III. PEROXIDE FORMATION BY PNEUMOCOCCUS AND ITS RELATION TO BACTERIAL OXIDATION-REDUCTION REACTIONS.

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AVERY AND MORGAN [1924] in confirming and extending the findings of M'Leod and Gordon [1922] have summarised the nature of the conditions which must be maintained in order that maximum amounts of peroxide may be produced in cultures of pneumococcus. No mention is made, however, of the effect of the various constituents of the culture media on the amount of peroxide formed, though experiments reported in a later paper [Avery and Neill, 1924, 2] show that peroxide is not formed by washed pneumococci, nor by extracts of washed organisms, unless there is present a substance of undefined character which may be obtained from cell washings, meat or yeast. The organism is usually grown in media which contain substances of animal or vegetable origin, so that, with an adequate supply of oxygen and in the absence of peroxide-destroying agencies, considerable amounts of peroxide have generally been obtained. Pneumococcus does not grow in peptone water, but it has been shown [Platt, 1927] that the addition of a small amount of "Gold Label" gelatin to peptone water provides a medium in which the organism will yield a growth of the same order as that obtained in the presence of meat extract, as, for example, in 3 % gelatin-bouillon. The amount of peroxide produced when pneumococcus is grown aerobically in the former medium is considerably smaller than when the same medium containing meat extract is used. This difference is apparently due to the absence from gelatin-peptone of the substances necessary to complete the peroxide-forming mechanism of the organism. A convenient medium is therefore available for the purpose of determining the nature of these components.

With reference to the function of the "complementary" substances, Avery and Neill [1924, 2] suggest that "the principal rôle may be the furnishing of easily oxidised or autoxidisable substances." In a later publication [Avery and Neill, 1924, 3] they show that the complementary substances are thermostable, and, like the thermolabile component in extracts of washed pneumococci, do not react with molecular oxygen or methylene blue. This opinion has been modified [Neill and Avery, 1925], for it is stated that the thermostable substances react slowly with molecular oxygen forming oxidising agents, and, in anaerobic conditions, methaemoglobin and methylene blue are reduced to

a slight extent. The extract of washed pneumococci is said to catalyse a reaction which is represented by the expression:

Neill and Hastings [1925] and Neill [1925, 2] find that, whilst both oxygen consumption and haemoglobin oxidation by the autoxidisable substances of alcoholic extracts of meat infusion and potato juice are greatly accelerated by the cellular catalyst (C), the reaction with other autoxidisable substances, *e.g.* turpentine, linseed oil and oleic acid, is not affected by the presence of (C). In view of the results of the present research it appears that the substances (RH) required to complete the oxidation-reduction system (C + RH) of the pneumococcus are not mainly the autoxidisable substances of extracts of animal and vegetable tissues of the type found in turpentine. Indeed, were they similar, one would expect that the reactions of both systems would be accelerated alike on the addition of component (C). The suggestion therefore is made that the extracts used, supposed to contain the substances (RH), also contain compounds capable of acting as hydrogen donators. In the presence of the pneumococcus substance (C), hydrogen may be donated to molecular oxygen, which may need to be activated, with the subsequent formation of hydrogen peroxide. Given appropriate conditions, haemoglobin may be oxidised to methaemoglobin and, in the absence of oxygen, methylene blue and methaemoglobin may be reduced. An important hydrogen donator in the case of peroxide-forming bacteria appears to be lactic acid, which may be present in media containing meat or yeast extracts or may be produced by the organism from fermentable substances in the medium.

EXPERIMENTAL.

Methods.

The estimation¹ of peroxide in the cultures was carried out by adding *M*/100 titanous sulphate in excess, the amount of reagent not oxidised being determined by titration with *M*/100 iron alum solution in the presence of potassium thiocyanate. The conditions specified by Thornton and Chapman [1921] for accurate estimations have been observed. It was found to be more convenient to store the titanous sulphate solution in 4*N* sulphuric acid under hydrogen than to adopt the method advanced by Russell [1926]. All the titrations were carried out in an atmosphere of carbon dioxide. The accuracy of the method for the present purposes was proved by estimating known amounts of peroxide in such culture media as would be employed. Small initial titrations, which remained unchanged on prolonged incubation, were observed in

¹ I am indebted to Mr C. R. Hoskins, B.Sc., of Leeds University, for his assistance with the technique of the peroxide estimations.

sterile media containing meat extract. The titration values for sterile control media are subtracted from those obtained in the following experiments.

