THE PURIFICATION AND CONCENTRATION
OF DIPHTHERIA TOXIN

III. SEPARATION OF TOXIN FROM BACTERIAL PROTEIN

MONROE D. EATON

Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis, Missouri

Received for publication February 13, 1937

Diphtheria toxin purified by a variety of methods has always contained bacterial protein. This raises the question whether the predominant protein in purified preparations is a fragment of the bacterial protoplasmin which carries the toxin, or is itself the toxin. In a previous paper (Eaton, 1936b) it was shown that in various purified preparations there is no relation between the titer of bacterial precipitinogen and the concentration of toxin. Furthermore, purification apparently reduces considerably the amount of bacterial precipitinogen, relative to toxin.

This paper will describe methods of iso-electric point fractionation used for the separation of toxin and bacterial proteins, and will present evidence that the residuum of bacterial protein left in purified toxin after fractionation constitutes a practically negligible part of the total protein present.

Small amounts of red porphyrin compounds are detectable by spectroscopic examination in purified and concentrated preparations. The possibility that a protein combined with these porphyrins may constitute another bacterial fragment in purified toxins must be considered. From studies of the properties of the porphyrins it appears that they are not firmly bound to any protein in the purified preparations but are strongly adsorbed to protein precipitates. A method will be described for the more complete separation of these colored substances from toxin.
On the basis of fractionation experiments described in the previous paper of this series it was concluded that the purified toxin contained several proteins differing in solubility. It is now evident that some of the protein then believed to be an impurity is actually slightly denatured toxin.

METHODS FOR SEPARATING TOXIN FROM BACTERIAL PROTEIN

Three different acid precipitation methods were applied to toxin purified by metal salt precipitation (Method III; Eaton 1936a). The toxin solutions used were quite concentrated, containing 300 to 600 Lf units per cubic centimeter.

1. Nucleic acid. An equal volume of 1 per cent nucleic acid solution neutralized with sodium hydroxide is added to the buffered toxin solution. The pH is adjusted to 5.3 with acetic acid and the resulting precipitate is centrifuged down, washed, and dissolved in dilute sodium bicarbonate solution at pH 8.4.

2. Phosphoric acid at 37°C. A phosphate buffer solution of the toxin is adjusted to pH 5.0 with phosphoric acid and incubated at 37°C for one to two hours. A slight denaturation of the toxin is purposely produced in this way in order to lower its solubility. The precipitated toxin is extracted with phosphate buffer at pH 7.0 to remove traces of bacterial protein. Most of the toxin remains undissolved but may be dissolved in very dilute alkali. The pH is then readjusted to 8.4 by adding the requisite quantity of acid sodium phosphate.

3. Acidified one-third-saturated ammonium sulphate solution. Ammonium sulphate to one-third of saturation is added to the toxin solution and the pH brought to 6.2. The small amount of colored precipitate is filtered off. The filtrate is then adjusted to pH 5.4 with sulphuric acid and placed in the ice box. The slow formation of a granular precipitate may continue for 48 hours. The precipitate is washed with one-third-saturated ammonium sulphate solution previously adjusted to pH 5.4, and dissolved directly in phosphate buffer. The whole procedure is then repeated.

If the toxin precipitate obtained as just described is suspended in water before adding buffer, denaturation occurs so that most of the toxin becomes insoluble at pH 7.0.
Purification of Diphtheria Toxin

Isolation of Bacterial Protein

Bacterial precipitinogen was detected and measured by means of the ring test using anti-serum obtained by injecting rabbits with washed diphtheria bacilli. Details have been given in the second paper of this series. All tests were read after four hours at room temperature when ring formation is at a maximum.

From the supernatant remaining after the precipitation of the toxin at pH 5.0 to 5.4 the bacterial protein may be salted out by adding ammonium sulphate to 0.8 of saturation. Any toxin remaining is also precipitated. The traces of toxin are separated from the bacterial protein fraction by acidifying the solution to pH 5.0 and incubating overnight. Complete removal of the toxin is indicated by failure of the bacterial protein fraction to bind antitoxin.

