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Electron Microscopy of the Toxin and Hemagglutinin of Type A *Clostridium botulinum*

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Electron micrographs of the toxin and the hemagglutinin of type A *Clostridium botulinum* showed the toxin to be either round or disclike particles of 4 to 4.5 nm. These particles could also be seen as arranged in long strands or tubules of 9 nm in width. The hemagglutinin appeared as a crystalloid monolayer of stacked particles of 9 nm forming regularly arranged structures of 20 nm. Seen in cross section, these structures appeared as tubules with a lumen of 9 nm. The regularity of the angle of 83° to the long axis of the structure in which the individual particles were arranged suggested that the hemagglutinins formed a helix with sufficient space within its coil to admit the strands of the toxins. A model of the possible arrangement of the toxin and the hemagglutinin in the native state is proposed.

To our knowledge no successful attempt has been made to observe the toxin of *Clostridium botulinum* by electron microscopy. The only electron micrographs of this toxin on record are those of type E toxin of these organisms reported by Kitamura and Sakaguchi (7). Type E toxin is elaborated in the culture in the form of a "protoxin" of relatively low toxicity, but which can be augmented as much as a thousand-fold by treatment with trypsin. Either the protoxin or the activated toxin can be dissociated into two components of equal molecular weight, one toxic and the other nontoxic. However, on electron micrographs both preparations appeared identical, and the morphology of the protoxin showed no evidence that it consisted of the above two components. The authors offered no conclusions as to the structure of the toxin molecules. Previous to the publication by Kitamura and Sakaguchi, Zacks et al. (10) reported the results of their experiments in locating the toxin of type B *C. botulinum* in the synaptic spaces of the myoneural junction of the cholinergic nerve-end organs. In these studies the toxin was conjugated to ferritin granules by the method of Smith et al. (9) with the aid of specific antibody to the toxin. No toxin was actually seen in the pictures, but its presence was inferred from the location of ferritin granules in the synaptic spaces. The dimension of the toxin molecules was then calculated by measuring the distances between the adjacent ferritin granules on the assumption that space was occupied by the toxin. Since no information as to the purity of their toxin preparations was supplied in the report and since evidence exists that even in crystalline form the toxin contains 80 to 90% impurity, the suggested dimensions for the toxin appear fortuitous. Furthermore, to assume that the toxin coupled with its homologous antibody will remain active and will still bind to the receptor sites of the nerve-end organs is wholly unwarranted.

The toxin of *C. botulinum* isolated and crystallized in our laboratory by the method of Duff (6) was dissociated into two biologically active fractions, one toxic which we named α component and the other named β, a powerful hemagglutinin. Both α and β were homogenous by all criteria employed (gel filtration, ultracentrifugation, and gel electrophoresis). The molecular weight of the toxin, as calculated from data obtained by ultracentrifugation of this component and by a method of gel filtration by Andrew, was 1.28×10^5 (5). Judging from its amino acid composition (2) and its behavior on chromatographic columns, α was a different protein from the hemagglutinin. The latter (β) had a molecular weight of about 7.5×10^5, and, when chromatographed on diethylaminoethyl (DEAE)-cellulose columns equilibrated and eluted with tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer at *p*H 8, β emerged as three components of molecular weight of 2.5×10^5, 5×10^5, and 7.5×10^5, respectively (3). All three components were active. It was, therefore, concluded that the hemagglutinin was an aggregate of three molecular species, the smallest of which was 2.5×10^5 daltons (4).

Botulinum toxin in the crystalline form is stable on storage in the range of *p*H from 1.0 to 9.5. Purified toxin is inactivated at *p*H 3 and rapidly deteriorates even if stored in the cold.

The availability of the pure toxin and hemagglutinin prompted an attempt to observe the two moieties in the electron microscope. It was hoped that results obtained would reveal data on the morphology of the toxin molecule and the physical relation of the two components.

MATERIALS AND METHODS

The materials used in this study were the crystalline preparation of the toxin of type A *C. botulinum* and the toxic and hemagglutinating components derived from it by column chromatography (3, 4).

The latter two substances in concentrations of 0.1 to 1.0 mg of protein per ml of solution were dialyzed against 0.1 M ammonium acetate buffer at the same *p*H as the stain to be used in the experiment. The crystalline toxin was kept in 0.03 M phosphate buffer, *p*H 6.8, containing 0.9 M $(NH_4)_2SO_4$ to prevent the crystals from solubilizing.

