CULTURE CONDITIONS AND THE DEVELOPMENT OF THE PHOTOSYNTHETIC MECHANISM

III. Influence of Light Intensity on Cellular Characteristics of Chlorella*

By JACK MYERS
With the Technical Assistance of James Johnston and Helen Davis
(*From the Department of Zoology and Physiology, University of Texas, Austin*)

(Received for publication, March 12, 1946)

Experimental material for studies on the photosynthetic process has been grown under a wide range of conditions. In the past little attention has been paid to differences in culture conditions since the photosynthetic mechanism was regarded as rigid and clearly defined. More recent work suggests that the various parts of the photosynthetic mechanism are distinct and separable. A possibility which now assumes added significance is that a range of experimental material with different photosynthetic characteristics may be obtained by control of previous conditions of growth. This would be a great aid to biochemical analysis of the mechanism.

A serious problem in any comparison of cells grown under different conditions lies in the selection of a reliable index of protoplasmic material to which metabolic rates may be referred. In previous studies on photosynthesis attempts express "absolute" rates have been limited in scope. Common procedure has been to compare the behavior of replicate samples of a single batch of cells. In metabolic studies on the algae and other microorganisms rates of metabolic processes have been expressed variously in terms of unit cell volume, unit dry weight, unit cell number, unit cell nitrogen, or unit chlorophyll. The present paper presents data describing the relationships between these different units of protoplasmic material for cells of *Chlorella pyrenoidosa* cultured under different conditions of light intensity. This study of effects of a single variable in culture conditions depends upon the use of an apparatus for the continuous culture of microorganisms described by Myers and Clark (1944).

EXPERIMENTAL

Chlorella pyrenoidosa (Emerson's strain) was grown in a modified Knop's solution in three units of the continuous-culture apparatus previously described. The Knop's solution contained 0.010 M $MgSO_4$, 0.012 M KNO_3, 0.009 M KH_2PO_4, 13.3 $\times$ 10^{-5} M $Fe_2(SO_4)_3$, 56.0 $\times$ 10^{-5} M sodium citrate, and 1.0 ml./liter each of the A_5 and B_6 solutions of Arnon (1938) which provide 0.5 parts per million B, 0.5 ppm. Mn, 0.05 ppm.

* Supported by a grant from the University of Texas Research Institute.

Zn, 0.02 ppm. Cu, and 0.01 ppm. each of Mo, V, Cr, Ni, Co, W, and Ti. The medium was prepared as described in a previous paper (1944) and adjusted to a pH of 5.0.

Constant temperature in the chambers was provided by rapid circulation of water from baths maintained at 25.00° and 25.05° ± 0.05°C. Illumination was provided by four pair of 40 watt clear lumiline tungsten filament bulbs mounted end-to-end on four vertical holders spaced symmetrically about each chamber. Lamps rated at 120 volts were operated at 115 volts ± 1 volt as secured through a voltage stabilizer. Light intensity was adjusted by variation of the distance of the lamps from the chamber and by use of 16 mesh galvanized iron screens placed around the chamber or lamps or both. One layer of screen has a transmission of 66 per cent; two layers, of 43 per cent. To obtain the lowest intensities used it was necessary to substitute three 10 watt, 120 volt, S14 bulbs mounted on each of the four vertical holders. By placing these 25 cm. away a uniform illumination on the chamber could be secured. Measurements of light intensity were made with a Weston No. 603 foot-candle meter which has a photronic cell with quartz window.[1]

Sterile precautions of culture were maintained throughout and checked by daily samples of ~1 ml. withdrawn into sterile flasks of glucose-peptone broth. Upon any evidence of contamination cultures were discontinued.

