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The Origin, Structure, and Pharmacological Activity of Botulinum Toxin*,†

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I. Introduction

BOTULINUM toxin is a term that is used to describe at least eight different biological substances. Although these substances are antigenically distinct, they have three important features in common: 1) they are synthesized by the same specie of bacterium, *Clostridium botulinum*; 2) they possess similar molecular weights and probably similar structures; and 3) they block acetylcholine release from cholinergic nerve endings. At least superficially, the

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eight types of botulinum toxin have a common origin, structure, and pharmacological activity.

For several decades investigators have known that all types of botulinum toxin depress neurogenic release of acetylcholine. In the recent past, however, this knowledge has been expanded greatly by two related areas of research. In the realm of pharmacology, advances have been made toward an understanding of the cellular and even molecular actions of botulinum toxin; in the realm of biochemistry, progress has been made toward determining the precise structure of the toxin molecule. It is gratifying to note that pharmacological studies on the cellular and subcellular effects of the toxin are closely compatible with biochemical studies on the gross structure and substructure of the toxin molecule. In short, a cogent picture seems to be emerging.

One of the purposes of the present review is to convey some of the emerging ideas about botulinum toxin. Uppermost among these ideas is the belief that botulinum toxin is not a simple receptor antagonist. It does not, for example, act on a single class of cell surface receptors and in so doing inhibit transmitter release. More likely, the toxin proceeds through a complex sequence of steps before its pharmacological effects are fully expressed. This sequence includes binding to a membrane receptor, subsequent internalization, and eventual inactivation of a process that is crucial to excitation-secretion coupling. In this same vein, the toxin itself is neither a small nor a simple molecule. To the contrary, it is a large molecular weight substance that can be broken by disulfide bond reduction and proteolysis into a number of stable fragments. Understandably, one of the most promising among current areas of research is that which seeks to link the individual steps in the pharmacological actions of the toxin with the individual fragments that can be obtained from the toxin.

Although progress certainly is being made in the field of botulinum toxin research, there are nevertheless substantive questions that remain unanswered. Therefore, a second purpose of the present review is to draw attention to issues not completely resolved and to problems not fully explored. The reasons for drawing attention to these matters are twofold. Firstly, a discussion that admits candidly that there are gaps in understanding and weaknesses in methodology tends to challenge workers; this is a necessary part of encouraging problem resolution. Secondly, a thoughtful examination of those areas in which problems do exist seems to reveal something that is quite exciting. There is reason to believe that techniques now being developed in molecular biology will have profound implications for pharmacology in general and for the field of botulinum toxin research in particular. Accordingly, the present review suggests several ways in which a merging of molecular biology and classical pharmacology could help to solve some of the remaining, unanswered questions about botulinum toxin.

II. *Clostridium botulinum*

A. Bacterial Taxonomy and Toxin Production

There are numerous strains of *C. botulinum*, and regrettably there are also numerous schemes for classifying the strains. These schemes are based on a variety of determinants, such as cultural properties, membrane antigens, and nucleic acid homology (258). Ironically, the two schemes that are most widely used have only nominal value to microbiologists, but both schemes are somewhat helpful to pharmacologists. According to one scheme, various strains of *C. botulinum* are classified on the basis of the type of toxin they produce (70, 258). At the moment, eight antigenically distinct toxins have been identified (types A, B, C_1, C_2, D, E, F, and G), so strains are labeled type A, type B, etc. According to the other scheme, the various strains are divided into two groups, these being proteolytic and nonproteolytic.

In the past, the technique of classifying strains on the basis of the types of toxin they produced was thought to be useful and uncomplicated. The presumed value of the scheme arose from a belief that individual strains could synthesize and release only one type of toxin. That belief is now known to be erroneous. There are at least three situations in which the "one strain-one toxin" rule does not apply. Most obviously, there are strains of clostridia that are nontoxigenic. Such strains may be naturally occurring or they may be the result of experimental manipulation. In either case, nontoxigenic organisms grow and multiply normally.

A more complex situation is that involving a strain of bacteria isolated by Gimenez and Ciccarelli (104). The toxin obtained from this strain was predominately, but not completely, type F. This conclusion was based on the observation that homologous (type F) antitoxin largely, but not completely, neutralized toxicity. When Gimenez and Ciccarelli tested type F antitoxin in combination with heterologous antitoxin, they found that a combination of type A and type F antitoxins neutralized all toxicity. A result such as this could mean that a single strain was producing two antigenically distinct toxins, or that a single strain was producing one type of toxin, but the toxin in question had a combination of type A and type F antigenicities. These two possibilities were examined by Sugiyama et al. (272), who used selective adsorption with type-specific antisera. They showed that type A toxicity could be precipitated from solution without altering residual type F toxicity, and vice versa. This finding indicated that two separate types of toxin were involved. Sugiyama et al. (272) estimated the relative proportions of the two toxins to be 99% type F and 1% type A, an estimate that differed slightly from that given earlier by Giminez and Ciccarelli (90% type F, 10% type A). In any event, the data show that a single bacterium is capable of producing two types of toxin.

The final and most complex situation involves *C. bo-*

tulinum types C_α, C_β, and D. As noted many years ago by Pfenninger (222), antiserum prepared against cultures of type C_α neutralized toxicity obtained from cultures of type C_β and type D. The reverse experiments did not produce the expected outcome; neither anti-C_β serum nor anti-D serum completely neutralized toxicity from type C_α cultures. These puzzling findings have since been explained, mainly by the work of Jansen (143). Apparently type C_α strains can produce three toxins, C_1, C_2, and D. Type C_β strains produce only C_2 toxin, and type D strains can produce C_1 and D toxins. These data on toxin production can account for the observations on cross neutralization.

The second scheme for classifying *C. botulinum* divides all strains into two broad categories—proteolytic and nonproteolytic. Knowledge of whether an organism is or is not proteolytic is essential to an understanding of toxin structure and activity. In keeping with this fact, a discussion of proteolysis will be deferred until a later stage of the presentation.

B. Bacterial Growth and Toxin Production

A substantial amount of work has been done on the growth and microbial physiology of *C. botulinum* (13–16, 94, 190). This research is pertinent to the discipline of microbiology, but it is largely beyond the scope of the present review. The only matters that can be considered here are those that relate growth and reproduction of the bacteria to synthesis and release of the toxin. Investigators who are concerned with these matters agree on the following points:

1. The toxin does not play an essential role in the growth and physiology of the bacteria. The most compelling evidence of this is that there are naturally occurring strains of *C. botulinum* that do not produce any toxin. Also, there are strains that do produce toxin, but which can be cured of toxin-producing abilities without impairing bacterial growth (see below).

2. Bacterial growth and toxin production can be manipulated separately and independently. For example, ethylenediamine tetraacetic acid can inhibit toxin production without inhibiting bacterial growth, and penicillin can inhibit bacterial growth without inhibiting toxin production (159).

3. Bacterial growth and toxin production have different nutritional requirements. For example, glucose is needed for toxin production, but it is not needed for bacterial growth (158).

4. Bacterial growth must occur before there can be toxin production, but the two phenomena can be dissociated in a temporal sense. Culture filtrates have little toxin during the anabolic or logarithmic phase of bacterial growth, but filtrates accumulate substantial toxin during the catabolic or autolytic phase (17, 159). As will be explained further in a later section, the titer of toxin can continue to increase even when cell growth has ceased.

The literature reveals that *C. botulinum* synthesizes and releases a remarkably potent toxin, but this toxin has no known role in the growth and physiology of the organism. Such findings suggest that although *C. botulinum* may synthesize the toxin, there is something other than the bacteria that governs synthesis. In at least two cases (C_1 and D toxins), there is evidence that toxin production is related to viral infection.

III. Viral Infection of *Clostridium Botulinum*

A. General Characteristics of Bacterial Infection

Stated simply, a bacteriophage is a virus that infects bacteria. Generally speaking, virus particles have an elementary structure; they are composed of a protein capsule that encloses a core of nucleic acid. The protein coat of most bacteriophages has a cubical head and a long, slender helical tail (25). Typically, it is the tail that attaches to specific cell surface receptors on bacteria, and it is through the tail that nucleic acid is injected into susceptible bacteria.

When phage particles infect bacteria there are two general categories of outcome, these being lysis and lysogeny (fig. 1). In the first case, the nucleic acid of the virus dominates that of the host bacteria. As a result, the synthetic apparatus of the bacteria is forced to produce large numbers of phage particles ($\sim 10^2$ to 10^3). There is, in addition, liberation of a lysozyme that degrades the bacterial cell wall. The culmination of these two events is lysis of the bacteria with an attendant release of fully formed phage particles.

In lysogeny, the nucleic acid of the phage becomes incorporated into that of the host bacteria. New phage particles are not formed; instead, when there is replication of the host nucleic acid there is simultaneous replication of the viral nucleic acid. This means that when a mother cell divides to form two daughter cells, both of the latter carry the viral infection. Because this type of infection does not ordinarily lead to cell lysis, it is referred to as being temperate. A bacteriophage that is temperate, i.e., involved in lysogeny, is called a prophage.

Temperate phages that are involved in lysogeny can revert to a virulent state and cause lysis. When this reversion occurs naturally, it is due to genetic mutation. In the context of laboratory research the reversion can be induced experimentally. Both physical (e.g. ultraviolet light) and chemical (e.g. acridine orange) techniques can be used to cause a prophage to become a lytic phage. In a manner of speaking the reverse can also occur. When a large number of bacteria are infected by a potentially lytic phage, a small number of bacteria will not undergo lysis. This small minority, due to spontaneous mutational change, will enter lysogeny.

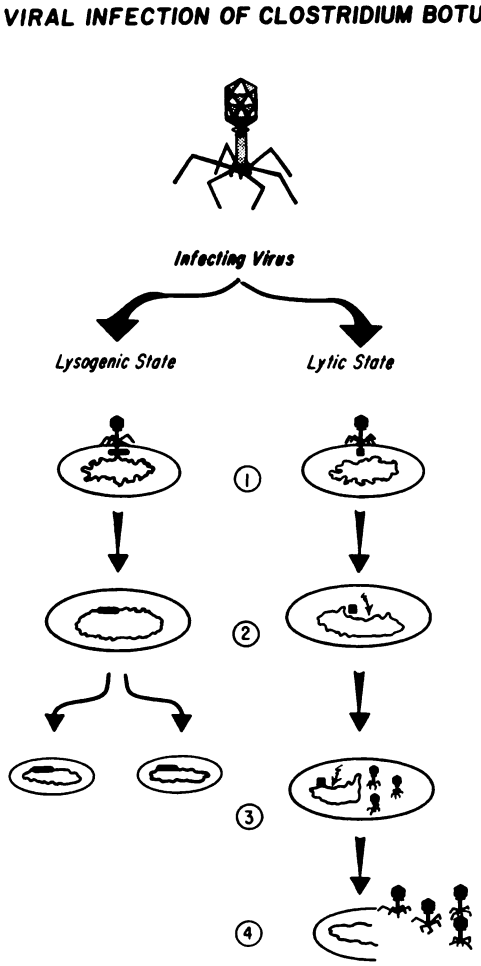

FIG. 1. When bacteria such as *Clostridium botulinum* are infected by virus particles, there are two general categories of outcome—lysogenic and lytic. In the lysogenic state, the virus particle binds to receptors on the bacterial cell membrane and then injects its nucleic acid into the host ①. This nucleic acid becomes fully integrated into that of the bacterium ②. As a result, replication of the host nucleic acid is accompanied by replication of the virus nucleic acid ③. In the lytic state, the virus also binds to cellular receptors and injects its nucleic acid ①, but the viral nucleic acid does not become integrated into that of the host ②. Instead, the viral nucleic acid directs the host to synthesize new phage particles ③. When synthesis of the phage particles is complete, the host lyses and fully formed viruses are released ④.

B. Specific Features of Clostridial Infection

Vinet and Fredette (291) were the first investigators to provide direct evidence (electronmicroscopy) that *C. botulinum* are infected by phage particles. Their study was soon followed by two others (89, 139), both of which considered the possibility that phage-mediated infection might be related to botulinum toxin production.

In 1970, Inoue and Iida published data that strongly suggested that there is a relationship between lysogeny and toxigenicity (140). Their study involved a sequence of observations and experiments that can be summarized as follows. Beginning with a toxigenic strain of type C *C. botulinum*, they used chemical techniques to induce lysis of all cells harboring prophage. The surviving cells, which presumably were not lysogenic, were nontoxigenic. When the nontoxigenic, nonlysogenic cells were infected with phage particles obtained from the original toxigenic line, virtually all nontoxigenic cells became toxigenic. This characteristic was stable and persisted through numerous cell divisions. When the converted cells were induced to lyse, the phage that were obtained could be used to infect and convert other nontoxigenic cells. In keeping with the previous literature on bacteriology and virology, Inoue and Iida (140) referred to the phenomenon they had demonstrated as phage conversion. The phenomenon was fully confirmed by Eklund et al. (92).

In addition to type C *C. botulinum*, type D *C. botulinum* can be converted from nontoxigenicity to toxigenicity by a virus (91, 141). The discovery that more than one type of *C. botulinum* can undergo lysogenic conversion prompted questions about interconversion. The most thorough study to address this question is that of Eklund and Poysky (88). These investigators showed that a type C toxigenic strain could be cured of its prophage and rendered nontoxigenic. This nontoxigenic strain, if infected with phage that mediate type D toxin production, became a type D strain. Likewise, if the nontoxigenic strain was infected with phage that mediate type C toxin production, the bacteria reverted to its type C status. The experiment could be continued by recuring and reinfecting the bacteria. Once again, the resulting nontoxigenic organisms could undergo lysogenic conversion to produce either type C or type D toxin.

In relation to the study just described, there is one point that requires clarification. The type C organism that was used in these studies produced mainly type C_1 toxin, although there was some synthesis of type C_2 toxin. Also, the type D organism produced mainly type D toxin, and only small amounts of type C_2 toxin. As reported by Eklund and Poysky (88), types C_1 and D toxins were subject to phage conversion. No evidence could be obtained that the C_2 toxin was associated with the lysogenic state. The same authors had previously reported that production of C_2 toxin was not phage-mediated (87).

Available evidence now indicates that phage particles play an integral role in the production of type C_1 and type D botulinum toxins. This situation is not unique. There are several species of bacteria that produce toxins and in which the production of toxins is governed by infecting phage (e.g. 217). These matters prompted Eklund et al. (90) to perform one of the more novel experiments yet reported. They began by converting a toxigenic strain of *C. botulinum* type C to a nontoxigenic strain, i.e. they cured it of its prophage. Next, they isolated phage particles from *C. novyi* that mediate production of an exotoxin that is peculiar to this organism. When the phage particles obtained from toxigenic *C. novyi* were incubated with the cured, nontoxigenic *C. botulinum*, the latter became toxigenic. Interestingly, the toxin that was produced was not the neurotoxin characteristic of *C. botulinum*, but rather the exotoxin characteristic of *C.*

novyi. In essence, the investigators had used phage particles to convert one specie of clostridium to another specie. By appropriately curing and reinfecting the converted species, Eklund et al. recreated the original, toxigenic type C *C. botulinum*.

The evidence that bacteriophage code for toxin production in type C and type D *C. botulinum* is quite strong. Nevertheless, there are two important questions that remain unanswered. Are all types of botulinum toxin production governed by viral infection? When infection does occur, what is the relationship between *C. botulinum* and its prophage? For both of these questions there are, at best, only incomplete answers.

It is presently known that types A to F *C. botulinum* harbor phage particles or plasmids (69, 139, 242). It is also known that for types C and D *C. botulinum*, there is a link between lysogeny and toxigenicity. Unfortunately, it is not known whether this link applies equally to types A, B, E, F, and G *C. botulinum*. Arguments by analogy are tempting, but such arguments cannot take the place of empirical findings. Definitive studies on strains other than those producing types C_1 and D toxin are required before any conclusions are drawn.

Also, the true nature of the relationship between phage particles and *C. botulinum* needs to be established. In the case of type C and type D organisms the relationship may be pseudolysogeny; this tentative conclusion is based on several lines of observation. Organisms that are cultured for many generations often lose their toxin-producing abilities (208). Invariably, the trend is from toxigenicity to nontoxigenicity; the reverse is not observed. A corollary observation involves the use of specific antibodies. The outer coat of phage particles is proteinaceous, so antibodies against phage coat antigens can be prepared. If toxigenic strains are cultured in a medium that contains antibodies to the infecting phage, toxigenicity is lost rapidly, much more rapidly than occurs naturally (92, 208). If the phage nucleic acid were fully integrated into that of the host bacteria, one would not expect toxigenicity to wane with successive generations. Also, antibodies to the phage coat should not interact with or neutralize incorporated nucleic acids. The data suggest that the phage are in a state of pseudolysogeny.

