STUDIES ON THE RESPIRATORY MECHANISM OF THE STREPTOCOCCI[1]

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There has been much uncertainty in the minds of investigators regarding the occurrence of known respiratory enzymes in the members of the Streptococcus genus. It has generally been assumed that they were absent. The present work was undertaken with the hope of clarifying the subject.

Warburg's theory (1924) assumes that any biological oxidation in which oxygen is the hydrogen acceptor is an activation of oxygen by an iron catalyst in the ferrous state. Wieland's theory (1913) states that the important process is the activation of hydrogen. Thunberg (1925) regards all biological oxidations as a series of dehydrogenations in which hydrogen is mobilized. Kluyver and Donker (1924–25), Szent-Gyorgi (1924), Fleisch (1924) and Oppenheimer (1926) have attempted to coördinate the Warburg and Wieland theories, adopting in each the part or parts that appear feasible. Keilin (1929) made a distinct contribution in demonstrating that the two theories function interdependently in the same organism, in the process of cell respiration. That cell oxidations may occur in the absence of Warburg's haemin catalysts has been demonstrated by various investigators.

In the belief that a fuller understanding of the respiratory mechanism of streptococci will eventually throw light on many questions as, for example, the mode of toxin production, and factors governing dissociation phenomena, a study was made of 22 representative strains of streptococci to determine their ability to produce respiratory enzymes.

[1] This paper covers in part the dissertation submitted to the Graduate School of Yale University by the author as a partial requirement for the Ph.D. degree.

STRAINS EMPLOYED

The twenty-two strains of streptococci and their origin are listed below. This group is comprised of eleven hemolytic, four non-hemolytic, three viridans, and four saprophytic organisms, two of which are thermophilic.

Strains of streptococci used, and sources

Serial number	*Organism*	*Source*
R-1	Hemolytic streptococcus	Scarlet fever, New Haven Hospital
R-1a	Hemolytic streptococcus	Scarlet fever, New Haven Hospital
R-2	Hemolytic streptococcus	Cellulitis, Birkhaug A 137
R-4	Hemolytic streptococcus	Mulford Laboratory No. 1045
R-5	Hemolytic streptococcus	Mulford Laboratory No. 1779
R-6	Hemolytic streptococcus	Mulford Laboratory No. 1048
R-36	Hemolytic streptococcus	Throat infection, recent isolation
R-37	Hemolytic streptococcus	Bovine udder origin, R 360
R-38	Hemolytic streptococcus	Bovine udder origin, R 359
R-39	Hemolytic streptococcus	Chicago sore throat epidemic. J. H. Brown, obtained from P. R. Edwards, × 40
R-40	Hemolytic streptococcus	Boston epidemic, J. H. Brown. Obtained from P. R. Edwards, E 41
R-11	*Streptococcus viridans*	Mulford Laboratory No. 13
R-12	*Streptococcus viridans*	Mulford Laboratory No. 14
R-13	*Streptococcus viridans*	Mulford Laboratory No. 16
R-7	Non-hemolytic streptococcus	Cardioarthritis *Small.*
R-8	Non-hemolytic streptococcus	National Type Culture Collection, Allen strain
R-9	Non-hemolytic streptococcus	National Type Culture Collection, Beattie strain
R-10	Non-hemolytic streptococcus	Mulford Laboratory No. 11
R-14	*Streptococcus liquefaciens*	National Type Culture Collection No. 799
R-15	*Streptococcus lacticus*	Laboratory culture, F strain
R-17	*Streptococcus fecalis*	J. H. Sherman
R-18	*Streptococcus bovis*	J. H. Sherman

In addition, a culture of *Escherichia coli* (strain R-21) was used as a positive control for many of the tests. The pathogenic streptococci were kept in duplicate on chocolate blood agar, one set at room temperature and the other in the ice box. The saphrophytic strains were kept on beef infusion agar.

MEDIA EMPLOYED

Since the major portion of this work was carried on with washed bacterial cells, large quantities of liquid media were utilized.

Hemolytic organisms were obtained in the largest numbers and in the shortest period of time by growing in Huntoon's (1918) hormone broth prepared without the addition of gelatin or laked blood, and containing 0.5 per cent, instead of 0.15 per cent, glucose; for the cultivation of organisms other than the hemolytic streptococci pneumococcus broth and agar prepared according to the method of Avery and Cullen (1920) was used.

CATALASE

Callow (1923), in a study of the volume of molecular oxygen produced from a weighed amount of cells, found that *Str. acidilactici*, *Str. hemolyticus*, and a cheese streptococcus formed no catalase.

McLeod and Gordon (1923) formulated their system of classification of bacteria according to catalase production and sensitiveness to hydrogen peroxide. While their work showed different sensibilities of streptococci to hydrogen peroxide, the cultures all failed to produce catalase.

Virtanen (1924), using damp masses and a dry preparation of "*Str. lactis*" under varying conditions of temperature and concentrations of H_2O_2, was unable to demonstrate catalase production.

In this investigation eighteen of the twenty-two strains of streptococci were subjected to tests for catalase. The heavy cell suspensions[2] obtained by centrifugation of broth cultures were tested for catalase by titrating with $KMnO_4$, a method evolved by Senter (1903) and modified by Tsuchihashi (1923).

The cell masses of streptococci and the uninoculated broth control both required 2.7 cc. of $KMnO_4$ to titrate the residual H_2O_2. As this is the amount of $KMnO_4$ necessary to titrate all of the H_2O_2 placed in the flasks, no catalase could have been present. The positive control organism, *Esch. coli*, on the other hand, required but 0.9 cc. to titrate the excess H_2O_2. It was concluded from these results that streptococci do not produce catalase, which is in harmony with the observations of the above-mentioned observers.

