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(Osborn Zoological Laboratory, Yale University.)

Studies on the oxidation-reduction potential of protozoan cultures.

II. The reduction potential of cultures of *Chilomonas paramecium.*

Von

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(With 4 figures in the text.)

Within recent years a number of papers have appeared concerning oxidation-reduction potential changes in cultures of various bacteria, and these observations have led to a more complete understanding of the factors which affect growth and fermentation rates in bacteriological cultures than was heretofore available (bibliography, JAHN, 1933; review, KLUYVER, 1933). However, to the knowledge of the writer, no electrical measurements of the oxidation-reduction potential changes in bacteria-free cultures of protozoa have been published. Some points concerning the importance of such studies have been mentioned previously, and a few preliminary colorimetric measurements of the potential of Chilomonas cultures have been made (JAHN, 1933). The present paper is the first of a series of studies on the potential changes in bacteria-free cultures of protozoa and contains measurements of electrode potential and p_H changes in the medium of bacteria-free cultures of *Chilomonas paramecium*, together with a description of the apparatus employed for use with glass and platinum electrodes. The method is an improvement over previous measurements on bacterial cultures in that the p_H as well as the electrode potential changes are followed. Inasmuch as these two factors are intimately related, simultaneous measurements are quite

desirable. The data obtained show that large changes take place in the oxidation-reduction potential of the medium during growth of the culture, and these findings support the theory proposed previously that changes in electrode potential may be related to the growth rate.

Material and methods.

The bacteria-free strain of *Chilomonas paramecium* used in these experiments was the same as that described in the previous paper of this series (JAHN, 1933). The medium used consisted of 1.0 % hydrolyzed casein, 0.12 % KH_2PO_4, and 0.08 % K_2HPO_4. In addition to these ingredients the cultures also contained either 0.20 % Na-acetate or 0.086 % NaCl. In this way the Na + concentrations were maintained approximately equal in all cultures, and the effect of the acetate ion could be determined.

Since bacterial (and presumably protozoan) cultures are rather poorly poised and are polarized by withdrawals of as little as 10^{-5} to 10^{-6} amperes (COHEN, 1931), it is necessary to measure the electrode potential while drawing much less than this amount of current from the system. It is also a decided advantage to be able to measure p_H without removing samples of the medium or disturbing the culture in any manner such as is involved in measurements with indicators or with hydrogen or quinhydrone electrodes. For this reason the glass electrode was used and all measurements of potential and of p_H were made with a vacuum tube potentiometer (DU BOIS, 1930). Since this instrument draws less than 5×10^{-15} amperes it is very well adapted for such measurements. The potentiometer here described is essentially the same circuit as that used by DU BOIS. However, several changes have been made, some at the personal suggestion of Dr. DU BOIS, and the whole apparatus has been adapted for measurements of protozoan and bacterial cultures.

The vacuum tube circuit contains one UX-232 screen grid tube which is operated at free grid potential. The wiring diagram and arrangement of apparatus are shown in fig. 1. The tube, one special switch (S), and a two pole double throw reversing switch (R) are enclosed in a grounded metal box (M) which is mounted immediately behind the bakelite panel (P). The screen grid is connected to the ground through potentiometer C and 1—4 dry cells so that it has a potential of —1.5 to —6.0 volts, dependent upon the sensitivity of the galvanometer used. The control grid is connected to a copper target with which either pole of switch S may be brought in contact. Switch S is a special single pole double throw switch of very low

capacity and with high insulation. One side of switch S (left in fig. 1) is conuected to a line from potentiometer B to switch R. The radio potentiometers A (5000 ohms) and B (200 ohms) are used to adjust this line to the free grid potential of the tube. The other pole of switch S is connected by a highly insulated and shielded line L to the electrode of which the potential is to be measured. The calomel half-cell is connected by a similar line (L') to switch R. The potentiometer K is the only calibrated potentiometer used and may be any high grade potentiometer calibrated to fractions of a millivolt. The wiring shown is for a Leeds and Northrup Type K, and all cannections to it are made in the usual manner except that the E.M.F. side (E) of the double throw switch is short circuited. Switch complex IHJ-I'H'J' is composed of one two pole double throw

