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Title: CHARACTERIZATION AND DETECTION OF CLOSTRIDIUM BOTULINUM TYPE A TOXIN

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The toxin of Clostridium botulinum 78A was found to make its first detectable appearance in the culture medium during the latter part of the log growth phase. The appearance of the toxin and a concomitant decline in pH were discussed in relation to the sporulation process. Toxin located intracellularly with ferritin-labeled antibody during this phase of the log period was mainly cytoplasm-associated.

Type A toxin was isolated by acid precipitation and purified electrophoretically. The 78A toxin was shown to possess a molecular weight of 81,000 by SDS-polyacrylamide molecular weight analysis. Also, using the relatively new technique of electrophoretic isoelectric focusing, the isoelectric point of the toxin was found to be near pH 5.23. Antitoxin produced against the toxin exhibited only one precipitin arc against culture supernatants of the type A organism; specificity was also denoted by the antitoxin's inability to cross-react in immunodiffusion with toxin types B, C, E, or F.

Serological methods of detecting toxin were examined in this study. The latex fixation method proved too insensitive, but the new and little

known method of electroimmunodiffusion was found to combine all the criteria necessary for a valuable in vitro detection system. With the combination of the specific rabbit antitoxin and electroimmunodiffusion, as little as 0.7 $LD_{50}$ could be detected in $1\frac{1}{2}$ hours.

# Characterization and Detection of *Clostridium botulinum* type A Toxin

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# CHARACTERIZATION AND DETECTION OF *CLOSTRIDIUM BOTULINUM* TYPE A TOXIN

## INTRODUCTION

At present the accepted method for detection and identification of *Clostridium botulinum* toxin is demonstration of toxicity in laboratory animals, usually mice, and neutralization of the toxicity with serologically specific botulinum antitoxins. The detection method, although specific and sensitive, is inadequate insofar as it is extremely time consuming in relation to the ominous consequences of botulinum poisoning. By the time laboratory confirmation occurs and treatment begins, 6-48 hours, the patient may already have succumbed or be seriously ill. The primary purpose of this investigation, therefore, was to develop new or adapt existing methods for detection of botulinum toxin that would decrease the time of diagnosis but would maintain the specificity and sensitivity of the mouse test.

The main problem involved in the development of such a detection technique is sensitivity, and it is for this reason as well as for the physiological bias of an *in vivo* response that the mouse test has been retained; botulinum toxin is unquestionably the most potent physiologically active toxin known. Therefore, any new detection method developed must be extremely sensitive. Since most serological tests meet combinations of the requirements of an *in vitro* test, a serological detection method which would meet all three requirements plus a fourth, quantitation, was sought. Serological methods such as immunodiffusion, bentonite flocculation and passive hemagglutination have proved inadequate on the basis of one or another of the criteria of speed, sensitivity or

specificity. Another reason for their inadequacy, however, has been that the present supplies of botulinum antitoxin have been developed primarily for the in vivo neutralization test. These antitoxins give rise to cross reactions in vitro because of the deficient methods of toxin isolation and purification presently used in their development. Also, because of the nature of equine sera, the antitoxins are unreliable for tests involving precipitation. Therefore, another purpose of this study was to develop a more specific antitoxin. The antitoxin was used in conjunction with electroimmunodiffusion, a little known, recently developed immunoelectrophoretic technique, which combines all four criteria necessary for toxin detection—specificity, sensitivity, expediency, and quantitativity.

Development of an antitoxin was contingent upon improvement of the methods of toxin purification. Present methods leave much to be desired in regard to purity, and this problem was attacked through the technique of electrophoresis.

Finally, although C. botulinum has been investigated for many years with respect to its role in food processing, characterization of the toxin it produces is limited. The molecular weight of the molecule remains in dispute, the physiological process of toxin production is yet unknown, and the mode of action of the toxin rests in dispute. Two of these problems, molecular weight and detection of the toxin in the bacterial cell, were investigated in this study.

# LITERATURE REVIEW

## Botulinum Epidemiology

The deadliest and fortunately rarest type of food poisoning is termed botulism. Botulism is caused by ingestion of a toxin produced by Clostridium botulinum, a gram-positive, anaerobic, spore-forming, rod-shaped, soil inhabiting bacterium. There are presently six recognized types of toxin, A, B, C, D, E, and F; Grimenez and Ciccarelli (1967) have, however, reported a new toxin type produced by a Clostridium botulinum isolated from an Argentine soil sample. The new toxin, reportedly, is not neutralized by types A, B, C, D, E, or F antitoxins. The toxins are differentiated solely on the basis of their serological specificity, but types A, B, E, and F generally have been involved in cases of human food poisoning while types C and D have been found primarily in cases of poisoning in lower animals.

Botulism is unique since the toxin is reported to attack the nervous system, in as yet an unproved fashion, rather than the gastrointestinal tract as do the more common agents of bacterial food poisoning, staphylococcal enterotoxin and salmonella enterotoxin. Similar symptoms are produced by all the serological types of botulinum neurotoxin which affect man and they may include vomiting, constipation, double vision, thirst, and difficulty in swallowing within 12-24 hours (Gordon and Murrell, 1967). Within 12-60 hours neurological symptoms appear including blurred vision, dilated pupils, and respiratory paralysis. In fatal cases death is caused by asphyxiation.

contamination. The foods incriminated in botulism outbreaks almost invaribly are given inadequate preliminary treatment such as heating, salting, smoking, drying, or pickling (Gordon et al., 1967). The food is then allowed to stand at a temperature that will permit the growth of C. botulinum with concomitant production of toxin and eaten without being cooked. Although the "botulinum cook" for low-acid, uncurred canned foods ensures a high degree of safety, newer methods of food processing such as freeze-dehydration, vacuum packaging, and pasteurization by irradiation may contribute to an increase in the threat of botulism. Even though these so-called "convenience foods" (Insalata et al., 1968) are generally frozen or maintained in a dry state until preparation and are, in all probability, quite safe when prepared and used as directed, mishandling can result in disaster if the food contains viable C. botulinum spores. And such is the case; Taclindo et al. (1967) and Insalata et al. (1968) have found incidences of C. botulinum spores in "boil-in-the-bag," vacuum packaged, pressurized, and dehydrated foods. Furthermore, pasteurization doses of irradiation have been found to be ineffective in eliminating type E spores (Segner and Schmidt, 1966; Graikoski, 1968), and Walls and Bell (1968) have even noted the appearance of increased levels of type F toxin in irradiated fish samples as compared with unirradiated controls. Even sterilizing doses of gamma irradiation have in one case (Fernandez, Tang, and Grecz, 1969) proved inadequate in ridding ground beef of spores or inactivating the type A toxin already present.

Botulism remains, and probably will be for quite sometime, the deadliest potential hazard of commercial food processors. Unfortunately

interest in it as a hazard increases and declines in direct proportion to its reported incidence, and suggestions for application of new information in sanitation and processing technology often go unheeded (Lewis and Hall, 1968). For these reasons quick and reliable methods for the detection of botulinum toxin "after the fact," so to speak, are necessary as a safeguard measure.

## Isolation, Purification, and Characterization of Clostridium botulinum Toxin

Maximal amounts of botulinum toxin have been shown to appear in the culture of C. botulinum after autolysis, from the eighth to the fifteenth day of incubation (Boroff, 1955; Bonventre and Kempe, 1960a), although mechanical disruption prior to natural autolysis results in a release of considerable quantities of toxin. These investigators suggested on the basis of their kinetic data that toxin production in C. botulinum is very closely related to the process of autolysis, a general and long known characteristic of the genus Clostridium. Also, from results of later investigations conducted to study the effect of the metabolic inhibitors, penicillin, chloramphenicol, versene, and dinitrophenol on toxin production, Bonventre and Kempe (1960b) found that culture growth and appearance of toxin in filtrates are not parallel processes, and only after autolysis of the culture has proceeded to an advanced stage is maximal toxicity of the filtrate obtained.

Investigations on the localization of toxin in situ have been meager. The report by Schantz (1964) that European workers had determined the toxin to be a lipoprotein lent credence to a hypothesis that the toxin

might be membrane-associated, but many investigators in the United States have consistently demonstrated the proteinaceous character of the toxin (Lamanna, 1959). Also, the finding (Gerwing, Morell, and Nitz, 1968) that the ribosomal pellet contained appreciable levels of toxin led to initial speculation that the toxin was associated with the ribosomes. However, sucrose gradient studies showed the major toxic portion not to be ribosome-associated. Nishida and Nakagawara (1965), having observed a relationship between sporulation and toxigenicity in Clostridium novyi and later in Clostridium perfringens (Nishida, Seo, and Nakagawa, 1969), postulate that this relationship commonly exists in C. botulinum. Nevertheless, as Schaeffer (1969) suggests, the question remains yet unanswered what the role of these toxic proteins is in the physiology of the cells which produce them.

Although not a documented fact prior to the experiments of Boroff (1955) and Bonventre et al. (1960a), the use of post-logarithmic culture filtrates had been a precondition on which rested early attempts at toxin isolation. With such filtrates Snipe and Sommer (1928) achieved the first concentration of C. botulinum type A toxin by acid precipitation at pH 4.0. Subsequently Sommer (1937) found that the toxin could be extracted from the precipitate in 0.075 M sodium acetate. The acid precipitation method was improved upon by Lamanna, Eklund and McElroy (1946) after Lamanna and his co-workers determined the toxin to be a heat-coagulable protein having the solubility properties of a globulin. They included shaking with chloroform, and crystallization from ammonium sulfate. Duff et al. (1957) suggested another method of preparation including acid precipitation at pH 3.5, extraction of the

toxin in 0.075 M calcium chloride, precipitation with 15% ethanol at -5 C, and finally crystallization from 0.9 M ammonium sulfate.

In 1948 Putnam, Lamanna and Sharp began investigations on the physical and chemical properties of the crystallized toxin. Putnam et al. (1948) reported electrophoretic homogeneity with the use of a Tiselius moving boundary apparatus. They also found that larger yields of toxin could be made by omitting their earlier suggested chloroform shaking step. Diffusion and sedimentation data were also tabulated and were used to calculate the apparent frictional ratio, 1.76, and the molecule was determined to have a molecular weight of 900,000. Quantitative precipitin studies also indicated that the toxin was serologically a single substance.

The high molecular weight of the toxin reported by Putnam et al. (1948) became something of a puzzle since diffusion through the gut or absorption via the oral canal had been the presumed mode of entry of the toxin in gaining access to the myoneural junction. The situation became more confused when Lamanna and Glassman (1947) reported the molecular weight of type B toxin as only 60,000; and at about this same time, Buehler, Schantz and Lamanna (1947) calculated the molecular weight of type A toxin to be 45,000 on the basis of cysteine content. It had seemed reasonable to assume that the active units of such toxicologically similar substances should be similar in molecular size, and the latter reports seemed to support this reasoning. Wagman and Bateman (1951) set about to elucidate the situation by trying to understand the behavior of the type A toxin in the ultracentrifuge in buffers at various pH's and ionic strengths. They found that over the range pH 3.98-

1.7 the toxin was monodisperse with a molecular weight of 940,000, while in the isoelectric region (assumed to be pH ~5.5) the material became polydisperse. At pH 7.5 a 7 S component (mol wt 160,000) was observed. However, in a subsequent paper Wagman and Bateman (1953) reported a different finding. They demonstrated that at pH 7.5, type A toxin exists in the form of two clearly distinguishable polydisperse components, one nontoxic hemagglutinin-associated component of average molecular weight of the order of magnitude 13 S, and another component with a molecular weight of at least 70,000. The 70,000 molecular weight component was tentatively identified as the toxic unit because of its highly toxic nature and because it was free of hemagglutinating activity previously shown by Lamanna (1948) to be associated with the early toxin preparations. The 7 S molecule discovered in Wagman's earlier paper (Wagman et al., 1951) was not mentioned. It is interesting to note at this point the failure of Tiselius electrophoresis in an earlier investigation (Putnam et al., 1948) to separate these components.

The possibility that the toxic moiety might be even smaller than 70,000 as reported by Wagman et al. (1953) was suggested in a study of the particle size of type A toxin in body fluids of injected rabbits (Hildebrand, Lamanna, and Heckly, 1961). They found that toxin which appeared in the lymph had a significantly lower sedimentation constant in the range between 4.4-11.4 S. This finding prompted Wagman (1963) to study the susceptibility of the proteinaceous toxin to proteolytic enzymes. He concluded that the 900,000 molecule whether normally produced by the C. botulinum organism or appearing as an artifact of the purification procedures is highly resistant to degredation by pepsin.

However, the smaller 158,000, 7 S, molecule could be degraded to fractions in the range of 4.1-4.6 S.

In 1964 Gerwing et al. developed a method whereby type E toxin could be isolated in a highly purified state by the use of acidified diethylaminoethyl (DEAE) cellulose. The toxin isolated in this manner appeared to be almost electrophoretically and ultracentrifugally homogenous; a small percentage of impurity was noted. The toxic component was also shown to possess a molecular weight of 18,000. Although type E toxin was known to be somewhat unique when compared with type A and B toxins because it undergoes an activation phenomenon when exposed to bacterial proteolytic enzymes (Sakaguchi and Tohyama, 1955) or trypsin (Duff, Wright, and Yarinsky, 1956), Gerwing and her co-workers (Gerwing, Dolman, and Bains, 1965) thought it logical to investigate type A toxin using DEAE cellulose chromatography. With this method, a 12,000 molecular weight type A component retaining 50% of the original toxic activity was isolated. Subsequently, authors of a series of papers from the Albert Einstein Medical Center, Philadelphia (DasGupta, Boroff, and Rothstein, 1966; DasGupta and Boroff, 1967; DasGupta and Boroff, 1968) have all refuted Gerwing's findings and report that the major toxic component isolated by DEAE cellulose has a molecular weight of 150,000. Most recently, Knox, Brown and Spero (1970) concluded from elution on DEAE cellulose and Sephadex G-75 that C. botulinum type A does not produce a toxic 12,000 molecule. They found no free toxic 12,000 molecular weight material in culture supernatants and obtained none in the isolative procedure. They suggest that the low molecular weight material of Gerwing et al. (1965) probably arises from proteolytic digestion

subsequent to release from the cell. The possibility of the presence of the small molecular weight unit probably should not be dismissed, however, since it has also been observed in studies involving agar diffusion. Schantz (1964), while measuring the diffusion rate of the toxin, found that the toxic component moved much faster than the hemagglutinin. And on the basis of its diffusion rate in agar he calculated the molecular weight of the toxin component to be between 10,000 and 20,000.

