GROWTH-INHIBITORY SUBSTANCES IN PNEUMOCOCCUS CULTURES.

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INTRODUCTION.

In the study of bacterial symbiosis and antagonism, considerable interest has centered in the presence or absence of growth when organisms are reinoculated into filtrates of cultures of the same or different species. The nature of the substances which favor or inhibit the simultaneous or subsequent development of bacteria in the same nutritive medium has provoked much discussion and stimulated considerable research. The inhibitory influence which different microorganisms exert on one another has been ascribed, for the most part, to an altered and unfavorable reaction brought about in the substrate by growth, or to exhaustion of the medium by the dominant species, these two factors either singly or together resulting in autoinhibition or in the suppression of the more susceptible variety. In addition, it has been assumed that certain deleterious waste products accumulate as a result of bacterial metabolism which react unfavorably on subsequent growth, or even cause mutual inhibition of both the homologous and heterologous organisms.

In the case of pneumococcus it is known that the reaction of the medium is of great importance (1, 2). It has been shown that growth cannot be initiated in plain broth more acid than pH 7, or more alkaline than pH 8.3, and that the optimum reaction for growth is pH 7.8. When pneumococcus is grown in broth containing a readily fermentable sugar, as dextrose, the reaction becomes more acid, due, in part at least, to the fermentation of the sugar. When the accumulation of acid has proceeded to the point pH 5, growth ceases. However, if the filtrate of a dextrose broth culture is readjusted to pH 7.8, and again seeded with pneumococci, growth will occur. It is evident, then, that the production of increased acidity is sufficient, in itself, to inhibit growth eventually. Acid production is, therefore, one factor in the autoinhibition of growth. On the other hand, when bacteria-

free filtrates of plain broth cultures are readjusted to the optimum reaction for growth (pH 7.8) and reseeded with pneumococcus, no growth occurs. If, however, to this same filtrate a small amount of sugar, as dextrose, is added, multiplication again occurs. Moreover, it has been found in the course of the present study that when a mixture of one part of broth and nine parts of pneumococcus culture filtrate is reseeded with pneumococcus, the organisms will grow. The addition, then, of 10 per cent of unmetabolized or "fresh" broth to a pneumococcus culture filtrate suffices to restore to the exhausted medium some substance essential for growth. Since this reactivation of a culture filtrate is effected by the addition of dextrose as well as by plain broth, this action is evidently due to the presence of some readily utilizable substance, possibly of the nature of a carbohydrate. These facts indicate that in addition to acid production, still another factor is concerned in the mechanism of autoinhibition; namely, the exhaustion of nutritive material in the medium.

Certain active substances contained in the cell bodies of pneumococci play an important rôle in the metabolism of these organisms. It has been shown that, in addition to the endohemotoxin, described by Cole (3), pneumococci contain a group of intracellular enzymes which hydrolyze peptones, split fats, and cause hydrolysis of saccharose, starch, and inulin (4–6). The action of these intracellular enzymes of pneumococci leads to the accumulation of metabolic products in the culture fluid, which may inhibit further growth. The action of the proteolytic enzyme of pneumococcus upon the peptone of the culture fluid results in the production of amino acids (7). The more active the growth of the culture, within certain limits, the greater is the concentration of amino acids in the culture fluid. The action of esterase, a lipolytic enzyme of pneumococcus, results in the breaking down of the esters to fatty acids. Finally, the carbohydrate-splitting enzymes are known to bring about the hydrolysis of such substances as sucrose, starch, and inulin to simpler sugars; the subsequent fermentation of these sugars results in the production of acid.

Recently attention has been directed to a hitherto unrecognized inhibitory product of pneumococcus growth, which accumulates in media under certain cultural conditions. This substance is a peroxide. In 1921, McLeod and Govenlock (8) reported the occurrence of a heat-labile substance in pneumococcus cultures which inhibited the further growth of this and other organisms. This substance was later identified as hydrogen peroxide by McLeod and Gordon[1] (9, 10).

[1] The evidence presented by these investigators is strongly in favor of the view that this substance is hydrogen peroxide rather than some organic peroxide. In the present work no attempt to determine the chemical identity of this peroxide has been made beyond the observations that it reacts with benzidine in the presence of a catalyst and that it is heat-labile and decomposed by the oxidases of plant tissue.

To the action of this compound they ascribe the pigment changes brought about in blood by pneumococcus. These observers found that hydrogen peroxide is also formed by other bacteria of the lactic acid group.