After precipitation of the bacterial protein with five per cent trichloracetic acid, the neutralized supernatant gives no precipitin test but the precipitate redissolved in dilute alkali and neutralized gives a good ring with the antibacterial serum.

Results of the Acid Fractionation Procedures

Fractionation of purified toxin by acid precipitation considerably reduces the titer of bacterial protein against the same antibacterial serum as may be seen from the results presented in table 1. The fourth column of the table gives the highest dilution of the preparation which will form a perceptible ring with the antibacterial serum; the last column, the number of Lf units per cubic centimeter at this titer dilution. A comparison of the Lf values at titer for fractionated and unfractionated toxins indicates that the acid precipitation methods reduce by five- to twenty-fold the amount of bacterial protein per Lf unit. The results of a less complete separation by fractional adsorption on magnesium hydroxide have been tabulated in a previous paper (Eaton, 1936b).

A slightly better separation of toxin and bacterial protein is obtained by one precipitation with nucleic or phosphoric acid than by two successive acid precipitations in the presence of ammonium sulphate. The chief objection to the nucleic acid method is
that it introduces a nitrogenous impurity. The procedure with phosphoric acid produces a denatured toxin showing a considerably increased flocculation time. In concentrated ammonium-sulphate solution the toxin is apparently less susceptible to denaturation and more completely precipitated at its iso-electric point. Acid precipitates produced in dilute solutions of electrolyte become partially or completely insoluble at pH 6.0, but toxin precipitated by acid from one-third-saturated ammonium sulphate solu-

<table>
<thead>
<tr>
<th>PREPARATION NUMBER</th>
<th>METHOD OF FRACTIONATION</th>
<th>LD UNITS PER CC</th>
<th>BACTERIAL PROTEIN TITER</th>
<th>LD UNITS PER CC AT TITER</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Unfractionated</td>
<td>420</td>
<td>1:32</td>
<td>13</td>
</tr>
<tr>
<td>2a</td>
<td>Nucleic acid toxin precipitate</td>
<td>360</td>
<td>1:3</td>
<td>120</td>
</tr>
<tr>
<td>2b</td>
<td>Supernatant from nucleic acid precipitate</td>
<td>10</td>
<td>1:20</td>
<td>0.5</td>
</tr>
<tr>
<td>2c</td>
<td>Phosphoric acid toxin precipitate</td>
<td>135</td>
<td>1:1</td>
<td>135</td>
</tr>
<tr>
<td>2d</td>
<td>Supernatant from phosphoric acid precipitate</td>
<td>28</td>
<td>1:16</td>
<td>1.7</td>
</tr>
<tr>
<td>1</td>
<td>Unfractionated</td>
<td>500</td>
<td>1:80</td>
<td>6</td>
</tr>
<tr>
<td>1a</td>
<td>Acid ammonium sulphate toxin precipitate</td>
<td>500</td>
<td>1:8</td>
<td>62</td>
</tr>
<tr>
<td>1b</td>
<td>Same as 1a</td>
<td>500</td>
<td>1:4</td>
<td>125</td>
</tr>
<tr>
<td>1c</td>
<td>Bacterial protein fraction*</td>
<td>0</td>
<td>1:100</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Unfractionated</td>
<td>800</td>
<td>1:80</td>
<td>10</td>
</tr>
<tr>
<td>3a</td>
<td>Acid ammonium sulphate toxin precipitate</td>
<td>360</td>
<td>1:8</td>
<td>45</td>
</tr>
<tr>
<td>3b</td>
<td>Bacterial protein fraction*</td>
<td>0</td>
<td>1:150</td>
<td>0</td>
</tr>
</tbody>
</table>

* Prepared from supernatant of toxin precipitate as described in the section entitled "Isolation of Bacterial Protein."

Estimation of the Amount of Bacterial Protein in Purified Toxin

When the actual concentration at titer of a single bacterial precipitinogen is determinable, it is possible to estimate by means of the precipitin test, under certain conditions, the amount of
this precipitinogen in purified toxin preparations. Since the bacterial precipitinogen and the toxin are apparently both proteins containing approximately the same percentage of nitrogen, the calculations may be based on measurements of the protein nitrogen.

