LIX. OXIDATION-REDUCTION POTENTIALS OF CULTURES OF HAEMOLYTIC STREPTOCOCCI. I.

By LESLIE FRANK HEWITT.

From the Belmont Laboratories (M.A.B.), Sutton, Surrey.

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Haemolytic streptococci can multiply over a wide range of conditions of oxygen supply from almost complete anaerobiosis to full exposure to the air, as on the surface of an agar slope. This ready adaptation to different conditions must involve changes in metabolic activity and biological behaviour. The measurement of oxidation-reduction potentials is the most direct method of following some of the effects of environmental conditions on the metabolic processes of the organisms.

When an "unattackable electrode" is immersed in a solution containing a reversible oxidation-reduction system a potential difference is set up at the electrode, and this potential may be shown, by simple thermodynamic reasoning, to be dependent on the proportion of oxidised and reduced forms of the substance studied. The potential of the normal hydrogen electrode is taken as the standard of reference and to this is ascribed the value of zero potential difference. An electrode potential referred to this standard is designated E_h and is measured in volts. The higher the oxidising intensity of a system the more highly positive is the E_h, and the more highly reducing a system the more negative is the E_h. An essential point to be remembered is that electrode potential, like temperature and p_H, is a measure of intensity level and not of capacity.

Bacterial cultures develop reducing tendencies during growth as shown, for example, by the decoloration of methylene blue. It would seem therefore that oxidation-reduction indicators might be employed for the determination of oxidation-reduction potential, in the way that p_H indicators are used for the determination of hydrogen ions. There are, however, certain disadvantages in the use of oxidation-reduction indicators, particularly in bacterial cultures. One disadvantage is that the oxidising capacity of the indicator is frequently great compared with the reducing capacity of the system, so that the bacterial culture is oxidised by the dye and the equilibrium is thereby displaced. Another disadvantage is the time taken for the dye to come into equilibrium with the system, which, in the case of bacterial cultures, is itself constantly changing. A further disadvantage is the participation of the dye in the

system and its possible toxic action on the organisms. Continuous readings cannot be made with a toxic dye; moreover, the killed organisms may have a different effect on the electrode potential of the medium. It is considered essential therefore to make direct determinations of the electrode potentials of bacterial cultures.

Gillespie [1920], who was particularly interested in the behaviour of water-logged soils, first noted the steady drift towards negative potentials in bacterial cultures. This was confirmed by Cannan, Cohen and Clark [1926], and by Fildes [1929] who found that a certain limiting reduction potential is necessary for the germination of tetanus spores.

The present communication is the outcome of some work carried out with Dr E. W. Todd in connection with the biological behaviour of haemolytic streptococci under different cultural conditions. The purely bacteriological aspect of the subject will be dealt with by Dr Todd in a communication shortly to be published.

The conditions of cultivation were found to have a marked effect on the electrode potential-time curves of the cultures and the results obtained are reported with the minimum of comment until it is felt that chemical interpretations and biological implications are substantiated.

Methods.

The oxidation-reduction potentials of bacterial cultures are generally not well poised, so that it is essential to avoid polarisation effects. For this reason and for rapidity of measurements the Lindemann electrometer proved an excellent instrument for determining the null-point of potentiometric balance. Measurements were facilitated by the use of a series of single-pole, double-throw knife switches for connecting each individual cell and a double-pole, double-throw switch for reversing the connections to the potentiometer.

