THE PRODUCTION OF PURPURA BY DERIVATIVES OF PNEUMOCOCCUS.

III. Further Studies on the Nature of the Purpura-Producing Principle.

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(Received for publication, December 17, 1926.)

INTRODUCTION.

It will be recalled from a previous (1) communication that pneumococcus extract was found to produce hemorrhagic purpura in laboratory animals.[1] The purpura-producing principle resists oxidation and heating to 100°C. for 10 minutes; it is filter-passing; it is present in the fraction of pneumococcus extract obtained by full saturation with $(NH_4)_2SO_4$ after the acetic acid-precipitable substances have been removed. The principle is not associated with type specificity or virulence of the organism and is distinct from the endohemotoxin always present in reduced extracts of Pneumococcus (2). The opinion was expressed at the time that the purpura material was probably a degradation product of pneumococci, since it is present only when cell disintegration accompanied by autolysis has taken place.

Subsequent study (3), established the fact that pneumococcus extract is thrombolytic both *in vivo* and *in vitro*, and that the development of purpura in mice following the injection of pneumococcus extract is associated with an excessive diminution in the number of blood platelets, the greatest decrease usually occurring within 24 hours. In addition the red blood cells also are greatly reduced numerically,

[1] Since our preceding paper, we have seen a reference of Carnot (Carnot, P., *Compt. rend. Soc. biol.*, 1899, li, 927) in which he describes reactions in rabbits to a "pneumococcus toxin." His descriptions indicate the development of purpura in these animals.

but the rate of their disappearance is slower than that of the thrombocytes. Although heat inactivates both the thrombolysin and hemolysin, it has no effect on the purpura-producing property of pneumococcus extract. Adsorption of extract with blood platelets reduces the activity of the thrombolysin, but does not influence the purpura-producing activity in animals.

In the present communication, further studies are reported upon the nature of the purpura-producing principle. Observations are recorded on (1) the immunological reactions, (2) the biological significance and (3) the further chemical fractionation of this substance in pneumococcus extracts.

I. Immunological Reactions of the Purpura-Producing Principle.

(*a*) *Antigenicity of the Purpura Material.*—The purpura-producing substance, fractionated as will be described later, was used as immunizing antigen in rabbits. It was noted that during immunization, purpura lesions which accompanied the earlier injections, were not induced by the succeeding inoculations of the material. After several weeks of immunization, the sera were tested for neutralization of purpura in white mice and for the presence of pneumococcus antibodies. Not only did these sera not possess the power of neutralizing the purpura substance, as is brought out below, but they contained no antibodies for the intact cell or its derivatives. Thus, precipitins were not demonstrated for the purified purpura principle; for the soluble specific substance of the homologous type of Pneumococcus, nor for either extract or protein of the cell. Similarly, it was not possible to show agglutinins for either the "S," encapsulated cell, or the "R," capsule-free cell; nor were the sera able to confer protection upon mice against infection with homologous or heterologous types of Pneumococcus. In this respect, the purpura principle differs markedly from the hemotoxin, since the latter is antigenic (2, 4).

(*b*) *Active Resistance to the Purpura Principle.*—The rabbits employed in the foregoing experiment indicated that notwithstanding the fact that the purpura substance is non-antigenic, an increased resistance may be acquired against purpura. In order to study more thoroughly whether resistance can be exhalted actively, mice were treated with heat-killed suspensions of Pneumococcus, or with

the protein (5), autolysate (1) or extract (6) of the bacterial cells. All the substances are known to stimulate the formation of antibodies (7) and the last two contain the purpura principle. Accordingly, mice received five weekly injections of the particular antigen either subcutaneously or intraperitoneally. 7 to 10 days after the final injection, the mice were tested for increased resistance to purpura.

TABLE I.

Increased Resistance to Purpura Acquired by White Mice Following Repeated Injections of Pneumococcus Extract.