The results of these measurements are presented in table 2. The Lf values and bacterial protein titers of these preparations have already been given by the corresponding numbers in table 1. The protein nitrogen values given in the third column of table 2

```
<table>
<thead>
<tr>
<th>PREPARATION NUMBER*</th>
<th>PREP. FRACTION</th>
<th>PROTEIN N</th>
<th>PROTEIN N PER Lf UNIT</th>
<th>PROTEIN N AT TITERS OF PRECIPITINOGEN (N/d)</th>
<th>RATIO BACTERIAL TO TOTAL PROTEIN (Pb/Pt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mgm. per cc.</td>
<td>mgm. per cc.</td>
<td>mgm. per cc.</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Unfractionated toxin</td>
<td>0.230</td>
<td>0.00046</td>
<td>0.0029</td>
<td>1/6</td>
</tr>
<tr>
<td>1a</td>
<td>Toxin</td>
<td>0.263</td>
<td>0.00053</td>
<td>0.033</td>
<td>1/60</td>
</tr>
<tr>
<td>1b</td>
<td>Toxin</td>
<td>0.250</td>
<td>0.00050</td>
<td>0.062</td>
<td>1/135</td>
</tr>
<tr>
<td>1c</td>
<td>Bacterial protein</td>
<td>0.046</td>
<td>&gt;0.01</td>
<td>0.00046</td>
<td>1/1</td>
</tr>
<tr>
<td>2</td>
<td>Unfractionated toxin</td>
<td>0.336</td>
<td>0.00042</td>
<td>0.0042</td>
<td>1/9</td>
</tr>
<tr>
<td>2a</td>
<td>Toxin</td>
<td>0.180</td>
<td>0.0005</td>
<td>0.023</td>
<td>1/50</td>
</tr>
<tr>
<td>2b</td>
<td>Bacterial protein</td>
<td>0.073</td>
<td>&gt;0.01</td>
<td>0.00049</td>
<td>1/1</td>
</tr>
<tr>
<td>3</td>
<td>Unfractionated toxin</td>
<td>0.190</td>
<td>0.00045</td>
<td>0.0075</td>
<td>1/15</td>
</tr>
<tr>
<td>3a</td>
<td>Toxin</td>
<td>0.095</td>
<td>0.00063</td>
<td>0.095</td>
<td>1/200</td>
</tr>
</tbody>
</table>
```

* Correspond to preparation numbers in table 1.

represent the nitrogen in precipitates produced by five-per-cent trichloracetic acid from known volumes of solution. The protein-nitrogen-Lf ratios in the fourth column indicate the purity of the toxin and will be discussed later. In the expression N/d, (N) represents the milligrams of protein nitrogen per cubic centimeter and (d) the precipitin-titer dilution of the various preparations tested against the same anti-bacterial serum.

If we assume that the dilution of the purified toxin preparation which just gives a perceptible ring with antibacterial serum contains the same concentration of bacterial precipitinogen as the
dilution at titer of the bacterial protein alone, then the ratio of bacterial protein to total protein in the toxin preparations may be stated thus:

$$\frac{P_b}{P_t} = \frac{N_b/d}{N_t/d}$$

In this equation,

- $P_b$ is the concentration of bacterial protein in purified toxin.
- $P_t$ is the concentration of total protein in purified toxin.
- $N_b/d$ is the concentration of bacterial protein nitrogen at titer in preparations 1c or 3b (bacterial protein).
- $N_t/d$ is the concentration of total protein nitrogen at titer (toxin plus bacterial protein) in the purified toxin preparations.

It is obvious from the figures in the fifth column of table 2 that the toxin fractions contain much more protein nitrogen per cubic centimeter at titer than do the corresponding bacterial protein fractions. From the ratio $P_b/P_t$ in the last column it appears that, in purified toxins before fractionation, bacterial protein represents 1 part in 6 to 1 part in 15 of all the protein. After fractionation the bacterial protein constitutes about 1 part in 50 to 200 or 0.5 per cent to 2 per cent of the total protein.

The error in the precipitin test is admittedly very large; but where not over 10 to 20 per cent of the total protein is bacterial precipitinogen, $P_b$ is small in relation to $P_t$ and the error in percentage is correspondingly small.

