OXIDATION-REDUCTION POTENTIALS IN RELATION TO THE GROWTH OF AN AEROBIC FORM OF BACTERIA[1]

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During the past few years a great many studies have been made on the electromotive properties of biological systems. In general, the studies indicate that bacterial cultures as well as sterile bacteriological media are active oxidation-reduction systems. The effect produced by the bacteria on the potentials of the substratum on the one hand, and the effect of the potentials of the substratum on the growth of the organisms on the other, involve relations of very definite significance.

In a previous communication, Allyn and Baldwin (1930) reported that marked effects on the growth of an aerobic form of bacteria were induced by certain changes in the oxidation-reduction character of the media. In continuation of these studies an endeavor has been made to study by electrometric measurements the oxidation-reduction potentials developed in certain bacteriological substrata and to interpret the same experimentally in terms of bacterial growth. Certain irregularities were encountered in these studies with aerobic organisms, rhizobia; yet some significant correlations, it is believed, were manifested between the potential-time curves of the substrata and the ability of the organisms to initiate growth therein.

EXPERIMENTAL

Methods

A simple potentiometer circuit was found unsatisfactory in making potential measurements in these poorly poised biological

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systems because of the polarization effects produced by a current of the magnitude characteristic of this circuit, passing through the system when finding balance. The polarizing effects were avoided by the use of a vacuum-tube potentiometer designed in this laboratory.[2] This circuit was found later to differ only in certain details from that used by Plotz and Geloso (1930) in their studies.

A diagram illustrating the principal features of the vacuum-tube potentiometer is given in figure 1. In the circuit shown in this diagram, when the switch S is thrown to the right, the plate current has a definite value, determined by the magnitude of the

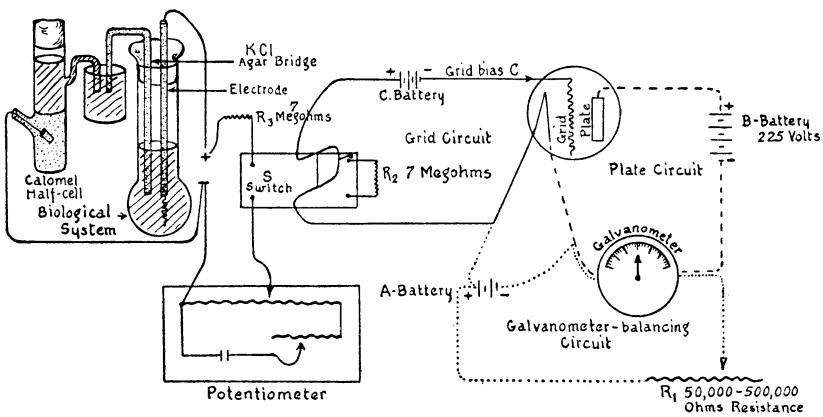

FIG. 1. DIAGRAM OF THE VACUUM-TUBE POTENTIOMETER

negative grid bias C. This current causes a deflection of the galvanometer in the plate circuit. By giving the resistance R_1 a suitable value, this current may be balanced out so that there is no deflection of the galvanometer.

Now when the switch S is thrown to the left, the E.M.F. to be measured becomes a part of the negative grid bias C. The plate current then is changed and again there is a deflection of the galvanometer. The potentiometer is connected also in the grid circuit so that any potential generated by it is a part of the

[2] Credit is due Mr. M. Johnson for the design of this circuit.

negative grid bias. If the potentiometer be adjusted so that the potential generated by it is equal and opposite to the potential to be measured, the net result is that the negative grid bias becomes the same as before and the galvanometer once more reads zero.

In brief then, the E.M.F. to be measured is brought into the system by throwing the switch S to the left. If the potentiometer be adjusted until the galvanometer returns to zero, it is evident that the reading on the potentiometer will be equal to the potential being measured.

The initial negative grid bias C is so adjusted that the grid current has a very low value (approximately 10^{-10} amperes). Such a current was found to have a negligible polarizing effect on the electrodes in the systems investigated. No drifting of the galvanometer obtained within the time necessary to complete the readings, and the readings were consistently reproducible.

The saturated calomel half-cell was used as a standard of comparison in actual operations. By interpolation from the tables of Clark (1928) the saturated calomel half-cell has been calculated to have a value of $E_h = 0.243$ volt at 28°C., the approximate temperature at which all the readings were made in these experiments. The data submitted have been corrected to the standard normal hydrogen electrode, $E_h = 0$, by simply adding 0.243 volt to the observed E.M.F.

The electrode vessels used in these studies were large test tubes (1 by 8 inches) blown out somewhat larger at the base in order to prevent the electrodes from coming in direct contact with the glass during operations (fig. 1). These tubes were of uniform size so that 50 cc. rendered the depths of the substrata in the different experiments comparable to those commonly employed in test-tube culture work. Two electrodes were inserted into each tube and in all cases at approximately the same level (2 inches) beneath the surface of the medium.

The electrodes consisted of bright platinum wire sealed in the end of small soft glass tubing, circuit contact being made by means of mercury. The difficulty arising from the cracking of electrodes during cleaning and sterilization was almost completely

avoided by the use of 22 gauge platinum wire and by flowing only a small amount of glass into the seal about the platinum. The electrodes were cleaned in chromic-sulphuric acid, rinsed thoroughly in distilled water, and flamed in an alcoholic flame before each experiment.

The electrodes, together with the tube for the KCl-agar bridge, were suspended firmly by a cotton plug in Pyrex test tubes (1 by 8 inches) and autoclaved at 15 pounds for twenty minutes. Subsequently the KCl-agar bridge was filled aseptically and the cotton plug with the electrodes and KCl-agar bridge, were bodily set over into a tube of the same diameter containing the system to be studied. After sterilization, the tubes holding the platinum electrodes were filled with mercury by means of a capillary pipette.

Some discrepancies were noted in the initial potential readings of comparable systems depending upon the method employed in the sterilization of the electrodes. Electrodes autoclaved while immersed in distilled water frequently indicated potentials approximately 50 millivolts more positive than similar set-ups in which the electrodes were autoclaved while merely suspended by cotton plugs in the empty Pyrex tubes. The electrodes sterilized under water were as a rule in agreement with each other and the same was true of those sterilized in steam, indicating rather definitely that the method of sterilization was responsible for the apparent differences in the observed readings.

These differences in potential readings were of greatest concern in attempting to make potential measurements on sterile bacteriological media. The electrodes sterilized by the two methods were brought into good agreement when oxidizing or reducing compounds, such as hydrogen peroxide or thioglycollic acid respectively, were introduced into the system, augmenting its electromotively active properties. In inoculated media, the electrodes sterilized by the two methods came into good agreement as soon as the bacterial growth was well under way.