Date of injection	Amount of extract injected	Number of mice injected	Number of mice developing purpura
	cc.		
Apr. 26	0.1	10	10
May 3	0.1	10	10
May 10	0.2	10	8
May 17	0.2	10	2
May 24	0.4	10	0
June 1	0.5	10	0

TABLE II.

The Protective Action of Immune Sera against Purpura in White Mice.

Sera	Number of mice injected	Mice developing purpura	
		Number	Per cent
Controls (no serum)	10	7	70
Normal serum	20	16	80
Antipurpura	10	10	100
Antipneumococcus	5	5	100
Anti-R	15	7	46
Anti-extract	25	13	52
Anti-autolysate	25	21	84
Antiprotein	20	14	70

It was found that repeated injections of pneumococcus vaccine or protein do not increase the tolerance of mice to purpura. Mice so treated developed purpura following the injection of the same amount of extract as was required for the production of purpura in normal animals. Immunization to pneumococcus extract or autolysate, on the other hand, definitely renders mice more resistant to pur-

pura. This fact has been demonstrated satisfactorily a number of times, and a protocol typical of the results (Table I) shows that ten mice were made refractory to purpura by repeated injections of increasing amounts of pneumococcus extracts. It is also of note that the purpura substance no longer causes a diminution in the number of blood platelets or red blood cells in mice whose tolerance to purpura has been raised.

(*c*) *Passive Protection against the Purpura Principle.*—Whether white mice can be passively protected against purpura, was studied by the use of a number of immune rabbit sera prepared by immunization with the purpura principle itself, with "S" and "R" strains of Pneumococcus and with cell extracts, autolysates and the nucleoprotein fraction of the organism. In determining the neutralizing action of the sera against purpura, mixtures of 0.5 cc. of serum and 0.1—0.2 cc. of extract were injected intraperitoneally either immediately on mixing or after incubation together for 1 hour at 37°C. The sera prepared by immunization with the purpura material did not prevent the occurrence of purpura in mice. In no instance, moreover, did any of the sera provide definite passive protection against purpura. The data in Table II show the variation in susceptibility of individual mice and consequently the difficulty of evaluating experiments of this kind. While in the present instance 70 per cent of the normal mice developed purpura, other observations have shown variations from 100 per cent (*cf.* Table I) to 30 per cent of normal mice reactive to a fixed dosage. However, it is sufficiently clear that the sera included in the study did not confer upon normal mice protection against purpura. The lower percentage of animals developing purpura when anti-extract and anti-R sera were utilized, is suggestive of a partial protection, but experience with the purpura substance makes more plausible the interpretation of variation in susceptibility.

The evidence obtained, therefore, is that the purified purpura principle is non-antigenic, in the sense that it produces no demonstrable antibodies. White mice injected repeatedly with substances containing the purpura material acquire an increased tolerance to purpura. On the other hand, the sera of rabbits immunized with the purpura principle, the intact cell or its derivatives confer upon white mice no passive protection against purpura.

II. Biological Nature of the Purpura-Producing Principle.

It was shown in a preceding paper (1) that the purpura substance was found only in those cell derivatives which represented degradation products of pneumococci. The evidence thus far indicates that the substance is not contained as such in the cell, but rather that the purpura principle is a product accompanying cell autolysis. The present experiments substantiate this point of view and confirm the autolytic origin of the purpura material.

Attempts to obtain the purpura substance by dissolution of the cell with bile were repeated, but again the bile solutes were found incapable of producing purpura. Pneumococci were dissolved also by means of sodium oleate, but the resulting solutions did not contain the purpura principle. Curiously enough, incubation of bile solutes to allow digestion by the autolytic enzymes present in the cell did not yield the purpura substance.

Extracts prepared by rapidly freezing and thawing heavy suspensions of live pneumococci are actively purpura-producing, and there is reason to believe that this activity is dependent upon the action of the enzymes of the cell. Since the enzymes of Pneumococcus are inactivated (8, 9) by heating, whereas the purpura principle is resistant to heat (1), it was possible to determine the rôle of the enzymes in the derivation of the purpura substance. Extracts were prepared by freezing and thawing pneumococci previously exposed to 100°C. for 5 minutes. The extracts prepared from heat-killed cells, however, were found to be incapable of producing purpura in white mice. This is further evidence that the purpura principle is not preexistent in the cell.

