

# STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

## I. PROTEOLYTIC ENZYMES.

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(Received for publication, June 11, 1920.)

Study of the biology of pneumococcus has led to a knowledge of certain biochemical characters which are common to the species as a whole, and to the recognition of fixed antigenic properties which serve to distinguish type differences within the species. The antigenic properties are inherent in the specificity of the bacterial protein and are detectable only by serologic reactions by means of which type relationships are recognized. The biochemical characters, on the other hand, are possessed in common by most pneumococci regardless of type differences, and are intimately associated with the life processes of the organism. These metabolic functions, upon which life of the cell depends, are in most instances referable to enzyme action. The presence or absence of a particular enzyme or group of enzymes determines largely the cellular activities of a microorganism. With the hope, therefore, of acquiring a better understanding of the way in which pneumococci adapt themselves to different environments, both in satisfying their nutritional needs and in exhibiting their invasive properties, the present study on the nature of the intracellular enzymes of this organism has been undertaken.

In the isolation and study of the enzymes of pneumococcus, apart from the living cell to which they are so intimately bound, use has been made of the fact that this organism undergoes rapid and complete solution in the presence of bile. Moreover, bile dissolves the bacterial cell with little or no change in the specific antigenic substance and with little or no injury to other demonstrable intracellular substances, such as the endohemotoxin. By dissolving the pneumococcus in bile and testing the cell-free solution on suitable substrates, enzymes are readily demonstrable. These enzymes have been found

to 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 demonstrating carbohydrate cleavage, however, bile was found to inhibit completely the hydrolysis of sucrose and starch, and another method of preparing the enzyme solution was necessary. This point will be emphasized in the paper on the intracellular invertase and amylase of pneumococcus (1).

The present paper concerns itself with the study of the proteolytic enzymes of pneumococcus. The intracellular nature of the enzymes, the influence of hydrogen ion concentration, the effect of age and concentration of the enzyme upon activity, and the relation of these enzymes to the virulence of the organism and to the mechanism of bile solubility will be discussed.

#### EXPERIMENTAL.

##### *Bacteriological Methods.*

*Media.*—The beef infusion broth containing 1 per cent peptone was prepared as previously described (2) except that (a) 2 gm. of dibasic phosphate per liter—anhydrous sodium phosphate or potassium phosphate—were used instead of 5 gm. per liter of sodium chloride, and (b) the medium was adjusted to a pH of 7.8.

*Bile.*—The ox bile used in preparing the pneumococcus enzyme solution was autoclaved, filtered, and again autoclaved as previously described (2).

*Sterility Controls.*—No antiseptics were used. The sterility of the enzyme solution was tested in broth and on blood agar plates. After addition of enzyme solution to the substrate, cultures of the mixtures were made by adding 0.1 cc. to 5 cc. of plain broth. In all the experiments recorded these controls remained sterile.

##### *Chemical Methods.*

*Preparation of Substrate Solutions.*—2 per cent solutions of peptone or protein were made in distilled water and the reaction was adjusted to the desired pH. The 2 per cent solution was then diluted with

an equal volume of 0.1 M phosphate solution of the desired pH. The final concentrations, unless otherwise stated, were therefore 1 per cent of substance in 0.05 M phosphate solution. The phosphate solutions were prepared from Merck's special reagents ( $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ ) according to Sørensen's (3) tables.

*Sterilization.*—Unless otherwise stated, sterilization was accomplished by autoclaving for 20 minutes at 15 pounds pressure.

*Hydrogen Ion Concentration.*—The pH values were usually determined colorimetrically, with the series of indicators outlined by Clark and Lubs (4). The solutions were diluted with two volumes of redistilled water, and the indicators used in such strength that one drop was required per 3 cc. of diluted solution. The readings were made by the comparator method (Walpole). Sørensen's standard phosphate and Walpole's (5) standard acetate solutions were used. These determinations were frequently checked by the electrometric method.

*Nitrogen Determinations.*—Total nitrogen determinations were made by the Kjeldahl method. Amino nitrogen was determined by Van Slyke's (6) nitrous acid method. With the peptone solution the determinations were made directly on 2 cc. samples, by means of the micro apparatus. Determinations of the amino nitrogen of the protein solutions were done by one of two methods: (a) 10 cc. samples were deaminized for 15 minutes in the large Van Slyke apparatus, and the nitrogen liberated was read in the micro burette calibrated to 0.002 cc.; and (b) the protein was precipitated with colloidal iron in the manner described by Van Slyke, Vinograd-Villchur, and Losee (7).

In determining the peptide nitrogen of the peptone solutions the peptides were split to amino-acids by acid hydrolysis (Van Slyke (8)). The peptide nitrogen was then calculated as the increase in amino nitrogen.

#### *Action of Intracellular Enzymes of Pneumococcus on Peptone (Fairchild).*

*Experiment 1. (a) Preparation of Enzyme.*—The washed bacterial residue from 2 liters of an 18 hour plain broth culture of *Pneumococcus* Type II (No. F 208) was taken up in 15 cc. of 33 per cent dilution of sterile bile (bile, 5 cc., + water, 10 cc.) and placed in the ice box over night. A portion of this bile solution of pneumococcus was inactivated by heat, as a control.

(b) *Preparation of Substrate.*—20 cc. portions of 1 per cent Fairchild's peptone in 0.05 M phosphate solution of various hydrogen ion concentrations were sterilized by the Arnold method on 3 successive days.

(c) *Sterility Control.*—No antiseptics were used. After the addition of enzyme solution to substrate, cultures of all digestive mixtures were made by adding 0.1 cc. of each to 5 cc. of plain broth. All cultures, including that of the bile solution of pneumococcus, were sterile.

