CXXII. OXIDATION-REDUCTION STUDIES IN RELATION TO BACTERIAL GROWTH.

I. THE OXIDATION-REDUCTION POTENTIAL OF STERILE MEAT BROTH.

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Several observations have been made on the oxidation-reduction (O/R) potentials of growing cultures of bacteria, but the O/R potential associated with the initiation of bacterial growth has been studied only by Fildes [1929, 1] in connection with the germination of spores of *B. tetani*.

Developing the views of Clark [1924] Fildes pointed out that "the term 'anaerobe' as applied to a micro-organism implies that it requires for growth a certain reducing intensity in its surroundings." Conversely, for organisms which will not grow under "strictly anaerobic" conditions, it may be assumed that the reducing intensity must not be too great if growth is to take place.

The relation of the onset of bacterial growth to the oxidation-reduction conditions in the surroundings is thus a subject which has a direct bearing on the biology of bacteria and, further, has been used by Fildes [1929, 2] to account in part for their pathogenic action.

In studying the effect of p_H, the hydrogen ion concentration of a culture medium can be adjusted to any value and maintained constant against the CO_2 of the air, etc., by means of buffers. In the case of the O/R potential, which depends, at constant p_H, on the degree of reduction of some electromotively active system, anything which tends to alter the degree of reduction will alter the potential. The interference of fortuitous oxidising or reducing agents with the normal behaviour of the oxidation-reduction systems of culture media must therefore be strictly controlled. The O/R potential of a culture medium has no definite significance until such factors as the casual access of the oxygen of the air are eliminated. An essential preliminary to any attempted control of oxidation-reduction conditions is, therefore, an investigation of the O/R potential of sterile culture media. Until this is done it is not possible to control the level of O/R potential in a medium.

There are obvious limitations and difficulties in the investigation of such problems by the O/R dyes used by Fildes and others. It was therefore decided to use electrometric methods.

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The ability of sterile meat broth and other bacteriological media to reduce various O/R dyes, notably methylene blue, has long been known. Only recently have attempts been made to measure this reducing intensity in terms of O/R potential. Dubos [1929] used Clark's oxidation-reduction dyes and found that plain broth under vaseline seal reduced potassium indigodisulphonate almost completely at p_H 7·8, corresponding to an E_h of about − 0·18 volt (E_h is the potential referred to that of the normal hydrogen electrode as zero). Fildes [1929, 1] made more extensive observations over a range from p_H 6 to 9 and showed that the p_H had an important effect not only on the rate of reduction but also on the final level of potential which the medium attained. His value at p_H 7·8 approximates to that of Dubos.

Coulter [1928] is the first to have applied electrometric methods to the study of the oxidation-reduction system of sterile broth. His experiments show clearly that there is an electromotively active system present in sterile broth which tends to establish an "inert" electrode at a definite level of E_h (at constant p_H) when access of oxygen is prevented. When a sample of broth was de-aerated with oxygen-free nitrogen the potentials of gold electrodes immersed in the broth steadily became more negative. The drift of potential was slower if the de-aeration took place only for a short time and the vessel was then sealed off. In either case Coulter found that the potential reached a final value of about E_h − 0·06 volt at p_H 7·6. This is about 0·1 volt more positive than the value found by Fildes and by Dubos, using dyes.

Unfortunately Coulter confined his observations to one p_H value. The present paper gives a more extended view of the electromotively active system of broth. The general behaviour of the system described by Coulter is fully confirmed. There is, however, a quantitative difference between his value of the potential at p_H 7·6 and the corresponding value found in the present work. This may be due to differences in the actual medium used.

Experimental.

The general technique for oxidation-reduction potential measurements described by Clark [1923], Clark and others [1928] has been used, with some alterations necessitated by the special nature of the system to be investigated. The electrode vessel consisted of a stout Pyrex vessel 4 cm. in diameter and 10·5 cm. long, having a capacity of about 100 cc. The vessel was closed by a large rubber bung carrying three electrodes, gas inlet and outlet tubes, and a saturated KCl-agar bridge for making contact with the standard half-cell. The half-cell used in these experiments was quinhydrone in $M/20$ phthalate. This was found to have a value of E_h + 0·445 volt at 38°, which was the temperature of the incubator enclosing the electrode vessel and half-cell.

