THE PURIFICATION OF TOXIN FROM CLOSTRIDIUM BOTULINUM TYPE A

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Until recently it could be said that the status of our knowledge of bacterial exotoxins had not changed since Roux and Yersin in 1888 (1) first obtained the filtrable diphtheria toxin. Indeed, Topley and Wilson in 1936 (2) stated, "We have not yet succeeded in isolating any bacterial exotoxin in a chemically pure state, although a considerable degree of concentration has been attained by various methods of fractional precipitation."

The same year Eaton (3) brought to light the first proof that a bacterial toxin (diphtheria) was a single protein-like substance by reporting the isolation of the toxin in a purified form. In 1937, Pappenheimer (4) isolated and characterized a toxic protein which appeared to be identical with diphtheria toxin. The properties of this protein were almost identical with those of the substance isolated by Eaton. With the protein nature of one bacterial toxin established, work on other bacterial toxins (streptolysin (5), scarlet fever (6, 7), tetanus (8), and Clostridium botulinum type A) has confirmed the validity of the concept that bacterial exotoxins are proteins.

When a toxin has been identified as a protein, proof of its purity presents many complex problems. Kekwick and McFarlane (9) present three criteria of a stable chemical compound: constancy of chemical composition, homogeneity of physical properties, and constancy of solubility. In work with a biologically active protein, the first criterion should of course include constancy of chemical composition and activity. A few of these criteria have been applied to bacterial toxins; e.g., diphtheria toxin (10) and scarlet fever toxin (7).

Previous work by Sommer (11) on Clostridium botulinum type A toxin has yielded a preparation containing 250,000,000 mouse m.l.d. per gm. This toxin was prepared in a peptic digest medium by precipitation at pH 3.5 to 4.0 and resolution in sodium acetate buffer. Since washed dried organisms were found to contain 100,000,000 mouse m.l.d. per gm., Sommer (11) assumed that the chemically pure toxin must have a potency many times greater.

2 This term is not defined by Sommer; in this paper, 1 m.l.d. is defined as the smallest amount of toxin which causes death within 4 days of all mice injected.
In the present study, Sommer's method of purification by acid precipitation and acetate buffer resolution has been extended according to the usual methods of alcohol and salt fractionation of proteins. The steps of fractionation have been followed by mouse m.l.d. titrations and nitrogen determinations and also by ultraviolet absorption spectra and electrophoresis. The fractionations, to be described in detail below, have resulted in preparations which are electrophoretically homogeneous, show characteristic protein properties, and have a maximum value of $220 \times 10^6$ mouse m.l.d. per mg. of nitrogen. Crystalline material has been obtained from these preparations. The variation of electrophoretic mobility with pH has been studied. Other physicochemical properties of the purified toxin are being studied and will be reported in a subsequent paper.

Methods

Strains—The Hall strain of Clostridium botulinum type A was used in preparing all cultures for toxin production. The strain was maintained in 1 per cent peptone, beef heart infusion broth, which contained chopped beef heart in the bottom of each tube. After an initial growth period of 24 hours at $34^\circ$, the cultures were stored at $4^\circ$ until needed. In the preparation of seed, the culture was grown 18 to 24 hours in chopped beef heart medium, then transferred to a 2 per cent pepticase medium for the same length of time. 2 ml. of the seed culture were added to each 100 ml. of medium, which was planted for toxin production.

Medium—The medium used for toxin production was prepared as follows: 2 per cent pepticase (a commercial tryptic digest of casein); 0.75 per cent corn steep liquor (containing approximately 60 mg. of N per ml.); tap water to volume; pH adjusted to 7.5; and 16 liters dispensed into each 5 gallon Pyrex bottle.

For more uniform toxin production the pepticase was treated with charcoal before being added to the medium. To each 100 gm. of pepticase, tap water was added to bring the volume to 500 ml. Solution was obtained by heating the mixture in the autoclave at 15 pounds pressure for 5 minutes. As soon as the pepticase solution was removed from the autoclave, 5 gm. of U. S. P. charcoal were added and the mixture was shaken intermittently.