The experiment was carried out as follows: Duplicate 20 cc. portions of the peptone substrate at reactions of pH 4.2, 5.3, 6.2, 7.0, and 7.6 were prepared. To one tube of each set, 1 cc. of enzyme solution was added, to the other 1 cc. of the inactivated enzyme solution. The tubes were then kept at 37°C. for 2 days. No antiseptic was used, sterility being maintained throughout by bacteriological technique. All cultural controls remained sterile, and after 48 hours the tubes were removed from the incubator for analysis. Duplicate amino-acid nitrogen determinations were made on 2 cc. samples by the Van Slyke method. The estimations of hydrogen ion concentration were determined colorimetrically on 5 cc. portions. The results are tabulated in Table I.

TABLE I.

*Determination of Peptone-Splitting Activity (Fairchild's Peptone).*

Tube No.	Final hydrogen ion concentration.		Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Inactive.	Active.	Increase.
	pH	pH	mg.	mg.	mg.
1	4.2	4.2	27.3	27.2	
2	5.3	5.3	27.5	32.3	4.7
3	6.2	6.2	27.6	45.3	17.7
4	7.0	7.1	29.5	49.8	20.3
5	7.6	7.6	28.2	47.9	19.6
Peptone.			28.2		

*Distribution of Nitrogen per 100 Cc. of Substrate.*

Total nitrogen.....	mg.	107
Amino " before hydrolysis.....	mg.	27.5
" " after " .....	mg.	78.6
Peptide " therefore equals.....	mg.	51.1

*Analysis of Experiment.*

The increase of 20.3 mg. of amino nitrogen per 100 cc. of substrate shows that 40 per cent of peptide nitrogen was split to free amino nitrogen.

In order to determine the proportion of the peptide that was digested, a nitrogen partition was done on the peptone solution. Total nitrogen on 2 cc. portions was determined by the Kjeldahl method. Peptide nitrogen<sup>1</sup> was computed as the difference between total amino-acid nitrogen before and after hydrolysis.

*Action of Intracellular Enzymes of Pneumococcus on Peptone (Witte).*

*Experiment 2. (a) Preparation of Enzyme.*—This was the same as in Experiment 1, except that the bacterial residue from 2 liters of Pneumococcus Type II (No. F 208) was dissolved in 15 cc. of 25 per cent solution of sterile bile in water.

*(b) Preparation of Substrate.*—The substrate was prepared as in Experiment 1, except that Witte's peptone was used instead of Fairchild's preparation.

*(c) Sterility Control.*—No antiseptic was used. All tubes including cultural control of the enzyme solution were proved sterile by subcultures as in the preceding experiment.

In Experiment 2 the peptone-splitting action of pneumococcus enzyme was tested on Witte's peptone. The experimental technique was the same as that described in the preceding protocol, except for the substitution of 1 per cent Witte's peptone for the Fairchild preparation. The Witte peptone substrate was analyzed for total nitrogen, and peptide nitrogen in the same manner. The results are given in Table II.

It is evident from Experiments 1 and 2 that pneumococcus contains within its cell an enzyme or enzymes capable of hydrolyzing peptides into amino-acids or simpler peptides. From 26 to 40 per cent of the peptide nitrogen present in the peptone substrate was split to amino nitrogen. The term "peptone" solution is used to indicate the mixtures of partially hydrolyzed protein products which are known commercially as "peptones." The considerable data available as to the chemical nature of these peptones show them to be mixtures of protein products of varying degrees of complexity. The amount of pep-

<sup>1</sup>By peptide nitrogen is meant nitrogen found in the peptide linkings, the —CO—NH— groups that link the different amino-acids together in peptides, proteins, or intermediate products. The process of hydrolysis consists in the splitting of these peptide groups, from each of which is generated a carboxyl group and an amino group. Thus



For further discussion of this point see Van Slyke, D. D., *Arch. Int. Med.*, 1917, xix, 56.

tide hydrolyzed was greater in the experiment in which Fairchild's peptone was used. The proportion of preformed amino nitrogen to total nitrogen in this preparation was greater than in the sample of Witte's peptone, indicating that, on the average, Fairchild's peptone consists of simpler intermediate protein digestion products than Witte's. This may be the reason that further digestion by the enzyme proceeded more rapidly in the Fairchild product, the enzyme attacking the simpler peptides of the preparation the more readily.

TABLE II.

*Determination of Peptone-Splitting Activity (Witte's Peptone).*

Tube No.	Final hydrogen ion concentration.		Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Inactive.	Active.	Increase.
	<i>pH</i>	<i>pH</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	4.4	4.4	15.5	15.6	
2	5.0	5.0	15.6	21.2	5.6
3	6.0	6.0	15.4	32.4	17.0
4	7.0	7.0	15.5	39.6	24.1
5	7.8	7.8	15.5	39.4	23.9

*Distribution of Nitrogen per 100 Cc. of Substrate.*

Total nitrogen.....	<i>mg.</i>	148
Amino " before hydrolysis.....		15.5
" " after " .....		108
Peptide " .....		92.5

*Analysis of Experiment.*

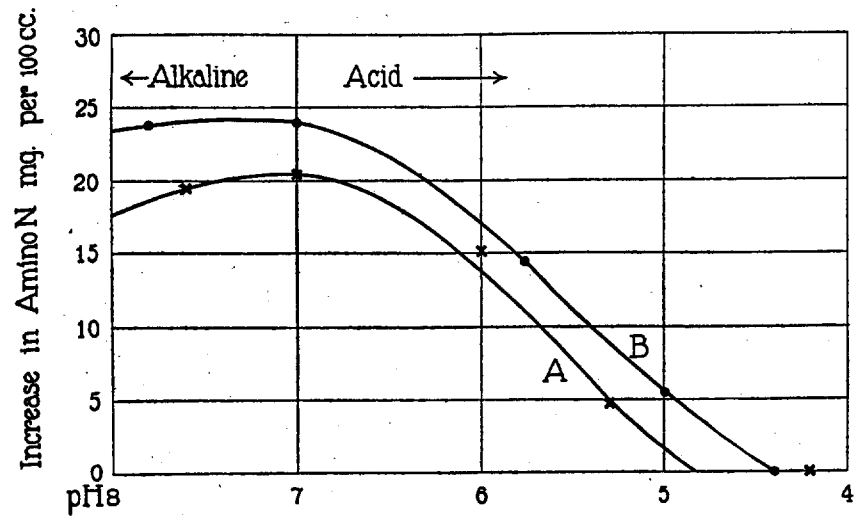
The increase of 24.1 mg. per 100 cc. of substrate shows that 26 per cent of peptide nitrogen was split to free amino nitrogen.

