SOME ECOLOGICAL CONSIDERATIONS OF THE TOXIN OF CLOSTRIDIUM BOTULINUM TYPE E IN THE LAKE MICHIGAN ECOSYSTEM

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ABSTRACT

SOME ECOLOGICAL CONSIDERATIONS OF THE TOXIN OF *CLOSTRIDIUM BOTULINUM* TYPE E IN THE LAKE MICHIGAN ECOSYSTEM

by Richard H. Monheimer

The possible involvement of type E botulism in Lake Michigan waterbird mortalities and factors influencing this botulism are considered. Various waterbirds were fed *Clostridium botulinum* type E toxin and it was determined that the birds can be affected by toxin produced by some strains of the organism. Type E botulinal toxin was found in dead alewives taken from the Lake Michigan ecosystem at levels sufficient to kill or sicken a bird. DDT in the culture medium in levels up to 500 parts per billion had no effect on the production of *C. botulinum* type E toxin. DDT and botulinal toxin, when present in white mice, interact when affecting the animals although little ecological significance can be concluded from the DDT-botulinal toxin interaction. Given a suitable medium, some strains of *C. botulinum* type E may be able to produce large amounts of toxin in the Lake Michigan ecosystem. The alewives in Lake Michigan may provide such a medium as well as providing a potential vehicle for the intoxication of fish-eating waterbirds.

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By

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INTRODUCTION

Clostridium botulinum is a rod-shaped, spore-forming bacterium whose optimum growth temperature is between 25 and 37 C (Bergey's Manual for Determinative Bacteriology, 1957). Although this organism is generally considered to be an anaerobe, it has been shown that the species can sometimes grow in an environment that is initially aerobic (Kaufmann and Marshall, 1965). As a culture of this organism grows, it produces an extremely potent exotoxin which can be lethal to selected vertebrates (Lamanna, 1959). Oral ingestion of this exotoxin rather than an infection by the culture is what makes C. botulinum dangerous.

There are six distinct antigenic types of C. botulinum which are classified as A through F. The toxin produced by each type is neutralized only by its type-specific antitoxin and it is by a neutralization test that the species is identified to type. All types of C. botulinum can cause both human and animal botulism, but types A, B, E and F are those usually associated with human botulism while C, D and E are the types that are most often associated with animal botulism. Type C botulism affects a large variety of animals but is particularly noted for causing large mortalities among wildfowl in the western prairies of the United States (Sciple, 1953). Botulism type D has occurred primarily in South African cattle but it has also been shown to be lethal to cattle in Australia (Simmons and Tsunemagi, 1964). Until recently, the animals most commonly affected by type E botulism were domesticated mink (Scholtens and Coebon, 1965).

A review of the literature (Dolman, 1960) shows that type E botulism has usually been associated with a marine environment. Cases of human botulism have usually been traced to improperly prepared marine foodstuffs, such as pickled herring, salmon eggs and whale meat. Pedersen (1955) concluded that C. botulinum type E is very prevalent on the sea bottom and several recent investigations have shown the organism to be present in both the Atlantic and Pacific Oceans (Ward et al., 1967; Craig et al., 1968). Although Prevot and Huet (1951) showed the presence of C. botulinum type E in fresh water perch in France, until recently there has been little reason to believe that C. botulinum type E could be a factor relevant to the ecology of the Great Lakes.

The first evidence that C. botulinum type E was present in the Great Lakes came in 1960, when an outbreak of botulism occurred in Minneapolis, Minnesota. Smoked "ciscoe" from the Great Lakes served as the vehicle for the food poisoning (Kautter, 1964). Another outbreak of human botulism occurred in 1963 which was attributed to smoked "chubs" taken from Lake Michigan (Scholtens and Coohon, op. cit.). In the fall of 1963, a large mortality of gulls (Larus argentatus; L. delawarensis), loons (Gavia immer) and several other species of waterbird occurred on Lake Michigan (Fay et al., 1965). Similar mortalities, of a greater or lesser extent, have occurred every year since 1963 (Fay, 1968). Examination of blood and tissues from a random sampling of these dead birds have shown the presence

of varying amounts of C. botulinum type E toxin (Kaufmann and Fay, 1964; Fay, 1966). Subsequent investigations have shown the spores of C. botulinum type E to be widespread throughout the Great Lakes (Foster et al., 1965; Bott et al., 1966).

The details involving the Lake Michigan waterbird mortalities and the discovery of C. botulinum type E microorganisms in the Great Lakes present good, but only circumstantial, evidence that type E botulism is the cause of the bird mortalities. To be reasonably sure that botulism is causing the deaths, a susceptibility level for the birds must be determined and then a source of the toxin in a form suitable for intoxication must be found in nature. Meanheimer (1965) studied the susceptibility of Ring-billed Gulls (L. delawarensis) to type E botulinal toxin and although botulism was induced in them, no definite level of susceptibility was determined. Also, no direct evidence of "naturally occurring" type E botulinal toxin in the Lake Michigan ecosystem has yet been recorded. This study, in part, thus provides the necessary evidence to conclude that the waterbird mortalities are indeed caused by type E botulism.

The second objective of this study is to examine ecological factors that may have influenced what appears to be the "sudden occurrence" of type E botulism in the Great Lakes. It is recognized that the lack of investigation, rather than the absence of the organism, may account for this apparent sudden occurrence. However, several changes in the Great Lakes ecosystem in the past decade could possibly account for this elevation into prominence of C. botulinum type E.

Two of these changes are the invasion of the originally marine alewife (Alosa pseudoharengus) and the accumulation of the chlorinated hydrocarbon 1,1,1-trichloro-2,2-bis(p-chlorophenol)ethane (DDT). Besides providing a new vehicle for intoxication of waterbirds, the alewives may provide a suitable medium in which C. botulinum type E may grow and produce toxin. DDT and/or the chemical make-up of alewives may affect the growth characteristics of C. botulinum type E so that it produces toxin more readily than under the previous conditions of the ecosystem. Also, DDT (O'Brien, 1964) and botulinal toxin (Brooks, 1964) both affect an animal's nervous system and there thus may be some sort of interaction between the two materials when both are present in an animal. This study, therefore, also examines these aspects of DDT and the alewives in respect to their possible role in the occurrence of type E botulism in the Great Lakes.

MATERIALS AND METHODS

Basic Laboratory Procedures. All cultures of C. botulinum type E produced in the laboratory during this study were grown in the same basic bacterial growth medium. This medium contained 5.0% Trypticase (Baltimore Biological Laboratories), 0.5% peptone (Difco), 0.2% sucrose (Merck) and 1.0% yeast extract (Difco). Anaerobic conditions were produced in the culture by the addition of 0.1% sodium thioglycollate. Prior to sterilization by autoclaving, the pH of the medium was adjusted to 6.8 by the addition of hydrochloric acid. Hereafter, this bacterial medium will be referred to as TPSY.