Probably the best analysis of the whole molecular weight question is given by Schantz (1967) in his discussion of the chemical and physical properties of C. botulinum type A toxin in culture. He reports that an analysis of the molecular weight distribution of toxin in the ultracentrifuge indicates that at least 80% of the toxicity is in the high molecular weight forms with the remainder having various degrees of molecular weights down to 10,000 or less. The smallest repeating unit containing cystine is 27,400 and the smallest containing cysteine, 26,700. From this information he concludes that a unit with a molecular weight significantly less than 15,000 could not be considered a subunit of a true polymer. However he did not exclude the possibility of mixed polymers with low molecular weight units. Since no definite knowledge is available on the size of the molecule formed in situ, aggregation or the reverse, disaggregation, may be greatly influenced by the nature of the culture medium and isolative procedures employed.

Although the molecular weight of the toxin is still in a state of discussion, several attempts have been made to assess the amino acid composition of botulinum toxin type A in hopes of explaining the extreme

toxicity of the molecule. After isolation of the toxin in crystalline form, Buehler et al., (1947) hydrolyzed the material and subjected it to microbiological assay for determination of the amino acid content; data were based upon a molecule of 900,000 molecular weight. No obvious explanation for the toxicity was found, although aspartic acid, tyrosine, and threonine were present in larger amounts than previously had been found in other proteins. The amino acid composition of crystalline type A toxin (900,000 mol wt) was investigated again later using more accurate and modern chromatographic methods now available (Stefanye, Schantz, and Spero, 1967). These workers confirmed Buehler's earlier data, although the abundance of the amino acids phenylalanine and glycine differed. Gerwing's group (Van Alstyne, Gerwing and Tremaine, 1966) also analyzed the amino acid content of their 12,000 molecular weight isolate and compared their results with Buehler et al. (1947). Although discrepancies existed between the two analyses, Van Alstyne et al. (1966) reported a remarkable similarity in the overall ratios between amino acids. One interesting finding not demonstrated by the other analyses was that few or no tryptophan residues were found in the molecule. A simple calculation, however, shows this finding to be possible. Buehler et al. (1947) demonstrated 82 moles of tryptophan per molecule on the basis of a 900,000 molecular weight unit. Therefore, approximately only one tryptophan residue should be expected for a molecule as small as 12,000.

Attention has been directed specifically toward the amino acid tryptophan and the correlation of fluorescence with toxicity. Early work on the formation of toxoid by treatment of the toxin with formalin demonstrated that the toxicity is lost with concomitant condensation of

two molecules of tryptophan in two molecules of toxin (Fraenkel-Conrat, Brandon, and Olcott, 1947). Another finding (Mager, Kindler, and Grossowicz, 1954) is that C. botulinum, to be toxigenic, requires in the culture medium ten times the amount of tryptophan needed for growth only. Work by Boroff (1959) in this area showed that all reagents and procedures which reduce fluorescence also destroy toxicity. Later, in an attempt to be selective for tryptophan without affecting other structures in the molecule or cleaving peptide bonds, Boroff and DasGupta' (1964) used a photooxidative method described earlier by Weil, Gordon, and Buchert (1951). The reaction involves photocxidation by visible light upon catalysis with methylene blue and was reported to modify the five amino acids, tryptophan, tyrosine, histidine, methionine, and cysteine (Weil et al., 1951). By this process Boroff et al. (1964) succeeded in modifying tryptophan and methionine in the toxin with a resultant 99% loss in toxicity. Since many methionine molecules could be selectively destroyed with $H_2O_2$ without loss in toxicity, the investigators concluded that tryptophan loss evidently damages the toxin molecule. No attempt, however, was made to assay for cysteine, another amino acid modified by this technique, nor were changes in secondary or tertiary structure taken into consideration.

## Detection of Clostridium botulinum Toxin

Probably the first recorded instance of a detection test for botulinum toxin was made by van Ermengen of Ghent in 1897 when he isolated an anaerobic spore-bearing bacillus and injected culture filtrates into various species of laboratory animals and observed fatal paralysis

(Dolman, 1964). No doubt he was conditioned by the already traditional examples of tetanus and diphtheria testing in animals, but his experimentation with detection techniques was also limited by the experimental tools at hand, namely animals known to be susceptible to bacterial toxins. For more than 70 years variations of this prototype bicassay have been accepted and have become so-called "standard procedures" as in the most recent NCDC handbook, Laboratory Methods in Anaerobic Bacteriology (Dowell and Hawkins, 1968) for the detection of botulinum toxin in food samples or in a patient's serum. Needless to say, no method of bioassay has ever been shown to be entirely satisfactory, and several distressing features of the botulinum mouse assay are poignantly discussed by Petty (1964). Although the test was originally designed for the examination of semi-purified toxin in culture filtrates, results are not as neat and clear when material to be tested consists of a slurry of food which may contain toxic materials other than the suspected botulinum toxin. This point has also been investigated recently by Segner and Schmidt (1968) in a study of nonspecific toxicities in the mouse assay test. Toxic amines, ammonia, and products of microbial degradation from other bacteria were listed as some of the reasons for nonspecificity of the test, but the majority of the nonspecific toxicities encountered were caused by bacterial infections initiated by intraperitoneal injection of the food supernatants. Petty (1964) further pointed out that such a bioassay is a very slow process, requiring almost an hour for preparation and eight to 48 hours for results. His final objection to the assay method is the inavailability of mice in a general laboratory. Many laboratories do not keep mice in stock, and mice

possessing the age and weight requirements of the bioassay do not have a very long shelf life.

Since workers (Hottle, Nigg and Lechty, 1946) early demonstrated that the toxin was antigenic, Lamanna and Doak (1947) investigated the titration of purified toxin with an antigen-antibody tube precipitin technique. The method was found to be very quantitative, but the investigators did not proceed to apply the technique to detection of toxin in food; their interest in the method was from a purely analytical viewpoint. No real progress was made in a rapid assay procedure until Lowenthal and Lamanna (1951) suggested utilization of the hemagglutinin reaction, based upon the earlier findings (Lamanna, 1948) that hemagglutinating activity and toxicity were associated. Unfortunately results of the effects of heat, storage, acid, and formalin treatment indicated that the hemagglutinin and the toxin were not one in the same entity. These findings limited the sensitivity and reliability of such a test. As mentioned previously in the review of the toxin's molecular weight, it was just at about this time that the toxin was shown by Wagman and Bateman (1953) to be composed of two unique and separable fractions, a hemagglutinin and the toxin.

Following the reasoning that goldfish have been used extensively in laboratories as animals for testing toxicity of chemicals, Crisley (1960) investigated the possibility of a goldfish botulinum assay. Although the goldfish succumbed to the toxin, the sensitivity of the assay was low and adoption of a 72-hour endpoint to obtain optimal sensitivity and reproducibility made such an assay prohibitive as a rapid detection device. The procedure also introduced the problem of goldfish maintenance in the laboratory.

Several other investigators, determined to keep a physiological bioassay, have experimented with a rapid *in vivo* method. After observation that intravenous injection of toxin into mice, rather than the classical intraperitoneal route, resulted in death of the animals in minutes, Boroff and Fleck (1966) concluded the method had little value in toxin detection, but was of more value in toxin titration. This opinion is also held by Japanese investigators of this method (Sakaguchi, Sakaguchi, and Misashi, 1968). They determined the precision of the method to be lower in food samples than in culture filtrates and results were made complex by the presence of other toxic substances in foods.

Although the fluorescent antibody (FA) technique had been utilized with great effect in other areas of bacteriology in the period between 1940 and 1960, it was not until the 1960's that attempts (Boothroyd and Georgalla, 1964; Walker and Batty, 1964; Midura *et al.*, 1968) were made to adapt the technique to the detection of *C. botulinum*. Each investigating group showed the technique was capable of detecting and differentiating either spores or intact cells in food specimens, but the method was of no value in detecting the soluble toxin which may remain long after the germination or disintegration of spores and the autolysis of cells. No assays of toxin in serum were conducted for this reason.

Serological detection methods other than the FA technique have also been investigated for their sensitivity and reliability in the detection of the toxin. These include the promising serological detection studies of types A, B, and E botulinum toxins by passive hemagglutination and bentonite flocculation (Johnson *et al.*, 1966); the

latter is least plausible. Bentonite particles sensitized with the respective antitoxins showed low sensitivity for detection of the toxin. However, formalinized sheep red blood cells sensitized with respective antitoxins detected as little as 0.75 to 1.3 $LD_{50}$ of type A toxin and 2.3 $LD_{50}$ of type B toxin in culture filtrates. The authors intended to conduct further studies to determine the applicability of the technique for detection of toxin in foods, but nothing has been reported in the literature to date. However, although nonspecific hemagglutination was not reported by these investigators, a major problem encountered in the use of formalinized, sensitized erythrocytes has been that of nonspecific hemagglutination (Butler, 1963).

Immunodiffusion has also been suggested for the detection of toxins by several workers (Anderson and Niedermeyer, 1968; Vermilyea, Walker, and Ayres, 1968). Anderson et al. (1968) combined a preparative technique with immunodiffusion and reported that detection of botulinum toxin could be obtained within 18 hours while a total time lapse of 48 hours was necessary for identification of the specific toxin; no quantitative analysis was conducted. Vermilyea et al. (1968) cut the detection and identification time down to 24 hours, but the method was found to be fairly insensitive. The least amount of toxin detectable was 630 $LD_{50}$ for type A toxin, 370 $LD_{50}$ for type B toxin, and 560 $LD_{50}$ for type E toxin.

It is clear from the above that much interest has been vested in obtaining a rapid assay method for detection of botulinum toxin to replace the time honored, but slower than time, mouse test. However, most, if not all, methods have met with inadequacy on the basis of the final tests of time, sensitivity, or reliability.

# MATERIALS AND METHODS

## Culture Conditions

### The Organism

Clostridium botulinum type A (Strain 78A) was used almost exclusively in this study since it was shown to produce spores necessary to initiate cultural growth and the desired toxin, both in relatively good yields. Preliminary investigations of other Clostridium botulinum type A stock cultures showed these organisms to produce low yields of either toxin or spores. C. botulinum 78A was obtained in a lyophylized state from the National Canners Association Western Research Laboratory, Berkeley, California. However, the organism was originally isolated from spinach at the G. W. Hooper Foundation Bureau of Chemistry, San Francisco, California. Stock cultures of this strain were subsequently maintained in Stock Meat Medium: 5.0% Trypticase (BBL), 0.5% Peptone (Difco), 0.5% glucose, 3 gm ground beef/10 ml liquid, pH 7.0 in the Food Microbiology Laboratory at Oregon State University.

Other Clostridium botulinum strains used for cross-reaction tests were obtained from the sources indicated in Table I, and each has been maintained in the Stock Meat Medium.

### Anaerobic Incubation

All cultures were incubated anaerobically in a Jasper P. Marsh Model 36501 anaerobic chamber (National Appliance Co., Portland, Oregon). The chamber was evacuated and flushed twice with 99.7% $N_2$:0.3% $CO_2$, and cultures were maintained in this gaseous environment at 30 C.

TABLE I. SOURCE AND TOXIN TYPE OF CLOSTRIDIUM CULTURES.

| *Clostridium botulinum* Toxin Type | Strain | Original Source |
|---|---|---|
| A | 33 | Natick Laboratories, Natick, Mass. |
| B | 115 | National Canners Association, Western Research Laboratory, Berkeley, Calif. |
| B | 213 | National Canners Association, Western Research Laboratory, Berkeley, Calif. |
| C | 11772 | University of California, San Francisco, Calif. |
| E | VH | Continental Can Company, Chicago, Ill. |
| F | Langeland | Communicable Disease Center, Atlanta, Ga. |

Growth Medium

The medium employed to initiate growth from stock meat cultures contained the following constituents: Trypticase, 5.0%; Peptone, 1.0%, Yeast Extract, 0.5% (Difco); glucose, 0.5%; Thioglycollate (Difco), 0.01%. The medium (pH 7.2) was prepared, 15 psi for 15 min, and immediately cooled to room temperature in an ice bath.

Sporulation Medium

This medium was used for preparation of spore stocks and contained the same components as the Growth Medium except the glucose level was reduced to 0.01% and sterile thiamine-HCl was added to effect a concentration of 0.01% after sterilization of the medium as suggested by Day and Costilow (1964).

Spore Stock Preparation

A volume (0.5 ml) of the culture in Stock Meat Medium was transferred to 100 ml Growth Medium; the culture was incubated anaerobically and allowed to attain early log culture phase (14 hr, $OD_{600} \sim 0.75$). A volume (0.5 ml) of this log culture was transferred to fresh Growth Medium and the new culture was incubated to early log phase (5 hr). Five milliliters of the second log culture was transferred to one liter of the Sporulation Medium. The sporulation culture was incubated anaerobically 96 hr with periodic phase microscopy sporulation checks. An incubation period of 72 hr was determined to give almost complete sporulation (93.4% spores in 20 microscope fields), but the spores continued to remain inside the sporangia even after 10 days of incubation and thus necessitated spore cleaning.

Spore Cleaning

Spores were freed from their sporangia by a modification of the method of Grecz, Annellis, and Schneider (1962). Crude spore suspensions containing spore-bearing vegetative sporangia, vegetative cells, and debris in 0.1 M potassium phosphate buffer (pH 7.0) were mixed with sterile enzyme solutions to give a final 1:10 dilution of the spore suspension and enzyme concentrations of 100 µg/ml trypsin and 100 µg/ml lysozyme. The mixture was incubated six hours at 45 C with periodic agitation and then centrifuged (2000 x g) and washed with chilled phosphate buffer of the same molarity and pH until a clear supernatant was obtained.

The spores, free of vegetative cell contamination when viewed with a phase microscope (Figure 2), were resuspended in the phosphate buffer

to effect a viscous solution and frozen in one milliliter aliquots for use in subsequent experiments.

Spore Assay

Prior to experiments involving C. botulinum 78A cultures, portions of the frozen spore stocks were defrosted, diluted and assayed for determination of a constant inoculum. Dilutions were made in 0.05 M potassium phosphate buffer (pH 7.0) and assays were conducted in shake cultures in Growth Medium containing 1.5% agar. A dilution containing 1-3 x $10^9$ spores/0.1 ml for inoculating 100 ml culture medium was generally sought. Spores were heat shocked 15 min at 80 C prior to inoculation into the Growth Medium.

