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Clostridium botulinum Neurotoxin

H. SUGIYAMA

Food Research Institute and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

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INTRODUCTION

Botulism is the syndrome resulting from the action of the toxin produced by *Clostridium botulinum*. This toxin blocks the passage of nerve stimuli at nerve endings and causes a bilateral neuromuscular weakness or paralysis in which impairments of cranial nerve functions (diplopia, dysarthria, and dysphagia) are prominent features. Respiratory failure is the usual cause of death. Gastrointestinal effects, such as diarrhea, may precede the development of neuromuscular paralysis, but constipation is common as the illness progresses. Convalescence may require months.

Until recently, the interest of microbiologists

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in botulism has been focused on the toxin and the food poisoning that follows ingestion of the toxin. Although its incidence is low compared with several other food poisonings of bacterial origin, botulism is important because the high toxicity and unique pharmacological action of the toxin cause severe illness. The recent identification of a different pathogenic form of botulism has produced additional interest.

The purposes of this review are (i) to discuss briefly the forms of botulism, which differ in the way that the toxin is acquired, with emphasis on a newly discovered form, infant botulism, and (ii) to discuss the structure and biological properties of the toxin. The similarities among the several antigenic types of botulinum toxin and between botulinum and tetanus toxins are considered. The growth of the organism and toxin production in foods are, for the most part, beyond the intended scope of this review. It has not been possible to cite individually all of the many publications; where omissions are made, the indicated references can be used to identify the original sources of information.

PATHOGENIC FORMS OF BOTULISM

The toxin that causes botulism can be acquired by one of three ways. The classically known botulism food poisoning is due primarily to toxin that is formed in and ingested with a food. In infant botulism, which may prove to be the most common form of botulism, toxin is produced in vivo during intraintestinal growth of the organism. Wound botulism also is caused by toxin formed in vivo, but in this case the toxin is formed in a wound.

Food Poisoning

Undoubtedly, some outbreaks of food poisoning, particularly those which occurred in earlier years, have not been entered into the compilation of outbreaks in the United States, but records list 766 outbreaks involving 1,961 cases between 1899 and 1977, with 289 outbreaks (680 cases) of these occurring after 1949. The overall case fatality rate is 50%, but this decreased to 19% for the period from 1960 to 1977. The toxin type responsible for more than 50% of the outbreaks is not known, but 26% of the outbreaks were due to type A, 8% were due to type B, and 4% were due to type E; one outbreak was due to type F, and two were due to a mixture of type A and type B toxins (31). The outbreaks involving the most cases are two recent occurrences; one in 1977 caused illness in 58 persons (210), and another in 1978 involved 34 cases (29). Home-prepared foods are more important than commercially processed foods. In this country, vegetables and fruits are more common vehicles of botulism than are meat or fish.

The incidences of botulism in other countries have been summarized previously (189). Most have occurred in the northern hemisphere, but cases have been reported from India, Argentina (D. F. Gimenez, personal communication), and Kenya (188). Since *C. botulinum* is found in the tropics and the southern hemisphere, the disease potential is worldwide. The apparent rarity of botulism in these areas may be because the disease is not recognized, because it is not reported, or because the foods used by the native populations are less subject to botulinogenic spoilage (189).

Wound Botulism

Wound botulism is the rarest of the botulism forms, with only 18 cases recorded in this country through 1977 (31). This illness results when *C. botulinum* by itself or with other microorganisms infects a wound and produces toxin, which reaches other parts of the body via the blood stream. A local lesion distinguishes this botulism from food poisoning, but the neurological effects are comparable. Only type A and type B cases have been reported.

Infant Botulism

Definite proof of clinical infant botulism was obtained in 1976 when *C. botulinum* and/or toxin was demonstrated in the feces of infants suffering from constipation and neuromuscular weakness (1, 128). More than 100 cases have now been reported from different parts of the United States (30). Although only two deaths are known, more would have occurred if emergency respiratory aid had not been available. The disease probably occurs worldwide since single cases have been reported in England (214), Australia (176), and Canada (28). As shown by the reclassification to infant botulism of an illness that occurred in 1931 (4), this is not a new disease but one whose true nature escaped identification. Only type A and type B cases have been reported.

Infant botulism gains even greater significance with the possibility that a fulminating version may be one of the causes of the sudden infant death syndrome (3). Among specimens from 211 sudden death cases, the bowel contents of 9 infants contained *C. botulinum*, and 2 of these also had the homologous type of toxin. These findings contrast with the occurrence of *C. botulinum* in only 1 fecal specimen from 160 age-

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matched healthy (control) infants.

Except for one case involving a 35-week-old child, infant botulism has occurred only in children between 3 and 26 weeks old (median age, 2.5 months). Type A cases are slightly more frequent than type B cases. The cases are epidemiologically independent events in infants fed formula or breast milk only or in infants receiving some supplements to milk (2). Since no case can be attributed to a food that contained toxin, the presence of *C. botulinum* and its toxin in the feces of acutely ill infants and convalescents indicates that the illness is a toxico-infection in which the organism colonizes and produces toxin in the intestinal tract (1, 53, 128). In the sense that the organism is most likely acquired by swallowing, infant botulism is a food poisoning. Spores are probably the infective form.

The concept that *C. botulinum* can multiply intraintestinally is not new. Russian investigators suggested in approximately 1960 that in vivo-formed toxin contributes to food poisoning, but the idea was not generally accepted because the supporting data were obtained by a procedure that was considered by others to be inappropriate (189). This skepticism has disappeared with the more recent finding of the organism and its toxin in the stools of a convalescent 32 days after the onset of illness, when the originally ingested materials would probably have been excreted or inactivated (54). However, there is as yet no proof that adults develop botulism by eating foods, such as raw vegetables, which may occasionally carry *C. botulinum* but not the toxin.

Observations made on adults of common laboratory animal species support the belief that inocula of small numbers of only *C. botulinum* spores do not readily colonize the intestinal tracts of human adults. When spore suspensions were treated to inactivate free toxin, only a few animals which were fed 10^7 to 10^9 spores developed botulism. In some instances, the toxin that caused the illness could have been released from the spores instead of being produced during vegetative multiplication in the host (189). In contrast to adults, infant animals can be infected enterically with much smaller challenge doses if colonization is followed by measuring production of toxin instead of overt botulism.

Intestinal botulinum colonization occurs when *C. botulinum* spores are inoculated intragastrically into conventionally raised mice 8 to 14 days old, but not when they are given to younger or older infants. Pups 8 to 11 days old at the time of challenge are the most susceptible. Colonization does not cause illness even though up to 2,000 adult mouse intraperitoneal 50% lethal doses (i.p. LD_{50}) are present in the colon and cecum and the toxin is detected from about 1 to 6 days postchallenge (199, 202). Since infant mice susceptible to infection are as sensitive as adults to the lethal action of parenterally administered botulinum toxin (D. C. Mills and HL Sugiyama, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, P11, p. 212), the continued well-being of botulinum-colonized infants is probably due to toxin being localized in the colon, where it would be poorly absorbed. The 50% infective dose of one type A culture strain is 170 spores per mouse. Essentially identical data have been obtained with rats (138a).

Toxin is not produced in conventional adult mice that are fed 10^6 spores, but all germfree adult mice are infected when challenged with 10 spores. When these highly susceptible axenic mice are exposed for 3 days to a colony of normal mice, they become as resistant as the conventionally raised adults. The formerly germfree mice have a variety of microbial forms in their feces when they are challenged with the spores (138).

The results obtained with the mouse and rat models support the proposition that the botulism of human infants has a microbial ecological basis (1, 53). The intestinal microflora of older individuals probably includes one or more species which, acting independently or in concert, prevent the growth of *C. botulinum*. Infants are susceptible because the microbial antibotulinum barrier has not yet developed or is easily displaced. Since germfree infants less than 1 week old cannot be infected, their resistance is due to factors which do not involve bacteria (138).

A toxico-infection is important in botulism of chickens. This infection differs in that susceptibility is not age dependent. The important growth of the organism takes place in the cecum (136).

Of the foods known to have been fed to infants who developed botulism, honey is the only one in which *C. botulinum* has been found (2, 129). A survey of samples from many parts of the United States suggests that this organism can probably be found in honey produced in every state if sampling is extensive enough (203). Since type B infant botulism cases are more common among infants fed honey, an unnecessary risk is taken when this supplement is fed to infants younger than 1 year (2). The source of the organism for cases who were not given honey is not known. *C. botulinum* of the type causing the illness has been recovered from vacuum cleaner dust, yard soil, and the soil of a potted plant, all of which were collected from homes of patients (2).

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CULTURES AND TOXIN TYPES

The property that separates *C. botulinum* from other clostridial organisms is the production of a neurotoxic protein which causes flaccid paralysis by acting at peripheral cholinergic junctions. The antigenically different toxins are identified as toxin types. The same neurotoxin is consistently produced by a particular culture, but toxins of different antigenic specificities are produced by different groups of culture strains.

Culture-Toxin Relationships

The established toxin types are A, B, C_1, C_2, D, E, F, and the type which was identified last, type G (33, 69). Some authors refer to seven toxin types, apparently considering C_1 and C_2 as subtypes. As discussed below, this interpretation is not justified since these two toxins are serologically completely different.

The one exception to complete antigenic difference between toxin types is the minor relatedness of types E and F. This was first shown by the neutralization of a few LD_{50} in a crude type F toxin sample by a large amount of anti-E serum that was obtained with crude immunizing agent (139), and a slight reciprocal cross-neutralization is also observed when the reactants are purified neurotoxins and antitoxins prepared with them (169, 230). Type B neurotoxin differs from the other toxin types in that the neurotoxic molecules of different type B cultures can be similar but not identical (see below).

Cultures are divided into types that correspond to their toxin types. Since many cultures produce only one type of toxin, culture typing is practical, but there are two categories of exceptions to the one culture-one toxin type generalization. One of these concerns cultures producing type C and type D toxins and is the reason for the apparent confusion about C_1 and C_2 toxins being subtypes. Type C cultures were separated into subtypes C_α and C_β when the antitoxins made with crude toxins did not protect against the toxic fluids of all cultures; anti-C_α serum neutralized the toxicities of all cultures, but anti-C_β serum neutralized only some (160). In addition, it was later shown that anti-C_α and anti-D sera partially cross-neutralize the toxicities in type C and type D cultures (26, 123).

These serological interrelations are explained by some cultures which produce several types of toxin. C_α cultures produce type C_1, C_2, and D toxins, with type C_1 as the dominant toxin; type D cultures produce the same mixture, but with type D toxin dominant; and C_β cultures produce only type C_2 toxin (62, 95). Some C_α and D strains do not produce C_2 toxin. Monospecific anti-C_1 serum does not neutralize the toxicity of C_2 toxin, and anti-C_2 serum does not neutralize C_1 toxin.