[2] The cell suspensions used in practically all of the tests made in this investigation were washed 2 to 3 times with saline solution and then vigorously aerated for one and one-half hours to remove any easily oxidizable organic matter.

PEROXIDASE

The peroxidase enzyme, like catalase, has received but brief consideration, in so far as the streptococci are concerned.

Stapp (1924) found the one culture of streptococcus used by him to be peroxidase-negative. Callow (1926) states that the one strain of streptococcus (*Str. acidi-lactici*) included in her list of organisms was faintly positive.

Anderson (1930), in her search for accessory growth substances in hemophilic organisms, used *Str. bovis* and *Str. liquefaciens* and concluded that streptococci neither produce nor utilize peroxidase. Loele (1929), using p-phenylenediamine as a reagent for the detection of peroxidase, found the one strain of *Str. pyogenes* employed to be negative. These few references to streptococci and peroxidase production appear to constitute the literary background of this subject.

Four reagents were used in this study for the detection of so-called peroxidase in bacteria. These were tincture of guaiac, a 50.0 per cent alcoholic solution of o-tolidin, a 50.0 per cent alcoholic solution of benzidine, and a 0.1 per cent aqueous solution of 2–7 diaminofluorene-HCl.[3] One drop of these reagents was added to a mixture of 0.5 cc. of cells, 2 drops of a 3.0 per cent solution of H_2O_2 (Merck's perhydrol) and 0.5 cc. of a buffer solution, pH 4.5.[4] The appearance of the characteristic color of these four dyes in the oxidized state was considered a positive test for peroxidase. Horseradish peroxidase (aqueous extract of horse radish) was used as a positive control, and mixtures of the reagents without bacteria as negative controls.

The presence of streptococcus peroxidase was demonstrated early in this work. The color that formed tended to fade quickly with all of the dyes except the 2–7 diaminofluorene-HCl. Since these findings of peroxidase in streptococci are in opposition to the results of previous investigators, a series of tests was made to

[3] Schmidt and Hinderer (1932) recently described this new reagent, which is a derivative of fluorene and phenanthren and has a chemical *structure similar* to the customary benzidine reagent.

[4] This buffer was prepared by using M/5 sodium hydroxide and M/5 potassium *acid phthalate*.

observe the influence of such factors as pH, concentration of bacterial cells, reducing intensity of the cells, and other more or less variable components of the test. The four peroxidase reagents were employed here in order to determine their relative sensitiveness as indicators of the presence of peroxidase.

pH range of the four dyes. Buffers were prepared according to Clark's (1928) method, using salt combinations to obtain a range between pH 3.0 and 8.8. The hydrogen ion concentrations were checked colorimetrically, and were correct within 0.2 pH, according to the indicator used. Employing R-37 as a test strain (before and after heating to boiling), color production was noted over the pH range, as will be seen in table 1.

TABLE 1

Showing the pH range for the four chemicals used in the detection of peroxidase

pH	GUAIAC	BENZIDINE	o-tolidin	2-7 DIAMINOFLUORENE-HCl
3.0	−	±	++	−
4.0	+	+++	+++	++
5.2	+++	+++	++	+++
6.1	++	±	++	+++
6.9	−	−	±	+
8.8	−	−	−	−

− indicates no color formation, ± doubtful, + definite color, ++ and +++ increasing density of color formed.

It will be noted from table 1 that a pH of 4.5 is a fair optimum for all reagents. This buffer strength was subsequently used for all tests. The results were the same for heated and unheated cells.

Sensitiveness of the reagents. To determine the sensitiveness of the four reagents, four cell suspensions were made up to various $BaSO_4$ turbidity standards and tested for the formation of color within a fifteen-minute period. In this experiment the dye was the only variable, the cell concentration, buffer, and H_2O_2 being identical. The results shown in table 2 are characteristic for the four color indicators employed.

Table 2 shows the sensitiveness of the four dyes that were used to detect peroxidase. It is evident that the 2–7 diaminofluorene-

HCl salt is far more sensitive than any of the other three dyes, and that it can be oxidized by one-third to one-fifth of the number of cells necessary to oxidize the others. This extreme sensitiveness is in agreement with the original claims of Schmidt and Hinderer (1932) for this chemical, when used in the detection of blood. o-tolidin at times appears more sensitive than benzidine and guaiac, although it will be shown later that the oxidation of this reagent is inhibited more by a reducing mechanism of the cell than any of the other reagents.

Time elapsing before the appearance of a positive color. Another factor to be considered in making the peroxidase test is the time of exposure. If the reducing action of the cell is very intense it may completely inhibit the oxidation of the color reagent (forma-

TABLE 2

Showing differences in sensitivity of the four reagents used for the detection of peroxidase, under identical conditions

	CELL CONCENTRATION*						HORSE-RADISH CONTROL	REAGENTS CONTROL
	1	2	3	3.5	4	6		
Guaiac	−	−	−	−	±	+	++	−
Benzidine	−	−	±	±	+	++	++	−
o-tolidin	±	±	+	++	++	+++	++	−
2-7 diaminofluorene-HCl	+	+	++	++	++	++	++	−

* Strain R-1 employed.

tion of color), or mask it for a long time. The necessary time which should elapse without color formation before the culture can be called negative is, therefore, important. The time required for the positive color to appear was determined for both living and killed (heated) cells.