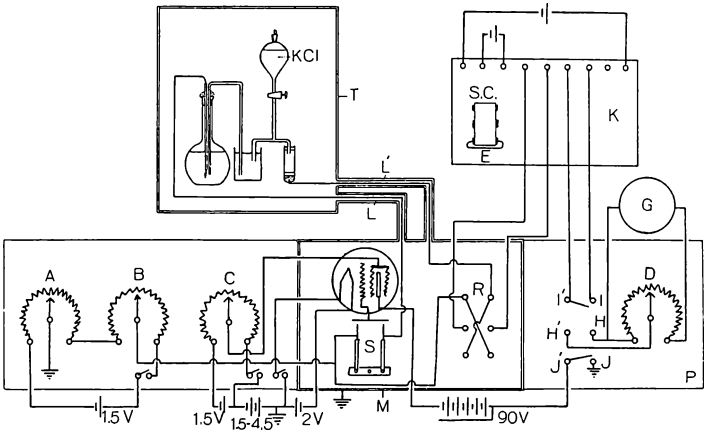

Fig. 1. Construction and arrangement of vacuum tube potentiometer for use with protozoan cultures. Explanation in text.

switch and two single pole single throw switches, one of which is connected across the terminals of each side of the double throw switch, all of which are mechanically interconnected so that the 90-volt battery-to-ground circuit and the potentiometer K-galvanometer circuits are always closed — either through the short circuit or through the galvanometer. Potentiometer D is a galvanometer shunt. In these experiments the galvanometer was a modified Leeds and Northrup Type R with a sensitivity of .0045 microamperes per mm.

When a measurement is to be made switch HH' is connected to the II' contacts, and current through the K potentiometer is adjusted in the usual manner. Then HH' is connected tho the JJ'

contacts; switch S is open. By means of potentiometer C the potential of the screen grid is adjusted until the galvanometer gives any convenient deflection ($\triangle$). The control grid is then at free grid potential. Then the battery line from B is connected by switch S to the control grid. A and B are adjusted so that the galvanometer returns to position $\triangle$. The control grid and the battery line are now both at the free grid potential of the tube, and the plate current is at a minimum. Then switch S is thrown in the opposite direction. This interposes potentiometer K, the calomel half-cell, and the culture to be measured between the control grid and the battery line B. Potentiometer K is then adjusted until the galvanometer deflection $\triangle$ is again reached. Since the control grid is still at free grid potential, polarization of the system being measured is negligible. The reading of the calibrated potentiometer K is equal and opposite to the potential of the system being measured as compared to the calomel half-cell. The position of the reversing switch R depends upon whether the potential of the electrode being measured is above or below that of the half-cell.

The platinum electrodes used were spirals of 24 guage platinum wire sealed in the end of glass tubes. If well annealed these electrodes can withstand repeated autoclaving. The glass electrodes were made from glass tubes over one end of which were sealed membranes of Corning .015 glass. These membranes were 0.5 to 2.0 mm in diameter, and some were thin enough to show interference bands. The tube was filled with .1n HCl. In this was immersed a platinum-silver-silver chloride electrode prepared by sealing an inch or more of platinum wire into the end of a glass tube wich had been drawn out as in fig. 2. The platinum wire was then plated with silver from a AgKCN solution at 3 milliamperes for 24 hours, washed 48 hours in distilled water, and chloridized by plating for 20 minutes in .1N HCl.

The arrangement of the electrodes as used is shown in fig. 2. The glass electrode, a platinum electrode, and a saturated KCl-agar bridge were tied into the cotton plug as in A. The ends of the agar bridge were immersed in vials filled with KCl-agar, and the electrodes were immersed in distilled water as shown in the diagram. Flasks fitted in this manner were autoclaved. Then the plug containing the bridge and electrodes was lifted out of the flask, loosened from the vial of agar, and placed into a flask of sterile broth of the same bore as the previous one (fig. 2 B). The flaks were then placed in the thermostat, and the glass electrode was tested for

leakage. The larger and thinner membranes showed a tendency to break in the autoclave. However, since the vacuum tube circuit used can measure the p_H with reasonable accuracy through membranes of 100,000 megohms resistance, the smaller membranes were found to be quite satisfactory for the present purpose. The sensitivity of the instrument decreases with increase in the resistance of the membrane; therefore, the thinnest membranes which would withstand autoclaving were used.

