STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

VI. The Oxidation of Enzymes in Sterile Extracts of Pneumococcus.

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INTRODUCTION.

Avery and Cullen (1, 2) have demonstrated that the pneumococcus cell contains the hydrolyzing enzymes (peptonase, lipase, sucrase, amylase, inulase) and a bacteriolytic enzyme. Cole (3) (1912), (4) (1914), has shown that pneumococci also possess a hemotoxin. These enzymes and the hemotoxin are endocellular, representing actual constituents of the cell, and consequently are demonstrable in sterile extracts prepared by disruption of the cell walls. In addition to these intracellular substances, the pneumococcus cell contains a number of oxidation-reduction systems. As shown in preceding papers, sterile extracts of pneumococci may be prepared in such a manner that some at least of these oxidation systems are preserved in an active state. Among the oxidation-reduction activities of these extracts already reported are the prompt formation of peroxide when the extracts are exposed to air (5), the consumption of molecular oxygen (6), the active reduction of methylene blue (7), and the rapid oxidation of hemotoxin (8) and of hemoglobin (6). The cell extracts are sterile; and all of these reactions are exhibited under conditions excluding the participation of living or intact cells.

In preceding papers it has been shown that the marked oxidation reactions which can be induced in these extracts are not without effect upon certain labile constituents of the pneumococcus cell. For instance, the hemotoxin, although itself non-reactive with molecular oxygen, is rapidly destroyed by oxidizing agents formed during the oxidation of other easily oxidized constituents of the extract. It seemed of considerable interest, therefore, to determine whether the hydrolyzing enzymes of Pneumococcus are destroyed in a manner analogous to that of the oxidation of the hemotoxin.

A number of examples of the inactivation of enzymes by oxidation have been reported in the literature. Sigmund (9) (1905) compared the effect of ozonized oxygen and pure oxygen upon the activity of a number of enzymes by passing the gases through enzyme solutions for 1 hour before adding the enzymes to their substrates. Treatment with pure oxygen frequently had some effect, but in all cases the ozone treatment caused a greater loss in enzyme activity. Starch-splitting enzymes and emulsin were destroyed to a significant degree, and pepsin also suffered some loss in activity. The effect upon sucrase was especially pronounced, dilute solutions of this enzyme being almost entirely destroyed. Kastle (10) (1906) has reported the destruction of lipase by ozone. Buchner and Hoffmann (11) have found that zymase and apparently also yeast sucrase are destroyed by ozone.

Hydrogen peroxide has been the oxidizing agent most frequently employed in studies of enzyme inactivation by oxidation. That catalase itself is destroyed by large amounts of hydrogen peroxide has been generally recognized since the time of the discovery of this enzyme (Senter (12), Vandevelde (13), and Vandevelde, Schoenfeld, and Leboucq, 1901 (14), and others). This occurrence is probably one of the factors which obscure the dynamics of the action of this enzyme. Peroxidase is also said to be susceptible to large amounts of hydrogen peroxide (Bach and Chodat, 1903 (15); Schönbein (16)), and to be destroyed by organic peroxides formed during the autoxidation of organic compounds (Bach and Chodat, 1903 (15); Bach, 1905 (17)). Hydrolyzing enzymes may be destroyed by hydrogen peroxide, although differences exist in the degree to which different enzymes of this type are inactivated by this chemical oxidizing agent. Vandevelde (1904) found that hydrogen peroxide in concentrations of 0.05 per cent destroyed most of the activity of starch-hydrolyzing enzymes and that concentrations of 0.2 to 0.5 per cent completely inactivated them. On the other hand, this author found that trypsin and pepsin were not destroyed and, indeed, concluded that the action of these enzymes was accelerated by the peroxide. Walbum (1911) (18) observed somewhat similar relations. Concentrations of 0.1 per cent completely destroyed ptyalin in 30 minutes, although very small amounts of hydrogen peroxide (up to 0.005 per cent) were without effect. Exposure of rennet to 0.5 per cent hydrogen peroxide for $\frac{1}{2}$ hour completely inactivated this enzyme, but pepsin and trypsin were not affected in the concentrations tested.

A number of other oxidizing agents of various natures have been found to inactivate enzymes (Senter, 1905 (19); Kastle, 1906 (10); see also review by Euler (20)). The fact observed by Falk, McGuire, and Blount (21) that a greater loss of enzyme activity occurred in vegetables dehydrated in the air than if the dehydration were done *in vacuo* is probably another example of destruction of enzymes by oxidation.

