

AD \_\_\_\_\_

CONTRACT NO: DAMD17-93-C-3118

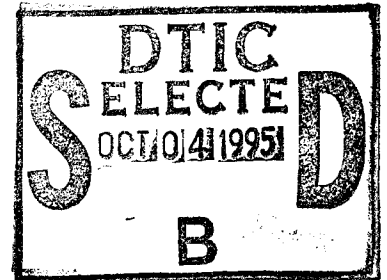
TITLE: Three-Dimensional Structure Determination of Botulinum Neurotoxin

PRINCIPAL INVESTIGATOR(S): Raymond C. Stevens, Ph.D.

CONTRACTING ORGANIZATION: University of California at Berkeley  
Berkeley, California 94720-1460

REPORT DATE: August 1, 1995

TYPE OF REPORT: Annual



PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;  
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

DTIC QUALITY INSPECTED 5

19951003 033

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> August 1, 1995	<b>3. REPORT TYPE AND DATES COVERED</b> Annual (August 1, 1994 - July 31, 1995)	
<b>4. TITLE AND SUBTITLE</b> Three-Dimensional Structure Determination of Botulinum Toxin			<b>5. FUNDING NUMBERS</b> DAMD17-93-C-3118	
<b>6. AUTHOR(S)</b> Ray C. Stevens, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of California at Berkeley Berkeley, California 94720-1460			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>  44123-2799	
<b>9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING/MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for public release; distribution unlimited			<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 words)</b> The immediate goals on structure and function relationship studies of botulinum neurotoxin are: 1) Determine the three-dimensional structures of botulinum neurotoxins isolated Heavy chain, Light chain and holo-neurotoxin at atomic resolution by x-ray crystallography. 2) Based on the structure of the isolated chains and holo-neurotoxin, understand the toxins mechanism of action. (1) In our continuing efforts to stabilize BT serotype A, we have determined that 1mM methionine as an anti-oxidant used from the initial stages of protein purification to protein storage, greatly increases the production of BT and increases the shelf-life of BT as determined by enzymatic assays and crystal growth experiments. (2) We have screened over 150 data sets for suitable additives (sugars, metals, buffers, salts) to improve the quality of the protein crystals and "lock" the protein into one conformation. (3) A large number of data sets have been collected at various wavelengths to optimize anomalous scattering and minimize problems due to non-isomorphism. (4) Suitable heavy atom derivatives are being located with Patterson methods. (5) We have determined optimal conditions for enzymatic assay with a 17mer peptide that mimics the 17 terminal amino acids to SNAP25. Using the enzymatic assay, a potential inhibitor has been synthesized to inhibit the proteolytic activity and stabilize the protein crystals into a single conformation. (6) A binding assay has been developed to determine the quality of the protein using surface plasmon resonance and locate inhibitors for the binding event.				
<b>14. SUBJECT TERMS</b> x-ray crystallography, 3-D structure, botulinum neurotoxin			<b>15. NUMBER OF PAGES</b> 19	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

RS Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, 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 Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

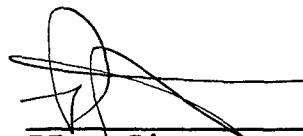
RS For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

RS In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

RS In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

RS In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Accession For	
NTIS GRA&I	<input checked="" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By _____	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	

 8/29/95  
\_\_\_\_\_  
PI - Signature Date

Three-Dimensional Structure Determination of Botulinum Neurotoxin  
Contract DAMD 17-93-C-3113

TABLE OF CONTENTS

FRONT COVER	
REPORT DOCUMENTATION PAGE	ii
FOREWORD	iii
TABLE OF CONTENTS	iv
INTRODUCTION	1
Nature of the Problem	1
Background of Previous Work	1
Purpose of the Present Work	2
Methods of Approach	2
BODY	
Experimental Methods	3
Relationship to Goals of Research	6
CONCLUSION	6
CRYSTAL DATA COLLECTION TABLE	7
APPENDIX	
Reporting Letter From Sub Contractor Univ. Wisconsin	A1
Updated Emergency Response Plan - Univ. Wisconsin, Madison	B1
Updated Emergency Response Plan - Univ. California, Berkeley	C1

## INTRODUCTION

### Nature of Problem

To determine the 3-dimensional structure of botulinum neurotoxins and their isolated domains. The following specifications are listed in the contract section C - Statement of Work:

Crystallization of the 150 kD holo-botulinum neurotoxins. Serotype A has been crystallized previously and crystallization conditions will be refined as necessary. Serotype B will be crystallized.

Determination of the 3-dimensional structure of serotype A. Heavy atom derivative screening is underway. Once 2 or 3 "acceptable" derivatives are known, data collection and processing of native and derivative data will be completed. The phases of the x-ray diffraction pattern will be phased, electron density maps calculated, and the structure will be determined.

