CULTURE CONDITIONS AND THE DEVELOPMENT OF THE PHOTOSYNTHETIC MECHANISM

II. An Apparatus for the Continuous Culture of Chlorella

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It has been a common experience that the photosynthetic behavior of algal cells may vary within rather wide limits depending upon previous conditions of culture. However, no attempt has been made to relate culture conditions to the subsequent type of behavior of the cells. A very great handicap to any such investigation is the internal variation which occurs with time within any one culture. In the first paper of this series (Myers, 1944) it has been pointed out that as a culture of Chlorella matures, the H^+ and NO_3^- ion concentrations of the medium, the conditions of carbon dioxide supply, and the effective light intensity and quality show marked variations. There are probably other variables as well; *e.g.*, the "inhibitors" of Pratt (1942). Consideration of the nature of these internal variables shows that all of them are functions of the population.

It has been a common practice in studies on photosynthesis to use algal cells from cultures harvested at some fixed period after inoculation. By using cells taken always at the same point on the growth curve a fair degree of reproducibility is to be expected, since all conditions which depend upon the population will be reproduced with some uniformity. Similar results may be obtained by periodically harvesting a part of the culture suspension and replacing it with fresh medium. Techniques of this kind have been reported by Felton and Dougherty (1924) for the culture of pneumococcus and by Ketchum and Redfield (1938) for the culture of marine diatoms. It would seem that if a culture could be continuously diluted so as to be maintained always at one point on its growth curve, then the effects of changing internal conditions might be eliminated entirely. This would

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afford at once (1) a source of experimental material of high uniformity and/or (2) a means of stablilizing internal variables so that relation of culture conditions to photosynthetic behavior might be systematically explored.

Following these considerations there has been developed an apparatus which maintains an algal[1] culture at a given density of population by automatically diluting the growing culture with fresh medium. This paper describes the apparatus and presents data illustrating its operation.

Description of the Apparatus

Fig. 1 is a diagrammatic, cross-sectional view of the apparatus. A number of parts have been distorted in position in order to place all of them in the same plane. The culture chamber[2] is made of three concentric glass tubes, affording an outer annulus, J, for circulation of constant temperature water and an inner annulus, A, in which the algal suspension is contained. Outside dimensions of the chamber are approximately 6.0 cm. diameter by 66.0 cm. long; the only critical dimension is the thickness of annulus A, which in all chambers used is 5 to 6 mm.

Any desired gas mixture is provided through the bubbler tube, B (shown in part) which simultaneously provides carbon dioxide and agitates the algal suspension. (The gas mixture is first humidified by bubbling through a column of liquid medium held at the same temperature as the chamber.)

Because of the requirement of a rather rapid air flow (2 to 6 cc. per sec.) the use of compressed gas mixtures has proved uneconomical. It has been necessary to devise a means of obtaining constant air-carbon dioxide mixtures. Outside air is delivered *via* a diaphragm pump at a constant pressure obtained by allowing the excess air to escape against a head of 8 feet of water. Carbon dioxide is delivered from a cylinder at about 4 pounds pressure by suitable mechanical reducing valves. The two gases pass through orifices so chosen that the resulting gas mixture has a composition of about 4.4 per cent carbon dioxide. The actual rates of flow of the two gases are indicated by calibrated Venturi flow gauges and occasional Haldane analyses have been made as over-all checks on the operation. The gas mixture is delivered at constant pressure by allowing the excess gas to escape against a head of about 6 feet of water.

Samples of the suspension are harvested as needed by opening a screw clamp and allowing the suspension to run out through the *withdrawal tube.* Fresh medium is added by activation of a solenoid valve, SV, which opens a rubber tube and allows the medium to run from a large aspirator bottle into the annulus A of the culture chamber.

By suitable precautions pure culture conditions can be maintained. The

[1] In principle it is applicable to other types of microorganisms as well.

[2] The first chamber was constructed by one of us (L. B. C.). Additional chambers have been obtained from E. Machlett and Son, New York City.

