APPARATUS FOR MAINTAINING BACTERIAL CULTURES IN THE STEADY STATE

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When cultures of bacteria are transferred in the usual way, both the character of the cells, and the composition of the medium are constantly changing.

If the cell concentration is held constant, on the other hand, the composition of the medium (including enzymes and viruses) in which the growth occurs, must also be constant and any change in the concentration of any substance indicates a change in the organism. This condition of a steady state is therefore the simplest to study, from a theoretical point of view.

A number of devices for the maintenance of cultures in continuous log growth have been described (Felton and Dougherty (1924); Novick and Szilard (1950); Anderson (1953); Myers and Clark (1944); Bryson (1952)). The present apparatus involves no new principles, but may be assembled from commercially available units. The flow of culture media is controlled by a photoelectric cell which is exposed to a light beam passing through the culture.

Electrical equipment required:

1. Klett photoelectric colorimeter, modified to take a 22 mm. tube.
2. Warner photoelectric relay. Model 5100 R.
3. Microscope illuminator, spherical, Bausch and Lomb.
4. Galvanometer, Leeds and Northrup, Number 2420, coil P. I. 102 B.S. coil resistance—20 ohms.
5. Allied Control Co. relay, 24 volts, P 45, CNSX-1, or General Electric solenoid, CR9503—209C.

Culture Cells.—Various types of cells may be used depending on conditions (Fig. 1). Cell A is used when the photoelectric colorimeter containing the cell can be put in a constant temperature room or incubator.

If this is not convenient, the cell may be water-jacketed (Fig. 1, cell B) and water circulated from a constant temperature water bath by means of a pump (American Instrument Co., model 4-603 P).

If the organism sticks to the glass, the cell may be treated with silicone (General Electric dri-film Number 87). If the bacteria still stick, a wiper may be attached to the cell (Fig. 1, A and C). The segments of rubber tubing are

cut through and the stainless steel stirring rod is forced through them (Fig. 1, C). The stirring rod is about 40 cm. long and is encased in a rubber tube which is tightly sealed to the rod at the upper end by forcing the rod through a Number 00 rubber stopper, which is then coated with rubber cement and forced into the end of the rubber tubing. This tube must be wet inside with glycerine before assembly, to prevent the rod from chafing the rubber.[1] The lower end of the rubber tube is attached to a glass tube, set in the rubber stopper, through which the rod moves. The stirrer should operate 150 to 200

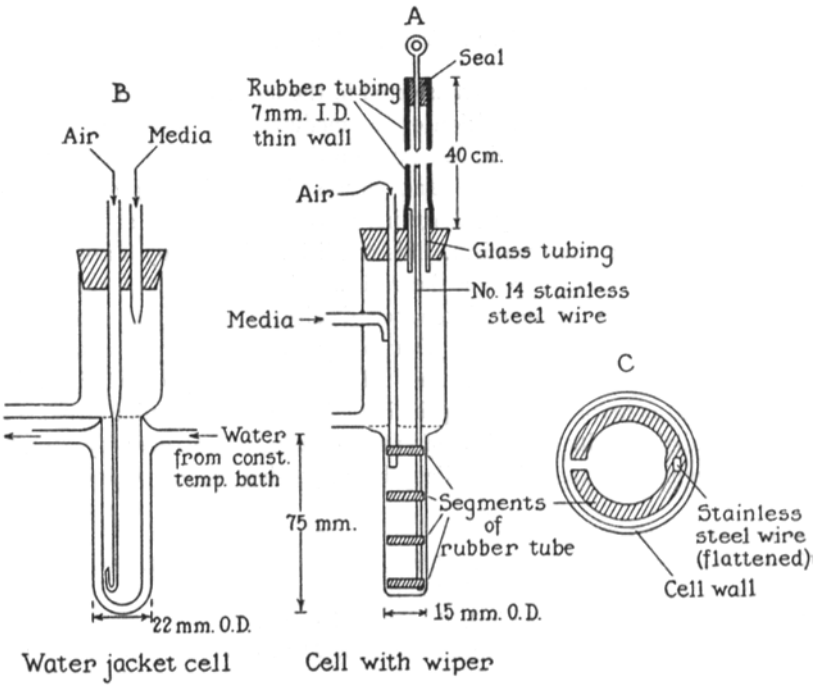

FIG. 1. Cells for steady state cultures.

times/minute through about 1.5 cm. A motor and crank, or a vacuum windshield wiper motor may be used to operate the wiper. Under these conditions, the whole surface of the cell is kept clean. The motion of the rubber segment in the light path does not affect the galvanometer if the latter is properly adjusted, since the motion is too rapid. The galvanometer should remain perfectly steady while the stirrer is in operation.