Given the structure of serotype A, the technique of molecular replacement will be used to determine the structure of serotype B.

Crystallization of Heavy chain neurotoxin.

Determination of the 3-dimensional structure of the isolated heavy chain. Molecular replacement using the structure of the intact holo-neurotoxin if the structure has not changed substantially. If not, isomorphous replacement will be used.

Crystallization of Light chain neurotoxin.

Determination of the 3-dimensional structure of the isolated light chain. Molecular replacement using the structure of the intact holo-neurotoxin if the structure has not changed substantially. If not, isomorphous replacement will be used.

Similar studies on other serotypes.

### Background of Previous Work

Before funding of the contracted work for the structure determination of botulinum neurotoxin serotype A, we determined crystallization conditions of botulinum neurotoxin serotype A (Stevens et al, *J. Mol. Biol.* **222**, 877 (1991)) and found the most suitable crystal form of serotype A for X-ray analysis are bipyramidal shaped crystals that crystallize in the hexagonal space group P321 (or P3221) with one dimer per asymmetric unit. The unit cell dimensions are  $a = b = 170.5 \text{ \AA}$ ,  $c = 161.7 \text{ \AA}$ . The crystals are composed of approximately 50% solvent/50% protein and diffract to  $3 \text{ \AA}$  resolution. Native and derivative data sets to  $3.0 \text{ \AA}$  resolution were collected in house (Room 405 Stanley Hall) at the University of

California, Berkeley using a shared (with 3 other research groups) Rigaku RU-200 rotating anode generator and R-axis Image Plate area detector system.

During year 1 of the contract, we reported that we had worked out the conditions to further purify the protein (isoelectric focusing and aggregation), stabilize the protein (zinc acetate, proteolytic inhibitors, ganglioside), and improve the crystal quality of the neurotoxin protein. We very carefully worked out condition to collect complete, accurate, and non-deteriorating x-ray diffraction data use flash freezing techniques to freeze the protein crystals at  $-170^{\circ}\text{C}$  during x-ray irradiation. We collected a large amount of both native (7 sets) and heavy atom derivative data (20 sets) that is used to phase the x-ray diffraction pattern to yield the 3-dimensional structure of the neurotoxin. As a feasibility study, we initiated crystallization trials of serotype B and we have obtained single crystals (completion of Contract Goal C2).

### Purpose of Present Work

To continue the contracted work on the 3-dimensional structure determination of botulinum neurotoxin and understand the toxins structure, function, and mechanism of action. Specifically we will locate the heavy atom positions of data collected and attempt to phase the diffraction pattern to allow us to trace the electron density of the protein molecule. We will also need to collect more x-ray data as we continue to improve our model of the protein structure.

### Methods of Approach

Using the technique of multiple isomorphous replacement, the 3-dimensional structure of botulinum neurotoxin will be solved using diffraction data from native and heavy atom derivative data sets that are collected in house or at synchrotron facilities. The technique requires one to:

- 1) Bind heavy atoms to derivitize the protein crystal and locate the position of the heavy atom using Patterson methods. The derivitized protein crystals must be isomorphous with the native protein crystals (except for the heavy atom itself) for the information to be interpretable.

- 2) Once the position of the heavy atoms have been located, approximate phases can be calculated to allow one to observe the electron density of the protein molecule. The program HEAVY (Terwilliger, 1981) and the CCP4 program suite (CCP4, 1979, The SERC (UK) Collaborative Computing Project No. 4, a Suite of Programs for Protein Crystallography, distributed from Daresbury Laboratory, Warrington, WA4 4AD, UK) will be used to combine and phase heavy atom derivative data. All computations will be conducted on a Silicon Graphics INDIGO graphics workstation.

- 3) Fitting of electron density will be made on a Silicon Graphics INDIGO graphics workstation using the program TOM and O (version 5; Alwyn Jones, 1990).

4) Upon completion of fitting the electron density, the experimental model will be refined using the program XPLOR (version 3.0; Brunger, 1992) installed on a Silicon Graphics INDIGO graphics workstation.

An alternative to isomorphous replacement is the method of molecular replacement which depends on the presence of related structures in different crystals. Proteins which are homologous and have closely similar structures are particularly useful. The near identity of the structures implies relations between different structure amplitudes and phases which are helpful in solving phase problems. Serotypes A, B and E are similar as well as dissimilar (pharmacologically similar, antigenically different). Hence, once a structure is obtained for one serotype, the analysis of crystals of other serotypes can be aided by the molecular replacement technique. This is based on the assumption that the overall tertiary structure of all three serotypes are similar. If this is not the case, then a search for heavy atom derivatives will have to be conducted for all three serotypes.