In a preceding paper (11), it has been shown that under favorable conditions peroxide appears in broth cultures of pneumococcus during the logarithmic phase of growth and that it persists for many days. The factors favoring peroxide production and accumulation are abundant oxygen supply and the absence of catalase. The peroxide behaves in many ways like hydrogen peroxide. It is unstable, sensitive to heat, and decomposes rapidly in alkaline solutions. Under certain conditions peroxide-containing filtrates of pneumococcus cultures possess the further property of converting oxyhemoglobin into methemoglobin.[1]

In the present paper are recorded the facts thus far ascertained in the study of the inhibition of growth of homologous and heterologous organisms by the peroxide formed by pneumococcus; and certain other factors concerned in the mechanism of growth inhibition of pneumococcus are discussed.

EXPERIMENTAL.

The accumulation of acid and the exhaustion of the medium are conditions brought about by growth of pneumococcus. Either of these conditions may so alter the medium as to render it unsuitable for further growth. The study of the inhibitory effect of hydrogen peroxide upon growth must, therefore, be approached indirectly so that its action may not be confused with that of other inhibitory factors which may develop simultaneously in the medium. This was done by making a parallel study of the effect of hydrogen peroxide upon the growth of *Staphylococcus aureus*, an organism not so susceptible as pneumococcus to the acid and exhaustion factors of growth inhibition. The growth of pneumococcus and *Staphylococcus aureus* in broth and in pneumococcus culture filtrates containing peroxide was therefore observed.

The Inhibitory Action of Commercial Hydrogen Peroxide on Growth of Staphylococcus aureus and Pneumococcus.

The effect upon growth of the addition of small amounts of commercial hydrogen peroxide[2] to broth was first investigated.

Experiment 1.—Varying quantities of a 1:100 solution of commercial hydrogen peroxide were added to known amounts of plain broth. The concentration of peroxide in the broth ranged from 1:300 to 1:1,500. Each dilution was tested in the manner described[3] for the presence of hydrogen peroxide. 5 cc. portions of the broth containing hydrogen peroxide in varying dilutions were tubed and seeded with one loop of an 8 hour broth culture of *Staphylococcus aureus*. A similar series was also inoculated with 0.05 cc. of an 8 hour broth culture of Pneumococcus Type II. The cultures were incubated at 37°C. and were observed for the presence or absence of macroscopic growth at the end of 24 and 48 hours. The results are given in Table I.

In Table I it is seen that 3 per cent hydrogen peroxide in plain broth, in dilutions of 1:1,200, inhibits the growth of *Staphylococcus aureus* for 24 hours. In concentrations of hydrogen peroxide of 1:1,000 or greater, no growth of *Staphylococcus aureus* occurred within the 48 hour period of observation. The inhibitory effect of hydrogen peroxide upon growth of pneumococcus in plain broth is not so pronounced. 3 per cent hydrogen peroxide in dilutions 1:400 inhibits the growth of pneumococcus for 24 hours. In concentrations of 1:300 no growth of pneumococcus occurred.

Since the inhibition of growth of *Staphylococcus aureus* and of pneumococcus in filtrates of pneumococcus cultures was to be investigated, and this with reference to the presence of preformed peroxide in the culture filtrates, it seemed advisable to determine the effect of adding known quantities of commercial hydrogen peroxide to filtrates of pneumococcus cultures in which peroxide had not formed. This was done in Experiment 2.

[2] Hydrogen peroxide Merck, U.S.P. IX, was the reagent used in all experiments. On titration this preparation contained 3 per cent hydrogen peroxide.

[3] Peroxide test: 0.5 cc. of fluid to be tested is placed in an agglutination tube containing a piece of fresh plant tissue (potato). 2 drops of freshly prepared saturated solution of benzidine in glacial acetic acid are added. The appearance of a blue color on the potato or in the surrounding fluid indicates the presence of peroxide (11).

Experiment 2.—150 cc. of broth were seeded with 0.05 cc. of an 8 hour plain broth culture of Pneumococcus Type II. Owing to the cultural conditions which limited the free access of air the accumulation of peroxide was inhibited so that at the end of 24 hours only a small amount could be detected. The culture was then passed through a Berkefeld filter. The filtrate was readjusted to pH 7.8. The readjusted filtrate was autoclaved to destroy the remaining trace of preformed peroxide. A 5 cc. fraction of this was seeded with 0.05 cc. of a broth culture of Pneumococcus Type II. To portions of the autoclaved filtrate varying amounts of 1:100 commercial (3 per cent) hydrogen peroxide were added. 5 cc. quantities of the filtrate containing these known concentrations of peroxide were then seeded with one loop of an 8 hour broth culture of *Staphylococcus aureus.*

TABLE I.