## Toxin Titration, Isolation, and Purification

Growth Curve

Several growth curve experiments were conducted with C. botulinum 78A to correlate physical and physiological events during growth with toxin production. Unfortunately constant removal of cultures from anaerobic incubation to check optical density and pH inhibited normal cell growth. This was overcome by running several discontinuous cultures with constant spore inocula and overlapping growth data to obtain a continuous growth curve. Optical density (600 mμ) was determined with a Bausch and Lomb Spectronic 20, and pH readings of the supernatant were obtained with a Corning pH meter and electrode throughout the growth curve. At selected intervals during the growth study, 5.0 ml samples were extracted from the culture for titration of toxin. The samples were filter-sterilized to obtain cell-free supernatants. These

samples were immediately frozen and were thawed at the time of toxin titration.

Toxin Titration

Toxin $LD_{50}$ titrations (Reed and Muench, 1938; Pizzi, 1950) were made with dilutions of the filtered supernatant in 20-30 gm mice (Swiss-Webster Strain). The mice were obtained from the Small Animal Lab, Oregon State University. Mice were injected intraperitoneally (ip) with 26 gauge, $\frac{1}{2}$ inch needles, and four mice were used for each injection dilution. Ten- and 20-fold serial dilutions were made in sterile gelatin phosphate buffer (0.1 M potassium phosphate, 0.2% in gelatin, pH 6.7) (Boroff et al., 1966). The $LD_{50}$ titration proper was conducted after a rough estimate of the toxin titer had been made in preliminary mouse injections. The $LD_{50}$ titration end point was death within a period of four days. Protein determinations (Lowry Method and $OD_{280/260}$) of the supernatant samples were made to correlate protein concentration with toxin titer. In certain experiments the MLD was used for toxin titration. The MLD is defined in this study as the last dilution of toxin which will kill all mice injected with that dilution.

Isolation of Toxin

Toxin was isolated by the acid precipitation method (Duff et al., 1957) from cultures determined to have stabilized in toxin content (96-hour cultures). The procedure is given in Figure 1. Electrophoretic analysis, toxin titration, and protein analysis were conducted at relevant steps during the isolation procedure to determine if the product being isolated maintained toxicity, if purity was being attained, and

Whole culture adjusted to pH 3.5 with 3 N $H_2SO_4$.
Precipitate allowed to settle 12 hr, 25 C.

First acid precipitate diluted 1:4 with distilled water and solution adjusted to pH 5.0 with 1 N KOH. Precipitate settled 24 hr at 4 C and centrifuged (7710 x g). Precipitate resuspended in water to 1/40 original culture volume and allowed to settle 8 hr, 4 C. Precipitate centrifuged (7710 x g).

Supernatant Discarded

Washed precipitate diluted four volumes with water and 1.0 M $CaCl_2$ to make 0.075 M $CaCl_2$ and adjusted to pH 6.5 with 1.0 N KOH. Solution filtered through Eaton & Dikeman #193 filter paper, 25 C.

Supernatants Discarded

Calcium chloride extract adjusted to pH 3.7 with 1 N HCl and allowed to settle at 4 C. Solution centrifuged (7710 x g).

Precipitate Discarded

Second acid precipitate dissolved to 1/160 culture volume in 0.03 M potassium phosphate buffer, pH 6.8. Solution clarified by centrifugation (7710 x g).

Supernatant Discarded

Solution brought to 15% EtOH concentration with 50% EtOH and placed at -5 C, 48 hr. Precipitate centrifuged (20,000 x g), -5 C.

Precipitate Discarded

(Because electrophoretic examination showed the product to be composed of no less than five distinct components, Figure 6, a second alcohol precipitation was conducted.)

Supernatant Saved

Precipitate from second alcohol precipitation dissolved in 0.03 M phosphate buffer containing 0.9 M $(NH_4)_2SO_4$ to 1/320 culture volume. Solution allowed to crystallize 15 da, 4 C.

Supernatant Saved

Final ammonium sulfate precipitated product resuspended in 0.05 M acetate buffer and dialyzed against the buffer 24 hr and frozen.

Supernatant Discarded

Figure 1. Acid precipitation scheme for type A toxin after method of Duff et al., 1957.

what proportion of the culture fluid protein was toxin.

Purification of Toxin

To check for purity of the toxin isolate, electrophoresis was conducted in both a 7½% and a 3% polyacrylamide gel matrix on a flat gel electrophoresis device. The electrophoretic apparatus was developed in the Food Microbiology Laboratory by Dr. Arthur W. Anderson and is described by Levant (1969). The toxin isolate was electrophoresed at 50 ma for 105 min, and the gel was subsequently stained in a 1% aqueous solution of Coomassie Brilliant Blue R 250 diluted 1:20 in 12.5% trichloroacetic acid (TCA) (Chrambach et al., 1967). The stained gel was clarified in 10% TCA until the stained protein bands were easily observed. Similar unstained gel segments were sectioned, and the sections were eluted in gelatin phosphate buffer. The eluates were injected into mice to test for toxicity; toxicity of an eluate indicated which stained band was toxin.

A purified crystalline type A toxin preparation supplied by Dr. Edward J. Schantz, U. S. Army Biological Center, Ft. Detrick, Maryland was subjected to electrophoresis and tested for toxicity in the same manner.

Antitoxin Production

Since electrophoretic analysis of the final toxin isolate showed the product to be composed of at least three protein bands, the electrophoretically separated component determined to be toxic by mouse assay was used for antitoxin production in rabbits. Polyacrylamide gel containing the toxin was macerated into very small pieces, and a small

volume of gelatin phosphate buffer was added to suspend the small particles in liquid. The suspension was mixed 1:1 with Freunds Complete Adjuvant (Difco), and a thick emulsion was prepared. After base bleedings, three rabbits were injected intramuscularly and subcutaneously with one milliliter volumes of the emulsion on days 1, 14, and 43. Sera of bleedings five and ten days subsequent to each injection were titered using both the alpha and beta methods of precipitin titration. Assays for neutralizing capacity were also conducted, and two mice were used for each neutralization step.

## Characterization of Toxin

### Sodium Dodecyl Sulfate Electrophoresis--Molecular Weight Analysis

Molecular weight determinations on the purified 78A toxin and the crystalline toxin supplied by Dr. Schantz were made by electrophoresis in SDS-polyacrylamide gels. The method followed was a modification of the methods reported by Weber and Osborn (1969) and Dunker and Ruechert (1969). Pepsin (2x crystallized, Nutritional Biochemicals Corporation), pancreatic ribonuclease (5x crystallized Type 1-A, Sigma Chemical Co.), trypsin (3x crystallized, Calbiochem), and bovine serum albumin (3x crystallized, Calbiochem) were used as molecular weight reference markers. The proteins (0.4 mg/ml) were incubated two hours at 37 C in 0.01 M sodium phosphate buffer (pH 7.0), 1% in SDS and 1% in B-mercaptoethanol. After incubation the protein solutions were dialyzed four hours at 25 C against a continuous flow of dialysis buffer (0.01 M sodium phosphate buffer, 0.1% in SDS and 0.1% in B-mercaptoethanol).

Molecular weight electrophoresis was conducted in both 5% and 10% polyacrylamide gels; the acrylamide and methylenebisacrylamide (BIS) contents of the 5% gel was 22.6 g/100 ml, 0.3 g/100 ml; the crosslinker was increased two fold in the 10% gel. Glass tubes 7 cm long and 6 mm in diameter were used for a typical run. The tubes were filled with the acrylamide mixture composed of 15 ml gel buffer (0.01 M sodium phosphate buffer, 0.2% in SDS, pH 7.0), 13.5 ml acrylamide solution, 1.5 ml ammonium persulfate (15 mg/ml), and 0.045 ml N,N,N',N'-tetramethylenediamine (TEMED), and the gel surface was overlayered with water. After 30 min the water was withdrawn, and the gel was overlayered with the protein solution composed of 3 µl of 0.05% Bromphenol Blue, 1 drop glycerol, 5 µl concentrated B-mercaptoethanol, 5 µl dialysis buffer, and 40 µl of the dialyzed protein preparation. The electrode compartments of the disc electrophoresis apparatus were filled with a gel buffer: water (1:1) solution, and electrophoresis was carried out at a constant current of 8 ma/gel for $4\frac{1}{2}$ hr. After electrophoresis the gels were removed from the tubes using the method of Davis (1964). The length of the gel and the tracking dye band were measured, and the gels were immersed in 20% sufosalicylic acid to remove the SDS and to fix the protein. The gels were stained in 0.02% Coomassie Brilliant Blue R 250 12 hr and clarified in 10% TCA. The protein band distances were measured after clarification, and the mobility computed according to the method of Weber et al. (1969). The calculated mobilities for all the proteins in both 5% and 10% gel were plotted, and the molecular weight of the toxin was subsequently determined.

Isoelectric Focusing of Type A Toxin

Because the presently accepted method of type A toxin isolation is not only extremely time consuming but also not specific as indicated by electrophoretic analysis, the feasibility of using electrophoretic isoelectric fractionation of the whole culture supernatant was pursued. The principle of isoelectric focusing in natural ampholyte pH gradients was originally reported by Svensson (1962) and has been adapted into a micro method by Catsimpoolas (1968) and Riley and Coleman (1968).

The gel medium is prepared by mixing 5 gm acrylamide, 0.05 ml TEMED, 0.2 gm BIS, 0.35 mg ammonium persulfate, and 5 ml of 40% stock ampholyte solution (pH 3-10, Ampholine, LKB Instruments), and sufficient deionized water to 100 ml; the solution was evacuated to remove entrapped bubbles. Toxin supernatant containing 0.2 mg protein, was dissolved directly in the ampholyte-containing polyacrylamide solution, and this mixture was used to fill 8 cm x 5 mm tubes as in disc electrophoresis. The gel was polymerized by exposure to light (Davis, 1964). Focusing was performed with the anode immersed in 5% (v/v) phosphoric acid and the cathode in 5% (v/v) ethylenediamine (Awdeh, Williamson, and Askonas, 1968) at a starting current of 5 ma/gel. The focusing was continued for 2 hours.

After focusing, the gels were immediately divided longitudinally. One half of the gel was stained in 0.2% Bromphenol Blue in ethanol:water:acetic acid (50:45:5) one hour and then destained in ethanol:water:acetic acid (30:65:5) until the background became clear (Awdeh, 1969). The other gel half was immediately sectioned into 3 mm wide sections, and the sections were each eluted in 0.15 ml deionized water 12 hr, 4 C.

The pH of each eluate was determined and correlated with its toxicity in mice.

Immuno-Electron Microscopy

Localization of the toxin within the cell proper was attempted by electron microscopy with ferritin conjugated globulin of equine anti-A antitoxin. Ethanol precipitation (Nichol and Deutsch, 1948) of the equine antitoxin obtained from the Science Resources Branch of the Communicable Disease Center, Atlanta, Georgia resulted in a product contaminated with albumin. The globulin was therefore purified on a DEAE cellulose column prepared according to the method of Peterson and Sober (1962). Elution of the globulin followed the method of Sober et al. (1956) for fractionation of equine serum.

The procedure employed for the conjugation of ferritin to globulin is derived from the method described by Singer (1959) but also includes modifications of Rifkind et al. (1963) and is described as follows: Horse spleen ferritin (6x crystallized, Calbiochem) was precipitated in 22% ammonium sulfate, and the precipitate was recovered by centrifugation (650 x g). The precipitate was resuspended in water and dialyzed against running water one hour and against a continuous flow of 0.05 M sodium phosphate buffer (pH 7.5) six hours, 25 C. Dialysis was discontinued and the preparation was centrifuged (100,000 x g). The resulting precipitate was suspended in 0.85% NaCl and 0.3 M borate buffer (pH 9.5) to achieve a protein concentration of 2.5% in 0.1 M buffer. Xylylene metadiisocyanate was added in the proportion of 0.1 ml/100 mg ferritin, and the mixture was stirred 45 min at 0 C. The mixture was centrifuged

(2500 x g), and the supernatant was carefully removed from the unreacted xylylene metadiisocyanate. One part globulin (20 mg/ml) was mixed with four parts ferritin (80 mg/ml) and stirred 48 hr, 4 C. The mixture was dialyzed against 0.1 M ammonium carbonate (pH 8.8) 12 hr, 0.05 M sodium phosphate buffer (pH 7.5) 6 hr and then subjected to low speed centrifugation (2000 x g) to remove any precipitate. Three sequential four-hour ultracentrifugations (100,000 x g) and resuspensions in 0.05 M sodium phosphate buffer (pH 7.5) were conducted to remove free globulin from the rapidly sedimenting high molecular weight conjugate. The final resuspension in phosphate buffer was made to effect a 2% protein solution. This preparation was filter sterilized and placed at 4 C.

Eighteen hour C. botulinum 78A cells were prepared for electron microscopy in the following manner. The cells were centrifuged (755 x g), washed twice in 0.1 M sodium phosphate buffer (pH 7.0) and resuspended in 2% glutaraldehyde for 4 hr, 25 C. The cells were centrifuged and washed twice with the phosphate buffer and resuspended in 1% $OsO_4$ for 6 hr, 4 C. The cells were centrifuged and washed twice in the phosphate buffer and imbedded in 2% ion agar. The agar was diced and run through the usual series of alcohol dehydrations and propylene oxide treatments prior to being embedded in the Epon plastic embedding medium for electron microscopy (Pease, 1964). The plastic was aged, and sections were cut on a Porter Blum type electric microtome.

Grids on which the sections were placed were: 1) immersed in ferritin conjugated antibody and subsequently washed in phosphate buffer, 2) immersed in phosphate buffer, 3) immersed in purified globulin and subsequently washed in phosphate buffer, or 4) immersed in ferritin conju-

gated antibody subsequent to immersion in purified globulin. The preparations were allowed to dry and were viewed with a Phillips 300 electron microscope. From observation of electron micrographs the ferritin conjugate was determined to contain large amounts of unconjugated ferritin; therefore, the conjugate was purified with electrophoresis, eluted, and applied to sections in the manner previously described. Excess ferritin contamination is evidently not uncommon and is generally eliminated by continuous flow electrophoresis of the contaminated conjugate (Boreck and Silverstein, 1961).

Cell sections were also stained with uranyl acetate and lead citrate (Pease, 1964) in an attempt to correlate stained sections with the ferritin conjugated globulin-treated sections.

