AN ABSTRACT OF THE THESIS OF

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Title: TOXIGENIC AND NON-TOXIGENIC STRAINS OF CLOSTRIDIUM BOTULINUM TYPE E

A. DIFFERENTIATION BY IMMUNODIFFUSION

B. ATTEMPTS TO INDUCE TOXIGENICITY BY MUTAGENIC AGENTS

Abstract approved: [redacted]
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In this study of toxigenic and non-toxigenic strains of Clostridium botulinum type E, attempts were made to induce toxin production in non-toxigenic cultures by exposure to mutagenic agents and to differentiate the organisms by immunodiffusion. First, cultures of non-toxic Cl. botulinum type E were exposed to various mutagenic agents in an attempt to produce toxigenic mutants. Next, an attempt was made to separate toxic and non-toxic Cl. botulinum type E colonies on the basis of their microscopic appearance by reflected and transmitted light. Finally, the use of micro immunodiffusion tests for the identification of Cl. botulinum type E toxin and consequent differentiation of toxic and non-toxic cultures was studied.

Nine non-toxic Cl. botulinum type E strains were exposed to a variety of mutagenic agents. These included 2-aminopurine, azaserine, acriflavin, 5-bromouracil and gamma ray irradiation. The degree of exposure was sufficient to kill 90% or more of the cells. After exposure, the survivors were transferred to fresh medium and the resulting cultures tested for toxigenicity. No toxin production was demonstrated in any of these cultures.

An irradiated culture of a non-toxic E-like strain was cultured on brain heart agar with sodium thioglycollate. The colonies which developed were examined microscopically by reflected and transmitted light. Of approximately 10,000 colonies observed, seven were picked because of their resemblance to the toxic colonies described by Dolman (1957b) and tested for toxicity. None proved to be toxic.

Two micro immunodiffusion methods were studied. Culture filtrates of 14 toxic and nine non-toxic strains of Cl. botulinum type E were concentrated approximately ten fold by dialysis against polyethylene glycol 4000. They were tested against type E antitoxin using the agar well micro immunodiffusion method. All toxic strains reacted with this antiserum giving a single line precipitin reaction visible in a strong beam of light. These strains did not react with type B antitoxin and gave a faint, fading reaction with type A antitoxin. The non-toxic filtrates did not react with types B or E

antitoxin and gave a faint, fading reaction with type A antiserum. Concentrated culture filtrates of nine other clostridial species were tested against types A, B and E antitoxin. None reacted with type E antitoxin. However, some did react with type A and B antitoxin, indicating the presence of antigens common to Cl. botulinum type A or B and some other clostridial species.

Using the template micro immunodiffusion method, concentrated culture filtrates of Cl. botulinum types A, B and E were tested against the same antitoxins. Again type E antitoxin proved specific and reacted with toxic type E cultures only; these cultures again failed to cross react with type A or B antitoxin. Because of the specificity of the reactions between type E culture filtrates and antitoxin, micro immunodiffusion methods appear to be very promising for differentiating pure cultures of toxic and non-toxic type E strains under laboratory conditions without the use of animals. Further work is needed to explore possible application to mixed cultures from fish and other specimens.

The sensitivity of the two micro immunodiffusion methods was compared by first determining the toxin concentration in a concentrated filtrate of a type E culture in terms of mouse LD_{50}/ml and then determining the minimum concentration of this toxin detectable by the two in vitro procedures. Visible precipitate lines developed only with the template micro method for this particular concentrate.

Thus, the template micro method appeared more sensitive. It was able to detect a minimum toxin concentration of 123 LD_{50}/ml or about 3.1 LD_{50} per 0.025 ml, the approximate volume used in the test.

Toxigenic and Non-Toxigenic Strains of *Clostridium Botulinum* Type E

A. Differentiation by Immunodiffusion

B. Attempts to Induce Toxigenicity by Mutagenic Agents

by

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TABLE OF CONTENTS

INTRODUCTION	1
HISTORICAL REVIEW	3
EXPERIMENTAL MATERIALS AND METHODS	16
Cultures	16
Media	17
Materials and Reagents	20
Methods	25
RESULTS AND DISCUSSION	43
Identification of non-toxic strains of Clostridium botulinum type E cultures	43
Mutagenic studies	46
Colonial characteristics	56
Immunodiffusion tests	60
SUMMARY	72
BIBLIOGRAPHY	77

LIST OF FIGURES

Figure		Page
1	Precipitin reaction between type E botulinal antitoxin and Clostridium botulinum type E by the agar well micro method	66
2	Precipitin reaction between type A botulinal antitoxin and Clostridium botulinum type A and Clostridium novyi by the agar well micro method	66
3	Precipitin reaction between type B botulinal antitoxin and Clostridium botulinum type A and Clostridium novyi by the agar well micro method	66
4	Precipitin reaction between type B botulinal antitoxin and Clostridium botulinum type B and Clostridium welchii by the agar well micro method	66

LIST OF TABLES

Table		Page
1	Biochemical properties of Clostridium botulinum type E non-toxic strains	44
2	Source of non-toxic strains of Clostridium botulinum type E used in mutagenic studies	47
3	Effect of 5-bromouracil on non-toxic Clostridium botulinum type E cultures	49
4	Effect of acriflavin on non-toxic Clostridium botulinum type E cultures	49
5	Effect of azaserine on non-toxic Clostridium botulinum type E cultures	50
6	Effect of 2-aminopurine on non-toxic Clostridium botulinum type E cultures	50
7	Effect of irradiation on four non-toxic Clostridium botulinum type E cultures	53
8	Characteristics of colonies of non-toxic Clostridium botulinum type E culture 066B-NT after exposure to irradiation	59
9	Concentrates of culture filtrates of toxic and non-toxic Clostridium botulinum type E tested by the agar well micro method against types A, B and E botulinal antitoxin	62
10	Concentrates of culture filtrates of clostridial species tested by the agar well micro immunodiffusion method against types A, B and E botulinal antitoxin	64
11	Concentrates of culture filtrates of Clostridium botulinum strains tested by the template micro immunodiffusion method against types A, B and E botulinal antitoxin	64
12	LD_{50} studies on toxic concentrated culture filtrate of Clostridium botulinum type E strain VH No 1 cultured in untrypsinized TPG	68

TOXIGENIC AND NON-TOXIGENIC STRAINS OF *CLOSTRIDIUM BOTULINUM* TYPE E

A. DIFFERENTIATION BY IMMUNODIFFUSION

B. ATTEMPTS TO INDUCE TOXIGENICITY BY MUTAGENIC AGENTS

INTRODUCTION

Outbreaks of the past few decades have made the public increasingly aware of *Clostridium botulinum* type E. From the microbiologist's viewpoint, this is an interesting organism due to its habitat, the thermolability of its spores, its production of toxin in a "protoxin" form in nature, and its tendency to become non-toxic.

Dolman (1957b), in Canada, has described three colonial types in type E botulinum strains: one, toxic and two, non-toxic. He separates them on their microscopic colonial morphology by reflected and transmitted light. Few people have published verification of this report. In this study, an attempt was made to pick toxic colonies from plates of non-toxic organisms using Dolman's method.

Dolman further states that the different phases of *Cl. botulinum* type E are due to mutation in the culture. If this is the case, it is of interest to determine whether the non-toxic variants will mutate back to the toxic phase. To this end, cultures of non-toxic *Cl. botulinum* type E were exposed to various mutagenic agents during the course of this study.

The time honored and time consuming method of separating the

botulism toxin types has been by immunological testing in mice. However, this is often a laborious task and in some cases laboratory animals are not readily available when needed. This study also includes an attempt to establish a quicker and more convenient method for the identification of type E botulinal toxin utilizing the slide method of agar-gel double diffusion.

HISTORICAL REVIEW

Clostridium botulinum type E was first described by Gunnison and Myer in 1936. The cultures they worked with were obtained from Russia. They found these organisms to be anaerobic, Gram positive, motile rods with oval, subterminal spores. The organisms were saccharolytic but not proteolytic. The spores demonstrated low thermal resistance, and toxin formation was variable with a tendency for cultures to become non-toxic. No protection from the toxin was given by Cl. botulinum type A, B, C or D antitoxin. Thus, a new botulinum type, E, was established.

Since the organism was described 30 years ago, there have been 84 identified outbreaks of type E botulism with 408 cases and 126 deaths. The major outbreaks occurred in Japan, the United States, Canada, Russia, Denmark, Sweden and Norway (Dolman, 1964). The first verified outbreak of type E botulism occurred in 1932 in Cooperstown, New York. The vehicle of toxin transmission was uncooked smoked salmon (Dolman, 1957a). Since then, fish and marine mammals have been implicated in most outbreaks of type E botulism. In 1953, Dolman and Chang isolated Cl. botulinum type E from the intestines of healthy fish. Dolman (1957a) stated that fish and marine mammals were potential sources of type E botulism as their intestines served as a reservoir for the organisms. He noted

that the organism may be present in other foods, but as the spores are thermally unstable, compared to other botulinal types, they are destroyed by less heating.

It was generally assumed that Cl. botulinum type E was found mainly in the waters off the northern land masses. Dolman (1957a) stated that type E spores were not generally found in soil or land areas due to the fact that they are thermally unstable and may be destroyed by temperature fluctuations. Dolman and Iida (1963) stated that type E spores have a terrestrial origin. According to Dolman and Iida, the spores are washed by rain or soil erosion into rivers and carried by currents to the off shore sea bottom. Here the spores germinate, multiply and are picked up by bottom fish in the area. They also add that they found very few spores in areas of the sea without washings from mountain areas. Ward and Carroll (1965) have challenged the "northern hemisphere" theory on distribution of the organism. They isolated it from mud samples at Galvaston Bay, Texas. They believe it is not limited to the northern land masses, as has previously been assumed, but rather has a world wide distribution.

Clostridium botulinum type E has been the major cause of cases of botulism from commercially prepared foods in the United States in the past two decades (Osheroff, Slocum and Decker, 1964). An outbreak occurred in 1956 involving white whale meat in seal oil;

two in 1959 involving salmon-egg cheese and seal or whale flippers in seal oil; one in 1960 involving commercially smoked fish in vacuum bags; one in 1961 involving salmon eggs and one in 1963 involving commercially canned tuna fish (Dack, 1964). Most of the implicated foods were unprocessed, processed at relatively low temperatures or air dried. The tuna fish outbreak involved post heat processing contamination due to improperly sealed cans. In all other cases, the low temperature used for the food preparation were especially suited to the survival of the heat labile type E spores.

The toxins of Cl. botulinum are not exotoxins in the true sense. They are liberated into the medium upon autolysis of the cells after the culture has grown and are not expressed from the intact cells. The formation of the toxins is not well understood, but they may be formed in combination with cell protein. In most cases they are activated by the cells' proteolytic enzymes; however, type E toxin is an exception as it needs to be exposed to an enzyme, trypsin, to become fully activated (Schantz, 1964).

Recently, the chararacteristics of type E toxin have been studied extensively. The molecular weight has been calculated to be 18, 600 (Gerwing et al., 1964). In 1966, Gerwing et al. found that cysteine side chains were involved in the active toxic site of the toxin molecule. They found this for type A and B toxin also and concluded

that the active region of the toxin molecule was not responsible for its immunological properties. Gerwing, Mitchell and Van Alstyne (1967) stated, however, that the cysteine residue is probably not solely responsible for the toxic activity of the protein molecule.

Type E toxin was found to have consistently low toxicity for laboratory animals inoculated intraperitoneally but was highly toxigenic in nature. It was also noted that laboratory cultures contaminated with bacillus species gave higher titers of toxicity. Duff, Wright and Yarinsky (1956) investigated this phenomenon and observed a 12-47 fold increase of intraperitoneal toxicity for mice after exposure of the toxin to trypsin. They postulated that the toxin was elaborated by the cell as an inactive precursor, and as the organism is not proteolytic, an outside enzyme must be used to convert the precursor to an active form. In nature, this phenomenon occurs in the gastrointestinal tract. In 1965, Gerwing, Dolman and Ko, through molecular weight analysis of the active and inactive toxin, showed that the active peptide lost at least 18 amino acid residues present on the inactive protein. They concluded that the tryptic activation involved the removal of amino acids from the toxin molecule.

