

AD635680

ANNUAL PROGRESS REPORT

Research Project Contract Number: DA 18-035-AMC-143A

Biochemistry and Mechanism of Action of Toxic Proteins

Report Period: 1 July 1965 to 30 June 1966

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The following report will summarize progress during the year July 1, 1965, to June 30, 1966, in work being performed under contract at the Pennsylvania Hospital. It should be noted that the final quarterly report of March 31, 1966, was delayed and therefore contains results obtained during the month of April.

I. Preparation of Toxin for Binding Studies:

Because of some reduction of personnel and funds, chemical studies were limited to those aspects relevant to the production of a material suitable for use in in vivo studies. Details are to be found in the previous quarterly reports. In brief, studies on the fragmentation of the toxin led to the following conclusion. All preparations of the toxin (except the crystal phase which is inaccessible to testing), at pH's varying from 7.0 to 9.2 and ionic strengths varying from 0.02 M to 1 M, appear to contain two principle components as well as numerous small fragments. Studies utilizing chromatograph separations show that even when these components are separated, they tend to reform equilibrium mixtures on standing for any length of time. In particular, toxin preparations kept at physiological conditions in a buffer at pH 7.4 (0.1 M NaCl, 0.05 phosphate) invariably contain the two components, no matter what the method of preparation used. Parenthetically we would add that toxin stored at 4°C in this particular medium retains at least 80% of its activity for more than a year. In view of this we have come to feel that whatever preparation of toxin is used it is probable that once the preparation has been injected into the animal it will form one of these equilibrium mixtures. As detailed in the last quarterly report, all fractions seem to be identical in all properties except size. Thus in the remainder of this report "toxin" or "labelled toxin" may be taken to refer to the mixture. Further studies on the components are

proceeding in order to obtain some idea of the type of equilibrium which exists. Regarding the smallest components, an estimate was made of their quantity by exhaustively dialyzing solutions of the toxin against the pH 7.4 buffer referred to above. Spectrophotometric and toxicity studies on the dialysate obtained indicated that only 0.1 to 0.2% of the toxin is found in components small enough (MW less than 20,000) to pass through the dialysis membrane used.

II. Fluorescent Toxin - Immunochemical Studies:

Progress in the successful labelling of the botulinus toxin and its application to the identification of binding sites in the mouse was made during the contract year. Binding of botulinus A toxin was studied with combined phase contrast and fluorescent microscopy using both fluorochrome labelled toxin and fluorochrome labelled antitoxin. Histochemical methods for localization of cholinesterases and employing fluorochromes were also used to identify endplate areas binding fluorescent antitoxin. Most of these methods had been successfully used in our studies of tetanus toxin and this experience enabled us to eliminate most of the artifacts which can occur. These techniques were employed to avoid possible confusion by mast cells which non-specifically bind fluorescent labelled antibodies.

Using fluorescein-labelled toxin, we found that binding was remarkably specific, being limited to the motor endplates. Thus botulinus toxin contrasts with tetanus toxin, which is more generally dispersed in the surface membranes of skeletal muscle and the cells of the nervous system. Direct binding of labelled toxin was confirmed by staining tissues from animals killed by the intravenous injection of botulinus A toxin with fluorescein-labelled botulinus antitoxin. Fluorescence was also observed

in capillaries in experiments using i.v. toxin. An important source of artifact, non-specific staining of mast cells, was eliminated. Our results have been recorded in color photomicrographs. This data confirms previous observations on the specific localization of botulinus toxin in motor endplates. The appearance of the fluorescent images suggest that the localization is in the primary synaptic cleft and/or the terminal axon. Ultrastructural localization is discussed below.

III. Studies with Ferritin-Labelled Botulinus Toxin:

In a series of experiments similar to those previously performed by Dr. Zacks and associates with botulinus B toxin, botulinus A toxin was labelled with ferritin and its successful binding was proven by immuno-electrophoresis. The labelled toxin was injected into mice. Ferritin particles were located in the basement membrane area of the subneural apparatus as previously shown with the B toxin. This confirms and extends the results of our fluorescent studies. Although we originally suspected that an increased number of ferritin particles were localized in the synaptic vesicles of the terminal axons as compared with B toxin binding, high resolution electron microscopy showed that this was not the case. The majority of the label was found in the subneural apparatus.

IV. Lead Binding by Motor Endplates:

A series of investigations during the year on the binding of lead by motor endplate structures in botulinus intoxicated animals was completed.

It has been known since 1958 that the subneural apparatus of the motor endplate specifically binds lead, and more recently, electron microscopy, performed by ourselves and others, has localized the lead in the muscle surface membrane and the overlying basement membrane of this area.

