OXIDATION-REDUCTION EQUILIBRIA IN BIOLOGICAL SYSTEMS.

II. Potentials of Aerobic Cultures of B. typhosus.

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Gillespie (1) was the first to observe that an indifferent metallic electrode placed in a culture of bacteria manifests a negative drift in potential. Potter (2) had found previously that an electrode in an inoculated portion of culture medium is negative to an electrode in an uninoculated portion of the same medium, when the two portions are separated by a porous diaphragm. Clark (3) and his collaborators have extended the observations of Gillespie and have aroused a widespread interest in the biochemical processes involved in the establishment of reduction potentials.

The hopes of the earlier investigators that the determination of reduction potentials would provide a measure of the intensity of oxidation or reduction in biological systems cannot yet be realized, since difficulties are apparent in the interpretation of such potentials in strict accordance with the theory of oxidation-reduction equilibrium as it has been developed for inorganic or relatively simple organic substances. The substances responsible for the observed potentials of biological complexes have not been identified; there is much to suggest, however, that the sulfhydryl bodies are involved. The work of Dixon (5) and Dixon and Quastel (4) and the recent careful study of Michaelis and Flexner (6) indicate that these bodies do not constitute a simple reversible oxidation-reduction system, as would be required for strict application of the theory of reduction potentials, although their solutions in the absence of oxygen yield definite potentials which vary with the C_H and the concentration of the reduced form of the substance. These substances are, therefore, electromotively active and the combined form glutathione is so generally present in biological preparations that it must be concerned in the production of electrode potentials. These and other electromotively active bodies may also actually intermediate the reduction potentials, acting as catalysts for the oxidation or reduction of organic constituents which are not themselves electromotively active. There is, however, at the present time no evidence that the reactions which may be catalyzed by such substances are subject to definition in terms of intensity values. Further data from biological systems

must be accumulated before a formulation of the mechanisms involved can be attempted.

No satisfactory explanation has been offered for the part played by molecular oxygen in the development of biological reduction potentials, although all investigators have noted that reduction potentials of considerable intensity are attained only when oxygen is removed from the system. This may be accomplished by deaeration by a stream of nitrogen or by a spontaneous oxygen consumption which may take place as in sterile bouillon (7) even when the system contains only inanimate materials. Before such anaerobic conditions are attained the potentials in any biological system are distinctly more positive than when oxygen is absent, and it is not possible to decide to what extent the electrode under these conditions acts simply as an oxygen electrode or measures the actual oxidation-reduction forces which prevail in the system or indeed to decide whether or not these two possible mechanisms are properly to be contrasted.

Limitations thus surround the investigation of biological potentials and the significance which may be attached to the results.

In the first paper (7) of this series the reduction potentials of sterile culture bouillon were examined, and it was found that sterile bouillon in the absence of oxygen yields rather definite and characteristic potential values. Although the significance of these values is limited it seemed possible that they might serve as comparison values in a study of the potentials of growing cultures. The subject of the present paper is the relation of the potentials of sterile bouillon to those of living cultures of *B. typhosus* in the same medium. It was the hope that such an investigation would throw light upon the reducing activities of bacteria and upon the mechanism by which oxidation-reduction potentials are produced.

Technic.

The arrangement of the electrode cell and the method of potential measurement was that described in the first paper of this series (7). The electrodes used were for the most part purified gold which had been employed in the earlier work.[1] An

[1] The electrodes were placed when not in use in chromic acid solution, and were immersed for several minutes in concentrated sulfuric acid before each experiment. It was found that if this precaution were not taken the electrodes were sluggish and did not agree with one another closely. It was believed that the concentrated acid removed traces of stop-cock lubricant which may have been carried into the cell and become attached to the electrodes, but it is possible that solution of a film of oxide on the surface of the electrodes may have been involved. See Michaelis and Flexner (6).

improvement was made in the method of setting up the agar bridge: instead of filling the tube with agar, sterilizing separately, and fitting it aseptically to the large stopper of the electrode cell, the tube was inserted in the stopper and its outer end connected by rubber tubing provided with a pinch-cock to a funnel containing the KCl agar. After sterilization of the completely assembled apparatus in the autoclave at 10 pounds for 10 minutes, the melted agar was allowed to flow, by opening the pinch-cock, into the bridge tube, while the apparatus was still warm. A drop of agar was allowed to form at the tip of the tube, at which time the pinch-cock was closed. After the agar had solidified the connection to the funnel was removed and replaced by a rubber tube, filled with saturated KCl, which was connected to a special form of calomel half-cell through a three-way stop-cock. During an experiment this cock was kept closed and the rubber tube was shellacked, so that the bridge was a closed system into which oxygen could not readily diffuse. Reliance was placed on careful shellacking of all exposed rubber surfaces for exclusion of oxygen which would otherwise diffuse through the rubber.