BODY

### Experimental Methods

Although botulinum neurotoxin serotype A crystallizes quite easily, the ability to collect x-ray diffraction data on protein crystals that are all "locked" into the same structure inside the crystal has become a hurdle in our efforts to determine the 3-dimensional structure. In layman's terms, botulinum neurotoxin molecules have the ability to conform to several different conformational structures caused by "floppy" or disordered regions of protein structure. This problem makes it difficult to merge together native data sets as well as heavy atom derivative data sets. This problem makes it difficult to use the techniques of isomorphous replacement with heavy atom derivatives since one data set is not similar enough to the next. To avoid this problem we have taken several different measures that are described below. These approaches have proven successful to other researchers that have encountered this problem. The problem of non-isomorphism is not uncommon in crystal structure determinations, particularly for neurotoxins (i.e. crystal structure determinations for cholera toxin, diphtheria toxin, pertussis toxin).

We have also been working with the DasGupta lab to stabilize the protein at the earliest possible stage by adding certain reagents that minimize heterogeneity in the protein sample. During the past year the DasGupta lab has supplied us with 197 milligrams of purified botulinum neurotoxin serotype A and 285 milligrams of type A complex with the heamagglutinin complex still intact. We have used this material to purify BT in house and to attempt to use protein that is even "fresher" than before in order to minimize aging effects that also add to the heterogeneity issue. We have noted that protein stored for a few days appears to be more difficult to crystallize than protein very recently purified (few days). Below is a list of experiments that have been conducted in the past year:

#### 1) Improved Stability of Botulinum Neurotoxin

Because the quality and stability of protein is critical to the success of this project, we have attempted several different conditions to stabilize the protein at the earliest possible stage. Of particular importance is the addition of 1 mM methionine to the protein purification and for the storage of the protein sample. By adding 1 mM methionine as an anti-oxidant, the protein molecule is protected against oxidizing agents that are present during protein purification. The addition of 1 mM methionine extends the shelf life of the neurotoxin for weeks instead of days based on experiments at the molecular level such as crystal growth, proteolysis assays, and binding assays. Based on assays developed in-house, protein that is stored for extended periods of time are not as efficient as protein stored for a few weeks (i.e. the protein is not as efficient as fresh/new protein at proteolysis of a 17 mer peptide that resembles the C-terminus of SNAP25).

#### 2) Neurotoxin Data Collection (Table I).

Data have been collected on approximately 150 data sets in house with a series of different transition metals, sugar molecules, gangliosides, inhibitors to zinc proteases, different pH conditions, and various salts to resolve the issue of the protein molecules packing in different orientations in the crystal lattice. Each different compound is used to collect data on two different crystals in order to confirm the effects that are observed. The addition of additives is to "lock" the protein molecules into one conformation. This will allow the technique of MIR to be used for the crystal structure determination.

#### 3) Heavy Atom Derivative Data Collection (Table I).

In addition to the numerous data sets collected in house, we have also collected data at two different synchrotron sources and tuned the wavelength to the absorption edge of the metal that we have bound to the protein. This approach allows us to determine the 3-D structure of the neurotoxin using anomalous dispersion of the heavy atom and maximizing the heavy atom signal. The additional data obtained from the anomalous signal can be enough to determine the crystal structure with a single derivative or at least increase the amount of usable data from one derivative. Of particular use in our study, are the Hg and Pb derivatives. The synchrotron source at Brookhaven national Laboratory (NSLS) has the capability to tune the wavelength next to the absorption edge and optimize the anomalous signal. Merging statistics in Table I include all data to the highest resolution bin without throwing away any data.

#### 4) Location of Heavy Atom Sites for Phasing

Interpretation of Patterson maps to locate the positions of the heavy atoms is currently underway on the data sets listed in Table I.

#### 5) Binding Assay of Binding Domain

To assay for binding and screen for potential inhibitors to the binding site in order to lock the binding site into one conformation for the crystal structure determination, we have developed an assay to rapidly screen for toxin binding to the ganglioside GT1b. The ganglioside is adhered onto a membrane surface that is fixed onto a gold surface. Using the techniques of surface plasmon resonance (Pharmacia BIAcore 2000), we are able to quickly determine the binding and dissociation of toxin to the ganglioside in-house. Inhibitors are

then quickly screened to see if they inhibit toxin binding to the ganglioside. The ganglioside GT1b is naturally a good inhibitor, but the long hydrophobic ceramide tail is not useful to the growth of large single crystals of the protein molecule, probably due to packing effects as well as the hydrophobic nature of the ceramide tail.