In crude toxin the protein nitrogen per Lf unit ($N/Lf$) has been found to be 0.0012 mgm. to 0.0035 mgm. If pure toxin contains about 0.0005 mgm. protein nitrogen per Lf unit then in crude toxin about 60 to 90 per cent of the protein nitrogen is in bacterial protein.

SEROLOGIC AND CHEMICAL IDENTIFICATION OF BACTERIAL PRECIPITINOGENS IN DIPHTHERIA TOXIN PREPARATIONS

Crude toxin contains several substances which give a precipitin reaction with antibacterial serum. These are in part complex carbohydrates (Hazen 1930). There are also present at least two
precipitinogens which appear to be proteins. These are characterized as follows.

Precipitinogen I is adsorbed from crude toxin solutions on calcium phosphate and is salted out by ammonium sulphate at one-third saturation.

Precipitinogen II is removed from solution by all of the reagents which have been used to precipitate or adsorb toxin. Like toxin it is salted out by ammonium sulphate at 0.4 to 0.7 saturation, but it differs from toxin in remaining in solution at pH 5.0 to 5.4.

Precipitinogen I may be almost completely separated from toxin in the first steps of purification. However, some of precipitinogen II is carried along with the toxin no matter what purification procedure is used. This precipitinogen may be most completely separated from toxin by acid fractionation.

The possibility that precipitinogens other than II are present in purified toxin must be considered. In order to determine this, tests were done with antibacterial serum treated to remove precipitins for II. The antibacterial serum was mixed with the bacterial protein preparation number 3b (table 1), incubated over night, and the resulting precipitate centrifuged down. The supernatant gave no precipitin test with the isolated bacterial protein which has been designated precipitinogen II.

The results of precipitin tests with serum treated in this way are presented in table 3. Corresponding tests with untreated serum

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>14 UNITS PER CC</th>
<th>REACTION WITH UN-TRATED ANTI-BACTERIAL SERUM</th>
<th>REACTION WITH ANTI-BACTERIAL SERUM ABSORBED WITH PRECIPITINOGEN II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude toxin</td>
<td>20</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>No. 1 unfractionated</td>
<td>500</td>
<td>+++++</td>
<td>+</td>
</tr>
<tr>
<td>No. 1a fractionated</td>
<td>500</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>No. 3 unfractionated</td>
<td>800</td>
<td>+++++</td>
<td>-</td>
</tr>
<tr>
<td>No. 3a fractionated</td>
<td>360</td>
<td>+++</td>
<td>-</td>
</tr>
</tbody>
</table>
are included for comparison. As expected, crude toxin gives a precipitin reaction with the absorbed antiserum due to the presence of precipitinogen I and probably other bacterial substances. One of the purified toxins before acid fractionation apparently contained traces of precipitinogen I so that it also gave a positive reaction.

It is evident that the other purified preparations in table 3 contain no detectable bacterial protein other than the one used to saturate the antiserum. This excludes the possibility that the precipitin tests obtained with the acid fractionated toxins are due to another bacterial protein which precipitates only at low dilutions with the antibacterial serum.

**PROPERTIES OF PURIFIED TOXIN AFTER SEPARATION OF BACTERIAL PROTEIN**

The toxic protein left after removal of most of the bacterial protein does not differ materially in its chemical properties from the purified toxin previously described. (Eaton, 1936b) The fractionation with acid removes traces of proteose and peptone so that practically 100 per cent of the nitrogen is precipitated by trichloracetic acid as protein.

It will be noted from the figures in table 2 that the protein-N/Lf ratios are slightly higher after acid precipitation than before. This is probably due to a slight alteration of part of the toxin by acid. In the preparation precipitated by phosphoric acid at 35°C. (preparation 2c in table 2) the protein-N/Lf ratio increased by about 35 per cent although some of the protein nitrogen had been removed as bacterial protein. The least increase in protein-N/Lf ratios occurred when the acid precipitation was done in one-third-saturated ammonium sulphate solution as with preparations 1a and 3a.