The strain of C. botulinum type E used throughout this study was isolated by Mr. Ralph Johnston (Food and Drug Administration, Detroit) from a fresh "whitefish chub" taken from Lake Michigan in 1963. This strain is identified by the Food and Drug Administration as 026-080x. A culture of C. botulinum type E was produced using the Vancouver Herring (VH) strain, which is a "common" laboratory organism, but this was used in only one phase of the gull feeding experiments.

At the beginning of this study, 15 ml screw-cap culture tubes containing TPSY had been inoculated with a spore suspension of the C. botulinum type E, incubated at 30 C for 15 hours and then frozen at -15 C. These frozen cultures, thawed just prior to use, were the stock used as the initial inoculum for all subsequent cultures.

Actively growing starter cultures were used for inoculating the medium for each culturing experiment. These starters were grown for

15 or 24 hours at 30 or 15 C respectively, depending upon the temperature at which the experiment was to be conducted. The length of time that the cultures of each experiment were held in the incubators at each temperature were determined by the results of a pilot study. Cultures for this pilot study were incubated in three-ounce prescription bottles at 15 or 30 C. One bottle was removed each day for 15 days from each temperature and the level of toxin determined to ascertain the time period for maximum toxin production.

Titration of the toxin produced by each culture was accomplished by intraperitoneally injecting Swiss-Webster white mice with various dilutions of the culture broth. The mice, which weighed between 13 and 21 grams, were raised specifically for this study by the Wildlife Pathology Laboratory of the Michigan Department of Conservation at the Rose Lake Wildlife Research Center. Dilutions of the toxic cultures were made with a buffer solution containing 0.2% gelatin and 0.4% dibasic sodium phosphate. The pH of the buffer was adjusted to approximately 6.2 with hydrochloric acid. All injections used for titrating toxin consisted of a 0.2 ml aliquot of the diluted culture. The results of the titrations are calculated in terms of mouse LD_{50} per milliliter of culture by the method of Reed and Muench (1938).

Type E botulinal toxin is known to have the property of becoming more toxic when subjected to the enzymatic action of trypsin (Duff et al., 1956). Because some form of self activation may occur in some cultures and not in others, a more accurate measure of the total

amount of toxin present in a culture may be the toxicity following trypsinization. Therefore, all cultures also were titrated after the incubation of equal parts of culture and a 1.0% solution of trypsin (Difco, 1:250) at 40 C for one hour. The trypsin was in a solution of Sorenson's phosphate buffer (Clark, 1928) at a pH of 5.9.

All cultures were examined microscopically to check for the occurrence of bacterial contamination. Also, a specific toxin neutralization test was employed to ascertain that the lethal agent in each culture was C. botulinum type E toxin. Mice were injected intraperitoneally with one unit (1,000 anti-MLD) of monovalent C. botulinum type E antitoxin (obtained from the Communicable Disease Center, Atlanta, Georgia) followed immediately by an injection of culture containing a lethal amount of toxin. This injection of culture, however, contained less than 1,000 mouse minimum lethal doses (MLD). Survival of the protected mice and deaths of unprotected mice identified the lethal agent as C. botulinum type E toxin.

Effect of alewives on toxin production. Various amounts of a sterile alewife homogenate were added to the basic TPSY medium to determine whether some aspect of the chemistry of alewives can affect the production of toxin by C. botulinum type E. Fresh-frozen alewives that had been taken by trawl from Lake Michigan were thawed, cut into pieces and added to an equal volume of distilled water. This mixture was then placed into a Sorvall Omni-mixer and homogenized. The supernatant which resulted from centrifugation of this homogenate at

40,000x g for 30 minutes was passed successively through millipore filters with membrane pores sizes of 3.0μ, 1.2μ, 0.45μ and 0.22μ. The fluid was filtered through each size membrane several times until it flowed freely. Final sterilization of this alewife extract was accomplished by passing it into a sterile disposable Nalgene filter with a membrane pore size of 0.20μ.

The sterile alewife extract was added aseptically to amounts of TPSY at a rate such that after the addition of one milliliter of starter culture, the extract made up either 2, 4, 8 or 16 percent of the total 100 ml volume. The containers used for this culturing were three-ounce prescription bottles. Five replicate cultures were run for each percentage of fish extract as well as for controls containing no extract. This experiment was carried out at both 15 and 30 C for incubation periods of ten and five days respectively.

Titration of the toxin in these alewife-TPSY cultures followed several schedules (the procedures were modified several times during this study because of the large numbers of mice and time involved in this type of bioassay). Those cultures grown at 30 C were assayed by injecting groups of ten mice. Dilutions of these cultures were made such that one group of mice had less than 50% but more than 0% mortality while another group had more than 50% but less than 100% mortality. The cultures incubated at 15 C were assayed by injecting groups of five mice. Dilutions used for this lot of cultures depended upon whether or not the culture was treated with trypsin. Non-treated

culture dilutions were undiluted, 1:10 and a two-fold serial dilution with the lowest being 1:50. Activated cultures were titrated using the same dilution scheme for these cultures containing less than 50,000 mouse LD_{50}/ml toxin but for cultures with higher titers, a two-fold serial dilution beginning at 1:1,000 was used.

Effect of DDT on toxin production. The effect of small amounts of DDT on the production of C. botulinum type E toxin was determined by adding low levels of DDT (p.p' isomer, 100%; City Chem. Corp., N.Y.) directly to 15 ml screw-cap culture tubes containing 10 ml TPSY. The DDT was first dissolved in acetone and dilutions prepared so that the addition of one milliliter of acetone would add 5, 50 or 500 parts per billion (ppb) DDT to the TPSY. A control set of tubes received one milliliter of DDT-free acetone. These tubes were then autoclaved to sterilize the medium and to drive out the acetone. The cooled tubes then received 0.05 ml of inoculum and were incubated at either 15 or 30 C for ten and five days respectively. Four replicates for each DDT level and the controls were run at each temperature.

The botulinal toxin produced in these DDT-TPSY cultures was assayed by injecting groups of three mice. A two-fold serial dilution was used, with the lowest dilution being 1:50 for non-trypsinized cultures and 1:1,000 for activated cultures.

Effect of successive botulinal toxin and DDT administration to white mice. Determination of the possibility of an interaction between type E botulinal toxin and DDT in animals was accomplished by injecting

DDT (p.p' isomer, 100%) dissolved in corn oil intraperitoneally into four groups of 60 white mice at the rates of 5.0, 7.5, 10.0 or 15.0 mg of DDT per mouse. A control group received an injection of corn oil only. Approximately 22 hours after the DDT treatment, the mice were injected intraperitoneally with dilutions of a pure culture of C. botulinum type E. Five dilutions of culture were used, ranging from 1:50 to 1:300. A control group received an injection of the gelatin-phosphate buffer diluent. All injections were of 0.2 ml.