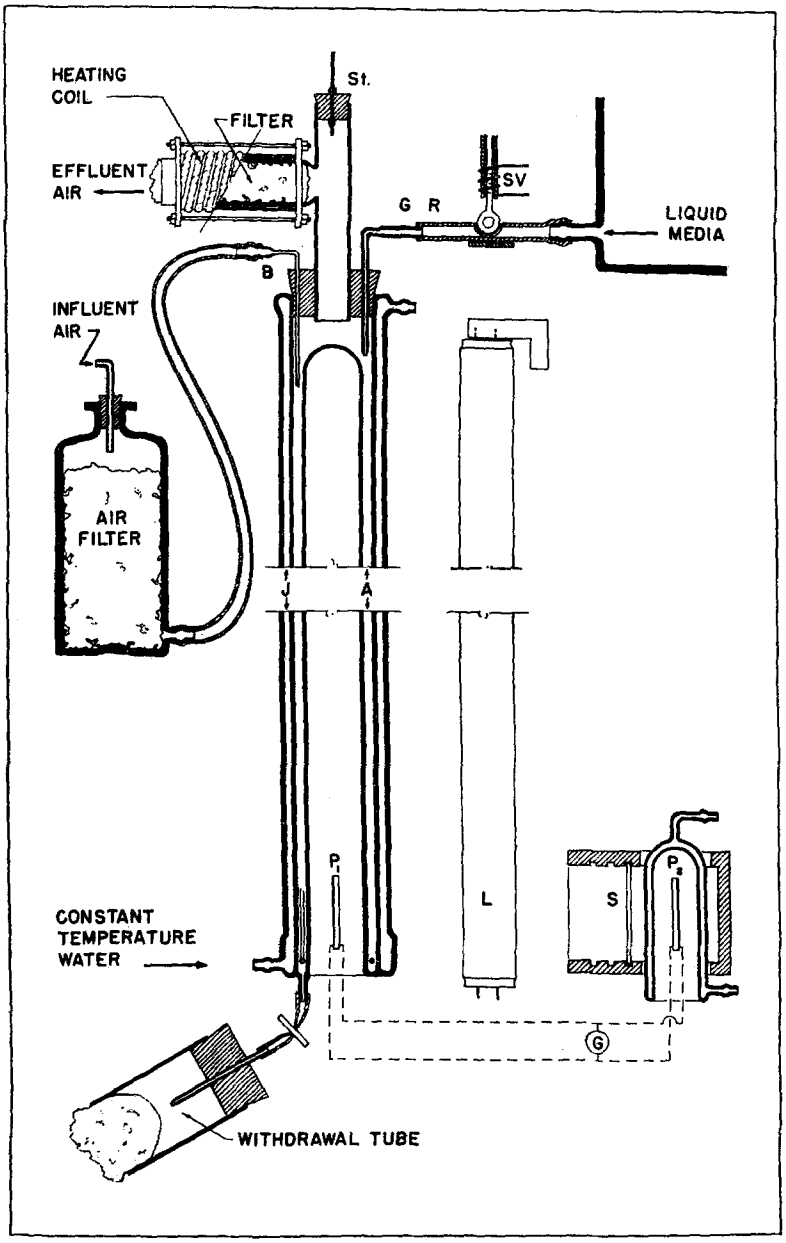

FIG. 1

withdrawal tube is protected by a glass skirt and cotton plug. After collection of a sample in a sterile flask a fresh cotton plug, previously autoclaved

in a large test tube, is inserted in the glass skirt. Influent air is filtered through a 500 ml. aspirator bottle packed with cotton. Effluent air passes out through a glass tube packed with cotton. The cotton filters must be kept warm to insure against condensation of moisture. A heating coil around the effluent filter is shown partly cut away. A heater (not shown) for the influent filter is provided by a simple tin-can and light bulb arrangement. Before setting up, the assembly consisting of the chamber, withdrawal tube, influent and effluent filters is sterilized by autoclaving. During this procedure a cotton plug is used in place of the rubber stopper, *St*, and the glass inlet tube for the medium is not connected to the aspirator bottle but is protected by a vial and cotton wrapping at *G*. The aspirator bottle is autoclaved separately, the end of the rubber tube, *R*, being protected by a vial and cotton wrapping.

After autoclaving, the chamber is placed in position, the clamp to the withdrawal tube closed, and a current of air passed through. An inoculating suspension of cells from an agar slant is introduced through the uppermost opening and the cotton plug replaced by a sterile rubber stopper, *St*, fitted with a glass rod for ease in handling. The rubber tube, *R*, from the aspirator bottle is attached to the glass tube, *G*, with aseptic precautions.

The whole procedure of sterilization is indeed cumbersome but it is required only at infrequent intervals of a month or more. New aspirator bottles of medium can be inserted by carefully removing the rubber tube of the old bottle, flaming the glass tube, *G*, and slipping on the sterile rubber connection of a new bottle. In operation a sample (about 1 ml.) is withdrawn daily into a flask of glucose-peptone broth as a check against bacterial or mold contamination. Many cultures have been run for a month or more without contamination.