The wiper may be operated intermittently, if desired, by putting an inter-

[1] The culture media may be led into the top of the rubber guard tube around the stirrer by means of a hypodermic needle. The media will then keep the stirring rod moist and prevent chafing.

mittent time relay in the motor circuit which will close the circuit at intervals, say 2 minutes/hour. If a vacuum windshield wiper is used to operate the wiper, it will stop at the end of the stroke automatically. Some types of electric windshield wiper motors also stop in a fixed position. Otherwise, a make and break switch must be placed in parallel with the relay controlling the motor, so that it will stop the motor at the end of the stroke, where the wipers will not interfere with the light beam.

Large Cells.—The apparatus may be used to collect any desired quantity of the culture, since the volume delivered depends only on the growth rate of the culture and the volume of the cell, *i.e.* the volume of culture media = $K \times$

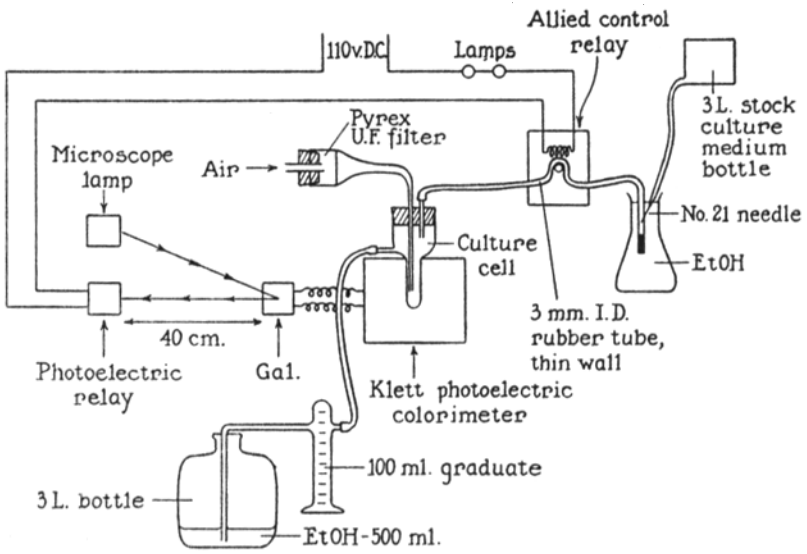

FIG. 2. Assembly diagram of steady state apparatus.

cell volume $\times$ time, in which K is the growth rate constant. If the cell volume is 1 liter, therefore, and the growth rate 2 per hour, then 2 liters of culture will flow from the cell per hour. In case such large culture cells are used, the outflowing culture is run through a tube in the photoelectric colorimeter, instead of placing the cell itself in the colorimeter.

Measurement of the Rate of Flow of the Culture Media Used.—The volume of culture media leaving the cell may be read directly from the graduate (Fig. 2) or in case of large volumes, from the flask. An automatic record may be obtained by a drop counter, described by Anderson (1953), or by attaching a time-recording instrument, such as a kymograph, to the relay which controls the flow of culture media. If the latter method is adopted, a Mariotte bottle is used to hold the culture media and the rate of flow is regulated by a long

(1 m.) capillary tube. If the Mariotte bottle is placed several feet above the cell, changes in level due to temperature or pressure fluctuation will be small.

Culture Media Connection.—The culture media is prepared and autoclaved in a large (3 liter) bottle to which is attached a rubber tube with a Number 21 hypodermic needle at the end. A rubber tube is also attached to the culture media intake of the cell, the end of this tube tightly sealed with a glass rod, and immersed in a beaker of alcohol or 1:3000 quaternal.[2] The needle on the end of the tube from the media bottle is thrust into the tube leading to the cell while under the solution and the connection left in the solution. No contamination has occurred since this method was adopted, although over one hundred such connections have been made.