Inhibition of Growth of Staphylococcus aureus and Pneumococcus by Commercial Hydrogen Peroxide.

Dilution of 3 per cent H_2O_2 in broth.	Benzidine test for H_2O_2.	Growth of *Staphylococcus aureus.*		Growth of Pneumococcus Type II.	
		24 hrs.	48 hrs.	24 hrs.	48 hrs.
1:300	+	0	0	0	0
1:400	+	0	0	0	+
1:500	+	0	0	0	+
1:600	+	0	0	+	+
1:700	+	0	0	+	+
1:800	+	0	0	+	+
1:900	+	0	0	+	+
1:1,000	+	0	0	+	+
1:1,200	±	0	+	+	+
1:1,500	±	+	+	+	+
Broth without H_2O_2.	0	+	+	+	+

Frequent observations were made during incubation to determine the presence or absence of macroscopic evidence of growth. The results are given in Table II.

From Table II it is evident that pneumococcus failed to grow when seeded into the homologous culture filtrate, even in the absence of peroxide. The failure of growth of pneumococcus under these conditions is therefore dependent upon factors other than peroxide. These factors have been referred to previously in this paper. The presence of small amounts of commercial hydrogen peroxide prevents growth of *Staphylococcus aureus* when this organism is seeded into

filtrates of pneumococcus cultures. A filtrate containing as little as 1:1,750 of 3 per cent hydrogen peroxide will inhibit growth of *Staphylococcus aureus* for 48 hours. As was shown in Experiment 1, in which the same lot of broth, unmetabolized, was used, a concentration of 1:1,000 of 3 per cent hydrogen peroxide is reached before this growth-inhibitory action upon staphylococcus is evident. It seems likely that this difference between metabolized and unmetabolized broth is due to the absence of certain nutritive elements in the former.

From Experiments 1 and 2 it seems clear that even small amounts of commercial hydrogen peroxide, when added to plain broth or

TABLE II.

Inhibition of Growth of Staphylococcus aureus in Pneumococcus Culture Filtrates by Commercial Hydrogen Peroxide.

Dilution of 3 per cent H_2O_2 in filtrate.	Growth of Staphylococcus R.					Growth of Pneumococcus Type II.
	12 hrs.	24 hrs.	48 hrs.	72 hrs.	6 days.	
1:1,000	0	0	0	0	0	0
1:1,200	0	0	0	0	0	0
1:1,500	0	0	0	0	0	0
1:1,750	0	0	0	++	++++	0
1:2,000	0	0	+	++++	++++	0
1:2,400	0	0	+++	++++	++++	0
1:3,000	0	+	++++	++++	++++	0
1:4,000	0	++++	++++	++++	++++	0
1:6,800	++++	++++	++++	++++	++++	0
Filtrate without H_2O_2.	++++	++++	++++	++++	++++	0

filtrates of pneumococcus cultures, exert an inhibitory action upon the growth of *Staphylococcus aureus*. On the other hand, pneumococcus grows in the presence of considerably higher concentrations of commercial hydrogen peroxide in broth than does staphylococcus, but fails to grow when reseeded into homologous culture filtrates, even in the absence of peroxide.

The Inhibitory Action of Peroxide Formed by Pneumococcus upon Growth of Staphylococcus aureus and Pneumococcus.

In order to determine whether or not the peroxide which develops in cultures of pneumococcus is concerned in the phenomenon of in-

hibition of growth which occurs when the same or other organisms are seeded into pneumococcus culture filtrates, the following experiment was performed.

Experiment 3.—0.5 cc. of a 6 hour plain broth culture of Pneumococcus Type II was seeded into 450 cc. of broth contained in a large Erlenmeyer flask. At the end of 27 hours incubation, a portion of the culture fluid was passed through a Berkefeld filter. The filtrate was readjusted to pH 7.8. The peroxide test was found to be positive. The filtrate was then divided. One part was autoclaved (120°C. for 10 minutes) in order to destroy the peroxide, and the other part left unheated. 5 cc. portions of the autoclaved and unautoclaved filtrates were seeded with 6 hour plain broth cultures of Pneumococcus Type II and *Staphylococcus aureus*, respectively. The inoculum, in the case of the pneumococcus, was 0.05 cc., and in the case of the staphylococcus, one 2 mm. loop. The specimens were incubated and observed first after 10 hours incubation, and then daily for 5 days. The results are given in Table III.

TABLE III.

Inhibition of Growth of Staphylococcus aureus and Pneumococcus in Heated (120°C. for 10 Minutes) and Unheated Filtrates of Pneumococcus Cultures.