The electrodes were plates of platinum foil, area 4 cm.^{2}, freshly gilded, electrolytically, for each experiment. The pieces of foil were welded to platinum wires and these fused into glass tubes, contact being made with mercury. Care must be taken to avoid minute cracks in the glass at the seal. These may

cause large discrepancies between electrode readings. The electrodes were cleaned in chromic-sulphuric acid before gilding. Care was taken not to finger the electrodes after they had been gilded and they were kept immersed in distilled water. Occasionally bright platinum electrodes were used. The bung carrying the electrodes, etc., was sterilised by autoclaving, supported loosely in a conical beaker with the electrodes under distilled water and the mouth of the beaker wrapped with cotton gauze. The electrode vessel, containing 50 cc. of buffer-broth, was autoclaved separately. The KCl-agar bridge carried by the bung was drawn off and sealed at both ends before autoclaving.

After autoclaving, the capillary ends of the agar bridge were cut off with a sterile glass-knife and the bung was fitted aseptically to the electrode vessel. The bung was painted with gold size when fitted, to make it gas-tight. The gas inlet tube contained a small plug of cotton wool to filter the gas. Oxygen-free nitrogen was used for stirring and de-aerating the electrode vessel and its contents. The nitrogen, from a cylinder of the compressed gas, was purified by passage through reduced copper gauze contained in a silica tube heated to a red-heat by an electric furnace. The rigorous precautions against leaks of oxygen recommended by Clark [Clark and others, 1928], including a copper tubing gas-line and elimination of rubber connections were adopted. A vigorous stream of nitrogen was used for about 20 minutes, and was then reduced to give a sufficient degree of stirring and de-aeration.

The potentials were measured by the valve potentiometer system described by Harris [1928]. This system has the advantage of avoiding polarisation of the electrodes when finding balance. This is a point of great importance in measuring the potentials of such a poorly poised system as that in broth.

Sterile buffered broth.

The broth used throughout these experiments was the standard laboratory medium: peptone-beef infusion (T. Morson and Sons' peptone). No glucose was added but native sugars were not fermented out. The broth was made of double strength for storage. Immediately after preparation, the batches of broth were tubed in 30 cc. lots, and while still hot from the autoclave placed for storage in anaerobic jars. For the experiments this broth was diluted with an equal volume of $M/10$ buffer [Clark, 1928] immediately before use. Several batches of broth were buffered with $M/10$ KH_2PO_4 + asparagine. With this the range p_H 6·5 to 9·5 could be conveniently covered by the one buffer mixture.

A hydrogen electrode titration was done on a sample of buffered broth from each batch. Samples of the broth (50 cc.) could then be made up to any required p_H by adding the required amount of standard alkali or acid read from the titration curve. The subsequent autoclaving altered the p_H a little. The exact p_H of each sample was, therefore, always determined by the hydrogen electrode after the drift experiment had been completed.

Character of the potentials.

Typical curves showing the change of potential with time when sterile broth is de-aerated are given in Fig. 1. When readings of the potential were

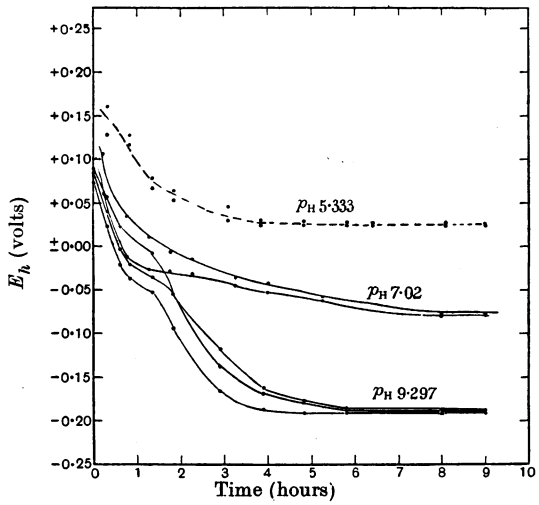

Fig. 1. Time-potential curves of sterile buffer-broth de-aerated with pure nitrogen.

made soon after the beginning of de-aeration, large differences between individual electrodes were observed. These tended to come together as the potential slowly became more negative. The electrodes never exhibited the precise agreement that is found with well-defined, well-poised systems. In general, with the electrodes prepared as described above, readings agreeing to within 10 mv. or less were finally obtained. Occasionally one electrode of the three was markedly different (this was generally found to be due to a cracked electrode); the closely agreeing values of the other two were then taken. The potentials of the three electrodes plotted against time gave individually smooth curves, tending to come together as the potential reached its limiting value after 6–8 hours.