It is evident from the above review that the activity of enzymes may be destroyed by oxidation and that differences exist in the susceptibility of various enzymes to oxidation. It is also significant

that, in most instances, the destruction of the enzymes has been due not to the action of molecular oxygen, but to oxidizing agents of which peroxides have been the most studied. The literature thus furnishes evidence that enzymes of cellular origin may be destroyed by oxidizing agents *in vitro*, and indeed, by oxidizing agents similar in nature to those which are formed during the oxidation of the "complete system" type of pneumococcus extracts. In none of the experiments in the literature, however, have the oxidizing agents used in enzyme destruction been derived from cellular oxidations.

Sterile pneumococcus extracts contain not only hydrolyzing enzymes, but also the oxidation-reduction systems which form peroxides when exposed to the air. This set of circumstances afforded an opportunity to demonstrate the destruction of enzymes by oxidizing agents actually formed by constituents of the same cell as that from which the enzymes themselves are derived.

Methods.

Bacteriological.—

Sterile Pneumococcus Extracts.—The sterile pneumococcus extracts used in this investigation were prepared in the manner described in a preceding paper (5). Two types of extract have been employed: the broth extract of unwashed cells and the phosphate solution extract of washed cells. The first type of extract contains "complete" oxidation-reduction systems which actively form peroxide when exposed to molecular oxygen and which actively reduce methylene blue. The second type of extract contains potentially active, but "incomplete" oxidation-reduction systems which form peroxide and which reduce methylene blue only if the system is "completed" by the addition of easily oxidized or autoxidizable substances which may be furnished by meat infusion or yeast extract. Because of these relations, the broth extract of unwashed cells and the phosphate solution extract of washed cells are at times referred to in the text as "complete" and "incomplete system" types of extract. Both types of extract contain approximately equal concentrations of the hemotoxin described by Cole, and of the enzymes described by Avery and Cullen.

Sterility Controls.—The sterility of all extracts has been controlled by cultural and animal tests. Unless otherwise stated, the broth extract of unwashed cells was filtered through Berkefeld candles. The phosphate solution extracts were not filtered, as filtration of this type of extract results in a great loss in the active concentration of all the enzymes. The sterility of the enzyme-substrate mixtures was controlled by cultural tests.

Chemical.—

Preparation of Substrates.—Unless otherwise stated, the substrates of the various enzymes were prepared as follows: The peptone solutions used consisted of 1 per cent solutions of Fairchild's peptone in 0.06 M phosphate solution (pH 7.8). The sucrose substrates consisted of 2 per cent solutions of sucrose in 0.06 M phosphate solution (pH 7.0). The starch, inulin, and raffinose solutions used as substrates were also prepared in phosphate solution of the same pH. The lipase substrate consisted of 2 per cent emulsions of Kahlbaum's tributyrin in 0.06 M phosphate solution (pH 7.5). All of these substrates were sterilized in the autoclave for 10 minutes at 120°C. The hemotoxin titrations were performed by the procedure previously described, with 2.5 cc. of a 1 per cent suspension of washed erythrocytes from sterile defibrinated rabbit blood.

Quantitative Analyses.—The hydrolysis of peptone was determined by amino nitrogen analyses by Van Slyke's method (22). The hydrolysis of tributyrin was determined by titration of the butyric acid produced; 5 cc. of the enzyme-substrate mixture were titrated to the original pH as determined by colorimetric comparison with the control; a micro burette was used. Qualitative tests of the hydrolysis of sucrose were carried out by the Benedict test; 0.4 cc. of enzyme-substrate mixture was overlaid on 3 cc. of Benedict's solution and boiled for 3 minutes. Quantitative estimations of reducing sugars were made by Benedict's volumetric method.

Oxidation of Extracts by "Exposure to the Air."—Although constant agitation of the extract seemed desirable for experiments on the rate of destruction of the enzymes by oxidation, it seemed necessary to exclude any possibility of the so called "*Schüttel-inactivierung*" as a factor in the observed enzyme destruction. For this reason, none of the extracts were shaken during oxidation. Oxidation was accomplished by exposing the extract to the air in shallow layers (less than 1.0 mm.) in Erlenmeyer flasks. During exposure to the air, the extracts were protected from light by wrapping the flask in black paper.

The extracts themselves were stored in the ice box at 2°C. under heavy vaseline seal. At the time of their use in the various experiments, the "age" of the extracts varied from 1 to 4 weeks. Stored under these conditions, no significant losses in activity of any of the enzymes were observed.

