# THE PEPTASE, LIPASE, AND INVERTASE OF HEMOLYTIC STREPTOCOCCUS.

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The study of the metabolic functions of cells and of more highly organized forms of life has shown that in many instances enzymes are responsible for the cleavage of complex molecules into their simpler components which may be utilized to support life and growth. While many of these enzymes are found in groups of plants or animals in which they accomplish the conversion of certain substances peculiar to individual species, others are more widely distributed. Among the latter group are those necessary for the disintegration of carbohydrates, fats, and proteins into the elementary molecules which are assimilated by the individual cell, so that it is not surprising that they have been found in bacterial extracts, because most bacteria are able to utilize complex organic materials for food.

It has been shown that all bacteria do not possess enzymes for the digestion of natural protein and that variations may be found in the heat resistance and action of peptolytic and sugar-splitting enzymes obtained from different bacteria. On account of these apparent differences in the identity of bacterial enzymes, the peptase, lipase, and invertase of the hemolytic streptococcus have been studied so that they may be compared with similar enzymes of other bacteria. It was hoped that further application of the methods employed would reveal differences sufficient to account for the variations in the pathogenicity of different strains of this streptococcus.

## *Methods.*

*Preparation of the Solution of Enzyme.*—The enzyme solutions used in the following experiments were prepared from broth cultures

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of a beta type hemolytic streptococcus. This strain was originally obtained from a fatal case of peritonitis. Since the objects of the experiments were to perfect a method by which active solutions could be obtained and to study the physical conditions optimum for the action of these solutions on various substrates, no other strains were studied. Active hemolysis could be obtained with this streptococcus on blood agar plates and the centrifugates or filtrates of actively growing broth cultures were hemolytic. The strain fermented lactose and salicin but did not produce acid in mannitol broth. The final hydrogen ion concentration of cultures in 1 per cent dextrose broth was pH 5.0.

The streptococci from which the enzymes were extracted were obtained by the sedimentation of broth cultures after 12 to 16 hours of active growth. The broth was prepared in the usual way except that it contained 2 per cent peptone instead of the 1 per cent solution ordinarily employed in bacteriologic work. Since the tap water used in the broth contained considerable inorganic material, the first lots of media prepared precipitated heavily during sterilization. It was impossible to obtain clean bacteria when this precipitation occurred. While the broth could be filtered and autoclaved a second time, bouillon which was first titrated to pH 0.9 and boiled a few minutes at this H ion concentration, then filtered and titrated to pH 8.0 gave no further precipitate. The media prepared in this way yielded a greater quantity of bacteria than lots which were twice autoclaved. The strain employed agglutinated spontaneously in this medium so that the streptococci settled to the bottom of the flask; the supernatant fluid could then be decanted, leaving the bacterial sediment in the remaining few hundred cubic centimeters. Due to the incomplete sedimentation and the scant yield from large flasks of media, 0.1 per cent dextrose was added before sterilization. Large quantities of bacteria could then be obtained within a few hours after the flasks were seeded; the final acid concentration was between pH 6.0 and 6.5 in the dextrose broth cultures. Dextrose was used in the preparation of all eyzyme solutions except in a few experiments designed to determine the effect of acid on the enzymes. It increased the growth and the acid developed was favorable for agglutination.

Large quantities of broth were required to obtain sufficient bacteria for one experiment. Usually a 6 or 12 liter flask of broth was seeded with 50 cc. of an actively growing culture. Sedimentation was complete after 12 to 16 hours incubation at 37°C., so that the supernatant medium could be siphoned off without disturbing the bacteria. The sediment was centrifuged, washed with distilled water, and transferred with a few cubic centimeters of phosphate solution ($\frac{M}{15}$, pH 7.0) to a sterile agate mortar. The mortar contained about 2 gm. of powdered glass. It was covered with thin rubber sheeting arranged so that the bacteria could be dried and ground without contamination. The streptococci were dried in a vacuum over phosphoric anhydride. When they were nearly dry the mortar was removed from the desiccator and the bacteria were ground with the occasional addition of several drops of distilled water until only a few cell bodies were sufficiently intact to retain the gentian violet in Gram-stained films. The ground material was next pipetted into a graduate and covered with a layer of toluene. This step was necessary to kill the few bacteria remaining alive after grinding. 12 hours later the bacterial suspension was pipetted from beneath the toluene and centrifuged until clear. The supernatant fluid was made up to the volume required in each experiment with $\frac{M}{15}$ phosphate solution (pH 7.0). This volume could not be more than 50 cc. without reducing the concentration of the enzyme beyond a point where decisive results were obtainable. All the glassware and solutions used in the procedure were sterile.

The enzyme solutions were opalescent at pH 7.0 but showed a flocculent precipitate when they were titrated to pH 5.0 with lactic acid. Ordinarily they varied in reaction from pH 6.8 to 7.2. Broth cultures of the final solutions were sterile. Only Gram-negative precipitate was found in the stained films of these bacterial extracts.

*Preparation of the Substrate Solution.*—The substrates in the majority of the experiments were peptone, cane-sugar, or esters of fatty acids. Ordinarily the solutions were prepared so that the final concentration would be 1 per cent. For the demonstration of lipolytic action the strength of the solutions of ethyl butyrate or triacetin were such that in some of the trials this final concentration was 2 per cent. Solutions or emulsions which would withstand temperatures above 100°C. were autoclaved.

Peptone solutions (either 2 or 4 per cent in distilled water) were adjusted to pH 7.0 with sodium hydroxide and sterilized by the Arnold method. The commercial bacteriologic peptone was so completely hydrolyzed that increases in amino nitrogen could not be determined after further digestion with active trypsin. The peptone[1] employed was prepared especially for these experiments. It contained 12.5 per cent nitrogen, 16 per cent of which could be determined as amino nitrogen by Van Slyke's method. After the solutions were sterilized the amino nitrogen was increased to 20 per cent of the total.