Studies on sporulation support the concept of pseudolysogeny (91). When toxigenic strains are induced to sporulate, they often lose their ability to synthesize toxin upon reentering the vegetative state. Of necessity, the spore must contain the full nucleic acid complement of the vegetative cell. The loss of toxigenicity during transition through the spore state suggests that the phage nucleic acid has either been lost or is no longer expressed.

To repeat, the link between lysogeny (or pseudolysogeny) and toxigenicity has been shown for type C and type D organisms. The host-phage relationship, if it exists, remains to be demonstrated for all other types of organisms. It is worth pointing out now, in anticipation of a later section, that lysogeny-mediated toxigenicity may be as important to pharmacologists as it is to microbiologists.

IV. The Structure of Botulinum Toxin

A. Gross Structure of the Molecule

Type A botulinum toxin is the only one of the eight types that has been crystallized (1, 178). Electrophoresis data indicated that the crystalline material was homogeneous. It was stable in acid solutions, unstable in alkaline solutions, and heat labile. As judged by diffusion data, the molecular weight was $\sim 1.13 \times 10^6$ (157); as judged by ultracentrifugal data, it was $\sim 9 \times 10^5$ (232). An amino acid analysis confirmed the protein nature of the crystalline molecule (28), but neither the number nor the nature of the amino acids present gave any hint as to why the molecule should be so potent. Studies on the physicochemical properties of the molecule did not reveal anything that would explain its pharmacological effects, although the investigators involved did speculate that the toxin might be an enzyme (232).

In spite of initial beliefs that the crystalline toxin was homogeneous, later work showed heterogeneity. Two different lines of research provided clues that the crystalline molecule could be fractionated, both functionally and biochemically. Shortly after the reports on isolation of the toxin, Lamanna found that the crystalline molecule possessed separable biological properties (174, 180). In addition to the well-known neurotoxin activity, the crystalline material also possessed hemagglutinin activity. Lamanna and his associates showed that neurotoxicity and hemagglutination were due to different molecules rather than being due to a single, bifunctional molecule. Thus, when the crystalline molecule was used to cause agglutination of red blood cells, and the agglutinated complex was removed from solution, there was no loss in residual toxin titer. Little subsequent work has been done to describe the structure and activity of the hemagglutinin (185, 186), although the molecule may have the binding properties of a lectin (7, 56).

In response to the work by Lamanna and his colleagues, Wagman published a series of studies on the ultracentrifugal behavior of botulinum toxin type A (293–296). The initial report confirmed that the type A crystals, when dissolved in mildly acidic solutions, were monodisperse (295). The sedimentation rate and diffusion coefficient substantiated previous work claiming a molecular weight of ~900,000 (232). However, Wagman and Bateman made the important observation that as the pH of a solution was raised to 7.5 and above, and the ionic strength was raised to ~0.1 and above, the dissolved crystalline molecule became polydisperse (293, 295, 296). The dissociation products obtained at mildly alkaline pH could be reassociated, but dissociation at a pH of 9.2 or higher was irreversible. In the final study in the series, Wagman (294) reported that the principal component

obtained when centrifuging a toxin solution at pH 9.2 was a molecule with a sedimentation constant of 7 S, i.e., a molecule whose molecular weight was ~150,000 to 160,000. Wagman referred to this substance as being dissociated toxin rather than intact (crystalline) toxin. As later work would show, Wagman had very nearly isolated the neurotoxin.

Several attempts were made to characterize the crystalline molecule further, but the first authentic success was reported by Boroff and DasGupta (52). A sample of crystalline toxin was applied to a DEAE-Sephadex column at a pH of 7.2. The column was then eluted with a chloride gradient (~0.05 to 1.05 M). When the gradient reached ~0.1 M, a single peak emerged that contained approximately 20% of the original protein and most of the original toxicity. At ~0.16 M, a second peak emerged that contained most of the original protein but only about 0.01% of the original toxicity. The initial peak, labeled α, was a reasonably pure preparation of neurotoxin; the second peak, labeled β, was hemagglutinin. As determined by the method of gel filtration (6), the neurotoxin had a molecular weight of ~150,000 and the hemagglutinin had a molecular weight of ~500,000. A revised estimate proposed that the hemagglutinin could exist in several states of aggregation, and the proposed weights of these aggregates were 290,000, 500,000, and 900,000 (50).

Ultracentrifugal analysis of the neurotoxin showed it to be homogeneous (24). The molecular weight, as estimated by Yphantis' method, was ~128,000. Other studies have confirmed that the neurotoxin has a molecular weight of ~150,000 (49, 50, 54, 119, 268). In addition, a variety of techniques (several chromatographic procedures, isoelectric focusing, ultracentrifugation, Ouchterlony gel diffusion, and polyacrylamide gel electrophoresis with sodium dodecylsulfate) indicate that the molecule weighing ~150,000 daltons is reasonably homogeneous.

The studies just described have, in the main, used the crystalline type A toxin as the starting material. However, comparable results can be obtained by fractionating the culture fluid obtained from growing organisms (48). Also, an alternative to ion-exchange chromatography has been developed by Moberg and Sugiyama (199). These workers have described an affinity column that uses *p*-aminophenyl-β-D-thiogalactopyranoside coupled to Sepharose beads. When a solution of crystalline toxin is applied to the column, the toxin-hemagglutinin complex adsorbs to the ligand. It is the hemagglutinin that binds to the ligands, so a one-step elution with phosphate-buffered saline can be used to isolate the toxin.

The neurotoxin is not always associated with an hemagglutinin, nor is hemagglutinin the only impurity with which the neurotoxin might be associated. The possibility of a neurotoxin-hemagglutinin complex exists only when a particular strain of clostridia synthesizes both (209). All eight types of *C. botulinum* are capable of producing a neurotoxin, but there has been uncertainty about whether all types can produce hemagglutinin. An absence of the latter substance could reflect either an inability on the part of the bacteria to synthesize hemagglutinin, or an inability on the part of investigators to detect the presence of hemagglutinin. In relation to the latter possibility, the literature provides a telling example. Lamanna and Lowenthal (180) reported that type C organisms do not produce a hemagglutinin that can act on chicken erythrocytes, but Boroff and DasGupta (19) found that type C organisms do produce a substance that can agglutinate human erythrocytes. Perhaps clostridial hemagglutinins, like clostridial neurotoxins (30), are relatively or even absolutely species-specific.

There are other substances with which the neurotoxin might complex, most notably unidentified proteins and nucleic acids. There is modest disagreement as to the number and nature of molecules with which the neurotoxin might complex and the state of aggregation of the molecules when released by clostridia (compare 118, 239, 268; and see review, 269). In spite of this disagreement, there is consensus that none of the impurities is covalently linked to the neurotoxin, and none of the impurities contributes to the paralytic actions of the toxin. Under physiological conditions the neurotoxin spontaneously dissociates from most, if not all, impurities. In essence, physiological solutions act like ion-exchangers to dissociate the toxin from loosely bound substances.

Several laboratories tried to develop procedures for isolating neurotoxins other than the type A, but none of the procedures successfully generated pure materials (80, 179, 295). However, the technique described by Boroff, DasGupta, and their associates for isolating type A neurotoxin proved to be applicable to other neurotoxins as well. In rapid succession, DasGupta et al. (51) and Beers and Reich (8) reported chromatographic procedures for isolating a homogeneous type B neurotoxin. In the first case, the reported molecular weight was ~165,000; in the second, ~167,000. Sakaguchi, Kozaki, and their associates have used comparable techniques and in so doing have obtained a comparable type B neurotoxin (167, 168). Type C_1 neurotoxin [MW ~141,000 (276)], type D neurotoxin [MW ~170,000 (198)], type E neurotoxin [MW ~147,000 (54); MW ~ 150,000 (162)], and type F neurotoxin [MW ~155,000 (309)] have since been isolated. Only the type G neurotoxin has not been described in a homogeneous state. Even so, the preceding data suggest that all eight botulinum neurotoxins are proteins of roughly equal molecular weights.

B. Substructure of the Molecule

Ideally, comparability of the type A to G neurotoxins would be established by a combination of studies on amino acid composition, amino acid sequence, and tertiary structure. Research on several toxins obtained from snake venom nicely illustrates this ideal approach (181). However, the botulinum neurotoxins are rather large molecules, and as such the study of their substructure is a formidable task. The work that has been done on the substructure of the molecules can be grouped conven-

iently into three categories: molecular weight determination and amino acid composition, reduction of disulfide bonds, and enzymatic degradation.

1. Molecular weight determination and amino acid analysis. A number of studies describe isolation of the various neurotoxins; in most cases the molecular weights of the neurotoxins fall within the range of 150,000 to 160,000 (see above). Only one study has made a specific attempt to compare two neurotoxins (types A and B) to determine whether there are real differences in molecular weight; the differences that were found were modest in proportion (58).

Only limited work has been done on the amino acid analysis of botulinum neurotoxin. An early study that relied mainly on microbial assays (28) found that the type A crystalline molecule was a simple protein composed of ordinary amino acids. Two later studies employed an amino acid analyzer (22, 260); these two studies are compatible with one another and with the study by Buehler et al. (28). An amino acid analysis of type B neurotoxin was reported by Beers and Reich (8), and an analysis of the type A neurotoxin was published shortly thereafter (22). None of the other neurotoxins has yet been analyzed. A comparison of the type A and type B neurotoxins indicates a large measure of similarity in terms of the nature and mole percent of the amino acids present. Both toxins contain a large number of hydrophobic residues.

2. Disulfide bond reduction. Type A neurotoxin contains four sulfhydryl groups and at least one disulfide bond (163). The type B neurotoxin similarly contains several sulfhydryl groups and at least one disulfide bond (8). Reduction of the disulfide bonds in types A, E, and F neurotoxins causes loss of biological activity (271). The full significance of these findings will be discussed momentarily.

3. Enzymatic degradation. Botulinum toxin is synthesized intracellularly and released into culture fluids when clostridia undergo autolysis (13, 14, 17, 159). As such, the toxin titer of culture filtrates is relatively low when cells are in the logarithmic phase of growth, but it increases dramatically when cell growth ceases and cell membranes rupture. The toxin titer may continue to increase even after complete autolysis has occurred and protein synthesis has stopped (159). These and other observations suggested that botulinum neurotoxin might be synthesized as an inactive, or relatively inactive, precursor. When this precursor was exposed to some activation phenomenon, such as selective cleavage due to proteolytic enzymes, the inactive precursor was converted into a fully active neurotoxin. Duff et al. (81) tested this hypothesis by using the type E toxin. Their selection was based on the fact that the potency of type E toxin in culture fluids is substantially less than that of toxins in the fluids obtained from type A and type B cultures. Duff et al. showed that treatment of type E toxin with trypsin caused the potency of the toxin to increase between one and two orders of magnitude. The potency of the trypsin-activated type E toxin approached that of the naturally occurring type A and type B toxins.

Bonventre and Kempe (13–16) demonstrated a similar phenomenon for the types A and B toxins, although the magnitudes of the increases in potency were less than those seen with type E toxin. This is the outcome that had been anticipated. Most type A and type B cultures of *C. botulinum* are proteolytic; most type E cultures are not. In keeping with their findings, Bonventre and Kempe (15, 16) proposed a general scheme to account for the production of botulinum toxin. According to them, all cultures synthesize an inactive precursor intracellularly. When these precursors are acted on by proteolytic enzymes, an active group is unmasked and the precursor becomes a fully active toxin. Cultures that are proteolytic can begin the unmasking process intracellularly and then complete it after autolysis. Cultures that are not proteolytic can produce only the weakly active precursor; exogenous enzyme must be used to generate a fully active toxin.

The ability of trypsin to activate botulinum neurotoxin has been shown for the type A (13, 14), type B (13, 14), type C (144), type D [only minimal activation (198)], type E (81), type F (130), and type G (104) molecules. It seems reasonable to assume that conversion of an inactive precursor to an active toxin, whether naturally occurring or experimentally induced, is a generalized phenomenon. The issue now at hand is to determine how proteolytic activation of the toxin occurs.

DasGupta and Sugiyama (54) have described one action of trypsin, and in so doing have contributed importantly to an understanding of the various botulinum neurotoxins. Working with the isolated type E neurotoxin, they showed that trypsin cleaved the single-chain molecule into a dichain molecule. Moreover, the two polypeptide chains were linked by at least one disulfide bond. When this disulfide bond was reduced, the two polypeptide chains were freed from one another. The two chains that were isolated have since been labeled heavy (H; MW ~100,000) and light (L; MW ~50,000). An examination of the isolated type A neurotoxin, i.e. toxin from a proteolytic strain, showed that it already existed as a dichain molecule, and that disulfide bond reduction released an H chain and an L chain. The pattern of dichain molecules from proteolytic organisms and single-chain molecules from nonproteolytic organisms, all of which can be separated into H and L chains by disulfide bond reduction, has been shown for type A (54), type B (8), type C (276), type D (198), type E (54), and type F (309) neurotoxins.

The picture that seems to emerge is that all botulinum neurotoxins are synthesized intracellularly as single-chain polypeptides with molecular weights of ~150,000 to 160,000 (fig. 2). Trypsin and other proteolytic enzymes can cleave these molecules into dichain structures. In the terminology originally applied to diphtheria toxin, this enzyme-induced cleavage is called "nicking" (103). When nicked, the various botulinum neurotoxins yield an H

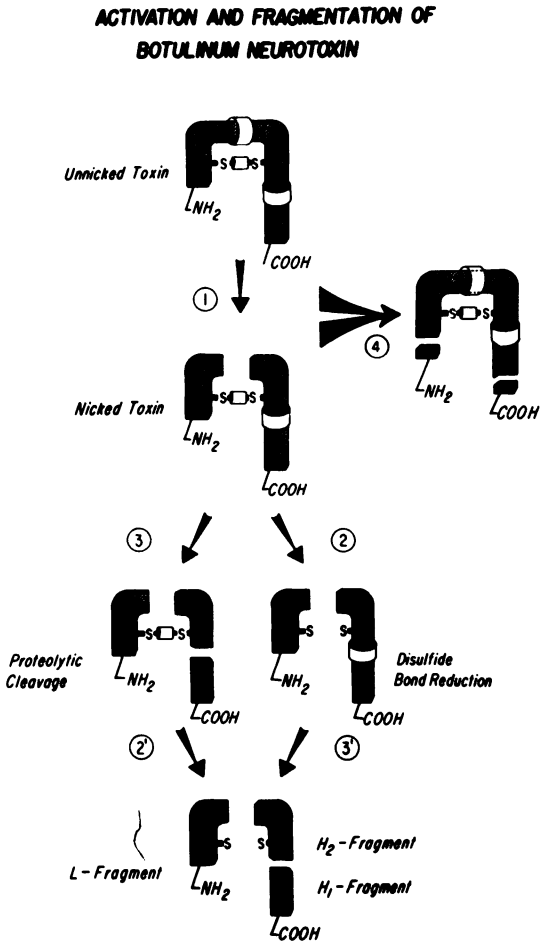

FIG. 2. Botulinum neurotoxin is a protein with a molecular weight of ~150,000 to 160,000. The molecule, which is synthesized intracellularly as a single chain or unnicked molecule, has at least three sites where it can be cleaved. When acted upon by endogenous or exogenous proteases ①, the toxin is nicked to yield a dichain molecule. If this molecule undergoes disulfide bond reduction ②, the nicked molecule can be separated into heavy and light chains. Conversely, if the molecule undergoes limited proteolysis ③, the nicked molecule can be separated into a larger fragment that contains two chains and an interchain disulfide bond, and a smaller fragment that contains only one chain. Theoretically, the molecule could undergo both disulfide bond reduction and limited proteolysis (2', 3') to yield a light chain and two fragments (H_1, H_2) from the heavy chain. In addition to the routes just mentioned, there may be another path for fragmenting the molecule. Endogenous or exogenous proteases may produce small alterations in addition to or independently of nicking ④. The precise nature of these alterations has not been established, but endgroup cleavage may be involved. Of the several molecules illustrated in the figure, only the nicked toxin is fully active. The unnicked toxin is weakly active, and the fragments resulting from disulfide bond reduction and/or proteolysis are atoxic.

and an L chain with a molecular weight ratio of ~2:1. When the nicked molecule is reduced, the H and L chains can separate. This similarity of structure provides the best evidence to date of homology among the several neurotoxins.

The foregoing studies greatly improve our understanding of the structure of botulinum neurotoxin, but they leave unanswered a number of questions. For example, what is the nature and identity of the enzyme that activates botulinum toxin? Also, what is the mechanism of action of the endogenous protease (e.g. esterase, amidase), and what are the linkages that are cleaved? And finally, what roles do nicking and disulfide bond reduction play in the pharmacological activity of the neurotoxin?

