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PHARMACOLOGICAL STUDIES ON CLOSTRIDIAL NEUROTOXINS

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**Pharmacological Studies on Clostridial Neurotoxins**

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Experiments have been done that compare the actions of botulinum toxin type A, tetanus toxin, diphtheria toxin and β-bungarotoxin on the cholinergic neuromuscular junction. All four toxins bind to tissues without producing adverse effects; each of the toxins has its own unique class of receptors. After tissue binding has occurred, botulinum toxin, tetanus toxin and diphtheria toxin are internalized; this process is antagonized by chloroquine, ammonium chloride and methylamine hydrochloride. β-Bungarotoxin is not internalized and is not antagonized by the drugs just listed. Diphtheria toxin acts intracellularly to ADP-ribosylate elongation factor 2. The intracellular actions of botulinum toxin and tetanus toxin have not been determined.

The actions of botulinum type C₁ toxin have been compared with those of type C₂ toxin. The former substance is a neurotoxin that blocks acetylcholine release at motor nerve endings. The latter substance is a binary toxin that is devoid of effects on the nervous system. Type C₂ toxin evokes a variety of systemic effects, including hemorrhaging in the lungs, secretion of fluids into the trachea, collection of fluids in the pleural cavity, and volume depletion-induced fall in mean arterial blood pressure. This constellation of effects causes pulmonary failure and subsequent death.
TABLE OF CONTENTS

1. Title Page ........................................ .... 1
2. Report Documentation Page .............................. 2
3. Summary .................................................. 3
4. Table of Contents ....................................... 4
5. Progress Report (11/1/81 to 8/31/82) ................... 5
   A. Comparative studies on toxins ...................... 5
   B. Comparative studies on nicked and unnicked molecules ............... 10
   C. Comparative studies on $C_1$ and $C_2$ toxins .......... 12
   D. Drug antagonism studies ............................. 15
6. Literature Cited ........................................... 20
7. Distribution List ........................................ 25
5. Progress Report

A. Comparative studies on toxins.

Diphtheria toxin is a substance that is produced by the organism *Corynebacterium diphtheriae* (Collier, 1977; Pappenheimer, 1977). The molecule is synthesized intracellularly as a single chain polypeptide with a molecular weight of ~61 kdal (Gill and Dinius, 1971; Collier and Kandel, 1971). When exposed to trypsin, the single chain molecule is cleaved ("nicked") to yield a dichain molecule in which a heavy chain polypeptide (~40 kdal) is linked by a disulfide bond to a light chain polypeptide (~21 kdal) (Gill and Dinius, 1971; Drazin et al., 1971).

Diphtheria toxin acts on a variety of cell types to inhibit protein synthesis, which in turn may cause cell death (Strauss and Hendee, 1959). The ability of diphtheria toxin to exert its pharmacological actions is related to a sequence of three events (Collier, 1977; Pappenheimer, 1977). First, the toxin binds to a specific class of cell surface receptors on vulnerable cells. Binding of the ligand to its membrane receptor is essential to the overall process of toxicity, but the binding step itself does not alter cell function. Next, receptor-bound ligand is internalized. The mechanism for translocation across the membrane has not been established, but the two most widely discussed possibilities are receptor-mediated endocytosis and ligand-mediated channel formation. Finally, the toxin acts intracellularly to ADP-ribosylate elongation factor 2; in doing so the toxin halts protein synthesis.

The structure of the diphtheria toxin molecule can be related to its pharmacological actions. The 40 kdal polypeptide contains a receptor binding moiety (Ittelson and Gill, 1973; Zanen et al., 1976; Middlebrook et al., 1978) and also possesses channel forming properties (Donovan et al., 1981; Kagan et al., 1981). The 21 kdal polypeptide is an enzyme that catalyzes the transfer of adenosine diphosphate from nicotinamide adenine dinucleotide to elongation factor 2 (Collier, 1967; Gill and Pappenheimer, 1971; Kandel et al., 1974). The entire (e.g., dichain) molecule is needed to poison intact cells, but only the light chain polypeptide is needed to inhibit protein synthesis in broken cell preparations.

The sequence of events that underlies the ability of diphtheria toxin to poison eukaryotic cells is not unique to this toxin (Gill, 1978). To the contrary, the two most potent pharmacological substances known - botulinum toxin and tetanus toxin - adhere to the same scheme. Botulinum toxin and tetanus toxin are microbial substances that are synthesized intracellularly as single chain polypeptides with molecular weights in the range 140 to 160 kdal (DasGupta and Sugiyama, 1977; Simpson, 1981). When exposed to trypsin, the single chain molecules are
nicked to yield dichain molecules in which two polypeptide chains (heavy chain ~100 kdal; light chain ~50 kdal) are covalently linked by a disulfide bridge (DasGupta and Sugiyama, 1972).