Albumin was obtained from sterile horse serum fractioned with ammonium sulfate. The precipitate was dialyzed and concentrated in a vacuum with sterile precautions. The concentration of the concentrated solution was determined by the Kjeldahl method and then adjusted to 4 per cent. This 4 per cent solution was heated at 56°C. for 1 hour. The casein was dissolved in a neutral phosphate solution and heated for 1 hour on 3 successive days in the Arnold sterilizer. Sterile horse serum was inactivated at 56°C. for 1 hour. 4 per cent solutions of the carbohydrates in $\frac{M}{200}$ phosphate mixture were sterilized in the autoclave. Cane-sugar was autoclaved at pH 7.2, dextrose at 6.8, and starch at 7.0.

*Chemical Methods.*—H ion concentrations were determined colorimetrically with mixtures of sodium citrate and hydrochloric acid or of phosphates prepared according to Sörensen (1). The readings were made by the comparator method with the Clark and Lubs (2) series of indicators.

Except in instances in which the macro Kjeldahl method is specified the total nitrogen and non-protein nitrogen were determined by the micro method of Folin and Wu (3). Trichloroacetic acid was used as a precipitant in the non-protein nitrogen determinations.

The amino nitrogen estimations were made with the micro apparatus described by Van Slyke (4). Solutions of alanine and leucine were first analyzed and the results were within 0.5 per cent of the theoretical values. The peptone substrates were not precipitated before analysis.

[1] This peptone was prepared by Fairchild Bros. and Foster, New York.

The percentage of inversion of the cane-sugar was determined by the colorimetric method of Folin and Wu (5). Preliminary precipitation of the small amount of protein in the mixtures of cane-sugar and enzyme was found to be unnecessary. The results have been expressed as milligrams of dextrose per 100 cc. of final solution.

The action of the lipase was determined by the method recently employed by Avery and Cullen (6). These results have been expressed in terms of cubic centimeters of $\frac{N}{50}$ alkali required to adjust the reaction of the active to that of the inactive enzyme tubes. They are based on 100 cc. amounts of substrate.

*Bacteriologic Methods.*—Aside from the toluene used for the sterilization of the enzyme solutions no antiseptics were required. In the preparation of the enzyme very little toluene was apparently carried over when the bacterial emulsion was pipetted into the centrifuge tubes because the digestion flasks were occasionally contaminated. Broth cultures of these flasks were made before chemical analysis; no experiments have been included in which the most rigid tests did not show sterility throughout. The purity of the cultures from which the enzymes were obtained was proven on blood agar plates.

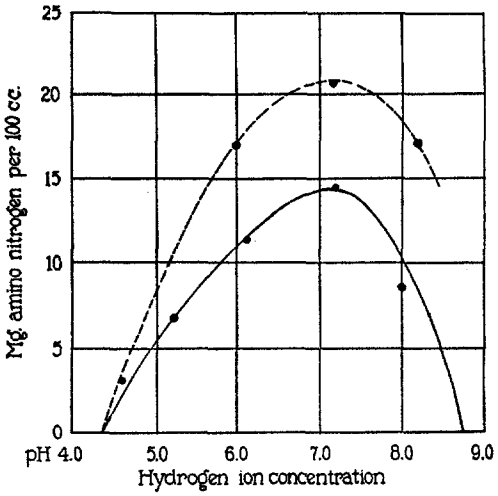
## EXPERIMENTAL.

### *The Effect of Acid Concentration on the Action of the Peptase, Invertase, and Lipase.*

Enzyme action is known to occur with the proper substrate under certain physical conditions which vary according to the nature of the enzyme. The range of acid concentration through which the reaction occurs and the optimum acid concentration are important in deciding the type of enzyme. Fuhrmann (7) noted that in a general way proteolytic bacterial enzymes responded to alkali and acid in a manner very similar to pancreatic trypsin. More recently Avery and Cullen (8) have shown that the peptase of pneumococcus has an optimum range of pH 7.0 to 7.8. This, together with the percentage of splitting obtained, led these authors to suspect that the enzyme was of an erepsin-like nature. They found likewise that the lipase (6) and invertase (9) of pneumococcus exhibited ranges similar to that of the peptonase.

*Experiments 1 and 2. The Effect of Acid Concentration on the Splitting of Peptone.*—Enzyme obtained from streptococci grown in 0.1 per cent dextrose broth was tested with a sterile solution of peptone as substrate. 10 cc. of the solution were pipetted into sterile 20 cc. volumetric flasks and sufficient HCl or NaOH was added to adjust the H ion concentration to the desired pH; 5 cc. of enzyme solution were added to each flask and the volume was brought up to 20 cc. with $\frac{M}{15}$ phosphate or citrate mixture of the pH desired.

Amino nitrogen determinations were done after digestion at 37°C. for 48 hours. Duplicate flasks prepared with boiled enzyme served as controls for each of the digestions. Flasks prepared without peptone served to control any increase in amino nitrogen due to the digestion of the protein in the bacterial extract. The




TEXT-FIG. 1. The effect of acid concentration on the peptone-splitting action of the enzyme solutions (Tables I and II).

object of the experiments was to determine the effect of acid concentration on the enzyme action within a sufficiently wide range to aid in establishing the identity of the enzyme. The final concentration of the peptone in the flasks was 1 per cent. Cultures made from the flasks immediately preceding analysis were sterile. The results are given in Tables I and II.

*Experiments 3 and 4. The Effect of Acid Concentration on the Inversion of Cane-Sugar.*—The details of these experiments are similar to those of Experiments 1 and 2 except that a solution of saccharose was used for substrate. A 4 per cent solution of cane-sugar was autoclaved in $\frac{M}{200}$ phosphate mixture at pH7.2; 5 cc. were pipetted into each 20 cc. flask so that the final concentration of the sugar was 1 per cent. The enzyme solution was divided into equal portions, one of which was boiled for 20 minutes. Duplicate flasks were prepared at the

TABLE I.