A series of studies of whole mounts of muscles from mice with acute botulinus intoxication revealed no detectable changes in lead binding at the light microscope level, although we suspected that in chronically poisoned animals the endplates were smaller and perhaps showed reduced lead binding. However, electron microscopic studies of acute and chronically poisoned animals (up to 26 days) showed no significant change in the amount or distribution of lead bound by these membranes. It seems therefore, that the rather characteristic binding site for lead at the post synaptic structures is not altered by acute or chronic botulinus intoxication. It is not known at this time whether these lead binding sites are also the acetylcholine binding sites. There is no evidence of any spread of the lead binding area of the muscle surface in acute botulinus intoxication.

V. Local Botulism:

A series of experiments showed that the injection of minute quantities of toxin (0.005 μ g in 1 μ g) into the gastrocnemii of mice produced a local flaccid paralysis with few signs of generalized botulism. Maximal signs were noted after 4 to 6 days and return of function began after approximately 15 days. The animals then rapidly returned to normal. No residual signs were noted. This experiment indicates that the action of the toxin can be circumscribed, that the binding reaction must be reversible and, since 15 days is too short to allow for regeneration of endplates, that the blockage of the endplates does not result in any permanent damage in this time interval. The ultrastructural morphology of the paralyzed muscle and endplates was studied and no abnormalities were found.

VI. Acute and Chronic Intoxication:

Electron microscopic studies have been made of muscle and endplate structures in mice with acute and chronic intoxication. The acute studies were largely a confirmation of previous work by ourselves and others and confirmed that there are no visible ultrastructural abnormalities in acute experimental botulism. Chronic intoxication has not however been well studied and therefore experiments were carried out in which animals were maintained for periods up to 26 days in a state of chronic paralysis. By adjustment of the dose, the animals were maintained in a state of severe generalized weakness without however requiring respiratory support. Tissues prepared from these animals were examined in the electron microscope and particular attention was paid to the motor endplates. In no case were any consistent abnormalities found in either the pre- or post-synaptic components of the motor endplates. We would particularly note that the synaptic vesicles were morphologically normal in all respects. The principle change noted was that after 12 days or more of chronic intoxication there was swelling of the mitochondria with an increase in the number of intra-mitochondrial dense granules. Such changes are non-specific and are interpreted as a result of chronic hypoxia.

VII. Absorption Studies from the Gastrointestinal Tract:

The problem of where and in what form botulinus toxin is absorbed in the gastrointestinal tract (the natural route of intoxication) has not been satisfactorily solved. We therefore attempted to study this problem by placing aliquots of labelled toxin in isolated segments of intestine and stomach in vivo. The fluorescent label could be traced on the surface of the intestinal mucosal cells and, 15 to 30 minutes later, on the epithelial

cells of the intestine and within the submucosal vessels. However, there was no fluorescence in the lacteals or in the lamina propria mucosa. These experiments were generally performed utilizing the first 4 cm. of the duodenum with quantities of toxin ranging up to 1 mg. in 0.2 ml. When labelled toxin was injected into the stomach, the fluorescent material was apparently destroyed within seconds and could not be localized in any portion of the stomach or its membranes. It should further be noted that animals which had received 1 mg. of the toxin directly into the stomach generally survived until sacrificed several days later. Animals which had received 1 mg. into the intestine died so long after the injection that well over 95% of the toxin must have been destroyed. These negative results led us to reconsider the route of administration of the toxin and in particular the rapid destruction of the toxin by the stomach indicated that absorption might be occurring primarily in the pharyngeal or esophageal regions. A careful study of the positioning of the tip of the stomach tube during attempts to administer toxin by this means lent additional weight to this hypothesis since it was noted that the tube usually stopped in the esophagus several mm. proximal to the stomach. Therefore experiments were devised (as detailed in the last quarterly report) in which toxin was administered directly into the mouth and also by "stomach tube". These experiments showed that although animals can be killed by administration of toxin via the stomach tube, an equally effective dose can be given directly into the mouth. In fact, if the volume is kept very small (so that an immediate swallowing reflex is not obtained) oral dosage is at least equally effective. The probability of oral absorption indicated by these results was confirmed by indirect ultraviolet microscope studies employing fluorescein labelled antitoxin. These experiments showed the passage of fluorescent

labelled toxin through the submucosal areas into the vessels. The demonstration of oral absorption of toxin helps to explain both the diminished toxicity found by so many workers studying absorption from the lower intestinal tract and the considerable variations of response by exposed individuals to contaminated food in natural outbreaks of intoxication.