Botulinum toxin acts on cholinergic nerve endings to block the release of acetylcholine (Burgen et al., 1949; Brooks, 1956). To exert this effect the toxin proceeds through a series of three reactions, including an extracellular binding step, a membrane translocation step, and an intracellular lytic step (Simpson, 1980). Tetanus toxin is generally known for its ability to act in the central nervous system to block the release of inhibitory transmitters (Bizzini, 1979), but the toxin also acts on peripheral nerves to block the release of acetylcholine (Habermann et al., 1980; Bigalke and Haberman, 1980). In producing blockade of cholinergic transmission, tetanus toxin proceeds through the same series of three reactions as does botulinum toxin (Schmitt et al., 1981).

Recent findings suggest that botulinum toxin and tetanus toxin are substances whose dichain structures can be related to their pharmacological actions. The heavy chain polypeptide of the clostridial neurotoxins have been shown to mediate tissue binding (e.g., Kozaki, 1979; Morris et al., 1980). The light chains are presumed to mediate intracellular toxicity (Simpson, 1981).

The data indicate that diphtheria toxin, botulinum toxin and tetanus toxin behave similarly in exerting their effects on target cells. This suggests that an effort should be made to determine whether the three bacterial toxins share a common receptor on cell surfaces, utilize the same mechanism for translocation across membrane, or produce the same intracellular lytic effect. The present research is the first to compare these three toxins at the cholinergic neuromuscular junction.

In addition to the three substances just described, β-bungarotoxin is an extremely potent polypeptide (~22 kdal) that is found in the venom of Bungarus multicinctus. The β-bungarotoxin molecule is composed of two subunits (~9 kdal and ~12 kdal) that are linked by a disulfide bond (Kelly and Brown, 1974; Abe et al., 1977). The relevance of nicking to the synthesis and biological activity of the molecule has not yet been studied.

β-bungarotoxin shares with botulinum toxin and tetanus toxin the ability to block acetylcholine release (Chang and Lee, 1963; Chang et al., 1973). In exerting this pharmacological action the toxin relies on two functional components, one of which is a binding moiety that fixes to nerve endings (Chang et al., 1973), and the other of which is an enzyme with phospholipase A2 activity (Howard, 1975; Wernicke et al., 1975; Strong et al., 1976; Abe et al., 1977). The relationship between the dichain
structure of the molecule and the bifunctional aspect of its pharmacological actions has not been determined. Nevertheless, the data that are available suggest that the actions of β-bungarotoxin should be compared with those of diphtheria toxin and the clostridial neurotoxins. The present research provides such a comparison.

The data that have been obtained during the present contract period support three conclusions: i.) the phrenic nerve-hemidiaphragm has receptors for at least five classes of toxins, these being botulinum toxin, tetanus toxin, diphtheria toxin, β-bungarotoxin and α-bungarotoxin, ii.) botulinum toxin, tetanus toxin and diphtheria toxin use the same or a similar mechanism for internalization, which may be receptor mediated endocytosis, but the bungarotoxins are not endocytosed, and iii.) diphtheria toxin, but not clostridial neurotoxins or bungarotoxins, ADP-ribosylate nerve tissue elongation factor 2.

Cell surface receptors. Most substances that block cholinergic transmission exert their effects by occluding membrane receptors. Botulinum toxin is fundamentally different. The binding of botulinum toxin to nerve membranes is essential to the development of toxicity, but the binding step itself does not block transmission (Simpson, 1980; and the present report). To inhibit the release of acetylcholine, the toxin must cross the membrane and exert an intracellular lytic effect (Simpson, 1980; and see below). Recent findings suggest that tetanus toxin also crosses the nerve membrane to produce blockade of cholinergic transmission (Schmitt et al., 1981).

The sequence of receptor binding, translocation across the membrane, and expression of an intracellular cytotoxic effect is not characteristic of most substances that act on the nervous system. However, there are many polypeptides and proteins that adhere to this scheme (Neville and Chang, 1978), and diphtheria toxin is generally regarded as a prototype for the group (Gill, 1978). Therefore, the actions of botulinum toxin and tetanus toxin have been compared with those of diphtheria toxin. β-Bungarotoxin has been included in the comparison because it shares with clostridial neurotoxins a presynaptic site of action.

The data reveal that botulinum toxin, tetanus toxin and β-bungarotoxin bind reasonably quickly and essentially irreversibly to the phrenic nerve-hemidiaphragm. The binding of these toxins to their membrane receptors does not alter cell function (and see Abe et al., 1977; Habermann et al., 1980; Simpson, 1980). This finding indicates that the neurotoxins do not occlude the nicotinic cholinergic receptor, and thus they do not share a common binding site with α-bungarotoxin.

