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STUDY OF TOXIC AND ANTIGENIC STRUCTURES
OF BOTULINUM NEUROTOXINS

Annual and Final Report

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February 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-80-C-0100

University of Wisconsin
Madison, Wisconsin 53706

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
	AD 7150 GVL	
4. TITLE (and Subtitle)		5. TYPE OF REPORT & PERIOD COVERED
Study of Toxic and Antigenic Structures of Botulinum Neurotoxins		Annual (1 Feb 84-31 Aug 84) Final (1 Aug 80-31 Aug 84)
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s)		8. CONTRACT OR GRANT NUMBER(s)
B. R. DasGupta		DAMD17-80--C-0100
9. PERFORMING ORGANIZATION NAME AND ADDRESS		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
University of Wisconsin Madison, Wisconsin 53706		62770A.3M162770A871.AB.067
11. CONTROLLING OFFICE NAME AND ADDRESS		12. REPORT DATE
U.S. Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701-5012		February 1985
		13. NUMBER OF PAGES
		14
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report)
		Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
15. DISTRIBUTION STATEMENT (of this Report)		
Approved for public release; distribution unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number)		
Botulinum neurotoxin, heavy chain, light chain, subunit chains, amino acid composition, amino acid sequence, structure-function relation, selective modification, mechanism of action, purification, toxicity, antigenicity, toxoid, neuromuscular junction.		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number)		
Amino acid compositions of botulinum neurotoxin (NT) types A,B, E and F were determined. The heavy and light chains of types A,B and E NT were separated, purified, and analyzed for amino acid compositions and partial N-terminal sequence. Selective modification of tyr, his and arg residues and also carboxyl and amino groups of types A and E NT demonstrated the role of these amino acid residues in toxicity and antigenicity. Type E NT completely detoxified following modification of his, tyr or amino groups has potential as a toxoid. Effects of types A and B NT on rat neuromuscular junctions appear non-identical.		

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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SUMMARY

The two broad goals of our research are to 1) determine the precise structure of botulinum neurotoxin (NT), and 2) establish the relationship between the structure and biological activities of the NT (namely its neurotoxicity and antigenicity). We have also initiated studies on the mechanism of its action.

I. Structure:

Amino Acid Composition: We have completed a rigorous examination of the amino acid composition of the NT types A, B, E and F.

These immunologically distinct NT types, now defined in terms of amino acid composition, can for the first time be compared on a chemical basis.

The heavy and light chains (mol. wt. ~100,000 and ~50,000, respectively) derived from types A, B and E NT were separated, purified and analyzed for amino acid composition. Sum of amino acid residues of the heavy and light chains of each of the three NT types agreed very well with the corresponding residues of a parent dichain. This affirms, for the first time on a chemical basis, that these NT types are composed of two chains.

Amino acid sequences of types A, B and E NT: The first 10-21 residues of the N-terminal of the heavy and light chains of types A, B and E NT and the single chain type B and E NT were determined.

Separation and isolation of subunit chains and fragments: Dependable ion exchange chromatographic procedures were developed to isolate the heavy and light chains of types A, B and E. Also, cleavage at the cysteine, methionine, arginine and lysine residues have yielded identifiable fragments.

II. Structure-Function Relationship: Selective modification of tyrosine, histidine and arginine residues and also carboxyl and amino groups of types A and E NT demonstrated role of these amino acid residues in toxicity and antigenicity.

III. Second Generation Immunogen (Toxoid): The type E NT completely detoxified by selective modification of histidine, tyrosine or amino groups appears to have the potential to be used as toxoid. Compared to the first generation toxoid (crude preparation of the NT detoxified with formalin), the second generation toxoid has several noteworthy features.

IV. Mechanism of Action: We have found different effects of types A and B botulinum NT on transmitter release at the rat neuromuscular junction. Both toxins decrease the frequency of miniature endplate potentials but type A did so to a greater extent. For example, at 3 days after toxin injection nerve-impulse-evoked transmitter release was reduced more for type A treated muscles than for type B. However, 3,4-diaminopyridine, an agent which increases nerve-evoked transmitter release by increasing Ca^{++} influx, was more effective in reversing the paralysis in type A than in type B-treated muscles. The results show that type B NT differs from type A, mainly by a shorter duration of action and by being less effectively antagonized by 3,4-diaminopyridine. These findings have implications for the medical treatment of the types A and B botulism.