The action of botulinal toxin has been well documented, although it is not completely understood. The toxin is distributed through the body by the blood stream. It blocks the acetyl-choline release

mechanism at cholinergic nerve synapses. This is an irreversible inactivation. Death is usually due to respiratory paralysis. When recovery occurs, it is slow. The central nervous system functions normally throughout the course of the illness (Brooks, 1964).

Non-toxic variants and colonial types of Clostridium botulinum type E

Early workers with the Clostridium genus noted the difficulties of getting pure cultures. Robertson (1916) noted that certain anaerobes are well adapted to live in close association with one another. She found that these cultures behaved in a symbiotic nature in the same way over a long period of time leading one to believe that the culture was pure. Reddish (1921) found that American strains of Cl. botulinum, often isolated from putrid material, were heavily contaminated with Cl. sporogenes. American strains often lost their toxigenic property. Reddish attributed this to heavy overgrowth with Cl. sporogenes. Gunnison and Meyers, in their original description, noted that type E cultures might change from a toxic to non-toxic form. Recent workers (Dolman, 1957b; Stiebrs, 1967; Craig and Pilcher, 1965) have noted that non-toxic colony isolates can be selected from toxic type E cultures. Several theories have been advanced to explain this phenomenon.

Dolman (1957b) stated that Cl. botulinum type E colonies exist

in three mutational phases: one, TOX, is toxin producing, and the other two, TP and OS, are non-toxin producers. He stated that the organism mutates between these stages, thus causing some cultures to become non-toxic and explaining why some colonies from a toxic culture are non-toxic. He believes this a more sensible solution to the variance in the cultures than the earlier belief that the cultures were all contaminated. Iida (1963) also observed Dolman's colony types. He found that the frequency and stability of the variation differed from strain to strain and noted that in some cases cultures lost toxicity without colonial changes. Lynt et al. (1967) studied some non-toxic type E strains serologically and found that they showed no greater antigenic differences from toxigenic type E strains than the latter strains showed among themselves. This helps support Dolman's argument that the non-toxic strains are variants of the toxic type E cultures.

However, other workers have begun to question Dolman's theories. Hodgkiss, Ordal and Cann (1966) studied the spores of a toxigenic type E strain and an "OS" non-toxic strain morphologically under an electron microscope. They found some differences in the spore types. Hobbs, Roberts and Walker (1965) have also questioned the validity of Dolman's statements. They were not able to isolate OS or TP colonies from toxic Cl. botulinum type E cultures and none of their toxic strains have lost toxicity. They did

biochemical tests on the OS variants supplied by Dolman and found them to differ substantially from type E. They also tried fluorescent antibody techniques and got no reaction by the OS variants against type E antibodies. They concluded that many of the non-toxic isolates labeled type E may actually be commensal clostridia found in marine sources which are difficult to separate from Cl. botulinum type E. Thus it can be seen that the status of non-toxic E like strains is still questionable.

Induced mutation in bacteria

Before the development of pure culture techniques, bacteria were regarded as highly variable entities. Workers following this trend of thought were called pleomorphists. Nageli, in 1877, was the champion of this cause stating that all morphological and physiological qualities of the bacteria may vary to a considerable extent.

In 1881, however, Robert Koch described a method for studying pure cultures of bacteria, i.e., cultures presumably arising from one bacterial cell. He used solid media to cultivate his organisms. He found that the organisms stayed together in discrete colonies. He was able to pick a colony, restreak it to new medium and obtain all of the same type colony as the original in the new streak. This beginning study of pure culture methods led to the belief that bacteria do not vary at all. Thus the monomorphist school of thought was established.

The monomorphic point of view persisted for quite some time. However, it became apparent that even the most scrupulously pure cultures of bacteria were able to change. This led to the investigation of these changes and the recognition of mutation in bacteria. The term mutation referred to sudden, unexplained heritable changes in bacteria which took place in the absence of recombination.

Spontaneous mutation was thought to occur in bacteria at a very low rate. Luria and Delbrück (1943) did some statistical studies on mutation rate. Up to the time of their work, there were two schools of thought on mutation. One school believed that mutation was directed by the environment, and the other believed that mutation occurred as a chance event. With their fluctuation test, Luria and Delbrück proved statistically that mutation occurs as a chance event and calculated the mutation rate to be .32 per 10^8 bacteria per generation.

In 1942, it was discovered that X-rays could induce mutation in Drosophila, a fruit fly. In 1947, Witkin described mutation induced in Escherichia coli by several chemical agents. Since then other agents have been used to induce mutations in bacteria. However, their action in causing mutation was not understood. In 1953, Watson and Crick pointed out implications of their structure of DNA for the nature of mutation. They found DNA to consist of a backbone of alternations of sugar and phosphate groups. To each sugar is

attached a base. The DNA molecule is a paired structure, each strand complementary to the other. The bases are joined by hydrogen bonds and only certain bases will fit the structure; i.e., a purine opposite a pyrimidine. They proposed that the sequence of the bases in the DNA molecule was the code to carry the genetic information. It was then concluded that any permanent alteration in the sequence of base pairs constituted a mutation. Alterations of base pairs can be of several types; base pair substitution, insertion and deletion, and rearrangement (Adelberg, 1966; Freese, 1963). Mutagenic agents are now classified, when possible, according to the above groupings.

5-Bromouracil is a structural analogue of thymine and is a pyrimidine base analogue. Zaminhof and Griboff (1954) showed that 5-bromouracil can be extensively incorporated into the DNA of bacteria replacing thymine residues. Litman and Pardee (1960), working with bacteriophage, found the mutations introduced by 5-bromouracil appear to involve small molecular changes. They theorized that when 5-bromouracil replaces thymine it should pair with adenine, but it may have a small probability of pairing with guanine causing an alteration in the base pairing and hence producing mutations.

2-Aminopurine is also a base analogue. It has been found to cause mutations in Escherichia coli by Zampiere and Greenberg (1966). 2-Aminopurine is incorporated to such a small extent in

the DNA that it has been impossible to determine which base it replaces (Freese, 1963). However, its mutagenic effect has been attributed to base-pairing mistakes as a consequence of its incorporation into DNA, substituting for a purine or pyrimidine.

Witkin (1947) first showed that acriflavin, an acridine dye, caused mutations in bacteria. Brenner et al. (1961) postulated that acridine dyes slide between adjacent base pairs forcing them apart, causing addition or deletion mutations, thus altering the amino acid code so as to yield a grossly altered protein or no protein at all. Lerman (1963) showed that acridine did stack between base pairs in the DNA molecule and could cause insertion or deletion mutants. It should be noted that the work was not done with acriflavin, specifically, and its action could be found to be different than is generally accepted now for the acridines.

The action of azaserine seems to be less well understood. Iyer and Sybalski (1959) found that azaserine induced mutation in E. coli. Handschumacher and Welch (1960) showed that azaserine interferes with the animation reaction in the synthesis of nucleic acid precursors, especially purines. Its primary site of action seems to be enzymes concerned with the donation of the amino acid groups of glutamine to a nucleic acid precursor. However, azaserine has been demonstrated to act in other ways in preventing synthesis of nucleic acid precursors. Freese (1963) noted that azaserine

is also an alkylating agent and may owe its mutagenicity to its alkylating ability rather than its inhibitory action.

The mutational effects of irradiation by gamma rays are not well understood either. Irradiation is thought to act on nucleic acids directly and indirectly. In its direct action, high energy radiation causes the breakage of the base, sugar and phosphate bridge bonds in DNA (Wacker, 1963). This may cause deletions in the bases leading to mutations (Freese, 1963). Zelle (1955) showed that irradiated oxygen is reduced to peroxide and stated that this may have some mutational effects on the bacteria. Wacker (1963) stated that the oxygen effect represents the indirect action of irradiation. Peroxides are formed which act on the components of nucleic acids within the cells. The chemical basis of irradiation effects can thus be seen to be manifold and rather unspecific.

Agar-gel double diffusion applied to bacterial toxin detection

Prior to 1948, immunodiffusion tests had been the simple single diffusion type. However, both Elek and Ouchterlony in 1948 devised the macro method of agar-gel double diffusion. Ouchterlony, working with diphtheria toxin and antiserum, showed that antigen and antibodies diffuse toward each other in a neutral gel and under some circumstances a precipitate formed in the gel at their common

meeting site. He stated that the reaction was of the same nature as an antigen-antibody reaction and the position of the band in relation to the diffusion center depended on the initial concentration and diffusion velocity of the two reacting substances. He also found that if different antigens and antibodies were mixed, multiple reactions appeared, and the systems reacted independently of one another. Elek also found that in a gel, diphtheria toxin and antitoxin diffusing toward each other, produced a line of visible precipitate at their meeting place.

Oakley and Fulthorpe (1953), using the tube double diffusion test, and Björkland and Beringo (1954), using the Ouchterlony plate method, found that the toxin of Clostridium welchii could be detected using the double diffusion technique. They both noted the formation of more than one precipitate line in the antigen-antibody reaction and concluded that the number of lines seen was equal to the minimum number of antigenic components in the system.

In 1958, Crowle developed a micro technique for the double diffusion test utilizing agar on a slide as the base for the diffusion. With this method, smaller amounts of reactants could be used. After the reaction had taken place, the slides could be stained. Thus, the method had the advantage of using smaller equipment, less reactants and providing a permanent record of the reaction.

Casman (1965) utilized this technique in the detection of

staphylococcus enterotoxin in food products. He used a constant-feed type apparatus of a plastix matrix with cone shaped holes similar to Crowle's. With this method, he was able to detect staphylococcus enterotoxin in a concentration of 1 μg/ml.

Double diffusion techniques have recently been applied to the identification of type **E** botulinal toxin. Sugiyama et al. (1967), using a macro method, were able to detect a line of visible precipitate in a reaction of type **E** toxin and antiserum. They noted that the sensitivity of the test decreased when the toxin was trypsinized. Vermilyea, Walker and Ayres (1968) used a micro method to detect type **E** toxin. They compared its sensitivity to the mouse test and found the mouse test more sensitive by a factor of 10^4. However, they noted that increased concentration of the toxin may increase the sensitivity of the procedure.

EXPERIMENTAL MATERIALS AND METHODS

Cultures

Clostridium botulinum

Type E		Type A	Type B
toxic	non-toxic		
Kalamazoo	GB-3	5A	115B
066B-TOX	805Bb	33A	113B
Tuna-can-402(6)	170C		
Seratoga	15ATi		
Isolate 1	8 ATi		
Isolate 2	170IX 12/31		
Isolate 3	900D		
VH No 1	34-1		
VH No 2	S-9		
Beluga	066B-NT		
Detroit	PMI-5		
Iwanai			
Alaska			
070			

Cl. sporogenes
Cl. histolyticum
Cl. tetani
Cl. novyi
Cl. welchii
Cl. septicum
Cl. bifermentans

Stock cultures of toxic and non-toxic *Cl. botulinum* type E were kept in cooked liver medium. Cultures of *Cl. botulinum* types A and B and *Cl. bifermentans* were kept in Robertson's cooked meat medium (Stiebrs, 1967). The other clostridia were obtained from the pathogenic collection of the Oregon State University

Microbiology Department. The stock cultures were kept at room temperature. In all cases, transfers were made to trypticase-peptone-glucose medium which were incubated at 28°C anaerobically and then kept at room temperature without special protection from the air. Routine inoculations were done from the TPG cultures. Anaerobiasis for growth of all cultures was obtained by placing them in Case anaerobic jars and evacuating and flushing the jars with nitrogen three times, leaving the jars filled with nitrogen.