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3 This work was considered to be of such an important nature that two groups working independently were assigned to it. The study reported here was undertaken as preliminary to an investigation into the nature of Clostridium botulinum type A toxoid.

4 Crystalline Clostridium botulinum type A toxin was previously prepared by Dr. Carl Lamanna and coworkers, working independently at Camp Detrick and using completely different methods.

for 30 minutes. The charcoal was then removed by filtration. This amount of pepticase was sufficient for preparing 5000 ml. of medium.

The corn steep liquor was treated with alkali and heated before it was added to the medium. To 100 ml. of crude corn steep liquor were added 200 ml. of cold tap water plus enough NaOH to bring to pH 9.0 to 9.5. Tap water was added to bring the volume to 400 ml. The mixture was heated in the autoclave at 15 pounds pressure for 5 minutes, then clarified by filtration. This filtrate was stored at 4° under toluene and used as needed. 30 ml. of this solution were used to prepare 1000 ml. of medium.

The bottles were autoclaved 1½ hours at 15 pounds pressure, commencing when the temperature of the exhaust line had reached 115.5°. The autoclave was opened 2 hours after the steam was turned off. The medium was allowed to cool in the autoclave overnight.

0.5 per cent dextrose was added when the medium had cooled to 40° (400 ml. of a sterile 20 per cent solution to 16 liters of medium).

Growth—As soon as the medium had cooled to 35°, each bottle was planted with 300 ml. of seed. All cultures were grown 4 days at 33-34°. A daily check of the pH was made and adjustment with 5 N HCl or 5 N NaOH was made as necessary to maintain the reaction at pH 5.7 to 6.0. At the end of the growth period each bottle was checked for purity by examining stained smears, and by subculture in nutrient broth, nutrient agar slants, and nutrient agar shake tubes.

Toxicity Test—The number of minimum lethal doses per ml. was determined by injecting intraperitoneally into 20 gm. mice 1 ml. volumes of toxin diluted in 0.2 per cent gelatin buffer (pH 6.8).2

Flocculation Tests (Lf)—Flocculation tests were carried out in order to determine the amount of antitoxin with which the toxin combined in vitro. The Lf value of a toxin is the number of units of antitoxin with which 1 ml. of toxin unites and flocculates in the shortest interval of time (12). All Lf determinations were carried out in a water bath at 42° with 1 ml. portions of diluted toxin and amounts of antitoxin6 (150 units per ml.) varying from 0.03 to 0.20 ml.

Chemical Determinations—Total nitrogen was determined by the micro-Kjeldahl method. Total phosphorus was determined by King's (13) method.

Electrophoresis—Electrophoresis investigations were carried out in the Tiselius apparatus (14), as modified by Longsworth (15). Toxin preparations were dialyzed statically at 2-5° against approximately 10 volumes of buffer, with change of buffer three times daily for 3 days, with measurements of conductivity to determine completion of dialysis (16). Relative concentrations were estimated by tracing the curves obtained under a

6 Globulin-modified antitoxin, Lederle.
photographic enlarger and determining areas under the traced pattern with a polar planimeter, the same specific refractive increment for all components having been assumed.

_Ultraviolet Absorption Spectra_—Determinations of the ultraviolet absorption spectra were made with the Beckman spectrophotometer with quartz cells of 1 cm. width.

**EXPERIMENTAL**

_Preliminary Study of Nature of Toxin; Stability of Toxin_—Experiments were carried out to confirm the reported inactivation of *Clostridium botulinum* type A toxin by alkali and heat (17). It was found that at room temperature the toxin was most stable between pH 1.0 and 6.0 with maximum stability between 4.0 and 5.0, while above pH 7.0 the toxin was rapidly destroyed. It was found also that a temperature of 60° at pH 5.0 was sufficient to destroy 100,000 m.l.d. in 5 minutes. In order to prevent inactivation of the toxin during the purification procedure, the pH was maintained below 7.0 by means of the buffers (1 per cent sodium acetate and 1 per cent potassium phosphate) and the temperature was maintained at 4° except during precipitation with Na₂SO₄, which was carried out at room temperature.