Demonstration of Destruction of Enzymes.—In the following tests, differences in concentration of active enzyme have been estimated by differences in the amounts of products of enzyme action over certain periods of time. Most of the experiments involved determinations of total destruction of enzyme activity, which made necessary the use of large amounts of enzyme solution and long digestion periods. The methods used, therefore, while theoretically not well adapted to estimations of the actual concentrations of active enzyme, serve the purpose desired in showing relative differences in the amount of enzyme which remained active after different oxidation and heating treatments.

Influence of Oxidation upon the Subsequent Activity of Pneumococcus Sucrase.

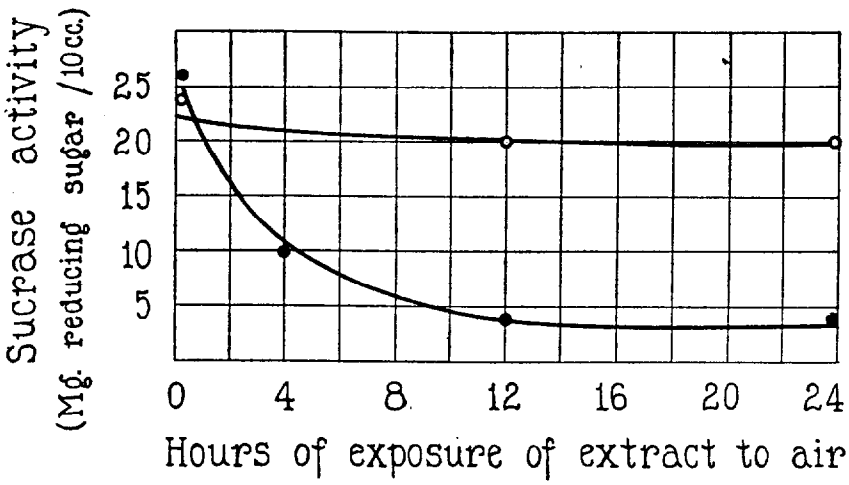
It may be recalled that when a broth extract of unwashed pneumococci is exposed to air, marked oxidative reactions occur; certain constituents of the extract are rapidly oxidized with the formation of detectable amounts of peroxide. During these oxidations, and probably through the agency of products formed by the union of molecular oxygen with the more reactive constituents of the extract, certain other substances may be oxidized, even though the latter substances themselves are non-reactive with molecular oxygen. Examples of this type of oxidation were furnished in preceding papers in the case of hemotoxin and of hemoglobin. Quite different results occur when the other type of extract, *i.e.* the phosphate solution extract of washed cells, is exposed to the air. Presumably because of the absence of easily oxidizable substances, no marked changes are evidenced and peroxide is not produced. In this type of extract, both the hemotoxin and hemoglobin prove relatively stable.

These differences are interpreted as due to the fact that, although neither hemotoxin nor hemoglobin is readily oxidized by molecular oxygen, both are rapidly destroyed by the oxidizing agents (presumably peroxides) which are formed during the oxidation of certain easily oxidized constituents of the broth extract of unwashed pneumococci. It now seemed of interest to determine whether the destruction of pneumococcus sucrase represented a similar type of oxidation. Sucrase, like hemotoxin, is contained in about equal concentrations in both types of pneumococcus extracts. Therefore, a comparison of the effect upon sucrase activity induced by aerating the two types of extract offers a means of investigating the nature of the oxidation reaction by which this enzyme is destroyed.

Accordingly, phosphate solution extract of washed pneumococci ("incomplete system" type of extract) and broth extract of unwashed pneumococci ("complete system" type of extract) were exposed to air at 37°C. After different periods of exposure, portions of the two extracts were removed and tested for sucrase activity. As controls on the effect of temperature itself upon the stability of the sucrase, portions of the two types of extract were held at 37°C., protected from air by a heavy vaseline seal, and added to the substrate at the same time as the oxidized extract.

A number of experiments of this type have been conducted. In all instances, the sucrase proved relatively stable when exposed to air in the washed cell extracts. The degree of destruction of the sucrase when the "complete system" type of extract is exposed to air varies with the initial sucrase activity and with the oxidizing (peroxide-forming) power of various extracts.

It is evident from these results (Text-fig. 1) that a rapid destruction of sucrase occurs during the oxidation of broth extracts of un-



° Phosphate solution extract of washed pneumococci.
• Broth extract of unwashed pneumococci.