Only three groups (53, 212, 281) have isolated proteases to sufficient purity to permit comparison of data, and their findings are in general agreement. The enzymes have molecular weights of ~35,000 to 50,000, and at least one of these enzymes possesses amidase and esterase activity, acts selectively on arginine and lysine residues, has sulfhydryl groups, and, most importantly, activates the botulinum neurotoxin precursor.

There is no unanimity of opinion regarding the site and mechanism of action of the endogenous protease(s). Arginine and lysine residues would be likely candidates, if an amide linkage is involved. However, it has not been established whether the endogenous protease activates the precursor by virtue of being an amidase or an esterase (55, 197, 212). Recent work tends to support the idea that the protease is an amidase, and that the site of action is an arginyl residue (59). When unnicked type E toxin was treated with a site-reactive agent that modifies arginine (1,2-cyclohexanedione), toxicity was lost and the molecule became resistant to nicking.

Probably the most difficult question to be answered is what relationship nicking bears to the pharmacological activity of the toxin. All workers agree that the toxin is synthesized as an inactive precursor and that proteases cause activation. It is also agreed that the toxin is synthesized as a single chain molecule and that proteases cause nicking. But are the two phenomena related? At present most investigators think so; that is, nicking seems to be necessary for, although it may not be sufficient for, activation to occur. Perhaps some molecular change in addition to nicking must occur to evoke full activation (55, 211). A counter opinion has been expressed by Ohishi and Sakaguchi (213), who believe that nicking and activation are separate and/or independent events. This conclusion is based on the finding that, under suitable conditions of pH and enzyme concentration, activation precedes nicking. This observation may contradict some established notions, but it does pose a clear experimental challenge. It invites the development of protocols that either achieve complete nicking in the absence of activation or achieve complete activation in the absence of nicking.

In contrast to the unsettled state of the literature on nicking, the literature on sulphur-containing groups is quite consistent. Sulfhydryl groups and/or disulfide bonds have been demonstrated directly (by assay) or indirectly (by reduction-mediated separation of H and L chains) for all but the type G toxin. The latter simply has not been examined. In all cases in which it has been tested, neurotoxin with reduced disulfide bonds has lost its pharmacological activity (e.g. 271).

Aside from the work on trypsin and other activators, there is another line of research on enzymatic digestion

that may hold promise. In essence, the work is a mimicry of that already done on tetanus toxin. Like botulinum toxin, tetanus toxin has a molecular weight of ~150,000 to 160,000, can be nicked to yield a dichain molecule with H and L chains and has intramolecular disulfide bonds (57). At least superficially, botulinum and tetanus toxins are quite similar. Helting and Zwisler (120) have reported that tetanus toxin can be cleaved by papain to yield two fragments, one weighing about 100,000 and the other weighing about 50,000. The larger fragment is a dichain molecule linked by disulfide bonds; the smaller fragment is a single chain molecule lacking disulfide bonds. The larger fragment appears to be the L chain plus approximately one-half of the H chain (H_2); the smaller fragment is the remainder of the H chain (H_1). Interestingly, the small fragment has been reported to bind to the tetanus toxin receptor and to inhibit the binding of native tetanus toxin (204). Botulinum neurotoxin is also digested by proteolysis, and the resulting chains are similar in weight to those obtained with tetanus toxin (47). The pharmacological activity of these fragments has not been tested.

C. Active Sites in the Molecule

The chemical nature of the antigenic and toxic sites in botulinum neurotoxin has not been established. No concerted effort to identify the amino acids that are involved in antigenicity has been reported. Efforts to determine the amino acids involved in toxicity have been reported, and the literature has been reviewed (19, 57). Although a number of studies have been published, all suffer from one or more serious defects. The studies, almost without exception, were done on impure preparations of neurotoxin. The reagents that were used to modify amino acid residues were not very specific. No study has examined all neurotoxins to ensure that a presumed active center was common to all neurotoxins, and no study has provided evidence to distinguish putative changes in the active site from possible nonspecific changes in three-dimensional structure. One must conclude that no compelling evidence has been advanced that would permit an identification of the active site(s) associated with toxicity.

It may be that the antigenic and toxic sites are composed of subunits. Sugiyama et al. (270) reported that the reduced toxin could not be precipitated by antitoxin to the intact molecule. This finding was questioned by Kozaki and Sakaguchi (167) and by Kozaki et al. (166). The H and L chains were reported to be antigenic, although less so than the native toxin. It remains to be determined where the antigenic sites are on the individual chains, and how or whether they interact in eliciting antibody formation to the native toxin. The toxic site may also be composed of subunits. Pharmacological data reveal that there must be a site that governs binding to target organs, and there may be another site that governs the actual phenomenon of toxicity. These possibilities are reviewed more extensively in a later section.

V. The Site of Action of Botulinum Toxin

A. Peripheral Cholinergic Transmission

In 1923 Dickson and Shevky published an extensive study that provided definitive evidence regarding the site of botulinum toxin action. In a two-part manuscript they reported a wide range of experiments, all of which implicated the cholinergic nerve ending as the target organ (66, 67). To appreciate the significance of this work, one must remember that Dickson and Shevky were working within the same historic time frame as were other workers who delineated the role of acetylcholine and certain catecholamines as neurotransmitters. Thus, very few substances preceded botulinum toxin in terms of being recognized as cholinergic blocking agents.

The work of Dickson and Shevky (67), as well as the nearly simultaneous work of Edmunds and Long (86) and Schübel (241), indicated that botulinum toxin interrupted transmission at the muscle end organ. Dickson and Shevky (67) found that transmission in the nerve trunk was not impaired, an observation later confirmed by Bishop and Bronfenbrenner (11) and Guyton and MacDonald (108). It was also found that muscle activity was not impaired, because direct electrical stimulation of muscle evoked responses in preparations in which neuromuscular transmission was blocked (67, 86, 241). In addition, Edmunds and Long (86) reported that nicotine elicited muscle responses in poisoned preparations. These combined findings narrowed the presumed site of toxin action to the presynaptic cholinergic nerve ending.

Later work has largely substantiated the classic studies that appeared in 1923. Guyton and MacDonald (108) showed that botulinum toxin does not alter receptor activity, nor does it possess a curare-like action. Unlike curare, botulinum toxin leaves voluntary muscle responsive to the administration of acetylcholine. Another distinction is that cholinesterase inhibitors appreciably antagonize curare, but they do little to antagonize botulinum toxin (30, 85, 108). These results were interpreted to mean that botulinum toxin acts at some site proximal to the release of acetylcholine.

In 1949, Burgen et al. (30) published a study that had two favorable effects on botulinum toxin research. Firstly, it introduced the isolated neuromuscular junction as a highly suitable preparation for studying botulinum toxin [the phrenic nerve-hemidiaphragm preparation described by Bülbring, (29)]. Previous work had relied on in vivo or in situ preparations. Secondly, it provided the seminal observation that paralysis due to botulinum toxin was accompanied by a decrease in neurogenic release of acetylcholine. Diminished output of acetylcholine was hypothesized to be due to one of three possibilities: the toxin blocked nerve impulse flow in the fine, terminal arborizations of the nerve, it blocked acetylcholine synthesis, or it blocked acetylcholine release. The first possibility has been rendered unlikely by the work of Brooks (27), Harris and Miledi (115), Stover et al. (266), and Thesleff (278). These investigators have pro-

vided either direct or indirect evidence that botulinum toxin does not impede impulse flow into nerve terminals of α-motoneurons. Instead, impulse flow is normal, but there is a progressive decrement in evoked release of acetylcholine. This outcome leaves either blockade of synthesis or blockade of release as a putative mechanism. In spite of one report claiming that the toxin inhibited choline acetyltransferase (285), most workers have found no evidence that inhibition of synthesis is a primary action of the toxin (e.g. 30). At the moment, the most widely held belief is that the toxin acts to block transmitter release (106, 175, 177, 308). This implies that the precise site of toxin action is at or near the nerve terminal membrane.

Localization of the toxin by histological techniques has not been fully accomplished. Work that has been done has involved localization of a ferritin-labeled toxin (311), localization of a fluorescent-labeled toxin or chaser antitoxin (312), and localization of an ^{125}I-labeled toxin (128). The first two studies did not use the isolated neurotoxin (i.e. the substance weighing ~150,000), whereas the third study did. Because of this, the first two studies are regarded as somewhat suspect. The third study illustrates autoradiograms of mouse diaphragms treated with the ^{125}I-labeled toxin, and the toxin appears to be localized in the region of the neuromuscular junction. The level of resolution of the autoradiograms does not permit a subcellular localization of the toxin in the synaptic region.

Aside from the neuromuscular junction there are at least three other sites in the periphery at which the toxin blocks acetylcholine release. Within the autonomic nervous system, the toxin blocks ganglionic nerve endings, postganglionic parasympathetic nerve endings, and those postganglionic sympathetic nerve endings at which acetylcholine is the transmitter. As was true of the neuromuscular junction, Dickson and Shevky (66) were pioneers in identifying the autonomic nervous system as being vulnerable to the paralyzing effects of botulinum toxin. They showed that numerous parasympathetically mediated responses were blocked by the toxin, including vagus nerve-induced inhibition of the heart, splanchnic nerve-induced intestinal motility, tympanic nerve-induced flow of saliva, pelvic nerve-induced bladder contraction and penis erection, and oculomotor nerve-induced constriction of the pupil. These studies showed blockade of parasympathetic transmission, but they did not distinguish ganglionic from postganglionic transmission.

That ganglia are susceptible, or at least partially susceptible, to botulinum toxin was demonstrated by Ambache [superior cervical ganglion (4)], Eccles and Libert [superior cervical ganglion (84)], Kupfer [ciliary ganglion (173)], Pumplin and McClure [superior cervical ganglion (230)], and Shankland et al. [sixth abdominal ganglion of the cockroach (243)]. The study by Eccles and Libet is especially noteworthy. By using the rabbit superior cervical ganglion, these investigators recorded postganglionic responses evoked by stimulation of the preganglionic trunk. In keeping with previous reports, they were able to record an early negative potential (excitatory postsynaptic potential, EPSP), a positive potential (inhibitory postsynaptic potential, IPSP), and a late negative potential (EPSP). These responses have been labeled the initial negative (N), positive (P), and late negative (LN) potentials. Eccles and Libet found that botulinum toxin blocked all three types of evoked responses. Furthermore, by combining the use of botulinum toxin with other drugs, they developed an anatomical-pharmacological scheme for ganglionic transmission. They hypothesized that the N potential was due to preganglionic release of acetylcholine that stimulated a nicotinic ganglionic receptor, and that the LN response was due to the preganglionic release of acetylcholine that stimulated a remote muscarinic ganglionic receptor. The P wave was believed to be due to preganglionic release of acetylcholine that stimulated an interneuron, and this cell in turn released a catecholamine that stimulated ganglionic receptors.

The study by Eccles and Libet is viewed as a milestone by students of autonomic pharmacology. It provided an heuristic model that has dominated two decades of research on ganglionic transmission. The study is also a milestone to investigators interested in botulinum toxin, because it represents one of the more sophisticated attempts to use the toxin as a pharmacological tool. Even so, the model cannot be viewed as complete or universally accepted. In relation to the IPSP, there is continuing uncertainty about its subcellular basis. The hypothesis of disynaptic transmission in which acetylcholine stimulates an intermediate cell, which in turn releases a catecholamine, has attracted much attention and generated much research. Amidst this research is that which proposes dopamine as the catecholamine transmitter (e.g. 183) and envisions cyclic 3′,5′-adenosine monophosphate as the mediator of the postsynaptic effects of dopamine (e.g. 192). Although these proposals are very interesting, they are not supported by entirely convincing evidence. Indeed, there are those who have argued that a monosynaptic event involving acetylcholine accounts for the IPSP (292) or that an electrogenic sodium pump accounts for the IPSP (164, 182, 206). Whichever explanation ultimately proves to be correct, it will not alter the validity of the observation that the IPSP, as well as the early and late EPSPs, are abolished when the ganglia are paralyzed by botulinum toxin.

No postganglionic site has been studied as carefully as have certain ganglia. However, the toxin does paralyze postganglionic parasympathetic transmission (2, 3, 10, 34, 116, 126) as well as postganglionic sympathetic transmission that involves acetylcholine (3). No one has published histological data on the localization of botulinum toxin at ganglionic or postganglionic sites.

B. Central Cholinergic Transmission

The literature on the effects of botulinum toxin on the central nervous system has experienced several oscilla-

tions. The original assumption was that the toxin acted in the central nervous system to produce the array of clinical problems that were seen. However, the work of Dickson and Shevky (66), Edmunds and Long (86), and Schübel (241) pointed to the peripheral nervous system as the area of poisoning. Their pharmacological and physiological findings were supported by histological data that demonstrated no toxin-induced neurological problems when the toxin was injected systemically or directly into the brain (45, 61). In response, the literature moved from a prevailing belief that the toxin acted centrally to a prevailing belief that it acted peripherally.

Another oscillation began in 1965 when Polley et al. (225) showed that i.v. administration of the toxin to monkeys caused a profound alteration of the electroencephalogram (EEG), characterized by a decrease and sometimes a disappearance of electrocortical activity. Similar results were obtained when toxin was administered i.v. to cats and guinea pigs (254). Unfortunately, these two studies were flawed by a serious methodological problem. In both cases a crude preparation of toxin was used, meaning that the rapid and profound central effects were probably due to some contaminant or dissociation product of the toxin. In a study in which homogeneous samples of neurotoxin and hemagglutinin were administered to animals, both molecules were devoid of EEG-altering effects (253). One histological study has shown that labeled neurotoxin collects in the blood vessels and parenchyma of the brain, but there was no evidence for significant amounts of toxin actually binding to nervous tissue (18).

A third cycle of central nervous system studies began during the past decade. Although relatively few in number, the studies are best viewed as falling into two groups, those that deal with the effects of botulinum toxin on acetylcholine metabolism, and those that deal with the binding of botulinum toxin to nervous tissue.

Molenaar and Polak (200) reported that cortical slices of rat brain, when incubated in vitro with potassium (25 mM), released acetylcholine. Treatment of tissue slices with botulinum toxin resulted in diminished release of acetylcholine. Normal synthesis of acetylcholine was not altered, but atropine-induced synthesis was inhibited. Analogous results have been reported for synaptosomes obtained from cerebral cortex (306). In synaptosomes, botulinum toxin did not alter uptake of choline, acetylcholine synthesis, or intracellular compartmentation of acetylcholine. It did, however, inhibit potassium-stimulated transmitter release, although it did not inhibit spontaneous release. Only one study has reported an effect of the toxin on the intracellular disposition of acetylcholine (107). According to this study, toxin-induced blockade of transmitter release caused retention of acetylcholine, compensatory changes in its subcellular compartmentation, and compensatory decreases in choline uptake. At least in part, these findings are at odds with those presented by other investigators (306). In a study of primary nerve cell cultures obtained from whole brain of embryonic rat, botulinum toxin inhibited acetylcholine release and inhibited its synthesis (9). Inhibition of synthesis was explained on the basis of compensatory decreases in choline uptake after toxin-induced blockade of acetylcholine release.

Haberman has described the binding of labeled botulinum toxin to the central nervous system (109, 110, 112). He found that ^{125}I-labeled toxin would bind to synaptosomes, and that binding could be prevented and/or reversed by unlabeled toxin or by antitoxin. In addition, he found that labeled toxin injected i.m. was accumulated by α-motoneurons and transported into the spinal cord. Toxin-binding sites on the nerve cell were reported to be partially sialidase-sensitive.

The binding of labeled botulinum toxin to synaptosomes (160, 165) and the ascent of labeled botulinum toxin from the injected muscle into the central nervous system have been confirmed (304). In addition, efforts were made to correlate neural ascent of botulinum toxin with blockade of transmission between α-motoneuron collaterals and Renshaw cells (e.g. 113). This junction is cholinergic in nature, and therefore might be expected to be susceptible to the toxin. It was found that Renshaw responses were not altered by toxin injected into muscle and allowed to ascend or by toxin injected into the ventral roots. Only direct injection of the toxin into the spinal cord produced altered responses, and the authors themselves noted that the meaning of this finding is hard to assess.

Potentially, central nervous system studies hold great promise. If the toxin does act to depress neurogenic release of acetylcholine from central nerve endings, this would mean that an additional tissue has been identified on which to conduct pharmacological and physiological studies. Indeed, the brain may offer quantitative advantages, because the number of cholinergic nerve endings centrally far exceeds that found in any peripheral tissue. But, at the same time, there are a number of unsettled points that need to be clarified. Some of the more problematic issues can be enumerated as follows:

1. Many of the studies on central cholinergic transmission employ high concentrations of toxin. Researchers need to determine whether these high concentrations are really necessary. If so, an attempt must be made to account for the differences between the toxin concentrations needed to block neuromuscular transmission and the concentrations needed to block central release of acetylcholine.

