RELATIONS BETWEEN PIGMENT CONTENT AND PHOTOSYNTHETIC CHARACTERISTICS IN A BLUE-GREEN ALGA*

By JACK MYERS and W. A. KRATZ‡

(From the Department of Zoology, University of Texas, Austin)

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Anacystis nidulans is a small blue-green alga which has many of the attributes desired in an experimental organism for photosynthetic studies. Reliable culture media and characteristics of its growth, photosynthesis, and respiration have been described (Kratz and Myers, 1955*a*, *b*). In exploratory studies it was noted that both the shape of the light intensity curve of photosynthesis and the visually observed pigmentation could be varied considerably by the culture conditions. It was found also that sonic treatment of aqueous cell suspensions gave quantitative breakage of cells to yield a clear aqueous extract. These observations led to the present study. Pigment characteristics and light intensity curves of photosynthesis are examined as related phenomena.

EXPERIMENTAL

Pure cultures of *Anacystis nidulans* were grown in a continuous culture apparatus (Myers and Clark, 1944) at 25 or 39°C., illuminated by tungsten lamps, and aerated with 0.5 per cent carbon dioxide in air. The cultures were maintained at low and constant population density by automatic dilution controlled by a photometric device. The culture chamber had the form of an annulus, about 6 mm. thick, between two vertical glass tubes. Samples were withdrawn periodically for the studies to be described below. The chamber was illuminated by parallel banks of lamps so that the radiation received per cell was independent of amount of suspension in the chamber. At any one temperature growth rate was controlled by light intensity. However, since the illumination was multidirectional and not easily interpretable, a more useful description of culture conditions is provided by the specific growth rate, k, which will be expressed in the convenient dimensions of $\log_{10}$ units per day.

The culture medium was medium C, described completely in a previous report (Kratz and Myers, 1955 *a*), containing per liter 0.25 gm. $MgSO_4 \cdot 7H_2O$, 1.0 gm. K_2HPO_4, 0.025 gm. $Ca(NO_3)_2 \cdot 4H_2O$, 1.0 gm. KNO_3, 0.165 gm. sodium citrate, 0.004 gm. $Fe_2(SO_4)_3 \cdot 6H_2O$, and 1.0 ml. of an A_5 trace element solution. The pH was 7.3–7.5 when aerated with 0.5 per cent carbon dioxide in air.

In preparation for experiments cells were centrifuged at 3300 *g* for 10 minutes

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‡ Present address: Trinity University, San Antonio, Texas.

in an angle centrifuge, washed once, and taken up in water or in the buffer solution to be used. Cell quantity was determined as routine in terms of packed cell volume obtained by centrifuging the cells out of the Warburg No. 9 buffer in capillary tubes for 1 hour at 2400 *g* in an International centrifuge. For cells grown under each set of growth conditions the relation between cell volume and dry weight was determined upon several different samples by washing twice in water, transferring to tared crucibles in a minimum quantity of water, and drying to constant weight at 105°C.

Photosynthesis was measured manometrically as rate of oxygen evolution in the Warburg No. 9 carbonate-bicarbonate buffer with a Na:K ratio of 1:1. It has been shown previously that this suspending fluid is equal to or better than any of the other common buffer systems used for manometric studies of photosynthesis. Cell quantities used were in the range of 0.05 to 0.4 mg. dry weight per vessel. The rectangular vessels had a cross-sectional area of about 12.5 cm.^{2} From the absorption curves presented below it may be calculated that the fractional light absorption by the cell suspension was never more than 5 per cent and usually less than 3 per cent, even for strongly absorbed spectral regions. The light source was a bank of 17 closely spaced 60 and 100 watt lamps operated at 120 $\pm$ 1 volts and located underneath the glass bottom of the bath. The total incident intensity could be varied by the use of screens inserted above the light bank. Rates in six vessels were measured simultaneously. Intensities upon the individual vessels were varied further by the use of Jena NG series neutral filters attached to the bottoms of the vessels by holders which also masked out stray light. Transmissions of the filters, which are not entirely constant over the visible region, were estimated from spectral transmission curves over the 550 to 700 mμ region which includes 75 per cent of the visible energy. The incident intensity upon the six filter-vessel combinations was measured before and after each experiment by a calibrated barrier-type photocell enclosed in an Erlenmeyer flask and immersed in the bath. By a slide and carriage arrangement the photocell could be moved to the mean position of each vessel.