TEXT-FIG. 1. Effect of exposure to air upon the subsequent sucrase activity of extracts of washed and unwashed pneumococci.

washed pneumococci. On the other hand, in the extract of washed cells, sucrase proves as stable in the presence as in the absence of air. These relations are analogous to those previously found in the study of the oxidation of hemotoxin and of hemoglobin by pneumococcus extracts. Hence, it would seem that sucrase destruction represents a similar type of oxidation. In the broth extract of unwashed cells, the destruction of the sucrase when the extract is exposed to oxygen may be assigned to oxidizing agents which are formed as products of the action of molecular oxygen upon other easily oxidized con-

stituents of the extract. In the case of the phosphate solution extract of washed cells, the stability of the enzyme seems to be explained by the fact that sucrase itself does not react readily with molecular oxygen; and that the "incomplete system" extract, unlike the unwashed cell extract, contains no easily oxidizable substances to form oxidizing agents by union with molecular oxygen.

Influence of Oxidation upon the Subsequent Activity of Other Carbohydrate-Hydrolyzing Enzymes of Pneumococcus.

It seemed probable that the pneumococcus enzymes which hydrolyze other carbohydrates might exhibit reactions similar to those

TABLE I.

Effect of Exposure to Air upon the Subsequent Activity of Pneumococcus Amylase, Raffinase, and Inulase.

Type of extract.		Enzyme activity of pneumococcus extract.		
		Amylase.	Raffinase.	Inulase.
Unwashed cells extracted in broth (complete system).	Reduced.*	+†	+++	+
	Oxidized.	−	+	−
Washed cells extracted in phosphate solution (incomplete system).	Reduced.	+	+++	+
	Oxidized.	+	+++	+

* Reduced extract stored under seal to protect from air for 24 hours at 37°C. Oxidized extract exposed to air in thin layer for 24 hours at 37°C.

† The concentration of reducing sugars represented by these symbols approximate the following values: −, less than 0.03 per cent; +, 0.10 per cent; +++, 0.30 per cent.

by which sucrase is destroyed by oxidation. To investigate this question, experiments similar to those previously described for invertase were carried out with pneumococcus inulase, amylase, and raffinase.

It is evident from the results of this experiment (Table I) that the active concentration of pneumococcus amylase, inulase, and raffinase is markedly decreased during the oxidation of the broth extract of unwashed pneumococci. Moreover, as previously observed in the case of sucrase, these carbohydrate-hydrolyzing enzymes are not

themselves reactive with molecular oxygen, since they proved stable during the exposure to air of the phosphate solution extract of washed cells.

The Effect of Oxidation upon the Subsequent Activity of Pneumococcus Lipase.

Avery and Cullen (1) have described the properties of the lipase contained in the pneumococcus cell. This enzyme was shown to split tributyrin with the production of large amounts of butyric acid. The literature reviewed presents examples of the oxidation of certain lipases by treatment with chemical oxidizing agents. Lipases from various organisms may exhibit strikingly different thermostability. In view of the known differences in resistance of various enzymes to chemical oxidizing agents, together with the variations in heat resistance of different lipolytic enzymes, it was determined to investigate the resistance of pneumococcus lipase to the oxidizing agents formed during the oxidation of pneumococcus extracts.

From the results of several experiments it is evident that the lipase activity of pneumococcus extract is not appreciably changed during the oxidation processes which occur when the "complete system" type of extract is exposed to the air. Although a series of tests involving different amounts of extract have been employed, no distinctions in the lipase action of "reduced" and "oxidized" extracts have been detected.

The results of the above experiments do not constitute evidence that pneumococcus lipase cannot be inactivated by oxidation, as subsequent experiments will reveal. However, it is obvious from them that oxidation processes which cause marked diminution in the active concentration of the sucrase, amylase, inulase, and raffinase do not have a comparable effect upon the activity of the lipase.

Influence of Oxidation of Extracts upon the Subsequent Activity of Pneumococcus "Peptonase."

Avery and Cullen (1) have shown that the pneumococcus cell contains certain constituents which hydrolyze intact proteins to some extent and split to a marked degree commercial "peptone" into simpler peptides and amino acids. As the maximum activity was exhibited in the further hydrolysis of peptide nitrogen, they applied the term "peptonase" to the enzyme (or enzymes) responsible for this proteolytic activity of pneumococcus.

From the literature reviewed, it is evident that enzymes which probably are more or less similar in nature to pneumococcus "peptonase," such as trypsin, have proved quite resistant to oxidizing agents. There are evident discrepancies in the reports of the various investigators upon the question of whether or not these enzymes are destroyed by oxidation at all, due probably to differences in experimental conditions. Evidently, however, these enzymes are among those most resistant to oxidation.

A number of experiments have been carried out to test the effect of the oxidizing agents formed in pneumococcus extracts upon the "peptonase" activity. In no instance did the oxidation of the broth extract of Pneumococcus have any detectable effect upon the activity of the "peptonase." Thus, the experiments presented have shown that the lipase and peptonase of pneumococcus are unaffected by exposure to concentrations of oxidizing agents which seriously diminish the active concentration of pneumococcus sucrase, amylase, raffinase, and inulase.