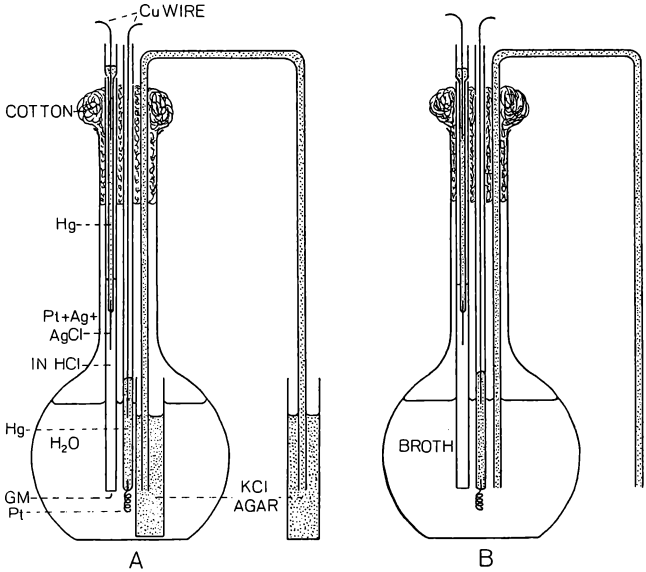

Fig. 2. Arrangement of glass and platinum electrodes. A, during sterilization in water. B, during experiments. The electrodes and bridge shown in A have been transferred to a flask of sterile broth in B.

The potential of a glass electrode in a solution may be affected by several factors other than the p_H of the solution, i. e., the strength of the HCl, the surface of the AgCl, and the assymmetry potential of the glass. This necessitates individual calibration of each electrode. As these factors may be affected by autoclaving, the electrode must be re-calibrated after it is autoclaved. This may be done under sterile conditions before the experiments are performed or else the experimental results may be recorded merely as voltage readings and the equivalent p_H values determined after the experiment is finished. The latter procedure was adopted. After the experiments the electrodes were washed in distilled water and re-calibrated with

Sorensen phosphate buffers over the range of the experiments. This calibration was repeated after each set of experiments.

The possibility of making continual p_H readings on a growing culture by means of sterile glass electrodes is a distinct advantage over previous methods used in bacteriology and protozoology. Also, the use of autoclave instead of chemical sterilization is to be recommended in that no foreign materials are introduced into the solution and in that sterilization is always positive. This latter point is extremely important in preventing wasted effort where readings are to be taken every few hours for several days. To the knowledge of the writer autoclave sterilization has not previously been used for glass electrodes.

The relative accuracy of the apparatus used for p_H measurements in buffer solutions is about $\pm$ .01 unit. However, the relative accuracy of the measurements on any culture in these experiments may be somewhat less (perhaps within $\pm$ .02 unit) for they are not corrected for any changes in the assymetry potential of the membrane which might have taken place during the experiment. The absolute accuracy depends upon the standard buffers used for calibration, and these were checked with quinhydrone electrodes to an accuracy of $\pm$ .03 p_H unit.

Experimental results.

The results of one series of experiments are shown in fig. 3. In these experiments the flasks of medium were inoculated eighteen hours after autoclaving. Flasks 1 and 2 contained casein peptone, phosphate, and NaCl; flasks 3 and 4 contained casein peptone, phosphate, and sodium acetate. One cc. inoculations were made from a culture without acetate, and the initial concentration of organisms in each flask was about 15 per cc. The thermostat was maintained at a temperature of 30° C. The initial Eh readings (compared to normal H_2 electrode at p_H 0) for the plain broth cultures were + 425 for flask 1 and + 380 for flask 3. The initial Eh readings for the acetate cultures were + 392 for flask 3 and + 420 for flask 4 The initial p_H was 6.5 in all flasks.