## Detection of Botulinum Toxin

### Latex Fixation

The use of innert carrier particles (latex particles) for the detection of botulinum toxin was attempted. Since optimum concentration of latex particles has been determined to be under 10% transmission at a wavelength of 650 mμ (Singer and Plotz, 1956), the latex particles (Difco, 0.81 u) were diluted to 6% transmission in borate-saline buffer (pH 8.5), glycine-saline buffer (pH 8.6), or acetate-saline buffer (pH 3.9). The pH range between 5.5 and 8.0 was avoided since spontaneous agglutination involving the latex particles and gamma globulin occurs in this pH range; pH's above 10.0 were not employed either since the reaction is totally inhibited beyond this pH (Singer et al., 1956). The latex particles and anti-A equine gamma globulin (260 μg/ml) mixture

was placed at 25 C one hour to allow fixation of the globulin to the latex surface (Oreskes and Singer, 1961). Other gamma globulin concentrations were tried, but 260 µg/ml gave the most consistent results and was not wasteful of the limited supply of gamma globulin. The reaction mixture was centrifuged (21,300 x g) 30 min, the supernatant containing excess gamma globulin was decanted, and the latex particles were resuspended in the respective buffer. One milliliter of the anti-A equine gamma globulin latex particle preparation was added to one milliliter volumes of 2-fold and 10-fold C. botulinum 78A and C. botulinum 115B culture supernatants to effect 4-fold and 20-fold diluted reaction mixtures. Appropriate controls were also prepared. The reaction mixtures were placed at 56 C for two hours and centrifuged 10 min at 770 x g. The tubes were gently agitated and examined for visible agglutination and for microscopic agglutination (40X).

Electroimmunodiffusion

Electroimmunodiffusion (EID) originally conceived by Laurell (1966) as a method for quantitation of precipitating serum proteins was adapted here for detection and quantitation of C. botulinum type A toxin. The method is based on the fundamental immunoprecipitin principles of Heidelberger and Kendall (1932) and is similar to the method of radial immunodiffusion introduced by Mancini, Carbonara, and Heremans (1965) in the principle of precipitation of a protein antigen in antibody-containing agar. However, EID makes use of an electrical field to induce a rapid, linear migration of antigen out of its well into the antibody-containing gel. Antigen-antibody aggregates are too large to migrate in

the gel after complexing, while the free antigen molecules migrate until they are precipitated. These complexes may dissolve in antigen excess but are reprecipitated after meeting more antibody further from the antigen well. The precipitin pattern assumes the form of a cone as the antigen is precipitated along its path of migration wherever the antigen-antibody equivalence zone is reached.

Anti-78A rabbit antitoxin and CDC anti-A and CDC anti-B equine antitoxins were used. The sera were dialyzed against a 0.025 μ borate buffer (pH 8.4) 24 hr at 4 C and were mixed thoroughly with 1.2% agarose. The agarose had previously been reconstituted in 0.025 μ borate buffer (pH 8.4) and stabilized at 45 C. To determine the desirable concentration of serum to incorporate into the agarose, several qualitative experiments were employed using microscope slides. No dilution attempted with the CDC anti-A equine antitoxin gave precipitation. A 1:20 dilution (1 ml antitoxin:19 ml agarose) of CDC anti-B equine antitoxin was determined to give precipitation, but since this represented a cross-reaction with the type A toxin supernatant the antitoxin was not used further. A 1:30 dilution of the anti-78A rabbit antitoxin gave good precipitation with type A toxin. Large 4" x 3¼" glass slides (projector slides) previously coated with 2% ion agar which had been allowed to dry were overlayered with 15 ml of the antitoxin-containing agarose. This volume was found to effect a gel depth of 2-3 mm. Gels were allowed to harden 15 min in a moist chamber. Sample wells were pulled by vacuum just prior to the use of the plates, and samples (2-5 μl) corresponding to known $LD_{50}$ quantities and known protein content were applied after the plates had been placed on the electrophoretic

apparatus and the electrical bridges had been constructed with Whatman paper wicks. Borate buffer (0.1 $\mu$, pH 8.4) was used as the electrolyte for both cathodic and anodic chambers. A current of 5 ma/cm was applied for one to four hours under constant cooling.

Slides were observed unstained and stained and the length of the visible precipitin cones were measured from the center of the antigen well to the tip to the nearest 0.1 mm. Two staining methods were used (see below), the second being somewhat less time consuming and also a better method if slides are to be photographed. Photographs were made of stained gels, and slides were dried and used as permanent records.

EID toxin detection tests of foods contaminated with known $LD_{50}$ quantities of C. botulinum 78A toxin were conducted as well as tests for the detection of the toxin in the blood of mice previously injected with known $LD_{50}$ dosages of the type A toxin.

Staining Method No. 1 (Lopez, Tsu, and Newton, 1969)

(1) Elute slides 6-8 hr in 0.05 M potassium phosphate buffer, 0.85% in NaCl, pH 7.4.

(2) Dry slides with hot air.

(3) Stain slides in 0.1% Buffalo Black (Amido Schwartz) in methanol: acetic acid:water (45:10:45) solution 5 min.

(4) Elute excess stain with same solvent; 7-8 10 min elutions.

(5) Redry slides in hot air.

Staining Method No. 2 (Crowle, 1958)

(1) Elute slides in buffer of Method No. 1.

(2) Immerse slides in solutions as follows:

(a) Distilled water, 10 min; twice,

(b) 0.1% Thiazine Red R in 1.0% acetic acid, 10 min,

(c) 1.0% acetic acid until background color fades.

(3) Dry slides in hot air.

# RESULTS AND DISCUSSION

The primary purpose of this study was to develop a sensitive, more reliable, and less time consuming method for botulinum toxin detection than the mouse assay currently in vogue. The development of a specific antitoxin to be used in such a technique, however, led to some interesting investigations of the physiology of toxin production and characterization of the toxin itself. The results and discussions concerning the results of these independent, but related investigations are presented in the following sections.

## Culture Standardization and Toxin Production

### Culture Standardization

There is a definite shortcoming in the botulinum literature with the failure of investigators, primarily investigators of chemical and physical properties of crystalline type A toxin but also with investigators of botulinum physiology, to report the strain of the organism which originally produced the toxin being investigated. In a sample of thirty-two papers from the early work of Snipe *et al.* (1928) to a more recent work of DasGupta *et al.* (1968) only nine authors, roughly 30%, reported the strain of *C. botulinum* used in their investigations. To compound this problem, there is also insufficiency in the information provided by authors on the culture conditions employed, i.e., temperature for growth, time of incubation, medium for cultivation, and gaseous environment. Although no work has yet been published on strain variation in regard to the chemistry of the toxin molecule, a good analysis of toxin production in different media conducted by Skulberg

(1964) demonstrated definite variation in toxin production in different media. Standardization of variables probably would aid a great deal in clarifying the disagreements which have arisen in the literature concerning the production and characterization of the toxin. For this reason, several attempts at standardization of culture methods were undertaken in this study.

Because spores are generally the agents of food contamination and toxic components appear as a result of their germination and cell metabolism, it seemed reasonable to begin cultures with spores in order that all products from germination through autolysis would be present in the medium to influence the toxin. Other investigators (Boroff, 1955; Duff et al., 1957; Bonventre et al., 1960a) who have made an attempt to standardize inoculation practices have simply employed serial transfers to obtain log phase seed cells with which to initiate a synchronous final culture. This type of culture method omits the important step in the organism's growth, spore germination, and any metabolic products produced therefrom. In the past, reason for reluctance on the part of investigators to use spores as a natural inoculum was that spores were generally produced in a complex meat or vegetable infusion-type medium containing tissue particles. Spores produced in such media were difficult to separate from both their sporangia and the tissue particles of similar size. Such difficulties have been overcome, however, because several investigators (Tsuji and Perkins, 1962; Krabenhoft et al., 1964; Day and Costilow, 1964) have developed aparticulate, clear hydrolysate media for spore production. An example of the spores of C. botulinum 78A produced in such an aparticulate medium and used in this study is

shown in Figure 2. The bright appearance of the spores as observed by phase microscopy indicates the refractile and thus viable nature of the spore; loss of refractility in a spore stock denotes loss of germination potential. Spores produced for this study and maintained in a frozen state have remained viable with very little decline in assay values for two years.

The growth curve of C. botulinum 78A initiated with spore inoculum is shown in Figure 3. Also included in Figure 3 is information on the pH of the culture medium and toxin content of the supernatant throughout the different culture phases. Because toxin content was greatest at 96 hours and generally declined thereafter, toxin was extracted from the 96-hour supernatant.

An interesting event to point out is the time at which detectable quantities of toxin began to appear in the supernatant. The event corresponded with the time at which the culture pH dipped to its lowest recorded value. Bonventre et al. (1960a) suggested that the hydrogen ion concentration did not change sufficiently, in an investigation of the correlation between autolysis and toxin production in which pH was measured at 12 hourly intervals, to cause a physiological disturbance great enough to contribute to autolysis. But information was not available at that time concerning the possible relationship of the sporulation process with toxin production in various members of the genus Clostridium (Nishida et al., 1965; Nishida et al., 1969) and in the genus Bacillus (Johnson and Bonventre, 1967). Although the change of pH does not effect a physiological disturbance great enough to explain an increase in toxicity, the change may be correlated with toxin production

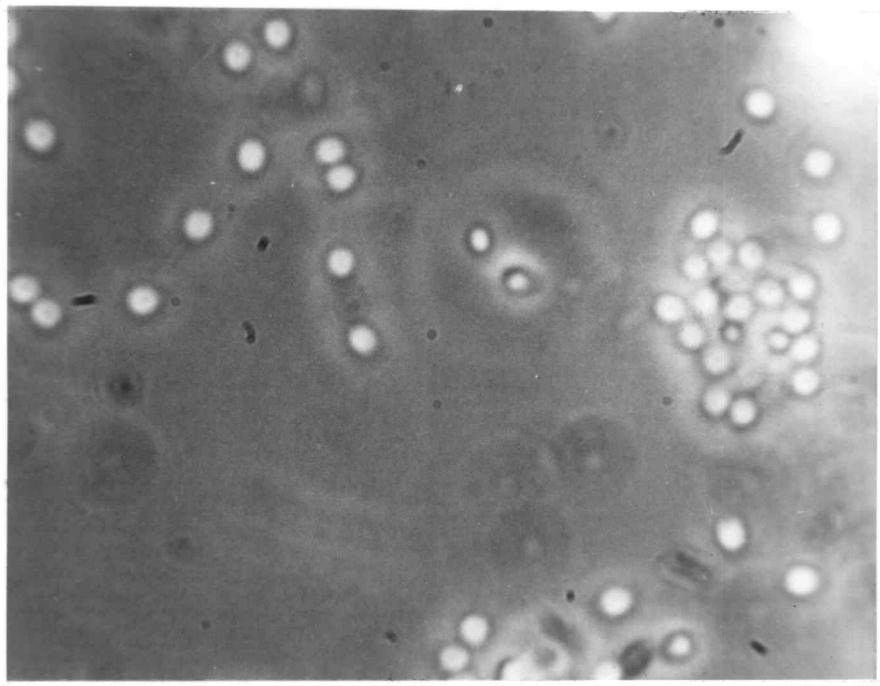


Figure 2. Refractile spores of Clostridium botulinum 78A. These viable spores were maintained in a frozen state 17 months.

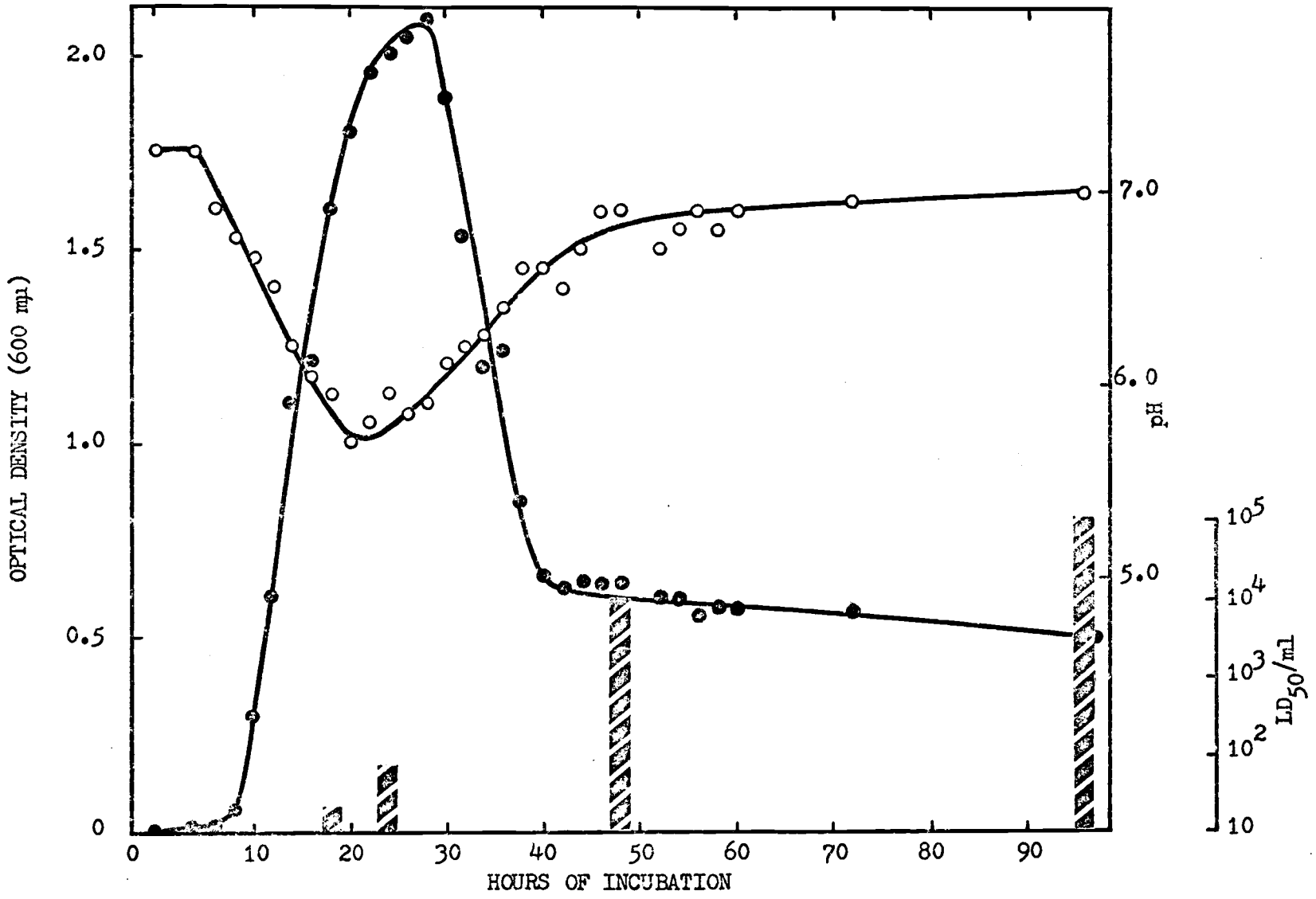



Figure 3. Growth curve of C. botulinum 78A including pH and toxin titration of culture supernatants. Symbols: o——o, pH; ●——●, optical density; ▨, $LD_{50}$.

in C. botulinum in another manner. Such a correlation has been found in Bacillus cereus (Johnson et al., 1967); a soluble, lethal toxin termed alpha toxin is produced when the pH of the culture medium begins to drop. The pH change is also shown to announce the onset of the sporulation process.