The electrode cell and its contents were maintained at 38°C. by means of a small heating coil which fitted closely about the cell. A thermometer was placed in and sterilized with the cell. The flow of electric current through the heating coil was controlled by sliding-contact rheostats; a low-range ammeter in the circuit permitted calibration of the current required to reach and maintain any desired temperature. Although a thermostat was not used the temperature was kept within the limits of ±1.0°C. variation by adjustment of the rheostats.

The bouillon used was standard meat infusion, adjusted to pH 7.6 and buffered by phosphate which was added to a concentration of M/15. A saturated solution of dextrose was sterilized by heating to 80°C. for 30 minutes; an amount of this sufficient to give a final concentration of 0.5 per cent was added aseptically, in those experiments in which a dextrose medium was used, after the bouillon had been autoclaved. For each experiment 25 cc. of bouillon were autoclaved in a small flask, at 10 pounds for 10 minutes, along with the electrode cell. After cooling to about 38°C. the bouillon was transferred aseptically to the separatory funnel of the cell assembly. While in the funnel the bouillon was inoculated with two loops of an 18 hour broth culture of *B. typhosus* and was then allowed to run into the electrode cell. At intervals during many of the experiments portions of culture were drawn off from the cell by means of the bottom outlet, for the purposes of measurement of pH and of enumeration of bacteria by dilution plate cultures.

The electrical measurements were made by means of the usual potentiometer assembly, and at the same time were followed in many of the experiments by a Leeds and Northrup recording potentiometer. This instrument was specially adapted for this work by the makers; the moving coil has a resistance of 2500 ohms and is delicately balanced. This instrument was used chiefly as a precaution against overlooking changes in potential during the hours when the electrode cell was not under actual observation. The potentials indicated by the automatic

recorder were in general within 5 to 10 millivolts of those determined manually. The numerical values referred to in connection with the description of the experiments represent independent readings taken with the more sensitive hand-operated potentiometer.

Measurement of oxidation-reduction potentials was made first on cultures in bouillon which contained only the small amount of dextrose naturally present. In such cultures the change in pH after 24 hours

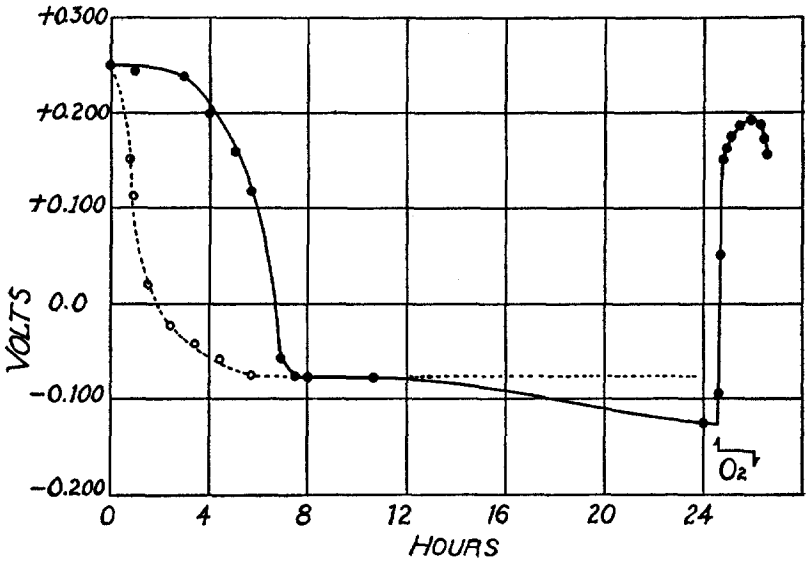

FIG. 1. The potentials of *B. typhosus* in bouillon without added dextrose are given by the solid line. The broken line gives the potentials of the bouillon in the sterile condition when deaerated with nitrogen. The rise in potential of the culture on passage of oxygen and the return to negative values on stopping the flow of oxygen are shown at the right of the figure. The culture throughout incubation had access to atmospheric oxygen. Ordinates represent *Eh* values at pH 7.6.