Effect of Oxidation of Extracts upon the Bacteriolytic Enzyme of Pneumococcus.

Avery and Cullen (2) have reported the presence of a bacteriolytic enzyme in Pneumococcus, which exhibits to a striking degree the property of causing lysis of pneumococcus cells which have been killed by heat, and to a less extent lysis of closely related bacteria as the *Streptococcus viridans* group. Since practically nothing is known of the nature or even of the identity of this enzyme, it seemed particularly desirable to compare its relative resistance to oxidation with that of other pneumococcus enzymes. Accordingly, comparisons have been made of the bacteriolytic activity of a broth extract of Pneumococcus which had been oxidized, with the bacteriolytic activity of an extract which had not been exposed to oxidation.

The results of these experiments cannot be given definite quantitative expression. However, repeated experiments of this nature leave no doubt of the susceptibility of the bacteriolytic enzyme to oxidation products formed during the oxidation of the "complete system" type of pneumococcus extract. In every instance, with dilutions of extract from 0.1 to 0.002 cc., the "reduced" broth ex-

tract manifested a greater bacteriolytic effect than did the same dilution of "oxidized" extract. Although differences in the activity of each corresponding dilution of "reduced" and "oxidized" extract were definite and unmistakable, the differences in no case (not even in dilutions of only 0.002 cc. of extract) approached the distinction between positive and negative bacteriolysis.

It is interesting to note the distinction between the bacteriolytic and hemolytic agents in pneumococcus extracts, which is afforded by the differences in resistance to oxidation. Although oxidation of the unwashed cell extract effects only a slight loss in the bacteriolytic activity, the hemolytic activity of the extract is completely destroyed under the same conditions.

The definite destruction of bacteriolytic activity which occurs during the oxidation of the "complete system" type of extract is manifested not at all when the phosphate solution extract of washed cells is exposed to the air. Hence, it seems that the bacteriolytic enzyme is oxidized by a reaction similar in type to those which effect the destruction of hemotoxin, sucrase, amylase, inulase, and raffinase.

The above experiments on the effect of oxidation of sterile pneumococcus extracts upon the activity of the various enzymes have demonstrated marked differences in their susceptibility to oxidation. Their resistance to the oxidation products formed when unwashed cell extracts are exposed to the air varies in the following order; peptonase, lipase, bacteriolytic enzyme, sucrase and other carbohydrate-hydrolyzing enzymes, and hemotoxin; the activity of the peptonase and lipase apparently is unaffected by oxidation reactions which seriously reduce the active concentration of other labile, pneumococcus cell constituents.

Relative Order of Heat Resistance of the Different Enzymes of Pneumococcus.

As a means of comparing the relative degree of heat resistance of the different enzymes with the relative order of their susceptibility to oxidation, tests were made of the stability of the enzymes at different temperatures, with the same pneumococcus extracts as those employed in the preceding experiments on oxidation. The degree of temperature, length of exposure, and pH of the solution are related

factors in the destruction of enzymes by heat. In these experiments the reaction of the extract (pH 7.1) and an exposure of 10 minutes were chosen as constant factors, and the degree of temperature alone was varied.

The different enzymes of Pneumococcus exhibit marked differences in heat resistance. The comparative rates of destruction of the enzymes at the different temperatures are reflected in Text-fig. 2. It is evident that those enzymes which proved more resistant to oxidation in the extract are also more resistant to heat. Thus, the known hydrolyzing enzymes of pneumococcus can be arranged in the same order of relative resistance, *i.e.* peptonase, lipase, sucrase, both from the standpoint of their resistance to heat and their resistance to the oxidation products formed during the oxidation of a broth extract of unwashed pneumococci.

Relative Resistance of Different Enzymes of Pneumococcus to the Action of Hydrogen Peroxide.

Hydrogen peroxide has been the oxidizing agent most frequently employed in studies of the destruction of enzymes by oxidation. It is evident from the literature that differences exist in the susceptibility of various enzymes to this reagent. The experiments presented in this paper make it equally evident that differences exist in the susceptibility of various enzymes of Pneumococcusto the action of the oxidizing agents which are formed during oxidation of the "complete system" type of sterile extract. If the destruction of pneumococcus enzymes under these conditions is due to the action of peroxides formed during the oxidation of pneumococcus extract, it might be expected that those enzymes the activity of which is least affected by the oxidation of the extracts, would prove most resistant to the action of reagent hydrogen peroxide.

