# PNEUMOCOCCUS HEMOTOXIN.*

By RUFUS COLE, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

It has been generally held that pneumococci or their products do not possess the power of producing lysis of red blood cells. On the other hand, it has long been known that the culture filtrates of certain bacteria, such as staphylococcus, certain races of streptococcus, and, above all, *Bacillus tetani,* possess this property to a marked degree. The properties of the hemolytic toxin produced by the last named organisms have been thoroughly studied by Madsen and others, and through this study many of the facts concerning hemolytic toxins have been discovered. Since such hemolytic toxins may be filtered, and since they may act as antigens, they may be considered true toxins in the Ehrlich sense. Old cultures of other bacteria, such as *Bacillus pyocyaneus* and *Bacillus anthracis,* may also be hemolytic, but the production of true hemolytic toxins by these organisms is considered doubtful. Indeed, very old cultures of practically all bacteria may produce hemolysis, but it is possible that this lytic effect, in certain of the cases at least, is due directly to changes in reaction of the old culture medium.

The property of certain races of streptococci of producing hemolysis has been considered by Schottmüller and others to be of great value in differentiating between the different varieties, and also between pneumococcus and the virulent streptococci, the so called *Streptococcus hemolyticus.* The usual method of determining whether bacteria possess this property is to grow them on agar plates containing blood, when in the case of hemolytic bacteria, such as *Streptococcus hemolyticus,* the colonies become surrounded by a transparent zone contrasting with the opacity of the rest of the medium, while in the case of non-hemolytic bacteria, such as pneumococcus, no such clear zones are seen.

* Received for publication, August 1, 1914.

While the power to produce hemolysis in culture medium is not possessed by pneumococci, or, if so, to a very slight degree, observations which we have made indicate that the bodies of pneumococci contain a substance or substances, which when set free are actively hemolytic, and that the serum of animals immunized to the bacteria or to the bacterial substance has increased power of neutralizing this lytic poison. These lytic substances differ from the so called bacterial hemotoxins in that they are contained within the bacterial cells and are only set free on the dissolution of the latter, but they may, nevertheless, have as great a pathological significance.

The first observations were made when studying the properties of a poison produced by dissolving washed pneumococci in dilute solutions of bile or sodium cholate.[1] The solution so obtained, which produces acute death in guinea pigs on intravenous injection, was also found to be hemolytic when added to an emulsion of red blood corpuscles in salt solution. At first this hemolytic property was thought to be due to the sodium cholate contained in the solution, but careful titration of the hemolytic power of the toxin showed that it was much greater than could be accounted for by the contained sodium cholate. In certain experiments, three hundred times as much sodium cholate in salt solution was required to produce hemolysis of 0.5 c.c. of an emulsion of sheep corpuscles as was contained in a minimal lytic dose of the toxin. It is not likely that the mere presence of bacterial substance increases the activity of cholate solutions, since the addition of protein to a cholate solution lessens its activity, and even peptone was found to have no intensifying action, but a slightly inhibiting action instead.

Later experiments have shown conclusively that cholate plays no important part in the reaction, since the lytic substances are present in extracts of pneumococci, obtained by allowing pneumococci to undergo autolysis in salt solution and also in extracts prepared by freezing and grinding the bacteria, in both cases without adding any cholate whatsoever.

## PREPARATION OF THE TOXIN.

In the study of the lytic effect of substances obtained from the bodies of pneumococci, the extracts have been prepared in one of the following ways:

(1) Pneumococci are grown for twenty hours in broth, removed from the broth by centrifugalization, and washed once in 0.85 per

[1] Cole, R., *Jour. Exper. Med.*, 1912, xvi, 644.

cent. salt solution. A test of the hemolytic power of toxins made from cultures grown for various lengths of time has shown that the toxins made from cultures twenty to twenty-four hours old are the most active. Toxins made from forty-eight-hour cultures possess little hemolytic power, while those made from seventy-two-hour cultures have no lytic power whatever. This difference is probably associated with the lysis of the bacteria which goes on in old cultures. The washed bacterial sediment is taken up in a small amount of salt solution, usually ten cubic centimeters for the bacteria from one liter of broth culture, and an amount of a 2 per cent. sodium cholate solution barely sufficient to cause solution of the bacteria is added. Usually one cubic centimeter is sufficient. After lysis has occurred, the solution is diluted with salt solution. If the bacteria were obtained from one liter of culture, the solution is usually made up to one hundred cubic centimeters. Different races of bacteria differ in the readiness with which they dissolve in cholate solution. Moreover, different solutions of bacterial bodies obtained in this way differ in their power to produce death in guinea pigs and also to produce hemolysis. Toxins, as above described, however, usually produce acute death in guinea pigs in doses of three to four cubic centimeters, and hemolysis of 0.5 of a cubic centimeter of sheep corpuscles in doses of 0.02 of a cubic centimeter or less, the whole mixture of toxin and corpuscles being made up to 2.5 cubic centimeters with salt solution.

