CXLII. THE REDUCTION POTENTIALS OF BACTERIAL SUSPENSIONS.

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(Received March 23rd, 1935.)

A NUMBER of observations have been recorded on the reduction potentials of growing bacteria [Gillespie, 1920; Cannon *et al.*, 1926; Aubel and Aubertin, 1927; Aubel and Genevois, 1927; Hewitt, 1930, 1, 2, 3]. It was considered that, in this earlier work, the continuous changes occurring in the medium during the growth of the organisms complicated the problem to an extent which made it impossible to obtain a clear idea of the cause of the production of the potentials recorded. In order to simplify the system, the following work was carried out with washed suspensions instead of with growing organisms.

In a few cases measurements on washed cells have already been carried out. Thus, Aubel *et al.* [1928] have recorded the potentials of kidney, liver and muscle tissue, and Cannon *et al.* [1926] measured the potentials of washed yeast cells. These latter authors find a slow fall of potential, which becomes much more rapid after the addition of succinate. If methylene blue is added after these low values have been reached, the potential is brought up to the range characteristic of the dye until the dye is reduced. This reduction is very slow, and the potential correspondingly does not soon fall, in the absence of succinate, whereas in its presence this "poising" action of the dye is soon overcome and the potential rapidly resumes its negative values.

In none of this work has the actual cause of the potentials been made clear. It is conceivable that they are due to the cells themselves influencing the electrode. In this case, the increased negativity observed on the addition of metabolites might be due to some combination between the enzymes of the cell and the metabolites which is capable of affecting the electrode to a greater degree than the cells alone. On the other hand, the electrode potential may be due to substances formed by the cell enzymes, which substances are themselves capable of affecting the electrode. The work to be described was undertaken with a view to obtaining some clearer conception of the nature of the reduction potentials of bacterial cells, as recorded both by the electrode and by the use of redox indicators. No attempt has been made to study redox equilibria of any particular enzyme system as has been done for example by Lehmann [1929] for the succinate-fumarate system.

EXPERIMENTAL.

The bacteria used in this investigation were *Bact. coli* (Escherich), *Bact. alkaligenes* and *Cl. sporogenes*. Of these, the first is a facultative anaerobe, the second a strict aerobe, whilst the last is a strict anaerobe. They were all grown on a tryptic digest of caseinogen. *Bact. coli* and *Bact. alkaligenes* were grown in Roux bottles for 15-18 hours and *Cl. sporogenes* was grown for 2 days anaerobically. The cultures were centrifuged and washed twice by centrifuging in distilled water, and uniform suspensions were made in water.

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Potentials measured at the electrode.

These were measured in an apparatus similar to that described by Green [1933] except that an extra side-tube was added to the reaction vessel by means of which solutions could be added to the fluid in contact with the electrode (Fig. 1). The nitrogen was freed from oxygen by passing over copper treated with palladium at a temperature of 380°. The potentials were measured at room temperature (which was observed at intervals) using a mirror galvanometer in series with which was a resistance of 50,000 ohms to minimise polarisation. Before and after the measurement of the reduction potential, a sample was taken for the determination of the p_H by the quinhydrone electrode. All the measurements of reduction potential are expressed as E'_0 at p_H 7.

Fig. 1. Apparatus for measurement of reduction potentials.

Fig. 2. *Bact. alkaligenes*. Electrode reduction potentials. ●—● Fresh suspension. ○—○ Suspension 2 days old.

The potentials were determined of bacterial suspensions in the presence of an equal volume of $M/20$ phosphate buffer at p_H 7.0, with or without the addition of other substances. In the absence of these additional substances, a slow continuous fall of potential was observed, which continued as long as readings were taken. These in exceptional circumstances were extended over a period of 3 days and no constant value was obtained. The rate of fall of potential was slightly more rapid in the case of *Bact. alkaligenes* than with the other two species, particularly if the suspension had been kept 1 or 2 days in the ice-chest (Fig. 2).

The effect on the potential of adding certain substances was next observed. With *Bact. coli*, the presence of a small amount of $M/10$ sodium succinate solution did not change the rate of fall of potential. With one suspension of *Bact. coli*, a potential of -0.25 v. was reached after 10 hours both with and without the addition of succinate. If glucose or sodium formate was added, on the other hand, a rapid fall of potential occurred, which was complete after 15 mins. to 1 hour. Usually this was followed by a gradual rise of about 0.03 v. extending over about 1 hour. The lowest potentials recorded are given in Table I.

