Enzymatic Action in the Presence of Some Common Antiseptics

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Many diversified studies of the influence of foreign materials on enzymatic action have been made. An attempt to compare the antiseptic properties of various compounds by yeast fermentation seems to have been made first by Dreser[1] in a study of colloidal silver preparations.

The effect of mercury compounds on yeast fermentation was studied in somewhat the same manner by Peterson[2]. As a result of his investigation, he was able to classify the mercurials, using mercuric chloride as a standard, into four groups depending upon their ability to inhibit the fermentative process. He concluded that the inhibitive effect of the yeast sugar fermentation, in most cases, is due to the mercuric ion concentration although, in some cases, other components of the solution were concerned.

By means of a simple device for quantitatively collecting the carbon dioxide produced by yeast fermentation, Branham[3] confirmed the principles of the yeast fermentation method of comparing antiseptics and also showed that the method had a general application.

The purpose of this investigation was three-fold: first, to study the influence of many common antiseptics on yeast sugar fermentation; second, to compare the effect of certain antiseptics on the enzymatic action of zymase with that of catalase, trypsin, and pepsin; and, third, to determine the influence of these antispetics on the action of the above enzymes in the presence of normal horse serum.

Experimental

In Table I are listed the antiseptics used in this investigation, showing their trade or common name, their chemical name, and the manufacturer or distributor from whom the antiseptics were obtained. The enzymes studied were yeast zymase, yeast catalase, pepsin, and trypsin.

The acriflavine, merthiolate, metaphen, pepsin, pancreatin, trypsin maltase, and the normal horse serum were supplied through the courtesy of Eli Lilly and Company, Indianapolis, Indiana.

The general method of procedure was to determine the concentration or quantity of antiseptic required to completely impede the activity of each of the enzymes studied.

Yeast Zymase

Ordinary cakes of Fleishmann's yeast were used in this work, and they were purchased every other day to assure fresh starting material. A 20% solution by weight of yeast was used throughout the study. This

[1]Dreser, 1917. Ztschr. f. Exper. Path. U. Therap. 19:285.
[2]Peterson, 1926. Journ. Amer. Med. Assoc. 87:223.
[3]Branham, 1929. Journ. Infect. Dis. 44:142.

TABLE I.—Antiseptics Used in This Investigation

Trade or Common Name	Chemical Name	Manufacturer or Distributor (U.S.A.)
Alcohol	Ethyl Alcohol	Commercial Solvents Corp.
Acriflavine	3.6 Diamino -10-Methylacridinium Chloride Monohydrochloride	Abbott Laboratories
Argyrol		A. C. Barnes Co.
Boracic Acid	Boric Acid	Coleman and Bell
Blue Vitriol	Copper Sulfate	Coleman and Bell
Carbolic Acid	Phenol	Mallinckrodt Chemical Co.
Corrosive Sublimate	Mercuric Chloride	Coleman and Bell
Ferric Chloride	Ferric Chloride	Coleman and Bell
Glycotanphene		Tannin Products, Inc.
Lavoris		The Lavoris Co.
Listerine		
Mercurochrome	Dibrom-Oxymercuri-Fluorescein	Hynson, Westcott and Dunning, Inc.
Merthiolate	Sodium Ethyl Mercurithiosalicylate	Eli Lilly and Co.
Metaphen	4-Nitro-Anhydro Mercuri Ortho-Cresol	Abbott Laboratories
Neo-Silvol		Parke Davis and Co.
Pepsodent		The Pepsodent Co.
Potassium Permanganate	Potassium Permanganate	Coleman and Bell
Silver Nitrate	Silver Nitrate	Daigger Chemical Co.
S.T. 37	Hexylresorcinol	Sharp and Dohme
Zinc Sulfate	Zinc Sulfate	Coleman and Bell

solution was prepared by dissolving or suspending 40 gm. of the yeast in enough distilled water to make the final volume 200 ml. This yeast suspension was stored in a refrigerator at a temperature of approximately 5° C.