*The Effect of Acid Concentration on the Splitting of Peptone.*

| Flask No. | Final acid concentration. | | Amino nitrogen per 100 cc. of solution. | | |
|---|---|---|---|---|---|
| | Active. | Inactive. | Active. | Inactive. | Increase. |
| | *pH* | *pH* | *mg.* | *mg.* | *mg.* |
| 1, 1a | 8.2 | 8.1 | 41.5 | 24.4 | 17.1 |
| 2, 2a | 7.2 | 7.2 | 45.6 | 25.1 | 20.5 |
| 3, 3a | 5.9 | 6.0 | 40.8 | 24.0 | 16.8 |
| 4, 4a | 4.6 | 4.6 | 27.9 | 24.9 | 3.0 |
| 5, 5a* | 8.0 | 8.0 | 3.3 | 3.6 | 0.0 |

* Flasks containing boiled and unboiled enzyme without substrate to control the autolysis of the protein in the enzyme solution.

TABLE II.

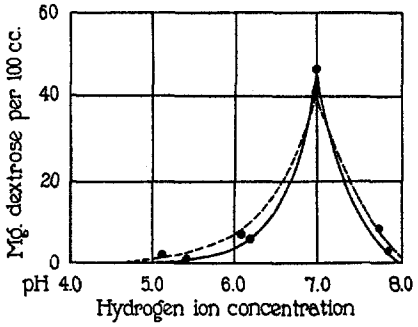
*The Effect of a Greater Acid and Alkaline Range on the Splitting of Peptone.*

| Flask No. | Final acid concentration. | | Amino nitrogen per 100 cc. of solution. | | |
|---|---|---|---|---|---|
| | Active. | Inactive. | Active. | Inactive. | Increase. |
| | *pH* | *pH* | *mg.* | *mg.* | *mg.* |
| 1, 1a | 8.8 | 8.8 | 27.7 | 24.3 | 3.4 |
| 2, 2a | 8.0 | 8.0 | 33.0 | 24.5 | 8.5 |
| 3, 3a | 7.2 | 7.1 | 38.5 | 24.3 | 14.2 |
| 4, 4a | 6.1 | 6.1 | 35.6 | 24.3 | 11.3 |
| 5, 5a | 5.2 | 5.2 | 30.0 | 23.2 | 6.8 |
| 6, 6a | 3.9 | 3.9 | 22.7 | 22.7 | 0.0 |
| 7, 7a | 3.2 | 3.2 | 28.6 | 28.9 | 0.0 |
| 8, 8a* | 7.0 | 7.1 | 3.5 | 3.1 | 0.4 |
| 9, 9a† | 7.0 | 7.0 | 26.6 | 26.6 | 0.0 |

* These flasks contained active and inactive enzyme without peptone.

† After the supernatant fluid was removed from the centrifuge tubes in the preparation of the enzyme solution the sediment consisting of ground streptococci was washed once and divided in half. One half was boiled. Digestion tubes were prepared with this boiled and unboiled sediment and peptone.

various acid concentrations with 5 cc. of active and 5 cc. of inactive enzyme. The invert sugar was determined as dextrose by the micro method of Folin and Wu after 48 hours of incubation. Cultures from the flasks were sterile. The invert sugar has been expressed as milligrams of dextrose per 100 cc. of final solution. Two experiments have been included in Table III on account of the peculiar type of curve obtained.




TEXT-FIG. 2. The effect of acid concentration on the inversion of saccharose (Table III).

TABLE III.

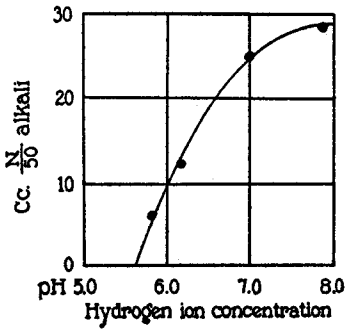
*The Effect of Acid Concentration on the Inversion of Saccharose.*

| Flask No. | Acid concentration. | | | Dextrose per 100 cc. of solution. | | |
|---|---|---|---|---|---|---|
| | Initial. | Final. | | Active. | Inactive. | Increase. |
| | Active. | Active. | Inactive. | | | |
| | *pH* | *pH* | *pH* | *mg.* | *mg.* | *mg.* |
| 1, 1a | 7.8 | 7.8 | 7.8 | 9.5 | 7.5 | 2.0 |
| 2, 2a | 7.0 | 7.0 | 7.0 | 53.2 | 7.5 | 45.7 |
| 3, 3a | 6.2 | 6.2 | 6.2 | 12.1 | 7.4 | 4.7 |
| 4, 4a | 5.4 | 5.4 | 5.4 | 8.1 | 7.5 | 0.6 |
| 1 | | 7.7 | * | 15.9 | * | 8.6 |
| 2 | | 7.0 | | 49.0 | | 41.7 |
| 3 | | 6.1 | | 12.8 | | 5.5 |
| 4, 4a | | 5.1 | 5.1 | 10.0 | 7.3 | 2.7 |

* In order to concentrate the enzyme in this experiment with the hope that greater inversion might be obtained, controls with boiled enzyme were omitted from the series with the exception of the control on Flask 4. This was chosen to rule out any possibility of interpreting as a result of enzyme action, increased amounts of dextrose which might occur purely from acid hydrolysis after long incubation at pH 5.1.

*Experiment 5. The Effect of Acid Concentration on the Hydrolysis of Ethyl Butyrate.*—The details of this experiment are similar to those of the preceding four experiments except that ethyl butyrate was used as substrate. 5 cc. of a 4 per cent emulsion of ethyl butyrate sterilized in the Arnold sterilizer were pipetted into each of eight flasks so that the concentration was 1 per cent after the volumes were made to 20 cc. Four of the flasks were prepared with boiled

and four with unboiled enzyme solution and the volume was made up with $\frac{M}{5}$ phosphate of the desired pH. After 48 hours of incubation the active enzyme mixtures were adjusted to the pH of the similar control containing the boiled enzyme. The results have been expressed in Table IV in cubic centimeters of alkali required to adjust the pH of 100 cc. of the solution after hydrolysis to that of the control flask containing the boiled enzyme.




TEXT-FIG. 3. The effect of acid concentration on the hydrolysis of ethyl butyrate (Table IV).

TABLE IV.