*Effect of Hydrogen Ion Concentration on the Activity of Pneumococcus Peptonase.*

Experiments 1 and 2 were planned to determine the relation of enzyme activity to hydrogen ion concentration. This relation is evident from the curves of Text-fig. 1, in which *A* represents Experiment 1 and *B* Experiment 2. The optimum activity of the peptone-splitting enzyme is from pH 7 to pH 7.8. With increase in acidity the

activity of the enzyme is increasingly retarded until complete inhibition results at pH 4.5.

These facts, that about 30 to 40 per cent of the peptide is hydrolyzed and that the optimum zone for activity of the enzyme is between pH 7 and 7.8, place it in the erepsin class of enzymes. Moreover, the fact that the curve of acid inhibition proceeds in a straight line to complete inhibition at a pH of about 4.5 indicates that the enzyme preparation used contains no pepsin and is, therefore, not a complex



TEXT-FIG. 1. Influence of hydrogen ion concentration on the activity of pneumococcus peptonase. Curve A, Experiment 1; Curve B, Experiment 2.

of erepsin- and pepsin-like enzymes. Since it seemed desirable to maintain an open mind on the question as to whether the enzymotic action in these experiments is more closely allied to trypsin or erepsin, and in view of its relative rate of action on native protein, the term "peptonase" has been used throughout the remainder of this report. It is significant that the optimum reaction zone for the intracellular peptolytic enzyme corresponds with the optimum for growth of pneumococcus (9).

*Use of Sodium Choleate in Demonstrating the Intracellular Enzymes of Pneumococcus.*

It is well known that pneumococci undergo solution in the presence of bile salts as completely as in the presence of bile itself. In order to determine whether the substitution of sodium choleate for bile in dissolving the organisms exerted any influence on the activity of the intracellular enzymes the following experiment was carried out.

*Experiment 3. (a) Preparation of Enzyme.*—Pneumococcus Type I (No. G<sub>2</sub>) was grown in 2 liters of plain broth for 18 hours at 37°C. The bacteria were removed by centrifugation, washed once in sterile isotonic salt solution, and suspended in 10 cc. of 5 per cent solution of sodium choleate. After 5 hours in a water bath at 37°C., the resultant solution of pneumococci was diluted with an equal volume of sterile distilled water. One portion of this solution was inactivated by heat and both were tested for activity in a peptone substrate.

*(b) Preparation of Substrate.*—20 cc. portions of 1 per cent Fairchild's peptone in 0.05 M phosphate solution were adjusted to the various hydrogen ion concentrations and sterilized by the Arnold method on 3 successive days.

This experiment was conducted in exactly the same manner as Experiment 1.

TABLE III.

*Determination of Peptone-Splitting Activity of a Solution of Pneumococcus Obtained by the Use of Sodium Choleate.*

Tube No.	pH	Amino nitrogen per 100 cc. of substrate.		
		Inactive.	Active.	Increase.
		mg.	mg.	mg.
1	5.0	37.9	42.9	5.0
2	5.4	37.9	50.7	12.8
3	5.8	38.7	53.4	14.7
4	6.2	40.4	55.2	14.8
5	6.6	41.6	59.2	17.6
6	7.0	42.2	60.3	18.1
7	7.4	39.6	58.9	19.3
8	7.8	40.7	58.3	17.6

From the data presented in Table III it is evident that when pneumococci are dissolved by sodium choleate there is liberated from the cell a peptone-splitting enzyme in the same manner as when solution of the organism is effected by the action of bile. It has also been



found that enzyme solutions obtained by disintegration of pneumococcus cells without the presence of bile or bile salts, by methods described in a succeeding paper (1), exhibit comparable activity. Therefore, bile salts are not essential to the action of the enzyme.

*Effect of Age on the Activity of the Intracellular Peptonase of Pneumococcus.*

*Experiment 4. (a) Preparation of Enzyme.*—The technique of preparing the enzyme solution in this experiment was similar to that described in preceding protocols, except that the bacterial residue from 2 liters of broth culture of Pneumococcus Type II (No. F 208) was dissolved directly in 10 cc. of undiluted bile. After 2 hours in the water bath at 37°C. and 1 hour at room temperature, the enzyme solution was stored in the ice box, and at intervals up to 43 days portions were removed and tested for activity.

*(b) Preparation of Substrate.*—1 per cent of Fairchild's peptone in  $\frac{M}{375}$  phosphate solution of pH 7 was prepared and sterilized in the autoclave at 15 pounds pressure for 20 minutes.

*(c) Sterility Control.*—No antiseptics were used. Cultures of the original enzyme solution and each digestion mixture at the time of carrying out the several tests proved sterile.

The results are presented in Table IV.

TABLE IV.

*Age Stability of Endopeptonase.*

Enzyme solution kept at about 4°C. 1 cc. added to 20 cc. of peptone (Fairchild), pH 7. 24 hours at 37°C.