In the continuous-culture apparatus a photometric device dilutes the culture suspension at such a rate that the density of population is maintained approximately constant. In the present study the population densities were arbitrarily adjusted at about 0.6 to 0.7 c.mm. cells per ml. In the chamber the algal suspension is contained in a 5 mm. annulus between two glass tubes. A rough check on the transmissions of several suspensions was made by illuminating with a narrow focused beam of light in a 5 mm. absorption cell placed just in front of a large photronic cell. This arrangement gives an approximate summation of the directly transmitted and forward scattered light. A suspension of maximum chlorophyll content (grown at 10 f.-c.) gave 73 per cent "transmission" as compared with the cell filled with water; a suspension of minimum chlorophyll content (grown at 360 f.-c.) gave 89 per cent "transmission." In the chamber an algal cell which is momentarily on the inside of the annulus gets

[1] Our experience with the calibration of the instrument should be cited. After a period of use of about 1 year the foot-candle meter was checked against a Bureau of Standards lamp operated through a carefully calibrated ammeter and found to read about 35 per cent high. It was returned to the manufacturer who corroborated the high calibration and inserted a new photocell unit and calibration. On return (and at the beginning of the experiments here reported) the instrument read about 4 per cent high against two different Bureau of Standards lamps; 6 months later (at the end of these experiments) it read 10 per cent low. Light intensity values reported here are meter readings without corrections which probably would not be significant. No accurate means of checking the linearity of the instrument were available. However, linear response with low resistance meters is a well known characteristic of this type of photocell.

much better than the above fractions of the incident flux since it is also exposed to the flux transmitted across the circle from the other side of the annulus. Because of the problems of scattering and the annular nature of the chamber the situation does not submit to precise analysis. However, it is clear that uniformity of illumination is good. Furthermore, the design of the apparatus insures that the effective light intensity is not changed by the growth or sampling of a culture. All factors considered, it is probably safe to estimate that no cell of a given suspension ever experiences a variation of light intensity of more than about 20 per cent.

Cells harvested from the apparatus have been examined for cellular characteristics. Parallel measurements of photosynthetic activity are reported in the following paper of the series.

Cell volume (cubic millimeters of packed cells per milliliter of suspension) was estimated by a centrifuging technique which in our hands has yielded good precision (±2 per cent). Duplicate 20 or 25 ml. aliquots of suspension are centrifuged out in conical centrifuge tubes of 50 ml. capacity cut down so that the capillary of a Van Allen thrombocytocrit (Van Allen, 1926) may be inserted to the bottom. The packed cells, resuspended in a little fresh Knop's solution of the same pH as the harvested suspension (pH 5.8), are sucked up into the bulb of the thrombocytocrit. A second small portion of the Knop's is used as a wash and also drawn up. The usual clip is placed over the thrombocytocrit capillary and the tube and duplicate centrifuged for 1 hour at 3300 R.P.M., 20 cm. radius, or a relative centrifugal force of 2420. As noted by others (Tang and French, 1933; Sargent, 1940) readings of the volume of packed cells approach a minimum value asymptotically. A minimum value is normally attained in less than 45 minutes. A 2nd hour of centrifuging produces less than 1 per cent decrease in reading. The reading remains unaltered for some time. The cells are tightly packed and at first can be expelled only by insertion of a wire. In time they loosen up and can be blown out. The capillary has a capacity of 30 c.mm. calibrated to 0.5 c.mm. and readable to 0.1 c.mm. Checks with weighed amounts of mercury show an accuracy of calibration of ±2 per cent in a series of six tubes. Other checks show that the cell volume so determined is independent of the choice of aliquot size.

As an index of cellular material the cell volume has been widely used as an approximate measure and variously criticized as an exact measure in photosynthesis work (*cf.* Crozier, Tang, and French, 1934; Sargent, 1940; Tang and French, 1933; van Hille, 1938). The cell volume obtained in our hands by the above technique is a rapid and precise estimate of cellular material. It requires a total time of about 75 minutes and a working time of only about 15 minutes. The cell volume per milliliter of suspension was therefore run as a routine and used as a common denominator for other measurements which could not all be made simultaneously.

Cell numbers were estimated by hemocytometer counts of about 1000 cells in a total of four drops of suspension as previously described (Myers, 1944).

Cell dry weights were obtained by centrifuging known aliquots of suspension, washing twice in distilled water, and transferring the cells in a little water to tared weighing bottles. Cells were dried 24 hours at 105°C. As a check on the method of drying, several duplicate samples were dried at room temperature *in vacuo* and at 105°C. The dry weights obtained by the two methods were not significantly different though the dried cells were quite different in appearance. Cells dried at 105°C. gave a hard, coherent film from which they could not be readily suspended in water; those dried *in vacuo* formed a light, fluffy powder, easily resuspended.

