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13. ABSTRACT ***(Maximum 200 Words)***

Botulinum Neurotoxin type E was purchased, and the protein was crystallized. The best diffracting crystals were obtained when the toxin was crystallized in the presence of a peptide from SNAP-25. An isomorphous tungsten derivative has been prepared and data have been collected to 3.2Å resolution. Three heavy atom sites were identified and used in phasing by the single isomorphous replacement with anomalous scattering method. The initial low resolution map clearly shows the protein envelop and also some protein features. Two additional heavy atom derivative candidates have been prepared.

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FOREWORD

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PI – Signature Date

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Comments on administrative and logistical matters.
As described in detail within the first progress report, due mainly to the need to find suitable replacements for personnel who had left the laboratory, the project actually did not start (and no funds were drawn) until March 1, 1999. Since the work is expected to require the full period asked for and approved in the proposal, we therefore would like to request an extension (at no additional cost) of the original termination date from 9/30/01 to 2/28/03. No additional funding would be required, however we would have to be allowed to carry the original funds into a new fiscal year.

Describe scientific progress for the period in terms of the tasks or objectives listed in the statement of work for this contract. Explain deviations where this is not possible. Include available data. Use additional pages, if necessary.

X-ray Crystallography of *Clostridium botulinum* Neurotoxins
Annual Progress Report for the Period Ending February 2000

Purified *Clostridium botulinum* neurotoxin type E (EC 3.4.24.69) precipitated with 60% ammonium sulfate was purchased from the Food Research Institute, Madison, Wisconsin, USA. It was stored at 10 °C until the toxin was prepared for crystallization. An SDS gel of the native toxin showed a single band of 150 kDa. All but about 5% of the protein was in the un-nicked form. The protein in ammonium sulfate solution was centrifuged in a refrigerated microcentrifuge for approximately five hours at 5000 rpm. The toxin was recovered by removing the supernatant and dissolving in 50 mM Hepes buffer, 100 mM NaCl at pH 7.2. This solution was then dialyzed against 50 mM Hepes buffer, 100 mM NaCl at pH 7.2 for two days to remove ammonium sulfate completely. The dialysate was changed four times during this period. The toxin was concentrated using Millipore concentrators to a final concentration of about 8 mg/ml. Our experience has shown that the above steps have to be carried out very carefully with minimal disturbance to the toxin. When the toxin was dissolved in a solution care was taken not to shake the vial but let it sit for awhile until the entire toxin goes into solution. For reasons not clear yet, this protein behaved differently from the toxin purchased from Porton town, UK and we had to change the crystallization conditions. However, the crystals obtained were of a different morphology and were very thin plates. The diffraction quality was not very good. Microseeding produced better crystals but still the diffraction quality was poor. We could collect native x-ray diffraction data to 3.3Å resolution only.

In our studies with *Clostridial botulinum* neurotoxin type B crystals we had found that when it is incubated with a VAMP 36mer peptide, the diffraction quality improved. We wanted to try a similar technique with BoNT/E by incubating the toxin with a polypeptide of SNAP-25, a substrate of this neurotoxin. Dr. James Schmidt of USAMRIID sent us a 46mer of SNAP-25 which we used for crystallization of BoNT/E. These trials gave better crystals with a different morphology . A picture of the new crystals follows (Fig. 1).

Figure 1: Crystals of Clostridium botulinum neurotoxin type E

These crystals diffract to at least to 2.5Å resolution and a native data set was collected at liquid nitrogen temperature. Crystals belong to the space group $P2_1$ with unit cell dimensions a = 81.4, b = 172.6, c = 137.3Å and β = 99.8°. In spite of flash freezing the crystal, we observed deterioration in diffraction quality over time due to crystal decay in the beam. A pseudo-precession picture of the h0l zone is shown in Fig. 2. The data set is 80% complete to 2.5 Å resolution, with the highest shell complete only to 25%. However, the data set is 98% complete to 2.74 Å. The overall R_{sym} is 0.086 with a redundancy of 3.5. Though we know that the presence of the SNAP-25 peptide improves the quality of the crystal, we have no explanation for this yet.