Washed aerated cells were divided into two lots, one of which was placed in boiling water for fifteen minutes. Horseradish peroxidase was included as a control reagent. The customary set-up for the peroxidase test was made with the heated and unheated suspensions. Table 3 presents the results obtained with four strains of streptococci; these are representative of all of the cultures used.

Table 3 clearly demonstrates the relative reducing ability of unheated and heated washed, aerated bacterial cells. The thermolabile reducing mechanism in the heated cells was apparently destroyed. The cell suspensions exert the greatest reducing action (inhibition of color formation) against o-tolidin, while the least interference is with the 2–7 diaminofluorene-HCl reagent. While the cell concentrations were not exactly alike, it is not believed that the number of viable cells differed in any degree comparable with that shown by the reduction time with the different organisms. The difference in the thermostability of bacterial peroxidase and horseradish peroxidase is evident, the heated horseradish extract failing to oxidize the dye after an hour's contact.

TABLE 3

Showing the time elapsing before the appearance of a positive peroxidase reaction with unheated and heated cells in the presence of the color reagents

STRAIN	TIME (MINUTES)							
	Guaiac		Benzidine		o-tolidin		2-7 diaminofluorene-HCl	
	Unheated	Heated	Unheated	Heated	Unheated	Heated	Unheated	Heated
R-1	10	4	6	2	18	2	4	2
R-8	12	8	6	2	25	2	2	1
R-14	30	8	30	5	30	5	4	2
R-37	9	4	6	2	18	2	2	1
Control (Horseradish)	5 sec.		10 sec.		15 sec.		5 sec.	

Action of respiratory poisons on streptococcus peroxidase. Warburg (1925) and Keilin (1929) have shown that KCN in high dilutions is a powerful inhibitor of cell respiration. Wieland and Sutter (1928) found that M/200,000 KCN inhibits the catalytic action of horseradish peroxidase to the extent of decreasing the purpurogallin number from 280 to 130.

An experiment was conducted by the writer to determine whether bacterial peroxidase is inhibited by low concentrations of respiratory poisons. The usual test for peroxidase was performed, with and without various dilutions of the inhibitive sub-

stances. Brom-acetic acid, iodo-acetic acid, sodium fluoride, arsenious acid and ethyl urethane, which apparently poison the dehydrase mechanism of certain cells, exerted no influence on the peroxidase test. Potassium cyanide and sodium pyrophosphate (so-called iron poisons) were much less active in inhibiting the bacterial peroxidase than was KCN in its action on horseradish peroxidase.

Burnet (1927) separated bacteria into two groups, according to their sensitiveness to KCN. He also showed that the organisms which produce catalase and supposedly contain Warburg's iron respiratory catalysts are inhibited by low concentrations of KCN, whereas others, including streptococci, are not inhibited unless the concentration of KCN is increased more than twenty-five times. Burnet's observations were in part confirmed by the present author in his study of the effect of KCN on 12 different strains of streptococci. Neutral solutions of KCN which were sterilized by filtration through filter candles were added directly to pneumococcus agar in varying amounts. The agar plates were streaked with broth cultures of the various streptococci, and examined after forty-eight hours incubation.

The streptococci were not markedly sensitive to the KCN when exposed under aerobic conditions, all of the 12 strains employed showing visible growth in the presence of 0.25 per cent of the reagent. In concentrations as high as 0.5 per cent it did not prevent growth in pneumococcus broth, and three out of four strains grew in the presence of 0.75 per cent.

That the growth of the streptococci, or the respiratory mechanism of these organisms as a functioning unit, is not inhibited by relatively high concentrations of KCN lends additional support to the findings that this iron poison does not interfere with the peroxidase test in streptococci except in high concentrations. These results indicate that so-called bacterial peroxidase differs from horseradish peroxidase not only in its thermostability, but also in that it contains no iron.

In an attempt to substantiate the positive qualitative findings of peroxidase by the color tests, certain quantitative procedures were employed.

Quantitative determination of peroxidase. Several procedures were followed, including the original purpurogallin method of Willstätter and Stoll (1918). Later Guthrie's (1931) test for peroxidase and Masumune's and Kodama's (1931–32) micromethod were employed.

The results obtained following the use of these tests were in the main negative. While the addition of a small amount of bacterial suspensions gave a slight increase in color production over the reagent control, it was not possible to demonstrate a linear function with increasing concentrations of cells, even when 50 cc. of a very dense suspension of living or dead cells was used. Penrose and Quastel (1930–31), using *Micrococcus lysodeikticus*, showed that peroxidase can be extracted unchanged from the intact cells by the use of lysozyme. A few preliminary experiments were conducted by the writer with cell extracts of streptococci obtained by lysis with egg-white lysozyme and with bacteriophage. The results were not conclusive.

Thermostability. The cause of the delayed positive color reaction was suspected of being a reducing oxidase in the cells. In order to follow this up and to determine the inactivation temperature, tubes containing several cubic centimeters of a suspension of various organisms were heated over a temperature range of from 45° to 70°C. for ten minutes. After heating, the usual peroxidase test was made and the time for the appearance and intensity of the color determined, using benzidine as the color reagent.

It will be seen from table 4 that the substance in the bacterial cell which is responsible for the delayed peroxidase reaction is inhibited increasingly with rise in temperature, until at 60°C. the delay before the appearance of the color is of the same duration as at higher temperatures. This indicates that a thermolabile mechanism is operative in delaying the oxidation of the dyes used in the detection of peroxidase. Such reducing action was not apparent, however, in strain R-37, which gave a positive color at all temperatures within as short an interval of time as ten seconds.