_Fractionation with Acid, Alcohol, and Na₂SO₄_—The whole culture containing both toxin and bacteria was adjusted to pH 3.5 and allowed to stand at room temperature for several days. The precipitate which formed was separated and washed by decantation several times with distilled water. The toxin was extracted by suspending the precipitate in one-fourth the original volume of 1 per cent sodium acetate solution adjusted to pH 6.5. After three such extractions the supernatants were combined and the insoluble residue was discarded. The extracts were adjusted to pH 3.5. The precipitated toxin was separated, washed twice with distilled water, and then redissolved in one-fourth the original volume of 1 per cent sodium acetate solution at pH 6.5. This concentrated toxin solution (Fraction A, Table I) was used for alcohol fractionation.

_Fractionation with ethyl alcohol was carried out at 4° at pH 6.5. Precipitates were centrifuged and washed with the same concentration of alcohol (buffered at pH 6.5) used in precipitation, and then taken up in distilled water. Fractions were obtained with 10, 20, 40, and 50 per cent ethanol. Table I summarizes the data obtained for each fraction._

_Fraction II, obtained with 20 per cent alcohol, was fractionated further with Na₂SO₄ at pH 6.5 at room temperature. The toxin was first precipitated by adding a half volume of a saturated Na₂SO₄ solution prepared_

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7 These determinations were carried out by Dr. E. C. Smith.

8 This residue may carry with it as much as 50 per cent of the toxin.
at 33°. The precipitate was removed by centrifuging and dissolved in distilled water. This toxin was then treated with Na$_2$SO$_4$ solution to yield two fractions, one precipitated at 0.18 saturation, the other at 0.4 saturation (Fraction II-S-2). The data on these fractions are also given in Table I.

**Table I**

*Results of Preliminary Fractionation of Crude Clostridium botulinum Type A Toxin*

<table>
<thead>
<tr>
<th>Product</th>
<th>Volume</th>
<th>N$_2$O</th>
<th>N:P ratio</th>
<th>Mouse m.l.d. in millions</th>
<th>Electrophoretic data at pH 6.52-6.82, relative concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml.</td>
<td>mg. per ml.</td>
<td></td>
<td>Per ml.</td>
<td>Total</td>
</tr>
<tr>
<td>Whole culture</td>
<td>44,000</td>
<td>2.18*</td>
<td>0.8</td>
<td>35,000</td>
<td>0.37*</td>
</tr>
<tr>
<td>Fraction A, concentrated toxin solution</td>
<td>295</td>
<td>1.37</td>
<td>8</td>
<td>2,400†</td>
<td>6.0</td>
</tr>
<tr>
<td>Fraction I, 10% alcohol</td>
<td>100</td>
<td>0.743</td>
<td>16.2</td>
<td>8</td>
<td>800</td>
</tr>
<tr>
<td>&quot; II, 20%</td>
<td>105</td>
<td>0.904</td>
<td>13.2</td>
<td>32</td>
<td>3,400†</td>
</tr>
<tr>
<td>&quot; III, 40%</td>
<td>65</td>
<td>0.288</td>
<td>5.1</td>
<td>2</td>
<td>130</td>
</tr>
<tr>
<td>&quot; IV, 50%</td>
<td>90</td>
<td>1.25</td>
<td>2.1</td>
<td>&lt;0.5</td>
<td>&lt;45</td>
</tr>
<tr>
<td>&quot; II-S-1, 0.18 saturated Na$_2$SO$_4$</td>
<td>40</td>
<td>0.740</td>
<td>15.0</td>
<td>4</td>
<td>160</td>
</tr>
<tr>
<td>Fraction II-S-2, 0.40 saturated Na$_2$SO$_4$</td>
<td>250</td>
<td>0.320</td>
<td>14.4</td>
<td>32</td>
<td>800</td>
</tr>
<tr>
<td>Fraction II-S-2', pH 5.0 supernatant</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 287-IV-A,† water-insoluble</td>
<td>15</td>
<td>0.300</td>
<td></td>
<td>60</td>
<td>100</td>
</tr>
</tbody>
</table>

* Value obtained from another experiment.
† The discrepancy in these values may be due to inaccuracies in mouse m.l.d. titrations in which 2-fold dilutions are used.
‡ Prepared from a different lot of crude toxin. The unusually low m.l.d. per mg. of N is explained in the text.