Pigment characteristics were examined in acetone and aqueous extracts. Two aliquots of the original harvested suspension were centrifuged and the cells washed once in water. The cells of one aliquot were suspended in 80 per cent acetone for 5 minutes and centrifuged; a second treatment gave quantitative removal of chlorophylls and carotenoids. The cells of the second aliquot were resuspended in water and broken by 30 minutes in a Raytheon 10 kc. generator; centrifugation at 3300 or 7200 *g* for 15 minutes in an angle centrifuge yielded a clear supernatant (pH 6.8–7.0) and a sediment of a few whole cells and cell debris amounting to less than 2 per cent of the original cell volume. Both extracts were made up to measured volumes and their absorption curves determined on a Beckman Model DU spectrophotometer.

The five types of cells used are described in Table I. Each type of cell is given a code letter for identification throughout the following sets of data.

Pigment Characteristics

Absorption curves for the acetone extracts and aqueous extracts of sonic-disintegrated cells are presented in Figs. 1 and 2. Each curve is based upon the

data of two experiments usually run on different days. In order to afford quantitative interpretation all data are calculated to optical densities for a 1.0 cm. layer containing 1.0 mg. dry weight per ml. For convenience complete curves are shown only for cells of types A and D and the other types are described only by the absorption peaks. Complete curves were obtained for all types of cells except B1 for the region 400 to 750 mμ. The exploratory pigment work and first absorption curves were run on cells of type B2 using lamps at a distance of 20 cm. For technical reasons this distance was changed to 30 cm. In the later routine of investigation pigment studies on type B1 cells were limited to absorption curves of the acetone extract over the red peak.

TABLE I

Characteristics of the Cells Used

Designation type	Growth conditions				Cell characteristics		
	Temperature	Light intensity	Growth rate	Density	Dry weight/cell volume	Respiration Q_{O_2}	
	°C.	watts*	k‡	mm.³cells/ml.	mg./mm.³	39° C.	25° C.
A	39	80	0.33	1.0–1.2	0.297	3.4	1.1
B1	39	320	1.3	1.2–1.3	0.271	4.7	1.5
B2	39	320§	—	1.5–1.7	0.271	—	—
C	39	960	2.5	1.6–1.8	0.266	7.5	2.5
D	25	320	0.55	2.3–2.5	0.278	4.5	1.5
E	25	960	0.85	2.5–3.0	0.260	17.7	9.3

* Total input watt rating of lamps used for illumination. Lamps were mounted on four vertical racks parallel to the chamber at a distance of 30 cm. (except B2).

‡ Specific growth rate defined by the relation $\log_{10} N/N_0 = kt$ in which t is measured in days. Light-saturated growth rates are 0.87 ± 0.03 at 25° C. and 3.3 ± 0.1 at 39°C.

§ Illumination differs from B1; lamps at a distance of 20 cm.

From the absorption curves concentrations of the chlorophyll, carotenoid, and phycocyanin pigments may be estimated. Critical values from the absorption curves and pigment contents calculated therefrom are given in Table II.

Chlorophyll *a* may be calculated from the data of MacKinney (1941) for 80 per cent aqueous acetone using the value of specific absorption coefficient $\alpha = 82.04$ at 663 mμ. If optical densities at 663 and 645 mμ are used to calculate concentrations of both chlorophylls *a* and *b* (*cf.* Arnon, 1949), chlorophyll *a* values differ by less than 1 per cent of those calculated at 663 mμ alone and chlorophyll *b* is always less than 4 per cent of chlorophyll *a*. This observation is taken as presumptive evidence of complete lack of chlorophyll *b* as previously inferred for another blue-green alga on similar grounds (Emerson and Lewis, 1942).