Age.	Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Increase.
<i>days</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
0	37.4	58.8	21.4
1	37.4	59.7	22.3
2	40.8	62.6	21.8
6	37.4	53.6	16.2
20	40.8	54.4	13.6
43	23.4	32.1	8.7

The length of time an enzyme may remain active is dependent upon the conditions of its preservation. The age stability of an

enzyme in dried form is greater than that of the same enzyme in solution. In the present instance, the intracellular peptonase of pneumococcus dissolved in undiluted ox bile retained about 40 per cent of its activity for over 6 weeks.

*Relation of Virulence of Pneumococcus to Enzyme Activity.*

*Experiment 5. (a) Preparation of Enzyme.*—Plain broth cultures (1,500 cc.) of a virulent and avirulent strain of Pneumococcus Type II (No. F 208) were centrifuged; the bacterial sediment was washed once in sterile isotonic salt solution, then dissolved in 15 cc. of undiluted bile, and held in the ice box over night. Portions of the enzyme solutions were inactivated by heating in the autoclave at 15 pounds pressure for 20 minutes.

TABLE V.

*Influence of Virulence of Pneumococcus upon the Activity of the Intracellular Peptonase.*

2 cc. of enzyme solution added to 20 cc. of peptone solution, pH 7. Incubated at 37°C. for 24 hours.

Pneumococcus Type II.	Minimum fatal dose.	Amino nitrogen per 100 cc. of substrate.		
		Inactive.	Active.	Increase.
	cc.	mg.	mg.	mg.
No. F 208 A	0.000001	39.8	68.6	28.8
" F 208 "B"	Greater than 1	39.2	67.8	28.6

(b) *Preparation of Substrate.*—1 per cent peptone (Fairchild) in  $\frac{M}{80}$  phosphate solution, pH 7, was sterilized in the autoclave.

(c) *Sterility Control.*—No antiseptics were used. Each tube in the experiment was tested for sterility by subculture and yielded no growth.

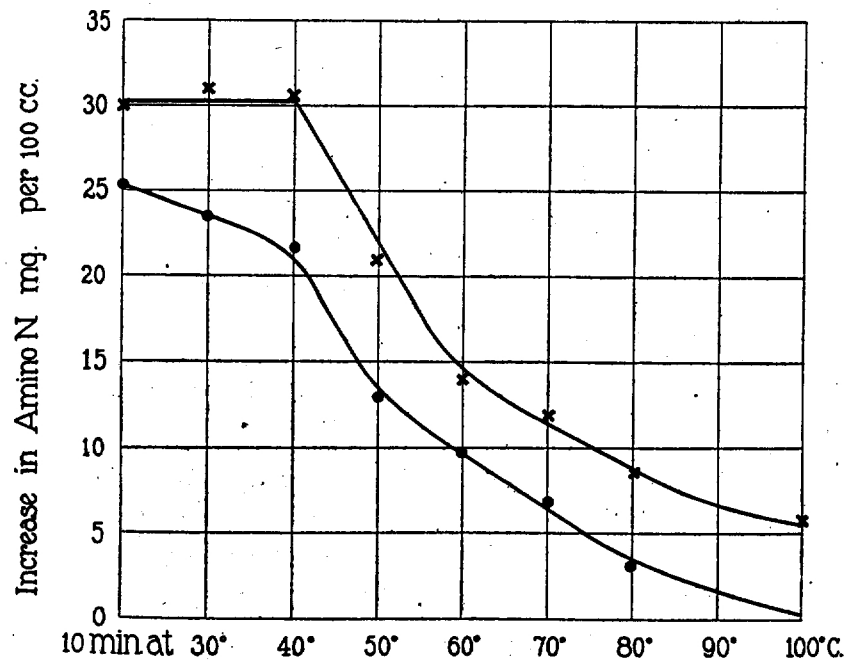
The development of a technique for the demonstration of endoenzymes made it possible to submit to experimental proof the question whether differences in virulence are in any way related to the activity of the intracellular enzymes. For this purpose a strain of Pneumococcus Type II (No. F 208) was chosen. This organism was originally isolated from the blood of a patient suffering from lobar pneumonia. The virulence of the strain, maintained by animal passage, was such that 0.000001 cc. of broth culture injected intraperitoneally into white mice proved fatal in 24 to 48 hours. A subculture of the same strain, the virulence of which had been attenuated by cultural methods, failed to kill mice in doses of 1 cc. Enzyme solutions from comparable amounts of bacteria were prepared from the virulent and avirulent cultures of this strain. The respective

enzyme preparations were tested for peptonase action by adding 2 cc. of each to substrates of 20 cc. of 1 per cent peptone solution adjusted by phosphates to pH 7. After 24 hours at 37°C. the degree of enzyme action was determined by measuring the increase of amino nitrogen as indicated in Table V.

From Table V it appears that loss of virulence is not associated with a corresponding loss of enzymotic activity. Under the conditions of this experiment at least, the amount of hydrolysis of peptone by the endoenzymes of the avirulent strain was equivalent to that of the virulent organism.

*Effect of Heat on the Intracellular Peptonase of Pneumococcus.*

Sensitiveness to heat is a biologic character of all enzymes. In determining the influence of heat upon dissolved enzymes, the degree of temperature, the length of exposure, and the reaction of the solu-



TEXT-FIG. 2. Heat stability of intracellular peptonase of pneumococcus. The lower curve represents the results after 24 hours incubation at 37°C., the upper curve after 48 hours at 37°C.

tion are closely interrelated. The optimum reaction for activity of the endopeptonase of pneumococcus, pH 7.4, and an exposure of 10 minutes were arbitrarily chosen, and the temperature alone was varied as shown in Text-fig. 2.