All weighing, accurate to the fourth decimal place, was done on an analytical balance, and all dilutions were made with a burette or pipette or both. All stock solutions were made by dissolving the antiseptic or enzyme in enough distilled water to give the desired concentration. For example, to make a so-called 1-100 solution, one gram of the antiseptic or enzyme was dissolved and diluted with enough distilled water to make the final volume of the solution 1,000 ml.

The test solutions were made up and mixed in the following order: 2 ml. of the desired antiseptic solution, 2 ml. of a 50% sugar solution, 6 ml. of distilled water or serum, 2 ml. of a 20% yeast suspension.

When those antiseptics which did not greatly retard enzymatic action were used, the ratio of the volume of water or serum to the volume of the antiseptic solution varied somewhat, but the total volume of antiseptic solution and water or serum was always maintained at 8 ml. The amount of sucrose solution and yeast suspension was never changed. The above portions were measured from freshly prepared solutions into test tubes by means of a 2 ml. pipette, thoroughly mixed, poured into saccharometer or fermentation tubes and incubated for two hours at a temperature of 38° C., plus or minus one degree.

TABLE II.—A Summary of the Data Obtained in the Study of Yeast Zymase

Antiseptic	End Point in Aqueous Sol. (Concentration)	End Point in a 50% Serum (Concentration)
Alcohol	15.8% (1-6.3)	17.4% (1-5.7)
Acriflavine	1-1200	1-400
Argyrol	1-15	No inhibition
Boric Acid	No inhibition	No inhibition
Copper Sulfate	No inhibition	1-180
Carbolic Acid	1-180	1-150
Corrosive Sublimate	1-18,000	1-6000
Ferric Chloride	No inhibition	No inhibition
Glycontaphen	1-1.7	1-1.5
Lavoris	No inhibition	No inhibition
Listerine	1-3	1-2.4
Mercurochrome	1-90	1-50
Merthiolate	1-240	
Metaphen	1-6000	1-4000
Neo-Silvol	No inhibition	No inhibition
Pepsodent	1-5	1-3
Potassium Permanganate	No inhibition	No inhibition
Silver Nitrate	1-18,000	1-120
S.T. 37	1-3500	1-2400
Zinc Sulfate	No inhibition	1-20

Discussion of data obtained with yeast zymase.—In performing the experiments using alcohol as antiseptic, it was found that, when the final concentration of the alcohol was greater than about 16%, a protein precipitate formed as the serum was added to the alcohol mixture and the quantity of precipitate increased with increasing alcohol concentration. No precipitate was observed in those tubes containing only water.

In both aqueous and serum media, it was found that low concentrations of argyrol enhanced the activity of zymase. In a serum medium, some repression was noticed when the concentration of argyrol was increased to about 1-10. No precipitation was observed in either medium within the limits studied.

A discoloration due to a precipitate occurred in the mixture containing the stronger concentrations of acriflavine and metaphen when the yeast and serum were added. The fact that the yeast gave a decidedly heavier precipitate might be due to the acid characteristics of the yeast.

In aqueous solution, boric acid repressed the yeast sugar fermentation but did not completely inhibit it. Apparently the retardation was proportional to the amount of acid added. In serum medium, however, the reaction started in the fermentation tubes containing boric acid before it did in those tubes containing the acid and those not containing it. In the more dilute solution, 1-100, the boric acid apparently increased the activity; in the tubes containing higher boric acid content, retardation occurred although the reaction was not completely stopped.

It is of interest to note that minimum activity of yeast zymase appeared near a copper sulfate concentration of 1-2,000 although complete inhibition was not obtained. The amount of carbon dioxide evolved in-

creased gradually on both sides of this interval. When the serum was added, precipitation occurred near a concentration of 1-900 and continued through a maximum at a concentration of between 1-60 and 1-200. During the first few minutes the activity was greatest at a concentration of about 1-1,000.

The serum was precipitated by the carbolic acid when its final concentration was made more than 1% with respect to phenol. In all dilutions greater than the end point of the experiment (1-80 for aqueous solution and 1-150 for serum solution), the tubes containing serum reacted first and more rapidly than those which did not contain phenol.

Within the range of concentrations studied, mercuric chloride did not precipitate the proteins of the serum.

