BACTERIAL GROWTH WITH AUTOMATIC pH CONTROL

(A) AN APPARATUS. (B) SOME TESTS ON THE ACID PRODUCTION OF LACTOBACILLUS ACIDOPHILUS

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From The Rockefeller Institute for Medical Research

Received for publication December 22, 1934

Since much modern bacteriological work requires large quantities of bacteria, and an important limiting factor in the growth of acid-forming bacteria is the decrease of the pH of the media, it has been considered desirable to design and test a device which will keep the pH constant at any chosen value during bacterial growth. Experiments on *Lactobacillus acidophilus* with the aid of this apparatus have yielded data on the rate of acid production at various pH values, and on the effect of dissolved gases on the bacterial growth. The following paper contains a description of the apparatus, and an account of some typical experiments.

A. THE APPARATUS

The device about to be described maintains a constant pH value by adding automatically at intervals the necessary amount of a solution of an alkali.[1] Glass electrodes are used for the measurement and control of the pH, since other types of electrodes require conditions and reagents not suitable in bacteriological media, and are rendered inaccurate, i.e., "poisoned," by such media. Glass electrodes, on the other hand, have no effect on the solutions in which they are placed, and their potentials respond, within their range of usefulness (about pH 1 to pH 9), only to the pH. The apparatus as a whole is shown diagrammatically in figure 1. The solution, the pH of which is to be controlled, is placed in the flask

[1] An apparatus for a similar purpose, but differing widely from ours in details, has been described by Whitnah, C. H., Ind. Eng. Chem. Anal. Ed., **5**, 352 (1933).

F. The pH is measured by the potential of a galvanic cell consisting of the glass electrode *G* and the reference calomel electrode *C*, both connecting with the solution in *F*. The glass electrode used is of the "durable" spiral type described by MacInnes and Belcher (1933). The calomel electrode, which is made with 3.5 N KCl, connects with the solution in flask *F* by means of a column of potassium chloride solution of the same concentration

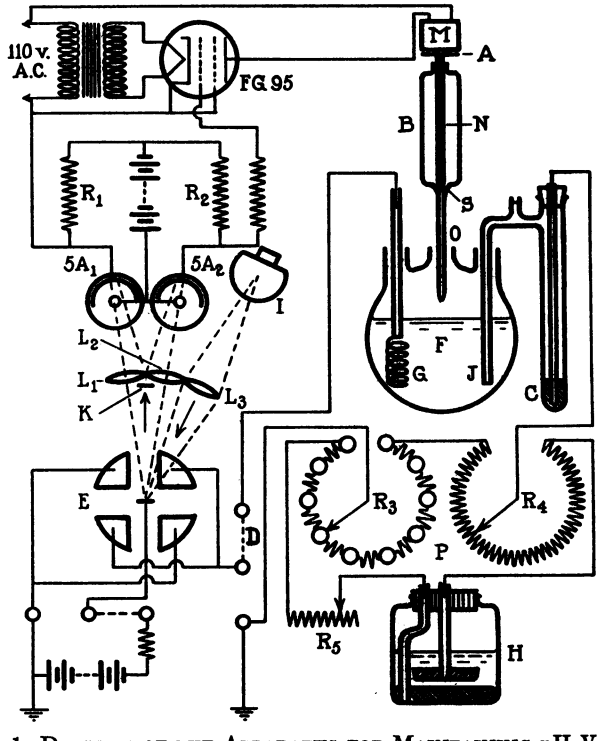

FIG. 1. DIAGRAM OF THE APPARATUS FOR MAINTAINING pH VALUES

and terminates in a very dense porous plug at the point *J*. The electromotive force of this galvanic cell is opposed by a known potential from the potentiometer *P*, and a condition of balance is indicated by the Compton electrometer *E*.