I. Structure

Until now botulinum neurotoxin types could not be defined in molecular terms. Some of these proteins are now precisely defined in terms of amino acid compositions and partial sequence.

Amino acid composition: To develop reliable data on the amino acid composition of botulinum neurotoxin (NT) types A, B, E and F we had planned to analyze three batches of each of the NT types. Each batch of a NT type was to be isolated from a separate toxin producing bacterial culture (using an inoculum from two laboratories, if available), and the NT was to be purified by at least two different methods, if available. Compared to triplicate analysis of a single batch of a NT type our approach is a more rigorous examination of (i) the amino acid composition of the NT, (ii) consistency of the purified NT, independent of source of bacterial culture inoculum and method of purification.

Type A: This study was completed (12).
 Type B: This study was completed (11).
 Type E: This study was completed (4).
 Type F: This study was completed (5).

Summary: (i) We found that each of the three NT types (A, B, and F) purified by two methods was comparable in purity and similar in amino acid compositions. Type E was purified by one available method. None of these NT types purified by two methods (developed independently in two laboratories) was previously compared under identical examination conditions. This observation, seemingly trivial, is indeed significant because the literature on botulinum NT includes claims of purification and characterization that are irreproducible and enigmatic.

(ii) The immunologically distinct botulinum NT types A, B, E and F are now defined in terms of amino acid composition. This allows, for the first time, a chemical basis to compare the NT types. Similarity between types A and E is closer than between any other two types. Five amino acids do not differ by more than 1 residue; e.g. number of residues in Type A/Type E are Thr 75/75; Pro 44/45; Tyr 71/70; His 14/15; Trp 17/16. The next best resemblance by this criteria is between types A and B; types A and F; as well as types E and C. In each pair 3 amino acids do not differ by more than 1 residue.

Our planned work on separation and purification of the H and L chains (mol. wt. ~100,000 and ~50,000, respectively) derived from types A, B and E botulinum neurotoxin (NT) and determination of the amino acid compositions of the subunit chains was completed following submission of a manuscript (20). Highlights of this major project are as follows:

1. This is the first report on the separation and purification of the H and L chains following controlled tryptic cleavage of the single chain type E NT.
2. This is the first report on ion exchange chromatographic purification of the two chains of type B NT.

3. Sum of amino acid residues of the H and L chains of a NT agreed very well with the corresponding residues of the parent dichain NT. This affirms, for the first time on a chemical basis, that the NT is composed of two chains.
4. This study also a wed for the first time a chemical basis to compare the H and L chains of (i) a NT type, and (ii) among NT types A, B and E. More similarity was found among the L chains than among the H chains of the three NT types.
5. An unexpected minor discovery with probable implication on the genetics of NT: Comparison of the number of amino acid residues between the H and L chains of each of the three NT types revealed that several amino acids in an H chain are double the number of corresponding residues of the L chain (e.g. number of serine residues in the H and L chains of type A are 55 and 28, respectively). Viewed in another way it appears that if the H chain was not double in mol. wt. of the L chain, but was equal, the H and L chains would have similar amino acid composition with respect to 6-9 amino acids. This may indicate that botulinum NT (mol. wt. 150,000) has arisen by gene duplication events involving an ancestral gene coding for 50,000 mol. wt. protein that now produces three different but related domains, one in L-chain (mol. wt. 50,000) and two in H chain (mol. wt. 100,000) - an idea originally proposed for tetanus neurotoxin by Taylor, C. F. et al. (Biochem. J. 209, 897, 1983).

Summary: (i) Knowledge of amino acid composition will enable us to plan enzymatic and chemical fragmentation of these subunit chains (e.g. number of Lys and Arg for tryptic cleavage, Met for CNBr cleavage). (ii) Quantitative comparison of the amino acid composition by the technique of Cornish-Bowden [J. Theor. Biol. 76, 369, (1979) and Methods Enzymol. 91, 60 (1983)], would allow to see what the chances are that similarities in the composition reflect similarities in sequence. Such analysis has revealed a very intriguing sequence homology between the L- and H chains of tetanus toxin [Taylor, C. F. et. al. Biochem. J. 209, 897 (1983)].