OXIDATION-REDUCTION PROPERTIES OF STERILE YEAST-WATER MANNITOL AND MANNITOL NITRATE LIQUID MEDIA

Two basic media were employed in these studies. The constituents of the two media were as follows:

1. Yeast-water mannitol medium

Distilled water	900 cc.
Yeast water*	100 cc.
Mannitol	10.0 grams
$MgSO_4 \cdot 7H_2O$	0.2 gram
K_2HPO_4	0.5 gram
NaCl	0.1 gram

2. Mannitol nitrate medium

Distilled water	1,000 cc.
KNO_3	1.0 gram
Mannitol	10.0 grams
$MgSO_4 \cdot 7H_2O$	0.2 gram
K_2HPO_4	0.5 gram
NaCl	0.1 gram

* The yeast water was prepared by autoclaving sterile, fresh, starch-free bakers' yeast in 10 times its weight of tap water, allowing to settle and decanting the clear supernatant liquid.

The media were prepared fresh and adjusted to pH 7.0 for each experiment. The freshly autoclaved media in tubes containing 50 cc. quantities were cooled rapidly to approximately 37°C. and the electrodes together with the KCl-agar bridge inserted at once. Care was taken not to shake the tubes during the operations. Some of the tubes of media were covered immediately with a $1\frac{1}{2}$-inch vaseline seal. The systems were then placed in a 28°C. incubator preparatory to making the potential measurements.

The potential-time curves of the two media with vaseline seals, under the conditions just described, are depicted in figure 2. It should be understood that the observations recorded here can represent only in a very general way the oxidation-reduction properties of these two sterile media. As previously pointed out the methods of sterilizing the electrode altered the initial readings of such systems. The results reported here were obtained with electrodes sterilized under distilled water. Electrodes auto-

claved in steam gave readings more negative by approximately 50 millivolts. This difference seemed to persist in the aerobic tubes, but in the media under vaseline seals the difference between electrodes sterilized by the two methods gradually disappeared as the potentials of the system dropped. Moreover, in the case of the yeast-water mannitol medium, different batches of the yeast-water used and even portions of the same batch taken at different ages seemed to influence the observed potentials of this medium.

In a general way, however, both of the freshly autoclaved media under aerobic conditions showed a positive drift of potential for

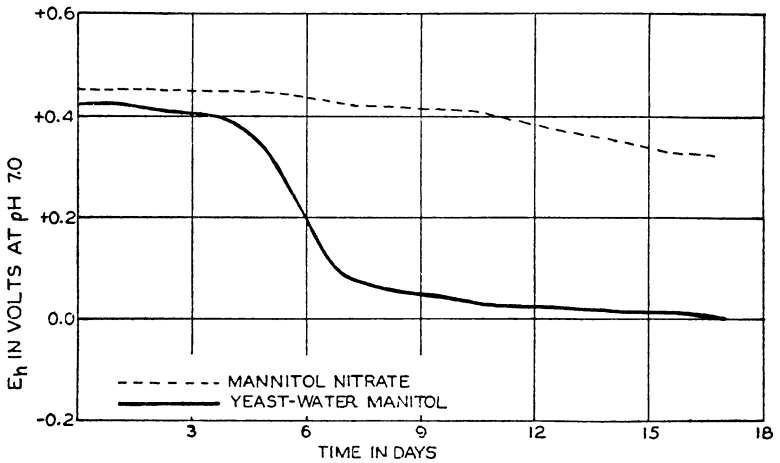

FIG. 2. POTENTIAL-TIME CURVES OF STERILE YEAST-WATER MANNITOL AND MANNITOL NITRATE MEDIA UNDER VASELINE SEALS

the first twelve to eighteen hours, during which time the media were approaching equilibrium with atmospheric oxygen. Thereafter the curves flattened, with a subsequent very slow positive drift over a period of several days. The mannitol-nitrate medium, under these conditions followed a course some 30 to 50 millivolts more positive than the yeast-water mannitol medium.

A very noticeable difference was observed, however, in the electromotively active properties of these two media when placed under a vaseline seal. It is recognized that vaseline seals may not be wholly impervious to atmospheric oxygen, yet it is prob-

able that diffusion through a $1\frac{1}{2}$-inch seal is very slight during the time alloted to the experiments. At any rate the conditions were comparable for the two media under investigation.

The mannitol-nitrate medium exhibited a negative potential drift of only about 12.5 millivolts over a period of seventeen days, whereas the yeast-water mannitol showed a negative potential change of some 425 millivolts (fig. 2). The striking contrast, in the reducing character of these two media when placed under vaseline seals is probably related to the difference in the abilities of the two media to support the growth of *Rh. trifolii*.

MEDIUM	DILUTIONS OF THE ORGANISMS, RH. TRIFOLII						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Yeast-water mannitol	+	+	+	+	+	+	+
Mannitol nitrate	−	−	−	−	−	−	−
Mannitol nitrate, plus 0.015 per cent $CaCl_2$	+	+	−	−	−	−	−

The yeast-water mannitol medium permits growth of *Rh. trifolii* (clover nodule organism, 202) in high dilutions, a 10^{-7} dilution of a five-day culture; whereas a 10^{-1} dilution of the organisms seldom manifests growth in the mannitol-nitrate medium.

Some evidence of improvement was obtained by changing the ion-balance of the mannitol-nitrate medium with the addition of 0.15 gram $CaCl_2$ per liter. In this case, the clover nodule organism was able to initiate growth in a 10^{-2} dilution, but only after a lag phase of some fourteen to seventeen days. It will be seen in data to follow, that when the potential of this mannitol-nitrate medium is properly reduced, growth in much higher dilutions results even without the addition of the $CaCl_2$; but greater improvement in growth is procured when both the $CaCl_2$ and a reducing agent are added.

OXIDATION-REDUCTION PROPERTIES OF CERTAIN MODIFICATIONS OF THE MANNITOL-NITRATE MEDIUM IN RELATION TO BACTERIAL GROWTH

It has just been indicated that the mannitol-nitrate medium under a vaseline seal showed only a very slight negative potential

drift as contrasted with the yeast-water mannitol medium. Further, the nitrate medium did not support growth of *Rh. trifolii* except when large inocula were used and the nutritive balance improved by the addition of $CaCl_2$. Even then growth occurred only after an extended lag phase.