It was possible by further fractionation to obtain a greater degree of purity of Fraction II-S-2. By adjustment to pH 5.0, a considerable amount of colored impurity precipitated out at 6°. This material was removed by centrifugation. The toxin was precipitated in 0.4 saturated Na$_2$SO$_4$ at pH 6.5 and redissolved in cacodylate buffer at pH 6.78 (Fraction II-S-2'). The data on this fraction are shown in Table I.

A new lot of toxin was prepared and purified in the same manner as
described above. One additional step in the fractionation procedure consisted of precipitation of the toxin by dialysis against distilled water. The toxin was separated and redissolved by dialysis against approximately 0.1 per cent NaCl at 4°. By this procedure a small amount of colored impurity became insoluble and was removed. The supernatant containing the toxin formed a white precipitate in 0.3 saturated Na₂SO₄, which dissolved in cacodylate buffer at pH 6.8 (Fraction 287-IV-A). The data on this fraction are shown in Table I.
The electrophoretic patterns of the four most active fractions above are shown in Fig. 1 and the ultraviolet absorption curves are shown in Fig. 2.

Results of Preliminary Fractionations—The data in Table I outline the progress in successive steps in the purification. The column, m.l.d. per mg. of N, gives an indication of the relative purity of each toxin fraction. Thus while the crude culture contained $0.37 \times 10^6$ mouse m.l.d. per mg. of N, the more purified preparations contained up to $100 \times 10^6$ mouse m.l.d. per mg. of N.

By means of fractionation with 20 per cent alcohol a separation was obtained between high phosphorus- and low phosphorus-containing substances. Further fractionation did not further reduce the N:P ratios.

The electrophoretic data, Table I and Fig. 1, illustrate the progressive increase of a single component, with gradual reduction of other components as the fractionation progressed. Although 20 per cent alcohol removed considerable nucleic acid, the electrophoretic pattern showed approximately 9 per cent of a substance with a mobility like that of nucleic acid.
Subsequent fractionation removed all of the fast moving component and finally yielded Fraction 287-IV-A, which was found to contain essentially one component.

By means of electrophoretic separation runs, Component A was isolated free of the other components in both Fraction II and Fraction II-S-2. It was found that in both instances Component A was the toxin.

The low value of $60 \times 10^6$ mouse m.i.d. per mg. of N of Fraction 287-IV-A, which is essentially homogeneous, is in sharp contrast to the $100 \times 10^6$ mouse m.i.d. per mg. of N for Fraction II-S-2', which contained only 63 per cent toxin (Component A). One explanation for the low m.i.d. value of Fraction 287-IV-A is a possible conversion of toxin to toxoid.

The ultraviolet absorption curve for Fraction II (Fig. 2) confirms the presence of nucleic acid by a wide absorption band (250 to 280 m$\mu$) which is typical of nucleoproteins, or a mixture of nucleic acid and proteins. Fraction II-S-2 and the electrophoretically isolated Component A from both Fraction II and Fraction II-S-2 gave sharp absorption maxima at 278 m$\mu$ with no evidence of nucleic acid absorption.

Isolation of Nucleic Acid from Culture Fluid—A nucleic acid was separated from the culture supernatant after precipitation of the crude toxin at pH 3.5. The supernatant was acidified further to pH 2.0 and the precipitate was removed and dissolved at pH 4.0. On addition of an equal volume of 95 per cent alcohol, precipitation took place. The precipitate was dissolved and reprecipitated twice more with 95 per cent alcohol and was finally washed with 95 per cent alcohol and dried. This product contained 13.6 per cent nitrogen and 7.65 per cent phosphorus, and had an N:P ratio of 1.78. The absorption spectrum showed a powerful absorption with a maximum at 258 m$\mu$ (Fig. 2, Curve I).