Glass double-limbed cells placed in wooden blocks were used, and, in some experiments, the cultures were kept in a state of agitation and aeration by using the three-limbed tube shown on the left in Fig. 1. The central tube was connected by rubber tubing to Ridley's [1928] mixing apparatus. In this way the cultures were alternately drawn up and depressed in the central tube, both the rate of movement and extent of excursion being readily adjusted. An agar-KCl bridge dipped into one limb of the culture-cell and into a vessel containing saturated KCl solution into which also a 3·5*N* KCl-calomel electrode dipped. Both gold wire and gold foil electrodes proved unreliable, so that platinum wires sealed into glass tubing were used and these gave steady reproducible readings. Some irregular results were explained by the discovery of a cracked electrode which caused direct electrical contact of mercury with the culture medium. Eight cells and the calomel electrode were placed in the incubator (at 37–38°) and the leads from these ran to the series of knife switches outside the incubator and thence to the potentiometer and electrometer. The glass apparatus was plugged with cotton-wool, sterilised in

hot-air and assembled with aseptic precautions. 8 cc. of medium were placed in each cell and the inoculum was 0·1 cc. of a 16-hour culture of the well-known "Aronson" strain of haemolytic streptococci. The purity of the cultures after experiment was confirmed by film preparations and agar plate cultures. Half-hourly readings were taken for the first 12–14 hours of incubation.

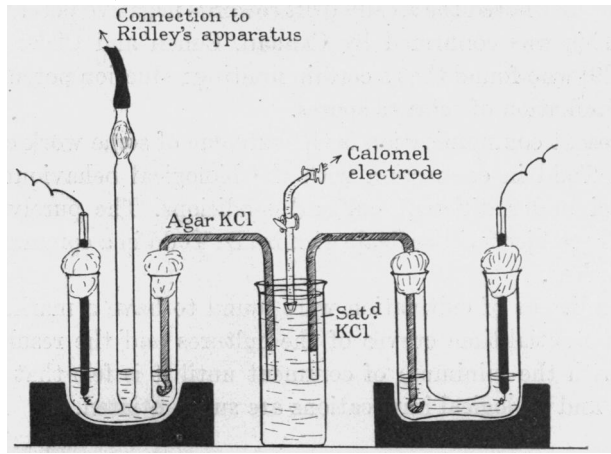

Fig. 1. Arrangement of apparatus in incubator.

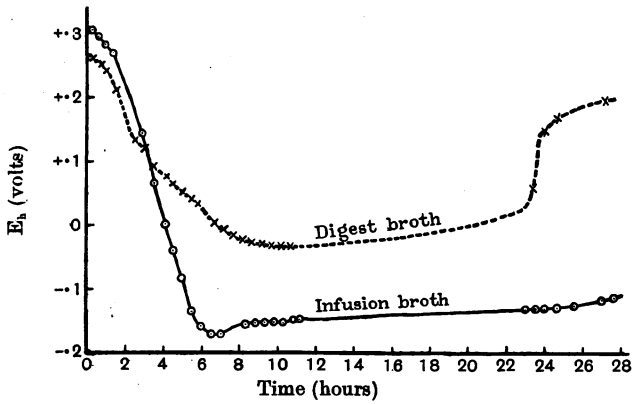

Fig. 2. Freshly boiled broths.

Results.

Tryptic-digest and peptone-infusion media (Fig. 2).

Horse-flesh infusion broth, containing 2 % of Difco peptone, and Douglas's tryptic-digest broth were used. The digest broth had a slightly lower initial electrode potential, but the potential of cultures in this broth fell more slowly and did not reach as low a level as in the infusion broth. The curves suggest more opposition to reduction processes in the digest medium and there is some indication of reduction in two stages. Further differences between the two kinds of broth will become apparent in later sections.

Effect of exclusion of air (Figs. 3 and 4).

When access to air of an infusion broth culture is prevented by sealing with melted vaselin the potential falls rapidly at first but subsequently more slowly, reaching eventually the same value as that reached in aerobic cultures.

In sealed tryptic-digest broth cultures the potential falls as rapidly as in aerobic cultures and reaches a lower level than that of aerobic cultures in the same broth.