Data presented in a previous communication by Allyn and Baldwin (1930) showed that growth was obtained with high dilutions of the organisms after the addition of cysteine or powdered agar to this nitrate medium. The cysteine introduced another factor, an organic nitrogen source, aside from a change in the oxidation-reduction potentials of the system. In the present studies, thioglycollic acid, a nitrogen-free reducing -SH compound, has been substituted for the cysteine. The potential-time curves of the various modifications of this substratum have been followed by electrometric measurements, and the effect of such potentials on the growth of the organisms in comparable systems determined.

Effect of thioglycollic acid and of agar. The effects of various concentrations of thioglycollic acid and of powdered agar on the growth of rhizobia in the nitrate medium without $CaCl_2$ are shown in the first part of table 1.

The medium was prepared fresh, and tubed in 8 cc. quantities. The thioglycollic acid, approximately 13 N, was found to be sterile. It was adjusted aseptically to pH 7.0 with sterile N/14 NaOH before being added to the medium.

The powdered agar was dried and autoclaved in a petri plate. From 0.2 to 0.4 gram was added to 10 cc. of medium after seeding the organisms.

A six-day culture of the organisms in a yeast-water mannitol medium was used as the source of the inocula for the various dilutions. It is recognized that some of the ingredients of the yeast-water medium were carried over with the inocula. As this procedure was uniformly followed, however, the conditions were comparable for all the experiments.

In these experiments none of the dilutions of *Rh. trifolii* was able to initiate growth in the control tubes containing the untreated mannitol-nitrate medium. With the addition of thio-

TABLE 1

The effect of thioglycollic acid and of powdered agar on the growth of Rh. trifolii 202 in the mannitol nitrate medium with and without the addition of $CaCl_2$

MEDIUM WITH NO $CaCl_2$	DILUTIONS OF ORGANISMS						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Check	−	−	−	−	−	−	−
Check +0.002 per cent thioglycollic acid	+?	−	−	−	−	−	−
Check +0.005 per cent thioglycollic acid	+++	−	−	−	−	−	−
Check +0.0075 per cent thioglycollic acid	+++	+++	++	+	+	+	−
Check +0.01 per cent thioglycollic acid	+++	+++	++	+	+	−	−
Check +0.025 per cent thioglycollic acid	+++	+++	+	−	−	−	−
Check +0.05 per cent thioglycollic acid	+++	++	−	−	−	−	−
Check + powdered agar 4 per cent	+++	+++	+++	+++	+++	+++	−
MEDIUM PLUS 0.015 PER CENT $CaCl_2$							
Check	+++	+++	−	−	−	−	−
Check +0.002 per cent thioglycollic acid	+++	+++	−	−	−	−	−
Check +0.005 per cent thioglycollic acid	+++	+++	+++	−	−	−	−
Check +0.0075 per cent thioglycollic acid	+++	+++	+++	+++	+++	+++	+++
Check +0.01 per cent thioglycollic acid	+++	+++	+++	+++	+++	+++	+++
Check +0.025 per cent thioglycollic acid	+++	+++	+++	−	−	−	−
Check +0.05 per cent thioglycollic acid	+++	+++	−	−	−	−	−
Yeast-water mannitol medium	+++	+++	+++	+++	+++	+++	+++

1. Readings recorded after seventeen days' incubation.
2. Inocula taken from six-day culture in yeast-extract mannitol medium.
3. +'s = amount of growth as judged by turbidity.

glycollic acid up to and including 0.0075 per cent, growth conditions were gradually improved as the amounts of the reducing compounds were increased. Progressive increases in the con-

centrations of the thioglycollic acid above 0.0075 per cent, produced a corresponding decrease in the number of dilutions showing growth.

Even though growth occurred in dilutions as high as 10^{-6} when the optimum amount of thioglycollic acid was added to the nitrate medium, much heavier growth occurred in the tubes receiving the larger inocula, as shown by the turbidity of the cultures. This would indicate that certain ingredients of the yeast-water medium carried over with the large inocula rendered the nitrate medium capable of supporting a larger growth of the organisms. Such nutrients alone, however, were ineffective in establishing suitable conditions in the nitrate medium for growth initiation, since no growth occurred in the control tubes to which no thioglycollic acid had been added (table 1).

The fact that heavy growth was obtained throughout in the tubes receiving the sterile powdered agar, lends some evidence to support the belief that certain inclusion materials of the commercial agar actually improved the nutritive balance of the medium. Other effects of the agar will be discussed presently.

Thioglycollic acid plus $CaCl_2$. In the first part of this paper it was pointed out that the mannitol-nitrate medium supported growth of the large inocula of rhizobia (10^{-1} and 10^{-2}) when 0.015 per cent $CaCl_2$ had been added to the medium; whereas under otherwise comparable conditions no growth occurred. This led to another experiment in which both the $CaCl_2$ and various amounts of thioglycollic acid (0.002 to 0.05 per cent) were added to the tubes of nitrate medium. The calcium chloride was sterilized separately in solution and added in proper concentrations to the tubes of sterile medium at room temperature to prevent the precipitation of the phosphates.

Under these conditions, the 10^{-1} and 10^{-2} dilutions of the organism were able to initiate growth in the tubes of nitrate medium to which the $CaCl_2$ but no reducing agent had been added. Visible signs of clouding did not appear with the 10^{-2} dilution however until after thirteen to fifteen days' incubation. The 10^{-3} dilution rarely showed visible signs of growth even after a period of one month (see table 1).

When the optimum amount of thioglycollic acid (0.0075 per cent) was added to this nitrate medium together with the $CaCl_2$, heavy growth obtained uniformly up to and including the 10^{-7} dilution of the organisms. Growth here was as good as that obtained in the yeast-water mannitol medium (table 1).

Electrode potentials of comparable systems. An attempt was made to follow, in a general way, the potential-time curves developed in the sterile media under the conditions just described. The procedure followed in making the electrometric determinations was as described in the discussion of methods.

In spite of certain irregularities encountered while working with media under aerobic conditions, definite correlations were manifested between the potentials of the substrate and the ability of the organisms to initiate growth under the conditions set forth in table 1. The fact that 0.002 per cent thioglycollic acid failed to render the nitrate medium favorable for growth, while, on the other hand, 0.0075 per cent made growth possible with high dilutions of the organisms may be explained, it is believed, in terms of the difference in potential levels established under these two conditions.

The 0.002 per cent thioglycollic acid produced a sudden, but temporary, fall in the potential of the medium, followed by a rapid rise within twenty-four hours back to some 374 millivolts positive to the normal hydrogen electrode. The curve then straightened with slow positive drift over a period of several days (fig. 3).

The addition of 0.0075 per cent thioglycollic acid (the optimum amount) caused a somewhat similar but lower dip in potential of the system followed by a much more gradual rise over a period of three to four days. After the fourth day the curve began to flatten but at a potential some 50 millivolts negative to the former (0.002 per cent). The lower region of potential maintained by the 0.0075 per cent thioglycollic acid would appear to account for the ability of the smaller inocula to initiate growth in this case and not in the other (fig. 3).