Assembly of the Apparatus

Electric Wiring.—The general arrangement is shown in Fig. 2. The wires in the Klett photoelectric colorimeter, connecting the photocell to the galvanometer, are cut and lead to the Leeds and Northrup galvanometer instead of to the one in the colorimeter. A damping resistance of about 1000 ohms is put in parallel to the galvanometer.

A 200 watt lamp is put in series with the microscope lamp and a 100 ohm variable rheostat in series with the lamp in the Klett colorimeter. The sensitivity of the apparatus is controlled by varying the intensity of the colorimeter lamp by means of this rheostat.

Two 50 watt lamp bulbs are put in series with the 24 volt Allied Control Co. relay.

Media Control Relay.—The Allied Control Co. relay has the spring passing through the coil. The spring is removed and replaced by a steel rod long enough to press against the armature and project an inch or more from the coil at the other end. This end of the rod is then attached to a sliding clamp so that, when the relay is closed, the clamp will pinch the rubber tube containing the culture medium.

Air Flow.—Air is bubbled through water and then through a U.F. grade, 50 mm. pyrex sintered glass filter.

Assembly.—All glass to rubber connections are coated with rubber cement before the connection is made.

The cell stopper must be wired firmly in place. All connections are coated on the outside with rubber cement after the apparatus has been autoclaved.

The cell and air filter, intake tube, and outlet tube including the graduate are autoclaved together.

The entire apparatus is then assembled as in Fig. 2, the culture media bottle attached to the cell intake tube by means of the hypodermic needle, the cell

[2] Quaternal DG, product of the Columbia Organic Chemicals Company, is a cationic germicide (10 per cent alkyl (C-8 to C-18) dimethyl benzyl ammonium chloride).

filled with culture media, and the air flow started. The cell is inoculated by injecting a suspension of the organism through the wall of the air intake tube with a hypodermic syringe.

Adjusting the Galvanometer and Photorelay.—The galvanometer is placed about 40 cm. from the photorelay and the microscope lamp adjusted so that the image of the lamp filament is reflected from the galvanometer mirror and focused on the photoelectric relay slit. The galvanometer is disconnected from the photoelectric colorimeter and moved until it just fails to cause the photoelectric relay to open the relay controlling the flow of culture media. The galvanometer is now connected to the photoelectric colorimeter with the culture cell filled with water in place, and the scale of this instrument set at about 10. The photoelectric colorimeter is now adjusted until the galvanometer just fails to operate the relay as before. The scale of the photoelectric colorimeter is then turned to the reading which corresponds to the turbidity at which it is desired to hold the culture. This may be determined by filling the cell with a suspension of the desired concentration, and determining the reading on the scale which just causes the photoelectric relay to operate. If a stirrer is used in the cell, this must be operating while the colorimeter is calibrated.

500 ml. of 1:1000 quaternal are placed in the 3 liter overflow bottle and the outflow tube from the graduate inserted.[3] The air flow is regulated so that a steady stream of bubbles comes from the capillary tube. The apparatus should now regulate the flow of media so as to keep the turbidity of the suspension constant.

In case a stirrer is used, the sensitivity of the galvanometer must be adjusted so that it is not affected by the motion of the stirrer. This is best accomplished by reducing the intensity of the light in the photoelectric colorimeter, until the galvanometer no longer swings as the stirrer moves. The galvanometer with full light intensity is unnecessarily sensitive. In case a kymograph record of the flow is made, the sensitivity is adjusted in the same way so as to give a record with flow-time intervals of 1 or more minutes. If the galvanometer is too sensitive, the time during which the culture medium flows will be too short; if not sufficiently sensitive, there will be too great a change in the concentration of bacteria.

Accuracy of the Turbidity Control.—The apparatus assembled as in Fig. 2, with decreased intensity of the microscope and photoelectric colorimeter lamps, and a 20 mm. cell, maintains the density within ± 5 per cent in the range of 20 to 200 scale divisions. If colorless culture medium is used, the sensitivity may be increased by removing half or all the red filter from the photoelectric colorimeter.