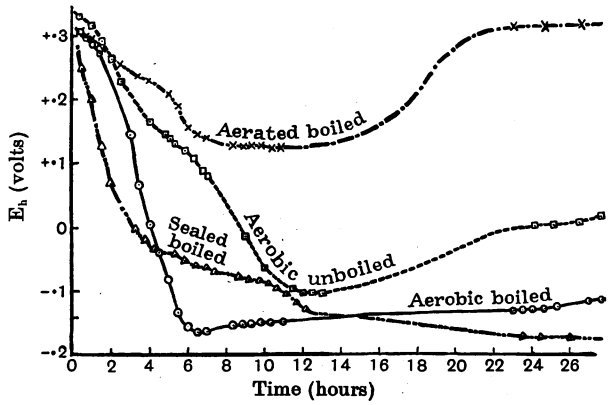

Fig. 3. Peptone-infusion broth.

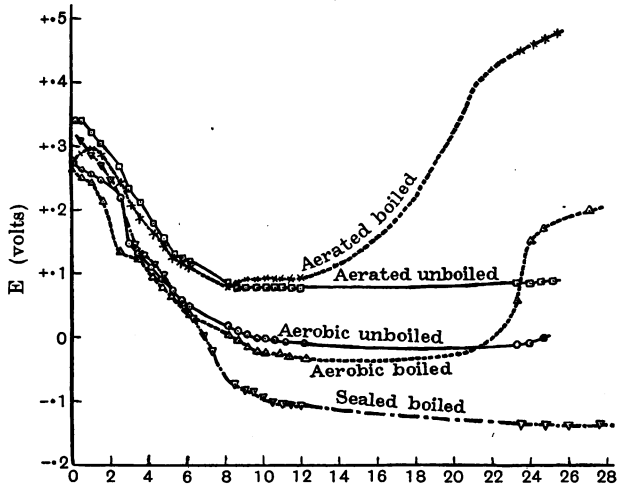

Fig. 4. Tryptic-digest broth.

Aerobically much lower potentials are reached in infusion broth but in the absence of air practically the same potentials are reached in infusion broth and tryptic-digest medium. This suggests an oxygen-carrying effect in tryptic-digest medium effected by substances liberated during tryptic digestion in the preparation of the medium. This phenomenon will be considered in more detail in a later section on the effect of serum.

Effect of freshly boiled medium (Figs. 3 and 4).

Tubes of broth were immersed in a boiling water-bath for 30 minutes and cooled immediately before inoculation. The electrode potential of the broth was reduced 30–100 millivolts by this treatment, and the potentials of cultures fell more rapidly and reached lower levels in freshly boiled broth. In a culture in freshly boiled infusion broth E_h − 0·1 volt was reached in 5 hours, whilst 11·5 hours were required to reach this potential in untreated broth. With tryptic-digest medium, boiling had less effect on reduction processes, but the potential rose much more rapidly after the phase of active proliferation in the freshly boiled medium (see Fig. 4). This facilitation of reduction effects and acceleration of oxidising effects by boiling the medium presents analogies to the expression of water from a sponge and the subsequent rapid re-absorption.

Effect of aeration (Figs. 3 and 4).

Aeration was effected by Ridley's [1928] apparatus, the cultures being alternately raised and depressed in cells of the type shown on the left-hand side of Fig. 1, fresh surfaces of the culture thus being constantly exposed to the air. When aerated in this way the potentials of cultures fell very slowly and did not reach the low levels observed in stationary aerobic cultures, and, after the phase of active growth, the potentials rose rapidly, in some cases reaching values corresponding to the formation of peroxide.

In freshly boiled broth the potentials rise much more rapidly after the phase of active growth, particularly in tryptic-digest media, thus providing a further example of the "sponge effect" already mentioned.

Effect of peroxide (Fig. 5).

