THE CHEMICAL NATURE OF SCARLET FEVER TOXIN*

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There is considerable confusion in the literature regarding scarlet fever toxin and "toxic or poisonous" substances obtainable from cultures of scarlet fever streptococci. In 1934, for instance, Hooker and Follensby (1) described an A and B toxin of scarlet fever; in 1936 Kodama (2) separated three substances from streptococci which gave skin reactions; and in 1938 Eaton (3) in a review rallied to the opinion of the plurality of the toxin.

The substance under discussion here is the specific soluble toxin of scarlet fever. There is no evidence thus far that more than one such substance exists and in order to demonstrate its presence the following tests should be made: (1) The substance should be destroyed by boiling; (2) suitable dilutions injected into the skin of numerous human subjects should give positive reactions whenever a positive reaction is given by one skin test dose of a standard scarlet fever toxin obtained from streptococci which have produced typical experimental scarlet fever in man, and give negative reactions when the reaction to the standard toxin is negative; (3) after giving a positive reaction in a human subject who gives a positive test with standard scarlet fever toxin, it should, in proper dilution, give a negative test in the same individual when mixed with a suitable amount of specific scarlet fever antitoxin; (4) it should produce nausea, vomiting, fever, malaise, and a typical scarlet fever rash in susceptible human subjects when given hypodermically in a single dose of 1000 to 3000 skin test doses.

The substance discussed here has been subjected to all of these

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Scarlet Fever Toxin
tests and is, therefore, specific scarlet fever toxin. It was prepared according to the method given by Dick and Boor (4). The toxin, a white, fluffy substance, dissolved readily in water, giving a yellow color in concentrated solutions, and contains from 20,000 to 30,000 skin test doses per mg.

From a study of the chemical properties of this purified toxin, as reported in this paper, the conclusion is drawn that scarlet fever toxin is a protein of small molecular weight, some amino groups of which are essential for its activity. Furthermore, a study of the electrophoretic mobility of the toxin at different hydrogen ion concentrations has shown that the toxin may be freed by electrophoresis of some nitrogenous impurities still present. In all the experiments the activity was tested with the skin test for susceptibility to scarlet fever as developed by Dick and Dick (5).

Effect of Temperature—It is generally agreed that scarlet fever toxin is very resistant to heat. To test the effect of temperature, the purified toxin (0.5 mg. per cc. dissolved in phosphate buffer of pH 7.4) was heated for 1 hour at different temperatures. Heating for 1 hour without shaking up to 90°, and for 45 minutes up to 100° (boiling water bath) caused no loss of activity. Bubbling either purified nitrogen or oxygen through the solution when heated in a boiling water bath resulted in loss of activity. Apparently, agitation at this temperature produced denaturation of the toxin as agitation at lower temperatures produces denaturation of proteins of higher molecular weight (for example, hemoglobin).

Effect of pH—It is generally believed that scarlet fever toxin is very unstable in alkaline solution, although no study of the influence of hydrogen ion concentration on the activity of the toxin has been reported. To test this influence, the universal buffer of Theorell and Stenhagen (6) was used from pH 2.08 to 11.77; by the use of this buffer, the chemical nature of the electrolytes was kept identical. The toxin solutions (1 mg. of toxin dissolved in 1 cc. of water plus 4 cc. of buffer) were kept for 24 hours at room temperature (±25°). They were then neutralized, diluted to the proper dilutions for skin tests, and filtered through Jena fritted glass filters for bacteriological filtration. The pH values of the solutions were measured by both the glass and hydrogen electrodes at 25°. As can be seen in Table I, the toxin was extremely re-
sistant to changes in the hydrogen ion concentration, the stability being greater in acid solutions than in alkaline solutions; it did not lose its activity after 24 hours in 0.1 N HCl (pH 1.08), but it was destroyed when kept in 0.1 N NaOH or in buffer solutions of a pH value of 11.77.

