STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

IV. Bacteriolytic Enzyme.

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(Received for publication, April 12, 1923.)

In preceding papers (1), studies on the proteolytic, lypolytic, and carbohydrate-splitting enzymes of pneumococcus have been reported. It has been shown that these enzymes are intracellular in nature and can be extracted from the bacterial cell by various methods. They possess to a remarkable degree the power of actively hydrolyzing peptones to simpler peptides and amino-acids, of converting carbohydrates to simpler products, and of splitting esters to fatty acids.

In addition to the enzymes previously described, it has been found that solutions of the cellular substance of pneumococci also contain an enzyme which is capable of exerting a lytic action upon other dead bacterial cells.

Methods.

Preparation of Enzyme.—In previous papers, the bacteriological and chemical methods used in these studies have been described in detail (1). The same methods have been followed in the present investigation. In the preparation of active enzyme-containing solutions from pneumococci the bacterial cells have been dissolved in bile, or the intracellular substances have been extracted by autolysis. When the former method is used the presence of bile does not obscure the effects of the bacteriolytic enzyme upon heat-killed organisms inasmuch as bile itself does not cause lysis of pneumococci which have been killed by heat.

Preparation of Substrate.—The substrates have consisted of suspensions of washed bacteria in phosphate solution of known hydrogen ion concentration. The bacterial suspensions were exposed to a temperature of 60°C. for 30 minutes (water bath), or 120°C. for 20 minutes

(autoclave). Antiseptics were not used as preservatives, and the sterility of all enzyme-substrate mixtures was proved by subculture.

Action of Intracellular Enzymes of Pneumococcus on Heat-Killed Bacteria.

Experiment 1.—Enzyme: The bacterial residue from 2 liters of a 10 hour plain broth culture of Pneumoccocus Type II was washed with physiological salt solution and resuspended in 10 cc. of 0.1 M phosphate mixture of pH 6.2. The bacterial suspension was immediately frozen and thawed seven times, and kept at ice box temperature for 10 days. During this period cytolysis occurred and the bacterial detritis settled to the bottom, leaving a clear, slightly opalescent supernatant fluid which was used in this experiment. 0.1 cc. of this solution plated on blood agar showed no growth after several days incubation.

Substrate: Bacterial substrates were prepared by suspending the washed cells in 0.1 M phosphate solutions of pH 7.8. The following organisms were tested: Pneumococci Types I and II, *Streptococcus viridans*, *Streptococcus hæmolyticus*, and *Staphylococcus aureus*. The bacterial suspensions were of about equal opacity and were autoclaved 20 minutes at 15 pounds pressure before use. 0.1 cc. of the active enzyme solution was added in each instance to 0.5 cc. of the bacterial substrate, and the total volume made up to 1 cc. by the addition of 0.1 M phosphate solution of pH 7.8. The tubes were then placed in a water bath at 37°C., and the degree of bacteriolysis was noted at varying intervals by observing the relative opacity of the tubes, and by microscopic examination of stained films.

After 15 minutes incubation at 37°C., tubes containing a mixture of active enzyme and heat-killed pneumococci showed marked disintegration of the bacterial bodies. After 2 hours incubation, complete dissolution occurred and stained films showed only Gram-negative detritis, and shadow forms of the organisms. Films prepared from the tubes containing pneumococcus enzyme and the dead bodies of *Streptococcus viridans* showed at this period many Gram-negative organisms in unevenly stained chains, with disintegration of over half of the bacterial cells. On the other hand, stained films of the tubes in which the active enzyme was in contact with substrates of hemolytic streptococci or staphylococci showed no visible change in the organisms either in form, size, or staining properties. The pneumococci used as substrate in this experiment were of another type than those from which the enzyme was derived. This fact would indicate that the bacteriolytic enzyme is not type-specific, since it exerts a lytic action on strains of pneumococci serologically unrelated. The bacteriolytic effect of pneumococcus enzyme on *Streptococcus viridans*, although distinct, is much slower, being complete only after the action is continued for several hours. On the other hand, the strains of *Streptococcus hæmolyticus* and *Staphylococcus aureus* studied in the present investigation were uninfluenced by the action of the pneumococcus enzyme, and even after prolonged exposure showed no visible changes morphologically or tinctorially.

In controls, in which no enzyme had been added to the suspensions of heated bacteria, the organisms were still of normal appearance and gave no evidence of changes in coloring or form even after incubation for 20 hours at 37°C. Pneumococci acted upon by enzyme for this length of time appeared only as Gram-negative amorphous material; the green-producing streptococci were at this stage undergoing further disintegration, in stained films only an occasional Gram-positive form was seen, and the majority of the organisms appeared as faint Gram-negative shadows. Even after prolonged action of the pneumococcus enzyme on the substrate, the hemolytic streptococci and staphylococci were well preserved, retained their staining properties, and showed no evidence of bacteriolysis. After incubation for 48 hours, the tubes in which active bacteriolysis had occurred, namely those containing pneumococci, and streptococci of the *viridans* type, showed evidence of clearing with disappearance of the bacterial whirl, and were much more translucent than the control tubes to which no enzyme had been added. In the case of hemolytic streptococci and staphylococci no noticeable change in the gross appearance of the digestion mixture was evident.