Growth Rate in Cell.—If the cell is inoculated with a few organisms, they

[3] Before a sample is collected, the tube is removed from the quaternal solution and rinsed thoroughly in alcohol.

will increase logarithmically until the concentration reaches the point at which the cell has been set to operate (provided this is within the log growth range). During this time the cells increase in accordance with the usual logarithmic equation: $K = \frac{2.3}{t} \log \frac{B}{B_0}$ in which K is the growth constant, B the total number of cells at time t, and B_0 the total number of cells at 0 time.

As soon as the automatic cell begins to operate, the number of organisms in the cell remains constant since the dilution rate exactly equals the growth rate.

The rate of growth of the organism in the cell is now: $\frac{dB}{dt} = KB_c$ in which B_c is the value of B in the cell at which the cell is set to operate. On integration this becomes

$$K = \frac{B - B_c}{B_c(t - t_c)} \tag{1}$$

in which t_c = time when $B = B_c$; *i.e.*, when the automatic operation began. Under these conditions, $B/V = B_c/V_c$ by automatic adjustment, or $B = B_cV/V_c$. Substituting the value of B in (1)

$$K = \frac{V - V_c}{V_c(t - t_c)} \tag{2}$$

V_c = volume of cell
V = total volume of media which entered cell
$V - V_c$ = volume of media which overflows from cell.

It is evident that this relation holds only if the concentration of organisms in the cell is the same as that in the overflow. If, therefore, any appreciable fraction of the organisms in the culture tube is attached to the wall or settles to the bottom, the growth rate as measured by the flow of media will be too high. It is necessary, therefore, to keep the entire surface of the cell free from adhering organisms, and not merely that part of the wall through which the light beam passes.

If the values for 2.3 log B/B_0 and $\frac{V - V_c}{V_c}$ are plotted (on the same scale) against time, two straight lines of the same slope result. The value of 2.3 log B/B_0 will increase with time until $B = B_c$ after which it remains constant. The value of $\frac{V - V_c}{V_c}$, on the other hand, is zero up to this point and then it increases at the same time rate as 2.3 log B/B_0 before the value of B_c was reached.

Curves plotted in this way are shown in Fig. 3.

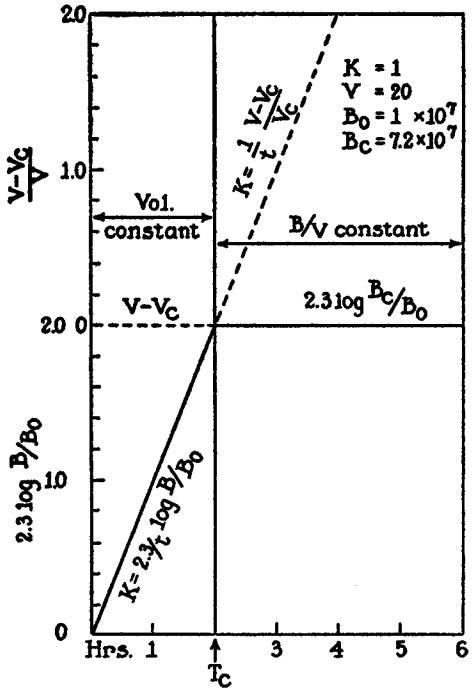

FIG. 3. Growth rate of cells in steady state apparatus at constant volume (1 to 2 hours) by turbidity, and at constant cell concentration by volume of culture media.

Growth Rate of Mixed Cultures.—Assume two organisms W and M, M growing at the rate K_M and W at the rate K_W.

Then
$$M = M_0 e^{K_M t}$$
$$W = W_0 e^{K_W t}$$

or
$$\frac{M}{W} = \frac{M_0}{W_0} e^{t(K_M - K_W)} \tag{3}$$

Evidently, if $K_M < K_W$, the ratio of M/W will decrease with time,[4] and under these conditions in the steady state cell, the M organisms will soon be washed out entirely.[5] If $K_M = K_W$, the ratio remains constant, while if $K_M > K_W$,

[4] It is not necessary to consider changes in volume due to addition of more culture media since the ratio of M/W cannot be changed by increasing the volume of the suspension by the addition of more culture media.

[5] Mathematically M/W approaches 0 as t approaches infinity but since less than one bacterial cell cannot exist, the number of M cells in the apparatus will be zero, as soon as the last cell is washed out (Hinshelwood (1946)).