Table I. *Reduction potential (E_0' at p_H 7.0 in volts) of washed suspensions of Bact. coli in the presence of:*

Glucose: -0.38 , -0.37 , -0.35 , < -0.33 , -0.36 , -0.36 , -0.35 , -0.37

Formate: -0.38 , -0.40 , -0.40 , -0.38 , -0.38

The effect of the substrates on the potentials is shown more clearly by following the decrease before and after the substrates are added. This was effected by attaching to the extra side-arm of the reaction vessel a tube containing the substrate solution. It was arranged that the nitrogen passing through the bacterial suspension also passed through this solution, thus ensuring that this was free from oxygen when it was mixed with the bacteria. A typical result is shown in Fig. 3. It will be seen that there is an immediate rapid fall of potential on the addition of glucose. When the minimum potential value has been reached, the addition of formate causes a further slight fall. If formate is added first, the low potential recorded is not influenced by the addition of glucose. This method of observing the potential showed that the addition of succinate has little or no effect on the rate at which the potential falls. Addition of formate after the succinate caused a rapid fall to -0.38 to -0.40 v. The observed potential of a mixture therefore appears to be that of the most negative system present.

Fig. 3. *Bact. coli*. Electrode reduction potentials.

The effect of toluene on the reduction potential of *Bact. coli* in the presence of glucose was studied in order to see what part glucose dehydrogenase, which is inhibited by toluene, plays in the development of the reduction potential.

Experiment showed that the addition of toluene to the bacteria before the glucose resulted in a very slow fall in potential which, after 24 hours, had only reached 0.0 v. On the other hand, if the toluene was added from the side-tube to the mixture of bacteria and glucose which had been reacting for some time, the rate of fall and the final potential were exactly the same as in the absence of toluene. These results recall those in which methylene blue, although not reduced by toluene-treated bacteria in the presence of glucose, is reduced if the toluene is added after the glucose [Yudkin, 1934, 2]. This was explained as being due to the production of substances from the glucose which, unlike glucose itself, are able to reduce methylene blue in the presence of toluene-treated bacteria. In the case of the reduction potential also it appears that the actual potential is due to the action of the bacteria on the products rather than the initial action of the bacteria on glucose.

The above results were all obtained with *Bact. coli*. With *Bact. alkaligenes* similar results were recorded as far as the absence of effect of succinate is concerned. The effect of glucose is different with the two species of bacteria since the potential of suspensions of *Bact. alkaligenes* is affected comparatively little by this substance. Formate has a more marked effect (Fig. 4). A final value of the potential with this organism was difficult to obtain owing to the continuous, though gradually slowing, fall of potential. At the end of 6 hours, however, the fall is almost immeasurably small and approximate values of the potential for the different substrates can be obtained (Table II).

Table II. Reduction potentials (E_0' at p_H 7.0 in volts) of suspensions of *Bact. alkaligenes* after 6 hours.

Bacteria alone or with succinate	-0.17
„ + glucose	-0.27
„ + formate	-0.30-0.32

Fig. 4. *Bact. alkaligenes*. Electrode reduction potentials. ●—● Bacteria alone. ▲—▲ Bacteria + glucose. ■—■ Bacteria + formate.

Only a few measurements of reduction potential were made with *Cl. sporogenes*. The fall of potential was very slow indeed, the potential even after 24 hours being only about 0.0 v. The rate of fall and final potential were unaltered by the addition of alanine, which this organism can readily activate as hydrogen donor [Stickland, 1934].

Potentials measured by dye reduction.

In Table III is given a list of the indicators used, together with their potentials at p_H 7.0 for 50 % reduction.

Table III.

Indicator	E_0' at p_H 7.0 50 % reduction	Indicator	E_0' at p_H 7.0 50 % reduction
1. Cresolindophenol	+0.210	7. Janus green	-0.252
2. Cresyl blue	+0.031	8. Phenosafranine	-0.256
3. Methylene blue	+0.011	9. Rosinduline	-0.281
4. Indigo carmine	-0.125	10. Neutral red	-0.340
5. Nile blue	-0.150	11. Benzylviologen	-0.359
6. Cresyl violet	-0.173	12. Methylviologen	-0.446

Dilute solution of these were placed in Thunberg tubes with the bacterial suspension and phosphate buffer p_H 7.0 and in some cases a solution of substrate. The tubes after evacuation were left at room temperature and observed after about 1 hour.