(2) Pneumococci are grown in broth, washed, frozen, and ground, and the powder is dissolved in salt solution. A diluted toxin so prepared, which kills a guinea pig acutely in doses of three to four cubic centimeters, also usually produces hemolysis of sheep corpuscles, with the technique above described, in doses of 0.02 of a cubic centimeter or less.

Toxins prepared in the above ways are identical in their reactions so far as studied, and in the following pages no mention will be made of the method of preparation in each individual experiment.

A few experiments have also been conducted with extracts prepared by allowing pneumococci to undergo autolysis in salt solution. A series of tubes were prepared, all containing equal quantities of washed pneumococci and equal quantities of salt solution. These

were kept at 37° C., and from time to time a tube was removed and the hemolytic power of the fluid tested. It was found that the hemolytic power of the emulsion rapidly increased and between six and eight hours was at its maximum. There was a very slight fall in hemolytic power up to eighteen hours, but at twenty-four hours it had markedly diminished and was entirely absent after forty-eight hours.

PROPERTIES OF THE TOXIN.

Toxins prepared by solution of pneumococci are lytic for rabbit, sheep, guinea pig, and human red blood corpuscles. Other corpuscles have not been tested. The hemolytic power is greatest for guinea pig corpuscles, less for sheep and human corpuscles, and least for rabbit corpuscles, but the differences are not striking. The rate of hemolysis depends upon the concentration of the toxin. If the concentration be sufficiently great, complete hemolysis may occur within five to ten minutes at 37° C.

Active toxins have been obtained from pneumococci belonging to all of the four immunological groups.[2]

An attempt has been made to discover whether or not any relationship exists between the virulence of organisms employed and the hemolytic power of the extract, and a larger number of races have been studied with this point in mind. Rosenow[3] has stated that the more virulent races of pneumococci autolyze most readily. This is generally true, but there are many exceptions. Also the more virulent races are more soluble in bile, though to this also there are apparent exceptions. When a series of cultures of pneumococci are tested, these two properties do not bear constant positions with reference to the virulence of the organisms. It may also be stated from our study that the most active hemolytic toxins are usually obtained from those races of pneumococci that have been most lately cultivated from the animal body and are most virulent. But here again there are sufficient exceptions to throw some doubt on the validity of the generalization. The attempt to increase the hemolytic power of the toxin produced from a given race by repeated passage through animals, testing the toxin production from time to time, does not

[2] Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.
[3] Rosenow, E. C., *Jour. Infect. Dis.*, 1912, x, 113.

yield results that are uniform and consistent. With a given race the hemolytic power of the toxin varies markedly from time to time, even though in each test the toxin is prepared in exactly the same way. This variation probably depends somewhat on the luxuriance of the growth, and this on many factors,—the exact composition of the medium, the exact temperature of the thermostat, etc. The amount of autolysis that has gone on in the culture before centrifugalization is also of importance. It is therefore impossible to predict with any given race and culture exactly the strength of the toxin that will be obtained. The conditions are quite different from those obtaining in the production of diphtheria toxin with different races of bacilli. Here races vary markedly in their power to produce toxin, but this property seems to be fixed in certain races, and no matter how long they are grown outside the body or under what unusual conditions, the power of producing toxin in large amounts is preserved.

As regards the relationship between the hemolytic power and the toxic power as tested by intravenous injection into guinea pigs, there is a much more constant relationship. Even here the parallelism is not exact, but actively hemolytic solutions have always been found to be toxic and, with a few exceptions, the reverse is true. During the past two years toxins from a large number of cultures have been tested, and from the results obtained the above conclusions are derived.

The same manipulations which affect the hemolytic power of the toxin also affect the toxic power. It is impossible to draw an absolute conclusion from this that the same substance which produces hemolysis is the cause of the fatal effect in guinea pigs, though this is strongly suggested. On the other hand, symptoms and pathological changes in the guinea pigs do not seem to indicate that the animals die from the effects of hemolysis alone. In these animals hematuria frequently occurs and focal hemorrhages are seen post mortem, but there are no indications of a wide-spread hemolysis either when death occurs acutely or only after several hours. It is possible that, as in tetanus toxin, the effects may be due to two substances occurring together.

