STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

II. Lipolytic Enzymes: Esterase.

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In the preceding paper (1) are recorded the facts so far obtained in a study of the proteolytic enzymes of pneumococcus. It has been shown that bile solutions of pneumococci, extracts obtained by disintegration of the organisms in phosphate mixtures, and sterile filtrates of autolyzing broth cultures possess the power to hydrolyze peptones and to a less extent certain intact proteins. In the present paper evidence will be presented that pneumococci possess also an endolipase of marked activity. The intracellular nature of this enzyme, the influence of age and hydrogen ion concentration on its activity, its thermostability, and relation to virulence and to the mechanism of bile solubility will be discussed.

In a monograph on bacterial enzymes, Fuhrmann (2) (1907) summarizes the work of earlier investigators on the occurrence of lipases in different species of microorganisms. In a review of the literature no reference has been found to work on the lipase of pneumococcus. Various methods for the demonstration of lipolytic activity of bacteria have been used. By a simple plate method, in which the zone of reaction about colonies of bacteria growing in the presence of certain test substances such as fat could be observed, Eijkman (3) detected lipolytic action in a number of organisms, including *Staphylococcus aureus*, *B. pyocyaneus*, *B. prodigiosus*, and *B. fluorescens*. Studying the lipase of *B. tuberculosis* and other bacteria, Wells and Corper (4), by testing the killed bacterial substance on esters and fats, demonstrated the presence of lipolytic enzymes in organisms which by the plate method apparently possessed no visible fat-splitting power. They further showed that sterile unheated emulsions of *B. tuberculosis*, while not actively lipolytic, possess enzymes capable of slowly hydrolyzing esters. Kendall, Walker, and Day (5) have demonstrated the occurrence of a soluble lipase in broth cultures of a variety of acid-fast organisms including tubercle bacilli of the human, bovine, and avian types. These authors found that the organisms, during the period of active growth, excrete a soluble lipase which occurs free in the medium.

Kendall and Simonds (6) have shown that sterile filtrates of plain and dextrose broth cultures of typhoid bacilli contain an esterase, capable of liberating acid from ethyl butyrate. The bacteria separated from the filtrates, however, showed but little esterase activity.

EXPERIMENTAL.

Methods.

Kastle and Loevenhart (7) have shown the advantage of using the lower esters, tributyrin and ethyl butyrate, in studying lipase activity. A preliminary experiment showed that when pneumococci are dissolved in bile the resultant solution contains an enzyme that splits both these esters. In the experiments to be recorded tributyrin has been used throughout as the substrate in the study of the intracellular lipase.

In determining lipase activity it has been customary to adjust the fat or ester substrate to approximate neutrality, with phenolphthalein as indicator, and then by titration to determine the amount of acid yielded by enzyme action. In the present study, however, it seemed more important to establish the optimum hydrogen ion concentration for action of the lipase, and then to maintain this reaction by the use of suitable buffer solutions. This buffered substrate maintains optimum conditions for enzyme action with a minimum of inhibition due to the acid products of hydrolysis. The amount of acid split off from the ester is determined by the amount of alkali required to readjust the digestion mixture to the initial reaction. It may also be calculated as the amount of acid required to change the buffered digestion mixture from the initial to the final hydrogen ion concentration. In the following experiments the ester was emulsified in 0.1 M phosphate solution of desired pH.

The method of preparing the enzyme solution, by dissolving the bacterial cells in sterile bile, was the same as that recorded in the experiments on the proteolytic enzymes of pneumococcus.

In no instances were antiseptics used as preservatives in the digestion mixtures. Sterility of all enzyme-substrate emulsions was proved by subculture.

Presence of an Intracellular Lipase and the Influence of Hydrogen Ion Concentration on Its Activity.

Experiment 1. (*a*) *Preparation of Enzyme.*—The bacterial residue from 4 liters of 18 hour plain broth culture of Pneumococcus Type II (No. F 208) was washed in isotonic salt solution, taken up in 20 cc. of sterile, undiluted ox bile, and held over night in the ice box. A portion of this enzyme solution was inactivated by heat.