Effect of Ketene—Acetylation with ketene has proved to be useful in determining the importance of amino groups for biological activity. It is a highly specific acetylating agent for aqueous solutions of proteins, and several investigators (Herriott and Nor-
of the toxin were dissolved in 10 cc. of 2 M acetate buffer of pH 5.06, put into a cellophane bag, and kept inside a 1 liter beaker containing the same acetate buffer to avoid changes in the hydrogen ion concentration inside the bag. Ketene was generated in the apparatus devised by Herriott (14). The ketene vapor, before reaching the toxin solution, was purified by being passed through a flask cooled with ether and CO₂ snow; the gas was bubbled at a rate of about 4 cc. per minute and samples were withdrawn at different intervals (5, 10, 20, and 40 minutes). The solutions were then dialyzed overnight in cellophane bags at 3° in running distilled water, made neutral, and tested at two dilutions, one 10
times stronger than the other. The toxin used as control was treated identically, except for the ketene treatment (Table II). The toxin, at a dilution of 1 X 8000 was completely inactive at the end of 5 minutes. There was some activity at a dilution of 1 X 800, but it was completely lost at the end of 40 minutes. This loss of activity, after a short treatment with ketene, may be taken as an indication that ketene destroys by acetylation the primary amino groups essential for the activity of the toxin.

Table II

Effect of Ketene on Activity of Scarlet Fever Toxin

50 mg. were dissolved in 10 cc. of 2 M acetate buffer, pH 5.06. Ketene was bubbled at the rate of 4 cc. per minute.

<table>
<thead>
<tr>
<th>Time after ketene treatment</th>
<th>Size of skin reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 X 8000 dilution</td>
</tr>
<tr>
<td>min.</td>
<td>mm.</td>
</tr>
<tr>
<td>Control</td>
<td>27 X 25</td>
</tr>
<tr>
<td>5</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>&quot;</td>
</tr>
<tr>
<td>20</td>
<td>&quot;</td>
</tr>
<tr>
<td>40</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Effect of Nitrous Acid—The action of nitrous acid on amino groups has been known for a long time, and was used by Levene and Van Slyke (15) for the determination of amino acids. As shown by Philpot and Small (16), nitrous acid resembles ketene in

1 We express our thanks to Dr. A. K. Boor for his kind loan of the ketene generator.
first attacking the primary amino groups in the protein molecule. If ketene destroyed the activity of scarlet fever toxin by acetylation of amino groups, nitrous acid would also destroy it. Such indeed was the case. The experiments were performed at pH 4.07 in acetate buffer. To 1.15 cc. of 1 N acetic acid were added 3.85 cc. of H₂O, 5 cc. of a toxin solution containing 4 mg. per cc., and 345 mg. of NaNO₂ (final concentration 0.5 M). After standing half an hour at room temperature, the solution was neutralized, dialyzed overnight as indicated, and tested. For control, a toxin solution was used in which the NaNO₂ was replaced by an equimolecular concentration of NaCl and the acetic acid replaced by acetate buffer with a final pH value of 4.06. Treatment with

<table>
<thead>
<tr>
<th>Substance</th>
<th>pH</th>
<th>Time of action</th>
<th>Size of skin reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.06</td>
<td>0.5 hrs.</td>
<td>20 × 18 (bright)</td>
</tr>
<tr>
<td>Sodium nitrite (0.5 M)</td>
<td>4.07</td>
<td>0.5 yrs.</td>
<td>Negative</td>
</tr>
<tr>
<td>Control</td>
<td>7.02</td>
<td>6.0 hrs.</td>
<td>30 × 32 (bright)</td>
</tr>
<tr>
<td>Iodine (0.002 N)</td>
<td>7.02</td>
<td>6.0 hrs.</td>
<td>Negative</td>
</tr>
<tr>
<td>’ (0.002 ‘)</td>
<td>4.63</td>
<td>6.0 hrs.</td>
<td>°</td>
</tr>
<tr>
<td>° (0.002 °)</td>
<td>3.01</td>
<td>6.0 hrs.</td>
<td>20 × 20 (bright)</td>
</tr>
</tbody>
</table>

Effect of Iodine—Iodine may react with proteins either as an oxidizing agent, or as a substitution agent, its activity as an oxidizing agent being increased as the hydrogen ion concentration increases, while its activity as a substitution agent diminishes under the same conditions. The effect of iodine (0.002 N) on scarlet fever toxin was studied at three pH values, 7.02, 4.63, and 3.01, the solutions being kept in the presence of iodine for 6 hours at 25°. At the end of this time the iodine was destroyed by the addition of cysteine, the solution was diluted, dialyzed overnight, and the skin tests performed (Table III). The toxin was destroyed by iodine at pH values of 7.02 and 4.63, while at a pH value of 3.01 it still retained activity (control, 30 × 32 mm.; with iodine, 20 ×
20 mm.). Doubtless, this iodine inactivation is due to action of the reagent on the amino groups of the toxin more than to strict oxidizing action, for when the oxidizing power was increased by making the solution more acid there was no inactivation.