Due to the very low initial cencentration of organisms only slight changes in Eh occurred during the first 24 hours. Also, the p_H changes were small, being only + .08 (to p_H 6.58) in all flasks. However, during the second 24-hour period the Eh dropped about 260 mv in all cultures; the p_H of the plain broth cultures rose to 6.69, and the p_H of the acetate cultures to 6.97. During the third

24-hour period the Eh of the plain broth cultures reached a minimum of about + 120 mv, and the readings of the electrode in flask 1 began to rise before the end of this period. The Eh of the acetate cultures continued to drop to about + 140 mv. The p_H of the plain broth cultures did not change appreciably, while that of the acetate cultures continued to rise, reaching p_H 7.30. During the fourth 24-hour period the Eh of the plain broth cultures rose to about + 275 mv, while that of the acetate cultures continued to drop, reaching Eh 0 at the end of the period. The p_H of the plain cultures rose slightly to p_H 6.75, while that of the acetate cultures rose to p_H 7.41. During

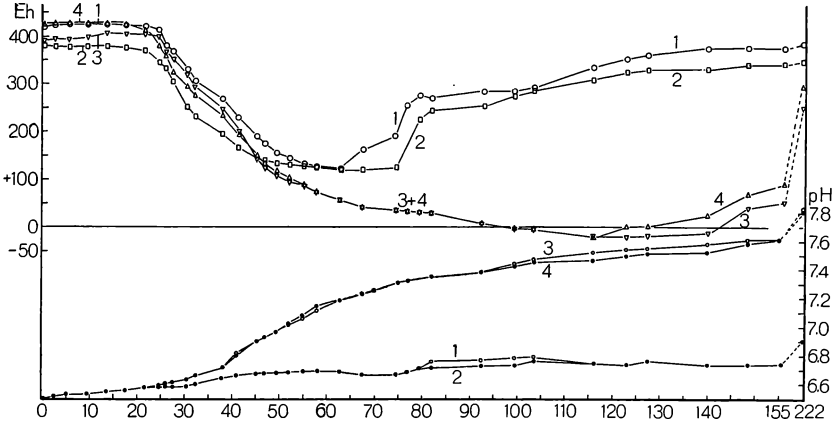

Fig. 3. Eh and p_H changes which occurred in four bacteria-free cultures of Chilomonas paramecium. Cultures 1 and 2 contained plain casein peptone broth; cultures 3 and 4 contained acetate casein peptone broth. Abscissa in hours; Eh in millivolts.

the fifth 24-hour period the Eh of the plain cultures continued to rise (to Eh + 330) while that of the acetate cultures reached a minimum at about Eh — 20. Changes in p_H were slight in all cultures. At the end of 222 hours the two plain broth cultures gave Eh readings of + 385 (flask 1) and + 340 (flask 2); the acetate cultures, + 250 (flask 3) and + 280 (flask 4). At this time the p_H values were 6.90 in the plain and 7.81 (flask 4) and 7.83 (flask 3) in the acetate cultures.

During the first 18 hours growth of the culture was hardly visible macroscopically. At 24 hours numerous organisms were visible and were scattered throughout the culture. At 30 hours a thin but quite definite layer of organisms was formed a few mm. beneath the surface of the liquid in all flasks. In the plain cultures this

layer continued to become denser until about the 60th hour, at which time the organisms began to sink to the bottom of the flask. After the 100th hour there was only a very slight subsurface layer visible in these cultures. In the acetate cultures the subsurface layer increased in density more rapidly between the 30th and 60th hours than in the non-acetate cultures, and it did not decrease visibly in density until after the 125th hour. However, the organisms began to accumulate in the bottom of the acetate flasks at about the 60th hour. At the 222nd hour most of the organisms in all flasks had fallen to the bottom. As observed macroscopically, growth in the plain cultures seemed to be very rapid until about the 60th hour while that in the acetate cultures seemed to continue at a high rate until about the 100th hour. The presence of acetate, then, seemed to increase both the growth rate and the length of time during which growth occurred. This increase of growth rate with acetate is in agreement with the data of LOEFER (1934).

In other cultures which were started with larger inocula than those described above the results are essentially the same except that the changes in Eh and p_H occurred earlier. In one series of cultures in which the initial concentration was about 600 organisms per cc. (instead of 15 as above) the Eh and p_H curves were very similar to those given above except that the sharp drop in Eh in all cultures and the sharp rise in p_H in the acetate cultures occurred 10—15 hours earlier. This is to be expected since the changes in the culture medium are functious of the number of organisms present as well as of the time after inoculation.