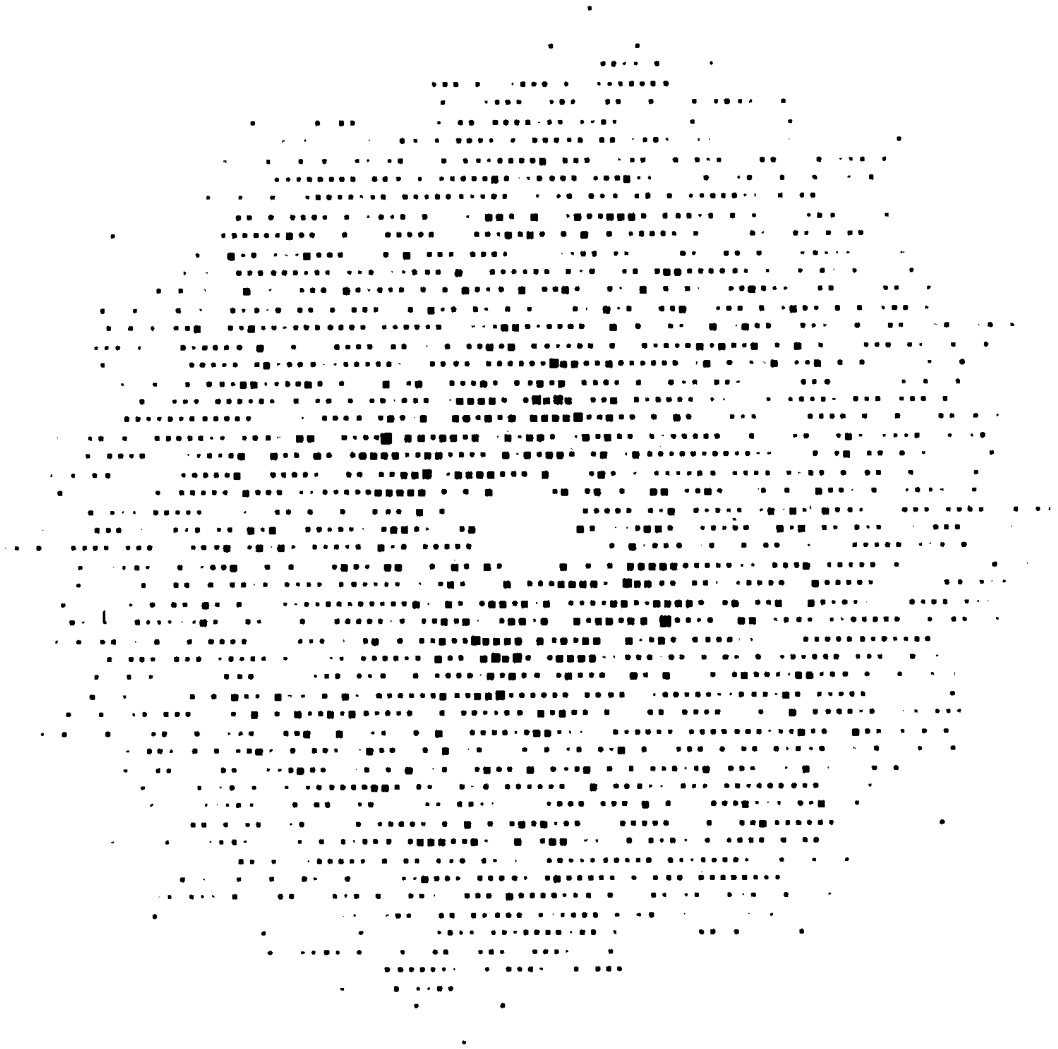

Figure 2: A pseudo precession picture of the h0l zone diffraction pattern from a BoNT/E native crystal. Data were collected at liquid nitrogen temperature from a flash frozen crystal at the NSLS beam line X25. The edge of the diffraction pattern corresponds to 2.5 Å resolution.

All *Clostridium botulinum* neurotoxins share significant sequence homology and are expected to have a similar fold. This prompted us to try the Molecular Replacement method to determine the structure using the recently determined high resolution crystal structure of BoNT/B as a search model. Various attempts with different software packages did not yield any reasonable solution. Splitting the BoNT/B model into three separate domains did not help. Using the crystal structure of BoNT/A (3.3Å resolution structure) also did not work. A probable reason may be that while BoNT/A and B are di-chain proteins, BoNT/E is a single chain protein, which might cause some differences in the conformation.

In view of the above, we have decided to use the Multiple Isomorphous Replacement with Anomalous Scattering method (MIRAS) and/or the Multiple Anomalous Dispersion (MAD) technique. Accordingly, we have started heavy atom derivative searches by soaking BoNT/E crystals in different heavy atom reagents. So far we have identified three derivatives, *viz.* platinum, osmium and tungsten. We have collected tungsten derivative data to 3.2Å resolution.

When compared with the native data it gave a R_{iso} value of 0.14. The Harker section for the anomalous difference Patterson map is given in Fig. 3. Three heavy atom sites have been located and are labeled as 1, 2 and 3 in the map.