Henneberg and Wilke (1902), using a culture of *B. xylinum*,

and Callow (1926), employing several different peroxidase-producing organisms, found the bacterial peroxidase to be thermostable. Streptococcus peroxidase was found in this study to be thermostable. It withstood autoclaving for at least one-half hour at 120°C.

It must be apparent from the numerous tests made by the writer for peroxidase in streptococci that a catalytic agent is present which gives a positive color reaction with the chemicals used in the detection of peroxidase.

TABLE 4

Showing the time (in minutes) elapsing before the appearance of a positive peroxidase reaction after the cells were heated at different temperatures

STRAIN	UNHEATED	45°	50°	55°	60°	65°	70°	BOILING
R-1	7	4	3	3	1	1	1	1
R-4	7	4	3	3	2	2	2	2
R-7	7	4	2	1	1	1	1	1
R-12	5	4	2	1	1	1	1	1
R-14	30	25	20	10	2	1	1	1
R-37	10 sec.	10 sec.	10 sec.	10 sec.	10 sec.	10 sec.	10 sec.	10 sec.
Horseradish	5 sec.	5 sec.	5 sec.	5 sec.	5 sec.	30 sec.	30 sec.	

CYTOCHROME

Peroxidase is believed by Keilin (1929) to be closely associated with cytochrome, and to be represented by one of the cytochrome spectroscopic bands, possibly band C. If bacterial peroxidase is actually represented by a specific band demonstrable with a spectroscope it should offer a means of corroborating the presence of peroxidase in streptococci. Keilin (1925) observed the presence of four bands in aerobic bacteria and the absence of bands in anaerobes. Yaoi and Tamiya (1929) examined a large number of bacteria for the presence of cytochrome and showed that aerobes possess four bands, facultative anaerobes, which included one culture of *Str. erysipelatis*, two, and anaerobes none. This is the only investigation of streptococci for cytochrome known to the writer. Kuhn, Hand and Florkin (1931) have shown that the active component of horseradish peroxidase is a ferroporphy-

rinic compound having a definite two-banded spectrum, at 520 and 550 mμ. The two bands in the horseradish peroxidase and the facultative anaerobes occur at the same wave lengths.

In this investigation a Zeiss microspectroscope was used. Illumination was furnished by means of a 500 watt bulb. Cell suspensions were prepared by centrifuging broth cultures and by scraping agar plates and taking up the cells in physiological saline solution. The turbidity of these suspensions was often increased until the cell concentration equaled 50 per cent. Sodium hydrosulphite (1.0 per cent solution) was added at times to the suspension under investigation, to make certain that the pigment, if present, would be in the reduced (visible) state; KCN and pyridine were also added in the hope of forming the hemochromogens which show the strongest absorption bands.

Most of the streptococci listed in this study and three strains of *Str. erysipelatis* were repeatedly examined for the presence of cytochrome. A top yeast and *B. subtilis* were used as known positive controls showing all four bands, while cultures of *Esch. coli* and *Sal. Schotmülleri* were employed as known positive facultative anaerobes showing two bands. While the four- and two-banded spectra were observed in these positive controls, no bands were seen at any time in the cultures or suspensions of streptococci.

INDOPHENOL OXIDASE

Schultz (1910) and later Kraemer (1912) demonstrated the presence of a thermolabile oxidase in bacteria. Gordon and McLeod (1928) modified the test by using paraphenylenediamine and eliminating a-naphthol. They state that streptococci do not react to the test. Bier and Reis (1930), using essentially the same technique in testing 26 different strains of streptococci, reported that some of the cultures gave this oxidase reaction. Loele (1929), in an investigation of a series of bacteria, and employing both p-phenylenediamine and indophenol blue, found that the one culture of streptococcus used gave a negative oxidase reaction.

Keilin (1929), with the use of an improved technique, successfully demonstrated the presence of a thermolabile oxidase in yeast

and in heart muscle, and has brought forth evidence to show that this oxidase is identical with Warburg's (1924) respiratory ferment.

In the present investigation eight different cultures of streptococci were tested for the presence of oxidase, Keilin's technique being used. Yeast and *B. subtilis* were employed as positive controls. No evidence was obtained of the presence of an indophenol oxidase in these streptococci.

OXYGEN UPTAKE

Callow (1924), in a study of the oxygen uptake of washed bacterial cells in buffer solution, found that the one strain of streptococcus (*Str. acidi-lactici*) included in her group of organisms took up practically no oxygen. In order to determine whether this inability to utilize molecular oxygen is characteristic of other members of the genus, oxygen uptake studies were made with the aid of the Warburg apparatus.[5]

The bacterial cells were obtained in the usual manner, that is by centrifugation of the broth cultures and washing twice with physiological salt solution. One cubic centimeter of the unaerated cells was placed in Warburg cups containing 0.25 cc. of 2N/1 NaOH and brought up to the required volume with saline solution, the amount of the diluent depending on the other substances added. Duplicate determinations were made in every instance. The manometers were read at hourly intervals. The oxygen consumption of a bacterial suspension of an hemolytic (strain R-1), a non-hemolytic (strain R-10) and a viridans culture (strain R-11) was measured over an eight-hour period. The oxygen uptake at the end of this interval was negligible. The experiment corroborates the findings of Callow with *Str. acidi-lactici.*

Keilin (1929) showed that the presence of indophenol oxidase in yeasts, as determined by the "Nadi" reagent, can be corroborated by oxygen uptake studies. The presence of this oxidase

[5] The writer wishes to express his appreciation to Mr. S. Kasdon for his valuable assistance in the use of the Warburg apparatus.

apparently greatly increases the amount of oxygen utilized in the presence of living cells.