Experiments were designed to ascertain if the order of susceptibility of the different enzymes is the same when treated with preformed hydrogen peroxide as when the enzymes are exposed to the oxidation products of pneumococcus extracts. It also seemed desirable to compare the relative susceptibility of the different enzymes to hydrogen peroxide with the relative heat susceptibility of the enzymes. With this in mind, in order that the results might be directly comparable with those already obtained in the preceding experiment, the present experiment was conducted with the same pneumococcus extract and with the same substrate preparations.

3.0 cc. quantities of the pneumococcus extract used in the preceding experiment were added to tubes containing 3.0 cc. of titrated "Dioxogen" diluted in sterile 0.06 M phosphate solution (pH 7.2). The final concentration of H_2O_2 in the extract-H_2O_2 mixtures was 150, 30, and 6 mM (approximately 0.5, 0.1, and 0.02 per cent H_2O_2). The tubes were sealed with vaseline and placed in a water bath for 24 hours at 37°C. The peroxide-treated extract, and untreated extract, were then added to the substrate mixtures to give the same concentration of pneumococcus extract as those used in the preceding experiment.

The results of this experiment (Table II) demonstrate that the enzymes of Pneumococcus differ in their relative susceptibility to the action of hydrogen peroxide. Furthermore, the peptonase and lipase enzymes which were unaffected by the oxidation products formed in

TABLE II.

Relative Resistance of Different Enzymes of Pneumococcus to the Action of Hydrogen Peroxide.

H_2O_2 to which enzyme had previously been exposed.	Peptonase.		Lipase.		Sucrase.	Bacteriolytic enzyme.	Hemotoxin.
	NH_2-N in 100 cc.		pH	N/14 acid in 10 cc.	Reducing sugar in 100 cc.	Bacteriolysis by 0.02 cc. extract.	Hemolysis by 0.10 cc. extract.
	Total.	Increase.					
mM	*mg.*	*mg.*		*cc.*	*mg.*		
0	65.1	12.1	5.7	4.10	26	++++	++++
6	57.9	4.9	6.8	2.0	3*	±	—
30	54.8	1.8	7.5	—	—	—	—
150	52.9	—	7.5	—	—	—	—

* Estimated by comparison with standard glucose solutions.

sterile pneumococcus extracts proved most resistant to the action of reagent hydrogen peroxide. The relative order of resistance to H_2O_2 exhibited by the peptonase, lipase, and sucrase of Pneumococcus also agrees with the order of heat resistance of the same enzymes. The lipase and peptonase enzymes of Pneumococcus in the above experiment are inactivated by considerably less drastic treatment with H_2O_2 than would appear from the literature to be the case with protein-splitting and lipase enzymes derived from higher organisms. Possibly the pneumococcus lipase and peptonase represent enzymes different in nature from the lipases and protein-splitting enzymes more commonly studied, as these pneumococcus enzymes are less resistant both to heat and to oxidation.

The Selective Destruction of Pneumococcus Enzymes by Oxidation and by Heat.

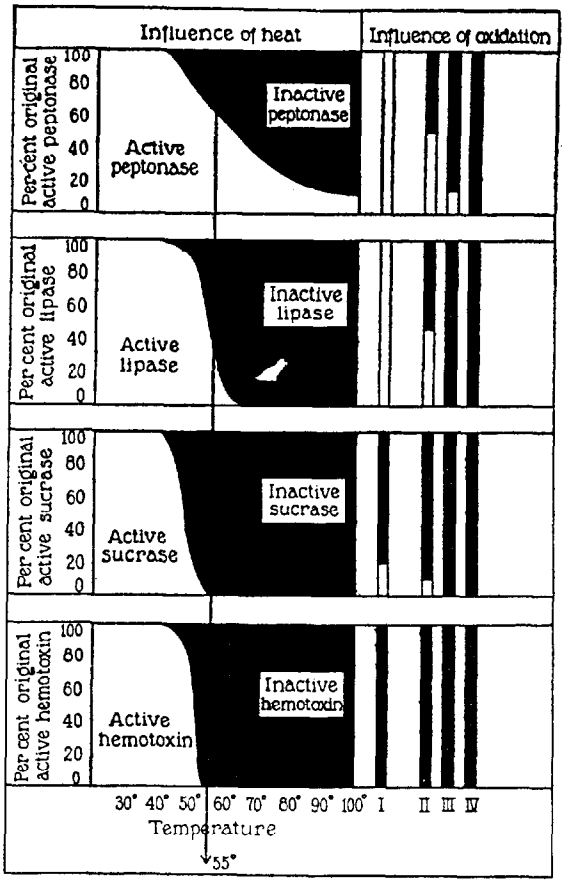
In a study of biochemical processes, mixtures of the constituents of certain cells are frequently employed. In such cases, the assignment of definite functions to the activity of a particular constituent demands the elimination or inactivation of other active substances present. One of the most frequent methods employed for this purpose is the "fractional inactivation" of the various constituents by heating at various temperatures. By a proper choice of temperatures, the more heat-stable constituents of the mixture may be retained in an active form, and the more heat-labile substances may be destroyed. In many cases, however, this method is limited by the fact that the fractionation is by no means sharp; temperatures which suffice for the complete inactivation of the more labile substances also effect serious loss in the active concentration of the relatively heat-stable constituents.