Effect of Porphyrindin and Other Oxidizing and Reducing Substances—Porphyrindin, a dye of highly positive oxidation-reduction potential ($E'_0$ at pH 7.0 = +0.57 volt) was recommended by Kuhn and Desnuelle (17) as a reagent for the rapid oxidation of $\text{-SH}$ groups in the protein molecule. As such it has been extensively used by Greenstein (18). The effect of porphyrindin, kindly supplied by Dr. L. Hellerman, was studied at pH 7.06 by treating the toxin with the dye (final concentration, 0.001 M) for half an hour, the skin tests being performed after dialysis and proper dilution. Porphyrindin, under such conditions, destroyed the activity of the toxin (Table IV).

The inactivation of the toxin by porphyrindin made it necessary

### Table IV

*Effect of Porphyrindin and Other Oxidizing and Reducing Substances on Activity of Scarlet Fever Toxin at pH 7.06*

<table>
<thead>
<tr>
<th>Substance</th>
<th>Time of action</th>
<th>Size of skin reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porphyrindin (0.001 M)</td>
<td>0.5</td>
<td>35 x 30 (bright)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>$\text{H}_2\text{O}_2$ (0.009 M)</td>
<td>4.5</td>
<td>28 x 13</td>
</tr>
<tr>
<td>$\text{CuCl}_2$ (0.00015 M)</td>
<td>4.0</td>
<td>22 x 20</td>
</tr>
<tr>
<td>Oxidized glutathione (0.033 M)</td>
<td>4.0</td>
<td>28 x 15</td>
</tr>
<tr>
<td>Iodoacetic acid (0.001 M)</td>
<td>4.0</td>
<td>35 x 30</td>
</tr>
<tr>
<td>Cysteine (0.023 M)</td>
<td>4.0</td>
<td>19 x 20</td>
</tr>
<tr>
<td>Glutathione (0.023 M)</td>
<td>4.0</td>
<td>28 x 21</td>
</tr>
<tr>
<td>$\text{Na}_2\text{S}_2\text{O}_4$ (0.02 M)</td>
<td>7.0</td>
<td>20 x 23</td>
</tr>
<tr>
<td>$\text{H}_2\text{S}$ (saturated)</td>
<td>5.0</td>
<td>28 x 20</td>
</tr>
<tr>
<td>$\text{Pt} + \text{H}_2$</td>
<td>6.0</td>
<td>29 x 22</td>
</tr>
<tr>
<td>Iodoacetamide (0.01 M)</td>
<td>4.0</td>
<td>35 x 30</td>
</tr>
<tr>
<td>Alloxan (0.05 M)</td>
<td>5.0</td>
<td>28 x 25</td>
</tr>
<tr>
<td>Caffeine (0.01 M)</td>
<td>4.0</td>
<td>33 x 35</td>
</tr>
<tr>
<td>Sulfanilamide (0.005 M)</td>
<td>0.1</td>
<td>35 x 25 (faint)</td>
</tr>
<tr>
<td>&quot; ultraviolet irradiation</td>
<td>0.1</td>
<td>30 x 20</td>
</tr>
</tbody>
</table>

Kuhn and Desnuelle (17) as a reagent for the rapid oxidation of $\text{-SH}$ groups in the protein molecule. As such it has been extensively used by Greenstein (18). The effect of porphyrindin, kindly supplied by Dr. L. Hellerman, was studied at pH 7.06 by treating the toxin with the dye (final concentration, 0.001 M) for half an hour, the skin tests being performed after dialysis and proper dilution. Porphyrindin, under such conditions, destroyed the activity of the toxin (Table IV).