Carotenoids are estimated with uncertainty since neither specific carotenoids

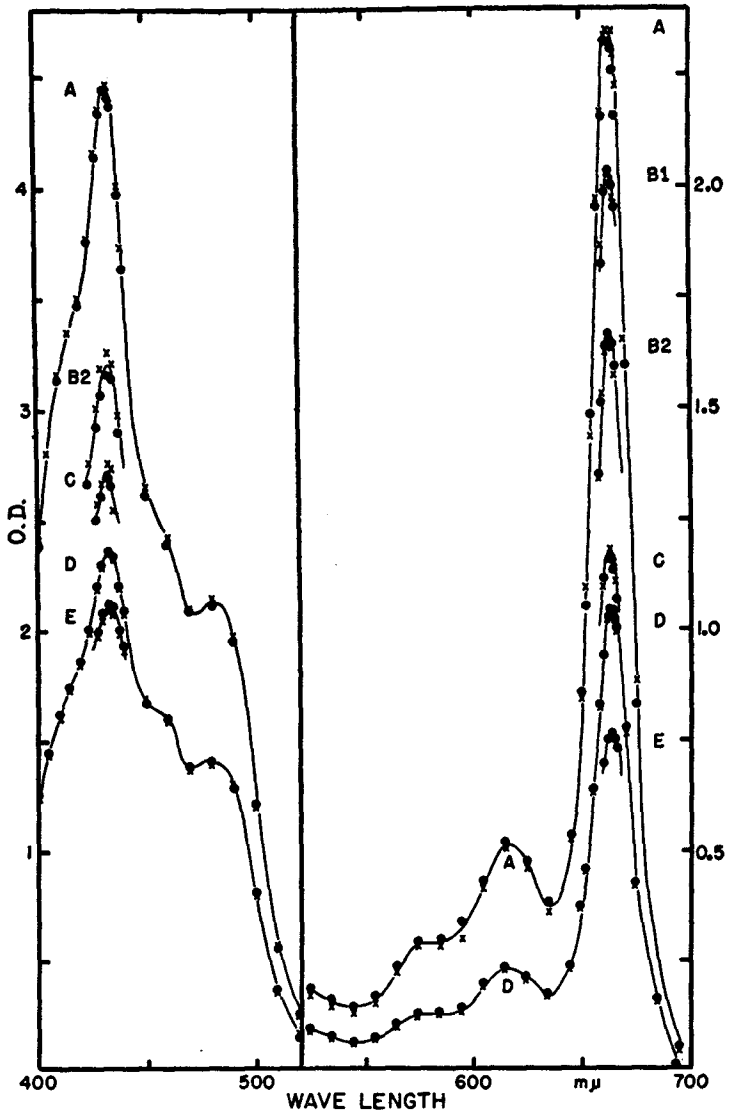

FIG. 1. Absorption curves for pigments of *Anacystis nidulans* extracted in 80 per cent aqueous acetone. O. D. represents optical density of 1.0 cm. of an extract containing the pigments of 1.0 mg. of cells per ml. Code letters refer to the cell types of Table I.

present nor their proportionate quantities are known. A rough calculation may be made following MacKinney (1941) by assigning a value of $\alpha = 200$ for total carotenoids in 80 per cent acetone at 460 mμ where absorption of chlorophyll *a* is negligible.