Media

1. Cooked liver medium

	Grams per liter
Beef liver	500
Peptone	10
Dipotassium phosphate	1

Cut beef liver into small chunks and soak in one liter of water overnight at 4°C. Skim off the accumulated fat. Heat in autoclave ten minutes at 121°C. Filter the meat and broth through cheese-cloth. Save the meat. To the broth, add peptone and phosphate and heat to 100°C. Adjust the pH of the broth to 8.4, filter it through paper and add enough water to make the broth up to one liter. To each test tube add a small amount of powdered $CaCO_3$. Then add one-half inch of beef liver chunks and cover with broth to a depth of two inches. Sterilize by autoclaving at 121°C for 15 minutes.

2. Trypticase-peptone-glucose medium (TPG)

A. Broth

	Grams per liter
Trypticase	50
Bacto-peptone	5
Glucose	4
Sodium thioglycollate	2

B. Agar

To broth base add:	
Bacto-agar	15

C. Indicator and agar

To broth base add:	
Noble agar	0.75
Methylene blue	0.002

Heat ingredients in distilled water to dissolve them, adjust the pH to 7.0 and sterilize by autoclaving at 121°C for 15 minutes.

3. Trypticase-peptone-glucose-yeast extract medium (TPGY)

To TPG broth base add:	
Bacto-yeast extract	20 gm/l

4. Brain-Heart Agar (BH)

A. Agar

	Grams per liter
Bacto Brain-Heart Infusion Powder	37
Bacto-agar	15

B. Agar with sodium thioglycollate added

To agar base add:	
Sodium thioglycollate	2

Sterilize medium by autoclaving at 121°C for 15 minutes.

5. Blood Agar (BA)

Bacto-Blood agar base	40 gm
Distilled water	1000 ml

Heat suspension to dissolve ingredients and sterilize by autoclaving at 121°C for 15 minutes. Cool to 45°C. To cooled base add, aseptically, 50 ml of sterile defibrinated sheep red blood cells. Pour plates and store at 4°C.

6. Litmus milk

Powdered milk	100 gm
Tap water	1000 ml
Blend in waring blendor	
pH	6.8
5% litmus solution	15 ml

Sterilize by autoclaving at 121°C for 12 minutes.

7. Carbohydrate medium

	Grams per liter
Trypticase	50
Bacto-peptone	5
Carbohydrate	5

Heat medium in distilled water to dissolve ingredients and adjust the pH to 7.0. Distribute the medium in test tubes and add Durham fermentation vials. Sterilize by autoclaving at 121°C for 15 minutes.

8. Nitrate reduction broth

	Grams per liter
KNO_3	5
Trypticase	10
Yeast extract	1
K_2HPO_4, anhydrous	2

Heat ingredients in tap water to dissolve them, adjust the pH to 7.0 and sterilize by autoclaving at 121°C for 15 minutes.

9. Salt medium

	Grams per liter
Trypticase	50
Bacto-peptone	5
Glucose	4
Sodium thioglycollate	1

Add appropriate percentage of salt and heat ingredients in distilled water to dissolve them. Adjust the pH to 7.0 and sterilize by autoclaving at 121°C for 15 minutes.

10. Trypticase Soy Broth (TSB) - Baltimore Biological Laboratory, Inc.

11. Tryptone broth - Difco Laboratories, Inc.

12. Spirit blue agar - Baltimore Biological Laboratories, Inc.

13. Nutrient gelatin - Difco Laboratories, Inc.

14. Cooked meat medium - Difco Laboratories, Inc.

Materials and Reagents

1. Filters

   A. Swinney

   These filters were assembled and sterilized by exposure to flowing steam for 30 minutes. 0.22 μ or 0.45 μ Millipore membrane filters were used. After use, the filters were decontaminated by autoclaving, washed with detergent and

rinsed well in tap and distilled water.

B. Seitz

Seitz filters were washed with 100 ml of 1% acetic acid, 100 ml of 1% sodium bicarbonate and rinsed with distilled water until the pH of the water coming through was neutral. They were sterilized by autoclaving.

C. Membrane filters

A Millipore filter apparatus was used. The membrane and filter assembly were sterilized separately by autoclaving. 0.22 μ filter membranes were used. They were assembled aseptically prior to use.

2. Toxicity testing and Lethal Dose 50 (LD_{50}) studies

A. Gel-phosphate buffer

Gelatin	0.2%
Dibasic sodium phosphate	0.4%

Adjust pH to 6.2 with HCl and sterilize by autoclaving.

B. Trypsin

Trypsin (1:250) - Difco	10 gm
Distilled water	100 ml

This solution was assumed to approximate a 10% solution. It was sterilized by Seitz filtration. Its sterility was checked before use.

3. Mutagenic chemicals

A. 2-aminopurine from Cyclo Chemical Corporation,

Lot R-4878 Grade II.

B. 5-bromouracil (5-Bromo-2, 4-Dihydroxypyrimidine); from Sigma Chemical Company, Lot 15B-0380.

C. Acriflavin, neutral NF C grade; from California Corporation for Biochemical Research, Lot 02120.

D. Azaserine; from Parke, Davis and Co., Lot X6730.

4. Irradiation studies

A. Cobalt 60 source

B. Glass vials, low potassium, 20 ml capacity, screw capped. Sterilized by exposure to flowing steam for 30 minutes.

5. Biochemical studies (see Manual of Microbiological Methods)

A. Kovac's reagent

B. Sulfanilic acid

C. α-napthylamine reagent

D. Brom thymol blue

6. Immunodiffusion

A. Polyethylene Glycol 4000, J. T. Baker Chemical Co.

B. Dialyzing tubing, cellophane, Van Waters and Rogers, Inc.

C. Scraper and Levelling table

D. Slide tray

E. Humidity chamber

Above three items from Universal Electrophoresis apparatus, Colab Laboratories, Inc.

F. Well pattern

Five wells were traced on cardboard in a cross shape. They were situated, measuring from edge to edge, two millimeters from the central well. The well diameter was 3 mm unless noted otherwise. The average volume held by these wells was 0.002 ml.

G. Templates

The templates were obtained from the Division of Microbiology, U. S. Food and Drug Administration. They consisted of plastic squares containing wells in a cross design and cone shaped with the smaller opening to be placed toward the agar. The distance between the center and peripheral wells was 4.5 mm. The wells held between 0.02 and 0.03 ml.

H. Electrical Insulating tape - Homart Plastic tape, 3/4 inch wide, Sears.

I. Sodium Ethylmercuriothioslaicylate, K & K Laboratories, Inc. One gram was dissolved in 100 ml of distilled water. It was diluted to 1:10,000 for further use.

J. 0.15 M phosphate buffer, pH 7.1

Solution I

Na_2HPO_4, anhydrous	21.7 gm
Distilled water to	1000 ml

Solution II

KH_2PO_4	20.4 gm
Distilled water to	1000 ml

Working solution

Solution I	67 ml
Solution II	33 ml

K. Agar

0.2% Ion agar

Ion agar, Colab	0.2 gm
Distilled water	100 ml

1% Ion agar

Ion agar	4 gm
0.15M phosphate buffer, pH 7.1	100 ml
Distilled water	300 ml
Merthiolate, 1% sol'n	1 ml

Noble agar

Base

Sodium chloride	0.85 gm
Merthiolate, 1% sol'n	1.0 ml
Sodium barbital	0.8 gm
Distilled water	100 ml
pH	7.4

To base add 1.2% Noble agar (Difco) and melt.

All agar was filtered through glass wool before use.

L. Antitoxins

Type A Botulinum Antitoxin, Equine serum, CDC Lot 1

Type B Botulinum Antitoxin, Equine serum, CDC Lot 3

Type E Botulinum Antitoxin, Equine serum, CDC Lot 1

M. Stain

0.01% solution of amido black made in the following solvent:

Absolute methanol	450 ml
Glacial acetic acid	100 ml
Distilled water	450 ml

Methods

Biochemical testing of non-toxic *Clostridium botulinum* type E cultures

Due to the controversy over the nature of "non-toxic" *Cl. botulinum* type E cultures, the strains used in this study were tested for biochemical agreement with *Cl. botulinum* type E. The organisms were tested for action on carbohydrates, hemolysis, lipolysis, indole production, reduction of nitrates, salt tolerance, reaction in litmus milk, gelatin liquefaction, reaction in cooked meat medium and possible aerobic contamination.

The organisms used were first tested for aerobic contaminants in TSB. The cultures were grown for 24 hrs in TPG at 28°C anaerobically. Where necessary, they were allowed to incubate longer to obtain good growth. Two-tenths of a ml of 24 hr culture grown in TPG was inoculated into 10 ml of TSB and incubated four days aerobically at 35°C. Cloudiness of the medium was indicative of aerobic contamination. Any cultures appearing contaminated were inoculated again from a different source and checked for aerobic

contamination before further use.

Two-tenths of a ml of 24 hr culture grown in TPG was inoculated into litmus milk (10 ml per tube) and incubated six days at 28°C anaerobically. A control of uninoculated medium was also incubated. At six days the tubes were read by comparing the inoculated tubes with the uninoculated control.

The organisms were tested for fermentation of maltose, lactose, sucrose, mannitol, salicin and dextrose. One-tenth of a ml of 24 hr TPG culture was inoculated into the various carbohydrate media in tubes with fermentation vials. Controls of the uninoculated media were included. These were all incubated anaerobically for three days at 28°C. Gas production was observed in the Durham vials. The production of acid was observed by adding five drops of brom thymol blue to the media and comparing the inoculated tubes with the uninoculated controls.

Indole production was determined by inoculating 0.1 ml of 24 hr culture prepared in TPG into tryptone broth. These were incubated anaerobically at 28°C for five days or until good growth occurred. Eight drops of Kovac's reagent were added to the tubes at the end of incubation. A red color in the reagent layer indicated indole production.

To determine the action of the organisms on nitrates, 0.1 ml of 24 hr culture was inoculated into nitrate reduction broth. These

cultures were incubated anaerobically at 28° C for one week, re-inoculated at the end of this time due to lack of growth and then tested for the production of nitrites after 48 hrs. Equal amounts of sulfanilic acid and α-napthylamine reagents were added to the tubes which were then allowed to stand approximately 15 min. If the tube was negative at this time, a few granules of granular zinc was added to determine whether the reaction was negative or had gone on to produce ammonia and therefore wasn't recorded by the reagents.

The organisms were tested for their ability to grow in TPG with various salt concentrations ranging from 2% to 5% at 0.5% intervals. The various salt concentrations were inoculated with 0.1 ml of 24 hr culture in TPG. One tube of basal medium, without salt, was also inoculated for each organism. These were incubated for 48 hr at 28° C anaerobically.

Blood agar plates were streaked with 0.1 ml of 24 hr culture in TPG to determine hemolytic action of the organisms. The plates were incubated anaerobically at 28° C for three days and then observed for the presence of hemolysis around the colonies formed.

The organisms were tested for their lipolytic action on Spirit blue agar. One-tenth of a ml of 24 hr TPG culture was spread on the agar and the plates incubated anaerobically at 28° C for five days. The presence of clearing and a deeper blue color under and around

the colonies was considered indicative of lipolysis.

To test their ability to liquefy gelatin, 0.2 ml of 24 hr TPG culture was stab inoculated into nutrient gelatin. These were incubated six weeks at 18°C anaerobically. An uninoculated control was incubated with the cultures. The presence or absence of liquefaction of the medium was observed.

The action of the non-toxic cultures on cooked meat medium was observed by inoculating 0.1 ml of 24 hr TPG culture in tubes of the medium and incubating them at 28°C for five weeks anaerobically. The action of the organisms on the meat particles was recorded.

At the end of the biochemical studies, the organisms were again tested for aerobic contamination by inoculating a drop of TPG culture into 10 ml of TSB and incubating them as previously described.

Antagonistic activity of cultures of non-toxic Cl. botulinum type E on toxin production

Kautter et al. (1966) described a bacteriocin-like substance active against strains of Cl. botulinum type E produced by certain non-toxic variants of the organisms. Because an attempt would be made to detect toxin production from mutants growing together with non-toxic organisms, it was necessary to test the available non-toxic strains for the presence of this substance. The spot test described by the above authors was used. The culture filtrates of vegetative

cells were tested undiluted. Only organisms showing no bacteriocin-like activity were used in the mutagenic tests.

