# STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

## III. Carbohydrate-Splitting Enzymes: Invertase, Amylase, and Inulase.

By O. T. AVERY, M.D., and GLENN E. CULLEN, Ph.D.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

(Received for publication, June 11, 1920.)

The two preceding papers (1, 2) on the enzymes of pneumococcus have dealt with the proteolytic and lipolytic activities of extracts of the bacterial cells, and of sterile filtrates of cultures. It has been pointed out that enzymes capable of actively hydrolyzing various substrates exist preformed within the cell and that by suitable methods they can be obtained free in solution and their action studied independently of the living organism.

The avidity with which pneumococcus attacks certain carbohydrates is manifest in the accelerated growth and increased acid production of organisms cultivated in the presence of these substances. Acids are produced in culture media from starches and glucosides, as well as from mono- and disaccharides. It has been customary to assume that these fermentation reactions are the summation of the action of several enzymes, first, the hydrolysis of the disaccharide or starch to monosaccharide, and secondly, the production of acid from the monosaccharide.

It was of interest, therefore, to determine whether the bile solutions of pneumococci which were known to contain protein- and fat-splitting enzymes also contained either or both groups of carbohydrate enzymes. Tests upon glucose, saccharose, starch, and inulin substrates were carried out with enzyme solutions containing peptonase and lipase prepared by dissolving pneumococci in bile.

The simple expedient of dissolving the bacterial cells in bile and testing the resultant solution for the presence of enzymes, a method admirably adapted to the determination of proteolytic and lipolytic activity, was found unsatisfactory for studying the enzymes attacking

carbohydrates, since bile in the concentration necessary to effect bacterial solution completely inhibits the activity of the enzymes converting sugar and starch. Consequently the method described below was adopted, by which disintegration of the pneumococcal cells is effected by suspending them in balanced phosphate solution at pH 6.2 and hastening physical disruption by repeated freezing and thawing of the bacterial suspension. The enzymes liberated from the bacterial bodies in this manner, as will be shown in the following experiments, are capable of hydrolyzing sucrose, starch, and inulin, the three test substances chosen for the typical reactions of invertase, amylase, and inulase.

## EXPERIMENTAL.

### *Bacteriological Methods.*

*Preparation of Enzyme Solution by the Acetone Method.*—Because of the fact that precipitation with acetone has been a satisfactory method for preparing and purifying many types of enzymes, an attempt was made to obtain an active carbohydrate-splitting enzyme from pneumococcus by the following procedure.

The residue of 2 liters of an 18 hour broth culture of pneumococcus was taken up in two 10 cc. portions of sterile distilled water, and each portion was poured into 150 cc. of acetone. After standing over night the supernatant solutions were removed from the precipitates which were then allowed to dry. Cultural examination showed that the organisms had been killed by acetone precipitation, although they remained intact and were Gram-positive.

One portion of the dry residue was shaken up with 10 cc. of 0.1 M phosphate at pH 7.4; the other was treated with 2 cc. of 0.1 N sodium hydroxide, allowed to stand for several hours, then neutralized with 0.1 N hydrochloric acid to pH 7.4, and diluted to 10 cc. Each portion was tested for lipase, for peptonase, and for invertase. The tests were all negative.

*Preparation of Enzyme Solution at pH 6.2.*—Previous work has shown that growth of pneumococcus cannot be initiated at a reaction more acid than pH 6.8 (3), and that disintegration of the bacterial

cell occurs most rapidly at about pH 6.2. Moreover, although the subsequent activity of the intracellular lipase and peptonase at optimum reactions is not materially influenced by temporary exposure to an acidity as great as pH 5, they show much less activity at pH 6.2 than at a neutral or slightly alkaline reaction. It seemed probable therefore, that disintegration of the pneumococcus cell, under conditions at which the autolytic processes are at a minimum and at which neither initial growth of the organism nor destruction of known enzymes occurs, might liberate the intracellular carbohydrate-splitting enzymes. Such conditions were obtained in the following manner.