The other exception to the one culture-one toxin rule is the cultures whose toxicities are neutralized completely only by a mixture of type A and type F antitoxins (68). Since type A or F antitoxin precipitates out the toxicity of only the homologous antigenicity, the dual antigenic specificities are not due to both type A and type F antigenic determinants being present on a single toxic molecule but occur because the cultures produce separate molecules of type A and type F antigenicities (204). The type F toxicity is estimated to be 1 to 10% of the total. These cultures, designated subtype Af, are stable, natural forms since other isolations have been made from soils from different parts of Argentina (70).

Culture Groups

C. botulinum cultures differ in important properties. If toxin production is not used as the justification for species determination and the taxonomic criteria applied to other species are considered, the cultures can be separated into four groups. Group I cultures are ovolytic (proteolytic) strains which produce toxin type A, B, or F; group II cultures are non-proteolytic strains which produce toxin type B, E, or F; group III cultures are weakly proteolytic or non-proteolytic strains which produce toxin type C_1, C_2, or D; and group IV contains the proteolytic but non-saccharolytic strain that produces type G toxin (189). Proteolytic strains that produce type E toxin have been isolated recently (142).

Cultures of one group are closely related among themselves but differ in important ways from cultures of another group, so that the cultural characteristics are not always evident when a strain is identified only by its toxin type. Except for the antigenicities of the toxins, a type B culture of group I is very similar to a type A strain but quite different from a type B culture of group II. Intragroup homogeneity and intergroup heterogeneity are shown by the antigens of vegetative cells (191, 219) and of spores (190), by homologies of deoxyribonucleic acid (119, 120) and ribosomal ribonucleic acid (96), and by susceptibility to bacteriophages (201).

All human botulism cases are caused by cultures of groups I and II. Cultures of these groups differ in the properties which influence the potential for causing food poisoning. Spores of group I cultures have high heat resistance relative to spores of group II cultures (122), and group II cultures can grow at a lower temperature (about 4 versus 10°C) than cultures of group I (192). Group III cultures are important causes of botulism in several animal species (189).

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As a rule, the toxin in culture fluids of group I strains is at or near its maximum possible toxicity, whereas the toxin of group II cultures must be activated with trypsin before it becomes fully toxic. This difference is due to the enzymes produced by the cultures and is reflected in the molecular form of the purified toxin (see below).

GENETICS OF TOXIN PRODUCTION

When subcultures are made of isolated colonies that are obtained by streaking a type C culture grown in acridine orange-containing medium, some are "nontoxigenic" in that they no longer produce the dominant C_1 toxin of the parent culture. This loss of toxigenicity is permanent since reversion to toxin production does not occur during repeated passages. When a mitomycin C-induced, cell-free lysate of the parent culture is added to an actively growing culture of a cured strain, cell lysis occurs, but many of the survivors produce C_1 toxin. Since the toxigenic cultures recovered by this procedure are not lysed by the mitomycin C-induced lysate, the loss and reacquisition of toxigenicity have been attributed to the curing of and reinfection by a lysogenizing bacteriophage, respectively (92).

Similar results have been obtained with a type D culture. Moreover, when a particular cured type D strain was grown with the lysate of a toxigenic type C culture, some of the subcultures recovered from the incubation produced type C_1 toxin. The cured strain used in this experiment was not reconverted to produce type D toxin (93).

The lysogeny-toxigenicity relationship was established more firmly when it became possible to purify the phages of the toxigenic cultures by forming plaques on lawns of nontoxigenic indicator cultures. Curing type C and type D cultures of their prophages made them nontoxigenic, but reinfection with a known phage restored toxin production. Two phages were isolated from a type C culture, but only one affected toxin production (64, 65). The culture-phage relationship is a pseudolysogeny. The tox^+ phage is lost when the culture is grown in the presence of anti-phage serum (93) or when the spores are heated at 70°C for 20 min (64). Cultures normally remain toxigenic by cells being infected during growth (146).

Type C and type D cultures can be interconverted with respect to the toxins which they produce (63). Although not possible with all strains (93), some cured type C and type D isolates are lysogenized by tox^+ phage of the heterologous culture type so that a type C culture becomes a producer of type D toxin and a type D culture becomes type C_1 toxigenic. When such cultures are cured of their tox^+ phage and are reinfected with the phage of the parent, they become indistinguishable from the starting cultures.

Cultures cured of prophages so that they no longer produce the dominant toxin continue to produce C_2 toxin. Interestingly, the elimination of the tox^+ phage for the dominant toxin results in a cured strain which does not produce the other minor toxin (C_1 toxin of type D cultures and D toxin of type C cultures). These observations (63) suggest that the same prophage controls two types of toxin, but the possibility of a common antigen in C_1 and D toxins apparently has not been tested.

Conversion to toxin production occurs only when a cured culture is paired with certain tox^+ phages. One reason for this specificity is the antigenic differences of the converting phages. Morphologically similar tox^+ phages for C_1 and D toxins fall into three antigenic groups, whose members productively lysogenize only those strains which have the receptors needed for phage attachment (150). However, other factors play a role since some antigenically dissimilar phages can convert the same cultures (78). tox^+ phage can absorb to but not convert certain cultures to toxin production (151). Some of these nonconverting adsorptions result because the cells carry a tox^- phage, which makes them immune to infection by the tox^+ phage (148).

Converted cultures produce different levels of toxin. Toxigenic cultures recovered from a lysogenizing incubation that results in extensive cell lysis produce less toxin than cultures from treatments resulting in minimum lysis (78). A culture converted by tox^+ phage that has been passed repeatedly through its nontoxic indicator culture produces less toxin than a culture converted by phage which has had fewer such passages (147).

Although many details remain to be understood, the evidence that lysogenic bacteriophages control production of C_1 and D toxins is convincing. A similar phenomenon has not yet been demonstrated for the other botulinum toxin types, although nearly all cultures carry temperate phages (52, 91). Some of these cultures carry plasmids (175), but a relationship to toxin production has not been reported. If C_2 toxin production depends on sporulation (140), this is not the case for types A and E since asporogenic L-form cultures of these types are toxigenic (25).

ASSAY OF TOXIN

Botulinum toxin is identified as the mouse lethal agent whose toxicity can be neutralized

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by one of the type-specific antitoxins. This animal has advantages other than cost. It is highly susceptible to all known toxin types, and individuals over a wide range in body weight are equally sensitive to the toxin (114).

In Vivo Quantitation

Toxicity is best titrated as mouse i.p. LD_{50} by determining the sample amount which, if injected i.p., kills 50% of a representative mouse population. However, the results obtained by laboratories can differ by as much as four- to fivefold. Such variation is reduced if the sample toxicity is determined in relation to the toxicity of a standard type A toxin preparation (171).

The i.p. procedure is cumbersome, and relatively large numbers of mice are needed. For some purposes, a substitute is the rapid intravenous method, which is based on the inverse relationship between the time to death and the number of i.p. LD_{50} injected intravenously (19). The toxicity of a sample can be obtained by reading from a standard curve the i.p. LD_{50} represented by the average death time obtained with two to four mice. The usable portion of the standard plot is the straight line portion between approximately 30 and 120 min (10^6 to 10^3 LD_{50}), depending on the type of toxin. The precision of this method is improved by a parallel line assay with a standard toxin preparation (168).

The standard curve must be made with not only the same toxin type but also the toxin form (see below) present in the sample; if different toxin forms are used, it must be known that intravenous injections of identical i.p. LD_{50} of the different toxin forms kill mice at similar rates. Otherwise, the i.p. toxicity determined with the intravenous method may be misleading (117).

Infant-Potent Toxin

Most samples of type A, B, and E toxins are about five times less lethal for adults than for 8- to 10-day-old mice (not corrected for differences in body weight) when injected i.p. However, the type B toxins of some cultures have unusual toxicity in that they are 500 to 5,000 times more lethal for infants than for adults (Mills and Sugiyama, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, P11, p. 212). The toxicity difference is also observed when these toxin preparations are given orally. Most of the cultures producing infant-potent type B toxin include isolates from infant botulism cases. Experimental intestinal botulinum colonization of infant mice by these cultures is not detected if adult mice are used to test for the toxin in the gut.

Serological Assays

All in vitro methods of assaying botulinum toxin are based on the quantitative reaction of the antigen (toxin) with its antibody (antitoxin). The earlier studies (189) do not need to be considered here since the quality of the antitoxins used in them makes it unlikely that the endpoints were due to toxin. Even the antitoxin which is raised with the toxoid of "crystalline type A toxin" is not suitable for these assays since the titer of the antibody for the nontoxic component of the crystals is higher than the titer of the antitoxic component (206).

Studies which avoid the non-specificity problem include a radioimmunoassay (15), a reversed passive hemagglutination assay (169), and an enzyme-linked immunosorbent assay (144, 145). Some of these methods reportedly detect toxin levels of slightly less than 50 mouse i.p. LD_{50}, but there may be a potential difficulty in that these LD_{50} values are based on comparisons of antigens in samples with mouse toxicity in a standard toxin preparation. These methods assume interconvertibility of antigenicity and toxigenicity, although the antitoxins are raised with toxoids and would react with at least some nontoxic but still antigenically active toxin molecules. The difference between antigenic and toxic determinants is also observed with activated type E toxin. A trypsinized preparation may be more than 100 times more toxic, but the smallest amount of antitoxin which neutralizes its toxicity is not much greater than the amount needed for the unactivated sample (103, 207).

The problem occurs when the toxicity and the amount of antigen in a sample are compared. During sample aging, toxicity always decreases more rapidly than the amount of serologically determined antigen, but the relative rate is not the same in all samples. In some cases, the amount of lethal toxin becomes significantly less than the amount of antigen. The difference would not be detected by the serological assays that are normally used (8). In vitro titrations of botulinum toxin should be in terms of antigen amount instead of toxicity, as is the convention for other toxins.

An antitoxin that might better measure toxicity in serological procedures would be the antitoxin that is obtained by immunizing an animal first with toxin that is neutralized with antitoxin and then with increments of active toxin. This with toxoid alone (Formalin-treated toxin) since repeated absorptions with toxoid leave a significant antitoxic titer. Since this antitoxicity, which is not removed by toxoid, reacts with active toxin, the antibody discriminates between toxin and toxoid (82).

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TOXIC COMPLEXES

The molecular nature of botulinum toxin is considered below from the aspects of (i) the purified neurotoxin and (ii) the association complexes that the toxin forms with nontoxic botulinum proteins. These complexes are present in culture fluids and are the natural state of botulinum toxin. In the attempts to purify toxin, these complexes were isolated first and, for a while, were considered to be the pure neurotoxin. Although discovered accidentally, the complexes are now known to be important to the pathogenesis of botulism food poisoning.

Complexes

Aside from the information that protein purification methods were applicable, the first real progress toward the purification of botulinum neurotoxin was the recovery by several procedures of highly toxic crystals from type A cultures. Physicochemical tests showed that the solutions of the crystals were homogeneous for entities of about 900,000 daltons. These observations suggested that the crystals represented pure toxin, but different interpretations became possible with other findings (17, 50, 113).