Conversely, the addition of 0.05 per cent thioglycollic acid obviously created conditions too reduced for optimum growth.

A pronounced lag phase was observed with the large inocula (10^{-1} and 10^{-2} dilutions) and complete inhibition with the smaller inocula. This amount of thioglycollic acid carried the potential of the medium to a very low level, 160 millivolts negative to the normal hydrogen electrode, and maintained the potential in this region for several days. Only a very gradual positive drift of the system was observed over a period of eight days due to the capacity factor characteristic of this concentration of the reducing agent (fig. 3).

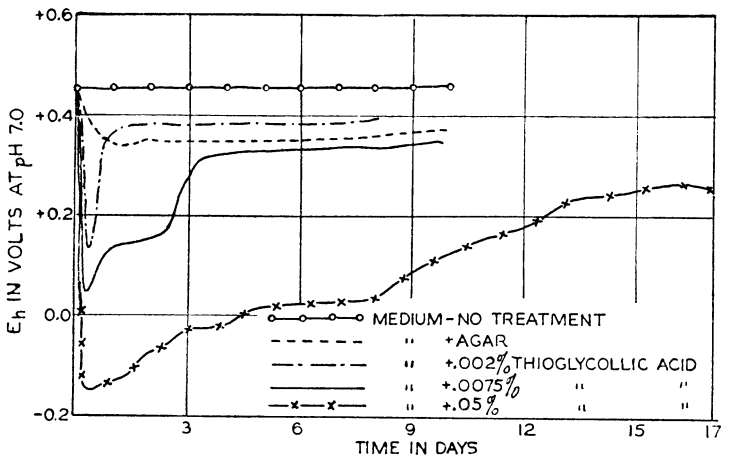

FIG. 3. POTENTIAL-TIME CURVES OF STERILE MANNITOL NITRATE MEDIUM WITH THE ADDITION OF AGAR AND THIOGLYCOLLIC ACID

The effect of sterile powdered agar. Sterile powdered agar, when added to the mannitol-nitrate medium, gave marked stimulation to bacterial proliferation. The electrode potentials of this medium before and after the addition of 0.2 to 0.4 gram of the agar per 10 cc. quantities of the medium, indicate that this substance is not inert as an oxidation-reduction system. The presence of this amount of agar caused a fall of approximately 100 millivolts in the potential of the medium, a condition which prevailed for several days (fig. 3). The coiled electrodes were held at the surface of the mass of powdered agar in the bottom of the tube.

In the light of previous observations, the effects of the agar on

bacterial growth in this purely synthetic medium would reasonably appear to be threefold: (*a*) the agar actually behaves as a mild reducing agent under these conditions; (*b*) certain impurities in the agar improve the nutritive balance of the medium, and (*c*) in a purely physical manner, due to its collodial nature, this material aids the organisms in making suitable oxidation-reduction adjustments themselves.

The last factor suggested is borne out in the experiments of Douglas, Fleming, and Colebrook (1917) in which it was found that porous substances such as cork, charcoal, sand, etc., when added to the medium greatly enhanced the growth of anaerobic bacilli.

Large versus small inocula. Unfavorable oxidation-reduction potentials seem to be less significant with large than with small inocula; and this is especially true when dealing with isolated individuals. The benefit derived from the associative action of microörganisms has been long recognized. Gillespie (1913), Webster (1925) Burnet (1925 and 1927), Valley and Rettger (1926), Quastel and Stephenson (1926), Dubos (1929) and others, working with a great number of different organisms, recognized that the organisms in some way assist each other in growth when in close proximity. To this beneficial association of microörganisms Churchman and Kahn (1921) applied the term "communal activity" and Robertson (1921), as a result of his studies on transplants of protozoa, introduced the term "allelocatalysis."

It is obvious that the bacteria themselves possess the faculty of making suitable adjustments of the potentials of the substratum within certain limits. It would naturally follow that the larger the inocula, the greater is this possibility. Under the conditions of the experiment referred to in table 1, the large inocula not only appear to be able to overcome conditions too oxidized but also conditions too reduced for optimum growth more effectively than do small inocula.

That large inocula can create more reduced conditions of their immediate environment by the respiratory activities of the protoplasm is not difficult to reconcile on a purely physiological basis. On the other hand, the benefit derived from this associ-

ative action under conditions too reduced for optimum growth is not so easily understood. The large inocula (10^{-1} and 10^{-2}) as shown in table 1 were able to withstand the low potentials produced by 0.05 per cent thioglycollic acid and proliferate, although growth was retarded for several days. Whether or not the large inocula were merely able to resist the reduced conditions and start growth after the potentials had risen to more favorable levels due to the auto-oxidation of the thio compound; or whether the bacteria themselves were able to exert some effect on a positive potential change is not definitely known.

The studies of Needham and Needham (1926) and Cohen, Chambers, and Reznikoff (1928) indicate that protoplasm is able to maintain a fairly constant potential within certain limits. More recently Plotz and Geloso (1930) have submitted some evidence to support the idea that bacterial cultures are able to exert an influence on a positive drift of potentials in substrata too reduced for optimum growth. It is well known that the cells of Nitrobacter are able to oxidize nitrite nitrogen as a source of energy and, at the same time, use the nitrite as a source of nitrogen in building protoplasm, a process which of necessity would involve a reduction of the nitrite before protein could be synthesized. This organism then must possess mechanisms for both oxidizing and reducing the nitrite compound.

Regardless of the theoretical explanations, it is seen experimentally that large inocula are able to withstand greater reduced conditions than small inocula. It would seem that the effect is in part due to the ability of the organisms to adjust the potentials in either direction, if not completely overtaxed by the adverse conditions of the system.

Limiting oxidation-reduction potentials. Recently several references have been made in the literature to the limiting oxidation-reduction potentials affecting bacterial growth. The studies of Fildes (1929), Knight and Fildes (1930) and Plotz and Geloso (1930) indicate that *Bacillus tetani* can initiate growth only within a definite potential range. Of course it is understood that these potential limits were operative for that substratum under investigation but not necessarily for all others, a point which should be properly emphasized.

A medium may be unfavorable for the best growth of the organisms for reasons other than an unfavorable oxidation-reduction potential. Under these conditions, it is reasonable to believe that slight deviations from the optimum oxidation-reduction range become of greater significance. This view is borne out in these studies by the data set forth in table 1.