*The Effect of Acid Concentration on the Hydrolysis of Ethyl Butyrate.*

| Flask No. | Acid concentration. | | | | Amount of N/50 NaOH required in titration. |
|---|---|---|---|---|---|
| | Inactive. | | Active. | | |
| | Initial. | Final. | Initial. | Final. | |
| | *pH* | *pH* | *pH* | *pH* | *cc.* |
| 1, 1a | 7.8 | 7.8 | 7.8 | 7.1 | 29.0 |
| 2, 2a | 7.0 | 7.0 | 7.0 | 6.6 | 25.0 |
| 3, 3a | 6.2 | 6.2 | 6.2 | 5.8 | 12.0 |
| 4, 4a | 5.8 | 5.8 | 5.8 | 5.6 | 6.0 |

The effect of acid concentration on the action of these enzymes is similar to the effect observed with the enzymes of other bacteria. In this respect they are nearly identical with the endoenzymes obtained by Avery and Cullen (6, 8, 9) from pneumococci, both with regard to the range of acidity through which they are active and the optimum acid concentration.

The peptolytic enzyme (Text-fig. 1) is not active below pH 4.4 and has an optimum action at about pH 7.2. The acid limit for

the inversion of cane-sugar was about pH 5.0 and the optimum action occurred at pH 7.0. According to Text-fig. 2 the curves showing the effect of acid on inversion differ from those for peptolytic and lipolytic action. Below pH 7.0 the type of curve obtained would appear to indicate that the optimum range was narrower for the invertase than for the other enzymes; while this part of the curve is established on three determined points, the curve above pH 7.0 in Text-fig. 2 is hypothetical because there are only two determinations within this range. It is possible that with more active preparations of invertase the curve would be similar to those obtained for the lipase and the peptase. The lipase of this streptococcus has an optimum reaction more alkaline than either of the other enzymes studied (Text-fig. 3).

*The Effect of Heat on the Action of the Peptase, Invertase, and Lipase.*

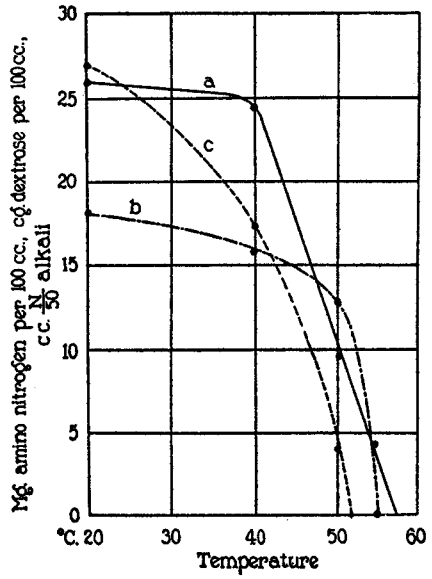
Susceptibility to heat is one of the characteristics of enzyme solutions. The temperature which destroys the enzyme varies with the source and type of enzyme and the conditions under which the heat is applied. Dry enzyme or enzyme in solution with substrate may resist temperatures which are destructive to simple aqueous solutions. The enzyme solutions in the following experiments were extracted from the streptococci without the addition of materials (with the exception of the toluene used in the procedure) foreign to bacterial growth and with a minimum amount of protein present. Neutrality was maintained during the extraction and heating. For this reason it is not possible to compare the results with those of previous workers too closely. The digestions were carried out at approximately the optimum acid concentration.

*Experiment 6. The Effect of Heat on the Peptolytic Enzyme.*—The enzyme solution was prepared as in the preceding experiments. It was divided among several thin walled tubes which were plunged into water of the desired temperatures for 10 minutes and afterward cooled quickly in ice water; 5 cc. of each of the heated portions were pipetted into flasks with 5 cc. of 4 per cent peptone solution. The volume of each flask was made up to 20 cc. with $\frac{M}{15}$ phosphate mixture (pH 7.0). Amino nitrogen determinations were made after incubation at 37°C. for 48 hours (Table V). The flasks were sterile after incubation. The initial and final pH of each flask was 7.0.

*Experiment 7. The Effect of Heat on the Invertase.*—This experiment is similar in every respect to Experiment 6 except that saccharose was used as the substrate.

Flask 1 contained enzyme which had been boiled 20 minutes. The substrate, saccharose, was autoclaved in 4 per cent solution at pH 7.2. The volume of each digestion was 20 cc. so that the final concentration was 1 per cent. The invert sugar was determined by the micro method after 48 hours incubation (Table VI). The initial pH and the final pH of each flask were 7.0. Cultures from the flasks were sterile.

*Experiment 8. The Effect of Heat on the Lipase.*—The procedure was similar to that in the preceding experiments. Ethyl butyrate served as substrate. A 4 per cent emulsion was sterilized in the Arnold sterilizer; 5 cc. were then pipetted into the digestion flasks with 5 cc. of enzyme solution which had been subjected




TEXT-FIG. 4. The effect of heat on the solutions of (*a*) peptase, (*b*) lipase, and (*c*) invertase (Tables V, VI, and VII).

to temperatures of 20°, 40°, 50°, and 55°C. for 10 minutes. The enzyme in Flask 1 was boiled 20 minutes. The volume of each flask was made up to 20 cc. with $\frac{M}{15}$ phosphate solution. The titrations were made with $\frac{N}{50}$ NaOH. The results are expressed in cubic centimeters of alkali required to adjust the reaction of 100 cc. of digest to that of the control flask prepared with boiled enzyme (Table VII).

The results obtained in the preceding experiments on the effect of exposing the enzyme solutions to heat have been summarized in Text-fig. 4. In order to correlate the various terms in which the results have been expressed considerable interpolation has been nec-

TABLE V.

*The Effect of Heat on the Peptolytic Enzyme.*

| Flask No. | Temperature at which enzyme was heated for 10 min. | Amino nitrogen per 100 cc. of solution. | |
|---|---|---|---|
| | | Analysis. | Increase. |
| | °C. | mg. | mg. |
| 1 | 100* | 26.9 | |
| 2 | 60 | 26.5 | 0.0 |
| 3 | 55 | 31.3 | 4.4† |
| 4 | 50 | 36.2 | 9.3 |
| 5 | 40 | 51.3 | 24.4 |
| 6 | 20 | 52.9 | 26.0 |

* Flask 1 was prepared with enzyme which was boiled 20 minutes.

