PHYSICOCHEMICAL PROPERTIES OF CRYSTALLINE CLOSTRIDIUM BOTULINUM TYPE A TOXIN*

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Interest in the chemical nature and mode of action of bacterial toxins has been renewed by the simultaneously announced crystallization of botulinal (1) and tetanal toxins (2) and the recent purification of several toxoids (3–5). Of these, crystalline Clostridium botulinum type A toxin alone has been submitted to an apparently complete elementary and amino acid analysis (6), as well as to preliminary physicochemical characterization (7–9) and immunological study (10). This toxin has been found to be a typical protein exhibiting no unique composition or physicochemical properties and is apparently devoid of a prosthetic group. A preliminary report of the molecular weight and homogeneity of botulinal toxin prepared by the method of Lamanna et al. (1) has already been made (9). Further details of the physicochemical characterization of this toxin and of the effect of the method of preparation on the molecular kinetic and serological properties of this substance are given in this report. Investigation of the botulinal toxin-antitoxin reaction has also been undertaken. Data to be published separately indicate that serologically the toxin acts as a single substance (10).

The original procedure for the crystallization of botulinal toxin (1) has been modified in several ways, including the omission of a step involving shaking of the crude acid-precipitated toxin with chloroform (11). However, much study had already been made of the properties of crystalline toxin of maximum potency prepared by the procedure first announced. A later method described by Abrams, Kegeles, and Hottle (7) differs from both the above procedures and also omits shaking with chloroform. In view of the suggestion (8) that partial denaturation may result from the use of chloroform in the purification of the toxin, some data on the physicochemical and serological characteristics of toxin prepared both by the original and the modified methods of Lamanna et al. are included in this communication.

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EXPERIMENTAL

The crystalline toxin used in this investigation was prepared by methods previously described (1, 11). Some of the material was later used for the amino acid analysis already mentioned (6). Several lots were studied, including one not subjected to shaking with chloroform (Batch S-5). The potency of the toxin varied somewhat from preparation to preparation but probably not significantly outside the errors of the mouse titration employed. The toxin contained about $220 \times 10^6$ mouse LD$_{50}$ per mg. of N as measured by a statistically valid titration (12) on 20 gm. white mice. The highest value recorded was $250 \times 10^6$ LD$_{50}$ per mg. of N (initial potency of Batch C). A single batch of toxin (Batch BT-3), characterized successively in electrophoresis, diffusion, and sedimentation behavior, retained a potency of $170 \times 10^6$ LD$_{50}$ per mg. of N upon recovery. It was observed, however, that even mild physical treatment of the purified toxin resulted in some loss of toxicity, especially at high dilutions.

Electrophoresis

The electrophoretic homogeneity of botulinal toxin was studied with the aid of the Tiselius apparatus (13). One apparatus used was equipped with the Philpot-Svensson optical system (14); another employed in later experiments utilized the Longsworth scanning system (15). All physical measurements were made on preparations equilibrated by long dialysis against buffer. All pH measurements were made with the glass electrode. Toxin of maximum potency prepared by shaking with chloroform is homogeneous in electrophoresis, as is illustrated by the diagrams of Fig. 1. However, the relatively small amounts of purified toxin available restricted the scope of these experiments. In this instance, only the lower middle section and bottom section of the two-compartment cell could be filled with the protein solution. In Fig. 1, photographs at two time intervals are given to illustrate the low degree of boundary spread with time. The upper diagrams represent migration for 11,400 seconds, the lower for 14,400 seconds. The mobility at $1^\circ$ in 0.1 N sodium acetate buffer, pH 4.38, calculated from the diagrams of Fig. 1, is $2.75 \times 10^{-5}$ cm.$^2$ volt$^{-1}$ sec.$^{-1}$.

No indication of a component other than toxin is to be seen in Fig. 1. It was regularly found that toxin freshly crystallized by the original method of Lamanna and coworkers (1) was electrophoretically homogeneous under these conditions. Occasionally, a small, fast moving shoulder was observed on the descending boundary, but this could always be removed by repeated crystallization. On long standing, one preparation (Batch C) yielded a sharp single ascending boundary of normal mobility but a split

1 The initial boundaries are slightly obscured because of light absorption by a detoxifying agent added to the external constant temperature bath.
descending boundary. However, at this time the preparation also appeared somewhat inhomogeneous, as judged both from diffusion studies and from light absorption photographs in the ultracentrifuge. On conversion to toxoid by treatment with formalin, the electrophoretic diagrams of this material were unaltered, although the mobility on the alkaline side of the isoelectric point was increased.