The circuit which controls the addition of alkali is arranged as follows. The light from a 32-candle power automobile headlight bulb *I* is focussed on the electrometer mirror with a lens

L_3. The reflected beam is divided between two lenses, L_1 and L_2, and is collected by two Western Electric vacuum phototubes, $5A_1$ and $5A_2$. These phototubes are placed in the opposite arms of a Wheatstone bridge. The bridge is completed by means of two fixed resistances, R_1 and R_2, which have values (10 to 12 megohms) comparable to the resistances of the illuminated phototubes. A 45-volt "B" battery supplies the bridge potential. The bridge terminals, normally joined to a galvanometer or other null point indicator, are connected, instead, to the control grid and filament of a double grid "thyratron" FG 95. The thyratron plate circuit includes the electromagnet M, which controls the flow of alkali from the buret B to the solution in the flask F.

The resistances, R_1 and R_2, are selected so that the thyratron grid potential is near the critical value when the phototubes have approximately equal illumination. A fine adjustment for bringing the grid bias very close to its critical value is obtained by shifting a small opaque screen at K. This screen may also be used to compensate for drifts in the natural zero of the electrometer needle.

A diagram of the magnetically operated buret is also included in figure 1. When current is passing, the magnet armature A lifts a glass rod, N, which extends the full length of the buret and carries a small rubber plug. The capillary outlet, O, is sealed through the lower end of the buret with a projecting shoulder, S, against which the rubber plug normally presses. When the plug is lifted from the shoulder the liquid flows, under gravity, from the buret. Since the magnet armature moves only 1 mm. in opening and closing the valve, a sufficiently strong pull can be obtained with a small current through the magnet. This magnet is also equipped with a small oil dashpot for the elimination of chattering. The valve avoids the lubrication necessary with ordinary stopcocks, a decided advantage in dealing with strong alkali, as in this work. The rate of flow from the buret may be adjusted by varying the length and bore of the capillary outlet.

The flask F is shown in more detail in figure 2. Alkali is added through the tube at U. The medium is agitated by the stirrer Z. A water seal for the stirrer is provided at W. Inoculation of the

medium and withdrawal of samples for study are carried out through tube X which is normally plugged with cotton wool. Gas can be bubbled through the medium by means of the tube Q–T and can escape through the cotton plug. The reference electrode dips into the upper part of the tube C'–J. Contamination by air-carried bacteria is minimized by making the ground glass joints of the skirted type shown in the figure. The flask,

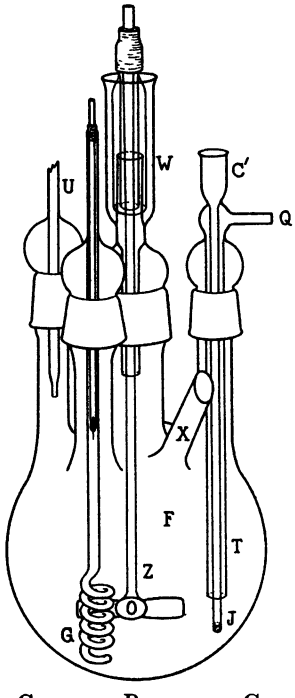

FIG. 2. SPECIAL FLASK FOR GROWING BACTERIAL CULTURES WITH pH CONTROL

and the medium in which the bacteria are grown, are sterilized in an autoclave.[2] Since, however, the glass electrode disintegrates rapidly and loses its calibration in steam or hot water, it is given a

[2] It was not considered necessary to autoclave the buret and its contents since it always contained a solution of sodium hydroxide at a concentration of at least 2 normal. The buret outlet was thoroughly flamed before connecting it to the flask F after the latter had been sterilized in the autoclave. If, however, it should be desirable to use, in the buret, solutions that need sterilization there is no reason why the buret and flask could not go into the autoclave as a unit.

chemical sterilization with 0.1 per cent mercuric chloride and is then rinsed several times, out of contact with laboratory air, in sterile water, before introduction into the flask. A direct test indicated that the mercuric chloride, in amounts sufficient to affect bacterial growth, is removed after two rinsings. Trials are now being made of an apparatus by means of which the chemical sterilization and rinsing of the glass electrode, and its insertion into the flask *F*, may be carried out with no possible contamination by air-borne organisms.

