AN ABSTRACT OF THE THESIS OF

James Morrison Craig (Name) for the Ph. D. (Degree)

in Microbiology (Major) presented on August 7, 1969 (Date)

Title: DISTRIBUTION OF CLOSTRIDIUM BOTULINUM TYPE E IN FISH SHELLFISH AND THE MARINE ENVIRONMENT OF THE PACIFIC NORTHWEST, AND PROTEIN PATTERNS OF THE TOXIGENIC AND NON-TOXIGENTIC STRAINS

Abstract Approved: (Major professor)

Interest in the distribution of Clostridium botulinum type E was heightened by the sudden outbreak of human botulism from smoked whitefish chubs and canned tuna fish in 1963. The question arose as to how widely the organism is distributed among fish and shellfish in the Northwest and what potential hazard exists for the consumer of fish products. This sporeforming anaerobic orgnism is heat sensitive and had eluded detection in other surveys where heat shock had been used to eliminate non sporeforming contaminants. More recent study using other techniques than heat to facilitate recovery has shown this organism to be widespread, especially in the marine environment.

This study was undertaken to find the incidence and

distribution of C. botulinum in the marine organisms and environment of the Pacific Northwest and the food products derived therefrom.

All species of fish were examined by incubating the gills and viscera individually in tryptone-peptone-glucose medium anaerobically at 28 C for four days and testing the culture filtrate for mouse toxicity by intraperitoneal injection. Toxic filtrates were typed by retesting them in mice protected by specific botulinal antitoxin of type A, B, E, or F.

Among salmonid fish the proportions of specimens of each species yielding toxic filtrates were as follows. Sockeye salmon from the Columbia River, 14 of 59 (23.7%); Chinook salmon from the Columbia River, 19 of 106 (18.0%); Chinook salmon from the Pacific Ocean, 1 of 18 (5.6%); Coho salmon from the Columbia River, 10 of 19 (34.4%); Coho salmon from the Pacific Ocean, 13 of 186 (7.0%); Steelhead trout from the Alsea River, 7 of 37 (19.0%). About one-third of the toxic cultural filtrates were successfully typed and proven to contain botulinal toxin. Most of them proved to be type E toxin but 3 were type A, 3 were type B and one, a comparatively new type, type F, was isolated from a Sockeye salmon in the Columbia River.

Pure cultures of Clostridium botulinum type E were isolated from 18 specimens and one specimen yielded a pure

culture of type F from a sockeye salmon. This was the second time this type had been isolated. In all of the experimental groups the proportion of fish producing toxigenic cultures was significantly higher in those taken in the two rivers than those of the same species taken from the ocean waters.

"Botton fish" represented by Cod, Sole, Grouper and members of the Sebastodes group were also tested in the manner described above. The number of specimens yielding toxic filtrates were 28 of 157 (17.8%). When grouped according to location at which the fish were caught, those near the mouth of the Columbia River produced a greater percentage of toxic filtrates than did those caught off the open shore line. The results were as follows: Bottom fish from Astoria, 23 of 70 (32.8%), Botton fish from Coos Bay, 5 of 87 (5.6%). Sturgeon specimens produced 3 of 24 (12.5%) toxic filtrates. Most of the species contained type E; however, one type A and one type B were found on typing, with about one-third of the toxic filtrates being successfully typed.

Environmental swab samples from the "deep sea" fillet processing plants produced 3 of 39 (7.7%) toxic filtrates. None of the 53 samples taken in the salmon processing plants produced toxic filtrates.

Shellfish were collected along the ocean beach and in the estuaries. Three to five shellfish were combined into a single

specimen and treated as described. All shellfish obtained from the estuaries demonstrated a higher percentage of toxic filtrates than those obtained from the ocean beach. The results were as follows: Razor clams, 11 of 75 (14.6%), Cockle clams, 12 of 15 (80.0%); Softshell clams, 8 of 12 (66.4%); Littleneck clams, 4 of 11 (36.2%); Horseneck clams, 1 of 3 (33.3%); Oysters, 6 of 19 (31.6%); Dungeness crabs, 17 of 24 (71%). Only the razor clams were collected exclusively from the ocean beach.

Loss of toxicity on holding mixed cultures at -15 C while awaiting typing was a continual problem. This accounts for only one-third of toxic filtrates being successfully typed.

Electrophoretic analysis of the total bacterial proteins was carried out on cell sonicates and cell free culture filtrates by first growing cells for four days at 28 C anaerobically. The cells were separated, washed and disrupted with ultrasonic energy. The cell free culture filtrate was concentrated 10 fold by dialysis against polyethylene glycol 4000. Both the toxigenic organisms and the toxic filtrate demonstrated an extra common protein band in the upper third of the electrophoretic pattern not present in the nontoxic spectra. This band might represent the type E toxin. Differences could also be noted in the number of protein bands in the lower third of the patterns in different nontoxigenic strains and also when the toxigenic and nontoxigenic strains were compared.

This could suggest an association with a phage in the toxigenic cultures.

DISTRIBUTION OF CLOSTRIDIUM BOTULINUM TYPE E IN FISH, SHELLFISH AND THE MARINE ENVIRONMENT OF THE PACIFIC NORTHWEST AND PROTEIN PATTERNS OF THE TOXIGENIC AND NONTOXIGENIC STRAINS

by

James Morrison Craig

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the

degree of
DOCTOR OF PHILOSOPHY

June 1970

APPROVED:

Redacted for Privacy

Professor of Microbiology
In Charge of Major

Redacted for Privacy

Chairman of the Microbiology Department

Redacted for Privacy

Dean of Graduate School

Date thesis is presented August 7, 1969

Typed by Mary Lee Olson for James Morrison Craig

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to the many persons who have given encouragement and help in fulfilling the requirements for this degree:

To Dr. K. S. Pilcher for his advice and guidance.

To Dr. A. W. Anderson for his support and encouragement.

To Mr. Sidney Hayes and Mrs. Wilda Layton for their technical assistance.

To my family, and especially my wife Reva, for their patience, understanding and continued support for yea these many years.

This study was financed in part under contract FDA 66-2 (neg) with the U. S. Food and Drug Administration, Department of Health, Education and Welfare and by NIH Grant 1-F3-AI-21-241-01.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

DISTRIBUTION OF CLOSTRIDIUM BOTULINUM TYPE E IN FISH, SHELLFISH AND THE MARINE ENVIRONMENT OF THE PACIFIC NORTHWEST AND PROTEIN PATTERNS OF THE TOXIGENIC AND NONTOXIGENIC STRAINS

INTRODUCTION

The 1963 outbreaks of botulinum food poisoning resulting from the ingestion of Clostridium botulinum type E toxin in smoked whitefish and smoked whitefish chubs from the Great Lakes (Anonymous 1964, Osheroff et al 1964) as well as earlier outbreaks, aroused interest in the incidence of Clostridium botulinum in other marine species such as salmon, tuna, shrimp and crab. The distribution of this organism in fish from the Great Lakes was reported by Bott (1966).

C. botulinum has been identified in marine sediments from the Gulf of Mexico (Ward and Carroll, 1965) and the Pacific Coast (Ecklund and Poysky, 1965); it has also been found in several species of fish (Dolman et al, 1953, Hinman 1966, Johannsen 1963) and shrimp in the Gulf of Mexico (Carrol et al 1966).

Eklund and Poysky (1965) reported the presence of the organism in a large percentage of Dungeness crabs collected in the Pacific Northwest.

This investigation was undertaken to study the incidence of C. botulinum type E in the most important species of fish and shellfish of the Pacific Northwest and to understand its ecological and physiological relationships in this region.

LITERATURE REVIEW

History and Background

Clostridium botulinum was first identified by von Ermengem in 1896. After a meeting of a music society in Ellezelles, Belgium, three persons died and twenty-three became ill. All had eaten raw ham. The ham was soft and pale in color and had a "rancid" odor. From this ham and from the spleen and the intestine of one of the victims Ermengem isolated an anaerobic organism which produced a very lethal toxin.

He compared the symptoms to the sausage-poisoning syndrome first described a century before, in 1770, which Müller had called botulism. Von Ermengem therefore proposed the name Bacillus botulinus for the newly isolated organism. This name was to last over sixty years.

Landmann (1904) traced an outbreak which occurred at Darmstadt, Germany, to beans used in a bean salad. From the bean salad Landmann was able to isolate a pure culture.

Leuchs (1910) produced antitoxins in horses against the two strains of Clostridim botulinum, the Ellezelles strain and the Darmstadt strain. On the bases of antitoxin protection tests, Leuchs demonstrated the occurrence of two different toxigenic types. The antisera of the Ellezelles strain would not protect animals injected with Darmstadt toxin and vice versa.

In 1917 Dickson of the Stanford Medical School in San Francisco, California, produced antitoxin in goats which were immunized against his strains III, IV, VI respectively. He found that strains III and IV, isolated from outbreaks of botulism in California and Oregon, were homologous. The toxin of strain VI however, which he had isolated from cheese received from Albany, New York, was not neutralized by the antitoxin against either strain III or strain IV.

Burke (1919) was able to separate twelve strains of Clostridium botulinum obtained from outbreaks in California and Washington by the use of antitoxin. The twelve strains fell into two distinct types as shown by toxin-antitoxin reaction in mice. The antitoxin of one type neutralized the toxin of its own type but not that of the heterologous type. Burke designated these as type A and type B. She also noted that type A usually produced a stronger toxin in broth than the type B strains, but their morphology and cultural characteristics were identical.

In the early 1920's three apparently additional types were described by Bengston (1922, 1923), Seddon (1922) and Theiler et al (1927). The Bengston strain, isolated from fly larvae implicated in a paralytic disease of chickens, was later designated as type C_a. The Seddon strain, isolated from beef carcasses, produced a toxin immunologically similar to the Bengston strain

and was designated as C_b. The strain which Theiler et al isolated from animal carcasses produced another immunologically distinct toxin called type D.

In 1935 a culture of Clostridium botulinum isolated from Red Fish caught in the Sea of Azov, was sent to Dr. K. F. Meyer of the Hooper Foundation, University of California, Berkley, California, by L. Bier of the Biological Institute of Dneipropetrowk, Ukraina, U.S.S.R. This was shown to be a new type by Gunnison, Cummings and Meyer (1936), who proposed for it the designation Clostridium botulinum type E. Later six different isolates from Dr. E. Kushnik of the above mentioned Russian institute were sent to Dr. Meyer and they also were confirmed as type E. Five of the cultures were from sturgeon (Acipenseridae) intestines (Dolman, 1953), Hazen (1937) reported a culture isolated in December 1934 from German canned sprats responsible for three cases of botulism, one fatal, as neither type A, B, or C. The clinical symptoms were typical for botulism however, and attempts at in vitro and in vivo neutralization of the toxin by monovalent botulinus antitoxin types A, B, and C or combined antitoxin failed. These were later confirmed as Clostridium botulinum type E.

Møller and Scheibel (1960) reported an outbreak of human botulism in 1958 on the Danish island of Langeland. Of the five

persons who consumed the contaminated liver paste, one died. The organisms isolated from the liver paste were similar to Clostridium botulinum in proteolytic and saccharolytic properties, but the toxin was not neutralized by antitoxin to types A, B, C, or D. The toxin was neutralized by excessive amounts of type E antitoxin, but apparently was not identical with type E, for it was not activated by trypsin. The culture was more extensively studied by Dolman and Murakami (1961) and designated as type F. Detailed studies on growth and toxin production of this organism was the topic of a doctoral thesis by Lillian Holderman (1964). Additional cultures of type F were isolated from mud samples of the California Coast by Eklund and Poysky (1964) and from sockeye salmon caught in the Columbia River by Craig and Pilcher (1966).

Distribution of Clostridium botulinum

Clostridium botulinum is distributed world wide, but the types are not evenly dispersed. The highest incidence of type A has been found in the Western United States and the Soviet Ukraine. Easton and Meyer (1924), Zlatogaroff and Soloviev (1927), Dolman and Murakami (1961).

Type B has been found most frequently in the Eastern United States, France and Norway (Prevot and Huet, 1951) (Dolman,

1964), although identifications have occurred in England (Haines, 1952), Holland, Switzerland, and Belgium (Meyer and Duboboki, 1922) as well as in British Columbia (Dolman, et al, 1960).

Type C has been isolated or identified in Australia (Grubb, 1964), Western United States (Boroff and Reilly, 1958) (Kahnbach and Gunderson, 1934) Canada (Avery et al, 1959) South Africa and South America (Carroll et al, 1966) and type C_b in North America, Australia, Europe and South Africa (Dolman and Murakami, 1961) (Carroll et al, 1966).

Type D seems to prevail in Africa and Australia and is more often involved with the death of cattle (Eales and Turner, 1952) (Dolman, 1964.)

Type E has been isolated or identified in British Columbia, Northwestern United States, Japan, Alaska, Sweden, Denmark, and Russia (Dolman, 1954, 1957, 1961) (Dolman and Iida, 1963) (Eklund and Poysky, 1965). It seems to have an affinity for the marine environment and to be the main etiological agent in outbreaks due to marine products (Dolman, 1957) (Cann et al, 1965) Bott et al, 1966) (Craig, Hayes, and Pilcher, 1968).

Type F has been isolated from an outbreak caused by food from Denmark (Møller and Scheibel, 1960), from marine sediments off the California Coast (Eklund and Poysky, 1965), North Dakota (Wentz, Scott, and Vennes, 1967), Argentina (Giminez and Ciccarelli,

1968), from a salmon in the Columbia River (Craig and Pilcher, 1966) and from crabs of the Atlantic Coast (Walls, 1969).

Incidence

Meyer and Eddie (1965) report the first case of human botulism in the United States and Canada as occurring in California in 1899. This incident involved one case from beef tamales but did not result in the death of the individual. Most of the outbreaks occurred in the Western United States and often with fatal results. Up until 1916 there were twenty-nine outbreaks of botulism reported in the United States, all except six occurring in states bordering on the Pacific Ocean (Meyer and Eddie, 1965). These outbreaks involved one hundred and ten persons of which eighty died resulting in a 73.6% fatality record.

The products involved in these early botulism outbreaks covered a wide range. Eleven involved vegetables, five of which had been comercially canned and six home canned. String beans were involved in six cases and were the single vegetable responsible for the largest number of botulism outbreaks. Six outbreaks were due to meat or meat products, two to seafood and another two to fruit. Cottage cheese was involved twice and the food product could not be determined in three other outbreaks.

Meyer and Eddie (1965) also list fifty-eight additional outbreaks occuring between 1916 and 1920. The majority of these involved home canned products with twenty-two being vegetables. However, ham, olives, home brew, and tuna appeared for the first time. During this same period, through 1920, Dickson and Burke cited cases in which domestic animals, mainly chickens with a few ducks and five burros, also became ill after eating contaminated food. One of the first outbreaks involving a large number of persons occurred at Stanford University in 1913 (Wilbur and Ophuls, 1914) from string bean salad in which twelve persons became ill and five deaths resulted. Not until 1919 were so many cases involved in a single outbreak. Individual outbreaks were reported prior to 1920 by Dickson (1915), Dickson (1917), Dickson and Burke (1918), and Dickson and Howitt (1920), Thom, Edmondson, and Giltner (1919), Debord and Thom (1920), and Kosher, Edmondson, and Giltner (1921), as well as others.

The relation of toxin and its physiological reaction to the disease was not appreciated at this time. Wilber and Ophuls (1914) believed that death was due to thrombosis of brain tissue.

Glancy (1920) reported the first outbreak in Canada occurring near Dawson City, Yukon, in 1919 which affected twenty-three persons with twelve fatalities. The cause of this outbreak was

first thought to be cold-storage beef, but on review by Geiger, Dickson and Meyer (1922) they concluded that commercially canned beets were the probably source.

Botulism continued in sporadic waves of three outbreaks involving five persons with three deaths in 1925 to a high of twenty-five deaths in 1947. From 1899 to 1963, one thousand, five hundred and sixty-one cases of botulism were reported in the United States (Osherhoff, Slocum, and Decker, 1964). The greatest number were in the decade 1930-1939, the second greatest, 1920-1929, and the third greatest, 1940-1949.

Home processed foods have been the most commonly implicated sources of infection since the early 1920's. Clostridium botulinum type A and B were most often responsible for the outbreaks arising from the noncommercially processed foods.

Type E became more prevalent as the cause of botulism in the late 30's and early 40's. In the United States in the past two decades, type E was the major cause of botulism from commercially prepared foods. The foods most often implicated were unprocessed or dried or smoked at relatively low temperatures.

In 1963, forty-six cases of botulism were reported in the United States. These cases, which included fourteen deaths in twelve outbreaks, represented the highest total in twenty years.

