EFFECT OF LYSOZYME ON THE RELEASE OF ERYTHROCYTE-MODIFYING ANTIGEN FROM STAPHYLOCOCCI AND MICROCOCCUS LYSODEIKTICUS

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Rantz and associates (1952, 1956) discovered an unusual antigen which is present in many species of gram-positive and absent in gram-negative bacteria, and referred to it as "non-species specific (NSS) antigen." This antigen becomes readily attached to erythrocytes and thus renders the latter specifically agglutinable by antibodies directed against it. Sheep red blood cells modified by Rantz antigen are lysed in the presence of antibody and complement. A similar or identical antigen common to staphylococcus and streptococcus was studied by means of hemagglutination or latex agglutination tests by Pakula and Walczak (1955), as well as by Znamirowski et al. (1959). Two modifications of the basic hemagglutination test were described recently, namely, the enzyme hemagglutination and Coombs hemagglutination procedures (Neter et al., 1959; Neter and Gorzynski, 1959). In the former method, erythrocytes are treated with one of several proteolytic enzymes (trypsin, pancreatic protease, ficin, or papain), either before or after modification with antigen of the Rantz type, and then tested with antibodies. In the latter method, hemagglutination is carried out with antigen-modified human erythrocytes and human serum or γ-globulin containing Rantz antibodies, followed by the addition of human γ-globulin (Coombs) antiserum. It was shown that these two procedures are considerably more sensitive than the basic hemagglutination method for the demonstration of antibodies of the Rantz type in human γ-globulin. Supernatants of cultures grown on brain veal agar were employed

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2 For the sake of brevity the terms Rantz antigen and Rantz antibodies are used here to connote antigens and antibodies of specificities as described by Rantz and associates (1952; 1956).

as antigen. Many, but not all, strains of Staphylococcus aureus and several strains of Bacillus subtilis yielded this antigen. In contrast, strains of Staphylococcus citreus and Micrococcus lysodeikticus did not.

Lysozyme, discovered by Fleming (1922), is a bacteriolytic enzyme widely distributed in nature. It is particularly effective against M. lysodeikticus and members of the genera Bacillus, Sarcina, and Staphylococcus. As reviewed by Salton (1957), this enzyme lysed susceptible bacterial cells and reduces turbidity of isolated cell wall structures; also, it liberates reducing groups and an acetyl-diamino sugar complex of glucosamine and the acidic hexosamine.

The present investigation was undertaken to determine (a) whether lysis of M. lysodeikticus by lysozyme results in the release of antigen of the Rantz type not detectable otherwise in cultures of this microorganism, and (b) whether lysozyme affects antigen liberation from staphylococci which either do or do not spontaneously release this material. Since lysozyme is present in secretions and certain cells of man and animals, such a study may contribute to a better understanding of lysozyme activity in vivo during natural and experimental infections.

MATERIALS AND METHODS

Several strains of coagulase-positive and coagulase-negative staphylococci, freshly isolated from clinical material, as well as strains of B. subtilis were utilized in this study. In addition, a coagulase-positive strain of S. aureus, and a strain of M. lysodeikticus were obtained from Difco Laboratories through the courtesy of Dr. C. W. Christensen. The microorganisms were grown on brain veal agar in Kölle flasks for 18 to 24 hr at 37 C. To each flask was added 25 ml of phosphate hemagglutination buffer (pH 7.3) (Difco). The resulting suspension was cen-
trifuged immediately at 24,500 × g for 20 min at 4°C. The supernatant in appropriate dilution was employed as antigen in the hemagglutination tests, and the sediment was washed and resuspended in lysozyme buffer (Difco). The latter contains dibasic sodium phosphate and monobasic potassium phosphate and has a pH of 6.2.

Crystallized lysozyme from fresh egg white was procured from Difco Laboratories. A stock solution containing 10,000 μg/ml in lysozyme buffer was kept frozen until used. Lysozyme in various dilutions was added to the washed sediment of the bacterial suspensions and incubated at 37°C for 30 min, unless indicated otherwise. For control purposes, samples of the suspensions were treated with lysozyme buffer. The suspensions were centrifuged at 24,500 × g at 4°C and the supernatants employed in the hemagglutination tests.