The number of viable organisms in a culture was determined by dilution of various amounts with 0.1 % gelatin-saline, several loopfuls of each dilution being plated out on a heated blood-agar medium.

For the preparation of the media Parke-Davis' peptone and Coignet's "Gold Label" gelatin were used. The bouillon was prepared by extracting lean beef with water at 100° for 2 hours (1 l. water to 500 g. beef) and adding 1 % Parke-Davis' peptone and 0.5 % sodium chloride. The media were all adjusted to p_H 7.8 and sterilised by autoclaving. The strain of pneumococcus (pn. 4) was isolated from pus from a case of empyema and was found to be bile-soluble.

Exp. 1. 240 cc. of 3 % gelatin-peptone and of 3 % gelatin-bouillon were placed in 3-litre Erlenmeyer flasks, the depth of the medium being about 12 mm. 10 cc. of a 10 hours' culture of a strain of pneumococcus (pn. 4) were used to inoculate each medium. Samples were taken out at intervals, under sterile conditions, for estimations of growth and peroxide. The results obtained are expressed graphically in Fig. 1, where the logarithm of the number of viable organisms per cc. of culture and the amount of peroxide (expressed as cc. $M/100$ H_2O_2) per 10 cc. of culture are plotted against the time of incubation at 37°.

Fig. 1.

It is evident from Fig. 1 that there is a marked difference in the amount of peroxide formed in the two media used, without corresponding differences in the amount of growth. Moreover, peroxide is not produced in gelatin-bouillon in appreciable amounts until near the end of the period of logarithmic increase of the bacteria. Another feature is more clearly demonstrated in the

following experiment when the cultures were incubated at a temperature lower than the optimum for growth.

Exp. 2. 10 cc. of 3 % gelatin-bouillon, inoculated with pn. 4 from an 18 hours' culture on heated blood-agar, were pipetted into 250 cc. Erlenmeyer flasks. After incubation at 30° for varying periods, 0.5 cc. was used for a growth estimation, the remainder being titrated with $M/100$ titanous sulphate and $M/100$ iron alum solutions. The results are given in Fig. 2.

Fig. 2.

The portion (A-B) of the time-peroxide curve given in Fig. 2 appears to be related to the period of stationary growth of the organism and may be due to the effect of the gelatin on the regulation of the oxygen supply to the organism. The organisms which develop before and at the stationary period will exhaust the supply of oxygen, so that there is insufficient for the formation of more peroxide, and there is also a tendency to reduction of that already formed. The latter tendency ceases with the active growth of the organism, whilst the respiration of the autolysed or autolysing bacteria proceeds until the maximum value is attained. That peroxide may be formed in cultures under conditions incompatible with active growth has been demonstrated by Avery and Neill [1924, 1]. Also, inhibitory amounts of peroxide are found in gelatin-bouillon cultures before the maximum value (about 0.01 %) is attained. (Morgan and Avery [1924] show that growth of pneumococcus may be inhibited for 24 hours in a concentration of peroxide of 0.006 %.) There is evidence that more peroxide is formed by the organism than that represented by the actual titration values.

Exp. 3. 150 cc. each of 5 % serum-bouillon and 5 % serum-3 % gelatin-bouillon, inoculated from a serum-bouillon culture of pn. 4, were incubated at 37° in 1-litre Erlenmeyer flasks. The ox-serum, which had been sterilised by filtration, was heated to 60° for 30 minutes before use. The data obtained for the rate of growth and peroxide formation are represented in Fig. 3.

From these results it appears that the chemical and physical properties of serum are such as to yield more immediate and regular production of peroxide. The addition of gelatin to serum-bouillon however results in a time-peroxide curve of the type obtained for gelatin-bouillon. It will be seen that the "step" in this curve appears earlier on account of the larger number of organisms produced, as shown in the growth curve.

Fig. 3.

Exp. 4. In Table I are given the results of some of a number of estimations of peroxide carried out on 10 cc. of 48 hour cultures of pneumococcus in 3 % gelatin-peptone containing sodium lactate. There was no marked difference in the amounts of growth in the various cultures after 12 hours' incubation at 37°.

Table I.