Immuno-Electron Microscopy

Advances in the study of labeled antibody led to the development by Singer (1959) of an electron-dense antibody conjugate which retains its specificity and can be used with great effect to visualize antigens in the contents of ultrathin sections of intact cells. It was with such a system that Rifkind et al. (1963) located influenza viral antigens in tissue and demonstrated their concentration at cell surfaces. Since the manner of toxin formation in C. botulinum is largely unknown, it was considered a possibility that the ferritin-conjugated antibody technique could aid in the establishment of a correlation between the toxin and some structural entity of the bacterial cell. Several experimental problems were envisioned, however. There are two possible methof applying the technique: 1) a pre-embedded staining method in which antigen is allowed to react with the labelled antibody before the cells are processed and sectioned in preparation for electron microscopy; 2) a post-embedded staining method in which labelled antibody is applied and is allowed to react with the antigen in suitably fixed and embedded thin sections. Almost all published investigations have been carried out using the pre-embedding technique because investigators believed the ferritin was specifically attracted to the embedding medium in the

second technique. The problem with the generally used method, however, is that although surface antigens are readily located, intracellular antigens are not. The technical difficulty of using the second method was overcome, however, by utilization of the suggestion of Borek and Silverstein (1961). They suggested the difficulty of non-specific ferritin attachment to embedding medium has been the result of the failure of investigators to purify their labeled antibody preparation. This failure results in a high "background" of the randomly distributed non-conjugated ferritin as is shown in Figure 4. After electrophoretic purification, the ferritin-conjugated antibody is observed primarily in the cell of C. botulinum 78A, Figure 5. In Figure 5 the dense ferritin-labeled antibody particles seem to be mainly associated with the cytoplasm; possibly they may be ribosome-associated. A similar study by Duda and Slack (1969) was located in the literature after the present study was initiated. Duda et al. (1969) observed the ferritin-labeled antibody to be arranged around the outer spore coats in protoplasts of older cells, but also aggregates of ferritin were found within the cytoplasm of younger cells. From the results, Duda suggested that the spore plays a role in toxin production, but no further explanation was offered. The relation between the appearance of the toxin in metabolizing cells and its later appearance in the spore may be that although a major portion of the toxin is liberated upon autolysis, some of it may inadvertantly be incorporated into the spore. Incorporation of small amounts of cell wall material between the invaginating membranes of the forespore has been observed (Walker and Short, 1969). The finding that toxin is incorporated into the spore is also supported by the observation

Figure 4. Ultrathin sections of C. botulinum 78A cells treated with unpurified ferritin-labeled antibody. The dense ferritin particles (f) are distributed randomly over the cells as well as the embedding medium; X 84,400.

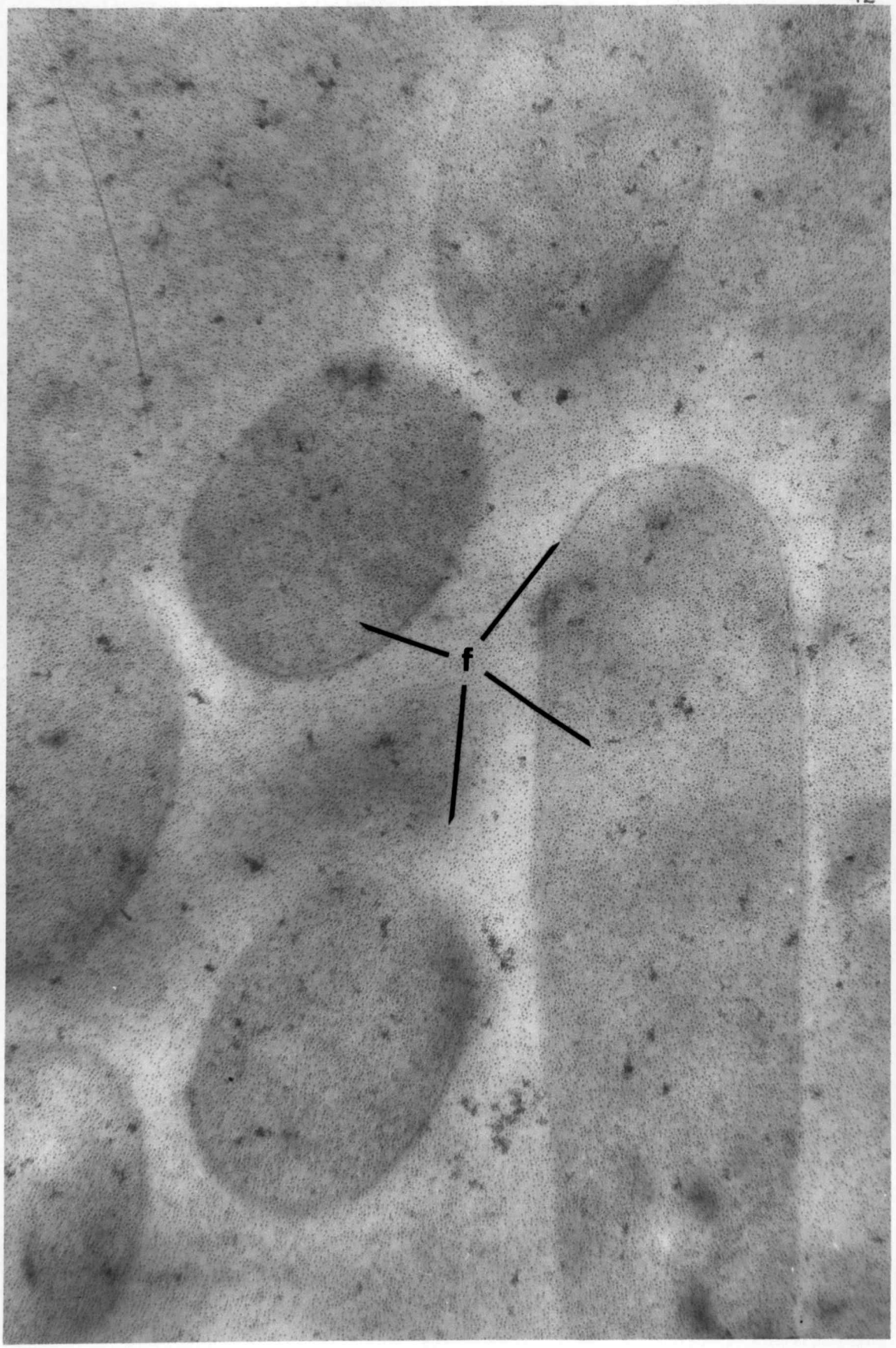
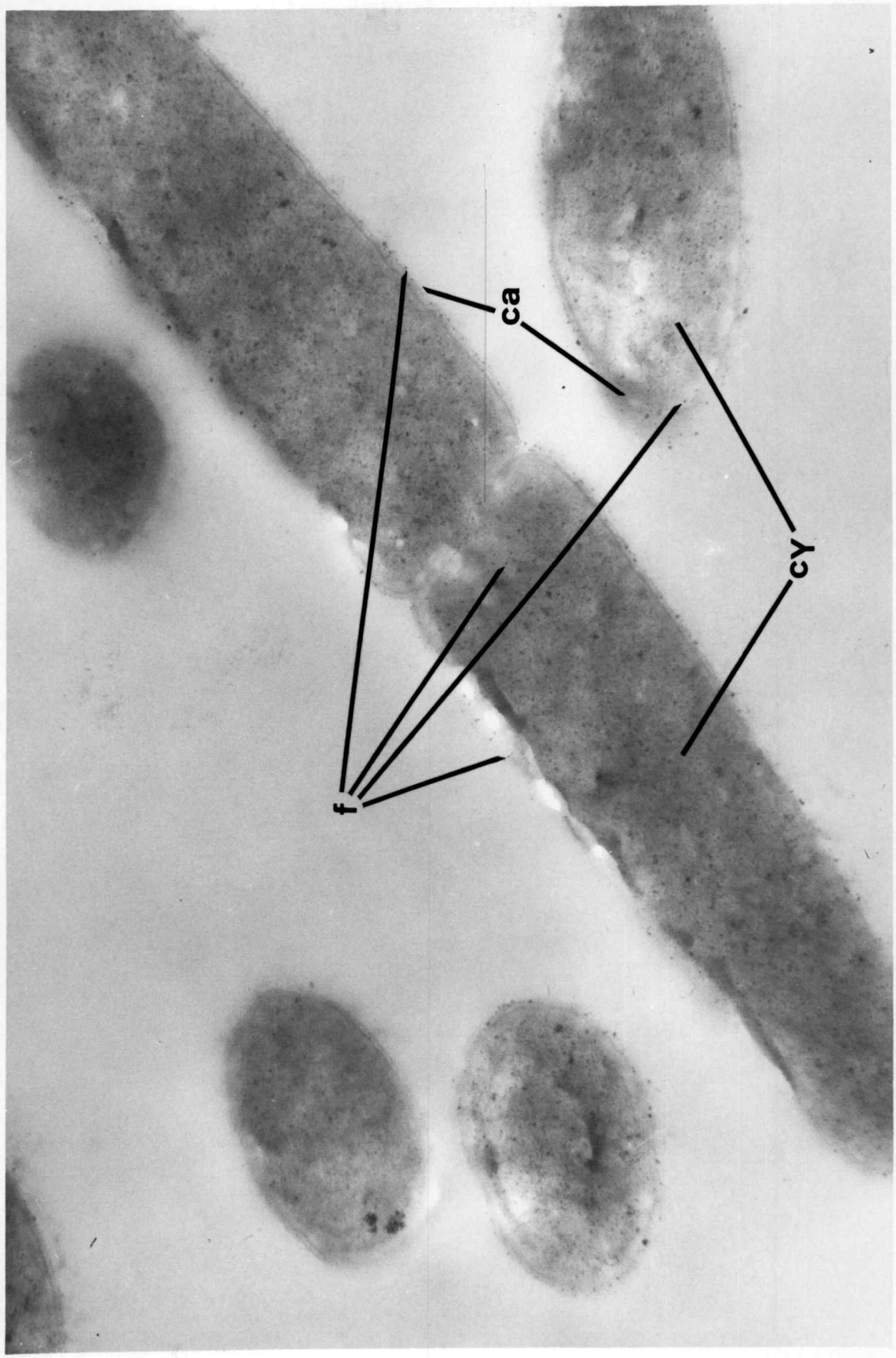

f

Figure 5. Ultrathin sections of C. botulinum 78A cells treated with purified ferritin-labeled antibody. The dense ferritin particles (f) are distributed primarily in the cytoplasm (cy), while some particles appear associated with the outer capsular material (ca); X 71,300.


ca
cy
f

of Grecz and Lin (1966) that each spore of C. botulinum 33A contains approximately 500 molecules of high molecular weight toxin. The observation in this study that ferritin-labeled antibody is cytoplasm-associated during the log phase and the similar observation by Duda et al. (1970) suggests that the antigenic component or components of the toxin are actively synthesized and are not breakdown products as has been postulated (Bonventre, et al., 1960b). This suggestion may be supported by the finding (Knox et al., 1970) that the low molecular weight components of a toxin preparation are high in nucleic acid content. Such components may represent fragments of nucleic acid plus a nascent peptide chain.

## Toxin Purification and Characterization

### Toxin Purification

Toxin, isolated by the method of Duff et al. (1957), was tested for purity by polyacrylamide electrophoresis. A comparison of the partially purified products from various stages in the acid precipitation method is shown in Figure 6. The final product isolated accounted for 0.0004% of the original supernatant protein. Electrophoretic separation of this product (No. 4, Figure 6) demonstrated that the product was impure; at least four protein bands were present. Although Duff et al. (1957) reported that their method yielded a homogenous product, their criterion of purity was a homogenous boundary at pH 3.8 with a sedimentation constant of 14.5 S; this was shown earlier by Wagman et al. (1951) to be composed of the hemagglutinin and the toxin. Tests for toxicity of eluates from horizontally sliced sections of the gel samples

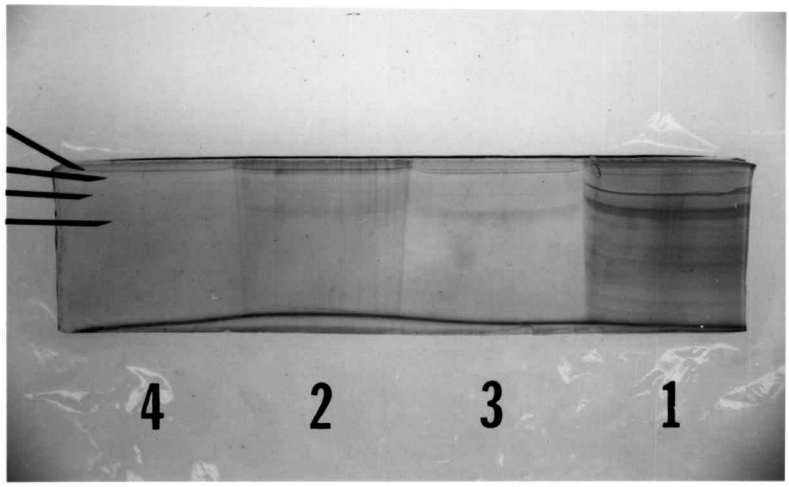



Figure 6. Electrophoretic separation of 78A toxin at different stages in acid precipitation procedure. Whole culture supernatant (1); first alcohol precipitation (2); second alcohol precipitation (3); ammonium sulfate precipitation (4). At least four separate protein bands (arrows) were observed in the final product isolated by the method of Duff et al. (1957).

indicated the eluates from the top gel area where two definite protein bands were present were the most toxic, $10^5$ MLD/0.1 ml. However, a low level of toxicity, 100 MLD/0.1 ml, was also associated with sections corresponding to an electrophoretic mobility of 0.45 where no definite staining band was observed. Tests for hemagglutination were also positive with eluates of the top protein banded area. Consequently, the 78A toxin prepared by acid precipitation was subjected to electrophoresis and elution until the toxin had been separated into a single migrating protein band, Figure 7, which exhibited extreme toxicity, $3.7 \times 10^6$ $LD_{50}$/1.0 ml.