Crystalline toxin, prepared by the modified method of Lamanna and coworkers (11) with the step of shaking with chloroform omitted, was likewise homogeneous in electrophoresis, as shown in Fig. 2. The mobility
at pH 4.38 was $2.69 \times 10^{-6}$ cm.$^2$ volt$^{-1}$ sec.$^{-1}$, the same within experimental error as for toxin prepared with chloroform shaking.

The small degree of boundary spread observed for both types of preparations indicated that the toxin was quite homogeneous in electrophoresis. Since there was some question that the chloroform treatment may have altered the toxin, the boundary spread for the two lots was analyzed statistically by a method already described (16). Unfortunately, the low solubility at the isoelectric point precluded boundary spread analysis in that pH range, the region best suited to this study. For a single moving boundary, the procedure of Sharp et al. (16) yields a value, denoted as the heterogeneity constant, which has the dimensions of mobility and increases with the electrical heterogeneity of the protein. The values for the two preparations compared favorably, being $1.4 \times 10^{-6}$ cm.$^2$ volt$^{-1}$ sec.$^{-1}$ for the salt-fractionated toxin, and $1.8 \times 10^{-6}$ cm.$^2$ volt$^{-1}$ sec.$^{-1}$ for toxin prepared by shaking with chloroform. These figures indicate a relatively low degree of reversible boundary spread (i.e., spread not attributable to diffusion alone).

**Diffusion**

The diffusion constant ($D$) of crystalline toxin, previously found to be homogeneous in electrophoresis, was determined at 25$^\circ$ by the refractive index scale method of Lamm (17), in the Neurath cell and apparatus (18). The values are given in Table I. The subscripts on $D$ indicate the several methods of calculation which allow different weight to various factors (18). For the first time interval given (84,300 seconds, or approximately 1 day) the boundary was yet too sharp for accurate measurement of the scale line displacements. With this exception, there is excellent agreement both among the values found at different time intervals and among those obtained at a given time interval when calculated by the several methods. The latter finding is a good criterion of molecular kinetic homogeneity, as measured by the somewhat insensitive method of diffusion, and may be expressed numerically by the Gralen index (19) given in the last column of Table I as $D_x/D_\infty^2$. The deviation of this constant from unity is a measure of the distribution of sizes in a substance.

Similar evidence for homogeneity in diffusion behavior is offered by the low value of the standard deviation for the diffusion constant calculated at points along the curves by the method of successive analysis (the sixth column, Table I). However, the best criterion of homogeneity in diffusion lies in transforming the actual diffusion curve to normal coordinates and making graphical comparison to the ideal Gaussian distribution curve. The rather good fit obtained by this procedure and the close merging of the maximum ordinates of the ideal and the calculated curves is shown in Fig. 3 (time, 354,840 seconds).
Sedimentation in Ultracentrifuge

The sedimentation characteristics of the crystalline toxin were studied in an air-driven analytical ultracentrifuge (20), the rotor of which carried a sector-shaped cell at a mean radius of 6.5 cm. The ultraviolet light absorption method was employed for preliminary analysis, the Lamm scale method (17) for quantitative studies. Sedimentation velocity scale diagrams obtained at a mean temperature of 32° and a mean centrifugal field of 48,000g have already been published (9). The diagrams revealed a single sharp sedimenting boundary and yielded a value for the sedimentation constant, $S_{2θ}$, of 17.3 Svedberg units at a protein concentration of 0.17 per cent, the lowest concentration studied. Although the base-lines of the sedimentation diagrams exhibited a small angular deviation from the horizontal, planimetric measurement of the area under each boundary revealed a constant concentration of sedimenting substance equal to the initial concentration (calculation by Equation 183a (21), including a correction for the wedge-shaped nature of the cell).

Comparison was made also between the experimentally obtained sedimentation diagrams and the theoretical sedimentation curves which would have obtained if the boundary spreading were due solely to normal diffusion.