Copper grids, 400 mesh, were coated with carbon films and were rendered hydrophilic by glow discharge. Two percent solutions of uranyl acetate, adjusted to *p*H 4.6 by ammonium hydroxide, and 3% of ammonium molybdate at *p*H 6.9 (also brought to this *p*H with ammonium hydroxide) were used as contrast agents. The negative-staining agents were filtered through membrane filters (Millipore Corp.), prior to use.

The specimens were examined at instrumental magnifications between 40,000 and 80,000 times with a Phillips 300 electron microscope, using an anticontamination device and a double-condenser illumination. The operation voltage was 60 kv. The magnification was established by the aid of a calibration grid.

RESULTS

The preparation of specimens of the crystalline toxin proved difficult to mount and stain. The crystals either dissolved in the buffer or disintegrated in the process of mounting and drying on the grid. Attempts to stabilize the toxin by fixing it with various concentrations of glutaraldehyde were not successful. When prepared in phosphate at *p*H 6.8 and in the presence of 0.9 M $(NH_4)_2SO_4$, conditions in which the crystals normally retain their structural integrity, the crystals were too large with too many superimposed structures to be satisfactorily viewed in the electron microscope.

The examination of preparations of the dissociated and purified toxin and the hemagglutinin components revealed the presence of two kinds of particles differing in size, morphology, and aggregate arrangement, as well as, penetrability to the contrast agent used.

The toxin particles observed in the electron micrographs appeared singly, in small groups, and in long, loosely formed double strands of about 9 nm in thickness (Fig. 1). The individual particles were irregular, disc-shaped structures measuring about 4.5 nm. The hemagglutinins, (Fig. 2) were likewise ringlike units of 9 nm in diameter either with what appeared to be a central opening or filled with material which retained uranil acetate stain. These units aggregated into evenly spaced double strands of roughly equal length, separated by a wider space from adjacent pairs. Each pair measured 19 to 20 nm laterally. On close examination it was observed that the lateral units of the hemagglutinin were stacked at a constant angle of 83° to the long axis of the aggregate, which in turn was arranged in a typical crystalloid monolayer. The hemagglutinin particles seen at higher magnifications were either singly or in groups of two and three particles and appeared to consist of thicker elements joined by thinner inter-connecting material (Fig. 3).

In our unsuccessful attempts to visualize the crystalline preparations, which served as the starting material from which the toxin and the hemagglutinins were derived, we noted the presence of structures which were different from the surrounding material (picture not shown). Magnified these structures were seen as rings of 20 nm in diameter, formed by smaller particles with projections making contact with an adjacent similar particle. The inner diameter of the rings measured 9 nm (see insert in Fig. 2.). No satisfactory measurement of the subparticles forming the ring could be obtained from this picture.

DISCUSSION

The aggregate arrangements of the toxin and the hemagglutinin as seen in the electron micrographs were rather surprising. It was the hemagglutinin and not the toxin which appeared in a crystal formation. It is thus conceivable that what has been hitherto regarded as crystalline toxin is in reality the hemagglutinin which crystallizes on purification, carrying the toxin in some manner down with it.

The regularity of the arrangement of the lateral element forming the paired hemagglutinin aggregate and the evenness of spacing separating the pair suggest a tubular structure, as such structure might appear in the electron microscope. The space between a pair of strands may then be its lumen.