The effect of heat, acids, etc., on the toxin as tested by its power

to produce hemolysis, corresponds with the previously reported effects of these agents on the poison as tested by injection into guinea pigs.[4] Heating to 55° C. for one half hour in our experience always destroys the hemolytic power of the toxin. Heating for one and one half hours at 45° C. usually has no effect, though in one experiment the hemolytic power was diminished after heating one half hour at 45° C. Rosenow[5] has stated that if the autolysate of pneumococci be plunged into boiling water and boiled for ten minutes, then quickly cooled in ice water, the toxicity for guinea pigs frequently remains. As far as the hemolytic effect is concerned, this statement does not hold good, for boiling for ten minutes and then plunging into ice water completely destroys its activity. The hemolytic power is slowly lost when the toxin is kept for some time on ice, though the change does not begin until after eighteen to twenty-four hours.

The hemolytic effect of the solution is markedly diminished or entirely lost after passing through a Berkefeld filter. However, even where controls have shown that the filters entirely prevent the passage of bacteria, the filtrate may still possess some hemolytic power, but it is diminished.

Digestion of the hemolytic toxin with trypsin destroys its activity within forty-five minutes. This is shown by the following experiment.

Fairchild's trypsin solution was employed, each cubic centimeter of which contains 300 units. Even dilute solutions of sodium hydroxide may cause hemolysis, so in making these tests a 5 per cent. solution of sodium carbonate was used to render the mixture alkaline.

The following mixtures were prepared, using toxin prepared from frozen and ground bacteria:

(*a*) 12 c.c. toxin.
3 c.c. 0.85 per cent. sodium chloride solution.

(*b*) 12 c.c. toxin.
1.5 c.c. trypsin solution.
1.5 c.c. 0.5 per cent. solution sodium carbonate.

Two series of tests were made (table I). In series 1 the mixtures were made up cold and the sheep corpuscles were added at once. In series 2 the mixtures were incubated at 37° C. for 45 minutes before the corpuscles were added.

[4] Cole, R., *Jour. Exper. Med.*, 1912, xvi, 644.

[5] Rosenow, E. C., *Jour. Infect. Dis.*, 1912, xi, 235.

TABLE I.[6]

*Series 1.*

| Tube No. | 1 c.c. of mixture in dilution. | Sodium chloride solution. | Emulsion of sheep corpuscles. | Results. Readings after 3 hrs. at 37° C. | |
|---|---|---|---|---|---|
| | | | | Mixture (*a*). | Mixture (*b*). |
| 1 | 1 : 2 | 1 c.c. | 0.5 c.c. | + | + |
| 2 | 1 : 4 | 1 c.c. | 0.5 c.c. | + | + |
| 3 | 1 : 8 | 1 c.c. | 0.5 c.c. | + | + |
| 4 | 1 : 16 | 1 c.c. | 0.5 c.c. | + | + |
| 5 | 1 : 32 | 1 c.c. | 0.5 c.c. | + | + |
| 6 | 1 : 64 | 1 c.c. | 0.5 c.c. | + | + |
| 7 | 1 : 128 | 1 c.c. | 0.5 c.c. | ± | ± |
| 8 | 1 : 256 | 1 c.c. | 0.5 c.c. | ø | o |
| 9 | 1 : 512 | 1 c.c. | 0.5 c.c. | o | o |
| | | | *Series 2.* | | |
| 1 | 1 : 2 | 1 c.c. | 0.5 c.c. | + | o |
| 2 | 1 : 4 | 1 c.c. | 0.5 c.c. | + | o |
| 3 | 1 : 8 | 1 c.c. | 0.5 c.c. | + | o |
| 4 | 1 : 16 | 1 c.c. | 0.5 c.c. | + | o |
| 5 | 1 : 32 | 1 c.c. | 0.5 c.c. | + | o |
| 6 | 1 : 64 | 1 c.c. | 0.5 c.c. | + | o |
| 7 | 1 : 128 | 1 c.c. | 0.5 c.c. | + | o |
| 8 | 1 : 256 | 1 c.c. | 0.5 c.c. | ± | o |
| 9 | 1 : 512 | 1 c.c. | 0.5 c.c. | ± | o |

At 37° C. for 45 min.