Botulinum toxin, tetanus toxin and β-bungarotoxin have different apparent affinities for tissues from mouse, rat,
hamster and guinea pig. When tested on a vulnerable tissue (mouse phrenic nerve), the three toxins have different apparent potencies, with botulinum toxin > tetanus toxin > β-bungarotoxin. Perhaps most importantly, the three toxins do not share the same membrane receptor. The C-fragment of tetanus toxin, which has been shown to bind to brain tissue (Morris et al., 1980) and to peripheral nerve (Simpson, submitted for publication), protected tissues from native tetanus toxin, but it did not protect tissues from botulinum toxin or β-bungarotoxin. In addition, tissue bound but catalytically inactive β-bungarotoxin did not protect tissues from the paralytic effects of clostridial neurotoxins. These data strongly indicate that botulinum toxin, tetanus toxin and β-bungarotoxin each have distinct receptors.

The findings on tetanus toxin and its C-fragment have one especially important implication. It has been proposed that clostridial neurotoxins are two component substances in which the heavy chain mediates tissue binding and the light chain mediates intracellular toxicity (Simpson, 1980; 1981). Morris et al. (1980) have shown that the C-fragment from tetanus toxin inhibits the binding of native toxin to isolated nerve membranes. The present research extends the work of Morris et al. (1980), and it provides the first demonstration that an atoxic fragment from a clostridial neurotoxin is capable of antagonizing the neuromuscular blocking properties of native toxin.

There is one point about the tetanus toxin data that requires clarification. Ledley et al. (1977) have reported that high concentrations of cholera toxin inhibit the binding of tetanus toxin to nerve membranes. Their study did not include any tests for tetanus toxin activity. In the present study, cholera toxin did not block neuromuscular transmission, and neither cholera toxin nor its binding fragment inhibited the paralytic action of tetanus toxin. The apparent discrepancy between the earlier and the present data may be explainable on methodological grounds. Ledley et al. (1977) reported that even high concentrations of cholera toxin only partially occlude receptor binding by tetanus toxin. This partial occlusion may be difficult to detect by bioassay procedures, such as monitoring onset of neuromuscular blockade. In addition, Ledley et al. (1977) used only small amounts of tetanus toxin in doing radio-receptor assays, whereas the present study used larger amounts of tetanus toxin to produce neuromuscular blockade. Taken together, the two studies suggest that if cholera toxin does have affinity for tetanus toxin receptors, that affinity is slight.

Diptheria toxin was shown to produce inhibition of protein synthesis in hamster and guinea pig diaphragm. The action of diptheria toxin was antagonized by CRM197, a serologically related protein that retains binding activity but which lacks catalytic activity (Uchida et al., 1973). CRM197 did not afford any protection against the neuroparalytic effects of botulinum toxin, tetanus toxin or β-bungarotoxin. When viewed in the context of the data discussed above, this finding
indicates that the diphteria toxin receptor is distinct from those for clostridial neurotoxins and for bungarotoxins.

**Internalization of toxins.** Diphtheria toxin must be internalized to exert its cytotoxic effect (Collier, 1977; Pappenheimer, 1977), whereas α-bungarotoxin acts at the cell surface (Lee, 1972). The precise site at which botulinum toxin, tetanus toxin and β-bungarotoxin exert their effects is less clear, although there is suggestive evidence that clostridial neurotoxins act in the cell interior (Simpson, 1980; Schmitt et al., 1981) and β-bungarotoxin acts at the cell surface (Howard and Wu, 1976).

There are a number of drugs that are known to inhibit the internalization and/or lysosomal processing of hormones and toxins that cross cell membranes (DeDuve et al., 1974; Goldstein et al., 1979). The most carefully studied of these substances are chloroquine, ammonium chloride and methylamine hydrochloride, all of which inhibit the action of diphtheria toxin on cell cultures (Kim and Groman, 1965; Leppla et al., 1980). In recent studies, chloroquine, ammonium chloride and methylamine hydrochloride were shown to be extremely effective antagonists of botulinum toxin (see part D below). This provides the strongest evidence to date that clostridial neurotoxins are internalized. By contrast, ammonium chloride and methylamine hydrochloride had no effect on the paralytic action of β-bungarotoxin.

The toxins that are internalized are too large to penetrate known pores or channels in the membrane. These toxins all bind to membranes, so the most likely mechanism for internalization is receptor mediated endocytosis. The fact that diphtheria toxin and the clostridial neurotoxins are antagonized by the same class of substances could mean that they are internalized by the same or a closely similar mechanism. On the other hand, β-bungarotoxin is either not endocytosed, or it is endocytosed by a mechanism that has not been pharmacologically characterized.

**Inhibition of protein synthesis and ADP-ribosylation of elongation factor 2.** As expected, diphtheria toxin inhibited protein synthesis in innervated diaphragms excised from animals (hamster, guinea pig) known to be sensitive to the toxin (Collier, 1977; Pappenheimer, 1977). Because of the magnitude of the observed effect, diphtheria toxin must have acted on diaphragm; it is merely presumption that the toxin acted on nerve endings.