Cell nitrogen was determined by slight modification of the method of Johnson (1941). Samples of suspension of 1 or 2 ml. were centrifuged out in test tubes and washed twice in water. Kjeldahl digestion and nesslerization were performed in the same tube. Nitrogen was estimated with a photoelectric colorimeter employing Corning filters No. 3387 and No. 5030 and calibrated with a standard curve. In our hands the precision of the method is estimated at ± 5 per cent.

Total chlorophyll was determined by extracting duplicate centrifuged samples (4 to 10 ml. of suspension) with boiling methanol, diluting to 10.0 ml., and reading in a photoelectric colorimeter employing a 1 cm. water cell, a sealed 1 cm. cell of M/3 copper sulfate, and an RG5 Jena filter. This gives a band extending from 640 to 710 mμ with a peak at 660 mμ. A calibration curve in relative units was obtained by quantitative dilution of a cell extract. Data are reported in these relative "chlorophyll units."[2] Inadequacies of the method (use of hot methanol, the broad wave length band of our colorimeter) are fully realized. However, as a relative estimate of total chlorophyll, it is rapid and reasonably precise ($\pm$ 2 per cent).

In practice samples of the culture suspensions were harvested at 24 hour (or other) intervals, leaving as an inoculum for the next interval a constant amount of suspension as indicated by a mark at an arbitrary height on the chamber. When a constant density of population is being maintained the relation between the amount of the inoculum and the amount of the sample is an index of rate of growth. From the characteristics of the continuous-culture apparatus perfectly logarithmic growth is to be expected, regardless of the population density maintained. Rate of growth is expressible in terms of the constant k in the equation $\log \frac{N}{N_0} = k\,t$. In applying the equation, N_0 is expressed in milliliters of inoculum at the beginning of each interval, N in total milliliters of

[2] A check against a chlorophyll preparation (American Chlorophyll Company, 5×) gave the result that 1 unit = 3.77 micrograms of chlorophyll. This would give chlorophyll analyses ranging from 4 to 20 per cent of the dry weight. In view of the values of 2 to 7 per cent reported by Sargent (1940) and calculated approximately from the data of Emerson and Arnold (1932) our values seem unreasonably high and we hesitate to report chlorophyll in absolute units. Cellular extracts and a methanol solution of the commercial preparation give nearly identical red absorption bands as checked by a Beckman spectrophotometer. A likely source of error not investigated is the purity of the commercial chlorophyll preparation.

suspension (milliliters of inoculum + milliliters of sample) at the end of a unit time interval, t, of 1 day.

RESULTS

The data are listed in Table I and presented graphically in Fig. 1. Each datum is an average of three determinations made on different days (in some cases weeks apart) over a period in which population density and growth rate were essentially constant. Population densities are presented as evidence that this is not a variable in the present experiments. Some experiments were run simultaneously at different light intensities; the general chronological order of

TABLE I

Light intensity	Relative growth rate	Dry weight	Cell nitrogen	Cell No.	Cell chlorophyll	Population density
f.-c.	k*	*gm.* $\times 10^{-6}$/ *c.mm.*	*γ/c.mm.*	$\times 10^{6}$ *cells/ c.mm.*	*units/c.mm.*	*c.mm./ml.*
360 (5)	0.840	27.0	20.3	6.3	2.7	0.60
92 (3)	0.830	24.2	21.9	13.2	5.3	0.70
55 (4)	0.730	23.7	20.7	15.3	6.9	0.70
35 (1)	0.576	22.2	21.3	26.0	9.0	0.64
19 (2)	0.336	—	—	—	—	—
(6)	0.362	—	—	24.0	10.0	0.69
10 (7)	0.180	21.4	21.5	31.2	11.6	0.65
6 (8)	0.103	—	—	28.2	12.7	0.70

* $k = 1/t \times \log \frac{N}{N_0}$, t being a time unit of 1 day.

experiments is indicated by the figures in parentheses after the light intensities in Table I.

The light intensity curve of growth in Fig. 1 is a typical light saturation curve and will be discussed in this connection in the following paper. It is presented here only for comparative purposes.