Oxidation of a mixture of biochemically active substances offers a further means of separation of certain of the active constituents. Recently, it has been shown that oxidation of cod liver oil results in the complete destruction of the fat-soluble vitamine A without any inactivation of the antirachitic vitamine (23). Inspection of data already presented in this paper reveals the fact that oxidation will also serve as a means of separating certain of the active constituents of a mixture of pneumococcus cellular substances. By properly regulated oxidation, the "fractional inactivation" of these various constituents is apparently quite as sharp as can be obtained by heating the extract at any temperature. To illustrate these relations, data of the preceding experiments are presented graphically in Text-fig. 2.

In this figure, there is plotted the relative amount of each of the original enzymes[1] which remains active when a mixture of pneumococcus cellular substances is heated at various temperatures or is exposed to

[1] The assumption is not made that pneumococcus hemotoxin is an enzyme. It is included in this study for purposes of comparison merely, as a known labile constituent of the pneumococcus cell.

The bacteriolytic enzyme is not included in this figure, due to the difficulty of obtaining a quantitative expression of the action of this enzyme.



TEXT-FIG. 2. Comparison of the influence of heating at different temperatures and the influence of oxidation, upon the subsequent activity of different enzymes of Pneumococcus.

The relative amount of the various enzymes which remains active after heating or after oxidation is calculated from data obtained in preceding experiments in this paper. (The data for the effect of heat upon hemotoxin are taken from Table II in a preceding paper (8).) It is assumed that the products produced by the untreated or original enzyme solution represent 100 per cent of active enzyme. The fact that this assumption is not theoretically correct (see under Methods) influences the slopes of the various curves, but does not affect in any way the interpretation given in the text.

The Roman numerals in this figure refer to the following: I, oxidized broth extracts of unwashed pneumococci; II, exposure for 24 hours to 6 mM H_2O_2 ("Dioxogen"); III, exposure for 24 hours to 30 mM H_2O_2 ("Dioxogen"); and IV, exposure for 24 hours to 150 mM H_2O_2 ("Dioxogen").

differing concentrations of oxidizing agents. The unshaded area in each case represents the percentage of original enzyme which is still active after heating or after oxidation; the shaded area represents the portion of the originally active enzyme which has been inactivated. A vertical line drawn from any point on the temperature axis is an indication of the percentage inactivation of the various enzymes after exposure for 10 minutes at that temperature. Analysis of the curves of the effect of temperature upon the activity of the several enzymes reveals the fact that a "fractional inactivation" by 10 minutes heating at different temperatures would by no means be distinct. Exposure of an extract for 10 minutes to 55°C., which suffices for the inactivation of hemotoxin and of sucrase (the most labile constituents of the mixture), results in a serious loss in activity of the more heat-resistant lipase and peptonase. On the other hand, it seems possible that, by the proper choice of oxidizing agents in regulated concentrations, some at least of the more easily oxidized constituents can be completely inactivated without a serious loss in activity of certain other constituents. This is evident in the case of the broth extract of unwashed cells which has been oxidized by exposure to air at 37°C. (I in Text-fig. 2). Owing to fortuitous conditions during the oxidation of these extracts, oxidizing agents are produced in concentrations sufficient to destroy all of the hemotoxin and most of the sucrase. Nevertheless, in spite of this degree of oxidation, the hemotoxin-free and sucrase-poor extract still manifests its original peptonase and lipase activity. The experiments with different concentrations of hydrogen peroxide did not furnish as sharp distinctions. Possibly analogous distinctions between these enzymes might be obtained by the requisite concentrations of this oxidizing agent, as later experiments have shown that certain more easily oxidized pneumococcus cell constituents are inactivated by concentrations of hydrogen peroxide which are without effect upon the above mentioned enzymes of Pneumococcus.

DISCUSSION.