The potentials of the system are extremely poorly poised, as shown by the ease of polarisation if a simple potentiometer circuit is used. With the valve potentiometer this was avoided. The potential to be measured was made to contribute to the negative grid bias of one of two balanced valves. The potential chain

− Au-plated Pt	Broth	KCl-agar	*M*/20 Phthalate, quinhydrone	Au-plated Pt +

was therefore effectively on open circuit during balancing. Even with this circuit, if the chain was left switched in, there occurred a very slow positive drift. The cause of this is unknown. It may be a further indication of the small

capacity and feeble poising of the electromotively active system in the broth. Possibly the charge on the electrode in the broth cannot quite be maintained by the small amount of active material available. An interesting side-light is thrown on this by the observation that this slow positive drift was much reduced if the area of the electrode was increased. Thus, while the drift was as much as 5 millivolts per minute with small pin electrodes of area 0·5–1 cm.^{2}, with plate electrodes of area 3–6 cm.^{2} the drift was less than 1 millivolt in 3 minutes. This was negligible during the time necessary to switch in the chain and take a reading. As a routine, the cells were left on open circuit until it was necessary to take a reading.

Time-potential curves were observed in a large number of experiments with sterile buffered broth from p_H 4 to 10.

Relation of E_h *to* p_H.

If the final levels of potential to which the broth drifts (Table I) are plotted against the corresponding p_H values, an interesting relation is obtained (Fig. 2).

Table I. *Relation of* E_h *of completely de-aerated buffer-broth to* p_H. *Observations with electrodes in absence of O/R dyes.*

(E_h is the mean of three electrodes.)

p_H	E_h (volts)	Buffer	p_H	E_h (volts)	Buffer
4·25	+ 0·070	Phthalate	7·084	− 0·099	Phosphate
4·82	+ 0·048	,,	7·31	− 0·092	,,
5·23	+ 0·023	,,	7·35	− 0·100	,,
5·96	+ 0·005	Phosphate-asparagine	7·37	− 0·110	,,
5·99	± 0·000	Phosphate	7·57	− 0·130	,,
6·05	+ 0·010	,,	7·62	− 0·130	Phosphate-asparagine
6·06	+ 0·015	Phosphate-asparagine	8·26	− 0·165	Phosphate
6·42	− 0·025	,, ,,	8·27	− 0·167	,,
6·59	− 0·003	Phosphate	8·45	− 0·185	Phosphate-asparagine
6·63	− 0·030	,,	8·46	− 0·166	Borate
6·82	− 0·055	Phosphate-asparagine	8·57	− 0·181	Phosphate-asparagine
6·89	− 0·048	Phosphate	9·30	− 0·190	Borate
6·92	− 0·041	Phosphate-asparagine	9·68	− 0·225	,,
7·00	− 0·050	Phosphate	10·47	− 0·258	,,
7·02	− 0·083	,,			

This curve at once recalls that given by a reversible system at any fixed degree of reduction in its relation with p_H. It may be said at once that the points on this E_h–p_H curve do not align with the precision that a well-defined reversible system gives. This lack of precision was particularly marked, in a few cases, with broths which had not been stored anaerobically, although these generally gave points on the E_h–p_H curve (Table I, phosphate-asparagine buffered broth). The discrepancy was as much as 40 mv. in three experiments. This was within the range p_H 6·5–7·5 (*i.e.* on the steepest part of the E_h–p_H curve); and further, since added dye was not reduced so much as usual, slight leaks of oxygen were suspected in these cases. This would have its greatest effect over the steep part of the curve.

The smooth curve drawn through the points has been given standard slopes in order to compare it conveniently with analogous curves. It is

striking that such a good fit is obtained in this way. The three sections of the curve are not due to the three different buffers used, since points obtained with phosphate and with phosphate-asparagine buffers occur on all sections of the curve.

For the broth system it is a fair assumption that this curve represents the relation with p_H at a high degree of reduction. Attempts to obtain a more negative potential level by reducing the broth with hydrogen and platinised asbestos have not succeeded. The potential level obtained was the same, within experimental error.