The inactivation of the toxin by porphyrindin made it necessary
to study the effect of substances which combine with either the
—SH or —S—S— groups of proteins, inasmuch as the presence of
these groups as the active constituents of certain hormones
(insulin, du Vigneaud (19)), enzymes (urea, Perlzweig (20), papain,
Hellerman and Perkins (21), cholinesterase, Nachmansohn and
Lederer (22)), and toxins (snake venoms, Micheal and Emde (23),
Binet, Weller, and Robillard (24)) has been recently established.
Of the compounds known to react easily with either —SH or
—S—S— groups under physiological conditions (at neutral reac-
tion and temperatures not above 38°) the following were used:
as reagents for —SH groups, CuCl₂ (0.00015 M), CH₃COOH (0.01
m), CH₂CONH₂ (0.01 m), oxidized glutathione (0.023 m), alloxan
(0.05 m), caffeine (0.01 m), H₂O₂ (0.009 m); as reagents for —S—
S— groups, cysteine (0.02 m), glutathione (0.023 m), Na₂S₂O₄ (0.02
m), H₂S (saturated), and Pt black plus H₂ (Table IV). Since none
of these substances had any effect on the activity of the toxin, it
must be concluded that no active —SH or —S—S— groups are
present. The inhibiting action of porphyrindin must therefore
be attributed to destruction of some active group other than a
sulphydryl group. To elucidate this question, a number of sub-
stances containing primary amine groups (glycine, phenylalanine,
histidine, tryptophane, tyrosine, tyramine, histamine, 1,4-
diaminobutane, diethylamine) were treated with porphyrindin at
pH 7.33, 25°, a ratio of 10 parts of amine to 1 of dye being used.
With tyrosine and tyramine the reaction was immediate, as shown
by prompt and complete reduction of the dye; with tryptophane,
the dye was half reduced at the end of 1.75 hours. Porphyrindin
was reduced by tyrosine and tyramine, presumably by oxidation
of their OH groups. From these experiments it is suggested that
tyrosine may be one of the active groups of the toxin, destroyed
by ketene by action on its NH₂ group and nitrous acid, by prophy-
ridin and ketene by action on its OH group.
Carpenter and Barbour (25) reported that the toxins from
Staphylococcus aureus and Clostridium welchii were inactivated by
treatment with sulfanilamide. Neither sulfanilamide nor the
oxidation product obtained by treatment with ultraviolet light
had any effect on the activity of scarlet fever toxin (Table IV).
Effect of Pepsin and Trypsin—Contradictory reports have been
published on the effect of trypsin on the activity of scarlet fever
toxin. Huntoon (26) states that the toxin is destroyed by trypsin, but Kodama (2) on preparing his toxin submitted the solution to the action of trypsin to hydrolyze the protein impurities. To test the effect of trypsin under optimum conditions for the activity of the enzyme, toxin solutions in 0.01 M Na₂HPO₄, containing 1 mg. per cc., were incubated at 38° with trypsin (0.13 mg. of active powder per cc. of toxin solution) for 3, 6, 9, 24, and 48 hours. At the end of the incubation period the solutions were neutralized with 0.01 M KH₂PO₄, diluted, and tested. When the incubation period was extended to 48 hours, more trypsin was added after 24 hours incubation.² In no case did trypsin destroy the activity of the toxin. The same results were found when pepsin was used as the proteolytic enzyme. The experiments were performed at a pH value optimum for enzyme activity, namely 3.01 (Table V).

**Table V**

*Effect of Pepsin and Trypsin on Activity of Scarlet Fever Toxin Incubated at 38°.*

<table>
<thead>
<tr>
<th>Substance</th>
<th>pH</th>
<th>Time of action</th>
<th>Size of skin reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.01</td>
<td>12</td>
<td>35 X 25 (bright)</td>
</tr>
<tr>
<td>Pepsin</td>
<td>3.01</td>
<td>6</td>
<td>20 X 20 &quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>3.01</td>
<td>12</td>
<td>30 X 25 &quot;</td>
</tr>
<tr>
<td>Control</td>
<td>3.01</td>
<td>24</td>
<td>15 X 10 (faint)</td>
</tr>
<tr>
<td>Pepsin</td>
<td>3.01</td>
<td>24</td>
<td>15 X 18 &quot;</td>
</tr>
<tr>
<td>Control</td>
<td>8.5</td>
<td>24</td>
<td>35 X 30 (bright)</td>
</tr>
<tr>
<td>Trypsin</td>
<td>8.5</td>
<td>24</td>
<td>38 X 22 &quot;</td>
</tr>
</tbody>
</table>

² In these experiments, trypsin and pepsin solutions were previously tested for skin reactions and were found to give negative reactions.