The light chain of diphtheria toxin is responsible for ADP-ribosylation. To exert this effect, the light chain must be released (disulfide bond reduction) or otherwise separated from the heavy chain. Unlike diphtheria toxin, neither the clostridial neurotoxins nor β-bungarotoxin ADP-ribosylated elongation factor 2. This outcome was obtained with native
toxins and with reduced toxins. This means that even if diptheria toxin and clostridial neurotoxins are internalized by the same mechanism, they exert different effects inside vulnerable cells. The intracellular substrate for clostridial neurotoxins remains to be identified.

B. Comparative studies on nicked and unnicked molecules.

Botulinum neurotoxin is synthesized intracellularly as a single chain (unnicked) polypeptide (DasGupta, 1981; Simpson, 1981). When these single chain molecules are exposed to trypsin, they are cleaved (nicked) to yield dichain molecules. The two chains in each molecule are linked by a disulfide bond.

The predominant specie of molecule in culture fluids of most strains of Clostridium botulinum is the dichain neurotoxin. This suggests that these organisms have an endogenous protease responsible for nicking. However, type E cultures are typically non-proteolytic, so the molecule that is released is the single chain neurotoxin. There are few reports (e.g., Duff et al., 1956; Sakaguchi and Sakaguchi, 1958) comparing the toxicity of trypsinized and untrypsinized molecules; those that have been published were done in vivo (mouse lethality studies) and they involved impure preparations of neurotoxins. The present research is the first to examine the pharmacological actions of pure unnicked (Eun) and nicked (En) type E toxin molecules on the isolated neuromuscular junction.

The data reveal that the Eun possesses, at most, only 1.0 percent of the activity of En. There are three ways in which these data can be explained: i.) Eun has no pharmacological activity; the apparent toxicity of Eun is due to trace contamination with En, ii.) Eun has a diminished ability to bind, but it possesses full capability to express toxicity (see below), or iii.) Eun has a normal ability to bind, but it has a diminished ability to express toxicity (see below). There is as yet no basis on which to choose one or another of these possibilities.

The data also reveal that cyclohexanedione (CHD), an agent that specifically modifies arginine residues (Patthy and Smith, 1975), exerts two major effects. It diminishes the toxicity of the dichain molecule, and it diminishes the ability of trypsin to nick and to activate the single chain molecule. These observations are consistent with results obtained in a study on the relationship between modification of arginine residues in type E neurotoxin and mouse lethality (DasGupta and Sugiyama, 1980). The fact that CHD diminishes the neuroparalytic activity of the dichain molecule makes evident that at least one arginine residue is involved in maintaining toxigenic structure. The finding that CHD antagonizes the ability of trypsin to nick and to activate the single chain molecule may seem to indicate that trypsin-induced nicking underlies trypsin-
induced activation. However, there are reasons to be cautious about accepting this causal relationship (DasGupta, 1981).

Firstly, trypsin could act by mechanisms other than nicking to activate the neurotoxin, and these putative mechanisms would not have been detected by the electrophoresis experiments. For example, trypsin could cleave a small fragment from the amino or carboxyl terminus. Secondly, Sakaguchi and his associates have published data that suggest that, under the appropriate conditions, the rate of nicking is not equivalent to the rate of activation (e.g., Ohishi and Sakaguchi, 1977). Taken collectively, the data show that at least one arginine residue is involved in maintaining toxigenic structure, but the data do not yet reveal whether the critical arginine residue is at the site of nicking and/or at some other site.

Trypsin activated type E neurotoxin interacts with the neuromuscular junction in a way that appears identical to that of type A neurotoxin (Simpson, 1980). En acts through a series of three steps, involving a binding, a translocation and a lytic step.

Botulinum neurotoxin binds irreversibly to a membrane receptor on the cholinergic nerve ending. Binding appears to require little or no energy, as judged by the fact that binding is little affected by changes in temperature. Perhaps the most important characteristic of binding is that it does not produce any adverse effect on neuromuscular transmission. Some event must occur after the binding step before there is blockade of transmitter release. This is in keeping with the concept that the process of binding occurs before and is separable from the process of toxicity (Simpson, 1981). By extension, this accounts for the possibility suggested above that Eun may be relatively inactive either because it lacks binding activity or because it lacks toxic activity.