† Cultures of the streptococcus in broth were not viable after they were heated at 55°C. for 10 minutes. They were not killed at 50°C.

TABLE VI.

*The Effect of Heat on the Invertase.*

| Flask No. | Temperature at which enzyme was heated for 10 min. | Dextrose per 100 cc. of solution. | |
|---|---|---|---|
| | | Analysis. | Increase. |
| | °C. | mg. | mg. |
| 1 | 100 | 7.2 | |
| 2 | 55 | 7.0 | 0.0 |
| 3 | 50 | 48.2 | 41.0 |
| 4 | 40 | 178.7 | 171.5 |
| 5 | 20 | 277.7 | 270.5 |

TABLE VII.

*The Effect of Heat on the Lipase.*

| Flask No. | Temperature at which enzyme was heated for 10 min. | Acid concentration. | | Amount of N/50 NaOH required in titration. |
|---|---|---|---|---|
| | | Initial. | Final. | |
| | °C. | *p*H | *p*H | cc. |
| 1 | 100 | 7.7 | 7.7 | |
| 2 | 55 | 7.7 | 7.7 | 0.0 |
| 3 | 50 | 7.7 | 7.4 | 13.0 |
| 4 | 40 | 7.7 | 7.3 | 16.0 |
| 5 | 20 | 7.7 | 7.3 | 18.0 |

essary to bring all the curves within a similar range. The temperatures required to destroy these enzymes were between 50° and 60°C. and corresponded closely with the thermal death-point of the streptococcus from which they were obtained. The results suggest that the death of the bacteria is due to the destruction of the enzymes. Such a conclusion, however, would not be in agreement with the previous literature. Repeated references are made to bacterial enzymes which are destroyed only by temperatures far in excess of that required to kill the bacteria from which they were obtained. Avery and Cullen found this to be true for the pneumococcus.

### *The Effect of Exposure to Acid.*

The length of time during which solutions of enzyme remain active depends largely on the physical conditions under which they are preserved. In the preceding experiments temperatures above 55°C. destroyed the enzymes when they were in solution at reactions optimum for their action. There was slight deterioration even at a temperature as low as 40°. In view of this sensitiveness to heat in neutral solution, a series of experiments was devised to discover the effects of weak acid. The enzyme was exposed to lactic acid by titrating to pH 5.0, incubating at 37°C. for 6 hours, and finally adjusting the solution to the optimum acidity. Lactic acid was chosen because it is one of the principal fermentation products of dextrose in broth cultures of streptococcus (10).

*Experiment 9. The Effect of Exposure to Acid.*—The bacterial sediment from 12 liters of 2 per cent peptone broth was extracted in the usual manner. Dextrose was not added to this broth because it was desirable to avoid exposing the bacteria to acid during their growth. The enzyme solution was divided into three equal portions to which 10 drops of methyl red and phenolsulfonephthalein were added. One lot was boiled for 20 minutes; the second portion was adjusted to pH 5.0 with lactic acid; the third served for a control. All three were incubated at 37°C. for 6 hours. The acid tube was titrated to pH 7.0 (the acid concentration which had been maintained in the other tubes during incubation) with NaOH at the end of this period. All three tubes were then made up to equal volumes with phosphate mixture (pH 7.0, $\frac{M}{15}$). The action of these lots of enzyme was observed with substrates of peptone, cane-sugar, and triacetin. The triacetin (8 per cent emulsion) was autoclaved at pH 7.0; the other substrates were prepared as in the preceding experiments. 5 cc. of substrate and enzyme solution were pipetted into

digestion flasks and made up to 20 cc. volume with phosphate solution. The analyses were made after incubation at 37°C. Cultures from these flasks showed no growth. The results are shown in Table VIII.

TABLE VIII.

*The Effect of Exposure to Acid.*

(*a*) *Peptolytic Enzyme.*

| Flask No. | Treatment of enzyme. | Acid concentration. | | Results per 100 cc. of solution. | |
|---|---|---|---|---|---|
| | | Initial. | Final. | Amino nitrogen. | Increase. |
| | | *pH* | *pH* | *mg.* | *mg.* |
| 1 | Boiled 20 min. | | 7.1 | 33.7 | |
| 2 | Incubated at 37°C. for 6 hrs. at pH 5.0 and adjusted to pH 7.0. | | 7.1 | 49.8 | 16.1 |
| 3 | Normal active enzyme. | | 7.1 | 63.9 | 30.2 |

(*b*) *Invertase.*

| Flask No. | Treatment of enzyme. | Acid concentration. | | Results per 100 cc. of solution. | |
|---|---|---|---|---|---|
| | | Initial. | Final. | Dextrose. | Increase. |
| | | *pH* | *pH* | *mg.* | *mg.* |
| 4 | Boiled 20 min. | 7.0 | 7.0 | 6.3 | |
| 5 | Incubated for 6 hrs. at pH 5.0 and adjusted to pH 7.0. | 7.1 | 7.1 | 6.4 | 0.1 |
| 6 | Normal active enzyme. | 7.1 | 7.1 | 44.0 | 37.7 |

(*c*) *Lipase.*

| Flask No. | Treatment of enzyme. | Acid concentration. | | Amount of N/50 NaOH required in titration. |
|---|---|---|---|---|
| | | Initial. | Final. | |
| | | *pH* | *pH* | *cc.* |
| 7 | Boiled 20 min. | 7.0 | 6.8 | |
| 8 | Incubated 6 hrs. at 37°C. at pH 5.0 and adjusted to pH 7.0. | 7.0 | 6.8 | 0* |
| 9 | Normal active enzyme. | 7.0 | 6.2 | 94.0 |

* On account of the partial hydrolysis of the inactive triacetin control the flasks were adjusted to pH 6.8. Controls without enzyme demonstrated that after triacetin had been autoclaved the reaction was more acid after 48 hours by pH 0.1 to 0.3.

*Experiment 10. The Effect of Exposing Weak Solutions of Peptolytic Enzyme to Acid.*—The preceding experiment was repeated with a weak solution of enzyme. The procedure was otherwise similar. The acid-treated enzyme was tested only with a substrate of peptone. The solution with which Experiment 9 was carried out yielded a heavy precipitate when it was acidified with acetic acid. The enzyme in this experiment did not show a precipitate but merely opalescence after the addition of weak acetic acid. The results of the experiment are shown in Table IX.