Oxygen toxicity

It was necessary to determine if non-toxic type E cultures could be held in 10 ml of culture medium in the atmosphere for various time intervals without dying. This information would be needed for interpreting results of exposure of the organisms to mutagenic agents. Twenty-four hr cultures were prepared in TPG. The cultures were centrifuged, the supernatant poured off and 10 ml of TPC with methylene blue indicator and agar were added to the pellet. The organisms were resuspended and counts made at intervals using TPG broth as diluting medium and TPG agar as plating medium. The count immediately after the addition of 10 ml of medium was taken to be the zero hour count and counts at other time intervals were compared with this to determine if the organisms were being killed by oxygen toxicity.

Testing for toxin production

Two methods were used in the testing of cultures for toxin production. One involved the use of TPG and the other, TPGY.

The organisms were incubated in TPG for five days anaerobically at 28°C. The cultures were then centrifuged. The supernatant

was divided into two parts and each part adjusted to pH 6.2 using HCl and pH paper. One portion was frozen, to be tested further if toxin was detected. Enough 10% trypsin was added to the other portion to give a 1% trypsin solution. The specimen was held at 37°C for 45 min. It was then filtered through a Swinney filter with a 0.22μ Millipore membrane into a sterile vaccine vial. One-half ml of this solution was injected intraperitoneally into each of two white mice. The mice were observed over a four day period. Death of the mice was taken as an indication of toxin production. A negative control of TPG and trypsin and a positive control of a known toxic strain were always included.

Using the other method, cultures were incubated anaerobically for three days at 28°C in TPGY containing 0.1% trypsin (Harmon and Kautter, 1967). The cultures were centrifuged and part of the supernatant filtered through Swinney filters. One-half ml of the filtrate was injected intraperitoneally into each of two white mice. The mice were observed over a four day period with death indicating toxin production. Again a portion of the supernatant was frozen for future use. A negative control of TPGY with 0.1% trypsin and a positive control of a known toxin producer grown in the same medium were always used. This method had the advantage of being simpler, faster and yielding greater toxin production than the TPG method.

Mutagenic studies, chemical

Non-toxic Cl. botulinum type E cultures were exposed to various chemical mutagenic agents. Twenty-four hour cultures in TPG or TPGY were prepared, centrifuged and the supernatant poured off. The chemical prepared as a sterile solution in TPG or TPGY was added to the organisms in a concentration sufficient to produce a 90% or greater kill in a suitable time interval. Plate counts were performed to determine the percent kill. The organism was spun down again, the mutagen poured off and the pellet was re-cultured and tested for toxicity utilizing the TPG or TPGY method.

Twenty-four hour cultures in TPG were exposed to 2-aminopurine in a concentration of 15 mg/ml. The chemical was dissolved in TPG and sterilized by membrane filtration. The organisms were exposed to 10 ml of the agent for one-half hr. Fresh medium was added to the survivors, and they were then recultured and tested for toxicity using the TPG method.

Azaserine was prepared at a concentration of 1 μg/ml in TPG and sterilized by membrane filtration. A 24 hr culture of the organism was resuspended in 10 ml of this solution. Exposure time was one-half hr. In one case a concentration of 0.25 μg/ml was used and in one case the time interval used was one hour to produce the 90% kill. Surviving organisms were tested for toxicity using the TPG

method.

Acriflavin was prepared at a concentration of 50 μg/ml in TPGY. It was sterilized by autoclaving at 121°C for 15 minutes and kept in the dark. Ten ml of the acriflavin solution was used to resuspend 24 hr cultures grown in TPGY. An exposure time of one-half hr was used for six cultures and one hr was used for two. The TPGY method for toxicity testing was used.

5-Bromouracil at a concentration of 50 μg/ml was dissolved in TPGY and sterilized by membrane filtration. Twenty-four hr cultures of non-toxic Cl. botulinum type E grown in TPGY were exposed to 10 ml of the 5-bromouracil solution for one-half hr. In two cases, this time interval was extended to one and one-half hr. The TPGY method for toxicity testing was used.

To determine the accuracy of the plate counts done above, it was necessary to determine the effect of the residual mutagen present in the plate on the growth of the organisms. Two or three cultures were tested for each mutagenic agent. The cultures and mutagens were prepared as described above. The highest concentration of mutagen utilized in plate counting and a dilution of the organism to give countable numbers were added to a Petri dish together with 15 ml of TPG agar. Counts were also made on control plates of the organism alone. The counts were compared to determine if the mutagen was inhibiting growth of the organism. Where the

mutagen exhibited an inhibitory action, the counts of the first experiment were considered unreliable as a measure of the bactericidal effect.

Non-toxic organism 066B-NT was cultured serially in maximum tolerated concentrations of acriflavin and 5-bromouracil to determine if continued growth in the presence of the mutagen might cause a mutation to the toxic form. 066B-NT was selected as it is a non-toxic variant of a well established toxic culture. The concentration of acriflavin which would just allow growth was determined by inoculating various concentrations of acriflavin in 20 ml of TPGY with a 1% inoculum from a 24 hr culture in TPGY and comparing growth obtained in 24 hrs visually and by plate counts. Utilizing the data obtained, a concentration of 10 μg/ml was selected. The same method was tried to obtain a concentration of 5-bromouracil to use. However, the results were not consistent and concentrations of 50 and 100 μg/ml were arbitrarily used. In both cases, the mutagens were prepared as described above and added to 20 ml of TPGY growth medium to effect the proper concentration. A 1% inoculum from a 24 hr TPGY culture was used to inoculate the first tube of acriflavin. A 0.5% inoculum was used in the 5-bromouracil. Each tube was allowed to grow 24 hrs anaerobically at 28°C, and then a 1% or 0.5% inoculum, as described above, from it was transferred to a new tube of the mutagen. The organism was transferred nine

times in acriflavin and ten times in the 5-bromouracil. Plate counts were done on the growth in each tube. A 1% inoculum from tube nine in the acriflavin series and 0.5% inoculum from the number 10 tube in the 5-bromouracil series were inoculated into TPGY with 0.1% trypsin and, after incubation, the cultures were tested for toxicity using the TPGY method.

Mutagenic studies, irradiation

Four cultures of non-toxic Cl. botulinum type E, 066B-NT, 34-1, S-9 and 805Bb, were exposed to doses of gamma rays from a ^{60}Co source to determine if toxic mutants would be formed. With one organism, cultures were irradiated in series over a period of days to determine if this would enhance the production of toxic mutants.

In order to obtain approximately uniform cultures a density standard was prepared. A 24 hr culture was prepared in TPG and the count determined. The culture was preserved with the addition of 3% formalin. Cultures to be studied were adjusted visually to the opacity of the standard by adding sterile medium or spinning down the cultures, removing some medium and re-suspending the organisms.

In preparing the bacteria for irradiation, 24 hr cultures were grown in TPGY. From these cultures, 12 hr cultures in TPGY were

prepared using a 10% inoculum. These were incubated anaerobically at 28°C. The 12 hr cultures were adjusted to the density standard. One-tenth ml was removed from the cultures and put into 9.9 ml TPG broth blanks and kept in the refrigerator on ice. These provided the zero hour counts. Five ml of each culture was placed into each of duplicate sterile vials and kept on ice for transportation and holding. Vials of 34-1 and 066B-NT were exposed to 125,000 rads and those of 805Bb and S-9 to 250,000 rads to obtain at least 80% kill. Plate counts were done on the zero hour blanks and both vials for each culture. One vial of each was then heat shocked at 60°C for ten minutes and the count done again to determine the number of spores present in the survivors. The remainder of the other vial (approximately 4.8 ml) was added to 15 ml of TPGY and enough trypsin added to make a 0.1% solution. After incubation, these cultures were then tested for toxicity using the TPGY method.

Culture 066B-NT was irradiated a total of three times in a separate experiment to determine if repeated irradiation might produce a toxic mutant. One percent of a 24 hr TPGY culture was inoculated to a fresh tube of TPGY and incubated 12 hrs. The culture was irradiated as described above. One ml of the vial which had not been heat shocked was transferred to 20 ml of TPGY and incubated anaerobically for 48 hrs at 28°C at which time the irradiation procedure was repeated. The culture was irradiated three times in this manner.

After the third irradiation, the total contents of the unheated vial was added to TPGY with 0.1% trypsin, and the resulting culture was tested for toxicity using the TPGY method.

Colonial characteristics

Several strains of toxic and non-toxic Cl. botulinum type E were streaked on various media to determine the feasibility of selecting toxic from non-toxic colonies using Dolman's (1957b) method. Twenty-four hr cultures in TPG were prepared. The cultures were diluted to give approximately 50 colonies on a plate. Thin plates of TPG agar and BH agar with and without sodium thioglycollate were poured, and the surface dried. One-tenth ml of the dilution of culture was placed on the plate and spread with a sterile glass spreader. The plates were incubated anaerobically for 48 hrs at 28°C. They were then removed from the jar, a few at a time, and observed by transmitted and reflected light under a dissecting microscope. Colonies selected were cut from the agar using a spatula sterilized by a flame and alcohol. They were transferred to TPG broth, incubated and tested for toxicity using the TPG method. At this time, they were restreaked on the above agars and after incubation observed microscopically again.

Having performed the above experiments, it was decided to try to utilize the colonial recovery method for selecting a toxic mutant

colony from among survivors of a non-toxic culture exposed to irradiation treatment. The non-toxic strain 066B-NT was exposed to irradiation and counts and heat shocking done as described previously. However, the culture in the unheated vial was held refrigerated. It was diluted over a period of days and spread on plates of BH agar with sodium thioglycollate as described above. Colonies were selected as previously described and tested for toxicity utilizing the TPGY method.

Immunodiffusion

A macro immunodiffusion method was attempted utilizing Elek's (1948) technique to try to detect type E botulinum toxin in vitro. Sterile filter paper strips, saturated with type E antitoxin, were placed in melted TPG Noble agar in small Petri dishes. The plates were dried for one-half hr at 55°C and then two organisms per plate streaked at right angles to the filter paper strips on the agar. The plates were incubated up to six days at 28°C anaerobically. Plates were removed at day four through day six and allowed to sit at room temperature in the atmosphere and observed for precipitate lines at various intervals up to 48 hrs. Three toxic and three non-toxic cultures were tested in this manner.

Two micro immunodiffusion methods for in vitro toxin detection were also attempted. The first method utilized wells punched

mechanically into agar poured over a slide (agar well micro method) and the second method utilized a continuous feed plastic template system described by Crowle (1958) and Casman (1965) to be called the template micro method. Utilizing these methods, several species of clostridia were tested against type A, B and E botulinal antitoxins. These included Cl. botulinum type A, B and E (toxic and non-toxic) and Cl. bifermentans, Cl. sporogenes, Cl. novyi, Cl. welchii, Cl. septicum, Cl. histolyticum and Cl. tetani. Several tests were run. The minimum well size to give a precipitin line was determined for the agar well micro method. In some instances the precipitin lines were stained. The sensitivity of both methods was evaluated by determining the toxin concentration in LD_{50}/ml of the highest toxin dilution that produced a precipitin line.

For both methods the slides used were prepared by boiling one hr in distilled water with a small amount of detergent. They were rinsed individually in distilled water and then boiled one-half hr in distilled water. They were air dried and stored in a dust free box.

Slide preparation for the agar well micro method

Washed slides were precoated by dipping the entire slide into 0.2% Ion agar. They were air dried and stored in a dust free box if not used immediately. The slides were coated by placing the precoated slides on a slide tray. This was placed on a levelling table

(previously levelled) and approximately 50 ml of 1% Ion agar at 50-60°C was poured over the slides. The scraper was passed over the slide tray, scraping excess agar off the slides. The agar was allowed to harden and, if desired, the slides were removed from the tray. Coated slides were stored up to 24 hr in a humidity chamber. Wells were cut in the agar by placing the cardboard well pattern under the coated slides, attaching a metal tube of 3 mm outside diameter to a vacuum pump and applying suction to the agar over the hole pattern.