Ferric chloride did not completely stop yeast sugar fermentation although for concentrations greater than 1-15 in both aqueous and serum media very little carbon dioxide was given off. In aqueous solutions maximum activity occurred at a final concentration of approximately 1-100. The serum formed a jelly-like mass with the iron which was difficult to remove from the fermentation tubes when the final concentration exceeded 1-50.

There was a slight precipitation when the serum was added to the glycotanphene at a final concentration of 1-2.5. The precipitation increased as the concentration of the antiseptic increased.

Pepsodent and listerine slightly precipitated the serum in concentration from 1-2 or 1-3, but there was no noticeable increase in the precipitation as the concentration of the antiseptics increased.

A curdy, flocculent precipitate formed in all of the fermentation tubes containing mercurochrome with a separation of the precipitate from the more dilute solutions. At a concentration of 1-40 a jelly-like mass was formed. When the serum was added, the precipitation seemed to be more pronounced between the concentrations of 1-60 and 1-120. No precipitation was noted from a solution of a concentration of 1-40.

Neo-Silvol did not inhibit yeast sugar fermentation. All tubes containing the antiseptic, within the concentrations studied, were more active than those which did not contain it.

Experiments using potassium permanganate as antiseptic, with concentrations varying from 1-100 to 1-9,000 in rather small intervals of dilution, were repeated several times. The datum cited in Table II is representative of the whole. Several of the various sets of data showed that the end point in aqueous solution is near 1-1,800; other sets seemed to be more active, the greater the concentration of salt. After 30 minutes, there was approximately two times as much carbon dioxide in the fermentation tube containing a concentration of 1-500 than there was in those tubes containing no antiseptic. There was more or less precipitation of manganese dioxide, increasing with the concentration in all dilutions. When serum was added to the potassium permanganate, precipitation occurred. The extent of precipitation increased with the antiseptic concentration in all tubes having a concentration of salt greater than 1-600. Several trials were made using solutions having a final strength varying from 1-100 to 1-500, and it was found that the tubes containing the salt were more active than those which did not con-

tain it. After 45 minutes, the tube containing a concentration of 1-600 had approximately twice as much gas in it as those containing no potassium permanganate, and the activity seemed to increase with increasing salt concentration.

Silver nitrate began precipitating the proteins of the serum in a concentration of 1-15,000 with respect to the salt, and the amount of precipitation increased as the concentration of silver nitrate increased. The degree of inhibition was also proportional to the amount of salt present.

Zinc sulfate was studied from a concentration of 1-4 to 1-15,000 inclusive. As the concentration of salt increased, the activity also increased. Within the range of the concentrations studied, the tubes containing zinc were more active than those which did not contain zinc. When the serum was added, precipitation started at a concentration of about 1-1,000 and increased as the zinc increased through a maximum near a concentration of 1-150 with no immediate precipitate from a solution of 1-15 with respect to zinc sulfate.

From the summary of the data obtained in the study of yeast zymase, Table II indicates, with the exception of zinc sulfate and copper sulfate, that more antiseptic is necessary to produce complete inhibition in a 50% solution of normal horse serum than is required to produce complete inhibition in an aqueous solution. Whether the serum increases the activity of the yeast zymase or decreases the activity of the antiseptic or both is problematical. Those antiseptics for which no end point was reached generally showed a greater activity in serum when the same concentrations were used in both aqueous and serum media.

Catalase

The general method of procedure was to determine the concentration or quantity of antiseptic required to completely inhibit the liberation of molecular oxygen from hydrogen peroxide by yeast catalase.

The yeast used was the ordinary small cakes made by the Fleischmann Yeast Company and secured every other day to assure fresh working suspensions. Two per cent solutions by weight were used throughout the study of catalase. It was made by suspending 4 gm. of yeast in enough distilled water to make the final volume 200 ml. This suspension was stored in a refrigerator at a temperature of approximately 5° C.

The following method of procedure was followed throughout the study of catalase. The desired amount of antiseptic was introduced into a test tube together with 2 ml. of a 2% yeast suspension and enough distilled water or serum added to make the final volume 10 ml. The tube content was then thoroughly agitated. After this mixture had been incubated for two hours at room temperature (about 23° C.), 2 ml. of 0.3N hydrogen peroxide was added. After mixing, the contents of each test tube were poured into a fermentation tube and incubated at room temperature for another hour. The smallest amount of antiseptic required to prevent the liberation of oxygen from the hydrogen peroxide was considered the end point of the experiment.