When bound to its receptor at the cell surface, the neurotoxin remains partially accessible to the neutralizing effects of antitoxin. With the passage of time, the neurotoxin becomes inaccessible to the neutralizing effects of antitoxin. This finding can best be explained by assuming that the neurotoxin is internalized. This idea is strongly supported by three related findings. Firstly, the ability of the neurotoxin to become inaccessible to antitoxin is an energy dependent phenomenon (e.g., retarded by low temperature). The neurotoxin is far too large to penetrate the membrane through any known channels or pores. However, an active process of translocation could account for membrane penetration and would also explain the energy dependence of the phenomenon. Secondly, there are numerous polypeptides and proteins that cross cell membranes (Neville and Chang, 1978), and there are also a number of drugs that inhibit internalization and/or lysosomal processing of these compounds (DeDuve et al., 1974; Goldstein et al., 1979). Two of the most extensively studied drugs are ammonium chloride and methylamine hydrochloride. Both of these drugs were found to delay the onset of paralysis due to En. The data indicate that ammonium chloride and methyl-
Amine hydrochloride were not inhibiting tissue binding of neurotoxin, nor were they reversing neurotoxin-induced paralysis. An intermediate step, such as translocation through the membrane, appeared to be affected. Thirdly, there was an interesting interaction between antitoxin and the antagonistic drugs. Both ammonium chloride and methylamine hydrochloride caused the neurotoxin to remain accessible to antitoxin for a longer period of time than occurred in the absence of antagonists. It is difficult to envision an explanation for these data other than that the neurotoxin is internalized.

In the presence or in the absence of antagonistic drugs, E₅₀ disappears from the neutralizing effects of antitoxin before there is full development of paralysis. This finding suggests that in addition to binding and translocation, there is at least one more step in which the neurotoxin is involved. It is the latter step that results in blockade of excitation-secretion coupling. The remarkable potency of the neurotoxin could easily be explained if the molecule acted intracellularly as an enzyme. Such an action would be closely analogous to that of several potent bacterial toxins (Gill, 1978).

C. Comparative studies on C₁ and C₂ toxins.

Both C₁ and C₂ toxins are synthesized by Clostridium botulinum, and in many cases they are synthesized by the same strain of bacteria (Sugiyama, 1980; Simpson, 1981). However, the nucleic acid sequences responsible for the synthesis of the toxins are not closely associated. The genome for production of C₁ toxin is carried by a bacteriophage (Inoue and Iida, 1970; Eklund et al., 1971), but the genome for production of C₂ toxin is carried by the bacterium itself (Eklund and Poysky, 1972; 1974).

C₁ and C₂ toxins have similar molecular weights, both being in the range of 150,000 to 160,000 daltons (Syuto and Kubo, 1977; Ohishi et al., 1980b). Also, both toxins are composed of two polypeptide chains that have a molecular weight ratio of 1:2 (Syuto and Kubo, 1977; Ohishi et al., 1980b). However, the substances differ in the sense that the two polypeptide chains of C₁ toxin are linked by a disulfide bond (Syuto and Kubo, 1977; 1981), but the two components of C₂ toxin are not covalently linked (Iwasaki et al., 1980).

The fact that the two toxins differ in relation to an interchain S-S bond has prompted experiments aimed at determining the effects of disulfide bond reducing agents and sulfhydryl group blocking agents. The presence of a disulfide bond was found to be essential for the neuromuscular blocking actions of C₁ toxin; when this bond was reduced, the toxin lost its potency. On the other hand, DTT did not inactivate the C₂ toxin. The latter substance either completely lacks disulfide bonds (e.g., intrachain), or it possesses such bonds at sites that are not
essential for expression of biological activity. By contrast, both toxins were vulnerable to sulfhydryl group blockade; treatment of either toxin with NEM caused loss of pharmacological activity.

The data just presented raise a provocative question. Is it possible that at an early stage of evolution the two components of C₂ toxin were covalently linked in a single molecule? An answer to this question might be obtained by cross-linking the two components with a bridge that possesses an easily reducible disulfide bond. Efforts to synthesize such a molecule and test it for pharmacological activity are currently underway.

C₁ toxin and the neuromuscular junction. It has long been assumed that C₁ and C₂ are neurotoxins, even though neither substance has previously been tested on an isolated neuromuscular junction. In fact, of the eight botulinum toxins, only four (types A, B, D and E) have actually been shown to block cholinergic transmission. The present research is the first to evaluate C₁ and C₂ toxins. The data reveal that both toxins are remarkably potent pharmacological substances, but they have differing mechanisms of action. C₁ is an authentic neurotoxin, whereas C₂ is a novel substance that lacks neurotoxicity.

A model has been proposed to account for the neuromuscular blocking properties of botulinum neurotoxin (Simpson, 1980; 1981). This model envisions the toxin progressing through a series of three steps, which includes binding, internalization, and subsequent expression of a lytic effect. Binding is little influenced by nerve stimulation or temperature, but it does leave the toxin accessible to inactivation by antitoxin; internalization is markedly influenced by drugs such as methylamine and ammonium chloride; the lytic effect is delayed by low rates of nerve stimulation and low temperature, and it does not leave the toxin accessible to antitoxin.

