LATEST DEVELOPMENTS IN RESEARCH ON BOTULISM[1]

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SUMMARY

Research on *C. botulinum* is proceeding throughout the world at a pace never before equalled, with greatest emphasis on type E. It is now clear that this organism is widley distributed in the aquatic environment and may be a frequent contaminant of fish and other marine animals. Much is still to be learned about its ecology and why it occurs in higher concentrations in some environments than in others. Although a large body of information has accumulated about the organism's characteristics, there is still much to be done before the public can be protected with certainty against further outbreaks.

Research on botulism received a dramatic stimulus in 1963. For the several preceding decades only 5 to 15 recognized outbreaks involving 20 to 25 cases occurred annually in the United States *(27)*. The great majority of these incidents were traced to under-processed home-canned fruits and vegetables. Commercial food processors, though generally aware of botulism's potential hazards, were not seriously concerned. Long experience had demonstrated the efficacy of modern food processing methods in protecting the consumer against this dread disease.

Our attitude of complacency was rudely shattered in 1963, when there were four distinct and well-publicized outbreaks of botulism for which the vehicles were commercially processed foodstuffs *(28)*. In fact, for the first time in 38 years commercially prepared foods were responsible for more cases of botulism in the United States than home-processed foods *(27)*.

The publicity surrounding these incidents alerted the public to the danger of botulism, and it also caused severe harm to certain segments of the food industry.

Although much was known about botulism and its causal organism in 1963, it soon became clear that more information was needed before we could confidently prescribe reliable preventive measures. The proceedings of a conference arranged by the Public Health Service in January, 1964, reviewed the state of our knowledge up to that time *(26)*. The succeeding years have seen a vast increase in the volume of research on botulism both in this country and abroad. The current interest in *Clostridium botulinum* centers around type E, undoubtedly because 3 of the 4 outbreaks of 1963 were caused by type E.

Type E botulism has been recognized as a problem associated with fish products in certain areas of the world for at least 20 years. Prior to 1964, however, only three or four laboratories in the United States were actively working with this organism. Notable among these were the groups of Dr. C. F. Schmidt of the Continental Can Company, Dr. L. L. Kempe of the University of Michigan, and the Food and Drug Administration in Washington, D. C. At the present time at least 24 groups in 21 universities, companies, or governmental installations in the United States alone are working on type E botulism. It is not possible to review all of the work being done since the results are only now beginning to appear in scientific journals. However, an overall view of the world's research effort was presented at a "Symposium on Botulism" held in Moscow, U. S. S. R. on July 20-22, 1966. Much of the substance of this review is from this symposium.

OCCURRENCE OF *C. botulinum* IN NATURE

The natural habitat of *C. botulinum* probably is the soil. Early surveys by K. F. Meyer and his associates in California revealed the spores of types A and B in soil specimens from many parts of the world. Later work by Dolman in Canada, Johannsen in Sweden, and several Japanese investigators similarly showed the widespread occurrence of type E, particularly in marine sediments and the intestinal contents of fish from northern waters. Johannsen *(19)* mentions especially British Columbia, Alaska, Japan, the Soviet Union, Scandinavia, and Western Europe. However, he also named Israel, the Mediterranean Sea, and the Gulf of Mexico as sources of the organism *(18)*.

Recent reports have extended and amplified our knowledge of the distribution of *C. botulinum* type E in nature. Nickerson and associates have demonstrated the organism in fish intestines and mud samples from the Gulf of Maine *(36)*. Cabelli and Richards found it in shellfish, mud and soil from marine estuaries in Rhode Island *(5)*. Ward and co-workers have found it in shrimp and bottom sediments from the U. S. Gulf coast *(36, 39)*.

In the Pacific Northwest, Craig and Pilcher have

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demonstrated type E in fish and mud from the Columbia River and along the coast of Oregon *(36)*. Eklund and Poysky found it in dungeness crab and in marine mud samples all the way from California to Alaska *(36)*.

