

THE RELATION OF PROTEOLYTIC ENZYMES IN THE
PNEUMONIC LUNG TO HYDROGEN ION
CONCENTRATION. AN EXPLANATION
OF RESOLUTION.

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The relation of the pneumococcus to the production of acid in culture media and the acid reaction of the pneumonic lung¹ suggest that crisis and recovery may be due to local biochemical changes in the course of which the acid death-point of the pneumococcus is reached. In this paper evidence is presented of certain factors influencing resolution in pneumonia. Dernby's work on the autolysis of animal tissue² suggested methods applicable to the investigation.

Three fatal cases (Cases 1, 2, and 3) of pneumococcus pneumonia in the stage of gray or gray-red hepatization furnished the material. The involved lung was passed through a nut butter cutter, the resulting fluid from the mash filtered through cheese-cloth, and the filtrate centrifuged. The grayish sediment was washed in normal saline solution and recentrifuged. The material thus obtained is spoken of below as cellular material. Microscopic examination showed numerous pus cells, many large mononuclear cells, and numerous pneumococci. Chloroform was added to this material to kill the bacteria. Cultures made after the addition of chloroform were sterile. For all the experiments the cellular material was diluted. The diluted mixture is called cellular suspension. This cellular suspension used as enzyme proved much more active than the clear fluid which separates on standing, suggesting that proteolytic action is due to a ferment liberated by the cells.

A special object of the investigation was to determine the influence

¹ Lord, F. T., *Tr. Am. Soc. Clin. Investigation*, 1916, 8; *J. Am. Med. Assn.*, 1916, lxxvii, 1981; 1919, lxxii, 1364.

² Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179.

of varying hydrogen ion concentration on the proteolytic activity of the cellular material.

Erosion of Blood Serum.—A proteolytic enzyme eroding the surface of Löffler's blood serum was readily demonstrated in the cellular material obtained from the pneumonic exudate in all three cases. The addition of chloroform to the cellular material excluded the action of living bacteria. The erosion of the surface of blood serum by the action of cellular material from various sources including the pneumonic exudate is a frequent observation in the laboratory. The relation of the proteolysis to varying degrees of acidity of the medium is an important matter.

Experiment 1.—Tubes of slanted blood serum at varying hydrogen ion concentrations were made as indicated in Table I.

TABLE I.

Method of Preparing Slanted Löffler's Blood Serum at Varying Hydrogen Ion Concentrations for Testing Proteolytic Action.

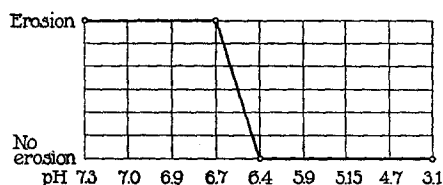
Blood serum 750 cc., dextrose bouillon 250 cc., glycerol 15 cc. To 30 cc. of this mixture was added the indicated amount of N hydrochloric acid or 0.1 N sodium hydroxide + water to 36 cc.

Flask No.	N HCl	0.1 N NaOH	Water,	pH
	cc.	cc.	cc.	
a	Slanted blood serum immersed in solutions of			3.1
b	known hydrogen ion concentration.*			4.7
1	2.0		4.0	5.15
2	0.9		5.1	5.9
3	0.7		5.3	6.4
4	0.5		5.5	6.7
5		2.0	4.0	7.0
6		4.5	1.5	7.3

* The creation of a higher acidity than 5.1 by the addition of acid to the serum prevented the inspissation of the medium and slants of coagulated blood serum already made were allowed to stand covered with standard solutions containing 0.2 M acid potassium phthalate or 0.2 M potassium dihydrogen phosphate and 0.2 M sodium hydroxide of hydrogen ion concentration varying from 4.6 to 2.2. Samples of the fluid were removed at intervals until the hydrogen ion concentration had become constant. By this means, after 3 days slants were obtained of Löffler's blood serum, the fluid covering which had a pH of 3.1 to 5.15.