An experiment was conducted to determine whether the oxygen uptake of streptococci would be greatly increased in the presence of p-phenylenediamine. For checking purposes, oxygen uptake

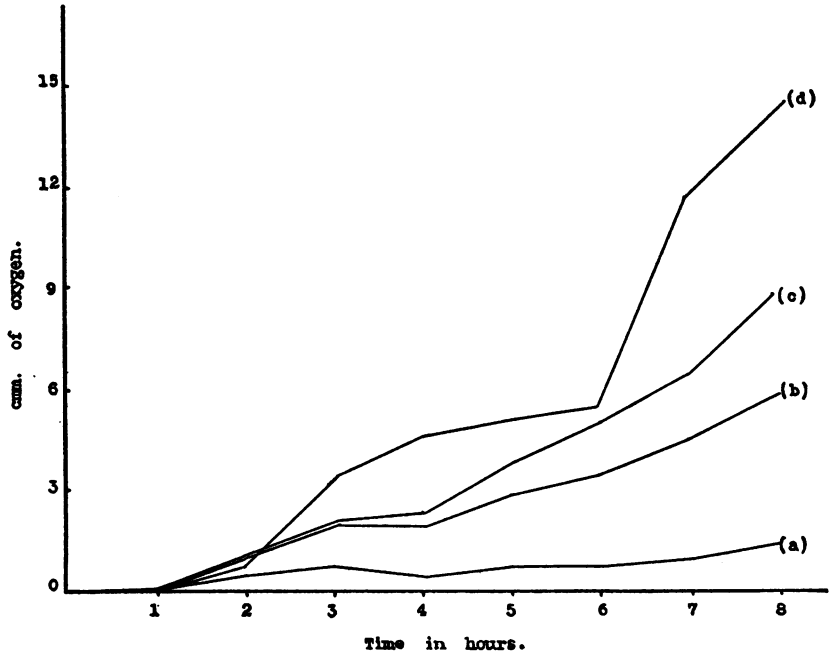

FIG. 1. SHOWING THE OXYGEN UPTAKE OF WASHED SUSPENSIONS OF STREPTOCOCCI OVER AN EIGHT-HOUR PERIOD

a, suspension of R-11 alone; *b*, suspension of R-11 plus p-phenylenediamine; *c*, suspension of R-11 plus p-phenylenediamine plus M/500 KCN; *d*, 1 cc. of p-phenylenediamine alone.

determinations were made on the bacterial suspension alone, on the suspension plus 1.0 cc. of p-phenylenediamine, on the cells plus p-phenylenediamine and 0.45 cc. M/500 KCN, and on 1.0 cc. of p-phenylenediamine solution alone. The results are shown in figure 1.

The *a* curve in figure 1 is representative of five duplicate tests.

The consumption of oxygen by streptococci, as shown here, appears negligible when compared with the large uptake of this gas reported by other investigators for other organisms.[6] It is apparent, therefore, that washed suspensions of streptococci do not possess the ability to utilize molecular oxygen in the absence of suitable metabolites. This inability to consume oxygen substantiates the negative findings in the search for indophenol-oxidase in these bacteria, an agent which is regarded as playing an important rôle in the oxygen uptake mechanism of some organisms.

The presence of p-phenylenediamine (curve *b*) caused a noticeable increase in the oxygen uptake, which increase was not hindered, but rather slightly accelerated, by the addition of KCN (curve *c*).

The oxygen uptake of p-phenylenediamine alone (curve *d*) is much greater than where the bacterial suspension was used, suggesting that the increased uptake may be due to autoxidation of this dye.

The "autoxidation" of p-phenylenediamine, when this reagent is added to bacterial suspensions, probably accounts for the oxygen uptake noted. This supports the view that indophenol oxidase is absent from streptococci. The finding that the iron poison (KCN) does not inhibit, but tends to increase slightly the oxygen taken up by this strain of streptococci, also lends added support to other findings reported in this paper, namely, that Warburg's haemin catalyst is absent from streptococci.

DEHYDRASES

Wieland (1912 to date) has held to the view that the mechanism of biological oxidation is a direct activation by the organisms of the substrate to be oxidized, rather than a process of intermediation or activation of oxygen. He considers the essential process a mobilization of hydrogen by the cell dehydrases.

Thunberg (1917) developed a technique for studying dehy-

[6] Hunt, G. A. (Jour. Bact., **26**, 341–60, 1933), using the same Warburg apparatus, showed an oxygen consumption by *Ps. pyocyanea* of approximately 275 cu. mm. of oxygen in three hours.

drases in muscle. This technique was applied to bacterial cells in an extensive series of researches by Quastel (1925) and his colleagues, and later by Kendall (1929). No studies on streptococci are known to the writer.

In applying Quastel's modification of Thunberg's technique in the present study of streptococci several changes were necessary. Methylene blue is toxic for streptococci, as was shown by Avery (1929) and others. Hence, this dye could not be used as an indicator of reduction. In my work higher dilutions of the dye were tried (up to 1/25,000) which, it was thought, might permit the use of methylene blue as a hydrogen acceptor, but these higher dilutions also were found to be toxic. Dubos (1929) showed that indigotetrasulphonate is tolerated by the streptococci. Preliminary tests made by the writer with eight strains showed that an M/1000 solution of this dye is not toxic for any of these organisms. For all subsequent tests a concentration of M/2500 was adopted, since it permitted clear-cut readings. Another modification was in the temperature, as 45°C. was found to be injurious to the organisms. In order to obtain information regarding the temperature at which cells do not proliferate, and also to determine the influence of anaerobiosis, a series of viability counts was made at various temperatures (41° to 45°C.) over a two-hour period. The counts were made by McCrady's (1915) dilution method, five tubes for each dilution being used, together with duplicate counts on blood agar plates as checks. A temperature of 43°C. appeared most practical.

