STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

II. The Production of Peroxide by Sterile Extracts of Pneumococcus.

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In the preceding paper (1) it was shown that pneumococci are able to form peroxide within pH zones and temperature limits, and under other conditions, that do not permit active cell growth and multiplication. The present paper is concerned with the production of peroxide by sterile extracts prepared by freezing and thawing the pneumococcus cell; most of these preparations were filtered through Berkefeld filters and all of them were proved sterile by cultural and animal tests. The following report, therefore, represents a study of peroxide production by sterile extracts of the cellular substances of pneumococcus entirely free from living and formed cells. In later papers, there will be reported other phenomena exhibited by the oxidation-reduction systems contained in these sterile extracts.

Peroxide Production by Bile Solutions of Pneumococcus.

The first attempts to study the formation of peroxide by pneumococcus extracts were made on bile solutions of the cells.

Broth cultures of Pneumococcus Type II (Strains R and G) were grown anaerobically. 0.5 cc. of sterile ox bile was added to 15 cc. of the anaerobic broth cultures in narrow tubes which were then sealed with vaseline and incubated at 37°C. for 12 hours. In some experiments, the bile-treated cultures were held several days in the ice box after incubation to bring about maximum solution of the cells. The clear, bacterial solutions were then centrifuged to remove any cells remaining undissolved. The supernatant fluid was removed from under seal and exposed to the air in Erlenmeyer flasks. Sterility tests were made by plating 0.1 cc. on blood agar and inoculating 0.2 cc. in blood broth.

Considerable difficulty was experienced in obtaining absolutely sterile preparations, although no difference in rapidity of peroxide formation was observed in solutions proving sterile and in those showing the presence of a few viable cells. The formation of peroxide could usually be detected after 20 to 30 minutes exposure of the bile solution of pneumococci to the air at room temperature. The peroxide, however, did not persist in the bile solution, apparently due to its rapid decomposition in this medium. Solutions giving positive peroxide reactions after aeration for 20 to 30 minutes, frequently became negative after 2 hours. Peroxide formation could be demonstrated in the case of bile solutions aerated at 2–4°C. although 2 to 3 hours aerobic exposure was required.

Although peroxide production was exhibited by the sterile bile solutions of pneumococcus, these did not lend themselves so readily to further study as did extracts prepared by repeating freezing and thawing of the cells.

Peroxide Production by Sterile, Filtered Cell Extracts of Pneumococcus.

Preparation of Extracts.—The method of preparing these extracts was as follows:

Anaerobic broth cultures of Pneumococcus Type II were centrifuged and the unwashed cells suspended in small quantities of the supernatant broth or in sterile phosphate solutions so that 1 cc. of the resultant suspension contained the bacteria from 35 cc. of culture, the actual concentration varying in different preparations. The suspensions of unwashed bacteria were then alternately and rapidly frozen and thawed and this process repeated six to nine times until maximum disruption of the cells occurred. Anaerobic conditions were maintained during the freezing and thawing process, by keeping the cell suspensions in long narrow tubes under vaseline seal. After the freezing process the suspensions were centrifuged 1 hour at high speed. The amorphous, Gram-negative detritus removed by the first centrifugation was practically devoid of formed cells. The supernatant fluid was removed as free as possible from cell residue. The centrifuging process was repeated, usually three times, until no visible detritus was thrown down. With sufficient care in removing the supernatant after each centrifugation, extracts could be obtained which were sterile, as judged by sterility tests in blood media. However, to remove any possible doubt as to the presence of living cells the extracts were filtered through Berkefeld candles.

The sterility of the filtered extracts was always controlled by plating 0.4 cc. in blood agar and by adding 0.4 cc. to blood broth. The filtered extracts were stored in the ice box in long, narrow tubes under vaseline seal.

These extracts reacted with the oxygen of the air with such avidity that preparations which were peroxide-free before filtration, gave strong peroxide tests immediately after the filtration process. Even if cooled to 2°C., the extracts could not be kept peroxide-free, although the whole filtration apparatus was packed in ice. However, the amount of peroxide formed during filtration was rapidly decomposed when the filtered extract was subsequently sealed with vaseline to prevent further oxidation. The extract rendered peroxide-free by its own reducing action under anaerobic conditions, was again able to form peroxide when exposed to the action of molecular oxygen.

Since the oxidizing-reducing activity of the extract might be impaired by even the transient presence of small amounts of peroxide, it seemed desirable to reduce to a minimum the contact of the extract with oxygen during filtration. In later preparations, the filtration procedure was modified to meet this end.