It became evident that these crystals contained more than one molecular species when solutions of the crystals were found to have a hemagglutinating activity that was adsorbed by erythrocytes without affecting the toxic titer (115). Although only a single boundary of a large unit was observed in the ultracentrifugation of acidic solutions of crystals, slower-sedimenting boundaries appeared when solutions at different alkaline pH's and ionic strengths were examined. A polydisperse fraction containing units of 40,000 to 100,000 daltons had two to three times the specific toxicity of the original sample but essentially no hemagglutinating activity, whereas a different solution contained a major fraction which had a sedimentation coefficient corrected to water at 20°C of 7 (158,000 daltons) and a specific toxicity close to that of the starting material (17, 50).

Conclusive evidence for the complex composition of the crystals was obtained when ion-exchange chromatographic methods became available. A diethylaminoethyl Sephadex column retained all of the protein in a solution of crystals in pH 7.2 buffer and resolved two protein peaks when eluted with an NaCl gradient in the same buffer. One peak (α fraction), representing about 20% of the applied protein, had high toxicity but little hemagglutinating activity, whereas the second peak (β fraction) had high hemagglutinating activity but low toxicity (42). The toxic peak was homogeneous for molecules of 150,000 daltons by gel filtration and for 7.2S molecules (molecular weight, 128,000 ± 12,800) by ultracentrifugation. In Ouchterlony immunodiffusion tests with antitoxin raised with a toxoid of the crystals, each fraction formed a single immune precipitate line which joined in a reaction of complete identity with a different one of the two lines produced by the crystals (22). A different chromatography resolved one toxic peak and distributed the hemagglutinin activity in several other protein peaks (41).

Importantly, since the specific toxicity of the α fraction is five times (42), or more likely three times (102, 117), higher than that of the crystals, the protein removed in the other fraction is not necessary for toxicity by the parenteral route. Since ultracentrifugation, chromatography, and immunodiffusion procedures would not normally break covalent bonds, type A toxic crystals are association complexes made of different molecular species. Electron micrographs of the crystals are interpreted as showing a core strand of neurotoxin within a coil of a hemagglutinin helix (21). Unless these facts are understood, the term crystalline type A toxin gives the misleading impression that the crystals represent only neurotoxic molecules.

Our understanding of type B and type E neurotoxins has evolved in a similar way (17, 50, 113). Preparations thought to contain type B molecules in a pure enough state for molecular weight determinations contained units of about 500,000 molecular weight (17), and an apparently homogeneous type E toxin preparation contained units of 350,000 molecular weight (101). The toxic units in these preparations are now known to be complexes in which a neurotoxic protein is associated with at least one nontoxic protein. In each case, ion-exchange chromatography of slightly alkaline samples separates the neurotoxic molecule from the other protein(s).

The natural state of botulinum toxin is the complexes. The sedimentation behavior of toxicities in cell-free culture fluids (types A through F) corresponds to units of 14 to 19S or 500,000 to 900,000 daltons (172), and gel filtration of extracts of type A cells separates the toxicity into units of 450,000 daltons and some of 150,000 daltons (80). Type E toxin extracted from a fish substrate behaves as molecules of 11.6S or 350,000 daltons (166).

Structure of Complexes

Growth fluids of some cultures have only one kind of complex, but some have more. These different complexes can be divided into three major groups (Table 1), with complexes in a

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TABLE 1. *Toxic complexes of botulinum toxin types and their specific toxicities*

Type	Toxicity ($\times 10^7$ mouse i.p. LD_{50} per mg of protein)[a]			Reference
	M complex[b]	L complex[b]	19S	
A	7.2–8.0	4.0–4.8	4.0–4.8	197
B (proteolytic)[c]	8.8–9.6	4.0–4.8	None	107
B (non-proteolytic)[c]	12.8–14.4	None	None	135
C_1	2.7[d]	None[d]	None	208
D (hemagglutinin)[e]	8.0	3.8	None	134
D (non-hemagglutinin)[e]	8.0	None	None	134
E	0.9	None	None	101
F	1.9	None	None	153

[a] Complexes were trypsinized when activation was needed for maximum toxicity; a 16% N content was assumed.

[b] M complexes: type F, 10S (235,000 daltons); type C_1, size not known; others, 12S (300,000 to 350,000 daltons). L complexes: 16S (450,000 to 500,000 daltons). 19S: 900,000 daltons. See Table 2 for sizes of neurotoxins.

[c] Toxic complexes of proteolytic and non-proteolytic cultures.

[d] M complex assumed from two lines in immunodiffusion tests. The toxicity was based on values in reference 209.

[e] Toxic complexes of cultures producing or not producing hemagglutinin.

given group having common features. M (medium) complexes are 10 to 12S bimolecular aggregates in which a neurotoxic molecule (5 to 7S) is associated with a similar-sized nontoxic protein which lacks hemagglutinating activity. L (large) complexes are trimolecular and contain the M complex plus a 7S, nontoxic molecule that is different from the nontoxic protein in the M complex. In the L complexes of type A and type D toxins and probably of type B toxin, this third component is a hemagglutinin. Type A toxic crystals are 19S.

There is an inverse relationship between specific toxicities and the sizes of the complexes of a particular toxin type (Table 1). The 19S type A toxic crystals seem to be an exception, but an explanation is available. Since L complexes (16S) are found when the crystals are dissolved in a buffer containing 1 M NaCl, the crystals are considered to be composed of dimers of L complexes (197). In such a situation, the specific toxicities of the 16 and 19S complexes would be comparable since the amounts of neurotoxin expressed as percentages of total protein would be the same.

The conditions of toxin production determine which of the possible complexes are present. When a type B culture is grown in a medium supplemented with 1 mM Fe^{2+}, toxicity is about equally distributed between M and L complexes, but if the supplement is 10 mM Fe^{2+}, the M complex dominates. The toxic entities in a food after growth of a type A or type B culture depend on the nature of the substrate. At least in foods, neurotoxin is not found in the uncomplexed state. Since M complexes in a type A culture fluid become L complexes during dialysis against a phosphate buffer, the degree of complexing also depends on the solvent (198).

Antigenicity and Reconstitution

Some of the nontoxic proteins in the complexes of the different toxin types are antigenically related. The hemagglutinin fraction isolated from type A toxic crystals is serologically related to the comparable portion of the type B L complex (49). The nontoxic proteins in the M complexes of type C_1 and type D toxins are antigenically indistinguishable. The nontoxic proteins of types E and F are not identical but are related, as are those in type A and F complexes (169).

The components of the complexes have a high affinity for each other. When the two proteins constituting the type E M complex are purified and then recombined in an equimolar solution, they form M complexes that cannot be distinguished from the starting bimolecular complex (99). The type B neurotoxin of a proteolytic culture forms M complexes with the nontoxic protein from the complex of a non-proteolytic type B toxin; the reverse combination is also possible (135).

Significance of Complexes

In contrast to the relative toxicities by the parenteral challenge routes, toxicity for mice by the intragastric route increases with each nontoxic protein that associates with the neurotoxic molecule. The toxin types differ in the quantitative relationship between toxicity increase and degree of complexing. The relative toxicities of the 7S-M complex-L complex-19S series of a type A culture are 1:12:20:360, whereas the relative toxicities of the 7S-M complex-L complex sequence of a type B culture are 1:20:16,000 (157).

Since the neurotoxic molecule in a complex series is the same, the greater potency of the larger complexes by the oral route is probably due to the associated proteins which protect the neurotoxin from inactivation in the gut and give greater opportunity for it to be absorbed before being inactivated (157). The appearance of toxic 7S units in the lymph and blood of animals fed complexes indicates in vivo dissociation (81, 101).

The resistance against inactivation when neurotoxin is in complexes is shown by in vitro tests. M complexes or 19S complexes do not lose much

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toxicity during holding for 30 min at pH 3 to 4, but purified neurotoxin loses most of its toxicity under these conditions. About 50% of the toxicity in a sample of 7S type E neurotoxin disappears during incubation for 30 min at 37°C, but the toxicity of an M complex is not greatly affected. Pure neurotoxins are more easily detoxified than those in M complexes by pepsin and pancreatin and by gastric and intestinal juices (195). In addition, when comparable i.p. LD_{50} of different-sized complexes are injected into the ligated duodena of rats, more active toxin passes into the lymph when the larger complexes are used (196).

The relationship between degree of complexing and toxic potency by the oral route has important implications for the botulism that results from ingesting preformed toxin. Foods containing equivalent mouse i.p. LD_{50} could differ in their botulinogenic potentials; those in which the toxin is mostly in L complexes would be greater health hazards than those which contain only M complexes.

Nomenclature

In this review the terms toxic complex and neurotoxin are used for the terms progenitor toxin and derivative toxin of some recent publications, respectively. These latter terms were advocated in a discussion of terminology (116) on the basis that progenitor toxin describes a parent form from which a toxic molecule is derived. It should be mentioned that progenitor toxin contains molecule(s) other than the neurotoxic protein when used in this context.

The reasons for not using the terms progenitor toxin and derivative toxin are (i) progenitor toxin has been used previously for the toxin form whose toxicity is activated by enzymes (43) and more importantly (ii) progenitor toxin gives the connotation that the complexes are formed with components that have a common origin, as would be the case if they were cleavage products of a single larger molecule. There is as yet no evidence that this is the case; at least in the case of the L complex of type D toxin, a contrary observation has been made. A type D culture which does not produce hemagglutinin has only M complexes, but when a related culture produces hemagglutinin, because it is lysogenized by a bacteriophage different from the one controlling synthesis of toxin (149), L complexes are formed by association of the M complex with the hemagglutinin (134).

The situation with bimolecular M complexes is less clear. Since these complexes form in mixtures made with pure neurotoxin and the complementary nontoxic protein, the constituents could be independently synthesized and then aggregate. On the other hand, if neurotoxic protein is never by itself in culture fluids (80, 198) but always in M complexes (or the M part of larger complexes), the two components are at least synthesized concomitantly. The demonstration of a toxic entity of M-complex dimensions which could not be dissociated into two equal parts without breaking a covalent bond would be evidence that the M complex is the product of a single gene.

NEUROTOXIN

All botulinum toxin types except C_2 and G have been recovered as highly purified preparations. When large culture volumes are to be processed, the preferred initial treatment is to precipitate the toxin by acidifying an uncentrifuged culture. The precipitation probably involves co-precipitation since type C and type D toxins do not precipitate unless ribonucleic acid is added before acidification (94). The usual purification of type E neurotoxin uses a different approach in that the starting material is an extract of harvested cells (100). More toxin is in the cells than in the culture fluid, and the intracellular toxin has a higher activation factor than the extracellular toxin (R. C. Heimsch, Ph.D. thesis, University of Wisconsin, Madison, 1973).