A strong test for ribose was obtained with Bial's orcinol-HCl reagent. The test for the desoxyribonucleic acid with diphenylamine was only slightly positive. This indicated that the nucleic acid was predominantly the ribose type.

Further evidence for the identity of the isolated nucleic acid with ribonucleic acid was obtained by comparing the absorption spectra of ribonucleic acid, desoxyribonucleic acid, and the Clostridium botulinum nucleic acid after reaction with Bial's orcinol-HCl reagent (Fig. 3). The spectrum of Clostridium botulinum nucleic acid followed that for ribonucleic acid.

Isolation and Crystallization of Toxin—By a study of the preliminary fractionation procedures, it was possible to devise a simplified method for the isolation of the toxin. The procedure is outlined in the accompanying flow diagram.

When the toxin had been sufficiently purified, as shown in the diagram, it was readily obtained in the form of fine needle-like crystals. In order
to get a water-clear solution of toxin before crystallization it was necessary to treat the cloudy, slightly yellow solution with 0.5 saturated ammonium sulfate. By re-solution in phosphate buffer at pH 6.8 and by prolonged centrifugation, it was possible to remove all insoluble material from the concentrated toxin solution. The crystallization was accomplished by dialyzing the concentrated toxin solution containing approximately 1 per cent protein against a low concentration of (NH₄)₂SO₄ (0.1 saturated) at pH 6.8 and 4°. The salt concentration was gradually increased until the solution became opalescent; it varied between 0.1 and 0.3 saturation, depending on the concentration of toxin. If precipitation occurred too rapidly, there resulted either minute crystals, an amorphous precipitate, or a mixture of both.

The crystalline toxin had the following properties: (a) The crystals were uniform in size and shape, and free of amorphous material when viewed under the microscope (Fig. 4); (b) the toxin was recrystallized from phosphate buffer, pH 6.8, on dialysis in 0.3 saturated (NH₄)₂SO₄ at 4°; (c) the solution of twice crystallized material had a higher toxicity (220 × 10⁶ m.l.d. per mg. of N) than any previously obtained; (d) the toxin was flocculated by antitoxin with a ratio of 110,000 m.l.d. perLf, which is slightly higher than the value of 80,000 m.l.d. per Lf found for crude toxin; (e)
Isolation of Toxin

Starting Material—192 liters crude toxin (whole culture) $1 \times 10^6$ m.l.d. per ml.; pptd. with acid at pH 3.5; acid ppt. washed 3 times with H$_2$O by settling and decantation; acid ppt. in 3 liters adjusted to pH 6.8 with 20% K$_2$HPO$_4$ and 5 N NaOH; saturated Na$_2$SO$_4$ solution added to 0.4 saturation; supernatant decanted and discarded.

Add water to 5 liters volume; adjust to pH 5.0; let stand 24 hrs.; decant supernatant

<table>
<thead>
<tr>
<th>Residue I</th>
<th>Supernatant I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add 1% phosphate buffer, pH 6.8, to 5 liters volume; adjust to pH 6.8; mix well; adjust to pH 5.0; allow to stand; decant supernatant</td>
<td>5 liters volume; m.l.d., $20 \times 10^6$ per ml.; 52% recovery</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Residue II</th>
<th>Supernatant II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treat same as Residue I</td>
<td>5 liters volume; m.l.d., $5 \times 10^6$ per ml.; 13% recovery</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Residue III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discard</td>
</tr>
</tbody>
</table>

5 liters volume; m.l.d., $1 \times 10^6$ per ml.; 2.6% recovery

Pool Supernatants I, II, and III; adjust to pH 3.5; ppt. forms; centrifuge

Supernatant

Discard

Ppt.