Illumination is provided by four tubular fluorescent or tungsten filament lumiline bulbs spaced symmetrically around the chamber. (Only one lamp is shown in the diagram.) Short period variations in light intensity are minimized by use of a voltage stabilizer which delivers 115 ± 1.0 volts to the lamps. Light intensity may be varied by changing the distance of the lamps from the chamber or by surrounding the lamps with sleeves made of wire screen.

Constant density of population is maintained by adding fresh medium as the culture grows. The solenoid valve, *SV*, is operated by a photometric device. Two photocells,[3] P_1 and P_2, are introduced into the current-balancing circuit recommended by Wood (1934) by connecting terminals of opposite polarity. The meter,[4] *G*, is a spotlight galvanometer which has a No. 922 RCA phototube mounted in place of the usual glass scale. The phototube

[3] General Electric rectangular, barrier-type photocells $\frac{29}{32}$ inch $\times$ $1\frac{45}{64}$ inch.

[4] Rubicon No. 3402; period 3.7 seconds; R = 335 ohms; CDRX = 3900 ohms.

feeds into an electronic relay employing a No. 2051 vacuum tube. The arrangement is such that an increase in light on the phototube energizes the relay. The relay operates the solenoid valve, SV.

The two photocells look at the same light bulb. P_1, the inside photocell, is screened by the annulus of algal suspension. P_2 is screened as desired by insertion of one or more uniformly exposed lantern slides, S. The outside photocell, P_2, is jacketed by a glass condenser through which is circulated the same constant temperature water used for the chamber; photocell and jacket are partially enclosed within a box arranged so that light enters only from the direction of the one light source. When low population densities (1 to 2 c.mm. cells per cc.) are desired, the inside photocell, P_1, is also screened with a piece of white paper so that its maximum output is about 50 microamperes. This decreases the sensitivity but greatly improves the stability of the photocell circuit.

In order to minimize ambient light effects on the photocells, the apparatus is partially enclosed in a box 30 x 30 x 36 inches high, painted white inside. The chamber just barely projects through a hole in the top of the box; and the air filters, solenoid valve, and aspirator bottle of medium are all mounted on top. The front of the box is a sheet of plywood with holes provided for ventilation and is easily removed when a sample is to be withdrawn.

In practice the original inoculum is allowed to grow until a desired population density is reached and the photocells are then balanced by inserting screens in front of the outside photocell. Thereafter a sample is withdrawn at a fixed time each day. The air flow is temporarily stopped and a small sample ($\sim$1 ml.) collected in a flask of sterile broth as a check against contamination. The rest of the culture is then withdrawn down to a mark placed a centimeter or so above the top of the inside photocell. This leaves an inoculum (50 to 100 ml. of suspension) for the next day's growth. As the algae multiply illumination on the inside photocell, P_1, is reduced. An off-balance current flows through the primary photocell circuit causing the galvanometer light spot to move across the phototube. The electronic relay then actuates the solenoid valve and allows new culture medium to flow in, diluting the algae and increasing the illumination on P_1 until a zero current again obtains through the galvanometer. In this way the algal suspension "grows" up the chamber. The design of the apparatus is such that conditions of illumination are independent of the total amount of the culture.

If samples are harvested from a culture at equal intervals, then the amount of the sample is an index of the rate of growth. It is also possible to harvest samples at any other desired time, provided that the culture is not allowed to run over. Daily samples of 200 to 300 ml. containing 200 to 600 c.mm. of algal cells are easily obtained.

Operation of the Apparatus

Satisfactory operation of the entire apparatus depends upon the stability and sensitivity of the primary photocell circuit. By maintaining the two photocells at constant temperature and keeping their illumination at a low level good stability may be obtained over a period of weeks. The over-all sensitivity is such that the solenoid never allows more than 1 per cent of the volume of the culture to flow in during one relay cycle.

One unit of the apparatus has been in operation for over a year and a second unit for about 6 months. Numerous difficulties have arisen. The present procedure of maintaining pure culture conditions is the result of a gradual improvement in technique. Development of an apparatus for producing a reliable gas mixture gave considerable trouble. In general the mechanical problem has been one of maintaining stability over long periods of time.

Several modifications in the preparation of culture media have been necessary. Originally we used a Knop's solution from which calcium was omitted (0.010 M $MgSO_4$, 0.012 M KNO_3, 0.009 M KH_2PO_4, 1.0×10^{-5} M ferric ion). Micro elements were provided by the addition of 1.0 ml. per liter each of the A5 and B6 solutions of Arnon (1938). These provide in the final medium 0.5 parts per million B, 0.5 ppm. Mn, 0.05 ppm. Zn, 0.02 ppm. Cu, and 0.01 ppm. each of Mo, V, Cr, Ni, Co, W, Ti. In this medium contained in a pyrex aspirator bottle there would develop in time a fine white precipitate. Concurrently, a culture provided with the medium would show a decreased rate of growth and lowered capacity for photosynthesis. Subsequently, we have purified the major salts by the adsorption procedure of Stout and Arnon (1939), increased the iron concentration to 13.3×10^{-5} M, and added sodium citrate to give 0.00056 M citrate as used by Hopkins and Wann (1927). With these modifications the culture media will remain clear indefinitely and rate of growth and capacity for photosynthesis are as great as obtained by any other method of preparation of medium.