In most purification methods M or larger complexes are isolated, and then the neurotoxin is recovered by chromatography of an alkaline solution of the complex. Purification of type A neurotoxin has been studied more than purification of the other toxin types. Since the type A toxic crystals are stable, they are used in pharmacological studies. These crystals can be obtained by using differential precipitations only (59), but crystals form more rapidly and are of better quality if smaller proteins and nucleic acid are first removed by diethylaminoethyl Sephadex chromatography (205). The neurotoxin can be isolated from the crystals by ion-exchange chromatography, but an affinity chromatography which uses galactose as the ligand for the hemagglutinin in the crystals is more efficient (137).

Molecular Weight

The antigenically different botulinum neurotoxins have molecular similarities that would be expected of molecules which have a common, unique pharmacological action. One of the similarities is in molecular weight (Table 2). The preferred molecular weight determination method is electrophoresis in polyacrylamide gels with sodium dodecyl sulfate, especially since the

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TABLE 2. *Botulinum neurotoxin types: dichain components and specific toxicities*

Type	Mol wt					Specific toxicity[a]	
	Neurotoxin ($\times 10^3$)	H chain ($\times 10^3$)	L chain ($\times 10^3$)	Ratio of H chain to L chain	Reference	Value ($\times 10^8$)	Reference
A	145–150	97	53	1.8	41, 42, 43	1.05–1.86	42, 43, 102, 155
B	152–170	104–112	51–60	1.8–2.0[b]	6, 47, 105, 155	0.98–1.14	6, 47, 105, 155
C_1	141	98	53	1.8	209	0.88	209
D	170	110	60	1.8	134	1.60[c]	134
E	147–150	102	50	2.0	43, 101	0.21–0.25	101, 155
F	128–155	105	56	1.9	154, 230	0.16–0.40[d]	154, 230

[a] The toxins were trypsinized when necessary for maximum toxicity. Toxicities are per milligram of protein, based on conversions using an N content of 16% or a 1% extinction coefficient (light path, 1 cm) when available. Minimum lethal doses were assumed to be one-half of the LD_{50} values.

[b] Calculated from H and L values found in individual studies.

[c] Based on a specific toxicity which was twice that of the M complex.

[d] Based on a 1% extinction coefficient (light path, 1 cm) of 1.63.

dichain nature of the neurotoxin can be studied at the same time.

Specific Toxicity

Lethalities for mice by the i.p. challenge route range from 10^7 to 10^8 LD_{50} per mg of protein, with types E and F at the lower end of the scale (Table 2). The differences in specific toxicity are probably not as important as the similarities; the values are obtained by different titration methods, and the relationships might not be the same in a different animal species.

Another possible reason for the differences in toxicity is that some of the toxicities reported were calculations from values that were determined per milligram of nitrogen or per unit of absorbance at 278 nm instead of directly (per milligram of protein). Since the toxins have different amino acid compositions, as shown by types A (20) and B (6), the use of a nitrogen content of 16% in some conversions may not be strictly correct. A similar kind of assumption was used to obtain a type F value (230) from a specific toxicity based on absorbance at 278 nm, although the absorbance at 278 nm of a 1% solution of type A is 16.3 (102) and that of a 1% type B solution is 18.5 (6). It is evident that even if the same protein unit were used in all determinations, exact relative toxicities would not be available. The very similar specific toxicities obtained with the calculations indicate that the different neurotoxin types are similar molecules.

One mouse i.p. LD_{50} represents only a few molecules. A calculation using a specific toxicity of 10^8 LD_{50} per mg of type A neurotoxin (102) of molecular weight 150,000 and Avogadro's constant shows that 1 LD_{50} consists of slightly less than 5×10^6 molecules. It has been suggested that humans develop botulism from a parenteral dose of 7 mouse i.p. LD_{50} and that ingestion of 7,000 such units is botulinogenic (113).

Small Toxin

As reviewed elsewhere (17, 50), neurotoxic molecules much smaller than 150,000 daltons have been reported. Some of these molecules, which are about one-half the generally accepted size, are probably artifacts of methodology, such as the use of low-ionic-strength buffer in the gel filtration procedure. Type A, B, and E toxins of 9,000 to 18,000 molecular weight have been reported but have not been confirmed by other laboratories. The significance of other reports of small units is difficult to assess since specific toxicity is often not given; when available, it is lower than the values shown in Table 2. Until the small units can be prepared consistently by different laboratories and in a quantity permitting other characterizations, their natural existence remains in doubt.

Disulfides

The changes in toxicity that accompany the modification of a particular amino acid species or chemical group have been studied. Some interesting results have been obtained, but it is not known whether the relationships are due to a change at the active site or to a nonspecific effect that alters the three-dimensional configuration necessary for maximum toxicity. These results are reviewed elsewhere (17, 50).

The amino acid compositions of type A (20) and type B (6) neurotoxins and of the M complex of type E toxin (167) do not give a clue as to why the proteins are toxic. However, the number of disulfide bonds is germane to the dichain structure of the toxic proteins. All toxin types have at least one disulfide bond that holds the subunits together. The data for type B neurotoxin (6) indicate a maximum of three disulfide bonds, whereas the data for type A toxin indicate that 1.65 mol of —SH are available to form one

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disulfide bond (102). A recent observation indicates that there should be at least two disulfide bonds in type A neurotoxin (see below).

Dichain Structure

Aside from similarities in molecular weight, in specific toxicity for mice, and in natural existence as M complexes or larger complexes, the different neurotoxin types are related also in having a common subunit structure. The proteolytic activity of a culture is important in determining the form in which a neurotoxin is purified, but all types are composed of two dissimilar polypeptide chains when isolated or after being trypsinized to activate toxicity.

The first evidence for the dichain nature and the importance of disulfide bonds to this structure was obtained when a type B neurotoxin from a proteolytic culture was treated with a protein denaturant and examined by polyacrylamide-gel electrophoresis. The sample treated with a disulfide-reducing agent resolved into bands representing units of 104,000 and 59,000 daltons, but the unreduced sample contained only 167,000-dalton units (6). The full significance of this observation was not apparent until it was found that type A and type B neurotoxins, which are at maximum potential toxicity, were disulfide-linked dichain molecules, whereas type E neurotoxin was a single-chain (unnicked) molecule unless activated with trypsin (43) (see below).

As shown by the ratios with values of 1.8 to 2.0 (Table 2), all botulinum toxin types are dichain molecules in which the larger subunit (H chain) is about twice the molecular weight of the complementary smaller unit (L chain). Noncovalent forces contribute to the integrity of the dichain structure since the chains cannot be separated by merely reducing the interchain disulfide bond. The nearly complete loss of toxicity when toxin solutions are reduced with dithiothreitol shows the importance of the disulfide bonds (200).

At concentrations of 1 mg/ml or higher, purified botulinum neurotoxins tend to precipitate when their disulfide bonds are reduced. Urea prevents the aggregation of reduced type B neurotoxin and helps in dissociating the chains, so that highly purified preparations of H and L chains are obtained by molecular sieve chromatography (105, 106). Except for a low toxicity which is probably due to trace amounts of intact dichains, neither preparation is toxic when injected into separate mice. Dichain molecules are reformed during dialysis of equimolar mixtures of the chains; this procedure regenerates about 40% of the theoretically possible toxicity. Thus, the expression of toxicity depends on the H and L chains being in a specific arrangement.

The use of urea to separate type B chains is similar to the procedure originally described for isolating the chains of tetanus toxin (124). This method is not suitable for type A neurotoxin since practically all of the type A protein precipitates during the reduction of disulfide bonds. However, its H chain can be isolated by reducing the neurotoxin in a buffer containing 1% reduced nicotinamide adenine dinucleotide and 0.5 N NaCl. A precipitate forms when the toxin is treated with dithiothreitol, but it is composed mostly of L chains, so that essentially only H chains are recovered when the supernatant is retreated one or two times. The type A H chain, like the type B neurotoxin H chain, has no intrinsic toxicity (109).

H and L chains are different antigens. Antisera made with them protect mice against the lethality of the intact toxin, but these antisera differ in effectiveness. For the same in vitro titer (determined serologically or by quantitative antigen-antibody reaction), anti-H serum is more potent than anti-L serum (105, 109). As might be expected, the usual antitoxin preparations have separate anti-H and anti-L molecules. The observations indicating that the H chain is part of the toxin that binds to nerve endings are considered below.

The L chains of type A and type B toxins are more resistant to degradation by trypsin or chymotrypsin than the complementary H chains. The first important change of dichain molecules during controlled digestion is the cleaving of the H chain at about the midpoint to separate one fragment (H_1) of about 50,000 daltons from another fragment of about 100,000 daltons. The latter piece is the L chain connected by a disulfide linkage to the remainder of the H chain (H_2 fragment) (Fig. 1). A disulfide loop is present in the H_1 fragment (B. R. DasGupta and H. Sugiyama, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B73, p. 25). It may be that anti-H serum has at least two antibody species, one for H_1 and the other for H_2 (see below).

Difference Among Type B Toxins

Antitoxins obtained with the toxins of proteolytic and non-proteolytic type B cultures neutralize the toxicity of either type of culture but differ in being less effective against the toxin of the heterologous culture (177). A quantitative difference is also observed in passive hemagglutination tests (169). The explanation of this is based on the fact that the toxins have identical L chains but antigenically different H chains. In gel immunodiffusion tests in which anti-H serum

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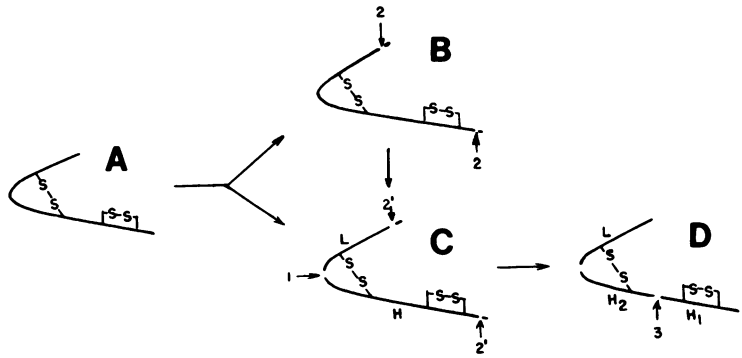

FIG. 1. *Known and suggested molecular cleavages of botulinum and tetanus neurotoxins. (A) Toxins synthesized intracellularly as unnicked molecules. (B) Possible action of SH-dependent protease (e.g., TLE). The scission of the peptide bond is at the N and/or C terminus or immediately subterminal (site 2). This results in partially activated botulinum toxin; no information concerning tetanus toxin is available. (C) Actions of the nicking protease and of trypsin. A dichain molecule is formed by cleavage of the peptide bond at site 1 so that the L chain is the N-terminal one-third of the unnicked molecule (data for tetanus toxin and suggested cleavage for botulinum toxin). The molecular weight of the H chain is about two times that of the L chain. If nicking is not related to toxicity activation, enzymes may act also at site 2', just internal to action site of TLE. This results in a molecule of maximum possible toxicity. (D) First change during digestion of botulinum toxin by trypsin or by chymotrypsin and during digestion of tetanus toxin by papain. The scission of the H chain at about the midpoint (site 3) yields H_1 and H_2 fragments.*

prepared with the H chain of the proteolytic culture is used, each of the H-chain preparations from the two toxins develops a single immune precipitate line, but these lines join in a partial identity reaction, instead of complete identity reaction (106).