The 940,000 molecular weight component isolated by Duff's method probably dissociates into its hemagglutinin and the toxin components at the alkaline pH of the electrophoretic buffer, pH 8.3. Unless dissociated, the large molecule would have difficulty moving into the 7½% polyacrylamide matrix which has been shown to exhibit extreme frictional resistance to proteins much over a molecular weight of 800,000 (Ornstein, 1964). Electrophoretic separation of a crystalline sample of type A toxin, purified by the method of Duff et al. (1957) and supplied by Dr. Edward Schantz, U. S. Army Biological and Physical Sciences Division, Ft. Detrick, is shown in Figure 8; two protein bands are also present in his preparation near the top of the gel along with diffuse, nonbanding but stainbinding material.

SDS-Polyacrylamide Molecular Weight Analysis

The development of the technique of molecular weight analysis of oligomeric proteins in SDS-containing polyacrylamide gel has made such an analysis, which heretofore has been accomplished by equilibrum

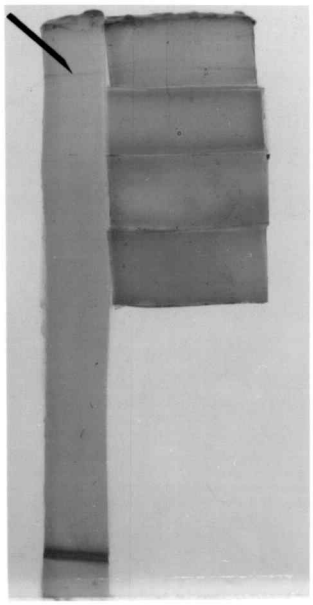


Figure 7. Purified C. botulinum 78A toxin protein band in polyacrylamide gel (arrow). The band at bottom of gel is Bromphenol Blue tracking dye.

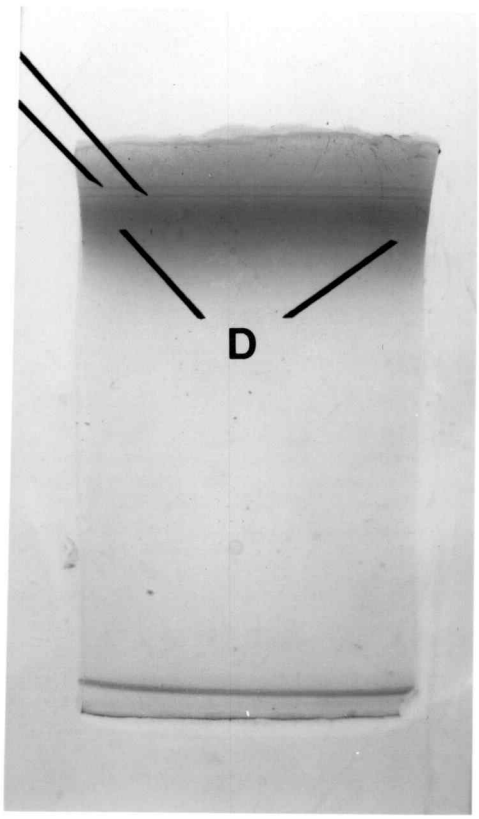



Figure 8. Electrophoretic separation of crystalline type A toxin supplied by Dr. Schantz, U. S. Army Biological and Physical Sciences Division, Ft. Detrick. Two protein bands appear at top of gel (arrows) along with a diffuse stainbinding area (D). The band at the bottom of the gel is the tracking dye.

centrifugation in guanidine-HCl, much less experimentally demanding but not at the loss of reliability. It appears that molecular weights may be determined with an accuracy of ±10% (Weber et al., 1969). As developed (Shapiro et al., 1967), the technique is rapid and requires very little protein sample, 5 µg or less. In solution, prior to electrophoresis, a protein after reduction somehow organizes the sodium dodecyl sulfate anions into a micellar complex, and the interplay of the anion binding and frictional resistance during electrophoresis acts to produce a relatively constant log size to mobility ratio. The effects of intrinsic molecular charge and conformation on electrophoretic behavior in the presence of the sodium dodecyl sulfate have been evaluated by Dunker et al. (1969) in studies on a set of model proteins and have been found to be negligible.

Figure 9 shows the separation of the polypeptide chains of type A toxin, pepsin, ribonuclease, and several fractions of bovine serum albumin in 10% gel. The protein placements in the photograph are not exactly relative since mobility is a function of both the gel length and of the distance the tracking dye moves. These variables must be measured for each individual gel in a single run. Since the tracking dye is eluted when the gels are processed for staining and the gels expand somewhat after being stained and destained, measurements must be taken at specific times in these processes. However, when the mobilities were calculated from the measurements and were plotted against the known molecular weights of the control proteins (molecular weights of the proteins were obtained from the papers of Weber et al., 1969 and Dunker et al., 1969) on a semi-logarithmic scale and a line drawn, an

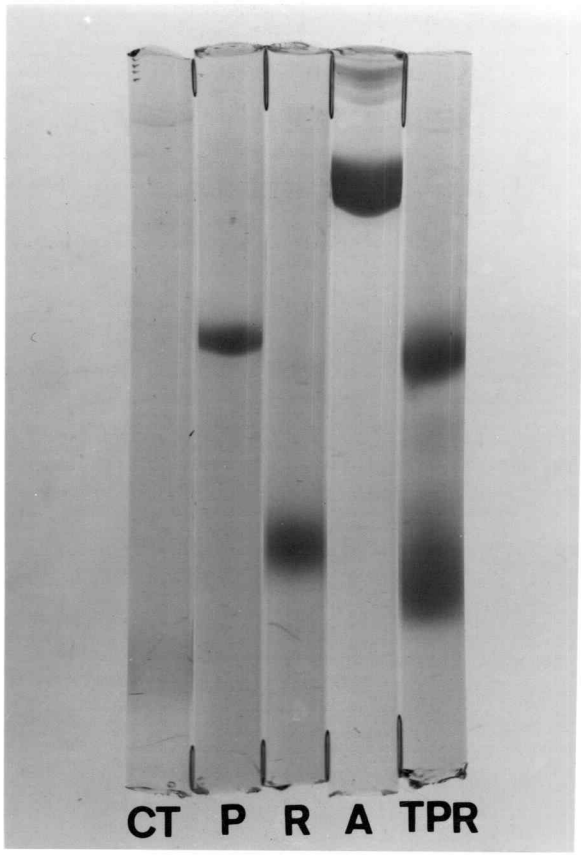



Figure 9. Electrophoretic separation of proteins in 10% SDS-polyacrylamide gel. Crystalline type A toxin supplied by Dr. Schantz (CT); pepsin (P); ribonuclease (R); bovine serum albumin (A); 78A toxin, pepsin, and ribonuclease (TPR).

estimation of the molecular weight of the protein in question, the toxin, was obtained. An example of this may be seen in Figure 10; each plot represents the average of five individual runs. Two components of the crystalline toxin supplied by Dr. Schantz were observed to have average molecular weights of 84,000 and 10,200; while the 78A component had an average molecular weight of 81,000. A component of low molecular weight was also observed in the 78A toxin preparation in several runs, but its appearance was inconsistent. Toxicity experiments of the two components were not undertaken because toxic activity of the protein is effectively destroyed by SDS and B-mercaptoethanol treatment.

The molecular weights obtained from the two components in the type A crystalline toxin and the molecular weight of the one component of the 78A toxin preparation seem to compare within 15% with molecular weights already reported in the literature, 70,000 (Wagman et al., 1953) and 12,000 (Gerwing et al., 1965). Since the hemagglutinin was still associated with the sample supplied by Dr. Schantz, the 12,000 molecular weight unit may be a subunit of the hemagglutinin moiety and not a subunit of the toxin moiety. However, the inconsistent appearance of a low molecular weight component in the 78A toxin preparation, supposed to be free of hemagglutinin, confuses this line of reasoning.

Isoelectric Focusing

The 78A toxin was also subjected to electrophoretic isoelectric-focusing. Figure 11 is a toxicity and pH profile of the eluates from an isoelectricfocusing gel. Several pH standards, 4.01, 7.00, and 10.01, were used over the wide pH range and the pH of the eluates

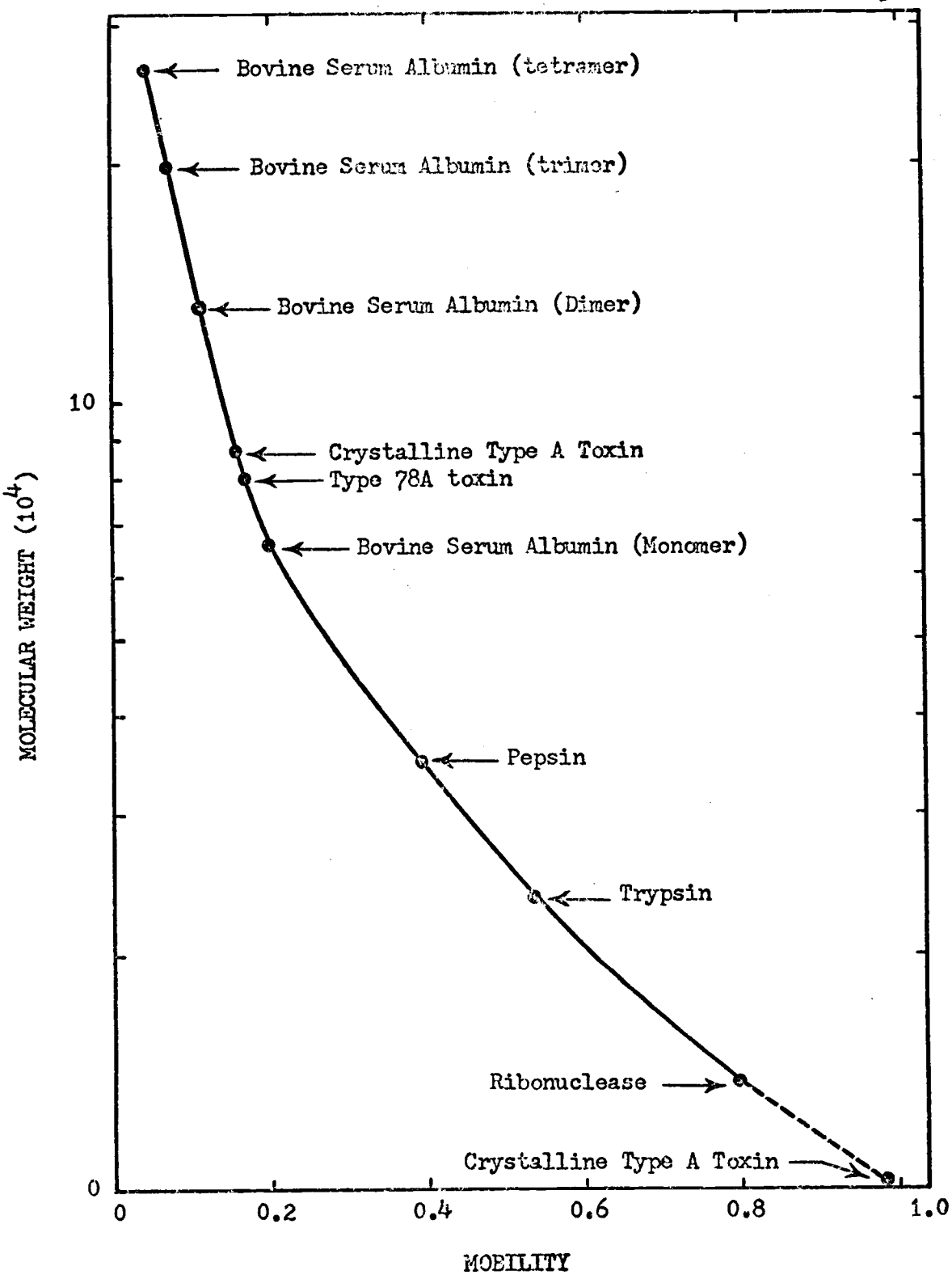



Figure 10. Determination of the molecular weight of crystalline type A toxin supplied by Dr. Schantz and the 78A preparation in 5% SDS gel. The molecular weights of the marker proteins were obtained from Dunker et al. (1969) and Weber et al. (1969), while the mobilities were obtained in this study.