The foods involved included chili peppers, string beans, corn, liver paste, tune fish, mushrooms, figs, and smoked whitefish. Commercially canned food products accounted for five cases with two deaths; commercially smoked products, for nineteen cases with seven deaths; home processed foods for twenty-two cases with the five deaths. The type of Clostridium botulinum toxin responsible as the etiological agent was identified in two outbreaks as four cases of type A; four of the twelve outbreaks involving eleven cases were type B, three outbreaks involving twenty-two cases were type E, and in the remaining outbreaks with nine cases, the type remained unknown. With the increasing number of outbreaks in which the etiological agent proved to be Clostridium botulinum type E, the food and Drug Administration instituted studies to determine the extent and distribution of type E organism in the United States. This study is an outgrowth of the desire to learn the extent of type E distribution.

EXPERIMENTAL MATERIALS AND PROCEDURES

Culture Media

Tryticase-Peptone-Glucose (TPG) Medium

The medium was prepared using 0.5 percent trypticase (BBL); 0.5 percent peptone (Difco); 0.4 percent glucose (Difco); and 0.2 percent sodium thioglycollate (Difco); made up to volume with distilled water. The pH was adjusted to 7.0 and sterilization for 15 minutes at 15 psi at 121 C followed. This is a modification of the procedure of Schmidt et al (1962) in which they added sterile glucose (0.2 percent) and sodium thioglycollate (0.4 percent) to a trypticase (5.0 percent)-peptone (0.5 percent) medium just prior to innoculation. They used the trypticase-peptone medium for the preparation of spore suspensions of proteolytic types A and B. Since type E strains are predominately saccharolytic they added glucose to grow Type E.

Liver-Veal Agar

The liver-veal agar base (Difco) was prepared by rehydrating 97 grams in 1,000 ml of cold distilled water. This suspension was solubilized by steaming followed by autoclaving for 15 minutes at 15 psi at 121 C. The basal medium was then allowed to cool to 45-50 C before the egg yolk suspension was added.

The fresh eggs were scrubbed and the surface disinfected by

by allowing them to set thirty minutes in each of two solutions: 1:10,000 mercuric chloride followed by 70 percent ethanol. After cracking, the eggs were separated aseptically by the use of a standard household separator and the yolks dropped into a sterile beaker, and mixed with an equal volume of 0.85 percent saline. The yolk and other membranes were then removed with sterile forceps. The egg yolk suspension was added to the cooled (45-50C) liver-veal agar base to a final concentration of 8-10 percent. Petri dish plates were poured immediately after mixing and stored at 4 C until set. It was found that better growth occurred if the surface was allowed to dry slightly by leaving the Petri dish lids ajar for one hour after pouring.

Blood Agar

The blood agar base (Difco) was prepared by rehydrating 40 grams in 1,000 ml of distilled water. The suspension was heated in steam until complete solution was obtained. After autoclaving for 15 minutes at 15 psi at 121 C, the sterilized medium was allowed to cool to 45-50 C and 5 percent defibrinated human blood was added. Plates were poured immediately after mixing and kept at 4 C until used.

Gel-Phosphate Diluting Solution

The diluent was prepared using 0.2 percent gelatin (Difco)

and 0.4 percent phosphate (Na_2HPO_4) as suggested by Duff, Wright, and Yarinsky (1956). The pH was adjusted to 6.2 using N/10 hydrochloric acid and then sterilized by autoclaving at 121 C for 15 minutes at 15 psi. This was used as a toxin diluent and as a solution for animal injection. Wentzel and Polsen (1950), Littauer (1951) and Duff, et al (1957) all cite the increase in toxicity and toxin stability by use of gelatin in buffered systems and by adjusting the pH below 6.8.

Trypsin

A concentrated trypsin solution was made by suspending 10 grams of trypsin (Difco 1:250) in 1,000 ml of distilled water and allowing the solution to remain overnight at room temperature to ensure maximum solubility. The solution was filtrated through a sterile Seitz filter using an EK pad. Sterile vaccine vials were filled and stored at -12 C until used.

Antitoxins

Clostridium botulinum, types A, B, C, and E antitoxin were all provided by the Food and Drug Administration of Washington, D. C. Type F antitoxin was provided by the United States Public Health Communicable Disease Center in Atlanta, Georgia.

All antisera were in a dehydrated form and when rehydrated

in the original volume of a sterile 1:1 glycerol-saline solution were equated at 10 units per ml. Types A, C, and E were equine serum while types B and F were rabbit serum.

Sampling Procedure

Samples of various species of marine fish, shellfish, smoked fish, sediment samples, and water samples were collected for testing.

Fish

Most fish were obtained either from the sport fisherman or commercial processing plants. Some of the steelhead were obtained from the fish hatchery traps on the Alsea River. A few were caught on hook and line or by net by the investigating personnel.

Each fish was placed on a sterile piece of paper and a 200 ppm solution of sodium hypochlorite was brushed over the surface to be cut out; the intestinal tract and the gills were removed aseptically. Sterile knives were used and the collector wore rubber gloves which were immersed in a sodium hypochlorite solution containing 400 ppm of chlorine, between specimens. The knives were also sterilized between specimens by allowing them to stand in a similar solution for 20 minutes and then dipping

in 70 percent alcohol and flaming before use. The gills and the viscera from each fish was placed in individual sterile plastic bags. After the addition of approximately 50 ml of TPG medium to each bag, most of the air was removed by squeezing. The bag was twisted and securely closed with a rubber band. The sample number and date was written on a card and placed under the rubber closure. The sample was them placed in a cooling chest in chipped ice. All samples were returned to the laboratory within twelve hours after they were obtained. Samples that could not be processed within twenty-four hours were frozen at -20 C. All samples were collected by the author or one of the team working on this project.

Shellfish

Clams and oysters were gathered from their natural habitat, washed free of sand in sea water, and three to five of the species were placed in a plastic bag. The bags were sealed with rubber bands and keps on ice until returned to the laboratory where they were refrigerated (4 C) until tested or frozen if testing was not possible within twenty-four hours. The contents of each plastic bag was considered as a single sample. On preparation for incubation the shell fish were scrubbed free of any attached sediments with a stiff brush and water, then dipped

in sodium hypochlorite solution, placed in a sterile pan, and opened with a sterile knife. The contents, including the liquid, were placed in a sterile plastic bag and approximately 50 ml of heated and cooled TPG was added. Most of the air was excluded by squeezing the bag and twisting the top after which it was sealed with a rubber band and incubated for four days at 28 C.

Crabs

Crabs were collected from the edge of the water, placed in plastic bags and held on ice or refrigerated (4 C) until tested.

Water Samples

A few samples of water were collected adjacent to or in the proximity of the commercial fish plants. These were collected in sterile bottles of approximately one gallon size. The samples were filtered under vacuum, first through a membrane filter of 0.45 micron pore size, followed by filtration through a filter of 0.22 micron pore size. The membrane filters were placed in 18 x 150 mm screw cap test tubes with 15 ml of TPG medium. Even with a two stage procedure, filtration was extremely difficult and the filters frequently plugged. Due to these difficulties water samples were not routinely processed.

Neutralization Tests for Typing Botulinum Toxin

Neutralization tests were performed by inoculating 0.5 ml of the rehydrated type A, B, E, or F antitoxin containing 5 units, intraperitoneally into each of four Swiss Webster strain white mice weighing 18 to 22 grams. The inoculations were made 30 to 40 minutes before the mice were challenged with the test sample.

Samples of the mixed cultures from specimens were sterilized by filtration through a 0.22 micron pore size membrane filter in a Swinney adapter. After filtering, the sample was tryspin activated by addition of 10 percent trypsin solution so as to give a final concentration of one percent trypsin. The trypsin-sample mix was allowed to stand for one hour at 37 C after which 1:10 and 1:100 dilutions were made in gelphosphate. Each dilution was then injected into two unprotected mice in a volume of 0.5 ml and into two protected by antitoxin as described above. The mice were observed over a 96 hour period for typical botulism symptons. They were under constant observation for four hours and observed at two hour intervals for an additional eight hours. After this period of rather close observation the interval was lengthened to four hours for the next twenty-four hours and four to six hours until the 96 hour time had passed.

When a sample was proven toxic after incubation, Type E antisera was used in the neutralization test for type determination

performed within twenty-four hours from replicate samples used in the toxicity test and frozen at -20 C immediately after use. If all animals died, protected and unprotected, the sample was then tested in a similar manner with A and B antisera. Often by this time the sample had lost toxicity. We then transfered 10 ml of the mixed culture from the sample to 50 ml of TPG medium and reincubated for four days, after which new neutralization tests were performed using A, B, and E antisera. Typing was determined by observing the deaths of the unprotected mice and the protected mice which did not live even though injected with antitoxin containing sera. The animal protected with a proper antisera would live thereby indicating the type of Clostridium botulinum present.

The rationale for this schedule is shown later.

Treatment of Sample

TPG medium was added to the sample after it had been placed either in a plastic bag, a bottle, or a test tube. The latter were closed with a screw top lid while the plastic bag was closed by twisting and folding the mouth of the bag after expelling most of the air and holding the bag by the use of a rubber band.

The sample was incubated at 28 C for four days after which an aliquot was withdrawn and centrifuged at 3,000 revolutions per

minute (rpm) in an angle head International Model V centrifuge for twenty minutes. The cleared supernate was drawn off and the centrifuge tube containing the sample was frozen at -20 C. Two ml was filtered through a 0.22 micron filter and used to test for toxicity and the balance of the supernate was frozen also at -20 C.

The sample was adjusted to pH 6.2 with N/10 hydrochloric acid using paper as a test for pH end point and 10 percent sterile trypsin (Difco) solution was added to give a final trypsin concentration of one percent. This was allowed to stand at 37 C for 45 minutes after which a 1:5 dilution was made with a gel-phosphate buffer at pH 6.2. Five tenths ml was injected into each of two mice, Swiss Webster strain weighing 18 to 22 grams. They were observed for 96 hours as described under "Neutralization Test".

Three to five gram samples of sediment or of smoked fish samples were placed in bottles, 50 ml of heated and cooled TPG was added and the bottles loosely capped. They were then placed in a Case anaerobic jar and the air evacuated and replaced with a mixture of 96 percent Nitrogen and four percent CO_2. This was repeated three times to insure evacuation of most air and its replacement with the inert gas mixture. The Case jar was then incubated at 28 C for four days after which the sample was centrifuged and processed as described before.

Sediments

Sediment samples were obtained from the beaches at the outfall of a stream or from the estuary at the stream terminus. These samples were collected from the area of the beach exposed between low and high tide. Three samples were usually collected from one transect; one foot below the water level, at water level, and one foot above the current tide level. These were scooped into sterile jars being careful contamination from the hands did not contribute to the sample. Some samples were taken in deeper water with a core sampler which contained a plastic liner. The core sampler was threaded on the end of a 1 1/2 inch pipe and plunged into the sand or mud below the water line. The plastic liner was filled with hypochlorite solution (400 ppm of chlorine) until just before use when the hypochlorite solution was dumped and the plastic liner placed in the core sampler. The samples were then placed on ice and upon reaching the laboratory were refrigerated at 4 C until tested.

Incubation Temperatures

Toxin production was dependent upon incubation temperature. Hazen (1937) demonstrated the great increase in toxin production by a strain of C. botulinum later classified as type E, at 30 C while little or no toxin was produced at 37 C. Dolman (1950)

likewise demonstrated toxin production in pickled herring at 30 C. Prevot (1951) noted that toxin was best produced at 30 C or below and chose to use 26 C. For this study, 28 C was chosen on the basis of the above investigations.

Incubation Time

The amount of time necessary to produce the maximum amount of toxin caused some concern. An insufficient time could mean missing some samples containing a low toxin producer while an excessive amount of time could cause a loss or destruction of toxin in the mixed culture. An experiment was then designed to assure a more complete knowledge of toxin production in relation to time of incubation.

Two samples were set up for testing. One, a flask of TPG medium and the other, a gill and viscera speciment removed from a salmon and placed in a plastic bag with 800 ml of TPG medium added. Both samples were seeded with 10 ml of a 24 hour culture of the VH strain of Clostridium botulinum type E. This strain was originally isolated by Dolman from pickled herring in British Columbia. Thus the first sample was a pure type E culture, while the second was a mixed culture.

The flask of TPG medium was provided with a vaccine type rubber stopper through which a needle could be inserted. In this

way samples were removed every 24 hours for testing. The fish gills and viscera were placed in a plastic bag, most of the air squeezed out, and the neck of the bag tied shut after placing a short piece of glass tubing through the neck. The glass tubing was fitted with a serum bottle closure through which a needle could be inserted for sampling at each 24 hour period.

The sample, when drawn off, was filtered through a 0.22 micron filter in a Swinny adapter to produce a sterile solution. This solution was then adjusted to pH 6.2 and a 10 percent sterile trypsin (Difco) solution was added to give a final trypsin concentration of one percent. This was allowed to stand at 37 C for 45 minutes after which 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions were made with sterile gel-phosphate buffer at pH 6.2. Five tenths ml was injected IP into each mouse used in the test.

The LD_{50} was calculated according to Reed and Munch (1938) using six mice per dilution for each sample tested. The results of the titration of the pure culture in TPG are shown in Figure 1. Here we see an increase in toxicity for about 48 hours with a nonsignificant decline, leveling off to a fairly constant value.

The toxin titration of the mixed culture from salmon viscera is shown in Figure 2. The maximum toxicity was reached in 60 hours with a two log degrease noted in the next 24 hours after which there was a slight nonsignificant decrease over the next

