRO-CROMATOGRAPHIC APPARATUS

This apparatus is being devised in this laboratory for the separation of proteins and polypeptides. The principal of electrophoresis and ion exchange chromatography is combined in order to achieve better and faster analysis (Fig. 22).

The apparatus consists of a chromatographic column (ion-exchange material packed inside). This column has two buffer inlets and two buffer outlets on either side with a specimen inlet in the centre. Two electrodes are fitted on either side and the whole apparatus is inserted in an outer thick pyrex glass tube.

The specimen is applied in the centre and the apparatus is placed horizontally on two fraction collectors. After the specimen is absorbed; an adequate current potential is passed through the column. The elution is then carried out with an adequate buffer by running the column on one fractionator and closing all the other outlets and inlets. Then the column is run on the other fractionator; the previous outlets being closed now.

The details of this apparatus are yet to be worked out as it is still in the preliminary stages of development.
Fig. 22. Electro-Chromatographic Apparatus
Molecular Structure of Derivative Aminoethyl Cellulose (Chloride Form)
PREPARATION OF D.E.A.E. CELLULOSE (OH FORM)

CELLULOSE

+ \[\text{Dichloro Diethyl Amino Ethyl Hydrochloride} \]

\[\text{Diethyl Amino Ethyl Cellulose} \]

(Selectacel OH Form)

Appendix VIII cont'
D.E.A.E. CELLULOSE (O₂⁻ FORM)

\[ + \text{NaOH} \]

(C) EQUILIBRATION \[ \frac{(0.01M) \text{CH}_3\text{COONa}}{\text{buffered at pH 4.5}} \]

D.E.A.E. CELLULOSE (CH₃COO, Ω⁻ FORM)

(d) WASHING \[ \text{NaCl} + \text{HCl} + \text{CH}_3\text{COOH} \text{ in } \text{HCl} \]

D.E.A.E. CELLULOSE (Cl· FORM)

\[ + \text{CH}_3\text{COOH} \text{ (acetic acid)} \]

APPENDIX VIII
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