Slide preparation for the template micro method

The plastic templates were cleaned by washing with moderately strong detergent in hot tap water using a non-abrasive cheesecloth. They were rinsed with tap water, 95% alcohol and dried again before use.

A double layer of plastic electrical insultating tape was placed on both sides of the middle two cm of the pre-cleaned glass slide which had been dipped in 95% alcohol and dried. The slide surface between the tape was coated with 0.2% Ion agar and dried in a dust free area.

A thin film of silicone grease (Dow Corning High Vacuum Grease) was spread on the side of the template with the smaller holes. In the area between the tape, 0.35 ml of the 1.2% Noble

agar (50-60° C) was placed on the slide. The silicone-coated side of the template was immediately placed on the melted agar and the edges of the bordering tape by putting one edge down first and then the other. When the agar solidified, the slides were placed in the humidity chamber.

Cell concentrate preparation

Cl. botulinum type E toxic and non-toxic cultures were cultured in 40 ml of TPGY with 0.1% trypsin at 28° C for three days anaerobically. The other clostridia were cultured in 40 ml of TPGY at 28° C for three days anaerobically. The cultures were centrifuged and the supernatant filtered through a Seitz filter. The filtrate was transferred to a graduated cylinder and the amount noted. Dialyzing tubing was loosely packed with PEG 4000, tied off with string and put in the cylinder with the filtrate. The fluid was dialyzed to effect a ten fold reduction in volume, approximately. The concentrated fluid was placed in a test tube and used immediately.

Addition of reagent to wells

(a) Agar well micro method

The central well was filled with antitoxin. Cell concentrates were added to the peripheral wells. Pasteur pipettes or 2.5 ml

disposable syringes with 5/8 inch 25 gauge needles were used to fill the wells. Opposite wells contained the same cell preparation. Duplicate slides were made. Well diameters of 1 mm, 1.5 mm, 2 mm and 3 mm were tested in an attempt to determine the minimum volume of material needed for a visible test. Slides were placed in a humidity chamber at room temperature and observed at intervals from 18-48 hrs over a strong light source.

(b) Template micro method

The central well was filled with antitoxin. The cell concentrates were added to the peripheral wells. Pasteur pipettes or 2.5 ml disposable syringes with 5/8 inch 25 gauge needles were used to fill the wells to convexity. Bubbles were removed from the wells by inserting small capillary tubing into the wells. Opposite wells contained the same cell concentrate. The slides were incubated three days in a humidity chamber at room temperature. To observe the precipitin lines, the templates were moved to one side and the slides held over a strong light source. The templates were decontaminated by heating to 75 °C for 30 min.

Staining method

Precipitin lines were stained 18 or 24 hr after preparation of the slides. The slides were immersed in a 1% sodium chloride

solution for six hours and then transferred to a fresh bath of the same solution for 16 hr. They were placed in a distilled water bath for one hr and then dried at 25 °C for at least 12 hr. The slides were stained 40 min in 0.01% amido black solution and rinsed in four changes (10 min each) of the methanol-acetic acid solvent (see page 25). They were drained and air dried.

LD_{50} determination

A toxic culture was grown for five days at 28 °C in TPG. It was spun down and the supernatant filtered and dialyzed as described above to give a seven fold concentration. The concentrate was divided into portions in sterile vials and held frozen at -10 °C. Dilutions of 10^{-1} through 10^{-4} were prepared in gel-phosphate buffer and 0.5 ml of each dilution inoculated intraperitoneally into each of ten white mice in the 25-32 gm weight range. A control set of ten mice injected with gel-phosphate buffer was also included. Deaths over a period of four days were recorded and the LD_{50} calculated according to the Reed and Muench (1938) method.

The concentrate, undiluted and in dilutions of 1:2, 1:4, 1:6 and 1:8 in distilled water, was tested using both micro immunodiffusion methods. Each method was done twice. By correlating the highest dilution at which a precipitin line appeared with the toxin concentration in LD_{50}'s, it was possible to determine the sensitivity of the immunodiffusion tests.

RESULTS AND DISCUSSION

Identification of non-toxic strains of Cl. botulinum type E

There has been a controversy over the nature of the non-toxic Cl. botulinum type E organisms. Some, as Dolman (1957b), state that the non-toxic isolates are mutational phases of the toxic organism. Others, as Hobbs et al. (1965), believe that many of these strains are other clostridia living commensally with the toxic organisms. Due to this controversy, the non-toxic cultures used in the mutagenic studies were tested to determine if they agreed with the description of Cl. botulinum type E in Bergey's Manual of Determinative Bacteriology (1957). In addition, the results were compared with those published by Kautter et al. (1966) and the strain GB-3 used in Kautter's study was run as a control.

The results of these tests are shown in Table 1. They agreed very well among themselves and with both Bergey's and Kautter's descriptions. There was a slight variation from the latter in carbohydrate action in that strains 805Bb and S-9 did not act on sucrose. The litmus milk was reduced, but after exposure to air, color was restored. The uninoculated control remained colored. Thus, the reduction of the litmus was probably due to the growth of the organism lowering the oxidation-reduction potential of the medium. The

Table 1. Biochemical properties of Clostridium botulinum type E non-toxic strains

Property	Reaction of strain									
	GB-3	15ATi	170C	066B-NT	8ATi	900D	170IX	805Bb	S-9	34-1
Fermentation of										
Glucose	AG[a]	AG	AG	AG	AG	AG	AG	AG	AG	AG
Maltose	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG
Lactose	NC[b]	NC	NC	NC	NC	NC	NC	NC	NC	NC
Sucrose	AG	AG	AG	AG	AG	AG	AG	NC	NC	AG
Mannitol	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Salicin	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Hemolysis	β[c]	β	β	β	β	β	β	β	β	β
Lipolysis	+	+	+	±[d]	+	+	+	+	±	+
Action on meat particles										
Digestion	-	-	-	-	-	-	-	-	-	-
Blackening	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	+	±[e]	-	-	±	±	+	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	-
Salt tolerance[f]	2.5	2.5	2.5	0	2.0	2.5	2.5	2.5	0	2.5
Litmus milk	red[g]	red	red	red	red	red	red	red	red	red
Aerobic growth	-	-	-	-	-	-	-	-	-	-

[a] acid and gas
[b] no change in medium
[c] Beta hemolysis
[d] some colonies lipolytic, some not
[e] slight gelatin liquefaction
[f] highest % NaCl at which organism would grow
[g] reduction of litmus

property showing the greatest variation among these strains and those described by Kautter was salt tolerance. Our strains varied in their tolerance from 0-2.5%. Kautter reported all strains tolerating up to 3.5%. However, a difference in basal medium could account for this discrepancy. The above mentioned variations seem to be very slight and the strains used fit the description of Cl. botulinum type E except for their failure to produce toxin.

The results of the above testing and also the serologic work done on some of the strains used, in another laboratory (Lynt et al., 1967), seem to indicate that these strains are closely related to toxic type E cultures. The strains differed substantially from other clostridia with which they might be confused, as Cl. sporogenes, Cl. parabotulinum and Cl. multifermentans, in that they showed no action on meat particles or milk. Non-toxic strains S-9 and 066B-NT have been compared to the toxic type E's serologically through agglutination by the above authors. 066B-NT appears to be identical to its toxic counterpart. Strain S-9, while lacking some antigens common to toxic type E, is similar in other respects and differs no more from the toxic strains than they differ from each other. Thus it seems that the strains used are probably, indeed, non-toxic Cl. botulinum type E cultures.

Mutagenic studies

Before mutagenic studies were attempted, the organisms to be used were all tested for bacteriocin-like activity. Kautter et al. (1966) reported a bacteriocin-like substance, active against toxic strains of Cl. botulinum type E, produced by certain non-toxic E-like organisms. Since an attempt would be made to detect toxin production from mutants growing together with non-toxic organisms, it was necessary to eliminate all non-toxic cultures producing this bacteriocin from the mutagenic studies. Seventeen strains were tested. Ten were found to produce no bacteriocin-like substance. One of the ten, 15CbTi, was eliminated as it colonially and biochemically did not resemble type E. Thus, nine strains, 15ATi, 170C, 066B-NT, 8ATi, 900D, 170IX, 805Bb, S-9 and 34-1, were found to be suitable for use in the mutagenic studies. The sources of these strains are given in Table 2.

Since Cl. botulinum type E is a strict anaerobe, it was necessary to determine if the cultures could be held in 10 ml of medium in the air for varying time lengths, as would be done in the mutagenic tests, without being killed by this exposure to air. This information would be necessary in evaluating the killing effect of the mutagens on the organisms. Samples of 24 hour cultures of strains 34-1 and PMI-5 in TPG broth were taken at half-hour intervals through 1 $\frac{1}{2}$

Table 2. Source of non-toxic strains of Clostridium botulinum type E used in mutagenic studies

Strain	Source
066B-NT	Toxic strain 066B-TOX, U.S. Food and Drug Administration
S-9	Viscera of Lake Erie smelt, U.S. Food and Drug Administration
170IX	Cockle clam, Yachats, Ore., Oregon State University, Dept. of Microbiology
170C	Cockle clam, Yachats, Ore., Oregon State University, Dept. of Microbiology
900D	Alsea River tidewater, Ore., Oregon State University, Dept. of Microbiology
8ATi	Eastern brook trout, Elk Lake, Ore., Oregon State University, Dept. of Microbiology
15ATi	Kokanee salmon, Elk Lake, Ore., Oregon State University, Dept. of Microbiology
805Bb	Haystack reservoir sediment, Ore., Oregon State University, Dept. of Microbiology

hours. Plate counts were made in TPG agar. The counts obtained for each organism were in close agreement with each other, leading to the belief that the organisms would not be seriously affected by this degree of exposure to oxygen. At this point, it was noted that the dilution tubes had to be shaken quite hard before each sample was taken causing oxygen to permeate the tubes and that the amount of agar in the diluting medium made it difficult to properly mix the pour plates. Thus, it was decided to leave both agar and indicator out of the diluting medium. Much later in the course of the experiments, however, it was noted that the cultures seemed to vary among themselves in viable counts suggesting variable degrees of oxygen toxicity. Therefore, the counts reported below cannot be considered to be, in all cases, due to the bactericidal effect of the mutagen alone.