TABLE IX.

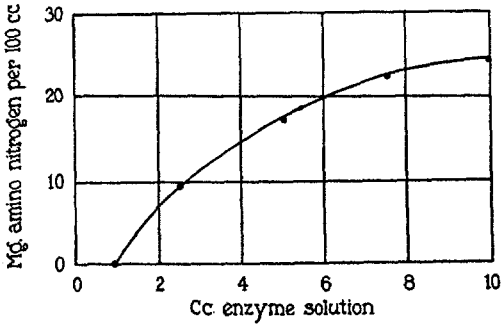
*The Effect of Acid on Weak Solutions.*

| Flask No. | Treatment of enzyme. | Final acid concentration. | Amino nitrogen per 100 cc. of solution. | |
|---|---|---|---|---|
| | | | Analysis. | Increase. |
| | | *pH* | *mg.* | *mg.* |
| 1 | Boiled 20 min. | 6.9 | 33.5 | |
| 2 | Incubated 6 hrs. at pH 5.0 and adjusted to pH 7.0. | 7.0 | 37.4 | 3.9 |
| 3 | Normal active enzyme. | 7.0 | 48.0 | 14.5 |

These experiments demonstrate that two of the enzymes are exceedingly susceptible to acid and that the third, the peptase, is partially destroyed at pH 5.0. It is difficult to account for the selective action of acid aside from the protective action which the protein in the solution might have for the peptase. Analyses show that the protein was far in excess of the percentage of reducing substances found in these solutions and for that reason it is logical to suppose that it protected the peptolytic enzyme. The invertase was probably more easily destroyed by acid in the absence of substrate or digestion products. The percentage of fat in the solutions was not estimated. In the second of these experiments the enzyme solution was less concentrated and contained but little protein; the deterioration of the enzyme was considerably greater. There is, of course, no objection to interpreting these results as due to actual differences in the character of the enzymes, since it was impossible to obtain purer solutions for further experiments.

*Experiment 11. The Effect of Enzyme Concentration on Peptolytic Digestion.*—The enzyme and substrate were prepared as in the preceding experiments. Flasks were prepared with 5 cc. of 4 per cent peptone solution and increasing

amounts of enzyme. The volumes were made to 20 cc. with $\frac{M}{15}$ phosphate mixture. Analyses were made after 48 hours of incubation at 37°C. (Table X and Text-fig. 5).




TEXT-FIG. 5. The effect of concentration of enzyme on the splitting of peptone (Table X).

TABLE X.

*The Effect of Concentration of Enzyme on the Splitting of Peptone.*

| Flask No. | Amount of enzyme. | Final acid concentration. | | Amino nitrogen per 100 cc. of solution. | | |
|---|---|---|---|---|---|---|
| | | Active. | Inactive. | Active. | Inactive. | Increase. |
| | *cc.* | *pH* | *pH* | *mg.* | *mg.* | *mg.* |
| 1 | 10.0 | 7.2 | 7.2 | 52.1 | 28.3 | 23.8 |
| 2 | 7.5 | 7.1 | 7.2 | 50.9 | 28.7 | 22.2 |
| 3 | 5.0 | 7.2 | 7.2 | 45.5 | 28.3 | 17.2 |
| 4 | 2.5 | 7.1 | 7.1 | 38.0 | 28.4 | 9.6 |
| 5 | 1.0 | 7.2 | 7.2 | 28.4 | 28.7 | 0.0 |

Table X shows that the weaker concentrations of enzyme are more effective in digesting peptone than are more concentrated solutions. Bayliss (11) has shown that the time element is important in concentration studies but concludes that weight for weight, regardless of the type of velocity curve which digestion with enzymes may show, higher concentrations are more effective than the lower ones. In this respect, it is apparent that this peptase follows the general law of enzymes.

*Experiment 12. The Proteolytic Action of the Enzyme Solutions.*—The solution of enzyme was prepared in the usual manner from 6 liters of 0.1 per cent dextrose broth culture of streptococci. The serum albumin was obtained by fractioning

sterile horse serum with ammonium sulfate, then dialyzing, and concentrating the dialyzed solution in vacuum; this procedure was carried out without bacterial contamination. The solution was heated to 56°C. for 1 hour before it was used. The casein solution was sterilized in the Arnold sterilizer. The horse serum was inactivated at 56°C. The solutions were prepared in 4 per cent concentrations; the peptone and casein were weighed but the albumin and horse serum were diluted until they contained 4 per cent protein reckoned on the basis of the total Kjeldahl nitrogen. This, of course, introduced a slight error in the protein concentration of the diluted horse serum. The solutions were adjusted to pH 7.0 with sterile acid or alkali.

5 cc. of enzyme solution, 5 cc. of substrate, and 10 cc. of phosphate solution were pipetted into sterile flasks. These mixtures were incubated at 37°C. for 3 days and analyzed by the micro Kjeldahl method after precipitation with trichloroacetic acid (Table XI). Duplicate and triplicate precipitations with 10 per cent trichloroacetic acid with solutions of various proteins demonstrated that the precipitation was equal and constant.

TABLE XI.

*The Proteolytic Action of the Enzyme Solutions.*

| Flask No. | Substrate. | Final acid concentration. | | Non-protein nitrogen per 100 cc. of solution. | | |
|---|---|---|---|---|---|---|
| | | Active. | Inactive. | Active. | Inactive. | Increase. |
| | | *pH* | *pH* | *mg.* | *mg.* | *mg.* |
| 1 | Casein. | 6.9 | 6.9 | 25.6 | 12.6 | 13.0 |
| 2 | Albumin. | 6.9 | 6.9 | 15.5 | 15.1 | 0.4 |
| 3 | Serum. | 7.0 | 7.0 | 21.1 | 19.3 | 1.8 |
| 4 | Peptone.* | 6.9 | 6.9 | 63.8 | 28.0 | 35.8 |

* The peptone digestion was included as a control on the activity of the enzyme solution. These results are expressed in milligrams of amino nitrogen per 100 cc. of solution.

