XLIV. PRODUCTION OF HYDROGEN PEROXIDE BY BACTERIA.

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A study of phenomena of auto-inhibition in bacterial cultures was published by McLeod and Govenlock [1921] in 1921 showing that a series of investigations on this subject had led to the demonstration of such phenomena in connection with the growth of many bacteria. C. Eijkman's results [1904 and 1906, 1, 2], which have been more questioned than accepted, were fully confirmed.

It was shown further that the Pneumococcus, a bacterium, which did not appear to have been investigated previously in this respect, produced in the course of growth a substance inhibitory to its own growth and to that of most bacteria. This inhibitory effect was more powerful than that observed in connection with the growth of any other bacterium examined and the inhibitory substance was found to be thermolabile (85°). The labile character of the substance produced suggested an analogy with toxins and ferments and for this reason the name "bactericidins" was suggested for such substances. Subsequent work has shown however that the substance produced by the Pneumococcus, at all events, is simpler in character and is in all probability hydrogen peroxide: but the question whether other bacteria produce specific inhibitory substances analogous to ferments remains an open one.

A short summary of the work done to show that the Pneumococcus produces hydrogen peroxide in culture has already been published as an abstract of a communication to the Pathological Society [McLeod and Gordon, 1922].

It is the purpose of this paper to give in some detail the experimental evidence for this conclusion together with some more recent observations on the subject.

Conditions necessary for the production of pneumococcal inhibitory substance in fluid media.

The original observations establishing the existence of a thermolabile bactericidal product in pneumococcal cultures had been made in solid media-serum agar—and such were obviously unsuitable for attempting to concentrate the bactericidal substance.

In trying to obtain evidence of the production of these substances by the Pneumococcus in fluid media, the Staphylococcus was used as a test micro-organism because it grows freely and with great constancy in all ordinary bacteriological media and because it had proved to be particularly sensitive to the inhibitory body produced by the Pneumococcus.

The experiment consisted in preparing a series of dilutions in peptone water of a 10 % serum bouillon culture of Pneumococcus, which had been heated for 30 minutes at 60° to kill off the Pneumococcus, and inoculating these with Staphylococcus. The inhibitory potency of the pneumococcal culture was judged by the extent to which it had to be diluted in order to permit of growth of the Staphylococcus.

By using such methods it was soon determined that aeration of the culture was of importance; thus little or no inhibitory effect would be produced by growing a Pneumococcus in 10 cc. of 10 % serum broth in a narrow test-tube and the Pneumococcus was always alive at the end of 48 hours, whereas the same organism grown in 10 cc. of the same medium spread out over the base of an Erlenmeyer flask gave rise to a markedly inhibitory culture in which the Pneumococcus had usually died at the end of 48 hours. It was found further that inhibitory cultures could be obtained more constantly if a current of air was kept bubbling through the culture during incubation, by connecting the flask containing the medium with a water suction pump. Control observations in which air was passed through sterile media kept for similar periods of time in the incubator gave negative results—the medium did not develop any inhibitory quality.

All the earlier observations were made with a bouillon medium prepared from meat extract and Parke Davis peptone, 1 %, to which 10 % of horse serum was added. The horse serum had been heated for an hour at 56° and was several months old. It contained very little catalase and subsequent observations have shown that it is important for constant production of the inhibitory body that the medium should contain little or no catalase. Some of our observations also tended to show that the amount of inhibitory substance produced was influenced by the kind of peptone used but the point has not yet been investigated carefully enough to permit of a definite statement.

Methods of concentrating the inhibitory substance.

Under the impression that the substance under investigation was probably a labile protein similar to toxin an attempt was made to obtain concentration by precipitating out the proteins of the culture with absolute alcohol at 0°, and redissolving the precipitate in a small quantity of water. The solutions so obtained showed little or no power of inhibiting bacterial growth. Further experiment showed, however, that the bactericidal substance dissolved in alcohol and passed into the filtrate. By concentrating such filtrates *in vacuo* at 35°–45° fluids of considerably enhanced bactericidal potency were obtained.

The average culture before concentration had an antiseptic potency sufficient to prevent development of *Staphylococcus aureus* in peptone water containing from 1 part in 3 to 1 part in 8 of pneumococcal culture, and by concentrating this culture to one-sixth or one-eighth of its original volume in the manner outlined above a residue was obtained at least four times as active as the original culture, *i.e.* capable of completely inhibiting growth of Staphylococcus in peptone water if present in the proportion of 1 part to 20 of peptone water. That is to say the fluid obtained was about equal to 4 % carbolic acid as regards its antiseptic effect on *Staphylococcus aureus*: it differed from the latter however in the marked instability of the antiseptic substance contained.