The washed bacterial residue from 1.5 liters of an 18 hour plain broth culture of pneumococcus was taken up in 15 cc. of 0.1 M phosphate solution of pH 6.2, and placed in the ice box until intact bacterial cells could no longer be found under the microscope. After the solution had been proved sterile, it was tested for the presence of known enzymes. The lipase activity of this solution was comparable to that obtained with the bile solution previously used.

*Preparation of Enzyme Solution by Cytolysis with Alternate Freezing and Thawing.*—This method differed from the preceding only in that the disintegration of the cell was hastened by repeated freezing and thawing. An ice-salt mixture of about −22°C. was used.

*Sterility Controls.*—In addition to the sterility tests on the enzyme solution, the final enzyme-substrate mixtures were tested by transferring 0.1 cc. to 5 cc. of broth and incubating for 48 hours.

### *Chemical Methods.*

*Preparation of Substrates. Sucrose.*—A 4 per cent sucrose solution was sterilized in boiling water for 20 minutes. 25 cc. of this sterile 4 per cent sugar solution were then added to 25 cc. of sterile phosphate solution of the desired pH.

*Glucose.*—Prepared in the same manner as sucrose.

*Starch.*—A 2 per cent and a 0.2 per cent suspension of Kahlbaum's rice starch in 0.1 M phosphate solution of pH 7.4 were autoclaved for 20 minutes at 15 pounds pressure.

*Inulin.*—A 2 per cent inulin solution in 0.1 M phosphate at pH 7.4 was sterilized as above.

*Phosphate Solutions.*—The 0.1 M phosphate solutions for the range pH 5 to 8.3 were prepared from potassium acid phosphate and sodium phosphate ($Na_2HPO_4$) according to Sörensen's tables. For the more acid range, mixtures of 0.1 M potassium acid phosphate and 0.1 N hydrochloric acid were used. In all the experiments the appropriate corrections were made for the effect on the reaction of the enzyme solution. All phosphate solutions were sterilized by autoclaving for 20 minutes at 15 pounds pressure.

*Hydrogen Ion Concentration.*—The hydrogen ion concentration was ordinarily determined by the colorimetric method. For the range of acidity greater than pH 4.5 and for frequent controls of the colorimetric standards, the electrometric method, using Clark's rocking electrodes (4), was employed.

*Determination of Reducing Sugar.*—Qualitative tests for reducing sugar were carried out by using 5 cc. of Benedict's qualitative solution and 0.5 cc. of test solution and boiling for 10 minutes.

Quantitative sugar determinations were made with either Benedict's quantitative titrating method, by determining the rotation of the solution, or by the gravimetric copper method.

*Carbon Dioxide.*—Determinations were made with Van Slyke's apparatus (5).

*Amylase Action.*—Hydrolysis of starch to dextrins was determined by means of the iodine color test. 1 cc. of the solution was diluted with 2 cc. of water and 0.2 cc. of a dilute iodine solution (about $\frac{N}{100}$) added. Hydrolysis of the starch or dextrin to reducing sugar was determined as indicated above.

*Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.*

*Experiment 1.*—A solution of Pneumococcus Type II prepared as outlined above was added to a series of tubes containing saccharose, inulin, starch, glucose, and glucose-peptone mixture. The glucose-peptone solution was used with the idea that the nitrogen of the peptone might accelerate glucose hydrolysis. The tubes were incubated for 48 hours at 37°C. The solutions were then analyzed as described. The results are given in Table I.

*Experiment 2.*—This experiment differed from the preceding one in that the suspension of pneumococci was frozen and thawed five times. The enzyme solution was held at ice box temperature (4°C.) for 16 days until culture controls in blood broth no longer showed the presence of viable organisms.

In addition to the substrates used in the preceding experiment two additional control tubes were included, one containing 10 cc. of a 2 per cent saccharose solution in 0.1 M phosphate solution plus 1 cc. of bile, and the second containing 0.2 per cent glucose. Since slight glucose fermentation might have been masked by an excess of the sugar, the dilute solution was included in this series. The results are presented in Table II.

TABLE I.

*Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.*

Enzyme solution prepared by cytolysis at pH 6.2.

| Substrate in 0.1 M phosphate solution. | Final hydrogen ion concentration. | | Qualitative determinations with Benedict's solution. | | Color with iodine. | | Rotation. | | Carbon dioxide content per 2 cc. | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Inactive. | Active. | Inactive. | Active. | Inactive. | Active. | Inactive. | Active. | Inactive. | Active. |
| | *pH* | *pH* | | | | | | | *cc.* | *cc.* |
| Saccharose, 2 per cent. | 7.4 | 7.4 | − | ++++ | | | 2.7° | 1.36° | 0.075 | 0.080 |
| Glucose, 2 per cent. | 7.4 | 7.4 | ++++ | ++++ | | | | | 0.075 | 0.075 |
| Glucose, 1 per cent, in 1 per cent peptone. | 7.4 | 7.4 | ++++ | ++++ | | | | | 0.075 | 0.090 |
| Inulin, 2 per cent. | 7.4 | 7.4 | − | ++ | | | | | | |
| Starch, 2 per cent. | 7.4 | 7.4 | − | + | Blue. | Lavender. | 0° | 0.2° | | |
| Starch, 0.2 per cent. | 7.4 | 7.4 | − | + | " | Vanishing light lavender. | | | | |
| Tributyrin,* 1 per cent. | 7.8 | 6.3 | | | | | | | | |

* Tributyrin was used as a control of enzyme activity.

It is evident from Tables I and II that pneumococcus contains enzymes that hydrolyze starch to dextrins (amylase), hydrolyze inulin (inulase), and invert saccharose to reducing sugars (invertase). The microorganism appears, therefore, to contain enzymes capable of hydrolyzing complex carbohydrates into simple sugars. Bile

completely inhibits the hydrolysis; whether this is due to destruction of the enzyme or to inhibition of its action has not been determined. This fact explains the failure to detect the carbohydrate enzymes in the bile solution used for the study of the peptonase and lipase of pneumococcus. On the other hand, the peptonase and lipase are as active in the solution obtained by the method described as in the bile solutions.

All attempts to demonstrate an enzyme capable of fermenting glucose or producing acid from glucose were unsuccessful.

TABLE II.

*Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.*

Enzyme solution prepared by alternate freezing and thawing.

| Substrate in 0.1 M phosphate solution. | Hydrogen ion concentration. | | Qualitative determinations with Benedict's solution. | Color with iodine. | Carbon dioxide content per 2 cc. |
|---|---|---|---|---|---|
| | Initial. | Final. | | | |
| | *pH* | *pH* | | | *cc.* |
| Glucose, 2 per cent | 7.3 | 7.3 | ++++ | | 0.09 |
| " 0.2 per cent | 7.3 | 7.3 | +++ | | |
| Starch, 2 per cent | 7.3 | 7.3 | ++ | Lavender. | |
| " 0.2 per cent | 7.3 | 7.3 | + | Vanishing lavender. | 0.07 |
| " 0.2 " " (control) | 7.3 | 7.3 | − | Blue. | |
| Inulin, 2 per cent | 7.3 | 7.3 | + | | |
| Saccharose, 2 per cent | 7.3 | 7.3 | ++++ | | 0.07 |
| " + 1 cc. of bile | 7.3 | 7.3 | − | | |
| Control | 7.3 | 7.3 | − | | |
| Tributyrin,* 1 per cent | 7.8 | 6.3 | | | |

* To control enzyme activity.

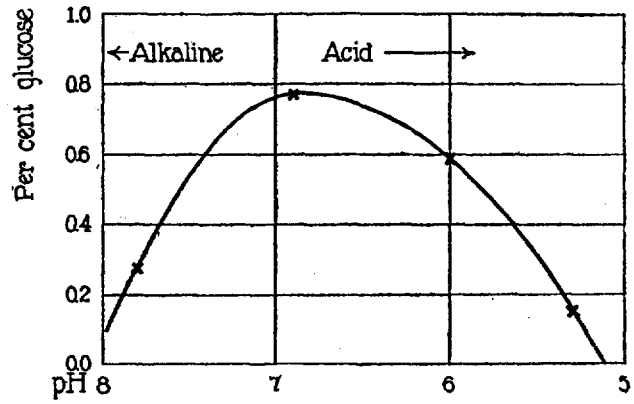
### *Intracellular Nature of the Carbohydrate-Splitting Enzymes.*