### Table I

<table>
<thead>
<tr>
<th>Time (sec.)</th>
<th>$D_M$</th>
<th>$D_A$</th>
<th>$D_{A1}$</th>
<th>$D_σ$</th>
<th>$D_S$</th>
<th>$\frac{D_σ}{D_A}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>84,300</td>
<td>2.08</td>
<td>2.10</td>
<td>2.12</td>
<td>2.19</td>
<td>2.05 ± 0.06†</td>
<td>1.03</td>
</tr>
<tr>
<td>189,480</td>
<td>1.99</td>
<td>1.96</td>
<td>1.96</td>
<td>1.97</td>
<td>2.06 ± 0.07</td>
<td>1.00</td>
</tr>
<tr>
<td>274,860</td>
<td>1.96</td>
<td>1.95</td>
<td>1.95</td>
<td>2.01</td>
<td>2.05 ± 0.12</td>
<td>1.03</td>
</tr>
<tr>
<td>354,840</td>
<td>1.98</td>
<td>1.94</td>
<td>1.89</td>
<td>1.95</td>
<td>2.04 ± 0.05</td>
<td>1.03</td>
</tr>
</tbody>
</table>

Average... 2.01 ± 0.07 $\times$ 10^{-7} cm.$^2$ sec.$^{-1}$

$D_{2θ}$... 2.14 $\times$ 10^{-7} cm.$^2$ sec.$^{-1}$

$D_{2θ}$... 1.87 $\times$ 10^{-7} cm.$^2$ sec.$^{-1}$

* $D_M$, $D_A$, $D_{A1}$, $D_σ$, and $D_S$ denote, respectively, the diffusion constant (with the dimensions cm.$^2$ sec.$^{-1}$) calculated by the maximum height, maximum height-area (unsquared), maximum height-area (squared), standard deviation, and successive analysis methods (18). $D_{2θ}$ and $D_{2θ}$ denote the diffusion constant corrected to the water basis at the respective temperatures. $D_S$ is given to permit comparison with the average value 2.02 $\times$ 10^{-7} cm.$^2$ sec.$^{-1}$, determined independently in the Tiselius apparatus by the Longsworth method on other batches prepared by both groups of workers (8).

† Protein concentration of 0.63 per cent in 0.1 N sodium acetate buffer, pH 4.38.

† Standard deviation from the mean of determinations at six or more points along the curve.
CLOSTRIDIUM BOTULINUM TOXIN

Fig. 3. Comparison of an ideal Gaussian distribution curve with the diffusion curve obtained on a 0.63 per cent solution of crystalline botulinum toxin. ○ indicates the position of the ideal curve; ○ represents the experimental scale line displacements, plotted in normal coordinates. Time, 354,840 seconds.

Fig. 4. Boundary spreading in a sedimentation experiment with crystalline botulinum toxin. The solid line indicates actual points obtained with the Lamm scale method; the dash line represents theoretical spreading due to diffusion alone. Time, 5400 seconds at 48,000 g.

of the toxin. The results are shown in Fig. 4, which gives the scale diagram for Batch BT-3 after 5400 seconds sedimentation at a speed of 25,400 R.P.M. The solid line represents the actual scale diagram; the dash line depicts the calculated curve (calculation by Equation 188 (21)), with the diffusion
constant obtained in separate diffusion experiments (see above). It may be seen that the boundary spread is somewhat greater than that attributable to diffusion alone. This is confirmed by the fact that the apparent diffusion constant calculated from the sedimentation diagrams, though variable, is greater than that obtained from the separate diffusion experiments.

The extraboundary blurring which occurs during the sedimentation of botulinal toxin may have been due to experimental conditions, such as possible slight vibration of the rotor or the difficulty of establishing the zero time of diffusion, or it may be indicative of a small degree of molecular heterogeneity. However, inspection of Fig. 4 reveals that the distribution of sedimentation rates does not differ significantly from the mean rate. If the increased boundary spreading is attributed to molecular heterogeneity, it could have originated either from a population of molecules differing but slightly in molecular size or from the presence of another substance of closely similar sedimentation properties. It was our experience that even mild physical procedures for study of this toxin induced some loss of potency, possibly by toxoid formation through surface denaturation. We thus attribute to this effect the small observed deviation of the actual sedimentation diagrams from theoretical curves calculated from diffusion data.

