THE PRODUCTION OF PURPURA BY DERIVATIVES OF PNEUMOCOCCUS.

I. General Considerations of the Reaction.

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During the course of studies on oxidation and reduction by pneumococcus made by Avery and Neill (1), Neill made the observation that when pneumococcus extract was injected into white mice hemorrhagic purpura developed. At a later date, we made a similar observation. This paper is a report of the study of the nature of this phenomenon.

In 1907, Heyrovsky (2) reported that he was able to produce purpura in white mice by injecting 24 hour filtrates of virulent pneumococci grown in dextrose broth. No other reference to this reaction has come to our attention.

We have found that if pneumococcus extracts, prepared by one of the various methods to be described later, are injected into white mice, after 4 to 6 hours the skin over the feet, tail, ears, snout, and genitalia take on a dark bluish purple color. This peculiar appearance is confined to the areas where the hair is either absent or scanty, but removal of the hair does not render the skin in the shaved area reactive. The intensity of the discoloration varies in the different regions and only one or several or all of the sites mentioned may be involved. Unless the amount of extract injected is very large the animals show no signs of intoxication and all recover. The lesion is at its maximum intensity usually in 24 to 48 hours following the injection and then slowly disappears, vanishing entirely in 5 to 7 days.

The development of the reaction may best be observed in the ear by transmitted light. It is then seen that the lesion begins as a small hemorrhage about the blood vessels and the extravasation extends into the surrounding tissues.

The microscopical changes[1] are best studied in sections made of the skin and viscera of animals autopsied immediately after they have died or been killed by chloroform vapor. It is then found that the changes are not confined to the skin but are also present in the lungs and voluntary muscles. The essential lesion consists of extravasations of blood into the loose tissues surrounding the blood vessels. In the skin the hemorrhages are usually found in the subcutaneous tissues, but may also be present in the superficial layers of the corium. The hemorrhages may be minute or may involve quite extensive areas. Frequently small collections of polymorphonuclear leucocytes are present in the hemorrhagic areas.

The degree of purpura is influenced both by the amount of the material introduced and the individual susceptibility of the experimental animal. Using a fixed quantity of extract for a series of mice there is considerable variation in the severity of the reaction in the individual animals. 0.2 cc. of extract usually produces purpura in half of the mice of a series. 0.4 cc. gives 90 per cent or more positive results with rather severe reactions. More than this amount proves too toxic and death usually occurs, although some mice have been able to withstand 0.6 cc. and, rarely, 0.8 cc. of the extract.

The reaction is produced equally well whether the extract is introduced subcutaneously, intraperitoneally, or intravenously. The intravenous method usually results in a more rapid reaction. Attempts to effect purpura by feeding have not, however, been successful. After being subjected to chloroform, mice were fed by stomach tube with amounts much greater than necessary to cause purpura if injected, but in these cases no purpura was evidenced. This is not surprising since trypsin destroys the activity of the purpura-producing principle.

Purpura was produced in guinea pigs and rabbits also. In the guinea pig the ears and the pads of the feet were affected; while the ears and scrotum of male rabbits,—which alone were used,—were chiefly involved.

Derivatives of Pneumococcus Causing Purpura.

In our study the development of purpura was first observed in white mice as a response to injections of pneumococcus extract pre-

[1] The pathology of the effect of pneumococcus extract in experimental animals is under study by Dr. Branch and a report upon it will be made subsequently.

pared according to the method of Avery and Neill (3). Purpura was also produced in white mice by Heyrovsky's method. Cultures of pneumococcus were grown in dextrose broth and a 1 day's growth was filtered and injected intraperitoneally. The reaction, however, was much less constant and less severe than that obtained by the introduction of extract. Moreover, when purpura did appear, as much as 1.5 to 2.0 cc. were required to produce the reaction. Filtrates of young cultures (8 to 16 hours) grown in plain beef infusion broth, however, have never yielded purpuric lesions. Filtrates from very old cultures grown in plain infusion broth for 3 weeks only rarely have given purpuric reactions and when they did the activity was slight.

Whole cultures, unfiltered, rarely cause the purpuric reaction. Young cultures usually cause an infection followed by rapid death of the mice. In hundreds of mice injected with young cultures of pneumococcus for one purpose or another never but once has purpura followed the injection. On this occasion the mouse had been injected with a virulent culture together with sufficient immune serum to afford passive protection. On the 5th day following the injection purpura was noted. It increased in intensity until the 7th day and then began to blanch. The infection itself, however, does not prevent the appearance of purpura, for purpura results when an active extract is injected into an animal together with a minimum infecting dose of culture.

Old cultures occasionally produce purpura. Two strains of Type III have given the most consistent results. After the original 24 hour culture in blood broth had been stored for a week or more in the ice chest, these strains were able to produce purpuric changes. Since the mice were not severely infected, it is probable that during storage the cultures had undergone sufficient autolysis to be partially transformed into extract-like substances.