*Experiment 3.*—Tests for invertase, amylase, and for a glucose-fermenting enzyme in the filtrate from a young actively growing culture of pneumococcus were carried out exactly as in the case of lipase and peptonase. Saccharose, starch, and glucose to make 1 per cent solutions were added to the sterile filtrate from a 5 hour culture of Pneumococcus Type II, and the mixture was incubated for 48 hours at 37°C. The mixtures were tested as in the preceding experiment and no evidence of enzyme action was demonstrable. Since in the solutions of pneumococci

obtained by the method described there are active carbohydrate-splitting enzymes, their absence in the filtrates of cultures during the early phase of growth, before cell disintegration has occurred, indicates that, like lipase and peptonase, these enzymes are intracellular in nature.

### *Influence of Hydrogen Ion Concentration on the Activity of the Intracellular Invertase of Pneumococcus.*

The preceding experiments have demonstrated the presence of several carbohydrate-splitting enzymes. It now seemed desirable to determine the influence of the hydrogen ion concentration on their activity.




TEXT-FIG. 1. Influence of hydrogen ion concentration on the activity of the intracellular invertase of pneumococcus.

*Experiment 4.* (*a*) *Preparation of Enzyme Solution.*—The solution was prepared as described in preceding experiments.

(*b*) *Preparation of Substrate.*—The substrates were prepared as outlined under Methods. 1 cc. of enzyme was added to 10 cc. of substrate solution; a duplicate tube without enzyme served as control for acid hydrolysis.

Both qualitative and quantitative reducing sugar determinations were made. The results are given in Table III and Text-fig. 1.

### *Optimum Hydrogen Ion Concentration for Amylase Action.*

These experiments were carried out as in the case of invertase. Although quantitative results were not carried out, the iodine color indicates that the optimum reaction is about pH 7.

TABLE III.

*Influence of Reaction on Invertase Action.*

| Enzyme preparation. | Hydrogen ion concentration. | Glucose determinations. | | |
|---|---|---|---|---|
| | | Qualitative determinations with Benedict's solution. | Quantitative determinations by Benedict's method. | Quantitative determinations by the gravimetric copper method. |
| | *pH* | | *per cent* | *per cent* |
| No. D 39 | 7.7 | ++ | 0.1 | |
| " D 39 | 6.9 | +++ | 0.51 | |
| " D 39 | 6.1 | +++ | 0.34 | |
| " D 39 | 5.2 | + | 0.1 | |
| " D 39 | 4.8 | − | 0.0 | |
| " F 208 | 7.8 | +≐ | | 0.29 |
| " F 208 | 6.9 | +++ | | 0.77 |
| " F 208 | 6.0 | ++ | | 0.59 |
| " F 208 | 5.3 | + | | 0.15 |
| " F 208 | 4.85 | − | | |

All controls negative.

TABLE IV.

*Influence of Reaction on Amylase Action.*

| Enzyme. preparation. | Hydrogen ion concentration. | Qualitative determinations with Benedict's solution. | Color with iodine. |
|---|---|---|---|
| | *pH* | | |
| No. D 39 | 8.2 | − | Deep, fading blue. |
| " D 39 | 6.9 | ++ | Red-lavender. |
| " D 39 | 6.1 | + | Blue-lavender. |
| " D 39 | 5.1 | − | Blue. |
| " D 39 | 4.8 | − | |
| " F 208 | 7.8 | ≐ | Very pale lavender, fading instantly. |
| " F 208 | 6.9 | ++ | Pale lavender, fading rapidly. |
| " F 208 | 6.0 | ++ | Deep " " slowly. |
| " F 208 | 5.3 | − | Faint change only. |
| " F 208 | 4.8 | − | Blue as controls. |

It is evident from Tables III and IV that the optimum hydrogen ion concentration for pneumococcus invertase and amylase is about pH 7.