The antiseptic solutions used in the study of catalase were prepared in the same way as those used in the study of yeast zymase.

The hydrogen peroxide used was commercial peroxide manufactured by Coleman and Bell. A 0.3N solution was employed to determine whether or not the catalase had been destroyed. It was prepared by first standardizing a potassium permanganate solution and then titrating this solution against the unknown hydrogen peroxide solution. After determining the normality of the peroxide, the proper dilution was made by means of a burette.

The test solutions were made up and mixed in the following order: 2 ml. of the desired antiseptic solution, 2 ml. of a 2% yeast suspension, 6 ml. of distilled water or serum.

When those antiseptics which did not greatly retard enzymatic action were used, the ratio of the volume of water or serum to the volume of the antiseptic solution varied somewhat, but the total volume of antiseptic and water or serum was always maintained at 8 ml. The concentration and volume of yeast suspension never changed.

Discussion of data obtained with catalase.—Acriflavine was studied within concentration intervals of from 1-50 to 1-1,500 inclusive. In Table III is recorded a summary of the data obtained in this study of yeast catalase. In aqueous solution, all of the concentrations evolved more oxygen than samples which did not contain any antiseptic. Qualitatively, the greater the concentration of acriflavine, the greater was the evolution of oxygen. In the more concentrated solutions, however, the amount of gas given off seemed to become more constant. When serum was added, precipitation occurred in all tubes containing acriflavine. Concentrations contained about the same amount of oxygen as the tubes containing no antiseptic.

TABLE III.—A Summary of the Data Obtained in the Study of Yeast Catalase

Antiseptic	End Point in Aqueous Sol.	End Point in Serum Sol.
Acriflavine	No inhibition	No inhibition
Alcohol	No inhibition	No inhibition
Argyrol	No inhibition	No inhibition
Carbolic Acid	1-50	1-50
Mercuric Chloride	1-1000	1-150
Metaphen	No inhibition	No inhibition
Neo-Silvol	No inhibition	No inhibition
Silver Nitrate	1-6250	1-70
S.T. 37	1-1250	No inhibition

In all concentrations studied below 60%, alcohol catalyzed the liberation of oxygen from hydrogen peroxide. Maximum activity appeared to be between 20% and 40% alcohol with a gradual decrease on both sides of this interval. When the serum was added, precipitation started near a concentration of 40% and increased as the amount of alcohol increased. Alcohol accelerated the reaction between the concentrations of 5% and 60% with maximum activity near 25% alcohol. Concentrations above 60% were less active than those tubes containing no alcohol.

All tubes containing argyrol were very active, denoting that the

colloidal silver which it contains promoted the liberation of oxygen from hydrogen peroxide. The reaction proceeded so rapidly, in aqueous solution, that it was practically complete before the solution could be transferred from the test tubes to the fermentation tubes. However, the argyrol was less active in serum medium than it was in aqueous solution. As the concentration of serum decreased and the concentration of argyrol increased, the activity increased accordingly. In a concentration of 10% argyrol and 40% serum, the reaction occurred almost instantaneously.

In aqueous solution, phenol acted catalytically between the concentrations of 1-75 and 1-1,000, the limits of this study. Maximum activity seemed to occur near a final concentration of 1-150. In serum medium, precipitation started at a concentration of approximately 1-75 and increased as the amount of phenol increased.

Mercuric chloride was studied in rather small intervals of concentration between dilutions of from 1-125 to 1-10,000. It was found, in aqueous solutions, that the salt increased the rate at which oxygen was liberated in solutions having concentrations between 1-4,500 and 1-10,000. In solutions stronger than 1-4,500, inhibition gradually increased with increasing concentration. The reaction was completely stopped near a concentration of 1-1,000. In serum medium, precipitation occurred with a bichloride concentration of about 1-750 and increased as the amount of salt was increased. Again the antiseptic increased the activity between the concentration 1-500 and 1-10,000.