From this experiment it appears that pneumococci possess an active intracellular agent which has the property of causing lysis of the bodies of heat-killed pneumococci, and, furthermore, that the lytic agent is not type-specific, since strains of pneumococci serologically different are equally affected. The bacteriolytic enzyme of pneumococcus also possesses to considerable degree the ability to attack streptococci of the *viridans* variety, cocci which biologically are more closely related to pneumococci than are hemolytic streptococci and staphylococci. Cocci of the last two varieties are apparently unaffected by the action of the enzyme, even after prolonged contact.

Influence of Hydrogen Ion Concentration on the Activity of the Intracellular Bacteriolytic Enzyme of Pneumococcus.

Under conditions similar to those described in Experiment 1, the effect of varying the hydrogen ion concentration on the bacteriolytic action of the enzyme was tested. In this experiment the enzyme solution was prepared from pneumococci of Type I and the bacterial substrate from pneumococci of Type II. The washed bacterial cells from 150 cc. of a plain broth culture of Pneumococcus Type II were suspended in 5 cc. of 0.1 M phosphate solution pH 7.4, and autoclaved at 15 pounds pressure for 20 minutes. After being killed by heat, the organisms were again centrifuged and the bacterial residue was washed and resuspended in 2 cc. of sterile distilled water. 0.1 cc. of active

enzyme solution and 0.1 cc. of bacterial substrate were now added to tubes each containing 1 cc. of 0.1 M phosphate solution. The tubes were adjusted to the desired hydrogen ion concentration by the addition, in the more acid ranges, of hydrochloric acid. The results of this experiment are shown in Table I.

TABLE I.

Influence of Hydrogen Ion Concentration on the Activity of the Bacteriolytic Enzyme of Pneumococcus.

pH	Enzyme solution of Pneumococcus Type I.	Substrate of heat-killed Pneumococcus Type II.	0.1 M PO_4 solution.	Lysis after 2 hrs. at 37°C.
	cc.	*cc.*	*cc.*	
2	0.1	0.1	0.8	−
	0	0.1	0.8	−
3	0.1	0.1	0.8	−
	0	0.1	0.8	−
4	0.1	0.1	0.8	−
	0	0.1	0.8	−
5	0.1	0.1	0.8	++
	0	0.1	0.8	−
6	0.1	0.1	0.8	+++
	0	0.1	0.8	−
7	0.1	0.1	0.8	+++
	0	0.1	0.8	−
7.8	0.1	0.1	0.8	+++
	0	0.1	0.8	−
8	0.1	0.1	0.8	+++
	0	0.1	0.8	−

+++ indicates complete lysis; ++, marked lysis; −, no lysis.

The lytic action of the enzyme was evident in the zone pH 5 to 8. At reactions more acid than pH 4 there was no evidence of lysis. At pH 3 a marked precipitation occurred. This may possibly be attributed to acid agglutination which for Type II pneumococci occurs at about this reaction. The optimum zone of hydrogen ion concentra-

tion, for the action of the bacteriolytic enzyme, corresponds closely to that already described for the peptonase, lipase, invertase, and inulase of pneumococcus.

Influence of Concentration of Enzyme on the Activity of the Intracellular Bacteriolytic Enzyme of Pneumococcus.

That the lytic action of solutions containing the intracellular substances of pneumococcus is enzymotic in nature, is evidenced by the following experiment in which it is shown that the rate and amount of lysis are a function of the concentration of the enzyme present. The conditions of this experiment were similar to those already described in previous experiments; the enzyme-containing solution was prepared

TABLE II.

Influence of Concentration of Enzyme on the Activity of the Bacteriolytic Enzyme of Pneumococcus.

Enzyme solution of Pneumococcus Type II.	Substrate of heat-killed Pneumococcus Type II.	0.1 M PO_4 solution pH 7.8.	Lysis after 1 hr. at 37°C.
cc.	*cc.*	*cc.*	
0.1	0.5	0.5	++++
0.05	0.5	0.5	+++
0.025	0.5	0.5	++
0.0125	0.5	0.5	+
0.006	0.5	0.5	+
0.003	0.5	0.5	+
.0	0.5	0.5	0

++++ indicates complete lysis; 0, no lysis.

from Pneumococcus Type II. This particular enzyme preparation had been kept in the ice box for 27 days. The bacterial substrate consisted of Type II pneumococci which had been prepared as already described.

From Table II it is evident that the action of the intracellular bacteriase is directly proportional to the concentration of the enzyme. It is interesting to observe that the activity of this enzyme, even after preservation in cold for 4 weeks, was still pronounced in quantities as small as 0.025 cc., and that traces of its action were detected in minimum amounts of 0.003 cc.

Influence of Heat on the Activity of the Intracellular Bacteriolytic Enzyme of Pneumococcus.

The following experiment was carried out to determine the effect of heat upon the bacteriolytic enzyme of pneumococcus.