Amino acid sequences of type A, B and E NT: We have determined sequence of the first 10-21 residues of the N-terminal of:

Type A	Light chain	(mol. wt. 53,000)
	Heavy chain	(mol. wt. 97,000)
Type B	Light chain	(mol. wt. 51,000)
	Heavy chain	(mol. wt. 104,000)
Type E	Light chain	(mol. wt. 50,000)
	Heavy chain	(mol. wt. 102,000)
Type B	Single chain	(mol. wt. 152,000) Unnicked
Type E	Single chain	(mol. wt. 147,000) Unnicked

These sequences (10,14,15,16,17,18,19) are presented in Fig. 1. Alignment of the N-terminal end of the L-chains of type A, B, and E, reveals extensive sequence homology. The same is also evident in the case of H chains of types A, B and E. This allows for the first time, at

Partial Amino Acid Sequence of Botulinum NT Types A, B, and E
(comparison with tetanus NT)

	1	5	10	15	20	
A, Single Chain L-Chain	not yet analyzed					
		<u>Pro</u> • <u>Phe</u> • <u>Val</u> • <u>Asn</u> • <u>Lys</u> • <u>Gln</u> • <u>Phe</u> • <u>Asn</u> • <u>Tyr</u> • <u>Lys</u> • <u>Asp</u> • <u>Pro</u> • <u>Val</u> • <u>Asn</u> • <u>Gly</u> • <u>Val</u> • <u>Asp</u>				ref. (i)
B, Single Chain L-Chain		<u>Pro</u> • <u>Val</u> • <u>Thr</u> • <u>Ile</u> • <u>Asn</u> • <u>Asn</u> • <u>Phe</u> • <u>Asn</u> • <u>Tyr</u> • <u>Asn</u> • <u>Asp</u> • <u>Pro</u> • <u>Ile</u> • <u>Asp</u> • <u>Asn</u>				(ii)
		<u>Pro</u> • <u>Val</u> • <u>Thr</u> • <u>Ile</u> • <u>Asn</u> • <u>Asn</u> • <u>Phe</u> • <u>Asn</u> • <u>Tyr</u> • <u>Asn</u> • <u>Asp</u> • <u>Pro</u> • <u>Ile</u> • <u>Asp</u> • <u>Asn</u> • <u>Asn</u>				(ii)
E, Single Chain L-Chain		<u>Pro</u> • <u>-----</u> • <u>Lys</u> • <u>Ile</u> • <u>Asn</u> • <u>Ser</u> • <u>Phe</u> • <u>Asn</u> • <u>Tyr</u> • <u>Asn</u> • <u>Asp</u> • <u>?</u> • <u>Val</u> • <u>Asn</u>				(iii)
		<u>Pro</u> • <u>-----</u> • <u>Lys</u> • <u>Ile</u> • <u>Asn</u> • <u>Ser</u> • <u>Phe</u> • <u>Asn</u> • <u>Tyr</u> • <u>Asn</u> • <u>Asp</u> • <u>Pro</u> • <u>Val</u> • <u>Asn</u> • <u>Asp</u> • <u>Arg</u> • <u>Thr</u> • <u>Ile</u> • <u>Leu</u> • <u>Tyr</u> • <u>Ile</u>				(iv)
Tetanus, Single Chain		<u>Pro</u> • <u>Ile</u> • <u>Lys</u> • <u>Leu</u> • <u>Asn</u> • <u>Phe</u> • <u>Arg</u> • <u>Tyr</u> • <u>Trp</u> • <u>Leu</u> • <u>Trp</u> • <u>Val</u> • <u>Gly</u> • <u>Phe</u>				(v)
A, H-Chain		<u>Ala</u> • <u>Leu</u> • <u>Asn</u> • <u>Asp</u> • <u>Leu</u> • <u>Cys</u> • <u>Ile</u> • <u>Lys</u> • <u>Val</u> • <u>Asn</u>				(i)
B, H-Chain		<u>Ala</u> • <u>-----</u> • <u>Pro</u> • <u>Gly</u> • <u>Ile</u> • <u>Cys</u> • <u>Ile</u> • <u>Asp</u> • <u>Val</u> • <u>Asp</u> • <u>Asn</u> • <u>Glu</u> • <u>Asp</u> • <u>Leu</u> • <u>Phe</u> • <u>Phe</u> • <u>Ile</u> • <u>Ala</u> • <u>Asp</u>				(ii)
E, H-Chain		<u>Lys</u> • <u>Ser</u> • <u>Ile</u> • <u>Cys</u> • <u>Ile</u> • <u>Glu</u> • <u>Ile</u> • <u>Asn</u> • <u>Asn</u> • <u>Gly</u> • <u>Glu</u> • <u>Leu</u> • <u>Phe</u>				(iv)