Type C₁ toxin appears to interact with the neuromuscular junction in a way that is compatible with the proposed model. Reducing the rate of nerve stimulation, reducing ambient temperature, or pretreating tissues with ammonium chloride or methylamine slow the rate of onset of C₁ toxin-induced paralysis. Additionally, type C₁ antitoxin is effective only if added simultaneously with or shortly after toxin; the antitoxin does not reverse toxin-induced paralysis.

Interestingly, the toxin is very potent in blocking acetylcholine release from motoneurons, but it has little potency in blocking transmitter release from ganglia. Animals that received the toxin intravenously developed neuromuscular blockade (e.g., flaccid paralysis), but they did not develop ganglionic blockade (e.g., low blood pressure or low heart rate). No effort was made to test the activity of C₁ toxin at postganglionic parasympathetic sites.
C2 toxin and the cardiopulmonary system. C2 toxin does not cause flaccid paralysis in mouse, rat, guinea pig or chick; it does not cause neuromuscular blockade in isolated phrenic nerve-hemidiaphragm or biventer cervixes; and it does not act as an antagonist of C1 toxin at the mouse neuromuscular junction. The data demonstrate convincingly that C2 toxin does not block the release of acetylcholine from motoneurons.

Aside from the neuromuscular junction there are several other neuroanatomical sites at which C2 toxin lacks activity. The toxin does not block ganglionic transmission or postganglionic sympathetic transmission at α-adrenergic sites, as evidenced by the fact that heart rate in intoxicated animals remains normal or slightly elevated. Furthermore, the toxin does not produce α-adrenergic blockade, as evidenced by the fact that aortic strips taken from intoxicated animals remain responsive to 1-norepinephrine. The large size and rapid onset of effect of C2 toxin make unlikely a central nervous system site of action. In short, there is no reason to believe that C2 is a neurotoxin.

Systemic administration of C2 toxin causes four prominent effects which, to varying degrees, may be interrelated. The toxin causes hypotension, hemorrhaging in the lungs, collection of fluids around the lungs, and collection of fluids in the trachea. C2 toxin does not cause relaxation of vascular smooth muscle, but it does cause an increase in vascular permeability (Ohishi et al., 1980a). These findings suggest that toxin-induced hypotension is not due to vasodilation, but instead is due to volume depletion. It is conceivable that the four prominent effects evoked by the toxin have a common subcellular mechanism, and that this mechanism is either an increase in cellular permeability or an increase in cellular secretion.

Because of the multiplicity of toxin effects, one cannot be certain about the primary cause of death. Indeed, death may be due to several interacting phenomena. The combination of hypotension, hemorrhaging into the lungs, and probable aspiration of fluids could act collectively to produce fatal pulmonary dysfunction. Thus, the outcome of both C1 intoxication and C2 intoxication is respiratory failure, but the underlying mechanisms are unrelated.

C2 toxin is a unique pharmacological substance. Without doubt, the most fascinating observation on C2 toxin is that its individual polypeptide components do not have to be administered simultaneously to evoke toxicity. The administration of either component, followed by a substantial interval and then administration of the other component, causes characteristic toxicity. This outcome is achieved with doses of the components that are individually atoxic.

One might argue that the two components reassociate in
plasma, and thus that toxicity can be produced only by the aggregate molecule. However, there is an experimental finding that makes this proposal unlikely. When animals received component II, and after an interval received component I plus antibody to component II, toxicity resulted. It is difficult to envision how one component can find and reassociate with the other component under conditions in which antibody cannot find and associate with its antigen. More plausibly, each chain exerts an effect that is not toxic, but the combination of effects is toxic. Perhaps the heavier component binds to tissues and alters them in a way that makes them vulnerable to the pharmacological actions of the lighter component.

There is a powerful motive for exploring the idea just proposed. C2 toxin appears to satisfy the criteria for being a binary toxin. By definition, a binary toxin is an entity whose individually administered components are atoxic, but whose collectively administered components are fully toxic. To date, only two binary toxins have been described, these being leukocidin, which has two components (Noda et al., 1980) and anthrax toxin, which has three components (Leppla, 1982). Thus, the data suggest that C2 toxin may belong to one of the rarest known classes of pharmacological substances. Beyond this, data on in vivo toxicity indicate that C2 toxin is the most potent binary toxin yet described.

Nomenclature. There is a semantic byproduct of the pharmacological data presented in this report. The current scheme of designating the botulinum toxins as types A, B, C1, C2 etc. is based on the premise that these substances form an homologous series. To qualify for inclusion, a substance should have the same structure, same site of action and same mechanism of action as other members of the group. C2 toxin fails to satisfy any of these criteria, and thus there is no reason to include it in the homologous series of neurotoxins. Therefore, the author proposes that the term botulinum neurotoxin be confined to the seven structurally similar substances that block acetylcholine release. These substances should be designated types A, B, C, D, E, F and G. The substance formerly identified as type C1 toxin may now be referred to merely as type C toxin. The substance formerly identified as type C2 toxin should now be called botulinum alpha toxin. Adoption of the term alpha toxin is in keeping with conventional practices for the naming of bacterial substances whose precise mechanism of action has not been determined.