(*b*) *Preparation of Substrate.*—2 per cent tributyrin (Kahlbaum) was emulsified in 0.1 M phosphate solution covering the range of pH values 4.9 to 7.8.

In carrying out the experiment 0.2 cc. of tributyrin was added to 10 cc. of sterile phosphate solution at the indicated reaction, and the mixtures were shaken until a fine emulsion was obtained. Three tubes were prepared at each reaction. 1 cc. of active enzyme solution was added to the first tube; 1 cc. of the same solution inactivated by heat to the second; and to the third 1 cc. of the undiluted bile used in preparing the bacterial solution. The tubes were then placed at 37°C. for 72 hours. The results of these experiments are given in Table I and are represented graphically in Text-fig. 1.

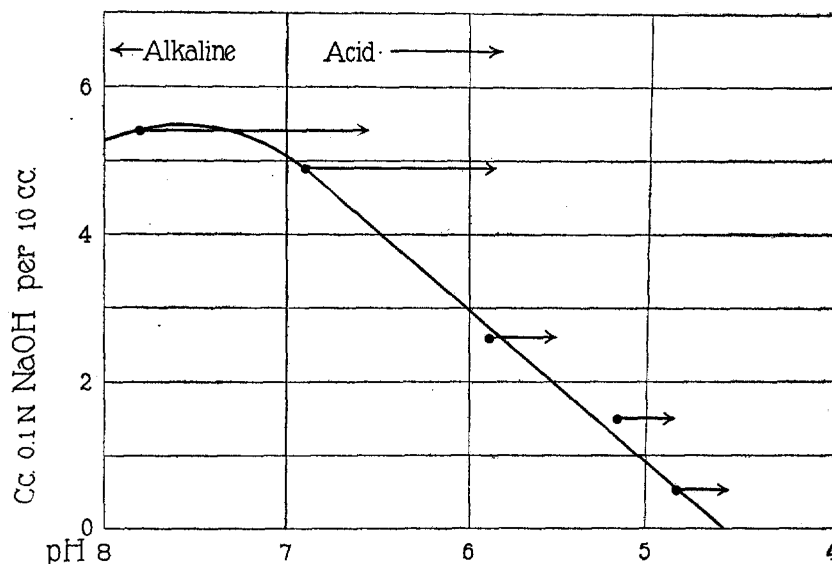
TABLE I.

Influence of Hydrogen Ion Concentration on the Activity of the Intracellular Lipase of Pneumococcus.

Initial reaction of digestion mixture.	Hydrogen ion concentration after 72 hrs. at 37°C.			10 cc. of mixture containing active enzyme readjusted with 0.1 N sodium hydroxide.	
	10 cc. of 2 per cent tributyrin + 1 cc. of				
	Bile.	Inactive enzyme.	Active enzyme.	To initial pH.	Amount required.
pH	*pH*	*pH*	*pH*	*pH*	*cc.*
4.9	4.9	4.9	4.6	4.9	0.3
5.2	5.2	5.2	4.9	5.2	1.5
5.9	5.9	5.9	5.5	5.9	2.3
6.9	6.9	6.9	5.8	6.9	4.9
7.8	7.8	7.8	6.5	7.8	5.4

The facts brought out in Experiment 1 demonstrate that within the pneumococcus cell there exists a markedly active lipase, or esterase. The acid formed from 10 cc. of 2 per cent tributyrin was equivalent to 5.4 cc. of 0.1 N alkali, or a normality of about 0.05 N butyric acid. The maximum activity of this esterase occurs at a reaction of about pH 7.8 and progressively decreases with increase in acidity. This

optimum reaction corresponds closely with that of the intracellular peptonase, and coincides with the optimum hydrogen ion concentration for growth of pneumococcus.



TEXT-FIG. 1. Influence of hydrogen ion concentration on the activity of the intracellular lipase of pneumococcus. The arrows indicate the extent of the reaction change.

Intracellular Nature of the Pneumococcus Lipase.