It has also been found that after insertion of a fresh bottle of medium the rate of growth and capacity for photosynthesis may be lowered for a day or so. It has been possible to demonstrate that the Knop's solution in contact with the rubber tubing leaches some toxic materials out of the rubber during autoclaving. This difficulty has been minimized by (1) using rubber tubing previously boiled in dilute alkali and leached out in distilled water and (2) running several hundred milliliters of medium from a fresh bottle out into a sterile flask before attaching the rubber tubing to the glass inlet tube of the apparatus.

Presented in Table I are typical data obtained on a culture of *Chlorella pyrenoidosa* (Emerson's strain) over a period of 3 weeks. The scanty data on the first eleven samples are omitted since rate of growth and capacity for photosynthesis were apparently limited by iron concentration. After the ninth sample a new bottle of medium containing 9.0×10^{-5} M iron was inserted.

From the twelfth sample on all data are presented, though in several cases data are believed invalid due to serious failure of the temperature control

TABLE I

Typical Data Obtained on Chlorella pyrenoidosa Grown in the Continuous Culture Apparatus

Temperature 25.05°C. Light intensity ~160 foot-candles as provided by four 20 watt "Daylight" fluorescent bulbs mounted 27 cm. from the chamber.

Sample No.	Sample size	Population		Maximum apparent rate of photosynthesis	
		Cells	Cell volume	Buffer 9	Buffer 11
	ml.	*10^9/c.mm.*	*c.mm./cc.*	*c.mm. O_2/min./c.mm. cells*	*c.mm. O_2/min./c.mm. cells*
12	216	—	1.33	—	—
13	—	0.0118	1.33	0.65	0.70
14	—	0.0120	1.33	0.62	0.66
15	—	0.0118	1.33	0.62	0.66
16	212	0.0121	1.33	0.64	0.68
17	216	—	—	—	—
(Added additional A_5 and B_6 to medium to give 1.5 ml./liter of each)					
18	224	—	1.33	0.63	0.69
(Added iron to give total concentration of 19. $\times 10^{-5}$ M)					
19*	211	—	—	—	—
20	226	0.0169	1.39	0.64	0.70
21	226	0.0168	1.33	0.66	0.71
(Inserted new bottle of culture medium)					
22*	123	—	—	—	—
23*	148	—	—	—	—
24	208	0.0141	1.33	0.64	0.68
25	198	—	—	—	—
26	213	—	—	—	—
27	200	—	1.31	0.63	0.67
28	205	0.0150	1.36	0.62	0.68
29	220	—	1.33	—	—
30	198	0.0180	1.36	—	—
31	202	—	1.39	0.62	0.68
32	210	—	1.39	—	—
33	198	0.0200	1.36	0.63	0.71
(Culture discontinued)					
Mean	210.5	—	1.346	0.633	0.687
Standard deviation	9.5	—	0.025	0.0125	0.017
Maximum variation	28.0	—	0.08	0.04	0.05

* Temperature failure; data for this sample not valid.

mechanism. Mean values, maximum variation, and the standard deviation are given at the foot of each column.

Samples were removed from the chamber at 24 hour intervals and 79 ml. of suspension left for inoculum each time. The mean sample size (omitting

samples 19, 22, and 23) of 210.5 ml. indicates a multiplication rate of 3.66 per 24 hours under the conditions employed. There is a slight downward trend in the data on sample size, probably reflecting a decay in light output of the lamps with time.

Densities of population of the samples were determined by means of hemocytometer counts (giving cells per c.mm.) and by centrifuging to give packed cell volumes (c.mm. cells per cc.). Hemocytometer counts were quite variable, probably reflecting to some extent the variations in culture media used. Each count represents the mean value from a sample of about 1000 cells so that the experimental error is about 5 per cent. Of all the data, these show the greatest variation.