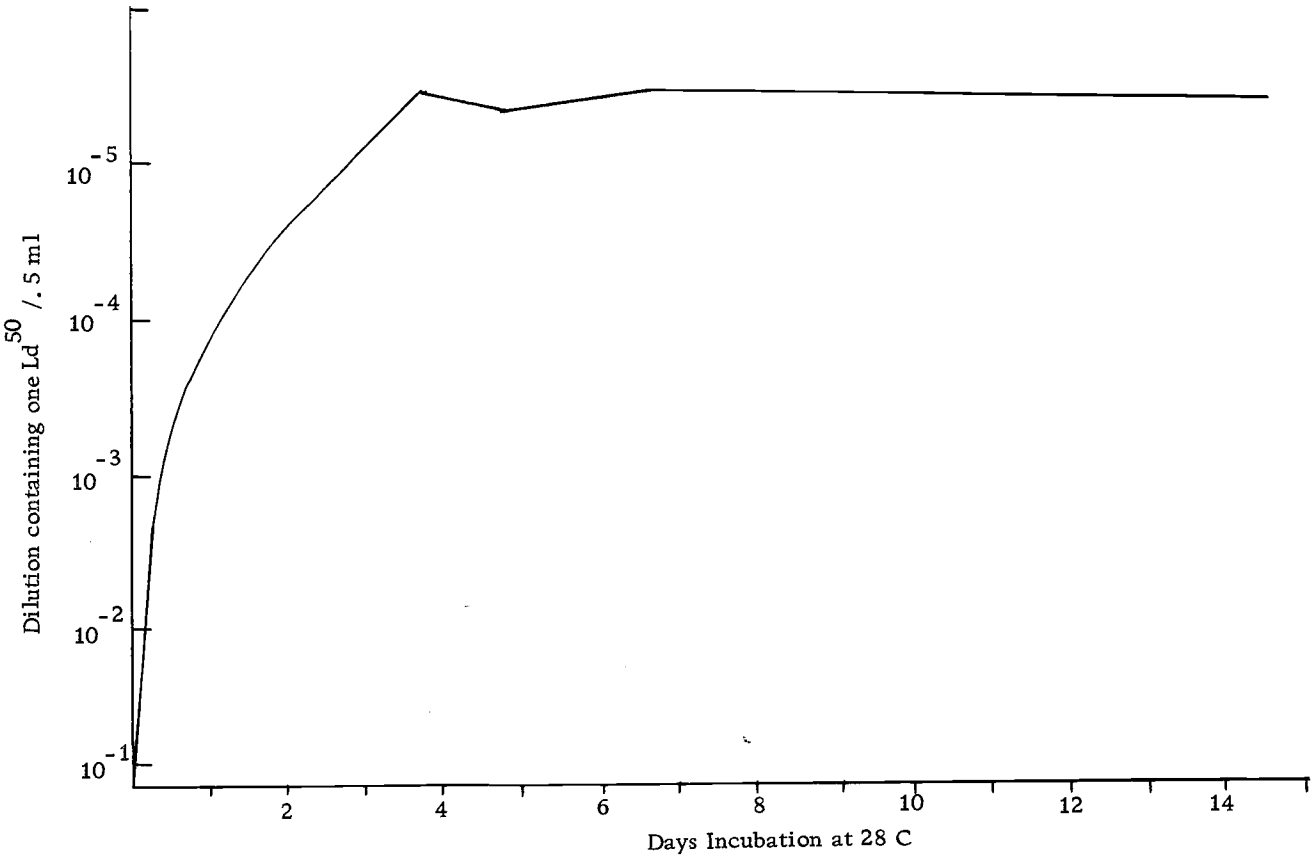


Figure 1. Toxicity of C. botulinum Type E in T. P. G. Medium

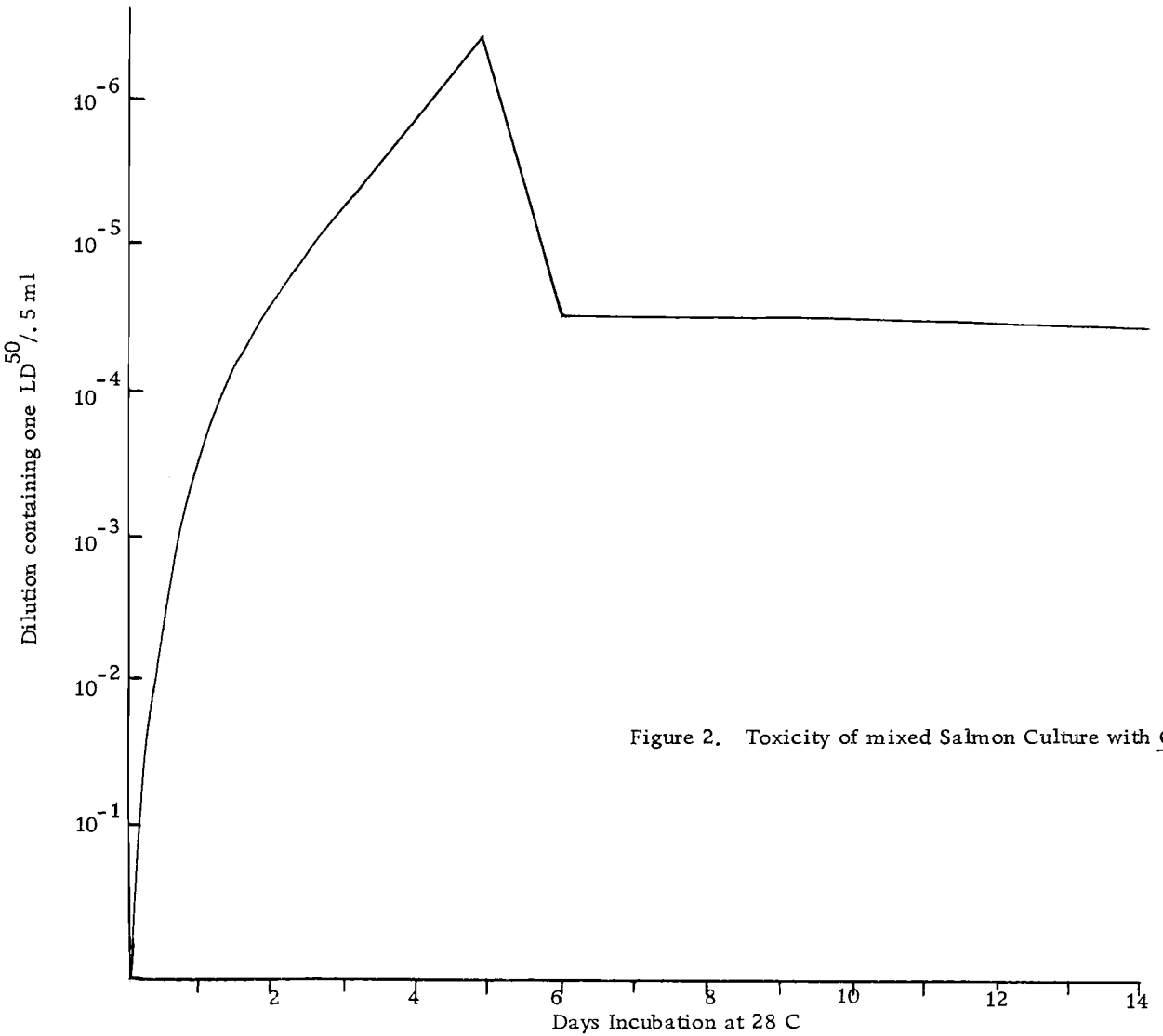


Figure 2. Toxicity of mixed Salmon Culture with C. botulinum Type E.

ten days. The maximum toxicity was about one log higher in the mixed culture than in the pure culture tested.

Both cultures were tested at the end of 28 days incubation and the slope of decline remained relatively steady with no further significant loss.

On the basis of these results the incubation time selected for testing all samples was 90-98 hours. This variation was due to our schedule in testing samples incubated.

Time of Death in Relation to Toxin in the Sample

It was observed that when a group of mice were injected with a toxin filtrate all the mice did not die at the same time. This raised several questions: How long should mice be observed after injection? How can one be sure of not missing a low toxin producer? What part does individual mouse resistance play in the relation of time of death to toxin concentration?

Two different tests were run with somewhat different results. TPG was seeded with Clostridium botulinum type E and incubated at 28 C for 96 hours. The fluid medium was then filtered through a 0.22 micron millipore filter in a Swinny adapter and the pH adjusted to 6.2 by use of a narrow range paper pH indicator. Trypsin was added to give a final concentration of one percent and the sample placed in a 37 C water bath for 45 minutes. Ten

fold dilutions were then made with gel-phosphate buffered solution and, for each dilution, 0.5 ml was injected intraperitoneally into each of six mice. The animals were observed carefully for typical symptons over a 160 hour period. The results are presented in Table 1. The second test was very similar except that an increased number of mice were used. The results are presented in Table 2. As a result of these tests continued observation of the mice was instituted for eight hours after injection and continued at about four hour intervals for 96 hours before being considered negative.

Isolation of Pure Cultures

After a sample had been proven toxic and the type determined by mouse protection with homologous antitoxin, isolation of the organism was attempted as soon as possible.

The spores of Clostridium botulinum type E are known to be heat sensitive and to approach the sensitivity of some of the vegetative cells which contaminate the sample. This therefore precluded the use of heat as a method of ridding the sample of these contaminants in order to isolate Clostridium botulinum in pure culture.

The procedure used was modified from that described by Johnson, Harmon, and Kautter (1964).

Table 1. Time of Death Related to Toxin Amount

Sample	Deaths Noted*	Mean Average Death Time
Undiluted	6 < 2 hrs.	1.5 hrs.
$10^{-0.5}$	6 < 2 hrs.	1.7 hrs.
10^{-1}	6 < 3 hrs.	1.7 hrs.
10^{-2}	6 < 12 hrs.	5 hrs.
10^{-3}	4 6-8 hrs. 2 12 hrs.	9.5 hrs.
10^{-4}	2 20-26 hrs. 1 36-40 hrs. 1 96-100 hrs. 2 survived	48 hrs.
10^{-5}	1 90-96 hrs. 5 survived	96 hrs.
10^{-6}	6 survived 160 hrs.	none

Table 2. Time of Death Related to Toxin Amount

Sample	Mean Time of Death	Mice Used
10^{0}	1-3 hrs.	12
10^{-1}	3-4 hrs.	12
10^{-2}	6 hrs.	48
10^{-3}	12 hrs.	48
10^{-4}	25 hrs.	48
10^{-5}	130 hrs.	12

* First figure represents mice, second is hours.

Two ml of the sample were mixed with 95% ethanol to give a final concentration of 50% ethanol. This mixture was allowed to stand at 25 C for one hour with occasional mixing, after which serial dilutions of 1:10, 1:100 and 1:1000 were made using sterile physiological saline. The sample-alcohol mixture was also subcultured into TPG broth. The 1:100 and 1:1000 dilutions were streaked onto plates of liver-veal agar containing four percent egg yolk, and onto blood agar plates. Plates were incubated in Case anaerobic jars under a nitrogen atmosphere for 48 hours at 28 C.

After incubation the plates were examined for typical colonies of Clostridium botulinum type E. Colonies were observed for surrounding opalescent zones (McClung and Toabe, 1947; Willis and Hobbs, 1958, 1959; Willis and Gowland, 1962), and for the pearly layer covering the colony. Beta hemolysis with a typical raised, rough, irregular colony on blood agar was also observed.

Colonies showing the type E characteristics were subcultured into TPG medium, incubated at 28 C for four days, and were tested for toxicity, by mouse inoculation, after sterile filtration and trypsin activation as described before.

Isolation was much facilitated by the serial dilution before

streaking. Direct plating of the original sample, without treatment with alcohol, did not result in isolation due to overgrowth of contaminats.

RESULTS

Cultural Examination of Marine Fish and Shellfish for C. botulinum

Cultural examination of a considerable number of the chief marine fishes, shellfish, sediment, and water samples of the Pacific Northwest was carried out to determine the relative frequency with which Clostridium botulinum type E or its specific toxin could be identified in cultures of freshly obtained samples. A total of 2,597 samples were collected for testing and no group was without problems in determination of toxicity, tpye of toxin present or isolation of pure cultures. One of the most frustrating conditions encountered was the loss of toxicity between testing for toxin and type determination. Occasionally a loss of toxicity was also noted when low levels of toxin were produced, so as to give a questionable agreement between the original test and retest. Samples in which toxin could not be clearly demonstrated were not included. Thus all reported numbers of toxigenic samples are minimal numbers.

The results obtained from three species of salmon:

Chinook	(Oncorhynchus tschawytscha)
Coho	(Oncorhynchus risutch)
Sockeye	(Oncorhynchus nerka)

as well as steelhead trout (Salmo gairdnerii); sturgeon (Acipenser transmontanus) and bottom fish as represented by sole, cod,

and grouper are presented in Table 3.

The location of the main sample collection points are shown in Figure 3.

A significant percentage of the specimens from each of the six groups gave rise to cultural filtrates which were toxic to mice on intraperitoneal injections. The percentage varied from 9.9 percent for the coho salmon to a high of about 24 percent for the sockeye salmon. The time of death for the injected mice varied from two to twenty-four hours in most cases. Only a few died between 24 and 60 hours which, except in unusual cases, was used as the maximal time for observation. Only animals that died with typical botulism poisoning symptoms were included in the study. The three species of salmon demonstrated a range of toxigenicity from a low of 9.9 percent in the coho salmon to 23.7 percent in the sockeye salmon. This difference is probably not due to differences in the fish species, but to other factors, such as the location at which specimens were collected. For example, when only those fish collected at Astoria, Oregon and up the Columbia River are considered, fourteen of fifty-nine of the sockeye specimens were toxigenic (23.6 percent) as were eight of the twenty-nine coho salmon specimens (27.8 percent).

Of the ninety-four toxic samples proved in the screening test, forth-eight were identified immunologically by retesting in mice

Table 3. Comparative Incidence of Clostridium botulinum Type E in Fish Taken at Different Sites Along the Washington and Oregon Coast.

Specimens	Number of specimens tested[a]	Toxigenic fish specimens[b]		Number of toxic filtrate specimens proven to contain botulinum toxin[c]
		Number	Percent	
Sockeye Salmon	59	14	23.7	4 type E 2 type A 1 type F
Chinook Salmon	124	20	16.1	5 type E
Coho Salmon	221	22	9.9	8 type E
Steelhead Trout	37	7	19.0	5 type E 2 type B
Bottom Fish (sole cod, & grouper)	157	28	17.8	16 type E 1 type A 1 type B
Sturgeon	24	3	12.5	3 type E
Total	622	94	15.1	3 type A 3 type B 41 type E 1 type F

[a] Each specimen tested by incubating gills and viscera of individual fish in TPG medium at 28 C for 4 days and testing the culture filtrate for toxicity in mice, as described under Materials and Methods.

[b] Yielding culture filtrates toxic for mice.

[c] Toxin identified by mouse protection with type specific antitoxin.

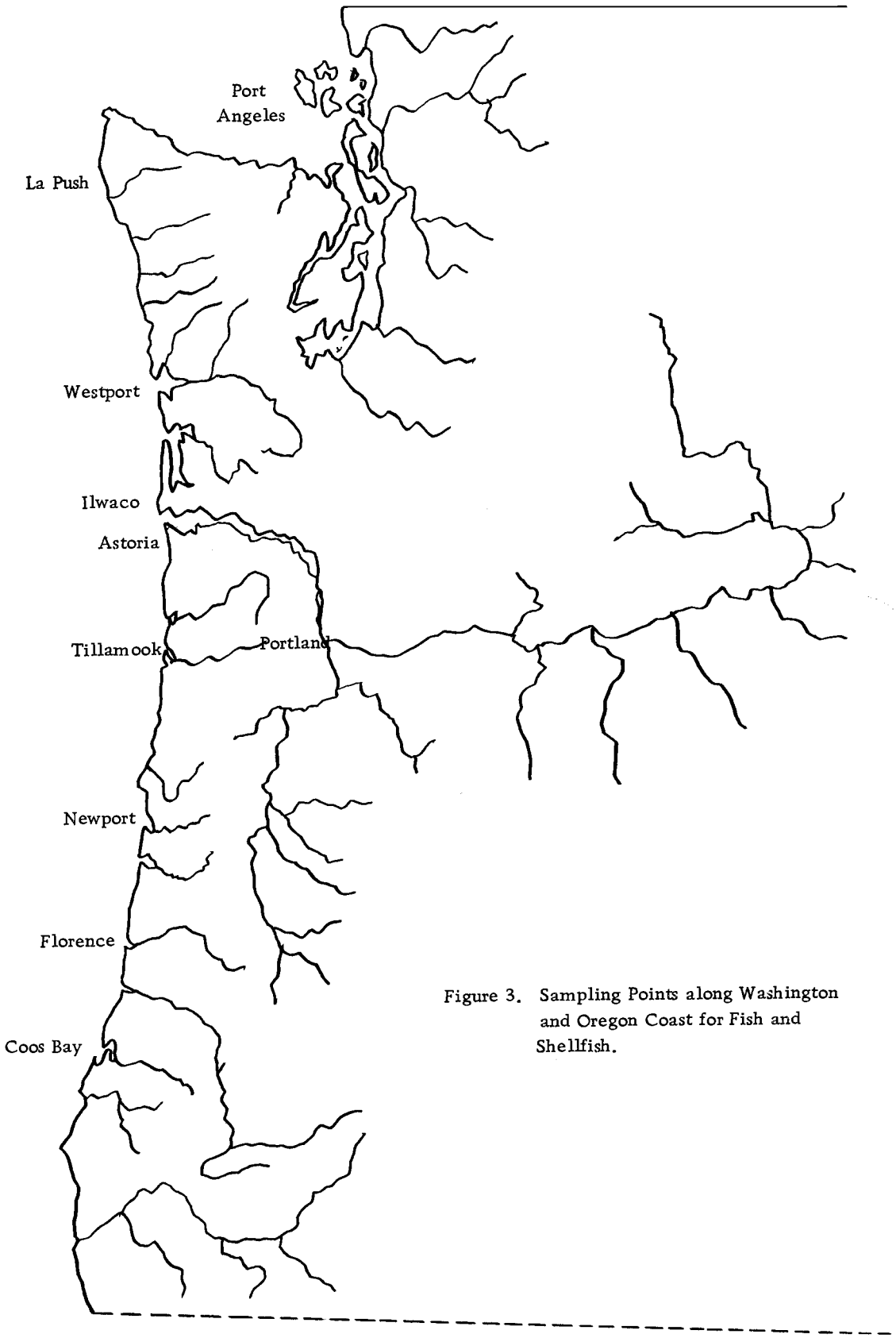


Figure 3. Sampling Points along Washington and Oregon Coast for Fish and Shellfish.

protected by type specific bolulinum antitoxin, as well as in unprotected mice. The remaining forty-six samples lost toxicity completely, or the results were doubtful and inconclusive. The change in toxicity occurred during the 24 to 30 hours the samples were stored at -15 C between the screening test and the typing test.