The water of condensation was removed before the experiment. Cellular material obtained from Case 3 was used. The cellular suspension was made by suspending four drops of cellular material in ten drops of standard solutions of known hydrogen ion concentration and the hydrogen ion concentration of the resulting cellular suspension was then determined. To the surface of the slanted blood serum³ of varying hydrogen ion concentrations a cellular suspension of nearly the same hydrogen ion concentration was added. After incubation erosion of the surface of the medium was observed on the slants with a pH of 7.3 to 6.7 (Text-fig. 1).

Other experiments confirmed these results and showed that the precaution of controlling the hydrogen ion concentration of the cellular suspension used as enzyme was unnecessary. The enzyme proved active at and below 6.7 and inactive at and above 6.4; *i.e.*,



TEXT-FIG. 1. Proteolytic enzyme eroding the surface of Löffler's blood serum. To slanted blood serum with water of condensation of hydrogen ion concentrations varying from 7.3 to 3.1 cellular material suspended in solutions of approximately the same hydrogen ion concentration was added. Erosion of the medium was noted in the slants with a pH of 7.3 to 6.7. No erosion occurred at a pH of 6.4 or higher.

on the more acid end of the scale. Chloroform was added to the cellular material and no growth of bacteria was observed on the surface of the medium.

Gelatin Method.—A stock solution was made by dissolving 350 gm. of gelatin in 625 cc. of hot water and the solution passed through cheese-cloth. 1 gm. of thymol suspended in water was added and the solution made up to 1 liter with water.

Before the experiments 200 cc. of this stock solution were taken and diluted to 500 cc. To 15 cc. of this solution hydrochloric acid or sodium hydroxide was added to vary the hydrogen ion concentrations and the volume was made up to 30 cc. with distilled water. Two series of gelatin solutions with hydrogen ion concentrations

³ Kept horizontal after the addition of the cellular suspension.

varying from 8.0 to 2.0 were thus prepared, one to serve as control and the other for the experiment.

Experiment 2.—To each flask in one of the two series three drops of the cellular suspension obtained from the pneumonic lung (Case 1) and used as enzyme were added. The cellular suspension was made from 0.5 cc. of cellular material (to which chloroform had been added) diluted with 10 cc. of sterile distilled water. Cultures from the cellular material showed no growth. Both control and experimental solutions were placed in the incubator. After 4½ and 22 hours 5 cc. samples were removed from each of the flasks in the two series, placed in thin test-tubes, and cooled for 15 minutes in ice water. On removal from the ice bath the degree of liquefaction of the gelatin in the two series was compared. For the purpose of recording the result the tubes found completely solid were classed as 0 and those completely liquid as 6, intervening numbers indicating a corresponding degree of liquefaction. Cultures from the experimental solutions at the termination of the experiment showed no growth.

Experiment 3.—An exactly similar experiment was performed with cellular material obtained from the pneumonic lung in Case 2.

The presence of an enzyme acting at both acid and alkaline ends of the scale was suggested by some degree of liquefaction of the gelatin in the experimental flasks, but the curve showed a tendency to rise progressively toward the ends of the scale, making the interpretation doubtful. The experiments with gelatin lose in value on account of an almost exactly similar behavior of the controls. Loeb⁴ has shown that gelatin at its isoelectric point ($C_R = 2.10^{-5}$) is practically insoluble, but when it is transformed into a salt by the addition of an acid (or a base) it becomes soluble provided it is in combination with a monovalent ion. Although the gelatin can be brought back to the critical hydrogen ion concentration by adding a calculated amount of acid or alkali before the samples are placed in the ice bath, this procedure further complicates the experiment and does not seem to offer more advantage than the use of the controls. The presence of an enzyme liquefying gelatin could not be demonstrated by this method.

Peptone Method.—Seven solutions of Witte's peptone with hydrogen ion concentrations varying from a pH of about 8.5 to 4.0 were prepared according to the method indicated in Table II. To por-

⁴ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 363.

tions removed from each of the seven flasks equal amounts of a homogeneous cellular suspension used as enzyme were added. The amino-acid nitrogen in the solutions was determined by Van Slyke's method⁵ with the micro-apparatus devised by him.⁶ The cells were first removed by centrifuging the solutions. The total nitrogen determinations were done in duplicate by Folin and Denis' method,⁷ by direct Nesslerization, or by the Kjeldahl method.