**Molecular Weight and Shape**

The sedimentation constant and diffusion constant taken together permit the calculation of the molecular frictional ratio \(f/f_o\), according to the Perrin theory (cf. pp. 41–43 (21)), yielding a value of \(f/f_o\), of 1.76. On the assumption that the molecules resemble prolate ellipsoids, this figure corresponds to a ratio of major to minor axis \(b/a\) of 14.6, hydration being neglected (21). Substitution of the sedimentation constant and the diffusion constant in the Svedberg equation (Equation 3a (21)), with a value of 0.75 (8) for the partial specific volume at 20°, yields a molecular weight of 900,000.²

In an effort to ascertain the particle size and shape of botulinal toxin directly, a few attempts were made to photograph a preparation shadowed with gold, by the use of the RCA electron microscope. Because of the small size of the toxin, relative to viruses and other biological substances usually studied in this apparatus, the results were inconclusive. It is hoped that

² The partial specific volume of chloroform-fractionated botulinal toxin calculated from the complete amino acid data is 0.736. This figure based on tentative values for the constituent amino acid molar volumes leads to a slightly lower molecular weight. (Cohn, E. J., and Edsall, J. T., Proteins, amino acids and peptides, American Chemical Society monograph series, New York, 370–375 (1943).)
resort to the newer techniques for the production of metal shadow coatings, with platinum or palladium on glass with subsequent replica stripping, will yield more decisive results in the near future.

**Immunological Homogeneity**

In addition to meeting the physicochemical criteria of homogeneity in electrophoresis and diffusion and sedimenting with a single sharp boundary in the ultracentrifuge, botulinal toxin has proved to be homogeneous in immunological behavior, for both toxin and antitoxin are completely precipitated in the equivalence zone (10). Moreover, quantitative comparison of the behavior in the precipitin reaction of batches of toxin prepared with and without chloroform shaking reveals no difference as a result of this treatment. When the ratio of antitoxin to toxin nitrogen in the precipitate in the zones of antibody excess and equivalence is plotted against the amount of toxin nitrogen added, according to the procedure of Heidelberger and Kendall (22), the curves for toxin prepared by the two methods coincide (cf. Fig. 5). However, for both substances a curvilinear relation obtains, even when the ratio of antitoxin to toxin nitrogen is plotted against the square root of the added toxin concentration. Botulinal toxin thus differs in precipitin behavior from ordinary protein antigens which usually yield a linear relationship between the antibody to antigen ratio and the amount of added antigen. A more complete discussion of the characteristics of the botulinal toxin-antitoxin reaction will be published separately (10).

**DISCUSSION**

Crystalline *Clostridium botulinum* type A toxin meets some of the usual criteria of protein purity, i.e. electrophoretic homogeneity, homogeneity in diffusion, sedimentation with a single boundary, and serological homogeneity in the toxin-antitoxin reaction. However, the toxin fails to meet a more rigorous test of protein purity, namely sedimentation without boundary spreading greater than that solely attributable to normal diffusion. In addition, preliminary phase rule solubility studies indicated the presence of more than one component. The solubility studies were discontinued because surface denaturation of the toxin was readily brought about by the shaking methods used to achieve equilibrium.

The apparent slight molecular inhomogeneity of botulinal toxin is attributed to the marked lability of this protein rather than to the presence of foreign components. Even ordinary handling at room temperature readily induces surface denaturation of solutions, with the formation of opalescence or visible aggregates, requiring clarification by centrifuging at a low speed in the angle centrifuge prior to physicochemical study. Spontaneous loss
of toxicity also occurs on long standing or at high dilution, necessitating the addition of protective agents at the extreme dilutions used in the estimation of biological potency. Rapid inactivation takes place in solutions above pH 7.

The lability and protein nature of highly purified bacterial toxins so far studied appears to be a general phenomenon. Crystalline tetanal toxin, originally electrophoretically homogeneous and apparently a single sub-

![Graph](http://www.jbc.org/)

**Fig. 5.** Composition of toxin-antitoxin precipitates in the zones of equivalence and antitoxin excess. Batch C was crystallized by the chloroform shaking method, Batch S-5, by a different method without shaking with chloroform.

stance serologically, undergoes spontaneous detoxification on standing at 0° without loss in antitoxin-flocculating power but with marked alteration in phase rule solubility properties (23). Moreover, this toxin shows two components in the ultracentrifuge, one apparently an atoxic dimer. Diphtherial toxin is essentially homogeneous by molecular kinetic criteria (24) but is rapidly inactivated above pH 6.0 (5). It has been reported that electrophoretically isolated, electrically homogeneous, erythrogenic toxin
of scarlet fever is nearly homogeneous in the ultracentrifuge as judged by comparison of the actual and theoretical sedimentation curves (25). However, the diffusion constant used in the latter analysis was not obtained by special precise diffusion experiments as in this investigation, but rather was a mean value obtained by calculation from a series of the same sedimentation curves. No bacterial toxin has yet been reported to exhibit a constant solubility. However, purified diphtheria toxoid does have a constant solubility and satisfies other criteria of protein purity (5).