Moving west, Japanese workers are still finding type E in Hokkaido and Northern Honshu (H. Iida, personal communication). Kravtchenko and Shishulina have reported that 10% of some 4,242 soil samples tested in the Soviet Union were positive for *C. botulinum (36)*. Type E was the most common, occurring in 62% of the positive samples, whereas type B was found in 28%, type A in 8%, and type C in 2%. Almost one third of 1365 fish intestines also proved to carry *C. botulinum*.

The only area that has consistently failed to yield type E is the British Isles. Soil and shorelines samples there have been uniformly negative, as have fish from the North Sea and shellfish from British coastal waters (Cann et al., *36;* G. Hobbs, personal communication).

In 1960 smoked fish from Lake Superior caused an outbreak of type E botulism in Minneapolis. Later, in 1963, two other outbreaks in this country were traced to smoked fish which probably came from Lake Michigan. These events strongly suggested that type E spores may occur naturally in the Great Lakes and constitute a previously unrecognized hazard to public health.

Since 1963 the people in our laboratory have been studying the ecology of type E in the Great Lakes. The organism has been found in fish from all four of the westernmost lakes, although its incidence was highest in fish from Lake Michigan and lowest in those from Lake Superior *(3)*. The presence of type E in these waters has been confirmed by Graikoski and co-workers at the Ann Arbor laboratories of the Bureau of Commercial Fisheries (J. Graikoski, personal communication) and by Pace and associates at the Milwaukee City Health Department *(36)*. Type E has been found in fish from Lake Cayuga in New York State *(7)* and in a lake of the Tennessee Valley *(16)*.

The work of Johannsen *(18)* revealed an unusually heavy concentration of type E spores in the Baltic Sea and particularly in the sound separating Denmark from Sweden. A similar heavy concentration exists in Green Bay of Lake Michigan *(3)*. Well over half the fish and practically all of the mud samples from this bay have proved to harbor type E. Currently we are trying to learn why certain bodies of water are more heavily contaminated than others.

There is no reliable way to quantitate type E organisms in samples from nature. The usual expression of concentration is a percentage of positive samples among the total tested. Unequivocal demonstration of type E toxin in an enrichment culture is sufficient evidence to indicate the presence of the organism, but a negative test for toxin is not necessarily proof that the organism is absent. Other microorganisms in the sample may inhibit the growth of type E or they may destroy the toxin as it is formed. Therefore, on the basis of evidence now available it must be assumed that *C. botulinum* type E may be a contaminant of fish from any waters whether fresh or salt. Therefore, protection against botulinum poisoning requires processing and handling methods that will insure either *(a)* destruction of the organism, or *(b)* prevention of its growth.

GROWTH OF *C. botulinum* TYPE E IN FOODS

Johannsen *(17)* was the first to report that type E grows poorly if at all in certain foodstuffs. It produced toxin readily in fresh or cooked herring and cod, but not in shrimp, crabmeat, and a variety of cured meat and fish products. Pivnick and Barnett *(36)* in Canada also inoculated several cured meats with type E spores and observed toxin formation only in one, a jellied ox tongue containing 1.5% salt. Folmer Nielsen and Pederson *(36)* could not obtain type E toxin in smoked salmon and attributed this to inhibition by formaldehyde absorbed during smoking. Likewise, Abrahamsson *(36)* did not find toxin in smoked eel after inoculation with spores and incubation at 20 C for 10 days. On the other hand, Cann et al. at the Torry Research Station in Scotland obtained toxin formation in several fish products, including smoked salmon, after inoculation and storage at 40 F *(6)*. In our experience smoked chub readily support growth and toxin formation if the salt content is minimal.

The occurrence of type E toxin in lightly salted smoked fish products naturally has led to consideration of possible hazards in cured meats. Under conditions of abuse many of these products will support the growth of *C. botulinum* types A and B (Pivnick and Barnett, *36*), yet billions of pounds of cured meats have been marketed in the U. S. without incident. The explanation may be the lack of contamination with spores of *C. botulinum*. Greenberg and co-workers at Swift & Company *(36)* recently examined 2,358 samples of beef, pork, and poultry from packing plants in the U. S. and Canada. Three-fourths of the specimens carried less than 3 putrefactive anaerobic spores per gram, and the most heavily contaminated sample of all contained only 115 P. A. spores per gram. Of 19,727 clostridial cultures isolated, the single *C. botulinum* identified was a type C.