INTRAMOLECULAR CHANGES AND ACTIVATION

The initial type E botulism cases were puzzling in that the clinical effects seemed more severe than expected from the mouse toxicities that were titrated in the responsible foods or in pure cultures. This inconsistency became understandable when it was found that toxicity is higher when toxin is produced in cultures contaminated with a proteolytic organism (170) and increases as much as 50-fold when culture fluids are trypsinized (60).

Although this activation of toxicity by proteases is most marked with the toxins of nonproteolytic cultures, the toxicities of toxins of proteolytic cultures also increase to varying degrees (50, 90). This activation is the result of intracellularly synthesized molecules (187) of low toxicity becoming extracellular and attaining greater activity when modified by enzymes of the culture (13, 14, 44, 90). This activation phenomenon is similar to the conversion of a zymogen into a molecular form having higher enzymatic activity.

Synthesis as Single-Chain Molecules

As deduced from sodium dodecyl sulfate-polyacrylamide gel electrophoresis, type E neurotoxin is a single-chain (unnicked) polypeptide of about 150,000 daltons and has at least one intrachain disulfide bond. During activation by trypsin, a peptide bond is cleaved so that it is nicked into the dichain form (Fig. 1). The bond cleavage is not evident unless the interchain disulfide (originally intrachain) bridge is reduced and other interchain attractions are minimized (43). The dichain form cannot be distinguished from the unnicked molecule by molecular weight (43), amino acid composition (167), or electrophoretic behavior (100). The toxicity of some preparations increases by several-hundred-fold (activation factor).

In contrast to the neurotoxin of a nonproteolytic type E culture, neurotoxin preparations from group I cultures may have only dichain molecules (43). However, mixtures of nicked and unnicked molecules can be recovered from type B and type F cultures by changing the conditions of toxin production (e.g., by shortening incubation time) (46, 48). The unnicked molecules in the mixtures have the same molecular weight as the naturally formed dichains and are converted by trypsin into dichains which are indistinguishable by sodium dodecyl sulfate-polyacrylamide gel electrophoresis from those already present. Nicking protease of the cultures and trypsin cleave the same peptide bond or bonds very close to each other in the amino acid sequence.

These observations support the interpretation that all cultures synthesize neurotoxin as unnicked molecules; the molecular form that is isolated depends on the proteolytic property of the culture and the conditions of toxin produc-

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tion. If a culture does not produce a nicking protease, only single-chain molecules are recovered; if this enzyme is produced, some or all of the single-chain molecules are converted into dichains.

In agreement with the results discussed above, there are only unnicked molecules in type A neurotoxin preparations that are purified from the extracts of cells from young cultures and thus are less exposed to extracellular proteases (E. Krysinski, Ph.D. thesis, University of Wisconsin, Madison, 1978). Only single-chain molecules are in purified type D toxin samples (134). A possible exception is the dichains which make up purified type C_1 toxin preparations (209). However, group III cultures can have proteases, and the toxin purification method uses alkaline conditions in an early step. These two circumstances could permit cleavage of the single-chain molecules by the nicking enzyme before it can be removed.

Nicking Site

Since the molecular weight of the H chain is about twice that of the complementary L chain, dichain formation involves scission of the single-chain molecule at a peptide bond that is about one-third along the chain, starting from either the N or the C terminus. If botulinum and tetanus toxins are homologous in this respect (see below), the L chain represents the N-terminal one-third of the unnicked molecule (Fig 1). This suggestion assumes that all botulinum toxin types are unbranched molecules and are nicked at comparable sites.

Based on the substrate specificity of trypsin, nicking occurs at the peptide bond formed by the carboxyl group of the arginine and/or lysine residue. A recent observation favors cleavage of the bond of arginine residues. When 1,2-cyclohexandione reacts with a protein in borate buffer, pH 8 to 9, arginine residues are specifically modified so that their peptide bonds are not cleaved by trypsin; the bonds of lysine residues remain susceptible. Single-chain type E neurotoxin treated with 1,2-cyclohexandione is resistant to nicking by trypsin (50a).

It is not known whether the peptide bond of more than one arginine residue is cleaved in the conversion into dichain molecules. If the scission site has several closely adjacent arginine residues, more than one bond can be cleaved. The multiple scissions would not be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, since only one or very few amino acids would be lost and the dichains would have a molecular weight similar to that of the starting unnicked molecules.

Activation Without Nicking

The toxin of even proteolytic cultures initially has relatively low toxicity, but during incubation for toxin production, toxicity is partially or completely activated by enzyme(s) of the cultures (50). A protease of a proteolytic type B culture, which activates toxin in young cultures of the same strain (40), is produced by other group I cultures but rarely by group II cultures. When the protease is not produced, the toxicity of such a culture can be activated by trypsin (45). Although it is more active on arginine than lysine bonds, the protease is not surprisingly a trypsin-like enzyme (TLE) in its substrate specificity. It is not affected by inhibitors of trypsin and is more like clostripain (EC 3.4.4.20) of *Clostridium histolyticum* in that it is a sulfhydryl-dependent enzyme (SH-protease) which is enzymatically active only in the reduced state (44).

The TLE activates single-chain type E neurotoxin but, unlike trypsin, does not nick the molecule; it also differs in that the activation factor is lower than that achieved with trypsin. The toxicity of the partially activated toxin that results from treating with purified TLE (45) or with crude SH-proteases from other cultures (152) increases further when the toxin is trypsinized. When a TLE-treated sample is trypsinized, the single-chain molecules are nicked into the expected dichain form. The final toxicity obtained by the sequential activations is lower than obtained by trypsinization alone, possibly because the experiment used purified neurotoxin, which can lose toxicity (156). The observations made with TLE suggested that complete natural activation may be a two-step phenomenon in which an SH-protease partially activates without nicking and another enzyme completes the activation by nicking the molecules.

Bond Cleavage of Activation

More recent observations indicate that nicking may not be related causally to the increase in toxicity. When a preparation of unnicked type B toxin is trypsinized at pH 4.5 instead of pH 6.0, which is usually used, maximum possible activation is attained at the time when only some of the single-chain molecules have been nicked (155). A possible difficulty in this kind of study arises if the sample is a mixture of single-chain and dichain molecules and the nicked form is assumed to be the most toxic molecule. If a solution has unnicked and nicked molecules in a molar ratio of 2:1, the nicking of all of the single-chain molecules would increase toxicity by a factor of only three. Such a small toxicity increment is not easily detected by toxin titration methods (46). However, in the work in

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which nicking and activation occurred at different rates, this difficulty was probably not encountered.

Other findings support the independence of nicking and activation. Toxicity in type D preparations, which are homogeneous for unnicked molecules, increases only minimally when the molecules are nicked by trypsin (134). The preparation of type A toxin that is purified from an extract made of cells in young culture has only single-chain molecules whose specific toxicity is comparable to that of the dichain isolated from toxic crystals (Krysinski, Ph.D. thesis).

Although the TLE of a type B culture is less effective than trypsin in activating type E toxin (45), a similar SH-protease purified from a proteolytic type F culture gives the same activation as trypsin if used at a molarity 150 times higher (156). Such a high SH-protease concentration activates type B toxin to only 20% of the trypsinized titer. The possibility that the high enzyme concentration might have nicked the single-chain molecules was apparently not considered.

The first attempt to explain the tryptic activation of type E toxin did not consider peptide bond cleavage to be the mechanism responsible since the size of the toxin molecule did not change. Activation was attributed to the esterase property of trypsin since, under the conditions of the activation procedure, the two activities were parallel (167). However, nicking would not have been recognized since the interchain disulfide linkage was not reduced.

Nevertheless, the esterase explanation of activation remains current because of other observations. An enzyme purified from pronase (PrE) has a ratio of lysine esterase activity to protease activity that is higher than that of trypsin. Since this enzyme is considered to be a better activator of type E toxin than trypsin (final activation, 2.08×10^6 versus 1.86×10^6 LD_{50}/ml), the two enzymes are believed to activate the toxin by acting on a lysine ester bond (133). The molecular form in the PrE-activated preparation was not determined. Other circumstantial evidence has been used to support the suggestion that activation is due to action on the ester bonds of lysine rather than arginine residues (141).

However, the toxic titers in the trypsinized and PreE-treated samples may not be significantly different since PrE was used at a slightly higher molar concentration than trypsin and the fiducial limits of the toxin assay method are ±30% (168). Also, ester linkages in activatable botulinum toxin have not been demonstrated.

The available observations do not eliminate the possibility that activation may be due to cleavage of peptide bonds. If such scission removes one or just a few amino acids from the N and/or C terminus, toxicity may increase without a detectable decrease in the molecular weight of the starting single-chain molecule (Fig. 1). This suggestion could be tested by determining whether there is a change in the terminal amino acids during activation. Provided that this suggestion has merit, a similar mechanism could explain the further toxicity increase that is observed when TLE-treated samples are trypsinized. If nicking is not the cause, some other molecular change must occur. This could be cleavage of a peptide bond(s) just internal to the bond cleaved by TLE (Fig. 1).

Discussions about the relationship between nicking and activation have not considered one possibility. If the molecules that are synthesized are modified by the enzymes of a culture so that the toxin can be nicked rapidly in vivo, the modified but still single-chain molecules could be as toxic as the molecules which are already dichains at the time of injection into an animal.

The difficulty in fully understanding the activation mechanism may be, in part, the result of assuming that identical changes occur with all of the botulinum toxin types. The properties of C_2 toxin suggest that this may not be the case. When present as a minor toxin in type C and type D cultures, this toxin can be activated with trypsin, although the major toxin is already fully activated (62). The complete activation of type E toxin but not type B toxin by high concentrations of SH-protease purified from a type F culture also suggests that toxins may differ (156).

Certain specifics are uncertain, but it seems reasonable to believe that all of the types are synthesized as single-chain molecules which undergo some molecular changes. Although it may not be related to toxicity activation, one natural or induced change is the conversion into the dichain form. Another molecular modification(s) activates toxicity. If the TLE is necessary for natural activation, a molecular change that is different from the TLE-induced change is needed for maximum activation. This enzyme may or may not be the enzyme that causes nicking. Trypsin is able to effect all of the changes. Several different proteases are produced by group I cultures (212), but the SH-protease class is the only one which has been shown to act on botulinum neurotoxin.