VIII. Nervous tissue Binding of Botulinus Toxin:

In a recent paper Hart et al, (1965), claimed that botulinus toxin can pass through the blood brain barrier since the feeding of brains from botulinus intoxicated rats to normal mice resulted in the deaths of the latter. We have never noticed significant localization in the brain and it therefore seemed worthwhile to repeat this observation in some detail. As a preliminary experiment, mice were poisoned with large doses of toxin and in the terminal phases of intoxication were killed by perfusion with saline. This perfusion represents a difference in technique as compared with the work of Hart et al. Brain and liver from these intoxicated animals were homogenized in small volumes of 7.2 buffer and clear supernatants obtained by spinning the homogenates at 20,000 X G. Aliquots of the supernatants were injected (i.p.) into mice. In no case did those animals receiving injection of brain supernatants show any signs of botulinus intoxication in contrast to animals receiving liver supernatants which died relatively rapidly. These results might indicate that if the brain contains any bound toxin it is not easily removable; but a more probable explanation of the difference between our results and those of Hart et al is that the total removal of blood from the brain also removed available circulating toxin. In liver, complete removal of blood by perfusion is virtually impossible and this residual blood probably contained the toxin.

We did additional studies on the possible detoxification or binding of botulinus toxin by brain "in vitro". These studies are still in progress and the results reported here should be considered as only a partial summary of our experiments. In brief, varying quantities of brain were incubated in Krebs-Henseliet buffer solution (pH 7.6) with and without toxin. Controls consisting of toxin incubated in buffer with and without glucose were also prepared. The results were contrary to expectation and are summarized in the table. In brief, it was found that the toxin alone is almost completely detoxified by incubation in oxygenated Krebs-Henseliet Ringer solution for 30 minutes. The addition of brain tissue to this results in the retention of some toxic activity which is however more nearly related to the quantity of brain used rather than to the quantity of toxin in the incubation mixture. Supernatants from brain alone showed some degree of toxic activity (0.1 mls. of incubation mixture causes death in 72 hours) when homogenates were incubated with glucose, but none in the absence of glucose. As yet no clear cut results have been obtained as to the effect of glucose on the amount of toxicity retained in the brain and toxin incubation mixtures. These results can be interpreted in one of two ways: (1) either the presence of substances, possibly such soluble components as gangliosides, stabilize the toxin, or (2) the presence of the toxin results in an increase in the previously minimal production of some unknown toxic substance. The latter hypothesis, that the toxin can cause the production of a toxic substance from selected sites is a very intriguing one and, we feel, deserving of further study. Experiments now in progress, utilizing fluorescent labelled toxin and dialysis of the incubation mixtures, will enable us to differentiate between the production by brain of a toxic substance and protection of the botulinus toxin during the incubation.

IX. Genetic Resistance to Botulinus Toxin in Mice:

As mentioned in earlier reports we have consistently found a small fraction (5%) of all random groups of mice tested to be partially or wholly resistant to botulinus toxin. In order to test whether this resistance is genetically determined or is a result of the physiological state of the particular animals concerned, we bred animals which had survived a large dose (0.1 μ g.) of the toxin through 3 generations. The final generation was then tested against a control group. The results show that the extreme resistance of the original ancestors was not inherited by the descendants as a whole; but that some resistance was bred into this group is equally certain. The results are shown in the figure which shows the death times of the control group as a histogram above the line, and the death times of the tested group below the line. It is fairly obvious that whatever inheritance of resistance is occurring it is neither sex linked nor a simple autosomal inheritance. We would like to carry out further studies utilizing larger groups. If the group of survivors with increased resistance could be further inbred, it is possible that offspring with greater resistance could be bred that would serve as valuable material for studies on the biochemical basis of botulinus intoxication. However, in view of the current situation of the contract, we cannot afford to utilize our time or personnel to pursue this problem now.

The Effect of Incubation With and Without Brain on the Toxicity of Botulinus Toxin

All incubations were carried out for 60 mins. in oxygenated Krebs-Henseleit Ringer solution, pH 7.4. Brains were obtained from normal white mice. The time of 360 mins. was chosen since it represents the median death-time

2 S.D. for a dose of 0.2 μ g. of toxin per animal. Glucose added to incubation as indicated.

Dose of Incubated Toxin per animal	No. of animals (out of ten) dying in 360 mins. in presence of:							
	No Brain		Whole Brain		Half Brain		$\frac{1}{4}$ Brain	
μ g.	Glucose	No Gl.	Gl.	No Gl.	Gl.	No Gl.	Gl.	No Gl.
5.0	15/20	14/20	10	10	10	10	9	9
2.0	3/20	1/20	10	10	8	8	8	9
1.0	0/20	0/20	10	10	10	7	10	10
0.5	0/20	0/20	8	7	5	8	9	9
0.2	0/20	0/20	8	7	4	3	9	7

BREEDING OF MICE FOR RESISTANCE TO BOTULINUS A TOXIN.

Distribution of death times of control and experimental animals following injection of 0.005 ug. of toxin.