A suspension of *Bact. coli*, with or without succinate, reduced 1-8 completely, 9 partially and did not affect 10, 11 and 12. With glucose, 1-9 were reduced completely, 10 was doubtful, 11 partially reduced and 12 not affected. With formate, even 12 was slightly reduced. The reduction potentials as recorded by indicators are therefore:

	E_0' in volts
<i>Bact. coli</i> alone or with succinate	-0.27 to -0.30
„ + glucose	-0.32 to -0.37
„ + formate	-0.39 to -0.41

These figures agree fairly well with those given by electrode measurements (p. 1132).

The results with *Bact. alkaligenes* do not show this agreement between the electrical and colorimetric methods. Dyes 1-11 are all reduced by the bacteria alone or in the presence of succinate or glucose (E_0' -0.36 to 0.38). With formate, 12 is also definitely reduced (E_0' about -0.42). Similarly, *Cl. sporogenes* shows potentials by this method which differ greatly from those shown by the electrode. With or without alanine, 1-11 are rapidly reduced at p_H 7.0, indicating a potential of about -0.38 [cf. Stickland, 1934].

Potentials measured at the electrode in the presence of indicator.

Series of electrode measurements were made with *Bact. coli* and *Bact. alkaligenes* in the presence of dyes. The results depended on the range of potential of the dye used. If this was very positive, *i.e.* if the dye was rapidly and completely reduced, then the effect was small. For example, if methylene blue was added to *Bact. coli* in the presence of formate, a pause in the falling potential occurred whilst the dye was being reduced (*i.e.* in the region of 0.0 v.) but the subsequent rate of fall and final potential recorded were the same as when no dye was present. Again, if the dye was added from the side-tube after the potential had reached a low value, a slight rise occurred which lasted for a minute or two if the quantity of dye added was small, and then the potential rapidly returned to its previous low value. These results recall those of Cannon *et al.* [1926] which have already been referred to.

If a dye is chosen which is only partially reduced by the system, the potential recorded is approximately that which would be expected from the range of potential of the dye. For example, *Bact. coli* and glucose in the presence of

benzylviologen give a reduction potential of -0.36 to -0.38 v. *Bact. alkaligenes* gives with benzylviologen the following reduction potentials measured electrically: alone, or with glucose -0.34 to -0.38 v.; with formate, -0.40 to -0.42 v. These values are those which would be expected from the amount of reduction of the dye used: that is, they agree with the values for the reduction potential obtained colorimetrically. The potentials recorded electrically in the presence and absence of these partially reduced dyes are, therefore, in agreement only in those cases where the electrical and colorimetric values are in agreement. This is shown in Table IV where the values obtained by the three methods outlined above are summarised.

Table IV. *Reduction potentials of bacterial suspensions E_0' in volts at p_H 7.0.*

Bacteria	Substrate	Potential measured		Electrometrically in presence of partially reduced dye
		Colorimetrically	Electrometrically	
<i>Bact. coli</i>	None	-0.27 to -0.30	< -0.25	—
	Succinate	-0.27 to -0.30	< -0.25	—
	Glucose	-0.32 to -0.37	-0.36 to -0.38	-0.36 to -0.38
	Formate	-0.39 to -0.41	-0.38 to -0.40	About -0.39
<i>Bact. alkaligenes</i>	None	-0.36 to -0.38	About -0.17	-0.34 to -0.38
	Succinate	-0.36 to -0.38	About -0.17	—
	Glucose	-0.36 to -0.38	About -0.27	-0.34 to -0.38
	Formate	About -0.42	-0.30 to -0.32	-0.40 to -0.42
<i>Cl. sporogenes</i>	None	About -0.38	About 0.0	—
	Alanine	About -0.38	About 0.0	—

The cause of the potential at the electrode.

The main problem was to decide whether the potentials were being caused by contact with the electrode of the bacteria, possibly with adsorbed reactants, or by contact with the electrode of the reactants themselves moving freely in the solution. A series of experiments to test this point was carried out with *Bact. coli*.

A bacteria-free filtrate of the mixture after the bacteria had acted on glucose for a long time was found to give a potential of about -0.20 v. which was almost certainly due to the glucose itself. However, it was found possible to obtain the low potentials recorded above in bacteria-free solutions by use of a thin collodion sac.

A small beaker with a well-fitting rubber stopper was used as reaction vessel. In the stopper were inserted two platinum electrodes, a tube through which the nitrogen bubbled into the solution, an exit tube for the nitrogen and a KCl-agar bridge making contact with the KCl half-element. Round one electrode was tied a collodion sac containing a mixture of buffer and substrate solution. In the beaker was placed the mixture containing bacterial suspension, buffer and substrate solution. Thus the electrode within the sac was in contact only with substances capable of diffusing through the sac.