In the preceding paper it was shown that in filtrates of broth cultures of pneumococcus proteolytic enzymes were demonstrable only in the later phases of growth. Their appearance free in the culture medium coincided with the dissolution of the bacterial cells. In the early stages of growth, however, when cell multiplication is occurring at a maximum rate no enzymes are demonstrable in the culture filtrate. These facts indicate the intracellular nature of the proteolytic enzyme. Similarly it is shown in the following experiment that during the early phases of growth no lipase is present in cell-free filtrates. That the organisms were actively growing is evidenced from the change in reaction of the culture from pH 7.8 to 7.4.

Experiment 2.—20 cc. of the Berkefeld filtrate of a 5 hour culture of No. F 208 (the same preparation that was used in Experiment 8 in the preceding paper) were divided into two 10 cc. portions, one of which was autoclaved; 0.1 cc. of tributyrin was then added to each and the tubes were incubated for 48 hours. The results are given in Table II.

TABLE II.

Absence of Lipase in Culture Filtrates of Pneumococcus during the Period of Active Growth.

Filtrates from a 5 hour broth culture of Pneumococcus Type II.

Sterile filtrate.	Tributyrin.	Hydrogen ion concentration of filtrate.	
		Before incubation.	After incubation.
cc.	*cc.*	*pH*	*pH*
10, unheated.	0.1	7.4	7.4
10, heated.	0.1	7.3	7.3

From Table II it is evident that the lipase, like the peptonase, is an intracellular substance liberated on disintegration of the bacterial cell.

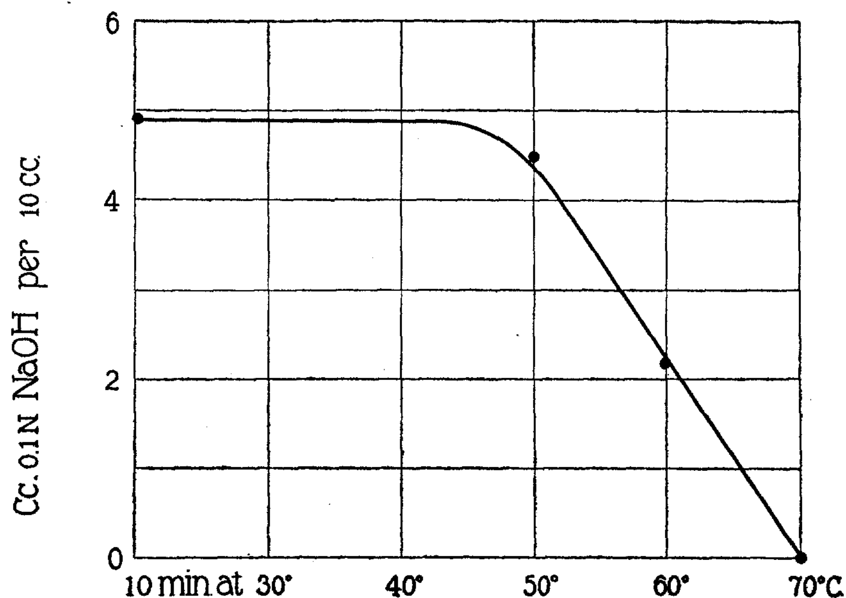
Effect of Heat on the Intracellular Lipase of Pneumococcus.

Experiment 3.—The thermostability of an enzyme in solution is influenced by the reaction of the medium in which it is dissolved and the length of exposure to the unfavorable temperature. In the present instance the enzyme was dissolved in bile, the solution adjusted to the optimum reaction for enzyme activity, pH 7.8, and subjected for 10 minutes in a water bath to the temperatures indicated.

The enzyme solution was the same as that used in Experiment 1 and was prepared from Pneumococcus Type II (No. F 208). 1 cc. portions of dissolved enzyme were carefully placed in sterile tubes and completely immersed in a water bath at the given temperature for 10 minutes. The tubes were immediately cooled to room temperature and to each were added 10 cc. of 2 per cent of tributyrin in 0.1 M phosphate solution, pH 7.8. After 24 hours at 37°C. the amount of acid split off from the ester by enzyme action was determined as in Experiment 1. The results are graphically presented in Text-fig. 2.