This experiment thus contained 30 sets of mice which had been injected with various levels of both DDT and botulinal toxin. Each set contained ten mice. Most of the sets contained five males and five females, but in several instances one mouse was eliminated from a given set due to leakage of injected material. The mice weighed between 13 and 18 grams and the sets were as homogeneous as possible with respect to the weights of the mice. Each set of mice was held in an individual box and was observed for six days following the DDT injection. Dead mice were removed from the boxes. The results are recorded as a percentage of mice affected, affected mice being those that died during the test and those that had typical DDT tremors at the end of the six day test period. This experiment was replicated a second time.

Effect of botulinal toxin on captive waterbirds. Gulls were chosen as the primary species of waterbird on which to test the effect of C. botulinum type E toxin because of the relative ease with which they

can be obtained and because of the previous work done with them (Meanheimer, op. cit.). Ring-billed Gulls and Herring Gulls (L. argentatus) which were three to five weeks old were taken from a gull nesting colony located on northern Lake Huron near Roger's City, Michigan. California Gulls (L. californicus) were captured as young on the Bear River Migratory Bird Refuge (near Brigham City, Utah) and shipped to East Lansing by air express. All gulls were held in pens at the Rose Lake Wildlife Research Center until they were full grown. Their diet consisted primarily of alewives supplemented periodically with canned dog food or raw meat. A dose of thiamine hydrochloride was also given periodically to prevent vitamin deficiencies caused by thiaminases in the alewives.

Toxin for feeding to the gulls was cultured in one-liter prescription bottles at 30 C for five days, then held at -15 C until used in the feeding trials. The toxin was fed to the waterbirds using a syringe and a blunted 1¼-inch 18 gauge needle. The beak was held open and the needle placed into the throat so as to avoid getting the fluid into the trachea. The toxin was slowly injected into the esophagus and the bird was then held with its neck outstretched for approximately one minute so that the fluid entered the gastro-intestinal tract and could not be regurgitated. Elimination of the possibility of oral irrigation due to large volumes of fluid was accomplished by feeding the larger doses in aliquots of 10 ml at intervals of one hour.

The differences in the susceptibility of waterbirds to toxin produced by different strains of C. botulinum type E, as Hansen (1942)

found for chickens, was determined by force-feeding Ring-billed Gulls with 40,000 mouse MLD toxin. Four birds received the VH strain of culture and four received the 026-080x strain. This culture was in a volume of 10 ml. Two groups of three California Gulls were also fed the different strains of culture; one bird in each group was fed 40,000, 80,000 or 120,000 mouse MLD in a volume of 10, 20 or 30 ml respectively.

C. botulinum type E strain 026-080x culture was used to determine the susceptibility level of Ring-billed Gulls. Four groups of 10 gulls were fed from 7,500 MLD to 30,000 MLD non-trypsin activated toxin. Four groups of four gulls were fed from 67,500 MLD to 250,000 MLD trypsin-activated toxin. All birds received a volume of 10 ml. Control groups included five gulls fed 10 ml of the uninoculated TPSY and five gulls fed 10 ml of the gelatin-phosphate buffer which was used to bring the toxin doses to their final volume.

Several other species of waterbird were fed strain 026-080x culture in doses ranging from 30,000 MLD to 150,000 MLD. These birds included 15 Herring Gulls, one Great Blue Heron (Ardea herodias), one Common Loon (Gavia immer), and one Horned Grebe (Colymbus auritus).

Detection of toxin in Lake Michigan. On June 16, 1967, eight dead alewives were collected from Lake Michigan just north of Ludington, Michigan. Two of the fish were collected from the bottom of the lake by scuba diving in 25 feet of water about 300 yards from the shore.

The other six fish were taken from the beach, two at the high-water line and four from the water's edge. The water temperature at the time of collection was approximately 17 C (62 F) while the air temperature was about 30 C (approximately 88 F). All fish were put into plastic bags which were placed on ice within 20 minutes after collection.

The fish were taken into the laboratory, held at 3 C overnight and tested the following day for type E botulinal toxin. Each fish was placed on absorbent paper to remove excess moisture and, following weighing, cut into several pieces. A potato ricer was used to press these pieces and the juices thus obtained were diluted with the gelatin-phosphate buffer. Since the undiluted juices of even fresh fish kills injected mice, the lowest dilution used was 1:10. Higher dilutions used were a two-fold series beginning with 1:100. The juices were also treated with trypsin and diluted in a similar manner. Testing for type E botulinal toxin was done by protecting the mice with specific antitoxin. Two mice were used for each dilution of each fish.

RESULTS AND DISCUSSION

Toxin in the Lake Michigan ecosystem. C. botulinum type E toxin was found to be present in the alewives collected from both the beach and water of Lake Michigan (Table 1). The toxin was present, therefore, in a vehicle suitable for the intoxication of fish-eating waterbirds. Increased toxicity was not found after treatment with trypsin and it can be concluded that the toxin was present in the activated form.

The fish which were collected from the beach appeared to have been washed ashore the preceding day. Some fungi were growing on the fish but they were not yet becoming dessicated. Both of the fish collected underwater were considerably decayed. Fish No. 1 was completely covered with fungus and the head was missing. Fish No. 2 was partially covered with fungus but was whole and less decayed.

It is now evident that C. botulinum type E toxin does exist in the Lake Michigan ecosystem and it is interesting to speculate about the ecology of this toxin. Although the toxin was found in only one location, there is no reason to believe that it is restricted to that geographical area or even the time of year at which it was found. The fact that the toxin was found both on a bathing beach and in the water raises the profound possibility of accidental human intoxication. Obviously, it would be desirable to have more investigation into the ecology of this naturally occurring toxin with respect to possible human involvement.

Table 1. *Clostridium botulinum* type E toxin in alewives taken from the Lake Michigan ecosystem.

Fish No.	Site collected	Weight of fish (gm)	Highest dilution to kill mice	MLD toxin per gm fish	Total MLD toxin in fish
1	Underwater	19	1:800	4,000	76,000
2	" "	26	1:100	500	13,000
3	Beach at waters edge	30	0	—	—
4	" "	24	0	—	—
5	" "	23	0	—	—
6	" "	32	0	—	—
7	Beach at high water line	30	1:100	500	15,000
8	" "	27	1:100	500	13,500

Effect of botulinal toxin on captive waterbirds. Although a small number of gulls were fed toxin produced by different strains of C. botulinum type E, it is highly probable that gulls do vary in their susceptibility to toxin produced by different strains. All of the California Gulls fed the 026-080x strain of toxin developed symptoms of botulism and died, while no signs of illness of any kind were observed in those that received the VH toxin (Table 2). Statistical analysis by the non-parametric Fisher Exact Probability Test indicates that, in doses from 40,000 to 120,000 MLD, the probability is only 3.0% that California Gulls will be killed in equal numbers by the two strains of toxin used. All of the Ring-billed Gulls that received 40,000 MLD of the 026-080x toxin became obviously ill and two of them died, while only one gull fed the VH toxin exhibited slight symptoms of botulism. None of the other Ring-billed Gulls fed the VH toxin became visibly ill (Table 3). The Fisher Exact Probability Test indicates only a 7.1% probability that the two strains of toxin tested have the same effect on Ring-billed Gulls.