It was found that if the sac was thin and not too wide, the final potentials recorded by the covered electrode were, within a few millivolts, the same as those recorded by the outside electrode. In one experiment with glucose for example a potential of -0.330 v. was recorded by the outside electrode and -0.327 v. by the covered electrode. Similar results were obtained with formate. It was necessary to leave the experiment for 6–8 hours before this agreement was obtained.

At the end of each experiment, the apparatus was dismantled carefully in order to observe whether the bacteria had passed into the sac through any small hole which had developed. In certain cases where this did occur it was very obvious, the liquid in the sac being quite milky and rapidly dripping out of the sac even when no visible hole existed.

DISCUSSION.

It seems fairly certain from the results given that the reduction potential of a bacterial suspension, as measured at an electrode, is produced by substances in solution in the suspension. The potentials recorded for suspensions in the absence of added substrate are probably due to substances exuding from the bacterial cells. This is supported by two observations; firstly, by the very slow and prolonged fall of potential, and secondly by the fact that older suspensions (of *Bact. alkaligenes*), in which presumably more of these substances have diffused out, show a more rapid fall of potential.

Of the substrates examined, two, namely glucose and formate, produce a potential more negative than the bacterial suspension alone. It may be inferred that the substances produced from other substrates, such as succinate, either have no effect at the electrode or have much less effect than the substances produced in the bacterial suspension itself. The equilibrium potential of the succinate-fumarate system is given as 0.00 v. at p_H 7.0 and 30° [Lehmann, 1929].

A possible explanation of the low potentials recorded in the presence of glucose and formate is that hydrogen is being produced, giving a partial pressure of $1-10^{-2}$ atmospheres. If this is correct then the reduction potential would be expected to depend on the rate at which the nitrogen was passed into the vessel since this would alter the amount of hydrogen left in the solution. This was found not to be the case. Also, a suspension capable of producing more hydrogen (*i.e.* containing a more active hydrogenlyase) might be expected to produce a more negative potential. The suspensions of *Bact. coli* as grown for this experiment contain no formic hydrogenlyase and very little glucose hydrogenlyase [Yudkin, 1932]. As has already been suggested [Yudkin, 1934, 1], it is conceivable that very small amounts of hydrogen are actually produced from formate in such suspensions but in quantities insufficient to be measured manometrically. These very small quantities might, however, be sufficient to affect the electrode. Suspensions of *Bact. coli* grown on glucose broth and hence containing active formic and glucose hydrogenlyases were prepared. The potentials of these suspensions in the presence of either glucose or formate did not, however, produce a more negative reduction potential than the other preparations.

The argument against hydrogen as the cause of the low potentials with glucose and formate in the case of *Bact. alkaligenes* is still stronger since in no circumstances is it possible to obtain measurable quantities of hydrogen by the action of this species on either glucose or formate.

We can also deduce from the experimental results that the substances responsible for the low potentials do not do so by virtue of being hydrogen donors in the presence of the bacteria. One might imagine that the activation of a substrate as a hydrogen donor involves a "loosening" of a hydrogen atom and that it is this which affects the electrode. But the results obtained with *Cl. sporogenes* in the presence of alanine show that this is not so.

We must, therefore, conclude that the potentials recorded by an electrode immersed in a bacterial suspension with or without added substrates are due to

unknown substances either coming out of the bacterial cells or being produced from certain of the substrates (*e.g.* glucose and formate). That these substances affect the electrode at all and so give a measurable potential by no means indicates that a definite oxidation-reduction equilibrium is being measured. In fact, the slow and continuous fall of potential in the absence of glucose or formate and the ease with which all the systems are polarised suggest that the effect on the electrode of some of these substances at any rate is quite fortuitous and that the values obtained for the potential by electrical measurement gives very little knowledge of the state of the cells.