An illustration of the results obtained from the mouse protection test with type specific antitoxin is shown in Table 4. The majority of the specimens successfully typed were shown to contain type E toxin, indicating the presence of the organisms in the mixed culture. Two specimens of sockeye salmon produced cultures containing type A botulinum toxin, and one produced type F, a relatively rare type (Craig and Pilcher, 1966). All the coho and chinook specimens produced type E botulinum toxin. The steelhead trout specimens yielded 19 percent toxic filtrates. Five out of seven contained type E toxin, whereas the other two were found to yield type B toxin. Twenty-six of the thirty-seven specimens were obtained from traps set near the fish hatchery on the upper Alsea River. Ten specimens were taken from the upper Columbia River and one came from the Sandy River. Of the ten specimens from the Columbia River no toxic filtrates could be demonstrated. This is in contrast to the salmon caught in the same location. In order to determine what organs or tissue might contain any

Table 4. Results of Successful Antitoxin Typing Tests on Toxic Samples

Filtrate Dilution Used	A Antitoxin		B Antitoxin		E Antitoxin		Unprotected		Type
	1/10	1/100	1/10	1/100	1/10	1/100	1/10	1/000	
Sample Identification No.									
1 266 Coho Columbia River	2/2; 2-6	1/2; 48-60	2/2; 2-6	2/2; 32-46 48-60	0/2	0/2	2/2; 2-6 6-20	1/2; 36-40	E
2 367 Sockeye Columbia River	0/2	0/2	2/2; 32-46	2/2; 32-46 48-60	2/2; 2-6 32-40	0/2	2/2; 2-6 32-40	0/2	A
3 394 Sockeye Columbia River	2/2; 6-14	0/2	2/2 6-14	0/2	0/2	0/2	2/2; 6-14	1/2; 90-96	E
4 598 Ling Cod	1/2; 6-10	0/2	2/2; 6-10	2/2; 6-10	0/2	0/2	2/2; 5-10	0/2	E
5 419 Sediment Alsea River	2/2; 2-4	2/2; 4-8	2/2; 6-10	2/2; 4-8	0/2	0/2	2/2; 4-8	2/2; 4-8	E
6 721 Sturgeon Astoria	2/2; 2-4	0/2	2/2; 2-4	0/2	0/2	0/2	2/2; 4-6	1/2; 4-6	E
7 752 Petrale Sole Astoria	0/2	0/2	1/2; 24-30	1/2; 24-30	2/2; 72-70	0/2	1/2; 24-40	0/2	A
8 161 Chinook Columbia River	1/2; 70-78 1/2; 6 days	0/2	1/2 20-26 1/2; 120-144	0/2	0/2	0/2	1/2; 20-26	0/2	E

Table 4. (continued)

Sample Identification No.		Filtrate dilution used: A Antitoxin 1/10	A Antitoxin 1/100	B Antitoxin 1/10	B Antitoxin 1/100	E Antitoxin 1/10	E Antitoxin 1/100	Unprotected 1/10	Unprotected 1/100	Type
9	10 Steelhead Alsea River	2/2; 24-30	0/2	0/2	0/2	2/2; 10-12 12-14	2/2; 14-24 22-24	2/2; <6	2/2; <22	B
10	21b Smoked perch; taken at Waldport	1/2; 4-5 7-14	1/2; <3 20-24	1/2; 4-5 5-7	2/2; 17-20	1/2; <3	0/2	2/2; <6	2/2; <6	E
11	232 Coho Columbia River	2/2; 7-15	0/2	1/2; 4-7	0/2	0/2	0/2	2/2; 4-7	1/2; 7-15	E
12	29c Steelhead Alsea River	2/2; 7-14	0/2	2/2; 7-14	0/2	0/2	0/2	2/2; 7-14	0/2	E
13	256 Coho Columbia River	1/2; 48-60	0/2	1/2; 32-46	0/2	0/2	0/2	1/2; 32-46	0/2	E*?
14	394 Sockeye Columbia River	-	-	-	-	0/2	0/2	2/2; 10-20	1/2; 20-24 1/2; 24-34	E
	(retest)	2/2; 6-14	0/2	2/2; 6-14	0/2	0/2	0/2	2/2; 6-14	1/2; 90-96	E
15	610 Sockeye Columbia River	2/2; 6-10	1/2; 6-10	2/2; 6-10	2/2; 6-10	2/2; 6-10	2/2; 6-10	2/2; 6-10	2/2; 6-10	?

Table 4. (continued)

Filtrate dilution used	A Antitoxin		B Antitoxin		E Antitoxin		Unprotected		Type
	1/10	1/100	1/10	1/100	1/10	1/100	1/10	1/100	
ALSO RUN USING C ANTITOXIN AND TETANUS ANTITOXIN 22.5/u/mouse									
	1/10	1/100							
	2/2 <10	2/2 <10							
					F Antitoxin		Unprotected		
					1/10	1/100	1/10	1/100	
	run with F antitoxin provided by CDC, Atlanta, Georgia				0/2	0/2	2/2; <4	2/2; <6	F

* Result questionable because only one unprotected mouse died after receiving the 1/10 dilution.

Fractions in table indicate number of mice dying of two injected. Figures following fraction show approximate time of death in hours.

spores or cells of Clostridium botulinum, samples of liver, muscle tissue, mid-intestine, gills, and viscera were tested on sixty-six salmon specimens and nine steelhead trout. No toxic filtrates were found among these special samples although two samples of intestine and one of liver were questionable. It seems probable, in view of these results and of the finding of other investigators with other fish (Bott et al, 1966) (Johannsen, 1963) that the intestinal tract was the chief source of the Clostridia. The exact relationship of Clostridium botulinum in the fish intestinal tract or other organs of the fish is not clear. Few, if any, detailed studies have been made of the interrelationship between fish covered by this study and the botulinum organism or toxin. Three specimens of twenty-four sturgeon tested produced toxic filtrates and all of these proved to be type E toxin giving a 12.5 percent toxicity rate. All of the sturgeon were collected from the Columbia River or from Astoria, located near the mouth of the river.

Seventeen and eight-tenths percent of the bottom fish yielded toxic filtrates. The vast majority of these proved to be type E toxin; however, one type A and one type B were demonstrated. Of the twenty-eight toxic cultures, ten again lost toxicity or were doubtful on toxin typing with specific antitoxin protected mice.

As the data for the fish collected from different locations along the coast and from the Columbia River became available, it became apparent that the proportion of the fish of a given species harboring toxigenic organisms varied with the location at which the fish were collected. Table 5 shows the percentage of toxigenic specimens of chinook and coho salmon caught in the Columbia River and various points along the Oregon and Washington Coast. It is evident that with both species of salmon, the proportion of the fish found to yield toxic filtrates was higher among those caught in or near the Columbia River than among those caught in ocean waters.

The same conclusion could be drawn from data obtained from the "bottom fish" collected. Table 6 gives the results from the "bottom fish" specimens collected at Astoria and compared with those specimens collected at Coos Bay on the southern coast of Oregon. "Bottom fish" include those species of fish found in the deeper ocean depths. The specimens collected at both Astoria and Coos Bay were obtained by commercial "drag boats" trawl fishing in fifty to eighty fathoms of water five to ten miles off shore. The Astoria fish specimens were obtained approximately five to six miles north of the mouth of the Columbia River while those collected at Coos Bay came from the ocean five to eight miles due west of Coos Bay, and include:

Table 5. Variation in Incidence of Toxigenic Specimens among Salmon Caught in the Columbia River and Those Caught in Ocean Waters

	Number fish[c]	Number toxigenic	Percent toxigenic	No. Typed
Chinook Salmon				
A. Columbia River				
1. River caught[a]	91	18	19.8	5 type E
2. Astoria[b]	15	1	6.7	1 type E
Total	106	19	18.0	6 type E
B. Coastal				
1. Westport	16	1	6.2	-
2. Port Angeles	2	0	0	-
Total	18	1	5.6	0
Coho Salmon				
A. Columbia River				
1. River caught[a]	19	4	21.0	3 type E
2. Astoria[b]	10	6	60.0	3 type E
Total	19	10	52.6	6 type E
B. Coastal				
1. Port Angeles	61	2	3.3	-
2. La Push	49	6	12.2	-
3. Westport	43	4	9.3	2 type E
4. Depot Bay Newport	33	1	3.0	-
Total	186	13	7.0	2 type E

[a] Indicates those fish caught between Astoria and The Dalles in the Columbia River.

[b] Indicates those fish landed at Astoria, but may be caught at the mouth of the Columbir River in the ocean.

[c] Gills and viscera of each fish cultured; see footnotes a and b in Table 1.

Table 6. Incidence of Toxigenic Specimens among "Deep Sea" Fish from Coos Bay and Astoria[a]

		Coos Bay			Astoria			No. typed
		Number tested	Number toxigenic	Percent toxigenic	Number tested	Number toxigenic	Percent toxigenic	
1.	Sole[b]							
	Dover	5	0	0	0	0	0	
	English	19	3	15.8	10	4	40	5 type E
	Petrale	14	0	0	12	2	16.5	1 type E 1 type A
	Rex	5	0	0	0	0	0	0
	Total	43	3	7.0	22	6	27.3	6 type E 1 type A
2.	Cod							
	True	1	0	0	10	1	10.0	1 type E
	Ling	7	1	14.3	10	1	10.0	2 type E
	Rock	4	0	0	9	6	66.6	6 type E
	Green	1	0	0	0	0	0	0
	Black	2	0	0	5	2	40.0	1 type E 1 type B
3.	Sebastodes sp.	20	0	0	14	7	50.0	0
4.	Whitefish	1	0	0	0	0	0	0
5.	Grouper	8	1	12.5	0	0	0	0
6.	Smelt	0	0	0	2	0	0	0
	Total	87	5	5.6	72	23	31.9	10 type E 1 type B

[a] Gills and viscera of each fish cultured; see footnotes a & b in Table 1.

[b] Local names are used.

Local name	Scientific name
Dover sole	Microstomus pacificus
English sole	Parophrys vetulus
Petrale sole	Eopsetta jordani
True cod	Gadus macrocephalus
Lind cod	Ophiodon elongatus
Rock cod	Sebastodes sp.
Green cod	Sebastodes sp.
Black cod	Anoplopoma fimbria
Whitefish	Theragra chalcogrammus
Grouper	Sebastodes sp.
Smelt	Hypomesus pretiosus

No specimens of Dover or Rex sole were obtained from Astoria. English sole had the highest toxigenic ratio both at Astoria and at Coos Bay. Five species of cod were collected. No specimens of Green Cod were collected from Astoria. The Ling Cod proved to be the exception in having a higher toxin percentage in the specimens collected at Coos Bay than those specimens collected at Astoria. However, this is not significant due to the low number of samples tested. No toxin could be demonstrated in the Sebastodes sp. collected at Coos Bay while those collected at Astoria demonstrated a 50 percent toxicity rate.

Table 7 is a summary of all "bottom fish" species collected at Coos Bay and Astoria and shows comparative total specimen data. Eighty-seven specimens from Coos Bay yielded 5.6 percent toxigenic specimens, whereas seventy specimens from Astoria gave 32.8 percent toxic cultures. This also may illustrate the

influence of the river as a source of the Clostridium botulinum and would add evidence to the concept of terrestrial origin and concentration by run off from the land as suggested by Johannsen (1963).

Table 7. Summary of Toxigenic Specimens among all "Bottom Fish" Species

	Number of Fish[a]	Number toxigenic[b]	Percent toxigenic
Coos Bay	87	5	5.6
Astoria	70	23	32.8

[a] Each specimen tested by incubating gills and viscera of individual fish in TPG medium at 28 C for 4 days and testing the culture filtrate for toxicity in mice, as described under Materials and Methods.

[b] Yielding culture filtrates toxic for mice.

Miscellaneous Fish

A small number of line caught species of fish were also tested. Three specimens of mackeral (Pneumatophorus japonicus) obtained at La Push, Washington, three specimens of smelt and 11 specimens of flounder (Atheresthes sp) obtained at Newport, Oregon, were processed for toxin production and all proved to be negative. The number of specimens tested was probably too small to be of

significant value and a greater number over a wider distribution should be obtained before the results could be used with much reliability.

Environmental Sampling of Fish Processing Plants

Twenty-one environmental swab samples were taken at plants producing fish fillets for the commercial market from "deep sea" species of fish and from salmon processing plants along the Oregon Coast. The comparative data is presented in Table 8. No toxin could be demonstrated from any of the swab samples obtained from the table, conveyor belts, hands or finished products from plants at Astoria. Three "deep sea" processing plants at Coos Bay were investigated and 26 samples were collected. Two of these produced toxic filtrates; and one proved to be type E toxin while the other lost its toxicity before it could be tested for type. Both of the proven toxic samples were obtained from the same processing plant. One sample of the "dip water", used to extend the shelf life of the fish fillets in the market, did yield a toxic filtrate after incubation. The number of samples tested was low and should be increased to be of significant value. Swab samples were also obtained from the salmon processing plants at Astoria, Portland, and Charleston. The data is presented in Table 8B. A total of 53 samples were obtained from the butcher tables, conveyor

Table 8. Results of Testing Environmental Samples from Fish Processing Plants for Toxin Production

	Number samples tested	Number samples toxigenic	Percentage toxigenic
A. Samples from "Deep Sea" Fillet Plants			
1. Coos Bay			
Swab samples[a]	26	2	7.7
"Dip" water[b]	4	0	0
2. Astoria			
Swab samples	9	0	0
"Dip" water	3	1	33.3
B. Swab Samples from Salmon Processing Plants			
1. Astoria	33	0	0
2. Portland	14	0	0
3. Charleston	6	0	0
Total	53	0	0

[a] Swab samples cultured by incubating in TPG medium at 28 C for four days.

[b] Dip water samples cultured by adding 8 ml to 15 ml of TPG medium and incubating at 28 C for four days.

belts. canning tables, and hands of operators, none of which gave any positive toxin production. Two samples were questionable but had lost toxicity upon retesting.

Smoked Fish Products

In order to assess the possibility of Clostridium botulinum spores contaminating fish products, especially smoked fish tissue, it was thought advisable to check the smoked fish offered for sale to the general public. Sixteen samples were collected from Oregon and Washington at the locations shown in Figure 4. Thirteen of the samples were smoked salmon and three were sea perch. Four of the samples produced toxic filtrates, and upon further examination, this toxin was identified immunologically as type E in two of the samples. One of the samples lost toxicity and one was inconclusive. No moisture or sodium chloride tests were taken on any of the samples. This information would have made the results more meaningful. The results do point out a potential health hazard for the unsuspecting housewife who wraps the smoked fish in saran wrap and places it in a refrigerator where anaerobic conditions could develop and toxin could be produced.

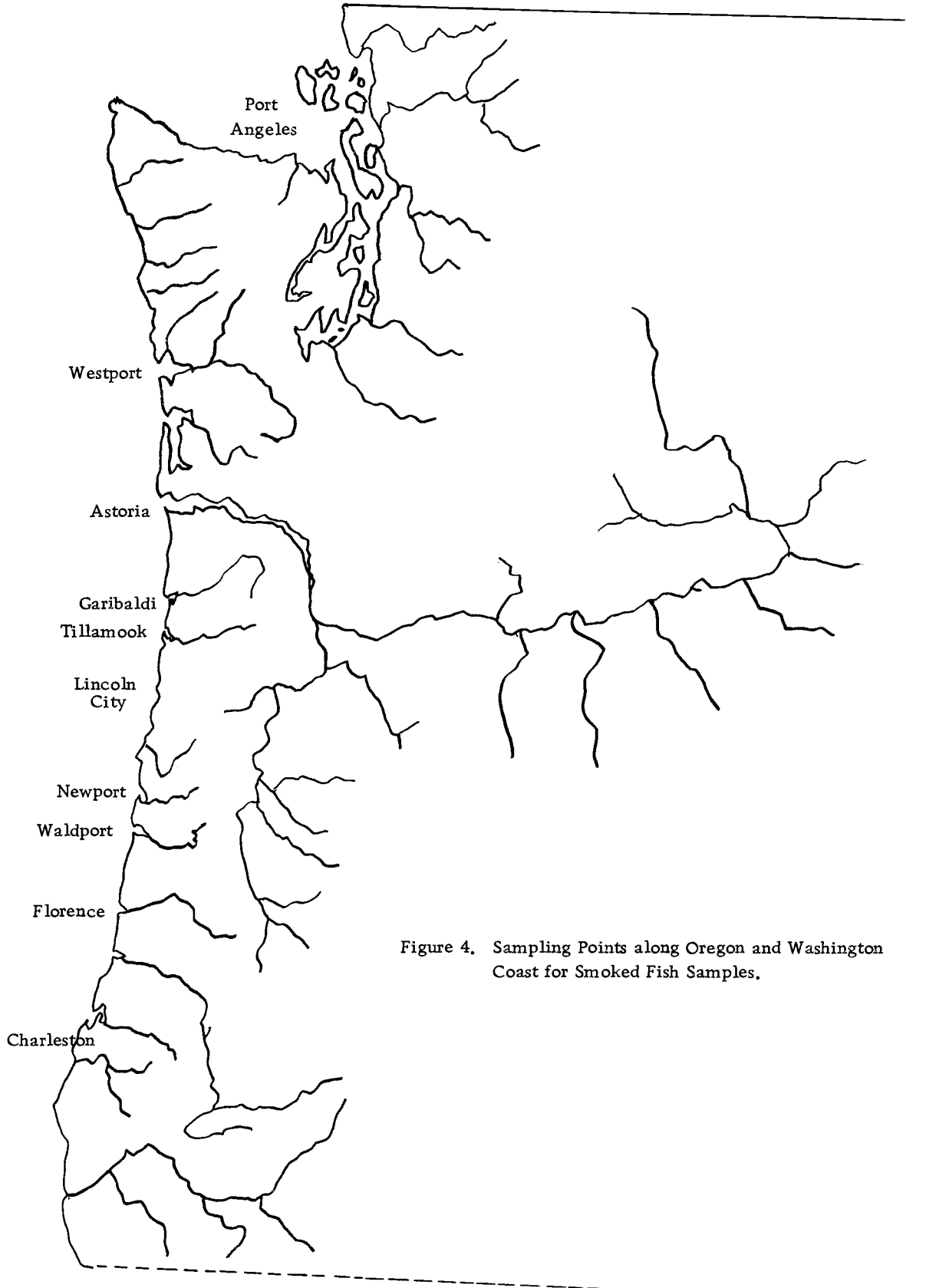


Figure 4. Sampling Points along Oregon and Washington Coast for Smoked Fish Samples.