A marked feature of the curves is the concomitant variation in cell number and chlorophyll per unit volume of cells. With higher light intensities of culture the cells become increasingly larger but with about the same chlorophyll content per cell. This is an entirely unexpected phenomenon. It appears that the adjustment of cell size and chlorophyll content are correlated by a cellular mechanism in such a way that there are always about the same number of chlorophyll molecules per cell. The question now arises whether there are any real changes in chloroplast size and structure that accompany the variation of chlorophyll concentration in terms of cell volume. This has important implications in relation to theories of the *photosynthetic unit.*

The intermittent light studies of Emerson and Arnold (1932) revealed that

for very short intense flashes separated by comparatively long dark periods the photosynthetic yield per flash per cubic millimeter of cells is an approximately linear function of the chlorophyll content per cubic millimeter of cells. The relationship was such as to indicate about 2500 chlorophyll molecules per molecule of carbon dioxide reduced per flash. This observation gave rise to the concept of a *photosynthetic unit*, a physical unit in which a large number of chlorophyll molecules somehow participate in the reduction of one molecule of carbon

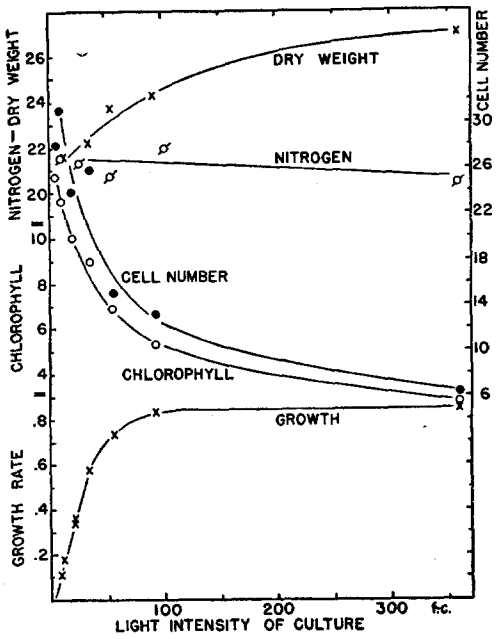

FIG. 1. Growth rate and cellular characteristics (cellular quantities/unit cell volume) as a function of light intensity of culture. Units are 10^{-5} gm. dry weight/c.mm., micrograms nitrogen/c.mm., 10^6 cells/c.mm., arbitrary units chlorophyll/c.mm.

dioxide. Alternative explanations have been proposed (*cf.* Emerson, 1937) and objections to the original theory have been made on various grounds. Our data offer a new point of view in interpretation of the observations of Emerson and Arnold.

When chlorophyll concentration is varied by light intensity of culture it appears that the effect is caused almost entirely by variation in cell size without much change in number of chlorophyll molecules per cell. It seems quite likely that a similar phenomenon occurs when chlorophyll is varied by the use of mercury or neon lamps as used by Emerson and Arnold. At least part of the difference between mercury and neon light must lie in a variation in effective intensity. Neon produced cultures which grew more rapidly and yielded cells

with lower chlorophyll concentration so that it seems comparable with our use of high intensity. If it be assumed that the same parallel between chlorophyll concentration and cell number occurred in the experiments of Emerson and Arnold as in ours, then carbon dioxide reduced per flash per cell (or per chloroplast) also becomes a constant value. In effect, the argument suggests a cellular or physiological type of the *photosynthetic unit*, which has quite different implications from those required of a physical type of unit.

Data on chlorophyll vary with light intensity in qualitatively the same fashion as previously reported for *Chlorella*. Sargent (1940) found chlorophyll contents of 3.3 per cent and 6.6 per cent respectively for cells grown under "high" and "low" light intensities. As a means of systematically varying chlorophyll concentration the fivefold range obtained here by control of light intensity may be compared with the sixfold range obtained by Emerson (1929) by control of iron concentration in *C. vulgaris*, the ten- to twelvefold range obtained by Fleischer (1934) by control of iron and nitrogen concentrations in the Cornell strain of *Chlorella*, and the sevenfold range obtained by Emerson and Arnold (1932) by use of illumination from mercury and neon gaseous discharge tubes for *C. pyrenoidosa*.

The dry weight per unit volume of cells changes more slowly with light intensity of culture, the total variation amounting only to about 20 per cent in the range of intensities studied here. Apparently cells grown at higher light intensities store less hydrated materials. In terms of dry weight and cell number our values are consistent with those cited by Tang and French (1933) for the same strain of *Chlorella*. Their data give 1 c. mm. cells = 18.7×10^6 cells = 23.2×10^{-5} gm.[3] Both of their values fit in between our values for cells cultured at 55 and 35 f.-c.