#### 6) Proteolytic Assay of Catalytic Domain

Because of the need for a means to evaluate the quality of protein used in crystal growth experiments in addition to the fact that we need to learn as much as possible about the behavior of the protein during crystal growth experiments and to help us understand the function of the neurotoxin once the structure is determined, we have initiated simple kinetic assays to evaluate the neurotoxins proteolytic activity. The substrate being used is a 17 amino acid peptide of the synaptic vesicle protein SNAP-25. After several months of attempting to optimize proteolytic activity, it was determined that the zinc concentration of the toxin must be 100 micromolar. If the concentration of any zinc salt is above the micromolar range, the neurotoxin activity is inhibited. If the concentration of any zinc salt is below the micromolar range, the neurotoxin activity is very low (less than 10% of full activity). With this information, we now routinely use 100  $\mu$ M zinc acetate in all crystallization conditions to help stabilize the neurotoxin. It cannot be overemphasized that micromolar concentrations of zinc are absolutely necessary for full activity. In similar zinc protease structures, it is observed that correct concentrations of zinc atoms can possess either structural/catalytic or both roles in aiding the proteolytic activity. In the case of botulinum neurotoxin, we believe zinc to play both roles since we have observed that zinc salts stabilize the crystal growth under the optimal conditions (micromolar).

Analogous to other crystal structure determinations, it has frequently been observed that inhibitors for enzymes aid in the stabilization of protein molecules by locking the enzyme into a single stable conformation. By using the above kinetic assay, we have been able to screen potential inhibitors of the neurotoxin to stabilize a single conformation. The inhibitors being investigated including peptide-like analogs synthesized by Professor Paul Bartlett in the Dept. of Chemistry of UC-Berkeley. Based on the protease recognition site, we are investigating the inhibitors Z-Ala-Gly-P-Phe-, Z-Phe-P-Leu-Ala-, Z-Gly-Ala-P-, and CbZ-Gly-P-Leu-Gly-. The "-P-" moiety is a phosphate backbone in place of the amide backbone that strengthens the peptide bond and does not allow cleavage by the protease. The inhibitor does however have the recognition elements that the neurotoxin binds.

#### 7) Recombinant DNA work on serotype A domains to produce protein for crystallography experiments.

High expression levels of the translocation domain of BT serotype A have been achieved and purified. The *E. coli* expressed protein is folded and appears to be composed of both  $\alpha$ -helices and beta-sheets based on CD (circular dichroism) experiments conducted in my laboratory. Since the present crystals of botulinum neurotoxin diffract to 3.0  $\text{\AA}$  resolution, we will be able to determine the 3-dimensional structure of the neurotoxin and observe secondary structure elements ( $\alpha$  helices, beta strands, and beta sheets). It will be difficult to observe detailed side chain interactions of the smaller side chains (the larger side chains should easily be observable). In order to obtain a more detailed picture of the protein structure, recombinant DNA work on isolated domains (binding, translocation, catalytic) have been initiated with the goal of crystallizing the domains and determining the 3-D structure by x-ray crystallography. The approach of

"divide & conquer" has been used in numerous examples to determine the structure of regions of protein molecules (i.e. SH2 & SH3 domains of tyrosine kinases).

All work on this aspect of the project has been conducted by personnel supported by the Department of Chemistry at UC-Berkeley. Proper authorization to conduct recombinant DNA work on fragments of botulinum neurotoxin were obtained from the Biosafety Officer, Office of Environment, Health and Safety (see Appendix B of Year 1 Annual Report).

This work is in line with goals C5 and C7 of the contracted work, to crystallize the isolated domains of botulinum neurotoxin. Large quantities of purified protein is required and a recombinant approach is being taken. The alternative approach is by purifying the light and heavy chains from one another. This approach would require twice or greater the amount of holo-neurotoxin plus purification. Secondly, it is impossible to separate the binding domain from the translocation domain in this fashion.

### Relationship to Goals of Research

All work to date focuses on the single goal of determining the 3-D structure. Although numerous side projects have emerged (kinetic assay, binding assays, recombinant DNA work), all of these projects are to aid in the crystal structure determination goal. Furthermore, the information obtained during these studies will be mandatory in understanding the function of the neurotoxin once the structure is known. All of the side projects listed above have been completed by personnel not supported by this contract. The individuals supported by the contract work only on the crystal structure determination.

### CONCLUSION

(1) In our continuing efforts to stabilize BT serotype A, we have determined that 1mM methionine as an anti-oxidant used from the initial stages of protein purification to protein storage, greatly increases the production of BT and increases the shelf-life of BT as determined by enzymatic assays and crystal growth experiments. (2) We have screened over 150 data sets for suitable additives (sugars, metals, buffers, salts) to improve the quality of the protein crystals and "lock" the protein into one conformation. (3) A large number of data sets have been collected at various x-ray wavelengths to optimize anomalous scattering and minimize problems due to non-isomorphism. (4) Suitable heavy atom derivatives are being located with Patterson methods. (5) We have determined optimal conditions for enzymatic assay with a 17mer peptide that mimics the 17 terminal amino acids to SNAP25. Using the enzymatic assay, a potential inhibitor has been synthesized to inhibit the proteolytic activity and stabilize the protein crystals into a single conformation. (6) A binding assay has been developed to determine the quality of the protein using surface plasmon resonance. The ganglioside GT1b is placed onto a membrane mimic gold chip. This approach is also being used to locate inhibitors to the binding event and lock the protein into one conformation.