Cultural Examination of Sediments Obtained from Coastal or Estuarine Locations for C. botulinum

Two questions arose soon after specimens were collected: At what temperature should the samples be incubated? And how should the mud specimen be sampled? In order to determine the temperature at which the sample should be incubated, three experiments were run using eight specimens in experiment number 1 and ten specimens in each of experiments 2 and 3. Each specimen was prepared in duplicate by adding 3 grams of mud to 50 ml of the TPG medium. The cultures were then placed in a Case jar which was evacuated three times and brought back to atmospheric pressure with a mixture of Nitrogen 96 percent and Carbon dioxide 4 percent. They were incubated at 12 C for sixteen days, 19C for nine days, or 28 C for five days. This procedure provided six samples from each sediment specimen. The results of these experiments are shown in Table 9.

The results of the three experiments are obviously inconclusive. In two cases 12 C appeared to identify toxic samples not identified by either the 18 C or 28 C samples. Even though the specimens were well mixed, when one sample was toxic, the duplicate at the same temperature was not. There was no significant difference between 28 C and 18 C. This comparison would have had more meaning if a known type E spore load had been

Table 9. Comparative Results of Toxicity Tests on Duplicate Split Sediment Samples Incubated at Three Temperatures, 28 C, 18 C, and 12 C.

Experiment No. 1				Experiment No. 2				Experiment No. 3			
Sample	Temp. (C)			Sample	Temp. (C)			Sample	Temp. (C)		
Number	28	18	12	Number	28	18	12	Number	28	18	12
1a	-	-	-	1a	-	-	-	1a	-	-	-
1b	-	+	-	1b	-	-	-	1b	?	-	-
2a	-	-	-	2a	-	?	-	2a	-	-	-
2b	-	-	-	2b	-	-	-	2b	+	-	-
3a	-	-	-	3a	-	-	-	3a	-	-	-
3b	-	-	-	3b	-	-	+	3b	-	-	-
4a[1]	-	-	-	4a	+	-	-	4a	+	-	-
4b	-	+	-	4b	-	-	-	4b	-	-	-
5a	-	-	-	5a	-	+	-	5a	-	-	-
5b	-	-	-	5b	-	+	-	5b	-	-	-
6a	+	-	-	6a	-	-	-	6a	-	-	-
6b	-	+	-	6b	-	?	-	6b	-	-	-
7a	-	-	-	7a	-	-	-	7a	-	x?	-
7b	-	-	-	7b	-	-	-	7b	-	-	-
8a	+	-	-	8a	-	-	-	8a	-	-	-
8b	-	-	-	8b	-	-	-	8b	-	x?	-
				9a	-	-	-	9a	+	-	-
				9b	-	-	-	9b	+	-	-
				10a	-	+	+	10a	-	-	-
				10b	-	-	-	10b	-	-	+

+ indicates culture toxic at either 1:10 or 1:100 dilutions

- indicates culture non-toxic in both dilutions

? = 1 of 4 mice died > 24; < 96 hours; result inconclusive

x? = 1 of 4 mice died > 6; < 24 hours; result inconclusive

inoculated into each sample. It was not certain that all of the split samples contained type E spores or a sufficiently high spore load to ensure outgrowth. Twenty-eight degrees C was selected as the temperature for incubating sediment samples in this study as 18 C and 12 C were not shown to give more valid results, and the incubation time at 28C was shorter. Furthermore this temperature did not tie up as much incubator space, did not require a BOD type incubator, and was identical to that used for incubation of fish samples. Also, it was close to the 30 C temperature found to be best by Bott et al (1965) in the study they conducted on Wisconsin sediments.

The methods of sampling the marine sediment was tested using the procedure proposed by Johannsen (1963) where three to five grams of sediment are placed into fifty ml of TPG medium, and the Japanese procedure (Nakamura et al, 1956) where ten grams of sediment are shaken in fifty ml of phosphate buffer (pH 6.4) for twenty minutes in a Burrell Shaker, allowed to settle for one minute to separate the heavy particulate material, and the supernatant fluid decanted into a fifty ml certrifuge tube. This is spun at 13,000 x g for thirty minutes and the supernatant decanted off and replaced with TPG medium. Both samples were then incubated in a Case jar as previously described. The results of this comparison are presented in Table 10. The Japanese

Table 10. Comparative Results of Toxicity Tests on Cultures of Duplicate Split Sediment Samples Using the Japanese Procedure and That Used by Johannsen

Sample Number	Johannsen Procedure	Japanese Procedure
1	-	-
2	-	+
3	+	-
4	-	+
5	-	-
6	-	-
7	-	-
8	-	-
9	-	+
10	-	?
11	-	-
12	?	+
13	-	?
14	-	?
15	-	-
16	-	-
17	-	-
18	+	-
19	+	+
20	?	-
21	+	+

+ indicates culture toxic at either 1:10 or 1:100.
- indicates culture non-toxic in both dilutions
? = 1 of 4 mice died> 24 hours; < 96 hours; result inconclusive
x? = 1 of 4 mice died > 6 hours; < 24 hours; result inconclusive

procedure, resulted in six positive specimens and three questionables, while four positives and two questionables were detected using the Johannsen method. This was not a significant difference.

Sixty-six sediment samples collected from the coastal areas of Oregon and Washington were tested for toxicity, using the Japanese method, and thirty-six were found to produce toxic filtrates while six were questionable. Of the toxic cultures fifteen proved to contain Clostridium botulinum type E toxin while eight were questionable and thirteen lost their toxicity. No other type was observed indicating the high incidence of type E in marine sediments, and the high percentage of toxigenic samples (55 percent).

Cultural Examination of Shellfish Samples

Crabs, clams, oysters, scallops, and shrimp were also included in this study. They were obtained from various locations along the Oregon and Washington coast. Results of the shellfish tests are shown in Table 11. All groups contained toxigenic samples except scallops and rock shrimp, where the numbers sampled were inadequate to be significant. The scallops were obtained from the "drag" net boats fishing for "bottom fish" five miles north of the Columbia River. Twelve specimens were obtained which were divided into two samples. The rock shrimp

Table 11. Incidence of Clostridium botulinum Type E in Shellfish from Oregon Coastal Waters

Identity of specimen	Source of samples	Number of samples tested	Number of samples toxigenic	Percent toxigenic	Number of toxic samples proven to contain C. botulinum
Clams, Razor	Winchester Bay, Seaside	75	11	14.6	3 type E 1 type A 1 type B
Cockle	Sequim, Wash., Yaquina Bay & Yachats, Ore.	15	12	80.0	10 type E
Soft-shell	Yaquina Bay, Reedsport & Florence, Ore.	12	8	66.7	8 type E
Little-neck	Yaquina Bay Florence, Ore., Nakcotta, Wash.	11	4	36.2	3 type E
Horse-neck	Florence, Ore.	3	1	33.3	1 type E
Oysters					
Pacific	Yaquina Bay, Tillamook, Charleston, & Bay City, Ore.	19	6	31.6	5 type E
Scallops	Astoria, Ore.	2	0	0	0
Rock Shrimp	Geribaldi, Ore.	1	0	0	0
Crabs					
Dungeness	Yaquina Bay, Winchester Bay, Ore.	24	17	71	4 type E 1 type B

* All clams, oysters, and scallops samples consisted of 3-5 shellfish; for the littleneck clams, 8-10 were used.

were collected from the beach at Garibaldi, Oregon, and represented 12 to 15 individual shrimp combined into one sample. All species of clams produced more than 33 percent toxic cultures except the razor clams which were collected from the open sandy ocean beach and not the estuary. Two species, cockles and soft-shell clams, exceeded 66 percent toxigenic samples. Type E Clostridium botulinum was the predominant strain of botulinum present; however, one type A and two type B were found. All clams were thoroughly scrubbed and washed so it does not seem likely that the organism was a carryover from the sediments in which they were found. The organism is more probably contained inside the shell of shellfish.

Dungeness crabs, picked from the intertidal zone on the open beach, showed a high percentage of toxic filtrates (71 percent). These typed as four type E and one type B. Thirty-seven of the fifty-nine toxic samples were able to be typed yielding one type A, two type B and thirty-four type E. Loss of toxicity and questionable results again accounted for the numbers unable to be typed after proven toxin production.

Isolation of C. botulinum from Toxic Specimens

Isolation of Clostridium botulinum was attempted from 39 specimens which had produced proven toxic filtrates and had been successfully typed. Thirty-eight were type E and one was type F.

Successful isolation of pure toxigenic cultures derived from a single "clone" was successful from 18 specimens of type E. A total of 44 toxigenic pure cultures of type E were obtained from these 18 specimens. Two toxic cultures of type F were also isolated and the type was confirmed by Dr. Lillian H. Holdeman of the Communicable Disease Center at Atlanta, Georgia, and Dr. Clarence F. Schmidt of Continental Can Company of Chicago, Illinois (Craig and Pilcher, 1966). Isolates were made using both blood agar and LVE agar; however, toxigenic isolates seemed to be more easily identified by the author from LVE. Differences in colonies of Clostridium botulinum type E as described by Dolman (1957) could be observed but consistency in recognition of his 'TOX', 'TP', or 'OS' forms could not be achieved. Many colonies with identical appearances were subcultured to TPG medium but the majority did not produce a demonstrable toxin. Cultures tended not to grow on subculturing from LVE or blood agar to TPG medium if standard procedure of picking colonies with a straight needle was used, even though exposure to air was less than ten

minutes after removal from the Case jar until again returned to anaerobic conditions. If a loop needle was used and the base of a well isolated colony was transferred by cutting a vertical section, so as to transfer a portion of the colony with its base, growth on TPG was successful. This was probably due to the need to transfer spores or cells located deep within the colony and may illustrate the extreme sensitivity of the vegeta tive cells to oxygen. The sources of Clostridium botulinum type E isolates were as follows: steelhead trout from the Alsea river; chinook salmon and coho salmon from the Columbia River; sturgeon from Astoria; English and Petrale sole and Ling cod from Coos Bay; Black and Red rock cod from Astoria; sediment samples from the beach at Newport and the tidewater areas of the Alsea River. These are listed in Table 12.

Toxin Levels Produced by C. botulinum type E Isolates

Toxin titrations were carried out on 11 of the isolated cultures. Fifteen ml of a 24 hour transfer culture of each organism in TPG were inoculated into 50 ml of this medium. The latter was incubated at 28C for five days and a portion was sterilized by millipore filtration and activated with one percent trypsin solution for one hour. Ten fold dilutions were prepared in gel phosphate buffer and 0.5 ml of each was injected IP into

Table 12. Samples from which Toxigenic Pure Cultures of Clostridium botulinum Type E and One Type F Were Isolated.

Culture No.	Type	Source
	Type E	
12	Steelhead	Alsea River
14	Steelhead	Alsea River
15	Steelhead	Alsea River
21	Smoked Perch	Waldport
161	Chinook Salmon	Columbia River
417	Sediment	Alsea River
427	Sediment	Newport
467	Smoked Salmon	Charleston
598	Ling Cod	Coos Bay
618	Coho Salmon	Columbia River
638	English Sole	Coos Bay
719	Sturgeon	Astoria
721	Sturgeon	Astoria
749	Petrale Sole	Astoria
786	Black Cod	Astoria
792	Rock Cod	Astoria
793	Rock Cod	Astoria
794	Red Cod	Astoria
	Type F	
610	Sockeye Salmon	Columbia River

into each of six mice. The results are shown in Table 13. The toxin levels of the type E isolates are not identical, which indicates the variability in the toxigenicity of these organisms.

Table 13. Toxin Concentrations of Clostridium botulinum type E Isolates

Culture Number	Dilutions of culture filtrate 10^{-2}	10^{-3}	10^{-4}	10^{-5}	No. of LD_{50} per 0.5 ml
15C	6/6	6/6	6/6	0/6	$10^{4.5}$
417D	6/6	6/6	0/6		$10^{3.5}$
598J	6/6	6/6	0/6		$10^{3.5}$
427C	1/6	0/6			$< 10^{2}$
719A	6/6	6/6	6/6	0/6	$10^{4.5}$

Fractions in Table Show $\frac{\text{no. of mice dead in 4 days}}{\text{no. of mice injected}}$

Loss of Toxicity in Mixed Cultures

Of one-hundred and ninety mixed toxic cultures for which typing was attempted, 130 either lost toxicity or the results of typing were questionable. The results are given in Table 14. This loss of toxicity was even more striking when questionable samples were repeated after the original screening. Colonies which produced the Nagler reaction (McClung and Toabe, 1947) and possessed a pearly layer did not all show toxicity. A total of three-hundred and eighty-four single colonies resembling type E

on LVE or blood agar, obtained by plating from 18 pure clostridial cultures isolated from toxic specimens, were tested for toxin production in TPG medium. Only 21.5 percent of these colonies proved toxigenic. For comparison, 214 colonies, isolated from ten known type E cultures obtained and identified by other laboratories were similarly tested. Results are presented in Table 15. Of this group 82.8 percent were toxigenic. The facts known about normal bacterial mutation cannot account for the large number of nontoxic "mutants" isolated from a supposedly pure toxigenic specimen. This may give some support to the "toxic phage" suggested by Iida (1968).

Reculture in Attempt to Clarify Questionable Samples or Those Showing Loss of Toxicity

In an attempt to revive lost toxicity or clarify questionable results, transfer from the original cultures, held at -15 C, to TPG media tubes was made. Five to eight ml of the thawed sample were transferred to 15 ml of TPG medium. The sample to be transferred was taken from the bottom of the tube, bottle, or flask to facilitate the transfer of the spores, if present. The transfers were incubated for five days and tested for toxicity in mice. Results are reported in Table 16. Four-hundred and seventy-two such transfers were made from cultures which demonstrated a loss of toxicity or questionable results. A total of

Table 14. Results of Antitoxin Typing Tests

No. of toxic samples where typing was attempted	No. of samples successfully typed	No. of samples which lost toxicity	No. of samples giving ques-questionable results
190	A B E F 3 3 53 1	70	60

Table 15. Toxicity Tests on Pure Cultures of Clostridia Isolated from Fish Specimens and on Known Cultures of Clostridium botulinum Type E.

	Number cultures tested*	Number cultures toxic	Number cultures non-toxic	Number cultures questionable	Percent cultures toxic
Clostridia isolated from specimens in this study	384	82	284	18	21.3
Clostridium botulinum type E cultures identified in other laboratories	214	177	37	0	82.8

* Each culature was a transfer of a single colony on LVE or blood agar to a tube of TPG medium.

Table 16. Retesting for Toxicity after Reincubation of Questionable Samples

Number samples tested by re-culturing	Number samples toxic after transfer	Number samples non-toxic after transfer	Number samples question-able	Percent samples toxic after transfer
472	22	390	60	4.6

22 samples were toxic after transfer or 4.6 percent of the samples tested. Three-hundred and ninety were non-toxic and 60 were again questionable. The value of retesting for recovery of lost or questionable toxin production seems open to doubt.

Electrophoretic Analysis of Toxic and Nontoxic Clostridial Cultures

In order to better understand the loss in toxicity and to investigate any possible differences that might have occurred, it was decided to investigate the protein fractions in the toxic and nontoxic Clostridium botulinum type E organisms. Only toxin producing organisms can be typed. The "nontoxic" type are often called "E-like" as their morphological, biochemical, and physiological characteristics seem to match those of the immunologically typed toxin producers. Study of the protein fractions by use of electrophoresis in a polyacrylamide gel matrix was decided upon. This procedure is a modification of that used by Ornstein (1964) and Davis (1964). The equipment to hold the gel was constructed to as to give a plate three by seven inches and three-sixteenths of an inch in thickness. This equipment was originally constructed by Dr. A. W. Anderson of Oregon State University. Illustration of this is shown in Figure 5.