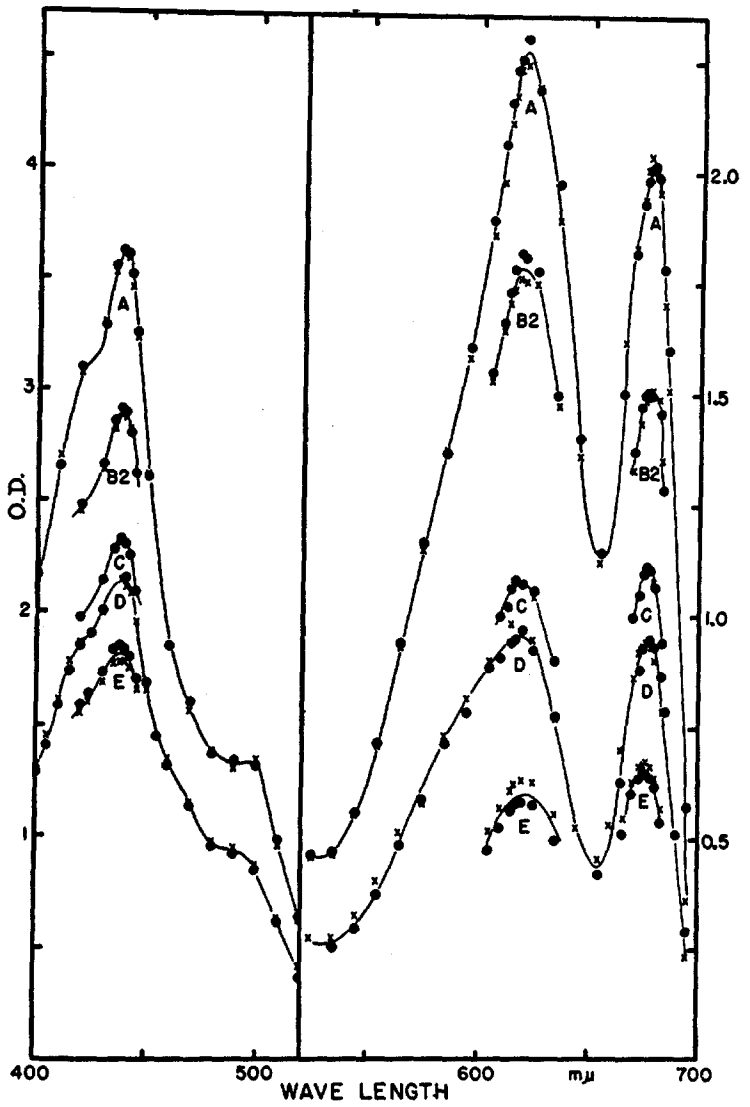

FIG. 2. Absorption curves for pigments of *Anacystis nidulans* in water after sonic cell disintegration. O. D. represents optical density of 1.0 cm. of an extract containing the pigments of 1.0 mg. of cells per ml. Code letters refer to cell types of Table I.

The phycocyanin peak in the aqueous extract at about 618 mμ corresponds closely in position to the 615 mμ observed by Svedberg and Katsurai (1929) in *Aphanizomenon*, 615 mμ observed by Emerson and Lewis (1942) in *Chroococcus*, and 620 mμ observed by Duysens (1952) in *Oscillatoria*. When *Anacystis* is extracted with acetone to remove chlorophylls and carotenoids and then dis-

integrated by sonic treatment, the resulting blue aqueous extract gives an absorption curve very close to those of Duysens (1952) and Emerson and Lewis (1942) with peak at 617 mμ. Extracts of this kind were not used as routine in the present study because of some difficulties in obtaining quantitative cell breakage. Phycocyanin content may be estimated from optical density of the total aqueous extract at 618 mμ but requires correction for the appreciable chlorophyll absorption at this wave length. The following procedure was adopted to estimate optical density at 618 mμ due to phycocyanin alone.

TABLE II

Critical Values from the Absorption Curves and Calculated Pigment Concentrations

Cell type	Absorption values*						Pigment concentrations		
	80 per cent acetone		Water				in per cent dry weight		
	D_{663}	D_{460}	D_{618}	D_{677}	D_p‡	D_c‡	Chlorophyll§	Carotenes ‖	Phycocyanin¶
A	2.34	2.40	2.27	2.02	1.90	1.87	2.85	1.20	24.1
B1	2.03	—	—	—	—	—	2.47	—	—
B2	1.67	1.92	1.78	1.51	1.50	1.39	2.04	0.96	19.0
C	1.17	1.66	1.08	1.11	0.87	1.04	1.43	0.83	11.0
D	1.04	1.60	0.96	0.95	0.78	0.88	1.27	0.80	9.9
E	0.77	1.65	0.61	0.66	0.48	0.62	0.94	0.83	6.1

* Optical densities of a solution which presents the extract of 1.0 mg. cells to 1.0 cm.^{2} of the light beam.