2. Reports on the labeling of botulinum toxin tend to use poor quantitative terms to describe residual toxicity. It is imperative that both the technique used to quantify toxicity and the results obtained be stated explicitly. There is no sensitive chemical assay for the toxin; estimates of toxicity are based on bioassay methods [e.g., time to death in mice (21)]. These techniques require large numbers of animals and careful methodology to detect differences in toxicity of 30% or less. Therefore, unless evidence to the contrary is presented, labeled toxin

could be a mixture of active and inactive molecules. This possibility poses serious problems for the interpretation of binding data.

3. Histological data have been published that show binding of labeled toxin to the presynaptic membranes of central nervous tissue (129). Curiously, the study does not illustrate any evidence of specificity of binding. Hence, it is unclear whether the labeled toxin binds nonspecifically to all presynaptic membranes or specifically to cholinergic membranes.

4. The studies on neural ascent of botulinum toxin (e.g. 109) raise two issues. The labeled material that was transported centrally did not bind to the neuromuscular junction. It is difficult to reconcile this observation with the known actions of botulinum toxin on cholinergic nerve endings. Also, the material that was transported was not isolated during transport to ensure that it was authentic botulinum toxin. Many macromolecules can be taken up at the nerve ending and entered into the flow of axonal transport. Such transport does not reveal where, or even whether, the transported substance can act to impair transmitter release.

As mentioned above, recent research on the central nervous system can be divided into two broad areas, these being studies that show toxin-induced blockade of acetylcholine release and studies that show toxin binding to nervous tissue. Data from the former area look increasingly encouraging, particularly the work by Wonnacott et al. (305, 307). Her data on synaptosomes indicate that there are many similarities between the central and peripheral actions of the toxin. By contrast, one must be more cautious about the data on toxin binding. It cannot be stated with confidence that a labeled toxin has been prepared that retains all the properties of native toxin and that binds specifically to the toxin receptor.

C. Acetylcholine Release from Nonneural Tissue

Thus far, attention has been focused on those studies dealing with storage and release of acetylcholine from nervous tissue. Two studies on nonnervous tissue have appeared. Stevenson (264) has reported that botulinum toxin does not block the release of acetylcholine from *Lactobacillus planterum*, a bacterium that can synthesize and release the amine. Ambache and Ferreira (5) have shown that injection of botulinum toxin does block the electrical discharge of *Electrophorus electricus*. In this creature, the electric organ is an anatomical analog of the neuromuscular junction, and as such it uses acetylcholine as a transmitter. The work on *E. electricus* could mean that botulinum toxin paralyzes the electric organ; but alternatively, it could mean that the toxin paralyzes the nerves that activate the organ, in which case paralysis of electrical discharge would be indirect. A reexamination of the effects of botulinum toxin on the electric organ may be timely. If the toxin does exert direct effects on the organ, then this tissue may prove valuable in studies aimed at isolating the brain receptor.

D. Release of Transmitters Other Than Acetylcholine

In their classic study, Dickson and Shevky (66) noted that botulinum toxin paralyzed what is now known as the parasympathetic nervous system, but it did not paralyze postganglionic sympathetic transmission. Although not so stated, their finding was that the toxin interrupted cholinergic transmission, but it did not affect adrenergic transmission. The selectivity of toxin action was verified in studies on organs that contain dual innervation, i.e. the eye (2, 3) and the sinoatrial node (290).

The apparent selectivity of toxin action has been questioned by Rand and Whaler (234) and by Westwood and Whaler (300). These investigators claimed that botulinum toxin could paralyze postganglionic sympathetic transmission. Before considering the data, it may be useful to consider the context in which these studies were performed. Burn and Rand have proposed that acetylcholine is involved in adrenergic transmission (31). Their concept is that nerve impulse flow in the sympathetic nerve triggers the release of acetylcholine, and this amine acts on the sympathetic nerve ending to evoke release of catecholamines. Thus, acetylcholine mediates release of norepinephrine, and norepinephrine mediates synaptic transmission. Much of the evidence supporting the Burn-Rand hypothesis comes from experiments in which drugs thought to act exclusively on neuromuscular or postganglionic parasympathetic transmission have been shown to act on postganglionic sympathetic transmission. It is in this context that the effect of botulinum toxin on adrenergic transmission was studied (234). The toxin was reported to block sympathetically induced contractions of the vas deferens, pendular movements of the ileum, and piloerection (234, 300). In one electrophysiological study, the toxin depressed nerve stimulation-induced excitatory potentials in the vas deferens, but it did not depress the frequency of spontaneous junctional potentials (131). In the only relevant study of central nervous system noradrenergic transmission, botulinum toxin did not diminish potassium-stimulated release of norepinephrine from synaptosomes (306).

It is difficult to assess the findings on adrenergic transmission. Unless there are marked tissue differences, the data on the eye and on the heart seem wholly contradictory to those on the vas deferens, ileum, and hair tufts. Even in cases in which the toxin did block adrenergic transmission, there were fundamental differences between this type of blockade and that which is typical of cholinergic transmission. For instance, the concentrations of toxin used to block adrenergic transmission were orders of magnitude higher than those commonly used to block neuromuscular transmission. Also, toxin-induced blockade of sympathetic transmission did not decrease the frequency of spontaneous postjunctional potentials, whereas toxin-induced blockade of neuromuscular transmission invariably decreases the frequency of spontaneous postsynaptic potentials (see section VI). Until work of a more definitive nature is done, it may be wise

to adopt a conservative explanation. The selectivity of botulinum toxin activity is a dose-dependent phenomenon; at low doses the toxin blocks only cholinergic transmission, but at high doses it may block other types of transmission.

The effects of botulinum toxin on noncholinergic and nonadrenergic transmission have scarcely drawn any attention. The inhibitory response of the guinea-pig fundus, which purportedly is a purinergic response (32), is not blocked by botulinum toxin (219). The release of adenosine triphosphate from cortical synaptosomes is not blocked by the toxin (302).

For sake of completeness, it should be mentioned that certain ganglia may have a response in addition to those described above (N, P, LN). This response, which is slow in developing and lengthy in duration (207), may be mediated by a peptide (83). The effects of botulinum toxin on the late, late negative potential have not been analyzed.

Several studies have already been cited that show that botulinum toxin does not block nerve transmission. Its actions seem to be localized to the nerve ending. The possibility that the toxin might block the function of sensory nerve endings has been examined and the results were negative (2, 108, 115).

VI. The Mechanism of Action of Botulinum Toxin

A. Cellular and Subcellular Actions of the Toxin

As reviewed in earlier sections, botulinum toxin acts to depress neurogenic release of acetylcholine. More precisely, the toxin exerts three specific effects that may or may not be manifestations of a single subcellular action. Firstly, the toxin blocks postsynaptic responses that are evoked by presynaptic nerve stimulation. At a cellular level this effect can be measured as a decrease in the muscle response (e.g. twitch) or the glandular response (e.g. secretion) that ordinarily follows nerve stimulation; at a subcellular level this effect can be measured as a decrease in the amplitude of endplate potentials (epp), EPSPs, and IPSPs. Secondly, the toxin diminishes the frequency of spontaneous miniature endplate potentials (mepp) at the neuromuscular junction. The toxin may likewise diminish the incidence of spontaneous potentials at cholinergic sites other than the neuromuscular junction, but this likely possibility has not been systematically examined. Thirdly, the toxin alters the amplitudes of spontaneous mepps. In normal preparations spontaneous mepps have a Gaussian distribution; in poisoned preparations the amplitude distribution is skewed to the left (low amplitude).

To some extent, the site of action of the toxin in exerting these several effects has been localized. The toxin does not block impulse propagation in the nerve trunk, nor does it block impulse flow into the terminal arborizations of motor nerves. On the other hand, the toxin does block release of transmitter, and it does so without altering postsynaptic responses to the transmitter or to appropriate agonists. These data pinpoint the cholinergic nerve ending as the target organ for the toxin.

It is a testimony to the complexity of the nerve ending that the subcellular site and molecular action of the toxin remain somewhat elusive. Nevertheless, those structures and processes known to exist in the cholinergic nerve have been examined to determine which might be vulnerable to paralysis. In the course of this research the toxin has been evaluated for its putative ability to alter synthesis and storage of acetylcholine, transmembrane flux of calcium, exocytosis of acetylcholine, as well as the membrane components that regulate these phenomena.

B. Experimental Approaches to Studying the Toxin

Experiments aimed at determining how botulinum toxin acts have been performed almost exclusively on the neuromuscular junction. The toxin does block ganglionic and certain types of postganglionic transmission, but autonomic preparations have not been employed by workers trying to describe the subcellular actions of the toxin. The electric organ may also be subject to the paralyzing effects of the toxin, but it too has not been used to any appreciable extent. Only those investigators who are interested in the central nervous system are beginning to use the toxin in a way that will permit them to compare their data to data that have already been obtained on the neuromuscular junction. Consequently, most hypotheses purporting to explain how the toxin acts stem mainly from work on neuromuscular transmission.

Given the fact that the toxin is an unusually potent substance, and that the toxin has a rather specific site of action, one might expect receptor binding studies dealing with the toxin and the neuromuscular junction to flourish. As it turns out, nothing could be less true! There are two obstacles that serve as serious impediments to the use of a labeled preparation of the toxin as a ligand for the toxin receptor. Both of these obstacles relate to the toxin itself. To begin with, the toxin is a remarkably potent pharmacological substance. In mice, a lethal dose is in the picogram range (114, 240), and mouse neuromuscular preparations are paralyzed by picomolar or lower concentrations of toxin (38, 111, 252). Such findings indicate that the toxin must bind with high affinity to a small number of tissue sites. As an extension, one can calculate that a usable ligand would have to be labeled to a high specific activity. If the number of tissue receptors is very small (cf. 114), even a toxin preparation labeled to a theoretical maximum specific activity might be barely usable. This brings us to a second obstacle. Although the toxin is very potent, the isolated substance (150,000 molecular weight) is somewhat unstable. As a result, procedures for labeling the neurotoxin typically cause inactivation. Not surprisingly, no one has successfully prepared a labeled neurotoxin of high specific activity and used it as a ligand to extract and isolate the receptor from neuromuscular tissues.

Alternative approaches such as a radioimmunoassay have not offered greater promise. Boroff and Shu-Chen (23) have described the preparation of antibodies to the type A neurotoxin and claim that their radioimmunoassay can detect as little as 100 mouse lethal doses of the toxin. When compared to other radioimmunoassays, the one developed by Boroff and Shu-Chen (23) has a respectable level of sensitivity. But when compared to the needs of investigators working on receptor isolation, the level of sensitivity simply is not adequate.

The state of affairs on botulinum toxin is not unique. No neurotoxin that acts presynaptically to block acetylcholine release has been radiolabeled and used as a ligand to isolate its receptor at the neuromuscular junction. This means that, in addition to botulinum toxin, no one has described isolation of a receptor for tetanus toxin, β-bungarotoxin, notexin, taipoxin, or any related neurotoxin. Apparently the isolation of the receptors for these substances will require the development of labeling procedures that generate stable toxins of high specific activity, the use of tissues especially enriched in cholinergic nerve endings, or both.

While the literature does not permit us to speak of successful biochemical approaches to characterizing the toxin receptor, published findings that stem from pharmacological and physiological studies do reveal something about the actions of the toxin. In a small number of cases, investigators have assayed tissues or tissue supernatants for acetylcholine and then related their findings to the action of the toxin. More often, investigators have monitored evoked responses in muscle (twitch, epp, mepp), and then used these responses to deduce what the toxin might be doing. Admittedly these approaches are indirect, but they have provided insights into the molecular pharmacology of botulinum toxin.

C. A Proposed Model for Botulinum Toxin Activity

Research on the pharmacology of botulinum toxin can best be understood by viewing published reports in two sequential lights. Initially, these reports can be examined for what they reveal about the phenomenology of toxicity. In other words, one can extract from the literature a reasonably clear picture of the general behavior of the toxin as it moves from the biophase to its ultimate site of action. As this review will show, there are a number of steps involved in the process. Next, when the individual steps have been identified, one can obtain from the literature some notion of the molecular events that underlie each step. As the reader will see, the phenomenology of toxicity is rather well understood, but the underlying molecular events continue to be the subject of vigorous inquiry.

Two reports that were published some years ago made valuable contributions to emerging thoughts about the interaction between botulinum toxin and the neuromuscular junction. Burgen et al. (30) noted that there was a distinction between binding of toxin and paralysis due to toxin. This group showed that the toxin became irreversibly bound to isolated rat phrenic nerve-hemidiaphragm preparations well before the onset of paralysis. Although not fully appreciated at the time, the data of Burgen et al. were among the first to suggest that binding of a bacterial toxin to a cell surface receptor did not cause toxicity. In a separate line of research, Hughes and Whaler (138) found that botulinum toxin activity was influenced by nerve stimulation. For any given concentration of toxin, paralysis occurred more rapidly when nerves were stimulated frequently. Preparations that were not stimulated were slow to paralyze, although paralysis eventually occurred. Hughes and Whaler hypothesized that their results could be interpreted in one of two ways. Either toxin activity hinged on the membrane events associated with the nerve depolarization-repolarization cycle, or it hinged on the membrane events associated with transmitter release.

These two possibilities were examined in a series of papers dealing with botulinum toxin, nerve stimulation, and calcium (245, 247, 248). In a sense, these studies formed a bridge between those of Burgen et al. (30) and of Hughes and Whaler (138). In particular, these papers confirmed that the pharmacological actions of the toxin can be divided into a binding phase and a paralytic phase; they established that excitation-secretion coupling rather than the depolarization-repolarization cycle influenced onset of toxicity; and they demonstrated that the influence of excitation-secretion coupling was on the paralytic phase and not on the binding phase. The finding that a particular experimental manipulation could exert differential effects on the two phases encouraged a search for other distinguishing features of binding and paralysis. This work provided evidence that, at nonsaturating levels of toxin, the binding phase was relatively rapid, it had a low Q_{10}, and it occurred in the absence of nerve stimulation and calcium. By contrast, the paralytic phase was relatively slow, it had a high Q_{10}, and it was retarded in the absence of calcium and nerve stimulation.

An unanticipated finding that arose from these studies related to the use of botulinum antitoxin (248). As expected, the antitoxin did not antagonize botulinum toxin that had produced paralysis; but unexpectedly, the antitoxin did antagonize botulinum toxin that had bound to nerve membranes. This indicated that toxin bound to surface receptors was exposed to antitoxin, but toxin that was causing paralysis was not exposed. From these data one may infer that the toxin is internalized before it exerts paralytic effects. This interpretation of the data obliges one to ask the following question: Does the toxin flip-flop from an external binding site to an internal paralytic site? An examination of the rate at which bound toxin disappeared from accessibility to antitoxin, and a comparison of this with the rate of onset of paralysis, indicates that a simple flip-flop motion cannot account for the data (251). Instead, there appears to be at least one step that intervenes between binding and paralysis.

A partial model that would account for the action of the toxin has been proposed (251). According to this

model, the pharmacological actions of the toxin should be envisioned as a sequence of at least three steps. The initial step involves binding of the toxin to a specific class of cell surface receptors. This binding does not require nerve activity or calcium, and it produces no obvious changes in nerve cell function. The next step involves transport of the toxin from an external location to an internal location, and hence is referred to as translocation. This step is similar to binding in the sense that it, too, produces no obvious changes in nerve cell function. Finally, there is a lytic step during which the toxin seriously alters nerve function; it is during this step that the toxin causes blockade of transmitter release.

Assuming that this model is correct, the task now is to clarify the molecular events that underlie each step. An effort must be made to isolate and characterize the toxin receptor, to identify and characterize the transport mechanism, and to identify and describe the subcellular process that is poisoned by the toxin. In each of these areas progress is being made.

D. Binding and Translocation Steps

The receptor for botulinum toxin has not been isolated or identified. The absence of such information can be attributed to the difficulties inherent in labeling the toxin and using it as a ligand for the receptor. As an alternative strategy, investigators have examined the ability of the toxin to interact with known components of the cholinergic nerve membrane.

Although there is relatively little information available on membrane proteins, virtually all of the lipid components of nerve ending membranes have been identified. In keeping with this fact, one study has tested the ability of individual membrane lipids to interact with botulinum toxin (256). The technique used was to incubate toxin and lipid under physiological conditions, and then to determine whether there were changes in residual toxicity. Marked decreases in toxicity were taken as presumptive evidence that the lipid in question interacted with the toxin. This in turn could mean that the interacting lipids possessed the properties of a receptor. Of numerous substances that were tested, only gangliosides inactivated the toxin in a way that seemed to be biologically meaningful. There were sharp differences among the gangliosides in their abilities to decrease toxicity; asialoganglioside was without effect, mono- and disialoganglioside were of intermediate potency, and trisialoganglioside was most potent.