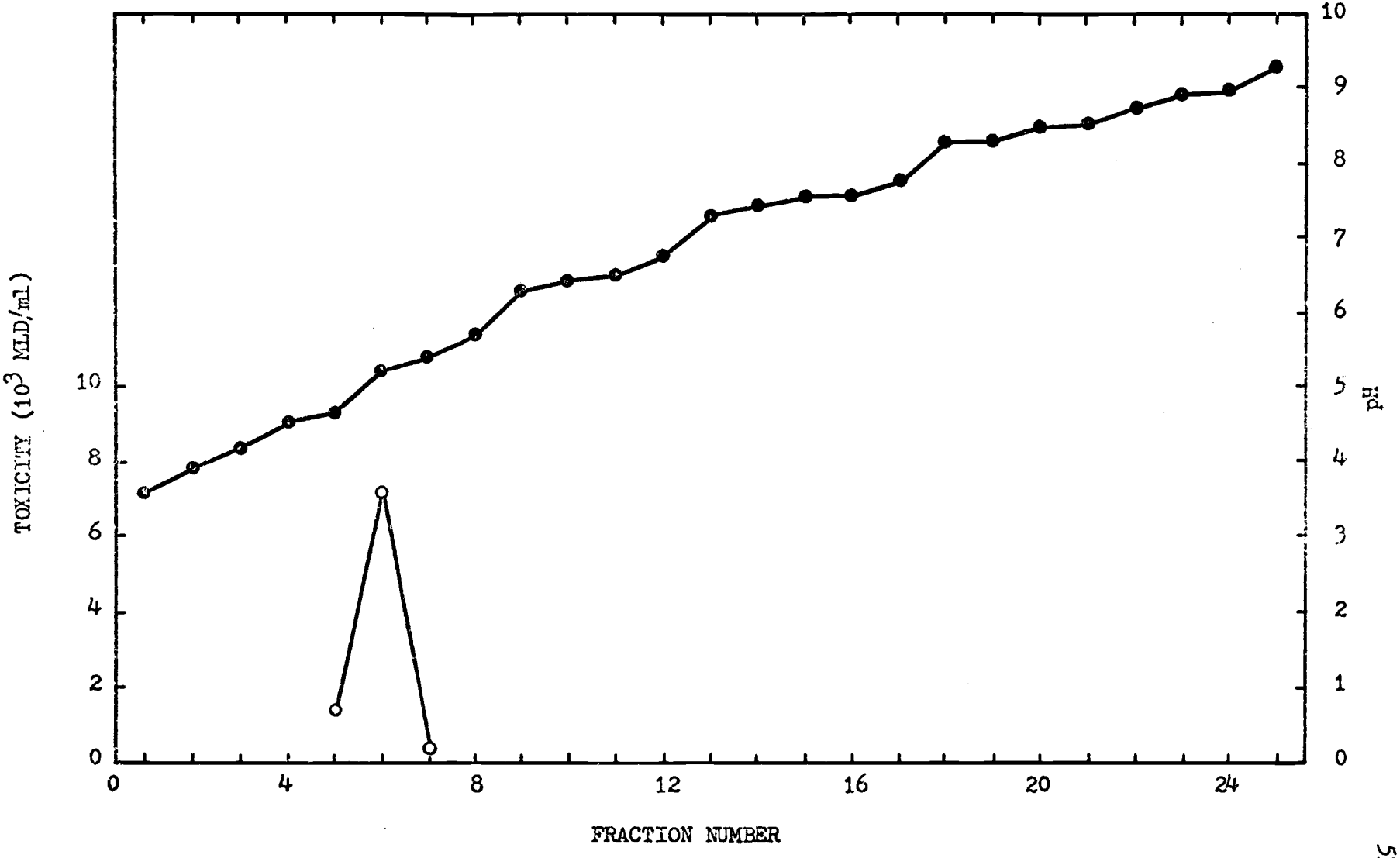



Figure 11. Toxicity and pH profile from an isoelectric focusing column. Symbols: ● , pH; ○ , toxicity.

corrected accordingly. Other investigators have isolated type A toxin by "isoelectric precipitation," but their methods were empirical. The first attempts by Snipe et al. (1928) involved precipitation at pH 4.0; in a later paper by Wagman et al. (1951) the isoelectric region was assumed to be approximately pH 5.5. In the present study the pH of the fraction containing the highest toxicity was 5.23.

Since electrophoretic isoelectric focusing selectively concentrates the toxin, the method could prove quite effective for large-scale toxin purification.

## Botulinum Toxin Detection Studies

### Latex Fixation

The use of carrier particles for adsorption of antigen or antibody and their subsequent application as serological reagents have been reported by several investigators. Oreskes et al. (1961) demonstrated that gamma globulin is adsorbed onto the surface of the latex particle and the interaction between antigen and antibody, which might otherwise not have effected a visible reaction, results in a readily observable agglutination.

The behavior of the adsorbed particles in various electrolytes has been discussed previously in "Materials and Methods." Specifically in this study, the pH range below 5.5 also had to be avoided since preliminary experiments demonstrated nonspecific agglutination below pH 5.5 in the controls. The agglutination presumably was the result of toxin precipitation, since the isoelectric point of the toxin falls below this pH. This necessitated the use of a narrow pH range, pH 8-9, in which

the latex test for botulinum detection could be adapted.

The gamma globulin fraction isolated from equine type A antitoxin, produced at the Communicable Disease Center, Atlanta, Georgia, was used in these experiments partly because it was originally hoped that existing botulinum antitoxins could be used for development of a detection technique. If this could be achieved, public and private health service laboratories could obtain the necessary sera easily should cases of suspected botulinum poisoning be referred to them. The latex test gave best results in the pH range 8.2—8.5, but the detection capacity was too low. The method was capable of detecting only as low as $1.2 \times 10^3$ $LD_{50}$. An example of the latex agglutination test is shown in Figure 12.

No really good explanation is offered for the poor detecting capacity of the system, since such a system is generally as good as the reacting capacity of the antiserum. Consequently, a possible explanation for the problem may be the reacting capacity of the equine globulin is poor. Although it exhibits a very high neutralizing capacity, the globulin molecules in this type of reaction may not be analogous to those in the latex reaction. As Boyd (1956) suggests, the same antigen can give rise to antibodies which exhibit differences in behavior; in fact antibody molecules in an immune serum are generally rather heterogeneous. Since the detection capacity of the system was low, even in the test reaction mixtures, no experiments were conducted with food samples contaminated with botulinum toxin to examine the practicality of the system.

Electroimmunodiffusion

Because of the simplicity of an immunodiffusion type reaction,

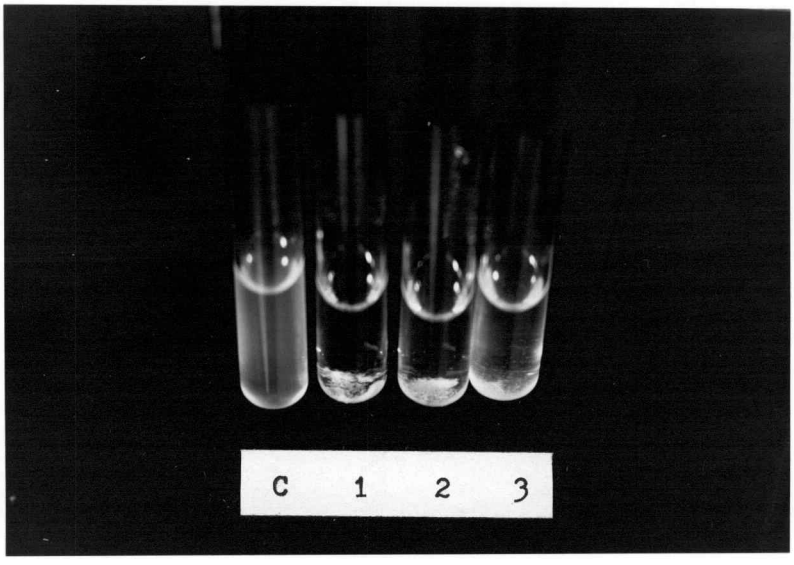



Figure 12. Latex agglutination. Control (C); 8000 $LD_{50}$ (1); 4000 $LD_{50}$ (2); 2000 $LD_{50}$ (3).

interest was returned to improvement of the technique for toxin detection. Historically there are essentially three different methods of application of the gel-diffusion principle: 1) the Cudin method, a single diffusion technique in which either antigen or antibody in solution diffuse into a gel medium containing the opposite component; 2) the Ouchterlony method, a double diffusion method in which antigen and antibody are deposited in solution into separate wells within a gel medium and are allowed to diffuse toward each other; 3) the Grabar and Williams method, a double diffusion method prefaced by electrophoretic separation of the antigen within the gel medium. These methods are limiting for botulinum detection with respect to time, since diffusion and precipitation will take from 16 to 48 hours (Anderson et al., 1968; Vermilyea et al., 1968). Immunodiffusion has also been shown to be approximately 300-600 times less sensitive than the mouse assay (Vermilyea, et al., 1968).

However, perusal of the literature turned up a new immunoelectrophoretic technique which offered solutions to both of the above stated limitations to immunodiffusion, time and sensitivity. In 1966 Laurell introduced a new technique for estimation of serum proteins by quantitative precipitation based on electrophoresis instead of diffusion of antigen into antibody-containing gel. The method requires no prior concentration of the test solution since as little as 0.125 µg protein is detected by the method (Laurell, 1966). The new technique is termed Laurell Electrophoresis or more commonly Electroimmunodiffusion (EID). This technique has gained little attention in the United States since its introduction in Sweden, and its application has been limited to

immunoglobulin quantitation. No investigators prior to this study have utilized the technique for detection and quantitation of biological fluids other than serum.

Although equine sera has been used extensively for development of neutralizing antitoxins, its flocculating nature has been its undoing as an agent of *in vitro* titration. In the precipitin reaction, precipitation occurs over a relatively narrow range of antigen concentration, and soluble complexes are formed in both the antibody excess and the antigen excess regions. Therefore in regions of excess antibody, in which detection systems generally work, no precipitin reaction will occur. In contrast to equine serum, precipitation experiments with rabbit serum show precipitation to occur over a wider range of antigen concentration; these include, to a certain extent, regions of antigen and antibody excess. Therefore, a rabbit antitoxin for use in electroimmunodiffusion was produced against the 78A toxin.

The *C. botulinum* 78A toxin prepared by electrophoretic elution was used to inject rabbits for antitoxin production. The antitoxin produced after three injections, including an anamnestic injection, had a precipitin titer of 1:120, but it had a low neutralization strength; 0.2 ml of the rabbit antitoxin neutralized only 9 $LD_{50}$. Continued injections would probably have produced a more potent neutralizing antitoxin, but interest in the serum was mainly in its precipitating capacity and the injections were therefore discontinued. Immunoelectrophoresis conducted with the antitoxin and culture supernatant fluid previously electrophoresed in 3½% polyacrylamide gel showed the antitoxin to be specific, i.e., only one precipitin band was observed,

Figure 13. Immunodiffusion conducted to test the antitoxin for cross-reactivity with other toxin types also demonstrated that the antitoxin was specific; no cross-reactivity was observed with types B, C, E, or F C. botulinum culture supernatants, Figure 14. Type D toxin was unavailable for testing; however, the most important type tested was type B toxin since this organism shares a common antigen with C. botulinum type A.

Electroimmunodiffusion was first studied to develop a quantitative relationship between the antitoxin and known lethal concentrations of the 78A toxin. In practice, the level of sensitivity of this technique has been shown to be limited by the requirement that precipitin cones must be well-defined and sufficiently large to be measurable (Lopez, et al., 1969). Cone size or length is a direct function of the antigen concentration, but the length can also be influenced by the amount of antiserum present in the gel medium; while the cone length can be increased by reducing the percentage of antiserum, this is accomplished at the cost of cone clarity through decreased amounts of immune precipitate. Well-defined cone lengths were obtained when the rabbit antitoxin was diluted 1:15 and 1:30; however, as shown in Figure 15, at a dilution of 1:30 the cone lengths were almost twice the length of cones at the 1:15 antitoxin dilution. An example of the precipitin cones using a 1:30 dilution of the rabbit antitoxin is shown in Figure 16. One may also observe from examination of Figures 15 and 16 the relationship of cone length to antigen concentration. Cone length increases as antigen concentration is increased. The relationship, however, becomes more subject to errors in measurements as the cones get shorter.

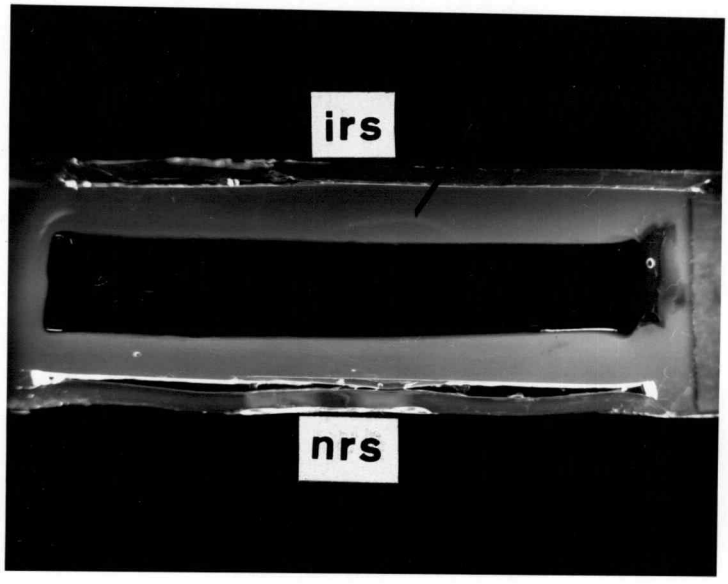



Figure 13. Immunoelectrophoresis of 78A toxin and rabbit antitoxin (IRS) and normal rabbit serum (NRS). The single precipitin arc (arrow) denotes purity and specificity of the 78A antitoxin.

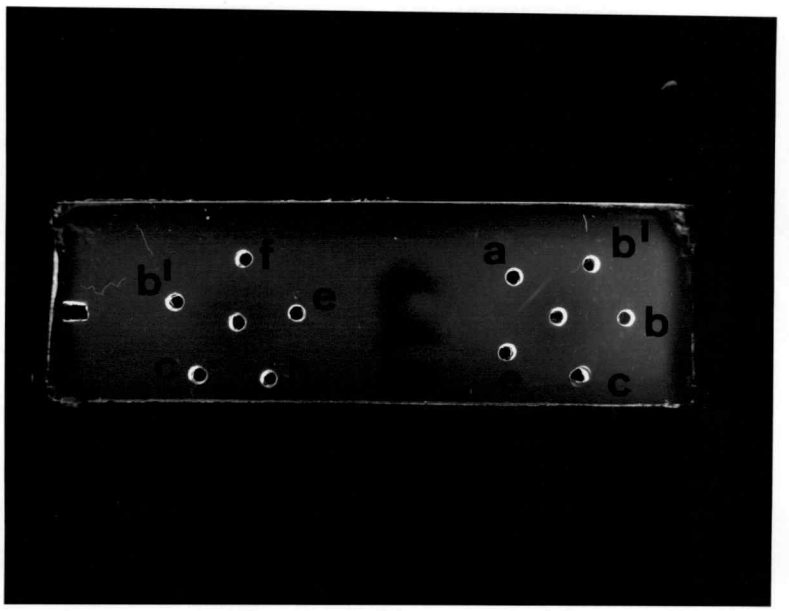



Figure 14. Immunodiffusion of rabbit antitoxin and supernatants of other C. botulinum toxin types, strain 33A (a), strain 115B ($b^1$), strain 213B (b), strain 11772 (c), strain VH (e), and the Langeland strain F (f). The presence of a single precipitin band between 33A and the antitoxin and absence of a precipitin band between other supernatants and the antitoxin demonstrates type specificity of the antitoxin.