growth is small, amounting to less than 0.3 pH; if 0.1 per cent or more of dextrose is present the acid resulting from fermentation of the sugar is sufficient to displace the pH of the medium in spite of the addition of phosphate buffer, and introduces a complication in the interpretation of the potentials observed. When the electrode vessel was half filled with bouillon, and the cocks of the gas tubes entering and leaving the cell were closed, the culture had access to an equal volume of air, and

the conditions were similar to those of the ordinary aerobic culture. The time-potential curves of cultures under these conditions have been obtained in a number of experiments.

The course of the potentials is shown for a typical experiment in Fig. 1. During the first few hours the potentials remain close to the initial values, which range as in sterile bouillon at the beginning of deaeration between +0.250 and +0.150 volt. The lower initial values have been given by bouillon which had been freshly autoclaved and rapidly cooled before transference to the cell. This is in agreement with the observation of Dubos (8) on the reducing power of sterile media for dyes. A positive drift in potential from a few millivolts to 0.040 volt has usually been observed beginning shortly after measurements were started, and lasting for 2 to 3 hours; it may be related to the equilibration of the freshly autoclaved bouillon with the air. Within 3 to 6 hours after inoculation there has been observed regularly a gradual decline in potential followed shortly by an abrupt drop to −0.040 to −0.070 volt, and a slower fall to −0.080 to −0.090 volt. This level is maintained for several hours and appears to represent an equilibrium value for the culture during its youth. It was not possible, as will be described later, to make frequent samplings of the culture because of the change in potential which these involved, but it is evident from the form of the growth curve in these and in other observations that the culture has passed through its lag period and is in the period of logarithmic increase in numbers of bacilli during the time when this level of potential is maintained. The potentials then undergo a further gradual decline and after 24 hours have been found very close to −0.125 volt. In one experiment in which observations were made over a longer period of incubation this decline continued and at the end of 53 hours the potential reached the value of −0.145 volt.

The time-potential curve of an aerobic culture during the first 24 hours of growth may thus be divided into 4 portions: (1) the potentials are close and slightly positive to the initial values; (2) a decline, in part abrupt, to −0.080 or −0.090 volt; (3) a period in which the potentials are maintained at this level; (4) a gradual decline to more negative values continuing over a period of several days.

It seems highly probable that the presence of oxygen in the culture

is of primary importance in the establishment of the potentials, and the following explanation has been evolved. In the first portion of the time-potential curve, the small numbers of bacteria introduced as inoculum are without effect on the conditions, which are such as to permit the solution of oxyen in the medium. In the second period characterized by the rapid decline in potential, the bacteria have begun to multiply and in their rapid respiration consume the dissolved oxygen of the medium. With logarithmic growth of the bacteria their respiration becomes sufficient to consume the oxygen more rapidly than it can dissolve into the medium, if the surface of the latter is undisturbed, and the actual concentration of oxygen in the system is reduced to a very low value. The potential at the electrode then shows a negative drift just as in sterile bouillon on removal of oxygen by deaeration, and the potential attained is the same as that found in sterile bouillon under anaerobic conditions.

In the earlier work the equilibrium potential of several specimens of sterile bouillon was found to be -0.060 volt. Different specimens vary somewhat in the value of the reduction potential under anaerobic conditions. Separate determinations have been made of the reduction potentials of the bouillon used in the present investigation, in the sterile condition; the values found are somewhat more negative than those previously reported and have fallen between -0.085 and -0.095 volt. Removal of oxygen by the respiration of the bacteria is thus sufficient to explain the attainment of this level of reduction potential in a growing culture.

That removal of oxygen and not the elaboration of reductive products by the bacteria is responsible for reduction potentials of this intensity in bouillon is indicated by the immediate response of the electrode to small amounts of oxygen. If a few bubbles of air or of pure oxygen are allowed to pass through the culture the potentials at once shift in the direction of more positive values, and if aeration is continued there is a return to the neighborhood of the initial values, or about Eh $+0.200$. On stopping the flow of oxygen there is observed again a negative drift which is more rapid than the initial fall that is observed when the culture contains smaller numbers of living bacteria. When small portions of the culture are withdrawn through the bottom outlet of the cell, the disturbance of the culture is apparently sufficient

to increase the rate at which oxygen is dissolved, since the potentials show a positive shift of 0.050 to 0.100 volt. This phenomenon is observed only when oxygen is available for solution by the culture medium, and does not occur when the medium has been deaerated and the space above the bouillon contains nitrogen.

