XXIII. OXIDATION-REDUCTION POTENTIALS OF PNEUMOCOCCUS CULTURES.

II. EFFECT OF CATALASE.

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In previous communications the electrode potential behaviour of haemolytic streptococci [Hewitt, 1930, 1], *C. diphtheriae* [1930, 2], staphylococci [1930, 3] and pneumococci [1930, 4] has been described by the author and it has been shown that the oxidation-reduction potentials established in bacterial cultures depend upon the cultural conditions and upon the organism studied. The potentials developed in streptococcal cultures are roughly similar to those in pneumococcal cultures, but are quite different from those in staphylococcal and *C. diphtheriae* cultures.

Haemolytic streptococci and pneumococci form peroxide in aerated cultures, whilst staphylococci and *C. diphtheriae* do not, and it is becoming increasingly evident that peroxide formation is an important factor in bacterial behaviour. McLeod and Gordon [1923] classify bacteria on the basis of peroxide formation and account for the phenomenon of anaerobiosis on the grounds of the toxicity of peroxide. In his recent work on the effect of oxidation-reduction conditions on bacterial variation Todd [1930] finds that peroxide may have a selective inhibitory effect on a particular variant and thus peroxide formation may lead to selection of another variant less sensitive to peroxide.

In view of the importance of peroxide it is of interest to eliminate its effect and to determine what other differences exist between different bacteria. Addition of catalase to a peroxide-forming organism such as the pneumococcus should inhibit peroxide formation and thus reveal other characteristics in its oxidation-reduction behaviour.

In this paper are described the effects of catalase on the electrode potentials developed in pneumococcus cultures.

Methods.

The general methods and apparatus for the measurement of electrode potentials were the same as those previously described [1930, 1]. An inoculum of 0·1 cc. of a 24-hour broth culture of pneumococcus type II was made into 8 cc. of medium in each case and the potential was observed from the time

of inoculation, at frequent intervals for the first 12 hours and then less frequently. The incubator temperature was 37°.

Catalase preparations.

Liver catalase. The liver catalase preparation was made by the general method of Batelli and Stern [1904]. Fresh liver was minced finely, extracted with water, the cell debris was centrifuged down and the supernatant fluid was treated with 2 volumes of 98 % alcohol. The flocculent precipitate was filtered off, dissolved in water and reprecipitated with alcohol. The precipitate was filtered off on a Büchner funnel, drained thoroughly and dried *in vacuo* over sulphuric acid. The yield was approximately 1 g. of dried powder from 1 lb. of fresh liver, and the preparation showed marked catalase activity.

Bacterial catalase. The bacterial catalase, kindly provided by Dr E. W. Todd, was prepared as follows. *Micrococcus lysodeikticus*, grown on agar slopes for 48 hours, was suspended in saline and rendered sterile by incubation with chloroform, the chloroform was removed and the killed organisms were then centrifuged down, the supernatant fluid being discarded. The freshly centrifuged cells had only a weak catalase activity but when allowed to stand overnight the catalase activity had increased enormously. Possibly the intact cells react sluggishly with hydrogen peroxide and catalase escapes slowly either by diffusion or by lysis of the cells.

Blood catalase. Sterile, washed red blood corpuscles suspended in saline were used as a third source of catalase.

Results.

Liver catalase.

0·1 g. of the liver catalase preparation described above was dissolved in 10 cc. of water and filtered through a sterile Seitz filter. 1 cc. of this sterile 1 % solution was added to each 10 cc. of peptone infusion broth. Catalase broth was prepared by heating broth in a boiling water-bath for 30 minutes, cooling and adding the liver catalase, and inactivated catalase broth was prepared by adding the liver catalase and then heating the broth for 30 minutes. The catalase broth had marked catalase activity whilst the inactivated catalase broth, used in control experiments, had no catalase activity.