Initial efforts to reproduce this work met with difficulties (e.g. 195, 289), but the negative findings can be explained. Those investigators who failed to obtain a positive result have, in the main, used species of gangliosides other than the trisialoganglioside, the one reported to be most effective in diminishing botulinum toxicity. Furthermore, the negative reports have commonly used the method of incubating gangliosides with auxiliary lipids, a procedure that counteracts the ganglioside effect. More recent efforts to reproduce the ganglioside effect have confirmed the original work; two prototype studies can be taken as illustrative examples. Habermann and Heller (112) have found that neuraminidase, an enzyme that cleaves sialic acid residues from certain gangliosides and sialoglycoproteins, nearly abolishes the subsequent binding of botulinum toxin to synaptosomes. Data of this kind suggest that botulinum toxin interacts with membrane-bound, sialic acid-containing molecules. Kitamura et al. (161) have reported that several species of ganglioside inactivate the toxin. Once again, the trisialogangliosides were found to be quite potent. There is, however, a distinction between the original study and the confirming study. The original study (256) reported that the trisialoganglioside commonly designated G_{T1} was most effective as a toxin antagonist. Since publication of that report, G_{T1} has been further analyzed and is now known to include at least two subspecies, G_{T1a} and G_{T1b}. Kitamura et al. (161) have found that G_{T1b} is the substance that is especially active, whereas G_{T1a} has little activity.

Irrespective of whether a particular ganglioside or other siliac acid-containing molecule inactivates botulinum toxin, this finding alone is not strong evidence that a receptor has been identified. Such findings are only a tentative clue that siliac acid-containing molecules should be regarded as receptor candidates. Convincing evidence on receptor identification will likely not arise until legitimate ligand binding studies are done. In the meantime, the ability of certain gangliosides to inactivate botulinum toxin remains the only known interaction between the toxin and a membrane component.

The mechanism by which the toxin molecule, or some portion of the molecule, moves from the cell surface to the cell interior is not established, but there is a basis for hypothesizing a specific mechanism. The neurotoxin in a homogenous state has a molecular weight of ~150,000. This is far too large to permit the molecule to penetrate any of the ion channels known to be present in nerve membranes. Translocation of the toxin seems to require either an active mechanism inherent in the molecule (e.g. enzymatic activity), an active mechanism inherent in the nerve membrane (e.g. endocytosis), or a combination of the two.

The first alternative is not supported by any data. If the toxin does possess enzymatic activity, that activity has yet to be discovered. The most plausible of enzymatic actions that would permit a molecule to cross a membrane is phospholipase activity. However, the toxin does not possess phospholipase C activity, and phospholipase C treatment of nerve membranes does not alter toxin activity (246). In addition, the toxin does not possess phospholipase A_2 activity (251), as do several other presynaptic toxins (e.g., β-bungarotoxin).

In the absence of data to implicate a mechanism inherent in the toxin molecule, one must consider the possibility that translocation depends on a mechanism inherent in the cell membrane, such as endocytosis. The general phenomenon of endocytosis is almost universal among cells (244), including those in the nervous system

(123, 124, 133, 279, 313). The major difference from cell type to cell type appears to be the rate at which the process can occur. Published values range from those that are tediously slow to those that are almost incomprehensively fast. In the latter category, one might consider certain macrophages that endocytose approximately 200% of their cell membrane surface per hour (261).

The characteristics of endocytosis that may be pertinent to translocation of botulinum toxin can be summarized in the following way. The rate of endocytosis is proportional to the rate of exocytosis (36, 62, 123, 238, 277). Nerves that are stimulated rapidly show vigorous endocytosis; quiescent nerves show little endocytosis. During the process of endocytosis, macromolecules in the synaptic cleft are taken up into the nerve cell. The process of uptake is enhanced when nerves are stimulated or exocytosis is evoked (36, 122, 134). Uptake is also enhanced when the macromolecule is membrane-bound rather than being free in the biophase (82, 97, 228, 265). Pharmacologically active substances are among the macromolecules that can be endocytosed, and their pharmacological actions can be expressed after endocytosis. For example, exogenous acetylcholinesterase (224) and ATPase (227) are taken up from the synaptic region, and their internalization leads to blockade of transmission.

Experimental findings from several sources indicate that drugs can enter nerve endings by endocytosis. There is no reason to believe that botulinum toxin is an exception. Even in the absence of morphological data showing labeled botulinum toxin inside an endocytic structure, the weight of evidence compels us to believe that the process can occur. Therefore, one could hypothesize that botulinum toxin is internalized by endocytosis. Even so, there remains the question of whether botulinum toxin that is endocytosed is the same botulinum toxin that causes blockade of transmission. Among other things (such as membrane recycling), endocytosis leads to lysosomes and/or to retrograde axonal transport. The possibility that internalized botulinum toxin could be fated for lysosomal destruction has not been studied, but there is evidence that internalized botulinum toxin can undergo retrograde axonal transport (109, 304). These provisos mean that if endocytosis is the mechanism for translocation, then there must be some way in which the toxin is spared from an irrelevant fate.

Aside from enzymatic or endocytic entry, there is another putative mechanism for internalization. This mechanism may not involve just the toxin or just the membrane, but instead may involve some synergy between the two. In considering the actions of a host of bacterial toxins, Gill (102) has advanced a provocative idea. He proposes that each toxin molecule has a fragment that can become inserted into the cell membrane, and this portion of the molecule, either by itself or in cooperation with the cell membrane, can create a protein channel or a protein carrier for the rest of the toxin molecule. There is little in the way of experimental evidence to support this concept, but the idea is intriguing and warrants attention.

E. Lytic Step

1. Acetylcholine synthesis and storage. Botulinum toxin does not block transmission of nerve impulses into the fine terminal arborizations of cholinergic fibers (115), but it does block the release of transmitter induced by nerve impulses or by potassium (27, 30, 115, 278, 306). These findings mean that the toxin either alters the metabolism of acetylcholine or alters the excitation-secretion coupling process.

Only rarely have investigators argued that botulinum toxin has a primary effect on synthesis or storage of acetylcholine. Torda and Wolff (285) claimed that the toxin could inhibit synthesis, but this claim has not been substantiated (30). Botulinum toxin does not exert any direct effects on the uptake of choline (9, 107, 306), the acetylation of choline (30, 93, 107, 284, 306), the levels of acetylcholine in peripheral tissues (34, 117), or the levels of acetylcholine in central nervous tissue bathed in normal medium (107, 200, 306).

In 1971, Harris and Miledi published a study (115) that significantly modified some of the existing concepts about the pharmacology of botulinum toxin. Many previous studies that had focused on the neuromuscular junction had shown that the toxin depressed nerve stimulation-induced release of acetylcholine. The techniques used to show this effect included measurement of acetylcholine in tissue supernatants, measurement of muscle twitch, and monitoring of mepp frequency (e.g. 27, 30, 278). In general, the findings had been that the toxin blocked acetylcholine release into supernatants, paralyzed neuromuscular transmission, and caused a complete cessation of mepps. The Harris and Miledi study differed from others in two important respects. Rather than witnessing a complete cessation of spontaneous mepps, they found that the frequency of these responses decreased sharply and then remained stable at the lower frequency. An associated finding was that there was a gradual change in the distribution of spontaneous mepp amplitudes. Control preparations had the customary Gaussian distribution, but poisoned preparations had a distribution that was skewed to the left (low amplitude). Both the persistence of spontaneous mepps and the change in amplitude distribution have been confirmed (20, 46, 259). In one case, the data were interpreted to mean that botulinum toxin had altered the storage of acetylcholine (20).

Boroff et al. (20) discussed several mechanisms that might account for the decreased frequency and amplitude of spontaneous mepps. They thought the most likely explanation to be that the toxin altered vesicle membranes to impede refilling with transmitter. Their proposal was presented in terms of an analogy, i.e. the toxin-vesicle interaction might be similar to the sperm-egg interaction. In the latter, the sperm causes a reaction

that alters the membrane properties of eggs. If the toxin were to act similarly, it might change the vesicle membrane in a way that impeded loading or storage of acetylcholine. Thus, spontaneous mepps of lower amplitude and frequency would be a reflection of poorly loaded vesicles.

Whatever the drawbacks to this hypothesis, and there are a number, it nonetheless is true that the idea was rather ingenious. However, there are several reasons for questioning the validity of the proposal. Three different types of experimental observations indicate that poisoned nerve endings contain a normal or nearly normal vesicular content of acetylcholine. In one direct test of the hypothesis, Wonnacott and Marchbanks (306) performed subcellular fractionation of synaptosomes that had been exposed to botulinum toxin; they could detect no evidence that the fractions enriched in vesicles were deficient in acetylcholine. Equivalent results have been obtained by using black (or brown) widow spider venom, a substance that evokes explosive release of acetylcholine (184). In a study designed to examine the effects of the venom on toxin-induced paralysis, Stern and Valjevac (262) reported that the in vivo toxicity of botulinum toxin was antagonized by black widow spider venom. A host of studies on the in vitro interaction between the two substances soon followed (46, 150, 229, 231). These studies have in common the finding that venom can still evoke explosive release of acetylcholine even if nerve endings have been paralyzed with botulinum toxin. In addition, the size of the quanta that are released are similar to those seen before paralysis. One final argument can be adduced that may negate the "impaired vesicle filling" hypothesis. When preparations are poisoned with botulinum toxin, there occasionally appear unusually large amplitude mepps (20, 26, 46, 282). These "super-mepps" persist even when there is fully developed neuromuscular blockade. Depending upon one's bias, it could be argued that the existence of super mepps contradicts any notion of impaired vesicle filling. But alternatively, a different bias could support the concept of heterogeneous vesicle populations, some of which are vulnerable to botulinum toxin and some of which are not (115, 229).

Aside from the issues just raised, there are conceptual arguments that make difficult the acceptance of a proposal that the vesicle is the subcellular target of the toxin. Botulinum toxin acts in small quantities, and its actions last for weeks or even months. It is generally estimated that the cholinergic nerve ending contains ~2 to 5×10^5 vesicles (35, 36, 184). When one considers the number of cholinergic nerve endings in a laboratory animal (e.g. mouse) and simultaneously considers the number of toxin molecules necessary to paralyze a mouse (e.g. 240), it is obvious that the number of vesicles far exceeds the number of molecules. The toxin could exert its lytic effect on the vesicle only if the toxin could act repeatedly and thereby inactivate many vesicles. But this idea is untenable. Estimates of the average turnover time for vesicles in nerve endings range from several hours to several days. For the toxin to exert a vesicle effect that lasted weeks or months, the toxin itself would have to survive inside the nerve ending for this length of time. There is no reason to believe that a eukaryotic cell will tolerate the sustained presence of a foreign protein. This renders unlikely any proposal that the toxin acts directly on vesicles to exert its lytic effect.

2. Calcium channels and other gates. If botulinum toxin were to block calcium channels in the nerve membrane, that could account for its ability to block both depolarization-induced and spontaneous release of acetylcholine. If the toxin were to block a gating mechanism for transmitter efflux, that could account for the two actions just mentioned and might also explain the skewed distribution in mepp amplitudes. The viability of these two proposals has made them the subject of continuing investigation.

To date, no one has demonstrated by a direct technique that the toxin blocks calcium channels. To the contrary, both Drachman and Fanburg (73) and Wonnacott et al. (307) reported that synaptosomes treated with botulinum toxin accumulated $^{45}Ca^{++}$ under resting conditions and when depolarized by potassium. However, by using an indirect technique, Hirokawa and Heuser (127) obtained morphological evidence that the toxin impaired transmembrane flux of calcium. They showed that intense stimulation of the cutaneous pectoris nerve-muscle preparation caused mitochondrial swelling and deposition of electron-dense granules in the mitochondria. These effects were attributed in part to the transmembrane flux of calcium that accompanies intense nerve stimulation. When preparations were poisoned with botulinum toxin, repetitive stimulation did not evoke the characteristic swelling or appearance of electron-dense particles. These results were thought to mean that the toxin had blocked calcium channels in the plasma membrane.

Because the techniques used by Hirokawa and Heuser (127) were indirect, the conclusions that were drawn are hard to assess. Nevertheless, there are at least four salient points that bear on the hypothesis that transmembrane flux of calcium is impaired. Firstly, the data of Hirokawa and Heuser do not allow one to distinguish primary from secondary effects. These authors argue that the toxin blocked calcium flux, and the absence of such flux protected the mitochondria during intense stimulation. There are other, perhaps equally acceptable, ways in which the data could be explained. Intense stimulation causes massive exocytosis, which in turn causes marked endocytosis. If the toxin acted at transmitter release sites to impair vesicle fusion, this would secondarily diminish any subsequent endocytosis. This point seems especially important, because the morphological experiments were done in solutions containing 10 mM calcium. Therefore, repetitive stimulation of control preparations caused nerves to engorge themselves on hypercalcemic solutions,

but this might not have happened in poisoned preparations. By virtue of blocking vesicle fusion, the toxin could secondarily have prevented endocytic uptake of the hypercalcemic medium. The absence of mitochondrial swelling and dense particle accumulation may have been due to the relative absence of endocytosis in poisoned preparations. There are other proposals that could also be advanced, but each of them has the same underlying premise. Botulinum toxin could act at sites other than the calcium channel, and in doing so still protect the mitochondria from the structural changes ordinarily associated with intense nerve stimulation.

A second point relates to the use of calcium ionophores. If the toxin were a calcium channel blocker, then some of the effects of poisoning should be reversed by inserting a calcium ionophore into the membrane. In particular, an ionophore should reverse the effects of the toxin on spontaneous mepps and on spontaneous release of acetylcholine. In testing this idea, Kao et al. (150) showed that the calcium ionophore X537A dramatically increased the frequency of mepps in control preparations, but it was without effect on poisoned preparations. Cull-Candy et al. (46) obtained substantially the same results with the ionophore A23187. They did, however, find that the ionophore effect varied depending on the extracellular calcium concentration. A23187 caused a massive increase in mepps in control muscles bathed in a calcium-free medium; it exerted no effect on mepp frequency in poisoned muscles bathed in the same medium, but it did cause a marked increase in mepp frequency in poisoned muscles bathed in medium with high calcium (5 mM). They interpreted their results to mean that the toxin did not block calcium channels, but it did diminish the effectiveness of intracellular calcium in evoking transmitter release. Only Wonnacott et al. (307) have found that a calcium ionophore (A23187) would restore transmitter release from poisoned preparations (synaptosomes) bathed in normal medium, but they were reluctant to assert that the toxin had blocked calcium channels.

A third point deals with the release of acetylcholine that is triggered by intracellular stores of calcium. Two studies have employed procedures that evoke increases in mepp frequency by displacing bound calcium from intracellular sites (46, 250). The reasoning was that, if the toxin did block calcium channels, this blockade should have no effect on transmitter whose release was triggered by intracellular calcium, i.e. calcium that did not have to penetrate the plasma membrane. Both studies reported that displacement of intracellular calcium sharply increased spontaneous mepp frequency in control preparations, but it had little effect on poisoned preparations.

The final point may ultimately prove to be the most telling one. An honest appraisal of the data so far published probably reveals that no study has provided overwhelming evidence to implicate or exclude the calcium channel as the site at which the toxin exerts its lytic effect. Before convincing data will become available, work of a quantitative nature will have to be done. It will be necessary to quantify the number of calcium channels in a nerve ending, and then compare this with the number of toxin molecules necessary to produce paralysis. If there is a rough equivalence in these numbers, then the calcium channel will be implicated; if there is a serious disproportionality (calcium channels >> toxin molecules), then any hypothesis calling for a direct action of the toxin on the channel will be unlikely. In fact, in vivo data already come close to achieving the second outcome. The LD_{50} for a mouse is $\sim 3 \times 10^{-11}$ g, or $\sim 2 \times 10^{-16}$ moles, or $\sim 1 \times 10^{8}$ molecules (240). These calculations place great restrictions on the number of calcium channels that can exist, assuming that the channels are the target for the toxin. If there were only 10^3 channels per nerve ending, there could be only 10^4 to 10^5 cholinergic nerves in the mouse periphery, a number that is clearly too low. To assume that there are fewer than 10^3 channels per nerve ending does not seem realistic. A quantitative assessment of the in vivo data suggests that the toxin does not act directly on the calcium channel, but this conclusion is tentative and must await more rigorous studies.