By reprecipitating the first concentrate with an excess of alcohol and reducing to a considerably smaller bulk it was possible to obtain fluids of higher antiseptic potency.

Chemical evidence of the presence of peroxide in the inhibitory cultures and concentrates.

The antiseptic concentrates from pneumococcal cultures had not been long under investigation before the observation was made that the addition of such fluids to emulsions of blood was followed by evolution of gas. We are indebted to Mr H. D. Kay of the Physiology Department, Leeds University, for suggesting that the effervescence was probably due to the presence of peroxide. This appears to be the correct explanation. Both cultures and concentrates give a strong blue colour when mixed with starch iodide paste containing traces of $FeSO_4$ (Schönbein's reagent). Wolff [1912] has pointed out that under certain conditions nitrites may give this reaction and cause confusion in regard to presence of peroxides in plant juices; but the absence of nitrites from the cultures is proved by failure to obtain any colour reaction on adding diphenylamine. Cultures so concentrated also give quite definitely a yellow or orange colour with titanium sulphate solution. Lastly a compound blue in colour and soluble in ether but disappearing rapidly if not extracted with ether immediately was obtained on adding 10 % chromic acid to a concentrate.

The chief evidences from the chemical standpoint for considering this substance to be hydrogen peroxide rather than some organic peroxide are:

(1) Its sensitiveness to decomposition by the catalase of blood and serum or by special catalase preparations from the liver [Morgulis, 1921]; Novy and Freer [1902] state that the various organic peroxides with which they worked differed from H_2O_2 in being relatively insensitive to the action of catalase.

(2) The fact that it tends to decompose on heating, but only does so very rapidly when the temperature is raised to 85°, in which respect it closely resembles H_2O_2, which distils at 84° under reduced pressure when relatively pure and concentrated but is rapidly destroyed about the same temperature in dilute and impure solutions [Baur, 1908].

(3) The gas evolved on adding catalase to a concentrate was proved to be oxygen since it was not absorbed by KOH solution but was absorbed to the extent of 90–95 % by alkaline pyrogallol solution and was capable of rekindling a glowing splint placed in it. Further the oxygen evolved corresponded very nearly to the amount calculated as available when the concentrate was titrated with potassium iodide, sodium thiosulphate and starch with a view to determining its H_2O_2 content.

Bacteriological evidence of identity of inhibitory substance in pneumococcal cultures with H_2O_2.

Two lines of investigation have been pursued.

First of all a number of bacteria have been compared as regards their varying sensitiveness to the inhibitory effect of (*a*) small concentrations of H_2O_2 in agar plates (*b*) the substances diffusing from deep plate cultures of Pneumococcus to superimposed layers of agar [McLeod and Govenlock, 1921]. It has been found that if bacteria are graded according to the greatest concentration of H_2O_2 in an agar plate which is compatible with their growth the order of their resistance to the H_2O_2 is as follows: *B. subtilis*, *B. prodigiosus*, some Streptococci, *B. coli*, other Streptococci, *Staphylococcus aureus*, Anthrax, Cholera, Shiga, Typhoid.

Among the bacteria which were observed often enough on inhibitory pneumococcal plates to give a reliable average result the order of resistances was: Streptococcus, *B. coli*, Staphylococci and Shiga. Although complicating factors such as favouring effect of other products of pneumococcal growth on certain bacteria may be present, the results are remarkably alike.

The second line was to determine how far the antiseptic effect of the concentrate of a pneumococcal culture corresponded to that of a dilute solution of H_2O_2 of similar strength, *i.e.* one which would give a similar figure for available oxygen when that was determined by titration with potassium iodide and thiosulphate solution in presence of starch. Four experiments of this kind were performed and the results are given in Table I.

Table I.

		Antiseptic potencies expressed as highest dilution in peptone water which inhibited growth of *Staphylococcus aureus* completely			
Experiment	H_2O_2 of concentrated culture calculated in "volumes" by KI, $Na_2S_2O_3$ + starch titration	(*a*) Concentrated culture	(*b*) Solution of H_2O_2 in saline. Strength as calculated for concentrate	(*c*) Concentrated culture inactivated by heat: $+H_2O_2$ of same strength as in (*b*)	(*d*) Concentrated culture inactivated by heat
1	0·5	1–34	1–34	1–24	—
2	0·8	1–24	1–50	1–50	—
3	0·4	1–12	1–32	1–18	—
4	0·38	1–25	1–50	1–25	nil

It will be seen that the figures for antiseptic potency are nearly identical for concentrates and for heat inactivated concentrates which had received

addition of H_2O_2 sufficient to restore them to the same titration figure for available oxygen as that of the concentrates.