Fig. 4 contains the corrected data for flasks 2 and 3 of fig. 3. The Eh values for appropriate 2.5 and 5 hour periods have been corrected for the p_H changes which occurred during the experiment. The standard correction for change of Eh with p_H has been used; that is:

$$Eh' = Eh - (60 \triangle p_H) \text{ (in mv)}.$$

The figure shows that the greater changes in p_H in the acetate cultures were partly responsible for the difference in Eh between the acetate and the plain media, but it also shows that only a smal' part of the difference can be explained in this manner. In the acetate cultures the Eh' minimum was lower (+ 45 mv as compared to + 132) and was reached after a longer period of time (ca. 100 hours as compared to ca. 60 hours) in the acetate than in the plain medium. Also, the Eh' value was maintained close to the minimum for a longer time (40 hours compared to 25 hours) in the acetate medium.

The slight initial rise in Eh in curve 3 (figs. 3 and 4) is rather unusual and migth be caused by an increase in oxygen tension after the medium was inoculated.

Electrodes 1 and 4 gave readings which were approximately 50 mv higher than those of electrodes 2 and 3 at times of high oxygen tension. However, in media of low oxygen tension (as at minimal Eh values, fig. 3) or in solutions of quinhydrone they checked within 1 mv of each other. This difference in readings of various platinum electrodes in the presence of poorly poised solutions was found to persist after boiling in concentrated nitric acid, and it is

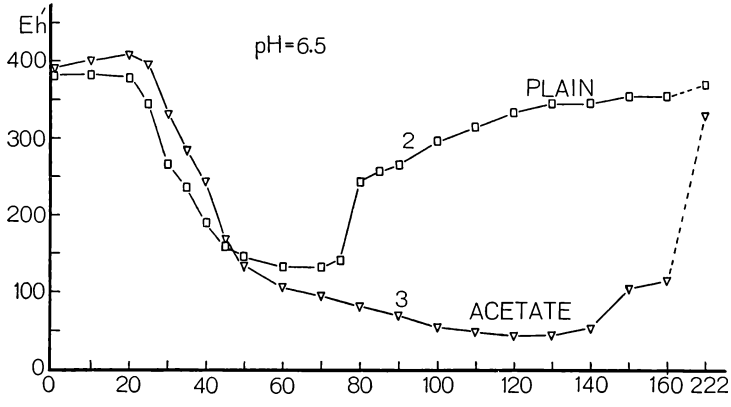

Fig. 4. Data from cultures 2 and 3 (Fig. 3) corrected for pH changes at appropriate 2.5 and 5 hour periods. This shows the changes in Eh which are independent of pH.

believed to be due to unknown qualities of the electrode surface. However, this peculiarity of the electrodes does not invalidate the above results; it is probably present in most experiments of this type and merely introduces an uncertainty as to what the true Eh value might be under conditions of high oxygen tension. In the present case the approximate magnitude of the changes and the value of the minimum rather than the initial potential are the data of most importance. A comparable discrepancy between the readings of different platinum electrodes is described by Allyn and Baldwin (1932).

Discussion.

The above data on *Chilomonas* are very similar to those which have been obtained by other workers on aerobic bacteria Hewitt, 1930; Allyn and Baldwin, 1930, 1932). It has been found that

various taxonomic groups of bacteria give Eh curves which are characteristic in the rapidity with which the Eh falls, the level of the minimum, and the length of time which this level is maintained. These characteristics of the curve are determined by the rate of growth and oxygen consumption of the bacterium, the constituents of the medium, the production of H_2O_2, of catalase, and of strong reductants such as H_2S, the relative degree of aerobiosis and anaerobiosis in which the organisms will live, and perhaps other and unknown factors. The potential of the medium, in turn, seems to affect the rate of growth of the organisms, and to determine the virulence and colony form of haemolytic streptococci (review, HEWITT, 1930), the direction of glucose fermentation by Aspergillus (KUSNETZOW, 1931), and perhaps other as yet undetermined metabolic reactions. It seems probable that similar relationships between the reduction potential of the medium and the metabolic activities of the organism might be present among the protozoa. However, it will be necessary to gather a considerable amount of data before definite conclusions regarding these relationships can be drawn. Inasmuch as this paper presents the first data of the kind for protozoa, and since the relative rates of division and O_2 consumption of Chilomonas and bacteria are unknown, and as the medium used for Chilomonas differs somewhat from that used in bacterial studies, it is not yet possible to compare closely the present curves with similar data for either protozoa or bacteria. Whenever such data becomes available it seems probable that very interesting comparisons might be made.