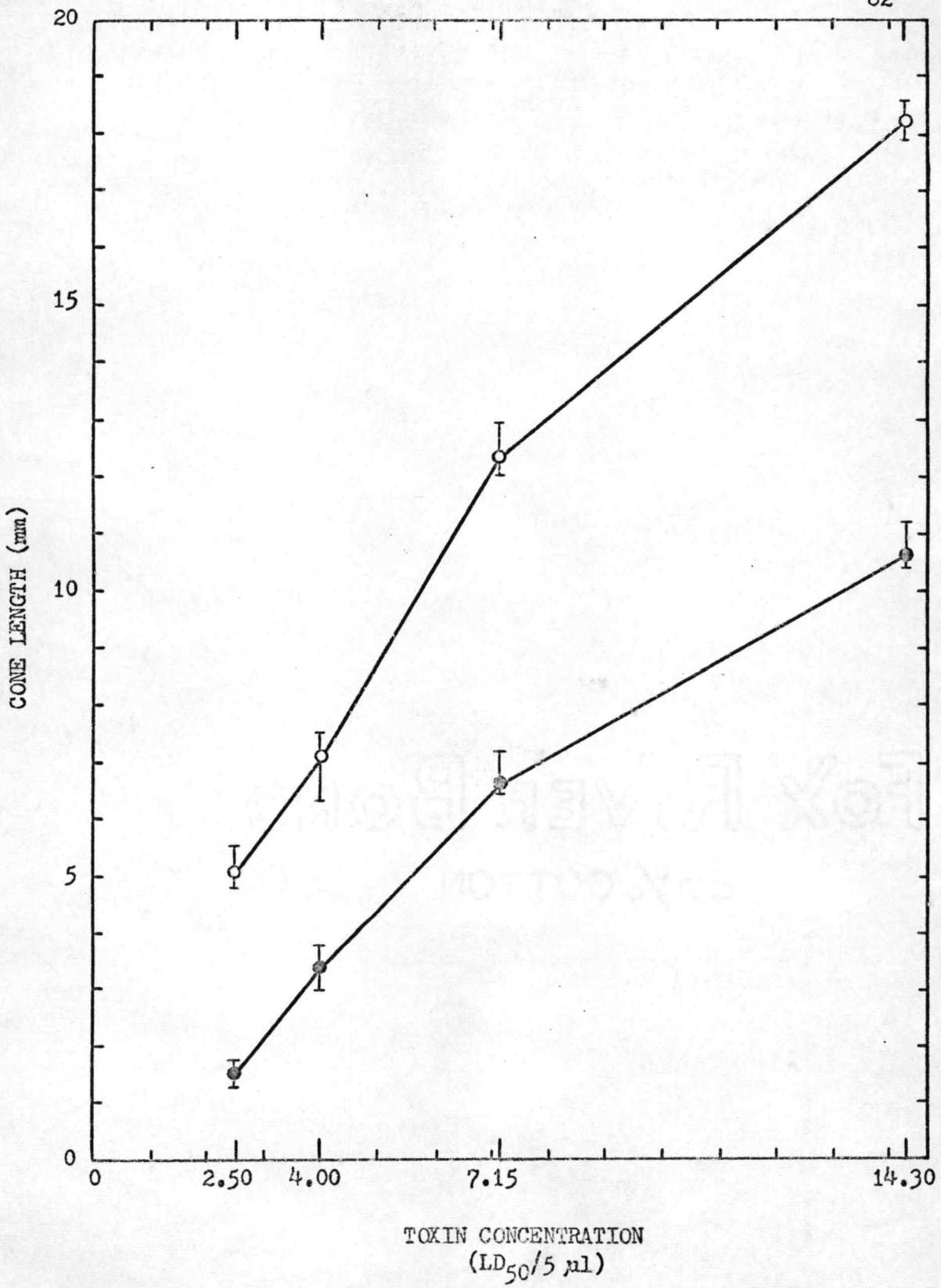



Figure 15. Comparison of precipitin cone lengths in gels containing 1:15 and 1:30 dilutions of rabbit antitoxin. Symbols for antitoxin dilutions: ● , 1:15; O , 1:30.

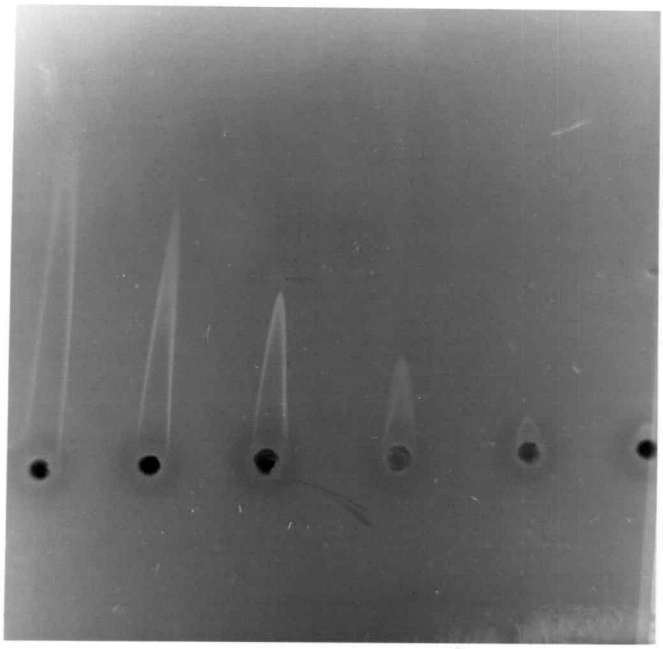


Figure 16. Electroimmunodiffusion of 2-fold dilutions of 78A culture supernatant in agarose containing 1:30 dilution of rabbit antitoxin.

The limit of sensitivity shown in Figure 15 is 2.5 $LD_{50}$; but the method was found to offer even greater sensitivity when an analysis of the sera of the individual rabbits used in this study was conducted. Results of this study, tabulated in Table II, demonstrated the necessity of developing a good precipitating antitoxin. Even greater sensitivity could be obtained by further diluting the serum of Rabbit No. 3. However, the time involved in detection was increased, since the precipitate could not be observed until the slides had been stained.

TABLE II. ANTITOXIN SENSITIVITY ANALYSIS.

| Rabbit No. | Antitoxin Dilution | Toxin $LD_{50}$ | Cone Length (mm) |
|---|---|---|---|
| 1 | 1:30 | 3.8 | 3.2 |
| 2 | 1:30 | 2.5 | 1.4 |
| 3 | 1:30 | 0.7 | 3.0 |

The results presented in Figure 17 show how the migration rate of the precipitin front changes with the duration of electrophoresis. The figure indicates essentially what has been observed by Laurell (1966) in his quantitative study of serum proteins. The antigen migrates rapidly during the early period of electrophoresis, except for the sample containing a larger concentration of antigen. Essentially then, detection time need take only one hour or less for electrophoresis and the time required to prepare the slide and sample. In this study, 15 minutes were required to prepare the agarose, cool it, and mix it with the antitoxin; approximately 15 minutes were required to allow the gel to set, pull wells, fill wells and set up the electrophoretic apparatus.

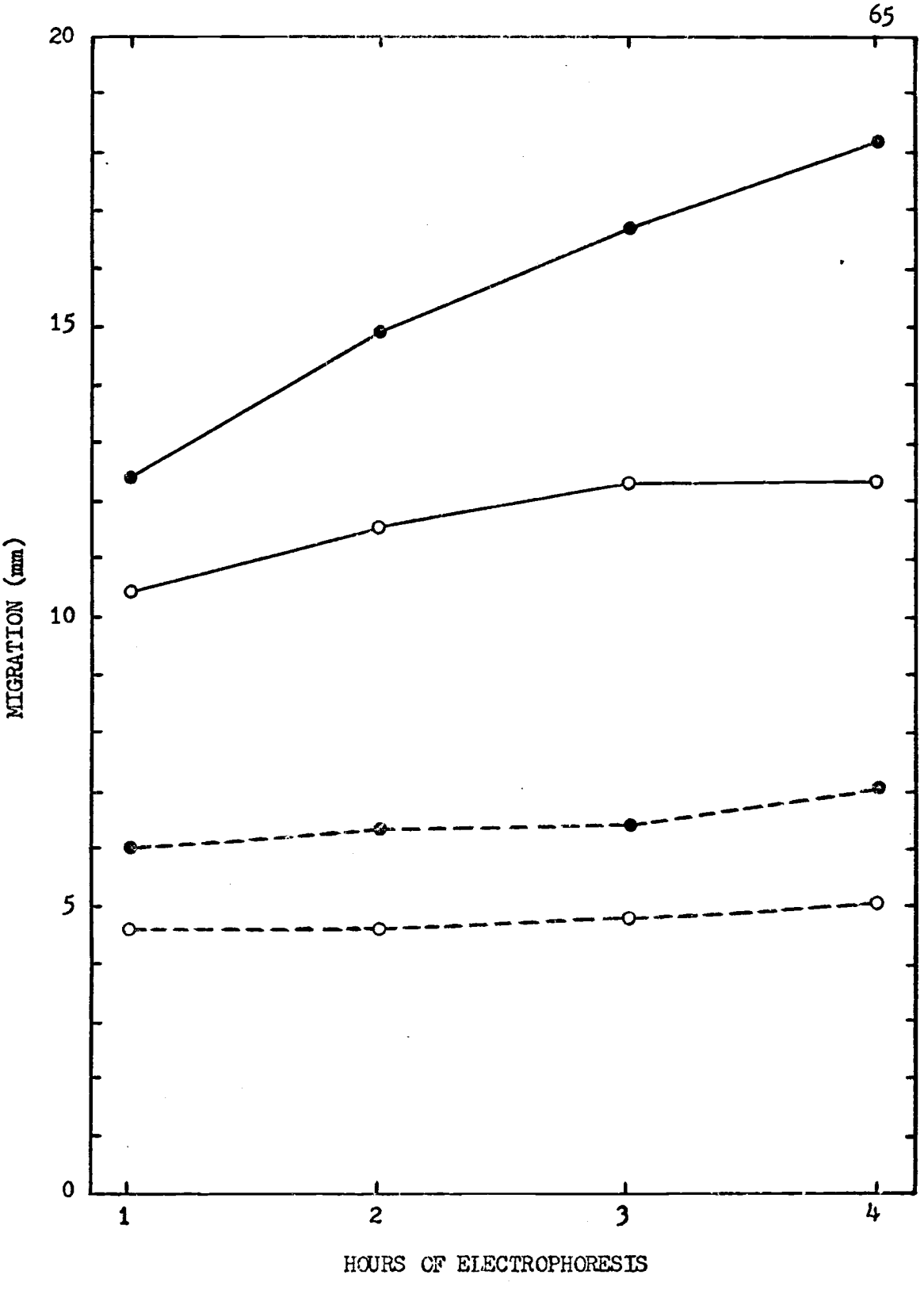



Figure 17. Relation between cone height and duration of electrophoresis. Symbols: ●—● , 14.30 $LD_{50}$; O—O , 7.15 $LD_{50}$; ●---● , 4.00 $LD_{50}$; O---O , 2.50 $LD_{50}$.

The entire duration of time necessary to detect the toxin takes approximately an hour and thirty minutes.

After the initial experiments to establish quantitative relationships, the EID method was used to detect toxin in food samples. Known lethal quantities of toxin were added to the foods, and 5 µl samples of supernatants of semi-liquid foods were placed in the antigen wells. In the case of solid food with little or no moisture, an aliquot of saline was added to obtain a slurry and 5 µl samples of this mixture were placed in the antigen wells. Results are shown in Table III.

TABLE III. EID TOXIN DETECTION IN FOODS.

| Food Sample | $LD_{50}$/5µl Food | Migration* (mm) | $LD_{50}$ Detected | % Recov. |
|---|---|---|---|---|
| Sausage | 15 | 9.0 | 12.9 | 86.0 |
| Canned Tuna | 15 | 6.0 | 8.6 | 57.3 |
| Canned Pumpkin | 15 | 3.5 | 4.1 | 27.3 |
| Canned Green Beans | 15 | 5.8 | 6.4 | 42.7 |
| Canned Spinach | 15 | 5.5 | 6.1 | 40.7 |

*Control Migration = 0.7 mm/$LD_{50}$

The technique was also used to detect toxin in the blood of mice previously injected with 100 $LD_{50}$ of the toxin. No toxin was detected at four hours in any of the group of ten mice, while at eight hours toxin was detected in the measurable range of 28 $LD_{50}$— 40 $LD_{50}$. These estimates were based on the lengths of precipitin cones of control samples.

The EID technique has been found to combine all the requirements

necessary for a botulinum toxin detection system. It is extremely sensitive and expedient, i.e., 0.7 $LD_{50}$ could be detected within 1½ hour. The method is also specific and quantitative. Finally, the simplicity of the method is one of its greatest assets for use in public or private health service laboratories.

## SUMMARY

The toxin of C. botulinum 78A was found to make its first extracellular appearance during the latter portion of the log phase of growth when the pH of the culture dipped to its lowest value. This parallelism was discussed with regard to the sporulation process. Intracellular toxin of cells from this phase of growth was located by means of ferritin-labeled antibody and was found to be primarily cytoplasm-associated.

Type A toxin was isolated by the acid precipitation method from a 96-hour culture supernatant which had stabilized in toxin content. The toxic product was further purified by electrophoresis and elution. Molecular weight analysis by the relatively new SDS-polyacrylamide gel method demonstrated that the major component of the toxin possessed a molecular weight of 81,000, and isoelectric focusing indicated the isoelectric point of the 78A toxin was very near pH 5.23.

Rabbit antitoxin produced as the result of injections of the electrophoretically purified toxin was shown to be specific by its immunoelectrophoretic reaction and its neutralization capacity. The antitoxin's specificity was also shown by its inability to cross-react in immunodiffusion with toxin types B, C, E, or F.

The latex fixation test was found too insensitive to be of any use in botulinum toxin detection. But the relatively new and little known method of electroimmunodiffusion was shown to meet all the criteria for a valuable in vitro detection method, specificity, sensitivity, expediency, and quantitativity. With this method, as little as 0.7 $LD_{50}$ could be detected in a controlled system in as little time as $1\frac{1}{2}$ hours. In foods, recovery of 15 $LD_{50}$ ranged from 27.3% in pumpkin to 86% in

sausage. The simplicity of the method is one of its greatest assets for use in public or private health service laboratories.

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