Aerobic cultures. In ordinary stationary aerobic cultures the potential fell to the same extent in the liver catalase broth and inactivated catalase broth. For the first 12 hours the catalase had no effect on the potential-time curve (Fig. 1). After the logarithmic phase of growth the potential of the culture in the inactivated catalase broth began to rise as is usual in pneumococcus cultures [Hewitt, 1930, 4] and the 48-hour culture had a potential 0·32 v. less negative than the 12-hour culture. In the catalase broth culture, on the other hand, the potential remained at the same low (*i.e.* reducing) level long after the logarithmic phase of growth and at the end of 50 hours had not

risen above the level of the 12-hour culture. The presence of catalase therefore prevents the disappearance of reducing conditions usually observed after the logarithmic phase of growth, although it has no effect on the initial establishment of reducing conditions. It seems evident that peroxide formation must be responsible for the disappearance of reducing conditions and the rise of potential since these are prevented by the presence of catalase. That peroxide formation is effective at such low (reducing) levels of potential is somewhat surprising and will be commented upon later.

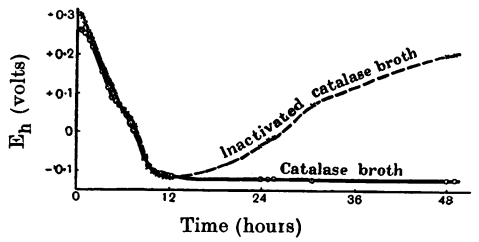

Fig. 1. Aerobic peptone infusion broth cultures of pneumococcus (liver catalase).

Aerated cultures. It was shown in a previous paper [1930, 4] that when cultures of pneumococci were subjected to vigorous aeration, the potential fell only to a slight degree and then rapidly rose to the high level of potential which corresponds with the formation of detectable amounts of peroxide. It was found that inactivated liver catalase had no effect on the electrode potentials of aerated pneumococcus cultures. In Fig. 2 the upper curve

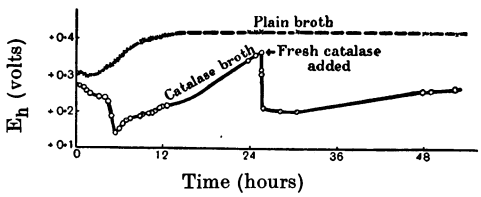

Fig. 2. Aerated pneumococcus cultures (liver catalase).

represents an aerated broth culture of pneumococci, and a similar culture containing inactivated catalase gave an almost indistinguishable curve. The potential fell only 0·007 v. then rose rapidly, and in 10 hours reached $E_h = + 0{\cdot}4$ v. at which level peroxide may be detected chemically in the culture. It was found, however, that in a similar culture containing active liver catalase the potential fell 0·13 v. and then commenced to rise. After 25·5 hours the potential had reached $E_h = + 0{\cdot}36$ v. At this stage a further quantity of liver catalase was added (equal in amount to that originally added). This resulted in an immediate rapid drop in potential amounting to 0·15 v. in 40 minutes. This effect was evidently due to decomposition of the peroxide present by the fresh catalase added, the original catalase having been destroyed by the continuous production of peroxide by the pneumococci.

A further interesting observation was made with regard to the effect of catalase. After 53 hours' incubation the aerated broth culture, the aerated inactivated catalase broth culture and the aerated catalase broth culture, were subcultured on blood agar. After 18 hours' incubation there was no growth from the two former inoculations but copious growth from the catalase broth culture. Peroxide formation had sterilised the broth cultures but the presence of catalase had protected the catalase broth culture. It is worthy of note that the aerated catalase broth culture was markedly turbid after 53 hours' growth showing that pneumococci flourish when the oxygen supply is abundant, provided that the harmful effects of peroxide are removed. This confirms the view previously expressed [1930, 1, 2, 3] that many bacteria grow most luxuriantly in the presence of a generous oxygen supply. This effect may be obscured with organisms which do not contain catalase owing to the toxic effect of the peroxide which may be formed when the oxygen supply is abundant.

Bacterial catalase.

0·05 cc. of a sterile killed preparation of *M. lysodeikticus*, centrifuged down to a thick suspension, was added to each 10 cc. of broth. For bacterial catalase broth the broth was heated for 30 minutes in a boiling water-bath and cooled before the addition of the preparation whilst for inactivated bacterial catalase broth, used as a control, the preparation was added to the broth before heating.

Aerobic cultures. In ordinary stationary aerobic cultures the effect of bacterial catalase was similar to that observed with liver catalase (Fig. 3).