The lowering of the Eh value during the course of the experiment is probably due to the lowering of the oxygen tension and perhaps also to the accumulation of reducing substances in the medium. If sufficient oxygen is available any reducing substances produced by the organism might be oxidized, so that the governing factor in the establishment of the potential is the equilibrium between the oxygen supply and the oxygen consumption of the cells and reducing substances. During the first 20 hours in the above experiments the diffusion of oxygen into the medium is rapid enough to maintain the potential approximately constant. From the 25 th to the 60—70 th hours in the plain medium and to the 120—130 th hours in the acetate medium the rate of oxygen consumption exceeded the rate at which the supply was replenished by diffusion. After this time the organisms ceased to divide, the oxygen consumption decreased, the oxygen tension rose as a result of diffusion, and the recorded Eh values rose to a much higher

level. The long time during which the minimum Eh level is maintained in acetate as compared to plain medium (Figs. 3 and 4) is at least partly due to the more rapid rate and longer continuance of growth in the acetate media. However, the possibility also exists that one or more of the products of acetate metabolism might be a strong reductant which was only slowly oxidized by molecular oxygen, but this possibility remains a subject for future investigation.

It would be extremely interesting to have quantitative data concerning the growth rates during different phases of the culture so that one might correlate slow or rapid growth with definite oxidation-reduction potentials. The solution of this problem, however, necessitates several changes in the technique which would permit mixing of the medium so as to obtain a uniform suspension of organisms without disturbing the reduction potential by introducing changes in the gas content of the medium. It seems as if such measurements might throw considerable light on the importance of the reduction potential for cell division. With anaerobic bacteria the reduction potential is generally regarded to be one of the most important factors affecting the growth rate. However, with aerobic organisms, the problem is greatly complicated by the presence of oxygen, and it has been suggested (Knaysi and Dutky, 1934) that for *Bacillus megatherium* oxygen tension is much more important as a limiting factor. In the experiments of Knaysi and Dutky the oxygen tension is obviously much more important than the reduction potential of the medium. However, in these experiments the difficulties of maintaining an oxygen tension capable of supplying the energy needs of the organism in a medium of low reduction potential were not completely overcome. Since the reduction of oxygen yields so much more energy than the reduction of other oxidizing substrates which might be present in the medium, one cannot draw many conclusions regarding the relative importance of reduction potential unless the oxygen tension of the medium or the oxygen consumption of the organism is maintained constant. So far neither of these experimental conditions has been attained, and we cannot say how important the reduction potential per se might be. However, it seems quite likely that the very large changes in oxygen tension and reduction potential which occurred during the course of the present *Chilomonas* experiments might have had some effect upon the growth rate of the organism. If so, then we would expect these factors to be operative in experiments on cell contiguity where the number of cells per unit volume is varied. This is well

recognized in bacteriology, and the present experiments support the contention that it must also be considered in experiments on protozoa. This and other explanations of allelocatalysis have recently been reviewed (JAHN, 1934). A possible mechanism whereby the oxidation-reduction potential of the medium might affect the oxidation-reduction potential of protoplasm and consequently the metabolic activities and rate of growth of the cell has also been discussed previously (JAHN, 1934).

Summary.

1. An apparatus is described by means of which continual measurements of the Eh and p_H of bacteria-free cultures of protozoa can be made. The method does not necessitate the removal of samples or disturbance of the medium in any way.

2. The Eh and p_H changes in bacteria-free cultures of *Chilomonas paramecium* have been measured. It was found that the Eh of plain casein peptone broth drops as much as 300 mv (Eh' change = 288 mv) and that the Eh of acetate casein peptone broth drops as much as 460 mv (Eh' change = 375 mv).

3. The most important factor in producing these changes is believed to be the removal of oxygen from the medium during growth of the organisms, but the possibility of the production of reducing compounds by the organisms is not eliminated.

4. The large magnitude of the changes indicates that they might be of importance as a causative agent of allelocatalysis.

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