When an abundant supply of oxygen is available, haemolytic streptococci may form peroxide, and this has a marked effect on the oxidation-reduction potential. In saline 0·02 % hydrogen peroxide has E_h + 0·52 volts. When peroxide was formed in aerated broth cultures (as detected by the benzidine-peroxidase test) the E_h registered between + 0·46 and + 0·5 volt according to the amount present. The upper curve in Fig. 5 shows the potentials observed in a tryptic-digest broth culture to which 0·007 % hydrogen peroxide was added before inoculation. It will be observed that there is a lag period before the potential commences to fall appreciably. After this period, when the peroxide has disappeared, the potential-time curve is almost identical with that of a plain tryptic-digest broth culture inoculated about 24 hours later than the peroxide culture. Incubation of the culture with the peroxide for some 8 hours appears not to have any appreciable effect on the organisms since the usual potential-time curve is obtained. This is in agreement with observations made on *B. sporogenes* [Quastel and Stephenson, 1926].

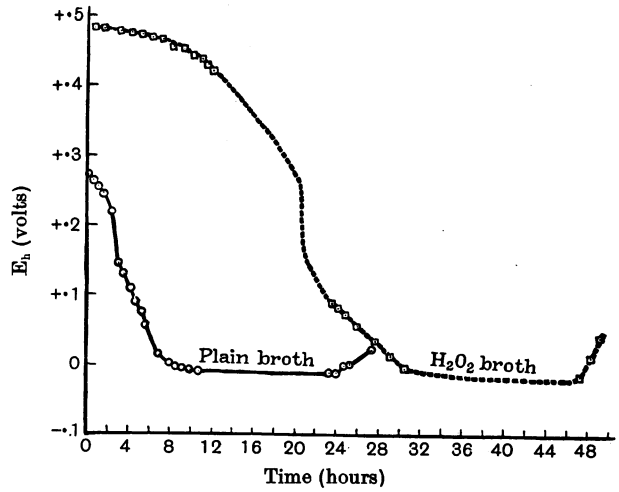

Fig. 5. Unboiled tryptic-digest broth.

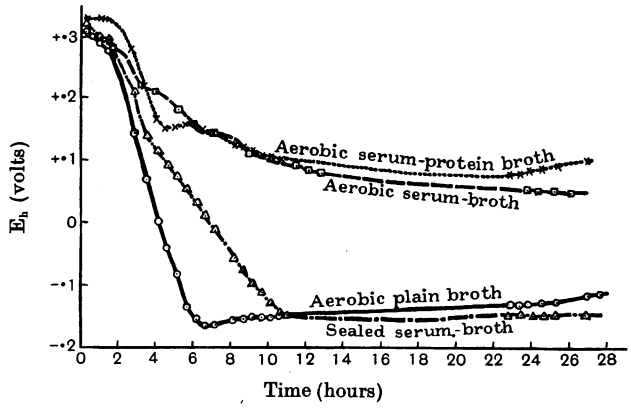

Fig. 6. Peptone-infusion broth.

Effect of serum (Fig. 6).

The presence of serum has a remarkable effect on the potentials reached by aerobic cultures of haemolytic streptococci. In aerobic 50 % serum-broth (peptone-infusion medium) the potential does not fall below $E_h + 0{\cdot}05$ volt despite the very luxuriant growth, whilst in plain peptone-infusion broth the potential drops to below $-0{\cdot}15$ volt. The same effect on the potential was obtained when separated serum-proteins were used instead of native serum. Serum-proteins were prepared by three precipitations by means of sodium chloride and acetic acid, dissolving in water, dialysing to remove excess salt, adjusting the reaction and making up to the original volume of serum.

When 50 % serum-broth cultures were sealed with vaselin the potential fell to the same level as that reached in aerobic plain broth cultures (*i.e.* some

200 millivolts lower than in aerobic serum-broth cultures). Evidently, then, serum has an oxygen-carrying effect in the presence of air, and this is due to the serum-proteins. This oxygen-carrying effect may be ascribed tentatively to the sulphur-containing groups in the protein molecule. The mechanism of oxidation of a substance, BH_2, in the broth in the presence of protein, PrS.SPr may be represented as follows:

$$PrS.SPr + BH_2 \rightarrow 2PrSH + B$$

and in the presence of air

$$2PrSH + \tfrac{1}{2}O_2 \rightarrow PrS.SPr + H_2O.$$

In a sealed culture without access to atmospheric oxygen this oxygen-carrying effect could not continue—thus conforming to the experimental results.