Further in the fourth experiment, where figures for concentrate and heat inactivated concentrate + calculated amount of H_2O_2 exactly correspond, a determination of volumes of gas liberated by catalase from 2 cc. of H_2O_2 of calculated strength was 0·7 cc. and that from 2 cc. of concentrate 0·67 cc.

That the antiseptic potencies of the concentrates should be usually less than those of solutions in 0·85 % saline of H_2O_2 of similar strengths is not surprising since in the strong solution of amino-acids etc. and suspended fatty bodies which constituted the concentrate the tendency to decomposition of the H_2O_2 is likely to be greater.

Changes in blood pigment effected by growth of Pneumococcus.

It has long been recognised that certain streptococci produce a green colour when grown on a blood agar plate, hence the name "viridans" [Schottmüller, 1903]. The Pneumococcus resembles this micro-organism in the appearance of its growth on blood agar plates. It has also been recognised for some time that pneumococci and certain streptococci produce methaemoglobin [Stadie, 1921].

Methaemoglobin is not however a green pigment and so far as we know no full investigation of the pigmentary changes associated with the development of the green colour has been made.

In recent years a medium called "chocolate agar" [Crowe, 1915, 1921; Neurin and Gurley, 1921], consisting of agar and heated blood mixed in varying proportions, has been much used. The blood here is no longer present in the form of haemoglobin or methaemoglobin and on such media the pigmentary changes produced by pneumococci etc. are much more striking than on unheated blood media. All gradations in colour between dark olive green and light yellow may be seen around pneumococcal colonies. If, however, small drops of dilute solutions of H_2O_2—$\frac{1}{4}$ to 1 "vol."—are repeatedly applied to the surface of such media a very similar range of colours is obtained. If the application of H_2O_2 is sufficiently powerful the medium is completely bleached. It would seem therefore that the varying colour changes which develop around pneumococcal colonies on such media are due to H_2O_2 formation in varying degrees of intensity. Also that the heated blood agar plate may reasonably be used to detect bacteria capable of forming H_2O_2.

Production of H_2O_2 by bacteria other than the Pneumococcus.

The following conclusions have been reached by using the "chocolate agar" plate as a primary indicator of H_2O_2 formation and then proceeding to confirm the fact that peroxide is produced by the bacteria which form green colonies, by growing them in bouillon or serum bouillon media in presence of abundant oxygen supply and testing their cultures with hydrogen peroxide reagents

such as Schönbein's or titanium sulphate solution. No bacteria have been met in the course of routine work or on going through a large series of stock cultures which produce a green colour on "chocolate agar," with the exception of various forms of cocci. Amongst the streptococci green-forming strains predominate but both amongst the haemolytic and the non-haemolytic forms strains are met which do not produce green on chocolate agar, and all such strains which have been examined have also failed to produce fluid cultures giving H_2O_2 reactions.

As a general rule, although with some distinct exceptions, the streptococci are differentiated from the pneumococci by producing a green colour later and with lesser intensity, similarly the presence of H_2O_2 is usually detected with the appropriate reagents at a later period in streptococcal than in pneumococcal culture, *i.e.* after 36 hours rather than after 18 hours.

In addition to the streptococci, bacteria from the urethra of sarcinal type and a coarse coccus unclassified have been found to produce a green coloration on "chocolate agar" and also to give H_2O_2 reactions in fluid media.

Amongst the many bacteria which do not produce green coloration on "chocolate agar" plates three were also tested for production of H_2O_2 in flasks of fluid media in which an air current was maintained. These were *B. coli*, Paratyphoid B. and Staphylococcus. All gave negative results and a number of different bacteria occurring from time to time as contaminations in such experiments have also given negative results.

Importance of catalase in connection with these phenomena.

It is an old finding in bacteriological work that most bacteria produce catalase [Gottstein, 1893].

If the production of H_2O_2 by bacteria as described above is a fact, certain observations may be reasonably expected to follow; amongst these are

(1) that bacteria producing H_2O_2 will not produce catalase;

(2) that such bacteria will grow much better in media containing catalase;

(3) that a medium containing some substance producing the catalase effect will be the best for maintaining stock cultures of such bacteria.