D. Drug antagonism studies.

Chloroquine inhibits the biological activity and/or cellular degradation of a variety of peptide hormones and protein toxins (DeDuve et al., 1974; Goldstein et al., 1979;
Leppla et al., 1980). In the case of certain toxins, investigators have proposed that inhibition of cellular degradation may be the mechanism that underlies inhibition of biological activity (Leppla et al., 1980). According to this hypothesis, protein toxins must be internalized and undergo lysosomal processing (e.g., proteolytic cleavage) before toxicity can be expressed. When lysosomal processing is inhibited, the internalized toxin is not cleaved to yield a biologically active molecule.

There are two points pertaining to this hypothesis that are relevant to the present research. Chloroquine is widely acknowledged to be an agent that antagonizes protein toxins that act intracellularly (Goldstein et al., 1979). Indeed, antagonism of any toxin by chloroquine is viewed as suggestive evidence that the toxin in question is internalized. Chloroquine is also known to be a lysosomotropic agent that inhibits degradation of some peptide hormones and protein toxins (DeDuve et al., 1974). However, the possibility that inhibition of degradation accounts for inhibition of pharmacological activity remains uncertain. In keeping with these points, the present research has examined the ability of chloroquine to antagonize botulinum toxin, tetanus toxin and β-bungarotoxin. Antagonism has been viewed as tentative evidence that the toxin in question is internalized, but not necessarily that the toxin undergoes lysosomal processing.

In preliminary experiments, both chloroquine and hydroxychloroquine were found to depress neuromuscular transmission. These drugs diminished muscle responses to potassium and to nicotinic cholinergic agonists. However, it is unlikely that the nicotinic cholinergic blocking properties of these drugs account for their abilities to antagonize toxins. Most cells that are vulnerable to internalized toxins do not have cholinergic receptors, so receptor blockade is irrelevant. In addition, blockade of nicotinic receptors by d-tubocurarine did not antagonize botulinum toxin.

A key pharmacological property of chloroquine and hydroxychloroquine is that, within certain time and concentration limits, their neuromuscular blocking effects are reversible. This has permitted experiments aimed at determining whether aminoguanolines antagonize the onset of cholinergic blockade caused by presynaptically acting neurotoxins. The drugs were found to antagonize botulinum neurotoxins types A and B, but they had little if any ability to antagonize tetanus toxin and β-bungarotoxin.

Two characteristics of the interaction between chloroquine and botulinum toxin seem noteworthy. One of these pertains to the sustained action of chloroquine, and the other pertains to the protective effects of type specific antitoxin. The data that were obtained show that chloroquine can exert a sustained effect. Tissues that were incubated in chloroquine
and botulinum toxin for two, three or four hours, then washed free of unbound drug and toxin, all were paralyzed in approximately the same length of time. There was no evidence that progressively longer intervals of incubation resulted in progressively shorter toxin-induced paralysis times. This finding indicates that the action of botulinum toxin was virtually arrested in the presence of chloroquine. If chloroquine does in fact arrest the toxin, then this drug would be the most effective antagonist ever described for delaying onset of toxin-induced neuromuscular blockade. An agent which shared with chloroquine the ability to arrest the action of botulinum toxin, but which differed from chloroquine in its ability to depress tissue excitability, would have a valuable therapeutic role.

It is interesting that antitoxin continued to exert a protective effect in the presence of chloroquine. Such data could be interpreted in one of two ways. The simpler explanation is that chloroquine causes the toxin to be trapped at an extracellular site. A more complex alternative is that the internalized toxin is not processed, so the toxin-receptor aggregate is reinserted into the membrane, i.e., reexposed to the extracellular environment. In either case, antitoxin would be expected to exert a protective effect. Once again, there are clear therapeutic implications.

Neither chloroquine nor hydroxychloroquine significantly antagonized tetanus toxin. In view of the similarity in origin, structure and pharmacological activity of botulinum and tetanus toxins (e.g., DasGupta and Sugiyama, 1977), it is surprising that antagonism was not observed. The observation is even more puzzling when one considers that tetanus toxin is known to be internalized by nerve cells when it produces in vivo spastic paralysis. One possible explanation for these data is that chloroquine acts at the neuromuscular junction to exert an effect not related to internalization of toxins. Or alternatively, chloroquine may exert its expected effect, but tetanus toxin may belong to a novel class of internalized poisons. In at least one respect the latter possibility is true. Most toxins that are internalized act in the immediate vicinity of cell entry. Tetanus toxin differs in that cell entry normally occurs at the nerve terminals of α-motoneurons, but drug action is normally expressed at a remote site (central nervous system inhibitory synapses). Whether this or some other mechanism accounts for the failure of aminoquinolines to antagonize tetanus toxin is unknown.