Fig. 1

ref. i) Schmidt, J. J., Sathyamoorthy, V. & DasGupta, B. R. *Biochem. Biophys. Res. Comm.* 119, 900 (1984)
 ii) Schmidt, J. J., Sathyamoorthy, V. & DasGupta, B. R. *Fed. Proc.* 43, 1954 (1984)
 iii) Sathyamoorthy, V., Schmidt, J. J. & DasGupta, B. R. *Fed. Proc.* 43, 1954 (1984)
 iv) Sathyamoorthy, V. & DasGupta, B. R. *Biochemistry* 23, 3361 (1984)
 v) Robinson, J. P. & Hash, J. H. *Mol. Cell. Biochem.* 48, 33 (1982)

this molecular level, comparison of the immunologically distinct and pharmacologically similar botulinum NT types A, B, and E. Significant sequence homology between tetanus neurotoxin (data from Robinson, J. P. and Hash, J. H. *Molec. Cell Biochem.* 48, 33, 1982) and botulinum NT is also evident.

Separation and isolation of subunit chains: We have developed dependable ion exchange chromatographic procedures to isolate the heavy and light chains of type B and E NT. The isolated chains appear pure in SDS-polyacrylamide gel electrophoresis. Two major problems that had to be solved were: (i) Type E NT (single chain) is first converted to the dichain form by cleaving a single peptide bond with trypsin. During this step secondary cleavages produce, in minor quantity, fragments other than the H and L chains; these had to be removed. (ii) Type B NT is isolated as a mixture of dichain (made of the H- and L chains) and its precursor, the single chain molecule. A method had to be developed to isolate the two subunit chains from the single chain molecule. The heavy (H, mol. wt. 97,000) and light (L, mol. wt. 53,000) chains of type A neurotoxin (mol. wt. 145,000) were separated according to a published method following reductive cleavage of the -S-S- bond(s) holding them together. But the isolated chains, particularly the H chain, remains contaminated with the dichain and single chain neurotoxin. (The purified type A neurotoxin is a mixture of nicked and unnicked molecules of identical mol. wt.). We developed a procedure to isolate the H chain from its parent single chain neurotoxin (20).

The amino acid sequence studies demonstrated convincingly the high degree of purity of the two chains as prepared in our laboratory. Until now "single band" in PAGE-SDS was our best criteria of purity.

II. Structure-Function Relationship

Role of tyrosine residues: Type A and E NT were treated with tetranitromethane at pH 7.9, reagent to protein molar ratio 50, 100 or 500:1. At each of the three levels of the reagent used (i) amino acid analysis of HCl hydrolysate showed reagent dose dependent formation of nitrotyrosine with loss of tyrosine, and modification of no other amino acids, (ii) serological reactivity and toxicity of type A were altered, although detoxification was not complete, and (iii) serological reactivity of type E was not damaged. Type E was completely detoxified at the highest reagent level. We deduce that tyrosine residue(s) are critical for toxigenic structures of type A and E NT and have different roles in the serological reactivities of these two types, of consequence for type A but not important for type E. Type E NT, completely detoxified by tyrosine modification, has potential as a toxoid (22).

Role of carboxyl groups: Type A and E NT were derivatized with water soluble carbodiimide in presence of norleucine methyl ester. The derivatized protein was acid hydrolyzed for amino acid composition to determine the number of carboxyl groups derivatized. Modification at three different reagent to protein concentration ratios yielded three incremental levels of derivatization and detoxification. No amino acid residues other than Asp and Glu were modified. Detoxification was not

complete and serological reactivity was damaged little, even after modification of 9 and 5 carboxyl groups in types A and E, respectively. Modification of 3 carboxyl groups of type A or E caused ~95% detoxification in both, but modification of 1 residue in type E did very little damage (21).