The strains mentioned above were exposed to 5-bromouracil, acriflavin, azaserine and 2-aminopurine and cultures of the survivors were then tested for the development of toxin. The results are shown in Tables 3-6. As can be seen, the organisms varied in their sensitivity to the mutagens, but for the most part 90% or greater kill was effected. Exceptions are noted in that 170C seemed resistant to the concentration of 5-bromouracil and 2-aminopurine used; 170IX had to be exposed to 5-bromouracil for an extended length of time to effect 90% kill; 805Bb and 34-1 had to be exposed to acriflavin and azaserine for extended time periods to effect the 90% kill; the

Table 3. Effect of 5-bromouracil on non-toxic Clostridium botulinum type E cultures

Strain	Exposure time (hrs)	Concentration (μg/ml)	Pre-exposure count (org/ml)	Post-exposure count (org/ml)	Toxin production in cultures of survivors after exposure
15ATi	0.5	50	4.8×10^6	1.8×10^5	no toxicity[a, b]
066B-NT	0.5	50	4.8×10^7	4.6×10^5	" "
8ATi	0.5	50	2.1×10^7	2.7×10^5	" "
900D	0.5	50	3.1×10^7	2.4×10^5	" "
805Bb	0.5	50	2.1×10^7	1.0×10^6	" "
S-9	0.5	50	3.7×10^7	7.0×10^5	" "
34-1	0.5	50	3.3×10^7	1.1×10^7	" "
170IX	1.5	50	$< 10^4$	2×10^3	" "
170C	1.5	50	2×10^4	9.3×10^4	" "

[a]Two mice injected intraperitoneally with 0.5 ml of culture filtrate remained healthy during four day observation period

[b]Toxic strain VH No 1, not exposed, tested for toxicity: two mice injected intraperitoneally with 0.5 ml of culture filtrate died in 24 hrs

Table 4. Effect of acriflavin on non-toxic Clostridium botulinum type E cultures

Strain	Exposure time (hrs)	Concentration (μg/ml)	Pre-exposure count (org/ml)	Post-exposure count (org/ml)	Toxin production in cultures of survivors after exposure
8ATi	0.5	50	62×10^6	2.3×10^5	no toxicity[a, b]
900D	0.5	50	36×10^6	2.2×10^5	" "
S-9	0.5	50	14×10^6	2.4×10^4	" "
170IX	0.5	50	28×10^6	4.1×10^5	" "
170C	0.5	50	11×10^6	2.2×10^5	" "
066B-NT	0.5	50	24×10^7	9.1×10^4	" "
805Bb	1.0	50	19×10^6	1.1×10^5	" "
34-1	1.0	50	47×10^6	1.1×10^6	" "

[a]Two mice injected intraperitoneally with 0.5 ml of culture filtrate remained healthy during four day observation period

[b]Toxic strain VH No 1, not exposed, tested for toxicity; two mice injected intraperitoneally with 0.5 ml of culture filtrate died in 24 hrs

Table 5. Effect of azaserine on non-toxic Clostridium botulinum type E cultures

Strain	Exposure time (hrs)	Concentration (μg/ml)	Pre-exposure count (org/ml)	Post-exposure count (org/ml)	Toxin production in cultures of survivors after exposure
8ATi	0.5	1.0	7.5×10^6	2.0×10^4	no toxicity[a, b]
900D	0.5	1.0	9.0×10^6	2.9×10^4	" "
S-9	0.5	1.0	1.6×10^6	5.0×10^4	" "
170IX	0.5	1.0	3.1×10^5	1.5×10^2	" "
170C	0.5	1.0	2.7×10^5	1×10^3	" "
066B-NT	1.0	1.0	4.7×10^7	6.1×10^4	" "
805Bb	0.5	1.0	3.5×10^6	4.6×10^5	" "
34-1	0.5	1.0	2.5×10^6	2.5×10^2	" "
PMI-5	0.5	0.25	1.1×10^5	1.8×10^2	" "

[a]Two mice injected intraperitoneally with 0.5 ml of culture filtrate remained healthy during four day observation period

[b]Toxic strain VH No 1, not exposed, tested for toxicity: two mice injected intraperitoneally with 0.5 ml of culture filtrate died in 24 hrs

Table 6. Effect of 2-aminopurine on non-toxic Clostridium botulinum type E cultures

Strain	Exposure time (hrs)	Concentration (mg/ml)	Pre-exposure count (org/ml)	Post-exposure count[a] (org/ml)	Toxin production in culture of survivors after exposure
15ATi	0.5	15	1.1×10^6	3.2×10^4	no toxicity[b, c]
8ATi	0.5	15	29.7×10^6	5.2×10^3	" "
900D	0.5	15	5.3×10^6	5×10^4	" "
805Bb	0.5	15	4.2×10^6	1.8×10^5	" "
S-9	0.5	15	2.6×10^6	4.6×10^5	" "
34-1	0.5	15	$> 4 \times 10^6$	1.3×10^3	" "
170IX	0.5	15	1.7×10^6	$< 10^3$	" "
170C	0.5	15	23×10^6	25×10^2	" "
PMI-5	0.5	15	1.6×10^5	4×10^4	" "
GB-3	0.5	15	6×10^5	2×10^4	" "

[a]Post-exposure count shown with figures corrected for residual effect of mutagen except for strains S-9 and 34-1

[b]Two mice injected intraperitoneally with 0.5 ml of culture filtrate remained healthy during four day observation period

[c]Toxic strain VH No 1, not exposed, tested for toxicity; two mice injected intraperitoneally with 0.5 ml of culture filtrate died in 24 hrs

concentration of 2-aminopurine used produced only a 80% kill of S-9; and the concentration of 5-bromouracil used only produced a 70% kill of 34-1.

Each mutagenic chemical was checked for its residual effects on the plate counts done above. The highest concentration of 2-aminopurine present in the plating medium was 1 μg/ml of the TPG agar. It was found in two out of three cultures that the residual mutagen reduced the count by half. Thus, the true counts after exposure were presumably about twice the values obtained from the plates; however, this correction did not cause any count to increase over the 10% survival figure. Table 6 for 2-aminopurine is shown with the corrected figures for all organisms except strains S-9 and 34-1 as a lower concentration of mutagen was present in the plates of these organisms. The highest concentration of azaserine utilized in plate counting was 0.01 μg/ml of agar. Three cultures were tested, and there was no inhibition evident from the residual mutagen. The highest residual concentration of acriflavin and 5-bromouracil in the plates counted was 0.33 μg/ml of agar. In the cultures tested the concentration of both mutagens gave no appreciable inhibition.

Non-toxic organism 066B-NT was cultured serially in the presence of acriflavin and of 5-bromouracil and then tested for toxin production. It was transferred nine times in TPGY broth containing a 10 μg/ml concentration of acriflavin and ten times in both 50 and

100 μg/ml concentrations of 5-bromouracil in TPGY broth. Counts done on each tube in the acriflavin experiment showed that the organism grew each time to approximately the same concentration. Counts done in both 5-bromouracil experiments, however, showed some variation in growth from day to day. No toxin production was detected in cultures inoculated from the last culture in each of the above series.

Four non-toxic strains, 34-1, S-9, 805Bb and 066B-NT, were exposed to gamma rays from a ^{60}Co source in an attempt to induce toxic mutations. Duplicate samples were exposed, and one of these was heat shocked at 60° C for 10 min and then counted to determine the approximate number of spores among the surviving population. It was desirable to have a low spore population as spores were considered to be fairly resistant to the effect of the irradiation and less likely to give rise to mutants. The results are seen in Table 7. In all cases, except with strain S-9, 90% or greater kill was effected. Exposure of S-9 to 250,000 rads produced 70% kill. In all cases, the majority of survivors were vegetative cells.

Strain 066B-NT was exposed to 125,000 rads, and the survivors were transferred to fresh TPGY medium. The resulting culture was again irradiated with 125,000 rads. This process was repeated for a total of three irradiations. Counts indicated that each time a 90% kill was effected, and the spore population among the survivors was low. Toxin production was not detected in a culture of survivors of

Table 7. Effect of irradiation on four non-toxic Clostridium botulinum type E cultures

Strain	Exposed to (rads)	Pre-exposure count (org/ml)	Post-exposure count (org/ml)	Post-exposure + heat shocking count[a] (org/ml)	Toxin production in cultures of survivors after exposure
066B-NT	125,000	6.9×10^7	4.8×10^6	7×10^3	no toxicity[b,c]
34-1	125,000	2.1×10^7	5.8×10^5	6.4×10^4	" "
S-9	250,000	1.0×10^5	3.5×10^4	3.4×10^3	" "
805Bb	250,000	3.8×10^7	3.6×10^6	7×10^3	" "

[a]Survivors heated at 60° C for 10 min

[b]Two mice injected intraperitoneally with 0.5 ml of culture filtrate remained healthy during four day observation period

[c]Toxic strain VH No 1, not exposed, tested for toxicity: two mice injected intraperitoneally with 0.5 ml of culture filtrate died in 24 hrs

the third irradiation.

As can be seen, no toxin production was detected in cultures of survivors after exposure of the non-toxic strains to the various mutagenic agents indicating that no detectable toxigenic mutation had taken place. This may be due to one or more of several reasons. First, the method of detecting a mutant toxin producer among a population of non-toxic organisms may not have been sensitive enough. Next, it is possible that the mutagens tried could not revert the non-toxic form back to a toxic form or that the mutation of a toxic to a non-toxic form is not revertible. Finally, it is possible that the change from a toxic to a non-toxic form is not a mutation and therefore not revertible by mutagenic agents.

In devising the mutation experiments, it was apparent that a satisfactory method of detecting the toxic mutants among a population of predominantly non-toxic organisms was badly needed. However, there is no good selective procedure available that favors the growth of toxigenic organisms in a mixed population. Dolman's (1957b) method for detecting colonies of toxin producing organisms, described later, was tried without success. Thus, it was decided to culture the entire population of survivors after exposure to the mutagenic agents. It was hoped that if mutation to a toxic form occurred, there would be a high enough mutation rate to cause these organisms to grow and produce enough toxin to be detected. Although it was

realized that this method was probably not very sensitive, it seemed to be the best, if not the only, method possible and practical.

Although the mutagenic agents used are thought to produce mutations by several different mechanisms, it is possible that none of these could induce a toxic form from non-toxic cultures. 2-Aminopurine and 5-bromouracil produce substitution type mutations. They cause base pairing errors during replication, chiefly adenine-thymine →guanine-cytosine errors. In rare cases, they may cause guanine-cytosine →adenine-thymine errors (Freese, 1963; Adelberg, 1966). Acriflavin, an acridine dye, is thought to cause mutation by insertion or deletion of DNA base pairs (Freese, 1963). Azaserine may owe its mutagenic ability to its alkylating action or its inhibition of purine synthesis possibly causing deletions, pairing mistakes or the incorporation of other natural base analogs (Freese, 1963). Gamma rays may cause mutations by splitting of the DNA base pairs causing deletions or by other indirect effects along the particle track (Wacker, 1963; Freese, 1963). It is possible that none of these mutagens are of the type to cause a reversion of non-toxic forms to toxic ones. It is also possible that the mutation causing a toxin producer to become non-toxic involves a long deletion in the chromosome and this is not revertible by any known method.

The above discussion presupposes that it is indeed a mutation that causes a toxic organism to become non-toxic. However, the

origin of and physiology of botulinum toxin production is not well understood. It is possible that mutation is not involved. The expected spontaneous mutation rate for bacteria, as established by Luria and Delbrück (1943), is three in 10^8 cells per generation. However, Craig and Pilcher (1965) found that in freshly isolated cultures of toxic type E organisms, only 21.4% of the colonies were toxigenic. In older, laboratory strains, 82.8% of the colonies were toxic. In either case, there is a much greater change from toxic to non-toxic forms than could be expected from established mutation rates of bacteria. And, although it does seem well established that at least some of the non-toxic organisms are closely related to the toxic type E's, it must be remembered that there is still controversy over whether or not the non-toxic organisms may be contaminants and never were or could be converted to toxic Cl. botulinum type E.

Colonial characteristics

Dolman (1957b) stated that Cl. botulinum type E toxic cultures consisted of three mutational phases identifiable by their microscopic appearance by reflected and transmitted light. In an effort to establish a good method of picking toxic mutants from among non-toxic organisms this colonial method was attempted. Several non-toxic and toxic strains of Cl. botulinum type E were grown on various media in an attempt to determine which media gave the best growth. Then an irradiated culture of non-toxic 066B-NT was cultured and

colonies picked in an attempt to isolate a toxic mutant.

Toxic strains Alaska and VH No 1 and non-toxic strains 805Bb and 34-1 were each spread on plates of TPG agar and on brain heart agar with and without sodium thioglycollate and cultured in an attempt to determine which media supported growth best. It was found that the cultures seemed to grow better on the media containing sodium thioglycollate. In an attempt to establish a relation between colony type and toxigenesis, these strains were plated out on the above three agars and then selected colonies examined for toxin production. Dolman described three colonial types. One, TOX, was toxic. Microscopically by transmitted light the colony had a mosaic pattern with alternating light and dark areas. Another, OS, was non-toxic. It appeared as an opaque colony on the plates and by transmitted light was coarsely granular with patches of brown opacity. The third type, TP, was non-toxic and appeared as a transparent smooth colony. By transmitted light it appeared homogeneous and minutely granular. The toxic and non-toxic cultures both contained colonies with and without the mosaic pattern. Colonies picked from the known toxic cultures proved to be toxic and those from the known non-toxic cultures were non-toxic regardless of their colonial type. In addition, when the colonies were recultured in broth and then restreaked on the agar, they all showed a mixed population again.