Materials and Methods

Ingredients

The ingredients used to prepare the gel were as follows:

1. N, N'-Methylenebisacrylamide, Bis (Eastman Organic Chemicals)
2. N, N, N', N'-Tetramethylethylenediamine, Temed (Eastman Organic Chemicals)
3. 2-Amino-2-Hydroxymethyl-1, 3-Propandiol, Tris (Eastman Organic Chemicals)
4. Ammonium Persulfate (Baker and Adamson)
5. Acrylamide Monomer (Eastman Organic Chemicals
6. Glycine, Ammonia free (Sigma)
7. Bromphenol Blue (Matheson, Coleman and Bell)
8. 1 N Hydrochloric acid
9. Kodak Photo-Flor 200 Solution
10. Riboflavin (Eastman)

The stock solutions prepared from the above ingredients are as follows:

(A) 1 N HCL 48 ml
Tris 36.3 g
Temed 0.23 ml
H_2O to make 100 ml

(C) Acrylamide 28.0 g
Bis 0.734 g
H_2O to make 100 ml

(G) $(NH_4)_2S_2O_8$ 0.14 g
H_2O to make 100 ml

(H) Buffer 10X (as used)
Tris 3.0 g
Glycine 14.4 g
H_2O to make 1000 ml

(J) 0.005% Bromphnol Blue in H_2O

(E) Riboflavin 4.0 g
H_2O to make 100 ml

Solution 1
1 part (A)
2 parts (C)
1 part H_2O
(pH 8.8-9.0)

The working solutions for polyacrylamide electrophoresis are as follows:

Buffered Wash Solution
NaCl 8.5 gms
KH_2PO_4 .227 gms
Na_2HPO_4 .710 gms
H_2O to 1000.0 gms
pH 7.4

Small Pore Gel
1 Part Solution 1
1 Part Solution G

Washing Gell Solution
1 Part Upper Gel (Canalco)
1 Part H_2O

Large Pore Gen
1 Part (H)
2 Parts (C)
1 Part (E)
3 Parts H_2O
(pH 6.5-6.8)

Cell Sonicate Preparations

The cell free extract was prepared by incubation of the organism for four days in TPG medium in which the glucose was increased to 1 percent. By so doing sporulation was retarded and vegetative cell mass could be harvested with few, if any, spores. Following anaerobic incubation for four days the cells were separated from the liquid medium by centrifugation at 10, 000 X G for thirty minutes at 4 C. The cells were then washed three times with sterile, cold,

0. 05 M phosphate buffer adjusted to pH 7. 2. After adequate washing, the cells were mixed with an equal volume of phosophate buffer and then were disrupted by sonification at 15, 000 to 23, 000 Kc with a Biosonik 111 (Bronson Instruments, Incorporated). Sonification was accomplished by holding the cell mixture in an ice bath and using short eight to ten second bursts and allowing to cool two to three minutes between bursts. Completion of sonification was checked using a Leitz phase contrast microscope to where less than five percent of the cells were left intact.

The sonicated material was centrifuged for 30 minutes at 13, 000 X G while held at 5 C. The cell free extraces were separated off and used for protein analysis.

Cell Free Medium Preparation

The medium was separated from the cells by centrifugation as previously described and was then concentrated by dialysis in cellulose tubing against a thirty-three percent Polyethylene Glycol 4000 slurry at 5 C. After a ten fold concentration, from 360 ml to 36 ml, the medium was transferred to a sterile 59 ml screw cap tube and stored at 5 C until used.

Gel Preparation

The small pore gel was prepared by mixing equal volumes

of solution 1 and solution G. This mixture was placed in the gel plate holder, shown in Figure 5, so as to make a sheet five centimeters deep. This was overlayed with water, to prevent formation of a meniscus, for about five millimeters in depth, being careful not to disturb the small pore gel and cause a mixture at the interface. The gel was then allowed to form, or polymerize, for 40 minutes at room temperature. After the gel had polymerized, the water was removed, along with any unreacted monomer solution, with a disposable Pasteur pipette. The surface of the small pore gel was then washed with washing gel solution, and after removal with a Pasteur pipette, large pore gel was added. The large pore gel was added to a depth of about one and five tenths centimeters and overlayed with water as before so as to prevent meniscus formation. The large pore gel was photopolymerized for 30 minutes with a 15 watt fluorescent lamp placed level with the gel and approximately six to seven centimeters away. Upon photopolymerization of the gel the water layer was removed again with a disposable Pasteur pipette. Samples were separated on the polyacrylamide sheet by insertion of dividers which consisted of plastic rod material inserted through the large pore gel to the small pore-large pore interface. The sample was placed on top of the gel by mixing with a 40 percent sucrose solution up to a 50/50 final volume mixture. The amount of sample used varied and was determined by trial run. Sample size ran from 20

to 100μl.

Tris-glycine buffer solution was diluted 1:10 with distilled water and both upper and lower reservoirs were filled with diluted buffer to within 10 mm of the reservoir tops. One-tenth ml of 0.005 percent bromphenol blue tracking dye solution was placed in the upper reservoir for every 100 ml buffer. Electrophoresis was performed in the upright position with the current adjusted to 50.0 millamperes and 300 volts. During electrophoresis cold water was circulated through the unit to prevent ohmic heating of the gel and to permit a more rapid electrophoretic separation. Electrophoresis was terminated when the tracking dye from the upper reservoir had migrated to within five millimeters of the bottom of the gel plate (between 45 and 60 minutes). The protein-laden polyacrylamide gel was removed from the trough by gently separating the two halves of the electrophoretic unit and immediately placed in a 20 percent trichloroacetic acid solution so as to fix the protein bands. It was allowed to remain in the fixing solution 30 to 45 minutes and then was placed in the staining solution.

Total Protein Staining

The procedure used to stain protein zones in the polyacrylamide gels was that described by Chrambach et al. (1967).

Following fixation the protein laden gel was immersed in

staining solution freshly prepared by adding 1 ml of a 1 percent aqueous stock solution of Coomassie blue (Colab Laboratories Incorportated) to 19 ml of a 12.5 percent TCA solution. The gel remained in the staining solution for one hour and then was transferred to 10 percent TCA. At this time the protein bands were visible and continued to gain in intensity over a 48 hour period. The bands were then photographed so as to maintain a permanent record.

Total Protein Electrophoretic Patterns

Six cultures were used in this portion of the study, four nontoxigenic "E-like" organisms and two toxigenic type E organisms all abtained from the U.S. Food and Drug Administration Bacteriological Laboratory at Washington, D.C. The organisms and their origins are listed in Table 17.

Three cell homogenates of the nontoxigenic strains, 28-2, 34-1 and PMI-5 were prepared by growing four days at 28 C anaerobically, separating and washing the cells and then disrupting them by ultrasonic energy as described in material and methods. These homogenates were used to determine their protein bands by electrophoresis using a gel plate on which all

FIGURE 5

Equipment to hold Flat Plate Gel.

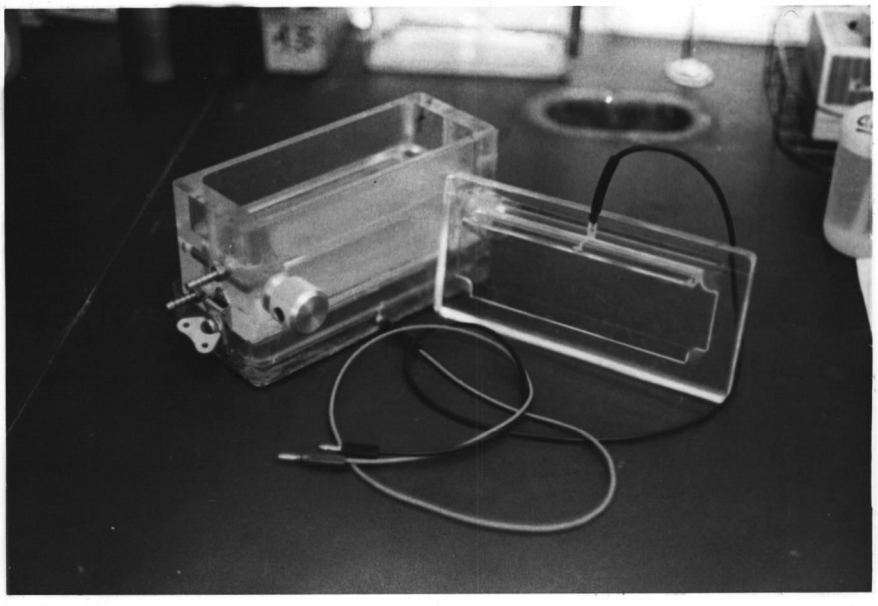


Table 17. Identification of Strains of Clostridia

Strain of C. botulinum type E	Source of Isolation	Geographical Area
VH Toxigenic	Pickled herring	Vancouver, Canada
066B Toxigenic	Smoked Whitefish Chubs	Great Lakes
066B Nontoxigenic	Derived from 066B Tox	Food λ Drug Administration
28-2 Nontoxigenic	Sediments	Great Lakes
PM1-5 Nontoxigenic	Sediments	Potomac River
34-1 Nontoxigenic	Sediments	Great Lakes

samples could be determined simultaneously using 70.0 milliamperes of current and a starting voltage of 300 volts. The results are shown in Figure 6.

The stained protein bands probably represent the enzymes, metabolic by products or the structural proteins of the cells. It is noted that the stained protein bands do differ in a minor manner. 34-1, the center sample, seems to lack the second top protein band and the intensity of all stained bands is a bit lighter in appearance than those of the other two. The 28-2 sample on the left possesses two bands which are very intense, and seems to lack at least one band in the lower half of the other two patterns. Part of the difference in intensity may be due to differences in the sample sizes used in the electrophoretic run. Minor differences may also be seen in Figure 7 where 28-2 and PM1-5 are compared. Culture No. 28-2 appears to lack the lowest band in the PM1-5 pattern. The top band is divided in the PM1-5 sample.

Comparison of the toxigenic and non-toxigenic cultures of C. botulinum type E shows more striking differences. Non-toxigenic 066B-nt strain is compared with the toxic VH strain in Figure 8. The toxigenic strain possesses an extra stained protein band in the upper half of the pattern which is entirely missing in the non-toxic variant. Some differences in the number of bands in the bottom third of the toxigenic culture may also be noted.

Figure 9 compares the toxigenic 066B strain with the identical non-toxic variant. We note here also a difference in intensity and possibly in the number of protein bands in the toxigenic culture.

The cell free filtrates from four day cultures of strains VH and 066B-nt, concentrated ten fold, were prepared and subjected to electrophoresis in the same way. The results of this are shown in Figure 10. Again the protein pattern of the toxic filtrate demonstrates an extra top protein band not seen in the nontoxic filtrate. This corresponds to the observations seen in washed cell sonicates of these two cultures.

The proteins in the cell free filtrates were mainly by-products or ectoenzymes which had passed through the cell membrane, as little if any cell lysis could be identified by microscope.

The extra top band, seen in both toxigenic cells and in toxic cell free filtrate, should be extracted and tested for toxicity by mouse injection. It may represent the type E toxin itself.

FIGURE 6

Total Protein Stained Gels of Cell Sonicates of Nontoxigenic strains C. botulinum type E

28-2 34-1 PM1-5

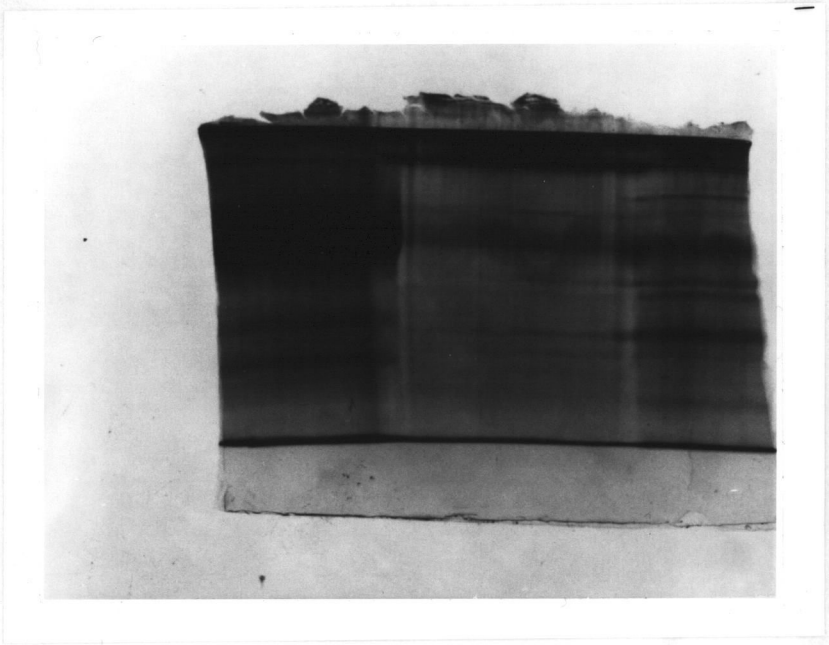


FIGURE 7

Total Protein Stained Gels of Cell Sonicates of Nontoxigenic Strains C. botulinum type E

28-2 PM1-5

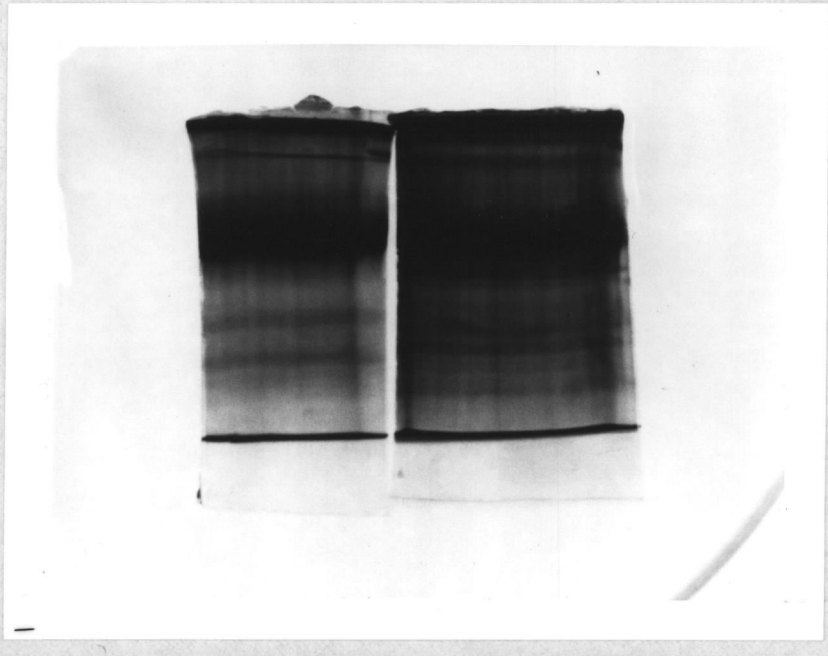


FIGURE 8

Total Protein Stained Gels of Cell Sonicates of C. botulinum type E Toxigenic Strain VH and Nontoxigenic Strain 066B-nt

066B-nt VH

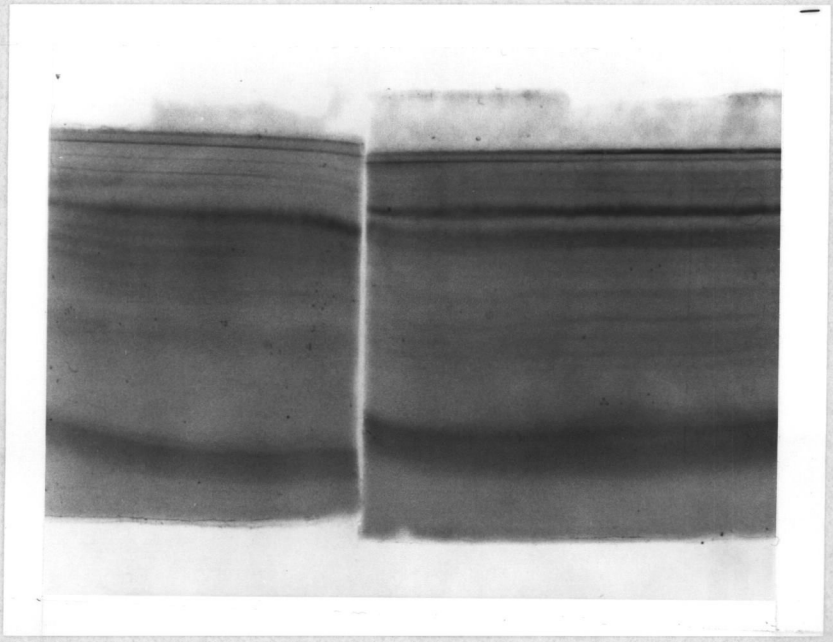


FIGURE 9

Total Protein Stained Gels of Cell Sonicates of C. botulinum type E Toxigenic Strain 066B and Nontoxigenic Strain 066B-nt