The operation of the apparatus is as follows. After calibration of the glass electrode and the calomel half cell with appropriate buffers, the potentiometer may be set at a value corresponding to the pH at which the system is to be maintained. Assuming that the potentials are now exactly balanced, the electrometer needle will be in its zero position and the light beam reflected from the mirror attached to this needle will be divided between the two phototubes in such a ratio that the thyratron is on the verge of operation. As acid is produced in flask *F* of figure 1 the pH of the solution drops and the electromotive force of the system is no longer balanced by that of the potentiometer. The result is that the electrometer needle rotates slightly from its zero position, thus causing a decrease in the light flux to one phototube and a corresponding increase to the other. The resulting shift in the balance of the Wheatstone bridge causes the thyratron to operate and the opening of the magnetic valve allows alkali to flow into the solution from the buret until a sufficient quantity has been added to restore the pH of the system to its preassigned value. This cycle of operations is repeated automatically and as often as necessary until the experiment is terminated.

With the present arrangement a change of 1 mv. in the potential of the glass electrode causes the thyratron to operate. This corresponds to about 0.02 of a pH unit. In actual operation, however, the deviations of the pH from a constant value are approximately ± 0.05 of a pH unit. Possible causes of deviations occurring while the instrument operates over long periods of time are as follows: (1) Fluctuations of the working current through

the potentiometer. (2) Variations in the intensity of the light source. (3) Shifts in the natural zero of the electrometer needle. (4) Variations of the glass electrode. (5) Variations of the liquid junction potential. These will be discussed in the order given.

1. Variations of the potentiometer current. The usual method for reducing errors from this source is to adjust the potentiometer frequently against a standard cell. However, if this method were adopted the apparatus would not be automatic. The necessity for such adjustments has been avoided by the use of a high resistance potentiometer and a Hulett (1903) standard cell, which can deliver a small current without measurably affecting its potential of 1.019 volts. The potentiometer consists of a decade unit, R_3 of figure 1, with 10,000 ohm steps and a 10,000 ohm variable unit, R_4, which is divided into 100 parts of 100 ohms each. A small supplementary unit, R_5, permits adjustment of the total resistance so that the drop across each division of R_4 is 1 mv. and that across each step of R_3 is 100 mv. The potentiometer is thus graduated in millivolts and can be read somewhat more closely than this, although the present application does not warrant the greater accuracy.

The Hulett standard cell, H, which supplies the potentiometer current is essentially a heavy-duty unsaturated Weston type standard cell. It is, however, designed to have a very low internal resistance and with the additional feature that electromotive force changes due to concentration polarization are largely eliminated. Tests have shown that the potential of this cell remains constant to better than 1 mv., even when furnishing current for the potentiometer, for an interval of several months. Within the accuracy desired errors due to variations in the working current of the potentiometer have thus been entirely eliminated.

2. Variations in the light source. The light source for the phototube bridge is operated from the 110-volt line through a step-down transformer. The fluctuations in the light are mainly due, therefore, to variations in the line voltage. The use of two phototubes in the bridge circuit, as has been described, largely eliminates the errors from this source since, when equally illumi-

nated, a variation in the light source affects the two tubes nearly equally and does not shift the bridge balance appreciably.

3. The zero shift of the electrometer. The apparatus will, obviously, regulate at the desired pH value only so long as the natural zero point of the electrometer does not shift. It has been our experience that the main, if not the only, cause of such shifts is slight variation in the level of the instrument. Such variations have been largely overcome by mounting the electrometer and the light system on a sturdy bracket attached to the outside brick wall of the laboratory. Any zero shifts, and changes in the operating characteristics of the thyratron, can be discovered by reversing the position of the double throw switch D, thus grounding the quadrants, and noting whether an adjustment of the screen K is necessary to cause the thyratron and magnet M to operate. This is the only adjustment found necessary on the apparatus and is made twice a day. If neglected during a run of 100 hours it may result in an error of the order of 0.1 pH unit.