Calcium channels that are activated by nerve depolarization represent only one of the gates that might be attacked by the toxin. Another possibility is that acetylcholine leaves the nerve through specialized channels, and these are the gates that are occluded by the toxin. The possibility of impaired gating of acetylcholine release has been raised by two groups (115, 176). Harris and Miledi (115) considered the gating hypothesis as one of several mechanisms that could account for the actions of the toxin, and particularly that of diminishing the frequency and amplitude of mepps. These authors themselves provided data and reasoning that seem to dispute the gating model. They point out that the concept of a gate is at odds with current thinking about exocytosis. Acetylcholine is not thought to be released through a gate; instead, it is thought that the vesicle membrane fuses and then becomes coextensive with the plasma membrane. This scheme does not include a gate that could be closed. Furthermore, a putative closing gate is not entirely compatible with experimental observations. The toxin causes the customary binomial distribution in mepp amplitudes to become skewed to the left. This is a relative and not an absolute phenomenon. That is, mepps of all amplitudes decrease in frequency, but small mepps disappear more slowly than large mepps. This is not the outcome that would be expected from a closing gate. If there were a channel through which acetylcholine was released, and if the duration of the open state determined the amount of acetylcholine released, then closing of the gate should produce an initial and absolute increase in the number of small mepps. As just explained, this expectation is at variance with experimental results. Finally, several investigators have found that the toxin-induced change in mepp amplitudes can be reversed, either transiently or permanently. Tetanic nerve stimu-

lation, long-term nerve stimulation, a combination of depolarizing concentrations of potassium plus calcium, and a combination of a calcium ionophore plus a high concentration of calcium all cause the skew in mepp amplitudes to revert to a normal, Gaussian distribution (46, 115). These data are hard to reconcile with the notion that a gate has been closed.

Lamanna and his associates have also proposed a gating mechanism, which has been called a "pipe and valve" model (114, 176). This hypothesis is not based on original experimental findings, but instead is a speculative model based on the reported findings of other investigators. The major premises underlying the hypothesis are that only a small number of toxin molecules are necessary to paralyze transmission, and that there are only a limited number of transmitter release sites in the nerve membrane. Quantitative considerations suggest that the limited number of release sites are the target for the toxin. If these sites encompass a gate ("pipe"), the toxin ("valve") might become inserted into it and physically obstruct transmitter release. Insofar as the hypothesis suggests that the toxin attacks a site critical to transmitter release, the model is in accordance with the thinking of virtually all investigators. Insofar as the hypothesis proposes a gate for acetylcholine efflux, it is subject to the same criticisms raised in the preceding paragraph.

3. Calcium and excitation-secretion coupling. Efforts to define the interaction between botulinum toxin and calcium began with the work of Thesleff (278). In studying the effects of botulinum toxin on motor innervation of the cat tenuissimus muscle, he found that mildly intoxicated preparations became more responsive when ambient calcium concentrations were doubled. He further reported that this effect was temporary, and that elevated calcium could not prevent paralysis. The fact that calcium can antagonize the onset of toxin-induced paralysis has since been confirmed (257). More recently, Thesleff and his associates have shown that botulinum toxin alters the process triggered by calcium, and the effect is to diminish the apparent sensitivity of that process to the actions of calcium (46, 187, 188). Their data involve the study of calcium alone, as well as the study of calcium in combination with ionophores (A23187) and other agents that enhance the flux of calcium (e.g. tetraethylammonium, guanidine, and 4-aminopyridine).

Studies on botulinum toxin and calcium ionophores have already been discussed. Work on the neuromuscular junction shows that an ionophore alone does not reverse the effects of the toxin on mepp frequency, but the combination of an ionophore plus elevated calcium overcomes mild paralysis (46, 150). Similar findings have been obtained with drugs that promote the inward flow of calcium, for which 4-aminopyridine is a prototype. This drug impairs potassium flux that is associated with the depolarization-repolarization cycle (201, 310). By doing so, it greatly enhances the voltage-sensitive inward flow of calcium (188). 4-Aminopyridine has a striking ability to reverse the effects of partial toxin-induced paralysis (187, 188), but it is less effective when tested on fully paralyzed preparations (111, 250).

Experiments with A23187 and 4-aminopyridine indicate that the toxin alters the calcium sensitivity of some intracellular process, a conclusion that is supported by a different line of investigation. Several years ago techniques were developed for generating a quantitative statement that relates the extracellular calcium concentration to the quantum content of an epp. When these two variables are plotted against one another they yield a straight line on logarithmic coordinates. Depending upon the type of preparation studied, the curve has a slope of ~2.5 to 4.0 (43, 65, 68, 137, 155). On a plot such as this, competitive antagonists shift the curve to the right without changing the slope but noncompetitive or irreversibly acting drugs shift the curve to the right and also depress the slope (233). Botulinum toxin shifts the curve to the right and depresses the slope to immeasurably low levels (46, 250). When preparations are only mildly paralyzed the addition of 4-aminopyridine shifts the curve back toward the control position (188). The interpretation of these results is that botulinum toxin causes preparations to become progressively less responsive to extracellular calcium. If the toxin does not block calcium channels, it must be decreasing the affinity or efficacy of calcium at some intracellular site.

Although the role of calcium in triggering exocytosis has not been well defined, morphologists are beginning to provide some rather vivid pictures of the probable site at which calcium acts. Since the pioneering studies of the 1950s, anatomists have known that the presynaptic nerve membrane is a heterogeneous structure (146, 216). Electronmicrographs of the nerve ending in profile show that the inner surface of the plasma membrane has regularly spaced, electron-dense protrusions. Each protrusion has its base on the membrane and a peak that extends several hundred Ångstroms into the cytoplasm. The major features of these protrusions are that they are surrounded by clusters of synaptic vesicles, and they tend to occur directly opposite those portions of the postsynaptic membrane that are enriched in receptor molecules. When originally seen in central nervous system synapses, the protrusions and vesicles were called "synaptic complexes" (216). Analogous structures have been described at the neuromuscular junction and have been called "active zones," a term meant to convey that these are the sites at which exocytosis occurs and from which transmitter substance is released onto postsynaptic receptors (44).

The application of freeze-fracture techniques has afforded even greater resolution of the active zones (74, 223). When viewed face-on, the presynaptic membrane specializations appear as a series of ridges, and these ridges are bordered on both sides by particles that may

extend through the membrane. It has been proposed that these intramembranous particles are the calcium channels associated with transmitter release (125). When synaptic vesicles fuse with the membrane, they appear as dimples that are usually juxtaposed to the intramembranous particles (125).

Pumplin and Reese (231) have applied the technique of freeze fracture to electronmicroscopy of cholinergic nerves paralyzed with botulinum toxin. In control preparations, electrical stimulation evoked vesicle fusion almost exclusively at the active zones. Preparations that were poisoned by botulinum toxin were largely devoid of evidence (dimples) of vesicle fusion. In addition to electrical stimulation, Pumplin and Reese examined the effects of brown widow spider venom. In the presence of calcium, the venom evoked transmitter release mainly at the active zones; in the absence of calcium, release occurred somewhat randomly on the nerve membrane surface. When preparations had previously been poisoned with botulinum toxin, the venom lost much of its ability to stimulate vesicle fusion in the active zones, but retained its ability to stimulate fusion elsewhere. These data suggest that the toxin acts at or near those sites in the active zone that regulate exocytosis. Parenthetically, the data do not offer much support for the idea that the toxin is a calcium channel blocker. The venom itself is thought to be a cation selective ionophore (100). Interestingly, the venom plus calcium evoked transmitter release at the active zones of control preparations, but the combination was less effective in exerting effects at the active zones of paralyzed preparations. If the venom can act as an ionophore to promote calcium flux and vesicle fusion in the active zones, why are its actions antagonized by botulinum toxin? The results do not indicate that the toxin blocks transmembrane flux of calcium, but rather that it blocks some step in excitation-secretion coupling that occurs after ion flux.

Kao et al. (150) have published electronmicrographs that are in accordance with those just discussed. They found that black widow spider venom caused massive vesicle fusion with the plasma membrane of untreated nerves, but its actions on botulinum intoxicated nerves were altered. Their pictures show vesicles "log-jammed" at the release sites when nerves were paralyzed with botulinum toxin. Accordingly, the site of action of the toxin was described as being the active zones, and the mechanism of action was described as being interference with the fusion of vesicle membranes and plasma membranes.

4. Subminiature endplate potentials and asynchrony. Of the several effects exerted by botulinum toxin, one has remained more or less a mystery. The ability of botulinum toxin to depress nerve stimulus-induced release of acetylcholine and to depress the frequency of mepps can be explained if the toxin is a calcium channel blocker or if it is a blocker of calcium-mediated membrane fusion. The ability of botulinum toxin to alter the amplitude of mepps is hard to explain on either of these bases. A different type of explanation has been advanced by Kriebel et al. (171), and it is one that calls for fundamental changes in concepts about transmitter release.

Traditional views about transmitter release resulted from a coalescence of morphological and electrophysiological research. On the one hand, Katz and his associates formulated the idea that acetylcholine was released in quantal units, and that the mepp was the electrophysiological response evoked by one quantum (152, 156). On the other hand, both DeRobertis and Whittaker obtained electronmicroscopic evidence that nerve endings contained vesicles, and that these vesicles contained acetylcholine (64, 303). These two lines of discovery merged into the vesicle hypothesis, according to which acetylcholine is stored in individual vesicles, and the release of acetylcholine by a single vesicle results in a single mepp.

In contrast to these traditional views, Kriebel and his colleagues have proposed that most spontaneous potentials are due to the synchronous release of several quanta of acetylcholine (169, 170, 171). According to this proposal, a single quantum evokes a response called a subminiature endplate potential, and multiples of the subminiature endplate potential are due to synchronous release of two or more quanta. Hence, the mepp is the response caused by simultaneous release of several quanta. Kriebel et al. (171) have reproduced the finding by Harris and Miledi (115) that botulinum toxin produces a leftward shift in the distribution of mepp amplitudes. In keeping with their novel ideas about transmitter release, they have suggested that the shift is due to toxin-induced desynchronization.

It is obvious that there are sharp differences of opinion in explaining the effect of botulinum toxin on mepp amplitudes. The more traditional view is that the size of individual mepps, which are due to individual quanta, decrease in magnitude. The opposing view is that the size of subminiature endplate potentials, which are due to individual quanta, do not change in magnitude, but the frequency of synchronized quantal release decreases. Resolution of this difference in opinion requires that the authenticity of synchronized quantal release be established or disproved. If the notion is proved correct, then the possibility that the toxin produces asynchrony deserves attention.

In relation to the validity of the model, several investigators of botulinum toxin have raised some pertinent questions. Both Spitzer (259) and Cull-Candy et al. (46) have reported that the shape of subminiature endplate potentials in botulinum-intoxicated muscles is different from the shape of potentials recorded in control muscles. In the former case, the rise times were occasionally depressed, which could mean that transmitter was being released from more distal sites than ordinarily occurs. In a different vein, Heuser (121) has proposed that quantitative studies on exocytosis and retrieval of membrane by endocytosis support the traditional view (1 quantum

~1 mepp) rather than the synchronization model (several quanta ~1 mepp). And finally, the arguments raised in an earlier section against toxin-induced blockade of an acetylcholine gate apply equally well to toxin-induced asynchrony. If the toxin acted to diminish the likelihood of simultaneous quantal release, then an initial effect of poisoning should be an increase in the number of unsynchronized events (i.e., subminiature endplate potentials). As already noted, this result is not obtained.

There are explanations other than asynchrony that are consistent with the phenomenon of diminished mepp amplitudes (20, 46, 115, 259). One of these is that there are different populations of vesicles, some of which are vulnerable and some of which are resistant to botulinum toxin. Another explanation is that there are optimal and suboptimal sites for transmitter release, and these sites are not equally paralyzed by the toxin. None of the various hypotheses holds a commanding experimental edge.

F. Known Limitations and New Perspectives

A full description of the molecular actions of botulinum toxin is not presently available, but there may be some clues and limitations that point the direction in which future research will go. Some of these clues are rather obvious and require only passing comment; others are more subtle or provocative and require thoughtful examination.

There are several limitations that establish the boundaries on any proposed mechanism of toxin action. Uppermost among these are the duration of action and the potency of botulinum toxin. This substance is capable of producing sustained blockade of transmitter release, both when used experimentally and when encountered clinically. This simple but often repeated observation has valuable implications. The duration of blockade, which can last many weeks or months, makes questionable any proposal that the toxin can be continuously present throughout paralysis. There is no evidence arising from the nervous system, or from any other tissue system, that a eukaryotic cell will permit a large foreign protein to reside freely in the cytoplasm for extended periods of time. Similarly, any proposal that calls for the toxin to be covalently linked to or intercalated in the membrane for weeks or months would be without experimental support and devoid of examples by analogy. To be sure, many molecules do, on a short time scale, bind tightly or even covalently to membrane receptors, and botulinum toxin may itself bind irreversibly or essentially irreversibly. However, there are no known pharmacological substances, and certainly no large molecular weight proteins, that bind to nerve membranes and remain bound for weeks or months. These matters suggest that a large, foreign protein like botulinum toxin does not produce sustained blockade of transmitter release by virtue of its own lengthy residence in cell membranes or cell interiors.

If botulinum toxin acted only for short periods of time, and if the concentrations of toxin needed to produce paralysis were high, then the number of sites in the nerve ending that would have to be tested for vulnerability to poisoning would be immense. But given the lengthy duration of toxin action, and given the small number of toxin molecules necessary to produce paralysis (see below), several exclusions can be imposed. The toxin cannot act on a one-to-one basis with any molecule that has a rapid turnover time, because there are not enough toxin molecules involved in paralysis. In fact, it may be that the toxin cannot act on a one-to-one basis with any molecule, irrespective of turnover time, because all species of molecules in the nerve ending may exist in excess of the minimum number of toxin molecules needed to cause paralysis.

Taken collectively, the foregoing remarks form the basis for advancing these two premises. Botulinum toxin exerts a poisoning effect that continues to be expressed after the molecule has left the nerve ending; the poisoning effect does not entail a one-to-one reaction with molecules or structures existing in large numbers or having rapid turnover times. These two premises rule out many putative mechanisms to account for where and how the toxin acts; the task that remains is to decide what putative mechanisms remain viable.

Few authors have expressed well-defined beliefs about the molecular actions of the toxin. Nevertheless, the literature does reveal that most workers entertain what might be called "one-hit" mechanisms. These mechanisms envision one toxin molecule reacting with one cellular molecule, and this reaction in and of itself causes paralysis. An example of such a mechanism would be that of botulinum toxin blocking calcium channels. A single toxin molecule could bind to and occlude a single calcium channel, and this occlusion could be the poisoning effect. An analogy would be that of tetrodotoxin binding to and occluding sodium channels.

For a one-hit mechanism to be acceptable it must satisfy at least two criteria. The susceptible molecule or structure must be present in numbers equal to or less than the minimum number of toxin molecules that cause paralysis. This criterion must be satisfied to explain the remarkable potency of botulinum toxin. Also, the susceptible molecule must be something that has a slow turnover time, thus explaining the long duration of toxin action. Of these two criteria, the quantitative one may be more instructive.

The minimum number of toxin molecules necessary to paralyze a mouse is ~10^8 (114, 240). The number of nerve endings in the mouse diaphragm is ~10^3 to 10^4. Assuming that every toxin molecule exerts a pharmacological effect, and assuming that the totality of effect is expressed at the phrenic nerve-hemidiaphragm, the number of toxin molecules causing paralysis would be several thousand per nerve ending. But these assumptions are patently false. No drug has a 100% efficacy, and botulinum toxin acts at many sites other than the diaphragm. Even the

most conservative estimates (e.g., highest efficacy, lowest number of target organs) yield a toxin to nerve ending ratio that cannot exceed several hundred. This estimate is compatible with as yet unpublished work on in vitro mouse preparations showing that the number of toxin molecules that will cause paralysis is several hundred per nerve ending, if not less.

Apparently the minimum number of toxin molecules necessary to cause paralysis is less than the number of any endogenous molecules or structures known to participate in transmitter release. It is smaller by several orders of magnitude than the number of molecules of transmitter (146); it is even smaller by orders of magnitude than the number of vesicles (35, 36, 184). The only structure of such limited numbers is the specialized release sites, and morphological data show that even these sites may be too numerous (124). The data do not seem to be in accord with any one-to-one mechanism for toxin-to-target interaction. Instead, the data suggest that there is a multiplicative mechanism. More plainly, the data suggest that botulinum toxin could act in small numbers to produce sustained paralysis if it were an enzyme whose substrate has a low turnover rate.

The proposal that botulinum toxin is an enzyme falls within the bounds of the several limitations just discussed. By virtue of possessing enzymatic activity, the toxin could react with many substrate molecules. Therefore, the extraordinary potency of the substance could be explained. By virtue of acting on a substrate of a low turnover rate, the toxin could exert a pharmacological action long after its own disappearance from the nerve ending. This in turn eliminates the need to conjecture that nerve cells harbor foreign proteins for extended periods of time.