W will soon disappear. The time required for the ratio of M/W to reach any required values follows from 3, by solving for t.

$$t = \frac{2.3\left(\log \frac{M}{W} - \log \frac{M_0}{W_0}\right)}{K_M - K_W} \tag{4}$$

The time required to reach any given ratio of M/W from a given value of M_0/W_0 is therefore inversely proportional to the difference between the two growth constants and directly proportional to the difference in the logs of the initial and final ratios.

TABLE I

Time for Ratio of M/W Growing at Rate K_W = 1 hour and K_M = 2 hours or 3 hours to reach Different Values

$$t = \frac{2.3\left(\log \frac{M}{W} - \log \frac{M_0}{W_0}\right)}{K_M - K_W}$$

$W_0 = 1 \times 10^7 \qquad M_0 = 1$

M/W	Time to reach corresponding value M/W if	
	$K_M = 2$	$K_M = 3$
10^{-7}	0 hrs.	0 hrs.
10^{-5}	4.6	2.3
10^{-3}	9.2	4.6
10^{-1}	13.8	6.9
1	16.1	8.2
10	18.4	9.2

The time required to reach various values of M/W starting from $M_0/W_0 = 1/10^7$ and with $K_W = 1$ and $K_M = 2$ or 3 is shown in Table I. If $K_M - K_W = 1$, the ratio increases 100 times in 4.6 hours, while if the difference is 2, the ratio increases 100 times in 2.3 hours.

Growth Rates of Mixed Cultures as Measured by the Dilution Rate.—If the cell is operating with organism W in the steady state, the dilution rate $\frac{V - V_c}{V_c\,(t - t_c)}$ will be equal to the growth rate $\frac{2.3}{t} \log \frac{W}{W_0}$. If now a second organism M appears in the cell and grows at a rate $K_M (K_M > K_W)$, the ratio M/W will increase as described above. Under the steady state conditions, $M + W = B_c$ and the growth rate of the culture as a whole will be

$$\frac{dM}{dt} + \frac{dW}{dt} = K_M M + K_W W$$

At first, $K_M M$ will be very small compared to $K_W W$ and the dilution rate will be equal to K_W; as soon as $K_M M$ reaches about 10 per cent of the value

of $K_W W$, the rate of dilution will increase and when $K_M M$ is large compared to $K_W W$ the rate will become constant again and equal to K_M.

The value of K_W is the slow dilution rate and K_M is the fast dilution rate. The time required for the change to occur, if the change actually is due to the overgrowth of W by M, is the time required for the value of M/W to change from about 1/10 to 10, since the apparatus is not capable of distinguishing between growth rates which differ by <10 per cent.

This is the time required to change the ratio of M/W one hundred times, with the values of K_W and K_M found from the dilution rate. This time may be

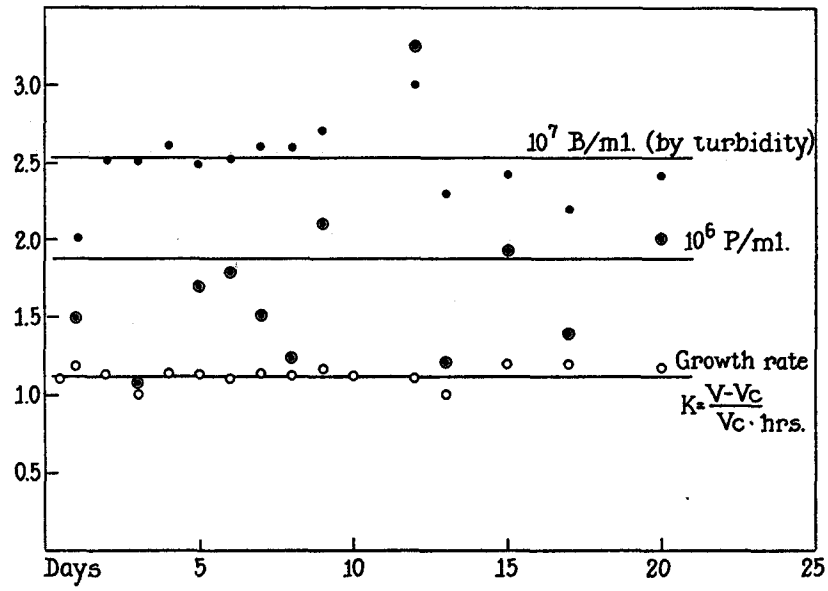

FIG. 4. Growth rate, cell concentration, and phage concentration of *B. megaterium* 899a growing in ammonium sulfate medium at 35°C. in steady state apparatus.