Indigotetrasulphonate, Hempel desiccators in place of Thunberg tubes, and a temperature of 43.0°C. were adopted as modifications of the original technique. The set-up to determine the activating powers of streptococci on different substrates was as follows: 3 cc. of buffer, 1 cc. of indigotetrasulphonate, 1 cc. of streptococcus suspension, and 1 cc. of the particular test agent were placed in small test tubes; these were placed in desiccator jars, and the jars evacuated and incubated at 43°C.

A series of chemical substances, 101 in number, including carbohydrates, fatty, amino and other organic acids, and other substances were used to test the activating powers of the streptococci.

M/50 to M/100 solutions of these materials were prepared, adjusted to pH 7.2 with normal HCl or normal NaOH, and sterilized either by autoclaving or by filtration. Observations were made at hourly intervals to note reduction of the dye. *Esch. coli* was used as a known control, on the strength of the work of the Cambridge University investigators with this organism, while bacterial suspensions added to the buffer and dye were used as negative controls. The concentration of cells used, while varying to some extent, corresponded to a $BaSO_4$ standard of 2–4 for the hemolytic organisms, and 5–8 for the other streptococcus suspensions.

M/50 solutions of thirty carbohydrates, alcohols and glucosides were employed in these experiments. The eleven hemolytic streptococci tested showed little dehydrogenating action, with the exception of strains R-36, R-39 and R-40, the last two being cultures of *Str. epidemicus*. These were able to activate only the hexose sugars. The four non-hemolytic organisms (strains 7 to 10 inclusive) activated the same three hexose sugars, while strain R-9 acted on disaccharides and glucosides in addition. The viridans cultures (strains R-11, R-12, R-13 and R-31) activated the hexoses and quite a number of other sugars. The remaining (saprophytic) streptococci (strains R-14, R-15, R-17 and R-18) showed greater activating power in the oxidation of a larger number of substrates than the pathogenic strains. The known control, *Esch. coli* (strain R-21), revealed a wide range of activation, which is in agreement with the work of Quastel and his colleagues.

Rate of reduction. To observe the influence of the cell concentration upon the speed of reduction of the dye, six tubes were set up in which the buffer, dye and amount of glucose were constant. To these tubes was added in series 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 cc. of a bacterial suspension of strain R-12 having a turbidity equal to a $BaSO_4$ standard of 5.0. The results are given in figure 2.

Figure 2 shows that the velocity of reduction is proportional to the concentration of cells. In the presence of a suitable hydrogen donor and hydrogen acceptor a dense suspension of cells appears unnecessary.

Effect of poisons and narcotic agents on dehydrogenations. The studies of Banga, Schneider and Szent-Gyorgi (1931a) on tissue respiration show that arsenious acid influences the hydrogen activation and total respiration to the same extent. Further work by these authors (1931b) showed that iodo-acetic acid also affects the respiratory function. Lundsgaard (1932) and Meyer

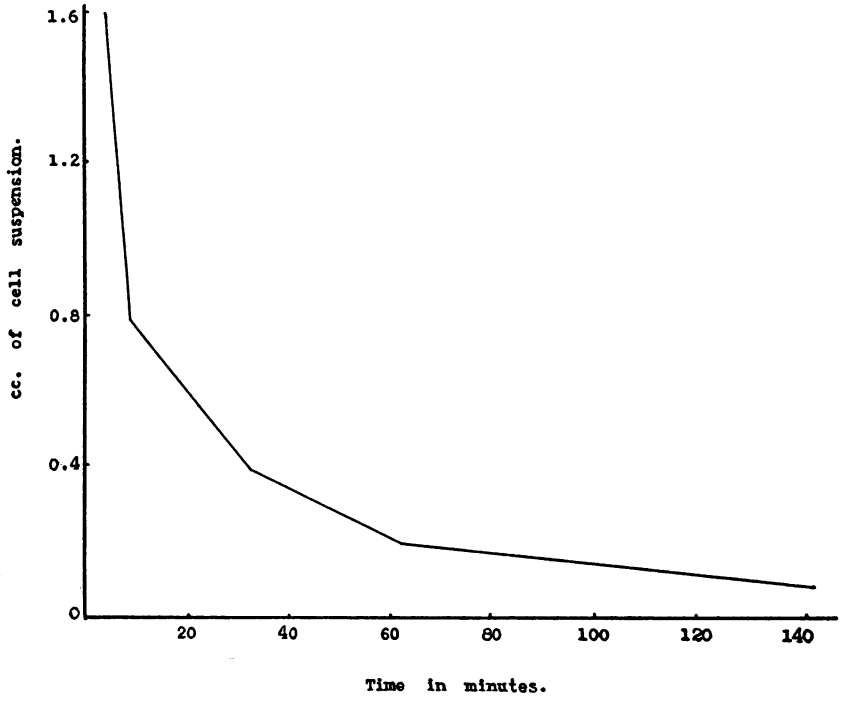

FIG. 2. SHOWING THE RATE OF REDUCTION OF INDIGO TETRASULPHONATE AS A FUNCTION OF THE CELL CONCENTRATION

(1932) further point out the specific inhibitive action of this substance.