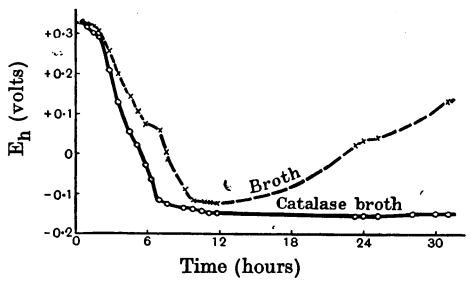

Fig. 3. Aerobic pneumococcus cultures (bacterial catalase).

After 30 hours' incubation the culture containing bacterial catalase had the same low potential reached after 12 hours' growth whereas in a similar culture not containing catalase the potential had risen 0·16 v. It may be worthy of note that whereas the boiled inactivated suspension of *M. lysodeikticus* had no effect on the establishment of reducing conditions, the unheated but killed bacteria appeared to facilitate reduction, since the potential fell rather more rapidly and to a slightly lower level in the bacterial catalase broth. This effect was not seen with liver catalase nor with blood catalase and may possibly be due to certain enzymes or accessory substances in the killed bacteria. The

point of inflection on the plain broth curve just below $E_h = + 0{\cdot}1$ v. has been observed on a number of occasions but its significance is uncertain.

Aerated cultures. Bacterial catalase had the same effect as liver catalase on the electrode potential behaviour of vigorously aerated cultures of pneumococci (Fig. 4). In the aerated broth and the aerated inactivated bacterial

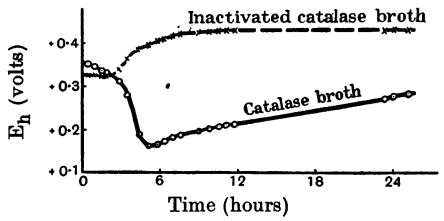

Fig. 4. Aerated cultures (bacterial catalase).

catalase broth cultures the potential fell only 0·004 to 0·007 v. and then rose fairly rapidly reaching the peroxide level ($E_h = + 0{\cdot}4$ v.) after 6 hours' incubation. In the bacterial catalase broth, however, the potential fell to the extent of 0·19 v. and then rose very slowly and no peroxide was formed even after 30 hours' incubation.

Blood catalase.

Blood catalase broth was prepared by adding 0·1 cc. of sterile washed red blood corpuscles to each 10 cc. of broth.

Stationary aerobic cultures. In stationary aerobic cultures containing blood catalase the potential fell in the usual way except that a flat portion of the curve occurred at a level of $E_h = + 0{\cdot}3$ v. (Fig. 5). The form of the curve suggested that some poising oxidation-reduction system had to be reduced before the potential could fall. It seems probable that some haemoglobin system may be responsible for this effect. In this connection it may be mentioned that only reversible oxidation-reduction systems can give a well-defined

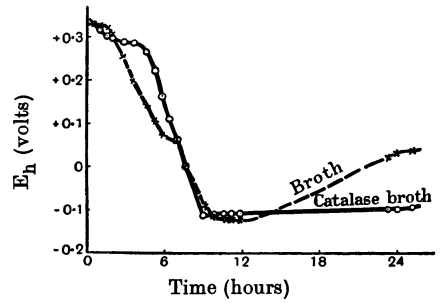

Fig. 5. Aerobic pneumococcus cultures (blood catalase).

potential at an unattackable electrode, but other systems may affect the potential-time curve of a culture and have a poising effect, since other reducing actions (which would be reflected in the fall in potential) cannot occur until the poising system is itself reduced.

The potential in a stationary aerobic blood catalase broth culture of pneumococci remained at a low level after 30 hours' incubation, whereas the potential in a plain broth culture had risen in the usual way. Blood catalase thus had the same effect in maintaining reducing conditions after the cessation of active proliferation as had liver and bacterial catalase.

Aerated cultures. The flat portion of the curve which was observed in stationary cultures was noticed also in vigorously aerated blood catalase broth cultures (Fig. 6). In the presence of blood catalase the potential of aerated

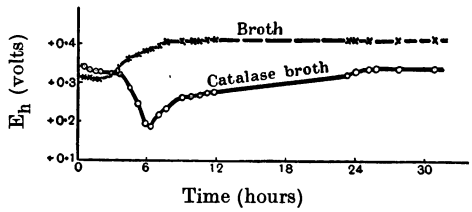

Fig. 6. Aerated pneumococcus cultures (blood catalase).

cultures fell to a much greater extent and rose more slowly than in plain broth cultures. Thus blood catalase had the same general effect on the potential as had liver and bacterial catalase preparations.