Chloroquine and hydroxychloroquine also failed to antagonize β-bungarotoxin, but this was an expected finding. β-Bungarotoxin acts extracellularly (Howard and Wu, 1976; Strong et al., 1977), so there would be no reason to expect a drug that alters internalization to inhibit onset of paralysis. Even if chloroquine exerts an extracellular effect at the botulinum toxin receptor, this would not be predictive of an interaction
with β-bungarotoxin. Two groups have reported that botulinum toxin and β-bungarotoxin do not share a common receptor site at the neuromuscular junction (Dolly et al., 1981; Simpson, see above).

The precise mechanism by which botulinum toxin causes blockade of transmitter release has not been established. However, one proposal is that botulinum toxin, like several other bacterial toxins, is internalized by target cells (Simpson, 1980). This proposal envisions the poisoning effect as one that is expressed intracellularly rather than extracellularly. This speculation must await direct proof that the toxin enters cells, or that the subcellular substrate for the toxin is inside cells. As explained elsewhere, this is a challenging task (Simpson, 1981a).

Suggestive proof for internalization could be obtained if drugs known to antagonize other internalized toxins were found to antagonize botulinum toxin. Chloroquine has been shown to inhibit the pharmacological actions or intracellular degradation of numerous peptide hormones and protein toxins (Goldstein et al., 1979). In this context, the ability of chloroquine to antagonize botulinum toxin strongly suggests that botulinum toxin is internalized.

The data on aminoquinolines do not support strong conclusions about their site of action. These drugs may act at the cell surface to inhibit tissue binding or membrane penetration by toxin, or they may act intracellularly to inhibit lysosomal processing of toxin. Whatever the site of action, the fact remains that chloroquine is the most effective pharmacological antagonist of botulinum toxin yet described.

In addition, it has been found that ammonium chloride and methylamine produce concentration-dependent antagonism of the onset of paralysis by botulinum toxin types A, B and C, but they do not antagonize other presynaptic toxins such as β-bungarotoxin or taipoxin. The concentrations of ammonium chloride and methylamine that antagonize botulinum toxin are equivalent to those that produce antagonism of other protein toxins (e.g., Kim and Groman, 1965; Ivins et al., 1975), yet they are lower than those that produce neuromuscular blockade.

The data indicate that the antagonists do not act simply by inhibiting the binding of botulinum toxin to membrane receptors. The antagonists exert a protective effect even when added 20 to 40 minutes after botulinum toxin. The toxin binds to tissue receptors with an apparent t½ of less than 20 minutes (Simpson, 1980). The fact that the antagonists can act after the toxin is tissue bound rules out receptor binding as the principal site of action.

The data also exclude the possibility that the antagonists act intracellularly to reverse the lytic effects of
botulinum toxin. In order to exert their effects, the antagonists had to be added to tissue baths before onset of paralysis. When added after onset of paralysis, neither ammonium chloride nor methylamine reversed neuromuscular blockade.

Apparently ammonium chloride and methylamine act at a step that occurs after toxin binding to membrane receptors but before toxin-induced blockade of transmitter release. Experiments with botulinum antitoxin support the concept that an intermediate step is involved. When control tissues were exposed to botulinum toxin, the toxin completely disappeared from the neutralizing effects of antitoxin within 30 to 40 minutes. However, when tissues were treated with ammonium chloride or methylamine and then exposed to botulinum toxin, the toxin did not completely disappear from accessibility to antitoxin for 80 to 90 minutes.

The antagonists seem to act by maintaining the toxin at an antitoxin sensitive site. The same outcome was obtained when chloroquine was used as an antagonist of botulinum toxin (see above). These findings could be explained on one of two bases. The antagonists could act at the cell surface to inhibit capping; if capping is essential to the process of internalization, then inhibition of capping will antagonize the onset of action of internalized peptides and proteins (e.g., Maxfield et al., 1979). Alternatively, the antagonists could act as lysosomotropic agents to inhibit lysosomal processing of complex molecules; if proteolytic cleavage is necessary to separate the toxin from its receptor, then inhibition of processing may cause the undissociated toxin-receptor complex to be reinserted into the membrane (e.g., Leppla et al., 1980). Either of these two proposed mechanisms could account for: i.) the ability of the antagonists to delay onset of toxin-induced neuromuscular blockade, ii.) the observation that the antagonists "trap" the toxin at an antitoxin sensitive site, and iii.) the proposal that the antagonists act after the step of toxin binding to receptors but before the step of toxin-induced paralysis.
6. Literature Cited


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