calculated by means of Equation 4 and compared with the time required to change from one dilution rate to the second, in an experiment. If the time agrees with that calculated from the two separate rates, then there is little doubt that the observed change in rate was due to the overgrowth of one organism with another. If the times do not agree, the conclusion is not so certain since it is assumed in the above calculations, that the growth of the two organisms is entirely independent. This may not be true.

EXPERIMENTAL RESULTS

The results of a preliminary experiment in which lysogenic *megaterium* was grown in the steady state in a minimal medium containing ammonium sulfate, glucose, phosphate, magnesium sulfate, and iron are shown in Fig. 4.

The growth rate, bacteria/ml., and phage/ml. all remained constant (within the error of the analytical methods used) for a period of 25 days.

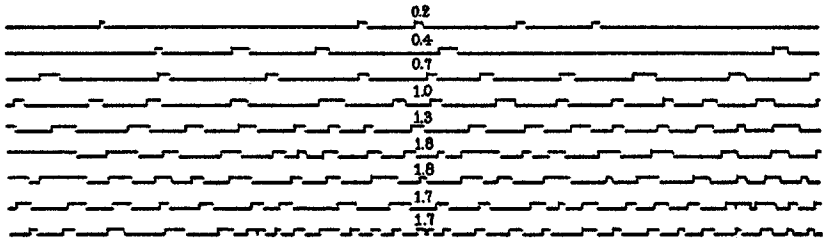

FIG. 5. Kymograph record of growth rate of lysogenic *B. megaterium* grown on peptone agar and suspended in ammonium sulfate culture medium. Cell density, 5×10^7/ml.; 35°. Flow rate of culture medium, 48 ml./hour; cell volume, 12 ml.; washout rate, 4/hour. Growth rate therefore $= \frac{4 \times (\text{time culture medium flows})}{\text{Elapsed time}}$.

The record has been cut in half so that only every alternate hour is shown. The record starts at the time the cells were added. The first line covers the period of 0 to 1 hour, the second line, 2 to 3 hours, etc. The kymograph traveled at the rate of 10 inches/hour.

The numbers written above the lines represent the growth rate for that 1 hour time interval.

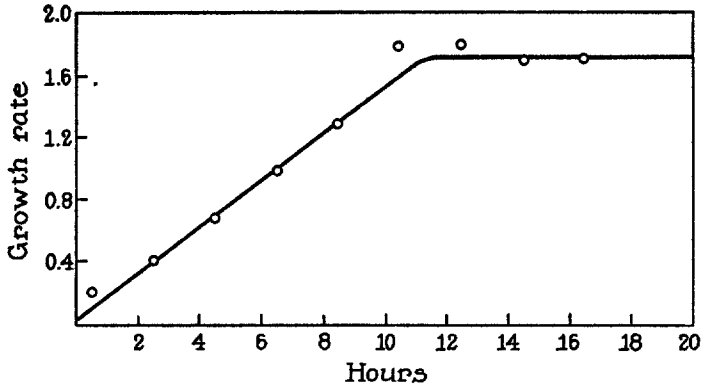

FIG. 6. Growth rate of lysogenic *B. megaterium* while becoming adapted to ammonium sulfate culture medium.

A kymograph record of the change in growth rate when a culture of lysogenic *B. megaterium* is transferred from peptone to ammonium sulfate medium is shown in Fig. 5. The figure covers a time interval of 17 hours. (Every alternate hour has been omitted to save space.)

The growth rates taken from this record are plotted against time in Fig. 6. (This strain of *B. megaterium* had previously been adapted to growth in ammonium sulfate medium and then transferred for 2 months on peptone agar.) The growth rate now increases to 1.7 instead of stopping at 1.2 as in Fig. 4. The cell density in this experiment was 5×10^7 at the beginning so that the conditions do not conform to those discussed in connection with Fig. 3.

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