Extracts were cooled to 2°C., the entire filtration apparatus was packed in ice, and the filtrate was collected in a long narrow tube instead of the usual flasks. With these precautions, the clear extracts could be filtered with minimum aeration at low temperature without the formation of significant amounts of peroxide.

To prevent contact of the extract with oxygen during filtration, an apparatus was devised which permitted the preparation to be filtered and collected in an atmosphere of pure nitrogen. The latter filtration procedure was employed in the preparation of all extracts used in the experiments reported in following papers of this series.

Peroxide Formation by Sterile Cell Extracts.—Sterile preparations of unwashed pneumococci, extracted in broth or phosphate solution in the manner described, contain a complete peroxide-forming system as shown by their ability to unite with or "accept" oxygen from the air with the prompt formation of peroxide. The sterile broth extracts, since they possessed greater activity than the extracts prepared in phosphate solution, have been used in the following experiments. The most active extracts are those which have been subjected to the minimum exposure to oxygen throughout their preparation.

Extracts which are exposed to the air for short periods and in which peroxide has been formed, may be rendered peroxide-free by the reducing action of the extract itself under anaerobic conditions. Such extracts, now peroxide-free, still possess the property of forming peroxide when again exposed to air. However, if exposure to oxygen, and to peroxide, is prolonged, the extract, after anaerobic destruction of the peroxide, shows a gradual diminution and final total loss of its former power of uniting with oxygen to form peroxide. The possible deleterious action of peroxide itself upon the activity of the system

complicates an analysis of the above phenomenon in terms of reversibility of oxidation and reduction. Experiments are planned to furnish data on this question.

Influence of Temperature.—The reduced extract shaken in the air at 20°C. for a few minutes gives a strong peroxide test. Peroxide is formed almost as readily when the extract is aerated at 2°C. The promptness with which peroxide formation takes place at these temperatures is indicative of the avidity with which the reduced extract unites with molecular oxygen.

Influence of H Ion Concentration upon Peroxide Formation by Sterile Extracts of Pneumococcus.

The following experiment was conducted to determine the influence of pH upon peroxide formation by the sterile extracts of the intracellular substances of pneumococcus.

TABLE I.

Influence of pH upon Peroxide Formation by Sterile, Cell Extracts of Pneumococcus.

pH at which extract was exposed to air.	Peroxide tests after aerobic exposure at 20°C. for 1 hr.
4.0	±
5.0	++
6.0	+++
7.0	+++
8.0	+++
9.0	+++

In Tables I to III − indicates that no peroxide was detected; ±, faint reaction; +, weak reaction; ++, moderate reaction; +++, marked reaction; and ++++, strong reaction.

0.5 cc. of sterile extract was mixed in small tubes with 1 cc. of sterile glycine-phosphate-acetate buffer solutions (2) at different pH. The tubes were sealed with vaseline and incubated at 20°C. for 1 hour as a further insurance of the absolute absence of peroxide in the adjusted extract before its exposure to air. The mixture was then transferred to another tube and shaken in the air. Aeration was continued by placing the tubes in a horizontal position in the dark.

At the intervals shown in Table I peroxide tests were made. Control tests of the extract at each pH were peroxide-free before exposure to the air.

Table I shows that detectable amounts of peroxide may be produced by the extracts within a reaction range of pH 5.0 to 9.0. However, as shown by the intensity of the reaction, the most active peroxide formation occurred in a zone between pH 6.0 and 9.0.

Influence of Heat upon the Peroxide-Forming Power of Sterile, Broth Extracts of Pneumococcus.

Influence of Heating at 55°C.—The following experiment is concerned with (1) the stability of the peroxide-forming system of cell extracts at 55°C., and (2) a comparison of the influence of heat upon the peroxide-forming ability of sterile extracts with that of the intact cell itself.

TABLE II.

Effect of Heating at 55°C. upon the Peroxide-Forming Activity of Sterile, Cell Extracts and of Suspensions of Whole Cells.

Extract or suspension heated at 55°C. for.	Peroxide-forming activity.	
	Filtered sterile cell extract.	Suspension of whole cells.
min.		
10	+++	++
20	+++	−
30	++	−
40	+	−
50	+	−
60	±	−
Unheated control.	++++	++++

Cell Suspensions.—A 12 hour anaerobic broth culture of Pneumococcus Type II (Strain G) was centrifuged and the cells were taken up in the supernatant so that 1 cc. of the fluid suspension contained the bacterial cells from 25 cc. of culture.