Dissolve in 1500 ml. 1% phosphate buffer, pH 6.8; dialyze against Na$_2$SO$_4$ to 0.4 saturation; ppt. forms; centrifuge

<table>
<thead>
<tr>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discard</td>
</tr>
</tbody>
</table>

Supernatant

Discard

Ppt.

Dissolve in 500 ml. 1% phosphate buffer, pH 6.8; adjust to pH 4.7; centrifuge

<table>
<thead>
<tr>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolve in 130 ml 1% phosphate buffer, pH 6.8; cloudy, slightly yellow solution; m.l.d. $500 \times 10^6$ per ml.; 34% recovery; total N, 2.25 mg. per ml.; m.l.d. per mg. N, $220 \times 10^6$</td>
</tr>
</tbody>
</table>
it was electrophoretically homogeneous (Fig. 5) with a mobility corresponding to that of other preparations of the toxin; (f) the ultraviolet absorption curve of twice crystallized toxin (Fig. 6) showed a single sharp maximum at 278 m\(\mu\).

Influence of pH on Electrophoretic Mobility—The influence of pH on the electrophoretic mobility of *Clostridium botulinum* toxin has been investi-

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**Fig. 4.** Photomicrograph of crystals of the toxin produced by *Clostridium botulinum* type A (first crystallization; 450 \(\times\)).

**Fig. 5.** Electrophoretic pattern of crystalline *Clostridium botulinum* toxin type A in acetate buffer of 0.06 ionic strength at pH 4.12 after 6630 seconds at 5.24 volts per cm.

gated over a wide range of pH values. The results are given in Fig. 7. Precise determination of the isoelectric point was extremely difficult, owing to the very low solubility of the toxin in the neighborhood of the isoelectric point at 1.0°. The protein concentrations ranged from about 0.1 per cent or less (near the isoelectric point) to 0.5 per cent, except for one run at pH 4.47 made with 1 per cent protein. The following uni-univalent buffers were used (18): 0.10 \(\mu\) HCl-glycine at pH 3.2; 0.06 \(\mu\) sodium acetate-
acetic acid from pH 4.0 to 5.5; 0.10 μ sodium cacodylate-cacodylic acid from pH 6.0 to 7.0; and 0.06 μ cacodylate buffer at pH 7.02. The mobility determinations were made on electrophoretically homogeneous or nearly homogeneous samples from three different preparations of toxin, with the
exception of two determinations made on a 68 per cent homogeneous preparation. Measurements of pH were made at room temperature with a Beckman glass electrode and pH meter, calibrated with 0.05 M potassium acid phthalate at pH 4.00. The correction of the pH values of the buffers to 1.0° was assumed to be negligible. Mobilities were determined from the rate of movement of the maximum ordinate, which in the case of the purified toxin preparations involved negligible error, because the refractive gradient curves were quite symmetrical (18).

The isoelectric point is estimated from our data to be at pH 5.6, with $(du/dpH)^o = 1.9$ near the isoelectric point (19).

The properties of the purified toxin are listed in Table II.

### Table II

<table>
<thead>
<tr>
<th>Activity</th>
<th>Toxicity per mg. nitrogen Mg. nitrogen per Lf unit</th>
<th>220 $\times 10^6$ mouse m.l.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>$0.00048$</td>
<td>$14.1%$</td>
</tr>
<tr>
<td>Phosphorus</td>
<td></td>
<td>$0.1%$</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td></td>
<td>Trace</td>
</tr>
<tr>
<td>Biuret test</td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Heat coagulability</td>
<td></td>
<td>pH 5.60</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td></td>
<td>$278 \mu$</td>
</tr>
<tr>
<td>Absorption maximum extinction</td>
<td></td>
<td>$0.1%; E = 1.67$ at $278 \mu$</td>
</tr>
</tbody>
</table>

* Determined on twice crystallized toxin unless otherwise indicated.
† Determined on a lyophilized sample of 90 per cent electrophoretically homogeneous toxin.
‡ Determined on toxin crystallized once.