Variations in heat susceptibility of bacterial enzymes have been observed by many investigators. Söhngen (8), in studying the process of fat-splitting by bacteria, describes a lipase which resists a tempera-

ture of 100°C. for 5 minutes. The thermostability of the lipase of acid-fast bacteria has been noted by Wells and Corper, and by Kendall, Walker, and Day. These authors point out that heating to 100°C. for 15 minutes had little effect on the activity of the enzyme. Resistance of lipases in general to high temperatures is unusual; the various plant and tissue lipases in solution are as a rule inactivated by temperatures of 60–70°C. From Text-fig. 2 which illustrates the



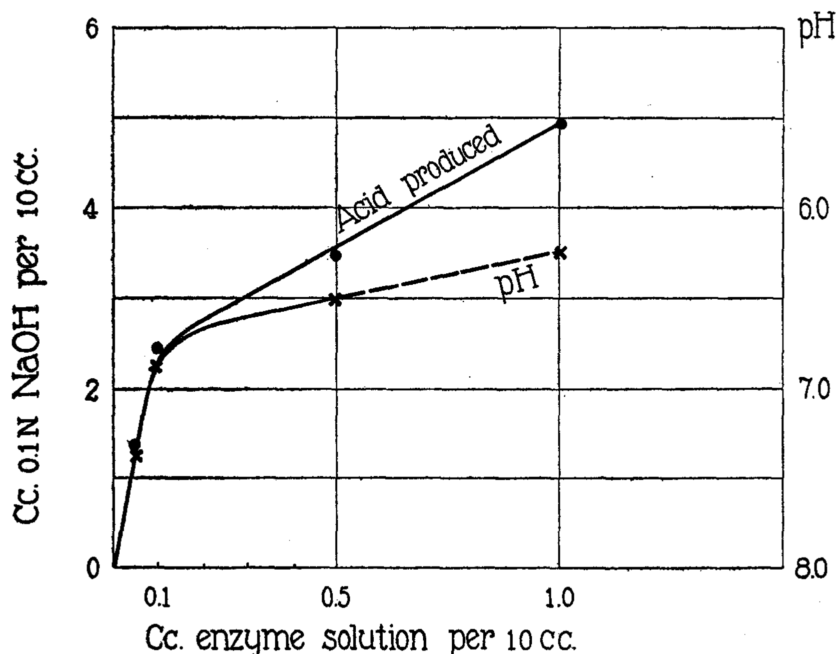
TEXT-FIG. 2. Heat stability of the intracellular lipase of pneumococcus.

effect on the pneumococcus lipase of exposure to various temperatures, it is evident that the dissolved enzyme suffers progressive loss of activity at temperatures above 50°C. and complete destruction at 70°C. for 10 minutes. According to Sternberg (9) the thermal deathpoint of pneumococcus is 52°C. for 10 minutes. The thermostability of the intracellular lipase under the conditions of this experiment, therefore, is somewhat greater than the heat resistance of the living organism.

Effect of Concentration of Enzyme on the Activity of the Intracellular Lipase of Pneumococcus.

If the ester-splitting property of bile solutions of pneumococci is enzymotic in nature, the rate of acid production should be proportional to the concentration of enzyme present. That this is the case is shown in the following experiment.

Experiment 4.—The enzyme solution, the same preparation used in the preceding experiment, was diluted with bile so that 1 cc. contained from 0.05 to 1 cc. of the original enzyme solution. These graduated quantities were added to 10 cc. of substrate consisting of 2 per cent tributyrin emulsified in 0.1 M phosphate solution of pH 7.8. The enzyme-substrate mixtures were incubated at 37°C. for 24 hours and the amount of acid hydrolysis was determined by the method outlined. The results are given in Text-fig. 3.



TEXT-FIG. 3. Influence of concentration of enzyme on the activity of the intracellular lipase of pneumococcus.

It is evident from the curves presented, that the rate of acid production by the action of the intracellular lipase on tributyrin is

directly proportional to the concentration of enzyme. Furthermore, as the enzyme concentration approaches the maximum, the amount of acid liberated by ester cleavage becomes so great that the resulting acidity is of itself sufficient to retard further enzyme action.