The molecular constants for crystalline botulinal toxins obtained in this investigation are in good agreement with those previously reported. Diffusion studies with the sintered glass disk technique afforded an estimate of about 1 million for the molecular weight (1). The minimum molecular weight calculated from amino acid analysis is approximately 45,000 (6), one-twentieth of the value, 900,000, determined by physical means and reported in this work. The molecular weight calculated by Kegeles (8) from diffusion and viscosity measurements, with the assumption that the toxin molecules approximate elongated ellipsoids, is 1,130,000. Using a different method the latter author obtained a diffusion constant for toxin prepared by the procedure of Lamanna et al. of $D_{20} = 1.79 \times 10^{-7}$ cm.$^2$ sec.$^{-1}$, in good agreement with our value of $1.87 \times 10^{-7}$ cm.$^2$ sec.$^{-1}$ (correction made to 20$^\circ$). However, for preparations made by the method of Abrams et al., Kegeles found a mean value of $D_{20} = 2.13 \times 10^{-7}$ cm.$^2$ sec.$^{-1}$. Since the ultracentrifuge and diffusion data reported in our study were obtained only on chloroform-fractionated toxin, the identity of size and shape of the toxin prepared by the several methods has not yet been clearly established.

Conclusions with regard to the apparent molecular shape of this protein must await further study. The frictional ratio derived from the sedimentation and diffusion constants in this investigation ($f/f_o = 1.76$) is markedly greater than that obtained from viscosity measurements (8) and the assumption of an elongated ellipsoidal molecular model ($f/f_o = 1.45$). In the latter study, fractions prepared by both methods were used in viscosity measurements, though one preparation subsequently proved to be electrophoretically inhomogeneous. Unless excessive hydration is assumed, neither value of the frictional ratio derived by these indirect physical methods is in accord with the report that electron micrographs of inactive formalin-treated toxin (toxoid) show particles nearly spherical in shape (11).

No explanation for the extreme toxicity of this protein can be deduced either from its composition or from its physical properties. However, the finding of a high molecular weight for botulinal toxin compared to other bacterial poisons poses two difficult problems: first, the explanation of the apparent absorption of the toxin through the gut in accidental botulism resulting from ingestion of spoiled foods; second, the elucidation of its mode
of action on administration by either the oral or intraperitoneal route. The singular oral toxicity of this protein is in accord with reports that it is relatively resistant to the proteolytic action of pepsin and trypsin (cf. (26)).

The suggestion has been made that tetanal, botulinal, and diphtherial toxins may act by interfering with the synthesis of some essential enzyme, and some evidence has been presented indicating that diphtherial toxin perhaps blocks the synthesis of cytochrome b or some closely related enzyme (26). However, botulinal toxin is far more potent than diphtherial toxin (26), and it may be calculated that on the basis of a molecular weight of 900,000 only 20,000,000 molecules are required to kill a mouse. This fact, together with the protein nature of the toxin, suggests that it may attain its potency indirectly by acting enzymatically to synthesize a cellular poison from some normal metabolite or otherwise break an indispensable link in some physiological reaction chain in nervous tissue.

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**SUMMARY**

1. Crystalline type A botulinal toxin is homogeneous in electrophoresis and in diffusion.
2. The toxin sediments with a single sharp boundary in the ultracentrifuge but with spreading somewhat greater than that attributable solely to diffusion. This behavior may indicate slight molecular heterogeneity attributable to the lability of this protein.
3. The molecular weight obtained from sedimentation and diffusion data is 900,000; the apparent frictional ratio is 1.76.
4. Quantitative precipitin studies indicate that botulinal toxin is serologically a single substance. The ratio of antibody to toxin in the precipitates in the zones of antibody excess and equivalence is not linearly related to the amount of antigen added.
5. Crystalline toxin prepared by several methods possesses identical electrophoretic and serological properties.
6. Its extreme potency, high molecular weight, and protein nature suggest that botulinal toxin may be an enzyme. However, no explanation of the mode of its pharmacological action is afforded by these studies.

**BIBLIOGRAPHY**

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