4. Variations of the glass electrode. It is also clear that the apparatus will maintain a constant pH value only if the glass electrode maintains its calibration throughout the period of use. Although the spiral type of electrode has large "asymmetry potentials" for which allowance must be made, these potentials attain steady values after the electrodes have been soaked in water for about a month, after which any further change takes place very slowly.[3]

5. The liquid junction potential. The liquid junction between the medium in the flask F of figures 1 and 2 and the 3.5 N KCl of the reference calomel electrode is made, as already stated, by means of the dense porous plug J. The tube C'–J shown in figure 2 is sterilized with the flask, after which it is filled with 3.5 N KCl, and the connecting tube of the calomel electrode is inserted at C'. The porous plug J is made from a sintered mixture of 30 per cent alundum powder and 70 per cent finely ground Pyrex. With a head of about 15 cm. of the potassium chloride solution a

[3] In the more recent work we have used the "membrane" type of electrode described by MacInnes and Dole (MacInnes, D. A., and Dole, M., Ind. Eng. Chem., Anal. Ed., **1**, 57 (1929); Jour. Amer. Chem. Soc., **52**, 29 (1930)).

few drops a day will pass through the plug.[4] Experiment has shown that, within the limits of accuracy of the operation of the apparatus, such a liquid junction does not differ from one in which the 3.5 N KCl solution comes into direct contact with the medium. However, difficulty may be encountered in experiments, such as the calibration of glass electrodes, in which large changes of pH are involved, if the plugs are not cleaned before introduction into each solution.

This survey of the possible causes for irregularities in operation of the apparatus indicates that the only adjustment necessary is for a slow shift of the zero of the electrometer. The apparatus has, as a matter of fact, functioned for 100 hours with no other attention.

We have found that some simplification is possible in the flask *F*, leading to increased ease in sterilization. Portability of the apparatus might be attained by the use of a vacuum tube electrometer, and we are investigating that possibility. However, our tests up to the present, and a survey of the literature, indicate that such electrometers have not yet been made with the required stability in operation.

B. TESTS ON THE ACID PRODUCTION OF LACTOBACILLUS ACIDOPHILUS

As is well known the growth of *Lactobacillus acidophilus* is accompanied by the production of much acid, principally lactic, and consequently a rapid lowering of the pH of the medium. This organism was chosen for study with the aid of the apparatus described in the previous section. The stock culture, which was obtained from a pure commercial strain, was carried in litmus milk with frequent subcultures. The medium used in all of the experiments to be described was 2 per cent "bacto"-peptonized milk and 3 per cent galactose. The experiments were carried out at 38° in an oil thermostat.

Quite unexpected difficulty was encountered in our early

[4] These plugs are, by several tests, less porous than the finest Berkefeld filters, so that they cannot be sources of bacterial contamination.

attempts to grow the bacteria in flask *F* of the apparatus. Since pH regulation by the addition of alkali cannot be carried out without stirring, it seemed probable that the difficulty was in some way connected with the agitation of the medium. In the undisturbed medium the organism itself will adapt the conditions to favorable growth. This is shown by the familiar phenomena observed when the bacteria develop in a test-tube of litmus milk. The medium, initially blue, is first bleached to white, and the dissolved oxygen presumably largely removed. This stage is followed by the formation of a top layer of pink solution, where the medium is in contact with the air, due to diffusion of oxygen and to re-oxidation of the litmus. The pink color results, of course, from the lowering of the pH by the acid-forming bacteria. It is evident that if air is constantly stirred into the medium the initial reduction cannot occur, at least to the same extent. On the basis of these observations it was decided to try to grow the bacteria in the stirred medium in the absence of oxygen. These experiments were, however, completely unsuccessful. Very slight or no growth was observed when the air in the flask *F* was replaced by a stream of purified nitrogen or hydrogen. It was, however, observed that growth was always obtained if the regulations of pH were made with sodium carbonate; and more difficulty was experienced if the adjustments were made with sodium hydroxide. This observation is in accord with the conclusion of Theobald Smith (1924; 1926) and of Valley and Rettger (1927) that carbon dioxide is necessary for the growth of bacteria. In our tests with purified nitrogen and hydrogen the carbon dioxide was apparently swept out of the medium. These early experiments, which are very briefly summarized above, indicate that although a high tension of oxygen is not favorable to growth of *Lactobacillus acidophilus* it will not completely discourage it, whereas carbon dioxide is absolutely necessary.