FACTORS AFFECTING GROWTH

One of the unique features of *C. botulinum* type E is its ability to grow at low temperatures. First Schmidt and his associates *(31)* and later Kempe *(24)* observed growth of type E at 37-38 F, well within the normal range for storage of refrigerated foods. These experiments were run with large inocula of spores in favorable culture media.

There is, of course, a relationship between size of inoculum and conditions necessary to prevent growth. Large numbers of spores may be expected to tolerate more salt or more acid than small numbers. Combinations of two or more unfavorable (but not necessarily limiting) conditions should be more effective than either one alone. Ample support for these assumptions has appeared recently.

Spencer *(36)* inoculated cured meat with 100 spores per gram of *C. botulinum* types A, B and E and varied the concentrations of curing ingredients. The amounts of salt and nitrite necessary to prevent outgrowth were lower than had been reported previously with larger inocula. Riemann *(36)* also showed that large numbers of type E spores can tolerate and grow in higher concentrations of salt than small numbers. Furthermore, large numbers of spores will grow at a lower pH than small numbers. Segner, Schmidt and Boltz *(32a)* observed growth down to pH 5.21 with 2 million type E spores per tube and pH 5.03 with 20 million.

Several workers have demonstrated a relationship between two or more environmental factors. Ohye, Christian and Scott *(36)* found the limiting concentration of salt for one strain of type E to be: 5.8% at 25 and 30 C; 5.1% at 20 C; and 4.3% at 15 C. According to Segner, Schmidt and Boltz *(32a)*, 5% salt was required to prevent growth at 16 to 30 C but only 4.5% was necessary at 8 to 10 C. Outgrowth time naturally is delayed as the salt concentration approaches the limiting value *(32a;* Pivnick and Barnett, *36)*. Riemann *(36)* found that type E could tolerate less salt as the pH approached 5.0. At 8 C Segner, Schmidt and Boltz *(32a)* observed growth at pH 5.9 but not at pH 5.7. However, at 30 C the organism grew at pH 5.2.

Attempts to find reliable chemical additives to prevent toxin production in foods—especially smoked fish—have received considerable attention. Those tried with some success include the antibiotic, Tylosin *(33)*, benzoate and nitrite *(32)*. Unfortunately it is not yet possible to assure uniform distribution of salt and other inhibitory chemicals in the tissues of intact foods such as fish *(40)*.

Vacuum packaging of food products has increased rapily during the past decade. The involvement of vacuum packaged smoked fish in two outbreaks of botulism has suggested to some that vacuum packaging may be responsible for the growth of *C. botulinum*.

Several groups of investigators have compared the rates of growth of *C. botulinum* in vacuum packaged and non-vacuum packaged cured meats *(8; 37;* Pivnick and Barnett, *36)*, smoked fish *(22)*, irradiated fresh fish *(1)*, and various other foods *(17)*. All reports are in general agreement that *C. botulinum* grows about the same whether the food is vacuum packaged or not. In other words, the composition of the food and other environmental conditions regulate growth, not the type of package.

It is true, as many have observed, that vacuum packaging prevents the growth of certain aerobic spoilage organisms and thereby may deny the consumer a possible warning sign that the product has been mishandled. It must be remembered, however, that non-vacuum packaged foods also may become toxic without showing obvious signs of spoilage. Therefore, the elimination of vacuum packaging would not guarantee safety from botulism.

Most of the work with type E is done with complex natural media, but a chemically defined medium is needed for nutritional studies of sporogenesis and toxigenesis. Several investigators have prepared synthetic media in which type E will multiply, but growth is sparse and morphology of the cells is atypical. Recently Snudden and Lechowich of Michigan State University have developed a chemically defined medium in which type E is said to grow with normal morphology, produce toxin and sporuate *(36)*. The medium is a modification of Difco's tissue culture formula No. NCTC109.