During the third portion of the time-potential curve the potential is maintained at the value characteristic of the sterile bouillon under anaerobic conditions. The duration of this period is uncertain; it appears to be several hours but probably does not exceed 18 hours from the time of inoculation of the culture. The uncertainty is due to the fact that even in bouillon to which dextrose has not been added there is observed an increase in acidity at the end of 24 hours of 0.2 to 0.3 pH, and the true potentials can be obtained only by a correction of the observed potentials for the change in pH. Frequent sampling for the purpose of following the course of the pH was impossible, because of the effect on the potentials which has been described above. For the same reason it was not possible to follow the growth curve closely during this period. It is known, however, that the growth curve of aerobic cultures of *B. typhosus* becomes flattened after about 18 hours of incubation and the rate of dying of the bacteria begins to approach the rate of multiplication. It is highly probable that autolytic processes occur at this time and liberate products of bacterial metabolism into the culture medium. The gradual fall in potential from about -0.085 volt to values between -0.125 and -0.145 volt which takes place following the brief period of maintained low potential may be regarded as a consequence of this autolysis and as evidence of the reductive nature of the substances set free. The data do not permit further characterization of these products; the potentials for which they appear to be responsible are considerably more negative than those observed in the bouillon when maintained in the sterile condition.

It was anticipated that the addition of dextrose to the bouillon would result in the attainment of more negative potentials by the culture than in a medium without added sugar.[2] This proved not to

[2] The sterile bouillon used in these experiments gave the same equilibrium potential (-0.085 to -0.095 volt) under anaerobiosis when dextrose was added, in the manner described above, to 0.5 per cent concentration as when dextrose was not added.

be the case. Cultures in 0.5 per cent dextrose bouillon given access to a fixed amount of air yielded time-potential curves very similar to those described above, if correction is made for the shift in pH occasioned by

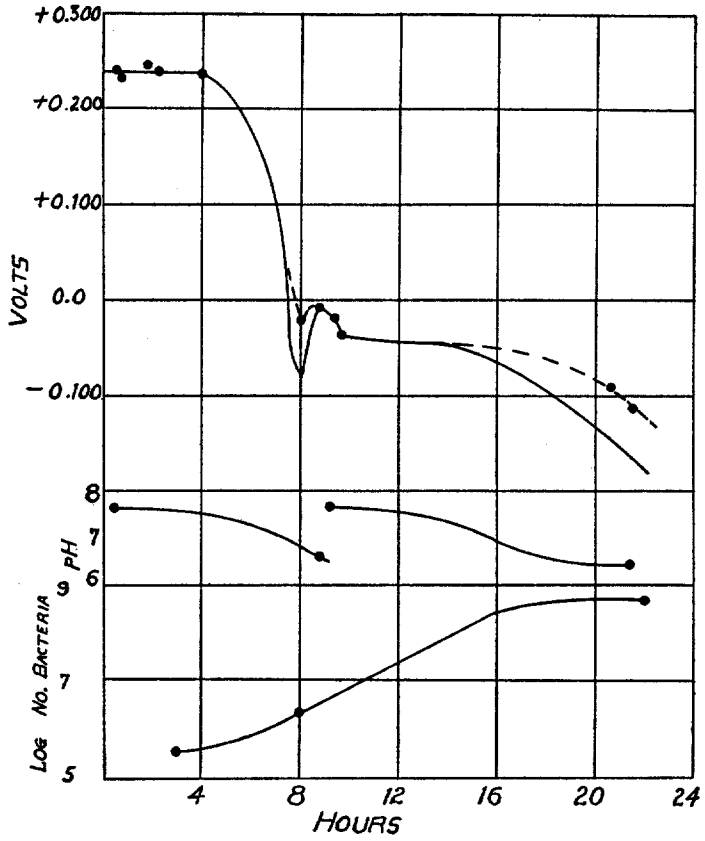

FIG. 2. The upper curve gives the potentials of *B. typhosus* in 0.5 per cent dextrose bouillon. The broken line shows the observed potentials, the solid line the potentials corrected for the change in pH. The positive shift occasioned by sampling of the culture is indicated. The curve of observed potentials represented in part by the broken line was drawn by the recording potentiometer; the experimental points represent values checked by the more sensitive galvanometer. The solid line gives the potentials as corrected for change in pH. The middle curve gives the approximate course of the pH during incubation. The lower curve gives the approximate growth curve. The culture throughout incubation had access to atmospheric oxygen.