Packed cell volumes were determined by centrifuging 15.0 ml. of suspension in a 15 ml. centrifuge tube. The packed cells were resuspended in a little of the same medium and transferred to a Bauer and Schenk tube of 3 ml. capacity and graduated in 0.004 cc. divisions. 1.33 c.mm of cells per cc. corresponds to 5.0 divisions which can be estimated to about 0.1 division. Constant values for the packed cell volume are obtained between 15 and 25 minutes centrifuging at a relative centrifugal force of 2150. It will be seen that the packed cell volume (c.mm. cells per cc.) is quite uniform with a maximum variation of about 6 per cent and a standard deviation of about 2 per cent.

Capacity for photosynthesis of the cells was determined by the Warburg technique, using the Warburg buffers 9 and 11 to provide approximately saturating concentration of carbon dioxide, a temperature of 25 $\pm$ 0.05°C., and a saturating light intensity. The light sources used were a grid of white fluorescent tubing ($\sim$1000 foot-candles) or two 60 watt lumiline bulbs ($\sim$450 foot-candles) immersed in the water bath just below the vessels. No differences in photosynthesis rates produced by the two sources can be detected when they are used at full intensity. For the manometric measurements a 5.0 ml. aliquot of the sample was pipetted into a 15 ml. graduated centrifuge tube. The cells were centrifuged out, suspended in distilled water, centrifuged out, suspended in Warburg buffer 9 (0.015 M K_2CO_3 + 0.085 M $KHCO_3$), centrifuged out, and suspended in buffer 9 to give 15.0 ml. 5 ml. of this suspension were pipetted into each of two rectangular Warburg vessels of about 10 ml. volume. By a similar procedure 5.0 ml. of a suspension of cells in buffer 11 (0.005 M K_2CO_3 + 0.095 M $KHCO_3$) were delivered to each of two other vessels. Rate of photosynthesis was determined graphically by plotting 5 minute readings taken over a period of about an hour. The rate in terms of Δmm. pressure per minute was multiplied by the vessel constant and divided by the volume of cells used to obtain c.mm O_2 per min. per c.mm. cells. Duplicate rates generally agree within a few per cent; the averages of the duplicates for each buffer mixture are the values listed in the fifth and sixth columns

of Table I. The data represent only the *apparent* rates of photosynthesis uncorrected for respiration. Respiratory rates were not measured.

Values of the apparent rate of photosynthesis per cubic millimeter of cells (fifth and sixth columns) show, for each carbon dioxide concentration, a maximum variation of 6 to 7 per cent and a standard deviation of about 2 per cent. The experimental errors in these values are contributed both by the errors of the manometric measurement and the error in determination of cell volume.

Additional data (not shown in the table) were obtained by occasional measurement of the pH of the suspension immediately after harvesting. Measurements with a Coleman glass electrode yielded pH values lying between 6.05 and 6.10. The fresh Knop's solution used had a pH of 5.0.

DISCUSSION

The data of Table I on maximum apparent rate of photosynthesis describe one arbitrarily chosen physiological characteristic of the cells. It is our experience that for a culture allowed to mature along the growth curve, the maximum rate of photosynthesis may decrease to a value as low as 0.05 c.mm. O_2 per min. per c.mm. of cells. Similar results were obtained by Sargent (1940, Table IV). Sargent's data (Table III) also illustrate the considerable variation in maximum rate of photosynthesis to be expected for cells of different cultures harvested at approximately the same age. It is evident that the maximum rate of photosynthesis is a characteristic which may vary widely between different batches of algal cells cultured by the usual procedures. The data (columns 5 and 6) of Table I illustrate the uniformity in maximum rate of photosynthesis to be expected of cells obtained from the continuous culture apparatus. Rate of growth also shows fair uniformity. Other physiological characteristics have not been examined. However, it is reasonable to expect that cells will be equally uniform in other characteristics since they are grown under highly uniform conditions.

In only one respect has the apparatus fallen short of theoretical expectations. A basic assumption is that the sample removed and the inoculum left in the chamber are identical as to concentrations of all components (*i.e.*, cells, inorganic ions, metabolites). This condition is not entirely attained. Because of the bubbling action, surface-active materials tend to accumulate at the upper liquid-gas interface and therefore do not distribute equally between the sample and inoculum. Foaming occurs, though a permanent foam is not formed until after several weeks of operation. However, no detrimental effects of the surface-active materials have yet been observed.

The data of Table I describe the operation of the apparatus as applied to one of the two purposes for which it was designed; *i.e.*, the production of uniform experimental material day after day. Application of the apparatus in studying

the relation of culture conditions to photosynthetic behavior will be considered in later papers of this series.

SUMMARY

1. An apparatus has been developed which maintains a constant density of population of *Chlorella* by automatic dilution of the growing culture with fresh medium.

2. Cells harvested from the apparatus in daily samples are highly uniform in rate of growth and rate of photosynthesis measured under arbitrarily chosen conditions.

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