‡ D_p and D_c designate optical densities contributed by phycocyanin and chlorophyll at 618 and 677 mμ respectively; calculated from D_{618} and D_{677} (see text).

§ Per cent chlorophyll = $D_{663}/0.8204$; based on value of MacKinney (1941).

‖ Per cent carotene = $D_{460}/2.0$; based on value of MacKinney (1941).

¶ Per cent phycocyanin = $D_p/0.079$; based on value of Svedberg and Katsurai (1929).

For the aqueous extracts adopt the following notation for phycocyanin and chlorophyll absorption:

	618 mμ	*677 mμ*
O. D. of phycocyanin absorption	D_p	d_p
O. D. of chlorophyll absorption	d_c	D_c
O. D. of chlorophyll plus phycocyanin absorption	D_{618}	D_{677}

From a replotting of the phycocyanin curves of Duysens (1952) and Emerson and Lewis (1942) and from our own curves of the aqueous extract of acetone-treated cells the relation between phycocyanin absorptions at the two wave lengths is estimated $d_p/D_p = 0.08 \pm 0.02$. The relation d_c/D_c must be evaluated from the acetone extracts with the assumption that it is the same in both solvents when wave length shifts due to the solvent are considered. The blue

chlorophyll peak occurs at 438 mμ in water and at 433 mμ in acetone, a frequency shift of 264 cm^{-1}. The red chlorophyll peak occurs at 677 mμ in water and at 663 mμ in acetone, a frequency shift of 312 cm^{-1}. Applying a shift of 300 $cm.^{-1}$ would move the 618 mμ of chlorophyll in water to 608 mμ in acetone. Hence d_c/D_c is evaluated as $D_{608}/D_{663} = 0.200 \pm 0.004$ from the five acetone extract curves, corresponding closely to the value of 0.199 taken from the data of MacKinney (1941). For the aqueous extract data D_p and D_c now may be evaluated:

$$D_p = 1.016\ D_{618} - 0.203\ D_{677} \text{ and}$$
$$D_c = 1.016\ D_{677} - 0.0813\ D_{618}$$

Calculated values of D_p and D_c are given in Table II. A check upon the calculation is afforded by comparing D_c, the calculated red peak absorption in water, with D_{663}, the observed red peak absorption in acetone. The ratio D_c/D_{663} is 0.84 ± 0.05 for the five types of cells in spite of a threefold variation in chlorophyll content.

Phycocyanin content of the cells may be estimated from the specific absorption coefficient of 7.9 at its peak value cited by Svedberg and Katsurai (1929). Unlike the absorption coefficients used for chlorophylls and carotenoids, the phycocyanin value includes the total chromoproteid. Estimates of the chromophore content are less certain (*cf.* Rabinowitch, 1951, p. 667) and therefore are not used. Comment is due on the remarkably high phycocyanin content of 24 per cent for type A cells. Since these cells contain 9.4 per cent nitrogen by elementary analysis, the phycocyanin must make up about 40 per cent of the total cell protein.

Examination of the chlorophyll contents of the different cell types given in Table II shows a declining trend throughout the series which is considered an effect of increasing light intensity enhanced at lower temperatures (*cf.* Table I). The phenomenon of decreasing chlorophyll with increasing light intensity is similar to that observed in *Chlorella* (Myers, 1946 *a*) and to the many observations on *sun vs. shade* plants. Adjustment of chlorophyll content to light intensity is of obvious ecological advantage to the organism but there is yet no biochemical explanation of the mechanism of adjustment.

The three fold range in chlorophyll content is paralleled by a fourfold range in phycocyanin content. A limited 1.4-fold variation in phycocyanin/chlorophyll ratio is disappointing; it presages no likelihood of experimental control of ratio for study of energy transfer mechanism. In another sense the relative constancy of the phycocyanin/chlorophyll ratio speaks for closely related mechanisms of synthesis of the two pigments. In contrast the carotenoid content shows rather small variation and little relationship to the other pigments Cells of type E with depressed chlorophyll and phycocyanin have a yellow-green appearance and might be mistaken for Chlorophyceae.