These observations have all been made; no evolution of oxygen over Pneumococcus colonies has ever been observed on pouring dilute solutions of H_2O_2 over cultures of these bacteria. The same holds for most streptococci. A more marked turbidity is developed in a serum bouillon culture of Pneumococcus than in a parallel culture where the serum bouillon has been heated to 65° for 30 minutes so as to destroy the catalase; that is if the cultures are incubated under conditions allowing of moderate access of oxygen. In fact it is very probable that the "vitamin" effects of fresh tissue fluids in promoting growth of Pneumococcus which Kligler [1919] describes are partly due to catalase.

Lastly it has been found that peptone bouillon containing 10 % of washed blood, a medium which retains to a considerable extent the power of de-

composing H_2O_2 even after heating for 20 minutes at 115°, is the most convenient for maintaining stock cultures of the Pneumococcus; no difficulty has been met in preserving various strains over long periods when subcultures have been made at 3–4 week intervals.

Possible importance of H_2O_2 formation by bacteria.

The most interesting possibility that arises in this connection is that H_2O_2-forming bacteria may under certain circumstances tend to kill themselves in the body by peroxide formation, just as they do in the test-tube. The most concrete case in which this possibility arises is the crisis in pneumonia. In the explanation of this there is admittedly a good deal of difficulty at the present moment, since the observed phenomena of phagocytosis, development of bactericidal quality in the serum, antitoxin formation etc. are inadequate. The chief difficulty in accepting such a theory of the crisis in pneumonia is that the pneumonic exudate is rich in catalase and it is most unlikely that any concentration of H_2O_2 similar to that which kills off the pneumococcus in culture can occur. There are two possible ways out of this difficulty. One is to suppose that something occurs to paralyse the catalase of the pneumonic exudate. Changes of reaction and enzyme reactions have been demonstrated in the pneumonic exudate which do not occur in the healthy tissues of the body [Lord, 1919, 1, 2; Lord and Nye, 1921, 1, 2]. Another is to suppose that a much smaller concentration of H_2O_2 may be effective in killing off the Pneumococcus in the lung than is required in cultural experiment on account of the oxidising ferments present in the exudate. Both however are subjects for extended experimental investigation.

Explanation of the formation of H_2O_2 by bacteria.

Wieland's [1912, 1, 2; 1913] theory of oxidation supposes that the essential phenomenon is the liberation of hydrogen and that the rôle of oxygen is that of an acceptor for the hydrogen liberated, while Wartenberg and Sieg [1920] show that, under certain conditions at all events, the first stage in the union of H and O is H_2O_2. Such a sequence of events would fit in well enough with the observed phenomena in pneumococcal cultures in which H_2O_2 is only formed as a bye-product where there is sufficient access of oxygen and little or no catalase.

The reason why H_2O_2 does not appear in the cultures of other bacteria may be either that they are catalase formers—the great majority of known bacteria; or that although not catalase formers they are too sensitive to the antiseptic action of low concentrations of H_2O_2 to grow sufficiently in the presence of oxygen to produce any recognisable traces of that substance—the anaerobes. When however certain strains of streptococci are considered which we have found to be incapable of producing catalase and relatively insensitive to H_2O_2, but which do not produce H_2O_2 in their cultures, it is obvious that other factors which await investigation must enter into the problem.

Conclusions and Summary.

1. The inhibitory substance developed in pneumococcal cultures to which there is abundant access of oxygen is H_2O_2.

2. This is proved both by chemical reactions and by the comparison of the antiseptic effects of the pneumococcal cultures and those of dilute solutions of H_2O_2 reckoned by titration etc. to contain a similar amount of available oxygen.

3. The early death of Pneumococcus in culture is usually due to accumulation of excess of H_2O_2.

4. The green or yellow colorations produced on heated blood media by certain bacteria are due to H_2O_2 formation.

5. In addition to pneumococci the only bacteria which have been shown to produce H_2O_2 are many streptococci, both haemolytic and non-haemolytic, and a few other coccal forms.

6. These findings can be utilised practically in putting bacteriological technique on a more definitely scientific basis in several respects.

7. The substance in fresh tissue fluids which specially promotes the growth of the Pneumococcus and which has been supposed to be of the nature of vitamin is most probably catalase.

In conclusion we have pleasure in expressing our indebtedness to Prof. M. J. Stewart in whose department this work was carried out and to Prof. H. S. Raper of Leeds University for valuable advice.

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