Five different concentrations (M/10 to M/100,000) of arsenious and iodo-acetic acids and of several other respiratory or fermentative poisons, were tested for their effect on dehydrases. A mixture of bacterial suspension of strains R-7, 12 and 14, 0.5 per cent glucose as a hydrogen donor, and 1.0 cc. of each of the graded

solutions of possible inhibitors was subjected to the usual Thunberg experiment. Controls, one containing no chemical and the other no hydrogen donor, were included. The results obtained with the three different organisms were essentially the same; none of the inhibitory agents except M/10 brom-acetic and iodo-acetic acids prevented the activation of glucose by the streptococci.

Thermostability of activators. A suspension of cells of strain R-12 which had been heated for fifteen minutes in boiling water was unable to activate glucose, thus showing the enzyme to be thermolabile. Tubes containing 2 cc. of the same suspension were heated for ten minutes at 50°, 55°, 65°, 71° and 80°C. and then tested for their ability to dehydrogenate glucose. The tube that was heated to 50°C. was the only one which gave evidence of reducing action; the others showed no reduction after six hours. The inactivation temperature for dehydrases closely corresponds to the inactivation temperature of the reducing substance which is responsible for the delayed peroxidase test, thus suggesting a direct relationship between dehydrases and peroxidases.

A series of fatty, amino, dibasic, hydroxy and keto acids and other chemicals,[7] totaling 71 in number, were used as substrates to determine the ability of the streptococci to activate them as hydrogen donors. No dehydrogenation was observed.

The inability of streptococci to activate these organic substances may be accounted for in several ways. First, it is possible that these substances are activated as hydrogen acceptors in the presence of a suitable donor, with the consequent liberation of energy. Again, in accordance with the recent extended investigations into the co-enzymes of dehydrases, it appears possible that the treatment of the bacterial cells (repeated washing and aeration) may so dilute or injure the co-enzyme as to prevent action on the substrate. The rather general activating powers of streptococci on carbohydrates, as contrasted with their inability to dehydrogenate the large number of other organic substrates used here, suggest that another mechanism may be active in the

[7] For a full résumé of materials employed, tables, etc. not included in this paper readers are referred to the author's dissertation in the Yale University Library.

breakdown of these substances, or a similar mechanism with different physical properties.[8]

HYDROGEN PEROXIDE PRODUCTION

Since McLeod and Gordon's (1922) discovery that hydrogen peroxide is the cause of the bleaching of blood agar by pneumococci, much attention has been given to the question of H_2O_2 production by streptococci (McLeod and Gordon, 1923; Avery and Morgan, 1924; Valentine, 1926; Todd, 1933; and others).

Other investigations dealing with H_2O_2 production by bacteria which tend to corroborate Wieland's and Thunberg's claims that the initial and final steps in oxidations are essentially dehydrogenations are those of Bertho and Gluck (1931), Davis (1933) and Sevag (1934). They experimented with lactic acid bacteria and demonstrated that the amount of H_2O_2 formed is equivalent to the oxygen uptake. If it could be demonstrated that one of the initial products of metabolism in all streptococci is H_2O_2, then it would be possible to state that this step is essentially a mobilization of hydrogen, according to Wieland's hypothesis.

Chocolate blood agar was used for the detection of H_2O_2 in the solid medium, while the reagents 2–7 diaminfluorene-HCl and the Kastle-Meyer reagent were employed in the liquid media. An aqueous extract of horseradish was used to furnish peroxidase. The procedure was as follows: A 300 cc. Erlenmeyer flask containing 40 to 50 cc. of pneumococcus broth was inoculated with 0.1 cc. of each of the strains which previously had been subcultured twice in blood-free broth. These were tested for H_2O_2 over a twenty-one-day period. Blood agar plates were streaked frequently as a check on the purity of the cultures. An untreated flask of broth and a flask of broth containing M/28,000 H_2O_2 were carried as negative and positive controls.

The results obtained indicate that a majority of the streptococci used produce H_2O_2 in pneumococcus broth when grown in shallow layers of the medium. Peroxide was not detectable in the hemolytic organisms, under the conditions employed in these

[8] The author is at present engaged in further research dealing with dehydrases in the streptococci.

experiments, before the second to the fourth day. Two strains of *Str. viridans* (R-8 and R-9) and three saprophytic strains (R-15, R-17 and R-18) produced H_2O_2 in from four to eight hours after inoculation.

TABLE 5

Showing the production of hydrogen peroxide by streptococci when grown on different media

STRAIN	PNEUMOCOCCUS BROTH	PNEUMOCOCCUS BROTH PLUS AMMONIUM LACTATE	GLUCOSE INFUSION	CHOCOLATE AGAR
R-1	+	−	−	+
R-1a	+	+	−	+
R-2	+	−	−	+
R-4	−	−	−	+
R-5	−	−	−	−
R-6	+	+	−	+
R-7	−	−	−	−
R-8	+	+	−	+
R-9	+	+	−	+
R-10	−	−	−	−
R-11	+	+	−	+
R-12	−	−	−	−
R-13	−	−	−	−
R-14	−	−	−	−
R-17	+	+	+	+
R-18	+	+	+	+
R-21	−	+	−	−
R-36	+	−	−	+
R-37	−	−	−	−
R-38	−	+	−	+
R-39	+	+	−	+
R-40	+	+	−	+
R-15	+	+	−	+
Broth control	−	−	−	−
M/28,000 H_2O_2	+	+	+	+

In an attempt to determine if the streptococci which did not produce detectable amounts of H_2O_2, when grown in plain broth, would do so in another medium, as Gordon (1933) showed, a series of flasks containing glucose pneumococcus broth and a series containing pneumococcus broth plus 0.5 per cent ammonium lactate were inoculated and tested daily over a sixteen-day

period for the production of hydrogen peroxide. The results of these tests are compared in table 5 with those obtained in plain broth and on chocolate blood agar.