The acid-precipitated preparations show a slight to marked increase in the flocculation time. Acid precipitation may reduce the toxicity by as much as 50 per cent but the acid fractionated toxins still have a toxicity of the order of 0.00005 mgm. of nitrogen per M.L.D. or about one hundred times the toxicity of crude toxin.
Measurements of the specific optical rotation of toxins purified by metal-salt precipitation gave values for $\alpha^2$ of $-45^\circ$ to $-47^\circ$. The preparation 1a obtained by acid fractionation showed a specific rotation of $-43^\circ$. The difference is within the limits of error in the measurements. Since the additional purification had little effect on the optical rotation the values given probably represent the approximate optical activity of the toxin.

PROPERTIES OF SLIGHTLY DENATURED TOXIN

Diphtheria toxin as a native protein is probably not precipitated in the presence of low concentrations of electrolyte at any pH. However, various toxin preparations contain different proportions of protein precipitable by acid under constant conditions. Since toxin denatured to various degrees will become precipitable at any pH up to 7.0, or even above, it is now evident that the acid-precipitable protein is denatured toxin and not a protein impurity as was formerly believed.

It has also been found that a sample of toxin which precipitates between 0.4 and 0.7 saturated ammonium sulphate becomes precipitable at 0.3 saturation after slight denaturation. These alterations in solubility are accompanied by an increased adsorbability on colloidal magnesium or aluminum hydroxide. Thus, a mixture of native and slightly denatured toxin may appear, from experiments on fractional adsorption or fractional precipitation with acid or ammonium sulphate, to consist of two or more proteins. The denatured toxin is the more easily mistaken for an impurity because slight denaturation will increase the N/Lf ration by as much as 30 per cent. 1

Since denaturation causes an increased flocculation time it is possible to obtain some indication of whether a certain preparation contains denatured toxin or a mixture of native toxin and an impurity by measuring the Kf. The relation of Kf to denaturation of toxin has been discussed in another paper (Eaton 1936c).

Probably even fresh crude toxin contains some denatured toxin. The fraction precipitated from crude toxin at one-third saturation

1 For a concise statement of the chemical changes in denaturation of proteins see Mirsky and Pauling (1936).
with ammonium sulphate shows an increased flocculation time which may be due to the denatured state of the toxin in this fraction, not, as stated in a previous publication, to the effect of admixed bacterial protein on the flocculation reaction.

**PROPERTIES OF THE Porphyrin Compounds IN DIPHTHERIA TOXIN**

The red porphyrin compounds described by Coulter and Stone (1931), which are present in crude toxin, may be mostly separated from the purified preparations by adsorption on charcoal or magnesium hydroxide. But traces difficult to remove are detectable in concentrated solutions of purified toxin. The close association of the porphyrins with the protein fractions indicated that these colored substances might be present as protein compounds analogous to hemoglobin.

Purified toxin contains two distinct substances showing absorption spectra. One of these, giving absorption bands with centers at 574 and 537 millimicra, corresponds to the complex porphyrin described by Coulter. The other with bands at 563 and 528 millimicra, in dilute phosphate buffer at pH 7.0, may be the copper compound of coproporphyrin said by Coulter to be a decomposition product of the complex porphyrin. These two substances are present in varying proportions and amounts in different toxin preparations.

The two porphyrins are apparently similar in their chemical properties. Both become extractable by ether from the toxin solutions upon bringing the pH to 5.6. Coulter and Stone extracted their complex porphyrin with ether from crude toxin acidified with acetic acid; probably at a pH near 4.0. The porphyrins will pass from the ether solution to an aqueous solution of sodium phosphate at pH 6.0. Both porphyrins are precipitated from aqueous solutions free of protein near pH 5.6. Coincident with the precipitation a shift of the absorption bands toward the red end of the spectrum occurs. On raising the pH to 6.0 or above the porphyrins redissolve and the absorption bands shift back to their original positions. This behavior indicates that the porphyrins are weak acids insoluble in water but forming
soluble sodium salts above pH 5.8 to 6.0. The fact that the porphyrins are precipitated near the iso-electric point of diphtheria toxin explains the difficulty in separating them from toxin by the purification methods used.