The results obtained in the preceding experiment indicate that the enzymes have but slight ability to bring about the digestion of higher proteins. The increase in non-protein nitrogen in the albumin-enzyme mixture is so small that it may have resulted either from autolysis of proteins in the enzyme solution or from an experimental error in the determinations. A series of determinations with solutions of enzyme showed increases of 0.1 to 0.2 mg. of non-protein nitrogen per 100 cc. of solution after active bacterial extracts were allowed to autolyze for several days. This increase, however, is so slight that

it need hardly be considered. The digestion of casein is definite since the increase was 13 mg. in 100 cc. of digest; this indicates that over 8 per cent of the protein was attacked. The failure of the enzyme to digest serum albumin might be due either to an antienzymatic action of this protein or to an inability of the enzyme to attack it. In view of the increase in the non-protein nitrogen of the serum after digestion, an increase which may be explained by the splitting of protease normally found in serum in small amounts, it seems probable that there is little inhibition of the enzyme by the serum albumin. Such action on casein and failure to digest albumin are characteristic of the erepsin-like enzymes previously found in many plants (12). From the tables given by Avery and Cullen in their study of the proteolytic action of the pneumococcus endoenzymes it is apparent that the "peptase" which they obtained acted similarly on casein and egg albumin, since they were unable to obtain mixtures beyond that due to the autolysis of the proteins occurring in the enzyme solutions alone.

*The Effect of Chloroform and Gentian Violet on the Peptolytic Enzyme.*

Early in these experiments it was found necessary to employ an antiseptic in the preparation of the bacterial extracts to kill the few bacteria which escaped grinding. Since toluene was known to have a definite bactericidal action and had been used generally to preserve solutions of organic material or to prevent bacterial growth during digestion with enzymes, it was used throughout these experiments in preparing the solutions. It was felt that other antiseptics might be used which would serve a similar purpose. A series of experiments was than carried out to study the effects of chloroform and gentian violet.

*Experiment 13. Comparison of the Effects of Toluene and Chloroform on the Peptolytic Enzyme.*—An emulsion of the bacteria was prepared in the usual manner. After the mass was well ground half was allowed to stand with toluene and half with chloroform for 12 hours. Each half was centrifuged after that period and the supernatant solutions were pipetted from the tubes without disturbing the layers of antiseptic. The solutions were sterile. Digestion flasks were prepared with substrates of peptone. Controls containing enzyme which had been boiled 20 minutes were included in the series. After 48 hours at 37°C. the amino

nitrogen was determined for each flask (Table XII). Cultures made in broth showed no growth.

TABLE XII.

*The Effect of Chloroform on the Peptolytic Enzyme.*

| Antiseptic. | Amino nitrogen per 100 cc. of solution. | | |
|---|---|---|---|
| | Active. | Inactive. | Increase. |
| | *mg.* | *mg.* | *mg.* |
| Toluene | 53.9 | 26.1 | 27.8 |
| Chloroform | 34.6 | 28.9 | 5.7 |

*Experiment 14. The Inhibition of the Peptolytic Action of Enzyme Solutions by Gentian Violet.*—Solutions of enzyme and peptone were prepared with toluene in the usual manner. Dilutions of gentian violet (Grübler) were made in $\frac{M}{15}$ phosphate solution (pH 7.0). 10 cc. of each of these dilutions were pipetted into sterile flasks with 5 cc. of enzyme and 5 cc. of 4 per cent peptone solution. Amino nitrogen determinations were made after 48 hours at 37°C. (Table XIII). Controls without gentian violet were prepared with boiled and normal active enzyme.

TABLE XIII.

*The Effect of Gentian Violet on the Peptolytic Enzyme.*

| Flask No. | Dilution of gentian violet. | Precipitate.* | Amino nitrogen per 100 cc. of solution. | |
|---|---|---|---|---|
| | | | Analysis. | Increase. |
| | | | *mg.* | *mg.* |
| 1 | 1:1,000 | ++++ | 29.8 | 3.0 |
| 2 | 1:5,000 | +++ | 37.9 | 11.1 |
| 3 | 1:10,000 | ++ | 43.8 | 17.0 |
| 4 | 1:20,000 | + | 43.6 | 16.8 |
| 5 | 1:50,000 | + | 56.8 | 30.0 |
| 6 | 1:100,000 | + | 57.0 | 30.2 |
| 7 | Active normal enzyme. | | 56.8 | 30.0 |
| 8 | Inactive enzyme. | | 26.8 | |

* Precipitate was formed in these flasks during incubation; the amount of precipitate has been indicated by plus signs.

Previous workers have observed that chloroform has an inhibiting action on digestion with enzyme; this has been noted frequently in the literature with respect to pancreatic trypsin. According to Fuhrmann, van Laer observed that solutions of diastase were inactive

if they were preserved with chloroform for more than 15 days. Fuhrmann (7) furthermore noted that chloroform was detrimental to bacterial enzyme action, while toluene was one of several antiseptics which might be used for the sterilization of cultures to demonstrate enzyme action. The results of Experiment 14 serve merely as confirmatory evidence of these facts. Although sterilization was accomplished with both of the antiseptics the use of chloroform resulted in considerable deterioration of the solutions.

The effects of the dilutions of gentian violet on the splitting of peptone were more surprising because Churchman (13) found that this dye had no effect on the digestion of proteins with trypsin. A very definite decrease in the peptolytic activity of these enzyme solutions was found in dilutions as high as 1:20,000. In 1:1,000 dilution the enzyme was practically inactive. Slight precipitation occurred throughout the series of flasks to which gentian violet had been added, but was most marked in dilutions more concentrated than 1:20,000. Since precipitates were not obtained with solutions of pure albumin or with the enzyme solution in similar dilutions of the dye, a series of tubes was prepared with 1 per cent of the peptone used in the experiment.