### DISCUSSION

One of the difficult problems in the isolation of a bacterial toxin is that of handling large volumes of fluid and recovering small amounts of active material. This problem was present in the purification of *Clostridium botulinum* toxin, since each liter of whole culture contained only about 20 mg. of toxin. By utilizing the acid precipitation technique at pH 3.5 it was possible to recover a portion of the toxin in a greatly purified state in a reduced volume. The data in Table I show that a 20-fold increase in purity of the toxin was attained by this one step, although only about 7 per cent of the toxin was recovered. Later work in which the toxin was extracted promptly from the acid precipitate showed recoveries approaching 70 per cent of the original toxicity of the whole culture.
The preliminary fractionation experiments with alcohol and Na₂SO₄ yielded information as to the nature of the impurities in the crude toxin (Table I). By means of 20 per cent alcohol it was possible to separate a highly active, low phosphorus-containing fraction (N:P = 13.2) from fractions containing more phosphorus (N:P = 5.05 and 2.08) and with much lower activity, thus showing phosphorus-containing impurities to be present. Much of this phosphorus-containing impurity was nucleic acid, since actual isolation from the culture fluid, ultraviolet absorption, and electrophoretic patterns showed it to be present in the crude toxin, whereas purified preparations showed no evidence of it. The ultraviolet absorption studies (Fig. 2) showed the transformation from nucleoprotein absorption (250 to 280 mμ) of the crude toxin to protein absorption alone at about 280 mμ, as the purification proceeded.

The presence also of a non-nucleic acid, phosphorus-containing impurity was indicated by an examination of the data on Fractions II and II-S-2. Although fractionation of Fraction II with Na₂SO₄ to give Fraction II-S-2 caused practically no change in the N:P ratio, the electrophoretic patterns showed a complete disappearance of the nucleic acid component (Fig. 1), and ultraviolet absorption data showed a sharp decrease in nucleic acid absorption (Fig. 2, Curves II and III).

The fractionation procedures which are described in the early parts of the experimental sections, and which were followed closely by ultraviolet absorption, electrophoresis, and toxicity, are presented to show how the conditions necessary for the best method of purification were developed. The fractionation procedures with alcohol were not studied further. The selection of Na₂SO₄ and (NH₄)₂SO₄ as fractionating agents does not imply that alcohol would not work just as well.

Maximum toxicity per mg. of nitrogen, electrophoretic homogeneity, and a single ultraviolet absorption band at 278 mμ have been used as criteria for following the various purification procedures. One fraction which fulfilled the latter two criteria had a toxicity of only 60 × 10⁶ mouse m.l.d. per mg. of nitrogen, which was much lower than the maximum value of 220 × 10⁶ mouse m.l.d. per mg. of nitrogen. On examination of the flocculating power of this toxin with antitoxin, it was found that 30,000 mouse m.l.d. constituted 1Lf unit of toxin, as compared with 80,000 m.l.d. per Lf unit which was obtained with fresh toxin. One explanation of the reduction in toxicity of this fraction is a possible spontaneous conversion of toxin to toxoid. The validity of this explanation depends upon the assumption (1) that the flocculating power of a toxin is not affected by this conversion process, (2) that the proportion of toxin to toxoid in crude toxin is fairly constant from one preparation to another, and (3) that toxoid has the same electrophoretic properties as toxin. In regard to the first two
assumptions, no evidence has been found to invalidate them. As to the third assumption, nothing is known regarding the properties of the spontaneously formed toxoid.

The final method of isolation of the toxin shown in the flow diagram was found to give a 34 per cent recovery of toxin. This procedure is a refinement and combination of procedures developed in the preliminary phase of this work. It was found to be very important to carry out the Na_2SO_4 fractionation at pH 6.8. In this way a more complete separation of toxin and nucleic acid-like substances was obtained. The colored impurities were best removed by precipitation at pH 4.7 to 5.0. By a repetition of these procedures at increasingly greater concentrations of toxin it has been possible to effect a remarkable degree of purification.