RESISTANCE OF BOTULINUM SPORES AND TOXIN

Another distinctive feature of type E is the relatively low heat resistance of its spores. Decimal reduction values (D values) at 80 C usually are less than 2 minutes *(29)* when the spores are heated in water, buffer or culture media. They are somewhat more resistant in fish and other protective materials, but still are far more sensitive than the spores of the other types of *C. botulinum*. The possibility that small numbers of resistant spores exist among a majority of relatively sensitive ones has been suggested *(13)* and has not yet been ruled out with certainty.

Spores of *C. botulinum* type A are among the most resistant of all microorganisms to ionizing radiations. Exposures on the order of 4.0 to 5.0 Mrad are required for destruction of 10^{12} spores (12 D), the commonly accepted baseline. Low intensity radiation of fresh fish to extend refrigerated storage life has shown considerable promise, but the low tem-

perature growth potential of *C. botulinum* type E has stimulated interest in the radiation resistance of both its spores and its toxin.

Radiation D values on the order of 0.12 to 0.17 Mrad have been obtained for type E spores *(29)*, placing their resistance close to that of types A and B. The radiation resistance of type E toxin also approaches that of type A toxin (D = 2.1 Mrad), suggesting the impracticality of detoxifying food products with ionizing radiation *(35)*. Miura and co-workers (G. Sakaguchi, personal communication) have shown that proteins and certain other nitrogenous compounds protected type E toxin against inactivation by radiation. The purer the toxin, the more easily it was inactivated.

Ito and others in California *(36)* have shown that chlorine is an effective lethal agent for *C. botulinum*. Exposure of the spores to 4.5 ppm free available chlorine in phosphate buffer at pH 6.5 caused 99.99% reduction of viable type E spores in 4 to 6 minutes and of types A and B in 3 to 8 minutes.

CHARACTERIZATION OF BOTULINAL TOXINS

As in the past, there is still much interest but uncertainty in the nature of the botulinal toxin, that unique "most poisonous poison" *(25)*. Type A toxin as originally crystallized is a protein of molecular weight 900,000. This toxin preparation is a complex in intimate association of the specific neurotoxin and a red blood cell agglutinating (hemagglutinating) factor, these being separable by appropriate procedures. In addition, the toxin can be dissociated into toxic particles of low molecular weight *(38)*. Recently, Boroff and associates who have presented evidence that tryptophane is necessary for toxicity *(2)* reported the separation of crystalline type A toxin by chromatography into 2 fractions of greatly different toxicities *(36)*. The possibility that the molecular weight of nearly one million of the crystalline type A toxin is the result of aggregation of smaller molecular weight units was presented on the basis that a different purification procedure gave essentially pure toxin molecules of around 12,000 molecular weight *(12)*. However, Schantz and Spero have recently calculated that the molecular size of toxin of type A (and other toxin types) as they are found in the crude toxic culture fluids is close to that found for the crystalline type A toxin *(36)*.

Type E toxin also is receiving attention, partly, no doubt, because of its unique property of activation by certain proteolytic enzymes. Treatment of the toxin as found in cultures with trypsin increases its lethality for mice by 10 to 100 fold.

Dolman's group in Canada *(11)* give the molecular weight of the purified toxin as 14,000 to 16,000, and that of the activated product after treatment with trypsin as 10,000 to 12,000. Sakaguchi's group in Japan, on the other hand, estimate the molecular weight of purified type E toxin as more than 200,000 *(30)*. Under their conditions, treatment with trypsin caused no reduction in the molecular size. Similar results were obtained both with toxin from pure cultures and from "izushi." Thus further work will be necessary to reconcile the differences and to clarify the mechanism of trypsin activation.

On the subject of toxin, Grecz and Lin *(36)* have reported the presence of heat resistant toxin in type A spores but not in types B and E. It was calculated that each spore contained about 500 molecules of toxin as compared with 500,000 molecules in a vegetative cell. As few as 50,000 young spores were lethal to mice, although their toxicity decreased on storage.

METHODS OF ISOLATING AND IDENTIFYING *C. botulinum*

Detecting *C. botulinum* in natural materials is complicated by the presence of other organisms, which may interfere with its growth. For this reason it once was customary to heat a sample to 80 C for 10 minutes to eliminate non-spore forming bacteria. However, this procedure cannot be used with type E because its spores are extremely sensitive to heat.