Controls made with toxin plus sodium carbonate and with sodium carbonate alone showed that the latter had no effect on hemolysis.

The fact that the action of trypsin destroys the hemolytic effect of the solution obviously does not prove that the toxin is of protein nature. It indicates, however, that the toxin is probably closely associated with the protein constituents.

Attempts to extract the toxic substance with ether have so far proved unavailing. The toxin has been extracted with large amounts of ether, the ether evaporated under a fan in the cold, and the oily residue taken up in a small amount of alcohol and made into an emulsion in salt solution. Such an emulsion, however, has not been found hemolytic. There is no evidence, therefore, that the hemolytic effects are due to fatty or lipoidal constituents of the bacterial cells.

The presence of blood serum is known to inhibit the action of certain hemolytic toxins, not only bacterial toxins but others as well. Experiments with normal serum showed that the action of the pneumococcus hemolytic toxin is also inhibited to some extent by the

[6] In the tables + indicates complete hemolysis; ± indicates partial hemolysis; ø indicates a trace of hemolysis; o indicates no hemolysis.

presence of normal horse serum, to a somewhat greater extent by normal sheep and normal human serum, and to a still greater extent by normal rabbit serum. This antihemolytic effect is also possessed by dilute solutions of egg albumen. The effect of mucus contained in the sputum of a patient suffering from pneumonia was also tested. The mucus was shaken in salt solution; this mixture was added to the toxin and after one half hour at 37° C., sheep corpuscles were added. It was found that the mucus also had marked inhibiting power. Noguchi[7] has brought evidence to show that the inhibiting effect of normal blood serum for tetanolysin is due, in part at least, to the presence of cholesterin. It was therefore important to determine whether or not the hemolytic effect of the pneumococcus toxin was inhibited by the presence of cholesterin.

A protocol of one of the experiments to determine the effect of cholesterin in inhibiting hemolysis is given below.

*Toxin.*—June 17, 1914. Toxin was prepared by adding the washed bacteria from 500 c.c. of a 20-hour broth culture of pneumococcus A69 to 2.5 c.c. of salt solution plus 1 c.c. of a 2 per cent. solution of sodium cholate, placing the mixture at 37° C. for 15 minutes and then adding salt solution to 50 c.c.

*Cholesterin Emulsion.*—Cholesterin crystals were dissolved in a small amount of warm ether, and then sufficient warm sodium chloride solution was added, shaking constantly, to obtain a 1 per cent. emulsion. This was heated over a steam bath for 30 minutes to drive off the ether and filtered.

The experiments (table II) have shown conclusively that exceedingly small amounts of cholesterin are able to inhibit the action of the toxin. It is probable that the inhibiting effect of serum is also due to its cholesterin content. It is unnecessary to discuss the theoretical aspects of this phenomenon here, since the problem has been thoroughly considered by Noguchi and others in connection with the inhibition of tetanus hemolysis by serum. As they have concluded, this inhibition by cholesterin probably indicates that the lipoidal constituent of the red blood cell plays an important part in hemolysis.

It also seemed of importance to determine the effect of lecithin on the hemolytic action of this toxin. Kyes[8] has shown that the presence of lecithin increases the hemolytic action of cobra venom,

[7] Noguchi, H., *Univ. Penn. Med. Bull.*, 1902, xv, 327.

[8] Kyes, P., *Berl. klin. Wchnschr.*, 1902, xxxix, 918.

TABLE II.

*Hemolytic Test with Toxin.*

| Tube No. | Experiments. | Hemolysis. | |
|---|---|---|---|
| | | 1 hr. at 37° C. | 24 hrs. on ice. |
| 1 | 1 c.c. toxin undiluted + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | + | + |
| 2 | 1 c.c. toxin diluted (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | + | + |
| 3 | 1 c.c. toxin diluted (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | + | + |
| 4 | 1 c.c. toxin diluted (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | + | + |
| 5 | 1 c.c. toxin diluted (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | + | + |
| 6 | 1 c.c. toxin diluted (1 : 32) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | + | + |
| 7 | 1 c.c. toxin diluted (1 : 64) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | + | + |
| 8 | 1 c.c. toxin diluted (1 : 128) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | ± | ± |