*Isoelectric Point of Scarlet Fever Toxin*—The electrophoretic mobility of the scarlet fever toxin was determined with Theorell's cataphoresis apparatus for test purposes (27) at 3.5° to avoid any danger of convection currents. The current intensity was kept at 5 milliamperes and the experiments terminated at the end of 3 hours. The toxin, dissolved in the buffer solution, was introduced in the U part of the apparatus, leaving five cells in both branches of the apparatus free for migration of the toxin. At the end of the experiment, the rubber disks were moved so as to
separate the different cells; the fluid of the cells was pipetted off, and the nitrogen content determined. After neutralization, dilution, and filtration, the skin tests were performed with the contents of each cell. The electrophoretic mobility of the toxin was studied at five pH values (Table VI), 4.56, 5.24, 5.76, 6.08, 6.98. (The pH values quoted here are those calculated from measurements made at 25°, and by the use of the temperature coefficients given by Clark (28).) The toxin migrated towards

**Table VI**

*Electrophoretic Mobility of Scarlet Fever Toxin As Determined with Theorell's Cataphoresis Apparatus*

\[ T = 3.5^\circ, + = \text{positive test for toxin activity}, - = \text{negative test for toxin activity}, U = \text{mg. per cent of N}_2 \text{ remaining in the U part of the cataphoresis apparatus}. \] The figures represent mg. per cent of N\(_2\) in the cell.

<table>
<thead>
<tr>
<th>pH</th>
<th>Anode</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Cathode</th>
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<tr>
<td></td>
<td>Cell 1</td>
<td>Cell 2</td>
<td>Cell 3</td>
<td>Cell 4</td>
<td>Cell 5</td>
<td>Cell 6</td>
<td>Cell 1</td>
<td>Cell 2</td>
<td>Cell 3</td>
<td>Cell 4</td>
<td>Cell 5</td>
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</tr>
<tr>
<td>4.56</td>
<td>2.94</td>
<td>2.45</td>
<td>1.94</td>
<td>2.02</td>
<td>1.56</td>
<td>1.48</td>
<td>0.7</td>
<td>0.31</td>
<td>0.24</td>
<td>0.13</td>
<td>0.19</td>
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</tr>
<tr>
<td>5.24</td>
<td>1.48</td>
<td>0.33</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>?</td>
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<td>1.48</td>
<td>1.38</td>
<td>0.62</td>
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<td>1.06</td>
<td>0.19</td>
<td>0.10</td>
<td>0.31</td>
<td>0.51</td>
<td>0.20</td>
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<td>1.94</td>
<td>2.00</td>
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<td>+</td>
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<tr>
<td>6.08</td>
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<td>0.17</td>
<td>0.15</td>
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<td>0.15</td>
<td>0.05</td>
<td>2.94</td>
<td>1.94</td>
<td>0.81</td>
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</tr>
<tr>
<td>6.98</td>
<td>0.73</td>
<td>0.38</td>
<td>0.11</td>
<td>0.31</td>
<td>0.25</td>
<td>0.13</td>
<td>3.56</td>
<td>1.84</td>
<td>1.3</td>
<td>0.71</td>
<td>0.48</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

the cathode from pH 5.76 on, and towards the anode from pH 5.24 to greater acidity. At pH 5.24, when the toxin migrated to the anode, some nitrogenous impurity migrated in the opposite direction, towards the cathode. At pH 5.24 and 4.56, when the toxin migrated to the anode, the average N content of the cells was 2.66 per cent, while the N content of the cells in the cathode branch, with no toxin, was 1.94 per cent. Electrophoresis of the toxin at pH 5.0 will therefore allow further purification of the toxin, for it will withdraw this nitrogenous impurity. Experiments in this direction are in progress.
The isoelectric point of the toxin, as determined by calculation of the ionic mobility from the data given in Table V was 5.55 (Fig. 1). This value is different from that given by Shinn (29), who reported it to be between pH 7.0 and 7.5; his experiments were carried out without proper control of the conditions necessary to perform electrophoretic studies, conditions studied carefully by Tiselius (30).

Ultrafiltration of Scarlet Fever Toxin—To estimate roughly the size of the scarlet fever toxin, the ultrafiltration of toxin solutions through graded collodion membranes was performed. The membranes were prepared according to the methods described by Elford (31) and Bauer and Hughes (32) in a room kept at constant temperature and humidity (24° and 60 to 65 per cent relative humidity); the degree of humidity was diminished to that existing in the room for the preparation of membranes of small pore size. The membranes prepared from parlodion manufactured by the Mallinckrodt Chemical Works were similar in property and size of pores to those described by Bauer and Hughes. The average pore size of the membranes was calculated by an application of Poiseuille's law governing the passage of water through a capillary tube. To test these membranes the following protein solutions of varied molecular weight were used: hemoglobin, prepared according to the method of Sidwell et al. (33), myoglobin, according

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Fig. 1. Isoelectric point of scarlet fever toxin. Temperature, 3.5°. Ordinate, ionic mobility × 10⁶; abscissa, pH values.