The sterile bacterial extracts used in this series of studies have been prepared by freezing and thawing anaerobically grown pneumococci. Owing to the method of preparation, the extracts contain all

of the intracellular constituents of Pneumococcus which are liberated by rupture of the cell wall. These extracts, therefore, include not only the oxidation-reduction systems reported in the immediately preceding papers, but also the endocellular hemotoxin and enzymes previously reported from this laboratory.

The literature furnishes evidence that enzymes may be destroyed, *in vitro*, by oxidizing agents similar in nature to those which are formed during the oxidation of broth extracts of unwashed pneumococcus cells. Sterile pneumococcus extracts represent systems containing not only a number of well known cellular enzymes but also reactive substances which form oxidizing agents when exposed to molecular oxygen. There was at hand, therefore, an opportunity to study the destruction of enzymes by oxidizing agents actually formed by constituents of the same cell from which the enzymes have been derived. That oxidizing agents formed by sterile extracts of pneumococci can oxidize hemotoxin, one of the labile cellular substances, has already been reported (Avery and Neill). The present investigation was designed with the object of furnishing examples of the fact that oxidizing agents (presumably of peroxide nature) may be formed from molecular oxygen by bacterial cells, and that these oxidizing substances may in turn destroy other labile, but important cell constituents.

The results of the present investigation reveal the fact that the oxidizing agents formed when the cellular substances are exposed to air do exert a destructive action upon certain of the enzymes of Pneumococcus. Differences are manifest in the resistance of the various enzymes to these oxidation reactions. The carbohydrate-hydrolyzing enzymes (sucrase, amylase, inulase, raffinase) prove the most susceptible to inactivation by oxidation. The bacteriolytic enzyme also suffers a loss in activity, while the peptonase and lipase are more resistant.

Enzymes of Pneumococcus which prove stable when exposed to the air in phosphate solution extracts of washed cells are oxidized during the oxidation of broth extracts of unwashed cells. Apparently, therefore, these enzymes are inactivated by oxidation reactions similar to those previously observed in the case of hemotoxin and hemoglobin. The enzymes seem to be relatively unaffected by molecular

oxygen itself, although they are oxidized by oxidizing agents formed by the action of molecular oxygen upon certain other, easily oxidized substances in the extract. The type of reaction may be represented as follows:

C represents the labile cellular constituent of a pneumococcus oxidation-reduction system; RH, a thermostable substance easily oxidized in the presence of C. It is evident from an analysis of preceding work that C is apparently present in both the "complete" and "incomplete" types of extract, while RH is apparently absent in the latter; that is, from the extract of washed cells.

(1) $[C + RH] + \text{sucrase} + O_2 \rightarrow ROOH + \text{sucrase} \rightarrow$ inactivated sucrase.
(2) $[C] + \text{sucrase} + O_2 \rightarrow$ sucrase not inactivated.

Whether the oxidizing agent is actually a peroxide cannot be demonstrated definitely, although this seems to be the case. In any event, the inactivation of the enzymes is due to oxygen of higher potential which is furnished during the oxidation of other substances in the same extract.

Experiments have also been made on the relative resistance of various pneumococcus enzymes to heat and to the action of reagent hydrogen peroxide. As a whole the results demonstrate that pneumococcus hydrolyzing enzymes may be arranged in the same order of relative stability, as concerns resistance to heat, to the action of hydrogen peroxide, and to the oxidizing agents formed when pneumococcus extracts are exposed to air.

SUMMARY.

1. Certain enzymes of Pneumococcus are destroyed by oxidizing agents formed when sterile extracts of the cellular substances are exposed to air. The carbohydrate-hydrolyzing enzymes (sucrase, raffinase, inulase, and amylase) are the most easily inactivated under these conditions, although the bacteriolytic enzyme is also reduced in activity. Similar treatment is without effect upon the active concentration of pneumococcus lipase and peptonase.

2. The enzymes which are destroyed during the oxidation of unwashed cell extracts are themselves non-reactive with molecular oxygen. The reactions by which they are destroyed seem to represent oxidations of a type similar to those proposed in previous papers for the oxidation of hemotoxin and of hemoglobin.

3. A study has been made of the relative resistance of different pneumococcus enzymes to heat and to the action of hydrogen peroxide.

4. The various enzymes may be arranged in the same order of relative resistance whether the rating be made from the standpoint of resistance to heat or of resistance to oxidation. Nevertheless, it appears that by a proper regulation of conditions of oxidation, certain labile constituents of a mixture of cellular enzymes may be inactivated with less effect upon the activity of other constituents of the mixture than when inactivation is brought about by heat.

CONCLUSION.

When sterile extracts of the cellular substances of Pneumococcus are exposed to air, oxidizing agents are formed which destroy many of the cell enzymes.

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