It will be recalled that tryptic-digest broth possesses this oxygen-carrying effect to some degree and it would seem possible that, during the tryptic digestion in the preparation of the medium, sulphur-containing compounds are brought into solution, thus conferring the oxygen-carrying effect on the broth.

Effect of sulphur compounds (Figs. 7 and 9).

In view of the participation of sulphydryl compounds in biological oxidation-reduction systems [Hopkins and Dixon, 1922; Meyerhof, 1923] and the oxygen-carrying effect of serum, the influence of two compounds containing the —S.S— linking on the potentials has been studied.

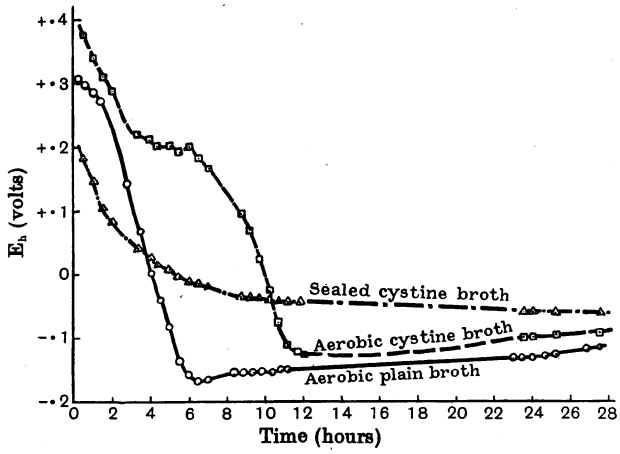

Fig. 7. Freshly boiled infusion broth.

In aerobic broth cultures containing 0·02 % (approximately) of cystine (see Fig. 7) the potential falls at first and then there is a very flat portion of the potential-time curve, after 4 to 6 hours' growth, just as with serum-proteins (Fig. 6). In sealed 0·02 % cystine-broth cultures there is a rapid initial fall of potential, but the level reached is not as low as in sealed serum-

broth cultures. A distinct similarity can be traced between the effect of serum and of cystine but the differences suggest the co-existence of some other effect.

Since cystine is relatively insoluble, the influence of thiodiglycollic acid was studied (Fig. 8). In 0·05 % thiodiglycollate-broth there is again the flat portion of the curve after 4 to 6 hours' growth in aerobic cultures and the rapid initial fall in sealed cultures. In addition, in the sealed cultures a

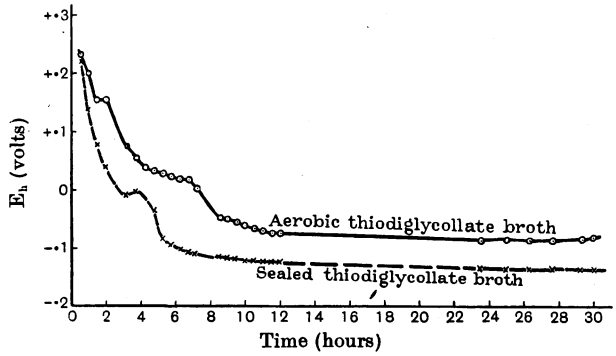

Fig. 8. Freshly boiled infusion broth.