Bile-dissolved cultures have never induced purpura, even when solutions prepared from young cultures of pneumococci, concentrated to the same extent as is done in preparing extracts, and dissolved in a minimum quantity of bile, are employed. That bile in itself does not inhibit the purpurogenic activity of an extract was shown by adding bile to an active extract in a concentration of 10 per cent. This mixture injected into mice produced purpura.

Pneumococci which had been killed by exposure to a temperature of 56°C. for 30 minutes,—a treatment sufficient to kill the pneumococci but not to destroy the activity of the purpura-producing material,—did not produce purpura in white mice. The soluble specific substance and nucleoprotein of pneumococcus (Heidelberger and Avery (4)) failed to produce purpura regardless of the quantity injected.

The observations so far recorded suggest that the substance, which on injection gives rise to purpura, is contained in the bodies of the pneumococci and is set free by the process of extraction employed. It is also found occasionally in the culture filtrate. It has been of importance to determine whether this substance exists preformed in the bacterial cell or whether it is a product of a digestive process occurring during the process of extraction.

Concentrated suspensions of pneumococci were prepared by centrifuging 1500 cc. of a 9 hour broth culture and suspending the sediment in 15 cc. of broth. When trypsin was allowed to act on a portion of this concentrated suspension the resulting fluid was not active. This was the case whether the bacteria in the emulsion were alive or had been killed by heat before exposure to the action of trypsin.

If, instead of trypsin, the native enzymes of the pneumococcus were allowed to act on the concentrated emulsion, the results were quite different, since the product in this case was highly active. In this experiment to 15 cc. of the concentrated pneumococcus emulsion, 10 cc. of an homologous extract containing the pneumococcus enzymes (Avery and Cullen (5)) were added and the mixture was incubated for 40 hours at 37°C. The mixture was then centrifuged and the supernatant fluid filtered. This filtrate was now found to be as active as the pneumococcus extract prepared by the Avery-Neill method.

The Nature of the Purpura-Producing Principle.

Aliquot portions of pneumococcus extract were heated at 60°C., 70°C., 80°C., and 100°C. for 10 minutes each. At the higher temperature exposures, coagulation occurred. The various specimens were injected individually into the peritoneum of white mice and it was observed that heating even at 100°C. for 10 minutes did not destroy the activity of the purpura-producing material. The results are appended in Table I.

Avery and Neill were able to destroy both the hemolytic and oxidation-reduction activity of extracts by exposure to air. It was of

interest to determine, therefore, the effect of oxidation on the purpura-producing principle of the extracts. Pneumococcus extract was oxidized until it was no longer hemolytic. It was still capable of producing purpura when injected into white mice. It can be said, then, that the purpura-producing principle is not associated with the hemolytic activity of the extracts, since the latter alone is destroyed by heat and oxidation.

Extracts of all groups of pneumococci contain the purpura-producing material. Experiments performed to correlate the purpura-producing activity with virulence showed no relationship between these two properties. An avirulent Group IV strain gave as good a purpura-producing extract as any of the most highly virulent strains in this laboratory.

TABLE I.

The Effect of Heat on the Purpura-Producing Material.

Each mouse was injected intraperitoneally wth 0.4 cc. of the respective extract.

Type of extract.	No. of mice in test.	No. of mice developing purpura.
Unheated extract	4	3
Extract heated 10 min. at 60°C.	2	2
" " 10 " " 70° "	2	2
" " 10 " " 80° "	2	1
" " 10 " " 100° "	6	5

Because it has been shown (6, 7) that an avirulent culture of pneumococcus may be a strain composed of both virulent and avirulent organisms, the experiment was extended to include a study of the extracts prepared from the R or purely avirulent strains. It was shown that the extracts possessed purpura-producing material.

Isolation of the Purpura-Producing Material.

For the isolation of the purpura-producing material from the extracts, saline extracts of pneumococcus were prepared according to the technique of Avery and Neill. These extracts were equivalent to the broth extracts in producing purpura. Normal acetic acid was added slowly to the saline extract until no further precipitation occurred. The material was centrifuged and all of the supernatant was withdrawn. The sediment was washed with normal salt solution, then dissolved in weak alkali and neutralized, and the volume was adjusted to the original

quantity. The supernatant no longer gave a precipitate with acetic acid. It was neutralized with normal NaOH.

The supernatant was next half saturated with $(NH_4)_2SO_4$ and a precipitate was obtained. This was centrifuged and the supernatant was collected and made up to full saturation with $(NH_4)_2SO_4$ and another precipitation was obtained.

The acetic acid precipitate, which contained essentially the nucleoproteins of pneumococcus (4) produced no purpuric lesions when injected into white mice. This was in accordance with the earlier observation on the nucleoprotein fraction. The supernatant from the acetic acid precipitation, on the other hand, was still able to produce purpura with apparently no loss in this property. The precipitate obtained by half saturation with $(NH_4)_2SO_4$ did not contain the purpura material while the supernatant was still purpura-producing. The precipitate obtained by full saturation with $(NH_4)_2SO_4$ was shown to contain the purpura-producing principle. This precipitate was washed free of salts by dialysis.