*Experiment 6. (a) Preparation of Enzyme.*—The bacterial residue from 4 liters of plain broth culture of Pneumococcus Type II (No. F 208) was dissolved in 20 cc. of sterile ox bile and held in the ice box over night.

*(b) Preparation of Substrate.*—1 per cent peptone solution (Fairchild) in 0.05 M phosphate solution adjusted to pH 7.4 was sterilized in the autoclave.

*(c) Sterility Control.*—Sterility was proved by subculture from each tube.

1 cc. of the enzyme solution with a pH of about 7.4 was placed in each of eight sterile tubes, and these in turn were immersed in water baths at 30°, 40°, 50°, 60°, 70°, 80°, 90°, and 100°C., respectively, for exactly 10 minutes. On removal the tubes were immediately cooled, and to each were added 10 cc. of the sterile substrate. After 24 and 48 hours incubation at 37°C. samples were removed for analysis. The results are plotted in Text-fig. 2. The heat sensitivity of the enzyme manifests itself in a progressive loss of activity after exposure for 10 minutes to increasing temperatures, until at 100°C. little or no activity remains.

#### *Effect of Concentration of Enzyme on the Activity of the Intracellular Peptonase of Pneumococcus.*

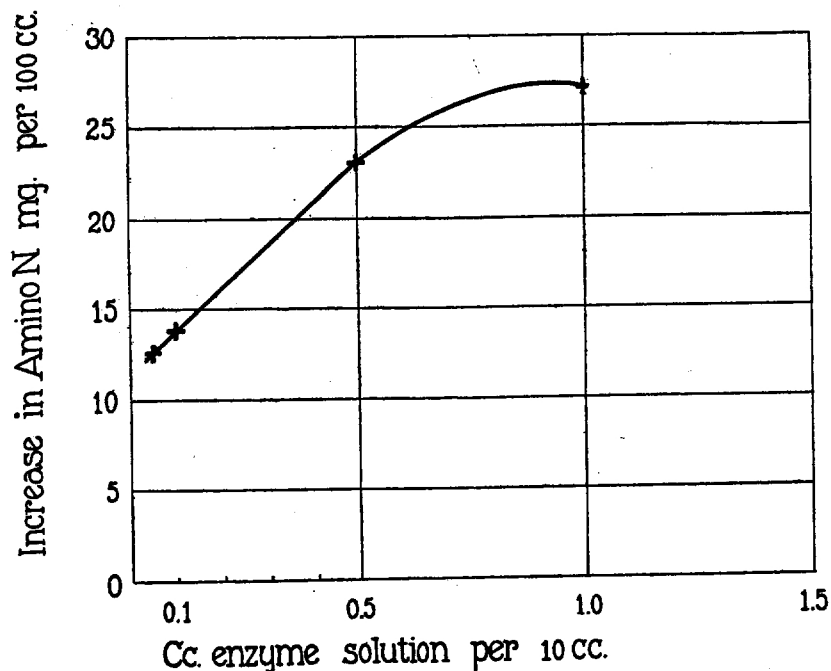
*Experiment 7.*—The same preparation of enzyme and substrate used in Experiment 6 was employed in this test, a bile solution of Pneumococcus Type II and a 1 per cent solution of peptone (Fairchild's) in 0.05 M phosphate mixture, pH 7.4.

The dissolved enzyme was diluted with bile so that 1 cc. of the final solution added to 10 cc. of peptone substrate contained 0.05, 0.1, 0.5, and 1 cc. respectively of the original enzyme solution. The results are plotted in Text-fig. 3.

For a more exact study of the dynamics of this enzyme it would be desirable to repeat this experiment with shorter digestion periods and smaller amounts of enzyme. It is evident, however, that under given conditions the rate of hydrolysis is proportional to the concentration of enzyme.

#### *Occurrence of Enzymes in Culture Filtrates of Pneumococcus.*

The experiments thus far have dealt entirely with intracellular enzymes. It was considered probable that the culture medium itself



TEXT-FIG. 3. Effect of concentration of enzyme on the activity of the intracellular peptonase of pneumococcus.

would contain similar enzymes which had either diffused out from the cell during growth or had been liberated by disintegration of the organisms in the culture fluid. In order to determine the validity of this assumption, the following experiment was undertaken.

*Experiment 8. (a) Preparation of Filtrate.*—100 cc. of an 18 hour plain broth culture of *Pneumococcus* Type II (No. F 208) was filtered through a Berkefeld candle N. The filtrate, pH 7, was tested for sterility by adding 5 cc. to 100 cc. of fresh broth and incubating at 37°C. In testing the sterility of culture filtrates of pneumococcus it is not sufficient merely to incubate the filtrate itself, but it is essential to inoculate fresh broth. This is probably due to the fact, recorded in a previous paper (Avery and Cullen (10)), that further growth cannot be initiated in the filtrates of a plain broth culture of pneumococcus even though the reaction is readjusted to the optimum hydrogen ion concentration. A portion of the enzyme-containing filtrate was inactivated by heat, to serve as a control.

*(b) Preparation of Substrates.*—These consisted of the sterile culture filtrate and a 1 per cent solution of peptone (Fairchild) in phosphate mixture adjusted

to pH 7, the same hydrogen ion concentration as the filtrate. The peptone solution was sterilized in the autoclave.

In determining the presence of enzyme in culture filtrates of pneumococcus the amount of cleavage was determined, (a) as the result of further action of the enzyme on the peptones present in the broth filtrates and (b) as the result of this action plus the action on additional peptones. This was accomplished by the procedure indicated in Table VI, three tubes of substrate being used, in all of which the final volume was 20 cc. The first tube contained 10 cc. of filtrate and 10 cc. of sterile water, the second, 10 cc. of filtrate and 10 cc. of 1 per cent peptone solution, and the third (the control), 10 cc. of 1 per cent peptone solution plus 10 cc. of sterile water. The determinations of amino nitrogen in the digestion mixtures before and after incubation are given in Table VI.