The crystalline material from which the two

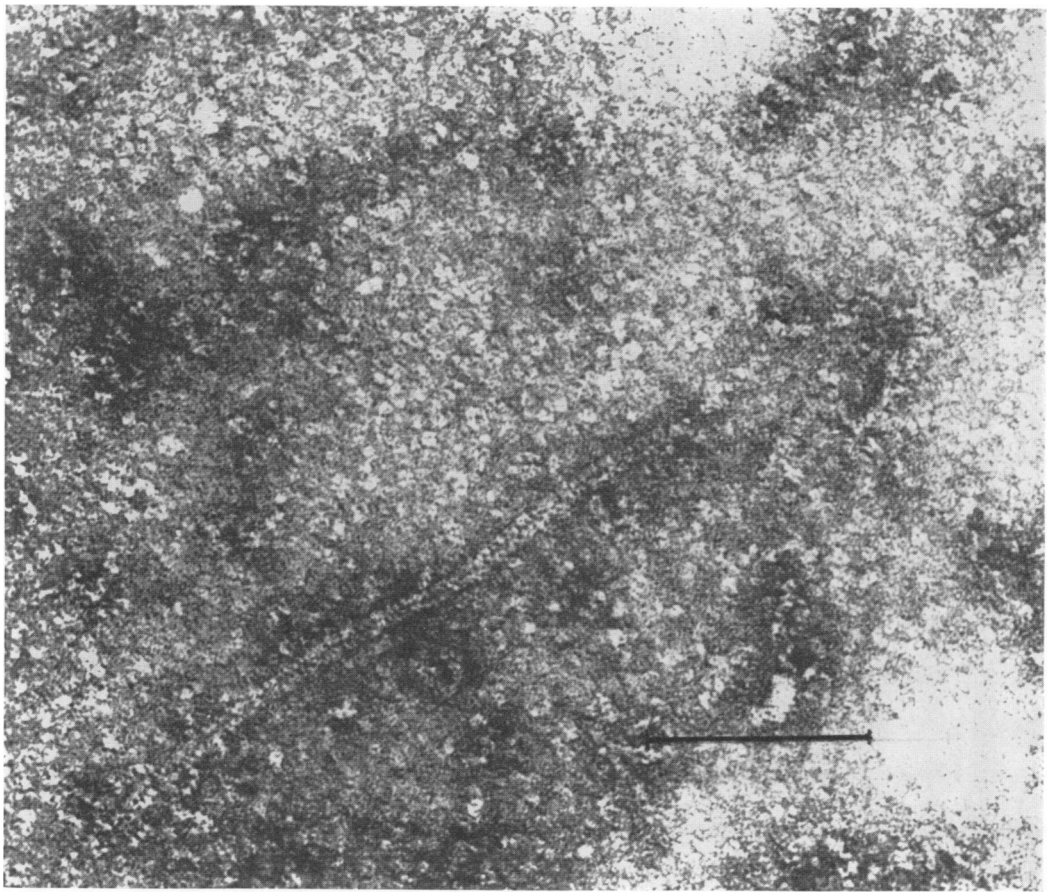

FIG. 1. *Toxin of C. botulinum type A. Negative staining with uranyl acetate after fixation with glutaraldehyde Individual particles aggregated into a long tubule formation with a diameter of 9 nm. Bar = 100 nm.*

components were isolated contained no other substances. The ringlike structures (Fig. 2 insert) seen in the electron microscope during our attempts to photograph crystalline preparation did not resemble the toxin; they may, therefore, be the hemagglutinins, perhaps in their cross section. The similarity of the width of the aggregates and the diameter of the ring support such a view. The constant angle at which the lateral elements are stacked to form the tubules, suggests a possible helical structure consisting of coils of 19 to 20 nm in diameter and forming an inner space of 9 nm. The toxin, on the other hand, which appears to form a long double strand, or perhaps also a tubule, is likewise 9 nm in width and consists of individual particles of 4.5 nm. This measurement is in agreement with the molecule size of the toxin as it was calculated by the method of Acker (1), from the elution rate of this substance through a Sephadex G-200 column (5). Thus, it would not be contrary to our observations to suggest that the toxin strand may fit within the coils of the hemagglutinin helix. We can thus design a model (Fig. 4) of the possible native structure of the toxin hemagglutinin complex as it is elaborated by *C. botulinum* into the medium. Such a concept is not in variance with what is known about the properties of these two substances: that the toxin invariably co-purifies with the hemagglutinin and resists dissociation, and that in association with the hemagglutinin the toxin is stable at a range of *p*H from 1 to 9.5 and can be stored at 4 C for months or years. Dissociated, that toxin is rapidly detoxified at *p*H 3.5 and becomes labile even in the cold. Freed from the toxin, the hemagglutinin remains as stable as it was before purification.