066B	066B-nt
Toxigenic	Nontoxigenic

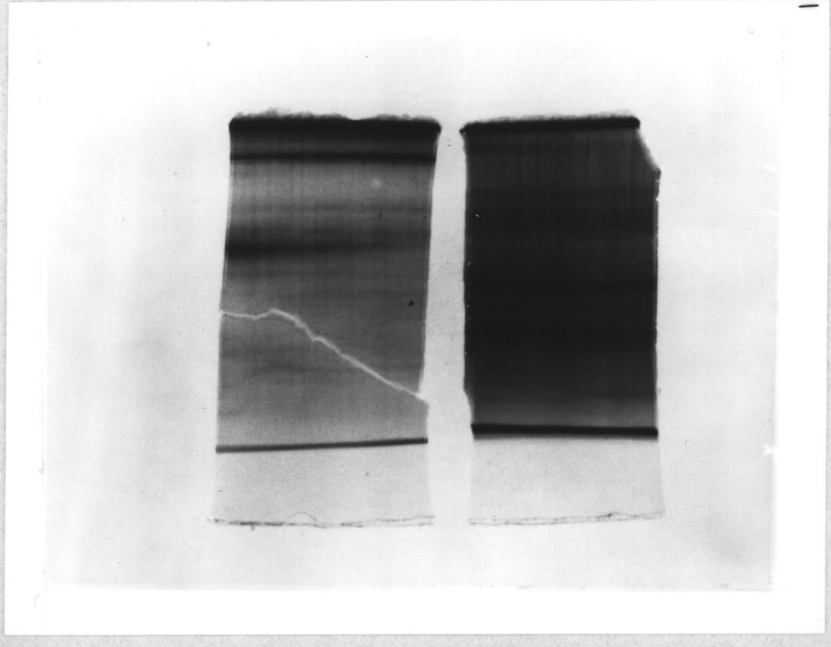
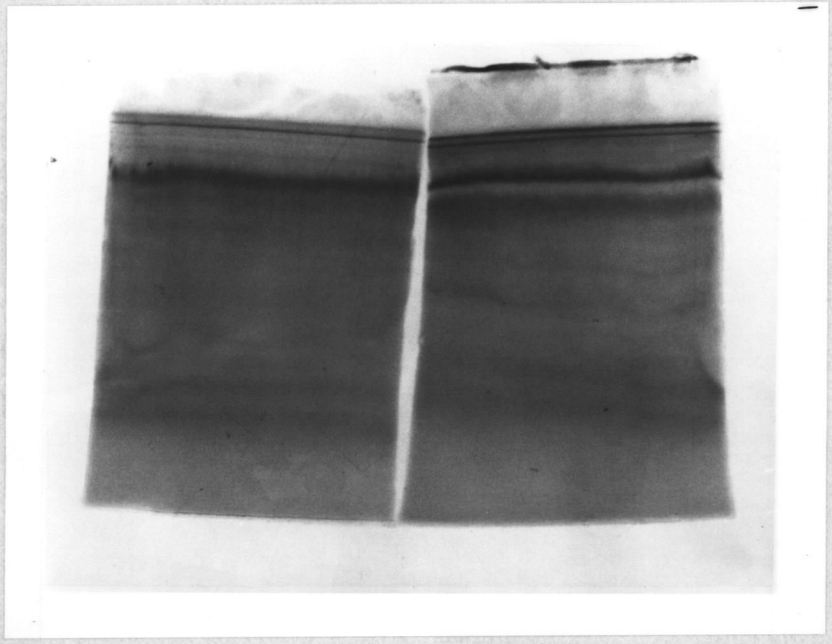


FIGURE 10

Total Protein Stained Gels of Cell Free Medium of *C. botulinum* type E Toxigenic Strain VH and Nontoxigenic Strain 066B

066B (nontoxigenic) VH (toxigenic)



DISCUSSION

The problem of demonstrating Clostridium botulinum in samples from nature is well known (Crisley, 1963; Johannsen, 1962; Meyer, 1956). No quick accurate method has yet been found to identify the presence of this organism. Many procedures and techniques are in the process of being tested including fluorescent antibody techniques, enzyme analysis, immunodiffusion, hemagglutination, and opsonic index (Petty, 1964). The classification of this organism now with respect to immunological type is tied to the production of toxin and the neutralization of the toxin by type specific antisera as demonstrated by animal inoculation. Lacking a selective medium for this organism, the usual procedure is to prepare an enrichment culture and then to look for botulinal toxin. Unequivocal demonstration of the toxin is adequate evidence that C. botulinum is present (Dolman, 1957b), although the evidence is strengthened by isolating the organism in pure culture. Failure to find toxin in the enrichment culture does not necessarily prove the absence of C. botulinum from the original sample. Other organisms may inhibit its growth or they may destroy its toxin (Johannsen, 1962) before it can be identified or typed as to strain. These organisms cannot, under our present system of classification, be identified as C. botulinum. The use of animals introduces another

variable factor which often can cloud and obscure clear cut reactions or results.

The physiological growth conditions under which the organism will vegetate and produce the maximum amount of toxin is also another variable factor which always causes concern as to whether the procedure used enabled the presence of a single spore to outgrow and produce toxin which can be detected and identified.

With these variable factors and conditions in mind this study attempted to minimize the problems and seek the optimal experimental conditions so as to give valid, reliable results. However, we did not always succeed.

The distribution of C. botulinum type E has indicated its close association with the aquatic environment (Bott, 1966; Cann, 1965; Dolman, 1964b; Hayes, 1966; Johannsen, 1962). While types A and B are occasionally found in the aquatic environment, the dominant type present is type E. It in fact has not been found prevalent very far from the water's edge and is believed by some to be located mainly below the high water mark of rivers and bays (Bott, 1966; Hayes, 1966; Johannsen, 1963). What are the factors in this habitat that are especially favorable to type E? Yet in some parts of the world type E has not been demonstrated or only occasionally (Cann. et al., 1965; Dolman, 1961; Ohye and Scott, 1957). Amongst the many factors which determine the growth and formation of type E

toxin are temperature, pH and buffering capacity, oxygen tension and redox potential, water vapor pressure and the presence of specific growth factors, essential amino acids and fatty acids, besides other synergistic or antagonistic agents, including antibiotic substance (Johannsen, 1965a; Kautter, 1966). Workers have reported all of these factors affecting growth in the laboratory but little if anything is known of any biochemical, biophysical or nutritional factors acting in the natural environment. Whatever they are, they are apparently factors not essential for or perhaps even inhibitory, to types A and B whose habitat is essentially in soils not associated with the aquatic environment. Only Ohye and Scott (1957) have even suggested any limiting factors and they postulate that temperature may be a major factor and that the temperature of the aquatic habitat of type E organism is lower than in the natural habitat of A and B strains.

This study also showed the influence of river waters from land drainage as influencing the incidence of type E in gills and viscera of fish. This is demonstrated by: the increased incidence of type E in salmon caught in the region of the Columbia River (23.2%) over that caught in the open ocean (6.9%); by the increased incidence in the steelhead trout from the Alsea River; by the increased incidence in "bottom fish" brought into Astoria (33.3%) over those landed at Coos Bay (7.7%); by the higher incidence of type E in bay clams (61.0%) over those dug from the open ocean beaches (14.6%). All of these

findings are in agreement and seem to be related to those in a survey of river sediments and ocean beach sediments (Hayes, 1966) where type E was found to be prevalent in the Columbia, Alsea, and Umpqua Rivers sediment samples but was found only infrequently in samples from the ocean beaches. These results all seem to indicate that the river and bay sediments are the chief sources of type E in contrast to ocean beach sediments and that the fish probably acquire the organism most frequently when they come in contact with the river waters or include in their food chain some organisms which do. The prime location of C. botulinum type E is undoubtedly the sediments and additional study of these should be undertaken.

Loss of toxicity, between the first mouse toxicity tests and the typing with specific antitoxin, in the mixed culture filtrate was a continual problem. Only 60 of 190 toxic cultures could be typed (31.6%). Low levels of toxin in the original toxin test also obscured clear cut results and gave rise to questionable samples. The mixed culture was quick frozen at -15 C between tests but this still did not alleviate the problem. Why should the toxin be destroyed, even in 24 hours and in a frozen state? Most authors agree that it could be proteolytic enzymes produced by other organisms in the culture. Many parameters have been tried such as the following. Other storage temperatures have been tried without any better success; -60 C produced about the same results as 4 C and -15 C. The addition of trypsin

inhibitors or antiseptics did not reduce the loss of toxin. Decrease in pH from 6.2 to 4.5 seemed to give some significantly better results when the sample was held for 23 days but no significant improvement when the sample was held for four days (Pilcher and Hayes, 1966).

D. Kautter has noted that loss of toxicity is reduced if trypsin is added to the medium at the beginning of incubation (personal communication). A significantly higher number of positive samples have been identified with a decrease in toxin loss if this procedure is followed. The exact reason for this is as yet unknown; however, it may be the toxin in smaller molecular fragment may not be susceptible to bacterial proteolytic enzymes produced in the mixed cultures by other bacteria or it may be that the toxin concentration is so increased in terms of mouse LD^{50} per ml, that even if some loss does occur in frozen storage, not all the toxicity is lost. It has been shown, by many investigators, that activation of the mixed culture with trypsin, 1% final mix at pH 6.2, can increase the mouse LD^{50} by 1000 fold.

When pure toxigenic cultures of old established type E strains are streaked out on LVE or blood agar and typical isolated colonies placed in TPG medium and incubated only 177 of 214 (82.8%) proved to produce toxin. This is also a question of interest. Why are not all the colonies toxic? Those nontoxigenic variants cannot be explained on normal or true mutation because of the great disparity in frequency of these variants compared to that of true mutants. A

possible explanation may be that the original cultures may have been a mixture of two Clostridia, one type E toxigenic and the other an "E like" nontoxigenic culture. Many workers (Dolman, 1957a; Kautter et al., 1966) have emphasized the difficulty of purifying Clostridia. If this were true, it would explain why the old established type E cultures from other laboratories gave rise to fewer nontoxigenic colonies (17.2%) than the freshly isolated cultures (78.7%) -- perhaps because they had gone through numerous plating and selection procedures and this selection had resulted in a culture of greater purity than those found in the new isolates.

The hypothesis that the toxigenic cultures are always phage infected needs to be considered. Recent work by Iida (1968) and Ecklund (1969) demonstrates phage particles with C. botulinum. We could then assume that the nontoxigenic variant colonies arise from cells that do not happen to be phage infected. The fact that nontoxigenic strains have never been demonstrated to give rise to toxigenic ones even when exposed to γ radiation or to many known chemical mutagens seems to fit this hypothesis. The true explanation remains unknown but the problem deserves attention.

The electrophoretic analysis of the protein fractions may shed some light on the "tox" - "nontox" problem associated with both culture isolation and lost toxicity of culture filtrates. Hobbs and Anderson (1967) and Dyer (1968) find that different strains of C.

botulinum demonstrate different enzymes responsible for some of the bands present. The dense band in the top portion of the toxigenic strain of type E could well be the toxin. This should be checked by immunodiffusion against antitoxin and then eluted and tested for toxicity. The bands probably represent structural protein, enzymes, or in the filtrate, the metabolic byproducts. The minor differences in the number and position of the bands could represent different systems not identical in all.

SUMMARY AND CONCLUSIONS

The distribution and incidence rate of Clostridium botulinum has been studied in the fish, shellfish, and sediments of the marine environment. The study extended over the coastal areas of Oregon and Washington. Smoked fish products were also examined for the presence of this sporulating anaerobe.

Samples were incubated anaerobically under the most favorable conditions known to facilitate outgrowth and metabolism of the organism and the production of its neurogenic toxin. The presence of toxin was demonstrated by injection into Swiss Webster strain whitemice. Determination of the type of C. botulinum was by specific antitoxin protection tests, again using white mice. When typed, isolation in pure culture of the organism was attempted and retesting and typing of the toxin from the isolate was repeated.

Clostridium botulinum type E was the major dominant strain isolated from the marine environment. All species of fish tested demonstrated the production of a toxic filtrate when gills and viscera were incubated in TPG medium at 28 C under anaerobic conditions. A few type A and B were found and a single culture of type F was isolated from a sockeye salmon in the Columbia River. The proportions of specimens yielding toxic filtrates were as follows:

Sockeye salmon 14 of 59 (23.7%)
Chinook salmon 20 of 124 (16.1%)
Coho salmon 22 of 221 (9.9%)
Steelhead trout 7 of 37 (19.0%)
Bottom fish 28 of 157 (17.8%)
Sturgeon 3 of 24 (12.5%)

Shellfish were also collected. Three to five shellfish were combined into a single specimen and treated as described. The proportions of specimens yielding toxic filtrates were as follows:

Razor clams 11 of 75 (14.6%)
Cockle clams 12 of 15 (80.0%)
Softshell clams 8 of 12 (66.4%)
Littleneck clams 4 of 11 (36.2%)
Horseneck clam 1 of 3 (33.3%)
Oysters 6 of 19 (31.6%)
Dungeness crabs 17 of 24 (71%)

Environmental swab samples from the "deep sea" fillet processing plants produced 3 of 39 (7.7%) toxic filtrates. None of the 53 samples taken in the salmon processing plants produced toxic filtrates.

When the results of specimen incubation were analyzed as to location collected, it was found that those collected near the Columbia River or in river estuaries produced a higher percentage of toxic filtrates than did those collected off the open ocean beaches. This was true for the coho and chinook samon, "deep sea" fish and clams.

The samples collected off or in the rivers compared to those collected off the open beach were as follows:

Coho Salmon 10 of 19 (34.4%) vs. 13 of 186 (7.0%)
Chinook Salmon 19 of 106 (18%) vs. 1 of 18 (5.6%)
"Deep Sea" Fish 23 of 70 (32.8%) vs. 5 of 87 (5.6%)
Clams 25 of 41 (61.0%) vs. 11 of 75 (14.6%)

This would lend support to the theory (Johannsen, 1963) that C. botulinum is of terrestrial origin and is being concentrated in the coastal areas by water runoff from the land mass.

Analysis of total protein patterns, in sonicates of bacterial suspensions, as demonstrated by electrophoresis through poly acrylamide gel sheets, differed in the two toxigenic and four nontoxigenic strains studied. A dense protein band was present in both the cell sonicate of the toxigenic strains and in the toxic cell free culture filtrate which was not found in the patterns of the cell sonicates of nontoxigenic cultures. This band in the upper third is the top one of the band spectrum, and has a relatively low electrophoretic mobility. It may be the actual type E toxin, but this remains to be determined. Minor differences were seen also in the lower third of the band spectrum.

This would suggest a difference in the genetic makeup in toxigenic and nontoxigenic cultures.

It is most important for the public and for the food industry to be aware of the geographical distribution of Clostridium botulinum and its prevalence in fish and shellfish in the Northwest and in food products made from them. They should also have a thorough knowledge of the conditions which govern the growth of the organism and the formation of toxin so due attention can be given to reducing or eliminating any health hazard which might be present.

BIBLIOGRAPHY

Avery, R. J., E. E. Dolman, P. L. Stovell and A. J. Wood. 1959. A natural outbreak of Clostridium botulinum type E intoxication in ranch mink arising from pork liver. Canadian Journal of Comparative Medicine 23:203-209.

Bengston, I. A. 1922. Preliminary note on a toxin-producing anaerobe isolated from the larvae of Lucilia caesar. Public Health Reports 37:164-170.

Bengston, I. A. 1923. Toxin producing anaerobe isolated from fly larvae. Public Health Reports 38:340-344.

Boroff, D. A. and J. R. Reilly. 1959. Studies of the toxin of Clostridium botulinum. V. Prophylactic immunization of pheasants and ducks against avian botulism. Journal of Bacteriology 77:142-146.

Bott, Thomas L., Janet S. Deffner, Elizabeth McCoy and E. M. Foster. 1966. Clostridium botulinum type E in fish from the Great Lakes. Journal of Bacteriology 91:919-924.

Burke, G. S. 1919. Notes on Bacillus botulinus. Journal of Bacteriology 4:555-565.

Cann, D. C., Barbara B. Wilson, G. Hobbs and J. M. Shewan. 1965a. The growth and toxin production of Clostridium botulinum type E in certain vacuum packed fish. Journal of Applied Bacteriology 28:431-436.

Cann, D. C., Barbara B. Wilson, G. Hobbs, J. M. Shewan and A. Johannsen. 1965b. The incidence of Clostridium botulinum type E in fish and bottom deposits in the North Sea and off the coast of Scandinavia. The Journal of Applied Bacteriology 28:426-430.

Cann, D. C., Barbara B. Wilson and J. M. Shewan. 1966. A comparison of toxin production by Clostridium botulinum type E in irradiated and unirradiated vacuum packed fish. Journal of Applied Bacteriology 29:540-548.

Carroll, Bobby J., Ephraim S. Garrett, Gladys B. Reese and Benjamin Q. Ward. 1966. Presence of Clostridium botulinum in the Gulf of Venezuela and the Gulf of Darien. Applied Microbiology 14:837-838.

Chambach, A., R. A. Reisfeld, M. Wyckoff and J. Zaccari. 1967. A procedure for rapid and sensitive staining of protein fractionated by polyacrylamide gel electrophoresis. Analytical Biochemistry 20:150-154.

Clifton, C. E. 1939. Utilization of amino acids by Clostridium botulinum. Proceedings of the Society for Experimental Biology and Medicine 40:338-340.

Clifton, C. E. 1940a. The utilization of amino acids and of glucose by Clostridium botulinum. Journal of Bacteriology 39:485-497.

Clifton, C. E. 1940b. Utilization of serine by Clostridium botulinum. Proceedings of the Society for Experimental Biology and Medicine 43:588-590.

Craig, James M. and K. S. Pilcher. 1966. Clostridium botulinum type F: Isolation from salmon from the Columbia River. Science 153:311-312.