TABLE VI.

*Presence of Peptonase in Culture Filtrate of Pneumococcus.*

Tube No.	Sterile filtrate unheated; pH 7.	1 per cent peptone solution; pH 7.	Water.	Amino nitrogen per 100 cc. of final solution.		
				Before digestion.	After digestion.	Increase.
	cc.	cc.	cc.	mg.	mg.	mg.
1	10		10	54.9	73.4	18.5
2	10		10	54.7	73.0	18.3
3		10	10	24.0	24.0	
4		10	10	24.0	24.0	
5	10	10		78.9	106.8	27.9
6	10	10		79.0	106.8	27.8

*Analysis of Table VI.*

Increase in amino nitrogen in filtrate alone.....	18.4 mg. per 100 cc.
“ “ “ “ “ “ + peptone....	27.9 “ “ 100 “
“ “ “ “ “ “ due to action of enzyme	
on added peptone.....	9.4 “ “ 100 “

From Table VI it is clear that under the conditions of the experiment the bacteria-free filtrate contained an active enzyme which continued to hydrolyze the excess of available peptide in the medium and in addition attacked the added peptone.

The demonstration of the presence of an enzyme in culture filtrates of pneumococcus after 18 hours incubation does not necessarily imply that the enzyme is a true secretory product which is elaborated during

growth and given off into the medium in the manner of exotoxins. Under optimum cultural conditions pneumococcus reaches its maximum growth relatively early (Chesney (11)), after which involution and disintegration of the bacterial cells soon begin. During this later period, the intracellular hemolysin of pneumococcus, which cannot be detected free in the medium during the early phase of active growth, is also released from the disrupted cell and can be demonstrated in the culture fluid. If the peptonase is an endoenzyme, its absence in filtrates during the period of active growth should be demonstrable.

In order, therefore, to test for presence of enzyme in the culture fluid before cell death and disruption with consequent liberation of intracellular enzyme occurred, the following experiment was carried out.

200 cc. of plain broth, pH 7.8, were inoculated with 0.5 cc. of a 5 hour culture of *Pneumococcus* Type II (No. F 208). After 5 hours incubation marked growth was apparent and the acidity had increased to pH 7.5. The culture was then centrifuged and the supernatant fluid was filtered through a tested Berkefeld candle N. This filtrate was kept in the refrigerator until its sterility was proved by culture. The sterile filtrate was then tested for enzyme activity as in the preceding experiment.

It was found that during the phase of active growth the culture fluid freed from bacteria possessed no peptolytic activity. Moreover, in this same culture fluid the intracellular hemolysin, known to be liberated by cell disintegration, was likewise not demonstrable, but the soluble substance shown by Dochez and Avery (12) to be elaborated during the earliest phase of cell multiplication was detectable in considerable concentration. The occurrence, therefore, of peptolytic activity in autolyzing broth cultures and its absence in the culture fluid during the early phases of active growth make it evident that the peptonase is a true endoenzyme.

*Effect of Exposure to Acid Reaction on the Intracellular Peptonase of Pneumococcus.*

It has been observed that cultures of pneumococci grown in sugar-containing medium reach a final hydrogen ion concentration of about pH 5 to 5.2. At this point the organisms quickly succumb, the acidity

produced by their own metabolic processes being sufficient in itself to stop growth. Lord and Nye (13) have found that pneumococci will not live for more than a few hours in a solution the acidity of which is greater than pH 5.1. Experiments in this laboratory have shown that pneumococci subjected to an acidity of pH 4.5 to 5 for 2 hours are no longer soluble in bile. Even when these organisms are removed by centrifugation, washed, and resuspended in phosphate solution at a pH 7.8, they remain insoluble in bile. The question arose whether this reaction of pH 4.5 to 5, which corresponds to the acid death-point of the bacterial cells, is also fatal to the intracellular enzymes, and whether this fact is of any significance in the phenomenon of bile insolubility.

To determine whether acid treatment injured the endoenzymes of pneumococcus the following experiment was carried out.

*Experiment 9.*—1 cc. of the enzyme solution used in Experiment 6 was treated with 4 cc. of 0.1 M potassium acid phosphate solution of pH 4.5. The reaction of the resulting solution was pH 5. After 2 hours at 37°C. the solution was readjusted to pH 7.4 by the addition of 0.3 cc. of N sodium hydroxide (calculated and verified on a separate sample), and 5 cc. of a 2 per cent peptone solution of pH 7.4 were added. As a control on activity, the untreated active enzyme was added to the same peptone substrate in similar proportions. The results are given in Table VII.

TABLE VII.

*Effect on Peptonase of Exposure to Acid Reaction.*

Final concentration of enzyme was 1 cc. per 10 cc. of 1 per cent peptone in 0.1 M phosphate solution of pH 7.4. 42 hours at 37°C.

Treatment of enzyme.	Amino nitrogen per 100 cc. of substrate.		
	Inactive. mg.	Active. mg.	Increase. mg.
Enzyme solution previously kept at pH 7.8.....	31.2	58	26.8
“ “ “ adjusted to pH 5.0 and after 2 hrs. at 37°C. readjusted to pH 7.4.....	31.2	58	26.8

The intracellular enzyme in solution suffered no loss of potency after being subjected for 2 hours to an acidity of pH 5, for upon readjustment to the optimum hydrogen ion concentration of pH 7.4, the



acid-treated enzyme exhibited an activity comparable to that of the untreated enzyme. The endopeptonase of pneumococcus is evidently little influenced by this reaction change. The bile insolubility of pneumococci at the acid death-point is, therefore, not associated with destruction of this enzyme, but is probably referable to coagulative changes in the cell protoplasm.