PHARMACOLOGY

Botulism is basically a flaccid paralysis that results from the toxin acting on the peripheral instead of the central nervous system. (Much of this discussion involves the neurophysiology at

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synapses [nerve-nerve or nerve-effector junctions]. Common terms and a simplified version of events are given in the Appendix.) Paralysis results because muscles of cholinergic innervation (mediated by acetylcholine [Ach]) become progressively unable to respond to stimuli that reach them via their motor nerves (indirect stimulation). Since the nerve trunk conducts impulses normally and the muscle contracts when directly stimulated, the toxin acts at or near the junction formed by the nerve and its innervated structure and not on the effector per se. The toxin blocks cholinergic transmission in the ganglia, the intestinal tract, and the few Ach junctions in the sympathetic nervous system. The neurotoxin is highly specific for cholinergic innervations; when injected into an organ having both types of innervations, the cholinergic but not the adrenergic (mediated by norepinephrine) elements are affected (229).

Presynaptic Site of Action

The observations made in the early studies with intact animals have been confirmed by in vitro experiments with nerve-muscle junctions (NMJ), such as the isolated phrenic nerve-diaphragm isolated from rats or mice. When the toxin is added to a bath fluid, the muscle progressively loses its responsiveness to indirect stimulation but is itself not affected since it contracts when Ach is added or when it is electrically stimulated (27).

These observations could be explained in several ways, but most possible explanations can be eliminated. Paralysis could result if toxin blocks transmission through the terminal nerve fibrils, where there is no myelin sheath, but this is unlikely since action potentials can be recorded from this part of the nerve (79). Unless stimuli pass through the fibrils, the poisoned preparation would not respond to closely spaced indirect stimulations (24, 194).

An NMJ becomes paralyzed from a lack of transmitter if Ach synthesis is inhibited, but observations show that botulinum toxin does not affect the availability of choline and acetyl-coenzyme A or their synthesis into Ach. The toxin does not inhibit Ach synthesis by choline acetyltransferase preparations (27), synaptosomes (227), or brain slices (71), and the diaphragms of poisoned and control animals have similar amounts of transmitter (50). Direct stimulations of paralyzed NMJ release as much Ach as indirect stimulations of controls (24).

Unless Ach reacts with postsynaptic receptors and is rapidly inactivated by acetylcholinesterase, the receptors do not respond to the transmitter that is released next. The suggestion that botulinum toxin inhibits the esterase has not been confirmed (185). Moreover, a poisoned NMJ still responds to exogenous Ach (27). The elimination of these possibilities leaves as the most likely explanation an action at the nerve ending itself, which prevents release of transmitter.

Release of Acetylcholine

When toxin acts on an NMJ being stimulated indirectly at a constant level, the amplitudes of the endplate potentials and muscle contractions which are evoked become progressively smaller; paralysis occurs when the nerve action potential no longer elicits a significant endplate potential (27, 78). The toxin reduces the frequency of miniature endplate potentials (mepp) even before it affects the endplate potential amplitudes and by the time that the full effects of the toxin have developed, mepp are at very low frequency (18, 36) or are completely abolished (32, 131). Since the postsynaptic Ach receptors are functional and nerve action potential passes through the nerve fibrils, these observations indicate a defect in the transmitter release mechanism.

The effects of spider venoms show that toxin-poisoned NMJ have a normal store of Ach but cannot release it by the usual physiological process. Black widow spider venom releases Ach from nerve endings by lysing synaptic vesicles; the result is a sudden and large increase in mepp frequency. When the venom acts on a botulinum toxin-poisoned NMJ, it elicits a burst of mepp that is similar to that evoked from control preparations (36, 97, 183). Brown widow spider venom has a similar action (164). Since the amplitudes of the venom-induced mepp are comparable to those of controls (36), botulinum toxin does not change the Ach quanta of the individual vesicles.

The coupling of the nerve action potential to the release of transmitter is mediated by Ca^{2+}, which acts in an unknown way. Botulinum-poisoned NMJ have some properties of a normal preparation that is in a bath of low Ca^{2+} or high Mg^{2+} (which is a Ca^{2+} antagonist). A further suggestion of the possible importance of Ca^{2+} was the brief improvement of indirectly stimulated release of Ach from a lightly poisoned NMJ when the cation concentration of the bath was doubled (211). The facilitation of transmitter release by tetanic stimuli or by closely spaced pairs of indirect stimuli is reasonable since Ca^{2+} uptake should be improved by these procedures.

Release of transmitter by poisoned NMJ improves when tetraethylammonium or a Ca^{2+} ionophore, both of which help increase the intraneuronal Ca^{2+} concentration, is added to the

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bath. Thus, the Ach release mechanism is still functional, but its operation requires an intracellular Ca^{2+} level higher than the Ca^{2+} level possible in a physiological situation (36, 183). Guanidine has some beneficial clinical effects as a means for increasing the cation level (163).

A striking in vivo effect is produced by 4-aminopyridine, which allows the development of a relatively high intraneuronal Ca^{2+} concentration even when extracellular Ca^{2+} is at the physiological concentration (2 mM). Rats paralyzed by botulinum toxin are able to move about within approximately 10 min after they are given 2 to 3 mg of 4-aminopyridine per kg of body weight. Paralysis returns in about 1 h, but a second dose of 4-aminopyridine again gives some relief (121). Whereas the Ach quanta released by a poisoned NMJ are low relative to the extracellular Ca^{2+} concentration, the quantum-Ca^{2+} relationship approaches that of a normal NMJ when a poisoned preparation is treated with this drug (121, 183). 4-Aminopyridine is less effective when paralysis reaches an advanced state. Theophylline has some anti-botulinum effect in delaying onset of paralysis and increasing survival of mice, but the mechanism of this protection is not yet clear (88).

Batrachotoxin, a frog venom, releases a barrage of mepp from normal NMJ by a Ca^{2+} dependent process that can, if necessary, use the cations already inside the nerve endings. Since this frog toxin does not elicit the mepp barrage from botulinum-poisoned NMJ, the effect of botulinum toxin is probably not on the influx of Ca^{2+}; botulinum paralysis is due to interference in the transmitter release step that occurs after Ca^{2+} is intracellular and before physical release (183). The cations taken up in response to a nerve action potential accumulate preferentially at the plasmalemma (presynaptic membrane) of the nerve terminus (5). The inhibition of transmitter release by the toxin has been demonstrated physically by freeze-fracture electron microscopy, in which the normal exocytotic release of Ach appears as deformations of the plasmalemma where synaptic vesicles fuse and particles, which are believed to be parts of vesicle membranes, concentrate. These deformations are observed very infrequently in poisoned NMJ (165).

A biochemical lesion could prevent transmitter release, but a strictly mechanical explanation is possible. The "pipe and valve" hypothesis postulates that toxin enters the channels through which Ach is released into the neuromuscular cleft when a valve opens during normal transmitter release. The nature of the valve is such that once toxin passes through, it cannot reverse direction; the toxin becomes a mechanical plug that prevents the normal outflow of transmitter (112). This hypothesis is supported by calculations which show a reasonable agreement between the number of transmitter efflux sites and the minimum number of toxin molecules that can cause paralysis of a diaphragm or a gastrocnemius (77). Some modification may be needed to incorporate the observations of the freeze-fracture experiments.

In addition to their reduced frequency, the mepp of partially paralyzed NMJ are mostly of small voltages so that, instead of the usual binomial form, the mepp amplitude distribution becomes skewed. It has been suggested that there may be two populations of vesicles and that toxin interferes with the packaging of transmitter in those vesicles which are the usual source of transmitter for normal, indirectly stimulated transmitter release. In this situation, the quanta released in the mepp would be small and could account for the skewed amplitude (18, 164). However, normal quanta are released when the poisoned preparation is treated with a Ca^{2+} ionophore plus a high cation concentration (36) or with spider venom. If normal mepp are the results of synchronous release of several quanta, the low-amplitude mepp of the poisoned NMJ could be due to release from fewer vesicles (108).

Synaptosomes and Other Test Systems

Early attempts to show binding of botulinum toxin and brain matter (Wassermann-Takaki phenomenon of tetanus toxin) were unsuccessful (229), but such binding has been demonstrated with radiolabeled toxin. Radiolabeled toxin also fixes to synaptosomes (73), which are nerve endings that are usually isolated from brain and have much of their structure and function intact. Although the affinity was not high, a synaptosome ligand has been used for the affinity chromatographic isolation of type A neurotoxin from its crystalline complex (74).

Botulinum neurotoxin reacts with synaptosomes as they exist in brain homogenates. More than 90% of the starting radioactivity is in the synaptosome fraction that is prepared from a rat brain homogenate which has been incubated with radiolabeled type A neurotoxin. Most of the radioactivity is associated with synaptosomal membranes, and about 50% of the radioactivity becomes free when the synaptosome preparation is treated with neuraminidase. Although the radioactivity is difficult to elute under physiological conditions, the radioactive units removed by a detergent have the molecular weight of native type A neurotoxin (98).

Synaptosomes synthesize Ach, and, when de-

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polarized by a high K^+ concentration, they release it. Botulinum toxin does not interfere with either synthesis or distribution of Ach between cytoplasm and vesicles. However, the amount of transmitter released by depolarization is reduced when a synaptosome preparation is first treated with botulinum toxin (227). A Ca^{2+} ionophore or a high concentration of cation reverses the inhibition (228). The toxin does not affect the uptake of Ca^{2+} by synaptosomes (55).

Botulinum toxin poisons brain slices in a similar way; it does not affect transmitter synthesis, but it reduces the amount of transmitter released when the slices are depolarized with K^+, in an action which mostly inhibits release of the newly synthesized transmitter instead of the "endogenous stores" (71). The toxin also poisons primary nerve cell cultures and reduces the amount of transmitter that is normally released by depolarization (9). In all three in vitro test systems, the principal action of botulinum toxin is inhibition of transmitter release.

Two-Step Kinetics

When toxin is added to the bath in which an NMJ is being stimulated indirectly at a constant rate, the dose is important in determining the time to paralysis (27). In addition, the time to paralysis with a given amount of toxin is a function of the rate at which the NMJ is indirectly stimulated after toxin is added; faster stimulations shorten the time to a specified degree of paralysis (89). The slow development of paralysis when an NMJ is in a bath having a low Ca^{2+} concentration or a high Mg^{2+} concentration indicates that release of transmitter is necessary before toxin can cause paralysis (178, 179).

Botulinum toxin binds rapidly to NMJ even when the development of paralysis is delayed by using a slow indirect stimulation rate. When a preparation in a Ca^{2+}-deficient bath is treated with toxin for 30 min and the preparation is washed thoroughly, paralysis is not prevented. However, antitoxin has a marked protective effect at this time (27, 179).

The belief that botulinum paralysis develops in two discrete steps is based on these and related findings (32, 180, 182). The first step is the fixing of toxin to nerve endings and does not require the NMJ to be actively releasing Ach. Binding is not greatly influenced by temperature and is difficult to reverse by washing, but it seemingly occurs externally, since antitoxin is protective. The binding itself has no obvious effect; paralysis is the consequence of the succeeding step, in which faster release of transmitter accelerates development of the toxic manifestation. The transmitter release apparently permits the bound toxin to translocate to a site at or from where it prevents further release of transmitter. In this temperature-sensitive translocation, the toxin moves to a site not reached by antitoxin. Whether the translocation involves the whole molecule or one of the subunit chains has not been determined (see below).