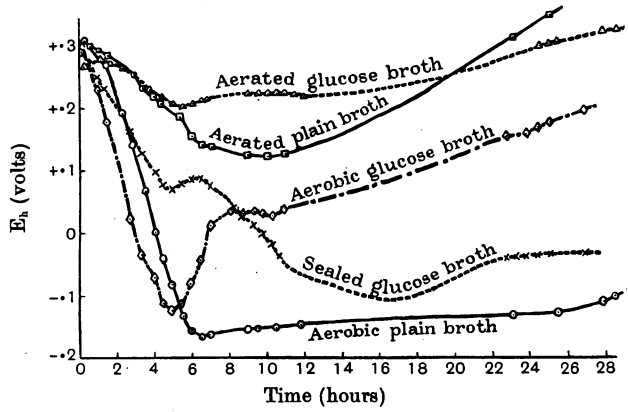

Fig. 9. Freshly boiled infusion broth.

potential some 50 millivolts more negative is reached than in the aerobic cultures. We have here, therefore, direct evidence of the oxygen-carrying effect of sulphur compounds and support for the hypothesis that these may account for the influence of serum on the potentials reached in cultures. That the effects observed are not absolutely identical may be due to the different range of effect of sulphur compounds when combined in the protein molecules, or to the effect of other constituents of the protein complex, *e.g.* lipins [Lepper and Martin, 1929].

Effect of glucose (Fig. 9).

In 1 % glucose-broth cultures the electrode potential falls very rapidly for about 5 hours and then suddenly rises some 100 millivolts. This is followed

by a slight fall in potential and then a gradual increase. Acid is produced in glucose-broth cultures about the time of the sudden rise in potential, and although the direct effect of p_H on the electrode potential is most probably insufficient completely to account for the phenomenon, this, together with the indirect effect of the acidity on the constituents of the culture may explain the effects produced. When air is excluded by a vaselin seal the potential in glucose-broth cultures falls more slowly than in aerobic cultures. This surprising result suggests that oxygen is required for the rapid development of high reduction potentials. Since the first stage of glucose fermentation (to lactic acid, etc.) proceeds anaerobically it would seem that the oxygen is required either for the utilisation of food-stuffs in the broth, or for utilising lactic acid, etc. if these are utilised by the streptococcus. When glucose-broth cultures are aerated vigorously growth is very luxuriant but the potential falls only to a very limited extent (some 100 millivolts compared with 450 millivolts in aerobic plain broth cultures). After the cessation of active proliferation the potential rises very slowly and does not reach the high oxidation potential reached in aerated plain broth; glucose or its breakdown products apparently opposing oxidation processes. Dubos [1929] has suggested that reductive conditions are necessary for active proliferation, but the high potential maintained in aerated glucose-broth cultures is accompanied by luxuriant growth, so that the reaching of a high reducing potential does not seem essential. Also in anaerobic sealed cultures, where there is little oxygen to oppose reduction processes, the potentials indicate a slower establishment of intense reducing conditions than in aerobic cultures, again suggesting that the organisms do not favour highly reducing conditions.

Effect of intermediate fermentation products (Figs. 10 and 11).

The effects of 1 % of the sodium salts of lactic acid, succinic acid and fumaric acid on the potentials of broth cultures of haemolytic streptococci are shown in Figs. 10 and 11. In aerobic cultures the potential falls rapidly—most rapidly with succinate and least so with fumarate broth cultures. When sealed with vaselin the potential falls very slowly in 1 % fumarate broth, somewhat more rapidly in broth containing both 1 % fumarate and 1 % succinate and still more rapidly in 1 % fumarate-, 1 % lactate-broth. The slow fall in potential in anaerobic cultures is probably due to the lack of oxygen for the utilisation of the acids. In the case of fumarate-lactate-broth the fumaric acid may act as hydrogen acceptor [Thunberg, 1921], as in the presence of muscle, thus oxidising the lactic acid to pyruvic acid which is probably readily utilised by bacteria.

$$\underset{\text{lactic acid}}{CH_3.CH(OH).COOH} + \underset{+\text{fumaric acid}}{COOH.CH:CH.COOH} \rightarrow \underset{\rightarrow \text{pyruvic acid}}{CH_3.CO.COOH} + \underset{+\text{succinic acid.}}{COOH.CH_2.CH_2.COOH}$$

In aerated cultures the potentials have a delayed fall followed by a slow rise in potential. This retarded rise in potential, also shown in glucose-broth, is in contrast with the observation of Platt [1927] that glucose and lactic acid accelerated the production of peroxide by pneumococci.