acid fermentation. The same effects on the potentials were produced by the admission of air into the culture either deliberately, or incidentally to the disturbance occasioned by sampling, so that the course of reduction potentials and the hydrogen ion concentration could not be followed simultaneously. A typical experiment is shown in Fig. 2. The broken line of the potential curves represents the potentials actually observed; the solid line gives the corrected potentials. The correction of -0.061 volt per unit pH has been applied, on the assumption that no critical points of ionization are uncovered within the range of pH encountered. The potentials show the same rapid drop to more negative values after 6 to 8 hours incubation as in the cultures without added dextrose. The subsequent course of the observed potentials is variable, and in the lack of knowledge regarding the changes in pH, the course of the true or corrected reduction potentials is uncertain. In the experiment recorded in Fig. 2, the pH at the end of 8 hours incubation was 6.6 and the corrected potential was -0.085 volt, which is the value observed at the corresponding period in cultures in which acid fermentation did not occur. At this time the pH of the culture in this experiment was restored to its original value of 7.6 by addition of NaOH. At the end of 22 hours incubation, continued acid production had reduced the pH to 6.7 and the corrected reduction potential was -0.165 volt. This value is 0.040 volt more negative than that observed in cultures without dextrose after the same period of incubation; it suggests that with the increased "turn-over" of bacteria in the presence of dextrose (although the numbers of viable bacteria at any time are no greater than in culture medium without dextrose) a larger amount of reductive substance is liberated from the typhoid bacilli.

The experiments which have been described above indicate that the conditions within an actively growing culture of typhoid bacillus in bouillon are essentially anaerobic, even when the culture has access to atmospheric oxygen. The oxygen which is constantly being absorbed by the culture has an effect on the rate of growth of the bacilli as is shown by experiments which will be reported later, but the actual concentration of oxygen within the medium must be very small. In order to observe the course of the potentials when oxygen is present in high concentration, an experiment was carried out in

which pure oxygen was allowed to bubble through the medium during the period of incubation. The result is shown in Fig. 3, in which the growth curve, and the changes in pH as well as the reduction potentials are recorded. The medium contained 0.5 per cent dextrose. Passage