Two abstracts on these two studies have been submitted for presentation of these studies at the Am. Soc. for Microbiology's annual meeting in March 1985.

Further bench work on these two projects, at this phase of the work, was completed just recently. We expect to complete analysis of the recent data and prepare manuscripts for publication in reviewed journals in the next 2-3 months.

Role of histidine residues: First phase of the studies on the role of histidine residues in the toxicity and antigenicity of types A and E NT was completed (9,13). Histidine residues of type E NT that are critical for toxicity are not important for serological activity and immunogenicity. In type A NT the situation is somewhat different; modification of histidine residues did not completely detoxify the NT, but produced some damage in its serological reactivity. The completely detoxified type E NT was used as an immunogen. Rabbits immunized with the toxoid (second generation immunogen, i.e., pure NT detoxified by selective modification of one kind of amino acid residues) produced antiserum that neutralized the toxin.

Role of amino groups: Selective modification of the lysine residues (and α -NH₂) of types A and E NT, with 2-methoxy-5-nitropropene, demonstrated that (i) type E NT could be completely detoxified without causing detectable damage to its serological reactivity, whereas (ii) type A NT could retain more than 10% of its original toxicity with severely diminished serological reactivity. These results indicate that lysine residues and/or the α -NH₂ group are (i) critical for the toxigenic structures of type A and E NT and (ii) have different roles in the serological reactivities of the two types, important for type A and apparently not important for type E (1).

Role of arginine residues: In dose-response experiments on the isolated neuromuscular junction (phrenic nerve hemidiaphragm preparation), the dichain type E NT was about 100 fold more potent than the single chain NT. This is the first demonstration of trypsin induced activation of type E using neuromuscular junction preparation. The single chain (unactivated type E NT, when modified at the Arg residues with cyclohexanedione, became refractory to the ability of trypsin to nick (conversion of the single chain to the dichain form) and activate the NT. Also the Arg specific reagent diminished the neuromuscular activity of dichain NT making it evident that at least one Arg residue is involved in maintaining toxigenic structure (6).

III. Second Generation Immunogen (Toxoid)

The type E neurotoxin completely detoxified by histidine modification appears to have the potential to be used as toxoid. Rabbits immunized with such a preparation produce antiserum that neutralizes the toxin. This study was completed (9,13). It is reasonable to expect that second generation toxoids can be produced by two other methods; by selective modification of amino groups (1) or tyrosine residues (22) of type E NT. In each case complete detoxification was achieved without detectable loss in serological activity.

Enzymatic and chemical fragmentation of NT: Through tryptic digestion and chromatography we have isolated a large fragment of the type A neurotoxin. The fragment (mol. wt. ~100,000) is composed of the L chain (mol. wt. 53,000) and approximately one half of the H chain, the two being linked by a -S-S- bond (probably the -S-S- that holds the L and H chain in the dichain neurotoxin of 145,000 mol. wt.). The fragment of the H chain was also isolated in essentially pure form (PAGE-SDS). It is of mol. wt. ~47,000 and serologically reactive to the antineurotoxin serum (reaction of partial identity in Ouchterlony plate). Therefore at least one antigenic determinant of the neurotoxin appears to be located on this half of the H chain. Cleavage of cysteine and half cystine residues of type A and B NT with 2-nitro-5-thiocyanobenzate, under described conditions (2) had yielded 8 fragments (mol. wt. range 93,000-13,800) and 11 fragments (mol. wt. range 93,500-16,600), respectively. CNBr cleaved the H or L chains (type A NT) at Met residues into 8-9 fragments. Higher yields of the fragments were of mol. wt. 30 and <28K from H chain; 8, 17 and 10K from L chain.