In all concentrations of metaphen studied, from 1-625 to 1-10,000, inhibition was not observed. In aqueous solution, all concentrations within this interval liberated more oxygen than those tubes containing no metaphen. It appeared that there was a small increase in the amount of oxygen near a final concentration of 1-2,000. In serum medium, all tubes containing metaphen as well as those tubes not containing metaphen evolved about the same amount of oxygen.

In aqueous medium, the volume of oxygen increased as the concentration of neo-silvol increased between the concentrations 1-7 and 1-300. In a serum medium, the volume of oxygen remained fairly constant throughout this interval.

Silver nitrate, in aqueous solution, did not accelerate the reaction within the limits of the concentrations studied. Beginning with the most dilute concentration of the salt, 1-30,000, the volume of the oxygen liberated became less as the concentration of silver nitrate increased. In a serum medium, precipitation occurred near a concentration of 1-1,600 and increased as the amount of silver increased. In a serum medium, precipitation occurred near a concentration of 1-1,600 and increased as the amount of silver increased. All tubes containing serum with a dilution of antiseptic greater than 1-50 liberated about the same volume of oxygen after one hour as those tubes having no antiseptic. Those tubes containing silver nitrate seemed to react more rapidly at the beginning.

The activity of all concentrations of hexylresorcinol between 1-2,000 and 1-20,000 seemed to be fairly constant although in aqueous solution there did seem to be a slight increase in the volume of oxygen near a concentration of 1-2,500.

A review of the data obtained in the study of catalase, Table III, indicates, with the exception of carbolic acid, that more antiseptic is necessary to produce complete inhibition in an approximately 60% solution of normal horse serum than is required to produce complete inhibition in an aqueous solution. The same was observed in the study of yeast zymase, Table II.

Pepsin

The pepsin used in this study was of the soluble type and furnished through the courtesy of Eli Lilly and Company, Indianapolis, Indiana. The solutions were prepared daily and stored in a refrigerator at a temperature of approximately 5° C. A 2% solution was used throughout which was made by dissolving 4 gm. of pepsin in sufficient distilled water to make the final volume 200 ml.

The other solutions used with pepsin were prepared in a manner similar to that employed in the study of yeast zymase and catalase.

Briefly, the general method of procedure consisted in determining the smallest amount of antiseptic that would prevent the digestion of "red" blood fibrin during a period of three hours.

The actual test solutions were prepared by mixing 2 ml. of the desired concentration of antiseptic, 4 ml. of distilled water, 2 ml. of buffer mixture, 2 ml. of a 2% solution of pepsin, and finally a few small pieces of "red" blood fibrin. The mixtures were then incubated for three hours at 38° C. The above portions were measured from freshly prepared solutions into test tubes by means of a pipette.

The buffer mixture was prepared according to the tables of Clark and Lubs by mixing 20.75 ml. of 0.2 N hydrochloric acid and 25 ml. of 0.2 N potassium chloride with enough distilled water to make 100 ml. This solution has a pH of 1.4.

The blood fibrin was dyed in a 2% aqueous solution of amaranth red for 30 minutes. All excess dye was then thoroughly removed by washing with water whose acidity had been previously adjusted to a pH of 3.0 with hydrochloric acid. This gave a red fibrin whose red color would not come out in acid media. Even the slightest digestion of the fibrin was made apparent by a noticeable red color in the test tubes. The dye and blood fibrin were obtained from Coleman and Bell, Norwood, Ohio.

When those antiseptics which did not greatly retard enzymatic action were used, the ratio of the volume of water to the volume of the antiseptic varied, but the total volume of the two was always maintained at 6 ml. Mercuric chloride and hexylresorcinol were the only antiseptics studied with pepsin.

A review of the data obtained in the study of pepsin indicated that for mercuric chloride the end point, in aqueous solution, is near a final concentration of 1-250. Hexylresorcinol was found not to inhibit pepsin activity.

Again it appeared that, in certain definite concentrations, mercuric chloride had some accelerating action. Apparently, more digestion of the fibrin took place near a concentration of 1-1,000 than at any other concentrations.