Toxin feeding trials using various doses of non-activated toxin strain 026-080x administered to the Ring-billed Gulls indicate that the LD_{50} is approximately 22,500 mouse MLD (Table 4). However, calculation by the method of Reed and Muench (op. cit.) gives a LD_{50} of just under 20,000 mouse MLD. The level which causes 50 percent of the gulls to become ill is calculated to be approximately 12,000 mouse MLD. One of the gulls fed gelatin-phosphate buffer as a control died, but it did

Table 2. Results from feeding California Gulls levels of toxin produced by two strains of C. botulinum type E.

Dose fed (MLD)	Strain of toxin fed*	
	Vh	026-080x
40,000	Lived	Died
80,000	Lived	Died
120,000	Lived	Died

* One gull received one level of one strain of toxin.

Table 3. Results from feeding Ring-billed Gulls 40,000 MLD of toxin produced by two strains of C. botulinum type E.

Strain of toxin fed	No. of gulls fed	No. exhibiting botulism symptoms	No. dead
VH	4	1	0
026-080x	4	4	2

Table 4. Results from feeding Ring-billed Gulls various levels of *C. botulinum* type E strain 026-080x toxin.

Dose fed (MLD)	No. of gulls fed	No. exhibiting botulism symptoms	No. dead
30,000	10	10	8
22,500	10	8	5
15,000	10	8	4
7,500	10	3	2
Uninoculated medium	5	0	0
Gelatin-phosphate buffer	5	0	1

not exhibit any symptoms of botulism. None of the gulls receiving the trypsin-activated toxin or the uninoculated TPSY became ill or died.

The results from feeding toxin 026-080x to the other waterbirds indicate that these species may vary in their susceptibility to the toxin (Table 5). A much larger number of birds would be needed to determine specific levels of toxin that are needed to produce specific effects.

The difference in susceptibility of the gulls to the different strains of C. botulinum type E toxin becomes of great interest when considering both the toxin found in the dead alewives and the calculated susceptibility levels of the Ring-billed Gulls to the 026-080x strain of toxin. Although the amounts of toxin found in the alewives should be sufficient to sicken or kill many of the gulls that might eat the fish, the question arises as to whether this strain of toxin is toxic to gulls. This specific toxin from the fish was not fed to gulls, but Fay (1966) reported that some dead alewives picked up along the beach of Lake Michigan did kill some of the captive gulls which ate them and that type E toxin was then detected in the blood from these gulls. This difference in susceptibility may also answer the questions arising from the fact that the toxin found in the fish was activated and that gulls appear to be less susceptible to trypsin-activated toxin. The toxin found in the fish is probably in some way different from the toxin used in this laboratory experiment and thus the two toxins may affect

Table 5. Results from feeding various waterbirds toxin produced by C. botulinum type E strain 026-080x.

Species	Dose fed (MLD)	No. of birds fed	No. exhibiting botulism symptoms	No. dead
Herring Gull	45,000	5	3	1
Herring Gull	90,000	5	4	0
Herring Gull	135,000	5	5	0
Great Blue Heron	150,000	1	1	1
Common Loon	150,000	1	1	1
Horned Grebe	30,000	1	1	0

gulls differently. The proteolytic processes of a gull's digestive system may denature the trypsin-activated toxin produced in the laboratory but not change the activated toxin found in the fish.

Relationship of toxin production as a function of time and temperature. The results illustrating the relationship of toxin production to time and temperature are shown in Figure 1 for non-activated samples. Figure 2 shows the curves for trypsin-activated samples. From these curves, which are drawn by inspection, it was decided that cultures grown at 15 C should be incubated for 10 days and those grown at 30 C should be incubated for five days. The dilutions for titrating the non-activated samples were a two-fold series while those for the activated samples were a ten-fold series; this ten-fold dilution series makes it difficult to compare the titers of the activated samples of each temperature.

Effect of DDT on toxin production. None of the cultures of C. botulinum type E grown in TPSY containing DDT were found to be contaminated by other bacteria and all of these cultures were typed positive for C. botulinum type E toxin. Statistical analysis of the toxicities of these cultures utilized only those LD_{50} values obtained from the trypsin-activated toxin (Table 6). A two-way analysis of variance of these data (Table 7) indicate that the levels of DDT used in this experiment did not affect the amount of toxin produced. Also, there was no interaction in the cultures between DDT and temperature when considering the titers of toxin produced. However, this analysis does indicate