Organism.	Growth in unheated filtrate.		Growth in autoclaved filtrate.	
	10 hrs.	5 days.	10 hrs.	5 days.
*Staphylococcus aureus.**	0	0	++	++++
Pneumococcus Type II.*	0	0	0	0

*When 5 cc. tubes of broth were seeded with these organisms, the inoculum being that used in Experiment 3, abundant growth occurred in 10 hours in each instance. In the case of the staphylococcus, the broth culture was distinctly heavier than that obtained when the autoclaved filtrate was inoculated with this organism.

This experiment shows that when staphylococcus is seeded into the filtrate of a pneumococcus culture containing preformed peroxide, no growth occurs. However, if such a filtrate is autoclaved prior to inoculation so that the peroxide is destroyed, good growth of staphylococcus is obtained within 10 hours. On the other hand, as was shown in the previous experiment, when pneumococcus is reseeded into the filtrate of pneumococcus culture, little, if any, growth occurs, regardless of the presence or absence of peroxide.

If the inability of staphylococcus to grow in the filtrate of a pneumococcus culture is due to the presence of peroxide, those filtrates which contain no peroxide should support growth of this organism. The

following experiment with filtrates of cultures in which the organisms grew under conditions such that peroxide could not form, bears upon this point. A comparative study was made of the growth of *Staphylococcus aureus* in a peroxide-containing filtrate and in similar filtrates in which, however, the accumulation of peroxide was prevented by cultural conditions.

Experiment 4.—50 cc. of plain broth in a 250 cc. Erlenmeyer flask were inoculated with 0.25 cc. of an 8 hour culture of Pneumococcus Type II. Another flask containing 50 cc. of 1 per cent dextrose broth was similarly inoculated. These two flasks were incubated at 37°C. under aerobic conditions. A third flask containing 50 cc. of plain broth was inoculated with 0.25 cc. of an 8 hour culture of Pneumococcus Type II, and incubated at 37°C. under anaerobic conditions (Brown's modified anaerobic jar). At the end of 18 hours the three cultures were filtered through Berkefeld candles. Tests for peroxide were performed upon the filtrates. 5 cc. portions of each filtrate were then tubed and seeded with one loop of actively growing broth culture of *Staphylococcus aureus*. At the end of 18 hours incubation the presence or absence of growth was noted. The results are given in Table IV.

TABLE IV.

Inhibition of Growth of Staphylococcus aureus in Unheated Filtrates of Pneumococcus Cultures in the Presence and Absence of Peroxide.

5 cc. of unheated filtrate of Pneumococcus Type II culture.	Peroxide test after 18 hrs. at 37°C.	Growth of staphylococcus in 18 hrs.
Plain broth (aerobic culture)	+	0
" " (anaerobic ")	0	+
1 per cent dextrose broth (aerobic culture)	0	+

The experiment shows that *Staphylococcus aureus* will grow in the unheated filtrates of pneumococcus cultures in which peroxide is not present in detectable amounts. In the filtrate of the anaerobic culture, in which the formation of peroxide was prevented by the exclusion of air, and in the filtrate of the dextrose broth culture, growth of *Staphylococcus aureus* occurred. On the other hand, in the filtrate of the aerobic cultures in which peroxide was demonstrated by the benzidine reaction, growth did not occur.

The Nature of the Inhibitory Action of Peroxide upon Staphylococcus.

The bacteriostatic influence of commercial hydrogen peroxide is strikingly shown in Experiment 2 (Table II). It is well known that

this substance is also bactericidal. In previous experiments the inhibitory action of the peroxide of bacterial origin has been demonstrated. It seemed of interest, therefore, to determine whether the peroxide formed by pneumococcus possesses bactericidal as well as bacteriostatic properties.

Experiment 5.—Pneumococcus Type II was grown in broth under conditions such that the ratio of surface area to total volume of culture fluid was large. These conditions were optimum for the production of peroxide (11). Fractions of the culture were removed after 13, 16, 19, and 27 hour periods of incubation, and passed through a Berkefeld filter. The filtrates thus obtained were readjusted to pH 7.8 by the addition of N/10 alkali. 5 cc. quantities of these filtrates containing peroxide were then inoculated with one loop of an actively growing broth

TABLE V.

The Bacteriostatic and Bactericidal Action on Staphylococcus aureus of the Peroxide of Pneumococcus Culture Filtrates.