*Action of the Endoenzymes of Pneumococcus on the Proteins, Casein, Gelatin, Albumin, and Fibrin.*

The data presented in the preceding protocols establish the fact that there is present within the bacterial cell an enzyme, or enzymes, capable of hydrolyzing peptones into amino-acids, and that this enzyme-complex manifests its optimum activity in a slightly alkaline medium. Simultaneous experiments were carried out with the same enzyme preparations to determine their action on the proteins, casein, gelatin, fibrin, and albumin.

*Preparation of Enzyme.*—In these experiments the enzyme solutions were prepared in the manner described in Experiment 6.

*Preparation of Substrates.*—2 per cent gelatin, albumin (egg), and casein solutions were adjusted to pH 7.4 and diluted with an equal volume of 0.1 M phosphate solution of the same hydrogen ion concentration and then sterilized in the autoclave. The fibrin substrate D was prepared by adding 0.2 gm. of dried commercial fibrin to 10 cc. of 0.05 M phosphate solution of pH 7.4 and was sterilized in the autoclave. The fibrin substrate F was freshly prepared from 10 cc. portions of sterile oxalated rabbit plasma. Sterile calcium chloride was added and the fibrin clot was washed in sterile salt solution and transferred to 10 cc. of sterile 0.05 M phosphate solution of pH 7.4.

*Experiment 10.*—To 10 cc. portions of the sterile protein solution 1 cc. of enzyme solution was added, and the mixture placed at 37°C. for 5 to 7 days. The degree of digestion was determined as in the peptone experiments by amino nitrogen determination with one of the following procedures: (a) 10 cc. portions of the digestion mixtures were placed in the deaminizing bulb of the original large type of Van Slyke apparatus. 15 minutes were allowed for deamination and the nitrogen evolved was measured in a micro burette graduated to 0.002 cc. (b) After removal of the proteins by colloidal iron, amino nitrogen determinations were made in the manner described in preceding experiments. The results are presented in Table VIII.

*Experiment 11.*—In following the extent of digestion of the peptone solution control amino nitrogen determinations on active enzyme alone had shown that

TABLE VIII.

*Proteolytic Action of Endoenzymes of Pneumococcus.*

Protein.	Duration of digestion.	Method of analysis.	Amino nitrogen per 100 cc. of substrate.		
			Inactive.	Active.	Increase
	<i>days</i>		<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Casein.	5	(b)	1.9	6.3	4.4
"	12	(b)	1.3	3.4	2.1
Gelatin.	7	(a)	6.3	6.7	0.4
	12	(b)	2.8	3.5	0.7
Fibrin D.	5	(a)	2.5	4.0	1.5
" F.	31	(b)	4.2	7.1	2.9

the increase in amino nitrogen due to autolysis of the bacterial proteins was negligible. However, because of the small concentration of free amino nitrogen in the protein experiment more rigid control of this protein digestion was carried out. Duplicate 10 cc. portions of casein, albumin, and 0.05 M phosphate, all at pH 7.4, were prepared. To one tube of each pair 2 cc. of active enzyme were added, and to the other 2 cc. of heated enzyme. Tubes were incubated at 37°C. for 5 days. Each dilution was then diluted to 25 cc., 5 cc. portions were removed for total nitrogen determinations, and the remaining 20 cc. were freed from protein by precipitation with colloidal iron. The non-protein nitrogen of the filtrate and washings was determined by the Kjeldahl method. The results are presented in Table IX.

TABLE IX.

*Proteolytic Action of Endoenzymes of Pneumococcus.*

Proteolysis measured by increase in non-protein nitrogen. Digestion for 5 days at 37°C.

Solution.	Total nitrogen per 100 cc. of solution.	Non-protein nitrogen per 100 cc. of solution.			Protein digested.
		Inactive.	Active.	Increase.	
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>per cent</i>
Enzyme.....	25.7	14.8	16.8	2.0	20.0
Casein*.....	14.6	7.0	19.4	12.4	8.9
Albumin*.....	12.3	6.4	6.5		

\* Protein figures corrected for nitrogen of enzyme solution added.

From Table IX it is evident that autolysis of the enzyme solution itself is occurring. This increase would, for the concentrations used in Table VIII, amount to about 0.5 mg. of amino nitrogen per 100 cc.; that is, the increase in the case of the gelatin experiment is due to the enzyme solution. The increase of 3 and 4 mg. in the case of casein and fibrin indicates a definite proteolysis.

The endoenzymes of pneumococcus are apparently able partially to hydrolyze the proteins, casein and fibrin, but not albumin or gelatin. This enzyme action on intact protein is distinctly less, however, than that which occurs in the presence of peptones.

#### DISCUSSION.

To present a critical review of the literature on the nature and action of bacterial enzymes would entail a task beyond the scope of this paper. Although extensive study of the enzymes of a large variety of different species of microorganisms has been made, comparatively little work has been done on the occurrence and character of the endoenzymes of pneumococcus.

Rosenow (14) demonstrated in extracts of virulent pneumococci and filtrates of broth cultures a proteolytic enzyme capable of hydrolyzing the proteins in meat broth, in ascites meat broth, and to a less extent the proteins of heated serum. He prepared extracts of pneumococci by suspending the bacteria from broth cultures in salt solution, adding ether, and allowing autolysis to proceed at 37°C. for 48 hours. The bacteria were removed by centrifugation or passage through a Berkefeld filter. Rosenow found that the degree of disintegration of the bacterial cells was directly proportional to the amount of proteolysis as measured by formol titration. He has shown further that the toxicity of broth, culture filtrates, and extracts of pneumococci is associated with proteolysis due to the dissolved enzymes.