X-ray Data collected between August 1, 1994 - July 30, 1995  
on Botulinum Neurotoxin serotype A (Crystal space group P321)

DataSet Name	X-ray Source	Resolution Å	Unit Cell (a,c) Å	#Reflections collected	#Reflections unique	R <sub>merge</sub> %	R <sub>mergetonative</sub> %
--------------	--------------	--------------	-------------------	------------------------	---------------------	----------------------	------------------------------

In house x-ray diffraction screening crystal conditions by checking diffraction quality on 2 crystals for each sample

- 1) Series of transition metal salts that are commercially available (approximately 60 data sets)
- 2) Series of sugar molecules (approximately 20 data sets)
- 3) Series of gangliosides (approximately 10 data sets)
- 4) Series of freezing conditions: ( final: 2.6M Sodium formate, 0.1M Imidazole maleate pH 6.5, 10%glycerol and 10% sucrose) series of freezing techniques: serial, quick& direct transfer, dialysis, and growth in cyroprotectant (2 months of various trials)
- 5) Buffer series from 4.0 to 10.0 including multiple buffers within pH 6.0 to 7.5 (approximately 20 data sets)
- 6) New crystal forms obtained with incomplete factorial I, II and magic 96 (approximately 20 data sets)
- 7) Others: Phe, Met, Cys, KF, Mn, Mg, Ca, Fe, Zn, Cd, Ni, Hg, Pb, Co, Cu (co-crystallized, check diffraction quality on 2 c's)

Synchrotron Radiation Data Collection Trips

(SSRL Aug 94)

btinati	SSRL	4.0	164.3, 159.4	68,859	15,423	8.4	-
btsd1	SSRL	4.0	164.6, 159.2	73,676	20,740	7.6	33.2
btsd7	SSRL	4.0	163.0, 160.3	46,433	14,030	12.0	22.2
bt6	SSRL	4.0	167.3, 159.2	65,967	20,887	8.9	22.0
btme2	SSRL	5.0	168.9, 161.6	27,258	7,446	6.0	40.4
bttn	SSRL	4.0	166.4, 159.2	56,885	18,125	6.3	30.0

(BNL May 95 - tuned wavelength to Hg and Pb absorption edge)

btonat5	NSLS	4.0	168.4, 160.3	67,115	21,650	6.6	-
btopbn	NSLS	4.0	167.7, 160.6	17,042	8,071	4.2	7.3
btopbn2	NSLS	4.0	168.9, 160.7	47,072	11,738	6.0	7.1
btohgt	NSLS	5.0	167.8, 160.6	36,061	11,419	10.6	10.7
btohg	NSLS	4.0	168.0, 160.8	68,893	18,414	11.2	13.7

(BNL June 95 - tuned wavelength to Hg and Pb absorption edge)

btat1b	NSLS	4.0	164.1, 160.2	100,213	20,054	10.0	-
btmhgcl	NSLS	4.0	164.1, 160.7	94,104	18,781	9.8	22.3
btpb	NSLS	4.0	164.8, 160.2	69,965	20,772	10.4	28.7
btsd1	NSLS	4.0	167.0, 160.4	121,296	21,098	9.5	25.5
btu2	NSLS	4.0	164.4, 160.2	58,013	19,021	12.0	10.1

## APPENDIX

Reporting Letter From Sub Contractor Univ. Wisconsin (A1)

Updated Emergency Response Plan - Univ. Wisconsin, Madison (B1-B3)

Updated Emergency Response Plan - Univ. California, Berkeley (C1-C3)

# FOOD RESEARCH INSTITUTE

608/263-7777  
Fax 608/263-1114

Department of Food Microbiology and Toxicology  
University of Wisconsin-Madison

1925 Willow Drive  
Madison, WI 53706-1187

**Michael W. Pariza**  
Director, Chair  
Professor  
263-6955

Annual Report on the Subcontract from  
DOD (DAMD 17-93-C-3118)

TO: R. C. Stevens, University of California, Berkeley

**Fun Sun Chu**  
Professor  
263-6932

FROM: B. R. DasGupta *BRD*

DATE: August 24, 1995

**Dean O. Cliver**  
Professor  
263-6937

Between August 16, 1994 and now, 197 mg of pure type A botulinum neurotoxin and 285 mg of complex of the neurotoxin and non-neurotoxin proteins was sent to Prof. Ray Stevens to prepare crystals.