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³ For the equations used to calculate the pore size of membranes, see Bauer and Hughes (32).
to the method of Theorell (34), cytochrome c, according to the method of Keilin and Hartree (35), clupein, purchased from The British Drug Houses, Ltd. Filtrations were carried out under a pressure of about 780 mm. of Hg, the filter being connected to a nitrogen tank. The toxin filtered through all the membranes that let hemoglobin (mol. wt. 68,000), carbon monoxide myoglobin (mol. wt. 17,500), and cytochrome c (mol. wt. 13,000) pass through; moreover, it filtered through a cellophane membrane with a pore size less than 1 μm. The toxin did not filter through

cellophane No. 600 although clupein (mol. wt. from 2000 to 4000) did (Table VII). We are well aware that the ultrafiltration method through collodion membranes, even if the membranes were of uniform pore size, can be applied only to molecules of spherical or globular configuration and to substances that are not adsorbed by the membrane. However, since the toxin passes through membranes that retain cytochrome c, while it does not pass through membranes which let clupein through, the evidence is strong in favor of the assumption that the scarlet fever toxin

* Care was taken to perform the ultrafiltrations at hydrogen ion concentrations optimum for non-adsorption by the membranes.
Scarlet Fever Toxin

is a small protein with a molecular weight less than that of cytochrome c (13,000) and perhaps more than that of clupein (2000 to 4000). It may be recalled that the molecular weight of diphtheria toxin is about 72,000 (Lundgren, Pappenheimer, and Williams (36)) and that of crotoxin about 33,000 (Slotka and Fraenkel-Conrat (37)).

Some Components of Scarlet Fever Toxin—Dick and Boor (4) reported that the purified toxin used in these experiments contained 11.83 and 1.04 gm. per cent of nitrogen by formol titration, i.e. free amino nitrogen. Korschun et al. (38), who prepared a toxin by alcohol precipitation, reported a total nitrogen content of 10.02 per cent and Stock (39) in a preliminary note reported a total nitrogen of 7 per cent. Undoubtedly, Stock was dealing with a substance not identical with the scarlet fever toxin as previously defined, since he found no free amino nitrogen, while the toxin as prepared by Dick and Boor (4), Korschun (38), and Veldee (40) does contain free amino nitrogen. As found by Korschun et al. (38), the toxin gave a red color with the biuret reaction, an indication that there is a peptide linkage and that the length of the peptide chain is small. It gave a positive Millon test, an indication of the presence of tyrosine in the molecule, and a faintly positive Hopkins-Cole test. It also gave a strongly positive Molisch reaction.

For the determination of the carbohydrate content, two methods were used: (1) titration with copper with the mieromethod of
Somogyi (41) after previous digestion for 3 hours with 5 N H$_2$SO$_4$ (see Lyman and Barron for the details of the procedure (42)), and (2) the colorimetric orcinol method of Sørensen and Haugaard (43) as modified by Hewitt (44). The carbohydrate content by the first method was 1.54 gm. per cent; by the second method, 1.43 gm. per cent. On measuring the extinction coefficient of the blue solutions obtained after heating the toxin with the orcinol reagent at different intervals of time, the curve obtained with Filters S47 and S53 of the Zeiss Pulfrich photometer was similar to that reported by Sørensen (45) for mixtures containing 3 parts of mannose and 1 of galactose (Fig. 2). The carbohydrate content of human serum is 3.2 per cent; that of horse serum, 2.4 per cent; of albumin, 1.08 per cent; of seroglycoid, 8.4 per cent; of ovomucoid, 20 per cent (Hewitt (46)). Thus the carbohydrate content of toxin is as low as that of proteins with the lowest carbohydrate content.