The basic hemagglutination and protease hemagglutination tests have been described in detail (Neter and Gorzynski, 1959). Briefly, a 2.5 per cent suspension of human red blood cells of blood group O was washed 3 times, and to the sediment was added the antigen in quantities sufficient to yield again a 2.5 per cent erythrocyte suspension. The mixtures were incubated in a water bath at 37°C. The red cells were washed 3 times and used in the basic hemagglutination procedure. For the protease hemagglutination test red blood cells were treated for 30 min at 37°C with a mixture of bacterial antigen in suitable dilution and pancreatic protease (250 μg/ml). The latter was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Commercial human γ-globulin in serial dilutions (volume 0.2 ml) was mixed with equal volumes of the treated erythrocyte suspensions. Appropriate controls were included. The mixtures were incubated in a water bath at 37°C for 30 min. The resulting hemagglutination following centrifugation at 300 × g for 1 to 2 min was recorded.

RESULTS

The effect of lysozyme on the release of antigen from bacterial cells was investigated. Selected for this experiment were one strain each of Staphylococcus aureus, S. citreus, and Micrococcus lysodeikticus; the supernatants of these bacterial suspensions did not contain Rantz antigen demonstrable by means of hemagglutination. Treatment with lysozyme (1000 μg/ml), or lysozyme buffer for control purposes, of the washed cell suspensions was carried out for 30 min at 37°C. Marked clearing occurred with M. lysodeikticus, but only minimal clearing with the strain of S. aureus. The supernatants of

### TABLE 1

**Antigen-liberating effect of lysozyme from bacterial cells**

<table>
<thead>
<tr>
<th>Human γ-Globulin Dilutions</th>
<th>Lysozyme</th>
<th>Lysozyme Buffer</th>
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<th>Lysozyme Buffer</th>
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<td>a*</td>
<td>b</td>
<td>c</td>
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<tr>
<td>1:20</td>
<td>4†</td>
<td>4</td>
<td>2</td>
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<tr>
<td>1:40</td>
<td>4</td>
<td>4</td>
<td>2</td>
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<td></td>
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<tr>
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<td>4</td>
<td>4</td>
<td>2</td>
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<tr>
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<td>4</td>
<td>2</td>
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<tr>
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<tr>
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<td>1</td>
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* a = Supernatant 1/10; b = supernatant 1/50; and c = supernatant 1/250.
† = No hemagglutination; 1 to 4 = various degrees of hemagglutination.
these suspensions were then used for modification of red blood cells. The results of the protease hemagglutination test with human γ-globulin as antibody source are presented in table 1.

Perusal of the table shows that lysozyme treatment resulted in the release of substantial amounts of antigen from the S. aureus strain, for supernatants in dilutions of 1:10 to 1:250 contained antigen demonstrable by hemagglutination. In contrast, the supernatant of staphylococci treated with lysozyme buffer did not contain this antigen. It may be noted, too, that neither S. citreus nor M. lysodeikticus produces this antigen without previous lysozyme treatment. It should be noted, too, that standing at 4°C for several (2 to 72) hours results in a substantial increase in the amount of antigen in the supernatants of these suspensions. It is conceivable that autolysis produces this effect.

Quantitative studies on the effect of lysozyme on the release of staphylococcal antigen were undertaken next. The results of a typical experiment are summarized in table 2.

Table 2 shows that substantial amounts of antigen were liberated from staphylococcal cells by lysozyme in concentrations of 100 to 1,000 μg/ml, and small amounts by as little as 10 μg/ml. In agreement with previous findings, it is seen that the protease hemagglutination test proved to be considerably more sensitive than the basic hemagglutination method. In additional experiments it was observed that still larger amounts of lysozyme (10,000 μg/ml) were strikingly less effective. Various amounts (1 to 10,000 μg/ml) were included in the table.
µg/ml) of lysozyme failed to cause release of antigen from S. citreus and M. lysodeikticus.

The enzyme lysozyme is remarkably resistant to heat at acid pH, but not at pH values higher than 7, when lysis of susceptible bacteria is used as indicator (Smolelis and Rantz, 1952). In order to determine the effect of heat and pH on the antigen releasing activity of lysozyme, the following experiment was carried out.

Lysozyme in a concentration of 100 µg/ml was mixed with equal amounts of 0.25 N NaOH, 0.25 N HCl, and lysozyme buffer, respectively. The preparations were heated in boiling water for 15 min, and samples were kept at 22 C. The materials were then neutralized with 0.25 N HCl and 0.25 N NaOH, respectively, and the effect of these preparations on the release of antigen from a strain of S. aureus was determined. It was found that lysozyme after heating at acid pH retained substantial activity, whereas heating at alkaline pH caused destruction. These data suggest that the known heat stability characteristics of lysozyme apply to its antigen releasing activity as well.