TABLE II.

Method of Preparing Peptone at Varying Hydrogen Ion Concentrations for Testing Proteolytic Action.

20 cc. of 4 per cent peptone (Witte's) + the indicated amount of 0.5 M phosphates + hydrochloric acid or sodium hydroxide + distilled water to 90 cc.

Flask No.	Chloroform.	0.1 N HCl	0.1 N NaOH	0.5 M phosphates.		pH indicated.	pH determined.
				KH ₂ PO ₄	Na ₂ HPO ₄		
	cc.	cc.	cc.	cc.	cc.		
1	1	16.0		10.0	0	4.0	4.0
2	1	8.0		9.0	1.0	5.2	5.2
3	1	3.0		5.0	5.0	6.8	6.6
4	1			2.0	8.0	7.3	7.3
5	1		3.0	1.0	9.0	7.7	7.8
6	1		8.0	0.5	9.5	8.0	8.0
7	1		14.0	0	10.0	8.5	8.5

The composition of the solutions used for the determination of peptone-splitting enzyme in the pneumonic lung was the same as that used by Dernby in his study of autolysis of animal tissue with the exception of a change in the amount of peptone from 40 cc. to 20 cc. and the water from 180 cc. to 90 cc.

Experiment 4.—To 0.5 cc. of cellular material obtained from a Pneumococcus Type II pneumonia (Case 1) chloroform was added and the material was allowed to stand over night to plasmolyze. Cultures made after the addition of the chloroform were sterile. A cellular suspension was made by adding to the 0.5 cc. 16 cc. of distilled water. 28 cc. from each of the seven flasks (Table II) were removed to separate flasks and to each were added 2 cc. of the homogeneous cellular suspension used as enzyme and four drops of chloroform. The remainder

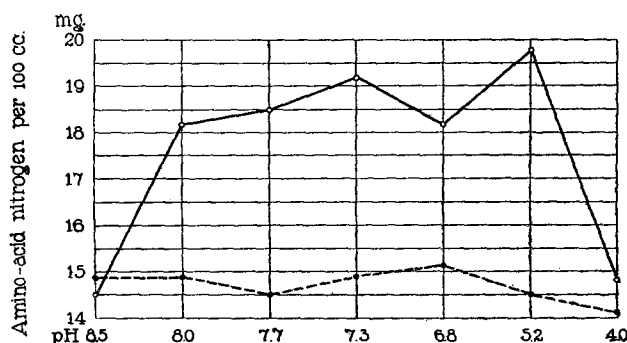
⁵ Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

⁶ Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxiii, 407.

⁷ Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.

of the original peptone solutions to serve as controls and the experimental solutions were allowed to remain in the incubator for 44 hours. The results of the determination of the amino-acid nitrogen are indicated in Table III and graphically recorded in Text-fig. 2.

As indicated in Table III the control solutions already contained from 14.3 to 15.1 mg. of amino-acid nitrogen per 100 cc., the slight variations in the different flasks indicating the experimental error which does not exceed 0.8 mg. per 100 cc. Though equal amounts of homogeneous cellular suspension were added to the experimental flasks the resulting increase in the amino-acid nitrogen varies at the different hydrogen ion concentrations and reaches a maximum in the



TEXT-FIG. 2. Peptone-splitting enzyme in the pneumonic lung from Case 1. The action of cellular material obtained from pneumonic lung in splitting Witte's peptone to amino-acid nitrogen after incubation for 44 hours is shown. The solid line indicates solutions with cellular material, the broken line the same solutions without cellular material (control).

flask with a hydrogen ion concentration of 5.2 pH, falling sharply from this point toward the more acid side and slowly toward the more alkaline side until a pH of 8.0 is reached when the curve again falls abruptly. The lack of variation beyond the experimental error in the control flasks under the conditions of the experiment and the much wider variations at the different hydrogen ion concentrations in the experimental flasks suggest that an enzyme of optimum activity at a pH of 5.2 is the cause of the increased liberation of amino-acid nitrogen. The experiment shows that the amount of amino-acid nitrogen in the peptone solutions without cellular suspension does not

TABLE III.