Avery and Cullen (8, 9) have shown that cell-free filtrates of Pneumococcus contain, among other enzymes, a potent bacteriolytic enzyme which is operative on a substrate of heat-killed pneumococci. It became possible therefore to analyze the relationships of autolytic digestion to the formation of the purpura principle. A solution of active pneumococcus enzymes prepared according to the method of Avery and Cullen was allowed to act on cells killed by heating for 5 minutes at 100°C. After digestion at 37°C., there was distinct lysis of the organisms and cell-free filtrates were obtained after 24, 48,

72 and 96 hours. The filtrates, when injected into white mice, were found to be actively purpura-producing. The enzyme-containing extract, when diluted to the same extent as the filtrates obtained from the digestion experiment, did not produce purpura.

A pneumococcus enzyme solution was inactivated by boiling. It was then allowed to act on heat-killed pneumococci as in the above experiment. No lysis was observed, however, and the filtrates obtained from these tests did not produce purpura. Similar tests conducted with active enzymes in the presence of bile showed that the purpura substance was not demonstrable under these conditions.

The evidence indicates definitely, therefore, that the purpura principle is not a preformed constituent of the cell, but that it is a product of autolysis.

Avery and Cullen (9) have shown that the enzymes of Pneumococcus cause lysis not only of Pneumococcus itself, but also of *Streptococcus viridans*. It was desirable, therefore, to determine whether purpura-producing properties could be obtained from streptococci by digestion with pneumococcus enzymes. Heavy suspensions of *Streptococcus viridans*, killed by heating at 100°C. for 10 minutes, were subjected to the action of pneumococcus enzymes as described in the preceding experiment. Filtrates obtained from the resulting bacteriolysis of streptococcus by pneumococcus enzymes were not capable of producing purpura in white mice.

Purpura is rarely seen in white mice during the course of experimental infection with Pneumococcus. This is possibly due to the fact that the virulent organisms are tremendously invasive and cause death rapidly. Since degraded and avirulent "R" cells yield as potent purpura-producing extracts as their virulent antecedents (1), a study of the disintegration of pneumococci provided a means for determining the occurrence of purpura following the injection of live pneumococci *in vivo*. Small and large amounts of concentrated suspensions of live "R" cells were injected both subcutaneously and intraperitoneally in mice. The majority of the mice survived but in no instance was purpura observed.

In summary, then, it can be said that the purpura-producing principle of Pneumococcus does not exist preformed in the cell. It definitely accompanies autolysis *in vitro* and, under the conditions

stated, purpura was not observed in animals injected with large amounts of live pneumococci.

III. Chemical Nature of the Purpura-Producing Principle.

Previous studies indicated (1) that the purpura substance was precipitated by full saturation with $(NH_4)_2SO_4$ after the nucleoproteins had been previously removed from pneumococcus extract. It was not clear, however, whether the purpura material was albumin or not. In the present study, further fractionation has been accomplished and the purpura principle has been separated from the original precipitate obtained with $(NH_4)_2SO_4$.

In the chemical purification, aqueous extracts of Pneumococcus were employed. Normal acetic acid was added to the extract until no further precipitation occurred. The acid precipitate was removed by centrifugation and the supernatant was withdrawn. The supernatant was heated to maximum coagulation, and the coagulum was whirled down. The materials removed by acid and by heat, as well as the supernatant after removal of both proteins, were studied.

As was previously shown (1) the acetic acid precipitate or nucleoprotein was not purpura-producing. The heat-coagulable proteins were irregular in their action and at best only faintly reactive. Since they were not washed, it seems likely that they were not entirely free from the supernatant. The supernatant remaining after both the acid-precipitable and heat-coagulable proteins had been removed, usually produced marked purpura in mice. The indications are therefore that the purpura principle is not present either in the acid-precipitable or heat-coagulable proteins.