## DISCUSSION.

It is generally accepted that in the utilization of carbohydrates by living bacteria, hydrolysis of these complex substances is brought about through the action of enzymes. The fact has been recognized that enzymes capable of converting sucrose and starch may be found in fungi, especially in yeasts and moulds. Studies on the carbohydrate-splitting processes of invertase-producing bacteria have also been reported by numerous investigators. The earlier work of Fermi and Montesano (3), particularly, lists a number of microorganisms, in sterile cultures of which invertase activity was demonstrable. The isolation and study of carbohydrate-splitting enzymes apart from the living cell have not, so far as we have been able to find in the literature, been attempted in the case of pneumococcus.

The demonstration of the intracellular agents concerned in carbohydrate cleavage by pneumococcus was accomplished by a method through which the release of endoenzymes from the intact organism was effected by breaking down of the cell structure under conditions not injurious to the reactive substances themselves. That physical disruption of the cell membrane through alternate freezing and thawing is subsequently followed by autolytic processes is indeed likely; that the chemical changes brought about by autolysis under these conditions, however, exert but slight influence on the activity of the enzymes studied is evidenced by the avidity with which hydrolysis occurs, and the length of time during which potency is preserved.

From the data presented in this and the two preceding papers, it may be concluded that within the cell bodies of pneumococci there exist in addition to the endohemotoxin described by Cole (6), a series of intracellular enzymes. The proteolytic and lipolytic functions of this endoenzyme-complex have already been described. In addition, there may now be added the activity of the endoenzymes causing hydrolysis of carbohydrate substances, such as sucrose, starch, and inulin.

The optimum hydrogen ion concentration for the invertase and amylase of pneumococcus is about pH 6.8 to 7. This represents a reaction slightly less alkaline than that shown to be optimum for the activity of the peptonase and esterase. The reaction most favorable

for the activity of enzymes that attack carbohydrates is not the same even for those having similar activities. Sörensen (7) has shown that the optimum hydrogen ion concentration for the invertase of beer yeast is about pH 4.5. In studying the influence of hydrogen ion concentration on the enzymic activity of three typical amylases of different origin, Sherman, Thomas, and Baldwin (8) found that the starch-splitting enzymes of malt and *Aspergillus oryzæ* are both most active at an acid reaction (pH 4.4 to 4.8), while the pancreatic amylase reaches its maximum at pH 7. The carbohydrate-splitting enzymes of pneumococcus function best at a neutral reaction and are operative over a zone which corresponds closely to the reaction range of the living organism when grown in the presence of fermentable substances (9). Attempts to determine the presence of an enzyme or enzymes capable of fermenting dextrose and producing acid, an action characteristic of the growing cell, have been unsuccessful.

The invertase, amylase, and inulase of pneumococcus, like the proteolytic and lipolytic enzymes, are intracellular in nature and are found free in culture fluids only after cell disintegration has begun.

## SUMMARY.

1. A method is described for the preparation of an active enzyme-containing solution of pneumococci, in which no living cells are present. These enzymes are capable of hydrolyzing sucrose, starch, and inulin.

2. The invertase and amylase of pneumococcus are active within the limits pH 5 to 8, with an optimum reaction of about pH 7. This reaction range corresponds closely with limiting hydrogen ion concentrations which define growth of the organism in the presence of carbohydrate.

3. These studies indicate that the enzymes described are not true secretory products of the living cell, but are of the nature of endoenzymes, since their activity can be demonstrated only when cell disintegration has occurred.

BIBLIOGRAPHY.

1. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1920, xxxii, 547.
2. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1920, xxxii, 571.
3. Fermi, C., and Montesano, G., *Centr. Bakt., 2te Abt.*, 1895, i, 542.
4. Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475.
5. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
6. Cole, R., *J. Exp. Med.*, 1914, xx, 346.
7. Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131.
8. Sherman, H. C., Thomas, A. W., and Baldwin, M. E., *J. Am. Chem. Soc.*, 1919, xli, 231.
9. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxx, 359.