Peptone-Splitting Enzyme in the Pneumonic Lung from Case 1.

Flask No.	Initial pH.	Control. Amino-acid nitrogen per 100 cc.	Experiment. Amino-acid nitrogen per 100 cc.	Cultures from flasks.	
				Before experiment.	At end of experiment.
		<i>mg.</i>	<i>mg.</i>		
1	4.0	14.3	14.7	No growth.	No growth.
2	5.2	14.5	19.9	" "	" "
3	6.8	15.1	18.2	" "	5 white colonies.
4	7.3	14.7	19.1	" "	No growth.
5	7.7	14.5	18.5	" "	" "
6	8.0	14.7	18.2	" "	" "
7	8.5	14.7	14.5	" "	" "

Control solution, total nitrogen (Folin and Denis method) 90 mg. per 100 cc. The five colonies obtained from the transplant from No. 3 were a contaminating organism, not pneumococci.

appreciably change. The experiment is open to the criticism that amino-acid nitrogen preformed in the cellular suspension may have been added to the peptone solutions, but other experiments have shown that there is too small an amount of preformed amino-acid nitrogen in the $\frac{1}{8}$ cc. of cellular material added to each flask to influence the individual readings. Any possible error from this source is eliminated in the following experiments.

Experiment 5.—An experiment was performed using cellular material obtained from another fatal case (Case 3) of Pneumococcus Type I pneumonia. The cellular material treated as before with chloroform was sterile. The cellular suspension was made by diluting 0.5 cc. of cellular material with 16 cc. of distilled water. 2 cc. of the cellular suspension were added to 28 cc. from each of seven freshly prepared solutions of Witte's peptone of the composition indicated in Table II. 0.3 cc. of chloroform was added to each flask.

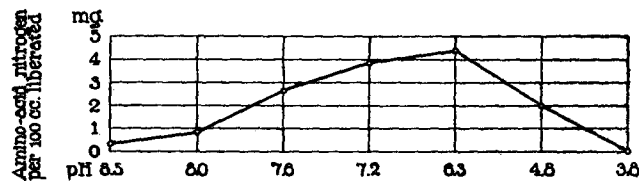
The amount of amino-acid nitrogen in each flask averaged 14.3 mg. per 100 cc. before the addition of the cellular material. The total nitrogen (Kjeldahl method) in the peptone solutions averaged 103.5 mg. per 100 cc. before and 106 mg. after the addition of the cellular suspension. The cellular suspension therefore added about 2.5 mg. of total nitrogen per 100 cc. This amount of total nitrogen in the cellular suspension is too small to account for more than a fraction of the amino-acid nitrogen liberated.

The amount of amino-acid nitrogen in each flask was determined before and after incubation for 42 hours. The results are indicated in Table IV. The increase in amino-acid nitrogen liberated is graphically shown in Text-fig. 3. The hydrogen ion concentration of the flasks changes very little during the experiment. Cultures from the flasks during the experiment were sterile. A peptone-splitting enzyme acting most strongly at a hydrogen ion concentration of 6.3 is indicated.

TABLE IV.

Peptone-Splitting Enzyme in the Pneumonic Lung from Case 3.

Flask No.	Chloroform.	Initial pH.	Final pH.	Amino-acid nitrogen per 100 cc.		Cultures during experiment.
				Before incubation.	After incubation.	
	cc.			mg.	mg.	
1	0.3	4.0	3.8	14.9	14.9	No growth.
2	0.3	4.9	4.8	15.6	17.6	" "
3	0.3	6.5	6.3	15.0	19.4	" "
4	0.3	7.1	7.2	14.6	18.5	" "
5	0.3	7.6	7.6	15.5	18.4	" "
6	0.3	8.0	8.0	15.2	16.5	" "
7	0.3	8.5	8.5	14.5	15.1	" "