Question may be raised regarding the dispersal of pigments in the aqueous

extract. A similar kind of preparation obtained from the blue-green alga *Synechococcus cedorum* was examined by Calvin and Lynch (1952). By grinding or ultrasonic treatment and centrifuging at 2000 *g* for 10 minutes they obtained a "blue-green supernatant having a strong Tyndall effect and a slight fluorescence." Further centrifugation at 36,000 *g* for 30 minutes gave a clear blue supernatant with characteristic phycocyanin absorption and only traces of the red chlorophyll peak; the particulate sediment showed a typical chlorophyll carotenoid absorption curve without evidence of phycocyanin.

Comparison with the preparation of Calvin and Lynch (1952) is provided by the following observations. Our preparations show less light scattering as evidenced by proportionately lower absorption at 740 mμ; D_{740}/D_{677} = 0.02–0.03 compared to a value of 0.1 estimated from the curves of Calvin and Lynch. One preparation was centrifuged at 52,000 *g* for 30 minutes in a refrigerated spinco centrifuge. The cleanly removed supernatant showed 81 per cent of the D_{618} and 60 per cent of the D_{677} of the original preparation. The sediment was very loosely packed and was removed only with appreciable contamination with supernatant. There is no uncertainty that the chlorophyll of our preparations is more finely dispersed than in the preparation of Calvin and Lynch.

The original extract and the 52,000 *g* centrifuged supernatant and sediment were observed in an intense focused cone of tungsten light with the aid of Corning blue filters number 4303 + 5030 ($\lambda < 600$ mμ) and red filter number 2403 ($\lambda > 620$ mμ). When viewed through the blue filter a Tyndall cone was observed with the original extract and sediment but not with the supernatant. When illuminated through the blue filter and viewed through the red filter a strong fluorescence was observed with the original extract and supernatant but not with the sediment. The chlorophyll concentrations in all three preparations, as indicated by the red absorption peak, had been brought to about the same value ($D_{677} = 0.30 \pm 0.03$ in a 1.0 cm. cell). It has been observed also that the original extract has only very slight and uncertain photochemical activity in dye reduction or in evolution of oxygen from quinone.

Light Intensity Curves of Photosynthesis

Light intensity curves of photosynthesis of the five types of cells at 25 and at 39°C. are presented in Fig. 3. The data are corrected for respiration according to values cited in Table I. Each curve is constructed from the combined data of two to four experiments and therefore contains any day-to-day variability in experimental material plus the errors of packed cell volume measurements as well as the errors of manometry. However, even within any one experiment the data obtained at 39°C. show greater scatter than expected of the manometric method. A search for unusual sources of error in the manometry was not successful.