The above discussion was confined entirely to potentials measured at the electrode in the absence of dyes. We have seen that colorimetric measurements of the reduction potentials often give results differing widely from those obtained by such electrical measurements, although they agree with electrical measurements made in the presence of partially reduced dye. The differences may be explained along the following lines. It has been pointed out by Green *et al.* [1934] that the reduction of dyes by bacterial cells depends on the existence in the cells of positive (oxidising) and negative (reducing) systems. Whether or not a certain dye will be reduced does not depend on the actual potential of the cell. A small quantity of redox indicator will be completely oxidised, completely reduced or partially reduced depending on the relative rates of oxidation and reduction of the particular dye by the positive and negative systems. In the case of a dye which is partly reduced, we may perhaps speak of "reduction equilibrium" of the dye in question, where the rate of reduction of the dye by the negative systems is equal to the rate of its oxidation by the positive systems. In the presence of partially reduced dye, an electrode will naturally take up the potential value of this mixture of oxidised and reduced dye since both forms are active at the electrode. In other words, colorimetric measurements of the reduction potential will agree with electrode measurements in the presence of partially reduced indicator, and both are measuring the "reduction equilibrium" of the dye.

On the other hand, as shown above, the potentials recorded at the electrode in the absence of dye are due to the existence in the mixture of certain diffusible substances which are able to affect the electrode. These are quite fortuitous and have nothing to do with the oxidising and reducing mechanisms of the cells.

There is, therefore, no method by which the reduction potential of a cell, in the sense commonly used, may be measured. The author is of the opinion that the conception of the reduction potential of the living cell as an equilibrium exhibited by the whole cell has no great meaning. It appears much more likely that different parts of the cell in which the various systems are differently proportioned will have very different reducing intensities. The use of the term reduction potential, if justified at all at present in biology, must be restricted to reversible systems in true equilibrium such as reduced and oxidised cytochrome or succinic dehydrogenase in the presence of fumarate and succinate.

SUMMARY.

1. The reduction potentials of suspensions of *Bact. coli* and *Bact. alkaligenes* were measured (1) electrometrically, (2) colorimetrically by redox indicators, and (3) electrometrically in the presence of indicators. A few measurements were also made with *Cl. sporogenes*.

2. Both *Bact. coli* and *Bact. alkaligenes*, either alone or in the presence of succinate, show a slow continuous fall of potential at the electrode, which never

reaches a constant value. Glucose and formate induce more negative potentials, which in the case of *Bact. coli* soon reach minimum values.

3. These potentials have been shown to be due to electromotively active molecules capable of passing through a collodion sac and not to the bacteria themselves or bacteria *plus* adsorbed substances.

4. The reduction potentials recorded by indicators, although sometimes in fair agreement with the electrode values (*Bact. coli*), often bear no relation to the latter (*Bact. alkaligenes* and *Cl. sporogenes*). The electrode measurements in the presence of appropriate dyes give values agreeing with those indicated by the colorimetric study.

5. It is pointed out that the methods involving the use of these redox indicators merely measure the "reduction equilibrium" of the dyes used, that is, the point at which the rates of oxidation and reduction of the dye are the same. On the other hand, measurements of the electrode potential without an appropriate indicator give no information beyond the fact that there are certain (diffusible) substances in the solution which can affect the electrode. We have, in fact, no method whereby the true reduction potential of cells can be measured.

6. There is no correlation between the mode of life of the organism and the "reduction potential" measured by these methods. *Bact. coli* is a facultative anaerobe, *Bact. alkaligenes* a strict aerobe and *Cl. sporogenes* a strict anaerobe, but the reduction potentials show no correlative differences.

I desire to thank both Prof. E. K. Rideal for suggesting this investigation and Dr D. E. Green for helpful discussion during the course of this work.

REFERENCES.

- Aubel and Aubertin (1927). *Compt. Rend. Soc. Biol.* **97**, 1729.
— — — and Mauriac (1928). *Compt. Rend. Soc. Biol.* **98**, 589.
— — — and Genevois (1927). *Compt. Rend. Acad. Sci.* **184**, 1676.
Cannon, Cohen and Clark (1926). *U.S. Public Health Reports*, 1926. Supplement 55.
Gillespie (1920). *Soil Science*, **9**, 199.
Green (1933). *Biochem. J.* **27**, 678.
— — — Stickland and Tarr (1934). *Biochem. J.* **28**, 1812.
Hewitt (1930, 1). *Biochem. J.* **24**, 512.
— — — (1930, 2). *Biochem. J.* **24**, 669.
— — — (1930, 3). *Biochem. J.* **24**, 670.
Lehmann (1929). *Skand. Arch. Physiol.* **58**, 173.
Stickland (1934). *Biochem. J.* **28**, 1746.
Yudkin (1932). *Biochem. J.* **26**, 1859.
— — — (1934, 1). *Biochem. J.* **28**, 1454.
— — — (1934, 2). *Biochem. J.* **28**, 1463.