Extract.—The sterile extract used in this experiment was prepared from 35 cc. of anaerobic broth culture of Pneumococcus Type II (Strain G).

Heating.—1.5 cc. of extract, or of cell suspension, were placed in a series of small agglutination tubes, sealed with vaseline, and incubated 20 minutes to insure anaerobic conditions during heating. One tube of each preparation was then immersed in a constantly stirred water bath at 55°C. for the period noted. Each tube was cooled immediately after heating and left standing under seal in the water bath at 15°C. until all tubes of the series had been heated. The contents of each tube were then transferred to another tube, shaken, and exposed to the air in the dark at 20°C.

Peroxide tests were made after 1 hour's aeration. Control tests proved the absence of peroxide in all cases before exposure to the air. Results are given in Table II.

As shown in Table II, the peroxide-forming activity of pneumococcus extract gradually diminished on heating at 55°C. However, after exposure of 1 hour at 55°C., the extract was still able to form small amounts of peroxide. On the other hand, suspensions of intact cells heated for 20 minutes or longer, lost all power of forming peroxide when exposed to air. This relation is especially interesting since in both instances the same strain of pneumococcus was used and the concentration of cells, from which the extract and the suspension had been prepared, was comparable. In the case of the suspension of bacteria the altered permeability of the cell membrane due to the action of heat may prevent the functioning of the peroxide-forming system even though the potential ability of cellular constituents to unite with oxygen and form peroxide has not been utterly destroyed.

Effect of Heating at Different Temperatures.—The preceding experiment has shown that continued exposure to 55°C. gradually destroys the peroxide-forming activity of sterile, pneumococcus extract. To obtain further information on the degree of thermostability of the peroxide-forming system, sterile extracts were exposed for a period of 5 minutes to temperatures ranging from 55° to 100°C.

1.5 cc. of cell extract were placed in narrow agglutination tubes, sealed with vaseline, and incubated 30 minutes at 37°C. The series of tubes was then immersed for exactly 5 minutes in a constantly agitated water bath at the temperatures shown in Table III. The tubes were cooled immediately in ice water and held under seal until all heating tests were completed.

The heated extract in each instance was then placed in tubes, shaken, and exposed to the air in a thin layer at room temperature. Samples were taken from these tubes for the peroxide tests. Results are given in Table III.

Table III reveals the fact that 5 minutes exposure to 55°C. has but slight effect upon the peroxide-forming activity of the extract. Indeed, as shown in the preceding experiments, these extracts may be subjected for an hour to 55°C. without complete loss of this function. 5 minutes exposure at 65°C., however, resulted in the

complete destruction of the system responsible for peroxide production. Subsequent experiments have shown that these extracts after 10 minutes exposure to 60°C., show marked ability to form peroxide. The critical temperature for the destruction of this activity, therefore, would seem to lie between 60° and 65°C.

TABLE III.

Effect of Heating at Different Temperatures upon Peroxide Formation by Sterile Extracts of Pneumococcus.

Cell extract heated for.	Peroxide formation after exposure to air for 1 hr.
5 min. at 55°C.	+++
5 " " 65° "	−
5 " " 75° "	−
5 " " 85° "	−
5 " " 100° "	−
Unheated control.	++++

Activation of Saline Extracts of Washed Pneumococci.

In the preceding experiments, broth and saline extracts of unwashed pneumococci have been shown to possess an active peroxide-forming system. In the following experiments, an attempt has been made to determine whether extracts possessing this same property could be prepared by the extraction of washed bacteria in salt or phosphate solution.

The technique employed in the preparation of saline extracts of washed cells was the same as that used in preparing broth extracts of unwashed pneumococci, with the following exception: the centrifuged cells instead of being extracted directly were washed several times in sterile water or salt solution and finally extracted in 0.05 M sterile phosphate solution, pH 7.5.

0.5 cc. of unfiltered sterile extract of washed pneumococci was added to an equal volume of meat infusion[1] or yeast extract[1] which had been adjusted previously

[1] Muscle infusion: The infusion was prepared by the routine procedure followed in the laboratory in the preparation of meat infusion broth. 500 gm. of lean beef were allowed to infuse in 1 liter of distilled water overnight at ice box temperature. After filtration through coarse paper, the infusion was sterilized for 20 minutes at 100°C. on 3 successive days. The reaction was adjusted to pH 7.5 before use.