The variation in nitrogen content per unit volume is small and probably not significant. The nitrogen content of the cells is surprisingly high (7.5 to 10 per cent of the dry weight). Check analyses by the Dumas method[4] on a sample of dried cells grown at 92 f.-c. yielded 8.3 per cent nitrogen as compared to 9.1 per cent calculated from the appropriate values of Table I. The latter calculated value is an indirect one since the nitrogen and dry weight were determined on separate aliquots and would be higher than a direct determination on the dry material if any nitrogenous compound is lost on drying. The possibility of loss of ammonia on drying was tested by evacuating, through a trap of dilute acid, a tube containing a considerable quantity of cells. Subsequent nesslerization revealed no ammonia in the acid trap. We are unable to explain the somewhat higher value obtained by our micro Kjedahl procedure than that obtained by

[3] The last value is corrected in its decimal point according to a personal communication from Dr. C. S. French.

[4] We are indebted to Dr. J. W. Melton of the Department of Chemistry for these analyses.

Dumas analysis. At any rate the nitrogen content of *Chlorella* is high and in the same range as the nitrogen content of 5.9 per cent to 9.6 per cent found for isolated chloroplasts of several higher plants by Menke (1938) and Neish (1939).

DISCUSSION

The results do not in themselves dictate the selection of any one particular cellular quantity as an index for metabolic acitvity in *C. pyrenoidosa*. Cell nitrogen has frequently been chosen on the theory that it provides the best measure of cellular machinery. Unfortunately the nitrogen analysis is too slow and (in our hands) of too low a precision for routine work. It closely parallels cell volume. This is not surprising if it be considered that the hydration of the cell is particularly influenced by its protein content. There is nothing to indicate any marked changes or instability in the water content of the cells. We have selected cell volume as a convenient index of cellular material in investigating the photosynthetic characteristics of cells grown under different light intensities. The data presented relate other cellular characteristics to cell volume so that metabolic rates may be converted to other indices at will.

SUMMARY

1. *Chlorella pyrenoidosa* has been grown in a continuous-culture apparatus under various light intensities provided by incandescent lamps, other conditions of culture being maintained constant. The harvested cells were analyzed for cell number, dry weight, nitrogen, and chlorophyll per unit cell volume.

2. Cell nitrogen and cell volume are parallel measures of cellular material over the range of light intensity studied.

3. The dry weight per cell volume increases slowly with light intensity, showing about a 20 per cent variation.

4. Chlorophyll concentration and cell number show a concomitant decrease with increasing light intensity, varying in such a way that there are always about the same number of chlorophyll molecules per cell. It is considered that this phenomenon has bearing on the interpretation of data which has led to the theory of the photosynthetic unit.

BIBLIOGRAPHY

Arnon, D. I., *Am. J. Bot.*, 1938, **25,** 322.

Crozier, W. J., Tang, P. S. and French, C. S., *J. Gen. Physiol.*, 1934, **18,** 113.

Emerson, R., *J. Gen. Physiol.*, 1929, **12,** 609.

Emerson, R., in Annual Review of Biochemistry, (J. N. Luck, editor), Stanford University, Annual Review of Biochemistry, 1937, **6,** 535.

Emerson, R., and Arnold, W., *J. Gen. Physiol.*, 1932, **16,** 191.

Fleischer, W. E., *J. Gen. Physiol.*, 1936, **18,** 573.

Johnson, M. J., *J. Biol. Chem.*, 1941, 137, 575.

Menke, W., *Z. Bot.*, 1938, **183,** 135.
Myers, J., *Plant Physiol.*, 1944, **19,** 579.
Myers, J., and Clark, L. B., *J. Gen. Physiol.*, 1944, **28,** 103.
Neish, A. C., *Biochem. J.*, 1939, **33,** 300.
Sargent, M. C., *Plant Physiol.*, 1940, **15,** 275.
Tang, P. S., and French, C. S., *Chinese J. Physiol.*, 1933, **7,** 353.
Van Allen, C. M., *J. Lab. and Clin. Med.*, 1926, **12,** 282.
Van Hille, J. C., *Rec. trav. bot. néerl.*, 1938, **35,** 682.