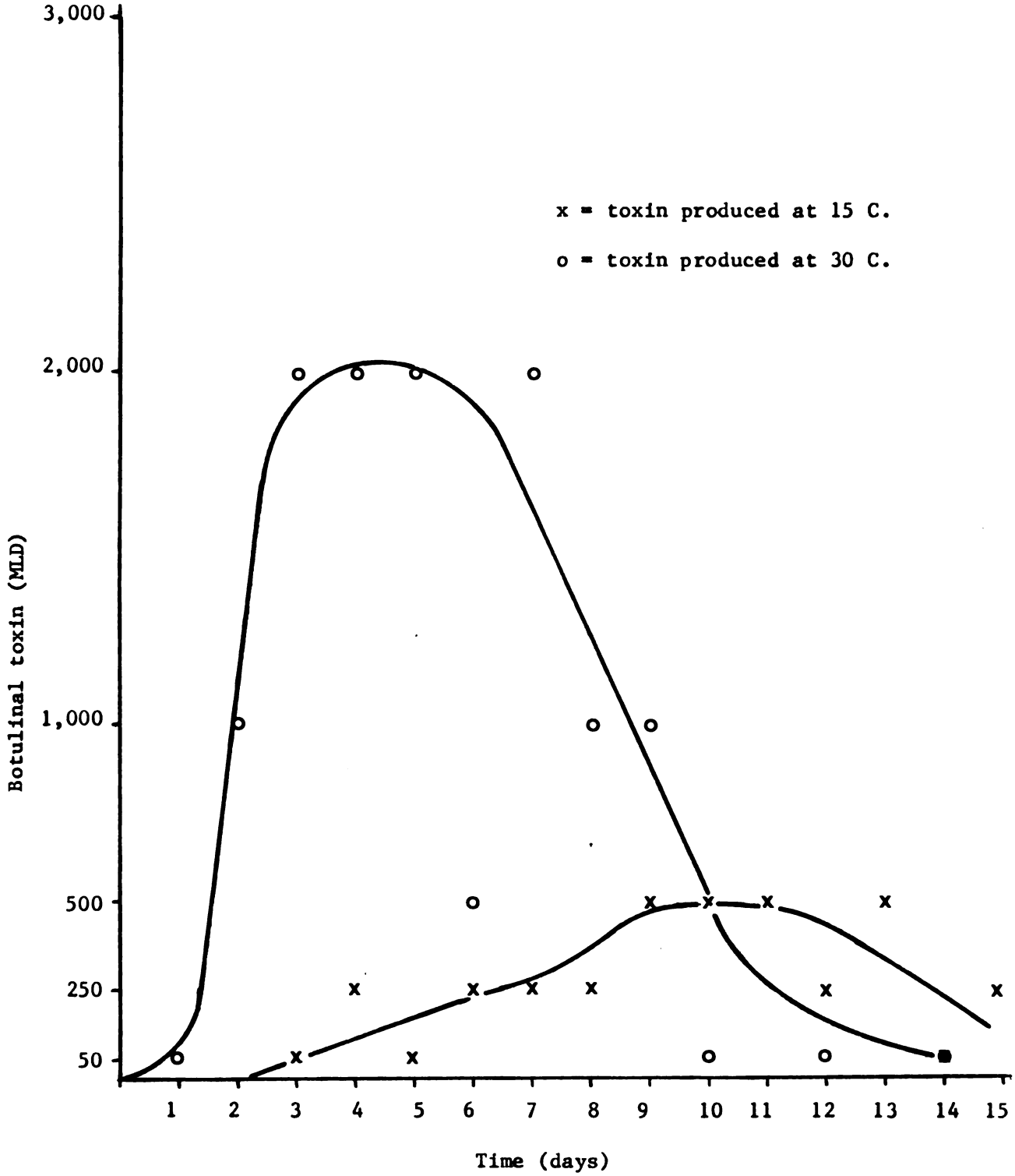

Figure 1. Curves showing the relationship of production of C. botulinum type E toxin (non-activated) to time and temperatures of 15 and 30 C.

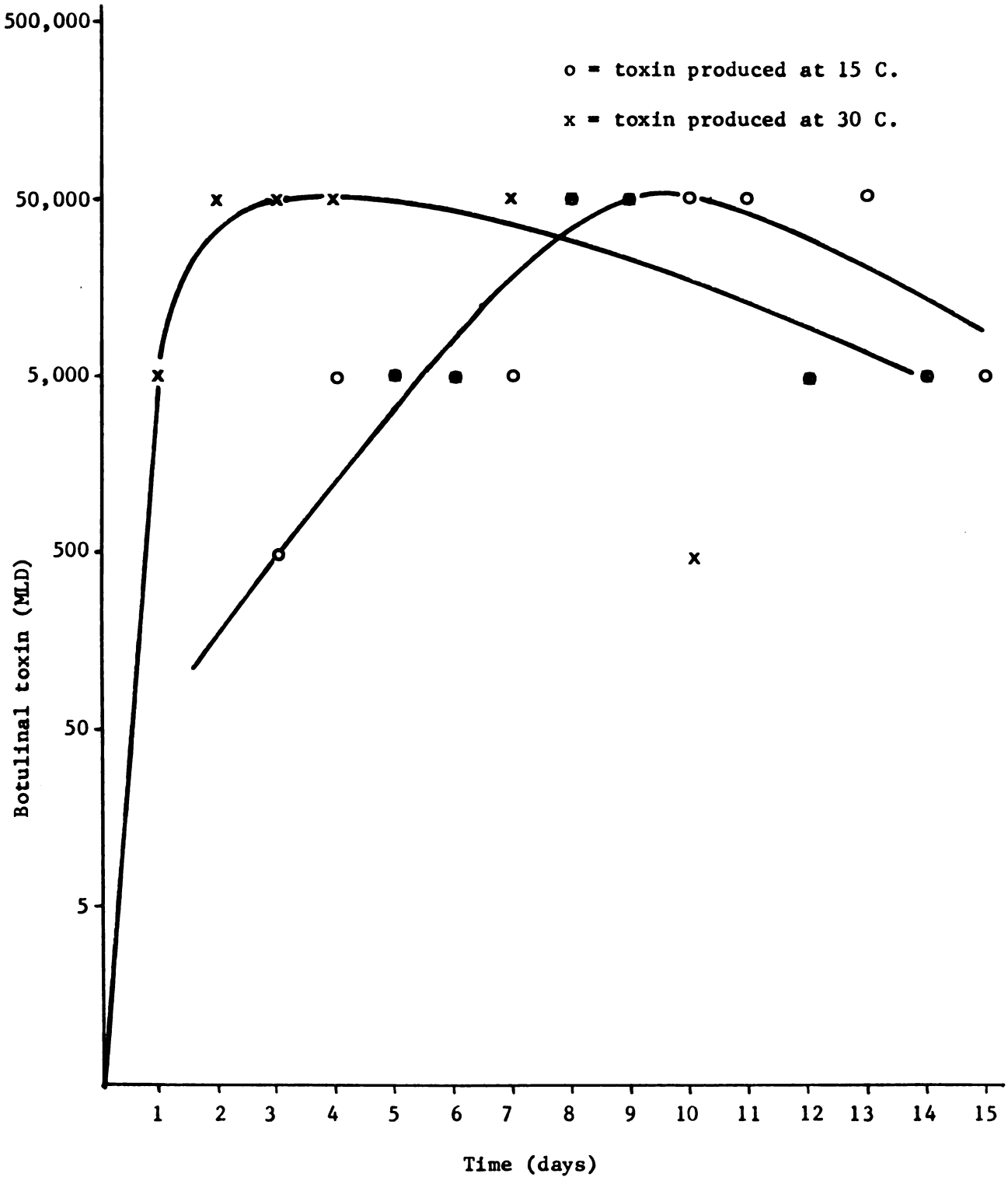

Figure 2. Curves showing the relationship of production of C. botulinum type E toxin (trypsin-activated) to time and temperatures of 15 and 30 C.

Table 6. Summary of the toxicities of C. botulinum type E cultures grown in TPSY containing DDT at 15 and 30 C.