Media		$M/100 \text{ H}_2\text{O}_2$
3 % gelatin-peptone		0.35 cc.
"	+0.02 % sodium lactate	0.78
"	+0.05 % "	1.10
"	+0.1 % "	1.22
"	+1.0 % "	1.10

It is noteworthy that no peroxide is obtained in gelatin-peptone cultures of pneumococcus when a small amount (0.2 %) of sodium thiolactate is present, even when positive tests for peroxide are obtained in control cultures. This substance did not appear to affect the amount of growth.

In a 48 hours' culture of the pneumococcus, containing initially 1 % glucose, a titration of 0.95 cc. $M/100 \text{ H}_2\text{O}_2$ was obtained. It will be observed that the peroxide formed on the addition of sodium lactate or glucose to gelatin-peptone cultures is not more than 50 % of the amount usually found in gelatin-bouillon cultures. Small increases in peroxide are found if 1 % glycogen or 0.02 % inositol is present in gelatin-peptone cultures. The meat extract no doubt furnishes other substances from which, directly or in the

course of metabolism, peroxide is produced. Quastel [1926] finds that the effect of mixtures of hydrogen donors is equal to the sum of the activities of the separate constituents. The probability is that a similar relationship exists for the constituents of a medium taking part in peroxide formation.

DISCUSSION.

In addition to the foregoing observations, there are other lines of evidence which point to the important part played by lactic acid in bacterial oxidation-reduction reactions [cf. M'Leod, Gordon and Pyrah, 1923]. A comparison of the curves in the above figures, which are typical of a number obtained, with that given by Morgan [1924] for the rate of change in p_H of a culture of pneumococcus also suggests that the peroxide and acid production are related. Further, it has been observed that aerobic cultures, in which there is active peroxide formation, invariably develop a more acid reaction than those in which only small amounts of peroxide are produced. This difference is not determined by the amount of growth. Valentine [1926] has recorded interesting variations in the properties of two strains of streptococci X and Y. Strain X produces much less peroxide and less acid than strain Y. The latter strain has also a much wider range of fermentation reactions.

There is undoubtedly a close parallelism between the reducing and peroxide-forming activities of extracts of pneumococcus [Avery and Neill, 1924, 3], so that it may reasonably be assumed that under appropriate conditions lactic acid will complete the reducing as well as the peroxide-forming systems. A comparable mechanism appears to exist in washed *B. coli* which, in the presence of lactic acid, has a marked reducing action [Quastel and Whetham, 1925, 1]. No peroxide formation however will be evident, on account of the presence of catalase in *B. coli*. It is significant that yeast reacts towards lactic acid in a similar manner and that both yeast and *B. coli* appear to metabolise lactic acid only when an ample supply of oxygen is available.

Glucose has been found to promote peroxide formation in gelatin-peptone cultures of pneumococcus. Now Neill [1925, 1] has shown that glucose does not activate extracts of washed pneumococci, but only completes the oxidation-reduction system of the intact cell. It therefore appears to be necessary that, before glucose can function as a hydrogen donor, fission of the glucose molecule must occur, probably with the formation of lactic acid. Quastel and Whetham [1925, 2] find that glucose has a high "reducing coefficient" in the presence of resting *B. coli*. Whether in this instance the glucose undergoes metabolism cannot be determined from available data.

In conclusion, it is suggested that, taking into account the relation of lactic acid to the type of bacterial reaction under discussion, less lactic acid would be formed in aerobic cultures of lactic acid-forming bacteria than in anaerobic cultures. This may explain the recent results of Rona and Nicolai [1926] and of Neuberg and Gorr [1926].

SUMMARY.

1. Cultures of pneumococcus have been obtained, which, though aerated and free from catalase, yet produce only small amounts of peroxide. A considerable amount of peroxide is formed when cultures contain meat extract. This difference in behaviour is not determined by the amount of growth in the two media.

2. The rates of growth of, and peroxide formation by, pneumococci are compared under various conditions. The effect of oxygen supply on the rate of peroxide formation is discussed.

3. An increase in the amount of peroxide formed is observed in pneumococcal cultures containing lactic acid or glucose. It is suggested that the lactic acid functions as a hydrogen donator in the presence of the pneumococcus, peroxide being formed when oxygen acts as hydrogen acceptor.

4. The influence of glucose and of lactic acid on some reactions of pneumococci is discussed and correlated with observations on other organisms.

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