Filtrate of pneumococcus culture after incubation at 37°C.	Peroxide test immediately after filtration.	Inoculum of *Staphylococcus aureus.*	Growth of *Staphylococcus aureus* after 7 days of incubation.	Peroxide test after 7 days of incubation.	Second inoculum of *Staphylococcus aureus.*	Growth of *Staphylococcus aureus.*		
						18 hrs.	42 hrs.	96 hrs.
hrs.								
13	+	1 loop.	0	0	1 loop.	+	+	+
16	+	1 "	0	0	1 "	+	+	+
19	+	1 "	0	Faint +	1 "	0	0	+
27	+	1 "	0	"	1 "	0	+	+

culture of *Staphylococcus aureus.* At the end of 7 days there was no evidence of growth. And subcultures on blood agar showed that no viable organisms were present. In these filtrates, in which the staphylococcus had not only failed to grow, but had been killed, peroxide was still present in demonstrable amounts in two instances. It is of interest to note that the filtrates in which peroxide persisted over a period of 7 days represented a fraction of the original culture after longer periods of growth and in which consequently the concentration of peroxide was presumably greater. The absence of peroxide in the other two filtrates is attributable to the destruction of this substance in culture fluids. The factors influencing the decomposition of this substance in media have been referred to in a preceding paper (11). The four filtrates, in all of which *Staphylococcus aureus* had previously failed to grow, and in two of which traces of peroxide were still demonstrable, were a second time inoculated with the same strain of *Staphylococcus aureus.* Growth promptly occurred in the two filtrates in which peroxide had

disappeared. In the other two filtrates, in which traces of peroxide still persisted, growth was delayed, and occurred only after a considerable period of lag. The results are recorded in Table V.

Experiment 5 shows that the peroxide in pneumococcus filtrates is not only bacteriostatic for *Staphylococcus aureus*, *i.e.* prevents cell multiplication, but also bactericidal. That the peroxide is actually the inhibitory agent is further shown by the fact that two of these same filtrates which, during the 7 days of incubation, had become peroxide-free, supported prompt and abundant growth of staphylococcus when again inoculated with this organism. In the two instances in which peroxide still persisted a considerable period of lag preceded active growth.

DISCUSSION.

The original studies of McLeod and his associates on the production of hydrogen peroxide by bacteria, and the subsequent confirmation by Avery and Morgan of the factors influencing the formation of peroxide by pneumococcus have led to the present observations on the nature of the phenomenon of growth inhibition in culture filtrates of pneumococcus. In an earlier publication it was pointed out that bacteria-free filtrates of *plain* broth cultures of this organism, even though readjusted to the optimal reaction, will not again support growth of pneumococcus unless small amounts of sugar (dextrose) are added. On the other hand, it was found that filtrates of *dextrose* broth cultures of pneumococcus, under optimal conditions of reaction, apparently contain sufficient unutilized carbohydrate to permit growth on reinoculation of the medium. The ability of pneumococci to multiply in a medium in which they have previously grown, was at that time thought to be dependent, in part at least, upon the maintenance of a suitable reaction and upon the presence of a residuum of fermentable substance left unmetabolized by previous growth.

The knowledge that pneumococci form peroxide and that this compound can be demonstrated in culture fluids, adds another factor in explanation of the phenomenon of autoinhibition of growth. The diffusion into the medium of this inhibitory substance is sufficient, in some instances at least, to account for the inability of certain

organisms to grow in pneumococcus filtrates. As pointed out in the present paper, growth of staphylococcus cannot be initiated in peroxide-containing filtrates of pneumococcus cultures. Evidence that peroxide formed during the growth is itself solely responsible for the lack of the subsequent growth of *Staphylococcus aureus* is found in the fact that staphylococci will grow in these same culture fluids after the peroxide has been destroyed by heat or deteriorated through age. Furthermore, staphylococci grow abundantly in filtrates of pneumococcus cultures in which the formation of peroxide has been prevented. In the case of *Staphylococcus aureus* the presence of peroxide is, therefore, the determining factor in the inhibition of growth, and the other two factors already referred to, namely altered reaction and exhaustion of the medium, are little, if at all, concerned in the inhibitory mechanism. In the case of pneumococcus, however, growth inhibition of this organism in its own culture fluid is conditioned primarily by the exhaustion of nutritive material and by reaction changes in the medium, and only secondarily by the presence of peroxide, since in readjusted culture filtrates, even in the absence of peroxide, a second growth of pneumococci cannot be initiated unless some readily utilizable nutritive material is added.

CONCLUSIONS.

The known factors concerned in the autoinhibition of growth of pneumococcus are:

1. The accumulation of acid products of metabolism, resulting in unfavorable reaction changes in the medium.
2. The exhaustion of the nutritive substances of the medium.
3. Under certain cultural conditions the formation and accumulation of peroxide in the medium.

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