The criteria which showed that the toxin is a single substance are (1) constancy of activity through two successive crystallizations at 220 million mouse m.l.d. per mg. of nitrogen and (2) electrophoretic homogeneity in

### Table III

**Comparison of Some Properties of Biologically Active Bacterial Proteins**

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Activity per mg.</th>
<th>Activity per Lf</th>
<th>N</th>
<th>S</th>
<th>Iso-electric point</th>
<th>Biuret test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diphtheria (4)</td>
<td>10,000 guinea pig m.l.d.</td>
<td>30 m.l.d.</td>
<td>0.46</td>
<td>16</td>
<td>0.75</td>
<td>4.1 Positive</td>
</tr>
<tr>
<td>&quot; (3)</td>
<td>10,000 guinea pig m.l.d.</td>
<td>20 to 35 m.l.d.</td>
<td>0.50</td>
<td>16</td>
<td>Negative</td>
<td>&quot;</td>
</tr>
<tr>
<td>Scarlet fever (7)</td>
<td>150 million skin test doses (2)*</td>
<td>15.2</td>
<td></td>
<td></td>
<td>0.71</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot; (6)</td>
<td>18 million skin test doses</td>
<td>30,000 skin test doses</td>
<td>0.23</td>
<td>14.0</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Streptolysin (5)</td>
<td>3,000 hemolytic units (2)†</td>
<td>16.8</td>
<td></td>
<td>2.34</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>Tctanus (8)</td>
<td>6.4 million mouse m.l.d.</td>
<td>12,000 m.l.d.</td>
<td>0.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium botulinum</td>
<td>30 million mouse m.l.d.</td>
<td>110,000</td>
<td>0.48</td>
<td>14.1</td>
<td>5.6 Positive</td>
<td></td>
</tr>
</tbody>
</table>

The numbers in parentheses are bibliographic references.

* The smallest amount of toxin that will, on the average, produce an erythematous reaction, 1 cm. in diameter, in the skin of a susceptible person in 24 hours.

† The amount of hemolysin which liberates 3.75 mg. of hemoglobin from a suspension of rabbit red cells containing exactly 7.5 mg. of hemoglobin, when 1.0 ml. of hemolysin solution at pH 6.5 is added to 1 ml. of red cell suspension and incubated for 30 minutes.
CLOSTRIDIUM BOTULINUM TYPE A TOXIN

the range tested from pH 3.2 through 7.0. That the toxin is a protein has been demonstrated by the properties listed in Table II.

The activity of Clostridium botulinum toxin places it among the group of highly active bacterial proteins (Table III). A recent preliminary report (8) places tetanus toxin in the same group. Scarlet fever toxin isolated electrophoretically is reported to be about 5 times as active as Clostridium botulinum toxin; however, its activity was measured by skin reactivity rather than by lethal power.

Since Clostridium botulinum type A toxin can now be prepared in pure form, the reaction of the toxin with formaldehyde, to produce toxoid, should be studied further. A toxoid produced in this manner would be pure enough for detailed study of its structure and composition. In addition such a toxoid should be free of bacterial protein. Preliminary work9 has shown that toxoid produced from purified toxin is highly antigenic in mice.

SUMMARY

A protein has been isolated from the Clostridium botulinum type A cultures and has been found to have the biological and immunological properties of the toxin. It behaves like a globulin with an isoelectric point of pH 5.6 and a total nitrogen of 14.1 per cent. It crystallized readily in 0.10 to 0.30 saturated \((NH_4)_2SO_4\) at \(4^\circ\), forming small needle-like crystals. Twice crystallized toxoid contained \(220 \times 10^6\) mouse m.l.d. per mg. of nitrogen.

The authors are greatly indebted to Dr. Norman Weissman, through whose cooperation the chemical analyses were made possible, and to Dr. Dennis W. Watson for many valuable suggestions.

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THE PURIFICATION OF TOXIN FROM CLOSTRIDIUM BOTULINUM TYPE A
Adolph Abrams, Gerson Kegeles and George A. Hottle


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