The porphyrins are strongly adsorbed to protein precipitates. The first small precipitate containing denatured toxin which is produced by one-third-saturated ammonium sulphate carries down the greater part of the porphyrin from the toxin solution. Because of this the ammonium sulphate and acid fractionation procedure not only serves to separate out bacterial protein but also frees the toxin of all but very small traces of porphyrin. The porphyrin with absorption bands at 563 and 528 is the most difficult to separate completely from the toxin.

**DISCUSSION**

The bacterial precipitinogen remaining in purified diphtheria toxin after acid fractionation is apparently identical with the bulk of the bacterial protein isolated by fractionation from the purified preparations. This bacterial protein gives a ring with the antiserum used at dilutions of 1:300,000 in terms of grams dry weight. The antibacterial serum, although reactive with very small amounts of bacterial protein, apparently contains no precipitin for about 99 per cent of the protein in the purified toxin preparations. If the toxic protein is derived from the bacterial cells it must be, (1) a substance that is readily soluble in normal saline and hence easily removed by washing the diphtheria bacilli used for production of the antiserum; or (2) a protein that does not produce precipitating antibodies in rabbits. Diphtheria toxin is a substance which dissolves out of the bacterial cells into the medium; and it has been reported that antitoxin produced in rabbits will not flocculate with the toxin. The Wadsworth-Wheeler medium used for the production of toxin contains no protein when sterile so that the protein under consideration could not have come from the culture medium.

These considerations do no exclude the possibility that a readily-soluble protein other than toxin is elaborated by the diphtheria bacillus. However, the close relationship between dena-
uration of the toxic protein and alterations in its reactivity with antitoxin, and the correlation of Lf values, toxicity, and protein nitrogen in the purified preparations make this explanation seem improbable. If the protein were merely a carrier of the toxin the relative amounts of protein, flocculating substance, and lethal toxin in different preparations would vary to a much greater extent than has been observed in the course of this work.

Attempts to crystallize the toxic protein have so far been unsuccessful. But the criteria of purity described in this and the previous papers could well be applied to a crystalline preparation.

The porphyrins are apparently substances of an acidic nature not combined with the negatively-charged proteins above pH 5.6. Below this pH they are insoluble in water. Since the porphyrins may be readily separated from proteins without any changes in their absorption spectra they are probably not decomposition products of a complex in the toxin preparations. Hosoya and his collaborators (1934) have made similar studies on the porphyrins in anatoxin. The question of whether diphtheria toxin itself shows absorption bands either in the visible or ultraviolet parts of the spectrum has been taken up in recent papers by Levaditi and his co-workers (1934), and by Ottensooer, Krupski, and Almasy (1935). The results were inconclusive. The porphyrins studied in the present work do not appear to be a part of the toxin molecule.

Locke and Main (1928) used iso-electric-point fractionation to separate toxin from bacterial protein but their preparations were considerably denatured, contained much pigment, and no measurements of the completeness of separation were reported.

SUMMARY

Methods for separating toxin and bacterial protein by iso-electric-point fractionation are described.

Measurements by the precipitin test of the amount of bacterial protein in purified toxin were based on titrations of the antibac-

---

1 In a recent paper Hosoya and his associates (1936) have described a method for the preparation of dried purified diphtheria toxin. In their preparations the M.L.D. is 0.01 mgm. which represents a toxicity only about five times as great as that of dried crude toxin.
Purification of diphtheria toxin

Bacterial serum against preparations containing known amounts of bacterial protein. The results indicate that the bacterial protein constitutes 0.5 to 2 per cent of the total protein in the most highly purified preparations.

Slightly denatured toxin is precipitated at a higher pH, and a lower concentration of ammonium sulphate, and is more readily adsorbed on magnesium or aluminum hydroxide than is undenatured toxin.

Evidence is presented that the porphyrins found in crude and purified toxins are not combined with protein but are precipitated near the iso-electric point of toxin and are easily adsorbed on protein precipitates.

The results of this work indicate that the purified protein, which has been freed of all but small traces of impurities detectable by the methods employed, is diphtheria toxin.

REFERENCES