**Eric A. Johnson**  
Associate Professor  
263-7944

The complex was sent for isolation of the neurotoxin in Berkeley with the hope that the neurotoxin freshly isolated (avoiding the shipment from Madison to Berkeley) may give beneficial results.

**Charles W. Kaspar**  
Assistant Professor  
263-6936

For one shipment (12/14/94) the neurotoxin was carboxymethylated to block the free -SH groups. The neurotoxin sent from 5/16/95 onward had 1 mM methionine added to the buffers during and after chromatographic isolation of the neurotoxin.

**John B. Luchansky**  
Assistant Professor  
263-7280

**Amy C. Lee Wong**  
Assistant Professor  
263-1168

**Robert K. Bush**  
Professor  
(also Medical School)  
262-7056

**E. M. Foster**  
Former Director  
262-4696

**John K. McAnelly**  
Senior Scientist  
Research Liaison  
263-6826

**Bibhuti R. DasGupta**  
Senior Scientist  
263-6806

	Mg/ml	Total mg	Date shipped	Batch no.
1	10.0	10.5	8/16/94	69
2	10.0	12.5	8/31/94	69
3	10.0	12.3	10/11/94	70
4	10.0	14.1	10/25/94	70
5	8.4	12.5	11/8/94	70
6	9.6	1.7	12/14/94	71
7	9.7	1.7	12/14/94	71
8	7.9	14.3	1/12/95	72
9	9.6	14.8	2/7/95	72
10	12.5	12.5	2/28/95	72
11	9.7	13.2	3/14/95	73
12	9.7	7.6	3/28/95	73
13	10.0	5.1	4/6/95	73
14	10.0	12.3	4/24/95	74
15	10.0	17.9	5/16/95	74
16	10.5	11.9	6/5/95	75
17	10.0	13.7	6/20/95	75
18	10.0	8.4	7/7/95	75
Total		197.0		

Type A complex	Date Shipped	Batch no.
75 mg	3/14/95	73
140 mg	4/24/95	74
70 mg	6/5/95	75
285 mg		

Office of Biological Safety

1552 University Avenue  
Madison, WI 53705  
608/263-2037  
FAX 608/262-3059

Biological Safety Committee

12 July 1993

Mr. Philip Vorlander  
City of Madison Hazardous Materials Response Unit  
325 W. Johnson St.  
Madison, WI 53703

Dear Mr. Vorlander:

We are presently seeking a contract with the U.S. Army to conduct research in furtherance of the Army's Medical Biological Defense Research Program (MBDRP). The MBDRP is a research and development program funded by Congress with the mission of providing medical strategies (vaccines, antidotes, treatments) for the defense of military personnel against the use of biological weapons.

Our work involves the use of *Clostridium botulinum* toxin, which is potentially fatal to humans. The investigator involved with this study is Dr. Bibuhti Das Gupta of the Food Research Institute, which is located on this campus at 1925 Willow Drive. Pursuant to Title 10, United States Code, Section 2370 (enclosure 1), we are required by law to coordinate annually with your agency for the purpose of providing information on the MBDRP and formalizing an agreement for emergency support.

*Clostridium botulinum* toxin is a bacterial toxin. It may be present in the following material located within our laboratory: *in vitro* bacteriological and biochemical materials. The primary routes through which disease occurs include: ingestion; skin, eye and mucous membrane absorption (including the respiratory tract); parenteral inoculation. Individuals affected by this agent may experience the following symptoms and potential adverse effects: neurological symptoms resulting in respiratory failure and death.

*Clostridium botulinum* toxin is considered to be a biosafety level two (BSL-2) agent. BSL-2 is one of four categories of agents, with BSL-1 being the least hazardous and BSL-4 being the most hazardous. BSL-2 agents include the broad spectrum of indigenous moderate-risk agents present in the community and associated with human diseases of varying severity. These agents can be safely used in activities conducted in an open laboratory area, provided the potential for producing aerosols is low. Where aerosols may be generated, research with these agents is performed in a biological safety cabinet or with other physical containment equipment. Primary hazards to personnel working with BSL-2 agents may include accidental autoinoculation, ingestion and skin and mucous membrane exposure.

When laboratory manipulations of *Clostridium botulinum* toxin involve production of purified toxin, BSL-3 may be indicated. BSL-3 procedures and pertinent facility design features are established for the laboratory in question.

Included among our laboratory safety program are the precautions recommended in the Department of Defense Safety Program (Federal Register 32 CFR Part 627 Friday 10 April 1992). This document describes the precautions taken to minimize hazards to personnel, the community and the environment and is summarized on the attached. In addition to these precautions, the personnel of Dr. Das Gupta's lab are annually examined for their titres against the botulinum toxin and are given booster shots as needed.