Although these attempts to differentiate toxic colonies from

non-toxic ones seems unsuccessful, it was decided to try to use this method on strain 066B-NT after it had been irradiated at 125,000 rads producing over a 90% kill. Brain heart agar with sodium thioglycollate was used for plating since Dolman had used BH agar and good growth was obtained with this medium. A total of 9,900 colonies were examined with a dissecting microscope by transmitted light. Seven of these were picked, transferred to TPGY and the resulting cultures tested for toxicity. The results can be seen in Table 8. The colonies chosen seemed to exhibit the mosaic pattern described by Dolman for the TOX colony, but none proved to be toxic.

Dolman reported his work in 1957. He theorized that the different colonial types represented mutational changes in the organisms. He found that the colonies could mutate from one type to another in the manner TOX $\longleftrightarrow$ OS $\longleftrightarrow$ TP. He also mentioned that all toxigenic colonies were mosaic, but that not all mosaic colonies were toxigenic. Iida (1963) supported Dolman's theories. However, Iida, working with Japanese strains, found that the frequency and stability of the colonial variation varied from strain to strain. He also found that some cultures could lose their toxicity without changes in their colonial forms.

From the studies reported here, it seems that the method of picking toxic colonies by appearance does not work as well in practice as it sounds in theory. This could be due to the technique

Table 8. Characteristics of colonies of non-toxic Clostridium botulinum type E culture 066B-NT after exposure to irradiation[a]

Colony appearance		Toxicity of colony	Prevalence on plates[b]
Macroscopic	Microscopic by transmitted light		
Medium size, opaque	Bright, coarse mosaic	non-toxic	4+
Small size, gray transparent	Bright, coarse mosaic	" "	3+
Large, lobate	Bright, coarse mosaic	" "	+
Medium size, irregular edge, opaque	Bright, coarse mosaic	" "	2+
Medium size, yellow opaque, elliptical, irregular edge	Bright, coarse mosaic	" "	2+
Medium size, gray, irregular edge	Bright, coarse mosaic	" "	2+
Gray edge with mucoid, yellow center	Mosaic periphery with no mosaic in center	" "	4+ (after 5 days)

[a]Culture exposed to 125,000 rads to effect at least 90% kill

[b]4+, most prevalent colony on plates
3+, next most prevalent colony on plates
2+, few colonies of this type on plates
+, rare colony of this type on plates

employed. However, other laboratories have also found this method unsuccessful. It may be that as the cultures are kept in the laboratory, their colonial nature changes. As Iida pointed out, cultures can lose their toxicity without colonial changes. The possibility must still be considered that some of the non-toxic colonies are contaminants, and the mutation theory would not apply in all cases. From the above considerations, the method does not seem to be very sensitive or practical for laboratory application.

Immunodiffusion tests

In an attempt to find an *in vitro* method of toxin detection, one macro and two micro immunodiffusion methods were attempted. The macro method was based on the test described by Elek (1948). One micro test utilized a thin layer of agar on a microscope slide with wells to hold the reactants punched into the agar mechanically; this method will be referred to as the agar well micro method. The other micro method was similar to the system described by Crowle (1958) and modified by Casman (1965). It will be referred to as the template micro method. The sensitivity of both methods was determined.

In the macro immunodiffusion test, three toxic and three non-toxic strains of *Cl. botulinum* type E were tested. Toxic strains VH No 1, VH No 2 and Isolate 2 and non-toxic strains 066B-NT, 34-1 and PMI-5 were streaked on plates of TPG Noble agar at right angles

to filter paper strips impregnated with type E botulinum antitoxin and embedded in the agar. These were incubated anaerobically at 28°C and observed over a period of four through six days. No visible reaction occurred on the plates. One reason for the failure of this method was thought to be the the fact that the organisms grew rather poorly when only inoculated in a small streak and not enough growth occurred to produce detectable toxin. It is also probable that even with good growth, the concentration of toxin would have been inadequate to give a precipitate.

Utilizing the agar well micro immunodiffusion method, concentrates of culture filtrates of 14 toxic and nine non-toxic Cl. botulinum type E strains were tested against type A, B and E botulinum antitoxins. The organisms were cultured anaerobically in TPGY with 0.1% trypsin three days at 28°C, filtered, and the filtrate was dialyzed against polyethylene glycol 4000 (PEG) to effect at least a ten fold concentration. The results are seen in Table 9. All the toxic strains gave a single line precipitin reaction with type E antitoxin (see Figure 1). They did not react visibly with type B antitoxin. None of the non-toxic strains reacted visibly with type B or E antitoxin. Both toxic and non-toxic strains gave a faint, fading reaction with type A antitoxin which disappeared after 48 hours. In addition, this reaction could not be stained with amido black. Concentrates of two toxic and two non-toxic strains were tested in different

Table 9. Concentrates of culture filtrates of toxic and non-toxic Clostridium botulinum type E tested by the agar well micro method against types A, B and E botulinal antitoxin

Strain	precipitin reaction with antitoxin type A	B	E
toxic			
Kalamazoo	1+[a]	-[b]	3+[c]
066B-TOX	1+	-	3+
Tuna-can 402-(6)	1+	-	3+
070	1+	-	3+
Seratoga	1+	-	3+
Isolate 1	1+	-	3+
Isolate 2	1+	-	3+
Isolate 3	1+	-	3+
VH No 1	1+	-	3+
VH No 2	1+	-	3+
Beluga	1+	-	3+
Detroit	1+	-	3+
Alaska	1+	-	3+
Iwanai	1+	-	3+
non-toxic			
170C	1+	-	-
S-9	1+	-	-
170IX	1+	-	-
15ATi	1+	-	-
8ATi	1+	-	-
34-1	1+	-	-
066B-NT	1+	-	-
805Bb	1+	-	-
900D	1+	-	-

[a] 1+, faint fading reaction

[b] -, no reaction

[c] 3+, sharp, strong single precipitin line

well sizes to determine the minimum volume of reactants that could be used. Wells with diameters of 1.0, 1.5, 2.0 and 3.0 mm were used. Only the 3.0 mm well size enabled one to see a visible precipitate. This well held a volume of 0.002 ml, approximately.

Two toxic strains of Cl. botulinum type A and type B and seven other species of clostridia were tested against types A, B and E antitoxin. The results are seen in Table 10. Several of these reactions were stained as described in the Methods section. These may be seen in Figures 2-4. In some cases, the number of lines seen in the unstained slides differed from those seen in the stained figures shown. This may be explained by the fact that lines not readily visible may stain and thus be seen. Also, what looked like a large, diffuse line unstained, may appear as two lines in a stained preparation. The reaction of type A antitoxin with type A toxin was seen after staining; it may be the fading line reported above as the slides were stained at 24 hours and the reaction never faded until 48 hours.

Four organisms were tested using the template micro immunodiffusion method. The results can be seen in Table 11. Again there were cross reactions seen with type A and B toxin and type B antitoxin, but none were seen with type E toxin or antitoxin.

The sensitivity of both micro immunodiffusion tests was measured by comparing the highest dilution of a concentrated culture filtrate at which a precipitin line appeared with the toxin concentration

Table 10. Concentrates of culture filtrates of clostridial species tested by the agar well micro immunodiffusion method against types A, B and E botulinal antitoxin

Organism	Number of precipitin lines/antitoxin type A	B	E
Cl. botulinum type A, 5A	1-[a]	1	0
Cl. botulinum type A, 33A	1-	2	0
Cl. sporogenes	0	1	0
Cl. histolyticum	0	0	0
Cl. tetani	0	1	0
Cl. novyi	1	1	0
Cl. bifermentans	0	0	0
Cl. welchii	0	2	0
Cl. septicum	1-	0	0
Cl. botulinum type B, 113B	1-	2	0
Cl. botulinum type B, 115B	1-	2	0

[a] 1-, faint, fading reaction

Table 11. Concentrates of culture filtrates of Clostridium botulinum strains tested by the template micro immunodiffusion method against types A, B and E botulinal antitoxin

Organism	Number of precipitin lines/antitoxin type A	B	E
Cl. botulinum type A, 5A	1	4	0
Cl. botulinum type B, 113B	0	3	0
Cl. botulinum type E			
non-toxic, 066B-NT	0	0	0
toxic, Beluga	0	0	1

Figure 1. Precipitin reaction between type E botulinal antitoxin and Clostridium botulinum type E by the agar well micro method. Reaction stained with amido black 18 hr after slide preparation. Central well contained antitoxin. Wells on the vertical axis contained concentrated culture filtrate of toxic strain Isolate 3. Wells on the horizontal axis contained concentrated culture filtrate of non-toxic strain 15 ATi. (4X)

Figure 2. Precipitin reaction between type A botulinal antitoxin and Clostridium botulinum type A and Clostridium novyi by the agar well micro method. Reaction stained with amido black 24 hr after slide preparation. Central well contained antitoxin. Wells on the vertical axis contained concentrated culture filtrate of type A strain 33A. Wells on the horizontal axis contained concentrated culture filtrate of Cl. novyi. (4X)

Figure 3. Precipitin reaction between type B botulinal antitoxin and Clostridium botulinum type A and Clostridium novyi by the agar well micro method. Reaction stained with amido black 24 hr after slide preparation. Central well contained antitoxin. Wells on the vertical axis contained concentrated culture filtrate of type A strain 33A. Wells on the horizontal axis contained concentrated culture filtrate of Cl. novyi. (4X)

Figure 4. Precipitin reaction between type B botulinal antitoxin and Clostridium botulinum type B and Clostridium welchii by the agar well micro method. Reaction stained with amido black 24 hr after slide preparation. Central well contained antitoxin. Wells on the vertical axis contained concentrated culture filtrate of type B strain 115B. Wells on the horizontal axis contained concentrated culture filtrate of Cl. welchii. (4X)

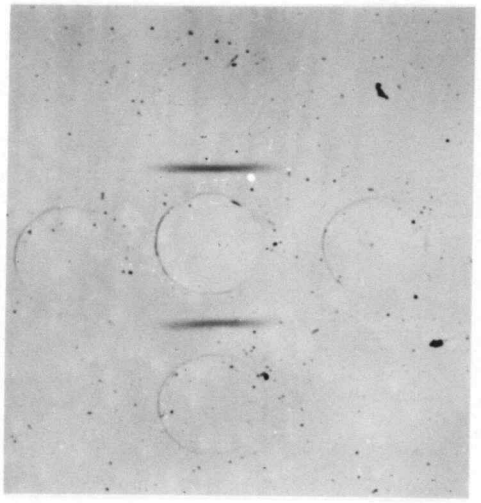

Figure 1.

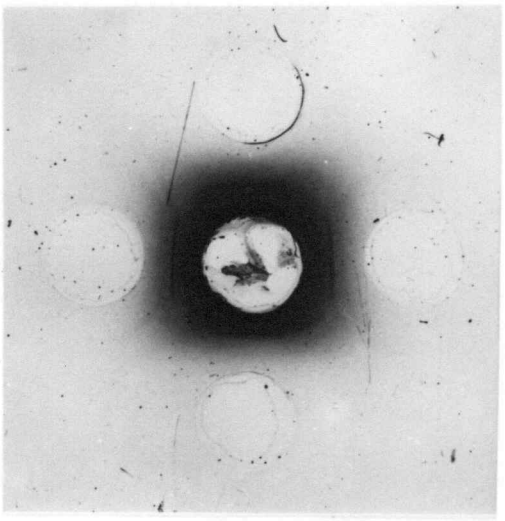

Figure 2.

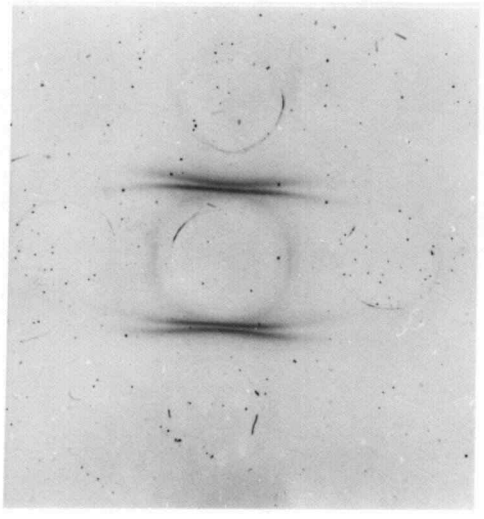

Figure 3.