*Test of Inhibition with Cholesterin.*

| | | | | |
|---|---|---|---|---|
| 1 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100) | 30 min. at 37° C. 0.5 c.c. sheep corpuscles added to each tube. | o | o |
| 2 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 500) | | o | o |
| 3 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 1,000) | | o | o |
| 4 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 5,000) | | o | o |
| 5 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 10,000) | | o | o |
| 6 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 50,000) | | ø | ± |
| 7 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100,000) | | ± | ± |
| 8 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. sodium chloride solution | | + | + |
| 1 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100) | 5 min. at 37° C. 0.5 c.c. sheep corpuscles added to each tube. | o | o |
| 2 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 500) | | o | o |
| 3 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 1,000) | | o | o |
| 4 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 5,000) | | o | o |
| 5 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 10,000) | | o | ø |
| 6 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 50,000) | | ø | ± |
| 7 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100,000) | | + | + |
| 8 | 1 c.c. toxin diluted (1 : 25) + 1 c.c. sodium chloride solution | | + | + |

the lecithin acting as an activator. He[9] has also been able to form a combination of the active constituent of cobra venom with lecithin. On the other hand, it has been shown that with other hemolytic toxins lecithin may have an inhibiting action.

The following protocols of experiments indicate the effect of lecithin on the pneumococcus hemolytic toxin (table III).

TABLE III.

| Tube No. | Experiments of July 2, 1914. | | Hemolysis. |
|---|---|---|---|
| 1 | 1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 10) | 30 min. at 37° C. 0.5 c.c. emulsion of sheep corpuscles added to each tube. | ø |
| 2 | 1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 50) | | ø |
| 3 | 1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 100) | | ø |
| 4 | 1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 500) | | + |
| 5 | 1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. sodium chloride solution | | + |
| 6 | 1 c.c. toxin A69 diluted (1 : 64) + 1 c.c. sodium chloride solution | | + |
| 7 | 1 c.c. toxin A69 diluted (1 : 128) + 1 c.c. sodium chloride solution | | + |
| 8 | 1 c.c. toxin A69 diluted (1 : 256) + 1 c.c. sodium chloride solution | | ± |

The tubes were kept for 1 hr. at 37° C. and 24 hrs. on ice.

The lecithin emulsion was prepared by making a 1 per cent. solution of Merck's lecithin in methyl alcohol, and of this a 10 per cent. emulsion was made in 0.85 per cent. salt solution.

This and other similar experiments have shown that lecithin in low dilutions has slight inhibiting action on the hemolytic effect of the toxin.

To learn whether non-hemolytic doses were rendered hemolytic by the presence of lecithin, experiments like the following were made (table IV).

From this and a number of similar experiments, it is evident that lecithin in no case increases the action of the hemolytic toxin. Except for the slight inhibiting action previously noted, therefore, lecithin has no effect on the hemolytic action of the toxin.

[9] *Kyes, P., Berl. klin. Wchnschr.*, 1903, xl, 956, 982.

TABLE IV.

| Tube No. | Experiments of March 31, 1914. | Hemolysis. |
|---|---|---|
| 1 | 1 c.c. toxin (1 : 10) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | + |
| 2 | 1 c.c. toxin (1 : 50) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | + |
| 3 | 1 c.c. toxin (1 : 100) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | ± |
| 4 | 1 c.c. toxin (1 : 500) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles | 0 |
| 5 | 1 c.c. toxin (1 : 500) + 1 c.c. lecithin emulsion (1 : 10) + 0.5 c.c. sheep corpuscles | 0 |
| 6 | 1 c.c. toxin (1 : 1,000) + 1 c.c. lecithin emulsion ( 1: 10) + 0.5 c.c. sheep corpuscles | 0 |
| 7 | 1 c.c. sodium chloride + 1 c.c. lecithin emulsion (1 : 10) + 0.5 c.c. sheep corpuscles | 0 |

The tubes were kept for 2 hrs. at 37° C.

## NATURE OF THE HEMOLYTIC TOXIN.

It is not the purpose of the present paper to discuss the chemical nature of the toxin or its mode of action. Sufficient study of it has not been made to render such a discussion profitable. From the properties already described it is evident that it is closely related to certain other vegetable and animal hemolytic toxins. It is extremely labile, is readily absorbed (as shown by the difficulty with which it passes through a bacterial filter), is destroyed by the action of trypsin, and its action is prevented by the presence of minute amounts of cholesterin and of larger amounts of lecithin.

To the view that it does not exist preformed in the bacterial cell, but is a product arising during self digestion or autolysis, the conclusive objection may be raised that it is present in the solutions prepared by freezing, drying, and grinding the bacteria. During this process there has been no opportunity for autolysis and the conclusion seems justified that the hemolytic substance is contained within the living bacterial cell.