DDT (ppb)	Incubation Temperature			
	15 C Toxicity (LD_{50})		30 C Toxicity (LD_{50})	
	Non-trypsinized	Trypsinized	Non-trypsinized	Trypsinized
0	1,145	109,890	1,520	102,565
0	1,600	160,000	1,600	92,595
0	1,200	205,340	2,290	123,000
0	1,145	175,840	1,600	102,565
5	5,405	487,805	2,185	87,720
5	575	205,340	2,670	87,720
5	2,185	205,340	2,290	87,720
5	2,290	184,500	2,290	102,565
50	1,335	205,340	2,670	102,565
50	1,600	205,340	1,600	105,820
50	1,600	205,340	1,600	105,565
50	1,200	205,340	2,670	102,565
500	1,145	205,340	1,335	87,720
500	2,670	184,500	2,290	54,055
500	2,290	246,305	2,290	123,000
500	1,335	175,840	2,290	87,720

Table 7. Results of the analysis of variance of the toxicities of the C. botulinum type E cultures grown in TPSY containing DDT at 15 and 30 C.

Source of variation	Degree of freedom	Sum of squares	Mean square	F
Temperature	1	1,029,411,281	1,029,411,281	33.43
DDT	3	96,476,311	32,158,770	1.04
Interaction	3	151,849,316	50,616,438	1.64
Error	24	738,946,078	30,789,419	—
Total	31	2,016,682,986	—	—

that, at the 99% significance level, there were differences in the amounts of toxin produced at the different temperatures. The mean value of the toxicities of the cultures incubated at 15 C was 210,462 LD_{50} while that of the cultures grown at 30 C was 97,029 LD_{50}. Therefore, it can be concluded that C. botulinum type E strain 026-080x produces significantly more toxin when incubated in TPSY at 15 C than at 30 C.

The fact that the strain of C. botulinum type E used in this experiment produces more toxin at 15 C than at 30 C holds significant ecological implications. The optimum growth temperature of this species is generally considered to be between 25 and 30 C, depending upon concentrations of the various nutrients in the growth medium (Solman, 1964). It has been found, however, that the species will produce small amounts of toxin at temperatures as low as 3.3 C (Schmidt et al., 1961). If large amounts of type E botulinal toxin can be produced in nature at 15 C, as is implied by this experiment, one can then infer that given a suitable medium, amounts of toxin can be produced in Lake Michigan (15 C is within the water temperatures achieved by Lake Michigan). This idea is in fact substantiated by the previously discussed finding of type E botulinal toxin in the dead alewives taken from the bottom of Lake Michigan. It is possible that the optimum growth temperature for C. botulinum type E differs from the temperature of optimum toxin production, although the production of toxin at the low temperature used in this experiment may be a quality

of the particular strain of C. botulinum type E. Strain 026-080x was isolated from the "cold" environment of Lake Michigan and it is not unreasonable to propose that this strain, as well as other strains present in Lake Michigan, produces "large" amounts of toxin at temperatures lower than is considered "optional" for the species.

Effect of alewives on toxin production. None of the cultures of C. botulinum type E grown in TPSY containing alewife extract were found to be contaminated by other bacteria and all of these cultures were positive for type E botulinal toxin. Statistical analyses of the toxicities of these cultures utilized the LD_{50} values from both trypsin-activated and non-activated samples, depending upon which treatment gave the higher value (Table 8). A one-way analysis of variance was used to analyze the toxin levels produced at each of the two temperatures because of the different methods used to titrate the two groups of toxin; if a two-way analysis of variance was used and a significant difference was found between the two temperatures, it could not be determined whether the difference was due to variations in toxin levels or to the variation of the titrating method. Due to lack of homogeneity of the variance terms of the LD_{50} values of the cultures grown at 15 C, these values were transformed to logarithms before statistical analysis.

The analyses of variance indicate that, at 30 C, there were no differences in the amounts of toxin produced at each level of alewife extract (Table 9). However, at 15 C, there was a significant difference, at the 97.5% level, between the amounts of toxin produced

Table 8. Summary of the toxicities of C. botulinum type E cultures grown in TPSY containing levels of alewife extract at 15 and 30 C.

Percent alewife	Incubation Temperature: 15 C Toxicity (LD_{50})		30 C Toxicity (LD_{50})	
	Non-trypsinized	Trypsinized	Non-trypsinized	Trypsinized
0	0	250	860	76,340
0	300	89,450	3,240	4,220
0	340	63,980	2,900	83,340
0	620	139,770	550	10,000
0	620	84,340	690	10,000
2	670	670	72,470	76,930
2	6,010	96,160	34,730	9,180
2	3,200	29,070	23,260	37,460
2	7,280	128,050	52,640	350
2	10	0	80,650	30,770
4	500	670	48,780	38,610
4	75	85	38,030	30,400
4	85	15	45,670	3,180
4	4,930	80,000	53,770	6,670
4	380	290	46,300	1,210
8	75	0	60,610	1,480
8	60	0	58,830	17,860
8	85	0	51,820	450
8	340	85	67,570	27,780
8	15	0	68,970	820
16	75	430	80,650	1,670
16	60	0	96,160	52,640
16	300	1,340	61,350	250
16	85	0	106,390	7,410
16	60	0	59,880	320

Table 9. Results of the analysis of variance of the toxicities of the C. botulinum type E cultures grown in TPSY containing levels of alewife extract at 30 C.

Source of variation	Degree of freedom	Sum of squares	Mean square	F
Alewife levels	4	5,598,995,780	1,119,799,157	2.11
Error	20	10,620,482,520	531,024,126	—
Total	24	16,219,478,300	—	—

at the different levels of alewife extract. Duncan's New Multiple Range Test (Duncan, 1955) indicates that the amounts of toxin decrease progressively as the levels of alewife in the culture increase (Table 10).

The data presented on the toxicity of the TPSY-alewife cultures tend to indicate that at warmer temperatures an alewife may be a suitable medium in which C. botulinum type E could grow and produce toxin. This is substantiated by the finding of toxin in the alewives which had been lying in the sun on the beach. However, the data also indicate that at lower temperatures toxin production is inhibited by the fish extract and extrapolation of these data to an alewife as the entire growth medium, leads one to expect very little toxin to be produced at low temperatures in an alewife. This, then, conflicts with the idea that toxin can be produced in an alewife in Lake Michigan, although toxin was found in such a fish.

Several possible explanations can be given as to why type E botulinal toxin was found in alewives taken from the bottom of Lake Michigan while this experiment indicates that toxin levels should be very low. This experiment was carried out using pure cultures of C. botulinum type E but it would be highly unlikely that a pure culture would occur in a fish in Lake Michigan. C. botulinum type E has been shown to have different toxin producing characteristics when grown in association with other bacteria (Valenzuela et al., 1966). Also, toxin may be produced by strain 026-080x or another strain in an alewife at low temperatures when incubated for a period longer than was used

Table 10. Results of the analysis of variance and Duncan's New Multiple Range Test for the toxicities of the C. botulinum type E cultures grown in TPSY containing levels of alewife extract at 15 C.