The active purpura-producing supernatant as such gave no qualitative color tests for protein. When it was concentrated to one-tenth volume however, several tests were obtained which aid in identification of the principle. With the biuret test, a pink to purple color was observed. The xanthoproteic test was negative, and Millon's reagent gave a yellow to brown precipitate. A precipitate was obtained with full saturation of $(NH_4)_2SO_4$; the precipitate first appeared at about 60–70 per cent saturation and it increased in intensity up to full saturation. Both picric acid and trichloracetic acid gave precipitates, but in both instances the precipitates vanished

on heating and returned on cooling. Since the concentrated solution, moreover, gave no further coagulation on boiling, it appears that the purpura-producing principle of Pneumococcus is identified with a primary proteose. It cannot be said whether it is actually a proteose or some closely associated substance. The purpura material is present in such small amounts and some is lost during fractionation, so that it becomes a difficult procedure to effect a further separation.

DISCUSSION.

The experiments recorded in this communication disclose that the purpura-producing principle of Pneumococcus does not stimulate the formation of demonstrable antibodies. The serum of animals immunized with either the purpura principle itself, with pneumococcus cells or with extract, autolysate or nucleoprotein of the bacteria does not afford white mice passive protection against purpura. In contrast to this fact, however, white mice and rabbits acquire an increased tolerance to purpura following successive injections of materials containing the purpura substance. It seems likely, therefore, that the "active immunity" in this instance is really an increased resistance to the toxic agent. This is supported by two facts: (1) the purpura-producing principle is not antigenic, and (2) passive protection against purpura is not transferred to normal mice by the serum of resistant animals.

The experiments on the biological nature of the purpura principle furnish direct evidence that it accompanies autolysis and that it is most probably a degradation product. It was not obtained as a preformed constituent of the cell, since, in the absence of enzyme action, cytolysis of live or dead cells did not yield the purpura substance. Dissolution of the live cell by bile or sodium oleate, neither of which inhibits the activity of the purpura principle when once formed, did not furnish the purpura material. Moreover, the presence of bile has been observed to inhibit definitely the formation of the purpura substance. In fact Jobling and Strouse have shown that a number of unsaturated fatty acids, including sodium oleate, actually do inhibit the action of proteases. Since bile does contain unsaturated fatty acid, there is some support for the belief that bile inhibits the digestive processes which give rise to the purpura prin-

ciple. Freezing and thawing heat-killed organisms in the absence of active enzymes did not liberate the purpura principle. On the other hand, the digestion of dead cells by active enzymes of Pneumococcus gave filtrates which were actively purpura-producing. In other words, the purpura substance appears to be an autolytic derivative of Pneumococcus.

Further fractionation of the purpura material indicates that it is closely related to a primary proteose. It has been definitely separated from both the acid-precipitable and heat-coagulable proteins of pneumococcus extract, but a more exact identification cannot be stated at the present time. In general proteoses have been found to be non-antigenic (10) although the observations of some investigators notably those of Fink (11) indicate that proteoses may be antigenic. That proteoses, moreover, may be toxic has been demonstrated by a number of investigators (12).

CONCLUSIONS.

1. The purpura-producing principle of Pneumococcus is non-antigenic in the sense that it does not stimulate the formation of antibodies.

2. White mice acquire an increased resistance to purpura as a result of repeated injections of toxic doses of the purpura substance.

3. The serum of rabbits immunized with the purified purpura principle, with "S" and "R" strains of Pneumococcus or with cell extracts, autolysates or the nucleoprotein fraction of the organism does not confer upon white mice protection against purpura.

4. The purpura principle does not exist preformed in the cell, but is rather an autolytic derivative; since it is formed only when pneumococci undergo autolysis, and it is not found when the autolytic ferments are inactivated.

5. The purpura substance is associated with the proteose fraction of active pneumococcus extracts.

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