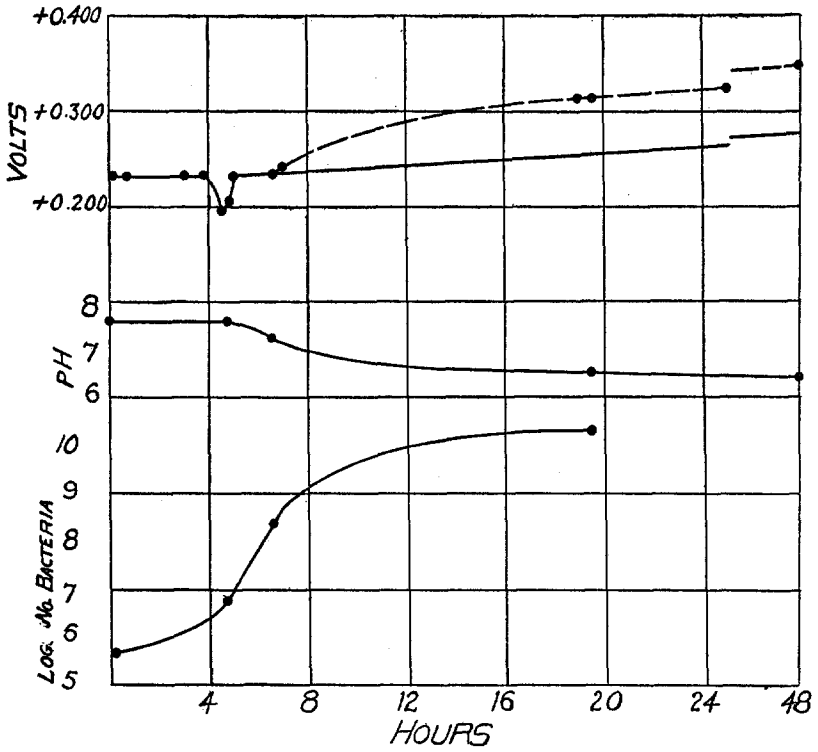

FIG. 3. The upper curve gives the potentials of *B. typhosus* in 0.5 per cent dextrose bouillon. Oxygen was passed through the culture from the time that the early negative drift was observed. The interrupted line represents the observed potentials, the solid line the potentials corrected for change in pH, which is shown in the middle curve. The lower curve represents the growth curve of the bacteria.

of oxygen through the culture was started immediately after the first rapid fall in potential, described above, had begun. The saturation of the medium with oxygen led to an immediate return of the reduction potentials to the initial values. The observed potentials then show a

gradual positive drift; when corrected for the change in pH, a shift to values more positive than those at the beginning of measurement is still evident, although the magnitude of the shift is smaller. The reduction potentials were maintained at a highly positive level throughout 50 hours of incubation. During the first 20 hours of growth the pH shifted from 7.6 to 6.6; the change is less than is observed in cultures which were not aerated. At the same time the numbers of viable bacteria increased enormously. The relations of the oxygen tension to rate of growth and character of metabolism are being made the subject of a separate investigation which will be reported later. In the present connection it is significant that in spite of the enormous numbers of living bacteria, there are not produced reductive substances capable of establishing in the presence of oxygen reduction potentials negative to those of the culture bouillon itself under like conditions. This is not what we should expect if molecular oxygen is inert with respect to reductive substances of bacterial origin. Deaeration at the end of 50 hours incubation, during which saturation of the medium with oxygen had been maintained, led to a rapid drop in reduction potential similar to that observed in cultures which had not been aerated. The equilibrium potential corrected for the shift in pH to 6.4, was − 0.080 volt; this is very close to that observed in the sterile bouillon under anaerobic conditions. Growth of the typhoid bacillus in a high concentration of oxygen appears not to lead to the production of substances of greater reducing intensity than those present in the medium before inoculation; in growth under very low oxygen concentration such substances apparently are produced and are liberated into the medium. Further investigation is required to establish the relation between the character of bacterial metabolism, with respect to the processes of respiration and fermentation, and the elaboration of reductive substances.

DISCUSSION AND SUMMARY.

The reducing properties manifested by bacterial cultures must be regarded as only one phase of a series of processes that come within the scope of the concept of oxidation-reduction phenomena. The importance of such processes is suggested in many phases of the metabolism of living cells, as in the accumulation of carbohydrate within

the cell, and the elaboration of other readily oxidized substances such as the hemotoxins studied recently by Neill (9). In these cases the energy of the living cell transforms substances in a state of relatively high oxidation into bodies that are in a condition of low oxidation. Conversely in respiration, as in the familiar oxygen-carbon dioxide exchange, a change in the condition of oxidation of carbon takes place in the opposite direction.

The scope of this concept is therefore very broad and includes other processes than the reduction of dyes and other substances such as nitrates and sulfhydryl bodies. Some definite aspect of the general problem must be chosen in order to bring the subject within the range of experiment. In the present series of investigations attention is centered upon those processes of oxidation and reduction which involve electromotively active substances, and are therefore subject to electrical measurement.

In the present paper the oxidation-reduction potentials yielded by cultures of the typhoid bacillus have been observed under the conditions which prevail in the usual aerobic culture in bouillon. Comparison of these potentials with those given by the culture medium in the sterile condition indicates that the living bacteria do not contribute the substances which are responsible for the observed potentials. On the other hand the respiratory consumption of oxygen by the typhoid bacillus appears to render possible the manifestation of characteristic potentials by the culture medium. Such potentials become apparent only when oxygen is removed from the system, and the rôle of the bacteria is the establishment of the anaerobic state.

The potentials of cultures in which the anaerobic condition was maintained from the beginning by artificial means will be reported in a later paper, but it may be said here that the potentials of such cultures confirm the idea of the importance of bacterial respiration in establishing a condition of anaerobiosis in cultures given access to air. The anaerobic cultures develop potentials of the same value as those in which oxygen is available, but attain the level characteristic of the culture medium more rapidly.