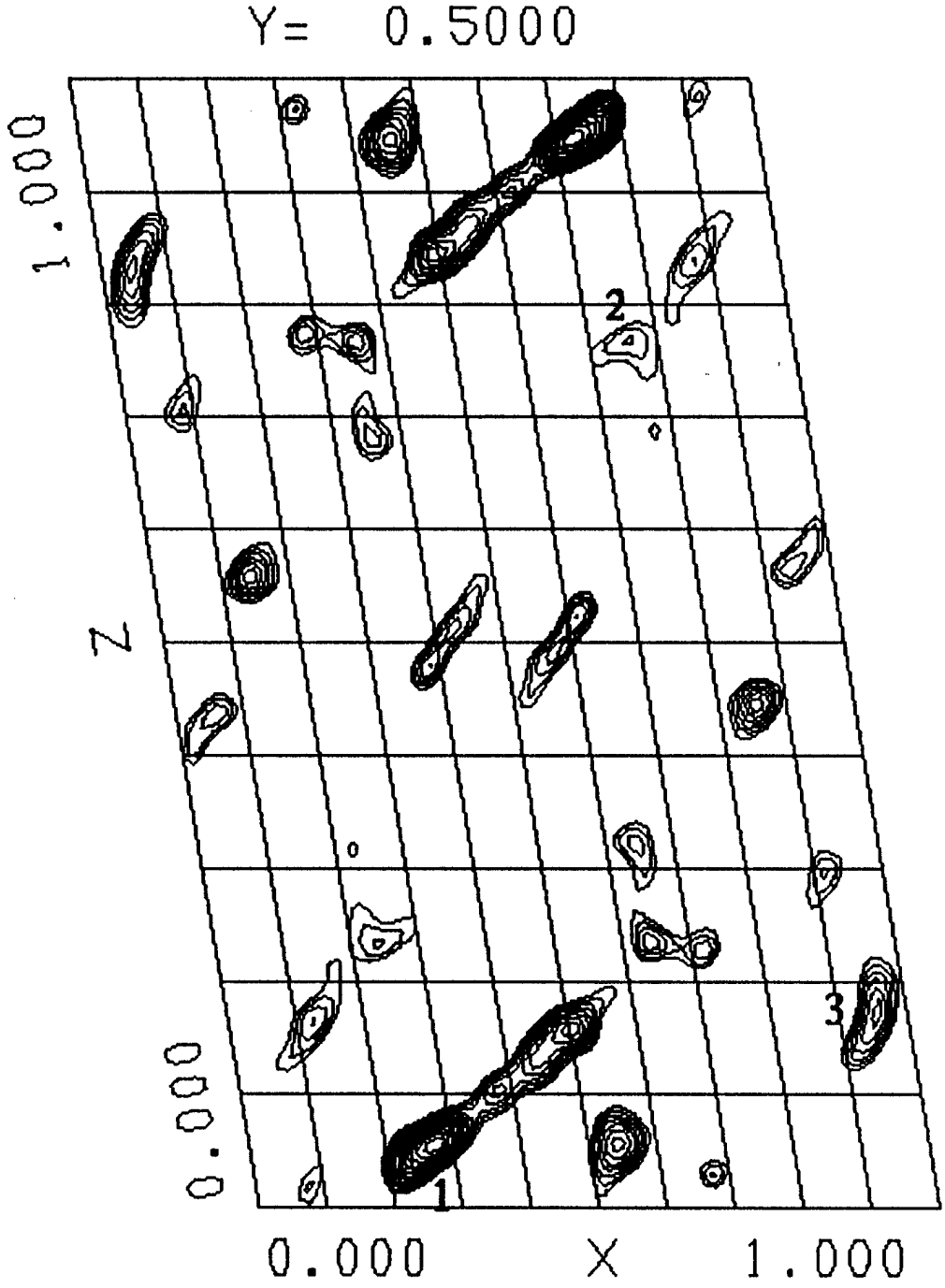

Figure 3. Harker section U 1/2 W.

We then computed phases using 4Å resolution data with these three sites by the SIRAS method (Single Isomorphous Replacement with Anomalous Scattering). The figure of merit and correlation coefficients are 0.80 and 0.81. The phases were further extended to reflections up to 3.0Å resolution. A 12Å slab of the solvent flattened map projected down the b axis is given in Fig. 4. The boundaries of the protein molecule are well defined and in the section shown we could also see long helices, probably belonging to the translocation domain.