Conclusions.

Readily reproducible potential-time curves, varying markedly with change of conditions, were obtained with cultures of haemolytic streptococci. By varying cultural conditions the electrode potential during growth may fall over 450 mv., or less than 80 mv., and, after the phase of active growth, the potential may remain at a very low level, corresponding to intense reducing conditions, or may rise rapidly to levels at which peroxide appears. These

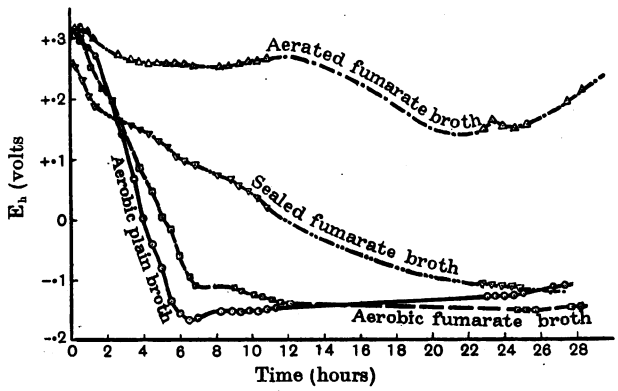

Fig. 10. Freshly boiled infusion broth.

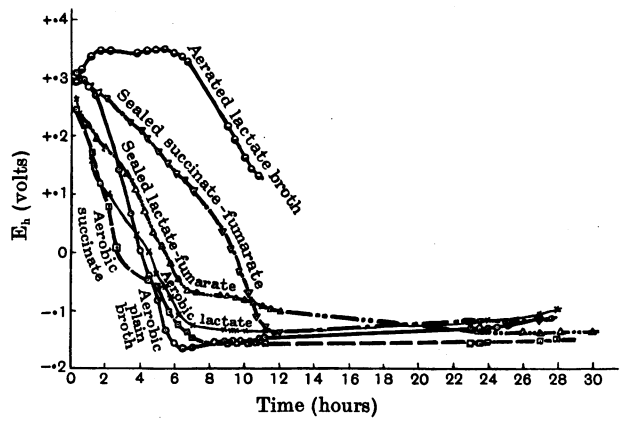

Fig. 11. Freshly boiled infusion broth.

very different potentials reached indicate the very wide range of oxygen tension at which the streptococci will grow readily, and must produce very different effects. Fermentation reactions, products of metabolism, morphology, virulence and toxin production can all probably be correlated with the oxidation-reduction potentials developed in the medium.

In aerobic 50 % serum-broth cultures the potential falls only 250 mv. during growth compared with a fall of 450 mv. in plain broth cultures. This oxygen-carrying effect, which is due to the serum-proteins and occurs only in aerobic cultures, is of interest from several points of view. With regard to

the mechanism of the effect, some evidence is presented that this may be due to the sulphur-containing units of the protein molecule. It seems possible that —SH groups in proteins may behave similarly to those in peptides such as glutathione. Serum-broth is a common culture medium for haemolytic streptococci and it seems probable that cultural conditions in serum-broth may approximate to those in the blood-stream. The special biological characteristics of organisms recently isolated from the body or obtained by animal passage may be due to the effect of serum-proteins, which, by regulating the oxidation-reduction potential, would control the metabolism of the streptococci.

Fildes [1929] has observed that the potential of subcutaneous tissue is too high for the germination of tetanus spores, and this again may be due to the maintenance of relatively high potentials through the agency of the oxygen-carrying effect of proteins.