The mannitol-nitrate medium, as such, did not support growth even when a 10^{-1} dilution of the organisms was used. Either of two changes in the medium made growth possible: (1) a change in the ion-balance by the addition of 0.015 per cent $CaCl_2$, or (*b*) the lowering of the potentials by the addition of thioglycollic acid. Either of these changes singly made growth possible whereas no growth occurred otherwise. On the other hand when the $CaCl_2$ was added and the system also reduced, an ideal situation seemed to be produced; good growth occurred throughout with high dilutions (10^{-7}) of the organisms. The limits of the potential range at which growth is possible with this organism seem to vary with the degree to which other factors are favorable to cell proliferation. A more suitable nutritive balance of the substratum appears to render it possible for the organisms to make wider adjustments of the potentials of the system.

Further, these studies indicate that lower potentials are required for rhizobia to initiate growth in the mannitol-nitrate medium than in the yeast-water mannitol medium. This is probably due to two factors: (*a*) the power of the nitrate medium to resist a change in potential when acted upon by the bacteria, and (*b*) the character of the nitrogen sources of the two media. In order to obtain results with the nitrate medium at all comparable with the yeast-water medium, the addition of 0.0075 per cent thioglycollic acid was necessary. This amount established a potential level in the nitrate medium well below that of any of the tubes of untreated yeast-water medium.

Greater reduction intensities seem to be required to reduce the nitrate preparatory to the synthesis of protoplasm than are required in the assimilation of the organic nitrogen of the yeast water. The transformation of NO_3 to NH_3 in the building of proteins would necessitate a reduction of the NO_3 by the organism.

THE OXIDATION-REDUCTION PROPERTIES OF CERTAIN MODIFICATIONS OF THE YEAST-WATER MANNITOL MEDIUM IN RELATION TO BACTERIAL GROWTH

The yeast-water mannitol medium, previously described, appears to be a very good medium for the growth of rhizobia. Growth is supported in high dilutions of the organisms and the total amount of growth is good. It is interesting to note the effects that certain modifications in the potentials of this medium have upon the ability of rhizobia to initiate growth as contrasted with the original untreated medium.

Effect of hydrogen peroxide alone. The hydrogen peroxide solution used contained 2.62 per cent actual H_2O_2. For the sake of convenience the various concentrations used were calculated as parts of this 2.62 per cent H_2O_2 solution per 1000 parts of medium. The hydrogen peroxide solution contained 0.20 grain of acetanilid per fluid ounce. This amount of acetanilid contained in the concentrations of hydrogen peroxide used was found to have a negligible effect upon bacterial growth.

A concentration of 1:3500 hydrogen peroxide solution was sufficient to inhibit the growth of a 10^{-1} dilution of rhizobia in the yeast-water mannitol medium. Further, it was found that the inhibitory effect produced by the peroxide in this medium was not dissipated rapidly under the conditions of these experiments.

Several sets of tubes of the yeast-water mannitol medium were prepared and divided into two series. A concentration of 1:3000 H_2O_2 was added to all the tubes of one series, and 1:3500 to the other at the beginning of the experiment. Subsequently, at intervals covering a period of eighteen days, one set of tubes of each series was inoculated with various dilutions of the organisms. Even eighteen days after the hydrogen peroxide had been added, these media failed to support growth of the organisms except in the case of the 10^{-1} dilution where the 1:3500 H_2O_2 had been added (table 2).

Electrode measurements on similar sterile systems indicate that a 1:3000 concentration of H_2O_2, when added to the yeast-water mannitol medium, produces a positive shift in the poten-

tials of the system to a level of 490 to 500 millivolts positive to the normal hydrogen electrode. This potential level is well above that observed in any of the experiments with the sterile untreated yeast-water medium. Further, this potential level persisted over a period of time comparable to the duration of the growth experiments just referred to above (fig. 4).

Any attempt to explain the lasting effect of the hydrogen peroxide in this system, both on bacterial growth and on electrode potentials, becomes a matter of conjecture. Whether or not the

TABLE 2

The permanency of the effect of H_2O_2 in yeast-water mannitol medium as related to the growth of Rh. trifolii 202

TIME OF INOCULATION AFTER H_2O_2 WAS ADDED TO MEDIUM	CHECK—NO H_2O_2					CONCENTRATION OF H_2O_2 (2.62 PER CENT ACTUAL H_2O_2)									
						1:3,000 H_2O_2					1:3,500 H_2O_2				
	Dilutions of organisms														
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
12 hours later	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
24 hours later	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
3 days later	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
8 days later	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
12 days later	+	+	+	+	+	−	−	−	−	−	+	−	−	−	−
18 days later	+	+	+	+	+	−	−	−	−	−	+	−	−	−	−

These data remained unchanged after one month's incubation of the tubes.

Original inoculum was taken from a five-day fluid culture of the organism in the yeast-water mannitol medium.

peroxide persists as such or forms some more stable organic compound in the medium, is not known.

Peroxide followed by thioglycollic acid. The following experiment was designed to determine whether or not the inhibitory effects of the concentrations of hydrogen peroxide, just referred to, were due to a poising of the medium at unfavorable potentials for cell multiplication, or to the killing of the organisms outright.

Several series of tubes of the yeast-extract mannitol medium were prepared and concentrations of hydrogen peroxide ranging from 1:3000 up to 1:4000 added to the tubes at the outset.

After a lapse of twelve hours all the series of tubes were seeded with various dilutions of the organisms. At subsequent intervals of twelve, twenty-four, forty-eight and eighty hours one set of tubes respectively of each series was reduced with 0.01 per cent thioglycollic acid (or 0.01 per cent cysteine). In some of the higher dilutions the organisms were killed, apparently by the hydrogen peroxide, during the course of the experiment. It was possible, however, to recover a number of the lower dilutions even after an exposure of eighty hours to these oxidized conditions (table 3).