Table 5 shows the diversity of the metabolic processes of streptococci, in so far as H_2O_2 production is concerned, when individual organisms are grown on different substrates. Pneumococcus broth, pneumococcus broth plus ammonium lactate, and chocolate blood agar revealed H_2O_2 production by a majority of the strains, and with but few discrepancies. Glucose pneumo broth, on the other hand, appeared to suppress completely H_2O_2 formation by almost all of the organisms, or to interfere with the reagents used in the detection of this substance. The addition of ammonium lactate to pneumococcus broth permitted the detection of H_2O_2 in two organisms which did not produce it in plain pneumococcus broth, while four strains producing peroxide in plain broth failed to show any in this medium. The chocolate blood agar appears to be a more sensitive indicator of H_2O_2 formation than the other reagents used.

DISCUSSION

The evidence presented in this paper indicates that streptococci do not produce catalase, cytochrome or indophenol-oxidase, from which one would conclude that Warburg's haemin respiratory system is absent from this group of bacteria.

The findings of a thermostable peroxidase in these organisms does not detract from the above claim, since it is not affected by low concentrations of potassium cyanide and because it was not possible to show that bacterial peroxidase is a component of either cytochrome or of indophenol-oxidase.

The findings of Kuhn, Hand and Florkin (1931) that the active component of horseradish peroxidase is a ferroporphyrinic compound having a definite spectrum with bands at 550 and 520 mμ, when contrasted with the author's failure to demonstrate bands at these wave lengths in streptococci found to contain peroxidase, suggests that the so-called peroxidases may differ from each other in certain respects. Opposed to the present author's observations are the findings of Yaoi and Tamiya (1929), who noted in one

culture of *Str. erysipelatis* the b and d bands of cytochrome, supposedly at the wave lengths mentioned above. In the present work in which repeated examinations were made of over a dozen strains of streptococci, three of which were strains of *Str. erysipelatis*, no bands were observed.

According to Keilin (1929), cytochrome is a carrier between the dehydrases and indophenol-oxidase in the systems which he studied. While it is easily conceivable that other systems and other oxidases are probably active in cell respiration, it is believed that the absence of indophenol-oxidase from streptococci brings added proof that the negative findings regarding cytochrome in this group of bacteria are correct.

Keilin (1929) evolved from his researches a scheme showing the inter-relationship between Wieland's dehydrase system and Warburg's oxidase system. In the present investigations on streptococci it has been shown that the Warburg mechanism is absent. The haemin respiratory system is present, however, in some facultative anaerobes, such as *Esch. coli* and *Sal. Schottmülleri*, while it is absent from others, including the streptococci. The lack of an iron system to complement the dehydrase system which is present in the streptococci leads one to inquire whether Warburg's haemin catalysts have a specific substitute or whether an entirely different mechanism may function in its absence.

Elliot (1932) has suggested that peroxidase may function as a counterpart of indophenol-oxidase in the presence of cytochrome, as in Keilin's scheme mentioned above. In the present investigation the reducing action of dehydrases and their inhibition of peroxidase indicates a relationship between dehydrases and peroxidases, which lends support to the present view. It is unlikely, however, that cytochrome would be present, since it and indophenol-oxidase apparently go hand in hand as an aerobic oxidase system. It is more plausible that a substitute hydrogen carrier similar to glutathione may function in a manner analagous to cytochrome, in the respiration of the type of facultative anaerobes represented by the streptococci, which were found in this study to be deficient in an aerobic oxidase system.

The question regarding the nature of the substance which acts

as a hydrogen acceptor in the streptococci must find an answer before a clearer knowledge of the mechanism of respiration in these organisms can be gained. The streptococci took up only negligible amounts of atmospheric oxygen when determinations were made in the Warburg apparatus. However, when the organisms were grown in shallow layers of broth, which permitted free aeration of the medium, H_2O_2 was detected in the majority of the cultures.

The formation of H_2O_2 in shallow layers of broth may be explained by assuming that when oxygen has reached a certain concentration it has a greater competitive affinity as a hydrogen acceptor for the mobilized hydrogen than have the other, unknown, hydrogen acceptors present in the cell or surrounding medium.

An inter-relationship between the dehydrase and peroxidase mechanisms in this investigation has been indicated. This dual system may be the complete respiratory unit in these bacteria, in which so-called iron-free bacterial peroxidase acts as a hydrogen acceptor.

SUMMARY

1. The enzyme catalase could not be demonstrated in the streptococci.

2. A thermostable peroxidase (which differed materially from plant peroxidase), was found to be present. Factors influencing its demonstration were studied.

3. The thermostable peroxidase appears to be intimately related to the thermolabile dehydrase mechanism in the cell; the latter tends to mask the peroxidase test, but is destroyed by heating.

4. Cytochrome and indophenol-oxidase (Warburg's respiratory system) could not be demonstrated in the streptococci.

5. The oxygen uptake of washed suspensions of streptococci was found to be negligible.

6. The ability of streptococci to activate 101 chemicals was studied. While it was possible to demonstrate the dehydrogenation of many carbohydrates, 71 other organic substances used in this test were not activated.

7. The respiratory mechanism of streptococci is discussed, and the conclusion drawn that the dehydrase-peroxidase system plays an important rôle in the respiration of the streptococci.

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