Trypsin

The trypsin used was manufactured by Fairchild Brothers and Foster and was furnished through the courtesy of Eli Lilly and Company A 2% solution was prepared daily by suspending 4 gm. of trypsin in sufficient distilled water to bring the volume to 200 ml. The suspension was stored in the refrigerator.

Briefly, the general method of procedure consisted in determining the smallest amount of antiseptic that would prevent the digestion of "blue" blood fibrin during a period of three hours.

The actual test solutions were prepared by mixing 2 ml. of the desired concentration of antiseptic, 4 ml. of distilled water, 2 ml. of buffer mixture, 2 ml. of a 2% solution of trypsin, and finally a few small pieces of "blue" blood fibrin. The solutions were then incubated for three hours at 38° C. The above portions were measured from freshly prepared solutions into test tubes by means of a pipette.

The buffer solutions were prepared according to the table of Clark and Lubs by mixing 46.8 ml. of 0.1 N sodium hydroxide and 50 ml. of 0.1 molar monopotassium phosphate with distilled water to make 100 ml. This solution has a pH of 8.

The blood fibrin was dyed in a 2% solution of aniline blue for 30 minutes. All excess dye was then thoroughly removed by washing with water whose basidity had been previously adjusted to a pH of 8 with ammonium hydroxide. This gave a blue fibrin whose blue color would not come out in basic media. Even the slightest digestion of the fibrin was made apparent by a noticeable blue color in the test tubes.

Phenol was the only antiseptic studied in connection with trypsin. A review of the data obtained in the study of trypsin indicates that for phenol the end point, in aqueous solution, is near a final concentration of 1-50.

Summary

A comprehensive and diversified study has been made concerning the influence of antiseptics upon enzymatic action.

The influence of alcohol, acriflavine, argyrol, boric acid, copper sulfate, carbolic acid, corrosive sublimate, ferric chloride, glycotanphene, lavoris, listerine, mercurochrome, merthiolate, neo-silvol, pepsodent, potassium permanganate, silver nitrate, hexylresorcinol, and zinc sulfate on yeast sugar fermentation, in aqueous media and in the presence of normal horse serum, has been studied. In all instances, with the exception of zinc sulfate and copper sulfate, it was found that more antiseptic was required to inhibit carbon dioxide formation in the presence of normal horse serum than was required to inhibit carbon dioxide formation in aqueous solution. The order in which the antiseptics prevented yeast sugar fermentation is given in descending order: mercuric chloride, silver nitrate, metaphen, hexylresorcinol, acriflavine, merthiolate, carbolic acid, mercurochrome, etc.

In the study of the influence of acriflavine, alcohol, argyrol, carbolic acid, mercuric chlorine, metaphen, neo-silvol, silver nitrate, and hexylresorcinol on catalase, in aqueous solution and in the presence of normal

horse serum, it was found, also, that, with the exception of carbolic acid, more antiseptic was required to prevent the liberation of molecular oxygen from hydrogen peroxide by the catalase in serum media than was required to prevent the liberation of oxygen from hydrogen peroxide by the catalase in aqueous solution. It was particularly observed that argyrol was much more active in an aqueous medium than in a serum medium.

In the study of the influence of mercuric chloride and hexylresorcinol on pepsin, it was found that much more antiseptic was required to enhance the action of pepsin than was required to inhibit the action of the other enzymes studied. A mercuric chloride concentration of 1-18,000 prevented yeast sugar fermentation; a concentration of 1-1,000 was required to stop the action of yeast catalase. It required a bichloride concentration of about 1-300 to inhibit the action of pepsin.

It was found that a carbolic acid concentration of 1-50 was necessary to prevent the digestive function of trypsin. The same concentration inhibited the liberation of oxygen from hydrogen peroxide; a concentration of 1-180 was required to stop yeast sugar fermentation.

From a survey of the data, it will be noted that, with two or three exceptions, much more antiseptic was required to inhibit the action of the enzymes in a serum medium than was required to prevent the action in an aqueous medium.

As a general rule, the antiseptics studied in this paper had a catalytic or promoting effect within a certain definite range of concentrations, and this effect was different for each enzyme.