This point of view is opposed to that which regards the potentials of bacterial cultures as a manifestation of reductive substances liberated by the bacteria. Since the potentials given by cultures in the

early part of their growth indicate only the level of reduction intensity which the sterile medium may attain, evidence is wanting from this source for the existence of such bacterial products.

In the later stages of growth when the rate of dying of the bacteria approaches the rate of multiplication, the potentials of cultures indicate a level of reduction intensity more negative than that of the culture medium itself. No attempt has been made to work out the exact relations between the growth curve of the bacillus and the reduction potentials since this would involve the detailed investigation proposed by Clark (3). It is certain however that reductive bodies are produced and stored within the cell, as is shown by the reduction of indicators within the living ameba in the experiments of Cohen, Chambers, and Reznikoff (10). The dissolution of cells which must occur in bacterial cultures that have passed the peak of the growth curve would be expected to liberate such reductive substances; if their oxidation is prevented, as in the anaerobic condition which results from respiration of the bacteria, and if in addition the substances are electromotively active, their presence might be indicated by change in the reduction potentials.

It seems possible from consideration of the potentials of cysteine studied by Michaelis and Flexner (6) that among the substances liberated on dissolution of the bacteria are products similar in nature to those already present in the culture medium, so that the more negative potentials may result from an *increase* in the concentration of an electromotively active substance. Other reductive products may be liberated, but nothing can be said at the present time as to their effect on the electrode potentials.

If the substances responsible for reduction potentials more negative than those of the culture medium itself were secreted during growth of the bacteria, rather than liberated by their dissolution, we should expect that their effect on the potentials would be observed during the youth of the culture. The gradual negative drift to values 0.040 to 0.080 volt more negative than those of the sterile bouillon which is observed in cultures after several days incubation indicates that distinct reductive phenomena are associated with the declining portion of the growth curve, in which death of the cells predominates over multiplication.

The failure to attain reduction potentials in cultures saturated with oxygen as negative as those given by cultures which were allowed to develop an anaerobic state may be due to the oxidation of any highly reductive bodies which had been liberated. On the other hand, the enormous numbers of living bacteria in the oxygenated culture suggest that there may be a less rapid "turn-over," and consequently a less extensive dissolution of bacterial cells in such cultures than in those provided with a less abundant supply of oxygen.

The behavior of the typhoid bacillus in the establishment of reduction potentials does not of course permit inference to be drawn as to the behavior of other bacterial species. The observations of Clark (3) show that different species run different courses of potential. It is probable however that any microorganism capable of consuming oxygen as completely as the typhoid bacillus would bring about in bouillon the establishment of potentials as negative as those yielded by the medium itself under anaerobic conditions, unless there is involved an oxidative mechanism which is not evident in the case of *B. typhosus*.

CONCLUSIONS.

1. The reduction potentials of *B. typhosus* in culture in bouillon which is given access to atmospheric oxygen show a negative drift that attains the values found in sterile bouillon when deaerated with nitrogen: *Eh* −0.085 to −0.095 volt at pH 7.6. The potential reaches this level after 6 to 8 hours incubation, and is maintained at this point for several hours. A slow decline to more negative values is then observed and continues for at least 48 hours, when a potential of −0.145 volt may be attained.

2. The bacteria influence the potentials in the first period of their growth by exhaustion of oxygen from the culture, thus permitting the characteristic potential of the culture medium to become manifest, and do not contribute the substances responsible for the observed potentials. The decline in potential to values more negative than those of the culture medium occurs during the time that the rate of dying of the bacteria approaches and exceeds the rate of multiplication; it is suggested that dissolution of bacteria liberates reductive substances.

3. Cultures in 0.5 per cent dextrose medium show a somewhat more negative potential after 18 hours growth than cultures in medium without dextrose. This may be due to the more rapid "turn-over" of the bacteria and the liberation of larger amounts of reductive material from dissolution of larger numbers of bacteria.

4. The potential of cultures through which oxygen is passed continuously does not show a negative drift at any time. This indicates that reductive substances of bacterial origin in the case at least of the typhoid bacillus do not influence the electrode potentials in the presence of oxygen and confirms the importance of bacterial respiration as the means for the removal of oxygen and the consequent establishment of characteristic reduction potentials in cultures.

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