The glucosamine content of the scarlet fever toxin was also very low, 0.73 gm. per cent. The toxin, dissolved in 4 N HCl, was subjected to 8 hours hydrolysis in a sealed tube in a boiling water bath. After neutralization, the determination of glucosamine was performed in a Zeiss Pulfrich photometer with Filter S53 by the colorimetric method of Elson and Morgan (47) modified by Hewitt (46). These low figures for carbohydrate and glucosamine content differ radically from Stock’s preliminary report (39) on the erythrogenic toxin of Streptococcus scarlatinae in which it is stated that his substance contained large quantities of carbohydrates and glucosamine.

Since the toxin contained no phosphorus (no color reaction was obtained when the determination of total phosphates was attempted by the colorimetric method of Whitehorn (48)), it may be concluded that no nucleoproteins exist in the toxin (Table VIII).

**Table VIII**

*Some Components of Scarlet Fever Toxin*

<table>
<thead>
<tr>
<th>Substance</th>
<th>Gm. per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total nitrogen (Kjeldahl)</td>
<td>11.33</td>
</tr>
<tr>
<td>Nitrogen (formol titration)</td>
<td>1.04</td>
</tr>
<tr>
<td>Total carbohydrate (copper titration)</td>
<td>1.54</td>
</tr>
<tr>
<td>&quot; (orcinol, colorimetric)</td>
<td>1.43</td>
</tr>
<tr>
<td>Glucosamine (colorimetric)</td>
<td>0.73</td>
</tr>
</tbody>
</table>
DISCUSSION

From a study of the chemical properties of scarlet fever toxin described in this paper, namely the nitrogen content, the presence of free amino nitrogen, the resistance to high temperatures and to wide changes in the hydrogen ion concentration, the precipitation by high concentrations of ammonium sulfate, the color of the biuret reaction, and the passage by ultrafiltration through collodion membranes which do not let cytochrome c through, it is logical to conclude that the toxin is a protein of small molecular weight. The toxin is not only resistant to heat and to changes of pH value, but also to the action of active proteolytic enzymes such as pepsin and trypsin. The loss of activity of the toxin by brief treatment with ketene, nitrous acid, and iodine in neutral solutions is good evidence in favor of the theory that the activity is related to the presence of amino groups in the protein molecule, amino groups which are destroyed by these agents. As porphyrinadin had been used for the estimation of certain mercaptans and of the sulfhydryl groups of certain proteins, the destruction of toxin activity by this reagent made it necessary to conduct a series of experiments with substances known to combine either with sulfhydryl or the disulfide groups. When none of these substances destroyed the activity of the toxin, it was found that porphyrindin was readily reduced by tyrosine, tyramine, and to some extent by tryptophane. Porphyrindin must therefore be rejected as a reagent for the detection of sulfhydryl groups of proteins, and the inactivation of the toxin by the dye may be attributed to its action on the OH group of tyrosine. (The positive Millon and Hopkins-Cole reactions in toxin are indicative of the presence of tyrosine and tryptophane in the toxin.)

The experiments on the electrophoretic mobility of the toxin are further evidence of its protein nature, for whether the toxin was made to migrate to the anode or to the cathode branch of the cell, a large portion of the nitrogen was retained in the active material. By assuming that the pure toxin contains 10 per cent nitrogen, it is calculated from these electrophoretic experiments that the material is 35 per cent pure. Since at pH values somewhat lower than those of the isoelectric point of the toxin (pH 5.55 at 3.5°) the protein impurity migrates towards the cathode
branch of the cataphoresis apparatus, there is here an easy method for further purification of the toxin.

The low carbohydrate and glucosamine content of the toxin, as well as the absence of phosphorus, speaks against considering it as a conjugated protein.

**SUMMARY**

Scarlet fever toxin is very resistant to the action of high temperatures (up to 100° for 15 minutes), wide variations in the hydrogen ion concentration (from pH 1.08 to 11.01), proteolytic enzymes (pepsin and trypsin), and a number of oxidizing and reducing agents. Its activity is destroyed by substances known to react with amino groups, such as ketene and nitrous acid. Evidence has been presented to support the view that inactivation produced by iodine and porphyrindin is also due to reaction with amino groups. From ultrafiltration with membranes of graded porosity it is concluded that the toxin is a protein of small molecular weight, between 13,000 and 4000. The isoelectric point of the toxin is 5.55 at 3.5°. As the carbohydrate and glucosamine content of the toxin is very low, and there is no phosphorus, it is concluded that the toxin is not a conjugated protein.

**BIBLIOGRAPHY**

Scarlet Fever Toxin