## ANTIHEMOLYTIC SERA.

In order to demonstrate that the hemolytic substance under discussion is of the nature of a true toxin, it is necessary to show that it possesses antigenic properties; that is, that its action is inhibited by the serum of animals immunized to it. For purposes of immuniza-

tion rabbits and sheep were employed. These were injected intravenously with increasing doses of the toxin every seven to eight days. The toxins for injection were prepared by dissolving the pneumococci in sodium cholate solution, according to the method previously described, and they were centrifugalized before injection. It is probable that with each injection a few living organisms were also introduced. Since the serum of these animals acquired no agglutinating power for the homologous organisms, however, it is not likely that the acquired properties of the serum were due to the antigenic properties of these few bacteria.

(*a*) *Immune Rabbit Serum.*—The protocol of one experiment is given below (table V).

*Rabbit 82-E.*—Immunization commenced June 14, 1912. Received 5 doses of toxic extract intravenously during a period of 4 months. The animal received no further injections until June 5, 1913. It then received 7 doses of toxin intravenously at intervals of 6 to 7 days, the last injection being made on July 19. Bled on July 31.

*Rabbit 285-A.*—Immunization commenced June 12, 1913. Received 7 increasing doses of toxin intravenously, the last one being given on July 19. Animal bled on July 31.

TABLE V.

*Sera Tested July 31, 1912. Toxin Prepared from Homologous Organisms.*

| Experiments. | | Hemolysis. | | | |
|---|---|---|---|---|---|
| | | Serum 82-E. | Serum 285-A. | Normal rabbit serum. | No serum. |
| Toxin diluted (1 : 8) + serum diluted (1 : 10) | ½ hr. at 37° C. | 0 | 0 | ± | |
| Toxin diluted (1 : 8) + serum diluted (1 : 50) | | 0 | 0 | + | |
| Toxin diluted (1 : 8) + serum diluted (1 : 100) | | 0 | 0 | + | |
| Toxin diluted (1 : 8) + serum diluted (1 : 500) | | ± | ± | + | |
| Toxin diluted (1 : 8) + serum diluted (1 : 1,000) | | ± | + | + | |
| Toxin diluted (1 : 8) + sodium chloride | | | | | + |

The experiments have been repeated many times with the sera of six rabbits immunized to this toxin. While in no case has the antihemolytic power of the immune serum been greater than 0.002 of a cubic centimeter to two hemolytic units of toxin, in all experiments the antihemolytic power of the immune serum has been considerably

greater than that of normal rabbit serum. Tests of the rabbit sera for agglutinins were made from time to time, but none of the sera acquired the power of agglutinating the homologous organisms.

(*b*) *Immune Sheep Serum.*—Three sheep have been given increasing doses of extracts of pneumococci. Two of these animals were treated with extracts of pneumococci of type I and one received injections of pneumococci of type III, the so called *Pneumococcus mucosus.* The results obtained from the study of the sera of these sheep were identical, and data concerning the serum of but one are given.

*Sheep A.*—Immunization was commenced May 29, 1913. The toxins for injection were prepared by dissolving pneumococci in sodium cholate solution, as previously described, and the injections were all made intravenously. During the period of about 6½ months the sheep received 18 injections of the toxin. The first injection of the toxin was prepared from the bacteria contained in 12.5 c.c. of a twenty-four-hour bouillon culture. The injections were gradually increased in size. On Dec. 2, 1913, the toxin injected was prepared from the bacteria contained in 1,900 c.c. of a twenty-four-hour bouillon culture. On Dec. 10, an injection was made of toxin prepared from the bacteria contained in 3,000 c.c. of a twenty-four-hour bouillon culture. This injection was apparently too large and probably contained a considerable number of living organisms. Following this injection the animal appeared sick, the temperature was elevated, and the respirations were rapid. After a few days' illness the animal appeared better, but a cough persisted for 2 months with gradual loss of weight and strength, and it died Feb. 10, 1914.

*Autopsy.*—The pleural and pericardial sacs showed extensive fibrous adhesions. The lungs were edematous. Smears and cultures from the heart's blood showed the presence of pneumococci and a gas-forming anaerobic bacillus morphologically like *Bacillus tetani.* Unfortunately, through an error the cultures were destroyed before the identity of the latter organism could be accurately determined.

TESTS OF SERUM OF SHEEP A.