Coordination of the following haz-mat emergency responses may be needed from your organization: Fire responders, police and security, emergency first aid, administration of antitoxin, assisted ventilation for respiratory failure.

Special equipment and training to both provide effective emergency support and reduce the potential hazards to emergency personnel include: Identification of agent and use of usual emergency room protective attire.

We request this support from your agency. If the provision of this support is acceptable to your agency, we request documentation of our agreement through an endorsement to this letter. You can endorse our agreement by signing the acknowledgement below\*\*. Any questions should be directed to Dr. Joseph Kanabrocki, Office of Biological Safety, 263-2037.

This is a continuing annual requirement that must be reported to Congress in September. Therefore, each July you will be consulted as to the status of this agreement. Our continued agreement, if unchanged, will be verified at that time. Any changes to our agreement will need to be formally executed.

Thank you for your assistance in helping us meet this statutory requirement. Your assistance allows us to perform research that is essential for the development of medical measures that will protect U.S. armed forces throughout the world.

Sincerely,



Robert Erickson, Director  
Research Administration-Financial

Enclosures

cc: Dr. B. Das Gupta  
Dr. J. Kanabrocki

\*\*Please return endorsement statement  
in the first block, next page to:

R. W. Erickson  
Rm. 446 Peterson Bldg.  
750 University Avenue  
Madison, WI 53706

I Official, an official of the Agency, having the authority to execute this agreement on behalf of the Agency, do hereby acknowledge its contents and agree to provide the emergency support identified above, if required.

Frank Orlando

Signature

Assistant Fire Chief

Title

29 July 1993

Date

Operations & Training

Position

#### Annual Review/Recertification

On Date, I consulted with Official, an official of the Agency, who has the authority to execute this agreement. This official has verified that our current agreement remains unchanged.

Joseph Kanabrocka

Signature

Director

Title

17 August 1994

Date

Office of Biological Safety

Position

#### Annual Review/Recertification

On Date, I consulted with Official, an official of the Agency, who has the authority to execute this agreement. This official has verified that our current agreement remains unchanged.

Joseph Kanabrocka

Signature

Director

Title

22 August 1995

Date

Office of Biological Safety

Position

UNIVERSITY OF CALIFORNIA, BERKELEY

BERKELEY • DAVIS • IRVINE • LOS ANGELES • RIVERSIDE • SAN DIEGO • SAN FRANCISCO



SANTA BARBARA • SANTA CRUZ

OFFICE OF ENVIRONMENT, HEALTH AND SAFETY  
UNIVERSITY HALL, 3rd FLOOR

BERKELEY, CALIFORNIA 94720

August 18, 1995

Headquarters  
U.S. Army Medical Research and Materiel Command  
Attention: MCMR-RCQ-S  
Fort Detrick, Maryland 21702-5014

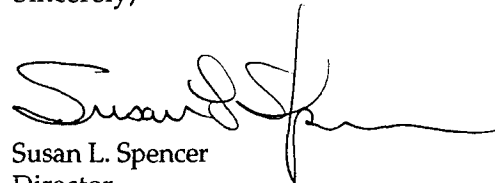
RE: **Emergency Response Plan**  
**Professor Raymond C. Stevens, College of Chemistry, University of California, Berkeley**  
**Contract No. DAMD17-92-C-2118**

Dear Sir/Madam:

The Office of Environment, Health & Safety of the University of California, Berkeley, has reviewed the Emergency Response Plan for the laboratory of Professor Raymond C. Stevens, who is under contract with the Army. Dr. Stevens' research remained the same this year and therefore, the Emergency Response Plan has not changed. The City of Berkeley continues to be the first responder for the campus and there is a long-standing cooperative relationship with the City's Office of Emergency and Toxics Management and the Berkeley Fire Department, as well as formal agreements.

Should you have any questions concerning the Emergency Response Plan, please contact Chris Carlson, Biosafety Officer, (510-643-6562).