Neurotoxicity of types A, B and E studied by methods other than mouse lethality assay: In the mouse lethality assay, death is a distant effect of the neurotoxin's singular action, blockage of acetylcholine release. The LD₅₀ is a measure of lethality, and indirectly of neurotoxicity. LD₅₀ is therefore inadequate to determine the similarities and differences in the neurotoxicities of various NT types. A better parameter of neurotoxicity should be a direct function of, and more proximate to, the effect of inhibition of neurotransmitter release. Therefore, we (in collaboration with Dr. L. C. Sellin and Prof. S. Thesleff) compared the neurotoxicities of types A vs. E, and A vs. B based on certain electrophysiological parameters. Such comparative studies of any two purified NT types were not reported before.

Type A vs. E NT: (7). Local blockade of transmitter release was produced by s.c. injection of pure NT types A or E above the tibialis anterior muscle of adult male rats. Extensor digitorum longus nerve-muscle preparation was examined for toxin induced alterations in single twitch and tetanic tension (in situ) or transmitter release (in vitro). For both single twitch and tetanic tension, muscle treated with low (56 LD₅₀) doses of type E recovered from initial partial paralysis or full paralysis (induced with 565 LD₅₀) 7 days after NT injection, while those treated with only 5 LD₅₀ of type A remained either fully or partially paralyzed through 10 days. Also, miniature end plate potential frequency and mean quantal content were reduced for a longer period of

time/or to a greater extent of muscles treated with type A (3 LD₅₀) than those treated with type E (20-282 LD₅₀). The type E used in this study was activated with trypsin. We concluded that 1) type E is less potent than type A, (consistent with the known higher specific toxicity i.e. LD₅₀/mg protein for type A than type E), and 11) type E is shorter in its duration of action than type A.

This and the accompanying study, noted below, are relevant to clinical observations made from a number of case studies; botulism from type A is more severe than type B, patients with type A needed more ventilatory support and were hospitalized longer.

Type A vs. B NT: (8). This study, done after comparing A vs. E, showed more interesting quantitative and qualitative differences between the effects of A and B NT. Blockade of neuromuscular transmission was produced in the lower hind limb of the rat by local injection of equipotent amounts (mouse LD₅₀) of type A or B NT. At 1, 3, 5 and 7 days after injection, the extensor digitorum longus nerve-muscle preparation was excised and analyzed in vitro for alterations in spontaneous and nerve stimulus-evoked quantal transmitter release. Type A NT treated muscles remained paralyzed up to 7 days. Muscles treated with type B NT although completely paralyzed at 1 and 3 days, twitched in response to nerve stimulation at 5 and 7 days after injection. Both NT types reduced spontaneous and nerve impulse quantal release; type A reduced more than type B, despite the fact that mouse i.p. lethality are similar (~10⁸ LD₅₀/mg neurotoxin) for type A and B. Unexpectedly, the drug 3,4-diaminopyridine, which increases nerve evoked transmitter release by Ca⁺⁺ influx, was more effective in reversing the paralysis in type A than in type B treated muscles. Although type B and type A NT are similar in mouse lethality assay (~10⁸ LD₅₀/mg protein), we concluded that type B differs from type A mainly by a shorter duration of action, and by being less effectively antagonized by 3,4-diaminopyridine. The efficacy of aminopyridines in the therapy of botulism would appear to depend on the NT type implicated.

An application (funding of new instruments) was prepared and submitted to DOD-University Research Instrumentation Program for three instruments (office of Naval Research, Arlington, VA 22217). Award was made (contract #DAAG 29-83-G-0063).

Another contract DAMD17-83-C-3034 "Exploration of binding and toxic site of botulinum neurotoxin" depends entirely on DAMD 17-80-C-0100 for biochemical support. Unactivated and activated (trypsinized) type E NT, type A NT and the heavy chain isolated from type A was provided to the "Exploration of binding and toxic site..." project. The project has yielded very encouraging and novel results (which will be reported separately as part of DAMD17-83-C-3034 report).

Collaborative investigations with USAMRIID investigators, Dr. L. C. Sellin and Dr. J. J. Schmidt: In collaboration with Dr. Sellin comparisons of the neurotoxicity of types A vs. B as well as A vs. E neurotoxins were made based on certain electrophysiological parameters (7,8). Collaborative investigations with Dr. Schmidt produced the first amino acid sequence determinations of botulinum NT (10,14,15,16,18).

Publications Supported by Contract DAMD17-80-C-0100

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