Discussion.

The presence of catalase has very marked effects on the oxidation-reduction conditions of pneumococcus cultures. By using catalase from different sources—from liver, bacteria and blood—it is possible to eliminate the interfering effects inherent in the use of enzymes which cannot be isolated in the pure state but which are always contaminated with other substances.

In ordinary aerobic pneumococcus cultures the presence of catalase appears to have no effect on the establishment of reducing conditions during the phase of active proliferation, but after this phase the electrode potential in a plain broth culture commences to rise, whilst in the presence of active catalase the potential remains at a low level for a considerable time. It seems evident therefore that the usual rise in potential, after the logarithmic phase of growth of pneumococci, is due to peroxide formation, which evidently occurs long before peroxide can be detected chemically. The hydrogen peroxide formed in oxidation-reduction processes is, therefore, immediately decomposed by catalase, before it has time to affect the electrode potential, which remains at a low (*i.e.* highly reducing) level. The electrode potential behaviour of an aerobic pneumococcus culture containing catalase is therefore roughly similar to that of cultures of staphylococcus or *C. diphtheriae* which produce their own catalase.

In vigorously aerated pneumococcus cultures proliferation occurs very slightly, if at all, and the potential shows only a slight tendency to fall (0·004 to 0·007 v.) and then rapidly rises to the level at which peroxide may be

detected chemically. When catalase is present, however, the potential falls appreciably (0·13 to 0·19 v.) and rises only slowly. As long as active catalase is present the level corresponding to peroxide accumulation is not reached but the catalase present is gradually destroyed by the active oxidising system. When fresh catalase was added to an aerated culture from which the catalase had disappeared there was an immediate rapid drop in potential.

50-hour aerated broth cultures of pneumococci are sterile but in aerated cultures containing catalase the bacteria still proliferate actively when sub-cultured after 50 hours' incubation. Growth is negligible in aerated plain broth cultures of pneumococci but in aerated catalase broth cultures growth is very good, showing that pneumococci flourish when the oxygen supply is abundant provided that the toxic effect of peroxide is eliminated.

Although there is a considerable fall in potential (*circa* 0·15 v.) in aerated catalase broth cultures of pneumococci, this fall is much less than that observed with staphylococci and *C. diphtheriae* (0·35 to 0·45 v.). This suggests that there are differences in reducing power of different organisms quite apart from the catalase- and peroxide-forming functions. It is by no means certain, therefore, that the failure of obligate anaerobes to grow aerobically is due solely to potential peroxide production in the absence of catalase. Not only the toxic effect of peroxide but also inability to effect oxidation-reduction processes at high levels of electrode potential may account for the phenomena of anaerobiosis.

Bacterial peroxide and hydrogen peroxide have a number of properties in common.

(1) They give the same chemical reactions (*e.g.* the benzidine-peroxidase reaction);

(2) display similar electrode-potential behaviour [Hewitt, 1930, 1];

(3) are both decomposed in the presence of catalase.

These facts suggest that bacterial peroxide and hydrogen peroxide if not identical must be very closely related compounds, and my experiments, so far, have failed to reveal any differences between them.

Apart from their catalase effect *M. lysodeikticus* and red blood corpuscles have other effects on the oxidation-reduction conditions developed in pneumococcus cultures. Killed *M. lysodeikticus* appeared to accelerate reduction effects. This reducing effect was destroyed by heating and may be due to the presence of reducing enzymes or accessory substances. Red blood corpuscles, on the other hand, exerted a poising effect possibly due to some haemoglobin system which itself had to be reduced before further reducing effects could be developed in the culture.

SUMMARY.

1. Some of the characteristic differences in behaviour between pneumococci and catalase-forming bacteria disappear when catalase preparations are added to the pneumococcus culture.

2. When catalase has been added the electrode potential of aerobic pneumococcus cultures remains at a low level long after the logarithmic phase of growth.

3. In aerated cultures the potential falls to a much lower level and growth is much more luxuriant when catalase has been added.

4. Bacteria differ in oxidation-reduction behaviour in respects other than that of peroxide formation.

5. Bacterial peroxide and hydrogen peroxide possess many properties in common.

6. Indications were obtained of an acceleration of reduction effects by killed aerobic bacteria (*M. lysodeikticus*) and of a poising effect by erythrocytes.

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