It is one thing to propose a model that falls loosely within the bounds of reason; it is something else to propose a model that looks adequately promising such as to stimulate research, or at least stimulate debate. The suggestion that botulinum toxin is an enzyme is based partly on empirical findings and partly on deduction. The argument would gain strength if there were relevant and compelling analogies based entirely on empirical findings. If it were true that other potent bacterial toxins have enzymatic activity, and if it were true that some of these toxins have structures similar to botulinum toxin, that should rightly attract notice. As it turns out, both expectations are easily met. There are so many bacterial toxins that are enzymes that this state of affairs tends to be the rule rather than the exception (102, 205). More specifically, two of the most potent bacterial toxins, cholera toxin and diphtheria toxin, possess enzymatic activity (42, 132, 217). Through an enzyme-mediated step cholera toxin causes pathological activation of adenylate cyclase (132), a substance found in many cell types (275). Diphtheria toxin also possesses enzymatic activity, the outcome of this activity being inhibition of protein synthesis (42, 217).

In the case of diphtheria toxin, the similarities with botulinum toxin are hard to overlook (compare 42 and 217 with the present review). Both botulinum toxin and diphtheria toxin are large molecular weight proteins that are synthesized by bacteria. Production of both toxins is governed by bacteriophage. The toxins are synthesized intracellularly as single-chain polypeptides, but they are nicked to form dichain molecules. The two chains are linked by disulfide bonds; reduction of these bonds causes loss of cellular toxicity. In the case of diphtheria toxin, one chain has a binding site that recognizes cell surface receptors, and the other chain has catalytic activity that causes cytotoxicity.

The two chains resulting from nicking and disulfide-bond reduction of botulinum toxin have not been isolated in substantial quantities and have not been tested for binding and enzymatic properties [but see a preliminary report (165)]. However, tetanus toxin, a clostridial toxin that is very similar in structure (57) and function (111, 148, 194) to botulinum toxin, has been fragmented, and the heavy chain has been isolated in quantity (120). This chain has binding properties that are indistinguishable from those of native tetanus toxin, but this chain does not possess the neurotoxicity of native toxin (204). Interestingly, it is the heavy chain of diphtheria toxin that binds to membrane receptors, and it is the other chain that has enzymatic activity.

Clearly there is danger in arguing solely by analogy. Even though botulinum toxin and diphtheria toxin have some features in common, the only truly acceptable evidence that botulinum toxin is an enzyme will be the direct demonstration of catalytic activity. Until this possibility is proved or disproved, the foregoing comments should be read solely for their heuristic value. A marshalling of data and deduction permit one to speculate that botulinum toxin possesses enzymatic activity, and this activity in turn could account for blockade of transmitter release. This speculation awaits experimental testing.

G. Synopsis of the Data

Botulinum toxin acts at the cholinergic nerve ending to block release of acetylcholine (fig. 3). Blockade does not result from inhibition of synthesis, storage, or metabolism of acetylcholine, nor does it result from inhibition of agonist-induced responses. The toxin acts on or in the nerve ending to antagonize those events that are triggered by calcium and that culminate in transmitter release.

A general model has been proposed to explain the actions of botulinum toxin on the nerve terminal (251). This model encompasses three discrete steps. Initially, the toxin binds to a specific class of cell surface receptors. The identity of these receptors remains unknown, but several characteristics of the drug-receptor interaction have been determined. Binding is rapid, essentially irreversible, but of a nature that leaves the toxin partially accessible to inactivation by antitoxin. The latter finding suggests that binding is extracellular.

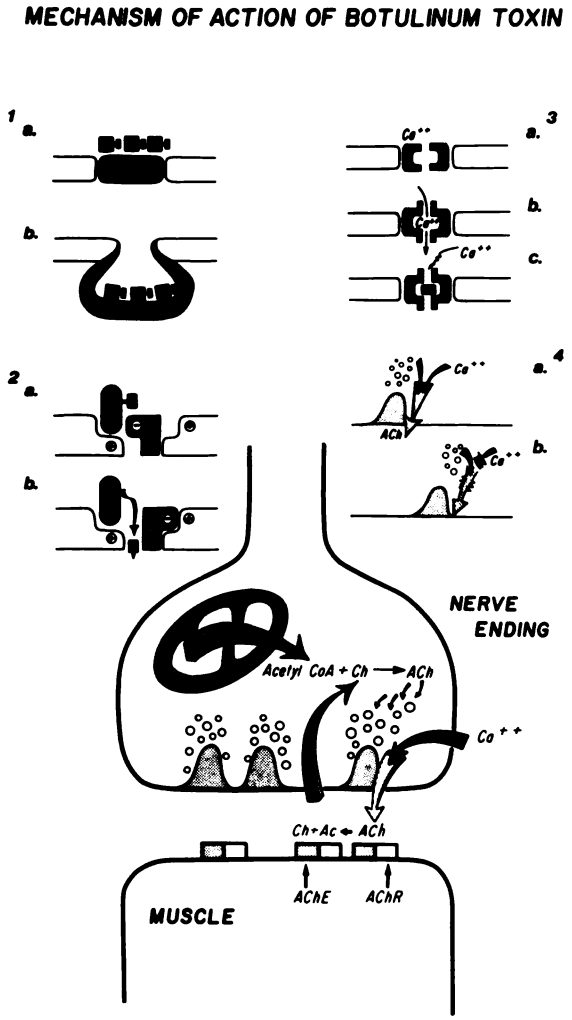

FIG. 3. Botulinum toxin blocks transmission at cholinergic sites such as the neuromuscular junction. The toxin has no direct effect on choline acetyltransferase, the enzyme that links acetate (Ac) from acetylcoenzyme A (Acetyl CoA) with choline (Ch) to form the transmitter substance acetylcholine (ACh). In addition, the toxin does not impair the ability of vesicles to become filled with transmitter, the ability of receptors (AChR) to interact with transmitter, or the ability of acetylcholinesterase (AChE) to degrade transmitter. Instead, the toxin appears to block the mechanism by which calcium triggers acetylcholine release. To exert its blocking effect the toxin must first bind to the membrane, after which it moves into or through the membrane. Various proposals have been advanced to explain these phenomena (1 to 4). For example, the toxin could bind to specialized parts of the membrane (1a) and then be internalized by adsorptive pinocytosis (2b). Alternatively, the toxin could bind to specialized sites in the membrane (2a), and then act alone or in cooperation with the membrane to create protein channels or protein carriers (2b). Neither the binding step nor the internalization step is known to alter cell function. To produce blockade of transmitter release, the toxin exerts an effect within the membrane or intracellularly. A putative membrane effect could be occlusion of calcium (Ca^{++}) channels. Under resting conditions the nerve membrane has cation channels that are in the closed state (3a). When the membrane is depolarized the cation channels open and calcium flows inward (3b). The toxin may act to occlude these channels (3c), thus impairing the ability of extracellular calcium to enter nerve cells and trigger exocytosis. A putative intracellular effect could be enzymatic in nature. Calcium ordinarily promotes the ability of vesicles to fuse with specialized membrane sites and secrete their stores of transmitter (4a). If this process involves a substrate that is vulnerable to enzymatic attack by botulinum toxin, then the ability of calcium to promote transmitter release would be inhibited (4b).

The second step is one in which the toxin disappears from accessibility to antitoxin, and does so without causing onset of paralysis. Of several interpretations that could be assigned to this finding, internalization of the toxin (or some fragment) seems most parsimonious. There are many examples of protein toxins binding to cell surface receptors and then being internalized (102, 205). The mechanism for membrane penetration has not been determined for any bacterial toxin, but investigators agree that passive diffusion is unlikely; an active process such as adsorptive pinocytosis or the creation of protein carriers or channels is more likely.

The final step is the one that results in blockade of transmitter release. As judged by data on the neuromuscular junction, evoked release is diminished, spontaneous release is diminished, and the characteristics of spontaneous release are altered. The toxin may act by a one-hit mechanism to produce these effects, or alternatively it may act like an enzyme. In either case, the toxin does not poison nerve endings at the cell surface. The toxic effect is intramembranal or more probably intracellular.

The terms "binding," "translocation," and "lytic" have been applied to the three steps. The term "binding" is common in pharmacology and requires no explanation. The term "translocation" has been borrowed from biochemistry, particularly that field of research dealing with the movement of proteins across membranes. The term "lytic" is common in toxicology and means, generally speaking, a destructive effect. As originally applied to botulinum toxin, the term had the added advantage of being a condensation of paralytic (251). But in view of speculations on enzymatic activity, the term might ultimately prove to be a condensation of catalytic.

VII. Drug Interactions

There are many reports indicating that a variety of drugs can interact with botulinum toxin. Unfortunately, most of these reports fail to examine interactions thoroughly and thus fail to provide complete explanations for the mechanisms that underlie the interactions. Exceptions to this general rule are the studies that deal with calcium, black widow spider venom, and gangliosides, all of which were discussed above. Other exceptions are studies that describe substances of immunological importance, such as antitoxin and toxoid, and substances that promote acetylcholine release, such as guanidine, tetraethylammonium, and 4-aminopyridine.

A. Substances of Immunological Importance

Botulinum toxoid is typically prepared by isolating and then formalin-inactivating the toxin (263). When appropriately administered in vivo, the toxoid confers immunity both to the living organism and to tissues obtained from the living organism. The former observation is to be expected, but the latter observation is somewhat surprising. Burgen et al. (30) reported that phrenic nerve-hemidiaphragms taken from immune animals were highly resistant to in vitro challenge with botulinum

toxin. The resistance observed was much greater than could be accounted for by any antiserum that might have been trapped in the extracellular space. This fascinating observation has not been reproduced since it was first reported. Nevertheless, assuming that the observation is valid, it raises an interesting possibility. Perhaps the toxoid elicited antibody formation in vivo, and then minute quantities of antibody entered nerves by the process of fluid phase pinocytosis (244). As a result, intracellular antibody could have antagonized the in vitro effects of the toxin. This proposal is highly speculative, but the implications of the proposal, both in terms of understanding how toxin acts and in terms of developing therapeutic strategies, should encourage closer examination of in vitro immunity.

Most of the published reports on botulinum antitoxin appear in the microbiology and immunology literature. These reports frequently note that antitoxin will neutralize unbound toxin, but it exerts no effect on toxin that has caused paralysis. An intermediate phenomenon was described in section VI.

Efforts to transpose immunological findings into pharmacological research now seem especially timely. Antibodies to the two polypeptide chains that are obtained by reducing the toxin have been isolated (e.g. 167), and attempts to prepare antibodies to the fragments obtained by limited proteolysis are underway. It is obvious that the availability of antibodies directed against specific regions of the toxin molecule will greatly aid research to determine where and how the toxin and its fragments act.

B. Substances that Promote Acetylcholine Release

There are numerous substances that promote acetylcholine release, some of which have been examined for their ability to antagonize botulinum toxin. The first such substance to gain wide attention was guanidine. In a brief clinical paper Cherington and Ryan reported that guanidine hydrochloride provided modest relief to a patient with botulism (40). In a follow-up study (237), guanidine was shown to relieve partially the neuromuscular blockade caused by botulinum toxin in laboratory animals. In spite of the initial and some later favorable reports (e.g. 41,210), most workers have found that guanidine provides little relief to botulism victims (98, 151, 299).

Guanidine promotes acetylcholine release at motor nerve endings, presumably by increasing the availability of calcium (60, 149, 215, 226). On this basis, the drug might be expected to antagonize botulinum toxin. However, the findings on guanidine should be compared with those on calcium ionophores (see section VI). Ionophores by themselves are poor antagonists of the toxin, but ionophores in combination with elevated concentrations of calcium act as antagonists. It is noteworthy that guanidine is more effective as a toxin antagonist if calcium levels are raised to 4 mM or higher (188).

Other drugs have been shown to promote transmitter release and to antagonize botulinum toxin (e.g. tetraethylammonium), but the one that may be of greatest interest is 4-aminopyridine. This agent greatly increases the amount of acetylcholine released by nerve stimulation, apparently by prolonging evoked depolarization (201, 310). Extracellular calcium enters nerves through cation-selective channels that are opened by nerve depolarization (136, 137, 154), so prolonging depolarization greatly enhances inward flow of calcium. 4-Aminopyridine deserves special consideration not only because it can antagonize botulinum toxin, but also because it can do so in the presence of normal calcium concentrations (188, 189).

Both in the context of clinical botulism and in the context of laboratory research there is one characteristic of 4-aminopyridine that must be emphasized. The drug can antagonize botulinum toxin only in the early stages of paralysis, or in cases of paralysis caused by small amounts of toxin. When preparations are paralyzed by large amounts of toxin, 4-aminopyridine exerts a lesser effect (111, 250). A clinically useful substance that will antagonize all concentrations of toxin in all stages of paralysis has not been discovered.

VIII. Consequences of Transmitter Blockade

Botulinum toxin is not believed to have any direct effects on the synthesis or storage of acetylcholine. Although Torda and Wolff (285) reported that the toxin could inhibit acetylcholine synthesis, other workers have not confirmed the finding. No evidence has been found that the toxin exerts any direct effects on choline acetyltransferase (30, 284) or on the high affinity uptake of choline (107, 306). Apparently the toxin does not alter either the availability of substrate or the activity of synthesizing enzyme.

Indirect evidence strongly suggests that paralyzed nerves can synthesize and store transmitter in a normal, or nearly normal, manner. This evidence has already been discussed, and it can be summarized as follows. When paralyzed nerves are exposed to black widow spider venom, there is explosive, but quantal, release of acetylcholine. In addition, several drugs or drug combinations (e.g. 4-aminopyridine) promote transmitter release from partially paralyzed nerves, and this release is similar to that which occurs in unpoisoned preparations.

Aside from synthesis and storage, there is the possibility that the toxin could alter acetylcholine metabolism. The only positive finding in this regard is that of Marshall and Quinn (191), who reported that botulinum toxin could inhibit acetylcholinesterase activity. Two other groups have failed to reproduce this finding (255, 274).

Even though the toxin does not exert any direct effects on acetylcholine turnover, it could exert indirect effects. For example, the toxin could cause changes in turnover that are compensatory in nature. Regrettably, the data

that bear on this problem have not been entirely consistent. Molenaar and Polak (200) presented evidence that the toxin could inhibit transmitter synthesis; Wonnacott and Marchbanks (306) provided evidence that the toxin did not inhibit synthesis; and Gundersen and Howard (107) found no evidence for marked inhibition of synthesis, but did obtain evidence that a small compartment of transmitter might be subject to inhibition of synthesis.

In contrast to the work on compensatory changes in acetylcholine turnover, work on other aspects of nerve and muscle physiology has been more consistent. Generally speaking, chronic treatment with the toxin evokes many of the same changes that are induced by axotomy or surgical denervation. Thus, the toxin alters the ultrastructure of nerve cells (273), the rates of nucleic acid and protein synthesis (297), anterograde axonal transport (26, 297), retrograde axonal transport (96, but see 172), axonal sprouting (79, 220), and many aspects of muscle structure and function (71, 76–78, 101, 145).

Probably the most fascinating change evoked by the toxin is an increase in the number and distribution of acetylcholine receptors. In 1947 Guyton and MacDonald (108) showed that injection of small amounts of toxin into the limbs of experimental animals produced local paralysis. This technique has been used by several investigators to study the chronic effects of toxin-induced synaptic and neuromuscular blockade. A repeated finding is that the toxin evokes the phenomenon of supersensitivity, i.e. an increased sensitivity to cholinergic agonists (33). This phenomenon can be demonstrated in fast muscle, in slow muscle, and in gland (95, 147, 278, 282). In experiments utilizing radioactive ligand to localize and quantify acetylcholine receptors, two groups have found that botulinum toxin causes an increase in the number and distribution of receptors similar to that found in surgically denervated preparations (221, 249).

In spite of the many similarities between surgical denervation and chronic toxicity, there are some quantitative differences. Botulinum toxin is less effective in increasing tissue sensitivity to agonists (221, 249), and the toxin is less likely to cause the development of tetrodotoxin-resistant action potentials (280). There are many likely explanations for these quantitative differences. Most obviously, local injection of botulinum toxin is a less precise technique than nerve crush or nerve sectioning. An investigator cannot reasonably expect local injection to produce complete or equal blockade of all nerve endings. Furthermore, surgical techniques that cause axotomy ensure loss of all neurogenic acetylcholine, but poisoned nerves continue to release minute amounts of transmitter (115). Yet another concern relates to the comparative effects of denervation and chronic toxicity on cholinesterase activity. Although both procedures cause the levels of enzyme at the endplate region to decrease, denervation exerts a quantitatively greater effect (72, 75, 267). This would contribute to the differences in supersensitivity caused by denervation and by toxin, because both an increase in receptor number and a decrease in enzyme activity contribute to the phenomenon of supersensitivity (193). Finally, the only substance whose release is known to be blocked by botulinum toxin is acetylcholine. If there are other substances whose release governs receptor number and distribution, and if release of these substances is not blocked by toxin, then toxin-induced effects will surely be less pronounced than those caused by axotomy.

The preceeding paragraphs describe a number of compensatory changes that occur in poisoned preparations. It may be worth stating that nerve and muscle are capable of the ultimate compensatory response; that is, they recover from the effects of poisoning. Whatever may be the lytic effect of the toxin, that effect is ultimately reversible. Given adequate time and suitable conditions, cholinergic junctions regain function (99, 283).