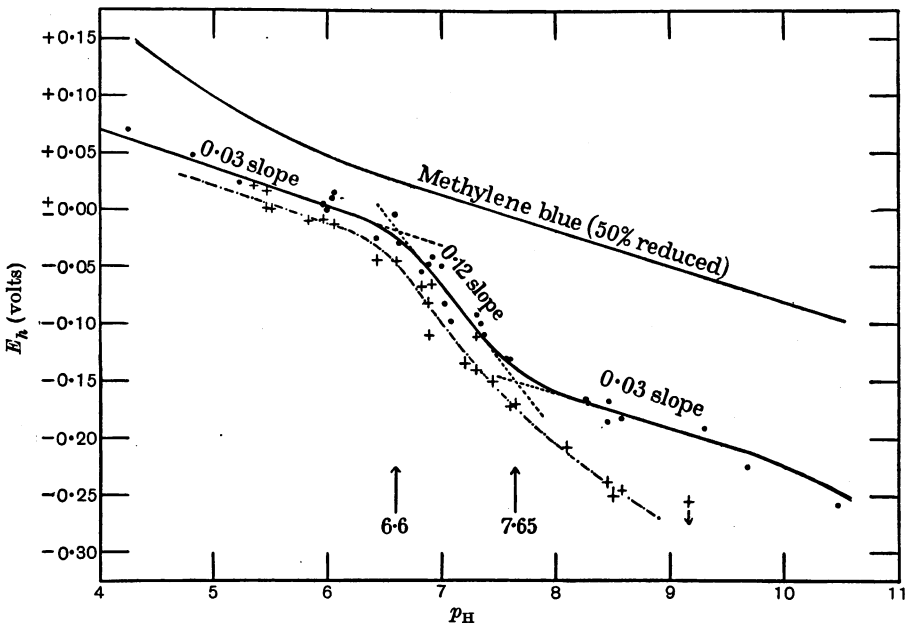

Fig. 2. Relation of E_h of completely de-aerated buffer-broth to p_H.
Full curve —•—•—•—•—: E_h by electrodes alone in broth.
Broken curve —+ - - - + - - - + - - -: E_h by dyes (colorimetrically and electrometrically).

Cannan, Cohen and Clark [1926] obtained a somewhat analogous curve by plotting certain potential levels of washed yeast suspensions against p_H.

If it be accepted that the curve of Fig. 2 does express a definite relation between p_H and some fixed degree of reduction of the electromotively active system of broth, the inflexions of the curve may be partially interpreted. By analogy with the behaviour of truly reversible systems [Clark and others, 1928], the inflexions indicate the dissociations of hydrions of the system. These dissociations may be approximately located at p_H 6·6 and 7·65.

The value of E_h at p_H 7·6 is −0·13 volt compared with Coulter's value of − 0·06 volt. It may be pointed out that the p_H at which Coulter was working falls within the zone in which I obtained most variation in the values for the final level of E_h.

The difference may be due to differences in composition of the media, or it is possible that the p_H of Coulter's medium was not exactly 7·6 after autoclaving. I have found that autoclaving even a buffered broth at slightly alkaline p_H tends to shift the p_H towards the acid side. The E_h–p_H curve shows that a slight change of p_H in the region 6·5–8 causes a considerable change of E_h, but the difference of E_h is almost too great to make the latter explanation fully satisfactory.

Correlation of E_h *determined by oxidation-reduction dyes and that determined electrometrically.*

The relative facility with which the oxidation-reduction dyes can be used has led to their adoption for measuring the reducing intensity of cells, tissues and culture media, but these measurements have seldom been correlated with electrode measurements on the same material in the absence of dyes. This has been done in the present case. Samples of the same batches of sterile buffered broth as used for the electrode measurements were de-aerated by the same technique in a duplicate apparatus. A quantity of dye to give a suitable colour for observation was added to the broth, and its degree of reduction determined colorimetrically. Colour standards were made by suitably diluting portions of this broth. In the case of dyes which had a noticeable colour when reduced in broth (*e.g.* the indigosulphonates) the colour of the partially reduced dye in broth was matched with the combined tint of two tubes, one of which gave the percentage oxidised tint and the other the complementary percentage reduced tint.

Arrangements were made whereby samples of the broth + dye, which was being de-aerated, could be drawn off from time to time, with exclusion of oxygen, and its colour matched with the standards. In this way, potential-time curves analogous to those observed electrometrically were obtained. At any p_H, if one dye became practically completely reduced, the next lower dye on the E_h scale was used as a check (Table II A). Ample time was allowed, so that the poising effect of the dye should not give an apparent final E_h level. The points thus obtained by dyes for the final level of E_h in de-aerated broth are plotted in Fig. 2. It is seen that the dye method gives, under controlled conditions of p_H and rigorous exclusion of oxygen, a curve sensibly parallel at first to the curve obtained by electrodes alone, but about 0·02 volt more negative. This holds, however, only over the range up to p_H 7·5. At more alkaline reactions than this the curves diverge, and a dye is reduced to an increasingly more negative E_h than would be indicated by an electrode alone in the same broth.