Because of its role in triggering Ach release, Ca^{2+} participates in botulinum paralysis in two ways. This cation makes it possible for the toxin to reach its action site. Ca^{2+} then becomes involved in a different sense in that the Ach release which it mediates is the physiological event that is blocked by toxin. At this stage an increased Ca^{2+} concentration can have an ameliorating effect.

Binding

Microscopic observations support the physiological hypothesis that botulinum toxin acts presynaptically. Labeled toxin injected into animals concentrates at neuromuscular junctions (85, 231). When type A neurotoxin is incubated with large synaptosomes and its distribution is determined with ferritin-labeled antitoxin, the toxin is found preferentially bound to presynaptic membranes, with little toxin on the postsynaptic structure (86). Although the experimental method may have been the reason, toxin was not found inside the nerve endings in these experiments.

The possibility that the affinity of toxin for nerve endings is due to the gangliosides of the nerve endings was suggested by the observation that most of the toxicity in a solution made with type A toxic crystals disappeared when a trisialoganglioside (GT_1) was added in an amount equal to the amount of crystals (186). These results were not confirmed when the test was repeated in the presence of a colloid that helps to prevent nonspecific inactivation of toxin (218), but the original finding is difficult to dismiss completely. Since treating the toxin-GT_1 mixture with neuraminidase recovered some toxicity, not all of the toxin was inactivated irreversibly. Moreover, a similar partial release of toxin (measured by radioactivity) occurred when the toxin-synaptosome complex was treated with the enzyme (98). It should be pointed out that for this kind of study type A toxic crystals may not be suitable; this neurotoxin could bind indirectly because the hemagglutinin with which it is complexed binds to galactose and some of its derivatives (49).

The H chain is probably the part of the dichain neurotoxin that binds to target sites, since synaptosome suspensions have greatly reduced capacities for binding radiolabeled type B toxin

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after they are treated with the H chain of type B neurotoxin. The much smaller reduction that is obtained with L chain preparations is attributed to the small amount of contaminating dichains. The H chain is as effective in this inhibition as unlabeled type B neurotoxin. Synaptosome preparations that are treated with intact type A or E neurotoxin bind less radiolabeled neurotoxin of the homologous and heterologous antigenic types. The absence of such cross-inhibition between type B and type E neurotoxins indicates that the synaptosomal receptors to which botulinum toxin binds may not be the same for all toxin types (104).

In Vivo Central Nervous System Action

Central nervous system effects produced by intracerebral injections of impure toxin samples are difficult to attribute to the neurotoxin itself. The responses that follow intravenous challenges with solutions of type A toxic crystals are more acceptable as being caused by toxin, but they are difficult to reproduce (184). Radioactive units with the antigenicity of toxin within 30 min of intravenous injection of labeled neurotoxin are found in the brain (16), but they could be in the blood vessels and not the parenchyma. Most workers believe that a blood-brain barrier prevents botulinum toxin from entering the brain tissue itself.

More relevant is the clinical finding of the H reflex (215), in which an afferent impulse returns directly as a motor stimulus. Since such a reflex is normally prevented in the spinal cord, some action of toxin inhibits or overcomes the preventive mechanism. This reflex is generally not mentioned in reports of botulism cases.

Botulinum toxin can enter the spinal cord if the radioactivity observed there represents undegraded neurotoxin. When radiolabeled toxin is injected intramuscularly, the radioactivity passes intraaxonally into the motor nerve and reaches the ventral root of the spinal cord half segment. Transport in the nerve is faster when the muscle is stimulated. A lower level of radioactivity appears in the contralateral half segment (66, 73, 224).

The toxin that enters the spinal cord could have some effects since neurotoxin binds to brain synaptosomes and inhibits the Ach release normally induced by a high K^+ concentration. Several reports describe neurophysiological changes in the spinal cord (76, 130, 225), but these changes are not necessarily from actions on cholinergic synapses. They appear to be effects at other sites, which are reflected in the activity of the motoneurons. Botulinum neurotoxin may be a more general poison for different kinds of neurons than indicated by the dominant peripheral action on Ach release. The initiation of these other toxicities may require toxin concentrations which are not reached in botulism cases (76).

Adrenergic Block

In the early literature there are some suggestions that botulinum toxin affects adrenergic junctions (229), but the best evidence for this has been obtained by in vitro tests with adrenergically innervated vas deferens. Such a preparation loses its ability to respond to indirect stimulation when treated with this toxin (222, 223). The toxin acts presynaptically since the poisoned preparation still responds to exogenous norepinephrine. Spontaneous junction potentials are not affected, but indirectly generated excitation potentials are reduced or abolished.

Development of an adrenergic block requires a longer test period or a more rapid indirect stimulation during latency than cholinergic NMJ (223). The relationship between the activity of the preparation and the time to paralysis is similar to that observed with cholinergic test objects. In terms of the number of mouse LD_{50} needed to elicit paralysis, adrenergic preparations are less sensitive (87). The clinical significance of this action is not known.

Chronic Effects

Acute effects are of great immediate concern, but the neuromuscular deficits of botulism can persist for months. Experimental chronic botulism is produced by injecting a small amount of toxin into a muscle so that the animal survives the systemic effects of the toxin that escapes from the injection site. Such a muscle remains paralyzed for weeks (213) and can be studied in situ or as an in vitro preparation.

The nerve endings of an acutely poisoned muscle show no morphological abnormality (56, 79, 211). However, as the paralysis becomes chronic, the poisoned muscle becomes similar to a muscle whose nerve has been severed surgically (56). Whereas only the endplate part of a normal muscle responds to Ach, the whole surface of a chronically poisoned muscle becomes responsive (211) because Ach receptors increase in number and appear over the surface (181). Hyperactivity starts disappearing as some neuromuscular function returns (213) and is coincident with the period when ultraterminal sprouts of motor neurons begin to form new junctions (56). Although some of the poisoned junctions may regain function, electrophysiological evidence supports the histologically suggested correlation between recovery of muscle function

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and development of new junctions (23).

An extra nerve implanted into a normal muscle fails to develop endplates, but it does so in a muscle which is denervated by surgery. Botulinum toxin substitutes for surgical denervation, so that the foreign nerve develops its own endplates (58). Thus, when some of the original endplates that were paralyzed by toxin recover function, a muscle fiber can have the functional synapses of two nerves (67). When toxin is injected into a muscle in which a foreign nerve has been placed, the implanted nerve, but not the original nerve, forms new junctions (58).

SIMILARITIES OF TETANUS TOXIN

Botulinum and tetanus (tetanospasmin) toxins are neurotoxic proteins produced by closely related clostridia. The similarities in structure and action of these molecules are considered below. To emphasize these similarities, the terminology used for botulinum toxin will be applied to tetanus toxin, although other terms are used in the original publications. More detailed discussions of tetanus toxin are available in several reviews (10, 12, 37, 50, 217).

Molecular Structure

Tetanus toxin was orginally determined to be a 4S molecule, but all recently determined values are closer to 7S. Its molecular weight, as determined in different laboratories, ranges from 127,000 to 174,000, with most values between 140,000 and 160,000 (10). These values are within the range of the molecular weights which have been determined for the botulinum neurotoxins.

Tetanus toxin has not been reported to form complexes comparable to those of botulinum toxin, but different purification methods may be the possible reason for this. Botulinum toxin complexes are found because only acidic conditions are used until the very last step, whereas protocols for tetanus toxin purification generally involve an alkaline solution in an early step. If tetanus toxic complexes are present, they would probably be dissociated and their existence would not be recognized.

The specific lethalities of toxins which produce different paralytic effects that may affect vital functions to varying degrees cannot necessarily be compared meaningfully. Despite this reservation, it is interesting that the mouse killing potencies of the two toxins are so similar. The values for tetanus toxin are often in the range from 1×10^8 to 3×10^8 minimum lethal doses per mg of N (10) (or 1.6×10^7 to 4.8×10^7, minimum lethal doses per mg of protein if a 16% N content is assumed). Since 1 LD_{50} can be expected to have less toxin than 1 minimum lethal dose, the specific lethality of tetanus toxin for mice is within the lower range of the specific lethality of botulinum toxins.

Tetanus toxin is like the toxin of proteolytic botulinum cultures in that it is synthesized as a single-chain protein and is nicked into a dichain molecule by an enzyme(s) of the culture after it becomes extracellular. This nicking was first noted in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis comparison of intracellular and extracellular toxins which had been subjected to oxidative sulfitolysis (35). Although the extracellular toxin resolved into two bands representing units of 95,000 daltons (H chain) and 55,000 daltons (L chain), intracellular toxin remained as an intact molecule of 150,000 daltons.

The effect of trypsin on intracellular tetanus toxin confirms that this toxin is synthesized as unnicked molecules. Intracellular toxin preparations contain only unnicked molecules, which are converted by trypsin into dichain molecules that are indistinguishable from the naturally formed dichains recovered from the culture fluids. Both dichains are 160,000-dalton units in which an H chain of 107,000 daltons is joined by disulfide linkage to an L chain of 53,000 daltons. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis resolves these subunits when the dichains are reduced specifically with dithiothreitol (124). Single-chain intracellular toxins and dichain extracellular toxins have the same toxicity (35).

Contradictory results were obtained in earlier studies of terminal amino acids (10), but the data obtained in a recent analysis of electrophoretically pure tetanus toxin (143) make it possible to understand the bond cleavage of nicking. Intracellular tetanus toxin has only one N-terminal amino acid (proline), but extracellular tetanus toxin, in agreement with its dichain nature, has one leucine in addition to the proline. This indicates that a peptide bond formed by the amino group of a leucine residue is cleaved when the single-chain molecule becomes a dichain. Since the L chain has the proline N terminus, the leucine residue is located one-third along the chain length of the unnicked molecule, starting from the N terminus (Fig 1). In view of the other similarities between tetanus and botulinum toxins, it would not be surprising if the L chain of botulinum toxin is also the N-terminal one-third of the unnicked parent molecule.

The separation of the H and L chains of tetanus toxin by molecular sieve chromatography of a reduced sample in urea (124) is very similar to the method used to isolate the chains of type B botulinum neurotoxin. The chains of

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both toxins are individually nontoxic, except for a residual activity that is attributed to contamination with some intact dichain molecules. When the isolated complementary chains are mixed in a 1:1 molar ratio and dialyzed, the chains recombine into dichains; in the case of tetanus toxin, the regenerated toxicity is about 90% of that theoretically possible (125).

A 50,000-dalton piece separates from a 100,000-dalton piece when dichain tetanus toxin is treated with papain. The smaller piece is the C-terminal one-half of the H chain (H_1 fragment [Fig. 1]), and the larger piece is the remainder of the H chain (H_2), which is still joined by a disulfide linkage to the L chain. The H_2 fragment separates from the L chain when the disulfide linkage is reduced (83, 126). A disulfide loop is present in the H_1 fragment. Although the fragments have not been isolated, a similar cleavage of the H chain is the first change observed in the digestion of botulinum dichain toxin by trypsin or chymotrypsin.