It is now clear that mud, soil and similar materials often contain organisms that strongly inhibit the growth of type E and interfere with its detection by existing methods. Kautter and co-workers *(23)* have described several bacteria that do this. In addition to their inhibitory property, some of these cultures exhibit all of the morphological, physiological and biochemical characteristics of type E except toxigenicity. We have encountered similar organisms, as have many other investigators *(4, 14;* K. Yamamoto, personal communication; H. Iida, personal communication).

The so-called "E like" organisms also complicate the isolation of toxigenic type E cultures from enrichment cultures in which the toxin has been clearly demonstrated. The "E like" colonies are indistinguishable from those of toxigenic type E organisms even on the liver veal egg yolk agar used in the "alcohol procedure" of Johnston and co-workers *(21)*. This procedure is excellent with certain materials, but it has not proved useful with mud and fish samples from the Great Lakes.

Many attempts have been made to adapt the fluorescent antibody technique to the identification to *C. botulinum*, especially type E, in mixed cultures. Thus far the method has not proved useful for this purpose.

A method of detecting botulinum toxin without employing test animals has just been described by Johnson and co-workers *(20)*. In this procedure, formalinized sheep red blood cells are sensitized with type specific antitoxin. When homologous toxin is added, hemagglutination occurs. As little as 0.75 to 1.3 mouse LD_{50} of type A toxin or 2.3 LD_{50} of type B toxin could be detected with this system. Further work on this procedure is under way in our laboratory with two main objectives: *(a)* adaptation to type E, and *(b)* detection of botulinal toxin in foods.

ANIMAL BOTULISM

Outbreaks of type C botulism in mink have occurred recently in Russia (Bulatova et al. *36)* and in Japan (H. Iida, personal communication). Skulberg *(34)* also reported outbreaks caused by type E in Norway and Denmark.

In the U. S. there has been considerable interest in the possibility that large "die offs" of gulls and other fish-eating birds in Lake Michigan might be caused by type E toxin. Kaufman and associates *(36)* were able to kill gulls by feeding 60,000 to 140,000 mouse intraperitoneal LD_{50} doses of type E toxin. Jensen and Gritman, on the other hand, were unable to intoxicate ducks and gulls by feeding as much as 3,000,000 mouse lethal doses *(36)*. They did, however, observe an adjuvant effect when types C and E toxins were fed simultaneously.

C. botulinum TYPE F

This type was recognized in 1959 as the cause of a single small outbreak in Denmark. There have been no other known incidents, although the organism has been isolated from marine mud taken off the Pacific coast *(10)* and in a salmon from the Columbia River *(9)*.

According to Walls *(36)*, type F will grow and produce toxin at temperatures as low as 4 C (39 F). The toxin is activated by trypsin like that of type E. The type F toxin and the "precursor" is apparently formed intracellularly since rupturing young cells results in their release *(15)*.

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ADVANTAGES CLAIMED FOR SANI-GUIDE SYSTEM

The Kendall Company, manufacturers of Kendall Sani-Guide Pipe-Line Inserts, claim that their Sani-Guide system offers a number of advantages as a practical bulk milk quality check.

In-line filtration has accompanied the growth of bulk tank milk handling and, according to the Company, this also has created problems. Too often large quantities of milk per unit of filtration overload the capacity of the product and high velocity pumps, introduced for in-place cleaning and used also for moving milk through filtering media, frequently cause breakage of single service filters. Usage of woven reusable filter media to solve the breakage problem has created a greater evil in terms of bacteria build-up. It was known that nylon had properties of catching hair, lint and insect parts and this led to experimenting with a proper nylon mesh mounted between paper gaskets, resulting in a device know as Sani-Guide Pipeline Inserts.

Among the advantages claimed is the fact that the producer can readily check his own operation by examining the inserts. Also, field and inspection personnel no longer accept excuses about tests being confused with those of neighbors or that extraneous material has blown into the tank truck during loading and delivery . There is proof whether or not the producers are maintaining adequate control by the material shown on the Sani-Guide.

Good results have already been achieved by segments of the dairy industry who have used the Sani-Guide program, the manufacturer states. A number of state and local health departments are using the program for spot-checking producers.