Source of variation	Degree of freedom	Sum of squares	Mean square	F
Alewife levels	4	22.20369	5.55092	4.28
Error	20	25.93703	1.29685	—
Total	24	48.14072	—	—

Percent fish*	8	16	4	2	0

* These levels of fish between which there are no significant differences are underlined.

in this experiment. Another possible explanation is that toxin may be produced in dead alewives while they are on the beach and that these fish are then washed back into the water during a storm. The colder lake water would tend to "preserve" the toxin. There are thus a number of factors that could influence toxin production in the Lake Michigan ecosystem.

An interesting phenomenon occurred in the TPSY-alewife cultures incubated at 30 C; the toxicities of the trypsin-activated samples decreased progressively as the level of fish extract in the cultures increased, while the toxicities of the non-activated samples increased as the level of fish extract increased. Statistical analyses of these data by the non-parametric Sign Test (Siegel, 1956) indicates that, at the 99% significance level, trypsin increases the toxicity of a culture when no fish extract is present but when 16% fish extract is present in a culture, trypsin decreases the toxicity. Examination of the mechanism of this phenomenon was accomplished by repeating the experiment at 30 C using three replicates each of the 0% and 16% levels of fish extract. However, prior to adding the fish extract to the culture, the extract was heated at 121 C for five minutes to denature any protein present. The results (Table 11) show that heated fish extract does not inversely affect the activation of toxin as does unheated fish. A two-way analysis of variance of these data (Table 12) indicates that there are no significant differences in the amounts of toxin produced between the 0% and 16% fish cultures or between the trypsin-activated and the non-activated samples. The non-parametric

Table 11. Summary of the toxicities of C. botulinum type E cultures grown in TPSY containing 0% and 16% heated alewife extract at 30 C.

Percent alewife	Toxicity (LD_{50})	
	Non-trypsinized	Trypsinized
0	670	22,865
0	335	2,860
0	335	13,335
16	335	2,860
16	400	13,335
16	600	13,335

Table 12. Results of the analysis of variance of the toxicities of the C. botulinum type E cultures grown in TPSY containing 0% and 16% heated alewife extract at 30 C.

Source of variation	Degree of freedom	Sum of squares	Mean square	F
Alewife levels	1	7,576,352	7,576,352	0.066
Trypsin	1	362,065,602	362,065,602	3.183
Interaction	1	53,583,067	53,583,067	0.471
Error	2	227,489,635	113,744,817	—
Total	5	650,714,656	—	—

test could not be used to analyse these data because of the small number of replications used. The small number of replications is also the most probable reason that significant differences were not found between the activated and non-activated toxin.

The conclusion that can be made from this culturing experiment using heated alewife extract is that the protein present in an alewife extract obviously affects, in some way, the toxin of C. botulinum type E. The protein probably affects the toxin at the molecular level and it is possible that it activates the toxin in a manner similar to trypsin. This is substantiated by the fact that the toxin found in the fish was activated. The mechanism of this "activation" cannot be exactly the same as trypsin because the molecule would not then lose its toxicity when treated with trypsin, as it did in this experiment.

Effect of successive botulinal toxin and DDT administration to the white mice. The results from injecting mice with both DDT and type E botulinal toxin are shown in Table 13 as the number of mice affected over the total number of mice injected with each level of toxin and DDT for both replications of the experiment. Inspection of these results indicated that males and females reacted similarly and so the data in this respect were combined. Statistical analysis of these results utilized a two-way analysis of variance, although the data were first changed to percent affected and then transformed with the Arcsin Transformation. This analysis (Table 14) indicates that, at the 97.5% significance level, there were differences between DDT levels as well as between toxin levels, and there was an interaction between

Table 13. Results from injecting mice with DDT followed 22 hours later by injections of C. botulinum type E toxin. Figures represent the number of mice affected over the total number of mice injected for each of the two replicates of the experiment.

DDT (mg/mouse)	Toxin Dilutions					Gelatin-PO_4 buffer
	1:50	1:100	1:150	1:200	1:300	
Oil only	9/10	0/10	0/10	0/10	0/10	0/10
	5/10	0/10	0/10	0/10	0/10	0/10
5.0	10/10	4/10	1/10	0/10	0/10	8/9
	8/10	6/10	3/10	1/10	0/10	7/9
7.5	9/9	8/10	4/10	1/10	0/10	7/10
	9/10	9/10	5/10	2/9	0/10	7/9
10.0	10/10	8/10	10/10	7/10	10/10	9/10
	9/10	8/9	9/10	5/10	0/9	8/9
15.0	10/10	10/10	10/10	8/10	9/10	9/10
	10/10	10/10	10/10	9/10	8/10	9/10

Table 14. Results of the analysis of variance of the mice affected by successive injections of DDT and type E botulinal toxin.

Source of variation	Degree of freedom	Sum of squares	Mean square	F
Toxin levels	5	19,534	3,907	18.19
DDT levels	4	32,886	8,221	38.27
Interaction	20	9,885	494	2.30
Error	30	6,445	215	—
Total	59	68,750	—	—

DDT and type E botulinal toxin when affecting mice as treated in this experiment. This analysis also indicates that the DDT treatment levels affected all mice similarly in the control group that received DDT only (F = 1.18).

The slope of the DDT-botulinal toxin interaction is obvious from the data; as the level of toxin decreases, it takes a greater amount of DDT to affect the mice. When an ED_{50} is computed by the method of Reed and Muench (op. cit.) for these mice affected on each level of botulinal toxin and each level of DDT, the slope can be graphically illustrated (Fig. 3). The slopes calculated by the least squares method for both DDT and botulinal toxin are virtually identical. It should be noted, however, that the 50% point computed for each level of DDT is dependent upon the level of toxin and vice versa.

To ascertain that it was the toxin and not some other material in the culture that caused the interaction, two groups of 50 mice were injected with the different levels of DDT. One of these groups was then injected with a 1:300 dilution of culture which had been treated with antiserum specific for C. botulinum type E toxin to inactivate the toxin from the culture. The results (Table 15) analyzed by the parametric paired t-Test show that, as opposed to the group of mice that received the 1:300 dilution of toxin not treated with antiserum (t = 2.768), a statistically similar number of mice at each level of DDT was affected in both groups (t = 0.521).