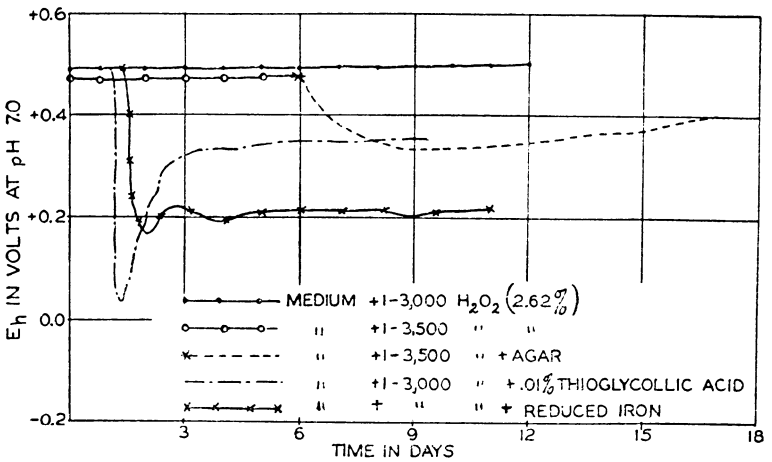

FIG. 4. POTENTIAL-TIME CURVES OF STERILE YEAST-WATER MANNITOL MEDIUM WITH THE ADDITION OF H_2O_2 AND SUBSEQUENT ADDITION OF THIOGLYCOLLIC ACID, REDUCED IRON, AND POWDERED AGAR

The potential-time changes produced by the introduction of the 0.01 per cent thioglycollic acid into the yeast-water mannitol medium containing the hydrogen peroxide were followed by electrode measurements under sterile conditions. Immediately after the addition of the reducing compound, a rather sudden low drop in the potential of the system was observed, followed by a rapid positive drift within thirty-six hours back to a region still some 150 to 175 millivolts negative, however, to that of original system. A slow positive change continued during the six days following (fig. 4).

It is evident in the light of these observations that the barely inhibitory concentrations of hydrogen peroxide exert their characteristic depressing effect on bacterial growth, in part at least, by poising the substratum at unfavorable potentials for cell multiplication.

Peroxide followed by other substances. In this experiment an endeavor was made to determine the effectiveness of certain other materials on the ability of the organisms to overcome unfavorable potentials induced by the addition of H_2O_2 to the yeast-water mannitol medium. Three concentrations of hydrogen peroxide

TABLE 3

The effect of H_2O_2 and the subsequent addition of thioglycollic acid to the yeast-water mannitol medium on the growth of Rh. trifolii 202

YEAST-WATER MANNITOL PLUS PARTS PER 1,000 OF H_2O_2	CHECK—NO THIOGLYCOLLIC ACID	0.0075 to 0.01 PER CENT THIOGLYCOLLIC ACID ADDED AFTER																								
		12 hours					24 hours					48 hours					80 hours									
	Dilutions of organisms																									
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
1:3,000 H_2O_2...	−	−	−	−	−	+	+	+	−	−	+	−	+	−	−	+	+	−	−	−	+	+	−	−	−
1:3,500 H_2O_2...	−	−	−	−	−	+	+	+	+	−	+	+	+	−	−	+	+	+	−	−	+	+	−	−	−
1:3,750 H_2O_2...	+	−	−	−	−	+	+	+	+	−	+	+	+	+	−	+	+	+	+	−	+	+	+	−	−
1:4,000 H_2O_2...	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−

The H_2O_2 solution (2.62 per cent actual H_2O_2), was added to the medium twelve hours before inoculation.

All tubes were inoculated at the same time and thioglycollic acid added at subsequent intervals as indicated.

were used: 1:3000, 1:3500, and 1:4000. The hydrogen peroxide was added to the tubes at the outset. Then, after twenty-four hours, the tubes were all seeded with the various dilutions of the organisms. After a lapse of another four hours, the materials such as sterile powdered agar, ground filter paper, fine sand, reduced iron and cysteine were added. Details of the set-up are given in table 4.

Some advantages were noted under the conditions of the experiments resulting from the addition of such rather inert materials as sand and filter paper. The materials, probably by trapping

small amounts of medium and preventing convection currents, render it possible for the organisms themselves to start growth within the interstices of these materials and spread to the surrounding medium.

The organisms seemed to derive greater benefit from the presence of the sterile powdered agar. This material not only affords greater physical aid to the bacteria in making their own adjustments due to its colloidal nature, but it also exerts a mild

TABLE 4

The effect of certain substances on the ability of the Rh. trifolii 202 to overcome unfavorable potentials induced by the addition of H_2O_2 to a yeast-water mannitol medium

YEAST-WATER MANNITOL MEDIUM + H_2O_2	PARTS OF H_2O_2 (2.62 PER CENT SOLUTION) PER 1,000 PARTS OF MEDIUM																	
	1:3,000 H_2O_2						1:3,500 H_2O_2						1:4,000 H_2O_2					
	Dilutions of organisms																	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Check	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
+ ground filter paper	+	−	−	−	−	−	+	+	−	−	−	−	+	+	+	−	−	−
+ fine sand	+	+	−	−	−	−	+	+	−	−	−	−	+	+	+	−	−	−
+ powdered agar	+	+	+	−	−	−	+	+	+	+	−	−	+	+	+	+	−	−
+ reduced iron	+	+	+	+	−	−	+	+	+	+	−	−	+	+	+	+	−	−
+ 0.01 per cent cysteine	+	+	+	+	−	−	+	+	+	+	−	−	+	+	+	+	+	−

The H_2O_2 was added to all the tubes; after twenty-four hours they were all inoculated; then after an elapse of some four hours, the various substances, powdered agar, etc., were introduced.

reducing effect upon the system itself under these conditions as shown by electrode measurements. Approximately 0.2 to 0.4 gram of the agar was introduced into 10 cc. of the medium (fig. 4).

The addition of the cysteine and reduced iron to the yeast-extract medium containing the hydrogen peroxide produced somewhat similar results on bacterial growth. In each case some of the smaller inocula were unable to initiate growth probably due to injury incurred by exposure to the hydrogen peroxide before the addition of the reducing agents (table 4).

Approximately 1.5 to 2 grams of reduced iron were added per 10 cc. of medium in the experiment above. Under comparable conditions electrometric measurements showed that this amount of reduced iron was capable of producing a negative shift of some 300 millivolts in the potential of this medium containing 1:3500 H_2O_2 and was capable of maintaining this potential level over a period of several days. In making the potential readings, the electrodes were inserted in the clear medium just above the reduced iron after it had settled to the bottom of the tube (fig. 4).

Attempts to follow the potential-time changes produced by the 0.01 per cent cysteine under these conditions gave erratic results. The instability of this compound at pH 7.0 and the rapidity with which it is auto-oxidizable made attempts at comparable results in these aerobic media futile. Its reducing properties, however, were obvious in all cases. The thioglycollic acid appears to be much more stable. It is not so readily auto-oxidizable and does not require sterilization due to its sterile liquid state at the outset, being approximately a 13 N acid.

OXIDATION-REDUCTION POTENTIALS DEVELOPED BY CULTURES OF RHIZOBIA

The potential-time changes developed by cultures of rhizobia in the yeast-water mannitol medium were followed by electrode measurements in a manner previously outlined in this paper (fig. 1). The cultures were all incubated at 28°C. The experiments were initiated at pH 7.0 and hydrogen-ion determinations made again at the close of the experiment. These bacteria produce slight pH changes during their development in the yeast-water medium. The potential-time changes of the systems during the growth of these aerobic cultures are plotted against time in figure 5.