Craig, James M., Sidney Hayes and K. S. Pilcher. 1968. Incidence of Clostridium botulinum type E in salmon and other marine fish in the Pacific Northwest. Applied Microbiology 16:553-557.

Crisley, F. D. 1963. The demonstration of botulinus toxin and Clostridium botulinum in foods. In: Examination of foods for enteropathogenic and indicator bacteria, ed. by K. H. Lewis and R. Angelotti. Cincinnati. p. 84-124. (U. S. Public Health Service)

Davis, B. J. 1964. Disc electrophoresis. II. Methods and application to human serum proteins. Annals of the New York Academy of Sciences 121:404-427.

DeBord, G. G., R. B. Edmondson and Charles Thom. 1920. Summary of chemistry investigations of poisoning due to ripe olives. Journal of the American Medical Association 74:1220-1221.

Dickson, Ernest C. 1915. Botulism, an experimental study: a preliminary report. Journal of the American Medical Association 65:492-496.

Dickson, Ernest C. 1917. Botulism: the danger of poisoning from vegetables canned by the cold-pack method. Journal of the American Medical Association 69:966-968.

Dickson, Ernest C. 1918. Botulism: a further report of cases occurring in the Pacific Coast States. The Archives of Internal Medicine 22:483-495.

Dickson, Ernest C. and Georgina E. Burke. 1918. Botulism: a method of isolating Bacillus botulinus from infected materials. Journal of the American Medical Association 71:518-521.

Dickson, Ernest C. and Beatrice M. Howitt. 1920. Botulism: a preliminary report of a study of the antitoxin of Bacillus botulinus. Journal of the American Medical Association 74: 718-722.

Dolman, C. E. 1953. Human botulism in Canada. Journal of the Canadian Medical Association 68:538-543.

Dolman, C. E. 1954. Additional botulism episodes in Canada. Journal of the Canadian Medical Association 71:245-249.

Dolman, C. E. 1957a. The influence of bacterial mutation upon type E botulinus toxin production. (Abstract) Canadian Journal of Public Health 48:27.

Dolman, C. E. 1957b. Recent observations on type E botulism. Canadian Journal of Public Health 48:187-198.

Dolman, C. E. 1957c. Type E (fish-borne) botulism: a review. Japanese Journal of Medical Science and Biology 10:383-395.

Dolman, C. E. 1961. Further outbreaks of botulism in Canada. Journal of the Canadian Medical Association 84:191-200.

Dolman, C. E. 1964a. Botulism. American Journal of Nursing 64:119-124.

Dolman, C. E. 1964b. Botulism as a world health problem. In: Botulism: Proceedings of a symposium, ed. by K. H. Lewis and K. Cassel, Jr. Cincinnati. p. 5-32. (U. S. Public Health Service. Publication no. 999-FP-1)

Dolman, C. E. 1964c. Growth and metabolic activities of Clostridium botulinum types. In: Botulism: Proceedings of a symposium, ed. by K. H. Lewis and K. Cassel, Jr. Cincinnati. p. 43-68. (U. S. Public Health Service. Publication no. 999-FP-1)

Dolman, C. E. 1964d. Remarks on open discussion: Problems of the future. In: Botulism: Proceedings of a symposium, ed. by K. H. Lewis and K. Cassel, Jr. Cincinnati. p. 241-242. (U. S. Public Health Service. Publication no. 999-FP-1)

Dolman, C. E. and H. Chang. 1953. The epidemiology and pathogenesis of type E and fish-borne botulism. Canadian Journal of Public Health 44:231-244.

Dolman, C. E., Helen Chang, Donna Kerr and A. R. Shearer. 1950. Fish-borne and type E botulism: two cases due to home-pickled herring. Canadian Journal of Public Health 41:215-229.

Dolman, C. E. and H. Iida. 1963. Type E botulism: its epidemiology, prevention, and specific treatment. Canadian Journal of Public Health 54:293-308.

Dolman, C. E. and Donna E. Kerr. 1947. Botulism in Canada with report of a type E outbreak at Nanaimo, B. C. Canadian Journal of Public Health 38:48-57.

Dolman, C. E. and L. Murakami. 1961. Clostridium botulinum type F with recent observations on other types. Journal of Infectious Diseases 109:107-128.

Dolman, C. E., M. Tomsich, C. R. Campbell and W. B. Laing. 1960. Fish eggs as a cause of human botulism. Journal of Infectious Diseases 106:5-19.

Duff, James T., George G. Wright and A. Yarinsky. 1956. Activation of Clostridium botulinum type E toxin by trypsin. Journal of Bacteriology 72:455-460.

Duff, James T., George G. Wright, Julius Klerer, Dorothy E. Moore and Richard H. Bibler. 1957. Studies on immunity to toxins of Clostridium botulinum. I. A simplified procedure for isolation of type A toxin. Journal of Bacteriology 73:42-47.

Dyer, John Kaye. 1968. A physiological approach to a rapid method for identifying C. botulinum. Ph.D. thesis. Corvallis, Oregon State University. 83 numb. leaves (Microfilm)

Eklund, M. W. and F. T. Poysky. 1965a. The significance of Clostridium botulinum type E in the application of radiation-pasteurization process to Pacific crabmeat and flounder, July 1965. Seattle. 72 numb. leaves. (U. S. Atomic Energy Commission. Division of Biology and Medicine). Final data report on U. S. Atomic Energy Commission Contract AT-(49-702442)

Eklund, M. W. and F. Poysky. 1965b. Incidence of Clostridium botulinum type E from the Pacific Coast of the United States. In: Botulism 1966: Proceedings of the fifth international symposium on food microbiology, ed. by M. Ingram and T. A. Roberts. London, Chapman Hall, p. 49-56.

Ecklund, M. W., F. T. Poysky and E. S. Boatman. 1969. Bacteriophages of Clostridium botulinum types A, B, E and F and nontoxigenic strains resembling type E. Journal of Virology 3:270-274.

Foster, E. M., J. S. Deffner, T. L. Bott and E. McCoy. 1965. Clostridium botulinum food poisoning. Journal of Milk and Food Technology 28:86-91.

Geiger, J. C., E. C. Dickson and K. F. Meyer. 1922. The epidemiology of botulism. Washington, D. C. 119 p. (U. S. Public Health Service. Bulletin no. 127)

Gimenez, Domingo F. and Alberto S. Ciccarelli. 1968. Clostridium botulinum type F in the soil of Argentina. Applied Microbiology 16:732-734.

Gordon, Milton, Mary Fiock, Allen Yarninsky and James T. Duff. 1957. Studies on immunity to toxins of Clostridium botulinum. III. Preparation, purification, and detoxification of Type E toxin. Journal of Bacteriology 74:533-538.

Grubb, W. B. 1964. Avian botulism in Western Australia. Australian Journal of Experimental Biology 42:17-26.

Gunnison, J. B., J. R. Cummings and K. F. Meyer. 1936. Clostridium botulinum type E. Proceeding of the Society for Experimental Biology and Medicine 35:278-280.

Hayes, Sidney Joseph, III. 1966. Occurrence of Clostridium botulinum type E in shellfish, lake fish, and aquatic sediments in the northwest. Master's thesis. Corvallis, Oregon State University, 106 numb. leaves.

Hazen, Elizabeth L. 1937. A strain of Bacillus botulinus not classified as type A, B, or C. Journal of Infectious Diseases 60: 260-264.

Hazen, Elizabeth L. 1938. Incitants of human botulism. Science 87:413-414.

Hinman, A. R. 1966. Cause, prevention and treatment of botulism. California Health 24:17-18.

Hobbs, G. and A. W. Anderson. 1967. Protein differences in strains of C. botulinum based on separation by disc electrophoresis. In: Botulism 1966: Proceedings of the fifth international symposium on food microbiology, ed. by M. Ingram and T. A. Roberts. London, Chapman, Hall, p. 417-421.

Holderman, Lillian Haldeman. 1964. Growth and toxin production of Clostridium botulinum type F. Washington, D. C., U. S. Public Health Service. 173 p.

Iida, Hiroo. 1968. Bacteriophages of Clostridium botulinum. Japan Electron Optics Laboratory News (JEOL News) 68:14-15.

Iida, Hiroo and Katsuhiro Inoue. 1968. Rhapidosomes of Clostridium botulinum type E. Japanese Journal of Microbiology 12:353-356

Inoue, Katsuhiro and Hiroo Iida. 1968. Bacteriophages of Clostridium botulinum. Journal of Virology 2:537-540.

Johannsen, A. 1962. Presence and distribution of Clostridium botulinum, type E, with special reference to the Oresund region. Sartryck of Nordisk Veterinaernmedicin 14:441-474.

Johannsen, A. 1963. Clostridium botulinum in Sweden and the adjacent waters. Journal of Applied Bacteriology 26:43-47.

Johannsen, A. 1965a. Clostridium botulinum type E in foods and the environment generally. Journal of Applied Bacteriology 28: 91-94.

Johannsen, A. 1965b. Clostridium botulinum type E I Rokta Fiskvaror. Sartryck ur svensk Veterinartidning, nr. 5, p. 7-19.

Johnston, Ralph W., John Feldman and Rosemary Sullivan. 1963. Botulism from canned tuna fish. Public Health Reports 78: 561-564.

Johnston, Ralph W., John Feldman and Rosemary Sullivan. 1964. Method to facilitate the isolation of Clostridium botulinum type E. Journal of Bacteriology 88:1521-1522.

Johnston, R., S. Harmon and D. Kautter. 1964. Method to facilitate the isolation of Clostridium botulinum type E. Journal of Bacteriology 88:1522.

Kaufman, Oliver and L. Dale Fay. 1964. Clostridium botulinum type E toxin in tissues of dead loons and gulls. Quarterly Bulletin of the University of Michigan 47:236-242.

Kautter, D. A., S. M. Harmon, R. K. Lynt, Jr. and T. Lilly, Jr. 1966. Antagonistic effect on Clostridium botulinum type E by organisms resembling it. Applied Microbiology 14: 616-622.

Koser, S. A., R. B. Edmondson and L. T. Giltner. 1921. Observation of Bacillus botulinus of canned spinach. Journal of the American Medical Association 77:1250-1253.

Landman, G. 1904. Ueber die Ursache der Darmstadter Bohnenvergiftung. Hygienische Rundschau 14:449-452.

Leuchs, J. 1910. Beitrage zur Kenntnis des Toxins und Antitoxins des Bacillus botulinus. (Zertschrift fur Hygiene und Infektionskrankheiten) 65:55-84.

Littauer, Uriel. 1951. Observations on type A toxin of Clostridium botulinum. Nature 167:994-995.

McClung, L. S. and Ruth Toabe. 1947. The egg yolk plate reaction for the presumptive diagnosis of Clostridium sporogenes and certain species of the gangrene and botulinum groups. Journal of Bacteriology 53:139-147.

Meyer, K. F. 1956. The status of botulism as a world health problem. Bulletin of the World Health Organization 15:281-298.

Meyer, K. F. and B. Eddie. 1965. Sixty-five years of human botulism in the United States and Canada. San Francisco. University of California. George Williams Hooper Foundation.

Møller, V. and I. Scheibel. 1960. Preliminary report on the isolation of an apparently new type of Clostridium botulinum. Acta Pathologica Microbiologica Scandinavica 48:80.

Nakamura, Yukata, Hiroo Iida, Kiyoshi Saeki, Kenzo Kanzawa and Takashi Karachimada. 1956. Type E botulism in Hokkaido, Japan. Japanese Journal of Medical Science and Biology 9: 45-48.

Nilehn, P. O. and A. Johannsen. 1965. Ett utbrott aviar botulism. Nordisk Veterinaermedicin 17:685-692.

Ohye, D. F. and W. J. Scott. 1957. Studies in the physiology of Clostridium botulinum type E. Australian Journal of Biological Science 10:85-94.

Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. Annals of the New York Academy of Sciences 121: 321-349.

Osherhoff, B. J., G. G. Slocum and W. M. Decker. 1964. Status of botulism in the United States. Public Health Reports 79: 871-878.

Petty, C. S. 1964. Botulism omnibus. In: Botulism: Proceedings of a symposium, ed. by K. H. Lewis and K. Cassel, Jr. Cincinnati. p. 177-183. (U. S. Public Health Service. Publication no. 999-FP-1)

Pilcher, K. S. and S. J. Hayes. 1966. Survey of distribution of C. botulinum type E. April 1 to Aug. 31, 1966. Corvallis. (Oregon State University. Department of Bacteriology. Quarterly report on U. S. Food and Drug Administration Contract FDA 66-2 (neg))

Prevot, A. R. and E. R. Brygoo. 1950. Recherches sur la toxine, l'anatoxine et l'antitoxine botuliques D. Annales de L'Institut Pasteur 79:1-13.

Reddish, George F. 1929. An investigation into the purity of American strains of Bacillus botulinus. The Journal of Infectious Diseases 29:120-131.

Reed, L. J. and H. Muench. 1938. A simple method of estimating fifty percent endpoints. The American Journal of Hygiene 27: 493-497.

Schmidt, C. F., R. V. Lechowich and J. F. Folinazza. 1961. Growth and toxin production by type E Clostridium botulinum below 40° F. Journal of Food Science 26:626-630.

Schmidt, C. F., W. K. Nank and R. V. Lechowich. 1962. Radiation sterilization of food. II. Some aspects of growth, sporulation, and the radiation resistance of spores of Clostridium botulinum type E. Journal of Food Science 27:77-84.

Seddon, Glenn G. 1922. Bulbar paralysis in cattle due to the action of a toxigenic bacillus with a discussion on the relation of condition to forage poisoning (botulism). Journal of Comparative Pathological Therapy 35:147-190.

Slocum, Glenn G. 1964. Problems of isolation and identification of Clostridium botulinum. In: Proceedings of a symposium ed. by K. H. Lewis and K. Cassel, Jr. Cincinnati. p. 83-88. (U. S. Public Health Service. Publication no. 999-FP-1)

Theiler, A., P. R. Viljoen, H. H. Green, P. J. Du Toit, H. Meier and E. M. Robinson. 1927. Lamsiekte (parabotulism) in cattle in South Africa. In: Twelfth Report of the Director of Veterinary Research, Durban. p. 821-1361.

Thom, Charles, Ruth B. Edmondson and L. T. Giltner. 1919. Botulism from canned asparagus. Journal of the American Medical Association 73:907-912.

Thompson, Luther. 1926. The blood agar plate for spore forming anaerobes. Journal of Bacteriology 11:305-321.

Tyler, H. Richard. 1963. Botulism. Archives of Neurology 9: 102-110.

Ward, B. Q. and B. J. Carroll. 1965. Presence of Clostridium botulinum type E in esturine waters of the Gulf of Mexico. Applied Microbiology 12:502.

Ward, B. Q., B. J. Carroll, E. S. Garrett and G. B. Reese. 1967a. Survey of the U. S. gulf coast for the presence of Clostridium botulinum. Applied Microbiology 15:629-636.

Ward, B. Q., B. J. Carroll, E. S. Garrett and G. B. Reese. 1967b. Survey of the U. S. Atlantic coast and estuaries from Key Largo to Staten Island for the presence of Clostridium botulinum. Applied Microbiology 15:964-965.

Ward, B. Q., E. S. Garrett and B. G. Reese. 1967. Further indications of Clostridium botulinum in Latin American waters. Applied Microbiology 15:1509.

Wentzel, L. M., M. Sterne and A. Polson. 1950. High toxicity of pure botulinum type D toxin. Nature 166:739-740.

Wilbur, Ray Lyman and William Ophuls. 1914a. Botulism: a report of food poisoning apparently due to eating of canned string beans, with a pathological report of a fatal case. The Archives of Internal Medicine 24:598-604.

Wilbur, Ray Lyman and William Ophuls. 1914b. Botulism: a report of food poisoning apparently due to eating of canned string beans, with a pathological report of a fatal case. Transactions of the Association of American Physicians 29:494-511.

Williams-Walls, N. J. 1968. Clostridium botulinum type F: Isolation from crabs. Science 162:375-376.

Willis, A. T. 1960. The lipolytic activity of some Clostridia. Journal of Pathological Bacteriology 80:370-390.

Willis, A. T. and G. Hobbs. 1958. A medium for the identification of Clostridia producing opalescence in egg-yolk emulsions. Journal of Pathological Bacteriology 75:290-305.

Willis, A. T. and G. Hobbs. 1959. Some new media for the isolation and identification of Clostridia. Journal of Pathological Bacteriology 77:511-521.

Willis, A. T. and G. Gowland. 1962. Some observations on the mechanism of the Nagler reaction. Journal of Pathological Bacteriology 83:219-226.

Winslow, C. E. A., Jean Broadhurst, R. E. Buchanan, Charles Krumwiede, L. A. Rogers and George Smith. 1917. The families and genera of the bacteria. Journal of Bacteriology 2:505-566.

Zlatogoroff, S. I. and M. N. Soloviev. 1927. Botulism in the Union of Socialist Soviet Republics. Journal of the American Medical Association 88:2024-2025.