IX. Molecular Biology, Classical Pharmacology, and Botulinum Toxin

During the course of research on the pharmacological actions of botulinum toxin two concepts have emerged that will have impact not only on the study of botulinum toxin but also on the study of nerve-ending function. The first of these concepts is that many of the remaining questions about botulinum toxin can be best answered by enlisting the principles and techniques of molecular biology. The second concept is that botulinum toxin and related substances, when modified in accordance with principles of molecular biology, could become exquisite tools for dissecting nerve function.

A. Molecular Biology and Botulinum Toxin

It is axiomatic that research on botulinum toxin cannot be complete until the full structure of the molecule is known. Only then will it be possible to identify active sites, localize these sites within the 3-dimensional structure of the molecule, and thus explain how the protein can bind to its membrane receptors. While no one would dispute the need for describing the molecule, many might see the task as too formidable to be within immediate reach. Fortunately, such a pessimistic view is not warranted. The structure, or at least the amino acid sequence, can be described without undue difficulty.

Traditional approaches to protein chemistry have serious limitations. For either sequencing or synthesis, molecules of $\sim 10^4$ daltons are challenging and molecules of $\sim 10^5$ daltons or greater are for all practical purposes impossible. These limitations mean that traditional techniques for determining the amino acid sequence or for synthesizing proteins can be applied to small toxins, or to reproducibly obtainable fragments from small toxins [e.g. the enzymatic fragment from diptheria toxin (63)], but they cannot be applied to large toxins or to large fragments. To analyze or synthesize these molecules, some other methodology must be employed.

The techniques of recombinant DNA research are well suited to a determination of the amino acid sequence of botulinum toxin. [For a review of recombinant techniques and their applications, see Wetzel (301).] With a combined knowledge of amino acid composition, which can be obtained by traditional methods, and a codon map, which can be obtained by recombinant methods, a presumptive linear structure for botulinum toxin could be described. As is well known, amino acid analyses are performed relatively easily. It may not be as widely appreciated that extensive codon maps can also be generated, and done so within reasonable lengths of time (e.g. 236). In fact, a knowledge of the origin of botulinum toxin strongly suggests that the problem is one for which nature has already carried out some preliminary steps. To establish a nucleotide sequence from which an amino acid sequence can be inferred, one must isolate the genome in question. Isolation is time-consuming if the genome comes from a eukaryotic cell, less time-consuming if the genome comes from a prokaryotic cell, and least time-consuming if the genome is in a phage particle or a plasmid. In at least two cases (C_1 and D toxin), the botulinum toxin genome is in a phage particle (91, 92, 140, 141). As such, the forces of nature have partially isolated the nucleotide sequence that must be mapped.

The development of codon maps, and from this a deduced primary structure for botulinum toxin, would have several values. A comparison of amino acid sequences in the eight botulinum toxins would show regions of homology and disparity. Such information would be indispensible to determinations of active sites that govern binding and that govern the actual pharmacological effect, i.e. blockade of transmitter release. Additionally, the information would help confirm the intuitively obvious expectation that the eight toxins have a common evolutionary origin. This mundane observation could be complimented by something more substantive. Botulinum toxin and tetanus toxin have numerous similarities in structure (57) and in pharmacological activity (111, 195, 252). Comparisons of their respective amino acid sequences could disclose whether these two clostridial neurotoxins had a common ancestor and could explain on a molecular basis the reasons for their differing tissue affinities.

From the perspective of toxinology and toxicology the most desired goals in botulinum toxin research are to isolate the tissue receptor and to identify the cellular process that is poisoned. Considering for a moment only the matter of tissue binding, the goal of isolating the toxin receptor is somewhat narrow in scope. However, if perspective is changed and focused instead on the nerve ending, the goal becomes much broader in scope. Neurobiologists would like to know whether nerve endings that release particular types of transmitters have unique membrane determinants. For example, do membranes of cholinergic nerve endings differ in any fundamental way from membranes of adrenergic or other nerve endings? As an extension of this, are there different and distinct populations of cholinergic nerve membranes? Such questions could be answered if there were drugs whose receptors were imbedded in cholinergic nerve membranes, and if these drugs had different affinities for various populations of cholinergic nerves. Botulinum toxin may satisfy these criteria.

No systematic effort to test all eight botulinum toxins and tetanus toxin on cholinergic transmission in diverse species and/or tissues has been reported. Nevertheless, a comparison of data from several studies shows that there are species and tissue differences. For instance, botulinum toxin type B is very potent in poisoning laboratory animals such as mice and guinea pigs, but it is much less potent in rats (30). Tetanus toxin paralyzes goldfish and mouse neuromuscular junctions in vitro, but it has negligible potency on rat and guinea-pig neuromuscular junctions (111, 195, 252). Even within a given specie individual toxins have variable potency in paralyzing different tissues. A common finding is that neuromuscular junctions are more sensitive than autonomic nerves or synaptosomes (compare 10, 38, 111, 252, and 306). These data could mean that the individual toxins have differing affinities for different cholinergic nerve endings.

The foregoing data suggest that cholinergic nerves have unique membrane determinants (viz. toxin receptors), or they have common determinants in unique microenvironments. This in turn implies that the toxins could be used as histological markers to localize cholinergic nerve endings, as affinity ligands to isolate cholinergic synaptosomes, and as ordinary ligands to extract and characterize membrane determinants. The only serious drawbacks to these proposals are the toxins themselves. The toxins have proved difficult to radiolabel and therefore have not been exploitable as ligands. Furthermore, the toxins are extremely potent, and this uncommon potency discourages many investigators from working with them. Difficult though they may seem, these obstacles can be overcome.

The study of toxin binding to membranes could be done in a way that poses little hazard to workers. The research that has been reviewed in the present article shows that the binding step and the poisoning step are pharmacologically separable. To the extent that research on other toxins, particularly tetanus toxin and diptheria toxin, is applicable to botulinum toxin, the binding and poisoning fragments are structurally separable. Should this prove true for botulinum toxin, the isolated binding fragment could be used to characterize cholinergic membranes and toxin receptors.

The difficulties that have been inherent in radiolabeling may also be solvable. Earlier attempts were initiated without knowledge of those portions of the molecule that must be left unperturbed to preserve biological activity. If the primary structure of the toxins were known, then presumed areas of importance could be established. The

areas (amino acids) thought not to be critical for biological activity would be chosen for modification during radiolabeling.

Turning once again to molecular biology, there is a methodology that can enhance efforts to obtain nontoxic binding fragments that could be radiolabeled. The methodology has the dual advantage of being easier than recombinant DNA research and of having an established precedent. In their study of phage-mediated diphtheria toxin production, Uchida et al. (286, 287) showed that phage mutants could be isolated that governed production of incomplete toxins. These substances were called "cross-reacting materials" (CRM), meaning that they were serologically related to the native toxin. CRM's were isolated that possessed binding activity without catalytic activity, and vice versa. There is no reason to doubt that mutants governing botulinum toxin production could be isolated and that some of these mutants would cause synthesis of incomplete toxins, i.e. molecules with binding but without poisoning effects. There is an especially strong motive for isolating botulinum toxin-like CRMs. Existing toxins afford only eight chances (nine counting tetanus toxin) that one molecule will be found with high affinity for a single and distinct class of cholinergic nerves. By isolating mutants, an investigator could call upon a virtually limitless source of testable ligands; among them will surely be substances that bind and that can be radiolabeled.

As mentioned above, the narrow goal of isolating the toxin receptor can be restated in terms of isolating membrane determinants that distinguish cholinergic nerves. The goal of isolating and characterizing the cellular process that is poisoned by the toxin can likewise be stated in broad terms. Neurobiologists would like to know whether the steps involved in excitation-secretion coupling are universal. In other words, do most chemically transmitting nerves use basically the same mechanism for storing and releasing neurotransmitters?

The most direct approach to determining whether there is commonality among nerves in the process of excitation-secretion coupling is to find a drug that is a universal uncoupler. Botulinum toxin does uncouple transmitter release at cholinergic nerves; its ability to uncouple the process at other nerves has not been firmly established. An apparent inability by the toxin to block transmitter release from noncholinergic nerves may be due to a lack of cell surface receptors. Were this to be the case, techniques arising from classical pharmacology and from molecular biology could be coalesced to solve the problem. To circumvent receptors, an isolated poisoning fragment (or a poisoning CRM) could be incubated with noncholinergic nerves at concentrations adequately high to allow for fluid phase pinocytosis, or the fragment could be injected directly into the cell. The former strategy is a mimicry of that already used with other pharmacological substances, such as acetylcholinesterase (224) and ATPase (227). The latter strategy has been used to introduce small molecules such as calcium into large nerve endings (196). To employ receptors, novel pharmacological substances could be synthesized that possess moieties that bind to nerves of choice; these moieties would be coupled to the poisoning fragment of botulinum toxin. (This strategy is discussed in the next section.) Whichever technique is used, a demonstration that the toxin has a universal effect, or even a semiuniversal effect, would argue for commonality of excitation-secretion coupling mechanisms. The reverse demonstration would support the reverse argument.

B. Molecular Biology and the Nerve Ending

Molecules on the cell surface or in the cell membrane are somewhat accessible to the machinations of scientists. Only occasionally are molecules in the cell interior accessible for study. The inherent difficulty in the latter situation is that the cell wall presents a barrier that must be penetrated. Unless one has a lipophilic agent that can diffuse across the cell membrane and attack the molecule of interest, study of an intracellular component can be problematic.

Neurobiologists, and especially those who study nerve endings, rely heavily on drugs that penetrate membranes. Attempts to analyze intranerve ending components are hindered when there are no substances that can reach and alter the intracellular components under investigation. Although neurobiologists have not developed many new techniques for promoting membrane penetration by drugs, scientists in other fields are developing techniques that can be borrowed and applied to the nervous system. Studies on cell deficiencies and cell neoplasms are particularly relevant.

When cells are genetically deficient in their ability to synthesize or store essential substances, retardation in cell growth or even cell death may ensue. A variety of procedures have been tested for promoting cell entry of deficient substances, but the use of liposomes has become somewhat popular (105). An illustration of this is the reported use of liposomes to introduce peroxidase into peroxidase-deficient phagocytes (298). Although the technique seems to work with phagocytes as well as some other cells, tissue targeting of liposomes has often been hard to achieve. Directing liposomes and their contents exclusively to cells of choice has been difficult.

In another area of investigation workers have tried to cause cell death by the introduction of exogenous substances. For persons who are interested in treating neoplastic diseases, there is a need to administer toxic agents that can locate and kill neoplastic cells. In response to this need, investigators have partially synthesized compounds that are called "educated cytotoxins" (218). Most of these cytotoxins have two components, one of which has high affinity for membrane determinants on target cells and the other of which has a poisoning effect that is lethal to target cells. There are many cytotoxins that can and have been tested, but the catalytic fragment of

diphtheria toxin has emerged as a favored choice. Tissue targeting of toxins has been achieved by various means and with varying success. As would be expected, drugs that bind to naturally occurring receptors have been used (37, 39), but often artificial approaches are more effective. Cytotoxins have been linked to antibodies directed against chemical antigens [e.g. dinitrophenyl hapten (202)] and biologically induced antigens [e.g. mumps virus (203); lactate dehydrogenase (235)]. More imaginatively, a cytotoxin has been enclosed within a virus coat and this novel assembly has been used to tissue target drugs (288).

There are powerful lessons that neurobiologists can learn from this work. To study the function of molecules inside the nerve ending, neurobiologists must design compounds that are tissue targeted for the nerve endings of interest. Ideally, the targeting moiety would be complexed with an active fragment that can inactivate a specific intracellular molecule. The goal would not be to cause abrupt and outright cell death, but rather to evoke observable changes in the behavior of otherwise viable cells.

For those who choose to study cholinergic nerve endings, botulinum toxin occupies a position of indisputable importance. The toxin, or more properly its binding fragment, is among the most tissue-selective drugs known to pharmacology. It will bind to cholinergic α-motoneurons at concentrations of 10^{-10} M and lower (38, 111, 252). This tissue specificity inevitably invites attempts to complex the binding fragment of botulinum toxin with pharmacologically active substances that have an intracellular substrate. The permutations of this scheme are endless, but some of the more promising come quickly to mind.

The binding fragment of one type of botulinum toxin could be linked to the poisoning fragment of other types, the point being to show the extent of homology in structure and function. More intriguingly, the binding fragment of botulinum toxin could be linked to the poisoning fragment of tetanus toxin, again to illustrate or decipher homology. Finally, the binding fragment of botulinum toxin could be linked to the catalytic fragment of diphtheria toxin or other cytotoxins. The partial synthesis of such compounds would result in innovative ways to produce cholinergic denervation. To date, no chimeric substances involving clostridial neurotoxins and nervous system targets have been described, but work on chimeric toxins with nonnervous system targets has been underway for years (214).

Another avenue of research might entail the use of botulinum toxin coupled with antibodies to intracellular antigens. Antibody studies on cell structure and function have a long history in pharmacology, but such work is normally confined to cells that are broken or sliced (12). This is due to the fact that antibodies, at least at reasonable concentrations, do not penetrate membranes. Nonbinding proteins can enter cells only by fluid phase pinocytosis, which is not an efficient entry mechanism. To achieve efficient entry, proteins must be subject to adsorptive pinosytosis for which membrane binding is an integral step (244). If antibodies to intracellular molecules could be complexed to the binding fragment of botulinum toxin, appreciable entry might be expected. Linking the binding fragment of botulinum toxin to antibodies against endogenous antigens could result in an entire class of novel pharmacological substances for the study of nerve-ending function.

Among the many substances that might be created, one specific compound deserves special notice. The treatment of botulism victims with botulinum antitoxin has no therapeutic value. The antitoxin can neutralize circulating toxin, but it cannot enter nerves to neutralize pharmacologically active toxin. Conceivably, a compound in which the binding fragment of botulinum toxin is linked to an antibody against the lytic fragment would have therapeutic utility. By means of adsorptive pinocytosis, the antilytic fragment might enter the nerve and arrest the action of native toxin.

The obverse of the experiments just proposed has already been alluded to. To determine whether various types of chemically transmitting nerves have a common mechanism for excitation-secreting coupling, the poisoning fragment of botulinum toxin could be linked to a molecule that recognizes the transmitting cell of interest. The identification of suitable molecules to tissue target the poisoning fragment may not be especially difficult. Most if not all nerve endings have receptors for transmitters and for modulators, and they have high affinity uptake systems for transmitters and transmitter metabolites. One or another of these receptors could bind the targeting moiety, after which adsorptive pinocytosis could lead to internalization of the poisoning fragment.

All of the ideas expressed thus far involve the use of naturally occurring substances. The potential usefulness of unnatural substances should not be overlooked. When the interaction between botulinum toxin and the cholinergic nerve ending becomes well understood, that knowledge should permit the design of ideal peptides or proteins that bind to nerve membranes. By virtue of knowing the desired amino acid sequence, investigators could synthesize a coding gene. When implanted into the proper host, this gene would promote large scale synthesis of the neuropharmacological agent being sought. This would be an ambitious project, but it is one that has been successfully accomplished in other areas of investigation (142). Neurobiologists, particularly those interested in botulinum toxin and the nerve ending, must begin to adopt these techniques and apply them to the study of the nervous system.

X. Concluding Remarks

There is a sense among researchers that a full description of the mechanism of action of botulinum toxin will be shortly forthcoming. If so, that event will mark a stage

of transition. Research in the past has focused on the toxin, and the nerve ending has merely served as a convenient tissue on which to do experiments. When the mechanism of toxin action is fully established a different perspective will be adopted. Research will concentrate on the nerve ending, and the toxin will become a powerful pharmacological tool. When the toxin ceases to be viewed as a poorly understood drug and instead is viewed as a valuable pharmacological tool, that will truly be a point of transition.

The work recently done by biochemists and pharmacologists has permitted a close look at the structure and function of the botulinum toxin molecule. Protein chemists have shown that the toxin is a dichain molecule that can be fragmented into two major components. Neuropharmacologists have shown that the toxin interacts with cholinergic nerves in a way that involves two major steps, an extracellular binding step and an intracellular paralytic step. When seen in the context of findings on other bacterial toxins, these data suggest that one toxin chain mediates tissue binding and the other chain causes cellular dysfunction.

The prospect of having a molecule with separable binding and paralytic fragments is potentially quite exciting for neurobiologists. The binding fragment could be linked to an assortment of pharmacologically active substances, and the products obtained would represent a novel class of drugs that are tissue-targeted for cholinergic nerves. Conversely, the paralytic fragment could be used to help unravel the excitation-secretion coupling process in chemically transmitting nerves. Should these prospects be realized, they would be a fitting culmination to decades of research on botulinum toxin.

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