300 cc. of plain broth pH 7.8 were seeded with a large inoculum (0.5 cc.) of a culture of Pneumococcus Type II. After 8 hours growth at 37°C., the culture was centrifuged and the bacteria were suspended in 20 cc. of 0.1 M phosphate solution at pH 7.2. To 10 cc. of this bacterial suspension, representing the cells from 150 cc. of culture, 0.2 cc. of undiluted ox bile was added. Solution of the organisms was marked after 30 minutes in the water bath at 37°C. and was complete after standing in the ice box over night. The remaining 10 cc. of the bacterial suspension to be used as substrate were autoclaved at 15 pounds pressure for 10 minutes in order to kill the organisms and to destroy the intracellular ferments.

TABLE III.

Influence of Heat on the Activity of the Bacteriolytic Enzyme of Pneumococcus.

Enzyme solution of Pneumococcus Type II, 0.5 cc.	Substrate of heat-killed Pneumococcus Type II.	Lysis.	
		After 2 hrs. at 37° C.	After 18 hrs. at 37° C.
	cc.		
Heated at 60°C. for 30 min.	0.5	−*	−
" " 80° " " 30 "	0.5	−	−
" " 100° " " 30 "	0.5	−	−
Unheated	0.5	++	++
No enzyme; phosphate solution and bile (1:50)†	0.5	−	−

* − indicates no lysis; ++, complete lysis.

† This shows that bile alone has no lytic effect on heat-killed pneumococci.

Aliquot portions of the solution containing the dissolved bacteria were heated in a water bath for 30 minutes at 60°, 80°, and 100°C., respectively. These heated solutions in 0.5 cc. amounts were now added to equal quantities of the suspension of heat-killed pneumococci. The results of this experiment are shown in Table III.

Analysis of Table III shows that exposure in the water bath to a temperature of 60°C. for 30 minutes destroys the activity of the bacteriolytic enzyme of pneumococcus. The resistance of the bacteriolytic enzyme of pneumococcus to heat is markedly less than that of the lytic agent of *Bacillus pyocyaneus* which, according to Emmerich (2), withstands steam at 100°C. for 2 hours.

DISCUSSION.

Evidence has been presented in this paper that pneumococci contain a bacteriolytic enzyme. This enzyme is associated with a number of other active intracellular agents which exert their effect upon various substances and which because of their enzymotic nature have been described as the intracellular peptonase, lipase, inulase, and invertase of pneumococcus. The bacteriolytic enzyme possesses the property of causing lysis of the dead bacterial bodies of pneumococci, and to a less extent the disintegration of closely allied organisms, such as *Streptococcus viridans*. However, it has no effect upon certain other Gram-positive cocci, as *Staphylococcus aureus* and *Streptococcus hæmolyticus*. The enzyme is not type-specific in its action, since an enzyme solution prepared from pneumococci of one type exerts a comparable action upon pneumococci of a heterologous type.

Emmerich, Löw, and Korschun (3) have demonstrated an enzyme in cultures of *Bacillus pyocyaneus* which possesses a remarkable lytic power. This enzyme, pyocyanase, in extraordinarily small amount, is capable of causing lysis of a number of other microorganisms such as *Bacillus diphtheriæ*, *Vibrio choleræ*, *Bacillus typhosus*, *Bacillus pestis*, streptococcus, and staphylococcus. In contrast with the pneumococcus enzyme, the enzyme from *Bacillus pyocyaneus* manifests its action on the living bacterial cell. Pyocyanase is remarkably heat-stable, resisting a temperature of 100°C. for 2 hours (2). This lytic agent is considered by Emmerich and his coworkers to be different from the peptonizing enzyme of *Bacillus pyocyaneus*. Löw and Kozai (4) have also demonstrated a bacteriolytic enzyme in cultures of *Bacillus prodigiosus*. Emmerich and Löw (5) include these bacteriolytic enzymes in the group of nucleases, which act on the nucleoprotein of the bacterial cell.

In the present instance, no assumption is made as to the identity of the pneumococcus bacteriolytic enzyme, since it is not known what particular constituent or constituents of the bacterial cell are acted upon. Whether lysis of pneumococci under these circumstances is the result of a single enzyme or the product of the interaction of more than one, and whether the enzyme or group of enzymes concerned in autolysis of pneumococci play any part in this form of lysis are questions at present undecided.

The fact that the pneumococcus bacteriolytic enzyme apparently has no effect on the living cell suggests that the living organism may possess a protective mechanism, possibly of the nature of antiferment. It may be only when this mechanism has been interfered with or destroyed that the bacterial cell is exposed to the action of its own enzymes.

SUMMARY.

1. Pneumococci possess an active intracellular enzyme which causes lysis of heat-killed pneumococci of the same and heterologous types and to a less degree of a closely related organism, *Streptococcus viridans*.

2. The optimum reaction for lysis lies between pH 6 and 8.

3. The bacteriolytic action is proportional to the concentration of the enzyme.

4. Heating the enzyme for 30 minutes at 60°C. destroys its activity.

5. The possible relation of the enzyme to autolysis is discussed.

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