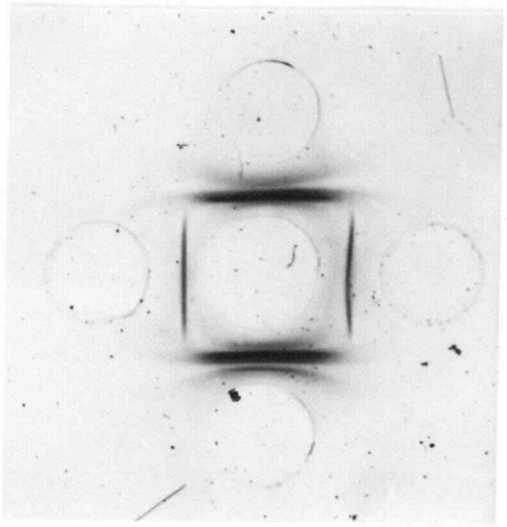

Figure 4.

in LD_{50}'s per ml. Cl. botulinum type E toxic strain VH No 1 was cultured five days in untrypsinized TPG. This medium was used as other workers (Sugiyama et al., 1967; Bulatova, 1965) found that although trypsinization increases the potency of type E botulinal toxin, it does not increase its antigenic activity in the precipitin reaction. The culture was filtered, and the filtrate was concentrated seven fold by dialysis against PEG. A series of ten fold dilutions of the toxic concentrate was made in gel-phosphate buffer and 0.5 ml was injected intraperitoneally into each of ten mice per dilution. The LD_{50} was calculated according to Reed and Muench (1938). The results are seen in Table 12. The toxic potency of the culture concentrate was found to be 246 LD_{50}/ml. The concentrate was tested undiluted and diluted with distilled water 1:2, 1:4, 1:6 and 1:8 using both immunodiffusion methods. No visible precipitate was formed with the undiluted concentrate and type E antitoxin by the agar well micro method. A visible precipitate was formed, however, using the template slide method with the undiluted concentrate and the concentrate diluted 1:2. Thus the template method seemed to be more sensitive than the agar well slide method. It would detect a concentration of 123 LD_{50}/ml. The wells of the plastic templates held an average of 0.025 ml of reactants. At the 1:2 dilution, then, this well held 3.1 LD_{50} of toxin.

Researchers have been attempting to find an in vitro method for

Table 12. LD_{50} studies on toxic concentrated culture filtrate of Clostridium botulinum type E strain VH No 1 cultured in untrypsinized TPG

Dilution of toxin in gel phosphate[a]	Infectivity	Dead	Not dead	Accumulated values: Dead	Accumulated values: Not dead	Infectivity: Ratio	Infectivity: Percent
10^{-1}	9/10	9	1	15	1	15/16	94
10^{-2}	6/10	6	4	6	5	6/11	55
10^{-3}	0/10	0	10	0	15	0/15	0
10^{-4}	0/10	0	10	0	25	0/25	0

Calculations:

$$\text{Proportionate distance} = \frac{(\%\text{ mortality just above 50}) - 50}{(\%\text{ mortality just above 50}) - (\%\text{ mortality just below 50})}$$

$$= \frac{55-50}{55-0} = .09$$

$$\text{Log of } LD_{50} \text{ titer} = \text{logarithm of dilution above 50\% infectivity} + (\text{proportionate distance} \times \log \text{dilution factor})$$

$$= 2 + (.09 \times \log 10)$$

$$= 2.09$$

$$LD_{50} \text{ titer} = 123/0.5 \text{ ml or } 246/\text{ml}$$

[a] 0.5 ml of dilution injected into mice

the detection of botulinal toxins. To this end, immunodiffusion methods have been studied. As seen in this study, type E toxin and antitoxin seem to be specific. Only a fading reaction with crude type E toxin and type A antitoxin was seen. This reaction was quite faint and easily distinguishable from the strong reaction of type E toxin and antitoxin. The results obtained indicate that the culture filtrates of other organisms tested against the type E antitoxin shared no antigens in common with type E botulinum toxin filtrates detectable by these methods. Another factor influencing the results is the purity and specificity of the antitoxin preparation. Type E toxin also does not seem to share any antigens with type A or B toxin.

In this study, cross reactions between culture filtrates of A and B botulinum strains and other clostridial filtrates with type A and B botulinal antitoxin were found. The culture filtrates of Cl. sporogenes, Cl. tetani, Cl. novyi and Cl. welchii were found to cross react with type B antitoxin. Cl. novyi cross reacted with type A antitoxin. In addition type A culture filtrate cross reacted well with type B antitoxin. Lack of good strong reactions with type A antitoxin, except in the case of Cl. novyi, is thought to be due to the hemolyzed state of this particular preparation. Other workers have also reported this phenomenon (Vermilyea, Walker and Ayres, 1968). The cross reactions between type A and B culture filtrates and the heterologous antisera have been attributed to a hemagglutinin

(Lamanna and Lowenthal, 1951). However, as more than one precipitin line was observed in these cross reactions, the culture filtrates must have more than one antigenic component in common. The studies here also indicate that the filtrates of Cl. sporogenes, Cl. tetani, Cl. novyi and Cl. welchii have at least one antigen in common with Cl. botulinum type B. Cl. novyi has at least one antigen in common with Cl. botulinum type A. Thus, as there are some cross reactions between type A and B botulinal antitoxins and other clostridia, immunodiffusion identification of A and B toxins in unpurified culture filtrates does not appear to be practical.

Recently, work has been done by other workers on the identification of type E toxin by immunodiffusion. This toxin-antitoxin system seems more specific. A simple single diffusion method was reported by Mardarowicz (1966) using the serum of a patient recovering from type E botulism as the antibody source. Sugiyama et al. (1967), using a macro plate method, reported detecting type E toxin in amounts as low as 1.2 LD_{50}/well. Their wells held 0.05 ml of reactants whereas the template wells of this study held 0.025 ml. Thus, the sensitivity figure of 3.1 LD_{50}/well found in this study was in fair agreement with their figure. Vermilyea, Walker and Ayres (1968) have also studied type A, B and E toxins by slide immunodiffusion methods. They reported no cross reactions between type E toxin and types A and B antitoxins or between A and B toxins and E

antitoxin. The present study is in agreement with their findings. They found, however, that the mouse test was still more sensitive than the immunodiffusion methods for detecting small amounts of type E toxin.

Tests reported here indicate that under laboratory conditions immunodiffusion tests on concentrated culture filtrates can be applied to distinguish toxic cultures of Cl. botulinum type E from non-toxic cultures, eliminating the need for animals in this area. However, further work needs to be done to explore other possible applications. The sensitivity of the method needs to be increased. This may be done by concentrating the toxin to a greater degree. Immunodiffusion methods should be tried in the testing of mud and food samples. The method, as tested here, seems to be specific for type E botulinal toxin in pure cultures. Whether it might also be applied to the mixed cultures obtained from mud and food specimens remains to be determined.

SUMMARY

In this study of toxic and non-toxic Clostridium botulinum type E, two major areas were examined. These included attempts to induce toxigenicity by mutagenic agents and attempts to differentiate the toxigenic and non-toxigenic strains by immunodiffusion. However, several supporting areas were also studied, such as the identification of the non-toxic strains used by biochemical reactions, the testing of the non-toxic strains for the presence of a bacteriocin, the trial of Dolman's (1957b) method of morphological identification of toxic and non-toxic colonies, and the attempted identification of toxin using a macro immunodiffusion method.

Available non-toxic strains were tested for their biochemical agreement with established descriptions of Cl. botulinum type E and for the presence of a bacteriocin. Nine strains were found to be suitable for use in the mutagenic studies. These strains resembled type E botulinum biochemically and morphologically and were found to have no bacteriocin-like activity against toxic Cl. botulinum type E strains as described by Kautter et al. (1966).

Several non-toxic strains were tested for the effect of oxygen toxicity during exposure to the mutagens. Results indicated that the organisms would be quite resistant to this degree of exposure to oxygen. However, later work showed that this was not always true, but

varied from organism to organism.

The non-toxic strains were exposed to a variety of chemical mutagenic agents. These included 2-aminopurine, azaserine, acriflavin and 5-bromouracil. They were exposed to these in a concentration and for a time interval previously found to give 90% kill. The survivors were recultured and tested for toxicity. No toxin was detected in any of the resulting cultures. Strain 066B-NT was cultured serially through ten transfers in maximum tolerated concentrations of 5-bromouracil and through nine transfers in acriflavin and then tested for toxin production. Again, no toxin was detected.

The effect of residual mutagen in the plates used in counting was determined. Only 2-aminopurine showed a marked inhibition of the organisms in the count. The figures were adjusted accordingly in this experiment. The percent of surviving cells was still in the desired range.

Four non-toxic strains were exposed to gamma irradiation from a ^{60}Co source to try to induce mutation to a toxic form. Eighty percent kill was effected by exposure to 125,000 or 250,000 rads. The spore population of the survivors was low. No toxin producers were apparent in cultures of the survivors. Strain 066B-NT was exposed serially to irradiation. Again the spore population of the survivors was low, and no toxin was detected.

In an attempt to differentiate non-toxic and toxic colonies, Dolman's (1957b) method was tried. Several toxic and non-toxic strains were cultured on various media to determine which agar gave best growth and selected colonies were cultured and their toxicity tested. All colonies tested from the toxic strains were toxic and all from the non-toxic strains were non-toxic regardless of colony type. Although these attempts were unsuccessful, an irradiated culture of non-toxic strain 066B-NT was cultured on BH agar with sodium thioglycollate. Approximately 10,000 colonies were examined through a dissecting microscope by transmitted light. Seven colonies were selected and tested for toxicity. Although they resembled Dolman's TOX colonies, none were toxic.

In an attempt to detect botulinum toxin by in vitro methods, a macro immunodiffusion method as described by Elek (1948) was tried. Three toxic and non-toxic strains were tested. No visible precipitate lines appeared in four to six days.

Two micro immunodiffusion methods were also attempted. By the agar well micro method, concentrated culture filtrates of 14 toxic strains of Cl. botulinum type E and nine non-toxic strains were tested against type A, B and E botulinal antisera. In addition, nine other clostridia species were tested against the same antisera. Only toxic Cl. botulinum type E strains reacted with type E antitoxin. They did not react with type B antitoxin. Non-toxic type E concentrates

did not react with type E or B antitoxin. Both toxic and non-toxic type E concentrates gave faint, fading reactions with type A antitoxin. None of the other species cross reacted with type E antitoxin, although some of them did with types A and B antitoxin. Using the template micro method, concentrates of toxic and non-toxic culture filtrates of Cl. botulinum type E and Cl. botulinum types A and B were tested against the various antitoxins. The toxic type E concentrate reacted with type E antitoxin and no cross reactions between type E antitoxin and concentrates of other clostridia were observed; nor were cross reactions between type E concentrates and type A or B antitoxins observed. These findings led to the belief that the micro immunodiffusion method could be used to differentiate pure cultures of toxigenic and non-toxigenic Cl. botulinum type E strains under laboratory conditions without the use of mice. However, work needs to be done in testing possible application of this method to mixed cultures from food and mud specimens.

The sensitivity of the template and agar well micro methods was compared. The highest dilution of a concentrated type E culture filtrate at which a precipitin line appeared was compared with the number of LD_{50} of toxin found per unit volume of the concentrate. The concentrate was found to contain 246 LD_{50}/ml. Only the template micro method would detect the toxin with a visible precipitate

line. Thus, it appeared to be the more sensitive method. This method would detect 123 LD_{50}/ml or 3.1 LD_{50} of toxin per well.

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