Yeast extract: 100 gm. of brewer's yeast in 400 cc. of distilled water were adjusted to pH 4.5 and boiled for 10 minutes. The cellular material was allowed

to pH 7.5. A double quantity of washed cell extract alone served as control. As further controls 0.5 cc. amounts of yeast extract or meat infusion without pneumococcus extract were added to 0.5 cc. of sterile phosphate solution. All of these mixtures were then aerated at 20°C. for 1 hour and then tested for the presence of peroxide. The results are recorded in Table IV.

The differences between the two types of extracts consist in the kind of medium used for extraction and in the removal of certain substances during the process of cell washing. Since sterile broth extracts of unwashed pneumococci form peroxide actively on exposure to molecular oxygen, they may be said to possess a complete peroxide-forming system which requires only the presence of molecular oxygen

TABLE IV.

Activation of Peroxide-Forming Power of Sterile Extracts of Washed Pneumococci.

Sterile extract of washed pneumococci.	Activating substance.		Phosphate solution pH 7.5.	Peroxide formation after 1 hr.'s aeration at 20°C.
	Meat infusion.	Yeast extract.		
cc.	*cc.*	*cc.*	*cc.*	
1.0				−
0.5	0.5			+
0.5		0.5		+
	0.5		0.5	−
		0.5	0.5	−

In this table − indicates negative peroxide reaction; +, positive peroxide reaction.

to initiate the process of peroxide formation. The saline extracts of washed bacteria, on the other hand, are by themselves unable to form peroxide when exposed to air (Table IV). The fact, however, that the addition of certain substances to these washed cell extracts serves to activate them in the presence of oxygen, indicates that they contain an incomplete peroxide-forming system. The behavior of these two types of cell extract is wholly analogous to that of the corresponding suspensions of living bacteria from which the extracts are derived. Suspensions of unwashed pneumococci in broth possess in common with the sterile broth extracts of unwashed cells this

to sediment at room temperature and the clear supernatant was pipetted off and tested for sterility. The extract was stored in the ice box and the reaction adjusted to pH 7.5 before use.

peroxide-forming function; similarly, saline suspensions of washed pneumococci, like the sterile saline extract of washed cells, lack this function unless reactivated by the addition of some accessory substance or substances.

Several substances have been found to serve as activators of peroxide-forming activity in cell suspensions and in sterile extracts in which this function has ceased. As might be expected, extracts of washed cells regain the property of forming peroxide by the simple addition of the cell washings. Moreover, sterilized broth such as is used for bacteriological purposes, heated meat infusion, and boiled yeast extract are also able to complement the otherwise deficient peroxide-forming system in saline extracts of washed pneumococci.

The nature of the activating substances in cell washings, meat infusion, and yeast extract is not wholly clear. However, the analogy between the completion of the peroxide-forming system of washed pneumococci and the stimulation by similar substances of the respiratory activity of washed tissue, suggests that here too the principal rôle of these activators may be the furnishing of easily oxidized, or autoxidizable substances.

SUMMARY.

In the present paper methods have been described for the preparation of sterile extracts of pneumococci. These extracts may be obtained by dissolving the bacteria in broth cultures by means of bile, or by extraction of the cellular substances by repeated freezing and thawing of broth or saline suspensions of unwashed cells. Under special precautions these extracts may be passed through Berkefeld filters without loss of potency. In this procedure, as in all other manipulations incident to their preparation, the extracts should be protected as far as possible from contact with air. All extracts were proved sterile by cultural and animal tests.

Sterile extracts of unwashed pneumococcus cells promptly form peroxide on exposure to air. Peroxide formation is almost as active in extracts aerated at 2°C. as in those exposed to the air at room temperature. Detectable amounts of peroxide may be produced by these cell extracts within the reaction range of pH 5 to 9, the optimal zone lying at reactions less acid than pH 6. The peroxide-

forming activity of the extracts is gradually diminished by prolonged exposure to 55°C., and is completely destroyed by heating at 65°C. for 5 minutes.

Cell extracts of pneumococci which have been thoroughly washed prior to extraction in salt or phosphate solutions exhibit no peroxide-forming activity. These extracts of washed cells may be activated by the addition of the cell washings, yeast extract, or muscle infusion.

CONCLUSIONS.

The peroxide-forming activity of pneumococcus is a function not dependent upon the presence of living cells.

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