The H_1 and H_2 fragments and the L chain of tetanus toxin are antigenically distinct (83, 126). A conformation of the dichain seems to make a fourth antigenic determinant since this antigen is not detected in isolated fragments. Other pieces, whose exact locations in the dichain are not well established, are also antigenic (11). The immunological data available for botulinum neurotoxin are not as detailed, but the different antigenicities of the L and H chains have been established.

Pharmacology

The first evidence that gangliosides of target cells might be the receptors of some toxins was obtained (217) when tetanus toxin was shown to have a high affinity for di- and trisialogangliosides (GB_{1b} and GT_1). This toxin also binds to synaptosomes (74) and to cultured nerve cells, which are rich in gangliosides (51). When other cells are present in a culture, tetanus toxin binds only to neuronal cells, but without preference for cells from a particular part of the nervous system (132). The toxin competes with thyrotropin for gangliosides on thyroid plasma membranes (118).

Previously inferred pathophysiological implications of toxin-ganglioside binding may require some changes if cells have different classes of receptors and biological effects are produced only by the toxin that binds to certain of these classes (12, 232). In any event, botulinum neurotoxin has comparable binding properties; it fixes to synaptosomes and cultured nerve cells so that they do not release the Ach amount normally induced by depolarization. The question of whether gangliosides are receptors for botulinum toxin is discussed above.

Tetanus toxin binds to gangliosides by its H chain (12, 84, 216), and some observations indicate the importance of the H_1 fragment. The likelihood that the H chain of botulinum toxin is the binding subunit has been shown for at least type B neurotoxin since isolated H chain of type B toxin competes against radiolabeled dichain type B toxin for sites on synaptosomes (104).

Tetanus results primarily from effects on the spinal cord, whereas botulism is due to interference at cholinergic junctions. Since tetanus toxin is normally acquired from a wound infection, its route to the spinal cord has been studied intensively. The hematogenous route has had its advocates (229), but it is now accepted that the toxin reaches the spinal cord by centripetal neural transport, particularly in motor nerves (193). The movement of toxicity (110) or, more recently, of radiolabeled toxin has been used to trace this migration. Tetanus toxin enters motor nerves at the presynaptic part of neuromuscular junctions, where radioactivity accumulates (161, 221), and it then moves intraaxonally to the spinal cord (66, 162). It then enters the spinal cord in the ventral root of the half segment. Stimulating the nerves results in faster movement of the toxin (220). Intramuscularly injected botulinum toxin also enters the spinal cord by a similar pathway.

From a functional viewpoint, a monosynaptic reflex is one in which an incoming stimulus passes directly to a motoneuron which, without being influenced by external factors, generates the action potential that it conducts to the effector (muscle). In a polysynaptic reflex, other neurons (interneurons) influence the generation of the action potential in the motoneuron and thereby modulate the reflex. Tetanus toxin does not affect monosynaptic reflexes, but it has an important action on polysynaptic reflexes (229) with Renshaw cell (RC) interneurons. An RC is activated at the synapse which it forms with a recurrent axon fiber of a motoneuron (or other RC), and it influences motoneuron function through its afferent position in the RC-motoneuron synapse. The disturbance of a polysynaptic reflex occurs when the toxin, which entered the spinal cord in the motoneuron, reaches the RC by transsynaptic retrograde movement across the RC-motoneuron synapse (61, 173).

Glycine is the transmitter of this synapse. When the original afferent impulse stimulates the motoneuron, the interneuron is activated via the feedback pathway (recurrent motoneuron fiber), and it releases glycine. The transmitter lowers the resting potential of the motoneuron

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so that the net response of the motoneuron to the original stimulus is a muscle-stimulating potential that is less than would be generated without the moderating influence of the RC. Tetanus toxin blocks release of glycine by the RC; it does not have a postsynaptic effect since the application of glycine elicits a postsynaptic response (38). Since the toxin reduces the inhibitory role of the RC (disinhibition), the muscular contraction of the reflex is exaggerated. The spindle reflex then becomes involved.

Tetanus toxin also blocks a different inhibitory synapse, which is thought to use γ-aminobutyric acid, by suppressing release of transmitter without a postsynaptic poisoning (39). Also, in the late stages of tetanus, the cholinergic synapse which activates the RC may be inhibited (7). These tetanus toxin actions which block release of transmitters in the spinal cord are similar to the inhibition of Ach release at neuromuscular junctions by botulinum toxin.

In agreement with its in vivo actions, tetanus toxin affects the release of glycine and γ-aminobutyric acid by a bed of synaptosomes during electrical stimulation. A bed prepared from the brains of rats injected previously with tetanus toxin releases reduced amounts of glycine and, to some extent, of γ-aminobutyric acid compared with controls. These effects do not occur when the toxin is added to a bed made with synaptosomes from normal animals (159). Likewise, botulinum toxin inhibits the depolarization-stimulated release of Ach by synaptosome suspensions. Although botulinum toxin is much more potent, tetanus toxin also acts on cultures of nerve cells to reduce the quantity of Ach that is released during depolarization (9).

Of the several observations indicating an action of tetanus toxin on peripheral cholinergic transmission (217), one of the most interesting is the flaccid, instead of spastic, paralysis of goldfish. If in situ pectoral fin muscles are injected with tetanus toxin and tested as isolated NMJ after they become paralyzed in approximately 1 day, the muscles fail to contract in response to indirect stimulation. Since the neuromuscular block results from a presynaptic effect that inhibits Ach release, the action of tetanus toxin on such a test preparation is similar to that of botulinum toxin (127).

A slow muscle (soleus) of mice loses its ability to respond to indirect stimulation within about 1 day after being injected with a small amount of tetanus toxin. The frequency of the spontaneous mepp of the muscle does not change over many days, but the skewing in the distribution of amplitudes by a disproportionate abundance of small mepp and other effects are effects of a presynaptic block of neuromuscular transmission. The legs may be spastic from the overactivity of the fast muscle. Chronically paralyzed muscles simulate chemically denervated ones in supersensitivity to Ach and formation of new endplates (57).

A block of transmission at the cholinergic neuromuscular synapses is obtained when muscles are injected with tetanus toxin and then removed from the animals many hours later. Such a block apparently does not occur when the NMJ are isolated first and then treated in vitro with toxin, as is normal in studies with botulinum toxin. The same phenomenon has also been observed in the effect of tetanus toxin on the release of glycine by synaptosomes. It is not known why the test objects for in vitro demonstration of tetanus toxin effects must come from toxin-treated animals.

CONCLUDING REMARKS

Botulinum and tetanus toxins are comparable in several respects. They are similar in size, are synthesized as unnicked proteins, and are later nicked by culture enzymes or experimentally by trypsin into dichains in which the larger unit is about twice the size of the smaller. They cause muscular paralysis by inhibiting transmitter release. The paralytic effects differ because the two toxins act at different sites with different kinds of synapses. When used experimentally at high concentrations or by direct application to selected sites, these toxins elicit effects that are not expected from clinical observations. At least some of the responses observed simulate actions that are important effects of the other toxin. Botulinum and tetanus toxins may have similar toxic potentials, whose expressions differ more in quantitative than qualitative aspects (9). The relative affinities of the H chains for different receptors may be a basis for the difference. Since a large amount of tetanus toxin fed to animals is toxic (111), it has a food-poisoning potential, but such illness does not occur, probably because the needed dose is not formed in foods.

Diphtheria toxin (34), ricin, and abrin (158) are toxins which inhibit protein synthesis. Like the two neurotoxins discussed here, they are proteins in which two dissimilar polypeptides are joined by disulfide linkages. When separated from each other, neither chain is toxic for animals, although the smaller chain can by itself inhibit protein synthesis in a cell-free system.

With these three other toxins, botulinum and tetanus toxins form a class of dichain molecules whose in vivo toxicities are due to a larger subunit binding to target cells and making it possible for a pharmacologically active smaller chain to reach its site of action. The evidence for the neurotoxins is less complete than that for the

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other toxins since there is no direct proof that the L chains are toxic in the absence of the complementary chain. Such evidence is difficult to obtain because a suitable test method is not known. In the case of botulinum toxin, the L chain might substitute for the dichain in preventing depolarization-evoked release of Ach from synaptosomes. However, its failure would not be surprising since inhibition by the dichain probably requires the H chain to attach to synaptosomes and correctly position the L chain. A suitable test preparation is likely to have membrane-free units which can be demonstrably poisoned with the dichains.

Nevertheless, the evidence is convincing that the neurotoxins are toxic in vivo because their complementary chains have functional roles that parallel those of the inhibitors of protein synthesis. Since neither chain is toxic when tested separately in mice, toxicity depends on the two chains being in a specific relationship to each other. The receptor-binding property of the H chains is indicated by the affinity of tetanus toxin H chain for certain gangliosides and the ability of botulinum H chain (type B) to prevent attachment of the intact dichain to synaptosomes. The L chains do not have receptor-binding activities.

Interest in botulinum neurotoxin started with the more practical aspects of its food-poisoning potential. However, with a better understanding of its pharmacology, this toxin has become an investigative tool and may even be useful in correcting medical problems. It may be an alternative to surgery for correcting strabismus. When the stronger of the two antagonistic eye muscles is injected with this toxin, it undergoes a slight atrophy during the time it is paralyzed; this gives the weaker muscle a chance to take up the slack and thereby improve alignment of the eye (175). The ability of this toxin to cause chemical denervation has been used to study the trophic effects of nerve ending regeneration. It can be used to study the mechanism of neuromuscular transmission when used in conjunction with agents that affect nerve endings by a different mechanism.

APPENDIX

The neurophysiology of Ach at the cholinergic synapse is as follows (Fig. 2). (i) A nerve stimulus (action potential) enters a nerve ending. (ii) This causes an influx of Ca^{2+}. (iii) Ca^{2+} acts so that the synaptic vesicles fuse with the limiting membrane. Vesicles are packets containing quanta of Ach which has been synthesized in the cytoplasm from choline and acetyl coenzyme A by choline acetyltransferase. (iv) Exocytosis of Ach from vesicles occurs. (v) Ach crosses the synaptic space and acts on postsynaptic receptors. The action of many Ach quanta released by the nerve stimulus produces an electrical potential change of the postsynaptic endplate (endplate potential), which causes a response of the innervated structure. Vesicles also release Ach spontaneously and randomly. These releases from one or a few vesicles at one time give rise to postsynaptic miniature endplate potentials which are insufficient to cause a response of the innervated structure. (vi) Ach is rapidly hydrolyzed by acetylcholinesterase.

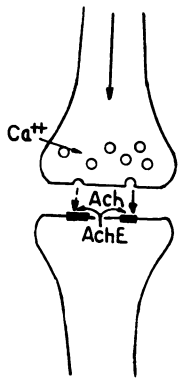

FIG. 2. *Neurophysiology of Ach at the cholinergic synapse. AchE, Acetylcholinesterase.*

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