Rh. japonicum followed a potential-time course in a region very positive to that of the other three cultures of rhizobia. Certain discrepancies were noted between comparable electrodes in cultures of this organism. The two electrodes in the same culture were in perfect agreement at times, whereas, often on subsequent days, a discrepancy of some 30 to 40 millivolts between the two

electrodes was observed. After growth had progressed for several days, one of the cultures was placed under a vaseline seal. The electrodes rapidly came into excellent agreement and remained so thereafter. The slow growth of these organisms and probably their uneven development accompanied by convection currents set up in certain regions of the tubes may have been responsible for the irregular behavior of the electrodes.

This positive zone of potential, characteristic of the *Rh. japonicum* culture, would appear to be due to the failure of the

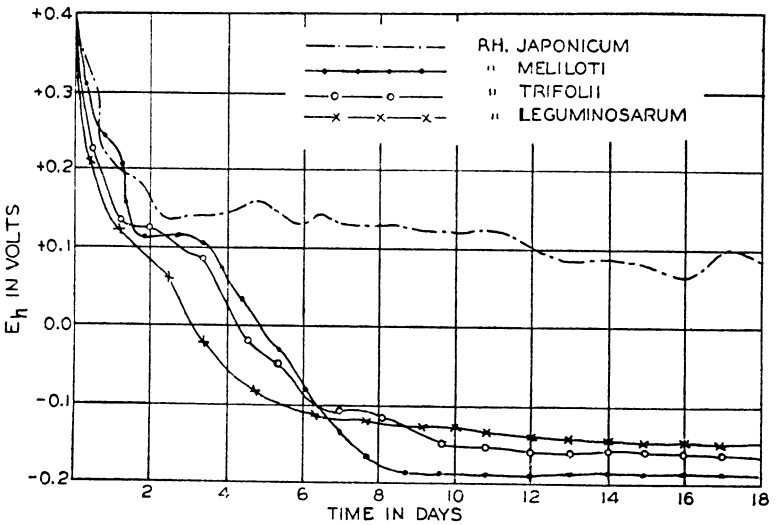

FIG. 5. POTENTIAL-TIME CURVES OF RHIZOBIA IN YEAST-WATER MANNITOL MEDIUM

organisms to exhaust the dissolved oxygen of the system. At no time was pellicle formation evident. The oxygen up-take by the organisms is apparently slow. After the addition of the vaseline seal referred to above, a negative drift of potential obtained, below that of the aerobic culture, as the oxygen became exhausted by the organisms under the seal.

Rh. trifolii, Rh. leguminosarum, and Rh. meliloti. These three aerobic cultures followed somewhat similar potential-time curves. The maximum low potentials attained, however, were all much

lower than that of *Rh. japonicum*. During the first day a rapid negative potential drift occurred in all cases, followed by a slow continued drift over a period of several days (fig. 5).

It will be noted that *Rh. leguminosarum* and *Rh. trifolii* follow courses somewhat negative to *Rh. meliloti* during the first few days of growth; but *Rh. meliloti* attains the lowest final potential. The first two organisms appear to be more aerobic than *Rh. meliloti*. They grew throughout the medium at first but soon formed dense pellicles while the base of the tube almost completely cleared. *Rh. meliloti* grew diffused throughout the medium during the entire period forming a very thin pellicle only after nine days' incubation.

The greater demand for oxygen and early dense pellicle formation may account for the more negative potentials characteristic of *Rh. leguminosarum* and *Rh. trifolii* in the early growth stages. On the other hand, the magnitude of the growth throughout the medium in the case of *Rh. meliloti* during the entire growth phase may account for the final low potential attained in this case.

The growth of the organisms in the nitrate-mannitol never resulted in potentials as low as those attained in the yeast-water medium. The electrodes were also held much less constant in the nitrate medium. The differences obtained in the potentials of the two systems may be due to inherent differences in the two media themselves or they may be due to a less vigorous growth of the organisms in the nitrate medium.

Factors responsible for potential changes in bacterial cultures. The attempt to analyze the factors responsible for the negative drift of potentials characteristic of bacterial cultures is as old as the observation of the phenomenon itself. The fact that the reduction of dyes took place in the medium some distance out from the zone of bacterial growth suggested to Spina and to Rozsahegyi, as early as 1887 that diffused products of bacterial metabolism were probably active in the reduction processes. Sterilization, however, seemed to destroy the reducing properties of such systems. Cahen (1887) in the same year added another link to the chain of thought by suggesting that these diffusion products may be in the form of a gas and readily escape from the

field of action, or may be readily oxidized upon being released from the bacterial cell. Hence, reduction of dyes would be evidenced only in the presence of living protoplasm, yet one of Spina's experiments showed very clearly that sterile nutrient gelatin itself was capable of reducing methylene blue.

More recently Barthel (1917) and Thornton and Hastings (1929) have emphasized the withdrawal of oxygen from the medium by the bacteria during the reduction of methylene blue in milk. The mere withdrawal of oxygen, however, will hardly account satisfactorily for all cases, especially where extremely low potentials approaching a hydrogen overvoltage are reached, far below the point where the last traces of oxygen have been exhausted.

Coulter (1928) and Coulter and Isaacs (1929) showed that cultures of *B. typhosus* developed potentials some 0.1 volt lower than were obtained in the sterile medium alone when deaerated with nitrogen. Plotz and Geloso (1930) contend that Coulter was unable to rid the medium of all the dissolved oxygen by deaeration with nitrogen, whereas the culture of *B. typhosus* carried the process to completion, thus accounting for the 0.1 volt more negative potential reached in the inoculated medium.

Plotz and Geloso (1930) devised a method for making electrode measurements in vessels hermetically sealed after deaeration of the system in order that the medium itself would dispense with the last traces of free oxygen. They conclude that bacterial growth has only a catalytic effect on the potential changes of the medium similar to that of platinum black in their experiments. The same low potentials were reached in all instances but more rapidly where bacteria were at work or when platinum black had been added to the medium. Working with seven different organisms they conclude further that the potential limits attained in bacterial cultures are not characteristic of the organisms developing therein, as all reach the same limit of reducing intensity. Hewitt (1930), on the other hand, showed that more highly reducing conditions were developed by *C. diphtheriae* than by cultures of haemolytic streptococci. Similar observations have been made with many other organisms.

It would appear that this situation is too complex to permit

sweeping generalities or to warrant the assignment of the reducing properties observed in bacterial cultures to any isolated factor. To contend that all bacterial cultures develop the same low reducing potentials would naturally entail an analogous statement that all compounds resulting from bacterial metabolism possess similar electromotively active properties. It is difficult to reconcile on this basis the action of such metabolic products as hydrogen sulfide and peroxide, the one a reducing compound and the other an oxidizing compound.