Relation of Virulence of Pneumococcus to Activity of the Intracellular Lipase.

Experiment 5. Preparation of Enzyme.—Two subcultures of the same strain of Pneumococcus Type II (No. F 208) were used. One of these, designated No. F 208 "B," the virulence of which had been greatly attenuated by cultural methods, failed to kill white mice in doses of 1 cc. of broth culture. The other, No. F 208 A, representing the original strain, the virulence of which had been preserved, was invariably fatal to these animals in doses of 0.000001 cc. injected intraperitoneally. The washed bacterial residues from 1,500 cc. of 15 hour plain broth cultures of these two organisms were collected and each was dissolved in 15 cc. of undiluted, sterile ox bile. The respective solutions were held over night in the ice box to ensure complete plasmolysis and were then tested for sterility on blood agar and in broth. The control enzyme solutions were inactivated by heat.

1 cc. portions of the active and inactivated enzyme preparations were added to tubes containing 10 cc. of 2 per cent tributyrin emulsified in sterile 0.1 M phosphate solution at pH 7.8. The digestion mixtures were then incubated for 24 hours at 37°C. Determinations of the hydrogen ion concentrations before and after incubation and of the amounts of acid produced in each instance are given in Table III.

TABLE III.

Relation of Virulence of Pneumococcus to Activity of the Intracellular Lipase.

Pneumococcus Type II.		Enzyme.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH.	Increase due to enzyme action.
Strain.	Minimum lethal dose for mice.		Initial.	Final.		
	cc.		*pH*	*pH*	*cc.*	*cc.*
F 208 "B"	Greater than 1	Active.	7.8	6.7	3.95	3.60
		Inactive.	7.8	7.7	0.35	
F 208 A	0.000001	Active.	7.8	6.7	3.75	3.45
		Inactive.	7.8	7.7	0.30	

Under the conditions of Experiment 5, in which were compared the relative potencies of the endoenzymes of an avirulent and of a virulent culture of the same strain of pneumococcus, loss of virulence was not associated with loss of enzyme activity.

Effect of Exposure to Acid Reaction on the Subsequent Activity of Pneumococcus Lipase.

Lord and Nye (10) have shown that pneumococci are rapidly killed at a hydrogen ion concentration of about pH 5.1. This acid death-point has been found both by these observers and by the present authors to correspond to the final reaction of broth cultures of pneumococcus when grown in the presence of sufficient carbohydrate. To determine whether this reaction is fatal to both organism and enzyme alike and whether this correlation has any significance in the mechanism of bile solubility the following experiment was performed.

Experiment 6.—1 cc. of active enzyme solution prepared as in Experiment 1 was added to 9 cc. of 0.1 M acid potassium phosphate solution of pH 4.6 (resulting reaction pH 5) and incubated at 37°C. for 2 hours. Then 0.83 cc. of N sodium hydroxide (calculated and verified on separate 9 cc. samples) was added to bring the acid enzyme solution to pH 7.8. After this readjustment of reaction 0.2 cc. of tributyrin was added as substrate, and the enzyme-substrate mixture incubated for 42 hours at 37°C. To serve as a control on activity, 1 cc. of the same enzyme solution, untreated, was added directly to 10 cc. of 0.1 M phosphate solution at pH 7.8 containing 2 per cent tributyrin (Table IV).

TABLE IV.

Effect on Lipase of Exposure to Acid Reaction.

Pneumococcus Type II.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH.
	Initial.	Final.	
	pH	*pH*	*cc.*
Untreated enzyme.	7.8	6.3	4.93
Acid-treated and readjusted.	7.8	6.4	3.80

From Table IV it is evident that after neutralization the activity of the pneumococcus lipase is little influenced by previous exposure,

to a reaction of pH 5. The bile insolubility of pneumococci after similar acid treatment is apparently not attributable to the death of the lipase, but is possibly referable to changes in the cell protoplasm.

Effect of Age on the Activity of the Intracellular Lipase of Pneumococcus.