Strangely enough, an attempt to grow these bacteria in the apparatus in an atmosphere of pure carbon dioxide at pH 7 resulted in a very slight growth. This result is also due to the fact that the stirred medium tends to come into equilibrium with the

atmosphere above it. The most important chemical equilibrium involved is

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

for which there is the mass action expression

$$\frac{(H^+)(HCO_3^-)}{(CO_2)} = K = 4.8 \times 10^{-7} \text{ (5) at } 38°$$

in which the parentheses represent activities of the substances enclosed. Thus, at a given hydrogen ion activity, or constant pH, there will be a constant ratio of the activities of the bicarbonate ion constituent and the dissolved carbon dioxide, and the latter is, of course, related to the partial pressure of gaseous carbon dioxide by Henry's law. Thus at pH = 7 and 1 atmosphere of carbon dioxide, the solubility under these conditions being 0.02433 mol per liter (Van Slyke, Sendroy, Hastings and Neill, 1928), the bicarbonate ion activity is 0.117. In the apparatus the titrating fluid was automatically added until a bicarbonate concentration of this activity was reached. The concentration obtained directly from the amount of alkali added was found to be 0.30, indicating a not improbable activity coefficient. The salt concentration was apparently too high for effective growth of the bacteria.

Excellent growth was, however, obtained when the gas passed through the medium was about 10 per cent carbon dioxide by volume, the remainder being nitrogen and about 0.2 per cent oxygen, the latter being due to a small proportion of that gas in the high pressure tank of nitrogen from which the mixture was made. Before inoculation the nutrient medium was brought to equilibrium with this gas mixture at the desired pH. This equilibration before inoculation was carried out for as long as sixteen hours without the appearance of turbidity or other evidence of bacterial growth due to contamination. Some typical measurements of the acid production by the bacteria with and without pH control are given in figure 3 where the amounts of alkali, in milli-equivalents per liter of nutrient medium necessary to titrate the acid are plotted as ordinates against the time in hours

as abscissae. For the regulated pH values the ordinates were computed from the readings of the buret *B*. In obtaining the data for the curve for "no pH control" the bacteria were grown, in the conventional manner, in flasks, 5-cc. samples being removed at intervals after gentle shaking and titrated, using phenolphthalein as indicator. Two such flasks were used and gave closely agreeing values. It will be observed that the amount of acid produced in a given time is greatly increased by keeping the pH value at 7, instead of leaving it uncontrolled. After 50 hours this increase is of the order of 100 per cent. Still greater acid

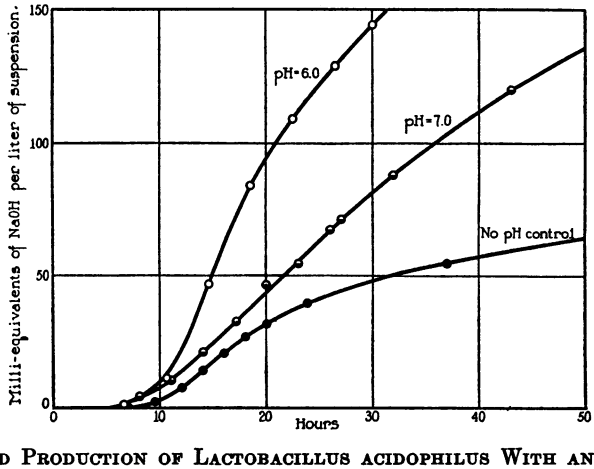

FIG. 3. ACID PRODUCTION OF LACTOBACILLUS ACIDOPHILUS WITH AND WITHOUT pH CONTROL

production is obtained by regulating the pH at a value of 6, the increase over no regulation being of the order of 200 per cent. The acid production curve for pH = 5.5 lies very close to that for pH = 6, the optimum being apparently near the latter value.

Figure 4 shows the change of pH with time when the organism was grown in flask *F* of the apparatus, but without pH control. The same medium was used as in the experiments with control, and the same rate of stirring. The pH, initially adjusted to a value of 7.25, dropped rapidly after inoculation and reached the apparent optimum value of 6 after 9 hours. This rapid drop continued to about pH 4, after which the change was slower.