It was found that the purified purpura-producing principle was coagulated on heating and was precipitated by trichloracetic acid, sulfosalicylic acid, and alcohol. It gave a positive biuret reaction, but did not give a xanthoproteic reaction and the Millon reaction was a doubtful one. The activity to cause purpura was destroyed by trypsin digestion.

The purified material acted like the extracts themselves and mice showed the same degree of variation in susceptibility to it that they did to the extracts. As small an amount as 0.04 mg.,[2]—a dosage of about 1/500,000 of the body weight,—was sufficient to produce purpura in some of the mice. It proved to be highly toxic for rabbits, and 0.16 to 3.2 mg. was fatal to 3 rabbits. The purpura-producing substance was not precipitated either by anti-extract or antipneumococcus sera.

Purpuric Responses to Extracts from Other Bacteria.

Experiments were conducted to determine whether the purpura-producing material was peculiar to pneumococcus alone. Extracts

[2] These quantities were calculated as protein on the basis of the organic nitrogen in the solution obtained by full saturation of the extract with $MgSO_4$, after precipitation with acetic acid.

were made of two strains of *Staphylococcus aureus*, three strains of *Streptococcus viridans*, one strain of hemolytic streptococcus, and one strain of *Bacillus coli*.

For these organisms[3] the preparation of the extracts was modified, since rapid freezing and thawing did not give solutions of the bacterial cells. The cultures were centrifuged and to the sediment was added a small amount of sterile sand. The bacteria were then dried *in vacuo* at a temperature not exceeding 37°C. and then ground in a ball mill at room temperature for varying periods of 48 hours or more until a fine powder was obtained. At this stage N/100 alkali was added and extraction was allowed to take place in the ice chest, in the ball mill. On the following day the material was removed and centrifuged and the supernatant was used in the experiments. Care was taken to keep the concentrations equivalent to that of the pneumococcus extract.

Extracts were prepared from four strains of meningococcus by freezing and thawing.

The extracts from these organisms were not capable of producing purpura in white mice. Amounts as great as 2.5 cc. were used for injection.

DISCUSSION.

Evidence has already been presented to show that pneumococcus extracts possess a powerful hemolysin and an oxidation-reduction system which is operative on hemoglobin (Avery and Neill). The present paper offers data to show that the extracts possess in addition a toxic principle which is capable of producing experimental purpura in animals. This activity is derived from extracts of avirulent as well as of virulent pneumococci. Extracts of the other bacteria tested either do not possess this activity, or the methods which were applicable for pneumococcus were inadequate to demonstrate it.

The purpura-producing material is obtained from the extracts by full saturation with $(NH_4)_2SO_4$. Whether it is an albumin or some closely related substance as a higher proteose, for example, for which albumin may act merely as a vehicle remains to be determined.

It is significant that bile solutions of pneumococci did not yield the purpura-producing substance. That, on the other hand, bile does not prevent its activity has also been shown. And those constitu-

[3] We are indebted to R. C. Lancefield for this method.

ents of the pneumococcus which exist preformed in the cell are recoverable by dissolution of the cell with bile. The method has been used successfully by Cole (8) to obtain the hemolysin, and by Avery and Cullen (9) to obtain the enzymes of pneumococcus. The purpuric substance was found to be present in those materials alone which represented degradation products of pneumococci. It appears probable, therefore, that the purpura-producing property is due not to a constituent of the living pneumococcus cell, but to a degradation product of pneumococcus. That it is a separate entity from the pneumococcus hemotoxin has been shown selectively. The hemotoxin can be eliminated by oxidation and heat, while neither of these means destroys the activity of the purpura-producing material. Autolysates of pneumococcus are definitely purpura-producing, yet, as prepared by our method, they contain no demonstrable hemotoxin. The fact that autolysates were purpura-producing while autolyzed cultures were rarely, and at best, poorly purpura-producing, is explainable on the basis of concentration, since the autolysates were 50 times more concentrated than the cultures.

It is well recognized that purpura accompanying pneumococcus infections in man is of extreme rarity. There are, however, occasional instances recorded in the literature. Bazan (10) and Nobécourt and Mathieu (11) have reported upon them. In their cases the purpura always preceded death. Whether there is any relation between the purpura-producing principle and the pneumococcus infections cannot be said at the present time. It is quite possible that the purpura substance may function in infections without necessarily producing visible signs of purpura. Experiments are planned to determine whether the purpura principle plays any part in pneumococcus infections.

CONCLUSIONS.

1. Pneumococcus extracts have been shown to be capable of producing hemorrhagic purpura in white mice, rabbits, and guinea pigs.

2. The purpura-producing principle resists heating to 100°C. for 10 minutes; it resists oxidation; it is filter-passing; its activity is destroyed by digestion with trypsin; it is obtained from pneumococcus extracts by full saturation with $(NH_4)_2SO_4$, after the acetic acid-precipitable substances have been removed from the extracts.

3. The purpura-producing principle is common to all four types of pneumococcus and apparently bears no relation to virulence.

4. The purpura-producing principle is probably a degradation product of pneumococcus.

5. This principle is not associated with the hemotoxin of pneumococcus, since the hemolytic activity of an extract may be destroyed without effect on the ability to cause purpura.

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