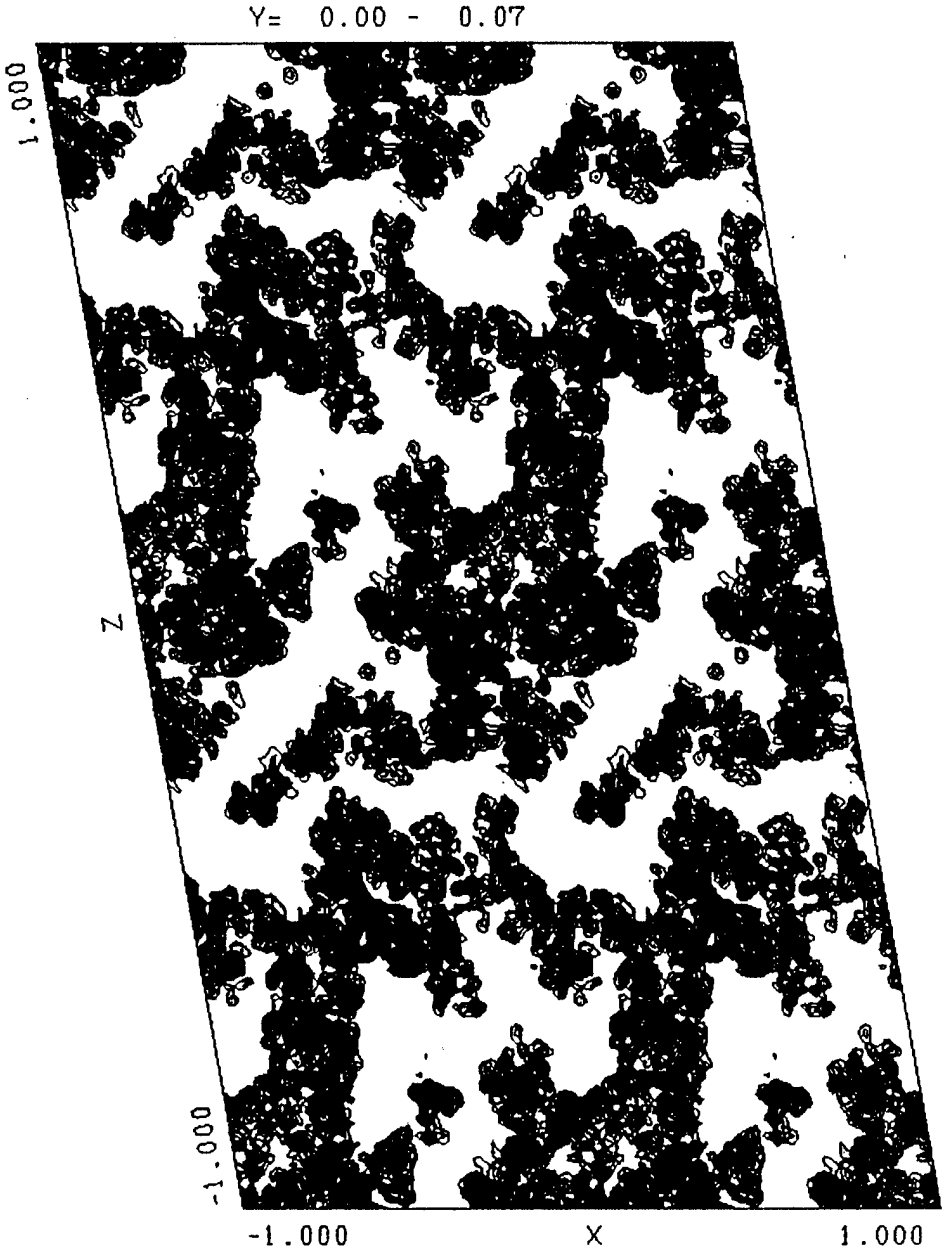

Figure 4. A 12Å projection of the map viewed down the b-axis.

Provide a brief statement of plans or milestones for the next quarter.

In the next quarter, we will be collecting derivative data from crystals soaked in osmium, platinum and other potential derivatives. Ideally, we would like to collect three wavelength data sets (or at least two wavelengths) from each derivative to use the MAD procedure to reduce errors due to scaling the native and derivative data. This was successfully done in the case of the BoNT/B structure determination. However, in the case of BoNT/E the crystal decay poses a problem. We are trying to improve the cryo-conditions and hope that the crystal will be more stable in the beam. When data from platinum and osmium derivatives are collected, we will determine the heavy atom positions by the cross Fourier technique and compute an electron density map to trace the chain.