Precipitate occurred in approximately the same amounts as had been found in the enzyme-peptone mixtures. From nitrogen determinations on the supernatant fluid obtained by centrifuging these peptone mixtures it was found that nearly one-tenth of the peptone was carried out of solution in a 1:1,000 dilution. The degree of precipitation in the various dilutions corresponded roughly to the inhibition of the enzyme in similar concentrations of the dye. In dilutions of 1:50,000 and 1:100,000, which will ordinarily inhibit Gram-positive bacteria, digestion was not hindered. These observations are not sufficiently comprehensive so that one may offer an entirely satisfactory explanation for the bacteriostatic action of gentian violet. It is known that violet-positive bacteria (14) will absorb sufficient dye during growth on agar containing almost imperceptible quantities so that the growing colonies acquire a deep purple color, and that greater percentages will entirely inhibit growth. These facts suggest that its bacteriostatic effect may be due to the absorption of sufficient quantities of dye to inhibit enzyme action.

Evidently, since the trypsin digestion of proteins is not hindered by gentian violet, the inhibition is not due to a direct action on the enzyme. Furthermore, only one-tenth of the peptone was carried out of solution by the dye so it is not probable that the digestion was inhibited to any great extent by a change in the concentration of the substrate. The most plausible explanation is an absorption of the enzyme by the precipitate which might occur in Gram-positive bacteria when exposed to gentian violet as well as in the peptone-enzyme mixtures.

## DISCUSSION.

The method used to extract the enzymes from the streptococci is a combination of various procedures which have been employed by previous investigators. Various innovations were introduced, such as the use of the phosphate mixtures during the grinding and sterilization of the cultures to protect the enzymes from deterioration. Further work showed that the three enzymes studied were exceedingly susceptible to heat, acid, and antiseptics, so that all the attempts made to protect them during extraction were justified. A method was desired which might be applied to other bacteria and would yield solutions of enzyme relatively free from cell debris. Although considerable protein was carried into solution the consistent results which were obtained throughout the experiments lead us to suspect that the physical conditions under which the various digestions were studied were not greatly modified by the material dissolved from the cell bodies. No attempt was made, however, to purify the solutions further.

The methods of study are similar to those previously employed by Avery and Cullen (6, 8, 9) in their work with pneumococcus, except that micro methods of analysis have been employed when possible in preference to methods requiring larger quantities of material for analysis. The enzymes resemble those which Avery and Cullen obtained from the pneumococcus, since they act through similar ranges of H ion concentration. The resemblance between the enzymes leads us to believe that similar enzymes exist in other bacteria.

The experiments on the effects of acid concentration showed that the enzymes studied were most active between pH 7.0 and 8.0. This

pH corresponds closely with the degree of alkalinity necessary for the optimum growth of streptococci. The lipase was most active at a pH more alkaline than that required for the other enzymes; this appears to be true for lipase obtained from other than bacterial sources. The acidity at which digestion might proceed varied. The lipase was found inactive at a point less acid than the final pH of cultures in dextrose broth, but the peptase, according to the curve constructed in Text-fig. 1 was active at an acid concentration slightly greater than pH 4.5. The limits of digestion in acid probably depend both on a union of substrate and enzyme and on the destruction of the enzyme itself; some of the later experiments on the effect of acid serve to elucidate this point. In Experiment 9 an acid concentration of pH 5.0 was found to be sufficient to destroy the invertase and lipase if solutions were exposed to the acid for 6 hours at a temperature of 37°C., while the peptolytic enzyme was still active after similar treatment. This suggests that the digestion obtained with the peptase in acid concentrations which completely inhibited the lipase and the invertase may be due to the resistance of that enzyme to acidities as low as pH 5.0.

Further work on physical conditions destructive to the enzymes was continued in Experiments 6, 7, and 8. A temperature of 60°C. continued for 10 minutes was found to be sufficient to destroy all three enzymes. According to the curves in Text-fig. 4, there was some deterioration even at 40°C., and the temperatures required for complete destruction were between 51° and 57°C. The streptococci from which the extracts were obtained were not viable after cultures were heated for 10 minutes at 55°C. The close agreement between these temperatures suggests that the destruction of the enzymes may be one of the factors causing the death of the organism by heat. The great number of previous observations on this point is hardly compatible with such a conclusion, since repeated observations by other investigators indicate that bacteria are ordinarily killed by temperatures which will not destroy their enzymes. Fuhrmann, however, observes that solutions of enzyme obtained from bacteria grown on media relatively free from protein are destroyed at lower temperatures than enzymes obtained from cultures on ordinary media. Other observations with enzymes from non-bacterial sources indicate that

products of digestion have a distinctly protective action. Throughout these experiments the peptase was the most stable of the enzymes. Analyses indicate that the solutions of enzyme contained considerable protein material; there was relatively little reduction of alkaline copper solutions, so we assume that there was a much smaller percentage of dextrose. It is probable that the amounts of protein and sugars in the solution account for the difference in stability of the enzymes. Such an assumption would explain the higher temperatures required to destroy the enzymes of pneumococci obtained by the complete solution of the bacterial bodies with the resulting autolysis and accumulation of digestion products.

Efforts were made to ferment dextrose by the addition of freshly ground bacteria and of yeast coenzyme to the digestion mixtures. No changes in acidity were observed after these procedures. Starch was not attacked by the enzyme solutions.

## CONCLUSIONS.

1. A method has been outlined by which the enzymes of hemolytic streptococcus may be extracted with comparative ease.

2. The peptolytic enzyme is active between pH 4.4 and 8.7 with an optimum action at pH 7.2. It is destroyed in neutral phosphate solution at a temperature of 57°C. continued for 10 minutes and at pH 5.0 deteriorates slowly at 37°C. Concentration experiments with solutions of the enzyme have shown that it resembles other enzymes. It is exceedingly susceptible to chloroform and its action is inhibited by dilutions of gentian violet. Casein is attacked but serum albumin is not digested after 3 days at 37°C.

3. The invertase is active between approximately pH 5.0 and 8.0 with an optimum of pH 7.0. It is destroyed by a temperature of 52°C. continued 10 minutes at an acid concentration of pH 7.0, or after 6 hours at 37°C. at pH 5.0. At this acidity it is more susceptible to heat than the peptase.

4. The lipase is active above pH 5.6. The greatest activity was observed at pH 7.9. It is completely destroyed after heating to temperatures over 55°C. for 10 minutes and resembles the invertase in its susceptibility to acid.

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