We realize that the above concept of the toxin

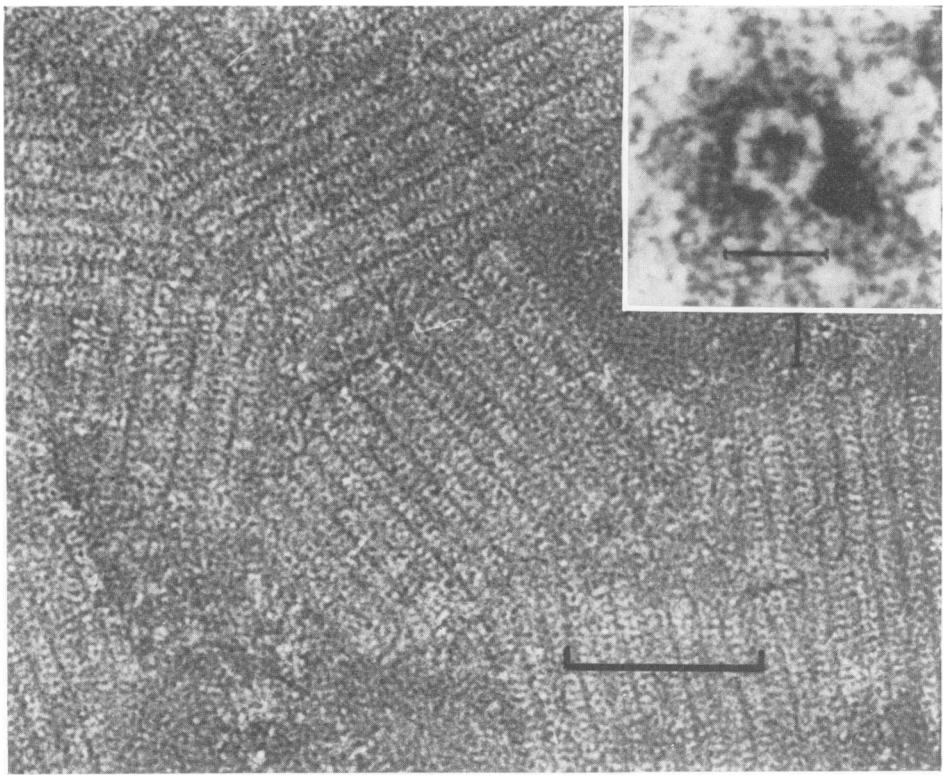

FIG. 2. *Hemagglutinin of C. botulinum type A. Negative staining after fixation with glutaraldehyde. Note typical crystalloid monolayer formation. Bar = 100 nm. Insert: structure forming hemagglutinin strands. Same staining method. Bar = 20 nm.*

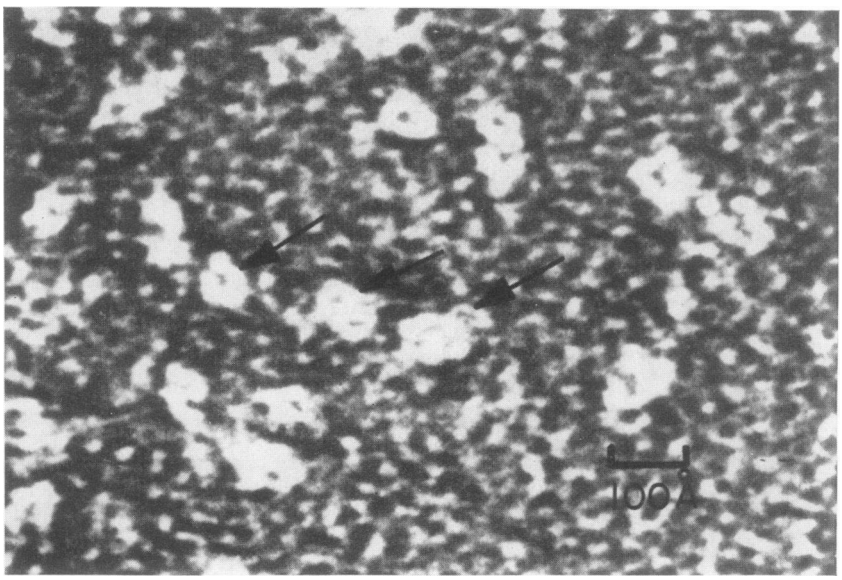

FIG. 3. *Individual hemagglutinin particles of C. botulinum type A. Negative staining with uranyl acetate. Note the particles singly and grouped in pairs and triplets. Bar = 10 nm.*

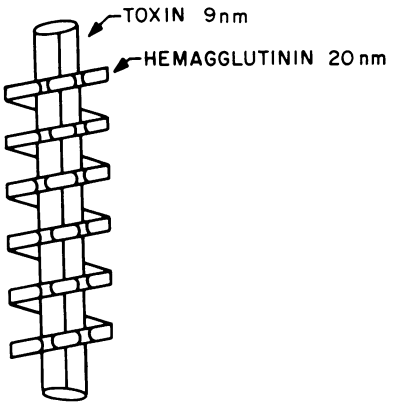

FIG. 4. *Possible native arrangement of the molecules of the toxin and the hemagglutinin of C. botulinum type A.*

structure still rests on the indirect evidence. Nevertheless, the proposed model offers an approach to the elucidation of the structure of these unique molecules and may also lead to the understanding of the basis of the biological activity of these two components; further studies are being directed by us for this purpose.

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