Evidence is presented in this paper of the existence of proteolytic enzymes within the cell body of pneumococcus. This enzyme or group of enzymes can be isolated from the living cell by dissolving the organisms in bile or allowing them to cytolize in phosphate solutions of pH 6.2. In the latter method alternate thawing and freezing of the bacterial suspension greatly facilitate the extraction process by disrupting the bacterial cells.<sup>2</sup> By these methods the intracellular

<sup>2</sup> See Avery and Cullen (1).

substances pass into solution in a medium in which they retain their activity for a considerable period. The proteolytic activity of these enzymes is manifest in their ability to hydrolyze, to some extent, intact protein and to split to a striking degree intermediate products, such as peptones, into simpler peptides and amino-acids. It has not been determined whether the two processes, proteolysis and peptolysis, are functions of the same enzyme or the result of the action of two separate enzymes.

It is evident that the action on the intact proteins, fibrin and casein, is distinctly less than on simpler substances such as occurs in peptone mixtures. For this reason the larger number of experiments has been carried out with a partially hydrolyzed protein, commonly known as peptone, as substrate. Because it exhibits its maximum activity in the further hydrolysis of peptide nitrogen, this enzyme is referred to as peptonase, a term indicative of its action on peptone.

The intracellular peptonase of pneumococcus hydrolyzes 30 to 40 per cent of the peptide nitrogen in peptone substrates to amino nitrogen. The peptonase activity of the bacterial substance is striking in its intensity. Weight for weight the substance hydrolyzes peptone several times as rapidly as the most active commercial samples of pancreatic preparations. The zone of its optimum activity is pH 7 to 7.8, similar to that of trypsin and erepsin, and corresponds to the optimum reaction for growth of pneumococcus. The absence of activity at a pH below 4.5 indicates the absence of pepsin. Bile salts, as well as bile itself, effect solution of pneumococci, and enzymes prepared by dissolving the cell bodies in solutions of sodium choleate manifest an equal degree of activity. The thermostability of the intracellular peptonase is greater than the heat resistance of pneumococcus. The enzyme is, however, sensitive to heat; an exposure of 10 minutes at 100°C. destroys its activity. Dissolved in undiluted ox bile the enzyme retained about 40 per cent of its activity over a period of 6 weeks. A direct proportionality has been shown to exist between the rate of hydrolysis and the concentration of the enzyme in the digestion mixture.

In bacteria-free filtrates of pneumococcus enzymes are demonstrable only when growth of the bacteria has progressed to the phase in which cell disintegration begins and liberation of the intracellular sub-

stances into the culture medium occurs. During the early stages of growth of pneumococcus, when the organisms are multiplying at their maximum rate and little or no cell death is occurring, enzymes cannot be detected in culture filtrates. This evidence indicates that the enzymes studied are intracellular in character and belong to the class known as endoenzymes.

As far as is known for bacteria, solubility in bile is peculiar to pneumococcus alone. The mechanism of this reaction is not fully understood. Pneumococci exposed to an acidity equivalent to or greater than pH 5 are not only rapidly killed but rendered completely bile-insoluble. The endoenzymes derived from pneumococcus, on the other hand, are little influenced in their subsequent activity by previous exposure for 2 hours to a reaction corresponding to the acid death-point of the bacterial cell. Similarly pneumococci rapidly succumb on short exposure to a temperature of 52°C. and the heat-killed organisms are no longer soluble in bile. Exposure of the proteolytic enzyme, however, to a temperature corresponding to the thermal death-point of pneumococcus, causes only slight retardation of its hydrolyzing power.

These facts, apart from their significance in a study of the nature of the endoenzymes, are of interest in interpreting the possible relation of these active intracellular substances to the mechanism of bile solubility of pneumococcus. From these limited observations, it does not appear likely that bile solubility is the result of the action of enzymes, of which bile serves as an activator, for agents, both chemical and physical, which render the cell insoluble in bile, exert in a similar concentration only slight inhibition on the intracellular enzymes.

Rosenow found that extracts of virulent pneumococci possessed the power to split foreign proteins such as those present in ascites meat broth, while extracts of non-virulent organisms showed no digestion. The observations recorded in this paper on the relation of virulence to enzyme activity of pneumococcus are too limited to warrant any final judgment. However, under the experimental conditions, loss of virulence of the organism was not associated with a corresponding loss of enzyme activity. Pneumococci with virulence differing by a ratio of 1,000,000 to 1 showed quantitatively identical proteolytic power. In further elucidation of this problem it would be of interest

not merely to compare differences in enzymic activity with variations in virulence of the same strain, but also to contrast the relative potency of enzyme preparations from pathogenic pneumococci of the disease-producing types with the activity of similar preparations from the more saprophytic varieties of little or no virulence.

#### SUMMARY.

1. Pneumococci contain an intracellular enzyme or enzymes which (a) hydrolyze to some extent intact protein and (b) hydrolyze with striking avidity peptones. The optimum reaction for hydrolysis is pH 7 to 7.8, which also represents the optimum for the growth of pneumococcus. For convenience the terms "protease" and "peptonase" have been used, but no assumption is made as to whether the two actions, proteolysis and peptolysis, are due to two separate enzymes or are two activities of the same enzyme.

2. Solutions of intracellular substance of comparable enzymic activity may be prepared by dissolving the bacteria in bile, in sodium choleate, or by mechanical and autolytic disintegration of the cell.

3. The rapidity with which peptone is hydrolyzed is proportional to the concentration of the enzyme.

4. Heating the enzyme for 10 minutes at 100°C. destroys its activity.

5. Increasing the acidity to pH 5, the acid death-point of pneumococcus, suspends activity but does not destroy the enzyme, for activity is restored by readjustment to pH 7.8.

6. Attenuation of virulence to 1/1,000,000 of the original virulence had no measurable quantitative effect on the enzyme activity.

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