*Agglutination.*—The serum was repeatedly tested for agglutination with the homologous organism, the last test being made with serum obtained on December 2, 1913. At no time did the serum possess any agglutinating power.

*Protection.*—Repeated tests of the protective power of the immune sheep serum for mice were made, employing the technique used in this laboratory for determining protective power.[10] It was found that the serum possessed fairly well marked protective power against pneumococci of type I, and some protective power against

[10] Dochez, A. R., *Jour. Exper. Med.*, 1912, xvi, 665.

pneumococci of other types, except type III, against which no protective power is ever present. The protective power, however, was never so high as that of the serum of horses immunized by injection of living organisms, nor was it so specific.

TABLE VI.

*Sheep Serum A, Obtained September 14, 1913. Inactivated. Tested September 15, 1913. Toxin 1.70.*

| Tube No. | Experiments. | | Hemolysis. | |
|---|---|---|---|---|
| | | | Normal sheep serum. | Immune sheep serum A. |
| 1 | 1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 10) ... | ½ hr. at 37° C. 0.5 c.c. emulsion sheep corpuscles added to each tube. | 0 | 0 |
| 2 | 1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 50) ... | | ø | 0 |
| 3 | 1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 100) .. | | ø | 0 |
| 4 | 1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 500) .. | | ± | 0 |
| 5 | 1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 1,000). | | + | 0 |
| 6 | 1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 5,000). | | + | ± |
| 7 | 1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 10,000) | | + | + |
| 8 | 1 c.c. toxin 1.70 + 1 c.c. sodium chloride ..... | | + | + |

The tubes were kept for 1 hr. at 37° C. and 24 hrs. on ice.

*Antihemolytic Power.*—(Table VI.) This and many similar experiments indicate that the serum of an animal immunized by the injection of toxin possesses an increased antihemolytic power. This antihemolytic action is not highly specific, however, as regards the type of organisms; a serum produced by the injection of toxin prepared from pneumococci of type I protects almost as well against a toxin prepared from organisms of type II as against one prepared from pneumococci of homologous type.

In immunization in this manner, the possibility cannot be excluded that in addition to the introduction of the toxin living organisms have also been introduced. It was therefore important to know whether or not an immune serum produced by the injection of living organisms would or would not have an effect on inhibiting the action of the hemolytic toxin. For this purpose the sera of horses immunized by the injection of living pneumococci were studied.

(*c*) *Immune Horse Serum.*—The horses from which these sera were obtained were immunized by injecting intravenously repeated and increasing numbers of living pneumococci previously washed

in salt solution. These bacteria were obtained by centrifugalization from bouillon cultures. Usually one or two doses of pneumococci killed by heat were injected before proceeding to the injection of living bacteria. The sera studied were those that have been used in the treatment of patients and were active in protection only against the organisms of the type used in immunization. The sera were actively and specifically agglutinating.

Serum I had such protective power that when 0.2 of a cubic centimeter of the serum was mixed with 0.1 of a cubic centimeter of a twenty-hour bouillon culture of organisms of type I and the mixture was injected into a mouse, the mouse lived; whereas 0.000001 of a cubic centimeter of the culture injected alone killed within twenty-four hours.

Serum II had a little less protective power, in that 0.2 of a cubic centimeter of serum protected against only 0.01 of a cubic centimeter of culture of which 0.000001 of a cubic centimeter injected alone killed within twenty-four hours.

Tests of the antihemolytic power gave results similar to those obtained in an experiment of which the following is the protocol (table VII).

The study of these sera showed that they possessed high neutralizing power for the hemolytic poison obtained from the bodies of pneumococci, even higher than that present in the serum of rabbits or sheep injected with the toxin. This antihemolytic power, however, is not very specific as regards type of organisms, serum I protecting against the toxin prepared from organisms of type II almost as well as against that prepared from organisms of type I and *vice versa*. That this protective action is not merely a non-specific reaction of all immune sera, however, is shown by the fact that an anti-influenzal serum, kindly supplied by Dr. Wollstein,[11] possessed little or no greater antihemolytic power than did normal serum. In the experiment above described, the anti-influenzal serum had a little greater effect than did normal horse serum, but it was no greater than that of other normal horse sera tested at other times.

(*d*) *Antihemolytic Action of the Serum of Patients Sick of Pneu-*

[11] Wollstein, M., *Jour. Exper. Med.*, 1911, xiv, 73.

TABLE VII.