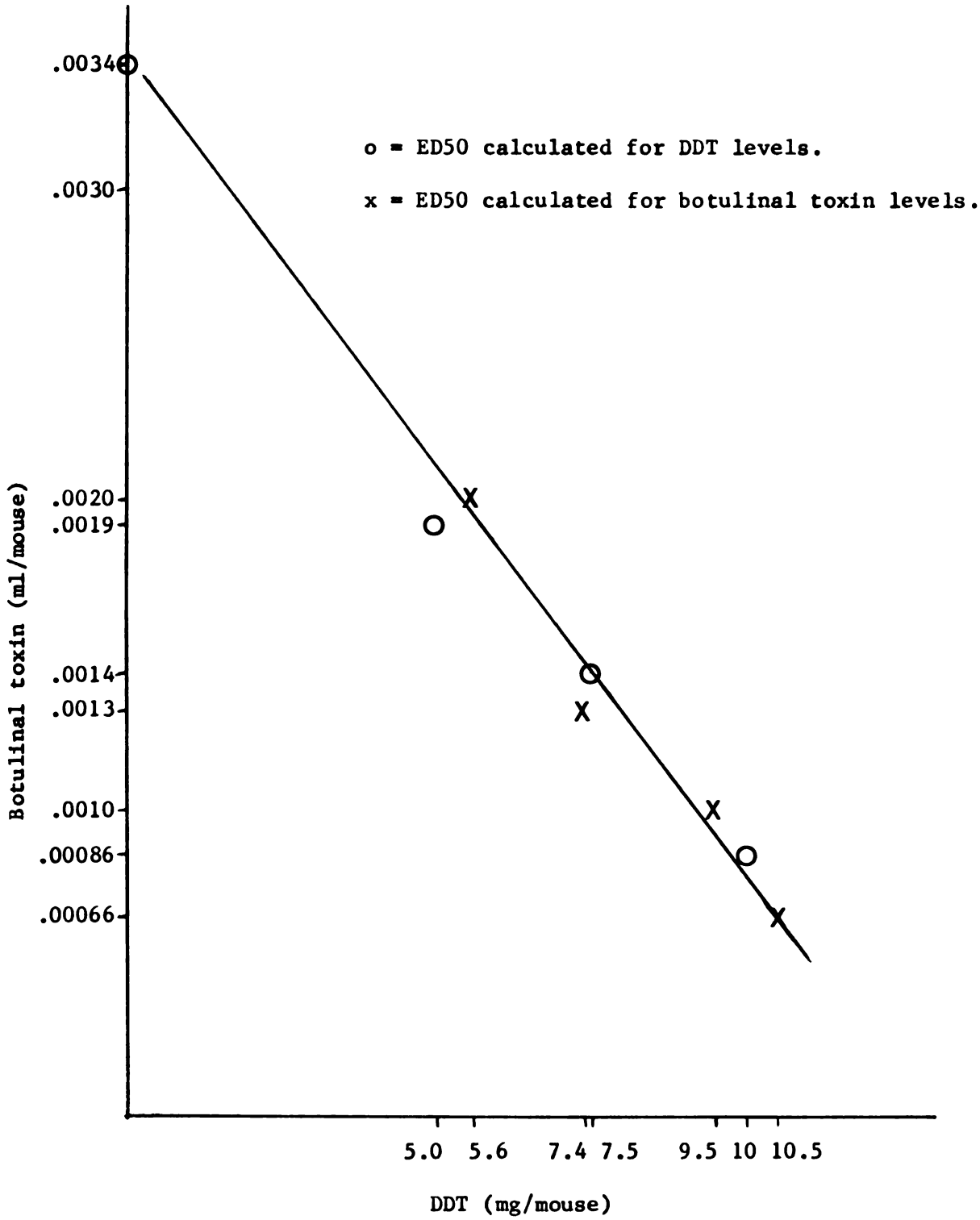

Figure 3. Points at which 50 percent of the mice become affected when injected with levels of DDT and C. botulinum type E toxin.

Table 15. Results from injecting mice with a 1:300 dilution of botulinal toxin protected with antiserum specific for type E toxin. Figures represent the number of mice affected over the total number of mice injected with each DDT level.

DDT (mg/mouse)	1:300 dilution of toxin treated with specific antiserum	Gelatin Phosphate Buffer
5.0	2/10	3/10
7.5	4/10	5/10
10.0	7/10	6/10
15.0	8/10	8/10

A possible explanation of this interaction lies with the modes of action of the two materials. The nervous system of DDT-poisoned animals responds to a single stimulus with a train of high-frequency impulses, resulting in tremors in the animal (O'Brien, 1967). Symptoms of poisoning have been correlated with the concentration of DDT in the central nervous system (Dale et al., 1963). The primary mode of action of botulinal toxins is preventing the release of acetylcholine at cholinergically activated nerve synapses (Brooks, 1964). Tyler (1963) noted that the toxin may affect the cholinergically activated inhibitory neurons of the spinal cord (Renshaw cells). Therefore, if the sublethal levels of the botulinal toxin used in this experiment inhibit the inhibitory cells of the spinal cord, the impulses caused by the DDT would be transmitted more freely through the central nervous system and thus elicit a greater than normal response. But, as the dosage of toxin decreases, the effect on these inhibitory neurons decreases although some effect may be present at the myoneural synapses, thus giving "protection" from the DDT-caused tremors.

Recognizing that caution must be exercised when extrapolating data from mice injected in the laboratory to animals as they occur in the wild, it does not appear that any ecological significance can be concluded when relating the results of this experiment to the Lake Michigan waterbird mortalities. At most, low levels of botulinal toxin may protect some individual birds from DDT, but it does not seem likely that large numbers of birds would obtain the precise quantities of the two materials that this experiment indicates is necessary for an antagonism.

CONCLUSIONS

The results of this study imply, quite conclusively, that the Lake Michigan waterbird mortalities are indeed caused by type E botulism. C. botulinum type E toxin was found in the ecosystem in a vehicle suitable for intoxication of waterbirds and the gull susceptibility experiments indicate that there was enough toxin in the dead alewives to affect many of the birds that might eat them. A remaining piece of information that would be desirable is an observation of a free-living waterbird actually consuming and then becoming affected by a naturally occurring toxic fish. This would be an extremely difficult task, to say the least. It would also be desirable to study the effects of various environmental stresses upon birds affected by sub-lethal levels of C. botulinum type E toxin.

The second objective of this study - to determine the reason for the apparent "sudden occurrence" of type E botulism in the Great Lakes - is, unfortunately, still not completely answered. This study indicated that DDT probably has not directly influenced the occurrence of the disease by either affecting the production of toxin or by acting synergistically with the toxin in affecting animals. However, DDT may be an indirect influence by killing fish which then may become a suitable medium in which C. botulinum type E can grow and produce toxin. But, no studies have yet proved DDT to be the cause of any fish mortality in the Great Lakes.

The role that the alewives play in the occurrence of type E botulism in the Great Lakes is still uncertain. This study has shown that at warmer temperatures an alewife can be a suitable growth medium for toxin production but, at colder temperatures, an alewife would not be a suitable medium for the production of toxin. However, the fact still remains that type E toxin was found in dead alewives taken from Lake Michigan. In any case, the recent advent of alewives in the upper Great Lakes does provide a potentially more available vehicle of intoxication of waterbirds than was present before their invasion. Another role that the alewives may have played in relation to type E botulism in the Great Lakes is the possibility that, since C. botulinum type E is considered to be of marine origin, the alewives may have introduced the organism into the Lakes when they invaded from salt water. This is indeed an interesting hypothesis.

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