The lowered acid production without pH control is obviously explained by this pH trend. It is of interest in this connection that Topley and Wilson (1929) say that *L. acidophilus* "grows best" at pH 7.6. It would appear to us that this is not a true optimum, but merely that this high initial pH gives the organism a large range of pH values to pass through before attaining the limiting value of about 4. On the other hand, Weiss and Rettger (1934) in a recent paper find the "average optimum" of this organism to lie between the pH values 5.8 and 6.6, in substantial agreement with our observations.

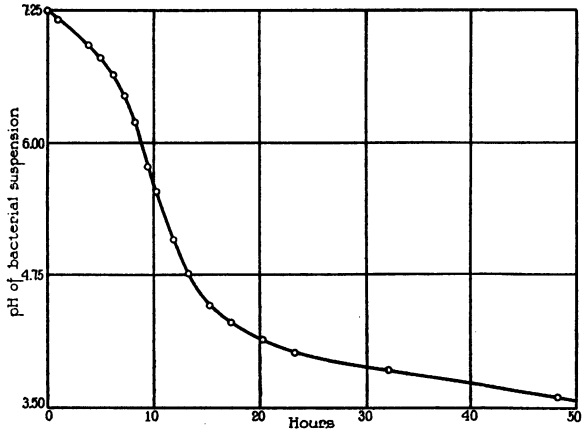

FIG. 4. CHANGE, WHEN NOT CONTROLLED, OF pH OF THE BACTERIAL SUSPENSION

In another series of experiments, which is still in progress, the measurements of acid production with no pH control and with control at various pH values have been accompanied by plate counts, photoelectric nephelometer readings, and determinations of apparent oxidation reduction potentials by means of an inert electrode. Such conclusions as are possible from our experiments as to the physiology of bacterial growth will be considered in a later paper.

SUMMARY

An apparatus is described by means of which the pH of a nutrient medium can be accurately and automatically controlled

during bacterial growth. The apparatus depends upon the measurement of the pH with a glass electrode, and the control of the addition of a solution of alkali by means of a photoelectric relay which is actuated by the deflection of a beam of light by the mirror of an electrometer. The apparatus functions for indefinite periods with a minimum of attention.

The apparatus has been used in the study of the acid production during the growth of *Lactobacillus acidophilus* at different controlled pH values and, for comparison, with no pH control. It has been found that the acid production can be greatly increased by maintaining the pH at relatively low values. It has, however, been found necessary to study the effect of the dissolved gases on the bacterial growth. Our studies have confirmed the conclusion of other workers that, at least for the organism under observation, carbon dioxide is necessary for such growth.

The authors wish to thank Drs. Oswald T. Avery, Walther F. Goebel, and Lillian E. Baker for advice and encouragement, and Drs. Clara Nigg and Kenneth Goodner for helpful suggestions concerning bacteriological technique.

REFERENCES

HULETT, G. A. 1903 Phys. Rev., **27**, 33.

MACINNES, D. A., AND BELCHER, D. 1933 Ind. Eng. Chem., Anal. Ed., **5**, 199.

SENDROY, J., JR., SEELIG, S., AND VAN SLYKE, D. D. 1934 Jour. Biol. Chem., **106**, 469.

SMITH, T. 1924 Jour. Exp. Med., **40**, 219.

SMITH, T. 1926 Jour. Exp. Med., **43**, 317.

TOPLEY, W. W. C., AND WILSON, G. S. 1929 The Principles of Bacteriology and Immunity. London, Edward Arnold and Co.

VALLEY, G., AND RETTGER, L. F. 1927 Jour. Bact., **14**, 101. (This article includes a bibliography.)

VAN SLYKE, D. D., SENDROY, J., JR., HASTINGS, A. B., AND NEILL, J. M. 1928 Jour. Biol. Chem., **78**, 765.

WEISS, J. E., AND RETTGER, L. F. 1934 Jour. Bact., **28**, 501.