*Toxin 1.70 Prepared from Pneumococci of Type I.*

| Experiments of October 7, 1913. | | | Hemolysis. | | | |
|---|---|---|---|---|---|---|
| | | | Immune horse serum I. | Immune horse serum II. | Normal horse serum. | Anti-influenzal serum. |
| Toxin (2 hemolytic units) + serum 0.1 c.c. | ½ hr. at 37° C. | Sheep corpuscles 0.5 c.c. | o | o | + | o |
| Toxin (2 hemolytic units) + serum 0.02 c.c. | | Sheep corpuscles 0.5 c.c. | o | o | + | ± |
| Toxin (2 hemolytic units) + serum 0.01 c.c. | | Sheep corpuscles 0.5 c.c. | o | o | + | + |
| Toxin (2 hemolytic units) + serum 0.002 c.c. | | Sheep corpuscles 0.5 c.c. | o | ø | + | + |
| Toxin (2 hemolytic units) + serum 0.001 c.c. | | Sheep corpuscles 0.5 c.c. | o | ± | + | + |
| Toxin (2 hemolytic units) + serum 0.0002 c.c. | | Sheep corpuscles 0.5 c.c. | + | + | + | + |
| *Toxin A69, Prepared from Pneumococci of Type II.* | | | | | | |
| Toxin (2 hemolytic units) + serum 0.1 c.c. | ½ hr. at 37° C. | Sheep corpuscles 0.5 c.c. | o | o | + | o |
| Toxin (2 hemolytic units) + serum 0.02 c.c. | | Sheep corpuscles 0.5 c.c. | o | o | + | + |
| Toxin (2 hemolytic units) + serum 0.01 c.c. | | Sheep corpuscles 0.5 c.c. | o | o | + | + |
| Toxin (2 hemolytic units) + serum 0.002 c.c. | | Sheep corpuscles 0.5 c.c. | o | o | + | + |
| Toxin (2 hemolytic units) + serum 0.001 c.c. | | Sheep corpuscles 0.5 c.c. | ø | ± | + | + |
| Toxin (2 hemolytic units) + serum 0.0002 c.c. | | Sheep corpuscles 0.5 c.c. | + | + | + | + |

The tubes were kept at 37° C. for 1 hr. and 24 hrs. on ice.

*monia and of Those Convalescent from That Disease.*—The sera of patients suffering with pneumonia and those of patients during convalescence from this disease have been tested for antihemolytic power against the pneumococcus toxin. It has been impossible, however, to demonstrate that these sera possess an increased antihemolytic action over the controls with normal human serum and the serum of patients suffering from other diseases.

From these studies of the antihemolytic action of immune sera, it is evident that by the injection into rabbits and sheep of a solution containing the bacterial substance of pneumococci, the serum of these animals acquires an increased power of inhibiting the hemolytic action of such a solution. This change in the serum occurs

when the fluid injected consists of solutions of the bacterial bodies in sodium cholate or of solutions prepared by freezing the bacteria and grinding them in salt solution. The antihemolytic power of these sera is not so great, however, as that of sera produced by the injection of living organisms. The latter sera, however, possess marked agglutinating properties, while the former sera have no power of agglutination. While in the production of antitoxic sera the possibility of the injection of a few living organisms cannot be excluded, the lack of agglutinating power renders it extremely probable that the development of antihemolytic properties is due to the injection of the hemolytic substance, and that, therefore, this hemolytic solution possesses antigenic properties and may be considered a true toxin.

## SUMMARY.

Solutions of the bodies of pneumococci, obtained by dissolving them in dilute solutions of sodium cholate, by permitting them to undergo autolysis, or by first freezing, drying, and then grinding in salt solution, are actively hemolytic for rabbit, sheep, guinea pig, and human red blood corpuscles. The substance on which this hemolytic property depends is very labile, much of its activity is lost on passing through a filter, and it is destroyed by the action of trypsin. In its properties it corresponds to the substance contained in such solutions which causes the death of guinea pigs on intravenous injection. Its activity is prevented by the presence of minute amounts of cholesterin.

Following the injection of this solution into rabbits and sheep, the sera of these animals acquire increased power of inhibiting its hemolytic action. It therefore possesses antigenic properties.

It may therefore be concluded that the bodies of pneumococci contain a toxin that is hemolytic for red blood corpuscles. This substance is not simply a product of autolysis but undoubtedly exists preformed in the bacterial cell. However, it is not given up to the surrounding fluid as long as the bodies of the bacteria are intact. It may therefore be considered a hemolytic endotoxin.