Additional experiments revealed that lysozyme in concentrations of 100 to 1,000 µg/ml neither destroyed nor enhanced the activity of Rantz antigen (supernatants of bacterial suspensions) for erythrocyte modification and interaction with homologous antibodies (hemagglutination), nor did this enzyme alter red blood cells with respect to their suitability for Rantz antigen attachment and immune agglutination.

The antigen released by lysozyme was identified as Rantz antigen in experiments which showed that this material specifically inhibits agglutination by γ-globulin of red blood cells modified by Rantz antigen obtained from either staphylococci or B. subtilis.

**DISCUSSION**

The supernatants of suspensions of many strains of S. aureus grown on brain veal agar contain Rantz antigen, demonstrable in hemagglutination tests. Other strains do not yield this antigen under identical conditions. The present investigation has revealed that the enzyme lysozyme affects the release of this antigen from cells of the latter strains. Lysozyme is effective in concentrations ranging from 10 to 1000 µg/ml. It was found, also, that still higher concentrations (10,000 µg/ml) were less active. Wilcox and Daniel (1951) observed reduced lysis of bacteria with high concentrations of lysozyme and suggested that this reduction in activity is due to the formation of a stable lysozyme-substrate complex. It is conceivable that in the present experiments similar conditions prevail.

In contrast to these strains of S. aureus, lysozyme treatment of S. citreus and M. lysodeikticus did not result in the release of demonstrable Rantz antigen. These results support previous findings to the effect that neither broth nor agar grown cultures of S. citreus and M. lysodeikticus contain this antigen. The observation that lysozyme caused only minimal lysis of certain strains of staphylococci and yet effected release of Rantz antigen suggests the extension of the present study to other antigenic or toxic components from various bacterial strains and species. It remains unknown why only certain strains of staphylococci readily and spontaneously release this antigen into the supernatant, conceivably by auto-enzyme action.

The present findings do not warrant discussion of the possible mechanisms of lysozyme action which results in antigen release. It is conceivable that the latter is a phenomenon secondary to the alteration of the cell wall. The properties of lysozyme and the chemical nature of cell wall components released by this enzyme were admirably reviewed recently by Salton (1957).

The chemical nature of the Rantz antigen remains to be determined. McCarty (1959) isolated polyglycerophosphate from several bacterial species known to contain the Rantz antigen and not from certain other species lacking this antigen. Preliminary experiments with polyglycerophosphate, kindly supplied by M. McCarty, revealed that this material, in amounts of 12 µg, inhibits Rantz hemagglutination. The possibility has to be considered, as suggested by M. R. J. Salton (personal communication, December 2, 1959), that Rantz antigen may be related to teichoic acid (inositol phosphate polymer). Further hemagglutination inhibition experiments with these compounds may aid in the chemical identification of Rantz antigen.

The question arises as to the possible activity in vivo of lysozyme on bacterial cells resulting in the release of antigenic components. It is well known that lysozyme, as originally observed by the discoverer of this enzyme, is present in body fluids, including nasal secretions and tears (Fleming, 1922). It has been shown that this enzyme is found also in rather high concentration in
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leukocytes. It is conceivable, then, that following phagocytosis lysozyme within the leukocytes alters certain bacterial cells resulting in the release of antigenic components, such as Rantz antigen. Similarly, lysozyme in plasma or tissue fluids may have the same effect. To what extent, if any, this activity of lysozyme contributes indirectly to noxious effects or antibody formation remains to be clarified by future investigations.

SUMMARY

Study on the effects of lysozyme on the release from bacterial cells of Rantz antigen, demonstrable by hemagglutination tests, revealed the following results.

Lysozyme in concentrations from 10 to 1000 \( \mu g/ml \) effects the release of antigen from certain strains of \textit{Staphylococcus aureus}, lysozyme in a concentration of 10,000 \( \mu g/ml \) is less effective. Lysozyme buffer, used for control purposes, does not effect liberation of antigen under the conditions of these experiments. Other strains of \textit{Staphylococcus aureus} spontaneously release this antigen into the supernatant.

Lysozyme treatment of \textit{Staphylococcus citreus} and \textit{Micrococcus lysodeikticus} fails to effect release of this antigen.

Heating at alkaline \( pH \) of lysozyme results in destruction of antigen releasing activity, whereas substantial activity is maintained after heating at acid \( pH \).

The significance of the data, with particular reference to the possible effects \textit{in vivo} of lysozyme on antigen release from bacterial cells, is discussed.

REFERENCES