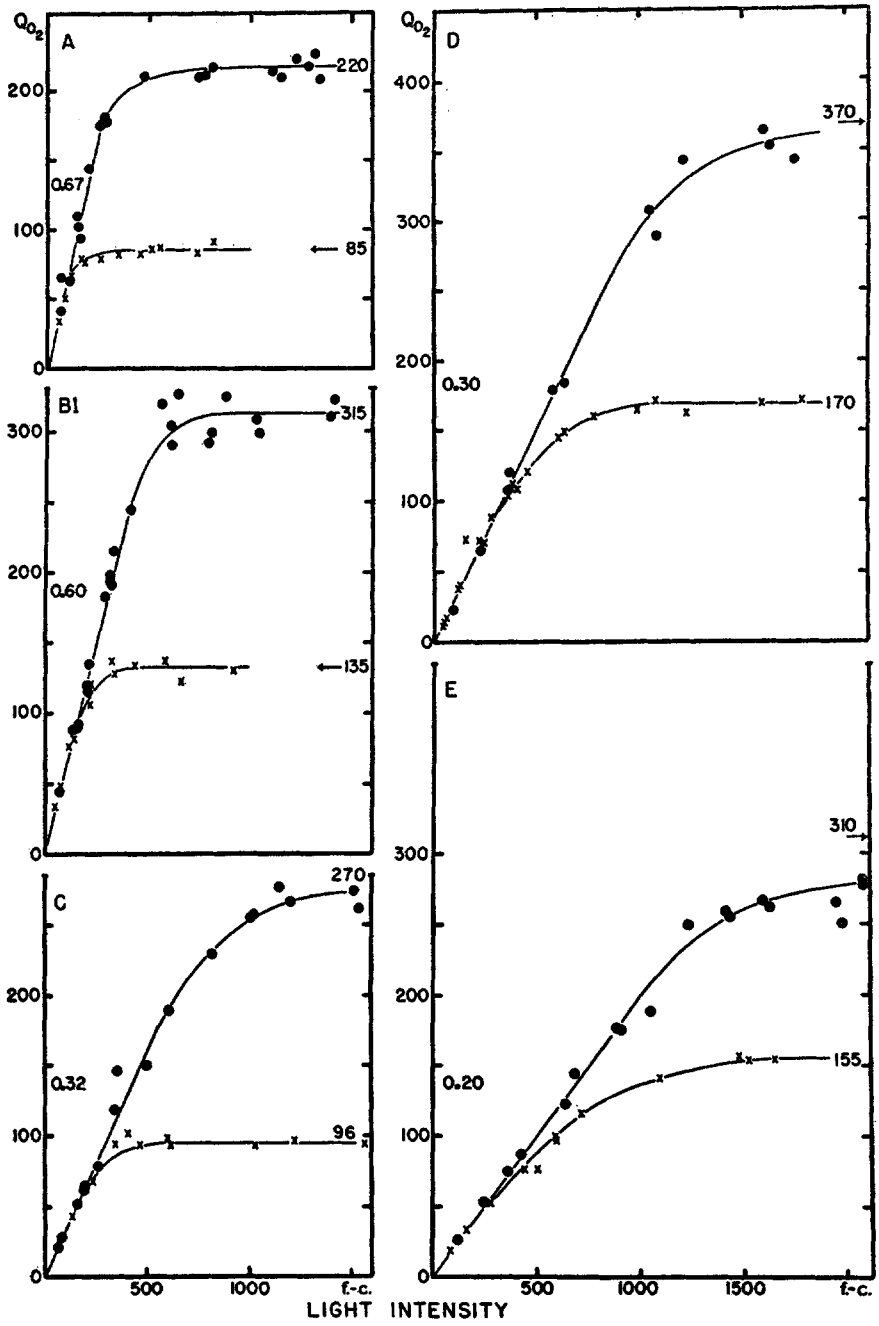

FIG. 3. Light intensity curves of photosynthesis of *Anacystis nidulans* at 25°C. (× × ×) and 39°C. (● ● ●). Code letters to different cell types and respiration corrections used are given in Table I.

The most interesting feature of the light intensity curves is the slope (α) of the light-limited portion designated by the figure to the left of each curve in dimensions of Q_{O_2}/f.-c. The slope may be considered a function of two characteristics of the cells: (1) their quantum yield and (2) their fractional light absorption as manifested by their pigment concentrations. In Fig. 4 α has been plotted as a function of chlorophyll and phycocyanin concentration. The plot against chlorophyll content has been drawn as a straight line extrapolating to