In the course of these studies on rhizobia certain data were obtained which, it is believed, lend some information concerning the factors pertinent to the reducing properties of growing cultures. After a growth period of three days, one of two comparable cultures of *Rh. meliloti* was placed under a vaseline seal. For a few days after the addition of the seal, this system fell in potential somewhat more rapidly than the aerobic culture. As growth progressed, however, the potential fall in the aerobic tube surpassed that of the anaerobic tube and continued more negative (some 120 millivolts) until the close of the experiment (fourteen days). If the change in potential were to be attributed wholly to the exhaustion of oxygen from the system, then the presence of the seal, it would appear, should have facilitated the development and maintenance of a more negative potential.

Data were obtained also which indicate that a bacteriological system is in a more reduced state subsequent to bacterial growth and sterilization than that of the original medium. Fresh tubes of medium were prepared and initial potential readings made. The tubes were then heavily inoculated with *Rh. trifolii*, *Rh. meliloti*, and *Rh. leguminosarum*. After eight days' incubation the cultures were autoclaved at 15 pounds for twenty-five minutes, rapidly cooled to 28°C. and clean electrodes again inserted. The sterilized cultures on an average tested some 100 to 150 millivolts more negative than the original sterile medium. The autoclaved culture underwent gradually a positive potential drift of approximately 20 millivolts daily for the first four days following sterilization, indicating the presence of some auto-oxidizable substance that had not been destroyed by sterilization.

The controversies arising from the various attempts to explain

the reducing properties of bacterial cultures may be reasonably compromised by remembering that bacterial cultures are specific entities. Even though many factors may be involved in the reducing properties of bacterial cultures as a group, the importance of any one factor or of a combination of factors for a given system must depend upon the specific character of the system in question.

THE BEHAVIOR OF BACTERIA TOWARD ATMOSPHERIC OXYGEN AS RELATED TO OXIDATION-REDUCTION POTENTIALS

Allyn and Baldwin (1930) reported that rhizobia in shake-agar cultures develop distinct growth zones at various levels in the tubes depending upon the oxidation-reduction character of the medium. Similar growth levels may be observed with rhizobia in liquid media, if care be taken not to disturb the cultures during growth. It was noted that, as the liquid media were made more reducing, the first signs of growth appeared correspondingly nearer the surface of the medium. The converse was true when the potentials were made more oxidizing.

In the experiments with the yeast-water mannitol medium to which hydrogen peroxide had been added in amounts just short of the inhibition of a 10^{-1} dilution of rhizobia, growth appeared first at the very bottom of the tube and diffused up as reducing conditions were set up by the bacteria. Ultimately, as the potentials of the system reached a low level due to bacterial growth, pellicle formation occurred and the bottom of the tube began to clear. The system, as shown by electrode measurements, passed through a potential range from $E_h = +0.490$ volt at the outset to $E_h = +0.050$ volt, at which time pellicle formation was evident with *Rh. trifolii*.

In the case of the mannitol-nitrate liquid medium, previously referred to, as the tubes of medium were reduced progressively with increasing amounts of thioglycollic acid, the first signs of growth appeared correspondingly nearer the surface of the medium. When 0.002 per cent of the reducing compound was added, growth appeared first at the bottom of the tube with a 10^{-1} dilution of *Rh. trifolii*. With the addition of 0.005 per cent

thioglycollic acid, growth first appeared about the middle of the tube; with 0.0075 per cent, about 1 cm. below the surface, and with 0.01 per cent, very close to the surface of the medium.

The smaller inocula initiated growth at zones somewhat lower than those just described for the 10^{-1} dilution under similar conditions. This difference is probably due to the lesser reducing properties of the smaller inocula themselves and the rise in the potential of the substratum by the auto-oxidation of the thio-compound during the greater lapse of time necessary for the smaller inocula to produce visible signs of growth.

In the case of rhizobia in the yeast-water medium, signs of surface growth could be predicted with surprising accuracy by means of the potentiometer. Signs of pellicle formation appeared with *Rh. leguminosarum* in undisturbed cultures at $E_h = +0.110$ to $+0.090$ volt; with *Rh. trifolii* at $+0.060$ to $+0.040$ volt; and with *Rh. meliloti* at $+0.010$ to -0.010 volt.

Rh. japonicum never formed pellicles, and as indicated in figure 5 this culture followed a potential-time course in a region very positive to the other three organisms. The oxygen up-take of *Rh. japonicum* is evidently not large. It appears evident that the phenomenon of pellicle formation with rhizobia is an expression of an oxygen relationship, or of the degree to which the organisms are aerobic in character.

SUMMARY

A vacuum-tube potentiometer circuit is described for avoiding polarization in making electrometric measurements of oxidation-reduction potentials of biological systems.

The studies indicate that the oxidation-reduction potentials of the medium exert a decisive influence on the ability of certain aerobic bacteria to initiate growth.

Two basic media were studied: a yeast-water mannitol and a mannitol-nitrate medium. The first medium was found to be the more reducing in nature and to support growth with high dilutions of the organisms. The mannitol-nitrate medium permitted growth in similar dilutions only after the potential was properly reduced. It was possible to poise the yeast-water man-

nitol medium with H_2O_2 at potentials sufficiently high to inhibit growth completely, and yet recover the organisms after eighty hours' exposure to these conditions, by reducing the potentials of the systems.

Commercial agar was found not to be an electromotively inert substance. The agar not only appears to afford physical advantages to the organisms in initiating growth due to its colloidal nature, but also to act as a mild reducing agent under the conditions of these experiments.

Large inocula possess greater ability to overcome unfavorable oxidation-reduction conditions than small inocula. This seems to hold true in media either too oxidized or too reduced for optimum growth.

The oxidation-reduction potentials which limit bacterial growth seem to be somewhat dependent on the other factors characteristic of the medium. A more suitable ion-balance or a more available nitrogen source, for example, appears to facilitate wider adjustments of unfavorable potentials by the bacteria themselves.

Potential-time courses developed by rhizobia have been followed by electrode measurements. The factors responsible for the negative drift of potentials in the bacterial cultures are discussed.

The behavior of the rhizobia toward atmospheric oxygen is determined largely by the oxidation-reduction character of the medium, as indicated by the growth levels established by the organisms in media possessing different oxidation-reduction properties.

Pellicle formation in liquid media seems to be definitely correlated with the oxidation-reduction potentials of the system in the case of rhizobia. The bacteria collect at the surface in a pellicle as the potential of the medium falls, due to their metabolic activities.

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