Electrodes in broth + dye indicate the dye potentials. Points obtained in this way are shown in Fig. 2 and afford a more precise measurement than the colorimetric method (Table II B).

A similar divergence between the indications of electrodes and dyes is seen, for example, with cysteine and other sulphydryl compounds. A cysteine

Table II. *Relation of* E_h *of completely de-aerated broth to* p_H. *Observations with O/R dyes.*

A. *Colorimetric observations.*

p_H	% reduction of dye (observed)	E_h (volts) (calculated)	Buffer
5·35	85 indigo 4*	+ 0·02	Phthalate
5·46	60 „ 3	+ 0·001	„
5·46	84 „ 4	+ 0·016	„
5·84	85 „ 4	− 0·01	„
6·60	80 „ 4	− 0·045	Phosphate
6·87	65 „ 3	− 0·082	„
6·87	35 „ 2	− 0·111	„
7·2	50 „ 2	− 0·134	„
7·3	50 „ 2	− 0·140	„
7·45	60 „ 2	− 0·150	„
7·65	77 „ 2	− 0·170	„
8·09	85 „ 2	− 0·207	„
9·16	98 „ 2	− 0·255	Borate

B. *Electrometric observations (electrodes + dyes).*

p_H	E_{h_1}. No dye (observed)	E_{h_2}. Dye present (observed)	$E_{h_2} - E_{h_1}$	% reduction of dye (calculated)	Buffer
5·96	+ 0·005	− 0·009	0·014	76 indigo 4*	Phosphate-asparagine
6·06	+ 0·015	− 0·013	0·028	77 „ 4	„
6·42	− 0·025	− 0·045	0·020	89 „ 4	„
6·82	− 0·055	− 0·067	0·012	76 „ 3	„
6·92	− 0·041	− 0·065	0·024	29 „ 3	„
7·31	− 0·092	− 0·111	0·019	9 „ 2	Phosphate
7·62	− 0·130	− 0·171	0·041	82 „ 2	Phosphate-asparagine
8·45	− 0·185	− 0·238	0·053	98·5 „ 2	„
8·57	− 0·181	− 0·245	0·064	99 „ 2	„

* Indigo 4 is indigotetrasulphonate.
„ 3 is indigotrisulphonate.
„ 2 is indigodisulphonate.

solution will reduce a dye to a value of E_h more negative than the cysteine solution alone would show. It is evident that much caution must be used in the interpretation of either electrode or dye measurements alone in any particular set of experiments.

In connection with this difference between the potential indicated by dyes and by electrodes, some experiments of Wurmser and Geloso [1928, 1929] are of interest. They investigated the potential of glucose solutions in buffers with electrodes, both in presence and absence of dyes. They found that the electrodes alone would eventually, after 5 or more days, reach approximately the same level of potential as was reached much more rapidly in the presence of dyes. This work has been only very briefly reported, without experimental details, and requires confirmation. In the present work, the potential had reached a definite level in 8–10 hours, after which time, in several prolonged experiments of 20–30 hours, no further change took place. From the regularity of the relation of E_h to p_H (Fig. 2) it is evident that the potential level attained in completely de-aerated broth after 8–10 hours is definite and significant and not purely arbitrary.

SUMMARY.

The object of the present investigation has been to establish some fundamental requirements of technique preliminary to the application of the ideas of oxidation-reduction potential theory to bacterial systems. To this end the behaviour of sterile meat broth has been studied.

The O/R potential of completely de-aerated, sterile, buffered meat broth has been measured over the range p_H 4–10·5.

The electrode potentials have been compared with those indicated by Clark's O/R dyes.

It is found that the two methods do not indicate the same potential level, a dye indicating a more negative potential than would be shown by an electrode in broth alone.

Quantitative data are provided that should assist the characterisation of the electromotively active system of broth.

I am deeply indebted to Dr P. Fildes for his interest in initiating this work. My best thanks are due to Prof. R. K. Cannan for much valuable advice and criticism.

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