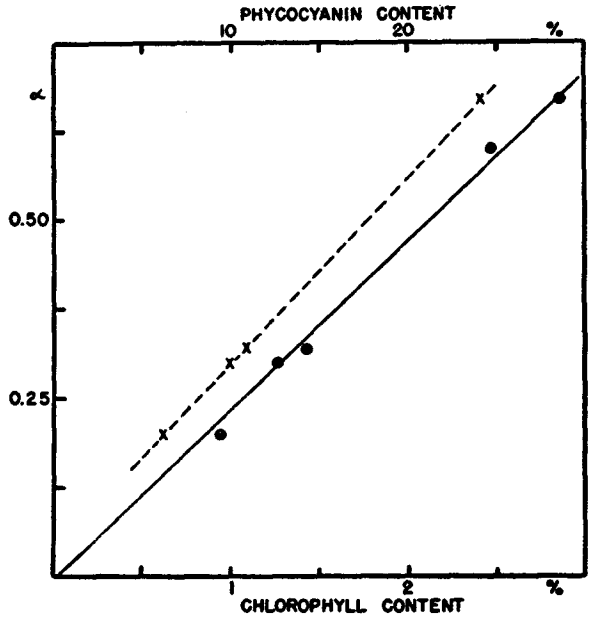

FIG. 4. The initial slope, α, of the light intensity curve as a function of chlorophyll (● ● ●) and phycocyanin (× × ×) content of *Anacystis nidulans*.

the origin although a slightly better fit is obtained by a straight line satisfying the experimental points only. The four points for phycocyanin content can be fitted to a straight line but not one which extrapolates to the origin. Because of small variations in chlorophyll/phycocyanin ratio and the limited precision of the method used, any special significance assigned to either pigment would be of doubtful significance. It may be inferred, however, that over the three- to fourfold range of pigment contents of the cells studied no marked variation in quantum yield is to be expected. An alternative statement of the case is that over the range of culture conditions studied there is no variation in rate of light-limited photosynthesis which is not explainable in terms of pigment concentrations.

Estimates of the light-saturated rates of photosynthesis (Q_{O_2}-max.) at 25

and 39°C. are indicated by the numbers at the right of each curve. For the 39°C. curves of C and D cells light saturation was not reached within the intensity range shown; approximate values (still containing some uncertainty) are shown for measurements under higher intensities obtained by raising the lamp voltage to 130 volts. If Q_{O_2}-max. for cells grown at 39°C. is plotted against either chlorophyll content or the previous specific growth rate (Table I), it is found to go through a maximum observed for type B cells. A similar phenomenon has been reported for *Chlorella* (Myers, 1946 *b*). Evidently the light-saturated rate of photosynthesis is controlled by factors which bear no simple relation to the development of pigments.

At light limitation all the curves of Fig. 3 show the expected lack of temperature effects. At light saturation curves A, B, and C for cells grown at 39°C. show a Q_{10} of 1.96 $\pm$ 0.13; curves D and E for cells grown at 25°C. show a Q_{10} of 1.69 $\pm$ 0.05. The latter value contains some uncertainty in attainment of light saturation at 39°C. but there is little doubt that the Q_{10} is greater for cells grown at the higher of the two experimental temperatures.

DISCUSSION

The data presented lead to no remarkable or unexpected result. The linear relation between chlorophyll concentration and the initial slope of the light intensity curve was anticipated but had not been demonstrated experimentally. The small variation in chlorophyll/phycocyanin ratio, in spite of a three- to fourfold variation in either component, is significant; it relates to the problems of phycocyanin synthesis on one hand and the mechanism of phycocyanin-chlorophyll energy transfer on the other. The data document further the usefulness of *Anacystis nidulans* as an experimental organism. The characteristics herein described recommend it for pigment, energy transfer, and quantum yield studies.

SUMMARY

1. The blue-green alga *Anacystis nidulans* was cultured under steady state conditions at 25 and 39°C. and under several different light intensities to give five different types of cells.

2. Cells were submitted to pigment analysis based upon acetone extracts and aqueous extracts obtained by sonic disintegration. The different cell types show a threefold range of chlorophyll content and a fourfold range of phycocyanin content with only minor changes in the chlorophyll/phycocyanin ratio. Cells of highest pigment content were estimated to contain 2.8 per cent chlorophyll *a* and 24 per cent phycocyanin, the latter on a total chromoproteid basis.

3. Light intensity curves of photosynthesis were obtained for each of the cell types at 25 and at 39°C. The slopes of the light-limited regions of the curves

are approximately linear functions of chlorophyll and phycocyanin contents. Maximum light-saturated rates of photosynthesis at 25 and 39° show no simple relation to pigment content.

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