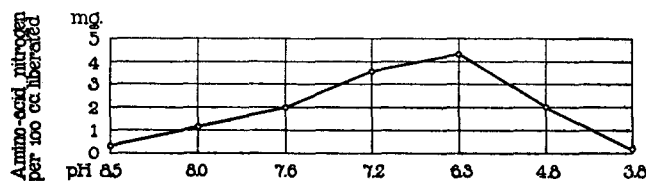
TEXT-FIG. 3. Peptone-splitting enzyme in the pneumonic lung from Case 3. The increase in amino-acid nitrogen at varying hydrogen ion concentrations in flasks containing Witte's peptone and cellular material from the pneumonic lung after incubation for 42 hours is shown.

Experiment 6.—0.5 cc. of cellular material similarly obtained from another fatal case of Pneumococcus Type I pneumonia (Case 2) and treated as before with chloroform was diluted with 16 cc. of distilled water. Cultures from the cellular material after the addition of chloroform were sterile. 2 cc. of the cellular suspension were added to 28 cc. from each of the seven original solutions of Witte's peptone used in Experiment 5. The amino-acid nitrogen in the peptone solutions was already 14.3 mg. per 100 cc.; the total nitrogen (Kjeldahl method) averaged 103.5 mg. per 100 cc. before and 105 mg. per 100 cc. after the addition of the cel-

lular suspension, indicating that the cellular suspension added about 1.5 mg. of total nitrogen per 100 cc. This amount of total nitrogen in the cellular suspension is too small to account for more than a small fraction of the amino-acid nitrogen liberated from the Witte's peptone. The amount of amino-acid nitrogen was determined in each flask before and after incubation for 42 hours, with the result recorded in Table V and graphically presented in Text-fig. 4. As in the preceding experiment a peptone-splitting enzyme acting most strongly at a hydrogen ion concentration of 6.3 is indicated. Cultures from the flasks before, during, and after the experiment were sterile.

TABLE V.
Peptone-Splitting Enzyme in the Pneumonic Lung from Case 2.

Flask No.	Chloroform.	Initial pH.	Amino-acid nitrogen per 100 cc.		Cultures before, during, and after experiment.
			Before incubation.	After incubation	
	cc.		mg.	mg.	
1	0.3	3.8	14.8	15.0	No growth.
2	0.3	4.8	14.7	16.7	" "
3	0.3	6.3	14.5	18.8	" "
4	0.3	7.2	14.8	18.2	" "
5	0.3	7.6	15.0	17.0	" "
6	0.3	8.0	15.0	16.1	" "
7	0.3	8.5	14.5	14.8	" "



TEXT-FIG. 4. Peptone-splitting enzyme in the pneumonic lung from Case 2. The increase in amino-acid nitrogen at varying hydrogen ion concentrations in flasks containing Witte's peptone and cellular material from the pneumonic lung after incubation for 42 hours is shown.

SUMMARY.

Evidence is given of the presence in the cellular material obtained from the pneumonic lung of a proteolytic enzyme digesting coagulated blood serum at hydrogen ion concentrations of 7.3 to 6.7 and inactive at higher; *i.e.*, more acid concentrations.

In addition, evidence is brought forward of the presence in the cellular material from the pneumonic lung of a proteolytic enzyme splitting peptone to amino-acid nitrogen. This enzyme is operative at hydrogen ion concentrations from 8.0 to 4.8, but most active at 6.3 or 5.2.

These findings may be regarded as having a bearing on resolution in pneumonia. During the course of the disease a gradual increase in the hydrogen ion concentration of the exudate probably takes place. With the breaking down of cellular material an enzyme digesting protein (fibrin) in weakly alkaline and weakly acid media may be liberated. With a gradual increase in the hydrogen ion concentration of the pneumonic lung the action of this enzyme probably ceases. An enzyme capable of splitting peptone to amino-acid nitrogen is probably active during the proteolysis of the fibrin and further activated when the hydrogen ion concentration of the pneumonic lung is increased to within its range of optimum activity at a pH of 6.3 and 5.2. By this means it may be conceived that the exudate is dissolved and resolution takes place.

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