The potential-time curves give some information regarding peroxide formation by haemolytic streptococci. In vigorously aerated cultures peroxide appeared after 16 to 24 hours' growth. In every culture in which peroxide was detected the electrode potential was high, *i.e.* between + 0·46 and + 0·52 volt according to the amount of peroxide formed. The same potentials were observed when hydrogen peroxide was added to saline or to broth. Bacterial peroxide is therefore closely similar to hydrogen peroxide. Low concentrations of hydrogen peroxide appeared not to have appreciable toxic effects on the streptococci but merely to produce a lag phase. In an aerobic tryptic-digest broth culture to which 0·006 % hydrogen peroxide was added the usual potential-time curve was obtained after the long lag period. In this stationary aerobic culture peroxide again appeared after 5 days' incubation. Glucose and lactic acid retarded peroxide formation whilst Platt [1927] found that with pneumococci they accelerated peroxide formation.

In describing the state of oxidation-reduction systems the electrode potential, E_h, is sometimes replaced by r_H, which, by analogy with p_H, is the logarithm of the reciprocal of the partial pressure of hydrogen in equilibrium with the system.

In the following table are summarised the minimum values of r_H reached in various cultures and the time required to reach these values.

System	Approximate minimum value of r_H	Time to reach r_H (hours)
Hydrogen peroxide in broth	30	0
Bacterial peroxide	30	0
Uninoculated broth	25	0
Aerated glucose broth cultures	21	5
Aerated plain ,,	17	8
Aerobic serum ,,	16	16
Sealed ,, ,,	9	12
Freshly boiled aerobic glucose broth cultures	10	5
,, sealed ,, ,,	10	24
,, aerobic digest ,,	13	12
,, sealed digest ,,	9	24
Unboiled aerobic infusion ,,	10·5	12
Freshly boiled aerobic infusion ,,	8·7	7
,, sealed ,, ,,	8·7	20

Dubos [1929] claims that reductive conditions are necessary for the rapid proliferation of small implants of haemolytic streptococci, but it has been found that in the present experiments the streptococci showed particularly luxuriant growth under conditions in which the potential remained at a high (oxidising) level and that anaerobically the fall in potential in glucose, lactate, and similar broth cultures was slow.

Distinct differences exist between the potential-time curves of different strains of haemolytic streptococci. In this communication results obtained with one strain only are reported, but most of the phenomena described have been observed with several different strains.

Summary.

1. Experimental details are described for obtaining reproducible oxidation-reduction potential-time curves of cultures of haemolytic streptococci. The potentials observed varied widely with different cultural conditions.

2. The presence of serum in broth inhibits the fall in potential during growth in aerobic cultures. This influence is due to an oxygen-carrying effect of the serum-proteins which has been tentatively ascribed to sulphur-containing units in the protein molecule. The biological significance of these phenomena is discussed.

3. The fall in potential during growth is less in tryptic-digest medium than in peptone-infusion broth.

4. In freshly boiled broth the potential falls more rapidly and to a lower level than in unboiled broth, and, particularly in tryptic-digest medium, rises more rapidly after the cessation of active proliferation.

5. When cultures are aerated the potential falls less during growth and rises more rapidly after the logarithmic phase of multiplication. Peroxide is frequently formed in aerated cultures and has a marked effect on the potentials, which are similar to those observed when hydrogen peroxide is added to broth or to saline. Glucose, lactic acid, etc. retard peroxide formation. When a low concentration of hydrogen peroxide is added to broth before inoculation there is a long lag period but after this the normal potential-time curve is obtained. The organisms, therefore, are apparently not injured by the peroxide in low concentration.

6. The potential falls rapidly in aerobic 1 % glucose-broth cultures but less rapidly in sealed cultures. A short phase of rapid increase of potential is observed in aerobic glucose cultures after a few hours' growth. In aerated glucose-broth cultures the potential falls very little despite the very luxuriant growth obtained.

7. No evidence was obtained that reductive conditions (low oxidation-reduction potential) favoured the growth of haemolytic streptococci.

The author is indebted to Dr R. G. White and Dr E. W. Todd for suggestions, assistance and helpful encouragement.

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