Sincerely,



Susan L. Spencer  
Director

SLS/crc:crp

cc: Nabil Al-Hadithy, Office of Special Community Services, Toxics Program, City of Berkeley  
Susan Aspray, Sponsored Projects Office  
Chris Carlson, Biosafety Officer  
Horace Mitchell, Ph.D., Vice Chancellor—Business and Administrative Services  
Steven Pedersen, Chair, Safety Committee, College of Chemistry  
Raymond C. Stevens, Assistant Professor, College of Chemistry



OFFICE OF THE CHANCELLOR

BERKELEY, CALIFORNIA 94720

June 16, 1993

Mr. Michael F. Brown, City Manager  
Martin Luther King Jr. Civic Center Building  
2180 Milvia Street  
Berkeley, CA 94704

Dear Mr. Brown:

The University is presently seeking a contract with the U. S. Army to conduct research in furtherance of the Army's Medical Biological Defense Research Program (MBDRP). The University is required by law (Title 10, United States Code, Section 2370) to coordinate annually with the City for the purpose of providing information on the MBDRP and formalizing emergency support. The MBDRP is a research and development program funded by Congress with the mission of providing medical strategies (vaccines, antidotes, treatments) for the defense of military personnel against the use of biological weapons. The work at the University involves the use of Botulinum neurotoxin, an agent which may have the potential to cause disease in humans.

The "Mitigation Implementation Agreement by and between the City of Berkeley and the Regents of the University of California" dated July 26, 1990, is a formal agreement between the University and the City and includes emergency services (fire and police). To facilitate approval of the MBDRP contract, we request documenting our emergency support agreement thorough an endorsement to this letter. The University is taking precautions to minimize the potential for any emergencies; however, in the event of an accident, the following emergency support services may be necessary from the City: fire services, emergency medical transport, emergency spill response, and police assistance. The University Police Department will provide primary police service; the Office of Environment, Health and Safety will provide emergency response assistance, and the Occupational Health Program (University Health Service) will provide emergency medical consultation. Should a case of botulism occur, the antitoxin is readily available through the Centers for Disease Control.

Following is information on Botulinum neurotoxin and the safety precautions implemented by Professor Raymond C. Stevens (College of Chemistry) for the research. Botulinum neurotoxin is a toxin of bacterial origin and is stored as a purified protein solution (seven milligrams per milliliter of solution); the maximum amount of solution employed at any one time is on the order of one milliliter. The primary routes through which disease occurs include: injection or ingestion. Individuals affected by this agent may have nerve paralysis.

Botulinum neurotoxin is a biosafety level two (BSL-2) agent. BSL-2 agents are one of four categories of agents, with BSL-1 being the least hazardous and BSL-4 being the most hazardous. BSL-2 agents include the broad spectrum of indigenous moderate risk agents present in the community and associated with human disease of varying severity. BSL-2 agents can be safely used in activities conducted in an open laboratory area provided the potential for producing aerosols is low. Where aerosols may be generated, the research with the agent is performed in a biological safety cabinet or with other physical containment equipment. Primary hazards to personnel working with BSL-2 agents may include accidental autoinoculation, ingestion, and skin and mucous membrane exposure.

Professor Stevens' laboratory safety program includes the following precautions taken to minimize hazard to personnel, the community, and the environment. All individuals working with the toxin are immunized against the toxin through the Occupational Health Program (University Health Service). The toxin is kept locked up at all times and Professor Stevens controls access. All individuals in the laboratory are instructed about the Standard Operating Procedure and go through a special training program.

Mr. Brown  
June 16, 1993  
Page 2

502 Stanley Hall has been assigned for the toxin work. Special equipment to provide both effective personnel protection and emergency support include a dedicated ventilation system with emergency power backup and monitor. The room is kept under negative pressure relative to the building pressure for containment purposes.

We appreciate your acknowledgment of the emergency service support the City provides the University through an endorsement to this letter and your assistance in helping us meet this statutory requirement. This is a continuing annual requirement which must be reported to Congress in September; therefore, for the duration of the contract, each July the University will contact the City and request an acknowledgment of the emergency services provided.

The Office of Environment, Health and Safety (EH&S) has been working with Professor Stevens, if you have questions please contact Susan Spencer (643-8965) or Jack Salazar (643-8676). They will be happy to coordinate a meeting with Professor Stevens.

Sincerely yours,



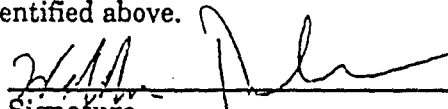
Daniel Boggan, Jr.  
Vice Chancellor - Business and Administrative Services

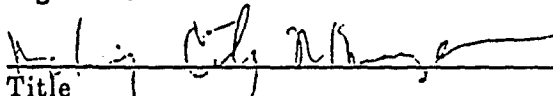
DB/SLS/jb  
Attachment

cc: Denise Johnston, Emergency and Toxics Management, City of Berkeley

Harry Chiladakis, College of Chemistry  
Dr. James Seward, Occupational Health Program  
Professor Raymond C. Stevens, College of Chemistry  
Susan L. Spencer, Environment, Health and Safety

I, William Rucker, an official of the City of Berkeley, having the authority to execute this agreement on behalf of the City of Berkeley, do hereby acknowledge its contents and agree to provide the health, fire, and police support identified above.

  
\_\_\_\_\_  
Signature

  
\_\_\_\_\_  
Title