Experiment 7.—Enzyme solutions prepared from pneumococci by extraction in phosphate solution as described in the preceding paper possessed lipase activity entirely comparable to that of enzyme solutions prepared by the bile method. These preparations were still active after preservation for 7 weeks, as is shown in Table V.

TABLE V.

Age Stability of Lipase.

Pneumococcus Type II.	Age.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH.
		Initial (0.1 M phosphate).	Final.	
	wks.	*pH*	*pH*	*cc.*
No. F 208	7	7.8	7.1	4.9
" II	3	7.8	6.9	6.5

DISCUSSION.

When pneumococci are dissolved in bile or extracted by the methods described, the resultant solution possesses, in addition to the proteolytic activity recorded in the preceding paper, a lipase (esterase) as measured by its power to split off acid from tributyrin.

Since a number of investigators (Hewlett (11), Magnus (12), and Loevenhart and Souder (13)) have shown that bile and bile salts not only do not interfere with lipase activity, but on the contrary accelerate the reaction, it was to be expected that the use of bile in effecting solutions of pneumococci would serve as an ideal method for demonstrating lipase activity. Bile, however, is not essential to the reaction, since extracts prepared by other methods exhibit comparable activity.

This esterase manifests its maximum activity at a hydrogen ion concentration of about pH 7.8, which is the optimum reaction for initiating growth of pneumococcus. The lipolytic activity of this enzyme progressively diminishes with increasing acidity until at about

pH 5 further hydrolysis ceases. That the point of acid extinction of esterase activity corresponds closely with the acid death-point of the living pneumococcus reveals another interesting correlation between cellular function and enzyme action. The rate of acid liberation from tributyrin during the initial stage of the action of the intracellular lipase is directly proportional to the concentration of enzyme. In the later phases of the reaction, the acid liberated by ester cleavage is sufficient in itself to retard further hydrolysis.

That the pneumococcus lipase is intracellular in nature is evidenced by the fact that it is present in maximum amount in bile solutions of washed bacterial cells, but cannot be demonstrated in culture filtrates during the period of active growth of the organism. The thermostability of the endolipase in solution is greater than the heat resistance of the living pneumococcus. After 10 minutes exposure in a water bath at temperatures greater than 50°C., the dissolved enzyme suffers progressive loss in activity until at 70°C. complete destruction results. Enzyme solutions preserved at refrigerator temperature retain their activity for weeks.

As to the possible relation of the activity of endoenzymes of pneumococcus to virulence of the living cell, the observations recorded in these studies are too limited to warrant discussion. Comparison of the relative potency of the endoenzymes of avirulent and virulent cultures of the same strain of pneumococcus has shown, however, that under the experimental conditions defined, loss of virulence was not associated with loss of enzymic activity.

Pneumococci exposed to a reaction corresponding to the acid death-point of the cell, that is, an acidity equivalent to or greater than pH 5, are thereby rendered insoluble in bile. This bile insolubility of pneumococci after acid treatment persists upon neutralization of the acid and even after the cells have been removed, washed, and resuspended in a neutral medium. In view of the possibility that the mechanism of bile solubility might in some way be related to the endolipase, in which instance the bile salts might function as coenzyme, or activator, it seemed pertinent to determine the effect of exposure to acid reaction on the subsequent activity of the enzyme itself. Since it has been shown, however, that upon neutralization the activity of the lipase is little influenced by previous exposure to a reaction of

pH 5, an acidity which kills the living cell, the phenomenon of bile insolubility of pneumococci after similar acid treatment is apparently not attributable to destruction of the endoenzyme.

SUMMARY.

1. Pneumococci contain an intracellular enzyme of marked lipolytic activity as measured by the acid liberated by its action on tributyrin.

2. Enzyme-containing solutions may be prepared by dissolving pneumococci in bile, or by extraction by other means.

3. The optimum reaction for maximum activity of the endolipase is about pH 7.8, which coincides with the optimum hydrogen ion concentration for growth of pneumococci.

4. Heating the enzyme for 10 minutes at 70°C. destroys its activity.

5. Attenuation of virulence of pneumococcus had no measureable effect on enzyme activity.

6. The possible relation of the endolipase to the mechanism of bile solubility is discussed.

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